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Title: Epigenetic modifiers as game changers for healthy ageing

Shikha Sharma*and Ramesh Bhonde*

Affiliations: Corresponding author: Dr. Ramesh Bhonde

Emeritus Scientist

Director Research

Dr D.Y. Patil Vidyapeeth University

Pimpri Pune 411018

India

Office :020 27805000 Ext 5046

Mobile: +91-9686010498

E-mail: rrbhonde@gmail.com

Alternate mail ID : ramesh.bhonde@dpu.edu.in

First author and corresponding author: Dr. Shikha Sharma

Affiliation: Institute for Stem Cell Science & Regenerative Medicine

GKVK Campus,

Bangalore-560065, India.

E-mail: shikha.ss19254@gmail.com

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Abstract

Epigenetic alterations during ageing are manifested with altered gene expression linking it to lifespan regulation, genetic instability, and diseases. Diet and epigenetic modifiers exert a profound effect on the lifespan of an organism by modulating the epigenetic marks. However, our understanding of the multifactorial nature of the epigenetic process during ageing and the onset of disease conditions as well as its reversal by epidrugs, diet, or environmental factors is still mystifying. This review covers the key findings in epigenetics related to ageing and age-related diseases. Further, it holds a discussion about the epigenetic clocks and their implications in various age-related disease conditions including cancer. Although, epigenetics is a reversible process how fast the epigenetic alterations can revert to normal is an intriguing question. Therefore, this paper touches on the possibility of utilizing nutrition and MSCs secretome to accelerate the epigenetic reversal and emphasizes the identification of new therapeutic epigenetic modifiers to counter epigenetic alteration during ageing.

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Introduction

Ageing is a progressive decline in the physiological process which leads to the onset of various diseases and disorders such as diabetes, cancer, and cardiovascular diseases and the risk of higher mortality rate. The probability of cancer increases after 50 years of age while heart diseases increase after 60 years of age. Thus ageing has the foremost impact on the social-economic welfare of an individual [1]. Hence, there is a dire need for rejuvenating factors that can delay ageing and extend lifespan. In the last few decades, life expectancy has increased widely due to the significant improvement in medical health facilities and health awareness among individuals [2]. However the mechanism underlying ageing is still unclear. Various studies are undergoing to delineate the cellular and molecular hallmarks of ageing. Epigenetic modification is considered to be the vital mechanism that influences the gene expression associated with ageing and age-related disorders such as heart failure and cancer [3,4]. Epigenetic factors influence gene regulation by regulating the binding of transcriptional factors to the DNA. These epigenetic factors include enzymes such as methyltransferase and demethylase that modify the methylation status of the DNA or core histone subunits H2A, H2B, H3, and H4 as well their variants H3.3, macroH2A, and H2A.Z. Emerging evidence support that ageing is associated with the gradual loss of histones, chromatin remodelling, DNA, and histone modifications [5, 6]. Due to the reversible nature of epigenomics, the current focus is on delineating the epigenetic changes involved in ageing to develop novel therapeutic targets to circumvent ageing and age-related disorders [5, 6]. This review covers the key finding in epigenetics related to ageing, age-related diseases and how epigenetic modification have an impact on lifespan and health span extension. It will further discuss epigenetic clocks and their implication in various age-related disease conditions including cancer. Finally, it will shed light on the role of nutrition including methionine, folate, caloric restriction, diet and MSCs exosomes as epigenetic modifiers in combating ageing.

Epigenetics and Aging

Ageing and Lifespan have been linked to epigenetic modifications in many organisms. Various studies suggest that epigenetic modifications either lead to the alleviation of ageing, delay age-associated disorders and increase the lifespan of organisms or promote

ageing and decrease the lifespan of organisms. Therefore understanding the mechanism underlying the epigenetic modifications in ageing will provide new insight into the development of strategies that could delay ageing and age-associated disorder and extend lifespan and health span.

Histone modifications

Histone modification at the post-translational level includes methylation, acetylation, phosphorylation and ubiquitination. Among these, histone modifications at lysine residue including methylation and acetylation are widely studied and known to influence the ageing process [7].

Influence of histone modifications on ageing and lifespan of different species

Histone methylation such as H3K4me3, H3K36me3, and H3K79me3 are associated with transcriptional activation while H3K27me3, H3K9me3, H4K20me3 are involved in the transcriptional repression and these modifications can drive the ageing process by regulating the genes related to the same [8]. A study showed that knockdown or inhibition of mRNA level of H3K4 mono- and di-demethylase LSD-1 increases the lifespan in *C. elegans* [9]. Also, it was seen in yeast that mutation in COMPASS (H3K4 methyltransferase complex) influences the expression of 500 genes and reduces the replicative lifespan [10]. It was found in *S. cerevisiae* and *C. elegans* that loss of H3K36me3 is associated with a shorter lifespan or deletion of K36me2/3 demethylase Rph1 in yeast is associated with increased expression of H3K36me3 and longer lifespan [7]. Knockdown of UTX-1 extends the lifespan of worms with an increase in the global expression of H3K27me3 [11]. It was observed in *drosophila* that hypomethylation of H3K27me3 is associated with longevity and a healthy life span by promoting glycolysis [12]. A study stated global reduced expression of H3K27me3 in the prematurely aged cells of patients with Hutchinson-Guildford progeroid syndrome (HGPS) [7]. Liu et al. observed increased expression of H3K27me3 in the quiescent satellite cells of mice [13]. Another study showed that loss of H3K9me3 in adult drosophila midgut was associated with intestinal stem cell ageing. [7]. It was observed that H3K9me3 methyltransferase SUV39H1 was downregulated in both human and mouse HSCs with age which corresponded to global decreased expression of

H3K9me3 and impaired heterochromatin function which is a marker for ageing [14]. It was found that the H3K4me3 level was upregulated in mouse hematopoietic stem cells (HSCs) with age [15]. However, H3K4me3, H3K4me1 and H3K27ac were found downregulated in human HSCs with age [7]. These studies indicate that mice and worms exhibit a different pattern of H3K27me3 during ageing as compared to human and drosophila while mouse and human HSCs showed distinct expressions of H3K4me3 during ageing indicating that histones marks may play an opposite role in controlling ageing across different species.

Acetylation of a histone lysine results in the neutralization of its positive charge which weakens the interaction between histone and DNA and leads to transcription activation [8]. Histone acetyltransferases (HATs) are known as transcription activators while histone deacetylases (HDACs) are regarded as corepressors and both play role in ageing and longevity [8]. It was found that aged mouse hepatocytes exhibit decreased expression of H3K9me3/H3K14ac bivalent marks suggesting that histone acetylation is associated with ageing. [7]. Sirtuins are class III HDAC that play role in the regulation of deacetylation of a lysine residue in an NAD^+ dependent manner [7]. Among sirtuins, SIRT1 was found to downregulated in various tissues of mice and humans including the heart, liver, kidney, and brain with age [7]. Sirt6 is an H3K9 deacetylase that was shown to inhibit senescence when overexpressed in rat and human nucleus pulposus cells and contributes to longevity [16]. It was found that the knockdown of H3K56ac, Hst3, and Hst4-related HDAC-encoding genes during yeast ageing is associated with a shorter lifespan [17]. SIRT2 level is essential for the rejuvenation of oligodendrocytes progenitor cells and is associated with remyelination in aged mice [18]. These reports suggest that increased expression of HDACs is associated with a longer lifespan and can be used as therapeutic targets to boost the lifespan of an organism.

