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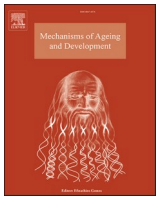

Counteracting aged DNA methylation states to combat ageing and age-related diseases

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ABSTRACT

DNA methylation (DNAm) overwrites information about multiple extrinsic factors on the genome. Age is one of these factors. Age causes characteristic DNAm changes that are thought to be not only major drivers of normal ageing but also precursors to diseases, cancer being one of these. Although there is still much to learn about the relationship between ageing, age-related diseases and DNAm, we now know how to interpret some of the effects caused by age in the form of changes in methylation marks at specific *loci*. In fact, these changes form the basis of the so called "epigenetic clocks", which translate the genomic methylation profile into an "epigenetic age". Epigenetic age does not only estimate chronological age but can also predict the risk of chronic diseases and mortality. Epigenetic age is believed to be one of the most accurate metrics of biological age. Initial evidence has recently been gathered pointing to the possibility that the rate of epigenetic ageing can be slowed down or even reversed. In this review, we discuss some of the most relevant advances in this field. Expected outcome is that this approach can provide insights into how to preserve health and reduce the impact of ageing diseases in humans.

1. Introduction

Ageing is the main risk factor for several causes of death, such as cardiovascular diseases and cancer. Ageing also increases susceptibility to chronic diseases, such as diabetes, neurodegeneration and metabolic syndromes (Brunet and Berger, 2014; Kennedy et al., 2014).

Several theories have been proposed to explain the mechanism underlying ageing and its connection with disease. Although no theory has been conclusively proven, growing evidence suggests that a change in epigenetic information over time, may underlie the age-associated cellular deterioration (Horvath and Raj, 2018; Liu and Zhu, 2021; Lopez-Otin et al., 2013).

In reality, there is now ample evidence that epigenetic variations are ubiquitous regulators of the ageing process in various organisms. Isogenic studies in animals have linked epigenetic changes with ageing phenotypes and lifespan (Jin et al., 2019; Kucharski et al., 2008; Treviño et al., 2020).

In humans, multiple studies on twins suggest that phenotypic differences that arise with ageing (Fraga et al., 2005; Poulsen et al., 2007), including differences in longevity (Herskind et al., 1996), can be traced back to divergent epigenetic signatures.

The cellular epigenetic state results from a dynamic interaction between several components, of which DNA methylation (DNAm) is just one and histone variants and histone post-translational modifications (PTMs) are also included. However, DNAm is the epigenetic modification where the knowledge of its link to ageing is the most advanced (Ciccarone et al., 2018; Zampieri et al., 2015). In fact, DNAm is currently the most promising molecular marker for monitoring ageing and predicting life expectancy in humans. This is particularly reinforced by the recent development of DNAm-based biological age estimators, the so-called epigenetic clocks (Bell et al., 2019; Horvath and Raj, 2018; Liu and Zhu, 2021). These clocks are emerging not only as simple and useful metrics to accurately monitor ageing and related health outcomes, but also a means of assessing the efficacy of interventions that attenuate ageing by modifying the epigenome.

While keeping a major focus on the most recent human studies, this article will initially review findings on age-related DNAm changes and DNAm clocks. Next, attention is focused on the putative links and commonalities of the methylation profile alterations, which have been observed in ageing with those that typify some major age-related

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diseases and conditions. Finally, the potential to counteract ageing and related conditions by interventions that slow down epigenetic ageing rates will be discussed, together with its limitations.

2. DNA methylation basics

DNAm is an epigenetic mark, present in practically all eukaryotic kingdoms, and is functionally associated with gene expression pattern and organism development (Aliaga et al., 2019; Skinner, 2011).

In this biological process, methyl groups are transferred to the C-5 position of DNA cytosines, culminating in 5-methylcytosine (5mC) synthesis at palindromic CpG dinucleotides (CG sites) (Greenberg and Bourc'his, 2019).

Despite forming a small fraction of the whole mammalian genome (< 2%), CG site-rich regions of roughly 1 kb, the so-called CpG islands, are closely embedded to over two-thirds of annotated mammalian gene promoters. Across the 28 million CG sites of the human genome, about 60–80% are consistently methylated in somatic cells, while the residual chunk of CG genome positions comprises unmethylated cytosines (Edwards et al., 2010; Illingworth et al., 2010).

Despite 5mC being predominantly detected at CG-rich sites, there are some exceptions such as human neurons and embryonic stem cells, which display intragenic non-CG methylation (Jang et al., 2017).

This reaction is catalysed by the activity of the DNA methyltransferase enzymes DNMT1, 3A and 3B (DNMTs), which covalently bind the methyl moiety to cytosines (Jones, 2012).

From an evolutionary point of view, the mechanism of cytosine methylation is not found in many eukaryotes, including several animals such as *Drosophila melanogaster* and *Caenorhabditis elegans* (Chang and Liao, 2017).

Mammal DNAm is determined and configured in three distinct phases: establishment (*de novo* DNAm), maintenance, and 5mC removal (Li and Zhang, 2014).

Firstly, DNAm establishment is determined by the activity of two methyltransferases, DNMT3A and DNMT3B, shaping the specific genomic methylation pattern for each germ cell or somatic cell (Fig. 1). This step is critical, as it coordinates the proper whole-genome DNAm after the global demethylation in primordial germ cells and early embryos, a process necessary for pluripotency return following germline cell specification and fertilisation (Jones, 2012; Messerschmidt et al., 2014).

As *de novo* DNAm can take place in any sequence or genomic situation, it is essential to preserve proper symmetrical CG methylation throughout DNA replications. The DNMT1 enzyme serves this crucial purpose, in combination with the E3 ubiquitin-protein ligase protein (UHRF1), to permanently safeguard the correct methylation maintenance of the genome (Gowher and Jeltsch, 2018) (Fig. 1).

The process of 5mC removal, defined as active DNA demethylation, relies on the activity of the ten-eleven translocation (TET) class of dioxygenases. These dioxygenase enzymes allow the elimination of 5mC by its progressive oxidation into cytosine metabolites. In fact, 5mC is chemically turned into the following cytosine oxidation intermediates: 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Li and Zhang, 2014).

All these nucleotides elicit DNA demethylation during replication. In the case of 5fC and 5caC, this process passes through a nucleotide removal mechanism determined by the activity of Thymine DNA Glycosylase (TDG) and the base excision repair pathway (BER) (Shen et al., 2013).

In contrast to active 5mC elimination which requires the activity of a series of enzymes, passive DNA demethylation is due to an incorrect functioning of the DNAm maintenance machinery during replication. When 5mC is not accurately preserved by the maintenance complex, it is eventually diluted during the physiological rounds of DNA replications

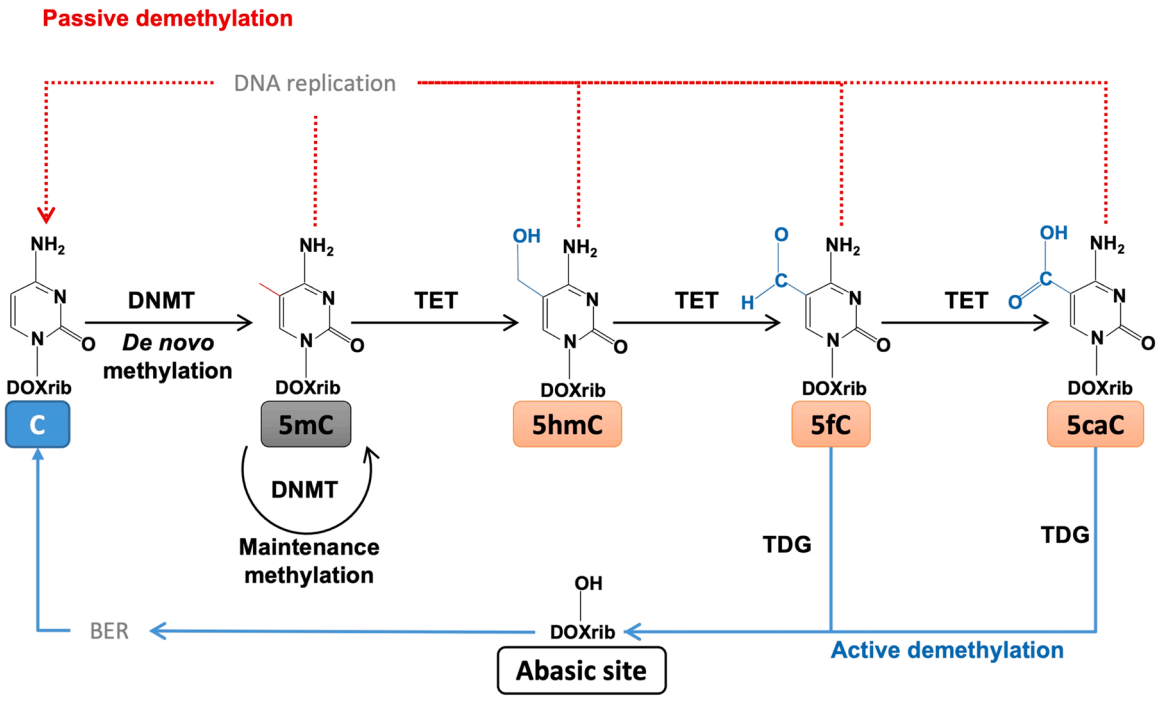

Fig. 1. DNA methylation/demethylation pathways. DNA methyltransferase enzymes (DNMT) form 5-methylcytosine (5mC) from cytosine (C). In *de novo* methylation, methyl groups are added to cytosine at unmethylated DNA. In maintenance methylation, unmethylated cytosine residues of hemi-methylated DNA (methylation on only one DNA strand of the CG dyad) are methylated after DNA replication. Iterative oxidations by TET dioxygenases (TET) produce 5- hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Passive DNA demethylation can occur by a reduction in activity, failed recruitment onto DNA or absence of maintenance DNMT during DNA replication. 5hmC, 5fC and 5caC are no longer recognised by DNMT enzymes and can induce passive DNA demethylation as well. Active demethylation can be achieved through the removal of 5fC and 5caC by the Thymine DNA Glycosylase (TDG), leaving an abasic site in the DNA. The abasic site is then removed by the base excision repair (BER) process and replaced by cytosine. DOXrib, deoxyribose.

(Li and Zhang, 2014) (Fig. 1).

