Chapter 7

Down Syndrome, Ageing and Epigenetics

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Abstract During the past decades, life expectancy of subjects with Down syndrome (DS) has greatly improved, but age-specific mortality rates are still important and DS subjects are characterized by an acceleration of the ageing process, which
affects particularly the immune and central nervous systems. In this chapter, we will first review the characteristics of the ageing phenomenon in brain and in immune system in DS and we will then discuss the biological hallmarks of ageing in this specific population. Finally, we will also consider in detail the knowledge on epigenetics in DS, particularly DNA methylation.

**Keywords**  Down syndrome · Ageing · Epigenetics · Epigenetic clock

**Introduction**

Down syndrome (DS) or trisomy 21 (OMIM #190685) is a complex genetic condition, caused by a chromosomal disorder, corresponding to a total or partial trisomy of the chromosome 21 (HSA21). It occurs in approximately 1 out of every 600–700 live births and it is the most common known genetic cause associated with moderate to severe intellectual disability. In the past decades, thanks to improvements in medical care for children and adults, life expectancy of DS people has rapidly increased (Bittles and Glasson 2004; Glasson et al. 2016; Leonard et al. 2000; Yang et al. 2002), but age-specific mortality rates are still important compared to other populations (Strauss and Eyman 1996). Subjects with DS appear to age differently to individuals without DS (Zigman 2013), and the increase in life expectancy does not follow the one observed in the general population or other groups with intellectual disability (Coppus 2013). DS is characterized by premature ageing, frequently described as a segmental progeroid syndrome (Martin 1982; Patterson and Cabelof 2012). DS subjects commonly display in middle adulthood health-related problems that do not normally arise in the general population before 70 years of age and that resemble those of the geriatric population: dermatological changes (skin wrinkling, hair loss and/or greying), presbycusis with prevalence of hearing loss of more than 90% after 50 years old (Picciotti et al. 2017), osteoporosis (Baptista et al. 2005; Carfì et al. 2014; McKelvey et al. 2013), early menopause onset, but most prominently, immune impairment and cognitive decline (Zigman 2013). Accelerated ageing in DS is atypical and segmental: it is associated with many, but not all, of the classical ageing signs (Esbensen 2010) and affects particularly the immune and central nervous systems. DS can represent a model to study events that occur with age, to identify molecular markers and potential therapeutic targets. As life expectancy in DS individuals increase, new health issues emerge with a great need of specialized therapeutic tools. In this chapter, we will review evidence on the premature ageing of the immune and central nervous systems in DS, as well as the presence of biological hallmarks of ageing, and finally, the epigenetics processes associated with this disease.
Ageing of the Central Nervous and Immune Systems in Down Syndrome

Brain Ageing

In addition to their congenital cognitive impairment, individuals with DS experience age-related cognitive decline and subsequent dementia (essentially Alzheimer’s disease, AD) more frequently and at an earlier age than people without DS. This represents a great health problem (Glasson et al. 2002) and has major implications from a care perspective. In the general population aged 60 and over, in Western Europe, prevalence rates of dementia are around 7% (“WHO|Dementia” 2012). These rates are higher in subjects with intellectual disability and specially in the population with DS (Coppus et al. 2006; Franceschi et al. 1990; Holland et al. 1998, 2000; Lai and Williams 1989; Margallo-Lana et al. 2007; McCarron et al. 2014, 2017; Oliver et al. 1998; Sekijima et al. 1998; Stancliffe et al. 2012; Tyrrell et al. 2001; Visser et al. 1997). In DS people aged 45 years and older, prevalence rates of dementia are reported between 15 and 45%, and approximately 50–80% of individuals with DS will develop dementia before they reach the age of 60–70 years old. The median age of dementia onset is below 60 years old, but with an important inter-individual variability in the age of the first clinical symptoms (Ballard et al. 2016; Lott and Dierssen 2010; Zigman and Lott 2007). Important variations are present in reported prevalence rates: discrepancies can be due to studies designs (longitudinal or cross-sectional studies), patient heterogeneity or choices in the instruments used to measure cognitive decline (Strydom et al. 2007).

Individuals with DS experience important cognitive deterioration, which follows frequently a course similar to the one seen in AD. Dementia is frequently preceded by changes in language skills and in executive functions (Ghezzo et al. 2014; Holland et al. 2000; Iacono et al. 2010; Kittler et al. 2006). Non-demented adult DS subjects over 40 years old have lower neuropsychological functions and adaptive skills as compared to younger ones, with a particular impact on language and short memory skills, frontal lobe functions, visuo-spatial abilities and adaptive behaviour (Ghezzo et al. 2014). Changes in cognitive abilities were age-associated, as performance at tests (semantic fluency, token test, phonemic fluency, Tower of London, Frontal Assessment Battery, etc.) were found to be inversely correlated with age (Ghezzo et al. 2014). Diagnosis of dementia in DS can be a major challenge, related to the presence of pre-existing congenital cognitive impairment, communication issues or difficulties in the choice of standardized tests in line with a limited capacity of individuals with DS to be assessed with traditional cognitive measures. In the youngest individuals, diagnosis can be delayed due to the initial presentation with atypical symptoms, like changes in behavior, in personality or psychological symptoms (Dekker et al. 2015). Finally, diagnosis is complicated by the presence of comorbidities affecting the neurological phenotype, such as epilepsy and depression, whose prevalence increase with age. Epilepsy has a particu-
larly important prevalence in DS subjects (McCarron et al. 2014, 2017), recently estimated at 77.9% in a prospective longitudinal study (McCarron et al. 2017) and among subjects with dementia, a substantial proportion are also diagnosed with depression (McCarron et al. 2017; Shooshtari et al. 2011).

Two neuropathological lesions are considered as hallmarks of AD: senile plaques and neurofibrillary tangles. While neurofibrillary tangles are aggregates of the abnormally hyper-phosphorylated protein tau (τ) within the cytoplasm of neurons, plaques are extracellular deposits in the cerebral cortex, containing the beta-amyloid (Aβ) peptide. Aβ is produced by sequential cleavage of the amyloid precursor protein (APP). Several peptides of different lengths are generated from APP by beta- and gamma-secretases and Aβ40 and Aβ42 are believed to be the most pathogenic. These neuropathological changes typical of AD start to develop in the childhood of DS subjects, decades earlier compared to aged control brains (Lemere et al. 1996; Leverenz and Raskind 1998; Lott and Dierssen 2010; Teller et al. 1996; Zigman and Lott 2007). Virtually all adults with DS over 40 years of age have sufficient senile plaques and neurofibrillary tangles for a neuropathologically based diagnosis of AD. The increase in Aβ charge with age, assessed in a longitudinal study, has been found to be related to the cognitive decline in the DS population (Hartley et al. 2017).

