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Epigenetic signature in persons with Down Syndrome

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Introduction

DOWN SYNDROME

Down Syndrome (DS) was first described by Edouard Onesimus Seguin (1812–1880), pioneer in France and in the United States of methods and systems for educating "those with mental retardation" (Neri and Opitz 2009). In 1866 John Langdon Down gave a new description of DS in his paper "Observations on an Ethnic Classification of Idiots". Down tried to classify patients with mental retardation on the basis of ethnic characteristics, recognizing four categories: the ''Ethiopian variety'', the "Malay variety", the ''American continent variety'' and the "Mongolian variety" today known as Down Syndrome (Down 1995).

Today we know that DS is the most common genetic autosomal disorder compatible with survival in humans (Biselli et al. 2010), affecting about 1/700 -800 newborns (Contestabile, Benfenati, and Gasparini 2010). DS is also the most common genetic cause of mental retardation (Biselli et al. 2010) and of intellectual disability in humans (Contestabile, Benfenati, and Gasparini 2010).

DS is a chromosomal disorder (Pueschel 1990; Roizen and Patterson 2003) that results from total or partial (region q22.1-22.3) trisomy of the chromosome 21 (HSA21) as demonstrated by Lejeune et al. in 1959 (Roat et al. 2008; Letourneau et al. 2014; Dekker, De Deyn, and Rots 2014). In particular DS phenotype can be associated to three types of chromosomal abnormalities: free trisomy 21, translocation or mosaicism (Newberger 2000). In over 95% of cases DS is a whole-chromosome trisomy due to meiotic non-disjunction of the chromosome 21 (Antonarakis et al. 2004; Lubec and Engidawork 2002). Many studies show that maternal age has a very strong effect on the incidence of trisomy 21. In about 86% of cases the nondisjunction event that leads to the trisomy occurs in the mother, with the maternal error being predominantly (75% of cases) in meiosis I. On the contrary paternal age has no effect on the incidence of trisomy 21. No environmental factors have been found to correlate with the occurrence of nondisjunction. (Valle 2006; Rachidi and Lopes 2008). In a minor fraction of cases (3-4%), DS arises from partial aneuploidy due to Robertsonian unbalanced chromosome translocation involving generally chromosomes 14 and 21 (Hook, Cross, and Schreinemachers 1983; Korenberg et al. 1994; Lyle et al. 2009; Biselli et al. 2010). In this kind of translocation the long arm of HSA21 is merged with another acrocentric chromosome in one of parents' cariotype. A subject with this kind of

translocation has a normal phenotype but has an high risk to produce gametes with a supernumerary chromosome 21. The risk of this type of Down Syndrome is not related to the mother's age (Cummings 2010). Finally in 1% of the cases DS can be caused by mosaicism, characterized by some cells containing 46 chromosomes and others with 47 chromosomes (Biselli et al. 2010).

Although clinical phenotype of each DS person is complex and variable in trait number and intensity (Rachidi and Lopes 2007), the main features of the disease are short stature, abnormalities in the morphology of neck and head (short neck, flattened nose, protruding tongue, almond shape to the eyes caused by an epicanthic fold of the eyelid), hyperlaxity of ligaments, frequent congenital heart disease, hearing defects, hypotonia, high risk of leukaemia, mental retardation at various degrees, endocrine disorders, early onset of Alzheimer disease, and immune disorders (Scriver 2001). Two exceptions to phenotype variability are the existence of mental retardation and neonatal hypotonia in close to 100% of individuals with DS (Korenberg et al. 1994). Anyway mental retardation remains the most invalidating aspect and the invariable hallmark disorder of DS with an hard impact on public health (Rachidi and Lopes 2007).

DOWN SYNDROME AND ACCELERATED AGING

In 1933 the mean age of death of DS children was 9 years (Earl 1934). Since then survival rates have risen drastically, but age-specific mortality rates are still increased in people with DS compared with typically developing population or people with other forms of intellectual disability (Bittles et al. 2002). Premature accelerated aging and the consequent development of age associated diseases like Alzheimer Disease (AD) seem to be the cause of higher mortality late in life of DS subjects (Zigman et al. 2007; Schupf and Sergievsky 2002). In DS we can observe signs of early aging in various body systems like integumentary, endocrine, sensory musculoskeletal, immunological and neurological systems (Zigman 2013), even if accelerated aging has been confirmed in some but not all body system of people with DS.

In the last decades life expectancy of DS subjects has considerably changed, increasing from 12 years in 1949 to nearly 60 years today (Penrose 1949; Bittles and Glasson 2004) and scientific community believes that it will increase in the future (Glasson et al. 2002). This means an increase in life expectancy of DS, which in Italy is estimated to be about 49,000 persons (Ghezzo et al. 2014).

This enhance in life expectancy is due to an improved access to medical treatment for those with learning disability, and a shift in public attitude during the past 20 to 30 years that has resulted in better care within the community (Arosio, Abbate and Zanetti 2004).

Despite the increase in life expectancy, DS subjects continue to show age-related diseases, especially regarding the immune and cognitive systems. In fact DS ranks among human "segmental progeroid syndrome" characterized by an acceleration of the biological aging process, affecting particularly some organ and tissue, such as immune system and brain.

Concerning immune system, subjects with DS are characterized by alterations of innate and adaptive immunity including modifications of B and T lymphocyte subsets and natural killer cells, defective phagocytosis and chemotaxis of polymorphonuclear leukocyte (Ugazio et al. 1990; Franceschi et al. 1990; Barrena et al. 1993; Cuadrado and Barrena 1996; Cossarizza et al. 1991; Franceschi et al. 1991). The precocious aging affecting the immune system of DS subjects is confirmed by the marked thymic involution and the rapid reduction of thymic output of virgin T cells, as a consequence of an increase in the levels of homeostatic cytokines, probably as a compensatory mechanisms (Nasi et al. 2006). Such features

determine an increased susceptibility to bacterial and infections, autoimmune phenomena such acquired hypothyroidism, celiac disease and diabetes mellitus (Kusters et al. 2009) and a 50-70 fold higher incidence of childhood leukaemia (Hasle, Clemmensen, and Mikkelsen 2000; Zipursky 2003). Leukemia and infections like pneumonia are still major causes of morbidity and mortality in DS subjects despite advances in treatment (Kusters et al., 2009). Since 1970 it has been hypothesized that Down Syndrome was associated with immune system's abnormalities due to the presence of an increased frequency of infections, autoimmune diseases and haematological malignancies (Burgio et al., 1975, 1978; Kusters et al., 2009; Levin et al., 1979; Oster, Mikkelsen and Nielsen, 1975). Between late 1980 and early 1990 (Kusters et al., 2009) several hypothesis have been formulated regarding DS as a form of abnormal precocious aging (Cossarizza et al. 1990; Cuadrado and Barrena 1996), observing differences between immune system of DS and non DS individuals. Recently on the basis of current (de Hingh et al. 2005) and past studies (Cossarizza et al., 1990; Lockitch et al., 1987; Ugazio et al., 1990), some authors (Kusters et al., 2009) suggest that immune system of DS subjects is intrinsically deficient from the very beginning . They argue that, in addition to thymus and T cells abnormalities, DS B-lymphocytes are also intrinsically different (Kusters et al., 2009). A profound B limphocytopenia in DS individuals has been observed, with absence of the normal enormous expansion in the first year of life (de Hingh et al. 2005) and a significant decrease of B lymphocyte (CD19+) is now reported also in DS fetuses (Zizka et al. 2006).

Regarding central nervous system, individuals with DS are more prone to age-related cognitive decline and are at high risk for developing dementia, which most commonly replicates features of Alzheimer's disease (AD). In fact, a presenile dementia syndrome that is clinically, neuropathologically (cholinergic, noradrenergic and serotonergic reduction), and histopathologically (senile plaques and neurofibrillary tangles) similar to Alzheimer's disease occurs in almost all DS subjects over the age of 40 years (Visser et al. 1997; Capone 2001; Head et al. 2001; Krasuski et al. 2002; Zana, Janka, and Kálmán 2007). In 1987 the gene for the Amyloid beta (Aβ) precursor that encodes for Aβ peptide was localized to the proximal part of the long arm of chromosome 21 (Robakis et al. 1987), in the obligate region of HSA21, that was supposed to be trisomic for the full expression of DS phenotype (Korenberg et al. 1990). The triplication of the *APP* gene that maps on chromosome 21 is

related to the deposition of Aβ in the brain across lifespan in DS subjects, promoting the development of AD in adults with DS (Zigman 2013).

Despite neuropathological characteristics of AD appear in adults DS over 35 to 40 years (Wisniewski, Wisniewski, and Wen 1985), there are some inter-individual differences between DS subjects, because some DS persons develop signs and symptoms of AD after 40 years, while other individuals reach 70 years without AD. This differences could be due to age, genetics, sex, estrogen, and presence of Metabolic Syndrome (Zigman 2013). In case of genetics, for example, numerous studies showed a significantly greater risk and an earlier age of AD onset in people with one or two ε4 alleles of *APOE* gene than in typically developing adults (Corder et al. 1993; Mayeux et al. 1993; Isbir et al. 2001b; Isbir et al. 2001a) and DS adults without any ε4 allele (Schupf et al. 1996; Prasher et al. 1997; Deb et al. 2000). On the contrary the presence of ε2 allele has been associated with a decreased risk of AD (Royston et al. 1994; Lai et al. 1999; Rubinsztein et al. 1999) and an increase in longevity (Royston et al. 1996; Frisoni et al. 2001). In cases of age and estrogen levels, increasing age and reductions in estrogen levels with menopause are linked to a greater risk for AD in DS (Bush and Beail 2004; Pike et al. 2009; Li et al. 2006), while results on the role of sex are ambiguous (Schupf et al. 1998; Lai et al. 1999). Peripheral hyperinsulinemia typical of Metabolic Syndrome (prevalence rate in DS of 14,6%) may inhibit brain insulin production causing an impaired Aβ clearance and high risk of AD (Zigman 2013; Braunschweig et al. 2004).

The combination of a pre-existing learning disability with superimposed age-related cognitive decline and dementia is difficult to treat, and is an expensive management problem. Many important medical, genetic and behavioral progresses have been made in understanding the phenotype associated with DS and also instrument to classify, diagnose AD in adults DS and to monitor long term function has been developed. Now it is also known that AD is not a certainty for this population, as originally thought (Zigman 2013). Despite all the progress that has been made over the years, it is hoped that in the future it will be fulfilled other advances such as prenatal treatments that may decrease the cognitive, health and functional limitations common to people with DS (Zigman 2013)

Human aging is a multi-factorial trait resulting from a combination of environmental,

genetic, epigenetic and stochastic factors, each contributing to a complex and heterogeneous overall phenotype. The aging process acts at several levels of complexity and with different dynamism, from molecule to cell, from organ to system, and finally to organism. As a consequence of these different aging rates, the aged body can be considered as an "aging mosaic" (Figure 1) composed by cells, tissues and organs showing varied levels of senescence (Cevenini et al. 2010; Cevenini et al. 2008). This is true not only for physiological aging occurring in "healthy" population, but also for age-related pathologies, including those described in persons affected by DS. Given its characteristics of accelerated aging, DS has been widely used as a model to study the biological basis of the aged phenotype (Biagi et al. 2010; Franceschi et al. 1991; Franceschi et al. 1992; Franceschi et al. 1995).

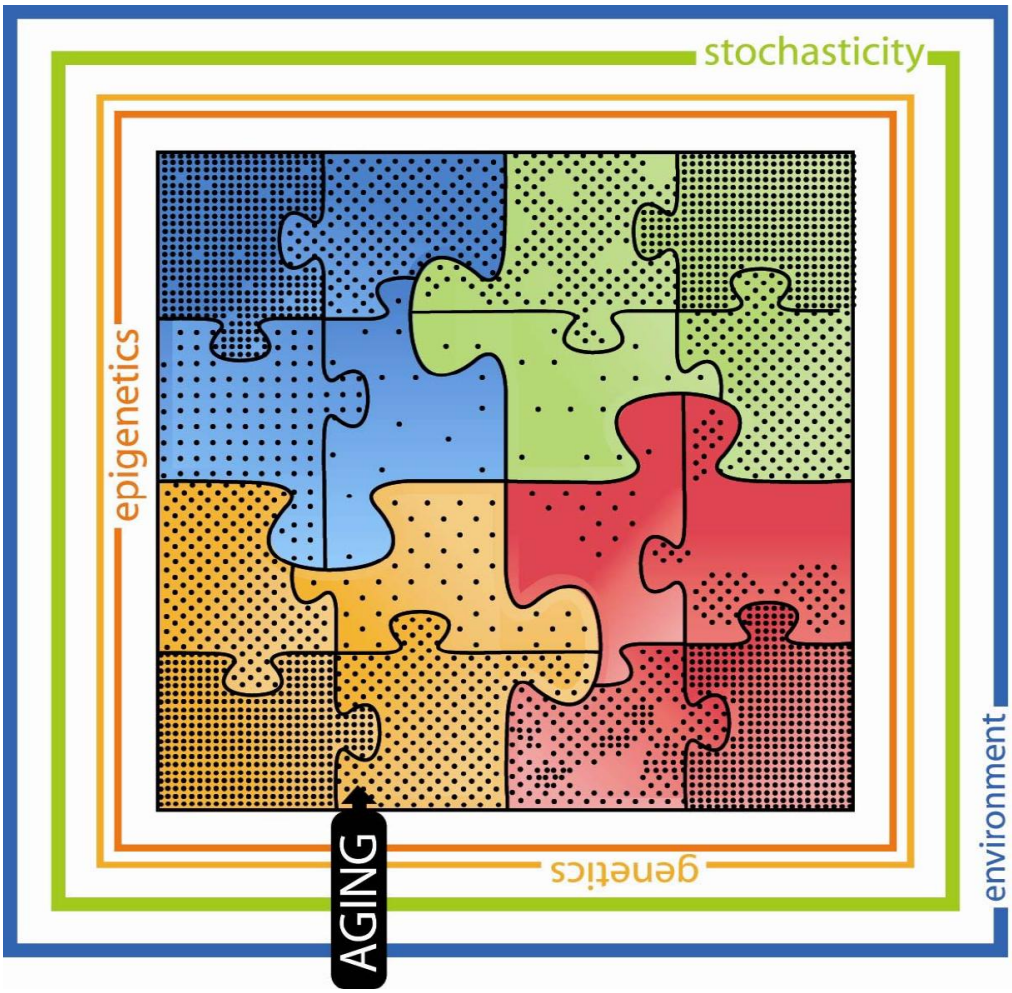


Figure 1. Schematic representation of the aging mosaic. Each organ of our body (indicated by a different color) is differently affected by aging process, whose degree and diffusion is indicated in the figure by the number and distribution of the black dots. This kind of mosaic depends on an interaction between several factors, environmental, stochastic and genetic-epigenetic variables, which differs in different organs but also in every tissue and cell type composing the organ (indicated as a pieces of the puzzle) (adapted from Cevenini et al., 2008).

PATHOGENETIC BASIS OF DOWN SYNDROME

Up to now two hypothesis exist to explain how DS can be led by triplication of HSA21 (Contestabile, Benfenati, and Gasparini 2010).

The "Reductionist" view (Epstein 1990), also known as the "Gene dosage effect" hypothesis, claims that individual phenotypic anomalies or features can often be assigned or mapped to specific regions of the genome, and conversely, phenotype can be a direct result of the cumulative effect of the individual genes imbalance located on the triplicated chromosome or chromosome region (Pritchard and Kola 1999).

The "Organicistic" view (Opitz and Gilbert-Barness 1990) or "Amplified developmental instability" hypothesis (Pritchard and Kola 1999) largely based on Waddington's views asserts that the phenotype of an aneuploid organism is due to general genomic imbalance, more than to the addition of the effects of individual genes (Neri and Opitz 2009).

It is widely assumed that the DS complex phenotype results from the dosage imbalance of the genes located on HSA21. Recent findings about the structure of the human HSA21 suggest that various genetic mechanisms may contribute to the phenotype of DS and its variability. Studies on the expression of mRNA in cells and tissues with trisomy 21 have shown that, while some genes on HSA21 are overexpressed, other subsets of genes on other chromosomes show an altered expression. This could be due to a variety of mechanisms including the activity of transcription factors encoded on HSA21 or elsewhere in the genome that are affected by the aneuploidy, as well the alteration of microRNA, RNA editing, histone modifications and DNA methylation machineries (Rozovski et al. 2007; Prandini et al. 2007; Li et al. 2006; Mao et al. 2005; Giannone et al. 2004; Patterson 2007).

EPIGENETICS

The term 'epigenetics' literally means 'outside conventional genetics' and it is now used ‘to describe the study of stable alterations in gene expression potential that arise during development and cell proliferation’ (Jaenisch and Bird 2003). In mammals epigenetic mechanisms involve postsynthetic modifications of either proteins that are intimately associate with DNA or of DNA itself: histone modifications like acetylation, ubiquitination, methylation, sumoylation and phosphorylation and DNA methylation respectively (Jaenisch and Bird 2003). Also miRNA have an important role in epigenetic modifications, as they are able to bind target mRNA, determining their degradation, and to promote chromatin modifications (Figure 2).

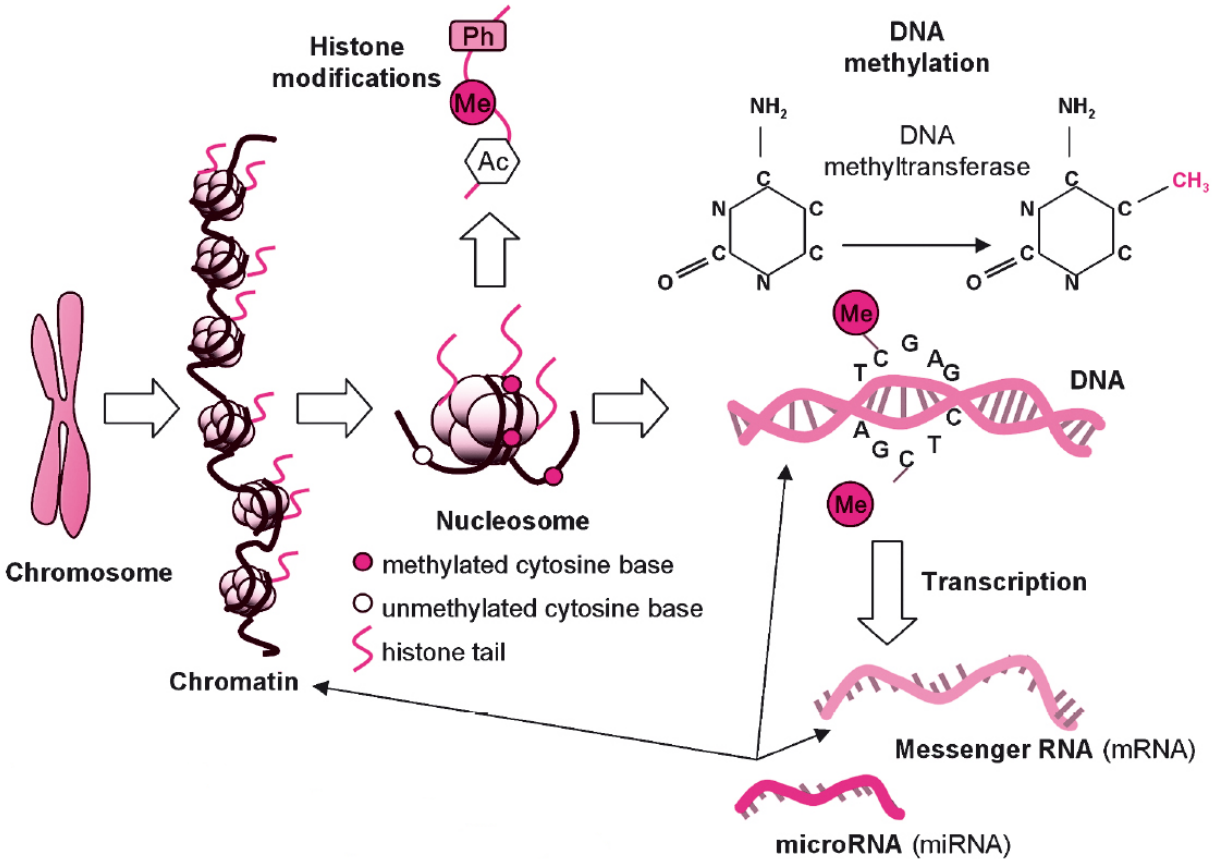


Figure 2. Epigenetic modifications (adapted from Stazi, Nisticò, and Serino, 2012)

In detail histone modifications and DNA methylation are involved in control of gene expression, X chromosome inactivation and genetic imprinting (Heijmans et al., 2007), but they also protect our body from viral genomes that would use the host cells for their own ends (Jaenisch and Bird 2003). Although epigenetic modifications occur during development and differentiation, and can be transmitted across cell divisions (Heijmans et

al. 2007), they can also be altered during human lifespan due to the influence of the environmental and stochastic factors, altering gene expression and determining significant phenotypic consequences (Jones and Baylin 2002).

