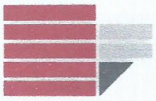
UNIVERSITÀ DELLA CALABRIA



UNIVERSITA' DELLA CALABRIA

Dipartimento di Biologia, Ecologia e Scienze della Terra

Dottorato di Ricerca in

SCIENZE DELLA VITA

CICLO

XXXI

DNA METHYLATION PATTERNS IN AGING AND AGING PHENOTYPES

Settore Scientifico Disciplinare BIO/18

Coordinatore: Ch.mo Prof. Maria Carmela Cerra

Firma _____

Supervisore/Tutor: Ch.mo Prof. Dina Bellizzi

Firma _____

Dottorando: Dott. Francesco Guarasci

Firma _____

Index

Summary 2

Chapter I.

General Introduction 4

Chapter II.

Original Research Work:
Epigenetic signature: implications for mitochondrial quality control in human aging 29

Conclusive Remarks 66

Appendix.

Published Works 68

Summary

During my PhD program, my work has been addressed to the study of the role of epigenetic modifications in aging and in age-related phenotypes.

Epigenetics is the study of changes in gene expression that do not involve changes to the underlying DNA sequence. These changes affect cellular phenotypic expression by regulating relative gene expression levels. They are a common and natural process in living cells and are tightly controlled by pre-programmed mechanisms. Epigenetics modifications can be influenced by multiple factors including environmental conditions, lifestyle, nutrition, use of drugs, disease state and age.

Patterns of DNA methylation, the best known and characterized epigenetic modification, change during aging; indeed, with increasing aging, genome-wide methylation levels decrease, meanwhile genomic regions, including CpG islands, become more methylated. Analyses of the above patterns provided new perspectives for establishing powerful biomarkers of human aging which have the potential to generate accurate prediction not only of the chronological but also of the biological age.

The first section of the PhD thesis consists in a comprehensive overview of the general features of DNA methylation and its implication in age and age-related diseases. The topic is addressed referring to the methylation patterns established not only at nuclear but also at mitochondrial genome level. In addition, the influence of a number of environmental factors on the above patterns is also discussed.

In the second section, an original research work, carried out in order to identify novel biomarkers of aging, is reported. In this work, methylation status of nuclear genes involved in mitochondrial fusion, fission, biogenesis and mitophagy, fundamental components of the mitochondrial quality control process, was investigated in subjects of different ages of the Calabrian population. The methylation levels of *RAB32* and *RHOT2* genes were significantly associated with age and, in particular, those of *RAB32* even with the risk of developing disability. The study, therefore, led to the identification of two new biomarkers for both chronological and biological aging.

In the Appendix, research works already published are reported. The first one concerns the correlation between DNA methylation and nutrition during lifetime. Global DNA methylation profiles were examined in different tissues of rats of different ages, fed with a standard and hypocaloric diet, and their association with aging and nutrition was evaluated. The results obtained have shown that tissue-specific variations in methylation levels occur during aging and that nutrition influences the state of global DNA methylation during the course of life. The hypocaloric diet seems to influence more strongly the epigenetic status of the offspring when administered during the maternal pre-gestational period compared to the gestation and lactation period. Therefore, changes in the global DNA methylation status represent an epigenetic mechanism by which age and nutrition intersect each other

and, in turn, influence the plasticity of aging. The second one is a review on the current advances in mitochondrial epigenetics studies and the increasing indication of mtDNA methylation status as an attractive biomarker for peculiar physiological and pathological phenotypes. It comes from the increasing evidence on the fact that, similarly to nuclear DNA, also mtDNA is subject to methylation and hydroxymethylation and these modifications are influenced by multiple environmental factors.

Chapter I.
General Introduction

The term epigenetics was introduced by the British embryologist and developmental biologist Conrad Waddington in the early 1940 to explain unclear features of development. He defined epigenetics as ‘‘the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being’’. Waddington was attracted in the interaction between environmental stimuli and genotype during development and proposed the concept of “epigenetic landscape” (Waddington 1942 and 1957; Goldberg et al., 2007; Allis and Jenuwein, 2016; Pinel et al., 2017).

Over the following decades, the meaning of the word epigenetics has changed and several definitions were formulated but, currently, it refers to mitotically or meiotically heritable phenotypic changes which are not derived from underlying DNA sequence change. Therefore, epigenetics indicates several changes influencing gene expression that are not wrote in the genome but can be inherited (Rakyan and Beck, 2006; Allis and Jenuwein, 2016). These changes are carried out by different mechanisms including DNA methylation, histone modifications and non-coding RNA (ncRNA) which are involved in gene transcription control.

Epigenetic patterns are established at pre-conceptional and gestational level but they undergo variations during life starting from intrauterine environment in response to internal, environmental and stochastic factors (Rakyan and Beck, 2006; Whitelaw and Whitelaw, 2006; Fraga, 2009; D’Aquila et al., 2013; Kanherkar et al., 2014; Meloni and Testa, 2014; Pal and Tyler, 2016). Precisely this flexibility makes the epigenome the means for the organism to adapt in response to different stimuli such as nutrition, seasonal changes, psychological state, social interactions, therapeutic drugs, physical exercise (Kanherkar et al., 2014). Therefore, epigenetic changes are often considered as bridge between genome and environment in the definition of phenotype (Norouzitallab et al., 2018).

In the last decade, many evidences have suggested that aging, which is deeply influenced by genetics, environment and by their interaction may be influenced by (and at the same time influences) epigenetics.

Here, DNA methylation and its involvement in aging and age related phenotypes are reviewed.

DNA methylation

DNA methylation represents the most prevalent epigenetic modification in all kingdoms of life and consists in a covalent transfer of methyl group to the aromatic ring of the DNA nitrogenous base (Barros et al., 2009; Illingworth and Bird, 2009; Kanherkar et al., 2014).

The C5-methylcytosine (5-mC) is the canonical methylated base in eukaryotes, the N6-methyladenosine (m6A) is the dominant modification in bacteria, meanwhile the N4-methylcytosine (4-mC) is very common in bacteria but absent in mammals. Albeit it has also been hypothesized the presence of 6mA in eukaryotic genomes, its minimal levels are detectable only by highly sensitive methods (Schübeler, 2015; Luo et al., 2015; Sánchez-Romero et al., 2015; Luo et al., 2016; Wu et al., 2016; Zhu et al., 2018).

In vertebrates, methylation mostly occurs at the cytosines followed by guanine residues (CpG methylation), although recent data report the presence of methylation in embryonic stem cells and neurons at sites other than CpGs (non-CpG methylation), mainly in CpA context, likely regulating cell type-specific functions (Lister et al., 2009; Patil et al., 2014; Pinney, 2014).

Notably, methylated CpGs are predominantly located into intergenic and intronic CpG-poor regions and repetitive sequences, such as interspersed and tandem repeats, most of which derived from transposable elements. Unmethylated CpG dinucleotides are, instead, concentrated in CpG-rich regions, termed CpG islands (CGIs), which are, on average, 1000 base pairs long and show an elevated G+C base composition and little CpG depletion (Gardiner-Garden and Frommer, 1987; Goldberg et al., 2007; Deaton and Bird, 2011; Moore et al., 2013). Approximately, CpG islands have been demonstrated to be associated with 70% of the annotated gene promoters, including all housekeeping genes, a number of tissue-specific genes and developmental regulator genes (Larsen et al., 1992; Saxonov et al., 2006; Zhu et al., 2008; Maunakea et al., 2010; Jones, 2012; Moore et al., 2013; Norouzitallab et al., 2018).

Methylation patterns come from the activity of enzymes belonging to the family of DNA methyltransferases (DNMTs), which transfer a methyl group from S-adenosyl-L-methionine (SAM) to deoxy-cytosine. In particular, DNMT1 is involved in the maintenance of the DNA methylation during cell division by acting on hemi-methylated CpG sequences, and DNMT3a and 3b are both responsible of the de novo establishment of DNA methylation (Okano et al., 1999; Dan and Chen, 2016; Lyko, 2018).

The dynamic regulation of the genome is determined by the balance between events of DNA methylation and demethylation (Figure 1). The latter process includes both the loss of 5mC during the replication (passive demethylation), induced by down-regulation of DNMT enzymes, inhibition of their activity or decreased levels of SAM, and the active removal of 5-mC (active demethylation) resulting in the formation of 5-hydroxymethylcytosine (5-hmC), considered to date the sixth base of DNA and a novel epigenetic mark (Branco et al., 2011; Guo et al., 2014; Saitou et al., 2012; Sadakierska-Chudy et al., 2015). Discovered for the first time in mouse and frog brain by Penn et al., recently the presence of 5-hmC has been reported in different tissues and cells and considered as an

intermediate of the oxidation of 5-mC by Ten-eleven translocation (TET)-family of methyl-cytosine dioxygenases (Hotchkiss, 1948; Griffith and Mahler, 1969; Penn et al., 1972; Naveh-Many et al., 1981; Waechter and Baserga, 1982; Tahiliani et al., 2009; Koh, 2011; Wu et al., 2017).

The earliest observations of the DNA methylation function date back to transfection experiments and microinjections of methylated sequences demonstrating that it induces gene silencing and that, in cultured cell lines, silent genes, can be activated following treatment with the demethylating agent 5-azacytidine (Jones 1985a and 1985b; Keshet et al., 1985; Yisraeli et al., 1988; Kass et al., 1993; Yan et al., 2014; Seelan et al., 2018).

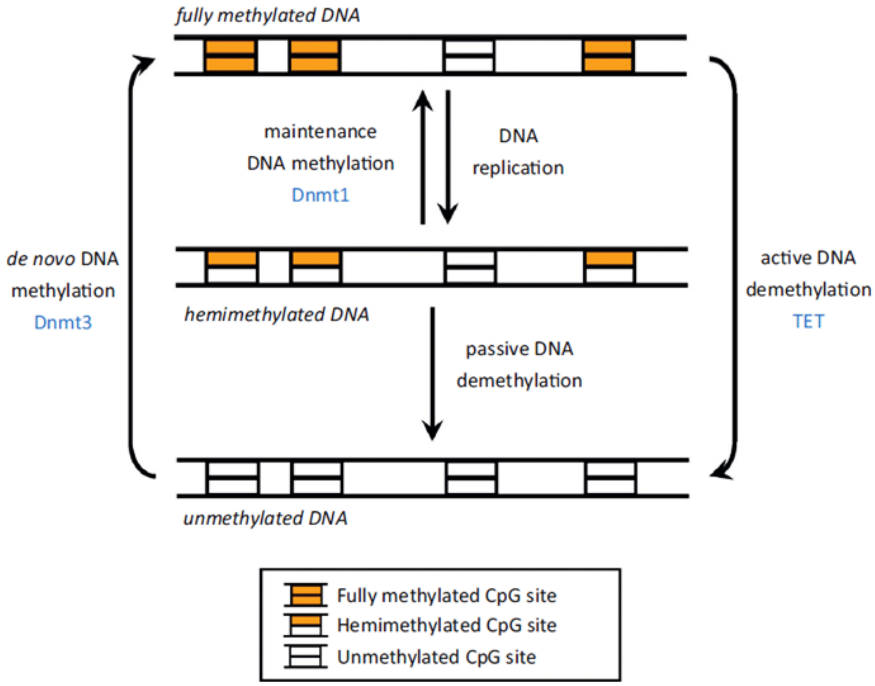


Figure 1. DNA methylation model. DNMT1 carries out its action during cell replication performing the so-called maintenance DNA methylation: the enzyme acts on hemimethylates CpG sites to restore DNA methylation status on the newly formed strand after cell division by copying pre-existing methylation patterns. On the other hand, DNMT3A and DNMT3B are de novo methyltransferases and function on unmethylated DNA by introducing methylation in only one of the two DNA strands at most CpG sites, generating the ideal hemimethylated sites for DNMT1. The active demethylation is performed by TETs (Jeltsch and Jurkowska, 2014).

Genome-wide studies of the methylome have highlighted that methylation patterns are cell-type specific and their effects are influenced by the position of methylated cytosines: if located adjacent to transcription factor binding sites, they block initiation, through either recruiting specific factors acting as gene expression repressors or by inhibiting the binding of activators, meanwhile in body gene they may either stimulate transcription elongation, thus hypothesizing a their role on splicing, or impede the alternative promoters activation (Watt and Molloy, 1988; Boyes and Bird, 1991; Singal and Ginder, 1999; Clouaire and Stancheva, 2008; Sasai and Defossez, 2009; Jones, 2012; Yin et al., 2017). Methylation in repeat regions such as centromeres is important for chromosomal stability, for

example chromosome segregation at mitosis, and is also likely involved in the suppression of the expression of transposable elements and thus to have a role in genome stability. Recently, the role of methylation in altering the activities of enhancers, insulators and other regulatory regions has been described. CpG islands methylation of the transcription start sites is associated with long-term silencing, see chromosome X inactivation, imprinting, genes expressed predominantly in germ cells and some tissue-specific genes (Moore et al., 2013; Huang et al., 2014; Allis and Jenuwein, 2016).
Recently, a more complex epigenetic landscape is emerging, as demonstrated by the role played by the mitochondrial genome (mtDNA) in regulating intracellular DNA methylation as well as by the evidence reporting that, similarly to nuclear genome, also mtDNA is subject to CpG and non-CpG methylation and hydroxymethylation. Although the first attempt to identify traces of methylation within mtDNA dates back to the early 1970, for many years the epigenetic modification of mtDNA was controversial. Only recently, the advent of more innovative and sensitive techniques has allowed the discovery of DNMTs members in mitochondrial protein fractions and unequivocally identified the presence of methylation within the mitochondrial control region (D-loop) and some genes (ND1, ND2, ND6, Cytb, COI, 12SRNA, 16SRNA). Several hypothesis have been formulated to explain the functional role of mtDNA methylation, including the processing of mitochondrial polycistronic primary transcript and the regulation of the affinity of TFAM binding (Bellizzi et al., 2013)

DNA methylation and aging

Aging is a slow and gradual decline process of functional abilities that makes individuals more susceptible to environmental phenomena and diseases, and leads to a reduction in the probability of survival and finally to death (Johnson et al., 1999; Kirkwood, 2005; Sebastiani et al., 2012).
Aging affects all living organisms but lifespan is characteristic of each species. Moreover, among the various populations and within them there is considerable variability as regards the way and the quality of aging. This heterogeneity has largely been described as resulting from a complex interaction among genetics, environmental e stochastic factors and, more recently, epigenetic alterations have been included (Montesanto et al., 2012; D'Aquila et al., 2013). These alterations, by regulating gene expression, influence not only most of hallmarks of aging (genomic instability, telomere attrition, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication) but, at the same time, themselves because they are subjected to dynamic changes during lifetime, a phenomenon described as epigenetic drift (López-Otín et al., 2013; Li and Tollefsbol, 2016; Pal and Tyler, 2016). During

early embryogenesis, genomic DNA undergoes reprogramming processes including genome wide demethylation and *de novo* methylation leading to the re-establishment of DNA methylation patterns in the progeny that will be maintained in the somatic cells throughout the lifespan. After birth, although global DNA methylation patterns are quite stable, stochastic and environmental stimuli (ROS, inflammation, diet, stress, trauma) as well as the failure of the epigenetic machinery may induce random changes at certain loci, leading to a loss of phenotypic plasticity among individuals (Jones et al., 2015; Zampieri et al., 2015). Indeed, during each cell division, aberrant DNA methylation patterns accumulates over time contributing to epigenetic drift and creating an epigenetic mosaicism that may allow for the selection of biological defects that may lead to cancer and other age-related diseases (Amodio et al., 2017). Support for these evidence comes mainly from studies carried out in mono- and di-zygotic twin pairs in which a gradual age-related divergence in epigenetic marks was observed in monozygotics (Martin et al., 2005; Lipman and Tiedje, 2006; Kaminsky et al., 2009; Bell and Spector, 2011; Tan et al., 2013; Mendelsohn et al., 2017). DNA methylation drift comes from non-directional changes occurring during aging and involves both hypermethylation and hypomethylation events. Recently, Slieker and coll. identified several age-related Variably Methylated Position (aVMPs) exhibiting high variability in their methylation status and are associated with the expression of genes involved in DNA damage and apoptosis (Slieker et al., 2016). During aging, epigenetic drift also deeply influences the function of aged stem cells by limiting their plasticity and their differentiation potential that ultimately results in the exhaustion of the stem cell pool and in the selective growth advantage in other stem cells, which leads to clonal expansion and local hyper-proliferation (Teschendorff et al., 2010; Issa, 2014; Li and Tollefsbol, 2016).

Recently, several studies reported the presence of directional and non-stochastic changes occurring over time within clusters of consecutive CpG sites throughout the whole genome, referred as age-Differentially Methylated Regions (a-DMRs) (Rakyan et al., 2010; Li and Tollefsbol, 2016; Bacalini et al., 2017). Hundreds of hyper- and hypo-methylated a-DMR have been identified in multiple tissues and replicated in independent samples. A number of these aDMRs were located within 500 bp of the transcriptional start sites. Literature data agree to consider them associated with biological mechanisms involved in aging and longevity. Ashapkin et al., assume that most hyper-aDMRs represent epigenetic perturbations inherent to the aging *per se*, while hypo-aDMRs may be correlated to modifications associated both with aging *per se* and age-dependent modifications in relative proportions of the blood cell subtypes (Bell et al., 2012; Ashapkin et al., 2017).

Candidate genetic loci undergoing profound epigenetic changes with age and in age-related diseases have been progressively characterized. Global genomic DNA hypomethylation is especially evident at repetitive sequences, to a greater extent at Alu and HERV-K sequences, contributing to the increase

of genome instability as well as at specific promoter regions of some genes including ITGAL (Integrin alpha-L) and IL17RC (Interleukin 17 Receptor C) (Vijg and Dollé, 2007; Bollati et al., 2009; Zhang et al., 2009; Jintaridth and Mutirangura, 2010; Wei et al., 2012). By whole-genome bisulfite sequencing (WGBS), Heyn et al. compared the DNA methylation state of more than 90% of all CpGs present in the genome between newborn and nonagenarian/centenarian samples. A significant loss of methylated CpGs was found in the centenarian vs newborn DNAs. This was observed for all chromosomes and concerned all genomic regions such as promoters, exonic, intronic and intergenic regions. Most of these changes were focal and the aged genome was consequently less homogeneously methylated with respect to the newborn due to the age-dependent epigenetic drift (Heyn et al, 2012).

Besides to this extensive hypomethylation, the promoter regions of specific genes are subjected to a gradual increase of DNA methylation across lifespan (Table 1). In most of cases, the observed hypermethylation was associated to the transcriptional silencing suggesting that with increasing age there is an epigenetic turning off of these genes.

Figure 2 represents DNA methylation variations occurring during aging within interspersed repeats and genes.

Function	**Gene symbol**	**References**
Angiogenesis	VASH1	Reynolds et al., 2014
Antigen processing and presentation	DPB1, DRB1, LAG3, TAP2, PSMB9, PSMB8, HLA-E, HLA-F, HLA-B, MICB, SLC11A1, HLA-DPA1, TAPBP, HLA-DMB	Reynolds et al., 2014
Cell adhesion	LAMB1, PCDHA1,2,3,4, PODXL, PCDH9, SORBS2	McClay et al., 2014
Development and growth	c-Fos, FGF8, FIGN, IGF2, HOXB5, B6, B7, B8, MEIS1, MYOD1, NKX2-2, TIAL1, UBE2E3	Choi et al., 1996 Issa et al., 1996 Ahuja et al., 1998 Christensen et al., 2009 McClay et al., 2014 Vidal et al., 2014
Genome stability and repair	MGMT, MLH1, OGG, hTERT, RAD50	Nakagawa et al., 2001 Matsubayashi et al., 2005

		Silva et al., 2008 Christensen et al., 2009 Madrigano et al., 2012
Ion channel	GRIA2, KCNJ8, RYR2	McClay et al., 2014
Metabolism	AGPAT2, ATP13A4, COX7A1, CRAT, ECRG4, ELOVL2, EPHX2, GAD2, LEP, MGC3207, MGEA5, SLC38A4, SLC22A18, SNTG1, STAT5A	Rönn et al., 2008 Bell et al., 2012 Madrigano et al., 2012 McClay et al., 2014 Gentilini et al., 2012
Immune response	CD4, INFG, TNF□□□NOD2, PTMS	Madrigano et al., 2012 McClay et al., 2014
Signal transduction	ARL4A, DLC1, GPR128, GRIA2, LAG3, MYO3A, PRR5L, PTPRT, TFG, TRAF6, TRHDE	Bell et al., 2012 McClay et al., 2014
Stress response	HSPA2	McClay et al., 2014
Transcription factors	ARID5B, BICC1, ESR1, FOXP1, HIPK2, LHX5, MLF2, NFIA, NOD2, POU4F3, RARB, TBX4, TBX20, TRPS1, WT1, ZBTB1, ZEB2, ZNF827	Gaudet et al., 2009 Christensen et al., 2009 Bell et al., 2012 Reynolds et al., 2014 McClay et al., 2014
Tumor suppression	APC, CASP8, CHD1, GSTP1, HIC1, LOX, LSAMP, N33, P16INK4A, RASSF1, RUNX3, SOCS1, TIG1, DAPK1, hMLH1, p16,	Ahuja et al., 1998 Fujii et al., 1998 Cody et al., 1999 Dammann et al., 2000 Virmani et al., 2001 Waki et al., 2003 Sutherland et al., 2004 So et al., 2006 Nishida et al., 2008 Yuan et al., 2008 Christensen et al., 2009 McClay et al., 2014

Table 1. List of genes displaying age-related DNA methylation changes in CpG islands located within their promoter regions.

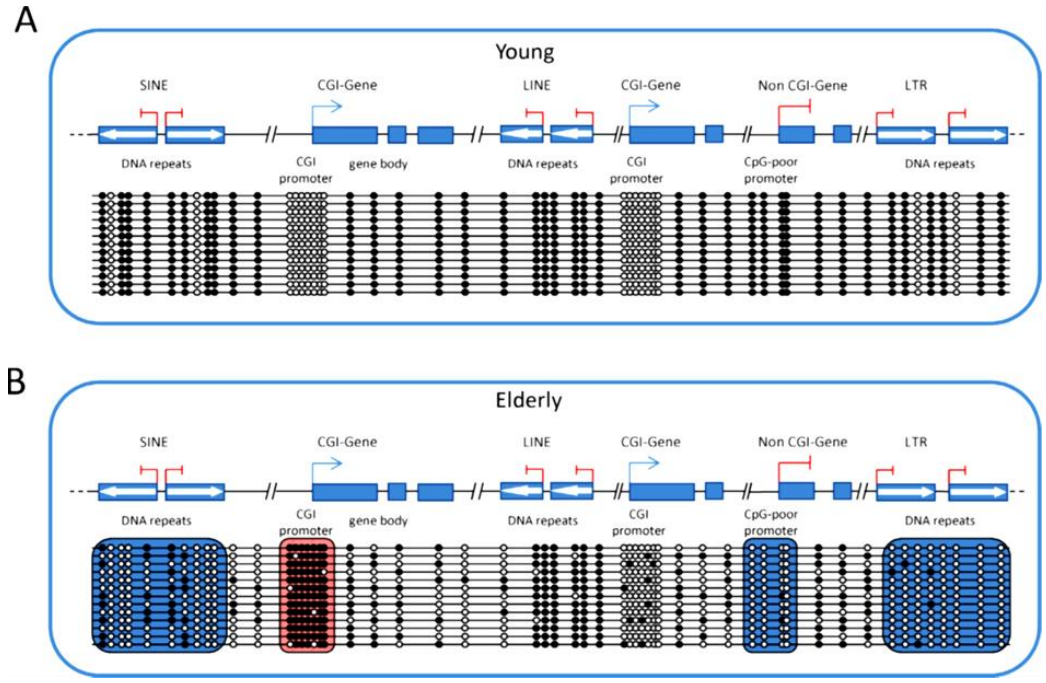


Figure 2. A Graphical representation of DNA methylation patterns changes in young (A) an old people (B). Active and repressed transcription at transcription start sites are indicated in ligth blue and red arrows respectively. Black lines indicate the methylation status of DNA from different people where white circles represent unmethylated CpGs, black circles represent methylated CpGs. Age-associated DMRs are highlighted in blue (hypomethylated regions) and red (hypermethylated regions), respectively. (Zampieri et al., 2015).

Epigenome-wide association studies (EWAS) identified the so-called "clock CpGs", namely a large set of CpG markers whose methylation status is measured in order to construct quantitative models effective in predicting the age of cells, tissues or organs, referred as epigenetic age or DNAm age. DNAm age not only reflects the chronological, but also the biological age and, thus, these biomarkers would, on one hand, facilitate the differentiation of individuals who are of the same chronological age yet have variant aging rates, on the other define a panel of measurements for healthy aging and, even further, predict life span (Bellizzi et al., 2012a and 2012b, D'Aquila et al., 2017 and 2018). Figure 3 depicts the existing relationship among chronological age, biological age and epigenome.

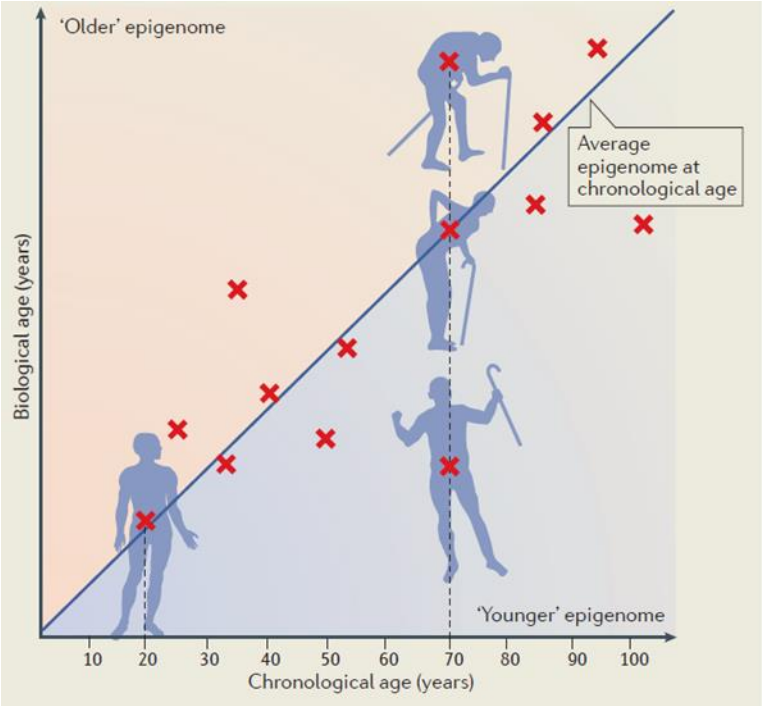


Figure 3. In the picture are indicated chronological age (x-axis) and biological age (y-axis), respectively. Individuals with the same chronological age may display different phenotypes due to their biological age (Benayoun et al., 2015).

Multiple candidate loci have been selected showing linear correlation of methylation status with biological age. Among these, the loci *ELOVL2* (ELOVL fatty acid elongase 2), *FHL2* (Four and a half LIM domains 2), *CCDC102B* (coiled-coil domain containing 102B), *C1orf132 (chromosome 1 open reading frame 132)* are the most thoroughly evaluated markers of age in various human tissues (Garagnani et al., 2012, Freire-Aradas et al., 2016). More recently, D'Aquila et al., reports the identification of *RAB32* and *RHOT2* genes as potential biomarkers of chronological and biological age. Being two genes involved in mitochondrial quality control, this evidence provide further confirm to the role of mitochondrial functions during age (in Chapter I: Original Research Work).

Starting from Bocklandt et al, which described the first age estimator model by using DNA samples from saliva, a series of epigenetic clocks were developed by analyzing DNA methylation marks in single and multiple tissues (Bocklandt et al., 2011). Currently, Hannum and Horvath clocks represent the most robust recognized models showing both a high age correlation (R>0.9) and low mean error of the age prediction (4.9 and 3.6 years, respectively). The first model was developed only in blood, while the second, designed matching data from 51 healthy tissues, such as blood, cerebellum, occipital cortex, buccal, colon, adipose, liver, lung, saliva, and cell types, including CD4 T and immortalized B cells, results compatible with different technological platforms and used in a wide range of studies (Hannum et al., 2013; Horvath, 2013).

Both models are also able to predict all-cause mortality independent of several risk factors including smoking, alcohol use, education, body mass index and comorbidities (Marioni et al., 2015; Christiansen et al., 2016; Perna et al., 2016). More recently, DNAmAge biomarkers which also consider clinical measures of physiological dysregulations have been developed. One of these, referred as DNAmPhenoAge (phenotypic age estimator), constructed by generating a weighted average of 10 clinical characteristic, such as albumin, creatinine, glucose and C-reactive proteins, and then analysed by regression analysis against DNA methylation levels in blood, proved to be effective in predicting mortality, health span, disease risk and in various measures of comorbidity (Levine et al, 2018). Age acceleration, that is an estimated DNAmAge higher than chronological age was registered for several age-related diseases including Down syndrome (Horvath et al., 2015a and 2015b), Alzheimer's and Parkinson's diseases (Levine et al., 2015; Horvath et al., 2015a and 2015b), HIV-infection (Boulias et al., 2016, Rickabaugh et al., 2015), frailty (Breitling et al., 2016), diabetes and cancer (Zheng et al., 2016; Bacalini et al., 2017).

Lastly, a significant number of reports also evidence a correlation between mitochondrial DNA methylation with aging. The first of them dates to 1983, when a decrease of mtDNA methylation was observed in aged cultured fibroblasts (D'Aquila et al., 2017). More recently, although methylation levels of the mitochondrial D-loop region resulted not associated with aging, high methylation levels (>10%) of one CpG site located within the *MT-RNR1* gene were observed more frequent in old women with respect to youngers (Bellizzi et al., 2013; D'Aquila et al., 2015). Furthermore, the non-canonical CpG methylation patterns, such as non-CpG and hydroxymethylation, are deregulated during aging, potentially leading to downstream changes in transcription and cellular physiological functions. A global non-CpG methylation decrease with age has been described. 5-hmC content significantly decreases in some tissues, including blood and liver, and is negatively correlated with aging, in association with low mRNA expression levels of TET1 and TET3 (Truong et al., 2015). In a contrasting, mouse cerebellum and hippocampus show an increase of 5hmC levels with aging which can be prevented by caloric restriction (Szulwach et al., 2012; Chouliaras et al., 2012). A decrease of mitochondrial DNA levels of 5-hmC during aging was observed in frontal cortex but not in the cerebellum (Dzitoyeva et al., 2012). An increase in 5hmC signals was observed in genes activated in old mice with respect to young ones demonstrating that 5hmC is acquired in developmentally activated genes (Szulwach et al., 2012). What is more, age-related non-overlapping 5-mCand 5hmC pattern have been observed (Kochmanski et al., 2018).

Considering that epigenetic marks induce profound changes in the gene expression and contribute to the cellular and organismal phenotypic plasticity during lifetime, it is evident that dysregulations of epigenetic patterns may contribute to age-related diseases, including cancer, diabetes, cardiovascular

and neurodegenerative diseases. These changes represent potential disease biomarkers. In all of these diseases, methylome-wide association studies (MWAS) have identified characteristic methylome signatures and brought to light as alteration in DNA methylation are hallmarks often coincident with those observed in aging. Example of this overlapping come from the observation that DNA hypomethylation, prevalently at repetitive DNA elements, and locus specific hypermethylation of tumour-suppressor genes (*p53*, *p21*, *p16*, *TIG1*, and *RB1*), oncogenes (*cMYC* and *TERT*), genes involved in type 2 diabetes (*COX7A1*, *PRDX2*, *IRS1* and *KCNJ11*), genes involved in Alzheimer's disease (*APP*, *PS1* and *BACE1*) generally occur in the above diseases as in aging thus causing aberrant gene expression (Brunet et al, 2014). Changes in the levels of 5mC and DNMTs, DNMT1 and DNMT3a have been detected in neuronal mitochondria from patients with amyotrophic lateral sclerosis (ALS) suggesting that motor neurons can engage epigenetic mechanisms involving DNMT upregulation and increased DNA methylation to drive apoptosis (Wong et al., 2013). Silva et al. found that patients with Alzheimer disease (AD) had a higher methylation frequency of *hTERT* compared to elderly controls (Silva et al, 2008). Repetitive LINE-1 elements were also reported to be significantly hypermethylated in AD patients with respect to healthy controls. AD patients have been further characterized by a decrease in brain SAM levels, and temporal neocortex neuronal nuclei were found to be hypomethylated in a patient with AD compared to his non-AD monozygotic twin (Mastroeni et al., 2009; Bollati et al., 2011). Guarasci et al. have recently observed that individuals affected by Down Syndrome exhibit dysregulated mtDNA methylation patterns in D-loop region with respect to healthy individuals (manuscript in preparation).

It follows that epigenetic based drugs which reverse aberrant DNA methylation profiles may be considered effective in the assessment and development of epigenetic-based treatments (Shenouda et al., 2009).

Environmental factors and epigenetics aging

Environmental factors, including chemicals, pollutants, diet, drugs, infectious, trauma, and psycho-social and socio-economic status have been associated with DNA methylation changes in aging and age-related diseases (Huidobro et al., 2013; Obata et al., 2015). These changes can be induced in the individual in each period of the life, from the in utero period to the elderly and are relevant with respect to the shift from the healthy to the diseased statuses of an individual with aging, due to the increased chance to encounter environmental insults or to accumulate their effects during aging. Epidemiological evidence suggests that maternal environmental exposure to stimuli result in

epigenetic changes, since DNA methylation patterns at DMRs are established before gastrulation, which occur early in development and play an important role in susceptibility to disease in later life. It was observed that energy-rich, protein-deficient, micronutrient-deficient and/or methyl donor-rich diets during pregnancy induce modifications of methylation profile in mothers which, in turn, can be transmitted to next generation thus regulating in offspring long term metabolic processes which contribute to age phenotypes and age-related disease (Vickers, 2014; Lillycrop et al., 2015; Park et al., 2017). An emblematic example of the trans-generational relationship between food and epigenetic modifications is represented by The Dutch Honger winter (1944-1945) family studies in which adult health outcomes in relation to exposure to famine prior to conception or at specific periods of gestation was analyzed. It emerged that prenatal exposure to the famine is associated with increased prevalence of overweight, hypertension, and coronary heart disease, meanwhile maternal famine exposure around the time of conception has been related to prevalence of major affective disorders, antisocial personality disorders, schizophrenia, decreased intracranial volume, and congenital abnormalities of the central nervous system (Lumey et al., 2007; Stein et al., 2009). More recently, Guarasci et al. reported that the differences in global DNA methylation among different tissues are magnified in 96 weeks old rats fed with low calorie diet. Moreover, the low-calorie diet appears to affect the offspring's epigenetic status more strongly if administered during the maternal pre-gestational period than the gestational and lactation time (Guarasci et al., 2018). Caloric restriction, the decrease in nutrient intake above the level of starvation and below what an organism would consume ad libitum, is one of the most consistent means of increasing life span across a spectrum of organisms. Both caloric intake and DNMT3A play a role in neuronal aging, in which DNMT3A contribute to the formation of memory and synaptic and neuronal plasticity (Miller and Sweatt, 2007). It was found that Dnmt3a-immunoreactivity increases with age in the CA3 and CA1-2 hippocampal regions of mice and that reducing caloric intake by 50% attenuates this increase. An age-related increase in 5mC occurs in these regions as well as the hippocampal dentate gyrus and is also attenuated by CR.63 Similarly, 5hmC content increases with age in all three of these regions, and CR opposes this age-related increase in the CA3 region, further implicating methylomics in hippocampal aging (Chouliaras et al., 2011; Johnson et al., 2012).

In this context, different studies have also demonstrated the role of nutrition in molecular mechanisms related to onset and progression of neurodegenerative diseases, such as Alzheimer and Parkinson diseases, psychiatric disorders and dementia. Transgenic mouse model of Alzheimer disease treated with a diet deficient of vitamin B12, B6 and folate showed a decrease of SAM/SAH ratio leading to impaired methylation potential, DNMTs inhibition and DNA demethylase stimulation, PSEN1 promoter hypomethylation, PSEN1 overexpression, increased amyloid processing and deposition in

senile plaques and, finally, cognitive impairment. The supplementation with SAM was able to restore control-like conditions in AD mice or even to partially revert the Alzheimer-like phenotype (Fuso et al., 2012).

Lastly, an increase of pollutant, metal and pesticides exposure, stress, trauma were significantly associated with increase in DNAm-age and in Horvath DNAm-age (Dihigra). Glucocorticoids, a class of endocrine signaling hormones which includes cortisol are a component of the biological response to stress. Notably, 85 of the 353 loci that comprise the Horvath epigenetic clock are located near glucocorticoid receptor elements, and 110 loci showed altered DNA methylation after exposure to dexamethasone, a glucocorticoid receptor agonist (Zannas et al., 2015). Intra-uterine exposure to arsenic alters DNA methylation in offspring which may result in a higher risk of disease in later life likely by influencing the generation of reactive oxygen species (ROS), which causes oxidative DNA damage, binding and inhibition of arsenic metabolites to enzymes, and perturbation of key signaling pathways (Rossman et al., 2011.).

Similar to nuclear DNA methylation, occurrence of the abnormal mtDNA methylation is often depending on different factors, such as diseases, environment, drugs, and food (Gao et al., 2017).

It was observed that fructose consumption induce metabolism disorders by stimulating hepatic mtDNA-encoded gene expression through epigenetic changes in mtDNA. Lower mtDNA D-loop methylation levels were found in the blood patients with late-onset Alzheimer's disease patients compared to the blood of normal controls. In addition, insulin resistance was associated with DNA methylation in mitochondrial NADH dehydrogenase 6 and D loop-region (Stoccoro et al., 2017). Alcohol exposure was reported modulates levels of DNMT enzymes (Mandal et al., 2017; Miozzo et al., 2018).