Regarding 5mC tasks, the literature reports that site-specific methylation patterns determine unique functions in mammals. The whole mammalian genome is intensively methylated in non-genic regions, coinciding with heterochromatin repetitive elements or intergenic regions. In such a scenario, DNAm mechanisms are crucial to the proper heterochromatin formation required for whole-genome stability, thus preventing the onset of several harmful processes for the cell such as X-chromosome activation, endo-parasitic repeated DNA expression/translocation/spreading and centromere and telomere instability (Li and Zhang, 2014).

Despite DNAm principally acting as an epigenetic silencing mark for gene expression, the functional cellular effects are specific and depend on the positioning and context of the DNAm. For example, the gene body methylation elicits gene expression and controls transcriptional elongation (Jjingo et al., 2012).

This repressive methylation state is largely confined to genes that need long-term silencing such as imprinted genes and genes in germ cells. In fact, both the distribution and methylation rate of CG sites influence transcription: while unmethylated CGI reside on housekeeping gene promoters and tumour suppressor genes, methylated ones are clustered on tissue-specific genes and oncogenes, to ensure their consistent and physiological suppression (Lu et al., 2014).

This precise pattern does not apply to genomic regions next to CGIs, known as CGI shores and shelves, and characterised by a lower CG amount. These regions showcase fluctuation in their 5mC content and patterns, a phenomenon associated with tissue-specific gene expression (Visone et al., 2019).

The 5mC formation in genic regions happens preferentially on exons over introns. DNAm configuration coordinates gene expression not only by acting on promoters but also on distal elements, such as enhancers, silencers, or insulators (Weaver and Bartolomei, 2014).

The full picture of molecular circumstances, that govern both local and temporal DNAm configuration, is not completely understood as yet. The discovery of a clear link between pre-established chromatin configuration and DNAm has shed some light on this subject, but this research area needs better clarification to help understand the physiological and pathological consequences of the fluctuations of the methylation pattern (Li and Zhang, 2014).

3. The ageing methylome

Studies investigating the link between DNAm and ageing in mammals date back to the 1980 s (Issa et al., 1994; Wilson and Jones, 1983). However, the age-dependent scenario of DNAm genomic patterns only started to become clear with the advent of genome-wide DNAm profiling technologies (Zampieri et al., 2015). We now know that age-induced DNAm changes occur in most human and mouse organs (Maegawa et al., 2010), as well as in those of several other species (Sandovici et al., 2011; Zeng et al., 2012).

Evidence from humans has been collected across the entire lifespan. Longitudinal studies in small children have shown that 5mC levels change as early as the first year of life (Martino et al., 2011). DNAm also starts to diverge very early in monozygotic twins (Martino et al., 2013, 2011). Significantly, DNAm differences between monozygotic twins become even more pronounced as they age, particularly in twins who have different habits and lived apart from each other (Fraga et al., 2005; Tan et al., 2016). This clearly supports the theory that DNAm patterns associated with ageing may result from the interaction between the environment and genetic blueprints. This phenomenon of epigenetic divergence is termed 'epigenetic drift', which claims that ageing results in a reduced epigenetic stability. This is manifested by a gradual departure of the genome from a shared baseline DNAm profile and by increased inter-individual epigenetic variability (Fraga et al., 2005; Issa, 2014; Jones et al., 2015; Tan et al., 2016) (Fig. 2).

However, some of the age-related DNAm changes correspond equally across individuals, regardless of sex or tissue type (Horvath, 2013; Horvath et al., 2012). In fact, high-throughput analyses have conclusively demonstrated that DNAm patterns in ageing are characterised by methylation changes shared between consecutive CGs, located in specific regions of the genome (Fig. 2). These regions are referred to as ageing-associated differentially methylated regions (a-DMR) (Fraga et al., 2005; Jones et al., 2015; Zampieri et al., 2015).

This suggests that, in parallel with stochastic changes causing DNAm divergence, the ageing genome acquires age-regulated DNAm changes in specific regions as part of an 'epigenetic programme'. For example, DNAm data from liver and blood tissues has shown that approximately 70% of hepatic a-DMR are also present in white blood cells and most of them show a positive correlation with age (Bysani et al., 2017). Among them are known age-associated CGs, corresponding to the ELOVL2 (ELOVL fatty acid elongase 2), FHL2 (four and a half LIM domains 2) and

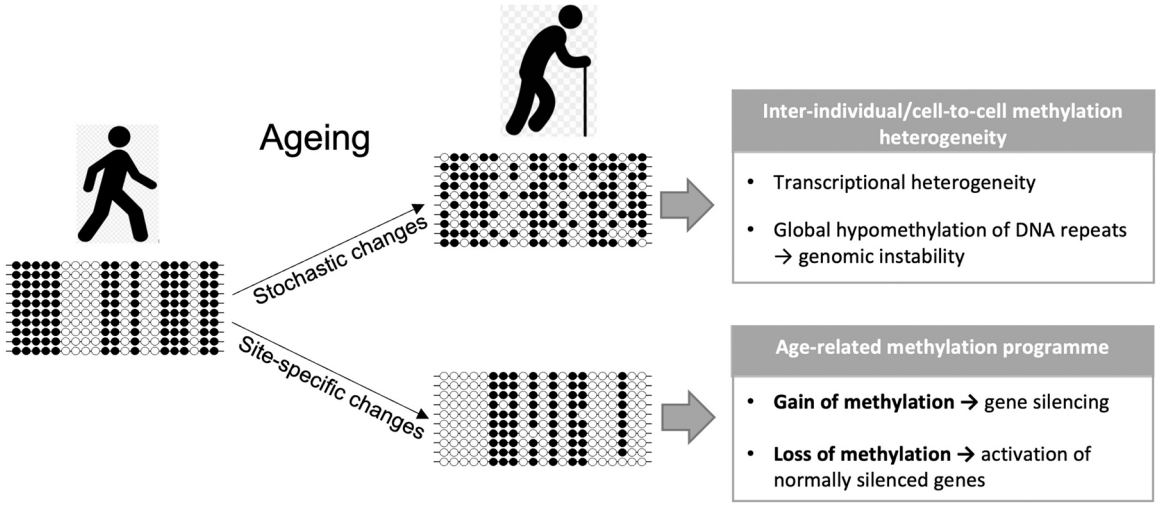

Fig. 2. General aspects of DNA methylation pattern changes caused by ageing. Schematic representation of the DNAm pattern in young and old people. The concordance between individuals and/or between cells in a tissue of the methylation profile is lost during ageing, due to stochastic or site-specific changes. Stochastic changes occur at largely unpredictable *loci* and generate divergent patterns among individuals and/or cells in a tissue. Site-specific changes occur at predictable CG sites and cause a profile that tends to match among individuals and/or cells. Horizontal lines represent generic genome tracts. Circles represent unmethylated (empty circles) or methylated (black-filled circles) CG sites.

KLF14 (Krüppel-like factor 14) genes (Garagnani et al., 2012; Steegenga et al., 2014).

a-DMRs correspond to regions of DNA that lose or gain methylation. These two opposing phenomena affect very different regions in terms of both location and functional role.

Loss of DNAm with age has been observed in most interspersed repetitive elements (e.g. Alu, HERV-K and LINE-1) (Bollati et al., 2009; Jintaridth and Mutirangura, 2010; Zhang et al., 2011).

Hypomethylation of repetitive elements was also confirmed by deep sequencing analyses comparing the DNAm profile of the whole genome of blood cells between newborn and centenarian individuals (Heyn et al., 2012). Notably, hypomethylation is not shared by other DNA repeats. For example, ageing is associated with hypermethylation of DNA repeats that are part of subtelomeres (Bacalini et al., 2021).

In addition to the demethylation of repetitive elements, age-associated hypomethylation occurs in megabase-long blocks of the genome that correspond mainly to gene-poor 'open sea' regions (Yuan et al., 2015), and also in gene regulatory regions such as CG-poor promoters (Heyn et al., 2012) and enhancers (Johansson et al., 2013). It is interesting to note that some of the DNA regions that undergo demethylation in ageing are pre-marked by specific histone PTMs, such as H3K4me1 (Fernández et al., 2015).

In addition to DNAm loss events, hypermethylation is another typical feature of the aged genome, in particular the hypermethylation of CGIs. When age-dependent methylation occurs at the level of the CGI promoters of transcribed genes, they can be silenced, thereby leading to an altered transcriptional programme. For example, a DNAm-mediated repression during ageing has been widely demonstrated for genes involved in immune cell response and differentiation, thus affecting immunocompetence in the elderly population (Tserel et al., 2015). However, hypermethylation events are not necessarily accompanied by changes in gene expression (Horvath, 2013). This is particularly evident for those genes that are already inactive due to repressive histone modifications (Rakyan et al., 2010; Teschendorff et al., 2010). In fact, many CG sequences that undergo hypermethylation with age are associated with chromatin regions showing the repressive histone marks H3K27me3/H3K9me3 in differentiated cells and the bivalent histone marks H3K27me3/H3K4me3 in stem cells (Rakyan et al., 2010; Teschendorff et al., 2010).

4. DNA methylation-based ageing clocks as predictors of biological age

In line with the unprecedented growth rate of the ageing world population (Harper, 2014; Kaeberlein et al., 2015; Moffitt, 2020), there is a clear need for biomarkers that can predict the functional capacity of tissues, organs and even the whole individual accurately. In addition, biomarkers should give an assessment of biological age, thus providing useful information for the assessment of an individual's rate of ageing and his risk of age-related diseases. However, despite the great efforts made in recent decades, no such marker has emerged to date (Jylhävä et al., 2017).

Biomarkers based on DNAm are among the most promising (Bell et al., 2019; Horvath and Raj, 2018; Liu and Zhu, 2021). They are allegedly associated with a broad spectrum of ageing outcomes, including mortality. These biomarkers can be used on cells, tissues and organs. Finally, they seem to have the ability to define biological age from prenatal tissues (Kerepesi et al., 2021) and paediatric samples (McEwen et al., 2020) up to tissues obtained from centenarians (Horvath et al., 2015).

Owing to developments in methylation array technologies, specific CGs have been localised in the genome, and based on this, the age of the DNA source can be estimated. The estimated age is often referred to as DNAm age, or epigenetic age, derived from the ticking of 'epigenetic clocks' (Horvath and Raj, 2018). Since epigenetic age is not only highly correlated with chronological age, but is also influenced by other biological factors and health status, it is considered to be a putative biomarker of biological age.