HISTONE MODIFICATIONS

The epigenetic modifications that have a greater effect on the chromatin structure are histone modifications (Figure 3). Chromatin is composed by a single molecule of DNA associated with a group of histonic proteins. Five histone types (H2A, H2B, H3, H4 and H1) interact with DNA to form spherical particles called nucleosomes. As mentioned before, the histone modifications consist of additions or subtractions of acetyl (COCH3), methyl (CH3), phosphate (PO4) groups and so on to the amino-terminal histone's tails protruding from the nucleosome and this kind of changes are mediated by numerous and specific enzymes such as for example histone acetyltransferase (Hat), histone deacetylase (Hdac), histone methyltransferase (Hmt). While histone acetylation and methylation reduce histone's affinity for DNA, leading to an open and functionally active chromatin conformation (euchromatin), on the contrary histone deacetylation and demethylation condense chromatin (heterochromatin) bringing it to a condition of transcriptional inactivity (Stazi, Nisticò, and Serino 2012). These changes in chromatin structure facilitate gene transcription, its inhibition or other important mechanisms.

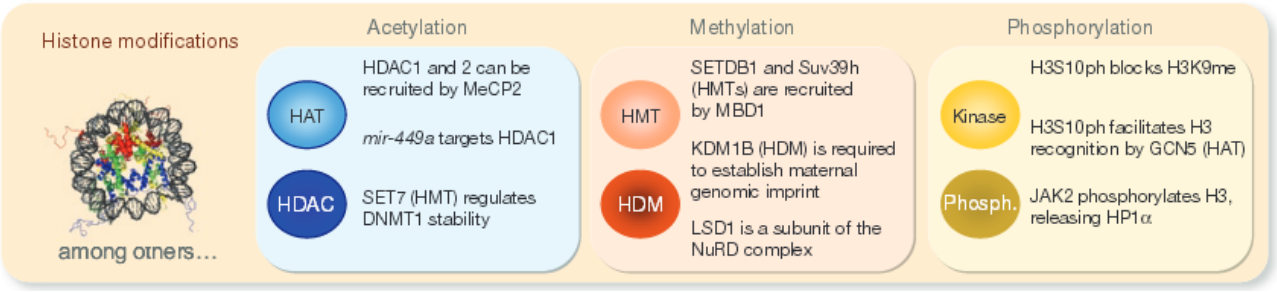


Figure 3. Histone modifications (Portela and Esteller 2010)

DNA METHYLATION

Beside histone modifications, also DNA methylation is an epigenetic mechanism that plays an important role in regulating gene expression and nuclear architecture (Jaenisch and Bird 2003; Talens et al. 2012).

More in details, in mammals DNA methylation targets are cytosine residues located in dinucleotide CpG sites (Bird 2002; Fazzari and Greally 2004). Methyl groups are typically attached at the 5-carbon position of cytosine in dinucleotide CpG and protrude from the cytosine nucleotide into the major groove of the DNA (Thompson, Fazzari, and Greally 2010a). The addiction of a Methyl group has two main effects: it prevents the binding of transcription factors that normally bind DNA and it recruits methyl-binding proteins, which in turn have an important role in gene silencing and chromatin compaction as reported in Figure 4 (Beck, Olek, and Walter 1999; Zweiger and Scott 1997). However the situation is not so well defined and the effects of DNA methylation may be different depending on the context: while methylation in proximity of a gene promoter cause gene silencing, the gene body methylation might stimulate transcription elongation; methylation in repeated regions such as centromeres is important for chromosomal stability and also have a role in genome stability by suppressing the expression of transposable elements (Jones 2012; Moarefi and Chédin 2011).

It is known that some methyl-binding proteins may even interact with multiple adjacent methylated cytosines (Filion et al. 2006) and these methylation-sensitive DNA bound factors may recruit additional complexes (e.g. histone deacetylases) that also have an important role in modulation of chromatin state and transcriptional activity (Jones et al. 1998).

In mammals DNA methylation is established during early development, when a general demethylation event is followed by genome wide *de novo* methylation (Jaenisch and Bird 2003). The enzyme's family that catalyzes the transfer of a methyl group from S-adenosyl methionine to DNA includes five members in mammals, indicated as Dnmts (DNA methyl transferase): Dnmt1, Dnmt2, Dnmt3a, Dnmt3b (Figure 5) and Dnmt3l. Only Dnmt1, Dnmt3a and Dnmt3b possess methyltransferase activity (Portela and Esteller 2010).

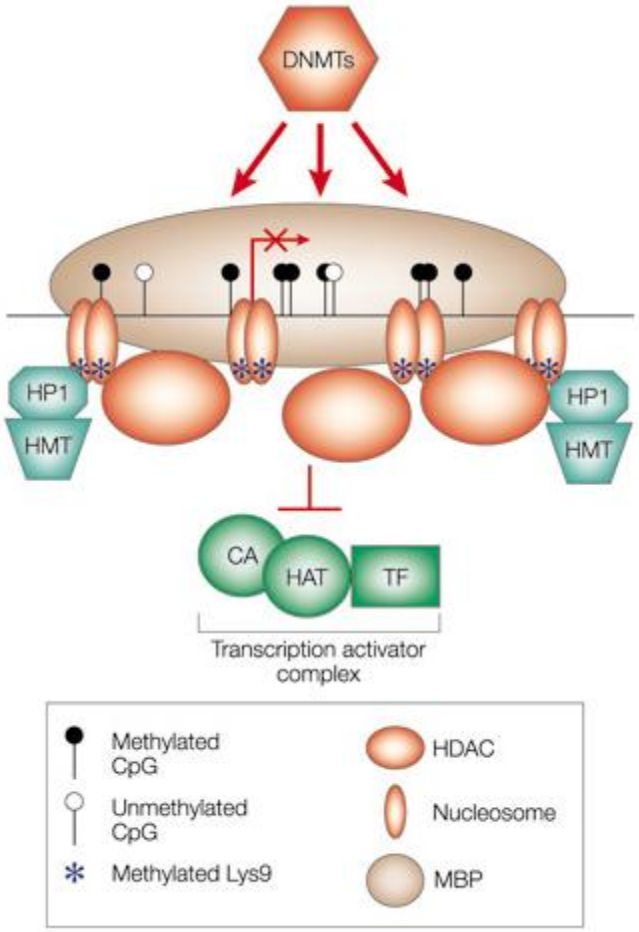


Figure 4. Heterochromatin configuration. The figure shows a transcriptionally silent DNA. In fact CpG sites are methylated and are bound by methyl-cytosine-binding proteins (MBPs), which are present in complexes that include histone deacetylases (HDACs). The histones are tightly compacted to nucleosome, because of their acetylation that together with the histone 'mark' of a methylated Lys9 residue on the tail of histone 3 (H3) makes chromatin transcriptionally inactive. H3 Lys9 methylation is maintained by a histone methyltransferase (HMT) that is recruited by the binding of the chromodomain protein HP1 (HP1) to the methylated H3 Lys9. Transcription activator complexes, composed by a transcription factor (TF), a co-activator protein (CA) and a protein with acetyltransferase (HAT) activity cannot access the area. On the contrary DNA methyltransferases (DNMTs) is able to reach DNA (adapted from Jones and Baylin 2002).

Cytosine methylation occurs symmetrically on both strand of DNA and it is maintained after DNA synthesis by the activity of the enzyme DNA methyltransferase 1, the maintenance, that promotes symmetric methylation of DNA, coping from the methylated to the unmethylated strand (Hermann, Goyal, and Jeltsch 2004).

The class of DNA methyltransferases that is responsible for *de novo* methylation includes Dnmt3a and Dnmt3b which are both strongly expressed in undifferentiated pluripotent embryonic stem cells and are downregulated upon cell differentiation (Okano, Xie, and Li 1998; Okano et al. 1999). Additionally, Dnmt3a is able to interact with its regulatory binding partner DNMT3L (Suetake et al. 2004), a DNA methyltransferase-like protein that

plays a central role in the establishment of maternal genomic imprinting but does not have catalytic activity on its own (Hata et al. 2002; Nimura et al. 2006). Dnmt3l binds unmethylated histone tails and activates *de novo* DNA methyltransferase activity in a tetrameric complex with Dnmt3a (Jia et al. 2007). Interestingly, the *DNMT3A* gene encodes two active isoforms with different tissue expression and subcellular localization patterns: Dnmt3a is associated with heterochromatin and Dnmt3a2 is concentrated on euchromatin (Chen et al. 2002).

Finally, regarding the Dnmt2, it contains all the catalytic signature motifs of conventional Dnmts, but it seems not to show any DNA methyltransferasic activity. However, it is reported that Dnmt2 methylates $tRNA.^{Asp}$ (Goll et al. 2006).

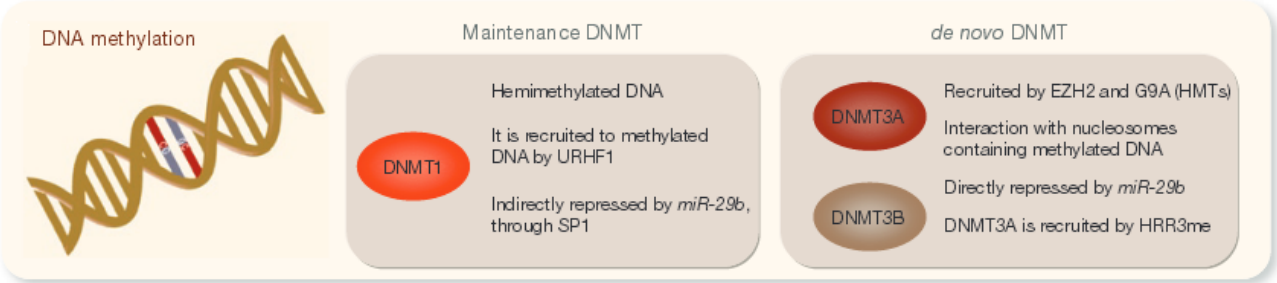


Figure 5. DNA methylation (adapted from Portela & Esteller, 2010)

CpG Islands

In normal conditions, around 80-90% of CpG sites within the human genome are methylated. Many CpG methylated are found in repeated sequences, most of which are derived from transposable elements (transposons). Methylation silences these sequences, preventing amplification events and new insertions in the genome. In some cases (1-2%) CpG dinucleotides tend to cluster in regions called CpG islands (Portela and Esteller 2010). CpG islands were identified almost 20 years ago as a result of whole-genome molecular studies, initially on the bases of the strikingly discordant patterns of digestion of genomic DNA by isoschizomeric restriction enzymes that differ for their sensitivity to cytosine methylation (Singer, Roberts-Ems, and Riggs 1979). Subsequently empirical genomic criteria were established (Gardiner-Garden and Frommer 1987) based on few sequence descriptors, a small number of HTF (HpaII Tiny Fragments) sequences and the 1985 GeneBank database, resulting in criteria that are still in use today (Fazzari and Greally 2004). CpG islands are defined as those regions that contain more of 200 nucleotides with a C+G content of at least 50% and an observed (within a given sequence) to expected (within the genome) CpG dinucleotides ratio of 0.60 or greater (Fazzari and Greally 2004).

Mammalian genomes contain CpG islands that can vary in size between 300 and 3000bp (Alvarado et al. 2014). In humans and mice, approximately 60% of all promoters colocalize with CpG islands, and they are usually unmethylated in normal cells, although 6% of them could become methylated in a tissue-specific manner during early development or in differentiated tissues (Straussman et al. 2009).

Hypermethylation of promoter regions of crucial genes (such as tumor suppressor, DNA repair and cell cycle control genes) can repress relevant gene expression and downregulate biological functions and it is associated with an increased risk of cancer. It is still an open question how CpG islands remain unmethylated in a globally methylated genome (Jaenisch and Bird 2003).

DNA methylation in CpG islands can cause gene silencing by several mechanisms. It can recruit methyl CpG binding domain (Mbd) proteins which in turn attract histone modifying and chromatin remodelling complexes to methylated sites (Esteller 2007; Lopez-Serra and Esteller 2008). DNA methylation can also directly inhibit gene expression and transcription by precluding the recruitment of DNA binding proteins from their target sites (Kuroda et al.

2009). On another hand unmethylated CpG islands generate a chromatin structure favorable for gene expression by recruiting CXXC finger protein 1 (Cfp1), which associates with histone methyltransferase Setd1, creating domains rich in the histone methylation mark H3K4 trimethylation (H3K4me3) (Thomson et al. 2010b).

DNA Methylation Patterns

It is known that DNA methylation does not occur exclusively at CpG islands but also in regions of lower CpG density that are situated in close proximity (~2 kb) of CpG islands, recently called CpG island shores (Irizarry et al. 2009). CpG island shelves are instead the 2 kb regions upstream and downstream of CpG island shores (Bibikova et al. 2011). Also methylation of CpG island shores is closely associated with transcriptional inactivation (Figure 6) and it has been demonstrated that most of the tissue specific DNA methylation seems to occur at the level of CpG island shores: for example 70% of the differentially methylated regions in reprogramming is associated with CpG island shores (Doi et al. 2009; Ji et al. 2010).

In some cases DNA methylation that occurs in gene bodies is coupled with transcriptional activation. (Figure 6). We can easily find gene body methylation in ubiquitously expressed genes and it is positively correlated with gene expression (Hellman and Chess 2007).

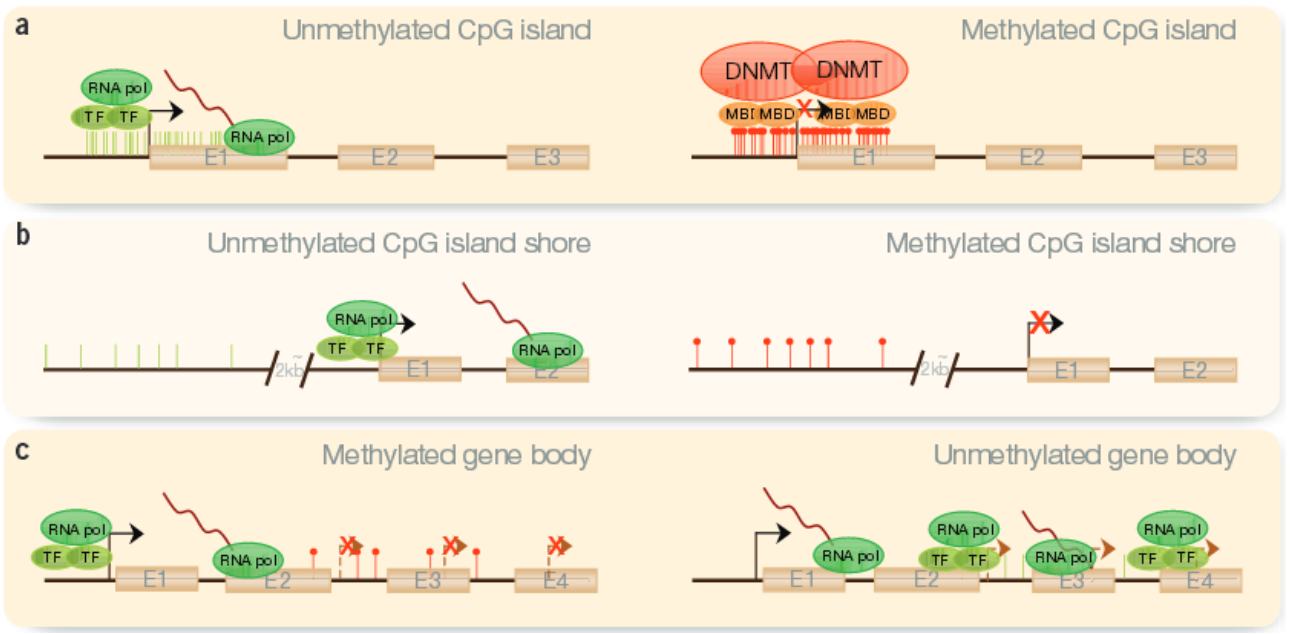


Figure 6. DNA methylation patterns. The normal scenario is represented in the left column while the alterations of this pattern are shown on the right for **(A)** CpG islands at promoters of genes; **(B)** CpG Island shores, **(C)** Gene body (adapted from Portela & Esteller, 2010).

METHYLATION ANALYSIS OF DOWN SYNDROME SUBJECTS

As mentioned before DNA methylation is an epigenetic mechanism that can be involved in altered gene expression of DS subjects.

Recently the epigenetic characteristics of Down Syndrome has been studied independently by four research groups who have described the DNA methylation patterns of different tissues at the genome wide level.

Two studies used the Illumina Infinium HumanMethylation27 BeadChip (Infinium 27k) to analyze the DNA methylation of persons with DS. In the first one the Illumina Infinium HumanMethylation 27k Bead Chip (Infinium 27k) microarray was used to investigate DNA-methylation levels of 27,578 CpG loci from chorionic villi and amniotic cell samples. Significantly different methylation levels between karyotypically normal samples and DS samples were observed (Eckmann-Scholz et al., 2009). The second study is a case/control study that, using the Infinium 27k microarray, analysed methylation levels in total peripheral blood leukocytes (PBL) of adults with and without DS, identifying a small group of genes, distributed across various chromosomes and not over-represented on chromosome 21, with consistently altered methylation (Kerkel et al. 2010). More recently, DNA methylation of DS placenta samples was analyzed by reduced representation bisulfite while DNA methylation of DS buccal epithelium samples was assessed by Illumina Infinium HumanMethylation450 BeadChip (Infinium 450k) (Jin et al. 2013; Jones et al. 2013). Both studies were concordant in showing marked DNA methylation alterations in DS cells and this kind of alterations were spread across the entire genome and were not enriched in HSA21.

Aim of the Study

Here we used a family-model of Down Syndrome (Figure 7) to better characterize DNA methylation of DS whole blood samples using the Infinium 450k and the EpiTYPER assay. Our model was composed by 87 subjects, in particular 29 persons with DS (DSP), their mothers (DSM) and their unaffected siblings (DSS).

In this way we obtained a trio-model that is important for two reasons: it allows us to control epigenetic changes, since genetics and environment are widely shared among members of the same family, and allows us to do a check in pairs between individuals who differ in disease, age, or the combination of the two.

