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# The methylation of nuclear and mitochondrial DNA in ageing phenotypes and longevity

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**Article highlights**

1. Epigenetic events contribute to the complexity of ageing process.
2. A global decline of genomic DNA methylation as well as nuclear gene-specific hypermethylation and hypomethylation events (epigenetic clocks) occur during ageing.
3. Stochastic or environmental factors contribute to the intra-individual and inter-individual DNA methylation heterogeneity at certain CpG sites (epigenetic drift).
4. Mitochondrial DNA methylation changes involving both regulatory and coding regions are emerging to play a role in ageing.

**Abstract**

An increasing body of data is progressively indicating that the comprehension of the epigenetic landscape, actively integrated with the genetic elements, is crucial to delineate the molecular basis of the inter-individual complexity of ageing process. Indeed, it has emerged that DNA methylation changes occur during ageing, consisting mainly in a progressive process of genome demethylation, in a hypermethylation of gene-specific CpG dinucleotides, as well as in an inter-individual divergence of the epigenome due to stochastic events and environmental exposures throughout life, namely as epigenetic drift.

Additionally, it has also come to light an implication of the mitochondrial genome in the regulation of the intracellular epigenetic landscape, as demonstrated by the being itself object of epigenetic modifications.

An overview of DNA methylation changes occurring during ageing process at both nuclear and mitochondrial level will be described in this review, also taking into account the recent and promising data available on the 5-hydroxymethylcytosine.

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**Keywords**: nuclear DNA methylation, mitochondrial DNA methylation, epigenetic clock, epigenetic drift, centenarians, ageing, longevity, ageing phenotypes

## Introduction

The study of ageing and longevity in humans is a complex task, since the quality of the ageing process and the probability to attain longevity are the result of the interaction between the individual genetic background and the lifelong exposure to chronic and acute stressors.

Owing to the high number of variables that are involved in the ageing process, the understanding of the mechanisms that determine the healthy versus unhealthy ageing is extremely difficult. Despite this complexity, in the seminal manuscript "Geroscience: Linking Ageing to Chronic Disease" (Kennedy, 2014) some of the most eminent scientists active in the field of ageing were able to reach a general consensus upon seven pivotal mechanisms (pillars), highly interconnected, that are central in determining the fate of ageing: mitochondrial metabolism, macromolecular damage, stem cells regeneration, proteostasis, adaptation to stress, inflammation and, last but not least, epigenetics.

Among the epigenetic modifications, the study of DNA methylation changes that occur during physiological and pathological age has increasingly attracted researchers' interest. The first reports in this sense date back to the 1990s, when gene-targeted approaches were used to investigate age-associated variations in specific tissues (Ahuja et al., 1998; Choi et al., 1996; Issa et al., 1996, 1994). Starting from 2006, the advent of microarray technologies for the study of DNA methylation has boosted the research in this field, both in terms of genomic coverage and of number of samples analyzed (Bibikova et al., 2006).

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In the last five years, multiple evidence about the presence of epigenetic marks, in terms of methylation and hydroxymethylation, have gradually emerged to also occur into mitochondrial DNA within D-loop and coding genes (Shock et al., 2011; Bellizzi et al., 2013; Ghosh et al., 2014; D'Aquila et al., 2015). It follows that, as for the epigenetic changes at nuclear level, also the mitochondrial epigenetic signature is being evaluated according to ubiquitous factors and correlated to peculiar phenotypes, including ageing and several diseases. As it is so often the case, the noteworthy amount of data generated by these analyses has provided some important hints on the epigenetic landscape of human ageing, but has also raised many questions that still demand to be addressed.

**Nuclear DNA methylation and hydroxymethylation in ageing**

Existing data on several human tissues support the view that during ageing three phenomena coexist: 1) a global decline of genomic DNA methylation; 2) systematic hypermethylation and hypomethylation events that involve specific genomic regions, referred as **age-Differentially Methylated Regions (a-DMRs)**; 3) an increase in intra-individual and inter-individual DNA methylation heterogeneity at certain CpG sites, referred as epigenetic drift (Bacalini et al., 2015, Zampieri et al., 2015, Jones et al., 2015).