By regressing epigenetic age on chronological age, epigenetic clocks can determine whether an acceleration or deceleration of biological age occurs in an individual. In other words, they can determine whether an individual is biologically older or younger than his chronological age. To date, more than 15 epigenetic clocks have been developed (Bergsma and Rogaeva, 2020). Here, our focus will be on some of the clocks which are most recurrent in the literature.

Most clocks share the feature of being built using machine learning techniques that estimate age in years by automatically selecting the most informative CGs.

The first clocks were reported by Bocklandt et al. (Bocklandt et al., 2011) and Hannum et al. (Hannum et al., 2013) and were based on the use of a single tissue. Subsequently, Steve Horvath demonstrated that it was possible to build a clock (often referred to as the Horvath clock) that could estimate epigenetic age from multiple tissues (Horvath, 2013). This clock was built using data from approximately 8000 DNAm microarrays, derived from over 30 different tissue and cell types and collected from patients ranging in age from children to adults. It is based on the methylation level of 353 out of a total of 27 K CG sites in the arrays.

However, although the first generation epigenetic clocks showed statistically significant associations with many age-related diseases and conditions (Chen et al., 2016; Dugué et al., 2018b; Horvath et al., 2015, 2014; Levine et al., 2016, 2015a, 2015b; Maierhofer et al., 2017; Marioni et al., 2015a), the effect sizes were small to moderate. Consequently, second-generation clocks were developed, incorporating not only CGs showing changes with chronological age, but also those representing differences in disease risk and physiological status between individuals. The rationale was that the use of chronological age only as a reference had a tendency to exclude the CGs whose ageing-related methylation reflects a deviation of biological age from chronological age.

On this basis, the Horvath lab recently developed the PhenoAge clock, focusing their predictor on phenotypic age rather than chronological age (Levine et al., 2018). Chronological age was replaced with a surrogate measure of phenotypic age, developed by using clinical data predicting mortality risk, including blood cell profiles and inflammatory markers. The resulting biomarker is based on measurements from 513 CGs. Although this biomarker was developed using data from whole blood, it correlated strongly with age in all the tissues tested. PhenoAge was found to be a powerful biomarker for measuring health and lifespan, which surpassed first-generation clocks in several aspects. For example, it predicted mortality significantly better than Horvath's pan-tissue clock (Levine et al., 2018).

More recently, the Horvath lab has defined an additional epigenetic age calculator specifically developed to be an effective predictor of lifespan, the GrimAge (Lu et al., 2019). This predictor is based on the combination of CG sites as a surrogate biomarker of smoking years, a selection of plasma proteins that had been previously associated with mortality or morbidity, and time-to-death.

The GrimAge was highly predictive of lifespan and risk of multiple health problems such as cardiovascular disease, hypertension and Type 2 Diabetes. It also surpassed the Pan-Tissue Clock, the PhenoAge clock and the Hannum Clock. In addition, the GrimAge surpassed other powerful DNAm-based predictors of mortality risk (Lu et al., 2019; Zhang et al., 2017).

These clocks typically capture directional and site-specific ageing-related changes, both in methylation gains and losses. For example, in the case of the Horvath clock, while 193 CGs sites correlate positively with age, 160 sites correlate negatively (Horvath, 2013). In fact, this clock does not include those CpGs that in isolation show a strong and robust correlation with age, such as those at the ELOVL2 *locus* (Garagnani et al., 2012; Slieker et al., 2018). Significantly, when considered individually, the methylation status for most of the CGs is only slightly

correlated with age (Horvath, 2013). This suggests that the age-related methylation variation of each CG contributes to epigenetic age in an additive manner and/or that the combination of CGs reflects the effects of ageing on the global DNAm pattern.

However, only a small proportion of CGs are common among the clocks and there is only a weak/moderate correlation between them (Bergsma and Rogaeva, 2020). This would suggest that the different clocks measure different aspects of ageing. Furthermore, the significance of the methylation of the clock CG sites in relation to ageing remains unclear.

With the perspective of building indices more directly related to known ageing-related methylation changes, other clocks are emerging and being developed. These are based on CGs located in regions of the genome that are relevant to ageing or on aspects of epigenetic ageing different from site-specific changes, such as stochastic epimutations.

Notably, Wang et al. (Wang and Lemos, 2019) developed an efficient clock based on DNAm of ribosomal DNA, a region that is missing from arrays used in previous analyses of age-associated DNAm, in spite of having a clear mechanistic link to ageing.

Gentilini et al. (2015) developed a promising biological age index based on the amount of stochastic epimutations that increase with age and are not shared between subjects, a salient feature of the ageing methylome that is largely unexplored.

Finally, a third generation of clocks called Pace-of-Aging was developed specifically to quantify the rate at which ageing proceeds rather than quantifying the extent of ageing accumulated up to the time of measurement, as the first and second generation clocks do. Measures of the Pace-of-Aging were developed by modelling multisystem physiological changes over time. These measures were carried out in the Dunedin Study birth cohort (Belsky et al., 2015). An initial Pace-of-Aging clock was based on analysis of DNAm changes at three time points, when Dunedin Study members were 26, 32 and 38 years of age, and was named DunedinPoAm (Pace-of-Aging from DNA methylation) (Belsky et al., 2020). A subsequent Pace-of-Aging clock was based on analysis of changes at four time points, when Dunedin Study members were 26, 32, 38 and 45 years old, and was named DunedinPACE (Pace-of-Aging Computed from the Epigenome) (Belsky et al., 2022).

All in all, despite being at an early stage, research on epigenetic clocks is shaping up to be a success. Their ability to estimate biological age in different tissues suggests that they are capable of capturing the effects of a pervasive mechanism, which acts at the system level (Jylhävä et al., 2017). Furthermore, the recent development of DNAm clocks capable of accurately estimating age in many mammalian species suggests that the clocks reflect the activity of specific and shared mechanisms, which underlie the ageing process (Study pre-print (CONSORTIUM et al., 2021)). However, much remains to be clarified. A very relevant limitation arises from the cross-sectional nature of most clock studies. The epigenetic age derived from these clocks does not yet provide direct insights into the dynamics of changes in an individual's biological age over time nor for definition of ageing rate and/or for prediction of age-related diseases.

5. Methylome ageing, clocks and their association with age-related diseases

Methylation changes that characterise an aged epigenome can adversely affect transcriptional processes and the structure and function of downstream proteins (Benton et al., 2017; Fraga et al., 2005; Jones et al., 2015). Not surprisingly, therefore, the rates of methylome ageing have an impact on the risk of age-related diseases, possibly through the loss of phenotypic plasticity in response to environmental stimuli (Li and Tollefsbol, 2016).

Among age-related diseases, cancer is the one where a role of epigenetic ageing is most evident. Cancer is a disease arising from the accumulation of genetic and epigenetic alterations, and for several cancers, the most important risk factor is age (Age and Cancer Risk, National Cancer Institute, www.cancer.gov).

There is a close similarity between the methylation profile of a cancer cell and that generated by ageing in normal cells (Fig. 3) (Dor and Cedar, 2018). Although neoplastic transformation can be triggered by multiple factors (e.g. mutations in key growth control genes), DNAm alterations play a key role in this process (Klutstein et al., 2016). The very similar methylation pattern of the cancer cell to that of an aged cell (gain of methylation in CGI promoters and overall loss of methylation), suggests that some of the age-related DNAm alterations were already present in the founder cell from which cancer cells descend, prior to its subsequent transformation (Klutstein et al., 2016). In fact, it is very likely that this basic age-related methylation alteration plays a role in cell growth that could participate in the early steps of cancer formation, along with other factors such as replication and clonal selection. The greater extent of DNAm alterations in the cancer cells compared to normal aged cells would then result from changes induced by other factors such as the cancer microenvironment and somatic mutations in genes that play a role in the DNAm management mechanism (Feinberg et al., 2016).

An age-associated methylation gain occurs in CGIs (Fig. 3) of genes that are crucial during development and play a tumour suppressor role in cancer. These same CGIs are also found to be hyper-methylated in cancer (Maegawa et al., 2010; Rakyan et al., 2010; Teschendorff et al., 2010). Significantly, some of these CGIs share the common feature of being Polycomb Group 2 (PCG2) binding sites, transcriptionally repressed in adult stem cells (Dozmorov, 2015; Teschendorff et al., 2010). This suggests a potential role of age-associated hyper--methylation in the molecular pathogenesis of cancer, where epigenetic ageing targets and silences specific sequences, which are also prone to be inactivated in cancer. This concept is supported by the observation that the lifetime risk of cancer in any tissue is directly related to the degree of age-related hyper-methylation of CGIs occurring in normal tissue (Klutstein et al., 2017). For example, the widespread hyper-methylation of CGIs seen in a healthy colon in the elderly can be partly identified in cancer tissue (Issa, 2004; Toyota et al., 1999; Weisenberger et al., 2006; Yamashita et al., 2003). In addition to the colon, the same relationship between age-related epigenetic changes and the risk of cancer could prevail in buccal mucosa in smokers (Wan et al., 2015), in the haematopoietic stem cells of the elderly (Sun et al., 2014) and in the skin (Raddatz et al., 2013).

Similarly, the loss of DNAm that occurs in ageing tissues seems to predict a similar, but more pronounced, profile in cancer. This applies to partially methylated domains associated with the nuclear lamina (Dmitrijeva et al., 2018; Zhou et al., 2018) and with the Alu and LINE1 DNA repeats (Fig. 3), whose hypomethylation has been associated with increased genomic instability. This instability, in turn, makes a tumour-promoting chromosomal arrangement more likely (Sartor et al., 2011; Zheng et al., 2017).