References

1. Ahuja N, Li Q, Mohan AL, Baylin SB, Issa JP. Aging and DNA methylation in colorectal mucosa and cancer. Cancer Research. 1998; 58(23):5489-94.
2. Allis CD, Jenuwein T. The molecular hallmarks of epigenetic control. Nat Rev Genet 2016;17: 487–500.
3. Amodio N, D'Aquila P, Passarino G, Tassone P, Bellizzi D. Epigenetic modifications in multiple myeloma: recent advances on the role of DNA and histone methylation. Expert Opinion on Therapeutic Targets. 2017; 21:91–101.
4. Ashapkin VV, Kutueva LI, Vanyushin BF. Aging as an Epigenetic Phenomenon. Curr Genomics. 2017; 18:385–407.
5. Bacalini MG, D'Aquila P, Marasco E, Nardini C, Montesanto A, Franceschi C, Passarino G, Garagnani P, Bellizzi D. The methylation of nuclear and mitochondrial DNA in ageing phenotypes and longevity. Mech Ageing Dev. 2017; 165:156–161.
6. Barros SP, Offenbacher S. Epigenetics: connecting environment and genotype to phenotype and disease. J Dent Res. 2009;88: 400–408.
7. Bell JT, Spector TD. A twin approach to unraveling epigenetics. Trends in Genetics. 2011; 27:116–125.
8. Bell JT, Tsai PC, Yang TP, Pidsley R, Nisbet J, Glass D, Mangino M, Zhai G, Zhang F, Valdes A, Shin SY, Dempster EL, Murray RM, Grundberg E, Hedman AK, Nica A, Small KS; MuTHER Consortium, Dermitzakis ET, McCarthy MI, Mill J, Spector TD, Deloukas P. Epigenome-wide scans identify differentially methylated regions for age and age-related phenotypes in a healthy ageing population. PLoS Genetics. 2012; 8(4): e1002629.
9. Bellizzi D, D'aquila P, Giordano M, Montesanto A, Passarino G. Global DNA methylation levels are modulated by mitochondrial DNA variants. Epigenomics 2012a; 4:17–27.
10. Bellizzi D, D'Aquila P, Scafone T, Giordano M, Riso V, Riccio A, Passarino G. The control region of mitochondrial DNA shows an unusual CpG and non-CpG methylation pattern. DNA Res 2013; 20:537–547.
11. Bellizzi D, D'Aquila P, Montesanto A, Corsonello A, Mari V, Mazzei B, Lattanzio F, Passarino G. Global DNA methylation in old subjects is correlated with frailty. Age. 2012b; 34:169–179.
12. Benayoun BA, Pollina EA, Brunet A. Epigenetic regulation of ageing: linking environmental inputs to genomic stability. Nat Rev Mol Cell Biol. 2015 Oct;16(10):593-610.
13. Bocklandt S, Lin W, Sehl ME, Sánchez FJ, Sinsheimer JS, Horvath S, Vilain E. Epigenetic predictor of age. PLoS One. 2011; 6:14821.
14. Bollati V, Galimberti D, Pergoli L, Dalla Valle E, Barretta F, Cortini F, Scarpini E, Bertazzi PA, Baccarelli A. DNA methylation in repetitive elements and Alzheimer disease. Brain Behav Immun. 2011 Aug; 25(6):1078-83.
15. Bollati V, Schwartz J, Wright R, Litonjua A, Tarantini L, Suh H, Sparrow D, Vokonas P, Baccarelli A. Decline in genomic DNA methylation through aging in a cohort of elderly subjects. Mechanisms of

Ageing and Development. 2009; 130:234–9.

16. Boulias K, Lieberman J, Greer EL. An Epigenetic Clock Measures Accelerated Aging in Treated HIV Infection. Mol Cell. 2016 Apr 21;62(2):153-155.
17. Boyes J, Bird A. DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. Cell. 1991; 64:1123–1134.
18. Branco MR, Ficz G, Reik W. Uncovering the role of 5-hydroxymethylcytosine in the epigenome. Nat Rev Genet. 2011; 13:7–13.
19. Breitling LP, Saum KU, Perna L, Schöttker B, Holleczek B, Brenner H. Frailty is associated with the epigenetic clock but not with telomere length in a German cohort. Clin Epigenetics. 2016 Feb 26;8:21.
20. Brunet A, Berger SL. Epigenetics of Aging and Aging-related Disease. J Gerontol A Biol Sci Med Sci. 2014; 69: S17–S20.
21. Choi EK, Uyeno S, Nishida N, Okumoto T, Fujimura S, Aoki Y, Nata M, Sagisaka K, Fukuda Y, Nakao K, Yoshimoto T, Kim YS, Ono T. Alterations of c-fos gene methylation in the processes of aging and tumorigenesis in human liver. Mutation Research. 1996; 354(1):123-8.
22. Chouliaras L, van den Hove DL, Kenis G, Dela Cruz J, Lemmens MA, van Os J, Steinbusch HW, Schmitz C, Rutten BP. Caloric restriction attenuates age-related changes of DNA methyltransferase 3a in mouse hippocampus. Brain Behav Immun. 2011 May; 25(4):616-23.
23. Chouliaras L, van den Hove DL, Kenis G, Keitel S, Hof PR, van Os J, Steinbusch HW, Schmitz C, Rutten BP. Age-related increase in levels of 5-hydroxymethylcytosine in mouse hippocampus is prevented by caloric restriction. Curr Alzheimer Res. 2012; 9:536–544.
24. Christensen BC, Houseman EA, Marsit CJ, Zheng S, Wrensch MR, Wiemels JL, Nelson HH, Karagas MR, Padbury JF, Bueno R, Sugarbaker DJ, Yeh RF, Wiencke JK, Kelsey KT. Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG island context. PLoS Genetics. 2009; 5(8): e1000602.
25. Christiansen L, Lenart A, Tan Q, Vaupel JW, Aviv A, McGue M, Christensen K. DNA methylation age is associated with mortality in a longitudinal Danish twin study. Aging Cell. 2016; 15:149–154.
26. Clouaire T, Stancheva I. Methyl-CpG binding proteins: specialized transcriptional repressors or structural components of chromatin? Cellular and Molecular Life Sciences. 2008; 65:1509–1522.
27. Cody DT, Huang Y, Darby CJ, Johnson GK, Domann FE. Differential DNA methylation of the p16 INK4A/CDKN2A promoter in human oral cancer cells and normal human oral keratinocytes. Oral Oncology. 1999; 35(5):516-22.
28. Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nature Genetics. 2000; 25(3):315-9.
29. Dan J, Chen T. Genetic Studies on Mammalian DNA Methyltransferases. Advances in Experimental Medicine and Biology. 2016; 123–945.
30. D'Aquila P, Bellizzi D, Passarino G. rRNA-gene methylation and biological aging. Aging (Albany NY).

2018; 10:7–8.

31. D'Aquila P, Giordano M, Montesanto A, De Rango F, Passarino G, Bellizzi D. Age-and gender-related pattern of methylation in the MT-RNR1 gene. Epigenomics. 2015; 7:707–716.
32. D'Aquila P, Montesanto A, Guarasci F, Passarino G, Bellizzi D. Mitochondrial genome and epigenome: two sides of the same coin. Frontiers in Bioscience (Landmark Edition). 2017; 22: 888-908.
33. D'Aquila P, Montesanto A, Mandalà M, Garasto S, Mari V, Corsonello A, Bellizzi D, Passarino G. Methylation of the ribosomal RNA gene promoter is associated with aging and age-related decline. Aging Cell. 2017; 16:966–975.
34. D'Aquila P, Rose G, Bellizzi D, Passarino G. Epigenetics and aging. Maturitas. 2013; 74:130–136.
35. Deaton AM, Bird A. CpG islands and the regulation of transcription. Genes Dev. 2011; 25:1010–1022.
36. Dzitoyeva S, Chen H, Manev H. Effect of aging on 5-hydroxymethylcytosine in brain mitochondria. Neurobiology of Aging. 2012; 33:2881–2891.
37. Fraga MF; Genetic and epigenetic regulation of aging. Curr Opin Immunol. 2009 Aug; 21(4):446-53.
38. Freire-Aradas A, Phillips C, Mosquera-Miguel A, Girón-Santamaría L, Gómez-Tato A, Casares de Cal M, Álvarez-Dios J, Ansede-Bermejo J, Torres-Español M, Schneider PM, Pośpiech E, Branicki W, Carracedo Á, Lareu MV. Development of a methylation marker set for forensic age estimation using analysis of public methylation data and the Agena Bioscience EpiTYPER system. Forensic Sci Int Genet. 2016 Sep;24:65-74.
39. Fujii H, Biel MA, Zhou W, Weitzman SA, Baylin SB, Gabrielson E. Methylation of the HIC-1 candidate tumor suppressor gene in human breast cancer. Oncogene. 1998; 16(16):2159-64.
40. Fuso A, Nicolia V, Ricceri L, Cavallaro RA, Isopi E, Mangia F, Fiorenza MT, Scarpa S. S-adenosylmethionine reduces the progress of the Alzheimer-like features induced by B-vitamin deficiency in mice. Neurobiol Aging. 2012 Jul;33(7):1482.e1-16.
41. Gao D, Zhu B, Sun H, Wang X. Mitochondrial DNA Methylation and Related Disease. Adv Exp Med Biol. 2017;1038:117-132.
42. Garagnani P, Bacalini MG, Pirazzini C, Gori D, Giuliani C, Mari D, Di Blasio AM, Gentilini D, Vitale G, Collino S, Rezzi S, Castellani G, Capri M, Salvioli S, Franceschi C. Methylation of ELOVL2 gene as a new epigenetic marker of age. Aging Cell. 2012 Dec;11(6):1132-4.
43. Gardiner-Garden M, Frommer M. CpG islands in vertebrate genomes. J. Mol. Biol. 1987. 196: 261– 282.
44. Gaudet MM, Campan M, Figueroa JD, Figueroa JD, Yang XR, Lissowska J, Peplonska B, Brinton LA, Rimm DL, Laird PW, Garcia-Closas M, Sherman ME. DNA hypermethylation of ESR1 and PGR in breast cancer: pathologic and epidemiologic associations. Cancer Epidemiology, Biomarkers & Prevention. 2009; 18(11):3036-43.
45. Gentilini D1, Mari D, Castaldi D, Remondini D, Ogliari G, Ostan R, Bucci L, Sirchia SM, Tabano S, Cavagnini F, Monti D, Franceschi C, Di Blasio AM, Vitale G. Role of epigenetics in human aging and longevity: genome-wide DNA methylation profile in centenarians and centenarians' offspring. Age (Dordr). 2013 Oct;35(5):1961-73.

46. Goldberg AD, Allis CD, Bernstein E. Epigenetics: A Landscape Takes Shape. Cell. 2007; 128:635–638
47. Griffith JS, Mahler HR. DNA ticketing theory of memory. Nature. 1969; 223:580–582.
48. Guarasci F, D'Aquila P, Mandalà M, Garasto S, Lattanzio F, Corsonello A, Passarino G, Bellizzi D. Aging and nutrition induce tissue-specific changes on global DNA methylation status in rats. Mechanisms of Ageing and Development. 2018; 174: 47–54.
49. Guo F, Li X, Liang D, Li T, Zhu P, Guo H, Wu X, Wen L, Gu TP4, Hu B, Walsh CP, Li J, Tang F, Xu GL. Active and passive demethylation of male and female pronuclear DNA in the mammalian zygote. Cell Stem Cell. 2014; 15:447–459.
50. Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sadda S. Genome-wide methylation profiles reveal quantitative views of human aging rates. Mol Cell. 2013; 49:359–367.
51. Heyn H, Li N, Ferreira HJ, Moran S, Pisano DG, Gomez A, Diez J, Sanchez-Mut JV, Setien F, Carmona FJ, Puca AA, Sayols S, Pujana MA, Serra-Musach J, Iglesias-Platas I, Formiga F, Fernandez AF, Fraga MF, Heath SC, Valencia A, Gut IG, Wang J, Esteller M. Distinct DNA methylomes of newborns and centenarians. Proc Natl Acad Sci. 2012; 109:10522–10527.
52. Horvath S, Garagnani P, Bacalini MG, Pirazzini C, Salvioli S, Gentilini D, Di Blasio AM, Giuliani C, Tung S, Vinters HV, Franceschi C. Accelerated epigenetic aging in Down syndrome. Aging Cell. 2015a Jun;14(3):491-5.
53. Horvath S, Pirazzini C, Bacalini MG, Gentilini D, Di Blasio AM, Delledonne M, Mari D, Arosio B, Monti D, Passarino G, De Rango F, D'Aquila P, Giuliani C, Marasco E, Collino S, Descombes P, Garagnani P, Franceschi C. Decreased epigenetic age of PBMCs from Italian semi-supercentenarians and their offspring. Aging (Albany NY). 2015b Dec;7(12):1159-70.
54. Horvath S. DNA methylation age of human tissues and cell types. Genome Biol. 2013;14(10): R115.
55. Hotchkiss RD. The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. The Journal of Biological Chemistry. 1948; 175(1):315-32.
56. Huang B, Jiang C, Zhang R. Epigenetics: The language of the cell? Epigenomics 2014; 6:73–88.
57. Huidobro C, Fernandez AF, Fraga MF. Aging epigenetics: Causes and consequences. Mol Aspects Med 2013; 34:765–781.
58. Illingworth RS, Bird AP. CpG islands–'a rough guide. FEBS Letters. 2009; 583: 1713–1720.
59. Issa JP. Aging and epigenetic drift: a vicious cycle. J Clin Invest 2014; 124:24–29.
60. Jeltsch, A and Jurkowska, RZ. New concepts in DNA methylation. Trends Biochem Sci. 2014 Jul;39(7):310-8.
61. Jintaridth P, Mutirangura A. Distinctive patterns of age-dependent hypomethylation in interspersed repetitive sequences. Physiological Genomics. 2010; 41:194–200.
62. Johnson AA, Akman K, Calimport SRG, Wuttke D, Stolzing A, de Magalhães JP. The Role of DNA Methylation in Aging, Rejuvenation, and Age-Related Disease. Rejuvenation Res. 2012 Oct; 15(5): 483–494.
63. Johnson FB, Sinclair DA, Guarente L. Molecular biology of aging. Cell. 1999; 96: 291–302.

64. Jones MJ, Goodman SJ, Kobor MS. DNA methylation and healthy human aging. Aging Cell. 2015; 14:924–932.
65. Jones PA. Altering gene expression with 5-azacytidine. Cell. 1985b; 40:485–486.
66. Jones PA. Effects of 5-azacytidine and its 2'-deoxyderivative on cell differentiation and DNA methylation. Pharmacol Ther. 1985a; 28:17–27.
67. Jones PA. Functions of DNA methylation: Islands, start sites, gene bodies and beyond. Nat Rev Genet. 2012; 13:484–492.
68. Kaminsky ZA, Tang T, Wang SC, et al. DNA methylation profiles in monozygotic and dizygotic twins. Nat Genet. 2009; 41:240–245.
69. Kanherkar RR, Bhatia-Dey N, Csoka AB. Epigenetics across the human lifespan. Frontiers in Cell and Developmental Biology. 2014; 2:49.
70. Kass SU, Goddard JP, Adams RL. Specific methylation of vector sequences inhibits transcription from the SV40 early promoter. Biochem Soc Trans. 1993; 21:9
71. Keshet I, Yisraeli J, Cedar H. Effect of regional DNA methylation on gene expression. Proc Natl Acad Sci U S A 1985; 82:2560–4.
72. Kirkwood TBL. Understanding the odd science of aging. Cell 2005; 120:437–447.
73. Kochmanski J, Marchlewicz EH, Cavalcante RG, Sartor MA, Dolinoy DC. Age-related Epigenome-wide DNA Methylation and Hydroxymethylation in Longitudinal Mouse Blood. Epigenetics 2018; 1-14.
74. Koh KP, Yabuuchi A, Rao S, Huang Y, Cunniff K, Nardone J, Laiho A, Tahiliani M, Sommer CA, Mostoslavsky G, Lahesmaa R, Orkin SH, Rodig SJ, Daley GQ, Rao A. Tet1 and Tet2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells. Cell Stem Cell. 2011; 8:200–213.
75. Larsen F, Gundersen G, Lopez R, Prydz H. CpG islands as gene markers in the human genome. Genomics. 1992; 13:1095–1107.
76. Levine ME, Lu AT, Bennett DA, Horvath S. Epigenetic age of the pre-frontal cortex is associated with neuritic plaques, amyloid load, and Alzheimer's disease related cognitive functioning. Aging (Albany NY). 2015 Dec;7(12):1198-211.
77. Levine ME, Lu AT, Quach A, Chen BH, Assimes TL, Bandinelli S, Hou L, Baccarelli AA, Stewart JD, Li Y, Whitsel EA, Wilson JG, Reiner AP1, Aviv A, Lohman K, Liu Y, Ferrucci L, Horvath S. An epigenetic biomarker of aging for lifespan and healthspan. Aging (Albany NY). 2018; 10:573–591.
78. Li Y, Tollefsbol TO. Age-related epigenetic drift and phenotypic plasticity loss: Implications in prevention of age-related human diseases. Epigenomics. 2016; 8:1637–1651.
79. Lillycrop KA, Burdge GC. Maternal diet as a modifier of offspring epigenetics. J Dev Orig Health Dis. 2015; 6:88–95.
80. Lipman T, Tiedje LB. Epigenetic Differences Arise During the Lifetime of Monozygotic Twins. MCN, Am J Matern Nurs. 2006; 31:204.

81. Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson JA, Ren B, Ecker JR. Human DNA methylomes at base resolution show widespread epigenomic differences. This was the first report of a human methylome at single-base resolution. Nature. 2009; 462:315–322.
82. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell. 2013; 153:1194–1217.
83. Lumey LH, Stein AD, Kahn HS, van der Pal-de Bruin KM, Blauw GJ, Zybert PA, Susser ES. Cohort profile: the Dutch Hunger Winter families study. Int J Epidemiol 2007; 36:1196–204.
84. Luo GZ, Blanco MA, Greer EL, He C, Shi Y. DNA N(6)-methyladenine: a new epigenetic mark in eukaryotes? Nat Rev Mol Cell Biol 2015; 16: 705–10.
85. Luo GZ, Wang F, Weng X, Chen K, Hao Z, Yu M, Deng X, Liu J, He C. Characterization of eukaryotic DNA N(6)-methyladenine by a highly sensitive restriction enzyme-assisted sequencing. Nat Commun. 2016 Apr 15;7:11301.
86. Lyko F. The DNA methyltransferase family: a versatile toolkit for epigenetic regulation. Nature Reviews Genetics. 2018; 19:81–92.
87. Madrigano J, Baccarelli A, Mittleman MA, Sparrow D, Vokonas PS, Tarantini L, Schwartz J. Aging and epigenetics: longitudinal changes in gene-specific DNA methylation. Epigenetics. 2012; 7(1):63-70.
88. Mandal C, Halder D, Jung KH, Chai YG. Gestational Alcohol Exposure Altered DNA Methylation Status in the Developing Fetus. Int J Mol Sci. 2017 Jul; 18(7): 1386
89. Marioni RE, Shah S, McRae AF, Chen BH, Colicino E, Harris SE, Gibson J, Henders AK, Redmond P, Cox SR, Pattie A, Corley J, Murphy L, Martin NG, Montgomery GW, Feinberg AP, Fallin MD, Multhaup ML, Jaffe AE, Joehanes R, Schwartz J, Just AC, Lunetta KL, Murabito JM, Starr JM, Horvath S, Baccarelli AA, Levy D, Visscher PM, Wray NR, Deary IJ. DNA methylation age of blood predicts all-cause mortality in later life. Genome Biol 2015; 16:25.
90. Martin GM. Epigenetic drift in aging identical twins. Proceedings of the National Academy of Science of the United States of America. 2005; 102: 10413–10414.
91. Mastroeni D, McKee A, Grover A, Rogers J, Coleman PD. Epigenetic differences in cortical neurons from a pair of monozygotic twins discordant for Alzheimer's disease. PLoS One. 2009 Aug 12; 4(8):e6617.
92. Matsubayashi H, Sato N, Brune K, et al. Age- and disease-related methylation of multiple genes in nonneoplastic duodenum and in duodenal juice. Clinical Cancer Research. 2005; 11(2 Pt 1):573-83.
93. Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, Fouse SD, Johnson BE, Hong C, Nielsen C, Zhao Y, Turecki G, Delaney A, Varhol R, Thiessen N, Shchors K, Heine VM, Rowitch DH, Xing X, Fiore C, Schillebeeckx M, Jones SJ, Haussler D, Marra MA, Hirst M, Wang T, Costello JF. Conserved role of intragenic DNA methylation in regulating alternative promoters. Nature. 2010; 466:253–257.

94. McClay JL, Aberg KA, Clark SL, Nerella S, Kumar G, Xie LY, Hudson AD, Harada A, Hultman CM, Magnusson PK, Sullivan PF, Van Den Oord EJ. A methylome-wide study of aging using massively parallel sequencing of the methyl-CpG-enriched genomic fraction from blood in over 700 subjects. Human Molecular Genetics. 2014 ;23(5):1175-85.
95. Meloni M and Testa G. Scrutinizing the epigenetics revolution. Biosocieties. 2014 Nov;9(4):431-456.
96. Mendelsohn AR, Larrick JW. Epigenetic Drift Is a Determinant of Mammalian Lifespan. Rejuvenation Res. 2017; 20:430–436.
97. Miller CA, Sweatt JD. Covalent modification of DNA regulates memory formation. Neuron. 2007 Mar 15; 53(6):857-69.
98. Miozzo F, Arnould H, de Thonel A, Schang AL, Sabéran-Djoneidi D, Baudry A, Schneider B, Mezger V. Alcohol exposure promotes DNA methyltransferase DNMT3A upregulation through reactive oxygen species-dependent mechanisms. Cell Stress Chaperones. 2018 Jan;23(1):115-126.
99. Montesanto A, Dato S, Bellizzi D, Rose G, Passarino G. Epidemiological, genetic and epigenetic aspects of the research on healthy ageing and longevity. Immun Ageing. 2012; 9:6.
100. Moore LD, Le T, Fan G. DNA Methylation and Its Basic Function. Neuropsychopharmacology. 2013; 38:23–38.
101. Nakagawa H, Nuovo GJ, Zervos EE, Martin EW Jr, Salovaara R, Aaltonen LA, de la Chapelle A. Age-related hypermethylation of the 5' region of MLH1 in normal colonic mucosa is associated with microsatellite-unstable colorectal cancer development. Cancer Research. 2001; 61(19):6991-5.
102. Naveh-Many T, Cedar H. Active gene sequences are undermethylated. Proceedings of the National Academy of Science of the United States of America. 1981; 78(7): 4246–4250.
103. Nishida N, Nagasaka T, Nishimura T, Ikai I, Boland CR, Goel A. Aberrant methylation of multiple tumor suppressor genes in aging liver, chronic hepatitis, and hepatocellular carcinoma. Hepatology. 2008; 47(3):908-18.
104. Norouzitallab P, Baruah K, Vanrompay D, Bossier P. Can epigenetics translate environmental cues into phenotypes? The Science of the Total Environment. 2018; 647: 1281–1293.
105. Obata Y, Furusawa Y, Hase K. Epigenetic modifications of the immune system in health and disease. Immunol Cell Biol. 2015; 93:226–232.
106. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell. 1999; 99:247–57.
107. Pal S, Tyler JK. Epigenetics and aging. Sci Adv. 2016; 2:1600584.
108. Park JH, Kim SH, Lee MS, Kim MS. Epigenetic modification by dietary factors: Implications in metabolic syndrome. Molecular Aspects of Medicine. 2017; 54:58-70.
109. Patil V, Ward RL, Hesson LB. The evidence for functional non-CpG methylation in mammalian cells. Epigenetics. 2014; 9(6):823–828.
110. Penn NW, Suwalski R, O'Riley C, Bojanowski K, Yura R. The presence of 5-hydroxymethylcytosine in animal deoxyribonucleic acid. Biochem J. 1972; 126:781–790.

111. Perna L, Zhang Y, Mons U, Holleczek B, Saum KU, Brenner H. Epigenetic age acceleration predicts cancer, cardiovascular, and all-cause mortality in a German case cohort. Clin Epigenetics. 2016; 8:64.
112. Pinel C, Prainsack B, McKevitt C. Markers as mediators: A review and synthesis of epigenetics literature. BioSocieties. 2018; 13, Issue 1:276–303.
113. Pinney SE. Mammalian Non-CpG Methylation: Stem Cells and Beyond. 2014;3(4):739–751
114. Rakyan VK, Beck S. Epigenetic variation and inheritance in mammals. Curr Opin Genet Dev. 2006 Dec;16(6):573-7.
115. Rakyan VK, Down TA, Maslau S, Andrew T, Yang TP, Beyan H, Whittaker P, McCann OT, Finer S, Valdes AM, Leslie RD, Deloukas P, Spector TD. Human aging-associated DNA hypermethylation occurs preferentially at bivalent chromatin domains. Genome Res. 2010; 20:434–9.
116. Reynolds LM, Taylor JR, Ding J, Lohman K, Johnson C, Siscovick D, Burke G, Post W, Shea S, Jacobs DR Jr, Stunnenberg H, Kritchevsky SB, Hoeschele I, McCall CE3, Herrington D, Tracy RP, Liu Y. Age-related variations in the methylome associated with gene expression in human monocytes and T cells. Nature Communications. 2014; 5:5366.
117. Rickabaugh TM, Baxter RM, Sehl M, Sinsheimer JS, Hultin PM, Hultin LE, Quach A, Martínez-Maza O, Horvath S, Vilain E, Jamieson BD. Acceleration of age-associated methylation patterns in HIV-1-infected adults. PLoS One. 2015 Mar 25;10(3):e0119201.
118. Rönn T, Poulsen P, Hansson O, Holmkvist J, Almgren P, Nilsson P, Tuomi T, Isomaa B, Groop L, Vaag A, Ling C. Age influences DNA methylation and gene expression of COX7A1 in human skeletal muscle. Diabetologia. 2008;51(7):1159-68.
119. Rossman TG, Klein CB. Genetic and epigenetic effects of environmental arsenicals. Metallomics. 2011 Nov; 3(11):1135-41.
120. Sadakierska-Chudy A, Kostrzewa RM, Filip M. A Comprehensive View of the Epigenetic Landscape Part I: DNA Methylation, Passive and Active DNA Demethylation Pathways and Histone Variants. Neurotox Res. 2015; 27:84–97.
121. Saitou M, Kagiwada S, Kurimoto K. Epigenetic reprogramming in mouse pre-implantation development and primordial germ cells. Development. 2012; 139:15–31.
122. Sánchez-Romero MA, Cota I, Casadesús J. DNA methylation in bacteria: from the methyl group to the methylome. Current Opinion in Microbiology. 2015; 25: 9–16.
123. Sasai N, Defossez PA. Many paths to one goal? The proteins that recognize methylated DNA in eukaryotes. Int J Dev Biol. 2009; 53:323–334.
124. Saxonov S, Berg P, Brutlag DL. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. Proceedings of the Natural Academy of Sciences of the United States of America. 2006; 103(5):1412-1417.
125. Schübeler D. Function and information content of DNA methylation. Nature. 2015; 517:321–326.
126. Sebastiani P, Solovieff N, Dewan AT, Walsh KM, et al. Genetic signatures of exceptional longevity in humans. PLoS One. 2012; 7:29848.

127. Seelan RS, Mukhopadhyay P, Pisano MM, Greene RM. Effects of 5-Aza-2'-deoxycytidine (decitabine) on gene expression. Drug Metab Rev. 2018; 50:193–207.
128. Shenouda SK, Alahari, SK. MicroRNA function in cancer: oncogene or a tumor suppressor? Cancer Metastasis Reviews. 2009; 28:369.
129. Silva PN, Gigek CO, Leal MF, et al. Promoter methylation analysis of SIRT3, SMARCA5, HTERT and CDH1 genes in aging and Alzheimer's disease. Journal of Alzheimer's Disease. 2008; 13(2):173-6.
130. Singal R, Ginder GD. DNA methylation. Blood. 1999; 93:4059–70.
131. Slieker RC, van Iterson M, Luijk R, Beekman M, Zhernakova DV, Moed MH, Mei H, van Galen M, Deelen P, Bonder MJ, Zhernakova A, Uitterlinden AG, Tigchelaar EF, Stehouwer CD, Schalkwijk CG, van der Kallen CJ, Hofman A, van Heemst D, de Geus EJ, van Dongen J, Deelen J, van den Berg LH, van Meurs J, Jansen R, 't Hoen PA, Franke L, Wijmenga C, Veldink JH, Swertz MA, van Greevenbroek MM, van Duijn CM, Boomsma DI; BIOS consortium, Slagboom PE, Heijmans BT. Age-related accrual of methylomic variability is linked to fundamental ageing mechanisms. Genome Biol. 2016; 7:191.
132. So K, Tamura G, Honda T, Homma N, Waki T, Togawa N, Nishizuka S, Motoyama T. Multiple tumor suppressor genes are increasingly methylated with age in non-neoplastic gastric epithelia. Cancer Science. 2006; 97(11):1155-8.
133. Stein AD, Pierik FH, Verrips GHW, Susser ES, Lumey LH. Maternal exposure to the Dutch famine before conception and during pregnancy: quality of life and depressive symptoms in adult offspring. Epidemiology 2009; 20:909–15.
134. Stoccoro A, Siciliano G, Migliore L, Coppedè F. Decreased Methylation of the Mitochondrial D-Loop Region in Late-Onset Alzheimer's Disease. J Alzheimers Dis. 2017;59(2):559-564.
135. Sutherland KD, Lindeman GJ. Choong DY, e Wittlin S, Brentzell L, Phillips W, Campbell IG, Visvader JE. Differential hypermethylation of SOCS genes in ovarian and breast carcinomas. Oncogene. 2004; 23(46):7726-33.
136. Szulwach KE, Li X, Li Y, Song CX, Wu H, Dai Q, Irier H, Upadhyay AK, Gearing M, Levey AI, Vasanthakumar A, Godley LA, Chang Q, Cheng X, He C, JinP. 5-hmC–mediated epigenetic dynamics during postnatal neurodevelopment and aging Keith. Nat Neurosci. 2012; 14:1607–16.
137. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science. 2009; 324:930–5.
138. Tan Q, Christiansen L, Thomassen M, Kruse TA, Christensen K. Twins for epigenetic studies of human aging and development. Ageing Res Rev. 2013; 12:182–187.
139. Teschendorff AE, Menon U, Gentry-Maharaj A, Ramus SJ, Weisenberger DJ, Shen H, Campan M, Noushmehr H, Bell CG, Maxwell AP, Savage DA, Mueller-Holzner E, Marth C, Kocjan G, Gayther SA, Jones A, Beck S, Wagner W, Laird PW, Jacobs IJ, Widschwendter M. Age-dependent DNA methylation of genes that are suppressed in stem cells is a hallmark of cancer. Genome Res 2010;

20:440–446.

140. Truong TP, Sakata-Yanagimoto M, Yamada M, Nagae G, Enami T, Nakamoto-Matsubara R, Aburatani H, Chiba S. Influence of Age-Dependent Decrease of DNA Hydroxymethylation in Human T Cells. J Clin Exp Hematop. 2015; 55:1–6.
141. Vickers MH. Early life nutrition, epigenetics and programming of later life disease. Nutrients. 2014; 6:2165–2178.
142. Vidal AC, Benjamin Neelon SE, Liu Y, Maternal stress, preterm birth, and DNA methylation at imprint regulatory sequences in humans. Genetics & Epigenetics. 2014; 6:37-44.
143. Vijg J, Dollé ME. Genome instability: cancer or aging? Mechanisms of Ageing Development. 2007; 128:466–468.
144. Virmani AK, Rathi A, Sathyanarayana UG, Padar A, Huang CX, Cunnigham HT, Farinas AJ, Milchgrub S, Euhus DM, Gilcrease M, Herman J, Minna JD, Gazdar AF. Aberrant methylation of the adenomatous polyposis coli (APC) gene promoter 1A in breast and lung carcinomas. Clinical Cancer Research. 2001; 7(7):1998-2004.
145. Waddington CH. The epigenotype. Endeavour. 1942;1:18–20.
146. Waddington CH. The Strategy of the Genes; a Discussion of Some Aspects of Theoretical Biology. Allen & Unwin, London (1957).
147. Waechter DE, Baserga R. Effect of methylation on expression of microinjected genes. Proc. Natl. Acad. Sci. U. S. A. 1982; 79:1106–1110.
148. Waki T, Tamura G, Sato M, Motoyama T. Age-related methylation of tumor suppressor and tumor-related genes: an analysis of autopsy samples. Oncogene. 2003; 22(26):4128-33.
149. Watt F, Molloy PL. Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter. Genes Dev 1988; 2:1136–1143.
150. Wei L, Liu B, Tuo J, Shen D, Chen P, Li Z, Liu X, Ni J, Dagur P, Sen HN, Jawad S, Ling D, Park S, Chakrabarty S, Meyerle C, Agron E, Ferris FL 3rd, Chew EY, McCoy JP, Blum E, Francis PJ, Klein ML, Guymer RH, Baird PN, Chan CC, Nussenblatt RB. Hypomethylation of the IL17RC promoter associates with age-related macular degeneration. Cell Reports. 2012; 2(5): 1151-8.
151. Whitelaw NC and Whitelaw E. How lifetimes shape epigenotype within and across generations. Hum Mol Genet. 2006 Oct 15; 15 Spec No 2:R131-7.
152. Wong M, Gertz B, Chestnut BA, Martin LJ. Mitochondrial DNMT3A and DNA methylation in skeletal muscle and CNS of transgenic mouse models of ALS. Front Cell Neurosci. 2013 Dec 25;7:279.
153. Wu TP, Wang T, Seetin MG, Lai Y, Zhu S4, Lin K1, Liu Y1, Byrum SD, Mackintosh SG, Zhong M, Tackett A, Wang G, Hon LS, Fang G, Swenberg JA, Xiao AZ. DNA methylation on N(6)-adenine in mammalian embryonic stem cells. Nature. 2016; 532:329–333.
154. Wu X, Zhang Y. TET-mediated active DNA demethylation: mechanism, function and beyond. Nature Reviews Genetics. 2017; 18:517–534.

155. Yan X, Ehnert S, Culmes M, Bachmann A, Seeliger C, Schyschka L, Wang Z, Rahmanian-Schwarz A, Stöckle U, De Sousa PA, Pelisek J, Nussler AK. 5-azacytidine improves the osteogenic differentiation potential of aged human adipose-derived mesenchymal stem cells by DNA demethylation. PLoS One. 2014; 9:90846.
156. Yin Y, Morgunova E, Jolma A, Kaasinen E, Sahu B, Khund-Sayeed S, Das PK, Kivioja T, Dave K, Zhong F, Nitta KR, Taipale M, Popov A, Ginno PA, Domcke S, Yan J, Schübeler D, Vinson C, Taipale J. Impact of cytosine methylation on DNA binding specificities of human transcription factors. Science. 2017; 356(6337).
157. Yisraeli J, Frank D, Razin A, Cedar H. Effect of in vitro DNA methylation on beta-globin gene expression. Proc Natl Acad Sci 1988; 85:4638–4642.
158. Yuan Y, Qian ZR, Sano T, Asa SL, Yamada S, Kagawa N, Kudo E. Reduction of GSTP1 expression by DNA methylation correlates with clinicopathological features in pituitary adenomas. Modern Pathology. 2008; 21(7):856-65.
159. Zampieri M, Ciccarone F, Calabrese R, Franceschi C, Bürkle A, Caiafa P. Reconfiguration of DNA methylation in aging. Mechanisms of Ageing and Development. 2015; 151: 60-70.
160. Zannas AS, Arloth J, Carrillo-Roa T, Iurato S, Röh S, Ressler KJ, Nemeroff CB, Smith AK, Bradley B, Heim C, Menke A, Lange JF, Brückl T, Ising M, Wray NR, Erhardt A, Binder EB, Mehta D. Lifetime stress accelerates epigenetic aging in an urban, African American cohort: relevance of glucocorticoid signaling. Genome Biol. 2015;16:1–12.
161. Zhang Z, Deng C, Lu Q, Richardson B. Age-dependent DNA methylation changes in the ITGAL (CD11a) promoter. Mech Ageing Dev. 2002; 123:1257–1268
162. Zheng YC, Ma JL, Liu Y, Liu HM. Writers and Erasers of Histone Lysine methylation with Clinically Applied Modulators: Promising Target for Cancer Therapy. Curr Pharm Des. 2016;22(39):5943-5947.
163. Zhu J, He F, Hu S, Yu J. On the nature of human housekeeping genes. Trends in Genetics. 2008; 24:481–484.
164. Zhu S, Beaulaurier J, Deikus G, Wu TP, Strahl M, Hao Z, Luo G, Gregory JA, Chess A, He C, Xiao A, Sebra R, Schadt EE, Fang G. Mapping and characterizing N6-methyladenine in eukaryotic genomes using single-molecule real-time sequencing. Genome Res. 2018 Jul;28(7):1067-1078.

Chapter II.

Original Research Work:

Epigenetic signature: implications for mitochondrial quality control in human aging

Epigenetic signature: implications for mitochondrial quality control in human aging

Patrizia D'Aquila*, Alberto Montesanto*, Francesco De Rango, Francesco Guarasci, Giuseppe Passarino[§], Dina Bellizzi[§]

Department of Biology, Ecology and Earth Sciences, University of Calabria, 87036 Rende, Italy

*these authors contributed equally to this work

[§]**Correspondence to:** Dina Bellizzi, Department of Biology, Ecology and Earth Sciences, University of Calabria, 87036 Rende, Italy. Phone: +390984492930, E-mail: dina.bellizzi@unical.it and Giuseppe Passarino, Department of Biology, Ecology and Earth Sciences, University of Calabria,87036Rende, Italy. Phone: +390984492932, E-mail: giuseppe.passarino@unical.it

E-mail addresses of all the authors:

PDA: d_patrizia2002@yahoo.it

AM: alberto.montesanto@unical.it

FDR: fderango@unical.it

FG: guarasci.francesco@gmail.com

GP: giuseppe.passarino@unical.it

DB: dina.bellizzi@unical.it

Keywords: DNA methylation, aging biomarkers, epigenetics biomarkers, mitochondrial quality control, geriatric parameters, chronological age, biological, age

Abstract

Maintenance of functional mitochondria is essential to prevent damage leading to aging and diseases. What is more, the research of biomarkers of aging is focusing on better predicting functional capability along the lifetime beyond chronological age. Aim of this study was to identify novel CpG sites the methylation of which might be correlated to the chronological and biological age. We performed methylation analyses of the CpG sites in candidate genes involved in mitochondrial biogenesis, mitophagy, fusion, and fission, all key quality control mechanisms to ensure maintenance of healthy mitochondria and homeostasis during aging, using DNA samples from two independent datasets composed by 381 and 468 differently-aged individuals, respectively. Twelve potential CpG predictors resulted associated with aging in the discovery dataset. Of these, two sites located within *RAB32* and *RHOT2* genes were replicated in the second dataset. What is more, individuals exhibiting methylation levels of the *RAB32* CpG site higher than 10% were observed more prone to disability than people with lower levels.

These results seem to provide the first evidence that epigenetic modifications of genes involved in mitochondrial quality control occur over time according to the aging decline, and may then represent potential biomarkers of both chronological and biological age.