From a neuroimaging perspective, as compared to AD patients in the general population, people with DS have reduced brain volumes, specially in the frontal and temporal lobes, and important cortical thickness (Lott 2012; Mullins et al. 2013; Pinter et al. 2001). Neuroimaging changes are observed before the onset of dementia (Krasuski et al. 2002; Sabbagh et al. 2015; Teipel et al. 2004) and are mostly associated with age (Beacher et al. 2010; Koran et al. 2014; Romano et al. 2016). Non-demented individuals with DS show an ‘accelerated ageing’ of some brain regions from a morphological point of view: they have significantly greater age-related reductions in volume of frontal, temporal and parietal lobes, and significantly greater age-related changes in ventricle volumes (Beacher et al. 2010; Koran et al. 2014). These neuroimaging changes are associated with the development of fibrillary β-amyloidosis (Annus et al. 2017) and correlated to cognitive and memory impairments (Koran et al. 2014; Krasuski et al. 2002). To directly measure by magnetic resonance imaging how brain structure changes with ageing, structural neuroimaging data have been used in a general population to generate indexes that predict ageing of brain. This model of ‘brain-predicted age’ was applied recently to DS subjects (Cole et al. 2017): brain-predicted age difference (equal to ‘brain-predicted age’ – ‘chronological age’) in DS participants was significantly greater than in controls, suggesting a premature structural brain ageing in DS subjects. The variability of the score was associated with the presence and the magnitude of Aβ deposition measured by positron emission tomography scans, and also with levels of cognitive performance (Cole et al. 2017).

Search for genetic risk factors for dementia in people with DS has been important during the past decades. As mentioned previously, Aβ, the major contributor to AD pathology, is produced from the precursor protein APP, which gene is located on the proximal part of the long arm of HSA21 and is thus present in trip-
licate in DS. This triplication yields to higher levels of APP and its splicing products, and was considered as the key contributor to strong increase in risk for AD in DS (Mao et al. 2003). However, APP is not the only gene present in triplicate in DS subjects and others appear to be also important determinants in the development of dementia by their impact either on Aβ pathology or on neuroinflammation, such as beta amyloid converting enzyme 2 (BACE2) or astrocyte-derived neurotrophic factor S100 beta (S100β) (Leclerc et al. 2010). Of particular interest is the gene for dual-specificity tyrosine phosphorylated and regulated kinase 1a (DYRK1A), which is involved in the appearance of AD-like pathology. It has emerged as a key determinant in neuronal loss, neurofibrillary degeneration and cognitive impairment, and is considered as a potential therapeutic target (De la Torre et al. 2014; Duchon and Herault 2016). It is worthy of note that in neurons derived from Induced Pluripotent Stem Cells (iPSC) of patients with DS, increased expression levels of genes such as APP and BACE2 have been reported, as compared to healthy controls, and have been associated with abnormal metabolism of Aβ \textit{in vitro} (Dashinimaev et al. 2017).

Additional genetic risk factors, not located on HSA21, have been investigated in dementia in DS subjects. One of the most consistent genetic risk factor found for AD dementia in the general population is the gene for Apolipoprotein E (APOE). ApoE is polymorphic with three different isoforms (ε2, ε3 and ε4) and is considered as a possible chaperone for Aβ deposition. APOEε4 allele has been associated with dementia in the general population (Liu et al. 2013). Also in DS subjects, the presence of one or two APOEε4 allele predisposes to a greater risk of AD, to an early-onset of symptoms (before 45 years old) and to a more rapid progression to death, as compared to individuals without DS or DS adults without any APOEε4 allele (Deb et al. 2000; Lai et al. 1999; Prasher et al. 2008). The APOEε4 allele is also considered as associated with earlier mortality in the DS population, independently of the risk of dementia (Rohn et al. 2014). Some authors (Prasher et al. 2008) recommend early screening for APOE genotype in individuals with DS, for early identification of individuals at increased risk of cognitive decline.

In the general population, multiple genome-wide association studies (GWAS) have identified at least 20 genes that are significantly associated with AD, but these genomic variants were not necessarily found in the specific DS population (Patel et al. 2014). In adults with DS, several studies have examined relations between single-nucleotide polymorphisms (SNPs) and dementia, using a candidate gene approach, identifying associations between AD in adults with DS and genetic variants in sortilin-related receptor 1 (SORL1) (Lee et al. 2007), estrogen-receptor 2 (ESR2) (Zhao et al. 2011) or APP and CST3 (Lee et al. 2017). SNPs in genes CAHLM1, IDE, SOD1 or SORCS1 were found to be associated with plasma levels of Aβ peptides (Aβ40, Aβ42, Aβ42/Aβ40 ratio) (Schupf et al. 2015). Finally, some genetic polymorphisms were found to be associated with the age at the onset of dementia in DS: APP locus (Margallo-Lana et al. 2004), PICALM and APOE loci (Jones et al. 2013a), HSD17B1 locus (Lee et al. 2012) or BACE2 (Mok et al. 2014).
In addition to neuropathology related to Aβ, several other types of pathologic processes, which could contribute to neurodegeneration and to the development of dementia, have been reported in DS subjects. Individuals with DS show higher levels of oxidative stress at all ages as compared to controls, notably in brains (Aivazidis et al. 2017; Cenini et al. 2012; Garlet et al. 2013; Jovanovic et al. 1998; Odetti et al. 1998; Reynolds and Cutts 1993). Cortical neurons from fetal DS exhibit an in vitro increase in intracellular reactive oxygen species (ROS) and elevated levels of lipid peroxidation, as compared to age-matched normal brains, leading to neuronal apoptosis (Busciglio and Yankner 1995). A critical process associated with oxidative damage in DS individuals with age is mitochondrial dysfunction (Arbuzova et al. 2002; Busciglio et al. 2002; Helguera et al. 2013; Valenti et al. 2011). In DS neurons, altered mitochondrial morphology and function have been reported, regarding in particular impairment of mitochondrial transport (Helguera et al. 2013). Mitochondrial dysfunction leads to an alteration in the metabolism of APP, resulting into the intracellular deposition of the insoluble form of Aβ (Busciglio et al. 2002). Finally, neuroinflammation, considered as a key contributor to neurodegenerative disorders, may lead to an increase in the vulnerability of DS neurons in the presence of senile plaques, neurofibrillary tangles and oxidative damage (Wilcock 2012; Wilcock et al. 2015).