Age-related changes also appear to contribute to several other age-related phenotypes. For example, the age-related hypermethylation observed in CGIs of genes associated with T-cell immune responses and differentiation may explain the decline in both immunocompetence and response to infection among aged individuals (Tserel et al., 2015). Age-related DNAm alterations in stem cells would reduce the efficacy of repair and regeneration of tissues, such as the gastrointestinal tract and leukocytes (Beerman et al., 2013; Sun et al., 2014). Age-associated epigenetic changes also affect the brain, probably contributing to structural and functional alterations that may account for a progressive cognitive decline and susceptibility to neurodegenerative disorders (Bishop et al., 2010; Lardenoije et al., 2015). Ageing leads to methylation changes in energy metabolism candidate genes, such as fatty acid elongase ELOVL (ELOVL2) and the transcription factor KLF14, which may play a role in the pathogenesis of common diseases including obesity and Type 2 Diabetes (Bysani et al., 2017). Finally, hypomethylation of the Alu and LINE1 elements during ageing has also been linked to a significant decline in lung function in the elderly (Lange

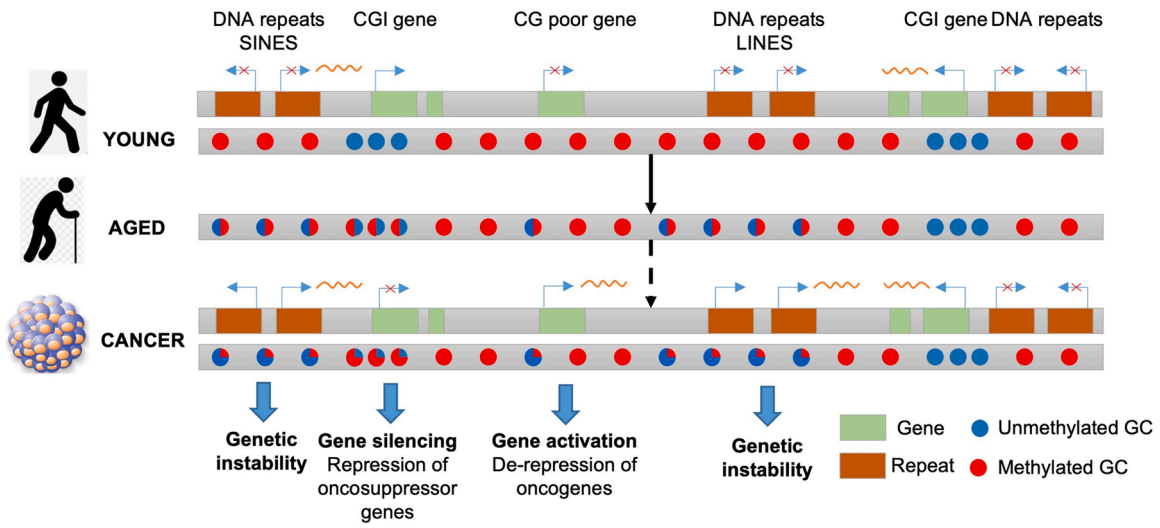

Fig. 3. DNA methylation pattern changes in ageing and cancer. During ageing, the genome undergoes progressive changes in the methylation profile. In young people, the profile is bimodal. Most of the genome is methylated, with the exception of CpG islands (CGI). As the genome ages, it undergoes demethylation involving regions associated with repeated sequences and non-CGI genes. Conversely, some CGIs undergo *de novo* methylation. These changes generate a distinct pattern, which increases in magnitude as a function of age. The methylation profile of the tumour is almost identical to that generated during ageing, but to a much greater extent. The upper box represents genomic DNA containing CGI-associated genes (CGI gene), CGI-less genes (CG poor gene) and different classes of interspersed repeats (SINES, Short Interspersed Nuclear Elements; LINES, Long Interspersed Nuclear Elements). Arrows indicate start sites for transcription, which is repressed when the cross is present. Each lower box represents the DNA methylation profile at CG sites (circles). The red filling of the circles indicates the degree of methylation.

et al., 2012).

The general similarity of hypo- and hyper-methylation dynamics between ageing and age-related conditions suggests that methylome ageing may provide a common background from which disease may arise and then diverge in epigenetic as well as phenotypic terms. It is therefore not surprising that a considerable number of recent studies reveals that epigenetic age acceleration, as a surrogate of the rate of epigenome ageing, is a powerful candidate biomarker for death from all causes and age-related diseases. The association with all-cause mortality risk (Lu et al., 2019; Perna et al., 2016; Wang et al., 2021; Wolf et al., 2018) and comorbidity count (Lu et al., 2019), suggests the ability of methylation clocks to capture the fragility that is acquired by the methylome during ageing and its resulting effect on health and survival. Furthermore, epigenetic age appears to measure the age-related decline of several physiological functions, including physical functions such as lung function (Marioni et al., 2015b), cognitive (Levine et al., 2015b; Marioni et al., 2015b), hormonal (e.g. menopause) (Levine et al., 2016; Lu et al., 2019), immunological (Carroll et al., 2017) and metabolic (fatty liver/excess visceral fat) (Lu et al., 2019) functions. In addition, a marked acceleration of epigenetic age seems to reflect methylome alterations that underpin an adverse ageing trajectory towards disease. This is suggested by the association of epigenetic age acceleration with cancer risk (Ambatipudi et al., 2017; Kresovich et al., 2019; Levine et al., 2015a; Lu et al., 2019; Perna et al., 2016; Zheng et al., 2016), cardiovascular disease (Horvath et al., 2016; Lu et al., 2019; Perna et al., 2016; Wang et al., 2021), Type 2 Diabetes (Lu et al., 2019), frailty status (Zhang et al., 2018), Alzheimer's disease (Levine et al., 2015b) and Parkinson's disease (Horvath and Ritz, 2015).

It is conceivable that epigenetic clocks do not capture the full epigenetic component of ageing and related health outcomes. For example, the results of Zhang et al. (Zhang et al., 2017) suggest that methylation changes associated with ageing-related phenotypes and diseases mostly differ from those associated with age.

However, epigenetic clocks represent an unprecedented tool in estimating biological age. The responsiveness of clocks to factors that accelerate or decelerate epigenetic ageing, even at younger ages where ageing is not yet manifested, provides an opportunity to evaluate strategies for intervention and for prevention of ageing and its pathological manifestations.

6. Epigenetic age reprogramming as a means to reverse ageing phenotypes

Reversal of ageing is widely described in the literature, especially in animal models. Pharmacological therapies and behavioural interventions can improve cellular communication and nutrient sensing, regulate transcription, slow down stem cell ageing and telomere attrition and prolong healthy lifespan (Flanagan et al., 2020; Kulkarni et al., 2020; Selvarani et al., 2021). Rejuvenation techniques such as heterochronic parabiosis, caloric restriction, cell reprogramming and treatment with senolytic substances are known to be particularly effective (Mahmoudi et al., 2019).

The pervasiveness of the roles of DNAm in the control of cellular functions makes it obvious that an epigenetic change often accompanies the rejuvenation caused by these techniques. However, a central question that has long remained unanswered is whether the observed change in methylation profiles is in fact the outcome of an epigenetic reprogramming aimed at reversing the ageing process.

In this context, the development of methylation clocks offers an analytical tool that can inform us if such epigenetic rejuvenation has occurred. The application of methylation clocks to known experimental rejuvenation protocols seems to show that there is correspondence between rejuvenation and epigenetic resetting. In other words, treatments that rejuvenate the body turn back the epigenetic clock, i. e. they reset the DNAm pattern to younger profiles.

Recently, at least three techniques have been applied to laboratory animals (mice and rats) that have effectively reversed the DNAm age and simultaneously provided evidence of a restoration of phenotypic characteristics of an older animal to levels comparable to those of a younger animal, including physical and behavioural performances. These include heterochronic parabiosis, caloric restriction and cellular reprogramming.

It will be necessary to translate these interventions to humans in order to counteract the increasing burden of age-related diseases (Harper, 2014; Kaeberlein et al., 2015; Moffitt, 2020).

It appears that the epigenetic clocks can be turned back in time, even

in humans. A growing body of evidence from population studies supports the possibility that this can be achieved through exposure to specific environmental factors (Fig. 4).

6.1. Heterochronic parabiosis

Decades of heterochronic parabiosis experiments, in which the circulation of old and young mice are linked to cause their blood to mix over a period of time, show that sharing the same blood leads to a rejuvenation of the old animal while the young mouse ages prematurely (Mahmoudi et al., 2019).

Rejuvenation through parabiosis also seems to involve epigenetic reprogramming and to be able to mitigate the appearance of age-associated epigenetic alterations. As a matter of fact, young blood restores TET2 expression and 5hmC levels in the brains of old mice, a mechanism required to compensate for the sharp age-related decline in neurogenesis thereby improving learning and memory (Gontier et al., 2018). These data raise the exciting possibility that rejuvenation, obtained by targeting epigenetic regulators associated with the ageing process, may be achieved through parabiosis.

The outstanding results of a pre-print paper (Horvath et al., 2020) are in line with this possibility. Based on previous observations that the beneficial effect of parabiosis is due to plasma-transported factors (Villeda et al., 2014), the authors developed a treatment they call 'Elixir'. The protocol is based on the principle of heterochronic plasma exchange (HPE), whereby the plasma of old rats is replaced by that of young ones. The transfer of young plasma into old rats was evidently very effective in rejuvenating various tissues and improving multiple clinical markers of ageing, including behavioural responses and cognitive functions. Significantly, the treatment more than halved the epigenetic age of old mice in multiple tissues. The particular significance of these results lies in the promise that ageing can be systematically controlled by rejuvenating the body through the circulatory system, with plasma as a vehicle. This might represent a huge advance in anti-ageing therapeutic strategies.

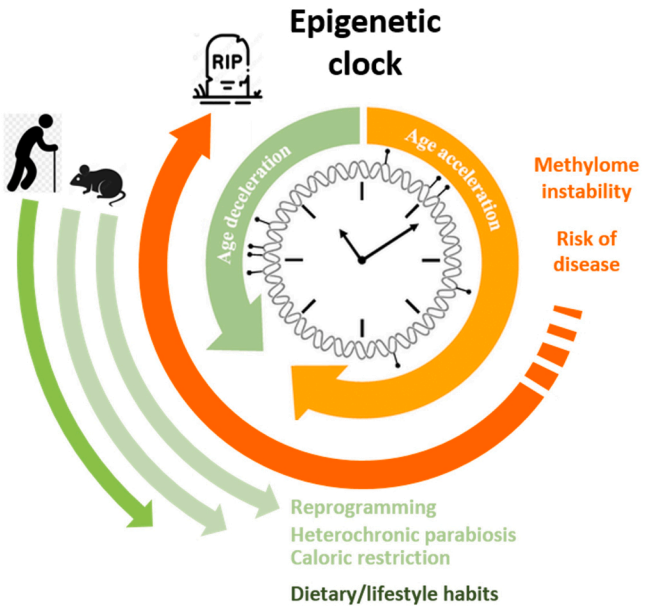

Fig. 4. Age-related disease risk can be counteracted by strategies that slow down the ticking of the epigenetic clock. Throughout life, a person's genomic DNA methylation profile (methylome) is permanently altered by multiple intrinsic and extrinsic factors. Their effects are recorded as a kind of epigenetic memory and propagated over time. The loss of methylome integrity may result in a progressive impairment of the body's homeostatic mechanisms, influencing disease burden and resistance. Loss of methylome integrity during ageing, and the consequent increase in disease risk, are reflected by increasing epigenetic age, as measured by the epigenetic clock. The risk of age-related disease could be lowered by strategies that reduce or reverse the rate of epigenetic ageing. In animal models, an epigenetic age-reducing effect has been reported for cellular reprogramming, heterochronic parabiosis and caloric restriction. In humans, a similar effect was shown following improvements in diet and lifestyle.