Global decrease in the genomic content of DNA methylation was described in multiple human tissues during ageing and it is **due** to loss of methylation at repetitive tandem and interspersed elements (Jintaridth and Mutirangura, 2010; Richardson, 2003). Age-associated genomic hypomethylation can significantly impair genomic stability by promoting the activation of transposable elements (Alexeeff et al., 2013; De Cecco et al., 2013). In addition, Yuan et al. (2012) found that large megabase-scale hypomethylation blocks occur in blood during ageing and that they significantly overlap with those observed in cancer, confirming an important link between the two processes (Yuan et al., 2015). Also

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in this case, hypomethylation blocks that occur during ageing can have an impact on the general structure of chromatin, as they tend to be associated with nuclear lamina-associated domains (Kelly et al., 2012).

In epigenome-wide association studies (EWAS) on ageing, regression analyses have been extensively used to identify the epigenetic clocks, that is those loci that are reproducibly associated with age across individuals (Jones et al., 2015). Several tissues were investigated, including blood, peripheral blood mononuclear cells (PBMCs), monocytes, T-cells, adipose tissue, saliva, breast, prefrontal cortex, skin, liver, kidney and muscle. These analyses identified tissue-specific age-Differentially Methylated Regions (DMRs), frequently located outside CpG islands, but highlighted also the existence of several concordant results between different tissues (Day et al., 2013). For example, the CpG island of *ELOVL2* gene, initially identified in blood (Garagnani et al., 2012), is an omnipresent result in the top ranking age-DMRs in almost all EWAS studies (Bacalini et al., 2015, Bacalini et al., 2016, Ronn et al., 2015, Steegenga et al., 2014, Reynolds et al., 2014, Giuliani et al., 2016). With a methylation status that in blood systematically increases with age from 0 to 100 years this locus appears a real swiss-clock in most of the tested tissues, and a replication-dependent process has been proposed to underlie the age-associated hypermethylation (Bacalini et al., 2016, Bacalini et al., 2015; Garagnani et al., 2012).

Prompted by the identification of so reproducible age-DMRs, researchers have attempted to investigate possible functional effects of these DNA methylation changes that can justify their association with the biological age of an individual. However, the effective contribution of age-DMRs to the ageing phenotype is still an enigma. In most cases age-DMRs resulted not associated with changes in expression of the corresponding genes (Steegenga et al., 2014; Yuan et al., 2015). Yuan et al. (2015) noted that in blood age-associated hypermethylation preferentially occurs at genes that are not expressed, while

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hypomethylation tends to involve genes that are expressed in this tissue. This observation suggests that age-associated changes in DNA methylation tend to stabilize baseline patterns of transcription and it can explain why age-DMRs are not associated to large alterations in gene expression (Yuan et al., 2015). On the other side, as age-DMRs are highly reproducible among different tissues, it is unlikely that they are affected by alterations in the tissue microenvironment that can occur during ageing (Weidner et al., 2014). We cannot exclude that a substantial fraction of age-DMRs is a “by-product” of the ageing process itself, for example a track of cell divisions that entail the accumulation of epigenetic mutations at permissive locations (Reynolds et al., 2014). However, some important exceptions exist. Recently methylomic and gene expression profiles on monocytes were evaluated in a very large cohort including 1224 individuals, and few age-DMRs that are associated with changes in gene expression during ageing were identified (Liu et al., 2013). In an another study, age-related hypermethylation of BDNF and SST, two genes implicated in several brain diseases, was associated to a decrease in their expression in orbital frontal cortex (McKinney et al., 2015).

Finally, epigenetic drift is an important component of DNA methylation remodeling during human ageing, although less easy to detect than directional changes. Epigenetic drift accounts for those variations in the epigenetic patterns that are not shared by individuals because they are stochastic or driven by specific environmental cues. The impact of epigenetic drift during ageing is particularly evident when methylomic profiles of twins are compared (Fraga et al., 2005; Heijmans et al., 2007; Martino et al., 2013; Pirazzini et al., 2012) and when longitudinal studies are performed (Bjornsson et al., 2008; Talens et al., 2012). These studies have shown that some regions of the genome are more prone to epigenetic drift than others and that the ability to maintain methylomic signatures is influenced by the genetic background of the individual (Bjornsson et al., 2008).

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Accordingly, in a very recent paper Slieker et al. (2016) identified several thousands of Age-related Variably Methylated Positions (aVMPs) that show an increased variability with ageing and that are associated in trans with the expression of genes involved in DNA damage and apoptosis (Slieker et al., 2016). Moreover, Gentilini et al. (2015) demonstrated that the number of stochastic epigenetic mutations, that is changes in DNA methylation levels that are subject-specific and not shared by the rest of the population, tend to increase exponentially during ageing (Gentilini et al. 2015).