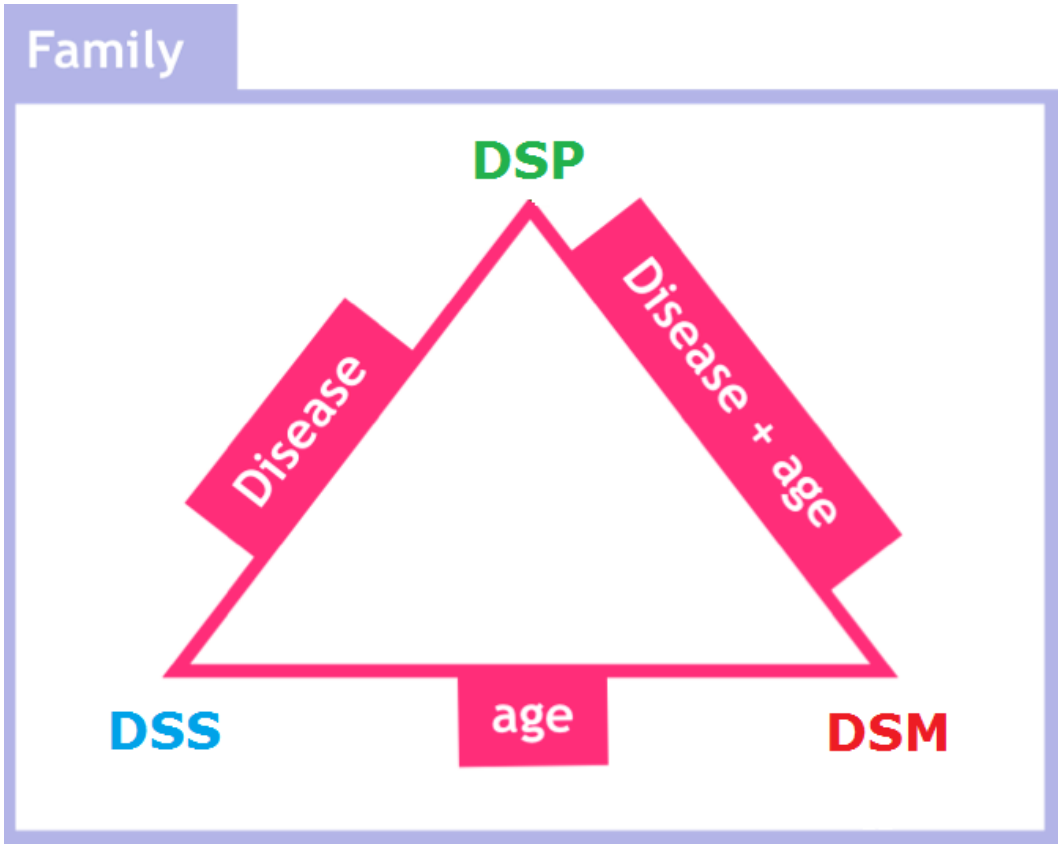


Figure 7. Family model of Down Syndrome.

More specifically we posed three questions:

1. Does an epigenetic signature of Down Syndrome exist?

2. Does this signature reflect a phenomenon of accelerated aging?

3. Is there a functional correlation of this signature?

Materials and Methods

SAMPLES

DSP, DSS and DSM were enrolled from 2008 in Emilia Romagna region (Bologna and Ferrara provinces), Italy, with the help of non-profit associations CEPS, OPIMM and ANFFAS. Participation to the study was totally on a voluntary basis, with no reward for the participants or their families. In particular a total of 29 DSP (12-43 years, 18 males, 11 females), 29 DSS (9-52 years, 7 males, 22 females) and 29 DSM (42-83 years) took part to the study (Table 1). Exclusion criteria were the following: acute illness, hepatic, renal or cardiac insufficiency, assumption of antioxidant or nutraceutical substances (vitamins, lipoic acid, acetylcysteine, omega 3 and 6 fatty acids, probiotics) within the last two months as detailed in the study by Ghezzo and co-workers (Ghezzo et al. 2014).

The study protocol was approved by the local Ethical Committee of the Sant'Orsola Hospital (University of Bologna) and an informed consent was obtained from relatives of DSP and from adult DSP.

	SUBJECTS	**MALE**	**FEMALE**	**AGE (years)**
DSP	29	18	11	12-43
DSS	29	7	22	9-52
DSM	29	-	29	42-83
TOTAL	87	25	62	

Table 1. Samples characteristics.

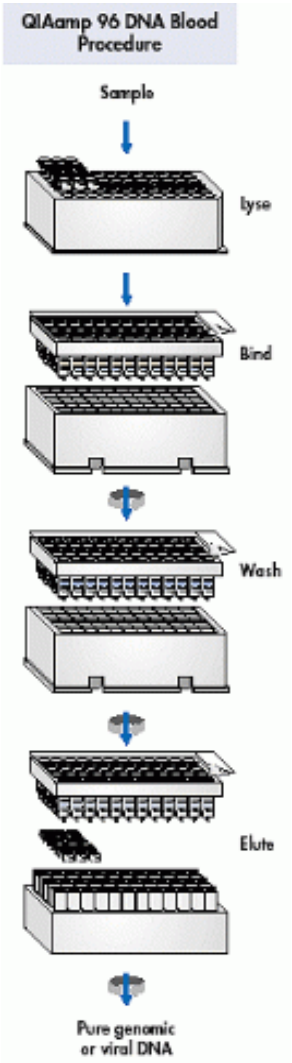
To confirm DNA methylation level of differentially methylated regions (DMRs) identified by genome-wide analysis we enriched the initial cohort of samples by recruiting other 49 persons affected by Down Syndrome and 33 age- and sex- matched unrelated controls. DMRs that showed striking correlation values between methylation levels and age of the subject in DSS compared to DSM, were replicated in a larger sex-balanced cohort (Time Serie). The analyzed samples included whole blood DNA from 494 individuals (245 men and 249 women) ranging from 9 to 99 years, plus 7 cord-blood DNA samples (3 males and 4 females).

DNA METHYLATION ANALYSIS

We studied DNA methylation using two different technologies: Infinium Methylation Assay and Sequenom MassARRAY. Both methods have in common 2 sample preparation steps: genomic DNA extraction and bisulfite treatment of DNA.

DNA extraction

Genomic DNA was extracted from whole peripheral blood using the QIAamp 96 DNA Blood Kit (QIAGEN, Hilden, Germany). This kit processes up to 200 µl of whole blood with a typical yield of 6 µg per 200 µl healthy whole blood (elution volume of 50–200 µl). The principle on which DNA extraction is based is the binding of DNA to the QIAamp silica-gel membrane, while contaminants pass through. The protocol consists of several steps (Figure 8):



- Sample lysis with K Protease and a specific buffer (AL), with incubation at 70 ° C and subsequent addition of ethanol.
- DNA binding to columns.
- Removal of PCR inhibitors, such as divalent cations and proteins, by two wash steps (buffer AW1 and AW2).
- Elution of pure nucleic acid in either water or a buffer (AE) provided with the kit.

Figure 8. Extraction steps with QIAamp 96 Blood Kit.

DNA Quantification

Quantification was performed using the Quant-iT™ PicoGreen® dsDNA Assay Kit, an ultra sensitive fluorescent nucleic acid stain for quantitating double-stranded DNA (dsDNA) in solution.

This kind of assay uses an advanced fluorophore, Picogreen, that becomes fluorescent upon binding to double strand DNA; the fluorescence intensity of the resulting complex depends directly on the amount of the target molecule in the sample. The quantification was performed with a robotic liquid handling system, Hamilton MLStar (Figure 9) that allows simple quantitation of hundreds of samples, using a 96- or 384-well format.

Figure 9. Hamilton ML Star.

Bisulfite treatment of DNA

For Infinium HumanMethylation450 BeadChip, sodium bisulfite conversion of 500 ng of DNA was performed using the EZ DNA Methylation-Gold Kit (Zymo Research Corporation, Orange, CA) according to manufacturer's instructions. For Sequenom EpiTYPER assay, 1000 ng of DNA were bisulfite converted using the EZ-96 DNA Methylation Kit (Zymo Research Corporation, Orange, CA) with the following modifications respect to manufacture's protocol: bisulfite conversion was performed with thermal conditions that repeatedly varied between 55°C for 15 min and 95°C for 30s for 21 cycles; after desulphonation and cleaning steps, bisulfite-treated DNA was eluted in 100 µl of water. Both kits are based on a three step reaction process between cytosine and sodium bisulfite, resulting in unmethylated cytosine being converted into uracil.

In this way, after amplification, changes in the DNA sequence, dependent on the state of methylation of the individual cytosines, are produced (Figure 10). These variations from C to T correspond to variations in G/A in the complementary strand.

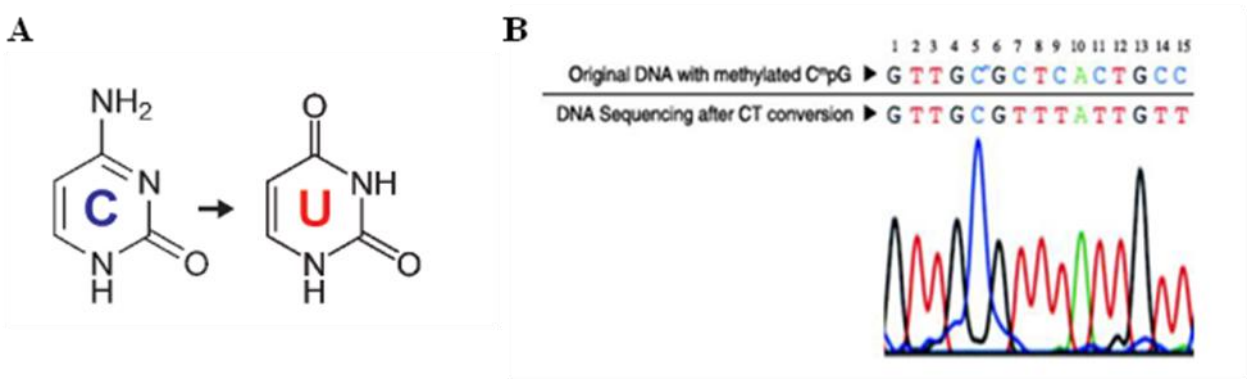


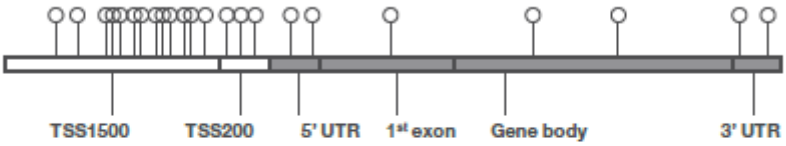
Figure 10. (A) C/U conversion by bisulfite treatment; (B) DNA sequencing results after bisulfite treatment. DNA with methylated CmpG (at nucleotide position #5) was processed using the EZ DNA Methylation-Gold™ Kit. The recovered DNA was amplified by PCR and then sequenced directly. The methylated cytosine at position #5 remained intact while the unmethylated cytosines (i.e., positions #7, 9, 11, 14 and 15) were completely converted into uracil following bisulfite treatment and detected as thymine following PCR.

GENOME-WIDE DNA METHYLATION ANALYSIS

Genome-wide DNA methylation of 29 families composed by a DSP, a DSM and a DSS was conducted using the Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA) following manufacturer's instructions, in collaboration with the Research and Biomedical Technologies Laboratory of Cusano Milanino (CRBT, Italy).

Infinium Human Methylation450 BeadChip

Infinium Methylation Assay allows to interrogate >485,000 methylation sites per sample at single-nucleotide resolution. The design of the array was developed under the guidance of a consortium of methylation experts. The 99% of RefSeq genes are covered, including those in regions of low CpG density and at risk for being missed by commonly used capture methods. Importantly, coverage was targeted across gene regions including promoter region, 5'UTR, first exon, gene body, and 3'UTR (Figure 11).



Feature Type	Genes Mapped	Percent Genes Covered	Number of Loci on Array
NM_TSS200	14895	0.79	2.56
NM_TS1500	17820	0.94	3.41
NM_5'UTR	13865	0.78	3.34
NM_1stExon	15127	0.80	1.62
NM_3'UTR	13042	0.72	1.02
NM_GeneBody	17071	0.97	8.97
NR_TSS200	1967	0.65	1.84
NR_TSS1500	2672	0.88	2.92
NR_GeneBody	2345	0.77	5.34

Feature Type	Islands Mapped	Percent Islands Covered	Average Number of Loci on Array
Island	26153	0.94	5.08
N_Shore	25770	0.93	2.74
S_Shore	25614	0.92	2.66
N_Shelf	23896	0.86	1.97
S_Shelf	23968	0.86	1.94

Figure 11. Coverage of HumanMaethylation450BeadChip throughout gene regions. The HumanMethylation450 BeadChip offers broad coverage across gene regions, as well as CpG islands/CPG island regions, shelves, and shores for the most comprehensive view of methylation state.

96% of islands were covered overall, with multiple sites within islands, as well as regions flanking islands, defined shores (2000 bp on the two sides of CpG islands) and shelves (2000 bp adjacent to each shore). Beyond genes and CpG islands regions, multiple additional content categories requested by methylation experts were also included: CpG sites outside of CpG islands, non-CpG methylated sites identified in human stem cells, differentially methylated sites identified in tumors versus normal tissues, *FANTOM 4* promoters, DNase hypersensitive sites, miRNA promoter regions.

Infinium Methylation assay protocol

After bisulfite conversion using the EZ DNA Methylation-Gold Kit, each sample is processed as follows:

- Whole genome amplification (WGA) that uniformly increases the amount of the DNA sample by several thousands fold, without introducing large amounts of amplification bias.
- Enzymatic fragmentation of amplified product by a controlled enzymatic process, using end-point fragmentation to avoid overfragmenting the sample.
- Isopropanol precipitation of the fragmented DNA and collection by centrifugation at 4°C.
- Resuspension of precipitated DNA in hybridization buffer.
- Application of bisulfite converted DNA-samples to the BeadChip (a single BeadChip accommodates 12 samples with 485577 measurements per sample).
- Hybridization of the samples onto the BeadChip and incubation in the Illumina Hybridization Oven overnight. During this step the amplified and fragmented DNA samples anneal to locus specific 50mers (covalently linked to one of over 500,000 bead types). In the Infinium I chemistry two bead types target each CpG locus: one bead type corresponds to methylated (C), another bead type to unmethylated (T) state of the CpG site. In Infinium II chemistry one bead type targets each CpG locus (Figure 12).
- Wash away of unhybridized and non-specifically hybridized DNA and chip preparation for staining and extension.
- Chip extension and staining in capillary flow-through chambers. Single base extension of the oligos on the BeadChip, using the captured DNA as a template, incorporates detectable labels on the BeadChip and allows to determine the methylation level of the query CpG sites.
- Array scansion by HiScan (Illumina), using a laser to excite the fluorophore of the single-base extension product on the beads. The scanner records high-resolution images of the light emitted from the fluorophores.

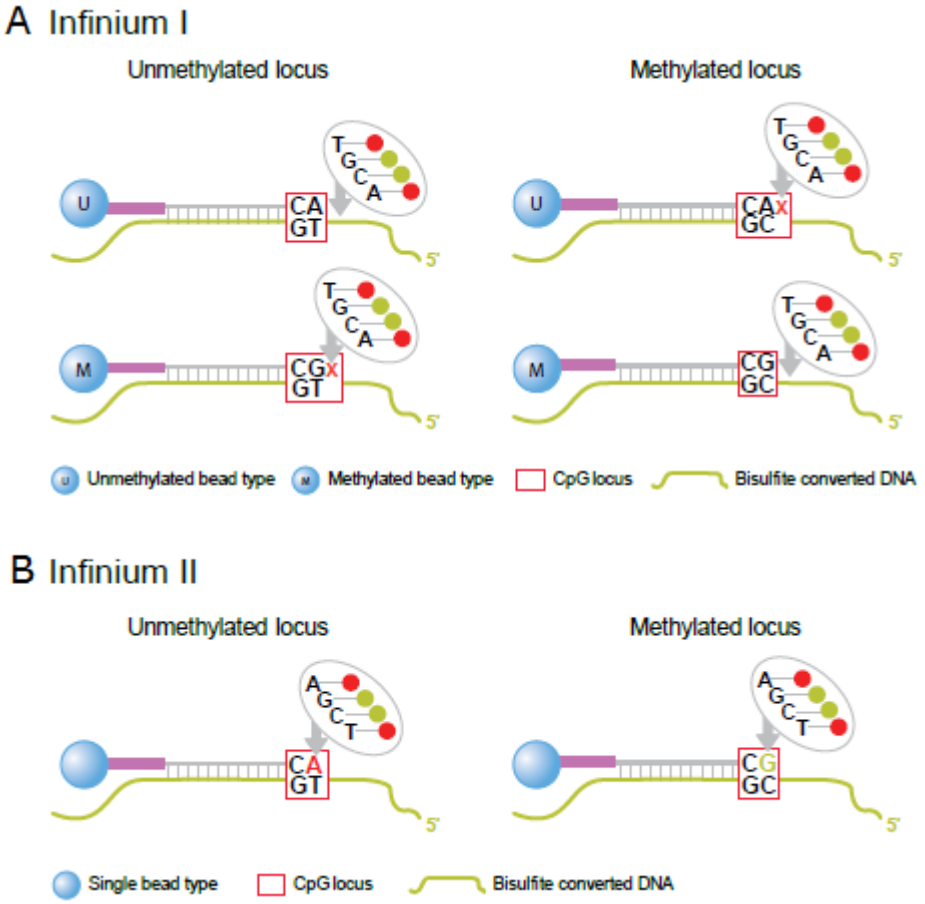


Figure 12. Infinium I and Infinium II assays. (A) Infinium I assay design employs two bead types per CpG locus, one each for the methylated and unmethylated states. **(B)** The infinium II design uses one bead type, with the methylated state determined at the single base extension step after hybridization.

Data Analysis

Methylation values from Infiunium450k array were expressed in term of β-values, that is the ratio of the methylated probe intensity and the overall intensity (sum of methylated and unmethylated probe intensities). Beta-value for an i^{th} interrogated CpG site is defined as:

$$Beta_i = \frac{\max(y_{i,methy}, 0)}{\max(y_{i,unmethy}, 0) + \max(y_{i,methy}, 0) + \alpha}$$

where y_i,methy and y_i,unmethy are the intensities measured by the i^{th} methylated and unmethylated probes, respectively (P. Du et al. 2010). Illumina suggests adding a constant offset α (by default, α=100) to the denominator to regularize Beta-value, when both

intensities of methylated and unmethylated probe are low. During analysis all negative values will be reset to 0, to avoid negative values after background adjustment.

The Beta-value can therefore assume a continuous value between 0 and 1 (or between 0 and 100% methylation): a value of zero indicates that all copies of the CpG site in the sample are completely unmethylated and a value of one indicates that every copy of the site is methylated (P. Du et al. 2010)

In this study GenomeStudio software (Illumina) was used to perform background subtraction, while IMA R package (Wang et al. 2012) was used to pre-process the β-values. All the samples were retained, as none had more than 75% of the probes with a detection p-value greater than $1e^{-05}$. 425 probes had a detection p-value greater than 0.05 in more than 75% samples and were removed, together with the probes containing missing values (23437) and those localized on sexual chromosomes. Based on these quality checks, 450981 out of 485577 CpG

Statistical analysis of Infinium 450k data

Infinium 450k was analysed using a recently developed statistical pipeline that privileges, when possible, a multivariate analysis on groups of adjacent CpG probes (Bacalini et al., 2015b). Indeed, when changes in DNA methylation involve a group of adjacent CpG sites the probability that they affect chromatin structure is higher compared to alterations that involve only one CpG site in a CpG-rich context (Beyan et al. 2012; Wessely and Emes 2012). Therefore, to identify DMRs that could have a relevant role in DSP phenotype, we implemented an original pipeline for the analysis of Infinium 450k data, which considers the concomitant variation of a group of adjacent CpG probes (region-centric approach), rather than the methylation level of a single CpG probe.