Histone methylation in age-related pathological conditions

Aberrant histone methylation directly contributes to age-related pathological conditions by dysregulating several signalling pathways which results in the development and progression of several pathological conditions including cardiac disorders, neurological disorders, osteoporosis, and cancer [19]. Lyu et al. showed that loss of H4K20me3 is

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associated with cardiac ageing and is regulated by TGF-ß signalling through miR29a [20] [19]. Global H3K79 hypermethylation was observed in the neurons of aged individuals which was inversely coupled to the energy metabolism suggesting its role in brain ageing and age-related brain disorders [21]. Zhang et al. found a decrease in the expression of H3K27me3 levels and an increase in the expression of H3K4me3 in neuroinflammation-associated genes of 22-month-old rats. Further, they noted that H3K27me3 was downregulated in intracerebral haemorrhage (ICH) induced acute brain injury of the young and old rats while H3K9ac levels were increased in 22 month old rats after ICH whereas H3K4me3 levels were unchanged in the young and old rat after ICH suggesting H3K27me3 and H3K9ac as the possible epigenetic target for the treatment of age-related brain injury and neuroinflammation [22]. It was found that aged mice exhibited reduced expression of SUV39H1 histone methyltransferase and upregulation of Mkp-1 in the hippocampus which was associated with a depressive-like phenotype [23]. Alzheimer's disease (AD) is observed mainly in the elderly and is related to dementia [24]. Lee et al., observed elevated levels of H3K9me3 in the cortical neurons of AD patients which were associated with abnormal heterochromatin structure and synaptic dysfunction [24]. These findings indicate distinct expression of H3K9me3 in the different regions of the brain which is associated with different pathological conditions. Spinal stenosis is an age-related degenerative disorder commonly found in the elderly population [25]. It was found that H3K4me3, H3K36me3, H3K9ac, and H3K18ac were highly upregulated in the fibrocartilage area adjacent to the degenerated irregular ligament in the ossification of ligamentum flavum (OLF) rat model [25]. Further, WNT5A and GDNF were found hypermethylated in non-OLF MSCs than OLF MSCs suggesting their role in the regulation of osteogenic genes including RUNX and BMP2 in spinal ligament ossification [25]. Various studies showed that aberrant histone modification and DNA methylation of critical genes including WNT5A, GDNF, ACSM5, miR-497 and miR-195 increases spinal ligament degeneration [25].

Histone acetylation in age-related pathological condition

Altered histone acetylation has been associated with large-scale chromatin modifications and thus can influence gene expression [26]. These modifications have been observed in ageing tissues and various age-related pathological conditions such as neurological

disorders, cardiac disorders, cancer and liver disorder [26]. It was found that sirtuins genes including sirt2, sirt3, sirt4, and sirt6 were differentially expressed in aged mice and were associated with loss of cochlear hair cells. Among these, sirt6 was found to be upregulated during ageing and suggested as a potential biomarker and therapeutic target for aged-related hearing loss [27]. It was found that sirt2 inhibition was associated with the amelioration of cognitive function and Aβ pathology in the AD mouse model [28]. A study reported that cognitive impairment can be prevented in aged mice by activating the AMPK-SIRT1-PGC1α pathway and antagonizing oxidative stress [29]. A ketone ester-rich diet mediated increased expression of sirt3 prevents degeneration of GABAergic neurons and seizure-related death in AD mice [30]. Song et al. reported that AMPK/Sirt1-mediated inflammation was increased in ageing rats and was positively correlated with myocardial fibrosis [31]. A study found reduced vascular expression of sirt1 in old obese individuals implicated in microvascular dysfunction through controlling mitochondrial ROS levels and proteins involved in the mitochondrial respiration chain [32]. Li et al., observed that sirt6 suppressed charged multivesicular body protein 2B (CHMP2B) in mouse aged hearts associated with ageing-related intolerance to ischemia/reperfusion (I/R) injury [33]. Zhu et al., observed that inhibiting HDAC3 in the hippocampus reduces spatial memory deficits and reduces amyloid plaque load and Aβ levels in the brains of AD mice model [34]. A study stated downregulation of acetylation of H3 histone at K9, K18 and K27 in human-induced pluripotent stem cells derived hepatocytes (hiHep) ageing. Further, they showed that increasing the acetylation of histones using histone deacetylase inhibitors including Nab and valproic acid resulted in increased proliferation of aged hiHep (>=40d) suggesting epigenetic modifiers as probable therapeutic targets for ameliorating ageing and age-associated disorders such as reduced hepatocyte plasticity [35]. These research reports suggest chromatin remodellers including SIRT1 and SIRT6 as important therapeutic targets for longevity and better health management for preventing the deterioration of various cells, tissues and organs of the body. Therefore it would be interesting to delineate the role of histone modifications in DNA repair, metabolism, inflammation, and oxidative stress in combating ageing. It would be worthwhile to explore the most prevalent histone marks among different age related disorder associated with ageing process and the epigenetic modifiers for the same.

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Influence of DNA methylation on ageing and age-related disorders

DNA methylation is a chemical modification that occurs mostly on the 5-carbon of cytosine island (CpG dinucleotide) identifiable by microarrays, deep sequencing, bisulfite conversion coupled to PCR, LC-MS/MS and pyrosequencing [36, 37, 38, 39]. DNA methylation/demethylation is associated with transcription repression/activation. DNA methylation is absent in yeast and appears limited in Drosophila melanogaster [40]. Instead of DNA cytosine methylation (5mC), N6 adenine methylation (6mA) has been observed in worms [41]. It is well known that transposable repetitive elements including Alu and LINE-1 harbour more than 90% of genomics 5-methylcytosines within their CpG islands and have been used to estimate the DNA methylation of the genome [42]. Various studies have shown that both Alu and LINE 1 repetitive element exhibit decreased methylation and variability with age suggesting ageing is associated with DNA hypomethylation [43]. Other studies found that DNA hypomethylation at specific regulatory and repetitive elements such as Alu elements (Alu) and long interspersed element-1 (LINE1) leads to genetic instability and subsequent tumor formation which is an age-related disorder [44, 45]. It was observed that IL-7 mediated a decrease in the expression of DNMT3a and DNMT3b and an increase in the expression of TET2 and TET3 led to the increase in the demethylation of Bcl-2 and c-Myc in aged mice which resulted in the increased population of DN3 cells associated with thymic involution [46]. It was found that individuals over 100 years of age exhibited whole genome DNA hypomethylation in $CD4^+$ T cells as compared to newborns [47]. A decrease in 5mC was also observed in the various organs including the liver, brain and small intestinal mucosa of old mice as compared to young mice [48].

It is well known that ageing predisposes organisms towards various abnormalities including cancer, type 2 diabetes and dementia. A recent report predicted altered DNA methylation status in several dementia-related genes including PON1, AP2A2, MAGI2, POT1, ITGAX, PACSIN1, SLC2A8 and EIF4E in healthy older adults > 70 years. Among these, some of them developed dementia in later life suggesting some of the age-associated abnormalities can be detected early and raises the possibility of their prevention with epigenetic modifiers [49]. Chinn et al. noted global DNA hypomethylation in ageing mice

and increased DNA methylation variability among male and female mice in the mouse dorsal hippocampus [50]. Hahn et al. observed that DNMT1 is associated with age-related loss of cortical inhibitory interneurons which is associated with cognitive decline [51]. Huang et al. demonstrated that loss of DNA methylation activity due to proteasomal degradation of DNMT3a variants drives the expansion of haematopoietic stem cells implicating the increased risk of age-related hematologic disease through epigenetic alterations [52]. Several reports have identified DNA methylation alteration in various tissues of T2D patients including blood, pancreas, skeletal muscle, liver, and adipose tissues indicating that epigenetic modification during ageing can prone individuals towards the development of type 2 diabetes [53]. These investigations signifies that DNA hypomethylation is associated with ageing and can lead to age-related abnormalities and therefore also raises the possibilities of their prevention through a reversal of epigenetic aberrations.