In most of the studies that we have described so far, the cohorts analyses for age-associated changes in DNA methylation included subjects with an age range from 20-30 to 80-90 years. Data on the epigenetic determinants of longevity and of extreme longevity (semi-supercentenarians: subjects who reached an age of 105-109 years, and supercentenarians: subjects who reached an age of 110 years) are still sparse, although they are of great interest to disentangle the basis of healthy ageing.

In the first study on the epigenome of centenarian subjects, Gentilini et al. analysed a cohort including blood samples from 21 female centenarians, their female offspring and unrelated female controls and demonstrated that age-associated decrease in global DNA methylation was delayed in centenarians' offspring, suggesting that the maintenance of the DNA methylation machinery can contribute to longevity (Gentilini et al., 2012). Xiao et al. used the methyl-DNA immunoprecipitation sequencing approach to characterize the whole-blood methylome of 4 Chinese female centenarians and 4 middle-aged controls (Xiao et al., 2016). According to gene ontology analysis, the identified DMRs were enriched in pathways associated to age-related diseases, like type-2 diabetes, cardiovascular disease and Alzheimer's disease, leading the authors to conclude that an epigenetic-mediated suppression of disease-related pathways contributes to a long-lived phenotype. More recently, whole blood methylomic predictors of old-age mortality were identified in a

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cohort a 111 nonagenarians. Interestingly, the identified CpGs were enriched in genes involved in the NF-κB pathway, supporting its role in the regulation of mammalian lifespan (Jylhava et al., 2016). It is worth to be noted that these studies share a common drawback: because, by definition, long-lived subjects do not have age-matched controls, it is difficult to distinguish age-related from longevity-specific DNA methylation patterns. In this sense the use of nonagenarians and centenarians offspring, that are a recognized model of healthy ageing, could be advantageous to identify epigenetic markers of human longevity (Bucci et al., 2016, Westendorp et al., 2009).

Finally, special attention has to be dedicated to the recent boost of studies analyzing the association between DNA methylation age (DNAmAge), estimated using the so called epigenetic clocks, and ageing phenotypes. With the term of epigenetic clock we refer to a mathematical model that, on the basis of the DNA methylation level of specific CpG sites, returns the estimated age of a subject. The first, and most successful, of the currently available epigenetic clocks was developed by Horvath, includes 353 CpG sites and performs well in most tissues in predicting the age of an individual (Horvath, 2013). Two additional age predictors, based on 71 CpG sites (Hannum et al., 2012) and just 3 CpG sites (Weidner et al., 2014) were further developed specifically for whole blood. Epigenetic clocks, in particular Horvath's clock and, to less extent, Hannum's clock, have proven to be informative not only of the chronological age of an individual, but also of his/her biological age, that is, of his/her health status in terms of physical and cognitive fitness (Horvath et al., 2014; Marioni et al., 2015). Age acceleration (that is, an estimated DNAmAge higher than chronological age) was concordantly (and impressively) registered for several age-related **conditions**, including Down syndrome (Horvath et al., 2015), Alzheimer's (Levine et al., 2015) and Parkinson's (Horvath et al., 2015) diseases, HIV-infection (Boulias et al., 2016, Rickabaugh et al., 2015) HIV-associated neurocognitive disorders (Levine et al., 2016),

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frailty (Breitling et al., 2016), liver obesity (Horvath et al., 2014), **menopause (Levine et al., 2016)** and cancer (Levine et al., 2015, Zheng et al., 2016). In addition, four studies reported an association between epigenetic age and mortality (Marioni et al., 2015, **Chen et al., 2016,** Christiansen et al., 2016, Perna et al., 2016). What about epigenetic clocks and **exceptional longevity**? To our knowledge, only one study has evaluated this topic so far (Horvath et al., 2015). Authors estimated DNAmAge in peripheral blood mononuclear cells from 82 semi-supercentenarians (mean age: 105.6 ± 1.6 years), 63 semi-supercentenarians' offspring and 47 controls age-matched with the offspring population. Semi-supercentenarians resulted 8.6 years younger than expected and, even more interestingly, their offspring had a DNAmAge in average 5.1 years than age-matched controls, sustaining the existence of epigenetic determinants of human longevity.