We grouped the CpG probes in clusters, defined "blocks of probes" (BOPs), taking into consideration not only their contiguity in DNA sequence, but also their genomic localization, which represents a critical aspect for data interpretation (Price et al. 2013).

Using Illumina probe annotation, we divided the probes in four classes (Figure 13 A): i) Class A: probes in CpG islands and CpG islands-surrounding sequences (shores and shelves) that map in genic regions (228,255 probes out of 448,628); ii) Class B: probes in CpG islands and CpG islands-surrounding regions (shores and shelves) which do not map in

genic regions (56,935 probes out of 448,628); iii) Class C: probes in genic regions which are not CpG rich (109,147 probes out of 448,628); iv) Class D: probes in non-genic regions which are not CpG rich (54,291 probes out of 448,628).

We evaluated the density of the probes in each class and we noted that only in Class A and Class B, that are the classes including CpG-rich regions, there were clusters in which multiple probes were in a range of 500-1000 bp (the range in which a co-regulation in methylation patterns was described (Li et al. 2010). Based on these analysis, we decided to group in BOPs only the probes belonging to Class A and Class B (Figure 13 B). In particular we defined as BOPs the groups of CpG probes localized in the same island, in the same shore or in the same shelf (73318 BOPs for Class A; 32013 BOPs for Class B). A region-centric statistical approach was used to compare Class A and Class B BOPs between DSP and DSS. On the contrary, for Class C and Class D probes a single-probe analysis was used.

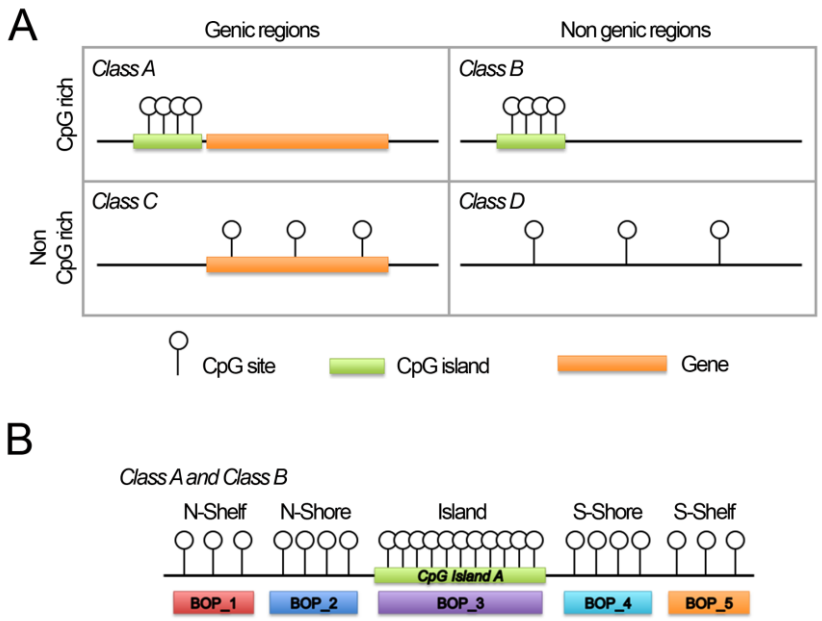


Figure 13. A region-centric approach to identify Down syndrome associated DMRs. In **(A)** are reported the 448,628 probes included in the Illumina HumanMethylation450 BeadChip that were divided in 4 classes on the basis of their genomic localization. **(B)** Graphic representation of probes grouped in BOPs. We grouped probes mapping in the island and in the surrounding regions of the same CpG island in 5 functional units: probes in the N-Shelf of the island, probes in the N-Shore of the island, probes in the island, probes in the S-Shore of the island, probes in the S-Shelf of the island. We grouped probes mapping in gene bodies on the basis of the gene in which they were located (adapted from Bacalini et al., 2015b).

We compared BOPs methylation values for Class A and Class B between DSP and DSS using the MANOVA function from the R package car. MANOVA test was applied on sliding windows of 3 consecutive CpGs within the same BOP (Figure 14), Among all p-values calculated for the different sliding windows, we choose the lowest for each BOP. In this way we identified the group of 3 CpGs most significantly differentially methylated between DSP and DSS within each BOP. All BOPs containing only 1 or 2 CpG probes were excluded from multivariate analysis. These CpG probes were compared between DSP and in DSS using the ANOVA function from the R package car, together with probes belonging to Class C and Class D.

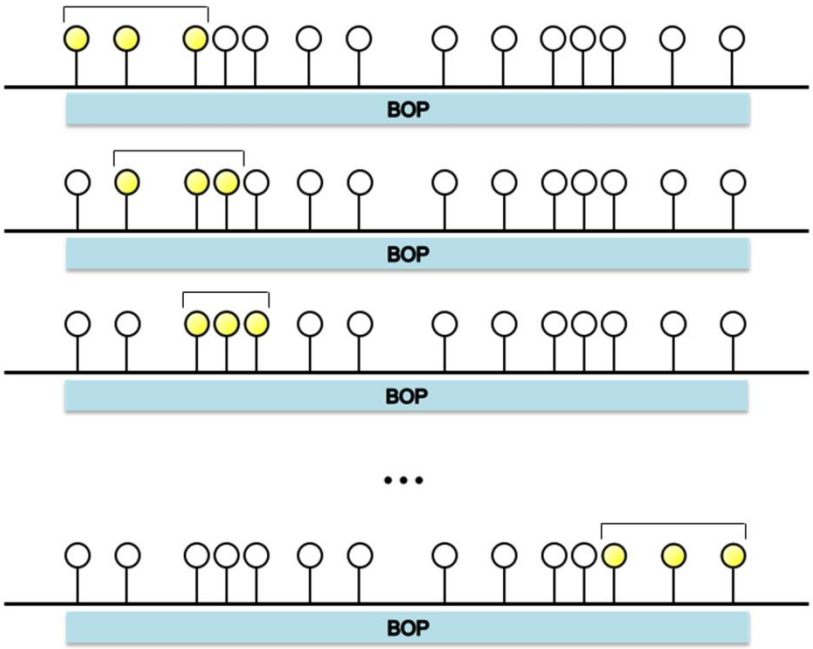


Figure 14. MANOVA Analysis. Graphical representation of the sliding window MANOVA used to normalize for BOPs lengths (adapted from Bacalini et al., 2015b).

A correction for sex, batch and cell counts (see the next paragraph) was performed both for MANOVA and ANOVA analyses. We also applied a Benjamini-Hochberg False Discovery Rate correction using the function *mt.rawp2adjp* from the R package *multtest*, to correct for multiple testing.

Using the list of 4648 class A BOPs differentially methylated between DSP and DSS we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and gene

ontology (GO) analyses. To conduct GO annotation of selected DMRs we used gene ontology enrichment analysis and visualization tool http://cbl-gorilla.cs.technion.ac.il/ (Eden et al. 2009), while to perform the significance analysis of KEGG pathways we used two tailed Fisher's exact test for each pathway (Francesconi et al. 2008).

Cells count inference from DNA methylation data

We used a recently developed algorithm (Houseman et al. 2012) to infer blood cell counts starting from DNA methylation.

This method starts from cell-specific DNA methylation signatures of purified leukocyte samples (validation dataset) and selects n CpG sites with the highest informativeness, with respect to blood cell types, using this information to predict leukocytes distribution in the target dataset. We used two validation datasets that are present in public databases. The first one (GEO Accession number GSE39981) reports DNA methylation profiles results of 46 samples (6 CD19+ B cells samples, 8 granulocytes samples, 5 CD14+ monocytes samples, 11 CD56+ NK cells samples, 8 CD3+CD4+T cells samples, 2 CD3+CD8+ T cells samples, 1 CD3+CD56+ NK sample and 5 CD3+ T cells samples) analyzed by the Infinium 27k. The second dataset (GEO Accession number GSE35069) includes data from the Infinium 450k from six healthy males on seven sorted cell populations (CD4+ T cells, CD8+ T cells, CD56+ NK cells, CD19+ B cells, CD14+ monocytes, neutrophils, and eosinophils).

We compared the predicted leukocytes distributions with flow cytometry results available for DSP, by testing the performance of both validation sets. We decided to use GSE39981 as validation dataset, because it was more effective in predicting specific cell types even if both datasets were successful in predicting experimentally measured cell counts. In fact GSE35069 was less effective in predicting specific cell types such as CD56+ NK cells but also in providing a major number of different sets of informative CpG sites: 100, 300, 500, 1000, 2000 CpG sites from the GSE39981 dataset; 100, 300, 500, 1000, 5000, 10000 and 20000 CpG sites from the GSE35069 dataset. By using GSE39981 as validation dataset we estimated the distribution of CD19+ B cells, CD3+CD4+ T cells, CD3+CD8+ T cells, granulocytes, CD14+ monocytes and CD56+ NK cells using the 500 most informative CpG sites, 453 of which were included in the Infinium 450k. Projections of cell type's distributions for DSP, DSS and DSM are reported in Figure 20 of Result section.

LOCUS-SPECIFIC DNA METHYLATION ANALYSIS

EpiTYPER assay

Locus-Specific DNA methylation analysis was performed using the EpiTYPER assay (Sequenom, San Diego, CA) following manufacturer's instructions.

EpiTYPER is a tool for the quantitative analysis of DNA methylation, implemented on the Sequenom MassARRAY platform.

The Sequenom MassARRAY system is a scalable platform that allows to analyse up to 384 samples for each working session. It is composed by three elements: MassARRAY Liquid Handler, MassARRAY Nanodispenser and MassARRAY Compact Analyzer. MassARRAY Liquid Handler is a 96 channels pipetting robot employed to optimize pipetting during preparation steps of 384 plate and together to minimize risks of contamination. MassARRAY Nanodispenser is an instrument that performs rapid transfer of 384 samples onto SpectroChip arrays. Finally MassARRAY Compact Analyzer is an automated bench top MALDI-TOF mass spectrometer which can analyze two Spectro CHIP® arrays per run (2x384 samples). The mass spectrometry analysis is conducted by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF MS) technology.

This technique involves the use of an analyzer TOF (Time Of Flight) and a source of MALDI (Matrix Assisted Laser Desorption Ionization) and provides accurate information on the structure and molecular weight of biomolecules such as peptides, proteins, oligonucleotides. The method MALDI-TOF-MS involves the use of a laser for desorption and ionization of analytes previously fixed to a solid matrix on a metallic target. This produces ions of the analytes in the gas phase, which are then accelerated by an electric field before entering the TOF analyzer. With this acceleration each ion will have a kinetic energy proportional to its charge, but independent of its mass. The ions then enter the TOF analyzer in which it is measured the time that each particle take to reach the detector placed at a known distance. Since the speed of the ions depends on the mass/charge ratio (the heavier particles travel at low speeds), the ions reach the detector at different times according to their mass.

Bisulfite treatment of DNA gives rise to G/A variations that result in a mass difference of 16 Da per CpG site, easily detected by the MassARRAY® system.

EpiTYPER assay protocol

The EpiTYPER protocol includes several steps (Figure 15):

- Design of PCR primers that target the region of interest; the length of the amplicon is usually in the 200-600 bp range, as bisulfite treatment leads to genomic DNA fragmentation. The bisulfite specific primers do not include CpG sites and therefore bind to both methylated and non methylated template. We designed bisulfite primers using the EpiDesigner software (Figure 16), specifically optimized for the EpiTYPER assay.
- Amplification of 10 ng DNA in 5µl total volume, using a 384-microtiter format: during this step a T7-promoter tag, which is necessary for the next step of *in vitro* transcription, is introduced.
- Dephosphorylation of unincorporated dNTPs and primers, by adding 2µl of Shrimp Alkaline Phosphatase (SAP) enzyme to each 5 µl PCR reaction.
- In Vitro Transcription and RNase A Cleavage, adding 5µl of transcription/RNase A cocktail to 2µl of PCR/SAP samples.
- Sample conditioning, using 20µl of ddH2O and 6µl of Clean Resin for each sample, with the aim to eliminate salts of sodium and potassium that with their mass may affect the analysis.
- Sample transfer of little samples volumes (20-25nl) from 384 PCR plate to a 384-element SpectroCHIP® bioarray.
- Sample analysis. During this step spectra from the two cleavage reactions are acquired using the MassARRAY® system.

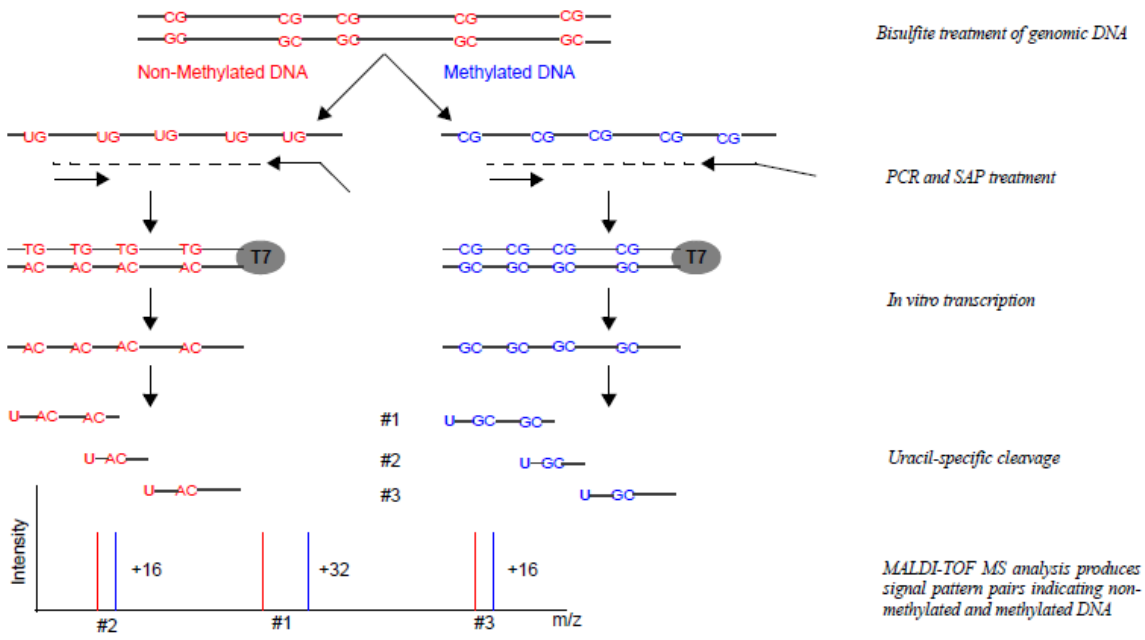


Figure 25. Overview of EpiTYPER process.

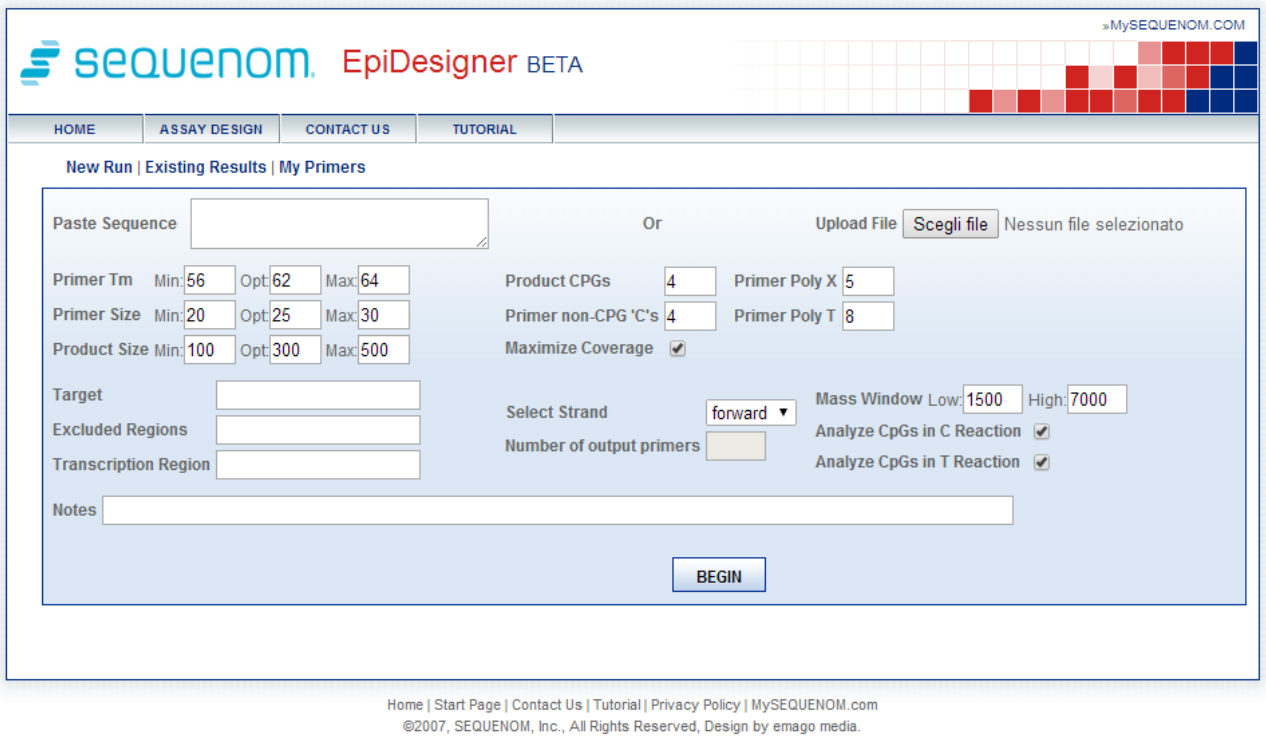


Figure 16. Epidesigner, a tool for designing quantitative DNA methylation experiments for Sequenom's EpiTYPER technology.

The EpiTYPER Analysis software was used to analyze results. MALDI-TOF MS analyzes the cleavage products, and a distinct signal pair pattern results from the methylated and non-methylated DNA template (Figure 17).

EpiTYPER generates quantitative results for each cleavage product analyzed. Each cleavage product encloses either one CpG site or an aggregate of multiple CpG sites. An analyzed unit containing one or multiple CpG sites is called a "CpG unit".
The obtained data were displayed using the software "Analyzer" (Figure 18), from the package EpiTYPER (Sequenom). This program allows comparing spectra, nucleotide sequences and data methylation in amplicon analysis.

The following genomic regions were analysed by EpiTYPER:

RUNX1 CpG island (chr21:36,258,992-36,259,453);
KDM2B N-Shore (chr12:121,973,796-121,974,353);
NCAM1 N-Shore (chr11:112,834,144-112,834,547);
the promoter of ribosomal gene *Ribo*;
5'sequence of 18S regions;
5'sequence of 28S regions;
ELOVL2 CpG island (chr6:11,044,680-11,045,053);
FHL2 CpG island (chr2:106,015,678-106,016,008);
PENK CpG island (chr8:57,360,525-57,360,938).