Introduction

Research over the past decades largely demonstrated that mitochondria have a crucial role in aging. The progressive production of ROS leads to an accumulation of somatic mitochondrial DNA (mtDNA) mutations and protein modifications with a consequent decline in oxidative phosphorylation (OXPHOS) activity, loss of bioenergetic capacity, and oxidative stress [1-4]. Mammalian cells adopt several systems in order to maintain mitochondrial integrity and function. This mitochondrial quality control includes pathways related to protein folding and degradation as well as systems involved in organelle turnover, shape, and movement [5-7]. An accurate and constant mitochondrial turnover is based on the coordination of biogenesis, occurring by growth and division of pre-existent mitochondria, and mitophagy, namely the self-destruction of damaged/dysfunctional mitochondria via the autophagic pathway. Multiple endogenous and exogenous factors, including nutrients, hormones, exercise, stress, cold exposure, and hypoxia, regulate mitochondrial biogenesis through ubiquitous transcription factors (SP1, YY1, CREB), nuclear respiratory factors (NRF-1, NRF-2) and coactivators (PGC-1α, PGC-1β, PRC)[8-11].In healthy cells, the elimination of damaged mitochondria in mammals by mitophagy is essential and is mediated by a pathway mainly composed of the PTEN-induced putative protein kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin [12-14]. Furthermore, mitochondria are highly mobile and rapidly change their size, shape, and distribution by continuous integration of events of fusion, the joining of two organelles into one, and fission, the division of a single organelle into two. All the above processes, mainly induced by cellular metabolic states, result in the maintenance of a healthy mitochondrial population, pivotal for cell survival and adaption to changing physiological conditions, and thus particularly important to forestall aging [7, 15, 16]. In fact, transgenic mice carrying specific mutations in mitofusin 1 (*MFN1*), mitofusin2 (*MFN2*) and optic atrophy (*OPA1*) as well as in dynamin-related protein 1 (*DRP1*) and fission 1 (*FIS1*) genes, the master mediators of mitochondrial fusion and fission, respectively, exhibit dramatic decrease in mtDNA content, mitochondrial function swelling, compensatory proliferation, severe

fragmentation of the mitochondrial network, suppression of content exchange between mitochondria, and production of interconnected mitochondria [7, 16, 17-
21]. Several findings highlighted that all the mitochondrial processes above described do not operate independently but they rather influence each other and are subject to concerted regulatory pathways [7].

Deregulation of mitochondrial quality control is one of the intrinsic causes of mitochondrial dysfunction which leads to aging and age-related diseases [22-25]. Several studies demonstrated that mitochondrial fusion is positively associated with the increase of survival in *C. elegans*, and with the exercise-induced longevity in both rodents and worms [26-28]. Conversely, the Mfn2-to-Drp1 ratio, named mitochondrial fusion index, was found significantly increased in muscles of aged rats compared to younger, suggesting an involvement of the fusion in inducing sarcopenia and muscle decay [29]. An age-correlated imbalance in fusion and fission events might increase the mitochondrial mass, if fusion is more prevalent, or mitochondrial number, when fission is more frequent, if extra mitochondria are not eliminated by mitophagy. Mitochondrial biogenesis should occur in order to compensate the decreased mitochondrial biomass resulting from mitochondrial degradation [30]. Consistently, *in vivo* and *in vitro* experiments demonstrated a decline of mitophagy pathway with age and an accumulation in aged cells of enlarged (giant) or highly interconnected mitochondria, with low ATP production, loss of cristae structure and swollen morphology [31].

Here, we aimed to disentangle the correlation between the above processes and aging from an epigenetic point of view. In fact, research identifying biomarkers of aging made extensive use of epigenetic changes of specific loci occurring during lifetime.

To this purpose, we performed a methylation analysis of 1437 CpG sites in candidate genes involved in mitochondrial biogenesis, mitophagy, fusion, and fission in order to evaluate the epigenetic variability of these sites in human aging and aging phenotypes. This analysis was performed by applying the Sequenom EpiTYPER technology to peripheral blood DNAs from two population

samples including 381 (discovery dataset) and 468 (replication dataset) differently-aged individuals, respectively.

RESULTS

CpG methylation profiling of mitochondrial quality control candidate genes in differently aged humans

We analysed DNA methylation profiles using Sequenom MassARRAY EpiTYPER, a bisulphite-based technology that relies on base-specific cleavage and mass spectrometry and measures the level of methylation in amplicons containing one or more CpGs. To assess potential epigenetic changes during aging, the profiles were investigated in bisulfite-treated DNA samples collected from 381 subjects (48- to 107-year-old) of the discovery dataset. Specifically, we investigated a total of 1437 CpG sites located within CpG islands falling in candidate genes involved in mitochondrial biogenesis, mitophagy, fusion, and fission processes and annotated in the UCSC genome browser. Following stringent quality control criteria, a total of 500 CpG sites entered further analysis. The sequences of the CpG islands containing these sites and their chromosomal localization are shown in Figure S1. The results of the univariate linear regression analysis are presented in Table 1 in which we observed that 54 out of the 500 potential predictors, underlined in Figure S1, are statistically significant after the Holm method to account for multiple testing. The methylation values of these 54 CpG sites are reported in Figure 1.

Starting from the set of 54 CpG units selected by the univariate analysis, we performed a stringent bootstrap-based stepwise selection procedure to remove potential spurious statistical associations. We found that among the most informative CpG markers (markers included in at least 80% of simulated models) there were BNIP3L_Amplicon1_CpG_10 (99.39% times), COX18_CpG_2 (85.16% times), COX18_CpG_15 (81.42% times), GABARAP_Amplicon2_CpG_7.8 (81.42% times), MARCH5_Amplicon1_CpG_2.3.4 (90.76% times), RAB32_Amplicon1_CpG_24 (86.6% times),

RHOT2_Amplicon1_CpG_16 (91.31% times) and TFB1M_CpG_12.13 (80.83% times). The model including all these variables explained about 43.06% of age variance of the study sample with a cross-validated mean absolute deviation (MAD) calculated for the entire discovery dataset of about 9.6 years. Figure 2 shows a plot with chronological age versus age predicted from the 8 CpG sites included in stepwise regression model.

In order to validate our findings, the CpG units selected by the permutation approach in the discovery dataset were tested in the replication dataset. Table 2 reports the results of the association obtained from the test. As it can be observed, we successfully replicated 2 out 8 significant associations and, in particular, those involving RAB32_Amplicon1_CpG_24 and RHOT2_Amplicon1_CpG_16 units.

Correlation of methylation profiles with geriatric components and survival at old age.

We verified whether the variability of the two validated CpG markers (RAB32_Amplicon1_CpG_24 and RHOT2_Amplicon1_CpG_16) might affect the quality of life in the 60-89 years old group of the discovery dataset. In particular, this age group was analysed for examining the association between the methylation level variability at the analysed units and physical and cognitive abilities measured by geriatric assessments, including Hand Grip (HG), Activity Daily Living (ADL), Mini Mental State Examination (MMSE) and Geriatric Depression Scale (GDS). As shown in Table 3 we found that RAB32_Amplicon1_CpG_24 epigenetic variability was correlated with the risk of disability. In particular, subjects with methylation levels higher that 10% were more than twice as likely to develop disability than people with low methylation levels at that site. A borderline association was also detected between higher methylation level (>10%) at the RHOT2_Amplicon1_CpG_16 and MMSE performances.

Expression of *RAB32* and *RHOT2* genes

To explore the functional relevance of the methylation of the CpG islands located within *RAB32* and *RHOT2* genes, quantitative real-time PCR assays were carried out in order to evaluate the expression levels of mRNA in the samples of different ages. Consistently with the methylation patterns above described, we observed that mRNA levels of *RAB32* and *RHOT2* genes decrease and increase, respectively, during age further confirming their potential role as biomarkers of both chronological and biological age (Figure 3A and 3B).

DISCUSSION

Recent progress in the field of DNA methylation has enabled the identification of numerous genes, and at the same time of a large set of CpG dinucleotides within them, showing a linear correlation of their methylation status with age. These "clock CpGs" can estimate the age of cells, tissues or organs and can predict mortality and time of death [32-37]. In our study, we aimed to reveal novel gene associated CpG sites which exhibit changes in their methylation status during the age. Considering that mitochondrial biogenesis, fusion, fission, and mitophagy contribute to ensuring the quality and the maintenance of healthy mitochondria during age, we explored the methylation levels of candidate genes involved in these processes, so far not investigated. For this purpose, we performed a methylation analysis of DNA samples from whole blood of differently aged human individuals displaying different phenotypes according to cognitive, functional and psychological parameters [38]. Of the 500 sites explored in the univariate linear regression analysis, we identified 54 CpG sites, belonging to 36 genomic regions, differently methylated between young and old subjects from which the highest 12 correlated with the age were selected as significant predictors by very robust bootstrap analysis. Of these 12, two CpGs sites, CpG_24 in *RAB32* and CpG_16 in *RHOT2* genes were confirmed by replication in an independent sample, thus supporting the usefulness of these genes as predictive biomarkers of chronological aging in humans. *RAB32* encodes a GTPase related to the oncogene RAS and plays a role in mitochondrial fission and mitophagy processes and in apoptosis as

well. *RHOT2* encodes a protein localized to the outer mitochondrial membrane and is involved in mitochondrial trafficking and fusion-fission dynamics. We detected a strong hyper- and hypo-methylation of *RAB32* and *RHOT2* genes, respectively, with age, in line with the several lines of evidence highlighting the complex nature of the relationship between DNA methylation and aging. In fact, if demethylation was largely described at the global level, hypermethylation and loss of methylation with aging were demonstrated occurring in gene associated CpG sites [39-43]. It is worth mentioning that the analysis of the methylation profile of the two genes in rats of different ages showed the existence of DNA methylation trends in blood similar to humans, but different patterns across heart, liver, kidney and brain tissues (data not shown).

The biological impact of the methylation status of *RAB32* and *RHOT2* genes is highlighted in the expression analysis in which we demonstrated a decrease and an increase, respectively, of mRNA levels of the two genes. It is noteworthy that the *in silico* analysis revealed that CpG_24 in *RAB32* and CpG_16 in *RHOT2* genes overlap with potential c-Jun and E2F-1 binding sites, respectively, which are widely reported being methylation-sensitive transcription factors [44-46]. Their role in regulating the activity of the above CpG sites remains to be further experimentally validated. Therefore, the expression levels of *RAB32* and *RHOT2* genes are regulated during aging by epigenetic mechanisms and it is plausible to hypothesize that they result in regulation of the mitochondrial quality control network, the effects of which, considering the role in this process of multiple molecular components, are not easily predictable. Future studies of overexpression or silencing of the two genes will be able to delineate their role in the above-mentioned network.

It is interesting to note that *RAB32* gene is also included in the Horvath's epigenetic clock, one of the most accurate and precise estimate of chronological age found in the human brain and other solid tissues. This evidence not only suggests the role of this gene as aging biomarker but also that our findings are not specific to blood tissue but are also valid for cells from other tissues. On the contrary, Horvath's epigenetic clock as well as other epigenetic models for age prediction completely neglected

ROTH2 gene making this gene a potential biomarker useful to improve the prediction accuracy of such model.

It is possible to assume that *RAB32* CpG site is not only a biomarker of chronological but also of biological age since we observed that individuals with methylation levels of this site higher than 10% are twice as likely to be prone to disability with respect to those with lower levels. This result is in line with multiple literature data demonstrating that individuals at the same chronological age may possess dissimilar biomarker signatures reflecting their biological age.

Our study emphasizes the existence of epigenetic regulation of genes coding for components of the mitochondrial quality control, highlighting the role that this control may play during age and the importance of methylated specific CpGs as promising biomarker for chronological and biological age.

MATERIALS AND METHODS

Population samples

The discovery dataset included 381 unrelated healthy individuals (185 men end 196 women) aged 48 to 107 years. The subjects older 60 years underwent a thorough geriatric assessment and a structured interview including the administration of a questionnaire validated at European level. The questionnaire collected socio-demographic information, anthropometric measures and a set of the most common tests to assess cognitive functioning, functional activity, physical performance, and depression. In particular, cognitive status was rated by Mini Mental State Examination (MMSE) [47]. Hand grip strength was measured by using a handheld dynamometer (SMEDLEY's dynamometer TTM) while the subject was sitting with the arm close to his/her body. The test was repeated three times with the stronger hand and the maximum of these values was used in the analyses. The management of activities of daily living (bathing, dressing, eating, independence in and out of bed)

was assessed by using the Katz' Index of activities of daily living [48]. Depressive symptoms were assessed using the 15-item Geriatric Depression Scale [49].

The replication dataset included 468 healthy subjects (206 men and 262 women) aged 18 to 108 years which were recruited at the INRCA Hospital, which is a reference point for the care of the aging people in the Calabria. Also this groups of subjects underwent through a geriatric assessment. Fully informed consent was obtained in writing from all the participants, and all the studies were approved by the Local Ethics Committee.

DNA samples

Six millilitres of venous blood were drawn from each human subject. Plasma/sera were used for routine laboratory analyses, while DNA was extracted from buffy coats following standard procedures.

Genomic DNA was obtained by phenol/chloroform purification. The DNA concentration and purity were determined spectrophotometrically.

Primer design for EpiTYPER assay

PCR primers for the genes of interest were designed using Sequenom's EpiDesigner software (Table S1). They do not contain CpGs, amplify both methylated and unmethylated sequences equally, and delimit amplicon of size below 300 bp to increase the amplification success rate, covering as many CpGs as possible.

A T7-promoter tag (CAGTAATACGACTCACTATAGGGAGAAGGCT) was added to the reverse primers for the *in vitro* T7 transcription and a 10-mer tag sequence (AGGAAGAGAG) was added to the forward primers to balance the PCR primer length.

Bisulfite treatment and PCR conditions

Bisulfite conversion of each DNA sample was performed using the EZ-96 DNA Methylation-Gold kit (Zymo Research, Euroclone, Milan, Italy), according to the manufacturer's protocol. Briefly, 1 μg of genomic DNA was added to 130 μl of CT conversion reagent in a final volume of 150 μl. The mix was incubated at 98 °C for 10 minutes and, successively, at 64 °C for 2.5 hours. After adding 400 μl of M-binding buffer to the wells of the silicon-A binding plate, each sample was loaded into the wells and centrifuged at 3000 g for 5 minutes. After adding of 400 μl of M-wash buffer to the wells and centrifugation at 3000 g for 5 minutes, 200 μl of M-desulfonation buffer was added to each well and incubated at room temperature for 20 minutes. Then, the solution was removed by centrifugation at 3000 g for 5 minutes and the wells were washed twice with 400 μl of M-wash buffer. Deaminated DNA was eluted in 30 μl of M-elution buffer. The PCRs were carried out in a total volume of 5 μl using 1 μl of bisulfite-treated DNA, EpiTaq PCR buffer 1X, 0.4 μM of each primer, 0.3 mM dNTP mixture, 2.5 mM of $MgCl_2$, and 0.005 U TaKaRa EpiTaq HS (TaKaRa, Diatech Lab Line, Milan, Italy). The thermal profile used for the reaction included a 4 minute heat activation of the enzyme at 95 °C, followed by 45 cycles of denaturation at 94 °C for 20 seconds, annealing at optimal temperature for each primer pair (Table S1) for 30 seconds, extension at 72 °C for 1 minute, then one cycle at 72 °C for minutes. 0.5 μl of each PCR product was electrophoresed on 1.5% agarose gel to confirm successful PCR and amplification specificity.

Dephosphorylation of unincorporated deoxynucleoside triphosphates and *in vitro* transcription and RNaseA cleavage

Unincorporated dNTPs in the amplification products were dephosphorylated by adding 1.7 μl DNase free water and 0.3 μl (0.5 U) shrimp alkaline phosphatase (SAP) (Sequenom, Inc., San Diego, CA, USA). Each reaction was incubated at 37 °C for 40 minutes, and SAP was then heat inactivated at 85

°C for 5 minutes. Subsequently, samples were incubated at 37 °C for 3 hours with 5 μl of T-cleavage reaction mix (Sequenom), containing 3.21 μl RNAse-free water, 0.89 μl 5X T7 polymerase buffer, 0.22 μl T-cleavage mix, 0.22 μl 100 mM DTT, 0.40 μl T7 RNA polymerase and 0.06 μl RNase A, for concurrent *in vitro* transcription and base-specific cleavage. The samples of cleaved fragments were then diluted with 20 μl water. Conditioning of the cleavage reaction was carried out by adding 6 mg of clean resin.

Mass spectrometry

10 nl of the resultant cleavage reactions were spotted onto silicon matrix preloaded chips (Spectro-CHIP; Sequenom) using the MassARRAY nanodispenser (Sequenom) and analysed using the MassARRAY Compact System matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer (MALDI-TOF) (Sequenom). The spectra's methylation ratios were calculated using EPITYPER software v1.0 (Sequenom). The method yields quantitative results for each of the sequence-defined analytic units referred as CpG units, which may contain either one individual CpG site or an aggregate of CpG sites. Triplicate independent analyses from sodium bisulfite-treated DNA samples were undertaken. The effectiveness of the entire experimental procedure was assessed by analyzing as control CpGenome Universal Unmethylated DNA (Chemicon) and CpGenome Universal Methylated DNA (Chemicon, Millipore, Germany) in serial mixtures of methylated and unmethylated products, with 10% methylation increments. Data quality control and filtering were carried out by the removal of the CpG dinucleotides whose the measurement success rate was <90%. Poor-quality and non-valuable data for the quantitative methylation of each CpG unit measured by MALDI-TOF-MS were excluded.

Expression profile analysis of the *RAB32* and *RHOT2* genes

The total RNA was extracted from the blood of individuals of various age (30–90 years old) using ReliaPrep RNA Tissue Miniprep System (PromegaCorp, Italy). Briefly, 2.5 ml of fresh whole blood was mixed to 7.5 ml of RNA red blood cell lysis solution and incubated at room temperature for 10 minutes. White cells were isolated by centrifugating samples at 3000 g for 10 minutes and lysed in 200 µl of LBA+TG buffer and 85% µl of isopropanol. The lysate was then transferred to a ReliaPrep minicolumn, and RNA was purified according to manufacturer's recommendation.

The RNA concentration was measured for each sample using a spectrophotometer and purity of the sample was evaluated using the 260/280 nm absorbance ratio. RNA samples were treated with DNA-free DNase to remove any residual genomic DNA contamination.

Reverse transcriptase-PCRs (RT–PCR) were carried out using the RevertAid RT Kit (Thermo Fisher Scientific, Milan, Italy). First, a RT mix including 500 ng of total RNA and 1 µl of Oligo(dT)18 primers was preheated at 65 °C for 5 minutes. Then, the reaction was carried out in a 20 µl final volume containing 1X reaction buffer, 20 U of RiboLock RNase inhibitor, 1 mm of dNTP mix, and 200 U of RevertAid M-MuLV RT reverse transcriptase. The mix was incubated at at 42 °C for 60 minutes and, successively, at 70 °C for 5 minutes to inactivate the reverse transcriptase. The cDNAs obtained were then used as a template for real-time PCRs carried out using the SYBR Green qPCR Master Mix (Promega) in a StepOne Plus machine (Applied Biosystems, Milan, Italy).

The final PCR mixture (10 µl) contained 1µl of cDNA, 1X GoTaq qPCR Master Mix, 0.2 µmoles of each primer, and 1X CXR reference dye. Forward and reverse primers were as follows: RAB32For 5'-CAGGTGGACCAATTCTGCAAA-3'; RAB32Rev 5'-GGCAGCTTCCTCTATGTTTATGT-3'; RHOT2For 5'-TGGAGCTGACTGCGGACTAT-3'; RHOT2Rev 5'-TCTGCACAAACTGGTAGC CAA-3'; GAPDHFor 5'-ATGGGGAAGGTGAAGGTCG-3'; GAPDHRev 5'-GGGGTCATTGAT GGCAACAATA-3'. The thermal profile used for the reaction included a 2 minutes heat activation of the enzyme at 95 °C, followed by 35 cycles of denaturation at 95 °C for 15 seconds and

annealing/extension at 60 °C for 60 seconds, followed by melt analysis ramping at 60–95 °C. All measurements were taken in the log phase of amplification. Negative controls (in which water instead of cDNA was added) were also run in each plate. StepOne Software V 2.0 was used to analyze data. Gene expression values were normalized to GAPDH gene expression, used as internal control. In addition, the normalized values measured in the 30-year-old human were used as reference values (relative quantification) for the other samples.

Statistical analyses

Linear regression analyses were carried out to evaluate the level of DNA methylation as a function of age. To evaluate the combined effect of the analysed CpG markers with respect to the age of sample under study, we used a stepwise variable selection procedure based on the Akaike information criterion (AIC) combined with a bootstrap re-sampling method. In brief, this algorithm firstly simulates a new dataset taking a sample with replacement from the original dataset; secondly, it runs a stepwise selection procedure based on AIC on this simulated dataset, and, finally, repeat the previous steps *n* times. At the end of this procedure, this algorithm records how many times each variable (i) was selected, (ii) the estimate of the regression coefficient was statistically significant, and (iii) changed signs. The final model was selected by retrieving the variables that were selected in at least 80% of the bootstrap samples using linear regression. The prediction accuracy of the developed prediction model was assessed using the adjusted R^2 parameter, which is a measure of the proportion of age variance explained by a particular set of CpG predictors and their combined effect. The final model was further validated using a Leave-One-Out Cross Validation (LOOCV) approach. In particular, LOOCV uses a single observation from the original sample (discovery dataset) as the validation data, and the remaining observations as the training data. This is repeated such that each observation in the sample is used once as the validation data. In this case, the mean absolute deviation (MAD) obtained in the discovery dataset was used as a performance measure.

Statistical analyses were performed using the R statistical language program (http://www.R-project.org/). In particular, the CpGassoc package was used to perform a linear regression analysis using the Holm method to account for multiple testing; the bootStepAIC package was used to perform stepwise variable selection procedure combined with a bootstrap resampling approach and, finally, *ggplot2* packages were used for graphics purpose.

Student's t-test was adopted to compare methylation profiles with respect to the analysed geriatric parameters.

AUTHOR CONTRIBUTIONS

PDA, AM, DB, GP: designed the study; PDA and FG: performed the experiments; AM and FDR: performed the statistical analysis; DB and GP: wrote the initial draft; PDA, AM, FDR, FG, DB and GP: participated in critical revision and approved the final manuscript before submission.

ACKNOWLEDGEMENTS

The work has been made possible by the collaboration with the nursing homes of SADEL S.p.A (San Teodoro, San Raffaele, Villa del Rosario, A.G.I srl, SAVELLI HOSPITAL, Casa di Cura Madonna dello Scoglio) in the frame of the agreement "SOLUZIONI INNOVATIVE PER L'INNALZAMENTO DELLA SALUTE E DELLA SICUREZZA DELLA POPOLAZIONE" with the University of Calabria.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

REFERENCES

1. Loeb LA, Wallace DC, Martin GM. The mitochondrial theory of aging and its relationship to reactive oxygen species damage and somatic mtDNA mutations. Proc Natl Acad Sci U S A. 2005; 102:18769-70.
2. Sanz A, Stefanatos RK. The mitochondrial free radical theory of aging: a critical view. Curr Aging Sci. 2008; 1:10-21.
3. Kong Y, Trabucco SE, Zhang H. Oxidative stress, mitochondrial dysfunction and the mitochondria theory of aging. Interdiscip Top Gerontol. 2014; 39:86-107.
4. Lightowlers RN, Taylor RW, Turnbull DM. Mutations causing mitochondrial disease: What is new and what challenges remain? Science. 2015; 349:1494-99.
5. Lopez-Lluch G, Irusta PM, Navas P, de Cabo R. Mitochondrial biogenesis and healthy aging. Exp Gerontol. 2008; 43:813-19.
6. Fischer F, Hamann A, Osiewacz HD. Mitochondrial quality control: an integrated network of pathways. Trends Biochem Sci. 2012; 37:284-92.
7. Yu SB, Pekkurnaz GJ. Mechanisms Orchestrating Mitochondrial Dynamics for Energy Homeostasis.Mol Biol. 2018; 430:3922-41.
8. Jornayvaz FJ, Shulman GI. Regulation of mitochondrial biogenesis. Essays Biochem.2010; 47:10.1042/bse0470069.
9. Scarpulla RC. Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network. Biochim Biophys Acta. 2011; 1813:1269-78.
10. Bragoszewski P, Turek M, Chacinska A. Control of mitochondrial biogenesis and function by the ubiquitin-proteasome system. Open Biol. 2017; 7: pii: 170007.
11. Callegari S, Dennerlein S. Sensing the Stress: A Role for the UPRmt and UPRam in the Quality Control of Mitochondria. Front Cell Dev Biol. 2018; 6:31.
12. Voigt A. Berlemann LA, Winklhofer KF. The mitochondrial kinase PINK1: functions beyond mitophagy. J Neurochem. 2016; 139 Suppl 1:232-39.

13. Yamano K, Matsuda N, Tanaka K. The ubiquitin signal and autophagy: an orchestrated dance leading to mitochondrial degradation. EMBO Rep. 2016; 17:300-16.
14. Villa E, Marchetti S, Ricci JE. No Parkin Zone: Mitophagy without Parkin. Trends Cell Biol. 2018; 28.882-95.
15. Westermann B. Mitochondrial fusion and fission in cell life and death. Mol Cell Biol. 2010; 11:872-84.
16. Prashant M, Chan DC. Metabolic regulation of mitochondrial dynamics. J Cell Biol. 2016; 2:379-87.
17. Chen H, Chomyn A, Chan DC. Disruption of fusion results in mitochondrial heterogeneity and dysfunction. J Biol Chem. 2005; 280:26185-92.
18. Chan DC. Mitochondrial fusion and fission in mammals. Annu Rev Cell Dev Biol. 2006; 22:79-99.
19. Hoppins S, Lackner L, Nunnari J. The machines that divide and fuse mitochondria. Annu Rev Biochem. 2007; 76:751-80.
20. Otera H, Wang C, Cleland MM, Setoguchi K, Yokota S, Youle RJ, Mihara K. Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. J Cell Biol. 2010; 191:1141-58.
21. Chen H, Chan DC. Physiological functions of mitochondrial fusion. Ann N Y Acad Sci. 2010; 1201:21-25.
22. Figge MT1, Reichert AS, Meyer-Hermann M, Osiewacz HD. Deceleration of fusion-fission cycles improves mitochondrial quality control during aging. PLoS Comput Biol. 2012; 8:e1002576.
23. Payne BA, Chinnery PF. Mitochondrial dysfunction in aging: Much progress but many unresolved questions. Biochim Biophys Acta. 2015; 1847:1347-53.
24. Diot A, Morten K, Poulton J. Mitophagy plays a central role in mitochondrial ageing. Mamm Genome. 2016; 27:381-95.

25. Srivastava S. The Mitochondrial Basis of Aging and Age-Related Disorders. Genes (Basel). 2017; 8:pii: E398.

26. Picard M, Shirihai OS, Gentil BJ, Burelle Y. Mitochondrial morphology transitions and functions: implications for retrograde signaling? Am J Physiol Regul Integr Comp Physiol. 2013; 304:R393-406.

27. Chaudhari SN, Kipreos ET. Increased mitochondrial fusion allows the survival of older animals in diverse *C. elegans* longevity pathways. Nat Commun. 2017; 8:182.

28. Distefano G, Goodpaster BH. Effects of Exercise and Aging on Skeletal Muscle. Cold Spring Harb Perspect Med. 2018; 8:pii: a029785.

29. Leduc-Gaudet JP, Picard M, St-Jean Pelletier F, Sgarioto N, Auger MJ, Vallée J, Robitaille R, St-Pierre DH, Gouspillou G. Mitochondrial morphology is altered in atrophied skeletal muscle of aged mice. Oncotarget. 2015; 6:17923-37.

30. Seo AY, Joseph AM, Dutta D, Hwang JCY, Aris JP, Leeuwenburgh C. New insights into the role of mitochondria in aging: mitochondrial dynamics and more. J Cell Sci. 2010; 123: 2533-42.

31. Dalle Pezze P, Nelson G, Otten EG, Korolchuk VI, Kirkwood TB, von Zglinicki T, Shanley DP. Dynamic modelling of pathways to cellular senescence reveals strategies for targeted interventions. PLoS Comput Biol. 2014; 10:e1003728.

32. Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sadda S, Klotzle B, Bibikova M, Fan JB, Gao Y, Deconde R, Chen M, Rajapakse I, Friend S, Ideker T, Zhang K. Genome-wide methylation profiles reveal quantitative views of human aging rates. Molecular Cell. 2013; 49:359-67.

33. Horvath S. DNA methylation age of human tissues and cell types. Genome Biol. 2013; 14: R115.

34. Weidner CI, Lin Q, Koch CM, Eisele L, Beier F, Ziegler P, Bauerschlag DO, Jöckel KH, Erbel R, Mühleisen TW, Zenke M, Brümmendorf TH, Wagner W. Aging of blood can be

tracked by DNA methylation changes at just three CpG sites. Genome Biology. 2014; 15: R24.

35. Jones MJ, Goodman SJ, Kobor MS. DNA methylation and healthy human aging. Aging Cell. 2015; 14:924-32.
36. Horvath S, Raj K. DNA methylation-based biomarkers and the epigenetic clock theory of ageing. Nat Rev Genet. 2018; 19:371-84.
37. Field AE, Robertson NA, Wang T, Havas A, Ideker T, Adams PD. DNA Methylation Clocks in Aging: Categories, Causes, and Consequences. Mol Cell. 2018; 71:882-95.
38. Montesanto A, Lagani V, Martino C, Dato S, De Rango F, Berardelli M, Corsonello A, Mazzei B, Mari V, Lattanzio F, Conforti D, Passarino G. A novel, population-specific approach to define frailty. Age (Dordr). 2010; 32:385-95.
39. Madrigano J, Baccarelli A, Mittleman MA, Sparrow D, Vokonas PS, Tarantini L, Schwartz J. Aging and epigenetics: longitudinal changes in gene-specific DNA methylation. Epigenetics. 2012; 7:63-70.
40. Johnson AA, Akman K, Calimport SR, Wuttke D, Stolzing A, de Magalhães JP. The role of DNA methylation in aging, rejuvenation, and age-related disease. Rejuvenation Res. 2012; 15:483-94.
41. Day K, Waite LL, Thalacker-Mercer A, West A, Bamman MM, Brooks JD, Myers RM, Absher D. Differential DNA methylation with age displays both common and dynamic features across human tissues that are influenced by CpG landscape. Genome Biol. 2013; 14:R102.
42. M Jung, GP Pfeifer. Aging and DNA methylation. BMC Biol. 2015; 13:7.
43. Goel N, Karir P, Garg VK. Role of DNA methylation in human age prediction. Mech Ageing Dev. 2017; 166:33-41.
44. Campanero MR, Armstrong MI, Flemington EK. CpG methylation as a mechanism for the regulation of E2F activity. Proc Natl Acad Sci U S A. 2000; 97:6481-86.

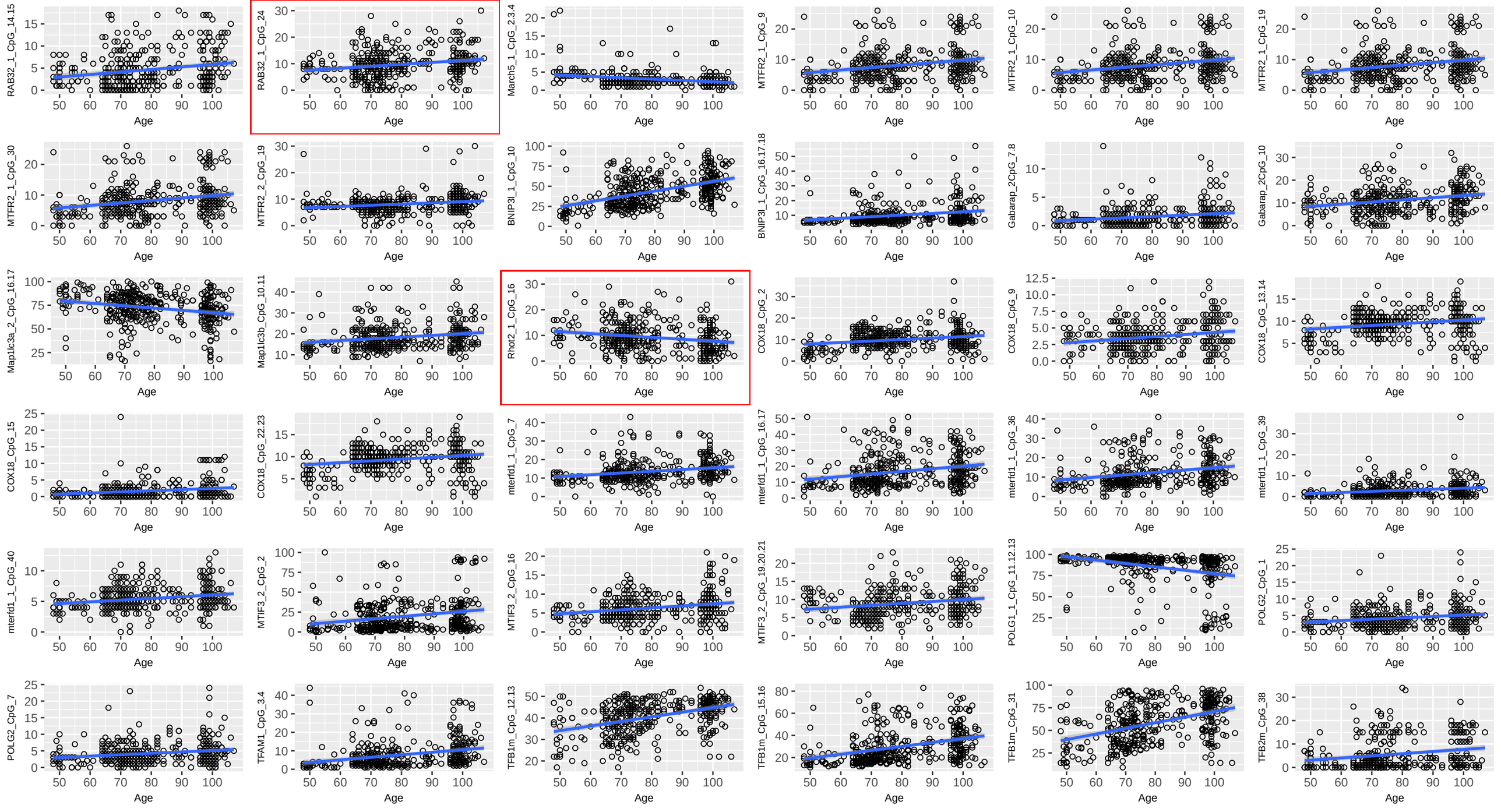
45. Zhu H, Wang G, Qian J. Transcription factors as readers and effectors of DNA methylation. Nat Rev Genet. 2016; 17:551-65.
46. Blattler A, Farnham PJ. Cross-talk between site-specific transcription factors and DNA methylation states. J Biol Chem. 2013; 288:34287-94.
47. Folstein MF, Folstein SE, McHugh PR. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. J Psychiatr Res. 1975; 12:189-98.
48. Katz, S., Ford, A.B., Moskowitz, R.W., Jackson, B.A., Jaffe, M.W. Studies of illness in the aged. The index of ADL: a standardized measure of biological and psychosocial function. JAMA. 1963; 1185:914-19.
49. Lesher EL, Berryhill JS. Validation of the Geriatric Depression Scale--Short Form among in patients. J Clin Psychol. 1994; 50:256-60.

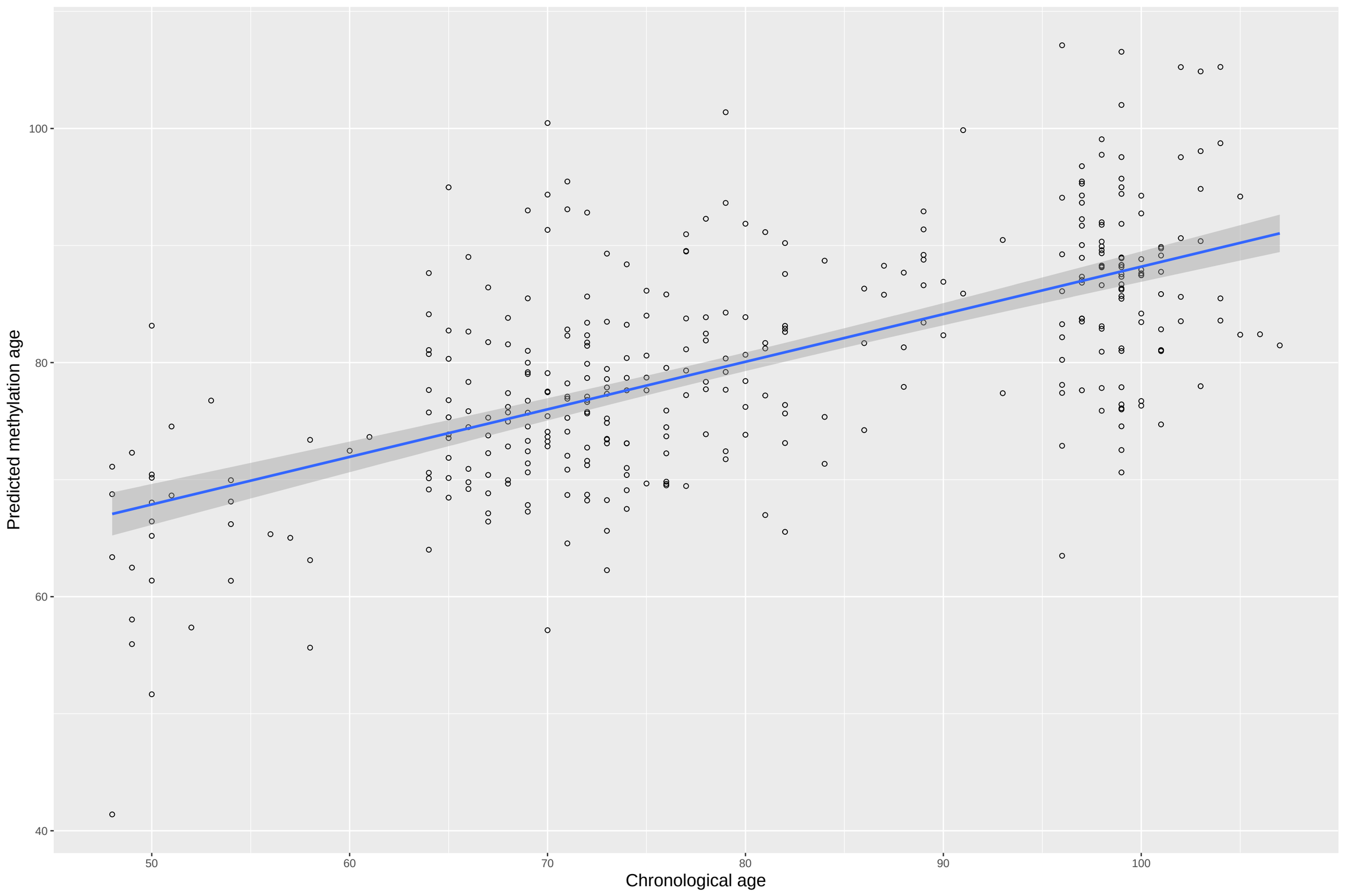
Figure legends

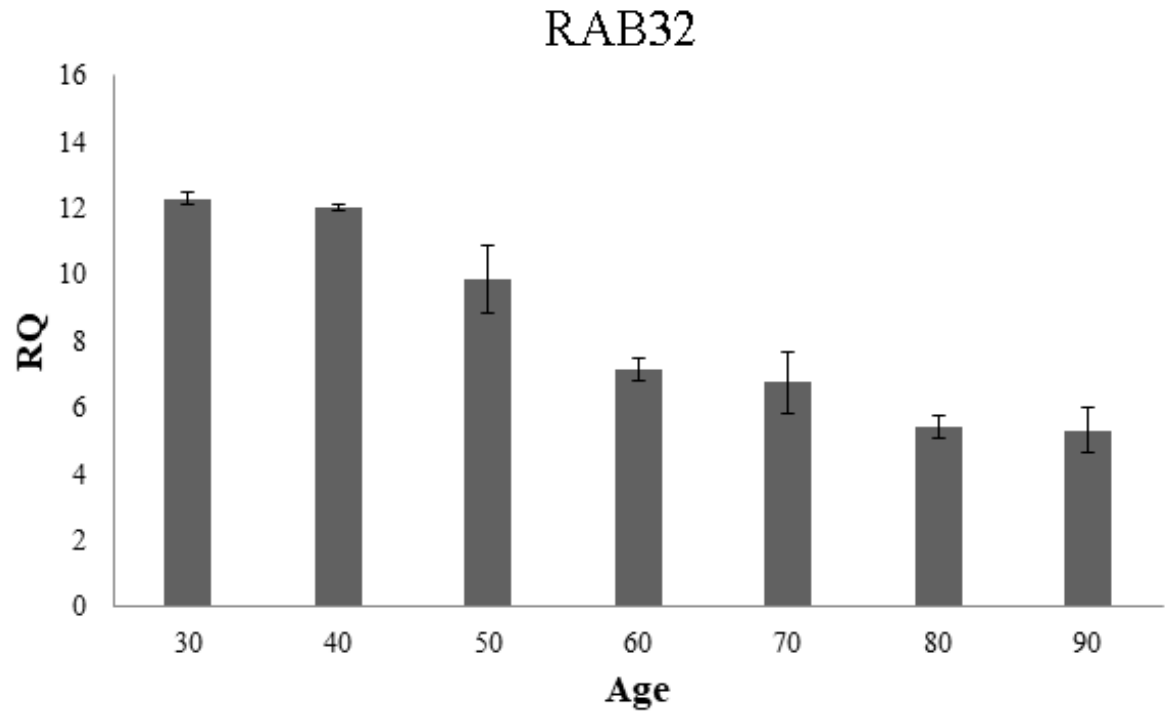
Figure 1. Scatter plot of DNA methylation values of the 54 CpG sites statistically significant after the Holm method as a function of human age. For each the blue straight line represents least-squares linear regression line.