**Ageing of the Immune System**

In the spectrum of the symptoms presented by DS subjects, several have led to the hypothesis that the syndrome is associated with impairment in the immune system: DS subjects have a higher susceptibility to bacterial infections and to develop hematological malignancies (Bruwier and Chantrain 2012; Goldacre et al. 2004) or organ-specific autoimmune disorders (hypothyroidism, celiac disease or insulin-dependent diabetes mellitus) (Guaraldi et al. 2017; Karlsson et al. 1998; Storm 1990). Immune abnormalities in DS have been described for more than 30 years and attention has been mainly focused on the immunologic impairment of the T cell compartment. DS subjects have reductions in the number of circulating CD4+ T cells as compared to age-matched controls (Barrena et al. 1993; Cocchi et al. 2007; Cossarizza et al. 1990; Joshi et al. 2011; Schoch et al. 2017; Trotta et al. 2011), with alterations in maturation and differentiation. Regarding CD4+ T lymphocytes subsets, an imbalance of subpopulations in the peripheral blood is noted (Barrena et al. 1993; Burgio et al. 1978; Guazzarotti et al. 2009), with reduction in naïve lymphocytes (Guazzarotti et al. 2009) or increased proportions of peripheral regulatory T cells with a defective inhibitory activity compared to age-matched controls (Pellegrini et al. 2012; Roat et al. 2008; Schoch et al. 2017). Recently, Schoch and colleagues reported higher percentages of Th1 and Th17 lymphocytes in children and adolescents with DS, in comparison to healthy controls, with similar percentages of Th2 cells (Schoch et al. 2017). The vast expansion of naïve helper (CD4+ CD45RA+) and cytotoxic (CD8+ CD45RA+)
CD27+) T lymphocytes, normally observed in healthy children, is lacking in DS subjects in the first years of life (Kusters et al. 2010). From a functional point of view, an impairment is also supported, with weak proliferative responses against specific mitogens (Franceschi et al. 1981; Karttunen et al. 1984; Lockitch et al. 1987; Park et al. 2000), as well as changes in cytokine production (Cetiner et al. 2010; Park et al. 2000; Roat et al. 2008). However, in their report recently published, Schoch et al. (2017) observed a normal functionality of lymphocytes in DS subjects and considered that they are able to mount effector T-cell responses with normal functional characteristics. Observed alterations in the peripheral T-cell compartment have led to numerous joint reports on thymic abnormalities in DS subjects (Fabris et al. 1984; Larocca et al. 1988; Murphy et al. 1990; Murphy and Epstein 1990, 1992; Musiani et al. 1990; Papadopoulos et al. 2003), with reduced thymic size, impaired intrathymic expansion of immature T cells and inefficient intrathymic maturation. Reduced thymic expression of large set of genes may be associated with the observed abnormalities (Lima et al. 2011). For example, AIRE (autoimmune regulator, located on HSA21) expression was found significantly reduced in DS thymic medullary epithelial cells, as compared to age-matched individuals.

Besides the T-cell compartment, the humoral immune system seems also disturbed. DS subjects have a lower number of total circulating B cells compared to healthy controls (Cossarizza et al. 1991; Schoch et al. 2017; Verstegen et al. 2010), with decreased transitional and naïve B lymphocytes (Verstegen et al. 2010) and also severe defects in memory B cells (Casetti et al. 2015; Joshi et al. 2011; Valentini et al. 2015; Verstegen et al. 2014), whereas germinal centers and plasma cells in tonsils appeared normal (Verstegen et al. 2014). Humoral immune responses after vaccination are altered in DS subjects (Kusters et al. 2011; Valentini et al. 2015). These impairments could be due to a true B cell defect, but also to a disturbance in T-lymphocyte help or a combination of both.

Some reports also pointed out modifications in the innate immune system. Bloemers et al. observed lower absolute total granulocytes counts in peripheral blood, lower absolute numbers of myeloid dendritic cells, but higher absolute numbers of pro-inflammatory CD14dimCD16+ monocytes (Bloemers et al. 2010). Regarding natural killer (NK) cells, literature results are contradictory. First reports identified an age-related expansion of CD57+ cells in comparison with age- and sex-matched healthy controls, associated with a low NK activity (Cossarizza et al. 1990, 1991), but more recent reports demonstrated that absolute numbers of NK cells were low (de Hingh et al. 2005) or normal (Bloemers et al. 2010) and discrepancies were attributed to differences of surface markers used. However, in the ultimate study published by Schoch and colleagues, they reported a significantly higher percentage of NK cells among lymphocytes of individuals with DS compared to controls (Schoch et al. 2017).

The picture is extremely complex, and it is still debated whether the immune system in DS is intrinsically deficient from the very beginning or another victim of a generalized process of precocious ageing. For some authors, observed alterations in immune system are related to a primary immunodeficiency, intrinsically present...
in DS subjects (de Hingh et al. 2005; Kusters et al. 2009, 2010). The findings of decreased naïve T and B cells are related to a deficient production from birth onwards, with absence of any vast expansion of lymphocytes during first years of life. Other authors favor the opinion of early senescence of the immune system (Barrena et al. 1993; Cossarizza et al. 1990; Cuadrado and Barrena 1996; Roat et al. 2008). Considering that important incidences of the conditions previously mentioned (higher susceptibility to bacterial infections, hematological malignancies, autoimmune disorders) are normally seen in elderly individuals, they supposed that DS is associated with a premature ageing in the immune system and that most of the phenotypic abnormalities observed are associated with an abnormal ageing of the thymus-dependent system and are reminiscent of immunosenescence seen in the elderly. Age-related alterations were thus reported: for example, progressive decrease in the values of CD4+ lymphocytes was observed in the first years of life in DS subjects (Cocchi et al. 2007) or levels of lymphocytes expressing the signal-joint T cell receptor rearrangement excision circles (sj-TREC+), that were lower in DS children as compared to controls, were strongly correlated with age (Roat et al. 2008).