Influence of epigenetic modifications on cancers

Ageing is associated with the increased development of most cancers [54]. Aberrant DNA modifications including DNA hypomethylation of open sea regions and DNA hypermethylation of promoter CpG islands have been observed in most of the cancers [54]. DNA hypermethylation on the promoter region of tumor suppressor genes including Rb, p14, p15, p16, p21, TIGI, RUNX3 and regulatory genes such as RAS association domain family 1A (*RASSF1A*) and retinoic acid receptor β (*RARB*), DNA repair mechanism genes BRCA1, MGMT can lead to their inactivation, genetic instability and subsequent cancer development [55]. On the contrary DNA hypomethylation is associated with the activation of oncogenes and tumor formation [55]. Although DNA hypermethylation is more common in cancers but DNA hypomethylation is also seen in various types of tumor including cervical, ovarian, prostate, liver, and B-cell chronic lymphocytic leukaemia [55]. Aberrant histone modification is also observed in many cancer types. It was found that cancer cells exhibit downregulation of histone acetylation at H4K16 and loss of histone methylation at H4K20me3 [56]. HDAC1 and HDAC2 expression was found to be increased in prostate and gastric cancers respectively [57, 58]. A deletion of EZH2 (H3K27 methyltransferase) results in the increased development of spontaneous T-cell leukaemia [59]. Chen et al. found that

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low levels of H3K4ac and elevated expression of H3K27me3 were associated with the progression of oral squamous cell carcinoma [60]. EZH2, SUZ12, and EED subunits are the components of methyltransferase Polycomb Repressive Complex 2 (PRC2) and are predicted to the valid targets for tumorigenesis via histone modification at H3 on lysine 27 residue (H3K27me3) [61]. PRC2 inhibitors (MAK683, EED226 and FDA-approved EPZ6438) reduced the proliferation of lymphoma cell WSU-DLCL2 (WSU), rhabdoid tumor (MRT) cells, pancreatic cancer cell Hs700T with *SMARCB1* deficiency, and ovarian cancer cell A2780 with *ARID1A*-deficiency through altering the methylation and acetylation of H3K27 of ECM, SASP, proteoglycan and cell cycle genes including GATA4, MMP2/10, ITGA2, GBP1 as well as cell cycle regulatory gene CDKN2A/p16 [61] suggesting chromatin modification as a key upstream pathway in balancing the cell cycle for controlling proliferation under stress conditions. However, these PRC2 inhibitors failed to reduce the proliferation of refractory RD, HeLa and mast cell activation syndrome (MCAS cells) although affecting the methylation process [61] hinting at the differential mechanism of chromatin remodelling in cervical cancer and other immune-related disorders. Another study reported that dual inhibition of H3K9 and H3K27 methylation led to reduced metastasis and proliferation of tumor cells such as breast cancer (MCF7, BT549, and MDA-MB-231), colon cancer (SW480 and HCT116), and prostate cancer (PC3 and DU145) using G9a inhibitor (UNC0642) and the EZH2 inhibitor (UNC1999, or UNC0642 + UNC1999) [62]. Chromobox family proteins (CBX2/3/8) were found upregulated in glioblastoma (GBM) suggesting the importance of epigenetic reprogramming in controlling the disease and disorders [63]. These findings indicate that aberrant DNA methylation of tumor suppressors and oncogenes and H3K27 methylation are associated with the development of most of the cancers.

Influence of epigenetic modifications on senescence and telomere during ageing

Senescence and ageing cannot be used interchangeably but the number of senescent cells increases during ageing and contribute to age-related dysfunction by reducing the regenerative capacity of tissues [64]. Decreased expression of histone methyl transferase nuclear receptor binding SET domain protein 2 (NSD2) was observed in the senescent bone marrow MSCs (BMMSCs) which suggests that epigenetic alteration in tissue-resident stem cells can contribute to ageing phenotype by driving them towards senescence and

reducing their regenerative property [65]. Giuliani et al., observed genome-wide DNA hypomethylation in replicative senescent BMSCs and HUVECs which is associated with different pathways including Wnt/β-catenin signalling, molecular adhesion, and insulin resistance [66]. Zhang et al. reported an increase in genome-wide H3K9 acetylation in flag leaf senescence suggesting its role in the sexual reproduction of cereal crops, crop growth, reducing photosynthesis and grain production [67]. It was shown that KDM4A-mediated H3K9me3 and DNMT3B promote senescence in nucleus pulposus cells (NPC) leading to intervertebral disc degeneration [68]. Histone demethylase KDM4B ablation is also known to cause a defect in the self-renewal of MSCs by promoting senescence-associated heterochromatin foci leading to skeletal ageing [69]. Moreover, replicative senescence is demonstrated as the causative agent of ageing in several cell types. Li et al. observed decreased expression of Ezh2 (Enhancer of zeste homolog 2) in the fibrotic left atrium tissue of aged mice and senescent atrial fibroblast due to replicative senescence through modulating the H3K27me3 level in the promoter region of CDKN2a (p16, p19) and Timp4 gene suggesting the role of histone marks in ageing by regulating replicating senescence [70]. A study reported maturation phase transient reprogramming (MPTR) where dermal fibroblast from middle-aged donors acquired their fibroblast identity possibly through the rejuvenation of the DNA methylation ageing clock suggesting full reprogramming is not required to reverse the ageing of somatic cells [71]. Ezh2 expression was also found to be associated with increased cellular senescence in Ang II-induced vascular smooth muscle cell ageing model through regulating H3K27me3 levels at ANXA6 promoter [72]. Histone H3 lysine 4 (H3K4) methyltransferase Smyd3 and elevated H3K4me3 modification were reported to promote senescence in rat endothelial cells [73]. These research reports advocate that H3K4me3, H3K9me9 and H3K27me3 histone marks are associated with senescence and can be used as possible therapeutic targets to rejuvenate ageing cells and tissue.

Senescence and ageing are also induced by telomere shortening. Telomerase is a ribonucleoprotein enzyme important for telomere elongation [74]. Telomerase is expressed at low levels in somatic cells and is constitutively expressed in stem cells and germ cells. However, telomerase activity was shown to be insufficient in these cells to

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maintain normal telomere length and thus gradual shortening of telomeres occurs over time and leads to replicative senescence which may contribute to ageing and age-related disorders [74]. Takasawa et al. observed increased methylation in telomerase reverse transcriptase (TERT) promoter differential methylated region of iPSCs during reprogramming which is associated with its increased promoter activity. Further, they noted decreased methylation in the TERT promoter region of parental somatic cells which also corresponds to generally low expression of telomerase in them [75]. This study indicates that the methylation status of TERT is important for its expression thus rendering telomerase susceptible to epigenetics changes during ageing and its expression. It was found that overexpression of telomerase can extend the lifespan of mice indicating its anti-ageing potential [76]. Another study demonstrated decreased expression of telomerase and relative telomere length in *Nipponia nippon*, *Colombia livia*, *Pelodiscus sinensis* and *Xenopus laevis* with age while no correlation was found in *Alligator sinensis* which indicates that telomerase and telomere shortening is not a single parameter to assess age [77]. It was found that DNAmGrimAge and DNAmPhenoAge acceleration were inversely related to the length of the telomere suggesting the degree of methylation at the telomere drive telomere shortening during ageing [78]. A study observed increased H3K4 and H3K79 methylation at telomere-proximal regions of replicative aged yeast cells suggesting that silencing of subtelomeric regions may have an impact on telomere length [79]. However, it is unknown whether epigenetic modification at the telomeric or subtelomeric end has any effect on the regulation of telomerase expression. Telomere length is also known to regulate the activity of human telomerase catalytic subunit hTERT by modulating the DNA methylation and histone marks on its promoter region during ageing [80]. Kim et al. observed that aged BJ human fibroblast cells with short telomeres exhibit increased expression of two active marks H3K4me3, and H3K9ac and one repressive mark H3K27me3 in TERT promoter as compared to young cells with long telomeres. Collectively, these results indicate that the hTERT promoter is more permissive in aged cells suggesting hTERT expression is influenced by the length of telomere and histone modifications [80]. Taken together, these data propound that telomerase can be used as an anti-ageing marker by modulating its TERT promoter methylation status using epigenetic modifiers.