Figure 2. Chronological age versus age predicted from the 8 CpG sites included in stepwise regression model in the discovery dataset.

Figure 3. Expression levels of *RAB32* (A) and *RHOT2* (B) in samples of different ages. These levels are reported as the mean of relative quantification values (RQ), measured in three independent triplicate experiments with standard error mean (SEM).


RAB32_1_CpG_14.15
RAB32_1_CpG_24
March5_1_CpG_2.3.4
MTFR2_1_CpG_9
MTFR2_1_CpG_10
MTFR2_1_CpG_19
MTFR2_1_CpG_30
MTFR2_2_CpG_19
BNIP3l_1_CpG_10
BNIP3l_1_CpG_16.17.18
Gabarap_2CpG_7.8
Gabarap_2CpG_10
Map1lc3a_2_CpG_16.17
Map1lc3b_CpG_10.11
Rhot2_1_CpG_16
COX18_CpG_2
COX18_CpG_9
COX18_CpG_13.14
COX18_CpG_15
COX18_CpG_22.23
mterfd1_1_CpG_7
mterfd1_1_CpG_16.17
mterfd1_1_CpG_36
mterfd1_1_CpG_39
mterfd1_1_CpG_40
MTIF3_2_CpG_2
MTIF3_2_CpG_16
MTIF3_2_CpG_19.20.21
POLG1_1_CpG_11.12.13
POLG2_CpG_1
POLG2_CpG_7
TFAM1_CpG_3.4
TFB1m_CpG_12.13
TFB1m_CpG_15.16
TFB1m_CpG_31
TFB2m_CpG_38
Age


Predicted methylation age
100
80
60
40
50
60
70
80
90
100
Chronological age


RAB32
RQ
16
14
12
10
8
6
4
2
0
30
40
50
60
70
80
90
Age

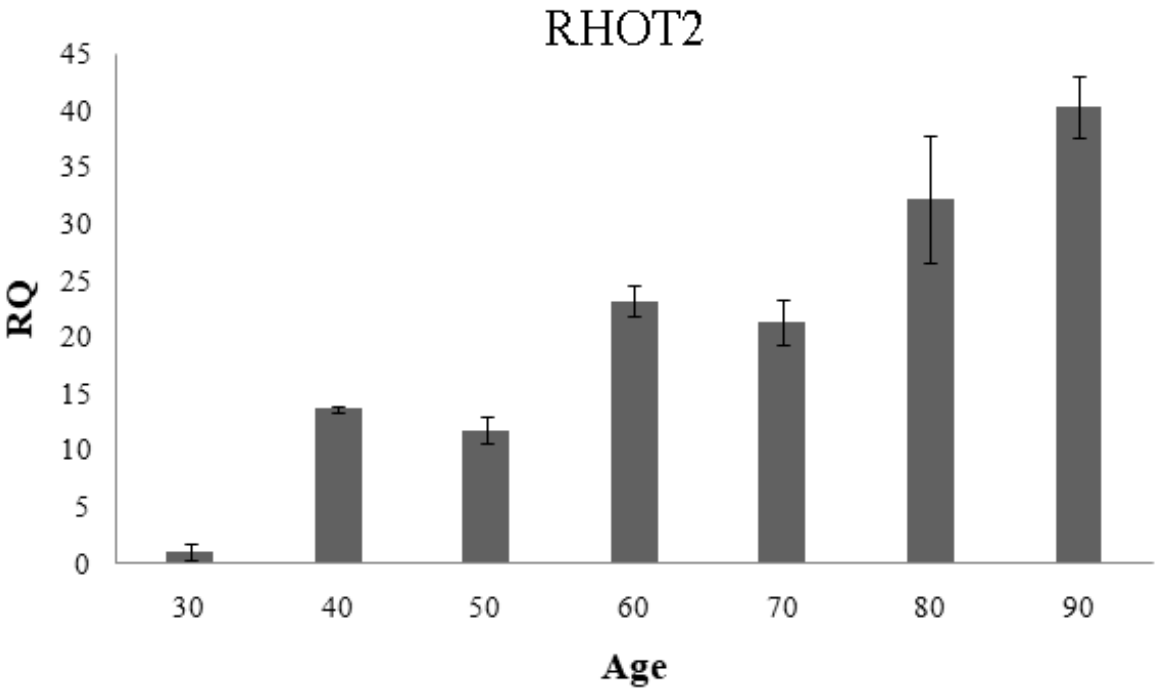

RHOT2
RQ
45
40
35
30
25
20
15
10
5
0
30
40
50
60
70
80
90
Age

Table 1. Univariate association analysis between CpG units and age of the sample under study

CpG unit	T-statistic	P-value	CpG unit	T-statistic	P-value
BNIP3L_Amplicon1_CpG_10	10.749	$1.113*10^{-23}$	MTFR2_Amplicon1_CpG_30	4.830	$1.986*10^{-6}$
BNIP3L_Amplicon1_CpG_16.17.18	4.664	4.31335*10-6	MTFR2_Amplicon2_CpG_19	3.877	$1.253*10^{-4}$
COX18_CpG_2	4.702	$3.606*10^{-6}$	MTIF3_Amplicon2_CpG_2	4.451	$1.125*10^{-5}$
COX18_CpG_9	4.252	$2.673*10^{-5}$	MTIF3_Amplicon2_CpG_16	4.767	$2.674*10^{-6}$
COX18_CpG_13.14	4.386	$1.496*10^{-5}$	MTIF3_Amplicon2_CpG_19.20.21	4.024	$6.951*10^{-5}$
COX18_CpG_15	4.034	$6.625*10^{-5}$	POLG1_Amplicon1_CpG_11.12.13	- 6.428	$3.896*10^{-10}$
COX18_CpG_22.23	4.374	$1.580*10^{-5}$	POLG2_CpG_1	3.888	$1.194*10^{-4}$
GABARAP_Amplicon2_CpG_7.8	3.966	$8.758*10^{-5}$	POLG2_CpG_7	3.888	$1.194*10^{-4}$
GABARAP_Amplicon2_CpG_10	4.774	$2.626*10^{-6}$	RAB32_Amplicon1_CpG_14.15	4.236	$2.880*10^{-5}$
MAP1LC3A_Amplicon2_CpG_16.17	- 4.411	$1.352*10^{-5}$	RAB32_Amplicon1_CpG_24	4.178	$3.669*10^{-5}$
MAP1LC3B_CpG_10.11	3.894	$1.167*10^{-4}$	RHOT2_Amplicon1_CpG_16	- 4.123	$4.604*10^{-5}$
MARCH5_Amplicon1_CpG_2.3.4	- 4.835	$1.938*10^{-6}$	TFAM_Amplicon1_CpG_3.4	5.527	$6.079*10^{-8}$
MTERFD1_Amplicon1_CpG_7	4.624	$5.201*10^{-6}$	TFB1M_CpG_12.13	8.692	$1.183*10^{-16}$
MTERFD1_Amplicon1_CpG_16.17	4.998	$8.881*10^{-7}$	TFB1M_CpG_15.16	7.571	$2.860*10^{-13}$
MTERFD1_Amplicon1_CpG_36	5.127	$4.744*10^{-7}$	TFB1M_CpG_31	9.324	$9.492*10^{-19}$
MTERFD1_Amplicon1_CpG_39	4.425	$1.269*10^{-5}$	TFB2M_CpG_38	4.117	$4.719*10^{-5}$
MTERFD1_Amplicon1_CpG_40	4.436	$1.204*10^{-5}$			
MTFR2_Ampicon1_CpG_9	4.830	$1.986*10^{-6}$			
MTFR2_Amplicon1_CpG_10	4.830	$1.986*10^{-6}$			
MTFR2_Amplicon1_CpG_19	4.830	$1.986*10^{-6}$			

Table 2. Results of the linear regression analysis carried out in the replication dataset

CpG unit	Beta	Standard Error	T-statistic	P-value
BNIP3L_Amplicon1_CpG_10	9.776	8.671	1.127	0.260
COX18_CpG_2	0.495	14.754	0.034	0.973
COX18_CpG_15	-2.961	24.346	-0.122	0.903
GABARAP_Amplicon2_CpG_7.8	-75.456	53.490	-1.411	0.159
MARCH5_Amplicon1_CpG_2.3.4	-38.731	54.953	-0.705	0.481
RAB32_Amplicon1_CpG_24	67.316	23.023	2.924	0.004
RHOT2_Amplicon1_CpG_16	-122.007	19.275	-6.330	$5.94*10^{-10}$
TFBIM_CpG_12.13	-9.401	9.502	-0.989	0.323

Table 3. Association between the methylation levels of *RAB32* and *RHOT2* CpG units and physical and cognitive abilities

CpG unit	HG (SE)	P-value	ADL OR (95%CI)	P-value	MMSE B (SE)	P-value	GDS OR (95%CI)	P-value
RAB32_Amplicon1_CpG_24	-0.465 (0.811)	0.567	2.019 (1.132- 3.601)	0.017	-0.303 (0.542)	0.576	1.240 (0.657-2.340)	0.507
RHOT2_Amplicon1_CpG_16	8.446 (6.863)	0.220	2.348 (0.015-357.763)	0.739	-8.166 (4.653)	0.081	0.053 (0.00-14.926)	0.308

Supplemental data

Figure S1. Localization of the 500 CpG sites (red) which passed quality control criteria. In particular, the 54 sites statistically significant after the Holm method are underlined. The organization of the sequence-analytic units performed by Sequenom software is also shown.

BNIP3L_Amplicon 1 (Chromosome 8)

GGCTTGTTGT GTTGCTGCCT GAGTGCCGGA GACGGTCCTG CTGCTGCCGC 26383109

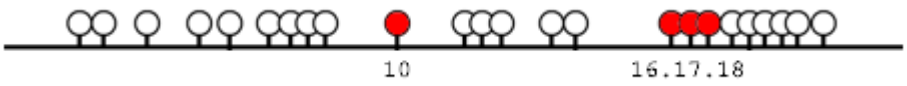
AGTCCTGCCA GCTGTCCGAC AATGTCGTCC CACCTAGTCG AGCCGCCGCC 26383159

GCCCCTGCAC AACAACAACA ACAACTGCGA GGAAAATGAG CAGTCTCTGC 26383209

CCCCGCCGGC CGGCCTCAAC AGTGAGTGCG GGGCCGAGGC TCTGTGAAGG 26383259

GGATGGGGGA GGAGGAGCAG CCCCGGCCGC CGCCACCGGC GCGGCGCGGG 26383309

AGGCGGGAGG AGAAGGCAGC TCATTGGCTC



COX18 (Chromosome 4)

GGATGTAGTG CTGGTAGGCT GCCAAAGGCA GCGTGACAGC ACCCCGTAAG 73069374

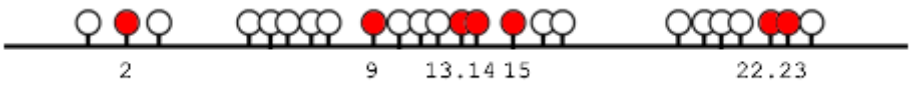
GCCACGGTGG AGAGCAGAAT GCTGCCCCAC CAGGGCAGGC CCGTGGCGGC 73069424

GTGCACGCCG AGCAGTACTT CCTCCGCAAC CCGCACCGGC GAAGACGCGG 73069474

CCAGGGCCTC GTACCAGCCG TTCGCATGTA CTGCAGAGAC TGGTGCCACT 73069524

GCCCACACTG GGAGAGTGGG GCGCTTGGCG CCGCTCGTAG GAACCGGCGC 73069574

AAGCGGCAGG TCCCTAGCCC AAAGCTGCAG GGC



GABARAP_Amplicon2 (Chromosome 17)

GTCCCCTCAA GGAAGCTGGG GCTGAAGAGG AGTAAAAGAT GGTAATCATC 7242731

ATACGAGACT TGGTTCTCCA AGTTCCTTTC GTTAACAACG TAGAGGAACA 7242781

GCAGGGACAA TTACAAGGTT AGCTATTCAC GAACCGTGTT GCTACGCTGA 7242831

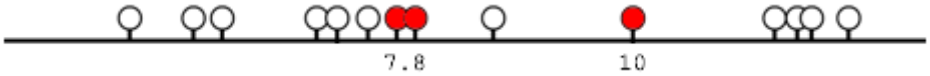
AGGCGGCCGT TGACACCAAA ACAAAGTAGT ACCCAAGTGG CGGAGATGAT 7242881

CTCCAGAAAT AAGAAGTCAA AAAGAAAACA GATGTTTGGA GAGATCTACA 7242931

GAACGCTTAA GTGCCAAAAT GAGTAGACCA ATAGGGACTG GAGAGGAGGT 7242981

TGGAAAGGTA GGGACTACGC CAGCGCGGAG GAACACTGCG GGACTTGAGT 7243031

TAAATCATGT GATCTC



MAP1LC3A_Amplicon2 (Chromosome 20)

GCTGTGGGGC CTGATGGCCC CGGGGGTGGG GGCTGGAGCT GGGGCGTGGC 34559128

CGGGGGCCGC CCCTCCGGGA CAGGCGGGGC GGACCTGGGC CGGCCCGACC 34559178

CGGCCTCACG GTCTGGCCGC TGTCCGCAGC CGACCGCTGT AAGGAGGTAC 34559228

AGCAGATCCG CGACCAGCAC CCCAGCAAAA TCCCGGTGAG TCCCGCACCC 34559278

CCAGCCCTGC CCCGCCCCCG CCTCGCGCGT TCCCGACACG ACCCCCTGCC 34559328

CGCCCGCCCT GCTCCCAGGT GATCATCGAG CGCTACAAGG GTGAGAAGCA 34559378

GCTGCCCGTC CTGGACAAGA CCAAGTTTTT GGTCC

MAP1LC3B (Chromosome 16)

GATGTGGGGC AGGCCTGGCA GTCGCCACAG ACGACCTAAC GGTAGGAAAA 87391589

TCTTACAGCC ACCAGGAGAG TTCCAGGCGC CGCGGCAGGG GGACTGGGAG 87391639

AGGGGACTGC GCCCAGAATG AAGGCTCGGG ACAAAAGCAG TTGCGCAAAC 87391689

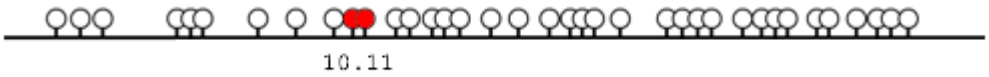
GCGCCAAGGC TGGGCGTCGA GTGACCGCGG GCGGAGGTCA CCAGCGGCCA 87391739

CTCCCCGGAA GCCACCCACG GACCACGCGC GCCCCTGCAC GCAGAGGGGG 87391789

CCAGGGCTCC ACGGGCGAGC GGCGACCCTG CCTCCCGGAG ACGGCGCGGC 87391839

CTGCCCTGCG CGCCTCAGCC CCGGGTGCCG GCGTCTCGGG CAGCACCACC 87391889

AAGTCTCTCT GGAGGGGAAA G



MARCH5_Ampicon1 (Chromosome 10)

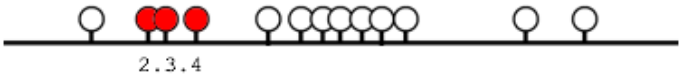
AGGTGGTGTA ATTCCCCCAA AATGGGCTCT GC**CG**CAGGAG AGGCTGGCTC 92290743

CAG**CG**C**CG**GG GGCTT**CG**GAA GGAGTTTCTG CCACCCCCAC TGC**CG**CCACT 92290793

GAC**CG**CCC**CG** A**CG**CCA**CG**GC **CG**GGGC**CG**GG GACCCTGATA AGAAATGGCC 92290843

CTTCAGCCCC CTCCCCTCAC CTGGCT**CG**GT CCCACCTGAG GGCAAGAG**CG** 92290893

GAGGCAAAAA CAAACAGGCA GGGAGGGCTG A



MTERFD1_Amplicon1 (Chromosome 8)

AACCTAAAGG CCCTTGAGGC CCTGGGG**CGC** **G**GTCTGGA**CG** **CG**GGTG**CG**C**C** 96261443

GGAACCTGAA CCCT**CG**CCAG GAG**CGCG**ATC CT**CG**TGCTGG GGCAGCTCCT 96261493

GCTTTACCTG TGG**CG**AGGCC TGCTTCC**CG**T AG**CG**GGTGAC CC**CG**GGAC**CG** 96261543

ACCAACT**CG**C TGGGC**CG**CA**C** **G**TCC**CG**TCC**C** **G**C**CGCG**C**CG**C A**CG**C**CG**GCTC 96261593

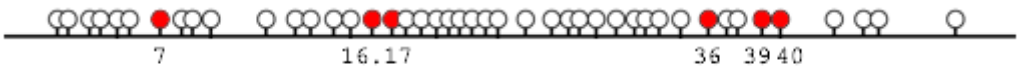
CTCAGCC**CG**C CCTACACAG**C** **G**CAGC**CGCG**C TCC**CG**GGCCC A**CG**TGGGC**CG** 96261643

CGCC**CG**GAGT GGG**CG**AGACC ATGTGC**CG**GG TTA**CGCG**GGG AGACAG**CG**GG 96261693

CTA**CG**TGCTC TGAAAAGAGC CAGCCAGGGG CCC**CG**GCTAC CCTTCTC**CG**G 96261743

CT**CG**CTATTC CTCTGAAGTC CTGTGGACTT GGAGTCTGAA AATTCCT**CG**A 96261793

GTGGTAGAGA TGCCTCAGCT AGGATGA



MTFR2_Amplicon1 (Chromosome 6)

GGTCACAAGC TTCCTGGTGC TCTCCAAACA CAG**CG**TGGTA GCCTTGCTCA 136249974

CCTTCCTC**CG** AAGC**CG**AGCC CC**CG**GGAATC ACCAGAACAT CAAG**CG**GAGG 136250024

G**CG**TTAGGAA TCCTCAATCA AATTCCAGAC TG**CG**CCACTC CTTGTGCAGA 136250074

T**CG**CTCAGA**C** **G**TCTACCCAG ACCT**CG**ACCC AGTTTCCACC **CGCGCG**CCT**C** 136250124

GGCTTGCAGC CACAGGCGGT AACGATGCCC TCAGCGAGGA ATAAGAGGTC 136250174

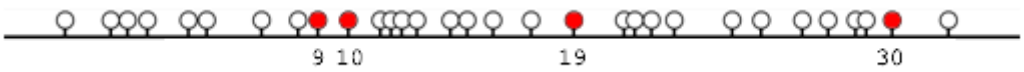
AACCCGGTGC CCAGCCCTGG AGCCTCACGC TCAGCCAGGG TAATGTTATG 136250224

GGAAGCGCGC CCCCGTCCTC CTGCGCCCAA CCAACCCACC TGCTAGTCCC 136250274

TAGGACCGGA CTGCTACTTC CGCATGACAG GACTGCTACT TCCGCATCCC 136250324

AGGACGAAAT CAAACTCCGC GGGCCAGGAC CCGTAACCAG CCTCATTGGG 136250374

CAAAACTGAA GATCGCTGCT TCTGATTGGT CATTGCATGA TGTCAGGCAA



MTFR2_Amplicon2 (Chromosome 6)

TGCTTCTGAT TGGTCATTGC ATGATGTCAG GCAATTCGGG AAGCGACTGC 136250440

TTTTCCTTCT CAAGAGGCGG GTCTTCCGAG AGTCAGCCAA TAGGAGCTGA 136250490

CGGGCAGGGC AGGTCGCGTT AGGACAGCGT CGCTCCCGCA GGGCAAGGTT 136250540

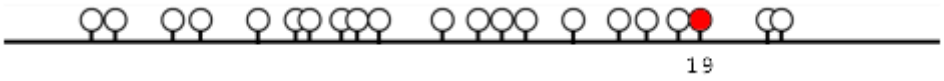
TTAGGTACAA ACTCGTGAGC TGGACTCGCA GTCCGGAGTT CGTACCCCAC 136250590

AGAACTCTTC GCAGGGAGTG TCTTACTCGC TACAGCCGGA CCCTAAGCGC 136250640

ATCCGCCTTC CCAAGTTCAT CTGACACAAA ATCGCGATTT ATTTTTTGGT 136250690

CATTTCATTT GTTCAGCTAA TATTAAAATA CTTGTGGAAC TGGCAGAAGC 136250740

CAAG



MTIF3_Amplicon2 (Chromosome 13)

AACTAGAAAA GCTCAGTGGG GCTGGCGGCA ACTCTAGGTC TAACATCCCT 27450291

CGGAAGTTCG TGCAGCGGCC TCGCTGAAAA TGGCTTTACA GCCCGGCAGA 27450341

ACCCTCCGAC TCCGCAGCAG GACCTGCGGA GCCTCTCCGC CCTCGGTACC 27450391

GGCGGGACGG GGTAGCCCTG ACCTTCCGGG TGCCTCCTCC ACAGGGGCGC 27450441

CCCCTAAGGC CACGCCCTCC CGATGCCGGT CCGCGCACCG CTCCGCACGC 27450491

CTCATATTTA GCATTACCTG TGCTGGGGCA AGCGATTGAC ATACTGTAGC 27450541

GGACGCAAGT ACAGCGGATC TGCGGCGAGT CCCCTTCGCT CTCCGTAGTG 27450591

GGCGGGGCTT CACCCCGCGT CCTTTAAAGG AAAGGTGATG GGCGGAGCCA 27450641

CGTTTTTCTA CTGTTGGGAT TCAGTTCCGC CGACAGAAAA TGCGGAGAAC 27450691

GCGGAGGAAG TCCCTGAGGG GAAATTGCTG CTCACCTGGC TCC

POLG1_Amplicon1 (Chromosome 15)

GGGGCAGCTG GGCCTGCAAC AGCAAGTTGG CCGCCTCCAG GTAGGGCAGG 89333314

CTCTGCTTCT GGGCCAGGAG GCGGAAGTGC TGGTCCAGGT TGTCCCCGTA 89333364

GAGGGGCGGC AGGCGCAGCT CCACGTCGGG CAAGGGCACG GCTGGCTGCC 89333414

CCCAGAGCCC GTGCTTCTGC AGGTGCTCGA CGCTGCGGCG CACCGCGGCC 89333464

TCGCCAGGCA TCTCCCCTCC TTGCCCGAAG ATTTGCTCGT GCAGCCCTCT 89333514

CGAGAGCATC TGGATGTCCA ATGGGTTGTG CCGCAGCTGC CCGCCCTCCG 89333564

AGGATAGCAC TTGCGGCTGC TGAGGCTGCT GTTGCTGCTG CTGCTGCTGC

POLG2 (Chromosome 17)

AACAGGCACC TGCAGACCTT ATGGCAGGCC CTGACGGCTA CACGAGAGCG 64496965

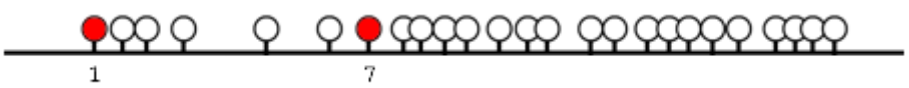
CATCTCTCTC CGAAGTTAAA GAGCACACTC TCCCATCACT CAACGGATCC 64497015

CAACAAGCCA CCACTACCGT TAACAGAATC CGGAGAGGCC ACGGCGCAGG 64497065

CGCAACGGAG GTGAGCGTGC TTGCGGGCGG CAGGCCCCAC CCCGGAAGCG 64497115

CATGTCTGCG TTCCGGCCGC AGCCGTCCCC CGCCCACCAT GGCGGACGCC 64497165

GGCTCGGATG GTTCTCTGCT AGCTTG



RAB32_Amplicon1 (Chromosome 6)

GGGAGAGGAA GTCCAGCTGG GCCCGGCCGG GCTTCGGAGG CGCCGCCCGG 146543563

GAGAGGAAGT CCAGTTGGGC CCGGCCGGGC TTCAGAGGCG CAGGGCGGGA 146543613

GCCGCCTCGC GCAGGGTCCT CCCCAAGCCG GCGCCAGGCC CTGCCCTCGT 146543663

CCGGCCCTGC CCTCGTCTGG CCCCGCCCGG GCCGCGAGCA CTGGCGGGTT 146543713

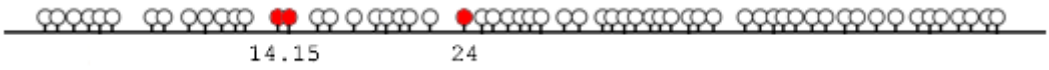
CTGGGTCCTG TGACCGGTCA GGCGGCGTCA GCGGGCGCGG CGGAGGGCTG 146543763

GCCGGCCTCG GGGGAGTTTC CGCGGCCGCC GGGGGCGCGG CGGCAGAGCG 146543813

CGAGGCCGGG CAGGGGGCCA GACTCGGAGT CGAGGCGCGC CCGACAGCCG 146543863

CAGCGCTCAT GGCGGGCGGA GGAGCCGGGG ACCCCGGCCT GGGGGCGGCC 146543913

GCCGCCCCAG CGCCCGAGAC CCGCGAGCAC CTCTTCAAGG TGCTGGTGA



RHOT2_Amplicon1 (Chromosome 16)

TGGAGTCTCT TTGTCCCCCT AGAAGCCGAG CAGACGGACG AGGAGCTGCG 668683

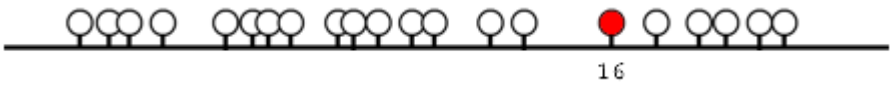
GGAGGAGATC CACAAGGTAC CCGTGGTGCG CGGGACGAGG GAGGGGCTGG 668733

GCGCGGGCTC GGCCTAATCC GCTTCGCAGC CTGGGGGATT GGACCGAGGT 668783

GCTCCGGGTG TCCTTGGCCC TGATAATTCT GTGACCTCCG CACTGAGGGT 668833

TGTCGGGGCC CCTACAGCGC ACCCCGCTGG GAGCCGGCAC CGCTCAGTCC 668883

AGTGGTGCTC CAGGGATAAC AGGACCC



TFAM_Amplicon1 (Chromosome 10)

GGGGTCCTGG ATGCAGGACT GTCTGTTACG TACAGCCCTT GTGACCGTCA 58385115

CGGGCGGACA CCGGCCAACG CCGGGTTGGG GTGAGGCCGC CGCCGCGGTC 58385165

CCTCCATCAC CCTCCTGGCC CGGCAGAGGA ACCCACTGCT CCGGGCGGCC 58385215

GGGGACAGAG GTGGCTCAAC AG

TFB1M (Chromosome 6)

CCTCAAGTCC AGGAGGAAAT TCTGTGATAG CTGCTTCGCT GCTTGCAGTC 155314352

TTAACAACTT AATGATTTCT CGAATCGTGG GCAACGGAGG GAGACGGCAA 155314402

GTGCTGAGTT TTCCGGAGGC AGCCATGATA CGCGGCAAGC ACCATCCAAC 155314452

CCTACCTCAC CCAGGACCTT CACCGCCGCT CCGAAAGAAA CGCGCAGGGG 155314502

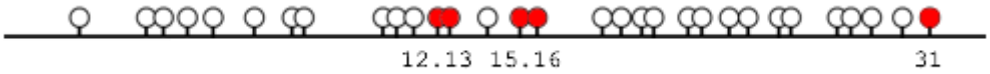
AGGAACCTGC GAGACCTAAG GCCCGCCTCG GAGTCAGCCC CATTGGTCAG 155314552

ACCTATCCCA CCGGAAGCGA TGACCGCGGA CAGGAAATTC CCGGCGTGCT 155314602

GAGAGCGCAT GCGCTAAGTC CTGCCGCGAG AAGGGCAGGC TGGGTGGTCG 155314652

GCGGCTTCCG CCTGTGAGAG CCGGGGGAGA GCCGGGTGGA CTAGGCTTCT 155314702

CCTGG



TFB2M (Chromosome 1)

TGGGATCCAC ATGTCCTTGT CTCTCAGGCC CGCTCCAAGA ATCACCTAGT 246566176

GCAGCTACTA CAGTGAACCC CACGCAGGGT ATCCCACGTG GAACATTTTC 246566226

TGGCGTCCGG GCCAGGTCAA GCGGAAGTAA ACACTAGAGC CTGCGCATGC 246566276

GAACAGCGGA GCCTTCCTGC TTTTCTCCCT CACTTCCGCT TCCGCCTCGG 246566326

CTCAGCCGCC CGAGGATTGT GAGTGGACCG TTGAGGAGAG CGACCGACCA 246566376

TCCGGCTGGT GTCCGGACTC GTACTCTATG GTTGTCCGCG CTCTGCGCTT 246566426

CCTCTCTAGC CGCCAGTGCT CTATGCTCCG CGGTCGCGGG CCGCCAGCCT 246566476

CCAGCCGGCC AGCCGCGAGG GGTGCGCAGA GGGAGGCGGG GCGGAAAGGC 246566526

GAGAGGTGTC TCCTCCACCG GAGCCAGGGG AGACCCGAGC AAGCTCCGTG 246566576

ACAGCACGTC GGCCGCCATG TCGCCGAGTG GGGCTGGAAA CAGACC

496

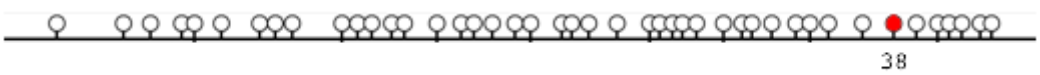


Table S1. Designed PCR primers of the amplicons analyzed in the study.

GENE	Target lenght (bp)	Primer sequences (5'-3') Forward	Reverse	Annealing (°C)
BNIB3L_Amplicon1	280	AGGGTTTGTTGTGTTGTTGTTTGAGT	AAACCAATAAACTACCTTCTCCTCC	62
BNIB3L_Amplicon2	179	AGATGGGGGAGGAGGAGTAGTTT	ACCCAAAACCCCAAAAAACA	62
BNIP3_Amplicon1	175	AGGGATTTGTAGGTAGTTGGAGTT	AAATTTTTATTTTTCTATTTTATTTATTCT	58
BNIP3_Amplicon2	275	GTTTTTTTGGTTTTTTTTAGGTTTT	CCCTACCCTATAAATTCCTCC	60
COX10_Amplicon1	363	AGGGAAGTTTTTTAGGTTTTAGATTTGTT	CCAAAAACTCCCCAAAATTACA	60
COX10_Amplicon2	229	TTGTGTTGGATATTATTATATGGATTT	TCCCAATACTCTCAACTAAAAAAAA	56
COX18	288	GATGTAGTGTTGGTAGGTTGTTAAA	TCCCTACCCTACAACTTTAAACTAAAAA	60
DNM1L	365	GTTTGTGGGAGGAGGGTTTTG	CAATCCCTAAACAAAACAAAAAAAA	58
ENDOG_Amplicon1	166	GGAGTTAGGTTTGATGATGGTGGTTAAT	TCCACTCACAAACACTACCAAAAA	58
ENDOG_Amplicon2	476	GGTTGGGTTTTGTTGTTTTTTTT	CAACTACTCCACCACCCAAAAC	60
FIS1	185	AGGGTTTTTATTTGATTTTTTTTAGGA	CCCCTACCACTAAACCATAA	60
GABARAP_Amplicon1	456	GTTGGATAGGGTTGGGTTGAG	AACCTTATAATTATCCCTACTATTCCTC	60
GABARAP_Amplicon2	366	AGGTTTTTTTAAGGAAGTTGGGGTTG	AAAATCACATAATTTAACTCAAATCCC	58
KIF5B	306	AGGGGAGAGTGGTTATTTTTTTT	CCTACCTCCCCAAAAACTTCTAC	60
MAP1LC3A_Amplicon1	414	AGTTTTTTTTAAGGAATGTTGTGATTT	AACCATCAAACCCCACAACC	58
MAP1LC3A_Amplicon2	335	GTTGTGGGGTTTGATGGTTT	AAACCAAAAACTTAATCTTATCCAAAA	58
MAP1LC3B	371	GATGTGGGGTAGGTTTGGTAGT	CTTTCCCCTCCAAAAAAACTTAATA	60
MARCH5_Amplicon1	231	GTGGTGTAATTTTTTTAAAATGG	TCAACCCTCCCTACCTATTTATTTT	60
MARCH5_Amplicon2	385	GGGTTGTGTAGTTTTTAGTGGAG	AACCAAACCCAAAAACAATAACACT	60
MARCH5_Amplicon3	106	TAGTGTTATTGTTTTTGGGTTTGGT	ATCCAACATCTACTATAAAACTTAATCC	60
MFN1	202	GTTTTGTGGGAAAGGAGAGAGTTAG	AACACCTACCTTAAAAAAAACCTCC	60
MFN2	187	AGGGAATTATAGTTTTTATGATGTAGTGGGA	AAACTAATAAACCCTAAACCCAACC	60
MTERF	154	GGTTTGGTAGGGGGTAGTAAGAGA	CCCCCTATAAAATCCCTATAAAATACC	54
MTERFD1_Amplicon1	427	AATTTAAAGGTTTTTGAGGTTTTGG	TCATCCTAACTAAAACATCTCTACCA	60
MTERFD1_Amplicon2	226	TTTTTTGTTTTTAGTTTTTGTTGGG	TAAAACAACCAACCCACTTCCT	60
MTFR1	404	AGGAGTTTGAGGAAATAGTTGATTGA	AATCTCAACCTTCAAAACAAATC	60

MTFR2_Amplicon1	500	GGTTATAAGTTTTTTGGTGTTTTTT	TTACCTAACATCATACAATAACCAATC	60
MTFR2_Amplicon2	354	TGTTTTTGATTGGTTATTGTATGATG	CTTAACTTCTACCAATTCCACAAAT	60
MTIF2	385	AGGGATTAAATGTATTTTGGAAAGGTTTT	CCTAAAAAAAAACCACAAATTAAACCA	60
MTIF3_Amplicon1	212	TTTTGTTTTATTTGTGTTAGGTAGTAAG	AACCCCACTAAACTTTTCTAATTCC	60
MTIF3_Amplicon2	493	AATTYAGAAAAGTTTAGTGGGGTTGG	TAAAACCAAATAAACAACAATTTCCC	56
MTIF3_Amplicon3	388	AGTTTTTTTATTGTTGGGATTTAGTTT	ACCCCAACTTTATTTTACCTCC	60
OPA1_ Amplicon 1	482	TGTTTAAGAAAGAAGGTAGGTAAATGTG	AAAATAACCCTCAACAACAAAAACA	60
OPA1_ Amplicon 2	209	GTTGAGGGTTATTTTTTGGGTTATT	TAAAAATAAAACAACCCCACTCTCA	60
PARKIN	212	AGGAGAGGTTGTATTTGGTAGGTATTT	ACTCCCAACAAACCCTAAACC	56
PINK1_ Amplicon 1	600	TAAAGTGTAAAGGGAAAGTTATTGT	CTCACCTAAATCTCCTAACAAACC	60
PINK1_ Amplicon 2	383	GGTTTGTTAGGAGATTTAGGTGAG	AAAACTTTCCTTCTCCATAAATTAAAA	60
POLG1_ Amplicon 1	350	AGGGGGTAGTTGGGTTTGTAATAGTAA	ACAACAACAACAACAACAACAACA	58
POLG1_ Amplicon 2	412	AGGTTTTTTGATTGGAGAGGGAG	AAACCAATCCACCTACTCCTTAAA	60
POLG2	276	AGAATAGGTATTTGTAGATTTTATGG	CAAACTAACAAAAAACCATCC	60
POLRMT_ Amplicon 1	478	AGGTTTGTTTTAGAATTTGAGTTTTTGTTT	CTTATACAACCTCCTAACCCCAAAT	60
POLRMT_ Amplicon 2	153	AGTTATATTTGGGGTTAGGAGGTTGTAT	TTTAACCTTTACCTCTTTACACCTAAAC	58
RAB32_ Amplicon 1	449	AGGGGAGAGGAAGTTTAGTTGGGTT	TCACCAACACCTTAAAAAAATACTC	60
RAB32_ Amplicon 2	302	AGGAGTATTTTTTTAAGGTGTTGGTGA	TCTTCTCCTAAAAATACCCAAACCAA	60
RHOT1	159	AGGGTGTTTTTGGTGAGAGGAGTTTA	ACAAAAACAAAAAAAAACAAAAACTCAA	60
RHOT2_ Amplicon 1	277	TGGAGTTTTTTTGTTTTTTTAGAAG	AAATCCTATTATCCCTAAAACACCACT	60
RHOT2_ Amplicon 2	365	TTTTAGGTTTTTAGATTAGGATTTGGA	AAACAAAACAAAAAAAACACCAAAC	60
TFAM_ Amplicon 1	172	AGGGGGTTTTGGATGTAGGATT	CTATTAAACCACCTCTATCCCC	58
TFAM_ Amplicon 2	200	AGGGGGATAGAGGTGGTTTAAT	CACTATAAAAAATCTACTAACATCC	60
TFB1M	405	GTTTTAAGTTTAGGAGGAAATTTTGTGA	CCAAAAAAAACCTAATCCACCC	60
TFB2M	496	TGGGATTTATATGTTTTTGTTTTTTAGG	AATCTATTTCCAACCCCACTC	60
TRAK1	260	GAGGAGGAGTAAGAGAGGAAGTTTTAG	TAAATCAAAAACCAAAAAAAATCCC	60
TRAK2_ Amplicon 1	257	AGGGATAGTTTATTTATTGGAGTGGTT	AAAAATAACTCTCCTTTAACTTCCCC	58
TRAK2_ Amplicon 2	232	AGTGGTAGTTTTTATATAATTGGGGAA	TTAAAACTCTACACCAATCCCAAAC	58

Conclusive Remarks

The analyses carried out during my PhD appointment have highlighted how epigenetic signatures changes across life and reinforced the evidence on the role of epigenetic modifications in the aging process.