**Biological Hallmarks of Ageing in Down Syndrome**

Ageing is a really complex and multifactorial process. Different conceptualizations have proposed sets of biological pillars of ageing, i.e. cellular and molecular processes that promote ageing in a highly interconnected way (Kennedy et al. 2014; López-Otín et al. 2013). DS individuals exhibit not only clinical accelerated ageing, but also an earlier appearance of these age-associated markers.

**DNA Damage Accumulation**

Genetic damages, which can be of endogenous or exogenous origin, accumulate throughout life and participate to the ageing process. Several lines of evidence suggest that DS is associated with a DNA repair defect, like many other progeroid syndromes (Patterson and Cabelof 2012). It has been observed that cells from DS donors (skin fibroblasts or peripheral blood lymphocytes) have higher basal endogenous levels of DNA damage, as compared to control cells (Franceschi et al. 1992; Maluf and Erdtmann 2001; Morawiec et al. 2008; Tiano et al. 2005; Zana et al. 2006). Moreover, DS cells were found to be more sensitive to DNA damaging agents as compared with the controls, with defective DNA repair processes (Morawiec et al. 2008; Necchi et al. 2015; Raji and Rao 1998; Zana et al. 2006). Loss of base excision repair phenomena, associated with a reduction in β-polymerase activity, were found in DS subjects (Cabelof et al. 2009; Raji and Rao 1998).
**Telomere Shortening**

Telomere attrition is considered as a contributor to cell senescence and ageing (Blackburn et al. 2015) and has been investigated for many years as a potential biomarker of ageing, with telomere length diminishing progressively with age (Jylhävä et al. 2017; Sanders and Newman 2013). Regarding DS, several teams have investigated telomere length in this syndrome. In the first report on telomere length quantification, a significantly higher rate of telomere loss was observed in blood lymphocytes from DS subjects (age 0–45 years) in comparison to controls (Vaziri et al. 1993). The same profile of acceleration of telomere loss, assessed by mean telomere restriction fragment length, was observed in another study published later where accelerated telomere shortening was associated with stem cell deficiency and was described as already present in fetal life (Holmes et al. 2006). Sukenik-Halevy et al. also investigated telomere length in pre-natal samples (Sukenik-Halevy et al. 2011). They studied telomere length and the *hTERC* gene copy number, which encodes the telomerase RNA subunit, in amniocytes of trisomy 21 conceptions and normal pregnancies. They observed a telomere shortening, associated with an increase in the copy number of *hTERC* in amniocytes of trisomy 21 fetuses compared to the control group with normal karyotype. Fetal DS fibroblast primary cell lines were also found to have more pronounced telomere attrition than their control counterparts (Gimeno et al. 2014).

Regarding DS newborns, Wenger et al. reported results of a small study on ten subjects: telomere length in newborns with DS was significantly shorter than in newborns with normal karyotype (Wenger et al. 2014). These results were in conflict with those published the same year by Nakamura et al.: telomere lengths were assessed by Q-FISH in a larger number of newborns (*n* = 31), with chromosomal abnormalities (trisomy 21 or trisomy 18) and controls with diploid karyotypes. These authors did not find marked differences of whole telomere length between the groups (Nakamura et al. 2014). Finally, it was recently observed that DS babies have longer telomeres than controls, with impact of maternal age on the length of telomeres (Bhaumik et al. 2017). These results were in accordance with those of Gruszczeka et al. who showed that blood leukocytes from juvenile DS patients (mean age = 4.5; range: 2–21 years) have longer telomeres than age matched controls (Gruszczeka et al. 2015).

Discrepancies in some published results could be attributed to different causes: differences in the age of the samples studied, differences in the cell type analyzed or differences in the technique for the measurement of telomere length. If the question of the presence of shorter telomeres at birth as an inherent trait is still debated, an accelerated rate loss during lifespan seems to be present in DS subjects. This acceleration of telomere erosion could serve as a clinical biomarker, informative on cognitive status. Jenkins et al. evaluated in several publications the relationship between telomere length in older DS subjects (>40 years old) and cognitive decline (Jenkins et al. 2006, 2008, 2010, 2012, 2016, 2017). They reported, in cross-sectional studies, that telomere length was associated with the presence of mild cognitive
impairment and dementia (Jenkins et al. 2006, 2008, 2010, 2012). T lymphocytes in DS subjects with dementia (age 45–60 years old) have shorter telomeres than those of DS subjects without dementia (Jenkins et al. 2006). In their latest publications in 2016 and 2017, these authors observed that longitudinal changes in telomere length were associated to transition to dementia (Jenkins et al. 2016, 2017). Telomeres became shorter with the declining of the clinical status (transition from ‘clinically normal ageing’ to mild-cognitive impairment or dementia) (Jenkins et al. 2016).

**Loss of Proteostasis**

Proteostasis, or protein homeostasis, referring to the processes associated with biogenesis, folding, trafficking and degradation of proteins in cells, is disturbed during ageing (Morimoto and Cuervo 2014). Cells (skin fibroblasts or frontal cortex) derived from DS individuals exhibit extensively remodeled proteostasis networks, as compared to controls (Di Domenico et al. 2013; Liu et al. 2017). Alterations in different key elements of these networks compared to euploid controls, such as chaperone systems, unfolded protein responses or proteasomal degradation have been observed (Aivazidis et al. 2017). When DS fibroblasts were exposed to a moderate heat stress, they were unable to cope with this increased proteomic stress, leading to diminished cell viability compared to controls (Aivazidis et al. 2017). This disturbance of the proteostasis network can participate to the accumulation of misfolded proteins, notably in the brain (Di Domenico et al. 2013).