Methods for the Detection of DNA Methylation

DNA methylation commonly occurs at 5 methylcytosine within CpG residues which plays an important role in the regulation of gene expression, cell proliferation, differentiation and genomic stability [81]. The reversible nature of DNA methylation demands an effective method for its diagnosis and treatment [81]. The various methods to detect DNA methylation changes are described below. Figure 1 depicts the comparison between these methods.

Bisulphite genomic sequencing

Bisulphite genomic sequencing is considered a gold standard method for the detection of DNA methylation at CpG sites at single base pair resolution [81]. In this method, sodium bisulphite treatment of DNA results in the deamination of cytosine to uracil in unmethylated DNA which gets converted to thymine in subsequent PCR amplification and detected by sequencing [81]. However, DNA methylation at 5 methyl cytosines remains unaffected by this conversion and is recognized as cytosine in subsequent PCR amplification and detection thus allowing the distinction between methylated and non-methylated DNA [81].

Reduced representation bisulfite sequencing (RRBS)

In this method, one or more restriction enzyme such as MsPI is used to cut the DNA to generate sequence-specific fragments and analyse DNA methylation at single nucleotide resolution [82, 83]. MsPI digestion cuts at CCGG sites and recognizes both methylated and non-methylated sites that result in the isolation of 85% of CpG islands [82]. The fragmented DNA is then treated with sodium bisulfite and sequenced [82, 83]. This method is effective where methylation level is high such as repeat regions and promoters [83].

Infinium human methylation beadchip design

Illumina has developed cost-effective and high throughput Infinium humanmethylation microarray assay for the detection of methylation across the genome [84]. These human methylation-based array platforms include HumanMethylation27 (27K) BeadChip, Illumina

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Human Methylation 450 K (450 K) array, and Infinium MethylationEPIC (EPIC) BeadChips which detects DNA methylation at single base resolution through measuring sodium bisulfite- treated DNA [84, 85]. These arrays are based on Illuminas beadchip technology and do not depend on polymerase chain reaction (PCR) and detect signals based on dye intensity [86]. In these arrays, DNA is first treated with sodium bisulfite and then subjected to whole genome amplification, fragmentation, hybridization and single base pair extension on the microarray chip [84]. In the 27K BeadChip, CpG site detection is based on two 50bp probes produced by two different bead types to hybridize methylated CpG site (M) and unmethylated CpG site (U) and generate the same color signal for both M and U while 450K BeadChip produces signal using two different probes (Illumina Infinium I and II) and employs two different color green and red to differentiate between M and U signals [84]. HumanMethylation27 (27K) BeadChip can assess 27,578 CpG sites including 14,495 protein-coding gene promoters while HumanMethylation450 (450K) BeadChip assays can interrogate 482,421 CpG sites and Infinium MethylationEPIC (EPIC) BeadChips can detect 935,000 CpG sites across epigenome [84, 87]. For Illumina beadchip arrays methylation levels are calculated as beta value $\beta = M/(M + U + \alpha)$ where α is arbitrary value usually 100 [84]. Moreover, the Epic array detects only 30% of epigenome rendering a large number of CpG sites unmeasured [86]. Another method known as methylation Capture bisulfite sequencing (MC-seq) can detect 3,708,550 CpG sites at single nucleotide resolution with more CpGs in the coding region and CpG islands at an affordable price utilizing a targeted next-generation sequencing approach [86]. Whole genome bisulfite sequencing is a next-generation sequencing technique and can annotate more than 28 million CpG sites but is associated with the high cost and large input genomic DNA requirement [86].

Bisulfite Pyrosequencing

Bisulfite pyrosequencing is a quantitative method to determine the methylation status of bisulfite-treated CpG sites at a single nucleotide level [88]. This method is regarded as the gold standard for the measurement of allele-specific methylation patterns [89]. In this method, DNA is first treated with bisulfite to generate either cytosine or thymine for the identification of methylated and non-methylated cytosines [88]. Further, it involves the addition of deoxynucleotide triphosphate (dNTPs) to the growing strand of DNA which

results in the release of pyrophosphate (PPi) that gets subsequently converted to ATP by an enzyme ATP sulfurylase [88, 89]. The ATP is utilized by the luciferase to convert the luciferin to oxyluciferin [88, 89]. The amount of light produced is detected and recorded as a peak which is proportional to the amount of nucleotide incorporated [88, 89]. The methylation percentage is calculated as the ratio of the cytosine peak to the sum of the thymine and cytosine peaks [89]. The main disadvantage of this method is that it analyses only smaller regions (350bp) [89].

Epigenetic Ageing clock

The epigenetic clock can be used as a diagnostic marker for the prediction of biological age. These biomarkers can be measured within tissues or cells or body fluid and thus can be used for disease detection or prognosis or therapy monitoring [90]. In 2013, Horvath analyzed approx.. 8000 samples from 51 healthy human tissues and cell types as well as approx. 6000 cancer samples and revealed exclusive 353 CpGs methylation sites called clock CpGs (also known as pan-tissue Horvath DNAm clock) accurately predicting age [91, 92]. Parallelly, Hannum et al. identified 71 CpGs sites (also known as Hannum DNAm clock) to predict ageing with high fidelity from the blood samples collected from individuals aged 19-101. This clock can be utilized to measure the ageing rate in age-related diseases such as tumors [93]. Further, Horvath lab developed the DNA methylation PhenoAge clock (also known as Levine clock) based on 513 CpG sites for the measurement of ageing and diseases such as cardiovascular disorder using DNA methylation values from whole blood [94]. Later, the same group released the DNA methylation GrimAge clock which is reported to predict mortality, coronary heart disease (CHD), and cancer [95]. Recently, Milicic et al. 2022, reported an association between age acceleration and hippocampal volume within Amyloid-β positive (Aβ) individuals using Hannum, Zhang and Phenoage epigenetic clocks. It is well known that Aβ plaques is the main cause of AD and accumulates with ageing [96]. In addition, several groups generated epigenetic ageing clocks in mice [97, 98, 99, 100, 101]. Furthermore, Knight et al. generated an epigenetic clock based on 148 CpG sites from cord blood to estimate the gestational age at birth [102]. Recently, McEwen et al. has identified Pediatric-Buccal-Epigenetic (PedBE) clock based on 94 CpG sites from buccal epithelial cells for measuring age in the pediatric population [103]. More recently, an