The primers corresponding sequences are:

RUNX1 Forward: aggaagagagGGTAGGAGTTGTTTGTAGGGTTTTAAT
RUNX1 Reverse: cagtaatacgactcactatagggagaaggctCCCACATCCCAAACTAAAAAAA
KDM2B Forward: aggaagagagGGGATTTTGATTATTTTATTGTTAGTTT
KDM2B Reverse: cagtaatacgactcactatagggagaaggctAAAACCCCTCCCTACCACTTAC
NCAM1 Forward: aggaagagagGGGAGGGTATTTTGGTAGGTATATTT
NCAM1 Reverse:cagtaatacgactcactatagggagaaggctAAAATTCCTAAACCTACAACTTCCAC
Ribo Forward: aggaagagagGTGTGTTTTGGGGTTGATTAGAG
Ribo Reverse: cagtaatacgactcactatagggagaaggctAAAACCCAACCTCTCCAAC
18S Forward: aggaagagagGTTTGTTGTTTTTTTTGGATGTGG
18S Reverse: cagtaatacgactcactatagggagaaggctCCTTACCTACCTAATTAATCCTACCAA

28S Forward: aggaagagagGGTATTTAGTTTTAGATGGAGTTTATTATT

28S Reverse: cagtaatacgactcactatagggagaaggctAAAAAAAACTAACCAAAATTCCC

ELOVL2 Forward: aggaagagagGTAAATTTGTAGGAATAGAGTTATTTTTTT

ELOVL2 Reverse cagtaatacgactcactatagggagaaggctCCCCTCTCCCACAAAAACC

FHL2 Forward: aggaagagagTTTTTTATGGTTATTTGTGGTGTTT

FHL2 Reverse: cagtaatacgactcactatagggagaaggctCCCTTTATTTACCAAAACTCCTTTC

PENK forward: aggaagagagAGGGGTTTTATGATGAAAAGAATTT

PENK Reverse: cagtaatacgactcactatagggagaaggctAAAAAAATCCCAAAAATTTCCAAAC

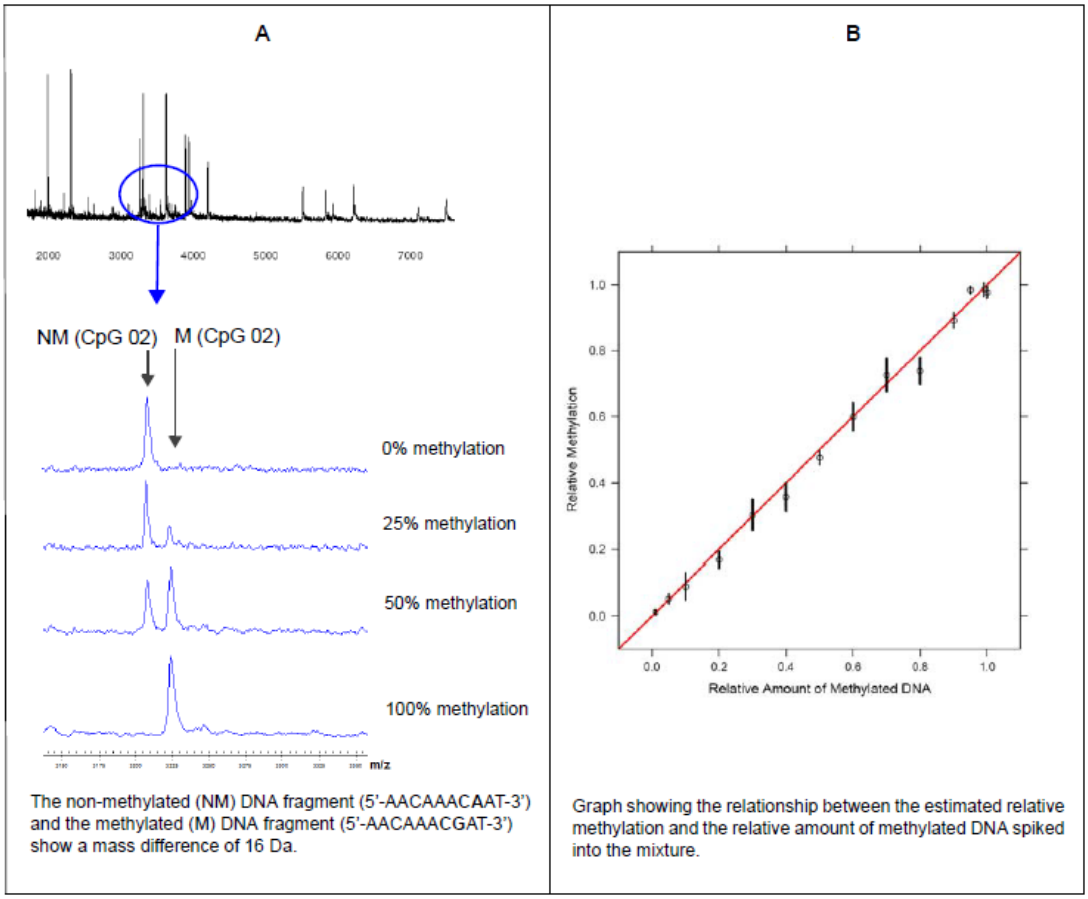


Figure 17. Results from a mixture experiment are shown above. It is an example illustration taken from a "zoomed in" view of a spectrum generated in EpiTYPER. In **(A)** it is reported an example of mass signal pairs for single CpG unit showing methylation ratios. **(B)** Representation of a graph of relative methylation levels derived from the same mixture experiment.

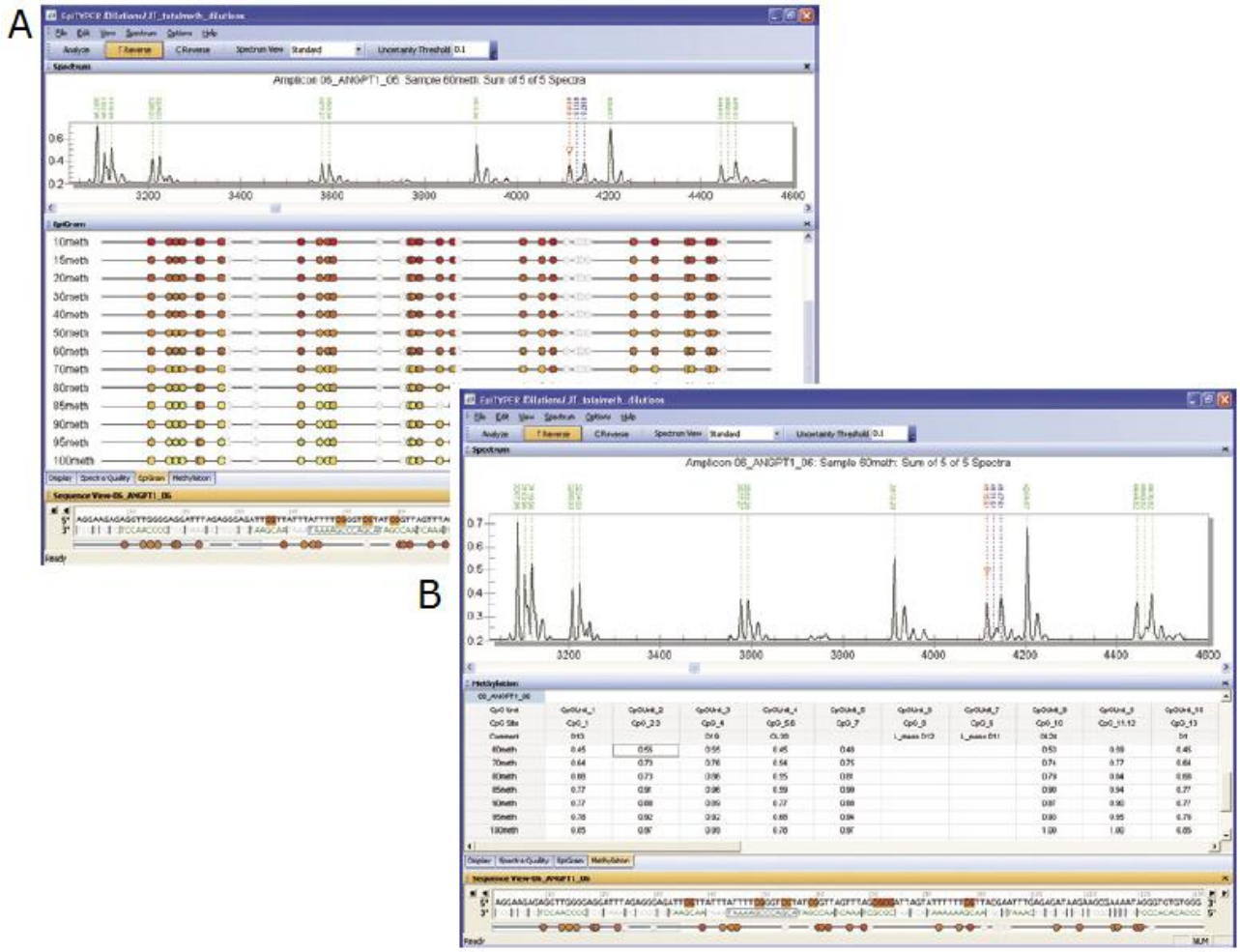


Figure 18. Software "Analyzer" from the package EpiTYPER (Sequenom). **(A)** This panel provides graphical representations of the CpG sites within the selected amplicon. Each is color-coded to the degree of methylation shown in the methylation tab panel **(B)** to provide a quick, reliable comparison between samples and CpG sites.

For each gene, CpG sites with missing values in more than the 20% of the samples were removed, as well as samples with missing values in more than the 20% of CpG sites. We used an R package to test if bisulpite conversion reaction run to completion (Thompson et al. 2009). For all samples analysed bisulphite conversion was from 98.9% to 100%.

Methylation data obtained by EpiTYPER assay were statistically analyzed using R software.

EXPRESSION ANALYSIS

Expression analysis were conducted using Real Time PCR in 4 DSP and 4 healthy sex- and age- matched controls, in collaboration with Prof. Lorenzo Montanaro (DIMES, University of Bologna).

RNA Extraction

RNA was extracted from peripheral blood using RiboPure™ RNA Purification Blood Kit (AMBION) following manufacturer's specifications. This kit allows the isolation of high-quality RNA directly from whole blood or from blood samples stored at -20°C in RNA*later*® Solution.
The RiboPure-BloodKit RNA isolation procedure consists of two parts:

- Cell lysis in a guanidinium-based solution and initial purification of the RNA by phenol/chloroform extraction.
- Final RNA purification by solid-phase extraction on a glass fiber filter.

The amount of blood used was between 300 μl and 500 μl.

RNA Quantification

RNA quantification was performed using the Thermo Scientific NanoDrop™ 1000 Spectrophotometer, an instrument able to measure 1μl DNA or RNA samples with high accuracy and reproducibility.
For each sample was carried out in a double quantification.

Real Time PCR

Total RNA was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR analysis was done in a Gene Amp 7000 Sequence Detection System (Applied Biosystems). For each sample, three replicates were analyzed.
Briefly semi-quantitative Taqman approach (taqMan Universal PCR master mix, Applied Biosystems) was employed to evaluate the expression of *KDM2B* and beta-glucoronidase (GUSB) as endogenous control. The expression of 45S pre-RNA was evaluated by Sybr Green (Power SYBR green PCR master mix, Applied Biosystems).

Each sample was exposed to the following cycling conditions:

- Denaturation step at 95°C for 10 min.
- 40 cycles of denaturation at 95°C for 15 s.
- Annealing and extension at 60°C for 1 min (1,5 min for 45S).

To inspect the specificity of Syber green PCR reactions we analyzed their melting curves carried out as follows: 60°C for 10s, with an increase of 0.5°C per cycle for 80 cycles.
Threshold cycles (Ct) in each triplicate were averaged and fold differences were calculated by the DDCt method as follow: N target =2 − (ΔCt sample − ΔCt calibrator), where ΔCt values of the sample and calibrator were determined by subtracting the Ct value of the endogenous control gene from the Ct value of each target gene (Penzo et al. 2014; Montanaro et al. 2010; Dussault and Pouliot 2006)

Results

DNA METHYLATION PROFILE OF PERSONS WITH DOWN SYNDROME

We used the Infinium 450k assay to investigate the DNA methylation profile of 87 whole blood samples from 29 families composed by a person with Down Syndrome (DSP), one healthy sib (DSS) and the mother (DSM).

All samples were analyzed on the BeadChip with a positive outcome. In particular each BeadChip carries internal quality controls that are able to evaluate bisulfite conversion efficiency. In our analysis, the efficiency has resulted higher than 99% for all samples analyzed, confirming the reliability of the method and the success of the preliminary steps (DNA extraction and bisulfite conversion).

After quality check we excluded chromosome X and Y data and we recovered 450.981 from 485.577 loci for subsequent analysis.

GLOBAL OVERVIEW OF THE DNA METHYLATION

We first compared the beta-values distributions of each chromosome between DSP, DSS and DSM to obtain a global overview of the DNA methylation patterns of DS. Significant differences were found between DSP and their relatives for many chromosomes, particularly in HSA21 (Figure 19) where DSP showed a decrease in the density of highly methylated loci with a concomitant enrichment in loci with methylation levels between 0.5 and 0.8.

Cells count interference from DNA methylation data

DNA methylation is tissue and cell specific, so difference in lymphocyte subpopulations could bias the discovery of differentially methylated regions (DMRs) when comparing whole blood DNA methylation from DSP against healthy subjects. In fact age-dependent defects in the innate and in the adaptive immune system of DSP, consisting in altered prevalence of the different lymphocyte subpopulations, are reported in many studies (Kusters et al. 2009). During statistical analysis we made a correction for lymphocyte subpopulations with the aim to identify intrinsic DNA methylation defects in DSP white blood cells. (WBC) Considering that we had cell type counts only for DS subjects, we overcome this lack of information using a recently developed algorithm able to infer blood cell counts starting from DNA methylation data (Houseman et al. 2012). Details of the cell

count estimation procedure are reported in Materials and Methods section. Our experimental data on DSP and the characteristic alterations in specific leukocytes populations are faithfully reproduced by the inferred cell counts (Figure 20). In detail , DSP showed a significant decrease in the number of CD19+ B cells (p-value <0.001) and CD3+CD4+ T cells (p-value <0.001) while they showed a significant increase in the number of CD3+CD8+ T cells (p-value <0.001). This situation leads to altered CD3+CD4+/CD3+CD8+ ratio, as previously reported (Cossarizza et al. 1990; Cossarizza et al. 1991). Taking this into account, we used inferred WBC counts as covariates in downstream analyses.

A

Chromosome	DSP *vs* DSS	DSP *vs* DSM	DSS *vs* DSM
1	2.23E-02	8.11E-03	1
2	1.46E-03	1.46E-06	1
3	2.85E-04	5.79E-06	1
4	2.99E-04	2.34E-04	0.001545
5	3.36E-02	7.82E-05	1
6	4.10E-03	8.09E-05	1
7	8.61E-03	1.10E-04	1
8	2.85E-04	1.43E-05	1
9	7.63E-03	1.38E-03	1
10	8.47E-05	1.71E-04	1
11	1.84E-01	3.29E-03	1
12	1.64E-02	7.73E-04	1
13	1.24E-03	1.08E-06	0.397274
14	4.34E-03	6.34E-03	1
15	1.82E-02	1.39E-04	0.643216
16	9.93E-03	2.62E-01	1
17	1.21E-01	4.90E-03	1
18	1.46E-08	5.07E-05	0.000001
19	1.09E-04	6.53E-09	1
20	4.09E-04	6.65E-03	0.108577
21	0.00E+00	0.00E+00	0.780454
22	3.76E-02	5.44E-03	1
21q22	0.00E+00	0.00E+00	0.265985

B

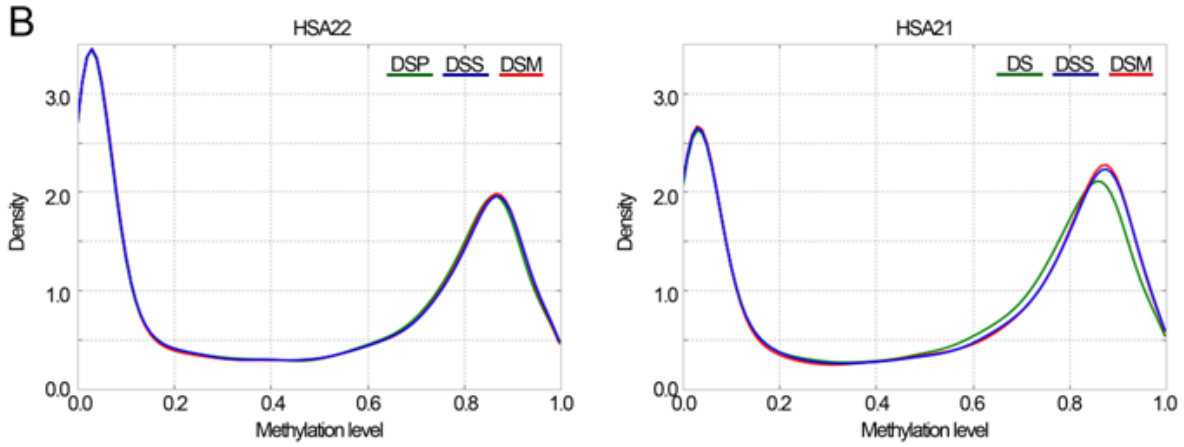


Figure 19. Comparison of DNA methylation distributions in DSP, DSS and DSM. (A) The table shows results of Kolmogorov-Smirnov test made by comparing, for each chromosome, the methylation distributions between DSP, DSS and DSM. The reported p-values are Bonferroni corrected. In **(B)** are reported probability density distributions of methylation values in HSA22 (8179 CpG probes) and in HSA21 (4055 CpG probes) (adapted from Bacalini et al,. 2015a).

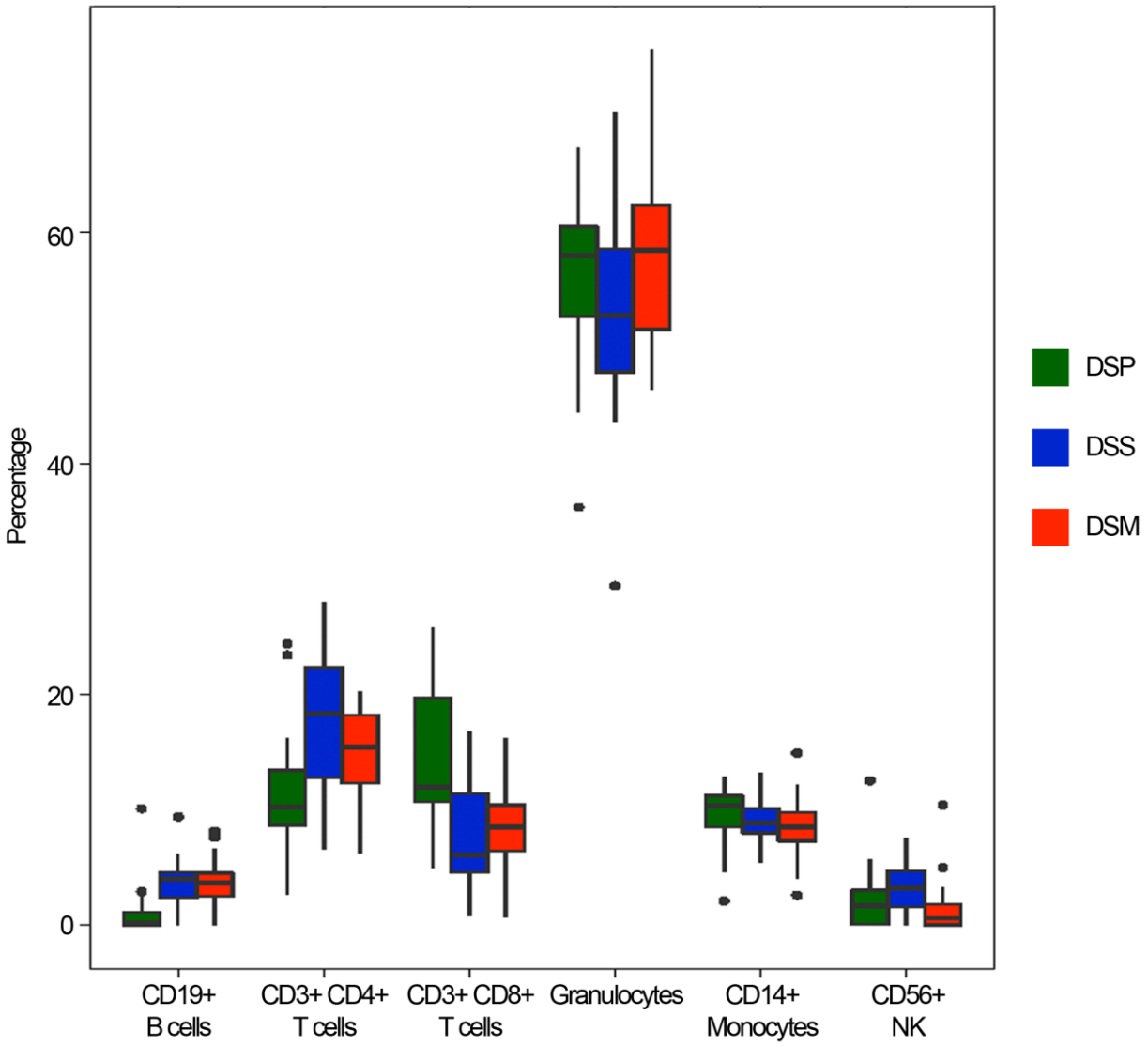


Figure 20. Estimation of cell counts. Distributions of estimated cell counts for DSP, DSS and DSM (adapted from Bacalini et al., 2015a).