**Oxidative Stress and Mitochondrial Dysfunction**

As previously mentioned, individuals with DS are characterized by higher levels of oxidative stress as compared to the general population, from a local and a systemic perspective (Aivazidis et al. 2017; Cenini et al. 2012; Garlet et al. 2013; Jovanovic et al. 1998; Odetti et al. 1998; Reynolds and Cutts 1993). They have increased basal levels of endogenous oxidative stress, which seems to occur early in life. An important number of genes coding for proteins relevant to oxidative damage are located on HSA21 and over-expressed in DS, among which superoxide dismutase 1 (SOD1) located in the Down Syndrome Critical Region 1 (DSCR1) seems to be the most relevant (Murakami et al. 2011). Increased SOD1 activity in DS subjects leads to a higher production of hydrogen peroxide (H$_2$O$_2$), not adequately compensated (Garlet et al. 2013). Iron metabolism dysregulation (Barone et al. 2017) and aberrant mammalian target of rapamycin (mTOR) pathway signaling (Di Domenico et al. 2017) are considered as potential contributors to the increase in oxidative stress observed in DS subjects.

The increased production of reactive oxygen species (ROS) in DS individuals is accompanied by mitochondrial dysfunction (Arbuzova et al. 2002; Busciglio et al.
Mitochondrial DNA (mtDNA) mutations, along with defective repair of mtDNA damage, are found at a high rate in DS subjects (Arbuzova 1998; Coskun et al. 2010; Druzhyna et al. 1998).

**Cellular Senescence**

Cellular senescence is a complex phenomenon, that is considered to be a contributor to the ageing process (López-Otín et al. 2013). Regarding DS individuals, literature data are not concordant. It was previously found that fibroblasts from DS subjects have a diminished rate of cellular proliferation than those from normal donors, but that there was no difference in terms of cumulative number of population doublings until replicative senescence or for the beta-galactosidase staining between the two populations (Kimura et al. 2005). More recently, Adorno et al. reported a strong proliferation defect, associated with a premature senescent phenotype according to beta-galactosidase staining in fibroblasts derived from DS subjects (Adorno et al. 2013).

**Stem Cells Ageing**

Evidence is now compelling on the presence of a decline in stem cell function during ageing, specially for hematopoietic stem cells (HSC) or neural stem cells (NSC) (Liu and Rando 2011). In DS individuals, some of the anomalies seem to be recapitulated earlier in life. A first report was published in 2002, regarding neuronal progenitors (Bahn et al. 2002). NSC derived from the central nervous system of DS or control post-mortem fetal tissues were investigated and authors reported abnormalities in neuronal proliferation and/or survival capacity in DS stem cells. Assessing gene expression, they identified a dysregulation of the network of genes regulated by the transcription factor REST (neuron-restrictive silencer factor). Regarding HSC, peripheral blood from fetuses and bone marrow samples from children with DS exempt of malignant hematological diseases were analyzed for their content in stem cells (assessed by the percentage of CD34+ cells) and compared to samples from non-trisomic controls (Holmes et al. 2006) and a marked stem cell deficiency was seen in both trisomic fetuses and children. To gain insights into the molecular mechanisms associated with the stem cell exhaustion, gene expression has been investigated and compared to those of non-trisomic controls (Cairney et al. 2009). HSC obtained from the iliac crest of DS children of 1–5 years old and NSC obtained from fetal DS cortex were studied and 430 genes were found differentially expressed. Analysis of these genes revealed an enrichment in pathways previously associated with cellular ageing. In particular, a down-regulation of DNA repair genes and an up-regulation of genes involved in apoptosis, inflammation and angiogenesis were observed (Cairney et al. 2009). Authors identified a dysregulation of
the Notch/Wnt pathway as a potential hub that may drive stem cell ageing. Finally, changes in DS stem cells were similar to those observed in stem cells of older people (individuals of 60–80 years old). The same trends were observed in HSC, suggesting the existence of shared molecular events between the two types of stem cells (Cairney et al. 2009). Adorno et al. took the advantage of the study of two murine models of DS and identified the USP16 gene, which is triplicated in DS, as an important contributor to the lack of self-renewal in DS stem cells (Adorno et al. 2013). An over-expression of USP16 in normal human fibroblasts decreased their proliferation, whereas its down-regulation in DS fibroblasts, which show proliferation defects and premature senescent phenotype in culture, promoted their proliferation. Finally, over-expression of the enzyme in human neural progenitor cells reduced their in vitro expansion potential and the formation of neurospheres (Adorno et al. 2013).

**Inflammation**

Ageing is characterized by a peculiar chronic inflammatory status, called ‘inflamm-ageing’ (Franceschi et al. 2000). As mentioned earlier, DS subjects exhibit markers of early immunosenescence and marks of chronic inflammation can be observed: PBMC of DS subjects are characterized by spontaneous higher production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF\(\alpha\)) and interferon (IFN\(\gamma\)), as compared to control cells in in vitro culture (Trotta et al. 2011). Serum levels of TNF\(\alpha\) and IFN\(\gamma\), associated with decreased levels of interleukin (IL)-10, were found to be higher in DS children as compared to healthy controls (Nateghi Rostami et al. 2012) and a similar pattern of pro-inflammatory cytokines was observed in adults with DS (Carta et al. 2002). However, all data do not tend to the same conclusion and some studies reported on the contrary an increase in anti-inflammatory cytokines: for example, besides the increase in TNF\(\alpha\) and IFN\(\gamma\), Trotta et al. observed also an increase in the production of IL-10 (Trotta et al. 2011). Levels of IL-4 and IL-10 were found significantly increased in children with DS, whereas IL-6 and TNF-\(\alpha\) levels were decreased (Cetiner et al. 2010). Recently, Zhang et al. performed a meta-analysis on 19 studies, demonstrating that patients with DS, despite great heterogeneity, have significantly increased circulating TNF\(\alpha\), IFN\(\gamma\) and IL-1\(\beta\), without differences in concentrations of IL-4, IL-6, IL-8 and IL-10 (Zhang et al. 2017).

**Biological Clocks in Down Syndrome: Evidence for a Biological Accelerated Ageing**

Twenty years ago, Nakamura and Tanaka estimated the biological age of 11 DS individuals according to a global index developed on 436 healthy subjects involving 14 clinical and biological variables (body mass index, systolic blood pressure,
diastolic blood pressure, total protein, ratio of albumin to globulin, concentrations of glutamate oxaloacetate transaminase, glutamic pyruvic transaminase, total cholesterol, triglyceride and blood urea nitrogen, white and red blood cell counts, hemoglobin concentration and hematocrit) (Nakamura and Tanaka 1998). They observed an increase in the biological age of the subjects with DS as compared to healthy subjects. It was also noted that important fluctuations of biological ages from year to year in DS subjects, a pattern that was not observed in controls, and could be in part due, according to the authors, to early senescence of the immune system (white blood cell counts were included in the index) (Nakamura and Tanaka 1998). More recently, new biomarkers of ageing, referred as biological ages or clocks, have been developed and applied to DS populations.