epigenetic clock based on ribosomal DNA is discovered and it is found to be evolutionary conserved in different species [104]. Few reports are suggesting the cost-effective generation of an epigenetic age clock based on a few CpG sites by bisulfite pyrosequencing method suggesting the feasibility of age estimation by a few biomarkers [105, 106]. Earlier, Weider et al. developed a cost-effective epigenetic clock based on three CpG sites related to ageing genes ASPA, ITGA2B, and PDE4C from human blood [105]. Later, Han et al. also generated an epigenetic clock by bisulfite pyrosequencing method in mice blood-based on three CpG sites in genes Hsf4, Prima1, and Kcns1 [106]. Recently, two dual-species human-opossum pan-tissue clock was developed to accurately measure chronological and relative age respectively in human and opossum [107]. Recently, it was reported that higher GrimAge is a predictor of old-age cognitive function and was shown to be associated with reduced cognitive and brain vascular age during later stages of life [108]. Sugden et al. observed that first-generation clocks including Horvath and Hannum clocks and second-generation clocks including GrimAge and PhenoAge were not effective in measuring the cognitive impairment in older adults while third-generation measurement of biological ageing using DunedinPACE was able to diagnose AD and risk of development of dementia [109]. A recent study using Horvath's clock demonstrates that higher BMI is associated with age acceleration in visceral adipose tissue (VAT) but not in blood, further suggesting that obesity leads to age acceleration in metabolic active tissues [110]. Recently, Caulton et al. developed a farm animal epigenetic clock using the "HorvathMammalMethyl40" methylation array for predicting the age of livestock animals including goats, cattle, Red and Wapiti deer and composite-breed sheep [111]. Using Illumina HumanMethylationEPICBeadChip, it was found that an increase in systolic blood pressure and pulse pressure accelerated epigenetic age [112]. Recently, Lam et al. developed epigenetic MRI (eMRI) that can measure DNA methylation changes in the living human brain paving the way to identify the onset of aberrant methylation and remedy for same [113]. Manco et al. developed duplex droplet digital PCR (ddPCR) assay to measure DNA methylation changes in the ELOVL2 gene as a biomarker for age prediction in forensic science [114]. These epigenetic biomarker clocks provide a platform to understand the epigenetic alteration during development, ageing, and age-related disorders and their comparison among species. These studies showed that most of the epigenetic clocks are

generated by utilizing reduced representation bisulfite sequencing (RRBS) or Illumina Infinium HumanMethylation450 BeadChip assay methods. HumanMethylation450 (450K) BeadChip assays is recently been replaced by Infinium MethylationEPIC (EPIC) BeadChips array due to the doubling of the number of CpG sites [115]. However, various studies have reported that a subset of 450K CpG sites is absent in Epic arrays thus raising the utility of EPIC arrays to accurately estimate the biological age [115, 116]. Hence further studies are needed to test the efficacy of EPIC array to measure the biological age. Table 1 represents the method used by different studies for biological age estimation. Recently, It was shown that the DNA hypermethylation profile of blood (108 markers) and frontal cortex (514 markers) depicted linear relation with ageing (8-96) while cerebellum (137 markers) showed non-linear relation with ageing due to the saturation of DNA methylation as age increased suggesting underestimation of age using cerebellum sample [117]. Pavanello et al. observed that the DNAmAge of the right and left kidney is older than blood DNAmAge and Age acceleration is faster in the right and left kidney than in blood from the same donor. Further, they noted that the telomere length of the right and left kidneys is longer than blood [118]. Therefore, further research is required for dissecting the correct tissues for age estimation under normal and disease conditions.

Epigenetic ageing clock for cancer

Ageing and cancer hold a complex relationship. Recent studies have been developed to measure biological ageing and predict cancer risk based on DNA methylation sites known as epigenetic clock and epigenetic age acceleration [119]. Dugue et al. assessed the association between age acceleration and various cancers using Human Methylation 450 K Beadchip assay. They observed that epigenetic ageing was associated with increased an risk of cancer with a five-year age acceleration leading to 4-9% cancer risk [119]. A study measured baseline blood DNA methylation of 2764 women and predicted age acceleration using three epigenetic clocks including Horvath, Hannum, and Levine. They found that five-year age acceleration was significantly associated with the development of increased breast cancer risk. Further, they noted using Levine's clock that five years of age acceleration corresponded to a 15% increase in the development of breast cancer [120]. Using HumanMethylation450 BeadChips it was found that GrimAgeAccel was weakly

associated with invasive breast cancer development mostly in postmenopausal women [121]. Durso et al. analysed a publicly available leukocyte methylation large dataset to demarcate the relationship between epigenetic age and cancer development. Using epigenetic clocks including Horvath, Hannum, Weidner; and two CpG specific: ELOV2 and FHL2 they observed a positive correlation between DNA methylation and breast and colorectal cancer respectively [122]. Zheng et al. found using the Horvath clock that epigenetic age acceleration is a predictor of colorectal cancer development, mortality, age, and tumor stage [123]. Chen et al., observed using Horvath, Hannum, and Levine clock that DNA methylation based biological age is positively associated with a risk factor for breast cancer including BMI and alcohol [124]. A study analysed 6000 cancer samples from 32 datasets and observed that some cancers showed a positive correlation with DNA methylation age while others showed a negative correlation [93, 94]. Using EAA Horvath and Hannum epigenetic clock, novel genetic variants in the SELP gene and HLA region was found to associate with age acceleration in childhood cancer indicating the potential drug target for preventing cancer development [125]. These investigations demonstrate that DNA methylation age acceleration is associated with the development and progression of various tumors and can be used as predictive markers for various cancers.

Epigenome targeted therapies

The emerging area of therapeutic intervention for the treatment of various diseases and disorders includes epigenetic drugs also known as “epidrugs” [126]. These epigenetic drugs are in different phases of clinical trials for the treatment of various disease conditions. They mostly act as inhibitors for DNA methyltransferases, histone-modifying enzymes such as histone methyltransferases, histone demethylases, histone acetyltransferases, histone deacetylases, and protein arginine methyltransferases while in some contexts they also act as activators. They act on the 3D architecture of chromatin encompassing a large network of signalling and metabolic pathways [126]. Recent research on epigenetic modifications has led to the development of new inhibitors such as histone methyltransferase (HMT), bromodomain and extra terminal protein (BET), lysine-specific demethylase 1A (LSD1/KDM1A), and protein arginine methyltransferase (PRMT) in addition to DNMT and HDAC inhibitors [127]. These epigenetic drugs are reported as potential therapeutics for

the treatment of various types of cancers such as skin, blood, colon, breast, and prostate [128]. Among these, several epigenetic drugs have been approved by FDA while many of them are in preclinical and different clinical stages. The DNA epigenetic modifiers include azacytidine, decitabine, EPG, SGI-110, DZNep, JQ1, EPG, and curcumin while azacytidine and decitabine are FDA approved. The five currently FDA-approved histone epigenetic modifiers are vorinostat (SAHA), valproic acid (VPA), romidepsin, belinostat, and panobinostat. Most of these drugs are either DNMT inhibitors or HDAC inhibitors [128]. These epigenetic compounds are known to exhibit immunomodulatory, cytotoxicity, antigrowth, and apoptotic effects either alone or in combination on different cancer cells [128]. In particular, these drugs inhibit the vital signaling molecules which are important for the reactivation of tumor suppressor genes and inhibition of the oncogenes; significant for tumor development and progression. Many epidrugs have been identified for the treatment of cardiac hypertrophy and heart failure and one promising candidate is HDAC. Various HDAC inhibitors (HDACi) are available for preventing and improving cardiac functions such as trichostatin A, scriptaid, suberoylanillide hydroxamic acid (SAHA, also known as Vorinostat), and SK-7041. These epigenetic drugs are involved in the suppression of cardiac hypertrophy, and fibrosis, reducing myocardial infarct size and preserving systolic function [129, 130, 131]. Givinostat, another pan-HDACi is associated with reduced inflammatory response, angiogenic effects, and cardiac fibrosis [132]. DNA methylation inhibitor, 5-azacytidine also implicated in T2D and cancer, plays an important role in the improvement of cardiac function and cardiac fibrosis [133]. It can be inferred from these studies that these epigenetic modifiers can be used for reversing the epigenetic aberrations of age and age-related disorders. Table 2 represents the epidrugs for different age-related pathologies.