Several genomic regions are subjected to DNA methylation dynamic changes throughout lifetime, including genome wide demethylation and *de novo* methylation, resulting in both hypo- and hypermetylated regions. Indeed, aged organisms have modified epigenomes, which underwent variations starting from intrauterine environment in response to internal (genetic), stochastic and environmental stimuli such as nutrition.

In the experimental research published in Guarasci et al. 2018 and reported in the Appendix we propose that the modulation of global DNA methylation status is among the mechanisms by which nutrition influences the aging process. In particular, we demonstrated that global DNA methylation level has tissue-specific variations during aging and is influenced by a low-calorie diet.

The identification of clock CpGs allowed to enhance the knowledge about age-related epigenetic modifications by constructing predictive models able to reflect not only chronological age of cells, tissues or organs but also the biological age. These models would differentiate individuals with the same chronological age but different rates of aging, in order to evaluate healthy aging, and possibly predict lifespan. In D'Aquila et al. 2019 (reported in Chapter II), we identified two genes (RAB32 and RHOT2) exhibiting changes in methylation status of specific CpG sites during age, which may represent novel potential biomarkers of both chronological and biological age involved in mitochondrial quality control processes. The involvement of these two genes in the mitochondrial quality control network provides new evidence about the key role of mitochondrial functions during the aging process. In fact, recently, a role of mitochondrial genome in the regulation of epigenetic landscape and the presence of epigenetic modifications on mitochondrial DNA itself is emerging (see review paper, D'Aquila et al. 2017, in the Appendix). Thus, our study points up that genes involved in mitochondrial quality control are regulated via epigenetic mechanisms and that such genes may have an important role during aging.

Appendix:
Published Works

Mechanisms of Ageing and Development xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Mechanisms of Ageing and Development

journal homepage: www.elsevier.com/locate/mechagedev

Aging and nutrition induce tissue-specific changes on global DNA methylation status in rats

Francesco Guarasci[a,1], Patrizia D'Aquila[a,1], Maurizio Mandalà[a], Sabrina Garasto[b], Fabrizia Lattanzio[c], Andrea Corsonello[b], Giuseppe Passarino[a,*], Dina Bellizzi[a,*]

[a] Department of Biology, Ecology and Earth Sciences (DiBEST), University of Calabria, 87036 Rende, Italy
[b] Italian National Research Center on Aging, 87100 Cosenza, Italy
[c] Italian National Research Center on Aging, Scientific Direction, 60124 Ancona, Italy

ARTICLE INFO

Keywords:
Global DNA methylation
Aging
Tissue specificity
Nutrition
Low-calorie diet
Maternal pre-gestational/gestational diet

ABSTRACT

A number of epigenetic studies have demonstrated that DNA methylation patterns exhibit a tissue specificity, but not much has been done to highlight the extent of this phenomenon. Moreover, it is unknown how external factors modulate the plasticity of the tissue specific epigenetic profile. We examined global DNA methylation profiles in tissues from rats of different age, fed with standard or low-calorie diet, and evaluated their association with aging and nutrition. Tissue-specific variations occur during aging with hyper-methylation taking place in all tissues except for liver. The expression of enzymes involved in methylation reactions (DNMTs and TETs) was consistent with the methylation patterns. Nutrition affects global DNA methylation status throughout lifespan. Interestingly, the differences among different tissues are magnified in 96 weeks old rats fed with low calorie diet. Moreover, the low-calorie diet appears to affect the offspring's epigenetic status more strongly if administered during the maternal pre-gestational period than the gestational and lactation time.

Therefore, we propose that changes in the global DNA methylation status may represent an epigenetic mechanism by which age and nutrition intersect each other and, in turn, influence the aging plasticity.

1. Introduction

Over the last ten years, the traditional idea that has considered the wide range of aging phenotypes as the results of genetically programmed processes and damage accumulation at both tissue-specific and whole-organism level has been progressively contextualized within a broader framework. Indeed, the sole contribution of genetics is not sufficient to elucidate the phenotypic divergence in health and adult-onset disease observed in genetically identical twins (Fraga et al., 2005). An epigenetic drift, consisting in significant changes in the overall content and locus-specific distribution of 5-methylcytosines, gradually arises during aging in human and organism models (Fraga et al., 2005; Bjornsson et al., 2008; Kaminsky et al., 2009; Wong et al., 2010; Bell and Spector, 2011; Tan et al., 2012; D'Aquila et al., 2013; Bacalini et al., 2017). More particularly, studies on DNA methylation dynamics across the lifetime has evidenced that genome-wide hypo-methylation of non-CpG islands and interspersed repetitive sequences (IRSs) are common age-related epigenetic events that cause genomic instability and contributing to cellular aging (Siegmund et al., 2007; Bjornsson et al., 2008; Bollati et al., 2009; Jintaridth and Mutirangura, 2010). In addition, by comparing DNA methylation profiles of newborns and nonagenarian/centenarians, Heyn et al. (2012) identified hypo-methylated CpGs in all genomic compartments such as promoters, exonic, intronic, and intergenic regions. On the other hand, robust, progressive, and peculiar hyper-methylation events across the lifespan occur at specific loci, to such an extent that the DNA methylation levels of discrete sites become predictive of both chronological and biological (frailty and cognitive decay) aging (Bellizzi et al., 2012; Horvath, 2013; Hannum et al., 2013; Weidner et al., 2014; D'Aquila et al., 2017; Stubbs et al., 2017).

Starting from pioneering experiments on nutritional modulation in model organisms, such as honey bee and rodents, the long-term effects of diet on gene expression variations are now increasingly recognized as major regulators that influence phenotypic plasticity as well as health and the lifespan *via* epigenetic modifications (Wolff et al., 1998; Kucharski et al., 2008; Wellen and Thompson, 2010; McKay and Mathers, 2011). In mammals, nutrient availability has been shown to induce epigenetic modifications at both global and locus-specific levels

* Corresponding authors.
E-mail addresses: giuseppe.passarino@unical.it (G. Passarino), dina.bellizzi@unical.it (D. Bellizzi).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.mad.2018.02.001
Received 6 October 2017; Received in revised form 31 January 2018; Accepted 6 February 2018

through a variety of molecular mechanisms which mainly involve mitochondrial activity (Anderson et al., 2012; Lim and Song, 2012; Hino et al., 2013; Jang and Serra, 2014; D'Aquila et al., 2015). More particularly, the bioavailability of S-adenosylmethionine (SAM), the substrate for the methyltransferase reactions, is regulated by the dietary intake of vitamin B2, B6, and B12 (Kim et al., 2009; Feil and Fraga, 2012). Therefore, studies carried out, mostly in rodents, revealed that a diet deficient in or supplemented with methyl donors, is responsible for global DNA hypo-methylation and hyper-methylation, respectively (Lee et al., 2005; Pogribny et al., 2006; Waterland et al., 2008; Pogribny et al., 2008; Pogribny et al., 2009; Mehedint et al., 2010; Craciunescu et al., 2010; Li et al., 2015; Farias et al., 2015; Zhang, 2015). In addition, calorie restriction (CR), namely the reduction of food intake widely recognized to extend longevity in different species, leads to aberrant DNA methylation patterns likely by modulating DNMT activities (Li et al., 2011; Maegawa et al., 2017).

As the epigenetic landscape is established during early embryogenesis, also early-life nutrition may cause epigenomic perturbations during aging and complex diseases, as hypothesized by the "developmental origins of health and disease" hypothesis (Barker, 2007; Chmurzynska, 2010; Vickers, 2014; Lillycrop et al., 2014). Several evidences reported that alterations in maternal nutrition, such as energy-rich, protein-deficient, micronutrient-deficient, and methyl donors-rich diets, induce modifications in methylation profile of the offspring in both human and animals (Li et al., 2010; Dominguez-Salas et al., 2012; Geraghty et al., 2015; Lee, 2015; Chango and Pogribny, 2015; Pauwels et al., 2017).

In our study, we explored in rats whether global DNA methylation profiles exhibit tissue-specific changes associated with age and are influenced by a low-calorie diet. In addition, the impact of this diet, administered during maternal pre-gestation and pregnancy/lactation, on offspring's epigenetic status was also investigated.

2. Material and methods

2.1. Animals

Experiments were performed on female Sprague-Dawley rats breeding locally in the animal care facility of the University of Calabria (Italy). Animals (n = 3 for each experimental condition) were housed in light (12:12 h light-dark cycle) and temperature (22 °C) controlled rooms with free access to food (ssniff diet V1535, German, metabolizable energy 3.057 Kcal/Kg) and water. The rats were divided into two groups: the first (control group) was fed with standard diet up to 27, 36, and 96 weeks old, the second (treated group) was fed with low-calorie diet (60% of the intake) for a total period of 24 weeks started at the age of 3, 12, and 72 weeks.

Female rats were also fed with standard and low-calorie diet for 24 weeks and then were coupled overnight and continued to be fed similarly during pregnancy and lactation. The resulting female offspring was divided into three groups and: 1) sacrificed at weaning (3 weeks); 2) fed with control diet for further 24 weeks and then sacrificed; 3) fed with low-calorie diet for further 24 weeks and then sacrificed.

Water and food intake were recorded every other day while body mass was recorded monthly. Animals were euthanized with inhalation of Diethyl ether followed by cervical transection, and immediately sacrificed. All procedures were conducted according to the European Guidelines for the care and use of laboratory animals (Directive 2010/63/EU) and in accordance with Italian law (Authorization number 295/2016-PR).

2.2. DNA and mRNA extraction

500 µl of rat peripheral blood was drawn by cardiac puncture and kept on ice in presence of DNA extraction buffer (10 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM EDTA). Heart, liver, kidney, brain, lung and a vessel (mesenteric artery) were excised, placed in cold HEPES-physiological saline solution (HEPES-PSS pH7.4, 141.8 mM NaCl, 4.7 mM KCl, 1.7 mM $MgSO_4$, 0.5 mM EDTA, 2.8 mM $CaCl_2$, 10 mM HEPES, 1.2 mM KH_2PO_4, 5 mM Glucose), weighed and thoroughly homogenized in presence of DNA extraction buffer. Then, 10% SDS and 10 mg/ml of proteinase K were added to all samples, which were then vigorously vortexed and incubated at 37 °C for 48 h with periodical mixing. Genomic DNA was obtained by phenol/chloroform purification. The DNA concentration and purity were determined spectrophotometrically.

0.05 g of frozen heart, liver and kidney were excised and homogenized in buffer RTL and total RNA was purified using RNeasy Mini Kit (Qiagen) according manufacturer's recommendations. RNA concentration was measured for each sample using a spectrophotometer and purity of the sample evaluated using the 260/280 nm absorbance ratio. RNA samples were treated with DNA-free DNase to remove any residual genomic DNA contamination.

2.3. Quantification of global 5-methylcytosine levels

Global DNA methylation levels were determined by using 5-mC DNA ELISA kit (Zymo Research) which exploits a unique anti-5-mC monoclonal antibody that is both sensitive and specific for 5-methylcytosine (5-mC). Briefly, 100 ng of genomic DNA extracted from all tissues of both standard and low-protein fed rats, as well as standard controls provided by the kit, were denatured at 98 °C for 5 min and used to coat the plate wells with 5-mC coating buffer. After incubation at 37 °C for 1 h, the wells were washed thrice with 200 µl of 5-mC ELISA buffer and then incubated at 37 °C for 1 h with an antibody mix consisting of anti-5-mC and a secondary antibody. Then, the antibody mix was removed from the wells through three consecutive washes with200 µl of 5-mC ELISA buffer and 100 µl of HRP developer were added to each well and incubated at room temperature for 1 h. The absorbance at 405 nm was measured using an ELISA plate reader.

In each experiment, the percentage of 5-mC was calculated using the second-order regression equation of the standard curve that was constructed by mixing equivalent molar concentrations at different ratios of full unmethylated and methylated control DNA (0, 5, 10, 25, 50, 75 and 100%), as suggested by the kit.

2.4. Dot blot hybridization

A total of 300 ng of genomic DNA were denatured (0.4 M sodium hydroxide, 10 mM EDTA at 100 °C for 10 min) and neutralized (6.6 M cold ammonium acetate, pH 7). Samples were then immobilized on a pre-wet Amersham Hybond-N membrane (GE Healthcare) using a gentle vacuum and then air-dried and hybridized by ultraviolet cross-linking for 10 min.

After blocking for 90 min at room temperature, the membrane was incubated for 90 min in primary antibody (1:2000 dilution of 5-mC and 5-hmC (Active Motif) and single-stranded DNA (ssDNA; EMD Millipore), washed, and incubated with anti mouse secondary antibody (1:2000 dilution; Active Motif) conjugated with horseradish peroxidase. Immunoreactivity was determined by means of the ECL chemiluminescence reaction (Amersham). As positive control of the procedure, a mixture of fully unmethylated and methylated DNA in a ratio of 0%, 20%, 40%, 60%, 80%, and 100% was used.

Quantitative evaluation of the blots of the samples was carried out by using densitometric analyses of band intensities (Kodak Electrophoresis Documentation and Analysis System 290, EDAS 290) and then normalized to the anti-ssDNA, used as the internal control.

2.5. Quantification of mRNA levels of DNMTs and TETs enzymes

Reverse Transcriptase-PCR (RT-PCR) reactions were carried out using the RevertAid RT Kit (Thermo Fisher Scientific). First, a RT mix

including 500 ng of total RNA and 1 µl of Oligo(dT)18 primers was preheated at 65 °C for 5 min. Then, the reaction was carried out in a 20 µl final volume containing 1 X Reaction Buffer, 20 U of RiboLock RNase Inhibitor, 1 mM of dNTP Mix and 200 of RevertAid M-MuLV RT reverse transcriptase. The mix was incubated for 60 min at 42 °C and successively at 70 °C for 5 min to inactivate the reverse transcriptase. The cDNAs obtained were then used as a template for real time PCRs carried out using the SYBR Green qPCR Master Mix (Promega) in a StepOne Plus machine (Applied Biosystems).

Forward and reverse primers used in the study were reported in Table S1. The final PCR mixture (15 µl) contained 1 µl of cDNA, 1X GoTaq qPCR Master Mix, 0.2 µM of each primer and 1X CXR Reference Dye. The thermal profile used for the reaction included a 2-minute heat activation of the enzyme at 95 °C, followed by 35 cycles of denaturation at 95 °C for 15 seconds and annealing/extension at 60 °C for 60 seconds, followed by melt analysis from 60 °C to 95 °C. All measurements were taken in the log phase of amplification. Negative controls (in which water instead of cDNA was added) were also run in each plate. StepOne Software V 2.0 was used to analyze data. Gene expression values were normalized to GAPDH gene expression, used as the internal control. In addition, the normalized values measured in 3 weeks-old rat samples respectively, were used as reference values (relative quantification) for the other samples.

2.6. Statistical analysis

Data is expressed as means ± SEM. Statistical analyses were performed using SPSS 20.0 statistical software (SPSS Inc., Chicago, Illinois). One-way analysis of variance (ANOVA) and Student's *t*-test were adopted, with a significance level defined as $\alpha = 0.05$.

3. Results

3.1. Global DNA methylation levels measurement in tissues from rats of different ages

5-methylcytosine (5-mC) levels were quantified by an ELISA assay in DNA samples extracted from various tissues of differently-aged rats (3, 27, 36 and 96 weeks old). As a quality control, in each assay was also evaluated the global methylation levels of samples obtained by mixing equivalent molar concentrations of the unmethylated (negative) and methylated (positive) controls at different ratios. A standard curve was generated by plotting the absorbance values for each mixture versus percentage of 5-mC levels. A logarithmic relationship was observed with a correlation of 0.996, thus confirming the effectiveness of the experimental conditions (Fig. S1). In Fig. 1 the age-associated changes of the DNA methylation status in the tissues analyzed are reported. In the heart, kidney, blood, and vessels, a loss of genomic methylation occurs in the period of life comprised between 3 and 27 weeks, meanwhile a progressive rise takes place from 27 weeks onwards. In the brain and lung, a linear increase of the methylation levels occurs across life. No differences are observed in the liver. All results were replicated by dot-blot hybridization experiments by using 5-mC antibody, (Fig. S2). What is more, 5-hmC evaluation revealed that this modified base is preponderant in the brain (Fig. S3).

3.2. Expression of DNMT and TET enzymes in tissues from rats of different ages

In order to evaluate the expression levels of DNMT and TET enzymes, quantitative real-time PCR assays were carried out in the heart, liver, and kidney from rats of different ages (Fig. 2A). Expression analysis revealed a positive correlation between the expression of DNMTs and global DNA methylation status. In the heart, the mRNA levels of DNMT1, the mayor enzyme responsible for maintaining methylation patterns following DNA replication, significantly increase during aging meanwhile those of DNMT3A, involved in *de novo* methylation, decrease in the period of life comprised between 3 and 27 weeks and then remain constant. No changes are observable for the mRNA levels of DNMT3B. In the kidney, all three enzymes exhibit a decrease in the period of life comprised between 3 and 27 weeks and a progressive increase from 27 weeks onwards. In the liver, no changes are present for all the enzymes. During aging, significant changes of the expression of TET enzymes, capable of oxidizing 5-mC into 5-hmC, are evident only in the kidney, in which, the levels of TET1 and TET2 increase in according with the 5-hmC pattern. On the contrary, mRNA levels of TET3 displays a decrease in liver and kidney (B).

3.3. Dietary effects on global DNA methylation levels in tissues from rats of different ages

In order to investigate whether nutritional effects on global DNA methylation during aging may be tissue-specific, 5-mC levels were quantified in DNA samples extracted from various tissues of differently-aged rats fed with control or six months low-calorie diet. In the heart and lung tissues of animals 27 weeks old fed with low-calorie, the diet started at weaning (3 weeks old) induces a hyper- and hypo-methylation in the tissues, respectively, compared to the control diet. Conversely, no remarkable methylation changes were observed in the other tissues (Fig. 3A). In animals of 36 weeks old, the effects of the low-calorie diet are appreciable only in the liver and in the brain (Fig. 3B). In animals of 96 weeks old, the effects of the low-calorie diet

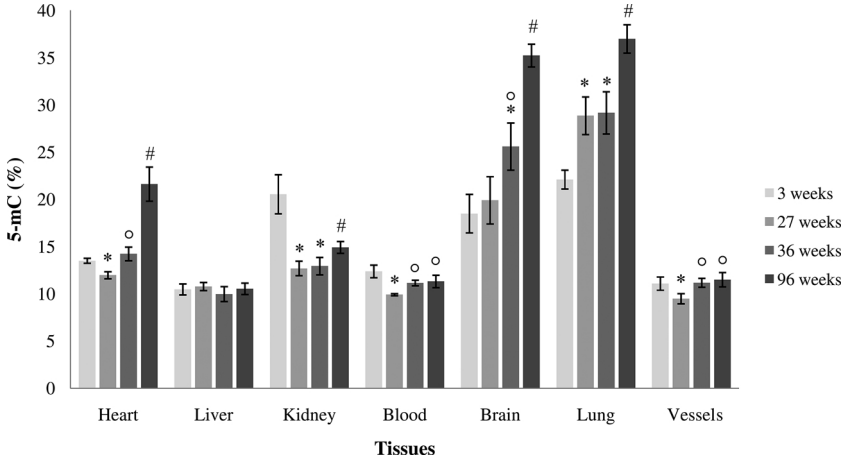


Fig. 1. Age-related DNA methylation changes in heart, liver, kidney, blood, brain, lung, and vessels of differently-aged rats. The values represent the main of three independent triplicate experiments with standard error mean.*P < 0.05 *versus* samples of 3 weeks.°P < 0.05 *versus* samples of 27 weeks. #P < 0.05 *versus* samples of 3, 27 and 36 weeks.

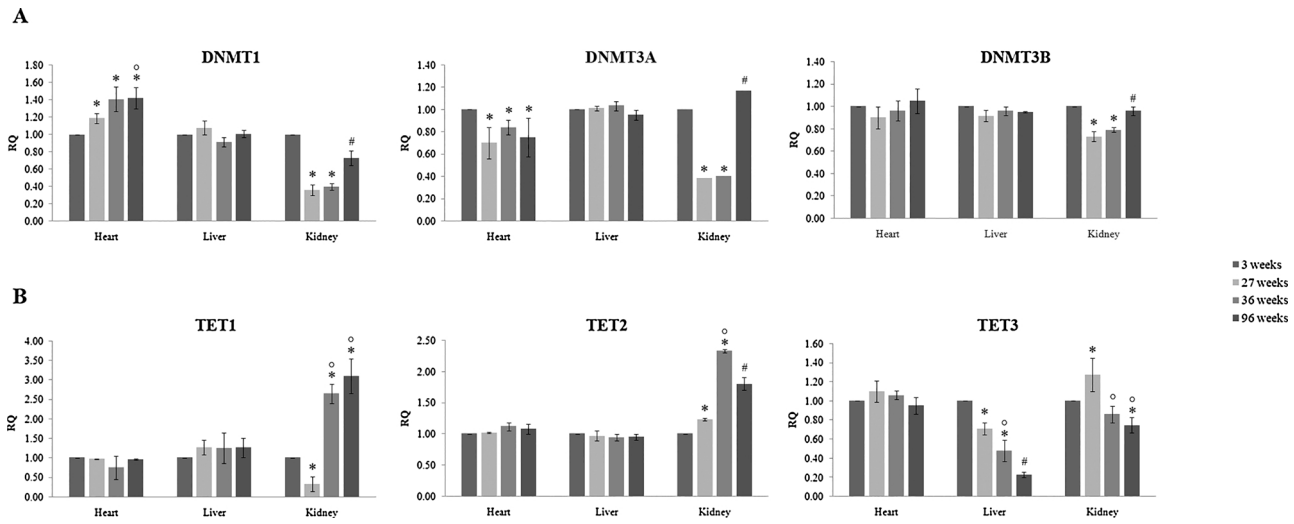


Fig. 2. mRNA levels of DNMTs (A) and TETs (B) measured in heart, liver, and kidney from rats of different ages. Levels are reported as the mean of relative quantification values (RQ), measured in three independent triplicate experiments with standard error mean (SEM). *P < 0.05 *versus* samples of 3 weeks. °P < 0.05 *versus* samples of 27 weeks. §P < 0.05 *versus* samples of 36 weeks. #P < 0.05 *versus* samples of 3, 27 and 36 weeks.

on global DNA methylation exhibit greater variability amongst tissues. Indeed, a hyper- methylation was observed in blood, a hypo-methylation in heart, kidney, brain, lung, and vessels, meanwhile no changes were observed in the liver (Fig. 3C).

3.4. Dietary effects on global DNA methylation during maternal pre-gestational and gestational time

We wanted clarify the effects of a six-months of low-calorie diet in female rats through pre-gestational and gestational time on global DNA methylation levels of offspring at weaning and after 24 weeks old. The low-calorie diet significantly increases the global methylation levels in both mother and offspring at weaning (3 weeks) independently of the tissues (Fig. 4). In addition, the offspring of the mother fed with low-calorie diet at weaning was divided into two groups: the first group was fed with control diet for 24 weeks and the second was fed with low-calorie diet for the same period. In both groups, the global DNA methylation levels were increased when compared to offspring from mother fed with control diet. In addition, differences in diet after weaning seemed to induce significant changes in methylation levels, in fact low-calorie diet after weaning displayed an increase in the methylation levels in blood and a slight decrease in kidney and vessels. No difference was observed in heart and liver between the two groups. Brain tissue was not available for these experiments.

Interestingly, the low-calorie diet through pregnancy/lactation did not influence the global DNA methylation status of the mothers (Fig. 5A). Indeed, a significant hypo-methylation induced by the diet is observed only in the blood tissue. In addition, it seems that the low-calorie diet has scarce effects on offspring since no significant difference were observed in heart, liver and vessels when compared to offspring of mothers fed with control diet (Fig. 5B). The only exceptions were kidney and blood in which a decrease in methylation occurs.

4. Discussion

The phenotypic plasticity of aging comes from the balance between the genetic background of each individual and the long-term effects of nutritional factors on health, which also act *via* epigenetic changes (Bellizzi et al., 2012; Feil and Fraga, 2012; Hino et al., 2013; D'Aquila et al., 2015; D'Aquila et al., 2017). If it is widely known that intra-individual tissue comparison exhibits significant variations in methylation levels at specific genes, little information on the global methylation status is available. The assessment of this status is particularly relevant to understanding whether the age-related changes observed are widespread or, conversely, peculiar of a definite tissue. In the latter case, the changes could reflect functional differences across tissues.

In the study here described, we quantified global DNA methylation levels in heart, liver, kidney, blood, brain, lung, and vessels from rats of different ages fed with control and low-calorie diet. Our findings indicated that DNA methylation changes are characteristic for each tissue which take place from weaning to old age. More particularly, the significant hypo- or hyper-methylation occurring in most tissues during the first period of life (3–27 weeks) could suggest that these changes play a role in growth and maturation. In fact, this is the stage of life in which the most significant processes of tissue-dependent transcriptional remodelling have been observed in both human and model organisms (Takasugi, 2011; Yu et al., 2014). The brain is quite a different matter. Its cell composition variability may explain much of the not observed variability in DNA methylation during this stage (Houseman et al., 2012; Guintivano et al., 2013). Nonetheless, starting from the 36th week, a gradual increase of methylation levels is observable in all above tissues, mostly notable in brain and lung in old animals. This is consistent with a recently published study by Stubbs et al. (2017), in which a positive correlation between age and DNA methylation was reported for heart, liver, lung, and cortex mouse tissues. Therefore, our findings change the perspective on the epigenetic remodelling during age. Indeed, if a global hypo-methylation associated with age was widely documented, to date the trend we observed provide new clues towards a global hyper-methylation so far reported only in human skin and murine hematopoietic stem cells (Beerman et al., 2013; Bormann et al., 2016; Sun et al., 2014; D'Aquila et al., 2017). This is in agreement with evidence reporting a significant rise in SAM:SAH ratios as a function of age (Poirier et al., 2001). We are confident in the reliability of our results since the global DNA methylation levels, determined by the ELISA assay, were confirmed by adopting an independent method on the entire study group. The explorative analysis on 5-hmC revealed the presence of this modified base in higher levels in the brain with respect to the other tissues and variable trends throughout life. Therefore, further studies are needed to better clarify its correlation with aging. Furthermore, gene-expression studies reported in this paper demonstrate that the observed epigenetic changes are correlated with the expression of the DNA methylation enzymes (DNMTs and TETs) which exhibit changes in their mRNA levels during aging. This is in line with literature evidence which, despite the existence of discordant cases, indicate that a transcriptional deregulation of DNMTs would probably accompany the aging process (Lopatina et al., 2002; Qian and Xu, 2014).

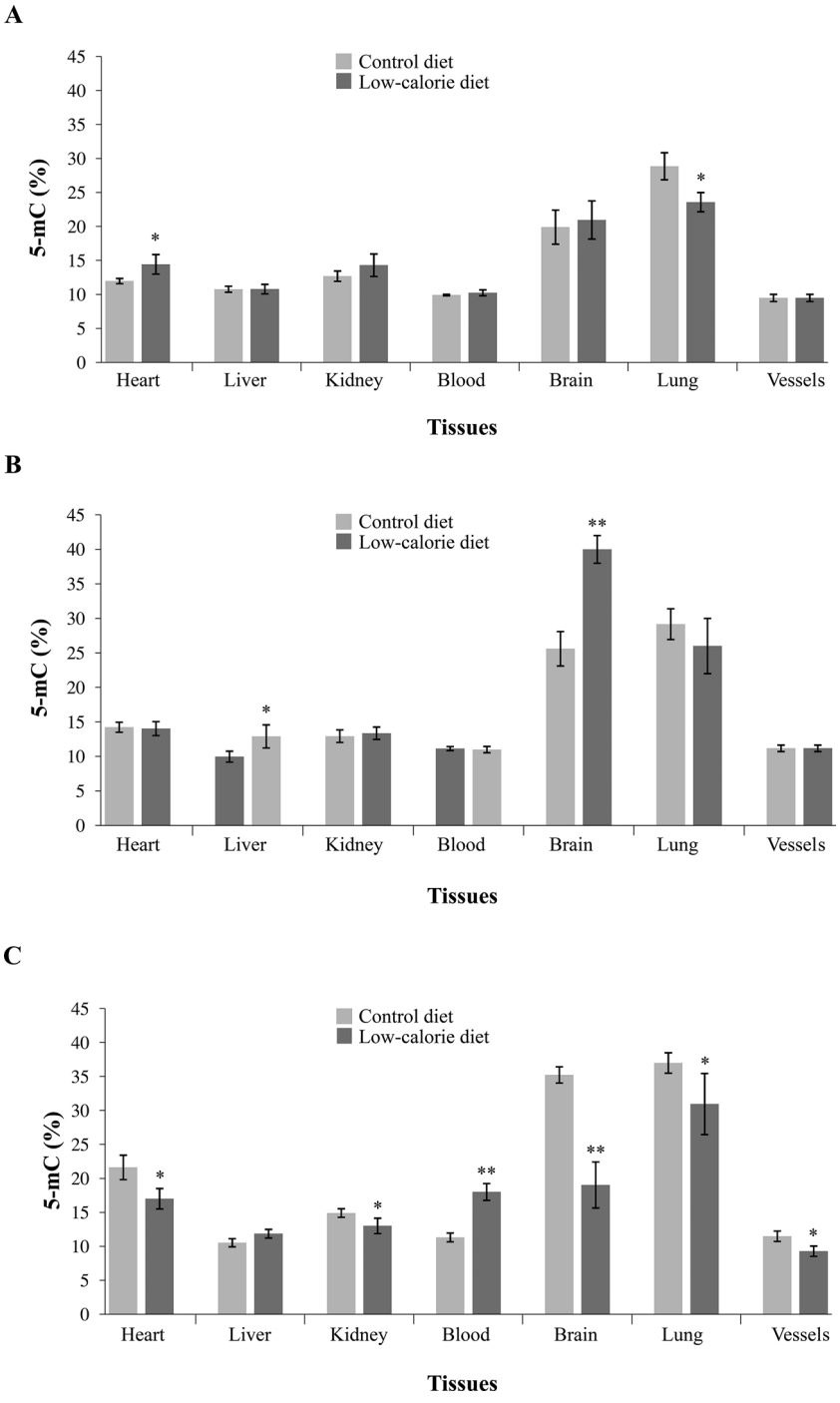


Fig. 3. Global DNA methylation levels in liver, kidney, blood, brain, lung, and vessels from rats of 27 (A), 36 (B) and 96 (C) weeks old fed with control and low-calorie diet. The values represent the main of three independent triplicate experiments with standard error mean.*P < 0.05 *versus* control diet. **P < 0.01 *versus* control diet.

By searching for the influence of a long term dietary switch on DNA methylation throughout life, we found differential tissue-specific responsiveness to the six months low-calorie diet. More particularly, by comparing the global DNA methylation levels between rats fed with control and restricted diets, although fluctuations are noticeable in juvenile and adulthood, the most significant effects were appreciable at old age. Not surprisingly, dietary content and calorie intake were inversely correlated with the availability of physiological SAM levels (Poirier et al., 2001).

In addition, an intriguing aspect, emerging from our study, is that a low-calorie diet seems to counteract the age-related DNA methylation changes. Indeed, 96 weeks-old rats fed with the low-calorie diet display methylation levels more similar to those of 27 weeks-old rats fed with standard diet. We can hypothesize that the reduced intake of calories favorably acts later in life as a buffer against those epigenetic changes which influence gene expression in aging. This is in line with the evidence reporting regulatory effects of caloric restriction on genes that contrast oxidative stress, inflammation, tumorigenesis, and neurodecay (Park and Prolla, 2005; Swindell, 2009; Wanders et al., 2014).

In the frame of the studies suggesting the impact of nutrients on the epigenome in the earliest stage of life, we designed a series of experiments aimed to clarify which maternal phase is more prone to feel the effects of nutrition, thus inducing long-term global DNA methylation changes in the offspring. In fact, in the last five years, the impact of

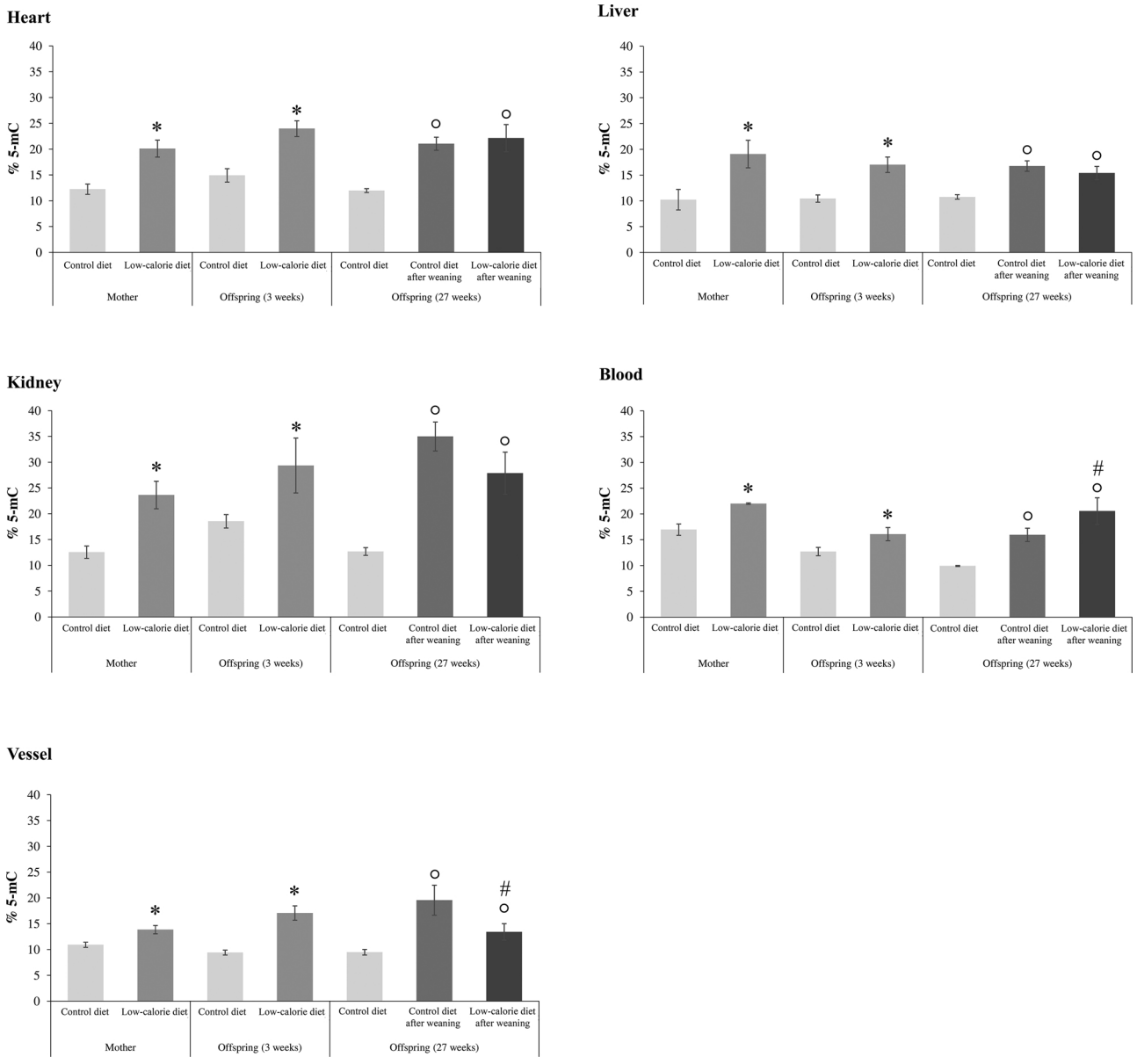


Fig. 4. Global DNA methylation levels in heart, liver, kidney, blood, and vessels from mothers fed with control and low-calorie diet for 24 weeks before pregnancy and their relative offspring sacrificed at 3 weeks and at 27 weeks after weaning. The values represent the main of three independent triplicate experiments with standard error mean.*P < 0.05 *versus* control diet; °P < 0.05 versus offspring from mothers fed with control diet; #P < 0.05 *versus* offspring (27 weeks) fed with control diet after weaning.

nutrients has been more frequently investigated in studies examining on the gene-specific level, such as *IGF2*, *LEP*, *RXRA* and *DNMT1*, rather than global DNA methylation in both human and model organisms (Park and Prolla, 2005; Buettner et al., 2006; Pauwels et al., 2017). Here we report that low-calorie diet appears to affect the offspring's pattern of global DNA methylation at weaning more strongly during the maternal pre-gestational period with respect to the gestational and lactation time. The relevance of diet during the pre-gestational period is further confirmed by the results obtained in 24 weeks- old offspring. Indeed, significant differences have been observed not so much in accordance with the type of diet administered along the 27 weeks of life, but depending on the mother's pre-gestational diet.

These results, on the one hand are particularly interesting, since they offer new clues in the field of the fetal reprogramming which will surely require further insight. On the other, they demonstrate that a process of global DNA methylation remodelling occurs according to the gestational timing of exposure to a specific diet. In this regard, an association study carried out on Dutch Famine at the end of WW II reported that the peri-conceptional period is a particularly sensitive phase for the establishment of specific methylation patterns of both imprinted (GNASAS and MEG3 genes) and non-imprinted (IL10, ABCA1 and LEP) genes (Heijmans et al., 2008; Tobi et al., 2009).

Given the general occurrence of the aging process across in mammals, we retain that data we obtained in rats can be extended to human beings.