IDENTIFICATION OF REGIONS DIFFERENTIALLY METHYLATED BETWEEN DSP AND DSS

As explained in the materials and method section, we used the analysis pipeline described by Bacalini et al. to identify DMRs between DSP and DSS subjects (Bacalini et al., 2015b). Briefly using Illumina probes annotation, we first divided our probes included in the array in four classes: i) Class A, including probes in CpG islands and CpG islands-surrounding sequences (shores and shelves) that map in genic regions; ii) Class B, including probes in CpG islands and CpG islands-surrounding regions (shores and shelves) which do not map in genic regions; iii) Class C, including probes in genic regions which are not CpG rich; iv) Class D, including probes in non-genic regions which are not CpG rich.
Then we grouped Class A and Class B CpG probes in clusters that we called "block of probes"(BOPs), containing the probes localized in the same island, in the same shore or in the same shelf. BOPs within Class A and Class B containing at least 3 CpG probes were compared between DSP and DSS using a multivariate statistical analysis (MANOVA) on sliding windows of 3 adjacent CpG probes. This multivariate approach is advantageous as it allows to identify general changes in the methylation status of the genomic region under consideration.
The remaining probes within Class A and Class B and the probes belonging to Class C and Class D were analysed using an univariate analysis (ANOVA). Sex, batch and cell types distribution were included as covariates.

In class A 4648 BOPs resulted able to discriminate DSP from DSS (q-value< 0.05; Figure 21 A and B). DSM methylation patterns were similar to those of DSS, confirming that the identified BOPs are characteristic of DSP and that these BOPs signatures distinguish the samples for the presence/absence of disease.

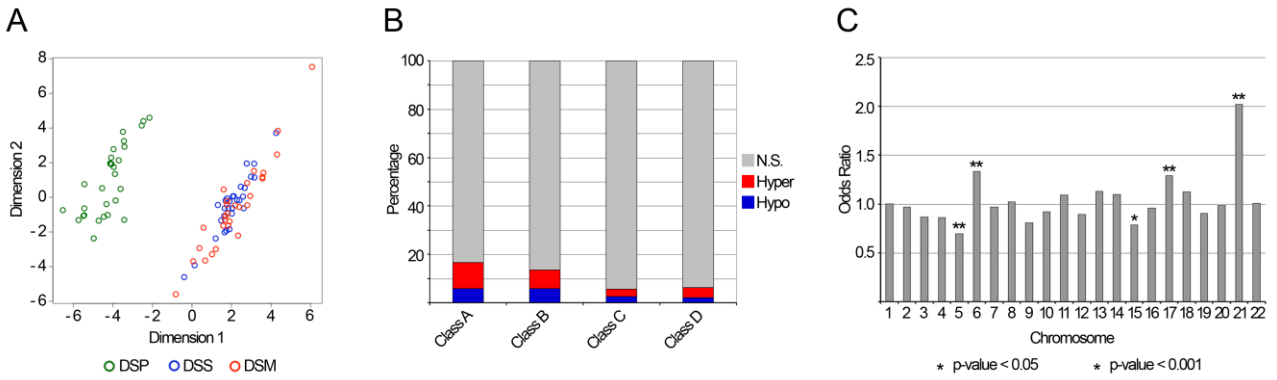


Figure 21. Down Syndrome associated DMRs. (A) The MDS plot reports a two-dimensional representation of the epigenetic distances between test samples, calculated using the methylation values of 4648 BOP selected as differentially methylated between DSP and DSS. **(B)** In the graph the percentage of identified DMRs is indicated for each of the four probes classes. Comparing DSP to DSS DMRs, they are distinguished between hypermethylated and hypomethylated.**(C)** Chromosomal enrichment of the identified DMRs. The Odds Ratio resulting from Fisher's exact test is reported for each chromosome and significant enrichments are indicates with asterisks (adapted from Bacalini et al., 2015a).

Observing Figure 22 we can note that the use of cell counts markedly reduced the number of identified DMRs.

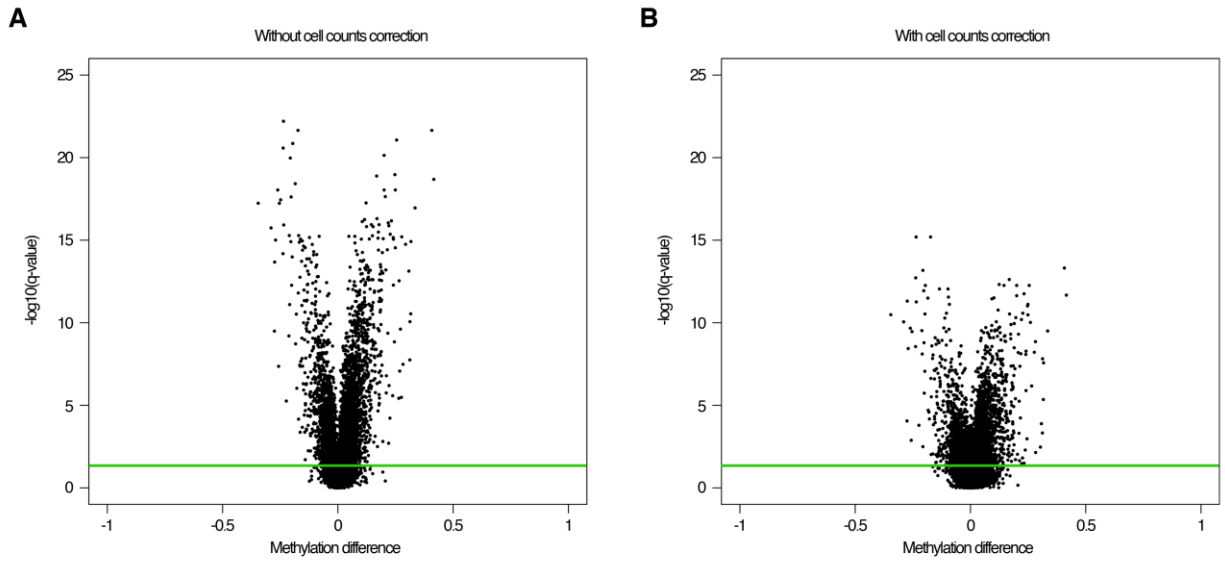


Figure 22 Effect of adjustment for estimated cell counts. The CpG probe most significantly differentially methylated between DSP and DSS was selected for each BOP. The volcano plot reports the –log10 (q-value) of each BOP against the methylation difference between DSP and DSS for the selected CpG probe, without **(A)** or with **(B)** correction for cell counts, in addition to correction for sex and batch effects. The green lines represent q-value=0.05 (adapted from Bacalini et al., 2015a).

Of the 6650 BOPs in Class B, 889 resulted differentially methylated between DSP and DSS (q-value < 0.05; Figure 21 B)

Comparing methylation values of Class C and Class D probes between DSP and DSS by ANOVA, 6051 CpG probes out of 109617 probes mapping in Class C and 3426 out of 54697 of probes mapping in Class D were differentially methylated between DSP and DSS (q-value < 0.05;Figure 21 B).

Since most of the Infinium 27k probes are also in the Infinium 450k array we considered the study of Kerkel and co-workers, that analyzed DNA methylation profiles in peripheral blood lymphocytes (PBL) from 29 DS adults and 20 age-matched controls using the Infinium 27k. They reported a list of differentially methylated genes distributed across various autosomes, with no specific enrichment for chromosome 21. We verified if the DMRs identified in Kerkel's study were confirmed in our cohort of samples. Kerkel et al. selected 7 probes as DMRs (cg07991621, cg08822227, cg09554443, cg05590257, cg14972143, cg00983520, cg21053323) that resulted differentially methylated also in our study (Figure 23), apart from cg05590257 that did not reach statistical significance, but this and the surrounding CpG probes showed a trend for differential methylation.

Looking at the distribution of Class A DMR across the chromosomes, we observed a significant overrepresentation of DMRs on chromosomes 21,17, 6 and 15, while we found a significant underrepresentation on chromosome 5 (Fisher's exact test, p-value < 0.05; Figure 21 C). We observed that DMRs spread on the entire length chromosome 17, while they were clustered in the HLA locus as regards the chromosome 6. Considering the fact that this locus is highly polymorphic, we wondered whether the differences in DNA methylation, observed between DSP and DSS could be due to the presence of SNPs in the probes of the array. To answer this question we clustered the samples on the basis of their methylation values in the selected HLA loci. We expected that members of the same family showed the same genetic/epigenetic variations and thus were clustered together (Figure 24). On the contrary, DSP tended to cluster together, suggesting us that the methylation profile of DSP in HLA loci was different from their relatives, independently from the genetic background.

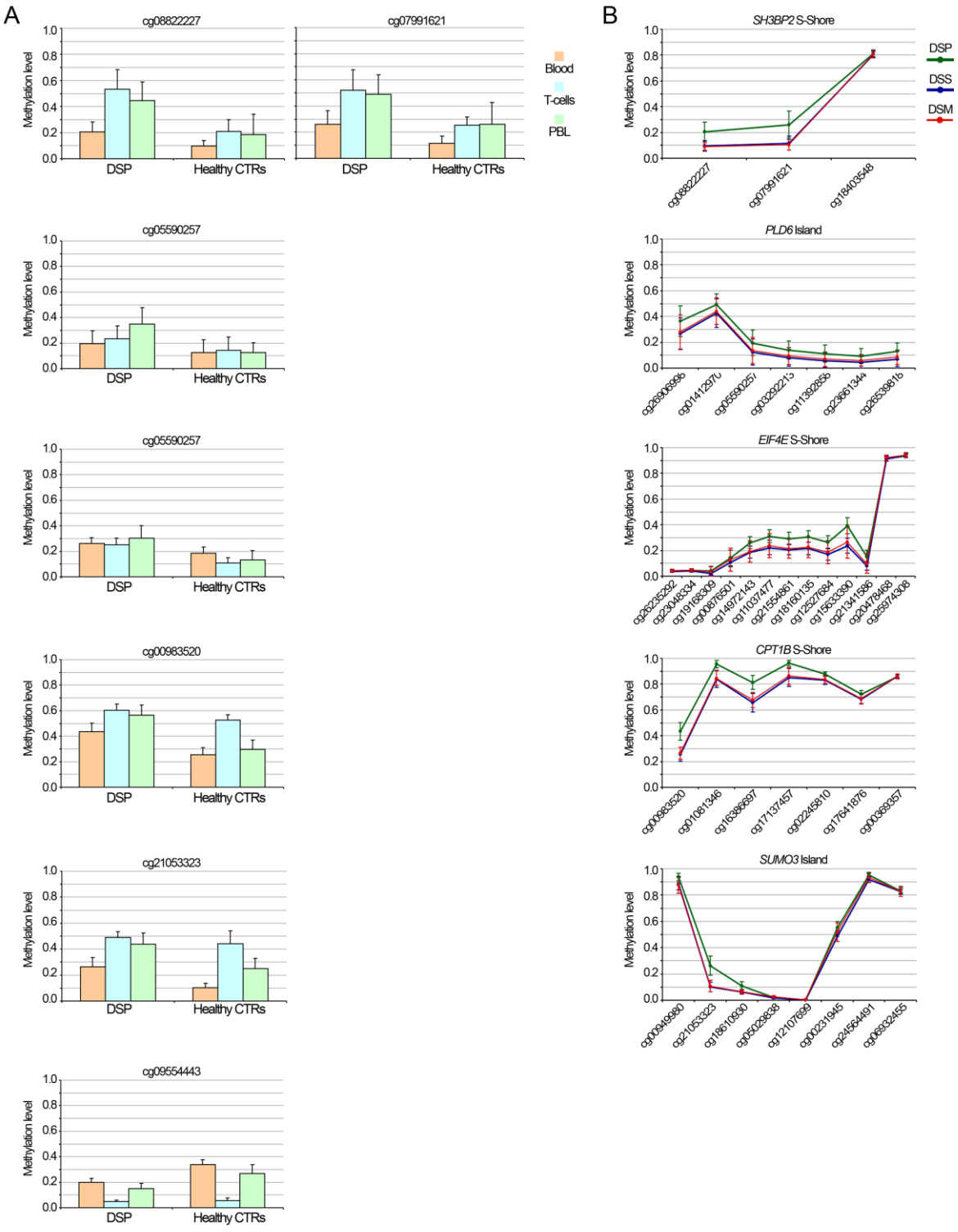


Figure 23. Confirmation of previous studies. **(A)** Here are indicated the DNA methylation levels of the CpG probes resulted differentially methylated in persons affected by DS compared to healthy controls in T-cells and PBL in Kerkel's study. DNA methylation values in whole blood for the same CpG probes measured in our study are also reported. **(B)** Graphical representation of the whole blood DNA methylation profile of the BOPs containing the CpG probes reported in **(A)**. The corresponding BOP of the probe cg09554443 is not reported because it maps to a not-CpG rich region in the CD247 gene (adapted from Bacalini et al., 2015a).

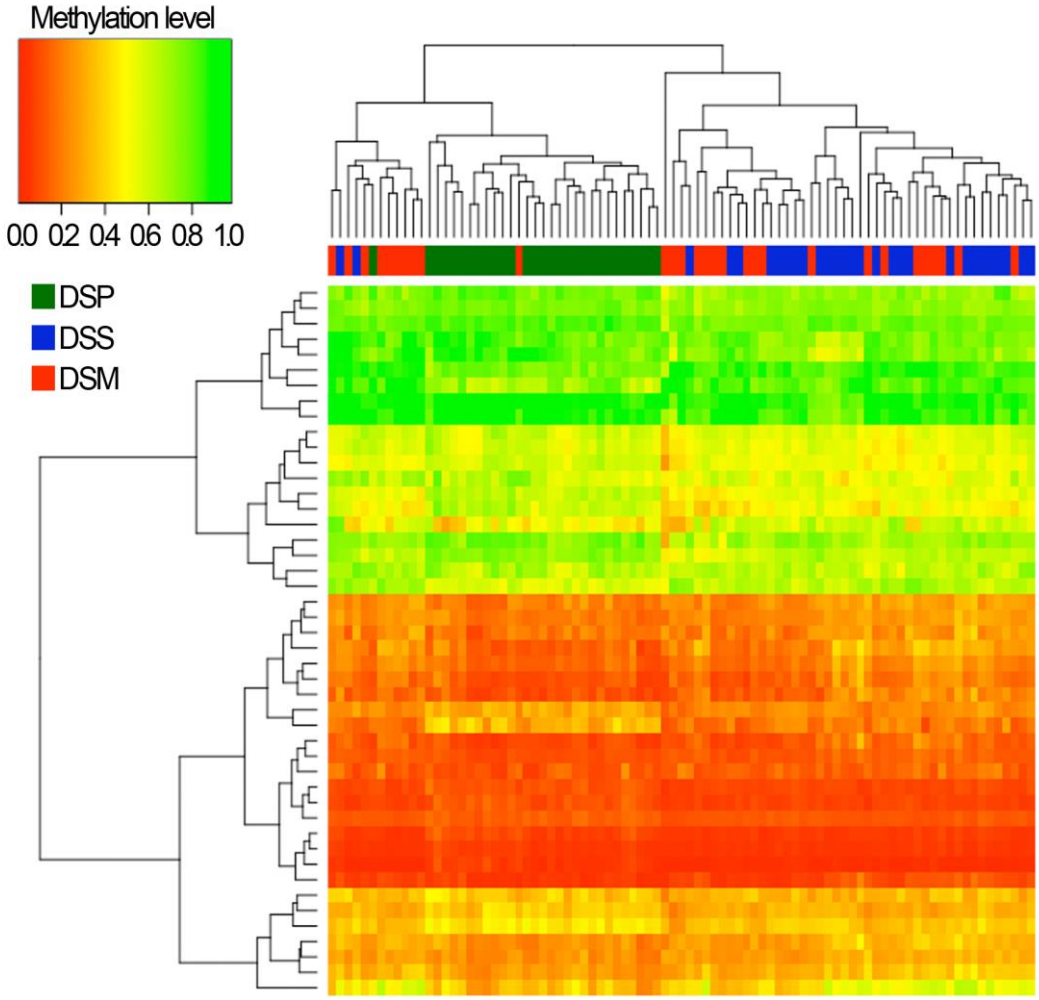


Figure 24. Hierarchical clustering of DSP, DSS and DSM for significative BOPs mapping in HLA loci. We selected the CpG probes differentially methylated between DSP and DSS for the 21 BOPs mapping in HLA loci.(ANOVA, q value <0.05; 46 CpG probes). In the heatmap are reported DNA methylation values for the selected CpG probes in rows (samples in columns and color-coded). Dendrograms depict hierarchical clustering of probes and samples (adapted from Bacalini et al., 2015a).