**Epigenetic Clocks**

During the last few years, DNA methylation (DNAm)-based biomarkers of ageing, called epigenetic clocks, have gained particular relevance in the field of ageing research and at present, three different models have been proposed (Hannum et al. 2013; Horvath 2013; Weidner et al. 2014). Horvath’s epigenetic clock, developed in 2013 (Horvath 2013), based on the DNAm levels of 353 specific CpG sites, was applied to four datasets (including 89 DS individuals in total) in which genome-wide DNA methylation levels were assessed in peripheral blood leukocytes, various brain regions, whole blood or buccal epithelium, using Illumina Infinium 27K or 450K platforms (Horvath et al. 2015). DS subjects exhibited a highly significant age acceleration effect (defined as a residual resulting from a linear model that regressed the calculated epigenetic age, also called DNAm age, on chronological age) in three independent data sets involving blood and brain tissues. Age acceleration in brain was estimated at 11.5 years, whereas it was at 4.25 years in blood, for an average of 6.6 years. Results were similar even after correction for blood cell type abundance measures. No significant age acceleration effect was observed in the dataset on buccal epithelium DNA. Cole et al. in their report on ‘brain-predicted age’ using neuroimaging methods in DS subjects, noticed that they observed, taking the same approach as Horvath et al., an effect of similar magnitude (7.4 vs 6.6 years of added ageing) (Cole et al. 2017).

In 2016, Obeid et al. investigated DNA methylation by pyrosequencing in three specific regions, named *ASPA*, *ITGA2B* and *PDE4C*, in 31 young subjects with DS and in controls (Obeid et al. 2016). These regions include the CpG sites that have been reported to predict ageing in adults according to Weidner’s clock (Weidner et al. 2014). They observed hypomethylation of *ASPA* and *ITGA2B* in subjects with DS, associated with a strong negative association with age. DNA methylation of *PDE4C* did not differ between the two groups but showed a positive correlation with age. According to the prediction of age using DNAm data of the three loci in combination with plasma concentrations of Aβ (1-42) measured by high sensitive ELISA assay, subjects with DS in their second decade were on average 3.1 (95% CI
1.5–4.6) years older than their predicted age based on regression extrapolated from their counterparts without DS.

**N-glycome Signature**

In recent years, plasma N-glycans have also emerged as biomarkers associated with ageing (Dall’Olio et al. 2013; Yu et al. 2016). Plasma N-glycome changes in 76 DS individuals of different ages have been investigated, as well as in controls (mothers and siblings), using two different methods (MALDI-TOF-MS and DSA-FACE) (Borelli et al. 2015). Glycomic changes associated with DS were identified, resulting in a specific plasma N-glycomic signature with 24 plasma N-glycans differentially expressed. GlycoAge, an index developed to monitor galactosylation changes that mark ageing, was evaluated in this cohort of subjects and increased values were found in DS as compared to their siblings, in particular at young age. Thus, from a glycomic point of view, DS individuals are older than their age-matched controls. Moreover, GlycoAge Test values were negatively correlated with the score of Performances IQ.

**Epigenetics in Down Syndrome**

In addition to the previously mentioned hallmarks of ageing, epigenetic mechanisms have emerged as playing a major role in ageing and age-related diseases (Kennedy et al. 2014; López-Otín et al. 2013). DNA methylation, histone modifications or non-coding RNAs are also involved in neurodevelopment, learning and memory, as well as neurodegenerative diseases (Day and Sweatt 2011; Della Ragione et al. 2014; Gräff and Tsai 2013; Saab and Mansuy 2014; Sanchez-Mut et al. 2016), and some importance has been attributed to epigenetics in the pathogenesis of DS over the past decade. Epigenetic influences have been investigated, particularly to explore their impact on the variability of the phenotypes observed in the disease, on the accelerated ageing process or on the occurrence of acute megakaryoblastic leukaemia (Malinge et al. 2013).

**DNA Methylation**

**Different DNA Methylation Patterns in Subjects with Down Syndrome**

One of the first reports on the presence of differential DNA methylation in DS individuals was published in 2001 (Pogribna et al. 2001): DNA extracted from lymphocytes of DS children was found globally hypermethylated, as compared to the DNA of
Their siblings, according to radiolabel incorporation. In agreement, in 2006, Chango et al. used a method based on a combination of methylation-sensitive arbitrarily primed polymerase chain reaction (MS-AP-PCR) and quantification of DNA fragments, to investigate relative levels of DNA methylation in peripheral blood lymphocytes: they identified six fragments that were hypermethylated in DS subjects as compared to controls (Chango et al. 2006). Some years later, using alternative techniques to assess DNA methylation levels, studies started to dissect in greater details the epigenetic characteristics of DS. DNA methylation patterns of samples from DS subjects were investigated at the genome-wide level, using Illumina Infinium platforms 27K and 450K (Eckmann-Scholz et al. 2012; Jones et al. 2013b; Kerkel et al. 2010) or reduced representation bisulfite sequencing (RRBS) (Jin et al. 2013; Sailani et al. 2015). Different tissues were investigated: peripheral blood lymphocytes from adults with DS (Kerkel et al. 2010), buccal epithelium (Jones et al. 2013a), skin fibroblasts (Sailani et al. 2015) or placenta samples (Eckmann-Scholz et al. 2012; Jin et al. 2013). Results were globally concordant, with shared common findings: marked DNA methylation alterations in DS cells with predominantly hypermethylation as compared to controls and genome-wide perturbation of DNA methylation without enrichment on HSA21.