5. Conclusion

In this study we propose that the modulation of global DNA methylation status is among the mechanisms by which nutrition influences the development and the aging process. Indeed, global DNA methylation status exhibits tissue-specific changes during age and is influenced by a low-calorie diet. A maternal low-calorie diet during the pre-gestational period results in greater changes with respect to gestational and lactation period. A future identification of genes and pathways subject to epigenetic modifications together with the reversibility of

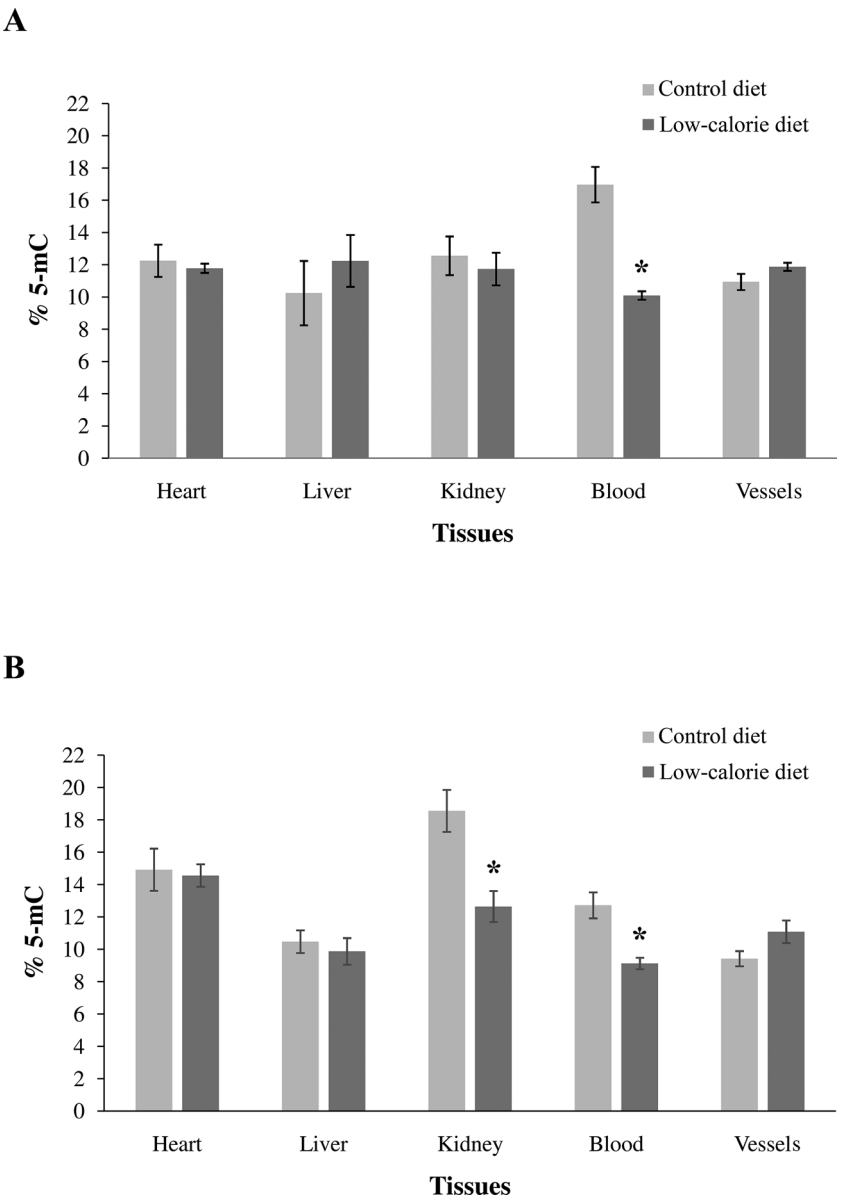


Fig. 5. Global DNA methylation levels in heart, liver, kidney, blood, and vessels from A) mothers fed with standard and low-calorie diet during pregnancy and lactation and of B) their offspring at the weaning. The values represent the main of three independent triplicate experiments with standard error mean.*P < < 0.01 *versus* control diet.

epigenetic marks will make it possible to fine-tune peculiar "finalized diets" that, with the appropriate mix of supplements, will extend life-span, improve health and development and prevent age-related diseases *via* epigenetic mechanisms.

Acknowledgements

The work has been made possible by the collaboration with the nursing homes of SADEL S.p.A (San Teodoro, San Raffaele, Villa del Rosario, A.G.I. srl, SAVELLI HOSPITAL, Casa di Cura Madonna dello Scoglio) in the frame of the agreement "SOLUZIONI INNOVATIVE PER L'INNALZAMENTO DELLA SALUTE E DELLA SICUREZZA DELLA POPOLAZIONE" with the University of Calabria. We thank Iona Thomas for the English revision of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.mad.2018.02.001.

References

Anderson, O.S., Sant, K.E., Dolinoy, D.C., 2012. Nutrition and epigenetics: an interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. J. Nutr. Biochem. 23, 853–859.

Bacalini, M.G., D'Aquila, P., Marasco, E., Nardini, C., Montesanto, A., Franceschi, C., Passarino, G., Garagnani, P., Bellizzi, D., 2017. The methylation of nuclear and mitochondrial DNA in ageing phenotypes and longevity. Mech. Ageing Dev. 165, 156–161.

Barker, D.J., 2007. The origins of the developmental origins theory. J. Intern. Med. 261, 412–417.

Beerman, I., Bock, C., Garrison, B.S., Smith, Z.D., Gu, H., Meissner, A., Rossi, D.J., 2013. Proliferation-dependent alterations of the DNA methylation landscape underlie hematopoietic stem cell aging. Cell Stem Cell. 12, 413–425.

Bell, J.T., Spector, T.D., 2011. A twin approach to unraveling epigenetics. Trends Genet. 27, 116–125.

Bellizzi, D., D'Aquila, P., Montesanto, A., Corsonello, A., Mari, V., Mazzei, B., Lattanzio, F., Passarino, G., 2012. Global DNA methylation in old subjects is correlated with frailty. Age (Dordr). 34, 169–179.

Bjornsson, H.T., Sigurdsson, M.I., Fallin, M.D., Irizarry, R.A., Aspelund, T., Cui, H., Yu, W., Rongione, M.A., Ekström, T.J., Harris, T.B., Launer, L.J., Eiriksdottir, G., Leppert, M.F., Sapienza, C., Gudnason, V., Feinberg, A.P., 2008. Intra-individual change over time in DNA methylation with familial clustering. JAMA 299, 2877–2883.

Bollati, V., Schwartz, J., Wright, R., Litonjua, A., Tarantini, L., Suh, H., Sparrow, D., Vokonas, P., Baccarelli, A., 2009. Decline in genomic DNA methylation through aging

in a cohort of elderly subjects. Mech. Age Dev. 130, 234–239.
Bormann, F., Rodríguez-Paredes, M., Hagemann, S., Manchanda, H., Kristof, B., Gutekunst, J., Raddatz, G., Haas, R., Terstegen, L., Wenck, H., Kaderali, L., Winnefeld, M., Lyko, F., 2016. Reduced DNA methylation patterning and transcriptional connectivity define human skin aging. Aging Cell. 15, 563–571.
Buettner, R., Parhofer, K.G., Woenckhaus, M., Wrede, C.E., Kunz-Schughart, L.A., Schölmerich, J., Bollheimer, L.C., 2006. Defining high-fat-diet rat models: metabolic and molecular effects of different fat types. J. Mol. Endocrinol. 36, 485–501.
Chango, A., Pogribny, I.P., 2015. Considering maternal dietary modulators for epigenetic regulation and programming of the fetal epigenome. Nutrients 7, 2748–2770.
Chmurzynska, A., 2010. Fetal programming: link between early nutrition, DNA methylation, and complex diseases. Nutr. Rev. 68, 87–98.
Craciunescu, C.N., Johnson, A.R., Zeisel, S.H., 2010. Dietary choline reverses some, but not all, effects of folate deficiency on neurogenesis and apoptosis in fetal mouse brain. J. Nutr. 140, 1162–1166.
D'Aquila, P., Rose, G., Bellizzi, D., Passarino, G., 2013. Epigenetics and aging. Maturitas 74, 130–136.
D'Aquila, P., Bellizzi, D., Passarino, G., 2015. Mitochondria in health, aging and diseases: the epigenetic perspective. Biogerontology 16, 569–585.
D'Aquila, P., Montesanto, A., Mandalà, M., Garasto, S., Mari, V., Corsonello, A., Bellizzi, D., Passarino, G., 2017. Methylation of the ribosomal RNA gene promoter is associated with aging and age-related decline. Aging Cell. 16, 966–975.
Dominguez-Salas, P., Cox, S.E., Prentice, A.M., Hennig, B.J., Moore, S.E., 2012. Maternal nutritional status, C(1) metabolism and offspring DNA methylation: a review of current evidence in human subjects. Proc. Nutr. Soc. 71, 154–165.
Farias, N., Ho, N., Butler, S., Delaney, L., Morrison, J., Shahrzad, S., et al., 2015. The effects of folic acid on global DNA methylation and colonosphere formation in colon cancer cell lines. J. Nutr. Biochem. 26, 818–826.
Feil, R., Fraga, M.F., 2012. Epigenetics and the environment: emerging patterns and implications. Nat. Rev. Genet. 13, 97–109.
Fraga, M.F., Ballestar, E., Paz, M.F., Ropero, S., Setien, F., Ballestar, M.L., Heine-Suñer, D., Cigudosa, J.C., Urioste, M., Benitez, J., Boix-Chornet, M., Sanchez-Aguilera, A., Ling, C., Carlsson, E., Poulsen, P., Vaag, A., Stephan, Z., Spector, T.D., Wu, Y.Z., Plass, C., Esteller, M., 2005. Epigenetic differences arise during the lifetime of monozygotic twins. Proc. Natl. Acad. Sci. U. S. A. 102, 10604–10609.
Geraghty, A.A., Lindsay, K.L., Alberdi, G., McAuliffe, F.M., Gibney, E.R., 2015. Nutrition during pregnancy impacts offspring's epigenetic status-evidence from human and animal studies. Nutr. Metab. Insights 8, 41–47.
Guintivano, J., Aryee, M.J., Kaminsky, Z.A., 2013. A cell epigenotype specific model for the correction of brain cellular heterogeneity bias and its application to age, brain region and major depression. Epigenetics 8, 290–302.
Hannum, G., Guinney, J., Zhao, L., Zhang, L., Hughes, G., Sadda, S., Klotzle, B., Bibikova, M., Fan, J.B., Gao, Y., Deconde, R., Chen, M., Rajapakse, I., Friend, S., Ideker, T., Zhang, K., 2013. Genome-wide methylation profiles reveal quantitative views of human aging rates. Mol. Cell. 49, 359–367.
Heijmans, B.T., Tobi, E.W., Stein, A.D., Putter, H., Blauw, G.J., Susser, E.S., Slagboom, P.E., Lumey, L.H., 2008. Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc. Natl. Acad. Sci. U. S. A. 105, 17046–17049.
Heyn, H., Li, N., Ferreira, H.J., Moran, S., Pisano, D.G., Gomez, A., Diez, J., Sanchez-Mut, J.V., Setien, F., Carmona, F.J., Puca, A.A., Sayols, S., Pujana, M.A., Serra-Musach, J., Iglesias-Platas, I., Formiga, F., Fernandez, A.F., Fraga, M.F., Heath, S.C., Valencia, A., Gut, I.G., Wang, J., Esteller, M., 2012. Distinct DNA methylomes of newborns and centenarians. Proc. Natl. Acad. Sci. U. S. A. 109, 10522–10527.
Hino, S., Nagaoka, K., Nakao, M., 2013. Metabolism-epigenome crosstalk in physiology and diseases. J. Hum. Genet. 58, 410–415.
Horvath, S., 2013. DNA methylation age of human tissues and cell types. Genome Biol. 14, R115.
Houseman, E.A., Accomando, W.P., Koestler, D.C., Christensen, B.C., Marsit, C.J., Nelson, H.H., Wiencke, J.K., Kelsey, K.T., 2012. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinf. 13, 86.
Jang, H., Serra, C., 2014. Nutrition, epigenetics, and disease. Clin. Nutr. Res. 3, 1–8.
Jintaridth, P., Mutirangura, A., 2010. Distinctive patterns of age-dependent hypomethylation in interspersed repetitive sequences. Physiol. Genomics 41, 194–200.
Kaminsky, Z.A., Tang, T., Wang, S.C., Ptak, C., Oh, G.H., Wong, A.H., Feldcamp, L.A., Virtanen, C., Halfvarson, J., Tysk, C., McRae, A.F., Visscher, P.M., Montgomery, G.W., Gottesman, I.I., Martin, N.G., Petronis, A., 2009. DNA methylation profiles in monozygotic and dizygotic twins. Nat. Genet. 41, 240–245.
Kim, K.C., Friso, S., Choi, S.W., 2009. DNA methylation, an epigenetic mechanism connecting folate to healthy embryonic development and aging. J. Nutr. Biochem. 20, 917–926.
Kucharski, R., Maleszka, J., Foret, S., Maleszka, R., 2008. Nutritional control of reproductive status in honeybees via DNA methylation. Science 319, 1827–1830.
Lee, W.J., Shim, J.Y., Zhu, B.T., 2005. Mechanisms for the inhibition of DNA methyltransferases by tea catechins and bioflavonoids. Mol. Pharmacol. 68, 1018–1030.
Lee, H.S., 2015. Impact of maternal diet on the epigenome during In utero life and the developmental programming of diseases in childhood and adulthood. Nutrients 7, 9492–9507.
Li, C.C., Maloney, C.A., Cropley, J.E., Suter, C.M., 2010. Epigenetic programming by maternal nutrition: shaping future generations. Epigenomics 2, 539–549.
Li, Y., Daniel, M., Tollefsbol, T.O., 2011. Epigenetic regulation of caloric restriction in aging. BMC Med. 9, 98.
Li, X., Sun, Q., Li, X., Cai, D., Sui, S., Jia, Y., Song, H., Zhao, R., 2015. Dietary betaine supplementation to gestational sows enhances hippocampal IGF2 expression in newborn piglets with modified DNA methylation of the differentially methylated regions. Eur. J. Nutr. 54, 1201–1210.
Lillycrop, K.A., Hoile, S.P., Grenfell, L., Burdge, G.C., 2014. DNA methylation, ageing and the influence of early life nutrition. Proc. Nutr. Soc. 73, 413–421.
Lim, U., Song, M.A., 2012. Dietary and lifestyle factors of DNA methylation. Methods Mol. Biol. 863, 359–376.
Lopatina, N., Haskell, J.F., Andrews, L.G., Poole, J.C., Saldanha, S., Tollefsbol, T., 2002. Differential maintenance and de novo methylating activity by three DNA methyltransferases in aging and immortalized fibroblasts. J. Cell. Biochem. 84, 324–334.
Maegawa, S., Lu, Y., Tahara, T., Lee, J.T., Madzo, J., Liang, S., Jelinek, J., Colman, R.J., Issa, J.J., 2017. Caloric restriction delays age-related methylation drift. Nat. Commun. 8, 539.
McKay, J.A., Mathers, J.C., 2011. Diet induced epigenetic changes and their implications for health. Acta Physiol. (Oxf.) 202, 103–118.
Mehedint, M.G., Craciunescu, C.N., Zeisel, S.H., 2010. Maternal dietary choline deficiency alters angiogenesis in fetal mouse hippocampus. Proc. Natl. Acad. Sci. U. S. A. 107, 12834–12839.
Park, S.K., Prolla, T.A., 2005. Lessons learned from gene expression profile studies of aging and caloric restriction. Ageing Res. Rev. 4, 55–65.
Pauwels, S., Ghosh, M., Duca, R.C., Bekaert, B., Freson, K., Huybrechts, I., Langie, S.A.S., Koppen, G., Devlieger, R., Godderis, L., 2017. Maternal intake of methyl-group donors affects DNA methylation of metabolic genes in infants. Clin. Epigenet. 9, 16.
Pogribny, I.P., Ross, S.A., Wise, C., Pogribna, M., Jones, E.A., Tryndyak, V.P., et al., 2006. Irreversible global DNA hypomethylation as a key step in hepato carcinogenesis induced by dietary methyl deficiency. Mutat. Res. 593, 80–87.
Pogribny, I.P., Karpf, A.R., James, S.R., Melnyk, S., Han, T., Tryndyak, V.P., 2008. Epigenetic alterations in the brains of fisher 344 rats induced by long-term administration of folate/methyl-deficient diet. Brain Res. 1237, 25–34.
Pogribny, I.P., Tryndyak, V.P., Bagnyukova, T.V., Melnyk, S., Montgomery, B., Ross, S.A., et al., 2009. Hepatic epigenetic phenotype predetermines individual susceptibility to hepatic steatosis in mice fed a lipogenic methyl-deficient diet. J. Hepatol. 51, 176–186.
Poirier, L.A., Wise, C.K., Delongchamp, R.R., Sinha, R., 2001. Blood determinations of *S*-adenosylmethionine, *S*-adenosylhomocysteine, and homocysteine: correlations with diet. Cancer Epidemiol. Biomarkers Prev. 10, 649–655.
Qian, H., Xu, X., 2014. Reduction in DNA methyltransferases and alteration of DNA methylation pattern associate with mouse skin ageing. Exp. Dermatol. 23, 357–359.
Siegmund, K.D., Connor, C.M., Campan, M., Long, T.I., Weisenberger, D.J., Biniszkiewicz, D., Jaenisch, R., Laird, P.W., Akbarian, S., 2007. DNA methylation in the human cerebral cortex is dynamically regulated throughout the life span and involves differentiated neurons. PLoS One 2, e895.
Stubbs, T.M., Bonder, M.J., Stark, A.K., Krueger, F., BI Ageing Clock Team, von Meyenn, F., Stegle, O., Reik, W., 2017. Multi-tissue DNA methylation age predictor in mouse. Genome Biol. 18, 68.
Sun, D., Luo, M., Jeong, M., Rodriguez, B., Xia, Z., Hannah, R., et al., 2014. Epigenomic profiling of young and aged HSCs reveals concerted changes during aging that reinforce self-renewal. Cell Stem Cell. 14, 673–688.
Swindell, W.R., 2009. Genes and gene expression modules associated with caloric restriction and aging in the laboratory mouse. BMC Genomics 10, 585.
Takasugi, M., 2011. Progressive age-dependent DNA methylation changes start before adulthood in mouse tissues. Mech. Ageing Dev. 132, 65–71.
Tan, Q., Christiansen, L., Thomassen, M., Kruse, T.A., Christensen, K., 2012. Twins for epigenetic studies of human aging and development. Age. Res. Rev. 12, 182–187.
Tobi, E.W., Lumey, L.H., Talens, R.P., Kremer, D., Putter, H., Stein, A.D., Slagboom, P.E., Heijmans, B.T., 2009. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. Hum. Mol. Genet. 18, 4046–4053.
Vickers, M.H., 2014. Early life nutrition, epigenetics and programming of later life disease. Nutrients 6, 2165–2178.
Wanders, D., Ghosh, S., Stone, K.P., Van, N.T., Gettys, T.W., 2014. Transcriptional impact of dietary methionine restriction on systemic inflammation: relevance to biomarkers of metabolic disease during aging. Biofactors 40, 13–26.
Waterland, R.A., Travisano, M., Tahiliani, K.G., Rached, M.T., Mirza, S., 2008. Methyl donor supplementation prevents transgenerational amplification of obesity. Int. J. Obes. (Lond.) 32, 1373–1379.
Weidner, C.I., Lin, Q., Koch, C.M., Eisele, L., Beier, F., Ziegler, P., Bauerschlag, D.O., Jöckel, K.H., Erbel, R., Mühleisen, T.W., Zenke, M., Brümmendorf, T.H., Wagner, W., 2014. Aging of blood can be tracked by DNA methylation changes at just three CpG sites. Genome Biol. 15, R24.
Wellen, K.E., Thompson, C.B., 2010. Cellular metabolic stress: considering how cells respond to nutrient excess. Mol. Cell. 40, 323–332.
Wolff, G.L., Kodell, R.L., Moore, S.R., Cooney, C.A., 1998. Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice. FASEB J. 12 949–257.
Wong, C.C., Caspi, A., Williams, B., Craig, I.W., Houts, R., Ambler, A., Moffitt, T.E., Mill, J., 2010. A longitudinal study of epigenetic variation in twins. Epigenetics 5, 516–526.
Yu, Y., Fuscoe, J.C., Zhao, C., Guom, C., Jiam, M., Qingm, T., Bannon, D.I., Lancashire, L., Bao, W., Du, T., Luo, H., Su, Z., Jones, W.D., Moland, C.L., Branham, W.S., Qian, F., Ning, B., Li, Y., Hong, H., Guo, L., Mei, N., Shi, T., Wang, K.Y., Wolfinger, R.D., Nikolsky, Y., Walker, S.J., Duerksen-Hughes, P., Mason, C.E., Tong, W., Thierry-Mieg, J., Thierry-Mieg, D., Shi, L., Wang, C., 2014. A rat RNA-Seq transcriptomic Body Map across 11 organs and 4 developmental stages. Nat. Commun. 5, 3230.
Zhang, N., 2015. Epigenetic modulation of DNA methylation by nutrition and its mechanisms in animals. Anim. Nutr. 1, 144–151.

[Frontiers in Bioscience, Landmark, 22, 888-908, January 1, 2017]

Mitochondrial genome and epigenome: two sides of the same coin

Patrizia D'Aquila[1], Alberto Montesanto[1], Francesco Guarasci[1], Giuseppe Passarino[1], Dina Bellizzi[1]

[1]Department of Biology, Ecology and Earth Sciences, University of Calabria, 87036 Rende, Italy

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. mtDNA features
3.1. The mitochondrial genome: structure, replication and transcription
3.2. Genetics of mtDNA
4. Mitochondrial DNA epigenetics
4.1. Mitochondrial DNA methylation and hydroxymethylation
5. mtDNA methylation as biomarker of aging and diseases
5.1. mtDNA methylation and environmental exposures
5.2. mtDNA methylation and aging
5.3. mtDNA methylation and diseases
6. Conclusions and future perspectives
7. Acknowledgement
8. References

1. ABSTRACT

The involvement of mitochondrial content, structure and function as well as of the mitochondrial genome (mtDNA) in cell biology, by participating in the main processes occurring in the cells, has been a topic of intense interest for many years. More specifically, the progressive accumulation of variations in mtDNA of post-mitotic tissues represents a major contributing factor to both physiological and pathological phenotypes. Recently, an epigenetic overlay on mtDNA genetics is emerging, as demonstrated by the implication of the mitochondrial genome in the regulation of the intracellular epigenetic landscape being itself object of epigenetic modifications. Indeed, *in vitro* and population studies strongly suggest that, similarly to nuclear DNA, also mtDNA is subject to methylation and hydroxymethylation. It follows that the mitochondrial-nucleus cross talk and mitochondrial retrograde signaling in cellular properties require a concerted functional cooperation between genetic and epigenetic changes. The present paper aims to review the current advances in mitochondrial epigenetics studies and the increasing indication of mtDNA methylation status as an attractive biomarker for peculiar pathological phenotypes and environmental exposure.

2. INTRODUCTION

Mitochondria are the only animal organelles to have their own genome. They comprise a circular, histone- and intron-free 'chromosome' of 16.6. kb of DNA, present in one or more copies, which encodes tRNAs, rRNAs, and a few subunits of the oxidative phosphorylation (OXPHOS) system (1,2). Traditionally the high mutation rate made the mtDNA an excellent tool for the reconstruction of human population history, similarly to what was described for other organelles (3). Lately, impressive evidence has expanded research in considering it as regulator of a wide variety of phenotypic physiological and pathological outcomes. This regulation takes place either directly by influencing the efficiency in energy metabolism or indirectly by interacting with nuclear genes and by increasing the penetrance of the nuclear mutations (4,5). Being semiautonomous organelles, mitochondrial functionality requires a coordinated expression of genes encoded by both the nuclear and mitochondrial genome, and this is accomplished through a close network of bidirectional signals between the two genomes balancing the mitochondrial status, in terms of biogenesis and function, and the energetic needs (6). In this context, it has also come to light that mitochondria and mitochondrial genomes impact the establishment and maintenance of the whole cellular epigenome by both controlling the availability of the co-substrates of epigenetics enzymes and being itself target of methylation changes (7-10). Indeed, the availability of high-throughput sequencing technology significantly improved the sensitivity of methods applied to mtDNA to detect methylated cytosines (5-mC), definitively clarifying the forty-year dispute about the possible existence of epigenetic modifications at mtDNA level (11). Not only methylated and hydroxymethylated (5-hmC) cytosines were noted at both CpG and non-CpG sites of mtDNA but also the intra-mitochondrial traslocation of DNMT and

TET enzymes and their dynamic regulation according to specific physiological (or pathological) conditions and in response to peculiar environmental changes were recently attested (12-19).

In this review, we summarize the most salient aspects relative to mitochondrial DNA genetics and epigenetics, giving special attention to those significant correlations between mtDNA methylation changes and peculiar phenotypes, diseases as well as environmental exposure.

3. mtDNA FEATURES

3.1. The mitochondrial genome: structure, replication and transcription

Mitochondria contain many copies (1000-5000) of their own genome, the mitochondrial DNA (mtDNA), documented for the first time in 1955 and indicated as non-chromosomal genetic element rho (20). These organelle share many features with prokaryotes and are commonly thought to originate by endosymbionts in the ancestral eukaryote. The historical "endosymbiosis theory" has been modified over the years and the revised theory has been labeled as the "hydrogen hypothesis" that postulates that the eukaryotic nucleus and the mitochondria were created simultaneously through the fusion of a hydrogen-requiring methanogenic *Archaebacterium* and a hydrogen-producing alpha-proteobacterium (the symbiont). A recent phylogenomic study suggests a common origin of mitochondria and the SAR11 clade of *Alphaproteobacteria* as a sister group to the Rickettsiales (21-23).

Human mtDNA is a covalently double-stranded closed circular molecule that is ≈5 μm long, has a molecular mass of 10^7 daltons and it is approximately 16.6. kb in size (2, 24). It was completely sequenced in 1981 and a revision of this sequence was later carried out by Andrews *et al*. (1, 25). Similar to bacterial chromosomes, mtDNA is organized into nucleoprotein structures called nucleoides, firstly reported in *Saccharomyces cerevisiae* and then observed in human cells. Nucleoids carry 1-2 molecules of mtDNA and are dynamic structures which can be associated with the inner mitochondrial membrane or also distributed through the mitochondrial network, as demonstrated by Garrido *et al*. by time-lapse fluorescence microscopy in cells lacking mtDNA (rho^0 cells) (26-30). Wang and Bogenhagen have identified a series of proteins forming nucleoids, including mitochondrial single-stranded binding proteins (mtSSB), TWINKLE, mtDNA polymerase (POLG), mitochondrial RNA polymerase (POLRMT) and mitochondrial transcription factor A (TFAM), which are directly involved in mtDNA replication and transcription, as well as ANT1 and prohibitin, which are proteins associated with mitochondrial membranes (31). According to some hypothesis, nucleoids replicate as discrete units, giving rise to daughter nucleoids identical to the parental, which can themselves segregate freely. Alternatively, nucleoids can reorganize, under nuclear genetic control, and dynamically exchange with each other mtDNA molecules (32, 33). It is likely that both the two models occur in cell and tissues in alternative conditions.

Human mtDNA has a compact structure characterized by very few intergenic spacers, except for one regulatory region, lacking of introns, overlapping genes and part of the termination codons are generated post-transcriptionally by polyadenylation of the mRNAs. It consists of two strands, a guanine-rich heavy (H) and a cytosine-rich light (L) strand, historically so named on the basis of the nucleotide composition and the separation in denaturing cesium chloride gradient. mtDNA contains 37 genes encoding for 13 essential subunits of the oxidative phosphorylation (OXPHOS) system, including seven subunits of complex I (ND1-6 and ND4L), one subunit of complex III (Cyt*b*), three subunits of complex IV (COI-III) and two subunits of complex V (ATP6-8), 2 rRNAs (12S and 16S) and 22 tRNAs. The remaining mitochondrial OXPHOS proteins, metabolic enzymes, DNA and RNA polymerases, ribosomal proteins and mtDNA regulatory factors are encoded by nuclear genes, synthesized on cytosolic ribosomes, usually with a N-terminal mitochondrial targeting sequence, and then imported into the organelle by translocases located in the mitochondrial compartments (34-37). The genetic code of human mtDNA differs from that of nuclear DNA. More specifically, in mitochondria TGA is not a termination codon but codes for tryptofan, ATA codes for methionine rather than for isoleucine and, finally, AGG or AGA code for a stop codon instead of arginine. Within mtDNA there is only one non-coding region, the displacement loop (D-loop), a region of about 1.1. kb, spanning between the phenylalanine and proline tRNA genes, that contains the origin of the H-strand replication (O_H) and the promoters of L- and H-strand transcription (LSP and HSP) as well as regulatory elements for both mitochondrial replication and transcription (38). The D-loop contains two hypervariable regions, HVRI (16024-16383 nt) and HVRII (57-372 nt), used especially in human population genetic and ancestry studies, and three highly conserved regions, CSBI-III, mainly involved in mtDNA replication. The loop is a stable triple-stranded structure that originated from the incorporation of a linear third DNA strand of approximately 650 nt, named 7S DNA, based upon its sedimentation properties, and originates from a premature termination of mtDNA replication from O_H (39). Beyond the historically recognized function to maintain an open structure that facilitates mtDNA replication, an involvement of the D-loop in maintenance of DNA topology, DNA recombination and structural association with membrane has been proposed (40).

MtDNA replicates independently of the cell cycle. To date, several models of replication have been proposed (41-43). Regarding the firstly characterized model, known as strand-displacement model (SDM), mammalian mtDNA molecules replicate unidirectionally from two distinct strand-specific origins, which are O_H and O_L, located in the D-loop region and in a cluster of five tRNAs, respectively. A round of replication begins at O_H and continues along the parental L-strand to produce a full H-strand circle. Only after the replication fork has passed the second replication origin, O_L,the synthesis of the L-strand initiates proceeding in the opposite direction (44-49). More recently, a second model, known as RITOLS (RNA incorporated throughout the lagging strand), has suggested a strand-coupled replication with a leading-strand synthesis proceeding with the simultaneous incorporation of RNA on the lagging strand and its following conversion to DNA when lagging-strand synthesis is started (50, 51).

MtDNA transcription occurs in opposite directions around the entire genome starting from the two strand-specific promoters, HSP and LSP, which are functionally independent, and generate poly-cistronic transcripts, subsequently processed to give mature mRNAs, rRNAs and tRNAs through a maturation process involving different enzymatic activities (38, 52-54). The H-strand encodes the majority of the genes through two transcription starting sites. The first is located 16 bp upstream of the tRNA phenylalanine gene and produces a transcript which terminates at the 3' end of the 16S rRNA gene, while the second is close to the 5' end of the 12S rRNA gene and produces a polycistronic molecule corresponding to most of the H strand. The L-strand, whose transcription start site is located within the L-strand promoter, encodes only eight genes including tRNAs and the ND6 subunit. Different *cis*- and *trans*- acting sequences and factors, respectively, involved in the replication and transcription processes, have now been identified and characterized (39, 55, 56). These factors include DNA polymerase gamma, mitochondrial RNA polymerase, mitochondrial SSB, TFAM, mitochondrial DNA ligase, RNA processing enzymes and different helicases and topoisomerases, TFB1M, TFB2M and mTERF. Some of these carry out their activity in both replication and transcription processes, such as the TFAM factor, that plays an overlapping role since it appears crucial for the regulation of genome copy number, although it is not a component of the basic replication machinery, and for mtDNA transcription, by unwinding the mtDNA helix and thus exposing the promoter region to the transcription machinery (57, 58). Other factors act as transcription regulators which can modulate the transcription of nuclear-encoded genes involved in various mitochondrial functions and biogenesis, such as nuclear respiratory factors 1 and 2 (NRF-1, NRF-2). Alternatively, they can be imported into mitochondria and alter transcription of the mitochondrial genome, for example the PPARγ coactivator 1α factor (PGC-1 gamma) (55, 59).

Mitochondrial DNA replication and transcription are constantly regulated by coordinated nuclear and mitochondrial pathways, during development and at both physiological and patho-physiological conditions, in order to ensure efficient biogenesis process, cell's energy demand, mitochondrial oxidative metabolism and proper cellular function (55, 59-62).

3.2. Genetics of mtDNA

Distinctive features of mtDNA make it particularly interesting for genetic studies as well as for the understanding of the aetiology of mitochondrial diseases. With rare exceptions reported in diverse eukaryotic taxa, it is inherited only maternally, through the oocyte cytoplasm, meanwhile mtDNA molecules in mammalian sperm, modified by ubiquitin labelling during spermatogenesis and degraded by the proteasomes and/or lysosomes after fertilization, are never transmitted to offspring, likely because they are highly damaged by ROS (Reactive Oxidative Species) produced during the spermatogenesis and the sperm swimming (63-68). For this reason, it has long been believed that mtDNA did not undergo recombination. However, over the last five years, some direct evidence concerning the existence of this process progressively emerging, taking into consideration that, in some cases, human paternal mtDNA may enter the egg and that mammalian mitochondria contain the enzymes necessary to promote homologous recombination (69-73).

Another form of mtDNA inheritance concerns the transfer of a large fraction of the mitochondrial genomic information to the nuclear genome, representing an important mechanism of genetic variation that helped to forge the prokaryote-to-eukaryote transition (74, 75). This transfer, that involves repair of double-stranded breaks by non-homologous end-joining, generates nuclear copies of mitochondrial DNA (NUMTs) which or are re-imported or, mostly, acquire novel functions. NUMTs account for a noticeable fraction of the nuclear genome. In the human genome at least 400 kb, in *Nasonia* 43 kb and in Apis over 230 kb of the nuclear DNA consists of NUMTs (76). In contrast, some genomes such as that of *Drosophila melanogaster* are nearly devoid of mitochondrial DNA (77). NUMTs appear to preferentially integrate into repetitive DNA sequences as well as into DNA regions with different GC content, thus suggesting that chromosomal structure might influence integration of NUMTs (78). Some NUMTs have accumulated many changes, and thus have resided in the nucleus for a long time, while others are similar to the reference human mtDNA, and thus must be recent. In humans, a dozen human loci are polymorphic for the presence of NUMTs, underscoring the rapid rate at which mitochondrial sequences reach the nucleus over evolutionary time. Overall, it is believed that

about a third of human-specific NUMTs is variable. In this context, NUMTs have been suggested as an interesting tool in primate phylogeny (79). Integration of NUMTs not only appears as neutral polymorphism but, more rarely, is also associated with human diseases, including severe plasma factor VII deficiency (bleeding disease), Pallister-Hall syndrome, mucolipidosis IV (80).

mtDNA has an evolutionary rate higher than that of the nuclear DNA, attributable to a high mutation rate due to both the lack of effective mitochondrial repair mechanisms and constant exposure of this DNA to oxygen free radicals which mostly derive from the mitochondrial oxidative phosphorylation system (OXPHOS). The accumulation of damages in mtDNA and the subsequent OXPHOS impairment over the course of life are at the basis of several aging and diseases models, in which mitochondria and mitochondrial genome play a central role (81-85).

Given the presence of multiple copies of mtDNA within each cell (polyplasmy), when a mutation occurs, both normal and mutated mtDNA can coexist in varying proportion, a condition known as heteroplasmy. The level of heteroplasmy can vary among cells, tissues, organs within the same individual, and among individuals in the same family as the result of a random distribution of both normal and mutant mtDNA molecules to the daughter cells (mitotic segregation). In human, many heteroplasmic mutations are associated to pathologies, in which biochemical defects in the respiratory chain can be detected only if the levels of mutations exceed a threshold value (about 60-80%), that is correlated to mutation type and to tissue's energy demand (86, 87). The occurrence of mutant and germinal mtDNAs in different organelles in the same cells may cause complementation, a process in which mitochondria fuse and mix their mtDNAs, so malfunctioning mitochondria can retrieve a wild-type mtDNA copy. This exchange should allow either the removal of the defective copy of the mtDNA or its repair by using a series of enzymes involved in this process. The mechanisms governing complementation, segregation and transmission of heteroplasmic mtDNA mutations depend on the dynamics of the mitochondrial compartment, the intra-mitochondrial organization and the mobility of mtDNA (33, 88, 89).

A substantial number of inherited mutations accumulate over time along radiating female lineages, that give rise to many types of mtDNA. These lineages differ with respect to mutations present in their ancestor and evolve independently. According to the presence/absence of specific variants at evolutionarily conserved positions, mtDNA types were categorized into haplogroups, which define a cluster of different mtDNA molecules sharing a common origin (90-93). The haplogroups have been identified by searching for population polymorphic sites, initially through the use of RFLP analysis and then by direct sequencing of both mtDNA coding and non coding regions. More recently, complete mtDNA sequencing allowed the subdivision of haplogroups into smaller groups, known as sub-haplogroups (94). The uniparental inheritance of mtDNA and associated lack of intermolecular recombination mean that these variants have remained restricted to specific ethnic groups and have been used by population geneticists to define the migration and colonization of the planet, supporting the 'out of Africa' hypothesis, which proposes that the human mtDNA had its origins in Africa before migrating out and populating the globe (87). The mtDNA variations were initially looked at as nearly neutral and used only for the reconstruction of human population history (95). The finding that these variations can have functional consequences and some of them are able to produce a spectrum of clinical symptoms in several mitochondrial diseases changed the view of their neutrality. In particular, such a view arose when it was shown that the different mtDNA lineages are qualitatively different from each other. The first evidence for this was provided by Ruiz-Pesini and co-workers, who reported that mtDNA molecules of H and T haplogroups displayed significant differences in the activity of OXPHOS Complex I and IV (96). More recently, significant associations have been found between mtDNA haplogroups and physiological phenotypes, including higher mitochondrial copy number, decreased reactive oxygen species production, mitochondrial metabolism, body fat mass, hearing loss, general cognitive ability, aging as well as with pathological traits, such as Parkinson and Alzheimer disease, diabetes, cardiovascular disease, schizophrenia, Leber's hereditary optic neuropathy and cancer, in which they were found to contribute to oncogenesis and metastatic spread (95, 97-103). In addition, specific mtDNA variants seem to contribute to climatic adaptation in human populations by regulating bio-energy processes (104-107). The above associations have been also explained considering that, although physically distinct, nucleus and mitochondria interact with each other through a bi-directional flow of information involving several signal transduction pathways (108, 109).