Furthermore, recent findings have shown that the process of 5-mC oxidation catalyzed by the TET family of methylcytosine dioxygenases leads to the 5-hydroxymethylcytosine (5-hmC), namely “the sixth base” (Tahiliani et al., 2009; Kriaucionis and Heintz, 2009). The dynamic distribution of this new mark, with a significant tissue-specificity that is in very high brain but almost scarce in liver, and the association with euchromatin and gene promoters and enhancers, is progressively outlying its involvement in the regulation of gene expression thus providing a new piece in the epigenetic modifications puzzle (Munzel et al., 2011; Jin et al., 2011; Szulwach et al., 2011a, b).

Only few data are available to date on the 5-hmC levels in ageing and almost all come out from studies on brain tissue. Chouliaras et al., by searching for changes of 5-hmC in the mouse hippocampus, observed an age-related increases in levels of 5- methylcytidine (5-mC) that was prevented by calorie restriction (CR), thus suggesting the geroprotective effects of CR may be exerted via epigenetic mechanisms such as methylation and hydroxymethylation of DNA (Chouliaras et al., 2012). In addition, the designation of the

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first genome-wide maps of 5-hmC in mouse cerebellum and hippocampus at postnatal day 7, 6 weeks and 1 year of age, revealed a progressive rise of 5-hmC in short interspersed nuclear element (SINE) and long tandem repeat (LTR) either during neurodevelopment, either with age in cerebellum. The depletion of 5-hmC on the X chromosome was observed in both males and females at all ages, with the sole exception of *Xist* and *Utx* genes characterized by 5-hmC enrichment in females. Consistent 5-hmC features were found in human cerebellum (Szulwach et al., 2011b). Furthermore, age-specific differentially hydroxymethylated regions (DhMRs) were found preferentially located within candidate genes for fragile X mental retardation protein (FMRP) and autism, thus suggesting that abnormal alteration of 5-hmC may contribute to the onset of neuro-developmental disorders (Wang et al., 2012). Likewise, 5-hmC levels were higher in the hippocampus of old than in young mice and in selected DNA sequences of the mouse *5-LOX* gene, a known target of ageing (Chen et al., 2012). Studies carried out on human post-mortem brain tissue samples of different age revealed an age-related raise of about 50% and 200% of 5-hmC levels in the cortex and in the white matter, respectively (Kraus et al., 2015). Conversely, a significant decrease with ageing of the global 5-hmC amount was more recently documented in human blood cells from healthy donors, in association with increased levels of 5-carboxylcytosine (5-caC) and partly ascribed to acquired mutations in *TET2* gene (Valentini et al., 2016; Buscarlet et al., 2016).

**Mitochondrial DNA methylation and hydroxymethylation and ageing**

Although it has been debated for a long time, and finally accepted only recently (Shock et al., 2011; Bellizzi et al., 2013; Hong et al., 2013; Gosgh et al., 2014; D'Aquila et al., 2015; Liu et al., 2016) the methylation of mitochondrial DNA (mtDNA) has been observed since 1973 (Nass, 1973; Cummings et al., 1974; Dawid, 1974; Groot and Kroon, 1979;

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Shmookler Reis and Goldstein, 1983, Pollack et al., 1984). In the last five years, with the development of more innovative and sensitive techniques, multiple evidences about mtDNA methylation and hydroxymethylation have gradually emerged so as to coin the term “mitoepigenetics” that indicates the complex bidirectional interaction between the nuclear and mitochondrial genomes relatively to epigenetic landscape (Manev and Dzitoyeva, 2013; Ghosh et al., 2015; van der Wijst and Rots, 2015). As for the epigenetic changes at nuclear level, also mitochondrial epigenetic signature is influenced by ubiquitous factors (airbone pollutants, metal-rich particulates, drugs, diet) and correlates to peculiar phenotypes, including ageing, and several diseases, including Down syndrome, amyotrophic lateral sclerosis, Alzheimer and Parkinson diseases, cardiovascular diseases, nonalcoholic fatty liver disease, polycystic ovarian syndrome and cancer (Infantino et al., 2011; Chestnut et al., 2011; Pirola et al., 2013; Byun et al., 2013; Baccarelli and Byun 2015; Blanch et al., 2016; Jia et al., 2016; Byun et al., 2016; Liao et al., 2016).