Along these lines, the findings of Mehdipour et al. (Mehdipour et al., 2021) offer an interesting alternative to plasma transfer. They show that old mice can be rejuvenated by simply replacing about 50% of their blood plasma with normal saline plus albumin at a physiological level. This would imply that the rejuvenation associated with the transfer of young plasma is not attributable to anything positive being carried by the young plasma, but from something negative in the old plasma being diluted by the procedure itself. It would be significant if these results were also confirmed in terms of a systemic slowing down of epigenetic ageing.

The widespread excitement generated in the general public by the research findings on young plasma and the obvious simplicity and low cost of plasma infusions (A controversial blood transfusion start-up called Ambrosia was already offering young blood plasma transfusions in the USA) prompted an FDA warning against the practice in February 2019. Reassurance about the significant risks (including infectious, allergic, respiratory and cardiovascular risks) potentially associated with this procedure requires further studies, which will most likely not be long in coming.

Meanwhile, a group of Russian biohackers, who are trying to break the mould, are performing plasma dilution experiments on themselves (https://rlegroup.net/rle_about/).

6.2. Caloric restriction

Caloric restriction (CR) is defined as the reduction of caloric intake without deprivation of essential nutrients (Speakman and Mitchell, 2011). It has been established that CR can attenuate molecular characteristics of ageing, reduce risk factors for many age-related diseases and extend healthy lifespan in multiple species, including non-human primates (Anderson and Weindruch, 2012; Colman et al., 2014; Flanagan et al., 2020; Mattison et al., 2012; Speakman and Mitchell, 2011). CR seems to work in humans also. Two clinical pilot studies have shown that dietary restriction lowers the levels of systemic biomarkers of ageing and also multiple risk factors of cancer and cardiovascular disease in healthy individuals (Brandhorst et al., 2015; Redman et al., 2018).

Critically, CR has profound effects on the epigenome by eliciting pervasive changes in DNAm, histone PTMs and chromatin remodelling.

In terms of DNAm, CR appears to be protective for age-related changes. An illustration of this is the evidence that the methylation age of macaques and mice subjected to CR is lower than expected, on the basis of their chronological age (Hahn et al., 2017; Maegawa et al., 2017). In addition, there is evidence of an impact of CR on the levels of some components of the DNAm/demethylation machinery. In mice, CR transcriptionally regulates the demethylation enzymes TET1 and TET3 (Unnikrishnan et al., 2018). Glucose restriction increased the activity of DNMT1 in human cell lines (Li et al., 2010).

Because CR, including dietary restriction, is a simple and non-invasive method of metabolic manipulation, clinical trials demonstrating an effect on epigenetic ageing rates, as well as on health span and lifespan are to be expected.

A pre-print paper (Waziry et al., 2021) derived from the multicentre, randomised controlled trial CALERIE (Ravussin et al., 2015) shows that two years of 25% CR has effectively delayed the epigenetic age, as measured by some DNAm clocks. Specifically, the effects of CR were strongest for the Pace-of-Aging clocks, in particular for the DunedinPACE measure, while the first-generation (Horvath and Hannum clocks) and second-generation (PhenoAge and GrimAge) clocks showed little or no slowdown in the acceleration of epigenetic age. Although the effect size appears to be relatively modest, confirmation of these data could significantly advance the concept that an intervention to extend

healthy lifespan in animals can slow the pace of biological ageing in humans.

6.3. Cellular reprogramming

Reprogramming techniques, such as somatic cell nuclear transfer (SCNT) and the creation of induced pluripotent stem cells (iPSCs) through the overexpression of the four transcription factors Oct3/4, Sox2, Klf4 and c-Myc (now referred to as the "Yamanaka factors" or "OSKM" factors), clearly demonstrate that the epigenetic information governing cellular identity can be removed to allow a new differentiation pathway (Mahmoudi et al., 2019).

Reprogramming through the induction of Yamanaka factors is receiving particular attention, probably because it is easier to pursue for intervention purposes. It is surprising to note that this reprogramming also leads to the reversal of multiple molecular hallmarks of ageing, thus representing a promising strategy to achieve rejuvenation (Mahmoudi et al., 2019).

The mechanisms by which pluripotency factors change the genomic patterns of DNAm are still largely obscure. However, there is evidence of an intricate interaction between central pluripotency factors (Oct4, Sox2 and Nanog) and DNMT and TET family enzymes that includes a mutual regulation of their expression (Shanak and Helms, 2020).

Apparently, during the cell differentiation process, when epigenetic handrails are reconfigured, epigenetic age is also reversed. In fact, Horvath's epigenetic clock confirms that iPSCs have an epigenetic age close to zero (Horvath, 2013; Olova et al., 2019). Furthermore, it has recently been demonstrated in mice that the epigenetic age of the germline is rejuvenated in the offspring during early embryogenesis and reaches its 'ground zero' state between E4.5-E10.5, a period that corresponds to gastrulation and includes the pluripotent state. Only after this period, the DNAm ageing of the offspring begins and then proceeds naturally (Kerepesi et al., 2021).

These observations confirm the intimate link between reprogramming and DNAm ageing, while giving it an illuminating natural context. However, the exploitation of reprogramming techniques poses considerable risks since the loss of cellular identity and the re-establishment of self-renewal capabilities invariably lead to a high risk of cancer.

To minimise this risk, attempts are being made to develop reprogramming strategies that separate de-differentiation from epigenetic rejuvenation. One promising approach is to limit the action of reprogramming factors as much as necessary to reach a kind of intermediate de-differentiation state where cells have started to rejuvenate epigenetically but are not fully de-differentiated.

The first evidence in this regard was provided using transient/periodic induction of reprogramming factors (Manukyan and Singh, 2014; Ocampo et al., 2016). Partial reprogramming ameliorated multiple signs of ageing and reversed the age-related trends of some epigenetic heterochromatin markers in senescent human fibroblasts (Manukyan and Singh, 2014) and progeroid mouse fibroblasts (Hutchinson-Gilford syndrome model - HGPS) (Ocampo et al., 2016). This occurred without any notable change in the differentiated phenotype of the fibroblasts.

Partial reprogramming was also applied in vivo to progeroid and naturally aged WT mice. Significant improvements of age-related phenotypes at the whole organism level were observed, including an increase in lifespan. Induction of OSKM was also capable of improving regenerative capacity of damaged organs such as muscle and pancreas. Significantly, these changes were not accompanied by the presence of any dysplastic lesion (Ocampo et al., 2016).

Further progress along this path is included in the works of Lu (Lu et al. (2020) and Sarkar et al. (2020).

Lu et al. (2020) demonstrated that ectopic expression of the transcription factors Oct4, Sox2 and Klf4 (OSK) can safely reverse the age of a complex tissue and restore its biological function in vivo. Local ectopic expression of OSK stimulated axon regeneration in a mouse model of optic nerve injury and led to improved vision in another mouse model glaucoma. In addition, OSK treatment in healthy but old mice led to vision recovery, comparable to that of young mice. Significantly, OSK induction also counteracted DNAm age progression based on a ribosomal clock. Moreover, these effects were largely dependent on active DNA demethylation, in particular the activity of the enzymes TET and TDG, thus supporting the idea that changes in DNAm profiles are involved in the ageing process as well as in its functional reversal.

Sarkar et al. (2020) transiently expressed OSKM together with the transcription factors LIN28 and NANOG (OSKMLN) in human primary cells (dermal fibroblasts, endothelial cells and chondrocytes) obtained from both young and old individuals. The treatment significantly reduced the DNAm age calculated by Horvath's clock (Horvath, 2013) of old cells and caused the regression of several ageing phenotypes. In addition, skeletal muscle stem cells from older humans and mice treated with OSKMLN showed a remarkable ability to regenerate new tissue in vivo when transplanted into damaged muscles of immunocompromised mice. As with the study by Lu et al. (2020), this work represents a significant step towards the development of potential therapies for ageing and age-related diseases based on counteracting the rate of DNAm age progression.

Finally, two very recent pre-print papers (Alle et al., 2021; Gill et al., 2021) confirm the rejuvenation capacity of reprogramming factors, their ability to epigenetically reprogramme cells and, above all, the apparent long-term stability of their effects.

Gill et al. (2021) show that transient reprogramming, achieved through the expression of reprogramming factors followed by withdrawal, effectively separates rejuvenation from reprogramming to pluripotency. Dermal fibroblasts from middle-aged donors can therefore also be rejuvenated in terms of DNAm age (Horvath, 2013), while retaining their cellular identity.

Alle et al. (2021) used a model of heterozygous mice for premature ageing carrying a single mutated Lamin A allele that produces Progerin. They report that a single, short phase of cellular reprogramming performed on two-month-old mice has an impact on both lifespan and health-span thereafter, protecting tissues and organs from age-related deterioration. The rejuvenating effect of the treatment is associated with a differential DNAm signature shared by all organs studied.

In conclusion, cellular reprogramming is rapidly emerging as a potential tool to combat ageing. Rejuvenation is clearly accompanied by extensive epigenetic remodelling and a reduction in DNAm age. However, it is not entirely clear whether this reflects a role of epigenetic mechanisms as a passive chaperone or driver.

The observation that rejuvenation by cellular reprogramming depends on the full functionality of epigenetic processes, such as histone methylation (Ocampo et al., 2016) and active DNA demethylation (Lu et al., 2020), provides initial evidence in favour of the second hypothesis.