A significant improvement in understanding the influence of specific mtDNA variants on cellular phenotypes and, therefore, the relationship between these variants and complex traits has come from *in vitro* studies using cybrid cell lines, made by the repopulation of rho^0 cells lacking of mtDNA with mitochondria derived from enucleated cells harbouring particular types of mtDNA molecules, i.e. mutated DNAs, on a common nuclear genetic background (110-112). More specifically, mtDNA and mtRNA levels, mitochondrial protein synthesis, cytochrome oxidase levels and activity, normalized oxygen consumption, mitochondrial inner-membrane potential and growth capacity were found to be different in cybrids with mtDNA molecules harbouring the H haplogroup, representing about 41% of mtDNA types

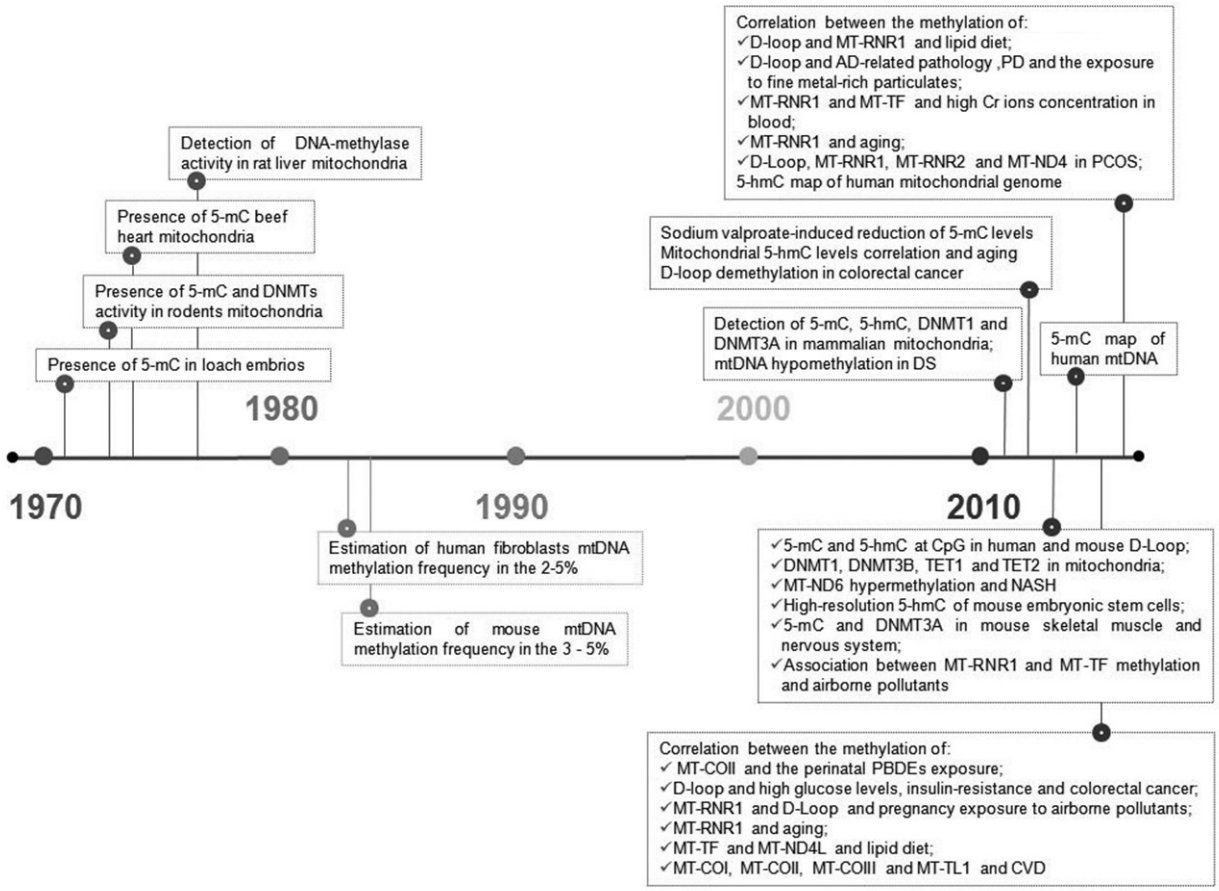


Figure 1. Timeline (years) of key discoveries supporting the existence of mitochondrial DNA methylation and hydroxymenthylation.

in our continent, when compared with those from the UK haplogroup, found in about 25% of Europeans (113). In addition, changes in expression levels of stress-responder genes as well as in intracellular ROS levels were ascribed to specific mtDNA sequences (114-116). It was also reported that mtDNA variants influence global DNA methylation most likely through a differential oxidative phosphorylation efficiency. In addition, Smiraglia *et al*. provided the first direct evidence that the absence/presence of mtDNA modulates the nuclear epigenetic modifications by influencing DNA methylation of several nuclear genes (7).

4. MITOCHONDRIAL DNA EPIGENETICS

4.1. Mitochondrial DNA methylation and hydroxymethylation

The possibility that the mitochondrial DNA can be the site of epigenetic modifications has long been the subject of intense discussion and controversies (Figure 1). The multicopy genome, the absence of canonical CpG islands as well as the lack of histones and their relative modifying enzymes prompted researchers to consider with skepticism the possibility that mitochondrial DNA could be a target of epigenetic modifications. Despite this, the under-representation of CpG dinucleotides within the mtDNA, with most of these co-locating with polymorphic variants, has suggested a susceptibility to mutation of these dinucleotides in the mitochondrial genome and, consequently, to methylation (117). The first attempt to identify traces of mtDNA methylation dates back to the early 1970s in loach embryos, beef heart and several mammalian mitochondria, that also showed the presence of DNA methylating enzymes within mitochondrial compartments (118-122). By contrast, roughly in the same period, there was no trace of cytosine methylation on mtDNA detected in yeast, *Ascomycete* fungi, rat, calf, frog, HeLa cells (123-125). A few years later, Shmookler Reis and Goldstein as well as Pollack *et al*. estimated a rate of mtDNA methylation equal to 3-5% in both human and mouse fibroblast (126, 127). Except for the failed attempt to find traces of mtDNA methylation in samples from gastric and colorectal cancer deployed by Maekawa *et al*. aimed at searching a mitochondrial epigenetic biomarker for cancer prediction and detection, the research in this field lapsed (128).

After nearly thirty years, the advent of more innovative and sensitive techniques, the discovery of DNA methyltransferase (DNMTs) family members in mitochondrial protein fractions and even more importantly the evidence proving the influence of mtDNA in nuclear epigenetics brought back the interest for mitochondrial epigenetics (7, 8, 13, 129-133). Initially, a mitochondrial isoform of DNMT1 was discovered in both human and mouse (13, 130, 134). Afterwards, also DNMT3A and DNMT3B were found localized within mitochondria, in a tissue-specific fashion (13, 131, 135). However, the persistence of methylated cytosines in the D-loop region of mouse ES cells that lacked for DNMT1, DNMT3A, and DNMT3B, although less marked than that of the wild type sample, suggested that a nonexclusive involvement of DNMTs in the establishment and maintenance of the mtDNA methylation patterns have to be regarded (13).

The 5-mC appeared to have a strand-specific role within the mitochondrial genome. Indeed, strand-specific bisulfite sequencing revealed that methylation is limited to the L-strand, with a prevalence in non-CpG sites (13). Moreover, asymmetrical effects on the transcripts expression from the heavy and light strands of mtDNA in mtDNMT1 overexpressed cells was observed, with increased levels of ND1, encoded by the H-strand and decreased levels of ND6, encoded by the L-strand (130). An inverse correlation between mt-ND2 and mt-ND6 expression was also independently observed in pathological phenotypes (14, 136).

An attractive hypothesis formulated to explain the functional relationship between methylation and gene expression of the mitochondrial genome has been detailed by Van der Wijst and Rots (137). These authors, indeed, suggested that mtDNA methylation regulates the affinity of TFAM binding and, thus, its action on mtDNA. This may result in an increased DNA compaction and in a reduced accessibility for POLRMT and TFB2B that may induce mitochondrial biogenesis rather that electron transport subunit transcription.

In addition, mtDNA methylation could be involved in the processing of mitochondrial polycistronic primary transcript (11,138-140). By alternative experimental approaches, such as 5-mC immunoprecipitation (Me-DIP), bisulfite sequencing, bisulfite pyrosequencing, bisulfite-next generation sequencing (NGS), Illumina MiSeq sequencing platform and liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS), the presence of methylated cytosines within the mitochondrial D-loop as well as in genes encoding for ND6, Cytb, COI, 12S rRNA, 16S rRNA, phenylalanine tRNA was evaluated (12-14, 134, 141-144). More recently, Ghosh *et al*., despite mitochondrial DNA polyploidy and its tissue- and developmental stage specific variable number, described a comprehensive mitochondrial methyl cytosine map. The authors pointing out that, except for some regions, such as *mt-ND6* and *mt-ATP6* that show methylation changes according to brain development, the pattern and the distribution of 5-mC across this genome appears quite constant (15). By contrast, only Hong *et al*. and Liu *et al*. have denied the existence of methylation within the mitochondrial genome, arguing this claim on the grounds that cytosine methylation within mtDNA is a very rare event and that such low levels cannot have any functional biological relevance (145, 146).

Next to cytosine methylation, cytosine hydroxymethylation (5-hmC) also recently raises significant interest and is regarded as a new mark. Although first discovered in 1972 in mouse and frog brain by Penn *et al*., a substantial boost in 5-hmC in evaluating this modified base has be provided the last 15 years, by experimental evidence reporting its presence in different tissues and cells and the occurrence of genome-wide changes during lineage commitment (147-152). In particular, the highest levels of 5-hmC has been detected in the central nervous system, comprising 0.6.% of the total nucleotides in Purkinje cells and 0.2.% in granule cells, while significantly variable levels were observed in other tissues as well as in cancer cells (149,153, 154). 5-hmC was initially considered as an intermediate of the oxidation of 5mC by the Ten-eleven traslocation (TET)- family of methylcytosine dioxygenases, in the active DNA demethylation pathway (148, 149, 155). However, considering its localization across the genome, mostly upstream of gene start site (GSS) regions and in gene body, its tissue-specific pattern as well as the deregulation of its levels, the 5-hmC appears to be closely implicated in the regulation of gene expression in both physiological and pathological conditions and in embryonic development (154, 156-159).

Recently, both mtDNA immunoprecipitation using antibodies directed against 5-hmC (hMe-DIP) and DNA-modification-dependent restriction endonuclease AbaSI coupled with sequencing (Aba-seq) showed a high density of 5-hmC within mitochondrial genome, indiscriminately at CpG and non-CpG sites (13, 130, 160, 161). These observations were supported by the finding of TET enzymes within the mitochondrial compartment (13, 160, 162). Very recently, Ghosh *et al*., through the use of genome scale datasets from 23 different cell lines and tissue types, designed a mitochondrial genome 5-hmC map. Interestingly, the mitochondrial hydroxymethylation profiles showed significant differences compared to the methylation once previously obtained by the same authors (15, 17). In particular, the 5-hmC density was lacking of conserved patterns across the mitochondrial genome, but they appeared mainly associated to cell and tissue-type, hinting towards a tissue-type specific role for 5-hmC. Moreover, similar to earlier reports, the regions comprising the gene start site (GSS) were characterized by a consistent

5-hmC density, with the highest levels in the GSS of tRNA genes of embryonic cell lines. Even though the latter observation suggests a possible involvement for this modified base during the developmental stages, the high density around the GSS did not show a significant correlation with the associate gene expression. Lastly, the presence of hydroxymethylation was evaluated in mtDNA control region (D-loop). In agreement with previous reports, this region is characterized by 5-hmC density, whose density appears to be closely linked to the cell and tissue type as well as to the developmental stage, with the embryonic cells displaying the highest density of 5-hmC (17). All the above described dynamic features of 5-hmC suggest that this mark may have a functional relevance, potentially regulating mitochondrial gene expression, but studies are still in infancy and many aspects have still to be elucidated.

5. mtDNA METHYLATION AS BIOMARKER OF AGING AND DISEASES

Once the existence of methylation/hydroxymethylation in the mitochondrial genome was confirmed, a significant number of reports have quickly provided that these two mitochondrial epigenetic marks exhibit significant correlation with environmental exposure, peculiar phenotypes and diseases, similar to what was observed for the methylation of the nuclear genome (Table 1).

5.1. mtDNA methylation and environmental exposures

A wide variety of environmental ubiquitous factors have been shown to influence epigenetic patterns in human and model organisms, resulting in both hypo and hypermethylation changes *in utero*, juvenile and adult life stages (11, 163-165). With regard to environmental-induced mitochondrial DNA methylation changes, interesting information has emerged from Byun *et al*. (12, 16) who analyzed the association between exposure to airborne pollutants and metal-rich particulates and blood mtDNA methylation. Authors observed that steel workers exposed to metal-rich particulate matter (measured as PM1) showed higher methylation levels of genes encoding for transfer RNA phenylalanine (*MT-TF*) and 12S ribosomal RNA (*MT-RNR1*) than low-exposed controls, while no significant differences were observed concerning the D-loop methylation. Conversely, the exposure to fine metal-rich particulates resulted in a significant reduction of the D-loop methylation levels and was significantly associated with markers of heart rate variability, but did not influence the *MT-TF* and *MT-RNR1* methylation. In addition, air benzene and traffic-derived EC exposure did not induce any effect on mtDNA methylation (12, 16). The analysis of chromate plating workers, displaying high concentration of Cr ions in their blood, were characterized by lower methylation levels in *MT-TF* and *MT-RNR1* genes (19). The apparent discrepancy between the two results was ascribed to the different exposure conditions, that might have different effects on mtDNA methylation.

It has been widely documented that the gestational environment stimuli received during prenatal life alter the global epigenome of placenta. Such stimuli could have both long-lasting effects on health span of an individual and transgenerational effects on fetal epigenomics reprogramming, according to still unclear mechanisms (166-169). In this context, a positive correlation has been demonstrated between placental mtDNA methylation of both *MT-RNR1* and D-loop, associated to a decrease in mtDNA content, and the pregnancy exposure to airborne particulate matter (PM) (170). Moreover, a reduction in the 5-mC levels of *MT-COII* was observed following the perinatal exposure of rats to Polybrominated diphenyl ethers (PBDEs), an organic chemical used as flame retardants in a variety of materials (171).

Beside the environmental pollutants, pharmacological agents were also found to have off-target effects on epigenetic signature (9, 172). Currently, the sole direct involvement of a mitochondrial epigenetics drug is represented by the anticonvulsant mood stabilizer sodium valproate (VPA), able to decrease the 5mC, but not the 5-hmC levels in mouse cultured cells (168). The potential role in mitochondrial epigenetics for a variety of drugs which regulate the intracellular epigenetic mechanisms and/or mitochondrial activity still needs to be clarified. A good candidate might be, for example, cocaine, which was associated on the one hand to the increase of both DNMT3A and DNMT3B gene expression, on the other to the d nuclear encoded mitochondrial genes (173-176).

Lastly, an mtDNA methylation involvement in the field of nutritional epigenetics studies has emerged very recently. Studies carried out on large yellow croakers (*Larimichthys crocea*) revealed effects on mtDNA methylation in fish fed different lipid sources. Indeed, in the liver, the methylation levels of *MT-TR* and *MT-ND4L* genes were significantly higher in fish fed with Perilla and olive oils, whereas those of *MT-RNR1* were lower in fish fed with olive oil with respect to the group fed with Fish and Sunflower oils. No changes in D-loop methylation were observed in all dietary treatments (177). In addition, with respect to the control group, fish fed a high- and a low-lipid diet were characterized by an increase of D-loop and *MT-RNR1* methylation, respectively (178).

5.2. mtDNA methylation and aging

The first evidence for a susceptibility to aging of mitochondrial epigenetic mechanisms was provided in 1983, with the observation of a hypermethylation of mtDNA in elderly cultured fibroblasts compared to those from younger donors (126). More recently, Dzitoyeva

Table 1. List of regions located within mitochondrial DNA displaying methylation changes according to specific phenotypes

Gene	Description	Methylation Change	Phenotype	Reference
D-loop	DNA control Region	↑	Pregnancy exposure to airborne particulate matter	159
		↑	Low-lipid diet in fish	166
		↑	AD-related pathology	17
		↑	Insulin resistance	171
		↑	High glucose	123
		↑	Polycystic ovarian syndrome	172
		↓	Parkinson Disease	17
		↓	Colorectal cancer	175
		↓	Exposure to fine metal-rich particulates	15
MT-RNR1	12S ribosomal RNA	↑	Exposure to high metal-rich PM1 exposure	11
		↑	Pregnancy exposure to airborne particulate matter	159
		↑	Low-lipid diet in fish	167
		↑	Aging	9
		↑	Polycystic ovarian syndrome	172
		↓	Aging	133
		↓	High Cr ion blood concentration	18
MT-RNR2	16S ribosomal RNA	↑	Polycystic ovarian syndrome	172
MT-TF	tRNA Phenylalanine	↑	Exposure to high metal-rich PM1 exposure	11
		↑	Perilla and olive oil diet in fish	166
		↓	High Cr ion blood concentration	18
MT-TL1	tRNA leucine 1	↑	Cardiovascular disease	173
MT-COI	Cytochrome c oxidase subunit I	↑	Cardiovascular disease	173
MT-COII	Cytochrome c oxidase subunit II	↓	Perinatal exposure to Polybrominated diphenyl ethers	160
		↑	Cardiovascular disease	173
MT-COIII	Cytochrome c oxidase subunit III	↑	Cardiovascular disease	173
MT-ND1	NADH dehydrogenase subunit 1	↓	AD-related pathology	17
MT-ND4	NADH dehydrogenase subunit 4	↑	Polycystic ovarian syndrome	172
MT-ND4L	NADH dehydrogenase subunit 4L	↑	Perilla and olive oil diet in fish	166
MT-ND6	NADH dehydrogenase subunit 6	↑	Nonalcoholic fatty liver disease	13

et al. analyzed different regions samples of brain from differently-aged mice, and observed not only the presence of both mitochondrial 5-mC and 5-hmC, but more interestingly, that progressive changes in these mitochondrial epigenetic marks occurs during lifespan in a region-specific manner (160). Particularly, Dzitoyeva *et al*. observed a decrease in 5-hmC but not in 5-mC levels and an increase in the expression of randomly selected mtDNA-encoded genes in the frontal cortex and in the cortex although no aging-associated changes in TET mRNAs, responsible for 5-hmC synthesis, were found. Conversely, no change in the levels of 5-hmc as well as in mRNA changes of mtDNA-encoded genes has been noted in the cerebellum, despite an increase of TET2 and TET3 expression. Furthermore, the expression of mtDNMT1 transcript in the brain and its modulation by aging was demonstrated (160). Moreover, D'Aquila *et al*., by analyzing human *MT-RNR1* and *MT-RNR2* genes,

encoding for 12S and 16S ribosomal RNA, respectively, revealed the presence of methylation at a CpG site of *MT-RNR1* (nucleotide position 932) as well as a positive increase of its methylation levels according to age and gender of analyzed samples (143). These findings, integrated by a survival analysis, reported that high methylation levels at the mtDNA 932 position significantly increase the mortality risk. This result suggested a still unclear functional role for *MT-RNR1* methylation that, ultimately may hamper individual survival chance. A significant role of *MT-RNR1* methylation in aging has also emerged from data obtained by Mawlood *et al*. which, by evaluating the methylation levels of 133 CpG sites in the mitochondrial genome by Illumina Sequencing, showed a stronger, negative correlation between two *MT-RNR1* CpG sites (nucleotide positions 1215 and 1313) and aging (144). The apparent discrepancy between the two previous results could be explained by the fact that the correlation between *MT-RNR1* methylation and aging could be site-specific. In addition the discrepancy could be strongly influenced by gender, environmental factors, nutrition and drugs, as also demonstrated for age-related nuclear epigenetic changes (12, 162, 179, 180).

5.3. mtDNA methylation and diseases

A mitochondrial involvement in the etio-pathogenesis of several diseases has been widely described and, only recently a role for mtDNA epigenetics is emerging. The first association between mtDNA methylation and disease was reported by Infantino *et al*., which observed a hypomethylation of mtDNA despite the increase of mtDNA content in Down Syndrome (DS) mtDNA (181). Afterwards, evidence from several publications has revealed that mitochondrial methylation may be regarded as a biomarker of neurodegenerative diseases. High levels of global 5-mC levels and of both DNMT1 and DNMT3A were detected in the mitochondria of neurons of patients with amyotrophic lateral sclerosis (ALS) (131). In addition, a significant abnormality in MT-RNR2 methylation and a severe loss of mitochondrial DNMT3A in skeletal muscle and spinal cords of ALS mouse models at presymptomatic or early symptomatic stages of disease were demonstrated (132). Similarly, alterations in mtDNA methylation were observed in Alzheimer Disease-related (AD) pathologies and Parkinson disease (PD). Blanch *et al*. observed, in both human samples and AD mouse models, increased methylation levels at both CpG and non-CpG sites in the D-loop region in AD-related pathology and a slight demethylation in the MT-ND1 gene, associated to an increase of ND1 expression. Conversely, a loss of D-loop methylation was observed in the substantia nigra of PD cases compared to the controls, whereas the 5-hmC levels in both AD-related pathology and PD remained unchanged (18).

It has also emerged that mtDNA methylation may be implicated in metabolic disorders. An *MT-ND6* hypermethylation, associated to a significant decrease in ND6 expression was observed in Nonalcoholic fatty liver disease (NASH) with respect to simple steatosis (SS) patients, thus suggesting an involvement of mtDNA methylation in the transformation from SS to NASH (14). A significant increase in D-loop methylation was also detected in obese and insulin-resistant individuals (182). In addition, compared to the control samples, high glucose in bovine and human retinal endothelial cells significantly increase the intra-mitochondrial DNMT1 levels and its binding to the mtDNA at both the D-loop and *Cytb* as well as the 5-hmC at the D-loop and *Cytb* regions of more than 3- and 2- fold, respectively. The increase in D-loop methylation was also associated to a decrease in mtDNA encoded gene expression, thus suggesting that in diabetes the mtDNA hypermethylation may result in dysfunctional mitochondria and promote capillary cell apoptosis (136). Lastly, by searching for the involvement of mtDNA epigenetics in the mitochondrial dysfunction occurring in Polycystic ovarian syndrome (PCOS), Jia *et al*. revealed that a hypermethylation in the D-loop, *MT-RNR1*, *MT-RNR2* and *MT-ND4* occurs in PCO oocytes in accordance with a down-regulated expression of mtDNA-encoded genes and impaired mitochondrial function (183).

The association between nuclear DNA methylation marks and cardiovascular disease (CVD) has been widely demonstrated. Analyses carried out in both CVD and healthy patients by bisulfite pyrosequencing demonstrated that cases displayed higher methylation levels than controls in *MT-COI*, *MT-COII*, *MT-COIII* and *MT-TL1* genes, meanwhile no significant difference in methylation was observed for *MT-ND6*, *MT-ATP6*, *MT-ATP8* and *MT-ND5* (184).

Limited data about the potential association between mitochondrial DNA methylation and cancer are currently available. A very low occurrence of mtDNA methylation in gastric, colorectal and cervix cancer was reported (141). In addition, a negative association between DNA copy number with cytosine methylation and hydroxymethylation was found in hepatocellular carcinoma cells (185). On the other hand, increased mtDNA copy number and ND2 levels associated with a markedly reduced methylation status of the D-loop were observed in colorectal cancer and associate to the clinic-pathological stages of the disease (136, 186).

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The identification of epigenetic changes in the mitochondrial genome has led to extreme revision of previous knowledge on phenotypic implications of mtDNA genetic variations. Indeed, the association between mtDNA methylation and several phenotypes suggests an original scenario about the role of mitochondria in the

cell life and sheds light on the identification of a series of biomarkers implicated in the above phenotypes. However, understanding of the molecular mechanisms by which mtDNA methylation operates is still in its infancy. The predominance of methylated and/or hydroxymethylated cytosines within the D-loop and in the upstream of gene start sites suggest a possible regulatory role in mtDNA expression, that requires further investigation. Once the role of epigenetic marks in mtDNA have been clarified and the reversibility of epigenetic modifications are elucidated, we predict that exciting advances will prompt the search for mitochondrial-specific therapeutic agents able to restore the altered epigenetic equilibrium.

7. ACKNOWLEDGEMENT

The work was supported by Fondi di Ateneo of the University of Calabria.

8. REFERENCES

1. S. Anderson, A.T. Bankier, B.G. Barrell, M.H.L. De Bruijn, A.R. Coulson, J. Drouin, I.C. Eperon, D.P. Nierlich, B.A. Roe, F. Sanger, P.H. Schreier, A.J.H. Smith, R. Staden, I.G. Young: Sequence and organization of the human mitochondrial genome. *Nature* 290, 457-465 (1981)
DOI: 10.1038/290457a0

2. J.W. Taanman: The mitochondrial genome: structure, transcription, translation and replication. *Biochim Biophys Acta* 1410, 103-123 (1999)
DOI: 10.1016/S0005-2728(98)00161-3

3. M. Lynch, B. Koskella, S. Schaack S: Mutation pressure and the evolution of organelle genomic architecture. *Science* 311, 1727-1730 (2006)
DOI: 10.1126/science.1118884

4. D. Mishmar, I. Zhidkov: Evolution and disease converge in the mitochondrion. *Biochim Biophys Acta*. 1797, 1099-1104 (2010)
DOI: 10.1016/j.bbabio.2010.01.003

5. D.C. Wallace, D. Chalkia D: Mitochondrial DNA genetics and the heteroplasmy conundrum in evolution and disease. *Cold Spring Harb Perspect Biol* 5, a021220 (2013)
DOI: 10.1101/cshperspect.a021220

6. G. Cannino, C.M. Di Liegro, A.M. Rinaldi: Nuclear-mitochondrial interaction. *Mitochondrion* 7, 359-366 (2007)
DOI: 10.1016/j.mito.2007.07.001

7. D.J. Smiraglia, M. Kulawiec, G.L. Bistulfi, S.G. Gupta, K.K Singh: A novel role for mitochondria in regulating epigenetic modification in the nucleus. *Cancer Biol Ther* 7, 1182-1190 (2008)
DOI: 10.4161/cbt.7.8.6215

8. D. Bellizzi, P. D'Aquila, M. Giordano, A. Montesanto, G. Passarino: Global DNA methylation levels are modulated by mitochondrial DNA variants. *Epigenomics* 4:17-27 (2012)
DOI: 10.2217/epi.11.109

9. D.T. Shaughnessy, K. McAllister, L. Worth, A.C. Haugen, J.N. Meyer, F.E. Domann, B. Van Houten, R. Mostoslavsky, S.J. Bultman, A.A Baccarelli, T.J. Begley, R.W. Sobol, M.D. Hirschey, T. Ideker, J.H. Santos, W.C. Copeland, R.R. Tice, D.M. Balshaw, F.L. Tyson: Mitochondria, energetics, epigenetics, and cellular responses to stress. *Environ Health Perspect* 122, 1271-1278 (2014)
DOI: 10.1289/ehp.1408418

10. P. D'Aquila, D. Bellizzi, G. Passarino: Mitochondria in health, aging and diseases: the epigenetic perspective. *Biogerontology* 16, 569-585 (2015)
DOI: 10.1007/s10522-015-9562-3

11. V. Iacobazzi, A. Castegna, V. Infantino, G. Andria: Mitochondrial DNA methylation as a next-generation biomarker and diagnostic tool. *Mol Genet Metab* 110, 25-34 (2013)
DOI: 10.1016/j.ymgme.2013.07.012

12. H.M. Byun, T. Panni, V. Motta, H. Hou, F. Nordio, P. Apostoli, P.A. Bertazzi, A.A Baccarelli: Effects of airborne pollutants on mitochondrial DNA methylation. *Part Fibre Toxicol* 8, 10:18 (2013)

13. D. Bellizzi, P. D'Aquila, T. Scafone, M. Giordano, V. Riso, A. Riccio, G. Passarino: The control region of mitochondrial DNA shows an unusual CpG and non-CpG methylation pattern. *DNA Res* 20, 537-547 (2013)
DOI: 10.1093/dnares/dst029

14. C.J. Pirola, T.F. Gianotti, A.L. Burgueño, M. Rey-Funes, C.F. Loidl, P. Mallardi P, J-S. Martino, G.O. Castaño, S. Sookoian: Epigenetic modification of liver mitochondrial DNA is associated with histological severity of nonalcoholic fatty liver disease. *Gut* 62, 1356-1363 (2013)
DOI: 10.1136/gutjnl-2012-302962

15. S. Ghosh, S. Sengupta, V. Scaria: Comparative analysis of human mitochondrial methylomes shows distinct patterns of epigenetic regulation in mitochondria. *Mitochondrion* 18, 58-62 (2014)
DOI: 10.1016/j.mito.2014.07.007

16. H.M. Byun, E. Colicino, L. Trevisi, T. Fan, D.C. Christiani, A.A. Baccarelli: Effects of Air Pollution and Blood Mitochondrial DNA Methylation on Markers of Heart Rate Variability. *J Am Heart Assoc* 5, e003218 (2016)
DOI: 10.1161/JAHA.116.003218

17. S. Ghosh, S. Sengupta, V. Scaria: Hydroxymethyl cytosine marks in the human mitochondrial genome are dynamic in nature. *Mitochondrion* 27, 25-31 (2016)
DOI: 10.1016/j.mito.2016.01.003

18. M. Blanch, J.L. Mosquera, B. Ansoleaga, I. Ferrer, M. Barrachina: Altered Mitochondrial DNA Methylation Pattern in Alzheimer Disease-Related Pathology and in Parkinson *Disease Am J Pathol* 186, 385-397 (2016)

19. L. Yang, B. Xia, Y. Yang, H. Ding, D. Wu, H. Zhang, G. Jiang, J. Liu, Z. Zhuang: Mitochondrial DNA hypomethylation in chrome plating workers. *Toxicol Lett* 243, 1-6 (2016)
DOI: 10.1016/j.toxlet.2015.11.031

20. B. Ephrussi, P.P. Slonimski: Subcellular units involved in the synthesis of respiratory enzymes in yeast*. Nature* 176, 1207–1208 (1955)
DOI: 10.1038/1761207b0

21. W. Martin, M. Müller: The hydrogen hypothesis for the first eukaryote. *Nature*. 392, 37-41 (1998)
DOI: 10.1038/32096

22. J.C. Thrash, A. Boyd, M.J. Huggett, J. Grote, P. Carini, R.J. Yoder, B. Robbertse, J.W. Spatafora, M.S. Rappé, S.J. Giovannoni: Phylogenomic evidence for a common ancestor of mitochondria and the SAR11 clade. *Sci Rep*. 1,13 (2011)
DOI: 10.1038/srep00013

23. J.M. Archibald: Endosymbiosis and Eukaryotic Cell Evolution. *Curr Biol*. 25, R911-21 (2015)
DOI: 10.1016/j.cub.2015.07.055

24. D. Bogenhagen, D.A. Clayton: The number of mitochondrial deoxyribonucleic acid genomes in mouse L and human HeLa cells. Quantitative isolation of mitochondrial deoxyribonucleic acid. *J Biol Chem* 249, 7991-7995 (1974)

25. R.M. Andrews, I. Kubacka, P.F. Chinnery, R.N. Lightowlers, D.M. Turnbull, N. Howell: Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. *Nat Genet* 23, 147 (1999)
DOI: 10.1038/13779

26. N. Garrido, L. Griparic, E. Jokitalo, J. Wartiovaara, A.M. van der Bliek, J.N. Spelbrink: Composition and dynamics of human mitochondrial nucleoids. *Mol Biol Cell* 14, 1583-1596 (2003)
DOI: 10.1091/mbc.E02-07-0399

27. X.J. Chen, R.A. Butow: The organization and inheritance of the mitochondrial genome. *Nat Rev Genet* 6, 815-825 (2005)
DOI: 10.1038/nrg1708

28. C. Kukat, C.A. Wurm, H. Spåhr, M. Falkenberg, N.G. Larsson, S. Jakobs: Super-resolution microscopy reveals that mammalian mitochondrial nucleoids have a uniform size and frequently contain a single copy of mtDNA. *Proc Natl Acad Sci U S A* 108, 13534-13539 (2011)
DOI: 10.1073/pnas.1109263108

29. D.F. Bogenhagen: Mitochondrial DNA nucleoid structure. *Biochim Biophys Acta* 1819, 914-920 (2012)
DOI: 10.1016/j.bbagrm.2011.11.005

30. V. Jayashankar, S.M. Rafelski: Integrating mitochondrial organization and dynamics with cellular architecture *Curr Opin Cell Biol* 26, 34-40 (2014)
DOI: 10.1016/j.ceb.2013.09.002

31. Y. Wang, D.F. Bogenhagen: Human mitochondrial DNA nucleoids are linked to protein folding machinery and metabolic enzymes at the mitochondrial inner membrane. *J Biol Chem* 281, 25791-25802 (2006)
DOI: 10.1074/jbc.M604501200

32. H.T. Jacobs, S.K. Lehtinen, J.N. Spelbrink: No sex please, we're mitochondria: a hypothesis on the somatic unit of inheritance of mammalian mtDNA. *Bioessays* 22, 564-572 (2000)
DOI: 10.1002/(SICI)1521-1878 (200006) 22:6 <564:AID-BIES9>3.0.CO;2-4

33. R.W. Gilkerson, E.A. Schon, E. Hernandez, M.M. Davidson: Mitochondrial nucleoids maintain genetic autonomy but allow for functional complementation. *J Cell Biol* 181,

1117-1128 (2008)
DOI: 10.1083/jcb.200712101

34. S. Kutik, B. Guiard, H.E. Meyer, N. Wiedemann, N. Pfanner: Cooperation of translocase complexes in mitochondrial protein import. *J Cell Biol* 179, 585-591 (2007)
DOI: 10.1083/jcb.200708199

35. J. Dudek, P. Rehling, M. van der Laan: Mitochondrial protein import: common principles and physiological networks. *Biochim Biophys Acta* 1833, 274-285 (2013)
DOI: 10.1016/j.bbamcr.2012.05.028

36. A.M. Sokol, M.E. Sztolsztener, M. Wasilewski, E. Heinz, A. Chacinska: Mitochondrial protein translocases for survival and wellbeing. *FEBS Lett* 588, 2484-2495 (2014)
DOI: 10.1016/j.febslet.2014.05.028

37. L.S. Wenz, L. Opaliński, N. Wiedemann, T. Becker: Cooperation of protein machineries in mitochondrial protein sorting. *Biochim Biophys Acta* 1853, 1119-1129 (2015)
DOI: 10.1016/j.bbamcr.2015.01.012

38. D.A. Clayton: Transcription and replication of mitochondrial DNA. *Hum Reprod* 15 Suppl 2, 11-17 (2000)
DOI: 10.1093/humrep/15.suppl_2.11

39. M. Falkenberg, N.G. Larsson, C.M. Gustafsson: DNA replication and transcription in mammalian mitochondria. *Annu Rev Biochem* 76, 679-699 (2007)
DOI: 10.1146/annurev.biochem.76.060305.152028

40. T.J. Nicholls, M. Minczuk: In D-loop: 40 years of mitochondrial 7S DNA. *Exp Gerontol* 56, 175-181 (2014)
DOI: 10.1016/j.exger.2014.03.027

41. S. Crews, D. Ojala, J. Posakony, J. Nishiguchi, G. Attardi: Nucleotide sequence of a region of human mitochondrial DNA containing the precisely identified origin of replication. *Nature*. 277, 192-198 (1979)
DOI: 10.1038/277192a0

42. D.P. Tapper, D.A. Clayton: Precise nucleotide location of the 5' ends of RNA-primed nascent light strands of mouse mitochondrial DNA. *J Mol Biol* 162, 1-16 (1982)
DOI: 10.1016/0022-2836(82)90159-0

43. G.L. Ciesielski, M.T. Oliveira, L.S. Kaguni: Animal Mitochondrial DNA Replication. *Enzymes*. 39, 255-292 (2016)
DOI: 10.1016/bs.enz.2016.03.006

44. H. Kasamatsu, J. Vinograd: Unidirectionality of replication in mouse mitochondrial DNA. *Nat New Biol* 241, 103-105 (1973)
DOI: 10.1038/newbio241103a0

45. B. Xu, D.A. Clayton: RNA-DNA hybrid formation at the human mitochondrial heavy-strand origin ceases at replication start sites: an implication for RNA-DNA hybrids serving as primers. *EMBO J* 15, 3135-3143 (1996)

46. J.N. Doda, C.T. Wright, D.A. Clayton: Elongation of displacement-loop strands in human and mouse mitochondrial DNA is arrested near specific template sequences. *Proc Natl Acad Sci* U S A 78, 6116-6120 (1981)
DOI: 10.1073/pnas.78.10.6116

47. D.A. Clayton: Vertebrate mitochondrial DNA-a circle of surprises. *Exp Cell Res* 255, 4-9 (2000)
DOI: 10.1006/excr.1999.4763

48. I.J Holt, A. Reyes: Human mitochondrial DNA replication. *Cold Spring Harb Perspect Biol* 4, pii: a012971 (2012)
DOI: 10.1101/cshperspect.a012971

49. J.P. Uhler, M. Falkenberg: Primer removal during mammalian mitochondrial DNA replication. *DNA Repair (Amst)* 34, 28-38 (2015)
DOI: 10.1016/j.dnarep.2015.07.003

50. E.A. McKinney, M.T. Oliveira: Replicating animal mitochondrial DNA. *Genet Mol Biol* 36, 308-315 (2013)
DOI: 10.1590/S1415-47572013000300002

51. Reyes, L. Kazak, S.R. Wood, T. Yasukawa, H.T. Jacobs, I.J. Holt: Mitochondrial DNA replication proceeds via a 'bootlace' mechanism involving the incorporation of processed transcripts. *Nucleic Acids Res* 41, 5837-5850 (2013)
DOI: 10.1093/nar/gkt196

52. N.D. Bonawitz, D.A. Clayton, and G.S. Shadel: Initiation and beyond: multiple functions of the human mitochondrial transcription machinery. *Mol Cell* 24, 813–825 (2006)
DOI: 10.1016/j.molcel.2006.11.024

53. T. R. Mercer, S. Neph, M.E. Dinger, J. Crawford, M.A. Smith, A.M. Shearwood, E. Haugen, C.P. Bracken, O. Rackham, J.A. Stamatoyannopoulos, A. Filipovska, J.S. Mattick: The human mitochondrial

transcriptome. *Cell* 146, 645-658 (2011)
DOI: 10.1016/j.cell.2011.06.051

54. Y.I. Morozov, D. Temiakov: Human Mitochondrial Transcription Initiation Complexes Have Similar Topology on the Light and Heavy Strand Promoters. *J Biol Chem* pii: jbc.C116.7.27966 (2016)

55. S. Leigh-Brown, J.A. Enriquez, D.T. Odom: Nuclear transcription factors in mammalian mitochondria. *Genome Biol* 11, 215 (2010)
DOI: 10.1186/gb-2010-11-7-215

56. A.P. Rebelo, L.M. Dillon, C.T. Moraes: Mitochondrial DNA transcription regulation and nucleoid organization. *J Inherit Metab Dis* 34, 941-951 (2011)
DOI: 10.1007/s10545-011-9330-8

57. C.T. Campbell, J.E. Kolesar, B.A. Kaufman BA: Mitochondrial transcription factor A regulates mitochondrial transcription initiation, DNA packaging, and genome copy number. *Biochim Biophys Acta* 1819, 921-929 (2012)
DOI: 10.1016/j.bbagrm.2012.03.002

58. Picca A, A.M. Lezza: Regulation of mitochondrial biogenesis through TFAM-mitochondrial DNA interactions: Useful insights from aging and calorie restriction studies. *Mitochondrion* 25, 67-75 (2015)

59. D.P. Kelly, R.C. Scarpulla: Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes Dev* 18, 357-368 (2004)
DOI: 10.1101/gad.1177604

60. E.S. Blomain, S.B. McMahon: Dynamic regulation of mitochondrial transcription as a mechanism of cellular adaptation. *Biochim Biophys Acta* 1819, 1075-1079 (2012)
DOI: 10.1016/j.bbagrm.2012.06.004

61. R.B. Vega, J.L. Horton, D.P. Kelly: Maintaining ancient organelles: mitochondrial biogenesis and maturation. *Circ Res* 116, 1820-1834 (2015)
DOI: 10.1161/CIRCRESAHA.116.305420