Epigenetic effect of Nutrition on ageing reversal

Nutrition is also linked to epigenetic modification and is known to impact the ageing rate. It is essential to understand the interaction between epigenetics, nutrition and ageing to unravel the factors including diet, caloric restriction, methionine, vitamins essential for promoting quality of life during ageing. A study has shown that mice and monkeys who are exposed to caloric restriction from the early phase of their life result in a reduction of the

age-related methylation drift which ensues at a younger biological age than their actual chronological age. It was found that age-related hypermethylation is more prominent in CpG islands while age-associated hypomethylation occurs at non-CpG sites [134]. In addition to this, other studies have also shown the association between caloric restriction and longevity through epigenetic modifications [135, 136, 137]. Minteer et al. observed that caloric restriction can slow down the epigenetic ageing process and identified EED and Polycomb group (PcG) as important chromatin regulators for cultural ageing through reprogramming of lung and kidney fibroblast to induced pluripotent stem cells (iPSCs) [138]. A recent report stated that both dietary restriction and rapamycin treatment can modulate DNA methylation at an early age than normal-aged mice indicating that preventing the DNA methylation changes associated with ageing at a younger age can increase longevity [139]. Gong et al., observed a decrease in the level of H3R2me2, H3K27me3, H3K79me3 and H4K20me2 in aged brain mice and their reappearance with dietary restriction and rapamycin [140]. These reports indicates a positive correlation between caloric restriction and longevity.

Methionine is the important precursor for S-adenosylmethionine (SAM) which in turn gets converted to S-adenosylhomocysteine (SAH) by Dnmt [141]. The ratio of SAM/SAH is known as the methylation index whose level regulates DNA methylation reaction by donating methyl group to CpG island [141]. Various reports suggested that supplementation of methionine resulted in DNA hypermethylation and decreased gene expression [141]. Several reports state that SAM/SAH ratio alters histone methylation including H3K4me3, H3K36me3, and H3K79me3 marks in yeast, C-elegans, and drosophila model systems [142]. Various studies have shown that methionine restriction or dietary restriction is associated with increased lifespan in various animal models including drosophila, c-elegans, mice and rat through modulating DNA and histone modifications [142]. Methionine can be obtained from various food sources including lamb, fish, beef, pork, egg, seeds, legumes, cereals, vegetables and fruits [143] indicating that a methionine rich diet has the potential to regulate DNA methylation reaction and thus can play role in delaying or reversing ageing or age-related disease conditions. However, it is unclear how ageing influences methionine metabolism in different tissue and organs. It is

important to understand how methionine affects the metabolic flux with age and influences ageing and age-related disorders. Moreover, further work is required to understand the influence of methionine on various methyltransferases and identify specific methyltransferases affected by the same. Thus additional studies are needed to study the role of methionine as an epigenetic modifier in reversing/delaying ageing or age-related disorders.

Folate is another metabolite whose supplementation positively correlates with SAM concentration and has a well-documented role in DNA methylation and less for histone methylation [144]. It is known to bind LSD1 demethylase of H3K4me1/2 preventing its inhibition by formaldehyde thus suggesting its role as an epigenetic modifier [145]. A study has reported that folic acid and vitamin B12 supplementation results in global hypermethylation in the unmethylated regions of CpG islands in 44 participants while monomeric and oligomeric flavanols (MOF) result in hypomethylation in 13 participants. They further found a reduction in the Horvath “epigenetic clock” with the MTHFR 677CC genotype in women following folic acid and vitamin-12 supplements [146]. It would be interesting to study the influence of the combinational effect of various nutritional epigenetic modifiers in increasing the age of various organisms.

Recently, Gensous et al. has demonstrated that a 1-year intake of a Mediterranean-like diet leads to epigenetic rejuvenation in elderly individuals with country, sex, and individual-specific effects [147]. Cavallucci et al. observed that β-Hydroxy-β-Methyl Butyrate (HMB) increases global histone acetylation while β-Hydroxybutyrate increased lysine β-hydroxybutyrylation (Kbhb) of histone tails in muscle cells highlighting that these compounds can be tested for ageing reversal [148]. A study revealed that soy-based food component genistein affected the binding properties of various epigenetic regulators including ATRX, SUV39H1/H2, and HP1BP3 resulting in reduced growth of cancer cells through the downregulation of proliferating genes [149]. It was found that the combined effect of dietary phytochemicals including sulforaphane (SFN), sodium butyrate (NaB) and Genistein has a more profound effect in downregulating HDACs (HDAC1, HDAC6, and HDAC11), DNMTs (DNMT3A and DNMT3B), histone methyltransferases (EZH2 and

SUV39H1) and histone acetyltransferases (GCN5, PCAF, P300 and CBP) and arresting the growth of breast cancer lines MDA-MB-231 and MCF-7 [150]. A recent study showed that polyamines metabolism influences DNA methylation activity and a longer intake of spermidine increases the longevity of mice [151]. These investigations indicate that diet management could be an alternative strategy to prevent/reverse epigenetic modification during age related disease conditions. However, it is yet to determine whether all epigenetic biomarkers reverse upon diet intake and slow down the progression of ageing and age associated disease conditions such as cancer, cardiovascular, diabetes and neuronal disorders..

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Mesenchymal Stem Cells exosomes for the treatment of ageing and age-related disorders

MSCs and their paracrine secretion hold great value for the treatment of ageing and age-related disorders. Sanz-Ros et al. observed that adipose mesenchymal stem cells derived extracellular vesicles were able to ameliorate various parameters associated with ageing including renal function, fatigue resistance, motor coordination, grip strength, and fur regeneration as well as able to decrease inflammation, oxidative stress and senescent markers in muscle and kidney [152]. Various studies have shown that MSCs and their exosomes were able to slow brain ageing by reducing/suppressing the secretion of inflammatory regulators, promoting angiogenesis, and neuronal cell repair [153]. A study found that co-culturing IMR-90 senescent cells with MSCs was able to reduce the expression of interleukin 6 (IL-6) and reverse the expression of various growth factors including growth differentiation factor (GDF11) and transforming growth factor (TGFβ1) as well as increased the number of mitochondria and telomere length. Moreover, they also observed that MSCs were able to reduce the symptoms related to ageing in aged mice [154]. Skin ageing is characterised by a decrease in the expression of extracellular matrix components including elastin and hyaluronic acid which leads to poor skin turgor and elasticity. Ong et al. found that condition media (CM) derived from red deer umbilical cord MSCs was able to upregulate the expression of hyaluronic acid by 83% and elastin by 56% in human dermal fibroblast suggesting the beneficial effect of CM on skin ageing [155]. MSCs and their secretome are also known to alleviate age-related disease conditions

including diabetic wound healing, immune dysfunction, bone repair and knee osteoarthritis through secreting various growth factors, and extracellular matrix components and inhibiting oxidative stress and inflammatory molecules [156, 157, 158, 159]. Various studies have shown that the rejuvenation of aged stem cells can be mediated by the secretome from young MSCs [160]. However, the mechanism underlying the same is unclear. Epigenetic modifications provide novel therapeutic targets for the treatment of ageing and age-related disorders. It is yet to find out whether MSCs mediate their action in ameliorating ageing and age-related diseases and disorders through modulating epigenetic mechanism. Recently, we hypothesized whether MSCs can act as epigenetic modifiers upon systemic or local transplantation. [161]. MSCs exosomes constitute various growth factors and miRNAs suggesting their suitability as epigenetic modifiers [162]. It would be worthwhile to check if it is possible to manipulate MSCs secretome for epigenetic modification through genomic modification and use as cell therapy to rejuvenate cells, tissues and organs. Moreover, MSCs exosomes can be used to replenish the lost miRNAs or epigenetic modifying enzymes or their activators/factors during ageing through genomic modifications. Since MSCs are also prone to undergo ageing under adverse conditions, it would be essential to use young MSCs condition media/exosomes as epigenetic modifiers rather than MSCs transplantation.