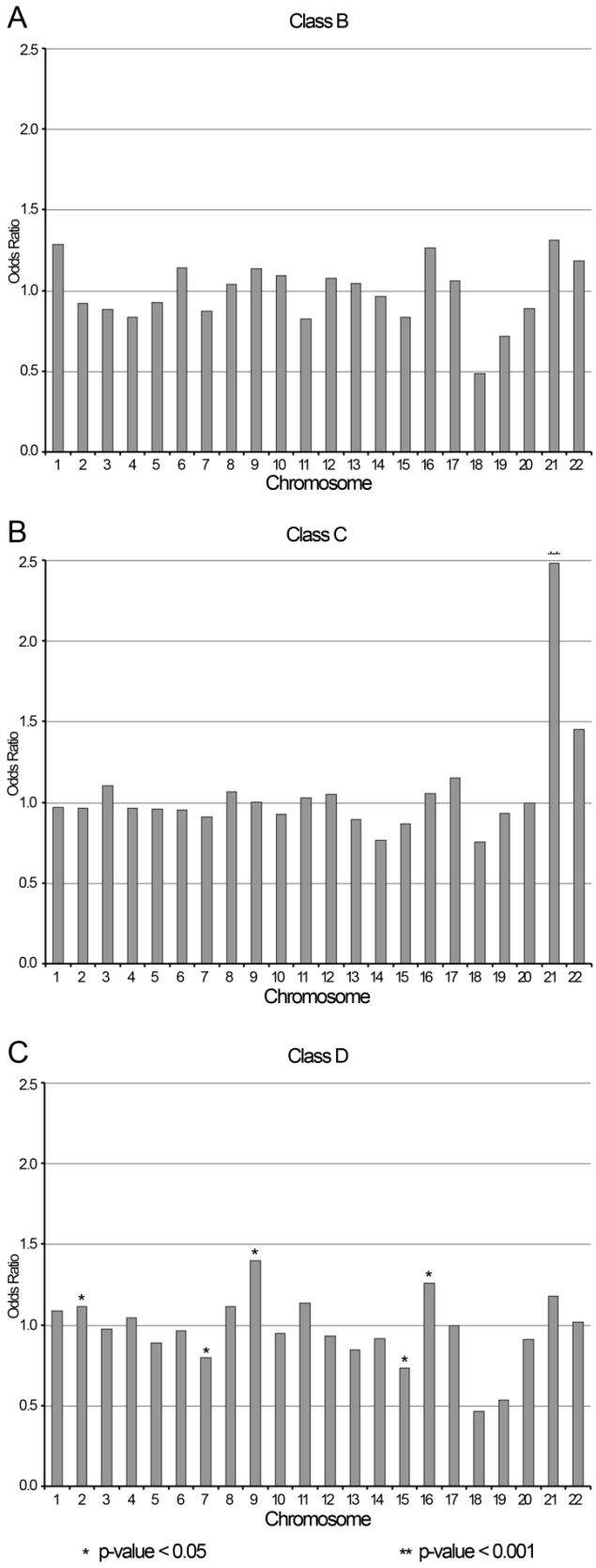


Figure 25. Chromosomal enrichment of DMRs in Class B **(A)**, Class C **(B)** and Class D **(C)** probes. For each chromosome, the odds ratio resulting from Fisher's exact test is reported. Significant enrichments are indicated with asterisks (adapted from Bacalini et al., 2015a).

Concerning chromosome 21 we observed DMRs enrichments also for CpG probes in Class C, but not for those in Class B and Class D (Figure 25).

We observed that almost all the other chromosomes contained genomic regions altered in terms of DNA methylation pattern, although HSA21 was the most affected by the aneuploidy. The result that we found can be compared with those obtained with the 27k array, apart from those related to chromosome 21. In fact Kerkel did not observe an enrichment on HSA21, probably because of the lower density of probes, especially in small chromosomes with low numbers of genes.

PATHWAY AND GENE ONTHOLOGY ANALYSIS

Using the list of the 4648 Class A BOPs differentially methylated between DSP and DSS, we performed Gene Onthology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (Table 2). GO analysis showed various enriched GO terms. In particular the selected DMRs mapped principally in genes involved in the morphogenesis of anatomical structures and developmental processes.

KEGG pathways, that reached statistical significance after FDR correction, were involved in ribosome, immune functions and type I diabetes.

Table 2. KEGG pathways and GO analysis 4648 BOPS resulted differentially methylated between DSP and DSS. The table reports the significantly enriched KEGG pathways and gene ontologies, resulted from the analysis with Fisher's exact test and Gorilla platform (as just reported in Materials and Methods section) (adapted from Bacalini et al., 2015a).

Description	**q-value**
Kegg Pathway	
Ribosome	0.013
Allograft rejection	0.013
Graft-versus-host disease	0.013
Cell adhesion molecules (CAMs)	0.013
Autoimmune thyroid disease	0.013
PI3K-Akt signaling pathway	0.013
Basal cell carcinoma	0.013
HTLV-I infection	0.034
Type I diabetes mellitus	0.040
Gene Ontology Process	
System process (GO:0003008)	0.027
Anatomical structure morphogenesis (GO:0009653)	0.032
Regulation of signal transduction (GO:0009966)	0.027
Multicellular organismal process (GO:0032501)	0.000
Single-organism process (GO:0044699)	0.015
Single-multicellular organism process (GO:0044707)	0.000
Positive regulation of biological process (GO:0048518)	0.027
Embryonic organ morphogenesis (GO:0048562)	0.006
Regulation of response to stimulus (GO:0048583)	0.035
Embryonic skeletal system morphogenesis (GO:0048704)	0.018
Anatomical structure development (GO:0048856)	0.017
Regulation of body fluid levels (GO:0050878)	0.038

IDENTIFICATION OF AN EPIGENETIC SIGNATURE OF DOWN SYNDROME

From the list of 4648 Class A BOPs resulted altered in DSP we defined an epigenetic signature of Down Syndrome by selecting a short list of DMRs whose DNA methylation status was remarkably different compared to healthy sibs. To this purpose as previously suggested by Du et al. (P. Du et al. 2010) we considered only BOPs containing at least 2 adjacent CpG sites for which DNA methylation difference between DSP and DSS was higher than 0.15. The BOPs that met these stringent criteria were found to be 68. DNA methylation profile of some of these BOPs are reported in Figure 26 A. In addiction Figure 26 B reports the results of the hierarchical clustering analysis that we performed using these loci. We found that the different methylation status of these 68 loci clearly separated DSP subjects from their relatives, while it did not distinguish DSS from DSM. We found a percentage of hypermethylated probes of 73% in DSP compared to DSS.

We took advantage of our family based model to understand if the epigenetic signature of DSP, that we obtained with our selection of CpG probes, was independent from genetic or environmental factors. In Figure 26 C it is graphically represented the difference in methylation values of the 68 BOPs (in row) between each DSP and his healthy sib. This hierarchical clustering does not clearly distinguish one family from another, suggesting that the epigenetic signature is not influenced by genetic and environmental factors, but only by the presence/absence of disease.

We performed a meticulous screening of the 68 differentially methylated loci included in the epigenetic signature to group them according to their principal main function, because they were in a too small number to perform ontology enrichment analysis. They were divided according to four main tasks: 1) hematopoiesis (*RUNX1*, *DLL1*, *EBF4* and *PRMD16*); 2) morphogenesis and development (*HOXA2*, *HOXA4*, *HOXA5*, *HOXA6*, *HHIP*, *NCAM1*); 3) neuronal development (*NAV1*, *EBF4*, *PRDM8*, *NCAM1*, *GABBR1*); 4) regulation of chromatin structure (*PRMD8*, *KDM2B*, *TET1*).

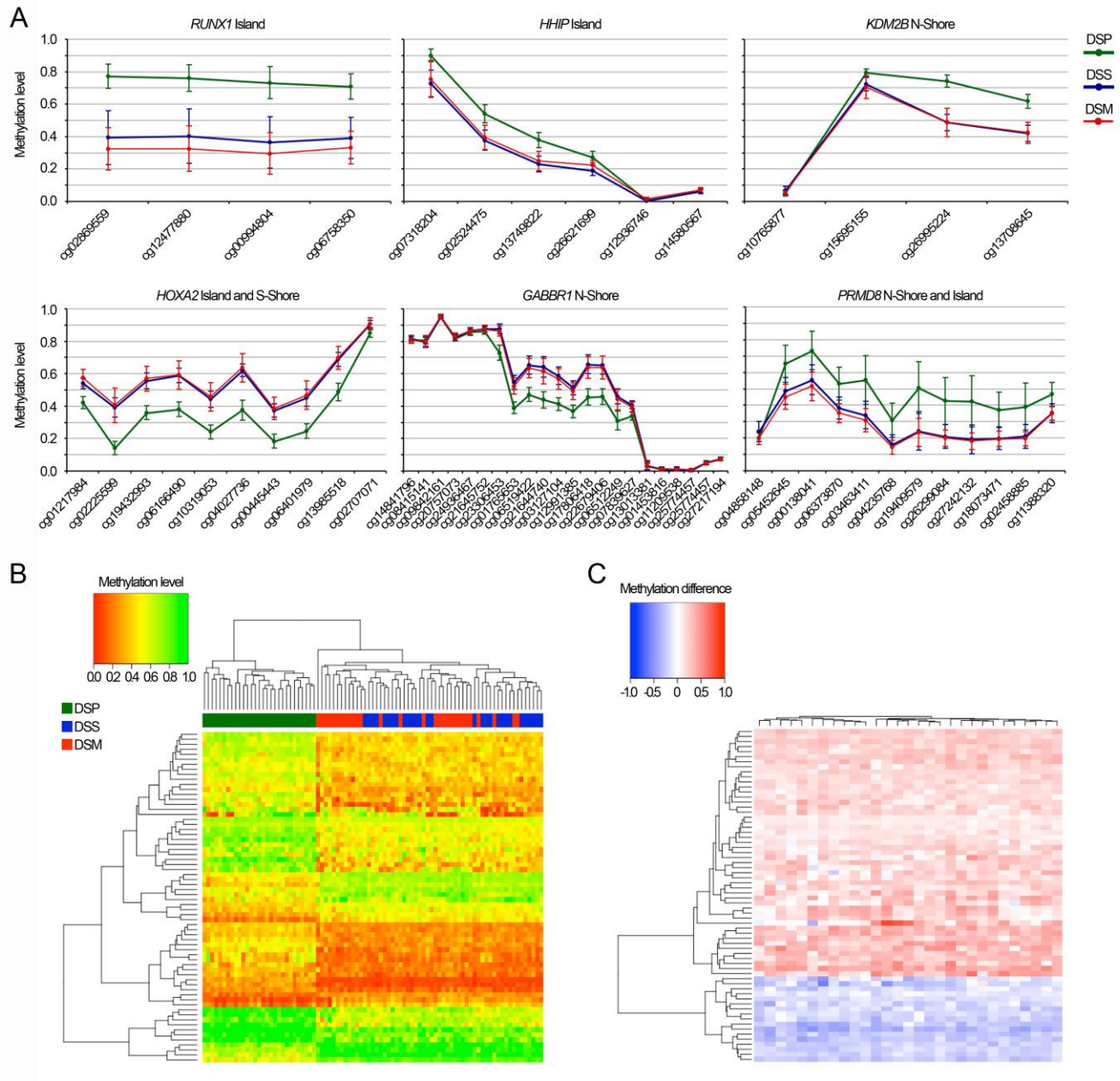


Figure 26. (A) DNA methylation profile of 6 of the 68 BOPs included in the epigenetic signature of Down Syndrome. **(B)** Heatmap reporting DNA methylation values for the 68 BOP included in the epigenetic signature of DSP. **(C)** Heatmap that reports differences in DNA methylation between each DSP and his/her DSS for the 68 BOPs included in the epigenetic signature. The methylation values of the most significant CpG probe within each BOP was considered both in **(B)** and in **(C)** (adapted from Bacalini et al., 2015a).

EPIGENETIC SIGNATURE OF DOWN SYNDROME: LOCUS SPECIFIC ANALYSIS

To confirm data obtained with Illumina assay we analyzed 3 DMRs included in the epigenetic signature: *RUNX1* island, *KDM2B* N Shore and *NCAM1* N Shore. This kind of analyses was conducted using an alternative method, the Sequenom's EpiTYPER assay. The analysis was performed on an enriched cohort of samples. In particular we tested the 29 DSP and DSS subjects of our family based model and in addition other 49 persons affected by Down Syndrome and 33 age- and sex- matched unrelated controls.

EpiTYPER analysis confirmed that CpG sites included in 450 BeadChip were differentially methylated between DSP and controls and showed that differences in DNA methylation extended also to the adjacent CpG sites (Table 3).

All the CpG sites within *RUNX1* and *KDM2B* amplicons were significantly hypermethylated in DSP respect to controls, while only 7/11 of the CpG tested in *NCAM1* island were significantly different between DSP and controls (Figure 27 A, B and C; Student's t-test).

Afterward we calculated correlation matrices of these 3 DMRs in DSP and controls to evaluate the reciprocal trend of CpG methylation in the examined amplicons. A high degree of correlation between the CpG may suggest the existence of mechanisms of co-regulation of their methylation status (Wessely and Emes 2012). The correlation is indicated by a continuous value between -1 (maximum inverse correlation) to +1 (maximum direct correlation). In Figure 28 the graphs representing these correlation matrices are reported. CpG correlation blocks included almost all the CpG probes in *RUNX1*, whereas in *KDM2B* and *NCAM1* it is possible to observe the presence of smaller correlation blocks which involves only few CpGs, but which did not show significant differences between DSP and healthy controls. This difference is probably due to the nature of the amplicon examined, as the *RUNX1* amplified region is located within a CpG island, while both for *KDM2B* and *NCAM1* the N-shores adjacent to the island were considered. These data suggest that methylation control in CpG island is greater than in shores.

Table 3. P-values relative to the differences in the indicated *KDM2B*, *RUNX1* and *NCAM1* CpG sites between persons affected by DS and healthy controls.

KDM2B CpG sites	**P value**
CpG_1	**$1{,}74\cdot10^{-19}$**
CpG_2.3.4	**$6{,}37\cdot10^{-23}$**
CpG_5	**$5{,}06\cdot10^{-14}$**
CpG_6	**$3{,}82\cdot10^{-16}$**
CpG_7	**$9{,}65\cdot10^{-21}$**
CpG_8.9	**$1{,}05\cdot10^{-18}$**
CpG_10.11	**$4{,}52\cdot10^{-19}$**
CpG_12	**$4{,}26\cdot10^{-22}$**
CpG_14	**$1{,}05\cdot10^{-8}$**
CpG_15	**$3{,}82\cdot10^{-16}$**
CpG_22	**0,002**
CpG_23.24	**$9{,}24\cdot10^{-10}$**
CpG_25	**$4{,}39\cdot10^{-7}$**
CpG_26	**$2{,}28\cdot10^{-6}$**
CpG_27	**$1{,}58\cdot10^{-7}$**

RUNX1 CpG sites	**P value**
CpG_1	**$8{,}49\cdot10^{-40}$**
CpG_3	**$4{,}51\cdot10^{-37}$**
CpG_5.6	**$7{,}89\cdot10^{-34}$**
CpG_7.8	**$6{,}09\cdot10^{-38}$**
CpG_9.10	**$1{,}59\cdot10^{-35}$**
CpG_20.21	**$4{,}16\cdot10^{-38}$**
CpG_22	**$1{,}38\cdot10^{-22}$**
CpG_23	**$1{,}21\cdot10^{-31}$**
CpG_24.25	**$1{,}78\cdot10^{-30}$**
CpG_26	**$6{,}89\cdot10^{-36}$**
CpG_30	**$8{,}37\cdot10^{-39}$**
CpG_31	**$1{,}47\cdot10^{-36}$**
CpG_32	**$1{,}19\cdot10^{-37}$**
CpG_34.35	**$3{,}13\cdot10^{-35}$**
CpG_36	**$1{,}19\cdot10^{-34}$**
CpG_37.38	**$3{,}29\cdot10^{-26}$**
CpG_39	**$1{,}69\cdot10^{-36}$**
CpG_40.41.42	**$1{,}55\cdot10^{-37}$**
CpG_43	**$3{,}97\cdot10^{-38}$**
CpG_44.45	**$8{,}23\cdot10^{-35}$**

NCAM1 CpG sites	**P value**
CpG_1.2.3.4	0,50
CpG_14	0,49
CpG_17	0,23
CpG_18	0,18
CpG_26	**$2{,}10\cdot10^{-10}$**
CpG_27.28	**$1{,}84\cdot10^{-4}$**
CpG_29	**$1{,}55\cdot10^{-5}$**
CpG_30	**$1{,}56\cdot10^{-8}$**
CpG_31.32	**$3{,}02\cdot10^{-38}$**
CpG_33	**$3{,}02\cdot10^{-38}$**
CpG_34	**$3{,}69\cdot10^{-35}$**

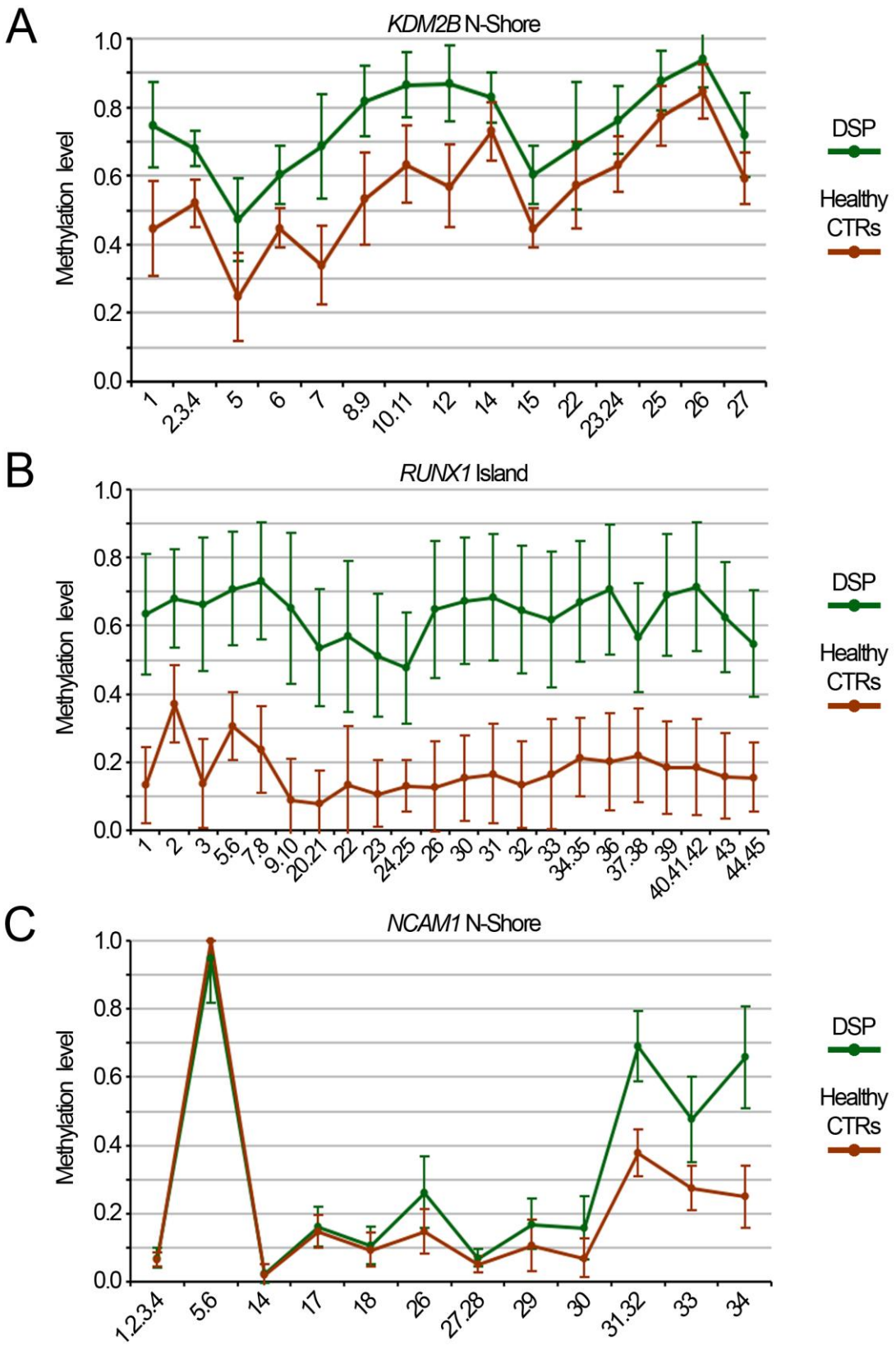


Figure 27. Validation of DSP DMRs by Sequenom EpiTYPER. DNA methylation values of *KDM2B* N-Shore **(A)**, *RUNX1* Island **(B)** and *NCAM1* N-Shore **(C)** measured in a cohort of 78 DSP and 62 age- and sex-marched healthy controls (adapted from Bacalini et al., 2015a).