The application of this technology on humans must wait until there is sufficient certainty about its safety. In addition, this application must overcome the technical challenges of genetic manipulation and difficult-to-produce biological products. The discovery that a combination of seven small-molecule compounds can generate pluripotent stem cells from mouse somatic cells could offer a useful alternative (Hou et al., 2013).

6.4. Environmental factors

Epigenetic mechanisms are viewed as molecular mediators of the long-term memory of the cumulative action of the environmental factors on individual health and ageing pathways (Martin and Fry, 2018).

There is a large and continuing amount of literature on the effects of the environment on DNAm, which now defines the specific research field of environmental epigenetics (Bollati and Baccarelli, 2010). The list of known environmental modifiers is long. It includes dietary factors, pollutants, behavioural and social factors. The effects include both global and site-specific changes in the genomic methylation pattern

(Martin and Fry, 2018).

The underlying mechanisms remain undefined and are intuitively heterogeneous in nature. Two frequently described hypotheses involve the direct action of certain environmental factors on the function of the DNMT and TET enzymes, and/or their action on SAM availability (Martin and Fry, 2018). If we consider only dietary factors, examples of the first of these two hypotheses include the dietary components epigallocatechin-3-gallate and caffeic acid, inhibitors of DNMT, and ascorbate, which increases the generation of 5hmC, most likely by acting as a cofactor for TETs. Examples of the second hypothesis are various nutrients present in the diet, including methionine, folate, choline, betaine, vitamins B2, B6 and B12, which influence SAM synthesis via the methionine cycle (Kadayifci et al., 2018).

As far as the epigenetic age is concerned, although very little is known about what controls the epigenetic clock, several papers have provided estimates of its heritability, which appears to be rather moderate, being frequently below 50% (Horvath, 2013; Levine et al., 2015b; Marioni et al., 2015a). Consequently, the action of the environment on the variation of the epigenetic clock seems to outweigh that of genetic factors. Recent work has actually claimed that epigenetic age variation across the lifespan relies entirely on environmental factors (Li et al., 2020). In any case, the apparent magnitude of clock heritability clearly leaves ample opportunity for intervention and /or prevention on the ticking rate of the epigenetic clock by the use of environmental modification. This fuels the need to identify environmental stimuli that slow down epigenetic age progression.

Multiple factors that have been found to be positively associated with epigenetic age acceleration are conterders for increasing the rate of epigenetic ageing. Among the most recurrent are lifestyles such as smoking and alcohol consumption, the biometric index BMI, psychological factors and socioeconomic factors such as low education and income (for review see (Oblak et al., 2021)). It is clear that a possible way to achieve a reduction in the rate of epigenetic ageing is by mitigating these factors. However, in this review we will focus on factors that are associated with slower acceleration of epigenetic ageing. These associations could in fact identify factors that are effective in guiding the ageing trajectory towards more favourable outcomes.

6.4.1. Environmental factors associated with a slower acceleration of epigenetic ageing by observational studies

Cross-sectional studies show that epigenetic age acceleration is negatively associated with multiple dietary and lifestyle habits that correlate with lower age-related morbidities.

As reported in multiple studies, vegetables and fruit are the food items whose higher consumption is frequently found to be associated with lower epigenetic age acceleration in the adult population (Dugué et al., 2018a; Kim et al., 2022; Levine et al., 2018; Lu et al., 2019; Quach et al., 2017). Significantly, this association also applies to plasma levels of diet-derived antioxidants such as carotenoids and tocopherols (Levine et al., 2018; Quach et al., 2017). The association of other dietary factors with lower epigenetic age acceleration, such as consumption of poultry, dairy products, fish and moderate alcohol consumption, shows less consistency across studies (Dugué et al., 2018a; Kresovich et al., 2021; Lu et al., 2019). The limited ability to replicate these findings could be due to multiple factors including the inaccuracy and poor long-term consistency of self-reported dietary habits, which could result in some important factors (e.g. early life exposures) being unidentified, as well as differences in the composition of study populations (e.g. demographic, cultural, genetic and health status differences).

There is only a small amount of data which describes the effect of food supplements and vitamins *status* on epigenetic age acceleration. However, these data are extremely relevant. For example, epigenetic age acceleration is reduced in individuals who supplement their diet with omega-3 polyunsaturated fatty acids, a supplement that is increasingly used to protect against cardiovascular disease (Lu et al., 2019).

In addition to the previously mentioned blood biomarkers of nutritional *status*, similar results were reported for vitamin D3, a vitamin known for its antioxidant and anti-inflammatory properties whose deficient levels have been proposed as a marker of ageing (Holick, 2006; Schöttker et al., 2019). Individuals with normal serum 25-hydroxyvitamin D levels have an average reduction in epigenetic age acceleration of 1 year compared to individuals with defective vitamin levels (Vetter et al., 2020). Again, however, this association did not emerge in a second, very similar cross-sectional study, probably due to methodological discrepancies (i. e. the epigenetic clock employed) and subtle differences in the age range of the cohort being analysed (Schöttker et al., 2019).

Although the association between epigenetic age acceleration and dietary factors is biologically plausible and highly relevant, in studies that take into account individual dietary components and nutrients, this association is apparently weak, as a large part of the acceleration variation observed in study populations remains unexplained.

Possibly, a stronger association may emerge from the study of dietary patterns, as supported by recent studies that use diet quality scores, already known to be associated with multiple chronic diseases and mortality (Kim et al., 2022; Kresovich et al., 2022). An example of such recommendation-based dietary indexes is the Dietary Approaches to Stop Hypertension (DASH) score (Fung et al., 2008). The DASH is a dietary pattern suggested by the 2010 Dietary Guidelines for Americans to promote health and reduce the risk of chronic diseases, which include diabetes, coronary heart disease and various types of cancer. The extent to which an individual adheres to the DASH pattern can be quantified by a score. High scores indicate a better quality of diet in terms of the combined intake of multiple items, including high intake of fruit and vegetables and reduced intake of meat, sweetened beverages and sodium.

Kim et al. (2022) analysed data from nearly two thousand individuals from the Framingham Heart Study Offspring Cohort and reported a cross-sectional negative association between the DASH score and multiple measures of epigenetic age acceleration. Significantly, all the epigenetic measures of ageing mediated the association between the DASH score and all-cause mortality.

Similar results were reported by Kresovich et al. (2022), extending the analysis also to multiple dietary patterns beyond the DASH. Similar to the DASH, these other scores – i.e., the healthy eating index (Krebs-Smith et al., 2018), the alternative healthy eating index (Chiuve et al., 2012), and the alternative Mediterranean diet (Fung et al., 2009) – incorporate foods which are found to be protective against heart disease and other chronic diseases.

Interestingly, both studies observed a modification effect on the healthy diet-epigenetic age association by specific lifestyle factors such as smoking and physical activity. This suggests that a high-quality diet is one of the modifiable factors that may result in a slower acceleration of epigenetic ageing and lead to a longer and healthier lifespan, even in individuals with an unhealthy lifestyle (e.g. smokers or sedentary individuals).

In addition to dietary factors, reduced epigenetic age acceleration has been found to be associated with higher physical activity in multiple studies (Kankaanpää et al., 2021; Kresovich et al., 2022; Levine et al., 2018; Lu et al., 2019; Quach et al., 2017; Stevenson et al., 2019). The importance of this association lies in the fact that inadequate physical activity is a major risk factor for non-communicable diseases and mortality (Lear et al., 2017), particularly for older individuals living in high income countries. As a matter of fact, elderly people are typically characterised by low or decreasing levels of physical activity (Lear et al., 2017). In addition, higher physical activity is a strong determinant of ageing trajectories towards healthier ageing (Arem et al., 2015; Daskalopoulou et al., 2017).

Of particular interest is the observation that the effect of physical activity on epigenetic ageing depends on the 'quality' of the physical activity that is performed, which may lead to opposite health effects. In fact, new evidence suggests a contrast between the health effects of physical activity in leisure time vs. that in the workplace, a phenomenon

referred to as the 'physical activity paradox' (Holtermann et al., 2012). While physical activity in leisure time, even of high intensity, has been associated with positive health outcomes, adverse consequences have been documented for physical activity in the workplace. Strikingly, while physical activity in leisure time is associated with negative epigenetic age acceleration, the opposite effect has been documented for physical activity in the workplace (Kankaanpää et al., 2021).

The mechanistic link between physical activity and slower epigenetic acceleration is unknown. Unexpectedly, physical activity does not appear to mediate the positive cross-sectional association between overall and central adiposity and higher epigenetic acceleration, suggesting that physical activity slows down epigenetic acceleration through avenues other than counteracting adiposity (Kresovich et al., 2022).

A possible reducing effect on epigenetic age acceleration also extends to social factors. An inverse association between epigenetic age acceleration and the level of education is recurrent in multiple studies (Dugué et al., 2018a; Fiorito et al., 2019; Gomez-Verjan et al., 2021; Hughes et al., 2018; Karlsson Linnér et al., 2017; Levine et al., 2018; Lu et al., 2019; Quach et al., 2017; Zhao et al., 2019). Out of these, the results of Fiorito, (Fiorito et al. (2019) are particularly promising as they confirm previous observations in a very large cross-cohort, cross-country sample. Although the level of education can be considered as an environmental factor biologically distant from DNA methylation, the impact of education on epigenetic age acceleration was comparable to and independent from other life-style factors such as smoking, BMI, alcohol and physical activity. This would suggests that an epigenetic ageing advantage can be gained by leveraging education beyond other modifiable environmental/lifestyle factors. Further support for this hypothesis comes from studies suggesting that individuals who experienced improvement of social context and education level throughout life, particularly during early childhood, have slower epigenetic ageing rates (Austin et al., 2018; Fiorito et al., 2017; Gomez-Verjan et al., 2021; Hughes et al., 2018).

This possible anti-ageing effect of environmental factors is also supported by recent cross-sectional analyses of migrant populations. A striking epigenetic age reduction matches the improvement of environmental context and health transition that are usually consequential to migration (Chilunga et al., 2021; Horvath et al., 2016). In fact, migration from a low-income to a high-income country is associated with changes in multiple environmental factors, including lifestyle and improved access to health services. According to the so called 'healthy migrant effect' (Wallace et al., 2019), these environmental changes explain the improved health status and the mortality advantage of migrant individuals over non migrants.