Conclusion and Future direction

Research over the last decade has greatly increased our knowledge about the epigenetic mechanism underlying ageing using various model systems including drosophila, c-elegans, yeast, mouse and humans (Table 3). These studies indicate epigenetic variance among species during ageing suggesting that epigenetic alterations need to be closely monitored in humans for the development of therapeutic targets for slowing or reversing ageing. Induced pluripotent stem cells (iPSCs) derived from the aged individual can be used as a human model system to identify epigenetic alteration and for the screening of epidrugs to reverse the epigenome aberrations. It is yet to prove if it is possible to demarcate ageing and different ageing-related abnormalities based on epigenetic marks. So far these studies show different age-related abnormalities harbour different epigenetics marks and can be distinguished based on different histone marks and epigenetics clocks (Table 4). These

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studies have shown that H3K9me3 is the most altered histone mark in various age-related pathologies. However, these studies also raise the possibility that epigenetic alteration may play a different role in different tissues and organs during ageing. Hence, it is essential to study the epigenetic modifications in different tissues and organs during ageing and the mechanism underlying the same for the development of therapeutic targets. It is also vital to understand that normal ageing and pathological ageing also share common epigenetic alterations. For e.g Normal ageing is associated with DNA hypomethylation whereas cancer is also associated with DNA hypomethylation which suggests that there may be other factors including lifestyle or environmental which predispose the organism towards pathological ageing. There could also be a possibility that extent of epigenetic alteration in whole tissue or organism may also be responsible for predisposing the organism towards pathological ageing. Hence there is a vital need to understand all the molecular pathways influenced by an epigenetic mechanism under normal and diseased conditions during ageing in particular tissue or organisms. From the foregoing account, it is clear that epigenetic changes initiate the ageing process. Hence, the reversal of such changes is likely to halt the progression of ageing and age-related disorders. However, it is not yet clear what causes the loss of epigenetic information during ageing. It is yet to prove whether epigenetic alteration during ageing is a cause or consequence of ageing. Yang et al showed that transient induction of genomic instability through non-mutagenic DNA breaks results in the alteration of chromatin changes including loss of epigenetic information and cellular identity along with the acceleration of epigenetic clock and cellular senescence suggesting epigenetic aberration is a cause of ageing in mammals [163]. High throughput sequencing, single-cell genomics, and CRISPR technology will greatly enhance our understanding of identifying the key epigenetic manipulations and mutations in epigenetic enzymes/factors that are casuals of ageing and age-related disorders. Identification of new epigenetic modifiers to reverse ageing and age-related abnormalities and to what extent/fast these epigenetic modifications can be reversed would pave a way for a healthy and longer lifespan. Therefore, the future of epigenetic therapy lies in the exploration of compounds that can act as epigenetic modifiers. MSCs exosomes exhibit the potential in reversing various related abnormalities however the mechanism is unclear. We have proposed the use of MSCs exosomes as epigenetic modifiers. MSCs exosomes harbour various growth

factors and miRNAs describing their potential as epigenetic modifiers. MSCs can be genetically modified to produce exosomes that can serve as cargo to replenish the lost miRNAs or factors or DNA and histone modifying enzymes during ageing. Other factors that can act as epigenetic modifiers include diet, methionine, folic acid and vitamins and bioactive compounds. It would be interesting to explore if epigenetic clocks which are set of CpG sites can estimate the influence of various epigenetic modifiers including methionine, folic acid, caloric restriction, diet and MSCs exosomes on increasing the lifespan of various organisms. In addition, Various studies suggest the efficiency of Yamanaka reprogramming factors for reversing the age-induced epigenetic changes in aged hematopoietic stem and progenitor cells, human senescent and centenarian fibroblasts as well as the fibroblast isolated from HGPS patients [164]. Interestingly, Strollo et al. (2018) have revealed that astronauts coming back from long-term space missions showed health problems which closely resemble the old age population indicating that ageing might be faster in space than on earth [165]. However, it remains to be seen to what extent ageing can be halted on earth as well as on spacecraft by reversing epigenetic signatures.

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Table 1: Methods for development of epigenetic clock and measurement of biological ageing

S.No	Sample used for biological age estimation	Epigenetic clock	Method used for biological age estimation	References
1	Publicly available individual data sets (Non-cancerous tissues and cancer cells)	Horvath Clock	Illumina 27K or Illumina 450K array platform	91, 92
2	Whole blood	Hannum DNAm clock	Illumina Infinium HumanMethylation450 BeadChip assay	93
3	Blood sample	PhenoAge clock (also known as Levine clock)	Illumina Infinium Human-Methylation450 BeadChip	94
4	Blood sample	GrimAge clock	Illumina Inf 450K array and the Illumina EPIC array	95
5	Whole blood sample	Hannum, Zhang and Phenoage epigenetic clocks	Infinium HumanMethylation EPIC (850 k) BeadChip array	96
6	Liver, lung, heart and brain (cortex) samples from newborn	epigenetic ageing clocks in mice	Reduced-representation bisulfite sequencing (RRBS)	97

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	to 41-week-old mice			
7	Whole blood of mice	epigenetic ageing clocks in mice	Reduced representation bisulfite sequencing (RRBS)	98
8	Mice liver	epigenetic ageing clocks in mice	Bisulfite sequencing on Illumina HiSeq-4000	99
9	Liver, lung, brain and heart samples of 6-, 10-, 12-, 20- and 30-month-old C57Bl/6 male mice	epigenetic ageing clocks in mice	Reduced representation bisulfite sequencing (RRBS)	100
10	mouse adipose, blood, liver, and kidney, muscle, and lung tissue samples	epigenetic ageing clocks in mice	Reduced Representation Bisulfite Sequencing (RRBS)	101
11	Umbilical cord blood or blood spots		HumanMethylation27 or HumanMethylation450 BeadChips	102
12	Saliva and Blood sample	Pediatric-Buccal-Epigenetic (PedBE) clock	BEC Illumina Infinium450 (450K) or BEC Illumina InfiniumEPIC (EPIC) microarray	103
13	Published data set of whole blood of mice		whole-genome or reduced representative bisulfite sequencing (WGBS or RRBS)	104

14	Blood sample		HumanMethylation27 BeadChip platform	105
15	Whole Blood		DNA Pyrosequencing	106
16	Liver, blood and tail were taken from opossums, Blood, muscle whole brain, liver, tail from mice, Ear samples from Tasmanian devil, blood samples for kangaroo and wallaby, human tissue samples (adipose, blood, bone marrow, dermis, epidermis, heart, keratinocytes, fibroblasts, kidney, liver, lung, lymph node, muscle, pituitary, skin, and spleen)	human-opossum pan-tissue clock	HorvathMammalMethylChip40	107