62. V.M. Pastukh, O.M. Gorodnya, M.N. Gillespie, M.V. Ruchko: Regulation of mitochondrial genome replication by hypoxia: The role of DNA oxidation in D-loop region. *Free Radic Biol Med* 96, 78-88 (2016)
DOI: 10.1016/j.freeradbiomed.2016.04.011

63. R.E. Giles, H. Blanc, H.M. Cann, D.C. Wallace: Maternal inheritance of human mitochondrial DNA. *Proc Natl Acad Sci U S A* 77, 6715-6719 (1980)
DOI: 10.1073/pnas.77.11.6715

64. W.E. Thompson, J. Ramalho-Santos, P. Sutovsky: Ubiquitination of prohibitin in mammalian sperm mitochondria: possible roles in the regulation of mitochondrial inheritance and sperm quality control. *Biol Reprod* 69, 254-260 (2003)
DOI: 10.1095/biolreprod.102.010975

65. M. Sato, K. Sato: Maternal inheritance of mitochondrial DNA by diverse mechanisms to eliminate paternal mitochondrial DNA. *Biochim Biophys Acta* 1833, 1979-1984 (2013)
DOI: 10.1016/j.bbamcr.2013.03.010

66. R.J. Aitken, K.T. Jones, S.A. Robertson: Reactive oxygen species and sperm function--in sickness and in health. *J Androl* 33, 1096-1106 (2012)
DOI: 10.2164/jandrol.112.016535

67. N. Nakamura: Ubiquitination regulates the morphogenesis and function of sperm organelles. *Cells*. 2, 732–750 (2013)
DOI: 10.3390/cells2040732

68. S. Breton, D.T. Stewart: Atypical mitochondrial inheritance patterns in eukaryotes. *Genome*. 58, 423-431 (2015)
DOI: 10.1139/gen-2015-0090

69. B. Thyagarajan, R.A. Padua, C. Campbell: Mammalian mitochondria possess homologous DNA recombination activity. *J Biol Chem* 271, 27536-27543 (1996)
DOI: 10.1074/jbc.271.44.27536

70. C. Wiuf: Recombination in human mitochondrial DNA? *Genetics* 159, 749-56 (2001)

71. M. Schwartz, J. Vissing: Paternal inheritance of mitochondrial DNA. *N Engl J Med* 347, 576-580 (2002)
DOI: 10.1056/NEJMoa020350

72. E.D. Ladoukakis, A. Eyre-Walker: Evolutionary genetics: direct evidence of recombination in human mitochondrial DNA. *Heredity* (Edinb) 93, 321 (2004)
DOI: 10.1038/sj.hdy.6800572

73. Y. Kraytsberg, M. Schwartz, T.A. Brown, K. Ebralidse, W.S. Kunz, D.A. Clayton, J. Vissing, K. Khrapko: Recombination of human mitochondrial DNA. *Science* 304, 981 (2004)
DOI: 10.1126/science.1096342

74. E. Hazkani-Covo, R.M. Zeller, W. Martin: Molecular Poltergeists: Mitochondrial DNA Copies (numts) in Sequenced Nuclear Genomes. *PLoS Genet* 6, e1000834 (2010) DOI: 10.1371/journal.pgen.1000834

75. M. Bernt, A. Braband, B. Schierwater, P.F. Stadler: Genetic aspects of mitochondrial genome evolution. *Mol Phylogenet Evol* 69, 328-338 (2013) DOI: 10.1016/j.ympev.2012.10.020

76. E. Hazkani-Covo, D. Graur: A comparative analysis of numt evolution in human and chimpanzee. *Mol Biol Evol* 24, 13–18 (2007) DOI: 10.1093/molbev/msl149

77. L. Viljakainen, D.C. Oliveira, J.H. Werren, S.K. Behura: Transfers of mitochondrial DNA to the nuclear genome in the wasp Nasonia vitripennis. *Insect Mol Biol* 19 Suppl. 1, 27–35 (2010) DOI: 10.1111/j.1365-2583.2009.00932.x

78. D. Mishmar, E. Ruiz-Pesini, M. Brandon, D.C.Wallace: Mitochondrial DNA-like sequences in the nucleus (NUMTs): insights into our African origins and the mechanism of foreign DNA integration. *Hum Mutat* 23,125-133 (2004) DOI: 10.1002/humu.10304

79. E. Hazkani-Covo: Mitochondrial insertions into primate nuclear genomes suggest the use of numts as a tool for phylogeny. *Mol Biol Evol* 26, 2175–2179 (2009) DOI: 10.1093/molbev/msp131

80. C. Turner, C. Killoran, N.S. Thomas, M. Rosenberg, N.A. Chuzhanova, J. Johnston, Y. Kemel, D.N. Cooper, L.G. Biesecker: Human genetic disease caused by de novo mitochondrial-nuclear DNA transfer. *Hum Genet* 112, 303-309 (2003)

81. R.X. Santos, S.C. Correia, X. Zhu, M.A. Smith, P.I Moreira, R.J. Castellani, A. Nunomur, G. Perry: Mitochondrial DNA oxidative damage and repair in aging and Alzheimer's disease. *Antioxid Redox Signal* 18, 2444-24457 (2013) DOI: 10.1089/ars.2012.5039

82. C.Guo, L. Sun, X. Chen, D. Zhang D: Oxidative stress, mitochondrial damage and neurodegenerative diseases. *Neural Regen Res*. 8, 2003-2014 (2013)

83. Y. Kong, S.E. Trabucco, H. Zhang: Oxidative stress, mitochondrial dysfunction and the mitochondria theory of aging. *Interdiscip Top Gerontol* 39, 86-107 (2014) DOI: 10.1159/000358901

84. F. Bonomini, L.F. Rodella, R. Rezzani: Metabolic syndrome, aging and involvement of oxidative stress. *Aging Dis* 6, 109-120 (2015) DOI: 10.14336/AD.2014.0305

85. F. Bozzo, A. Mirra, M.T. Carrì: Oxidative stress and mitochondrial damage in the pathogenesis of ALS: New perspectives. *Neurosci Lett* S0304-3940, 30287-30287 (2016)

86. Y. He, J. Wu, D.C. Dressman, C. Iacobuzio-Donahue, S.D. Markowitz, V.E. Velculescu, L.A. Diaz Jr, K.W. Kinzler, B. Vogelstein, N. Papadopoulos: Heteroplasmic mitochondrial DNA mutations in normal and tumour cells. *Nature* 464, 610-614 (2010) DOI: 10.1038/nature08802

87. J.B. Stewart, P.F.Chinnery: The dynamics of mitochondrial DNA heteroplasmy: implications for human health and disease. *Nat Rev Genet* 16, 530-542 (2015) DOI: 10.1038/nrg3966

88. F. Legros, F. Malka, P. Frachon, A. Lombès, M. Rojo: Organization and dynamics of human mitochondrial DNA. *J Cell Sci* 117(Pt 13), 2653-2662 (2004) DOI: 10.1242/jcs.01134

89. S. Vidoni, C. Zanna, M. Rugolo, E. Sarzi, G. Lenaers: Why mitochondria must fuse to maintain their genome integrity. *Antioxid Redox Signal* 19, 379-388 (2013) DOI: 10.1089/ars.2012.4800

90. Torroni, K. Huoponen, P. Francalacci, M. Petrozzi, L. Morelli, R. Scozzari, D. Obinu, M.L. Savontaus, D.C. Wallace: Classification of European mtDNAs from an analysis of three European populations. *Genetics* 144, 1835-1850 (1996)

91. V. Macaulay, M. Richards, E. Hickey, E. Vega, F. Cruciani, V. Guida, R. Scozzari, B. Bonné-Tamir, B. Sykes, A. Torroni: The emerging tree of West Eurasian mtDNAs: a synthesis of control-region sequences and RFLPs. *Am J Hum Genet* 64, 232-249 (1999) DOI: 10.1086/302204

92. Torroni, A. Achilli, V. Macaulay, M. Richards, H.J. Bandelt: Harvesting the fruit of the human mtDNA tree. *Trends Genet* 22, 339-345 (2006) DOI: 10.1016/j.tig.2006.04.001

93. M. van Oven, M. Kayser: Updated comprehensive phylogenetic tree of global human mitochondrial DNA variation. *Hum Mutat* 30, E386-94 (2009)
DOI: 10.1002/humu.20921

94. Achilli, C. Rengo, C. Magri, V. Battaglia, A. Olivieri, R. Scozzari, F. Cruciani, M. Zeviani, E. Briem, V. Carelli, P. Moral, J.M. Dugoujon, U. Roostalu, E.L. Loogväli, T. Kivisild, H.J. Bandelt, M. Richards, R. Villems, A.S. Santachiara-Benerecetti, O. Semino, A. Torroni: The molecular dissection of mtDNA haplogroup H confirms that the Franco-Cantabrian glacial refuge was a major source for the European gene pool. *Am J Hum Genet* 75, 910-918 (2004)
DOI: 10.1086/425590

95. D.C. Wallace: Mitochondrial DNA variation in human radiation and disease. *Cell* 163, 33-38 (2015)
DOI: 10.1016/j.cell.2015.08.067

96. E. Ruiz-Pesini, A.C. Lapeña, C. Díez-Sánchez, A. Pérez-Martos, J. Montoya, E. Alvarez, M. Díaz, A. Urriés, L. Montoro, M.J. López-Pérez, J.A. Enríquez: Human mtDNA haplogroups associated with high or reduced spermatozoa motility. *Am J Hum Genet* 67, 682-696 (2000)
DOI: 10.1086/303040

97. E.M. Byrne, A.F. McRae, D.L. Duffy, Z.Z. Zhao, N.G. Martin, M.J. Wright, G.W. Montgomery, P.M. Visscher: Association study of common mitochondrial variants and cognitive ability. *Behav Genet* 39, 504-512 (2009)
DOI: 10.1007/s10519-009-9276-x

98. G.J. Tranah, T.M. Manini, K.K. Lohman, M.A. Nalls, S. Kritchevsky, A.B. Newman, T.B. Harris, I. Miljkovic, A. Biffi, S.R. Cummings, Y. Liu: Mitochondrial DNA variation in human metabolic rate and energy expenditure. *Mitochondrion* 11, 855-861 (2011)
DOI: 10.1016/j.mito.2011.04.005

99. Chen, N. Raule, A. Chomyn, G. Attardi: Decreased reactive oxygen species production in cells with mitochondrial haplogroups associated with longevity. *PLoS One* 7, e46473 (2012)
DOI: 10.1371/journal.pone.0046473

100. T. Kato, N. Fuku, Y. Noguchi, H. Murakami, M. Miyachi, Y. Kimura, M. Tanaka, K. Kitamura: Mitochondrial DNA haplogroup associated with hereditary hearing loss in a Japanese population. *Acta Otolaryngol* 132, 1178-1182 (2102)
DOI: 10.3109/00016489.2012.693624

101. P.G. Ridge, T.J. Maxwell, S.J. Foutz, M.H. Bailey, C.D. Corcoran, J.T. Tschanz, M.C. Norton, R.G. Munger, E. O'Brien, R.A. Kerber, R.M. Cawthon, J.S. Kauwe: Mitochondrial genomic variation associated with higher mitochondrial copy number: the Cache County Study on Memory Health and Aging. *BMC Bioinformatics* 15 Suppl 7, S6 (2014)
DOI: 10.1186/1471-2105-15-S7-S6

102. M.J. Keogh, P.F. Chinnery: Mitochondrial DNA mutations in neurodegeneration. *Biochim Biophys Acta* 1847, 1401-1411 (2015)
DOI: 10.1016/j.bbabio.2015.05.015

103. S.R. Atilano, D. Malik, M. Chwa, J. Cáceres-Del-Carpio, A.B. Nesburn, D.S. Boyer, B.D. Kuppermann, S.M. Jazwinski, M.V. Miceli, D.C. Wallace, N. Udar, M.C. Kenney: Mitochondrial DNA variants can mediate methylation status of inflammation, angiogenesis and signaling genes. *Hum Mol Genet* 24, 4491-4503 (2015)
DOI: 10.1093/hmg/ddv173

104. D. Mishmar, E. Ruiz-Pesini, P. Golik, V. Macaulay, A.G. Clark, S. Hosseini, M. Brandon, K. Easley, E. Chen, M.D. Brown, R.I. Sukernik, A. Olckers, D.C. Wallace: Natural selection shaped regional mtDNA variation in humans. *Proc Natl Acad Sci U S A* 100, 171-176 (2003)
DOI: 10.1073/pnas.0136972100

105. D.C. Wallace, E. Ruiz-Pesini, D. Mishmar: mtDNA variation, climatic adaptation, degenerative diseases, and longevity. *Cold Spring Harb Symp Quant Biol* 68, 479-486 (2003)
DOI: 10.1101/sqb.2003.68.471

106. P.E. Coskun, E. Ruiz-Pesini, D.C. Wallace: Control region mtDNA variants: longevity, climatic adaptation, and a forensic conundrum. *Proc Natl Acad Sci U S A* 100, 2174-2176 (2003)
DOI: 10.1073/pnas.0630589100

107. Y.T. Cheng, J. Liu, L.Q. Yang, C. Sun, Q.P Kong: Mitochondrial DNA content contributes to climate adaptation using Chinese populations as a model. *PLoS One* 8, e79536 (2013)
DOI: 10.1371/journal.pone.0079536

108. U. Cagin, J.A. Enriquez: The complex crosstalk between mitochondria and the nucleus: What goes in between? *Int J Biochem Cell Biol* 63, 10-55 (2015)
DOI: 10.1016/j.biocel.2015.01.026

109. S.M. Jazwinski: The retrograde response: when mitochondrial quality control is not enough. *Biochim Biophys Acta* 1833, 400-409 (2013)
DOI: 10.1016/j.bbamcr.2012.02.010

110. M.P. King, G. Attardi: Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science* 246, 500-503 (1989)
DOI: 10.1126/science.2814477

111. D. Pye, D.S. Kyriakouli, G.A. Taylor, R. Johnson, M. Elstner, B. Meunier, Z.M. Chrzanowska-Lightowlers, R.W. Taylor, D.M. Turnbull, R.N. Lightowlers RN: Production of transmitochondrial cybrids containing naturally occurring pathogenic mtDNA variants. *Nucleic Acids Res* 34, e95 (2006)
DOI: 10.1093/nar/gkl516

112. H.M. Wilkins, S.M. Carl, R.H. Swerdlow: Cytoplasmic hybrid (cybrid) cell lines as a practical model for mitochondriopathies. *Redox Biol* 2C, 619-631 (2014)
DOI: 10.1016/j.redox.2014.03.006

113. Gómez-Durán, D. Pacheu-Grau, E. López-Gallardo, C. Díez-Sánchez, J. Montoya, M.J. López-Pérez, E. Ruiz-Pesini: Unmasking the causes of multifactorial disorders: OXPHOS differences between mitochondrial haplogroups. *Hum Mol Genet* 19, 3343-3353 (2010)
DOI: 10.1093/hmg/ddq246

114. D. Bellizzi, P. Cavalcante, D. Taverna, G. Rose, G. Passarino, S. Salvioli, C. Franceschi, G. De Benedictis: Gene expression of cytokines and cytokine receptors is modulated by the common variability of the mitochondrial DNA in cybrid cell lines. *Genes Cells* 11, 883-891 (2006)
DOI: 10.1111/j.1365-2443.2006.00986.x

115. D. Bellizzi, D. Taverna, P. D'Aquila, S. De Blasi, G. De Benedictis: Mitochondrial DNA variability modulates mRNA and intra-mitochondrial protein levels of HSP60 and HSP75: experimental evidence from cybrid lines. *Cell Stress Chaperones* 14, 265-271 (2009)
DOI: 10.1007/s12192-008-0081-x

116. P. D'Aquila, G. Rose, M.L. Panno, G. Passarino, D. Bellizzi: SIRT3 gene expression: a link between inherited mitochondrial DNA variants and oxidative stress. *Gene* 497, 323-329 (2012)
DOI: 10.1016/j.gene.2012.01.042

117. L.R. Cardon, C. Burge, D.A. Clayton, S. Karlin: Pervasive CpG suppression in animal mitochondrial genomes. *Proc Natl Acad Sci U S A* 91, 3799-3803 (1994)
DOI: 10.1073/pnas.91.9.3799

118. B.F. Vanyushin, G.I Kiryanov, I.B. Kudryashova, A.N. Belozersky: DNA-methylase in loach embryos (Misgurnus fossilis). *FEBS Lett* 15, 313-316 (1971)
DOI: 10.1016/0014-5793(71)80646-4

119. B.Vanyushin, M.D. Kirnos: The nucleotide composition and pyrimidine clusters in DNA from beef heart mitochondria. *FEBS Lett* 39, 195-199 (1974)
DOI: 10.1016/0014-5793(74)80049-9

120. B.Vanyushin, M.D. Kirnos: Structure of animal mitochondrial DNA: nucleotide composition, pyrimidine clusters, and methylation character. *Mol Biol* (Mosk)10, 715-724 (1976)

121. B.Vanyushin, M.D. Kirnos: Structure of animal mitochondrial DNA (base composition, pyrimidine clusters, character of methylation). *Biochim Biophys Acta* 475, 323-336 (1997)
DOI: 10.1016/0005-2787(77)90023-5

122. M.M. Nass: Differential methylation of mitochondrial and nuclear DNA in cultured mouse, hamster and virus-transformed hamster cells. *In vivo* and *in vitro* methylation. *J Mol Biol* 80, 155-175 (1973)
DOI: 10.1016/0022-2836(73)90239-8

123. D.J. Cummings, A. Tait, J.M. Goddard: Methylated bases in DNA from Paramecium aurelia. *Biochim Biophys Acta* 374, 1-11 (1974)
DOI: 10.1016/0005-2787(74)90194-4

124. I.B. Dawid: 5-methylcytidylic acid: absence from mitochondrial DNA of frogs and HeLa cells. *Science* 184, 80–81 (1974)
DOI: 10.1126/science.184.4132.80

125. G.S. Groot, A.M. Kroon: Mitochondrial DNA from various organisms does not contain internally methylated cytosine in -CCGG- sequences. *Biochim Biophys Acta* 564, 355–357 (1979)
DOI: 10.1016/0005-2787(79)90233-8

126. R.J. Shmookler Reis, S. Goldstein: Mitochondrial DNA in mortal and immortal human cells. Genome number, integrity, and methylation. *J Biol Chem* 258, 9078-9085 (1983)

127. Y. Pollack, J. Kasir, R. Shemer, S. Metzger, M. Szyf: Methylation pattern of mouse mitochondrial DNA. *Nucleic Acids Res* 12, 4811-4824 (1984)
DOI: 10.1093/nar/12.12.4811

128. M. Maekawa, T. Taniguchi, H. Higashi, H. Sugimura, K. Sugano, T. Kanno: Methylation of mitochondrial DNA is not a useful marker for cancer detection. *Clin Chem* 50, 1480-1481 (2004)
DOI: 10.1373/clinchem.2004.035139

129. D.C. Wallace, W. Fan: Energetics, epigenetics, mitochondrial genetics. *Mitochondrion* 10, 12-31 (2010)

130. L.S. Shock, P.V. Thakkar, E.J. Peterson, R.G. Moran, S.M. Taylor: DNA methyltransferase 1, cytosine methylation, and cytosine hydroxymethylation in mammalian mitochondria. *Proc Natl Acad Sci U S A* 108, 3630-3635 (2011)
DOI: 10.1073/pnas.1012311108

131. B.A. Chestnut, Q. Chang, A. Price, C. Lesuisse, M. Wong, L.J. Martin: Epigenetic regulation of motor neuron cell death through DNA methylation. *J Neurosci* 31, 16619-16636 (2011)
DOI: 10.1523/JNEUROSCI.1639-11.2011

132. M. Wong, B. Gertz, B.A. Chestnut, L.J. Martin: Mitochondrial DNMT3A and DNA methylation in skeletal muscle and CNS of transgenic mouse models of ALS. *Front Cell Neurosci* 7, 279 (2013)
DOI: 10.3389/fncel.2013.00279

133. Castegna, V. Iacobazzi, V. Infantino: The mitochondrial side of epigenetics. *Physiol Genomics* 47, 299-307 (2015)
DOI: 10.1152/physiolgenomics.00096.2014

134. M. Mishra, R.A. Kowluru: Epigenetic Modification of Mitochondrial DNA in the Development of Diabetic Retinopathy. *Invest Ophthalmol Vis Sci* 56, 5133-5142 (2015)
DOI: 10.1167/iovs.15-16937

135. L.J. Martin, M. Wong: Aberrant regulation of DNA methylation in amyotrophic lateral sclerosis: a new target of disease mechanisms. *Neurotherapeutics* 10, 722-733 (2013)
DOI: 10.1007/s13311-013-0205-6

136. S. Feng, L.Xiong, Z. Ji, W. Cheng, H. Yang: Correlation between increased ND2 expression and demethylated displacement loop of mtDNA in colorectal cancer. *Mol Med Rep* 6, 125-130 (2012)

137. M.G. Van der Wijst, M.G. Rots: Mitochondrial epigenetics: an overlooked layer of regulation? *Trends Genet* 31, 353-356 (2015)
DOI: 10.1016/j.tig.2015.03.009

138. L. Davenport, R.H. Taylor, D.T. Dubin: Comparison of human and hamster mitochondrial transfer RNA. Physical properties and methylation status. *Biochim Biophys Acta* 447, 285-293 (1976)
DOI: 10.1016/0005-2787(76)90051-4

139. V. McCulloch, B.L. Seidel-Rogol, G.S.Shadel: A human mitochondrial transcription factor is related to RNA adenine methyltransferases and binds S-adenosylmethionine. *Mol Cell Biol* 22, 1116-1125(2002)
DOI: 10.1128/MCB.22.4.1116-1125.2002

140. J. Cotney, S.E. McKay, G.S.Shadel: Elucidation of separate, but collaborative functions of the rRNA methyltransferase-related human mitochondrial transcription factors B1 and B2 in mitochondrial biogenesis reveals new insight into maternally inherited deafness. *Hum Mol Genet* 18, 2670-2682 (2009)
DOI: 10.1093/hmg/ddp208

141. C. Sun, L.L. Reimers, R.D. Burk: Methylation of HPV16 genome CpG sites is associated with cervix precancer and cancer. *Gynecol Oncol* 121, 59-63 (2011)
DOI: 10.1016/j.ygyno.2011.01.013

142. R.D. Kelly, A. Mahmud, M. McKenzie, I.A. Trounce, J.C. St John: Mitochondrial DNA copy number is regulated in a tissue specific manner by DNA methylation of the nuclear-encoded DNA polymerase gamma A. *Nucleic Acids Res* 40, 10124-10138 (2012)
DOI: 10.1093/nar/gks770

143. P. D'Aquila, M. Giordano, A. Montesanto, F. De Rango, G. Passarino, D. Bellizzi. Age- and gender-related pattern of methylation in the MT-RNR1 gene. *Epigenomics* 7, 707-716 (2015)
DOI: 10.2217/epi.15.30

144. S.K. Mawlood, L. Dennany, N. Watson, J.

Dempster, B.S. Pickard. Quantification of global mitochondrial DNA methylation levels and inverse correlation with age at two CpG sites. *Aging (Albany NY)* 8, 636-641(2016)
DOI: 10.18632/aging.100892

145. E.E. Hong, C.Y. Okitsu, Smith AD, Hsieh CL. Regionally specific and genome-wide analyses conclusively demonstrate the absence of CpG methylation in human mitochondrial DNA. *Mol Cell Biol*. 2013 Jul;33(14):2683-90
DOI: 10.1128/MCB.00220-13

146. B. Liu, Q. Du, L. Chen, G. Fu, S. Li, L. Fu, X. Zhang, C. Ma, C. Bin: CpG methylation patterns of human mitochondrial DNA. *Sci Rep* (2016) 6, 23421
DOI: 10.1038/srep23421

147. N.W. Penn, R. Suwalski, C. O'Riley, K. Bojanowski, R. Yura: The presence of 5-hydroxymethylcytosine in animal deoxyribonucleic acid. *Biochem J* 126, 781-790 (1972)
DOI: 10.1042/bj1260781

148. M. Tahiliani, K.P. Koh, Y. Shen, W.A. Pastor, H. Bandukwala, Y. Brudno, S. Agarwal, L.M. Iyer, D.R. Liu, L. Aravind, A.Rao: Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 324, 930-935 (2009)
DOI: 10.1126/science.1170116

149. S. Kriaucionis, N.Heintz: The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science* 324, 929-930 (2009)
DOI: 10.1126/science.1169786

150. S.G. Jin, X. Wu, A.X. Li, G.P. Pfeifer: Genomic mapping of 5-hydroxymethylcytosine in the human brain. *Nucleic Acids Res* 39, 5015-5024 (2011)
DOI: 10.1093/nar/gkr120

151. S.M. Kinney, H.G. Chin, R. Vaisvila, J. Bitinaite, Y. Zheng, P.O. Estève, S. Feng, H. Stroud, S.E. Jacobsen, S.Pradhan: Tissue-specific distribution and dynamic changes of 5-hydroxymethylcytosine in mammalian genomes. *J Biol Chem* 286, 24685-24693 (2011)
DOI: 10.1074/jbc.M110.217083

152. M. Kim, Y.K. Park, T.W. Kang, S.H. Lee, Y.H. Rhee, J.L. Park, H.J. Kim, D. Lee, D. Lee, S.Y. Kim, Y.S.Kim Dynamic changes in DNA methylation and hydroxymethylation when hES cells undergo differentiation toward a neuronal lineage. *Hum Mol Genet* (2014) 23, 657-667
DOI: 10.1093/hmg/ddt453

153. M. Münzel, D. Globisch, T. Brückl, M. Wagner, V. Welzmiller, S. Michalakis, M. Müller, M. Biel, T. Carell: Quantification of the sixth DNA base hydroxymethylcytosine in the brain. *Angew Chem Int Ed Engl* 49, 5375-5377 (2010)
DOI: 10.1002/anie.201002033

154. L. Wen, X. Li, L. Yan, Y. Tan, R. Li, Y. Zhao, Y. Wang, J. Xie, Y. Zhang, C. Song, M. Yu, X. Liu, P. Zhu, X. Li, Y. Hou, H. Guo, X. Wu, C. He, R. Li, F. Tang, J. Qiao: Whole-genome analysis of 5-hydroxymethylcytosine and 5-methylcytosine at base resolution in the human brain. *Genome Biol* 15, R49 (2014)
DOI: 10.1186/gb-2014-15-3-r49

155. R.M. Kohli, Y. Zhang: TET enzymes, TDG and the dynamics of DNA demethylation. *Nature* 502, 472-479 (2013)
DOI: 10.1038/nature12750

156. C.E. Nestor, R. Ottaviano, J. Reddington, D. Sproul, D. Reinhardt, D. Dunican, E. Katz, J.M. Dixon, D.J. Harrison, R.R. Meehan: Tissue type is a major modifier of the 5-hydroxymethylcytosine content of human genes. *Genome* Res 22, 467-477 (2012)
DOI: 10.1101/gr.126417.111

157. W. Li, M. Liu: Distribution of 5-hydroxymethylcytosine in different human tissues. *J Nucleic Acids* 2011, 870726 (2011)
DOI: 10.4061/2011/870726

158. C.G. Chapman, C.J. Mariani, F. Wu, K. Meckel, F. Butun, A. Chuang, J. Madzo, M.B. Bissonette, J.H. Kwon, L.A.Godley: TET-catalyzed 5-hydroxymethylcytosine regulates gene expression in differentiating colonocytes and colon cancer. *Sci Rep* 5:17568 (2015)
DOI: 10.1038/srep17568

159. M. Zhao, J. Wang, W. Liao, D. Li, M. Li, H. Wu, Y. Zhang, M.E. Gershwin, Q. Lu: Increased 5-hydroxymethylcytosine in CD4(+) T cells in systemic lupus erythematosus. *J Autoimmun* 69, 64-73(2016)
DOI: 10.1016/j.jaut.2016.03.001

160. S. Dzitoyeva, H. Chen, H. Manev: Effect of aging on 5-hydroxymethylcytosine in

brain mitochondria. *Neurobiol Aging* 33, 2881-2891 (2012)
DOI: 10.1016/j.neurobiolaging.2012.02.006

161. Z. Sun, J. Terragni, J.G. Borgaro, Y. Liu, L. Yu, S. Guan, H. Wang, D. Sun, X. Cheng, Z. Zhu, S. Pradhan, Y. Zheng: High-resolution enzymatic mapping of genomic 5-hydroxymethylcytosine in mouse embryonic stem cells. *Cell Rep* 3, 567-576 (2013)
DOI: 10.1016/j.celrep.2013.01.001

162. H. Chen, S. Dzitoyeva, H. Manev: Effect of valproic acid on mitochondrial epigenetics. *Eur J Pharmacol* 690, 51-59 (2012)
DOI: 10.1016/j.ejphar.2012.06.019

163. J.C. Mathers, G. Strathdee, CL. Relton: Induction of epigenetic alterations by dietary and other environmental factors. *Adv Genet* 71, 3-39 (2010)
DOI: 10.1016/b978-0-12-380864-6.00001-8

164. D. Hala, D.B. Huggett, W.W. Burggren: Environmental stressors and the epigenome. *Drug Discov Today Technol* 12, e3-8. (2014)
DOI: 10.1016/j.ddtec.2012.05.004

165. S. Ghosh, K.K. Singh, S. Sengupta, V. Scaria: Mitoepigenetics: The different shades of grey. *Mitochondrion* 25, 60-66 (2015)
DOI: 10.1016/j.mito.2015.09.003

166. F. Pacchierotti, M. Spanò: Environmental Impact on DNA Methylation in the Germline: State of the Art and Gaps of Knowledge. *Biomed Res Int* 2015, 123484(2015)
DOI: 10.1155/2015/123484

167. N.P. Evans, M. Bellingham, J.E. Robinson: Prenatal programming of neuroendocrine reproductive function. *Theriogenology* 86, 340-348 (2016)
DOI: 10.1016/j.theriogenology.2016.04.047

168. D.M. Silberman, G.B. Acosta, M.A. Zorrilla Zubilete: Long-term effects of early life stress exposure: Role of epigenetic mechanisms. *Pharmacol Res*109, 64-73 (2016)
DOI: 10.1016/j.phrs.2015.12.033

169. Constantinof, V.G. Moisiadis, S.G. Matthews: Programming of stress pathways: A transgenerational perspective. *J Steroid Biochem Mol Biol*160, 175-180 (2016)
DOI: 10.1016/j.jsbmb.2015.10.008

170. B.G. Janssen, H.M. Byun, W. Gyselaers, W. Lefebvre, A.A. Baccarelli, T.S. Nawrot: Placental mitochondrial methylation and exposure to airborne particulate matter in the early life environment: An ENVIRONAGE birth cohort study. *Epigenetics* 10, 536-544 (2015)
DOI: 10.1080/15592294.2015.1048412

171. H.M. Byun, N. Benachour, D. Zalko, M.C. Frisardi, E. Colicino, L. Takser, A.A. Baccarelli: Epigenetic effects of low perinatal doses of flame retardant BDE-47 on mitochondrial and nuclear genes in rat offspring. *Toxicology*328, 152-159 (2015)

172. Sadakierska-Chudy, M. Frankowska, M. Filip: Mitoepigenetics and drug addiction. *Pharmacol Ther* 144, 226-233 (2014)
DOI: 10.1016/j.pharmthera.2014.06.002

173. J.B. Dietrich, R. Poirier, D. Aunis,J. Zwiller: Cocaine downregulates the expression of the mitochondrial genome in rat brain. *Ann N Y Acad Sci* 1025, 345-350 (2004)
DOI: 10.1196/annals.1316.042

174. Q. LaPlant, V. Vialou, H.E. 3rd Covington, D. Dumitriu, J. Feng, B.L. Warren, I. Maze, D.M. Dietz, E.L. Watts, S.D. Iñiguez, J.W. Koo, E. Mouzon, W. Renthal, F. Hollis, H. Wang, M.A. Noonan, Y. Ren, A.J. Eisch, C.A. Bolaños, M. Kabbaj, G. Xiao, R.L. Neve, Y.L. Hurd, R.S. Oosting, G. Fan, J.H. Morrison, E.J. Nestler: Dnmt3a regulates emotional behavior and spine plasticity in the nucleus accumbens. *Nat Neurosci* 13, 1137-1143 (2010)
DOI: 10.1038/nn.2619

175. K. Anier, K. Malinovskaja, A. Aonurm-Helm, A. Zharkovsky, A. Kalda: DNA methylation regulates cocaine-induced behavioral sensitization in mice. *Neuropsychopharmacology* 35, 2450-2461 (2010)
DOI: 10.1038/npp.2010.128

176. Z. Zhou, Q. Yuan, D.C. Mash, D. Goldman: Substance-specific and shared transcription and epigenetic changes in the human hippocampus chronically exposed to cocaine and alcohol. *Proc Natl Acad Sci U S A* 108, 6626-6631 (2011)
DOI: 10.1073/pnas.1018514108

177. K. Liao, J. Yan, K. Mai, Q. Ai: Dietary Olive and Perilla Oils Affect Liver Mitochondrial DNA Methylation in Large Yellow Croakers. *J Nutr* 145, 2479-2485 (2015)
DOI: 10.3945/jn.115.216481

178. K. Liao, J. Yan, K. Mai, Q. Ai: Dietary lipid

concentration affects liver mitochondrial DNA copy number, gene expression and DNA methylation in large yellow croaker (Larimichthys crocea). *Comp Biochem Physiol B Biochem Mol Biol* 193, 25-32 (2016) DOI: 10.1016/j.cbpb.2015.11.012

179. M.B. Terry, L. Delgado-Cruzata, N. Vin-Raviv, H.C. Wu, R.M. Santella: DNA methylation in white blood cells: association with risk factors in epidemiologic studies. *Epigenetics* 6, 828-837(2011) DOI: 10.4161/epi.6.7.16500

180. L. Delgado-Cruzata, N. Vin-Raviv, P. Tehranifar, J. Flom, D. Reynolds, K. Gonzalez, R.M. Santella, M.B. Terry: Correlations in global DNA methylation measures in peripheral blood mononuclear cells and granulocytes. *Epigenetics* 9, 1504-1510 (2014) DOI: 10.4161/15592294.2014.983364

181. V. Infantino, A. Castegna, F. Iacobazzi, I. Spera, I. Scala, G. Andria, V. Iacobazzi: Impairment of methyl cycle affects mitochondrial methyl availability and glutathione level in Down's syndrome. *Mol Genet Metab* 102, 378-382 (2011) DOI: 10.1016/j.ymgme.2010.11.166

182. L.D. Zheng, L.E. Linarelli, L. Liu, S.S. Wall, M.H. Greenawald, R.W. Seidel, P.A. Estabrooks, F.A. Almeida, Z. Cheng: Insulin resistance is associated with epigenetic and genetic regulation of mitochondrial DNA in obese humans. *Clin Epigenetics* 7, 60 (2015) DOI: 10.1186/s13148-015-0093-1

183. L. Jia, J. Li, B. He, Y. Jia, Y. Niu, C. Wang, R. Zhao: Abnormally activated one-carbon metabolic pathway is associated with mtDNA hypermethylation and mitochondrial malfunction in the oocytes of polycystic gilt ovaries. *Sci Rep* 6, 19436 (2016) DOI: 10.1038/srep19436

184. A.A. Baccarelli, H.M. Byun: Platelet mitochondrial DNA methylation: a potential new marker of cardiovascular disease. *Clin Epigenetics* 7, 44(2015) DOI: 10.1186/s13148-015-0078-0

185. F. Shen, W. Huang, J.H. Qi, B.F. Yuan, J.T. Huang, X. Zhou, Y.Q. Feng, Y.J. Liu, S.M. Liu: Association of 5-methylcytosine and 5-hydroxymethylcytosine with mitochondrial DNA content and clinical and biochemical parameters in hepatocellular carcinoma. PLoS One 8, e76967 (2013) DOI: 10.1371/journal.pone.0076967

186. J. Gao, S. Wen, H. Zhou, S. Feng: De-methylation of displacement loop of mitochondrial DNA is associated with increased mitochondrial copy number and nicotinamide adenine dinucleotide subunit 2 expression in colorectal cancer. *Mol Med Rep* 12, 7033-7038 (2015)

Abbreviations: 5-hmC: Cytosine hydroxymethylation, 5-mC: Cytosine methylation, AD: Alzheimer Disease, ALS: Amyotrophic Lateral Sclerosis, COI-III: Cytochrome c oxidase subunits I-III, CSBI-III: Conserved Sequence Blocks I-III, CVD: CardioVascular Disease, Cytb: Cytochrome b, DNMT: DNA MethylTransferase, DS: Down Syndrome, ES cells: Embryonic Stem cells, GSS: Gene Start Site, hMe-DIP: h5-mC immunoprecipitation, HSP: Heavy-Strand Promoter, HVRI-II: Hyper Variable Region I and II, LC-ESI-MS: Liquid chromatography-electrospray ionization tandem mass spectrometry, LSP: Light-Strand Promoter, Me-DIP: 5-mC immunoprecipitation, MT-ATP6: ATP synthase F0 subunit 6, MT-ATP8: ATP synthase F0 subunit 8, mtDNA: Mitochondrial DNA, mTERF: Mitochondrial Transcription Termination Factor 1, MT-ND1: NADH dehydrogenase subunit 1, MT-ND4: NADH dehydrogenase subunit 4, MT-ND4L: NADH dehydrogenase subunit 4L, MT-ND5: NADH dehydrogenase subunit 5, MT-ND6: NADH dehydrogenase subunit 6, MT-RNR1: 12S Ribosomal RNA, MT-RNR2: 16S ribosomal RNA, mtSSB: Mitochondrial Single-Stranded Binding proteins, MT-TF: tRNA Phenylalanine, mitochondrial- encoded, MT-TL1: tRNA Leucine 1, mitochondrial- encoded, NASH: Nonalcoholic fatty liver disease, NGS: Next Generation Sequencing, OXPHOS: Oxidative Phosphorylation, PCOS: PolyCystic Ovarian Syndrome, PD: Parkinson Disease, PM: Particulate Matter, POLG: Mitochondrial DNA Polymerase Gamma, POLRMT: Mitochondrial RNA Polymerase, RFLP: Restriction Fragment Length Polymorphism, RITOLS: RNA Incorporated Throughout the Lagging Strand, ROS: Reactive Oxidative Species, rRNA: Ribosomal RNA, SDM: Strand-Displacement Model, SS: Simple Steatosis, TET: Ten-Eleven Traslocation (TET) enzyme, TFAM: Mitochondrial Transcription Factor A, TFB1M: Mitochondrial Transcription Factor B1, TFB2M: Mitochondrial Transcription Factor B2, tRNA: Trasfer RNA, VPA: Sodium Valproate

Key Words: mtDNA Genetics, mtDNA Epigenetics, mtDNA Methylation, mtDNA Hydroxymethylation, Aging, Diseases, Environmental Factors, Review

Send correspondence to: Dina Bellizzi, Department of Biology, Ecology and Earth Sciences, University of Calabria, 87036 Rende, Italy, Tel: 390984492930, Fax: 39098493601, E-mail: dina.bellizzi@unical.it