In 2010, performing microarray-based genome-wide DNA methylation profiling of white blood cells and T-lymphocytes from adults with DS and normal controls, Kerkel et al. observed consistent hypo or hypermethylation in 118 genes in DS subjects, with corresponding differential expression for some of them (TMEM131, TCF7, NPDC1) and without specific enrichment for genes on HSA21 (Kerkel et al. 2010). Many of the differentially methylated genes are involved in lymphocyte development and function. The alterations in methylation were generally stable in a given individual and were independent of the differential cell counts. In samples derived from buccal epithelial cells of adults, 9982 probes were found differentially methylated between DS and control samples, with 3300 of them having an absolute difference between means of methylation of more than 10% (Jones et al. 2013b). Here also no enrichment for HSA21 was observed. Within the differentially methylated CpG sites, authors identified a number of genes known to be involved in the pathology of the disease and several probes overlapped with the ones identified in other published works (Jin et al. 2013; Kerkel et al. 2010). Interestingly, cognitive function was assessed with Dalton Brief Praxis Test and correlations with epigenetic data were investigated: five CpG sites were found to be correlated with cognitive impairment, including two probes located in the TSC2 gene, a component of the mTOR pathway that has previously been associated with AD pathology (Jones et al. 2013b). Recently, Sailani et al. investigated DNA methylation profiles of skin fibroblasts of monozygotic twins (MZ) discordant for DS (Sailani et al. 2015): they observed 35 differentially methylated gene promoter regions, that were also concordant with data obtained by comparing unrelated controls and DS individuals and that are mostly related to embryonic organ morphogenesis and development. Here again no enrichment for HSA21 was found and global higher levels of methylation were observed in the twin affected by DS, compared to his unaffected sibling. Interestingly, the differentially methylated regions observed were stably maintained in iPSCs generated from fibroblasts obtained from the twin pair discordant for DS.
Apart from samples derived from children or adults with DS, Eckmann-Scholz et al. and Jin et al. investigated DNA methylation patterns in placenta samples and observed a general hypermethylation across all chromosomes in placentas with trisomy 21, compared to normal ones. The first team used the Illumina Infinium 27K BeadChips on chorionic villi samples and identified 464 loci corresponding to 404 genes differentially methylated in samples with trisomy 21 as compared to samples with a normal karyotype (Eckmann-Scholz et al. 2012). 387 Genes, significantly enriched in developmental processes, were found hypermethylated in the trisomic samples and three of them are located on HSA21 (COL6A2, H2BFS, RUNX1). Jin et al. used an improved version of RRBS to quantify DNA methylation levels in 17 placenta villi samples (11 DS and 6 control samples) and performed also RNA-Seq analysis in five normal and four DS placenta villi samples (Jin et al. 2013). A global hypermethylation was observed in all genomic regions in the samples with trisomy 21, with a predominance in promoter regions. Out of the 589 sites found hypermethylated, significant down-regulation of gene expression was observed in 207 genes. Interestingly, three genes (TCF7, FAM62C and CPT1B), that were found differentially methylated between DS and controls in the study published by Kerkel et al. on adult samples (Kerkel et al. 2010), were also found similarly differentially methylated in the placental samples in this study, suggesting a possible conservation of the methylation patterns in different tissues and across the life course.

Three recent reports analyzed DNA methylation patterns in developing fetal cortex (El Hajj et al. 2016; Lu et al. 2016; Mendioroz et al. 2015). They all observed hypermethylation in the brains with trisomy 21 as compared to controls. In the report by Lu et al. four sites overlapped with the ones found by Kerkel et al. in adult peripheral blood lymphocytes (Kerkel et al. 2010) and 88 probes were also shared with the adult buccal epithelium (Jones et al. 2013b). Mendioroz et al. evaluated DNA methylation in cerebrum of fetuses as well as cerebral and cerebellar cortex of adults: some genes that were differentially methylated in fetal brains were also in adult brain cells, suggesting again an early onset of the epigenetic changes (Mendioroz et al. 2015). Differentially methylated sites found in samples with trisomy 21 were enriched in CpG in or near specific transcription factor binding sites and some of them were also found differentially expressed. In their study, Lu et al. identified an alteration in the signaling pathway of several genes involved in ubiquitination and suggested this pathway as a possible key player in the development of DS neuropathology (Lu et al. 2016).

Using Illumina Infinium 450K platform, our team analyzed whole blood samples from a family-based model of DS, chosen in order to minimize confounding genetic and environmental factors, with 29 trios composed by the DS person, his mother and his non-affected sibling (Bacalini et al. 2015b). Data were processed through a pipeline specifically tailored to identify differentially methylated regions (DMRs) (Bacalini et al. 2015a). Our analysis confirmed a prevalent hypermethylation, as well as the majority of DMRs previously identified by Jones et al. (2013b), and our gene ontology analysis identified an enrichment in genes involved in morphogenetic and developmental processes (HOXA family, RUNX1, EBF4, NCAM1) as well as
regulation of chromatin structure (*PRMD8*, *KDM2B*, *TET1*). However, unlike other published studies, we observed an enrichment in HSA21 for the DMRs identified in DS subjects as compared to their controls. We selected a short list of 68 DMRs whose DNA methylation status was remarkably different between DS subjects and healthy siblings (methylation difference greater than 0.15), which constituted an epigenetic signature of DS.

Five-hydroxymethylcytosine (5hmC) is also an epigenetic modification that occurs on cytosine-bases and that is produced by the activity of TET enzymes. 5hmC acts as an intermediate during the DNA demethylation but has been also considered as acting as a stable epigenetic marker. During ageing, 5hmC content was found to be negatively correlated with age in blood or PBMC (Buscarlet et al. 2016; Valentini et al. 2016) and its importance in brain development and ageing has been recently investigated (Kraus et al. 2015). Alterations in DNA hydroxymethylation in DS were evaluated by Ciccarone et al.: the content of 5hmC was measured by dot-blot assay on DNA extracted from PBMC and levels were found lower in PBMC from DS subjects as compared to controls (Ciccarone et al. 2017).

Finally, it is important to note that DNA methylation patterns are used in prenatal diagnosis of DS. Studies have been investigating differentially methylated regions that could differentiate fetuses with trisomy 21 and fetuses with normal karyotypes, in order to develop non-invasive pre-natal diagnosis techniques analyzing free fetal-specific DNA methylation in maternal blood (Hatt et al. 2015, 2016; Jin et al. 2013; Lee et al. 2016; Lim et al. 2011; Papageorgiou et al. 2009, 2011; Sifakis et al. 2012; Yin et al. 2014).