The first evidence about an association between mitochondrial epigenetic marks and ageing was provided in 1983, with the observation that, in cultured fibroblasts, methylation of mtDNA genomes decreases with culture age (Shmookler Reis and Goldstein, 1983). More recently, Dzitoyeva et al., (2012) by analyzing brain samples from differently-aged mice, observed that, during ageing, the 5-hydroxymethylcytosine levels decreased in the frontal cortex and not in the cerebellum, although no ageing-associated changes in TET mRNAs were found. The authors also reported an increase in the expression of selected mtDNA-encoded genes in the frontal cortex, that could be ascribed to the 5-hydroxymethylcytosine increase since the 5-methylcytosine levels remained unchanged during ageing. Conversely, no changes of the 5-hydroxymethylcytosine status as well of mRNA changes of mtDNA-encoded genes have been found in the cerebellum, despite an increase of TET2 and TET3 expression. Thus, it seems that progressive changes in these mitochondrial epigenetic marks

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occur during lifespan in a region-specific manner (Dzitoyeva et al., 2012). In addition, an age-dependent modulation of mtDNMT1 expression was reported in the brain (Dzitoyeva et al., 2012). Moreover, D'Aquila et al. (2015), by analyzing human mitochondrial genes encoding for 12S (*MT-RNR1*) and 16S (*MT-RNR2*) ribosomal RNA, revealed the occurrence of methylation at a CpG site of *MT-RNR1*. High methylation levels of the above site (>10%) were more frequent in old women with respect to younger controls (D'Aquila et al., 2015). Based on differential methylation within this gene, a 9-year long follow-up survey showed that subjects with high methylation levels exhibit a mortality risk significantly higher than subjects with low levels suggesting a still unclear functional role for *MT-RNR1* methylation (D'Aquila et al., 2015). What is more, Mawlood et al. (2016) by evaluating the methylation levels of 133 CpG sites in the mitochondrial genome by Illumina sequencing, further confirmed the role of MT-RNR1 methylation in ageing, although they showed a negative correlation between two *MT-RNR1* CpG sites and ageing (Mawlood et al., 2016). The two contradictory results about the correlation between *MT-RNR1* methylation and ageing could be explained by the fact that this correlation could be site-specific and strongly influenced by gender, environmental factors, nutrition and drugs, as also demonstrated for age-related nuclear epigenetic changes (Byun et al., 2013; Chen et al., 2012; Terry et al., 2011; Delgado-Cruzata et al., 2014).

The functional role of methylation and hydroxymethylation of the mitochondrial genome remains still poorly elucidated. Their arrangement in some genes and in peculiar regions, such as promoter regions and conserved sequence blocks, their occurrence in non-CpG sites, the low levels of mtDNA methylation observed in some analyses, the methylation limited to the L-strand might imply a functionality of both epigenetics processes. Asymmetrical effects on the expression of mtDNA in mtDNMT1 over-expressed cells were reported, with increased levels of ND1 (encoded by the H strand) and decreased levels of

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ND6 (encoded by the L strand) (Shock et al., 2011). An inverse correlation between mt-ND2 and mt-ND6 expression was also observed in pathological phenotypes (Pirola et al., 2013, Feng et al., 2012). Van der Wijst and Rots suggested that mtDNA methylation regulates the binding of TFAM and, consequently, its activity, to mtDNA either directly or indirectly by acting on proteins that post-translationally modify TFAM and, thus, modulate its affinity for DNA. This regulation could result in an increased DNA compaction and in a reduced accessibility for POLRMT and TFB2B factors therefore inducing mitochondrial biogenesis rather that electron transport subunit transcription (Van der Wijst and Rots, 2015). Other hypothesis suggest that mtDNA methylation could be involved in the processing of mitochondrial polycistronic primary transcript (Iacobazzi et al., 2013, Cotney et al., 2009; McCulloch et al., 2002; Davenport et al., 1976).

**Conclusions**

A massive amount of studies have showed that profound changes in DNA methylation and hydroxymethylation patterns, both at nuclear and mitochondrial level, occur during human ageing. Nevertheless, our knowledge about the intimate connection between epigenetic changes and ageing is still at the beginning. For example, in many cases is still not clear if epigenetic changes are a "readout" of the aged phenotype, or if on the contrary they play an active role in driving this complex process. At the same time, the relationship between nuclear and mitochondrial changes in DNA methylation/hydroxymethylation has not been exhaustively investigated yet, although they certainly exist as suggested by the effect of mtDNA variability on nuclear epigenetic profile (Bellizzi et al., 2012). Further studies are urgently need to uncover the role of epigenetics in human ageing and longevity, with the ultimate aim of identifying drugs and interventions that, by modulating DNA methylation and hydroxymethylation patterns, could foster healthy ageing worldwide.

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