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17	Whole blood	DNAm GrimAge	Illumina 450 K methylation array	108
18	Whole blood	Horvath and Hannum clocks, GrimAge and PhenoAge DunedinPACE	Illumina 450k arrays and HumanMethylationEPIC BeadChip Array	109
19	Human blood, liver, muscle, and adipose tissue	Horvath's clock	Illumina Infinium 450K and 27K arrays	110
20	Ear tissue punches of deer, cattle, goat, and sheep	Farm animal epigenetic clock	HorvathMammalMethyl40	111
21	Peripheral venous blood samples		Illumina HumanMethylationEPIC BeadChip	112
22	Pig brains		Epigenetic MRI (eMRI)	113
23	Peripheral blood		Duplex droplet digital PCR (ddPCR) assay	114
24	Whole blood and brain samples		Illumina Infinium 450k Human DNA methylation and Illumina 27k Human DNA methylation profiles	117
25	Kidney samples		Bisulfite conversion and DNA pyrosequencing	118

Table 2: Epidrugs for age related pathologies

S.No	Epigenetic inhibitors	Target	Pathology	Reference
1	Azacytidine	DNMT inhibitors	Cancer, T2D, and cardiac fibrosis	128, 133
2	Decitabine	DNMT inhibitors	Cancer	128
3	EPG, SGI-110, DZNep, JQ1, EPG, and curcumin	DNMT inhibitors	Cancer	128
4	Combine effect of SAHA and Olaparib	HDAC inhibitors	Cancer	128
5	Valproic acid (VPA), romidepsin, belinostat, and panobinostat	HDAC inhibitors	Cancer	128
6	trichostatin A, scriptaid, suberoylanillide hydroxamic acid (SAHA, also known as Vorinostat), and SK-7041	HDAC inhibitors	Cardiac hypertrophy	129, 130, 131
7	Givinostat	pan-HDAC inhibitor	Cardiac fibrosis	132

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Table 3: Epigenetic modifications among different species during ageing

S.No	Epigenetic Modification	Expression	Organism	Consequence	References
1	H3K36me3/ K36me2/3 demethylase Rph1	Loss	*S. cerevisiae*	Shorter lifespan/longer lifespan	7
2	H3K36me3	Loss	*C. elegans*	Shorter lifespan	7
3	H3K4 mono- and di-demethylase LSD-1	Knockdown	*C. elegans*	Increased lifespan	9
4	COMPASS (H3K4 methyltransferase complex)	Mutation	Yeast	Reduces lifespan	10
5	UTX-1/ H3K27me3	Knockdown/ overexpression	*C. elegans*	Longevity	11
6	H3K27me3	Hypomethylation	*Drosophila*	Longevity	12
7	H3K27me3	Reduced expression	Human (Hutchinson-Guildford progeroid syndrome (HGPS))	ageing	7
8	H3K4me3	upregulated	*Mouse HSCs*	ageing	15
9	H3K4me3, H3K4me1 and H3K27ac	downregulated	*Human HSCs*	ageing	7

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10	H3K9me3	Loss	*Drosophila*	ageing	7
11	H3K9me3 methyltransferase SUV39H1/H3K9me3	Decreased expression	*Human/mouse*	ageing	14
12	H3K9me3/H3K14ac	Decreased expression	Mouse hepatocytes	ageing	7
13	SIRT1	downregulated	Mouse and humans (heart, liver, kidney, and brain)	ageing	7
14	Sirt6	overexpressed	rat and human nucleus pulposus cells	longevity	16
15	H3K56ac, Hst3, and Hst4-related HDAC-encoding genes	Knockdown	Yeast	shorter lifespan	17

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Table 4: Epigenetic modifications during age related pathologies

S.No	Epigenetic Modification	Expression	Pathology	Organism	References
1	H4K20me3	Loss	cardiac ageing		19
2	H3K79	Hypermethylation	Brain disorders	Neurons of aged individuals	20
3	H3K27me3/ H3K4me3	Decrease/Increase	neuroinflammation	neuroinflammation associated genes of 22 months old rats	21
4	H3K9ac	Increased	intracerebral hemorrhage	22 months old rat	21
5	SUV39H1 histone methyltransferase	Reduced expression	depressive like phenotype	Aged mice	22
6	H3K9me3	Elevated level	synaptic dysfunction	cortical neurons of AD patients	23
7	H3K4me3, H3K36me3, H3K9ac, and H3K18ac	upregulated	Ligament degeneration	ossification of ligamentum flavum (OLF) rat model	24
8	sirt2, sirt3, sirt4, and sirt6	Differential expression	loss of cochlear hair cells	Aged mice	25

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9	sirt2	Inhibition	amelioration of cognitive function and Aβ pathology	AD mouse model	26
10	Sirt1	activation	Prevention of cognitive impairment	Aged mice	27
11	Sirt3	Increased expression	prevents degeneration of GABAergic neurons and seizure-related death	AD mice	28
12	Sirt1	Reduced expression	microvascular dysfunction	Obese rat	30
13	HDAC3	inhibition	reduces spatial memory deficits and reduces amyloid plaque load and Aβ levels	AD mice model	32
14	acetylation of H3 histone at K9, K18 and K27	Downregulation	human-induced pluripotent stem cells derived hepatocytes (hiHep) aging	human	33
15	Alu and LINE 1 repetitive elements	hypomethylation	Ageing	human	41

15	DNMT3a and DNMT3b	Decreased expression	thymic involution	Aged mice	44
16	TET2 and TET3	Increased expression	thymic involution	Aged mice	44
17	DNMT3a	Proteosomal degradation	age related hematologic disease	human	50
18	DNA hypomethylation and hypermethylation of oncogenes and tumor suppressor genes respectively		cancer	human	51

Rejuvenation Research
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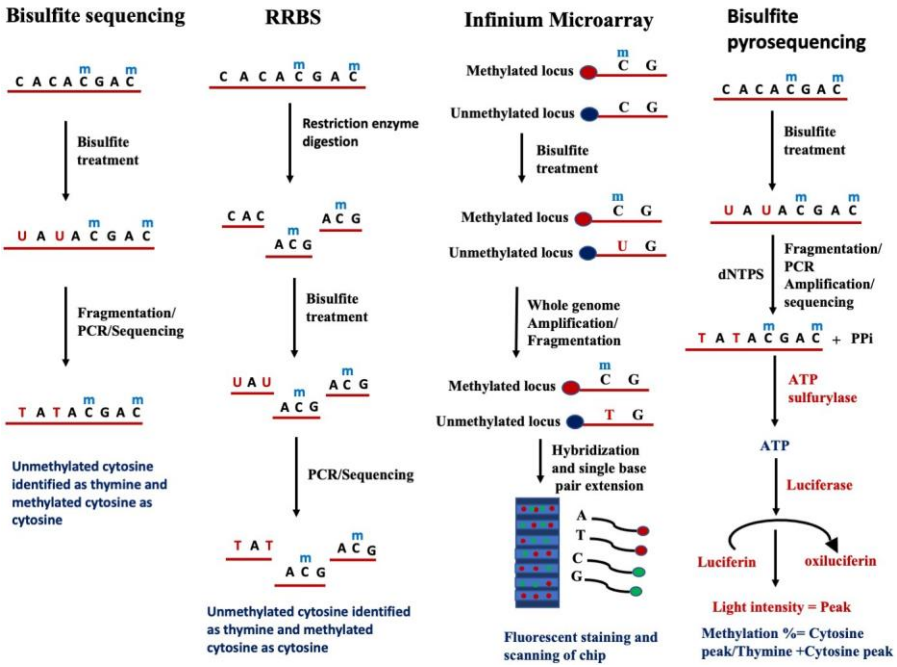

Figure 1 legend: Methods for the detection of DNA methylation