**Regulation of DNA Methylation in Down Syndrome**

Several proteins are involved in the regulation of DNA methylation patterns, which result from a balanced state between methylation and demethylation processes. In mammals, DNA methylation is related to the addition of a methyl group to cytosine bases, generally located in CpG dinucleotides, by the DNA methyltransferase (DNMT) family of enzymes (DNMT1, DNMT3A and DNMT3B), whereas demethylation is mostly catalyzed by TET enzymes (TET1, TET2 and TET3). In order to understand potential mechanisms driving the epigenetic changes seen in DS, levels of these enzymes were investigated. Expression levels of *DNMT1* and *DNMT3B* were found similar between DS subjects and controls, whereas *DNMT3A* was found down-regulated in PBMC from DS subjects compared to controls (Ciccarone et al. 2017). On the contrary, *DNMT3L*, found on HSA21, encodes a protein that has no catalytic function on its own, but assists DNMT3A and DNMT3B in establishing *de novo* DNA methylation marks. *DNMT3L* was found over-expressed in DS neural progenitors (Lu et al. 2016). Regarding TET enzymes, they were all previously found down-regulated in DS (Jin et al. 2013) and reduced levels of *TET1* and *TET2* expression in DS subjects as compared to controls were confirmed recently (Ciccarone et al. 2017). This down-regulation may lead to hypermethylation through decreased DNA demethylation.
DNA methylation reactions need a universal methyl donor, represented by S-adenosylmethionine (SAM), which is generated in a metabolic network called one-carbon metabolism. DS subjects are characterized by perturbations in this metabolism, related to the over-expression of cystathionine beta-synthase (CBS) gene located on HSA21. CBS is a central enzyme in this specific metabolic pathway, catalyzing the conversion of homocysteine into cystathionine. In 2001, plasma levels of homocysteine, methionine, S-adenosylhomocysteine (SAH) and SAM were found decreased in children with DS, whereas plasma levels of cystathionine and cysteine were found increased (Pogribna et al. 2001), consistent with an increase in CBS activity (Chadefaux et al. 1985). Discordant results were published later: cystathionine, cysteine, SAH and SAM were found at higher levels in DS compared to the controls, whereas levels of methionine did not differ significantly (Obeid et al. 2012; Obermann-Borst et al. 2011). There are also discordant results regarding homocysteine levels, that were found higher (Song et al. 2015) or lower (Meguid et al. 2010) in DS subjects as compared to controls.

**Non-coding RNAs**

Another epigenetic mechanism implicated in ageing, neurodevelopmental disorders and neurodegenerative diseases is represented by non-coding RNAs (ncRNAs) (Jung and Suh 2014; Tan et al. 2013). In recent years, different research groups have studied the potential contribution of micro-RNAs (miRNAs) to the regulation of DS transcriptome and in fine, to DS phenotypes and phenotypic variability. Various miRNAs are encoded on HSA21 and therefore likely over-expressed in DS, and some of them have been implicated in the development of some DS-related pathologies. Principal HSA21-encoded miRNAs that have been implicated in DS and found over-expressed in the disease or adequate models are miRNA-125b-2, miRNA-155, miR-99a, let-7c and miRNA-802 (Siew et al. 2013). Using real-time quantitative PCR to study the expression of miRNA-155 in fibroblasts from a MZ twins discordant for DS, Sethupathy et al. observed that miR-155 was over-expressed in the fibroblasts from the twin with DS (Sethupathy et al. 2007). miR-155 is also over-expressed in DS brain, spleen and liver (Li et al. 2012), as well as in iPSCs generated from human DS amniotic fluid cells (Lu et al. 2013). miR-155 have been associated to brain pathology, as altered expression has been found in AD: miRNA-125b and miRNA-155 are significantly up-regulated in sporadic AD and have emerged as key contributors to the sporadic AD process (Zhao et al. 2015). Up-regulation of miR-125b and miR-155 have been associated to the pathogenic mechanism of complement factor H deficiency that drives inflammatory neurodegeneration in AD and in age-related macular degeneration (Lukiw et al. 2012). Finally, miR 125b-2 has been also identified as a potential onco-miR associated with megakaryoblastic leukemia in DS individuals (Klusmann et al. 2010).
Two studies have investigated miRNA expressions in DS placentas, without significant match between the two datasets (Lim et al. 2015; Svobodová et al. 2016). In 2015, Lim et al. identified 34 differentially expressed miRNAs in DS placenta samples compared to normal ones (16 up-regulated and 18 down-regulated miRNAs), distributed on various chromosomes, without inclusion of any HSA21-derived miRNA (Lim et al. 2015). In 2016, Svobodová et al. identified 7 miRNAs over-expressed in DS placentas as compared to euploid samples and three of them were located on HSA21 (let-7c, miR-125b and miR-99a) (Svobodová et al. 2016). Analyzing of genome-wide expression of miRNAs in cord blood mononuclear cells from fetuses with DS, Xu et al. observed that most of the mRNA targets of differentially expressed miRNAs were associated with immune modulation (Xu et al. 2013).

Resembling miRNAs, long noncoding RNAs (lncRNAs) have been also demonstrated to have various regulatory roles in gene expression and to contribute to neurological diseases (Qureshi et al. 2010). A large number of lncRNAs were identified differentially expressed in iPSCs generated from DS subjects as compared to normal iPSCs. Most of the differentially expressed lncRNAs were closely associated with mitochondrial functions and they could thus be associated to the dysfunction of mitochondria observed in DS (Qiu et al. 2017).

**Conclusion**

For many decades DS has been described as a progeroid syndrome, as subjects affected are characterized by the appearance early in life of many typical age-related conditions, involving particularly neurological, immune, endocrine, musculoskeletal and sensorial systems. As DS subjects now live longer than before, we observe more and more age-related diseases in this specific population. Notwithstanding the recent advances in the characterization of the biological basis underpinning DS age-related phenotype, several aspects are still to be elucidated. One of the unsolved questions is whether DS atypical ageing is precocious or accelerated. Some data from literature are in favor of the presence of a phenomenon of precocious ageing in DS, present from fetal life and birth and which could be considered as an intrinsic characteristic of the syndrome. On the contrary other reports, using the latest generation of biological markers of ageing, consider DS as an accelerated-ageing condition. The combination of both phenomena is a possibility that should be further explored, also in the framework of the research for early anti-ageing interventions in this population.

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