Chilunga et al. (Chilunga et al., 2021) show that health transition in Ghanaian migrants, who have entered and resided in Europe, is associated with lower epigenetic age acceleration compared with non-migrants residing in Ghana.

Similar results were obtained in second-generation British-Bangladeshi women raised in the UK, who show a lower epigenetic age acceleration than their counterparts women who spent their childhood in Bangladesh. In this case, exposure to an adverse environment during a childhood spent in Bangladesh appears to alter the pace of epigenetic ageing and also results in altered reproductive function, despite subsequent migration to the West as young adults (Study pre-print (Stöger et al., 2020)).

All in all, the results of these studies suggest that the levels and rates of change of health trajectories during life and old age may be modified by a mixture of health-related environmental factors, including dietary habits, behavioural factors (e.g. physical activity), and contextual social factors (e.g. education and social participation), which may slow down epigenetic ageing.

However, from the perspective of using these results for interventional purposes, one must bear in mind that they are limited by their cross-sectional origins. The cross-sectional association of a factor with a slower rate of epigenetic ageing describes an instantaneous and possibly transient characteristic of the individual. However, it does not mean that there is a real interaction between the environmental stimulus and epigenetic age acceleration, nor does it identify the time frame in which this interaction may have occurred. Is the slowing effect due to an interaction in the present or in the past? We also cannot discern whether these interactions are stable over time, nor can one predict what the outcome would be on the quality of ageing and longevity. In fact, many of the available studies do not analyse more than one time period and, moreover, there is still a lack of longitudinal studies.

The possibility that the epigenetic clock is particularly susceptible to environmental factors at a specific developmental stage or age range is of importance because it can define time windows useful for intervention. Studies on this topic are very few in number. However, a broader look outside the research area of ageing provides some insight into potential developmental differences (Gluckman et al., 2009; Mitchell et al., 2016). For instance, early stages of prenatal development have received particular attention. Foetal programming by environmental factors, better known as the hypothesis on the developmental origins of adult health and disease, has become a major focus of environmental epigenetic research (Hales et al., 1991). There is substantial evidence that *in utero* exposure to different environmental factors has a major impact on later phenotypes via epigenetic mechanisms (Drake et al., 2012; Ferland-McCollough et al., 2012; Gluckman et al., 2008, 2005; Heijmans et al., 2008). In contrast, the sensitivity to environmental influence observed at later time periods is not as strong as during the foetal period (Guintivano and Kaminsky, 2016; Heim and Binder, 2012). In line with these observations, some studies indicate that the rate of progression of the epigenetic clock is largely established before adulthood (Kananen et al., 2016; Li et al., 2020). This is particularly significant as many of the associations found at later ages could be related to early exposures. In fact, a longitudinal study of a large cohort of older adults shows that the association between DNA methylation-age and many phenotypes (including blood, physical, cognitive and lifestyle phenotypes, as well as mortality) depends largely on both the general cognitive ability measured at the age of 11 years and the number of years in education. In fact, adjustment for cognitive ability at the age of 11 years attenuated most of the cross-sectional associations found at older ages (Stevenson et al., 2019).

The temporal stability of the relationship between an environmental factor and epigenetic age acceleration would ensure that the action of the environmental stimulus would be long lasting and would predict its long-term effect. In this respect, the available longitudinal studies are discouraging. A longitudinal analysis of African-Americans, although conducted on a limited number of cases, finds that only a few cross-sectional associations with epigenetic age acceleration are significant in longitudinal terms. These are limited to the positive associations with BMI and smoking while the associations of other factors, such as alcohol consumption and level of education, are not confirmed (Zhao et al., 2019). One possibility is that the interaction of the clock with environmental factors changes over time. A longitudinal study of twins actually suggests that new sources of person-specific environmental influences on the clock emerge with age. Epigenetic clocks may therefore be sensitive to new environmental stimuli throughout life (Jylhävä et al., 2019).

Finally, the consequence of having an epigenetic age younger than the chronological age is suggested by the positive relationship between epigenetic age acceleration and the risk of mortality and age-related diseases, a link that recurs in many studies. The negative association with longevity is less clear, however, and is described in a few and relatively small case-control studies, in terms of the size of the population analysed. Specifically, the observation that exceptionally long-lived Italians (who have reached an age of 105–109 years) are epigenetically younger than expected on the basis of their chronological age (Horvath et al., 2015) is not confirmed by the analysis of a population on the Nicoya Peninsula in Costa Rica, which has one of the highest old-age life

expectancies in the world (McEwen et al., 2017).

6.4.2. Epigenetic rejuvenation by the control of diet and lifestyle - interventional studies

Even though ageing and related conditions and diseases are inevitable, preliminary population studies are trying to prevent age-related DNA methylation changes in humans by the use of control of diet and lifestyle (Table 1).

Initial evidence of an effect on epigenetic age, as a result of modification of environmental factors, comes from dietary intervention studies using supplementation with nutrients known to have an effect on the DNA methylation pattern.

Sae-Lee et al. (2018) analysed methylation data, available in the public domain, from an intervention study with folic acid + vitamin B12. The study was small in size and included 44 elderly participants (over 65 years), randomised to folic acid (400 μg/d) and vitamin B12 (500 μg/d) supplementation for 2 years. Both of these vitamins are known to modify epigenetic profiles as they contribute to the production of the methyl donor SAM through the one carbon metabolism.

The study identified an association between dietary supplementation and decreased Horvath epigenetic age (Horvath, 2013). However, this association was of low magnitude and was limited to women carrying the MTHFR wild type genotype 677CC. This suggests that in men higher doses of folate may be required to produce the same biological effect.

Later, Chen et al. (2019) conducted the first randomised controlled clinical trial evaluating the effect of a nutritional intervention on epigenetic ageing. Based on evidence associating vitamin D supplementation with health improvements and methylation changes (Zhu et al., 2016, 2013), the authors hypothesised that vitamin D supplementation may also slow down epigenetic ageing. They measured the epigenetic by Horvath (Horvath, 2013) and Hannum (Hannum et al., 2013) clocks in 51 overweight African-American adults with suboptimal vitamin D status, before and after 16 weeks of supplementation. Three different vitamin D dosages were administered (600 IU/d, 2000 IU/d and 4000 IU/d). Compared to placebo, both the 2000 IU and 4000 IU groups showed decreased epigenetic ageing by one of the two clocks (a decrease of 1.85 and 1.90 years in Horvath and Hannum epigenetic age, respectively).

Unfortunately, the cohort size of these studies is relatively small. Therefore, further studies are required to confirm the effects of dietary supplements on epigenetic age acceleration.

More recently, some studies have evaluated the epigenetic rejuvenating effect of more complex dietary patterns.

A non-controlled pilot study (Gensous et al., 2020) evaluated the impact of a 1-year Mediterranean-like diet in a small cohort of 120 healthy elderly subjects, half Italian and half Polish. Changes in their epigenetic age were assessed by using the Horvath clock (Horvath, 2013). The dietary pattern involved increased consumption of fruit, vegetables, legumes, unrefined cereals and olive oil, accompanied by a low intake of meat, dairy products and alcohol consumption, as is typically observed in the Mediterranean area. In addition, the dietary strategy included daily supplementation with a low dose of vitamin D (10 μg or 400 IU/d).

The results show epigenetic rejuvenation following the intervention, especially in subjects who were epigenetically older at baseline. Higher levels of adherence to the diet were associated with greater epigenetic rejuvenation. However, the effect size on epigenetic age was relatively small. In addition, the reduction in epigenetic age was statistically significant only in Polish subjects and, in particular, in females suggesting that the protective effect of diet on epigenetic age is country and sex-specific.

The importance of this study lies on the connection between epigenetic age and the effects of the Mediterranean diet, one of the best lifestyle strategies for prolonging longevity and avoiding the common disorders of ageing, including heart disease and cancer (Estruch et al., 2018; Samieri et al., 2013; Shikany et al., 2021). Therefore, the effects of the Mediterranean diet on epigenetic ageing deserves revaluation in a larger survey.

Subsequent studies have evaluated the effect of combining diet with physical activity and other lifestyles. Unfortunately, they also share the limitation of a relatively small sample size, plus the inclusion of mainly one gender, making it impossible to investigate possible sex-specific effects.

Meir et al. (Yaskolka Meir et al., 2021) conducted an 18-month randomised non-controlled trial in about one hundred sedentary adults, predominantly males with abdominal obesity and/or dyslipidaemia. The study explored the relationship between epigenetic age (calculated from a 240-CpG-based prediction formula (Li et al., 2018)) and intrahepatic and abdominal fat deposits following a dietary and lifestyle regime aimed at producing weight loss. The intervention consisted of an isocaloric low-fat or Mediterranean/low-carbohydrate regime with the addition of nuts and with/without the addition of moderate physical activity.

After 18 months of this regime, the epigenetic age remained significantly correlated with the chronological age. However, the median increase in epigenetic age among those who responded successfully to weight loss was significantly lower (0.6 years) than among those who failed in weight loss (1.1 years). This was particularly evident in older participants, i. e. those with a median age of over 48 years, where the regime attenuated the epigenetic age by 7.1 months.

A similar but controlled study was conducted on approximately 200 postmenopausal women (aged 50–69 years) (Fiorito et al., 2021). The experimental group underwent a dietary intervention, a physical activity intervention and/or a combination of both. The dietary intervention consisted of adopting a diet based on plant foods, with a low glycaemic load, low in saturated fats, trans fats, and alcohol, and rich in antioxidants. The physical activity intervention consisted of daily moderate recreational physical activity combined with occasional strenuous activity. Before and after the interventions, lasting two years, the epigenetic ageing was quantified both in terms of GrimAge (Lu et al., 2019) and stochastic epigenetic mutations load (SEM) (Gentilini et al., 2015).

The results show that the effects of the interventions depend on the type of clock. Compared to controls, the dietary intervention led to a significant reduction in GrimAge (delta of −0.66), whereas the physical activity intervention caused a significant reduction in the SEM (delta of −2.06). It is notable that these results indicate that diet quality and physical activity influence epigenetic ageing through complementary molecular mechanisms, suggesting that their effect is potentially cumulative rather than interchangeable.

Finally, Fitzgerald et al. (2021) sought to evaluate more comprehensively how a regime of diet and lifestyle interventions can positively impact epigenetic ageing. They conducted a randomised controlled clinical trial in 43 healthy adult males aged 50–72 years. The intervention group underwent an extensive eight-week programme that included sleep, exercise and relaxation in addition to a plant-centred diet with probiotic and phytonutrient supplements that modulate DNA methylation. The rationale for including relaxation and sleep amelioration was based on previous evidence associating changes in DNA methylation profile and epigenetic age with stress (Moore et al., 2017; Wolf et al., 2016; Zannas et al., 2015), stress-reduction practices (Pavanello et al., 2019) and sleep quality (Carroll et al., 2017; Carskadon et al., 2019; Nilsson et al., 2016). The analysis of epigenetic ageing was conducted using the Horvath epigenetic age (Horvath, 2013).

The results show a reduction in epigenetic age in the experimental cohort of 3.23 years compared to the control group. Furthermore, individuals in the experimental group, despite the limited duration of the intervention, reached an epigenetic age almost two years younger than the baseline age. In contrast, epigenetic age tended to increase in the controls.

This last study effectively demonstrates how the use of a multimodal intervention has clear advantages. A significant set-back of biological

Table 1
Major intervention studies on epigenetic rejuvenation by the control of diet and lifestyle.

Intervention		Study design	Cohort	Clock	Main findings	Reference
Vitamins supplementation	Supplementation with *folic acid + vitamin B12* (2 y) or oligomeric flavanols (MOF) (8 weeks)	Nonrandomised trial	**MOF**: 13 males (mean age 25 y; age range 18–30 y) **Folic acid + Vitamin B12**: 44 subjects (mean age 27 y; age range 27–30 y), 43% males	Horvath (Horvath, 2013)	Significant reduction in EA solely among women with the MTHFR 677CC genotype after folic acid and vit. B12 supplementation	Sae-Lee et al. (2018)
	Supplementation with *vitamin* D_3 (16 weeks); placebo vs. ~600, ~2000, ~4000 IU/d	Randomised controlled trial	51 overweight/obese African Americans, mean age 26 y, 16% males	Horvath (Horvath, 2013) Hannum (Hannum et al., 2013)	Horvath EA was decreased by 1.83 y and 1.62 y in 2000 IU/d and 4000 IU/d groups after the treatment, respectively. Changes in Hannum EA were not significant. Serum vit. D concentrations were significantly associated with decreased Horvath EA only	Chen et al. (2019)
Diet	1-y *Mediterranean-like diet* (increased consumption of fruit, vegetables, legumes, unrefined cereals and olive oil, accompanied by a low intake of meat, dairy products and alcohol consumption)	Randomised trial	60 Italian subjects, mean age 72 y, 82% males; 60 Polish subjects, mean age 71 y, 67% males	Horvath (Horvath, 2013)	A trend towards EA reduction was observed. The effect was statistically significant in Polish females and in subjects who were epigenetically older at baseline	Gensous et al. (2020)
Diet & lifestyle	18-month *lifestyle intervention* (an isocaloric low-fat or Mediterranean/low-carbohydrate regime with the addition of nuts and with/without the addition of moderate physical activity)	Randomised trial	120 abdominally obese subjects, 92% males, mean age: 50 y	Li (Li et al., 2018)	Attenuation of EA increase was observed in successful weight losers (> 5% weight loss) vs. weight-loss failures (0.6 y vs. 1.1 y) and in participants who completed the trial with healthy liver fat content vs. participants with fatty liver (0.6 y vs. 1.8 y)	Yaskolka Meir et al. (2021)
	Dietary intervention (diet based on plant foods, with a low glycaemic load, low in saturated fats, trans fats, and alcohol, and rich in antioxidants). *Physical activity intervention* (daily moderate recreational physical activity combined with occasional strenuous activity). *A*	Randomised controlled trial	219 postmenopausal women subdivided into four groups (CT, dietary intervention, physical activity intervention, dietary + physical activity intervention), mean age ~ 56 y	GrimAge (Lu et al., 2019) SEM (Gentilini et al., 2015)	Dietary intervention had a significant slowing of the GrimAge clock (delta of 0.25 and −0.41 in the control and dietary intervention groups, respectively), whereas increasing	Fiorito et al. (2021)

(*continued on next page*)

Table 1 (*continued*)

Intervention		Study design	Cohort	Clock	Main findings	Reference
	combination of both. Two y duration				physical activity led to a significant reduction of SEM (delta of 1.82 and −0.23 in the control and physical activity intervention groups, respectively.	
	Eight-week *dietary and lifestyle treatment* programme that included sleep optimisation, exercise and relaxation in addition to a plant-centred diet with probiotic and phytonutrient supplements	Randomised controlled trial	43 males (21 control group, 22 treatment group), age range 50–72 y	Horvath (Horvath, 2013)	Compared to the control group, the treatment group was 3.23 y younger at the end of treatment according to the clock.	Fitzgerald et al. (2021)
Growth hormone	Administration of *recombinant human growth hormone + dehydroepiandrosterone and metformin.* One y duration	Randomised trial	9 males, age range 51–65 y	Horvath (Horvath, 2013) Hannum (Hannum et al., 2013) GrimAge (Lu et al., 2019) PhenoAge (Levine et al., 2018)	The mean EA was approximately 1.5 y less than baseline after the treatment. The GrimAge showed a 2–y decrease in epigenetic vs. chronological age that persisted six months after discontinuing treatment.	Fahy et al. (2019)

d = day; y = years; EA = epigenetic age.

age was achieved within only eight weeks.

All in all, these works suggest that changes in lifestyle behaviour, through non-invasive and otherwise generally beneficial interventions, which are known to have mechanistic plausibility in the influencing of methylation pathways, may lead to a moderate slowing of epigenetic ageing. Efficacy appears to be measurable in both normal people and relatively old or health impaired individuals. Nevertheless, it is not yet fully established whether this slowdown is sufficient in intensity or time stability to have any significant outcome in terms of longevity, risk of developing age-related diseases and quality of life in old age.

Alternative strategies could yield greater reductions in epigenetic age. This is suggested by a small clinical study (Fahy et al., 2019) showing a loss of as much as 2.5 years of epigenetic age (the Horvath) after just one year of treatment with the growth hormone to produce immune rejuvenation (by restoring the thymus gland tissue which degenerates with age). However, potential side effects of such an invasive treatment had to be taken into consideration. The fact that the growth hormone can also promote diabetes made it necessary to add a cocktail of two widely used anti-diabetic drugs, dehydroepiandrosterone (DHEA) and metformin.

In conclusion, whilst epigenetic age does not measure all characteristics of ageing and is not a proxy for ageing itself, the use of epigenetic clocks seems to be a valid strategy to assess the effectiveness of putative anti-ageing treatments, based on environmental control. However, the results obtained so far need to be verified in controlled, large-scale and long-term clinical trials. The small sample size and its homogeneity for critical factors such as age, gender and ethnicity, limits the statistical power of the studies undertaken to date and do not allow for stratified statistical analyses to test additional hypotheses. Longer follow-ups will produce a better assessment of the perspective of a realistic and more general improvement in ageing, a perspective that for now remains more of a promise than a reality.

Human rejuvenation with Yamanaka factors or the “Elixir” plasma treatment must successfully overcome many regulatory obstacles and may require years of testing. Plasma dilution can in principle be done very early on in existing blood treatment facilities, but only on the condition that the procedure can be proved to be safe.

Modification of environmental factors is apparently less effective, but possible new combinations of factors could be investigated together with time periods of particular susceptibility. Safety issues are likely to be limited although some epigenetically active dietary supplements may cause adverse outcomes, e. g. folic acid (Patel and Sobczyńska-Malefora, 2017).

7. Conclusions and prospects

Our understanding of DNA methylation in ageing is proceeding very rapidly, aided by the widespread application of genome-wide techniques and the discovery of epigenetic clocks.

Epigenetic age is emerging as a useful risk metric that incorporates both genetic and environmental experiences. However, in addition to providing data to inform prediction, it is expected that the clocks may pave the way for gaining new aetiological insights. Investigating the regulators and drivers of age-related methylation changes and the mechanisms behind the clock could reveal ageing-related genes and epigenetically deregulated pathways, which may clarify the biology of ageing and its pathological correlations.

Furthermore, the identification of the molecular mechanisms of epigenetic ageing could provide new and more effective targets for intervention. At the moment, the methylation resetting observed in intervention studies is more a confirmation of a change at a molecular level than a direct target of the strategy itself.

The majority of current studies on the relationship between methylation, epigenetic clocks and ageing are correlative. Therefore, the possibility remains open that the methylation changes seen with age and ageing phenotypes do not actually drive these very same phenotypes, but simply carry out the job of third-party mechanisms.

In addition, methylation measurements were mainly performed on tissues. The clock measures tissue ageing and may be susceptible to changes in cellular composition. Single-cell analyses might be needed to understand how changes in individual genomes feed into the epigenetic age of the whole tissue and how this translates into phenotypic changes of cells and of the tissue of which they are a part. An enlightening example of this approach is observed in the results of Hernando-Herraez et al. (2019), showing that the DNA methylation drift causes a transcriptional drift that increases in murine muscle tissue stem cells as a consequence of ageing, leading to a progressive decline in muscle regeneration.

Finally, since DNA methylation is just one component of the epigenetic regulatory system, the observed age-related changes might simply represent the work of other genomic control mechanisms, such as histone modifications (Ciccarone et al., 2018). Furthermore, the discovery of 5hmC and active DNA demethylation mechanisms could contribute to the intricate landscape of epigenetic events occurring during ageing (López et al., 2017). In this context, the fact that the techniques used in DNA methylation ageing studies generally do not discriminate between 5mC and 5hmC hinders detailed understanding of the function of 5mC and the contribution of 5hmC to ageing.

Therefore, caution is needed in the use of interventions that have an impact on epigenetics, especially for strategies that affect cell differentiation or employ methylation-modifying compounds/nutrients. This is because epigenetic processes and changes are potentially all-pervasive. To be successful, epigenetic treatments need to be more selective towards the molecular target and the tissue/cells involved. Otherwise, the treatments could cause the same disorders they are trying to counteract, e.g. cancer.

Finally, establishing the remediation of DNA methylation profiles of ageing as surrogate endpoints for geroscience, will require trials that include long-term follow-ups in order to establish the impact of the intervention on the primary endpoints of healthy ageing and on its safety.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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