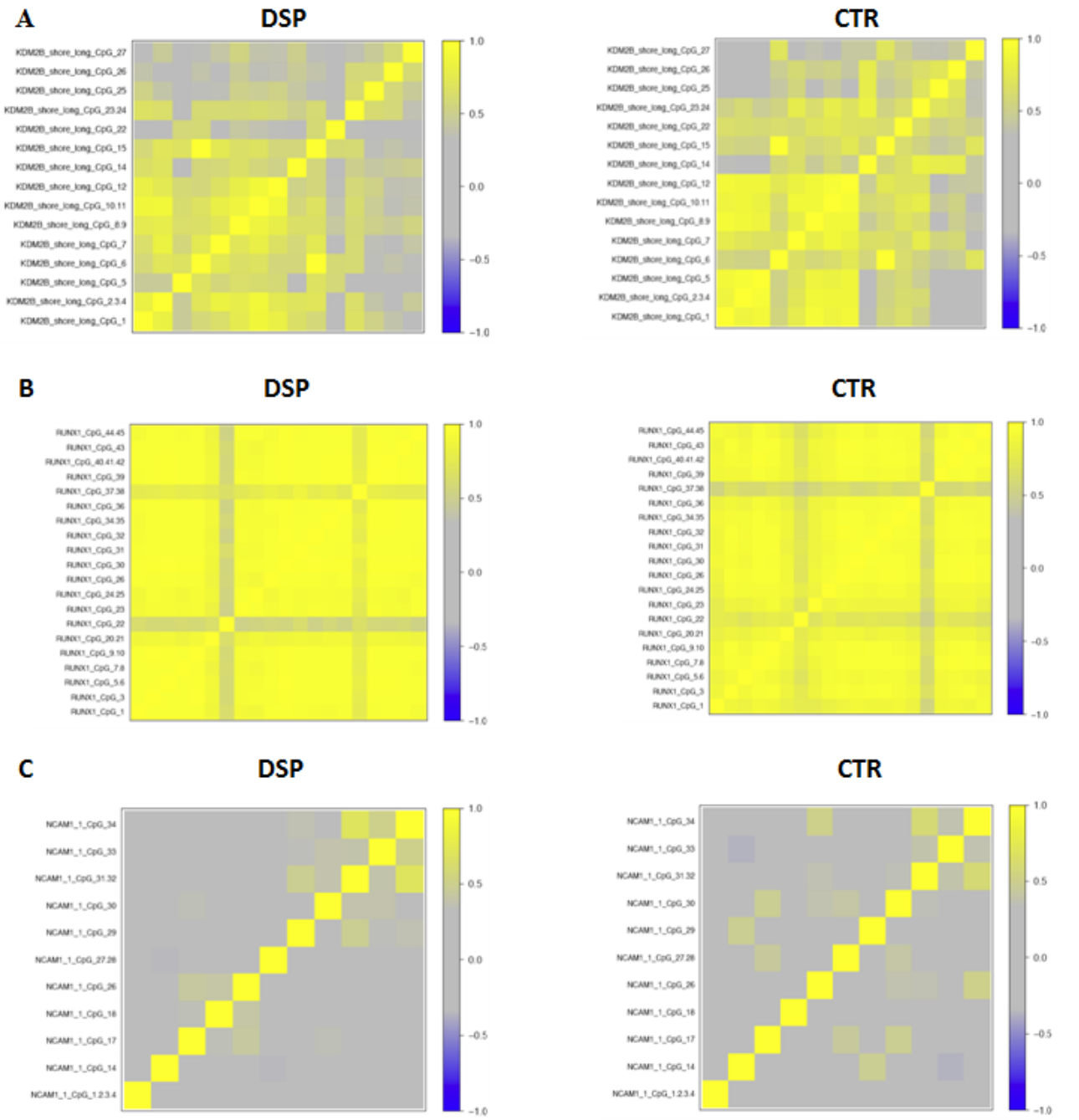


Figure 28. Correlation matrices between CpG units within each CpG island/island Shore of *KDM2B* **(A)** *RUNX1* **(B)** and *NCAM1* **(C)** in DSP and controls.

It is also possible to observe that *KDM2B* correlation level is lower in DSP compared to controls. Accordingly, the characteristic of many diseases is the loss of CpG correlation that reflects a defect in the control of methylation levels.

METHYLATION OF RIBOSOMAL GENES

The EpiTYPER assay was used also for the quantitative analysis of DNA methylation of 3 regions belonging to the sequence of the ribosomal gene (*rDNA*), which is completely excluded from the Infinium 450k design: the gene's promoter (*Ribo*) and the regions at 5' of *18S* and *28S* subunits (Figure 29). These analyses were conducted on the enriched cohort of samples.

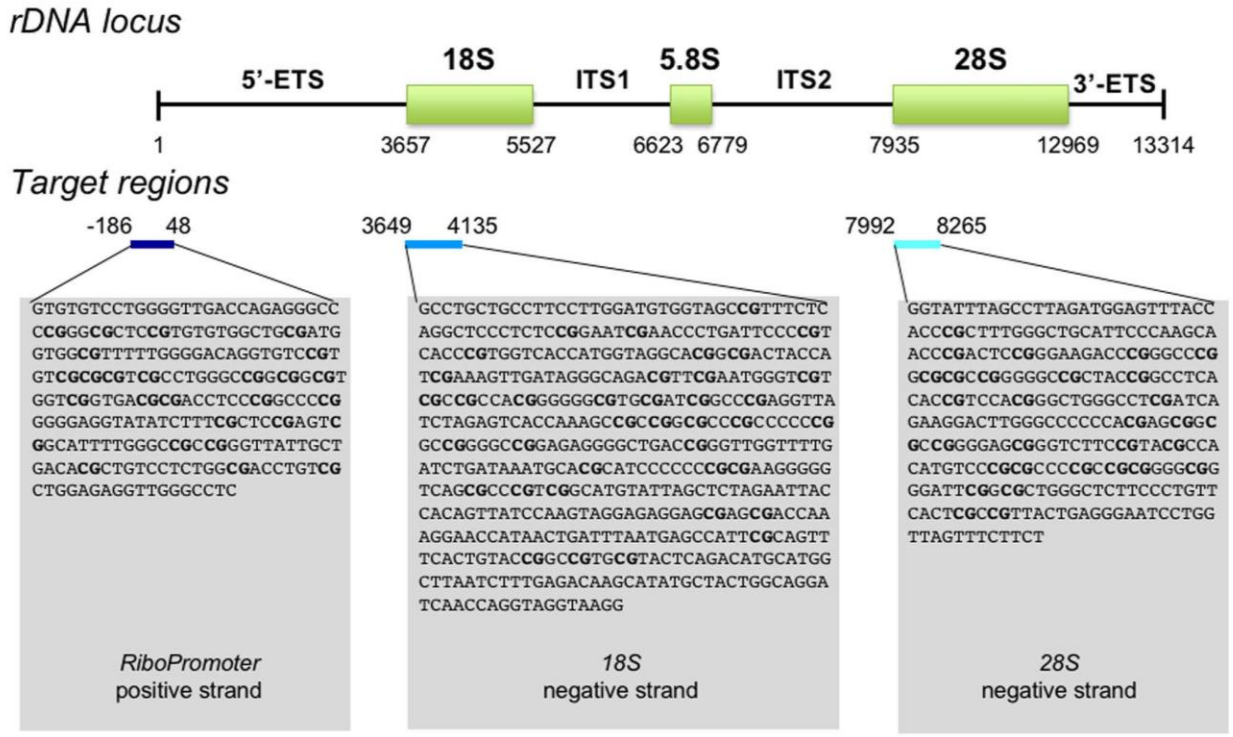


Figure 29. *rDNA* locus and target regions selected for DNA methylation analysis (adapted from Bacalini et al. 2014).

Referring to *Ribo* and *18S* amplicons it has been necessary to conduct a preliminary analysis before comparing their methylation level between the two groups of interest (DSP and controls). Indeed while all samples of *RUNX1*, *NCAM1*, *KDM2B* and *28S* amplicons were analyzed in the same EpiTYPER experiment (same experimental batch), for technical reasons *Ribo* and *18S* amplicons were analyzed in two different experimental sessions, respectively comprising 68 samples (34 DSP and 34 controls) and 72 samples (44 DSP and 28 controls). The average methylation values of CpG sites in DSP and controls in the two experimental batches are shown in Figures 30 and 31.

In these graphs methylation values of some CpG sites appear to be different in the two batches. By performing a Student's t test it has been possible to confirm that the observed variations are significant for the following CpG sites:

• CpG 15:16 and CpG 26 for the amplicon *Ribo*;
• CpG 2, CpG 9:10, CpG 17:18, CpG 28:29 and CpG 35 for the amplicon *18S*.

This kind of difference takes the name of "batch effect" and it is due to a technical artifact due to differences (not always identifiable and/or controllable) between the two experimental sessions. Therefore batch effect is not linked to biological differences between samples analyzed in the two sessions, that were equally balanced for the number of DSP and controls. As the batch could be a confounding factor, when comparing the DNA methylation values between DSP and controls it was included as a covariate with age in the ANOVA analysis.

Table 4 and Figure 32 show that a subset of CpG sites resulted differentially methylated between DSP and controls. In detail we observed significant differences in methylation values in 3/8 CpG of *Ribo* promoter, 3/13 CpG in *18S* amplicon and 6/10 CpG in *28S* amplicon, with an hypermethylation in DSP compared to controls.
We calculated correlation matrices also for these 3 DMRs of ribosomal genes in DSP and controls (Figure 33). Considering the spatial proximity of the three amplicons we reported in the same matrix all the correlation levels of *Ribo, 18S* and *28S* sequences. This analysis showed that CpGs of the three amplicons studied are highly correlated both in DSP and in controls, suggesting that the disease does not affect the ability to control the methylation status of this region.

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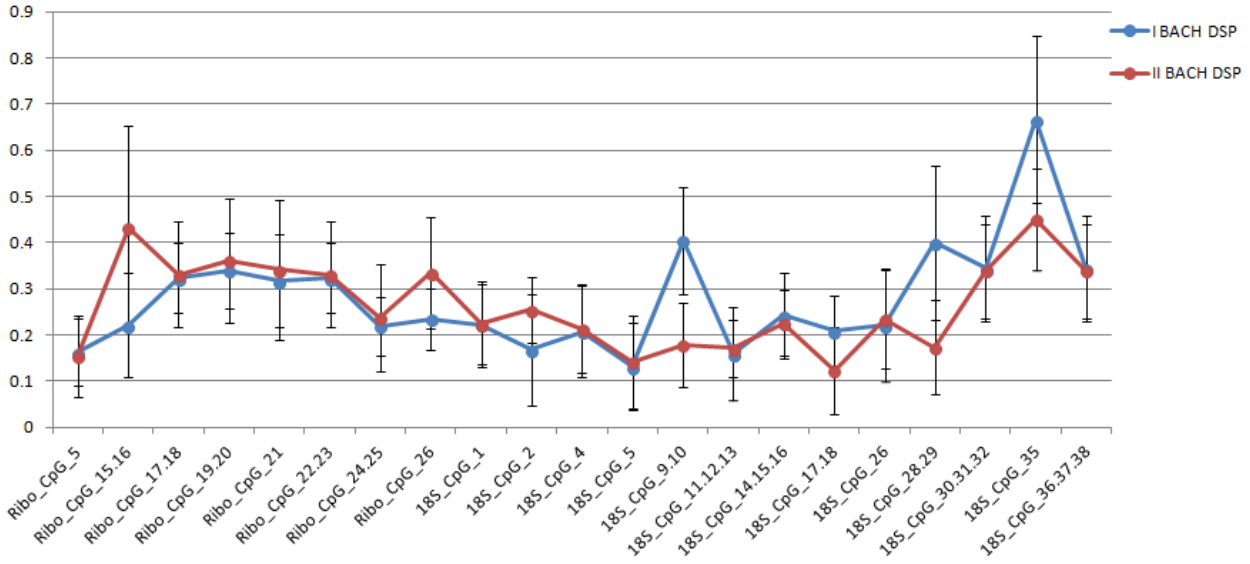

Figure 30. Average of methylation values and relative standard deviations in DSP in the first and second batch.

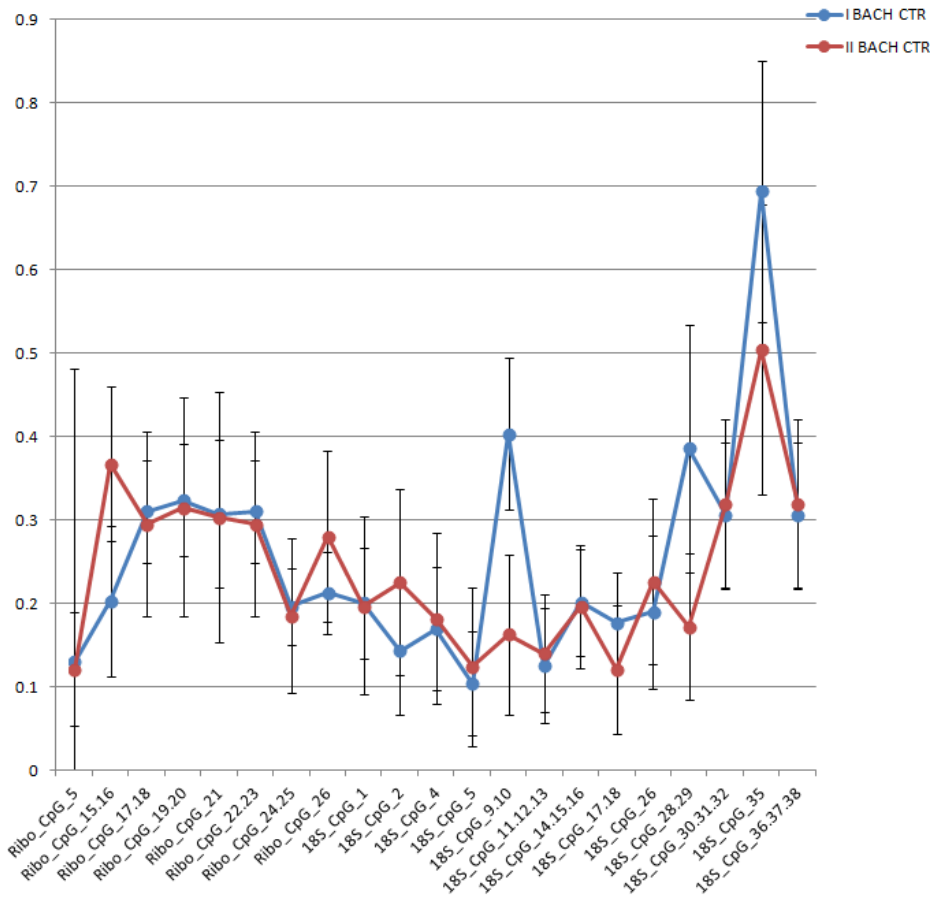

Figure 31. Average of methylation values and relative standard deviations in controls in the first and second batch.

Tabel 4. P-values of *Ribo*, *18S* and *28S* sequences CpG sites.

Ribo promoter CpG sites	**P value**
CpG_5	**0,012**
CpG_15.16	0,178
CpG_17.18	0,135
CpG_19.20	0,099
CpG_21	0,286
CpG_22.23	0,135
CpG_24.25	**0,014**
CpG_26	**0,013**

***18S* CpG sites**	**P value**
CpG_1	0,126
CpG_2	0,141
CpG_4	**0,048**
CpG_5	0,140
CpG_9.10	0,735
CpG_11.12.13	**0,021**
CpG_14.15.16	**0,014**
CpG_17.18	0,178
CpG_20	0,283
CpG_28.29	0,793
CpG_30.31.32	0,126
CpG_35	0,154
CpG_36.37.38	0,126

28S CpG sites	**P value**
CpG_1	**0,008**
CpG_2	0,136
CpG_3	0,068
CpG_10	**0,011**
CpG_11	**0,003**
CpG_12.13	0,053
CpG_15.16.17	**0,023**
CpG_18.19	**0,014**
CpG_20	0,136
CpG_29.30	**0,032**

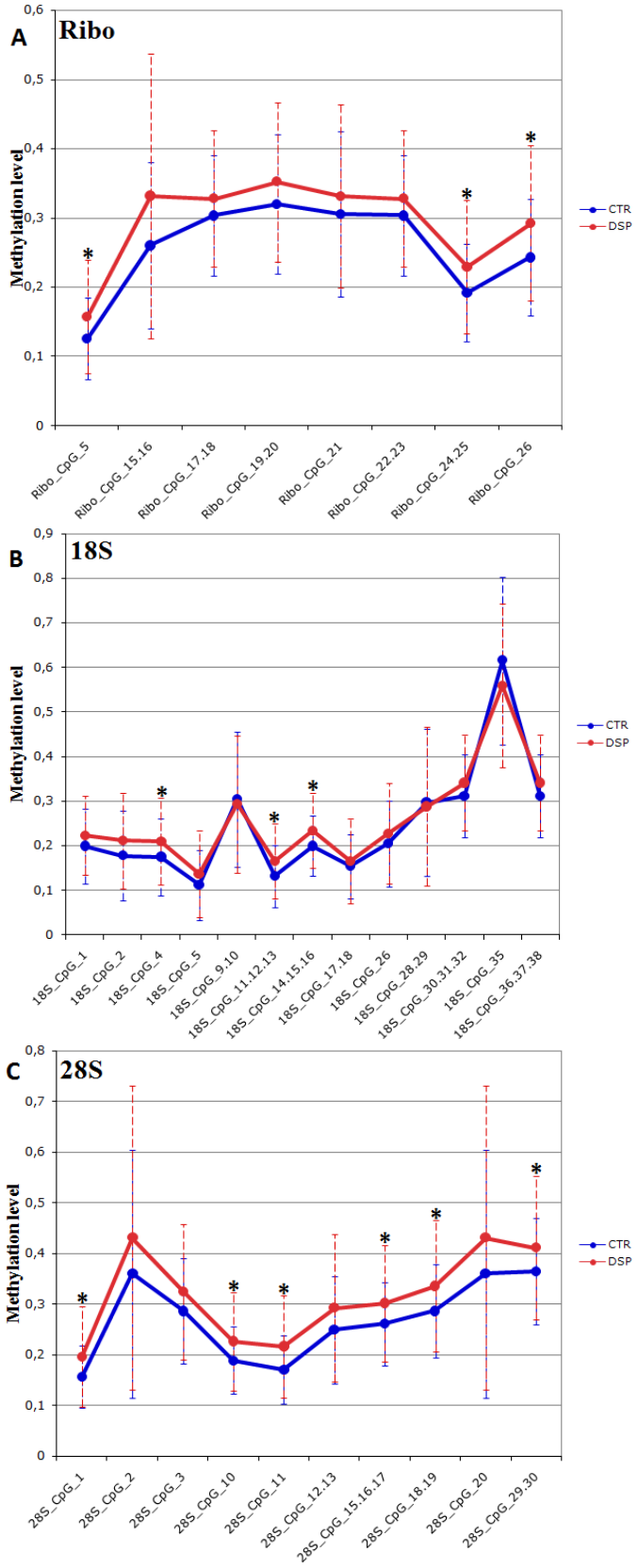

Figure 32. Validation of Down Syndrome DMRs by Sequenom EpiTYPER. DNA methylation values of *Ribo* **(A),** *18S* sequence **(B)** and *28S* sequence **(C)** measured in a cohort of 78 DSP and 62 age- and sex-matched healthy controls**.**

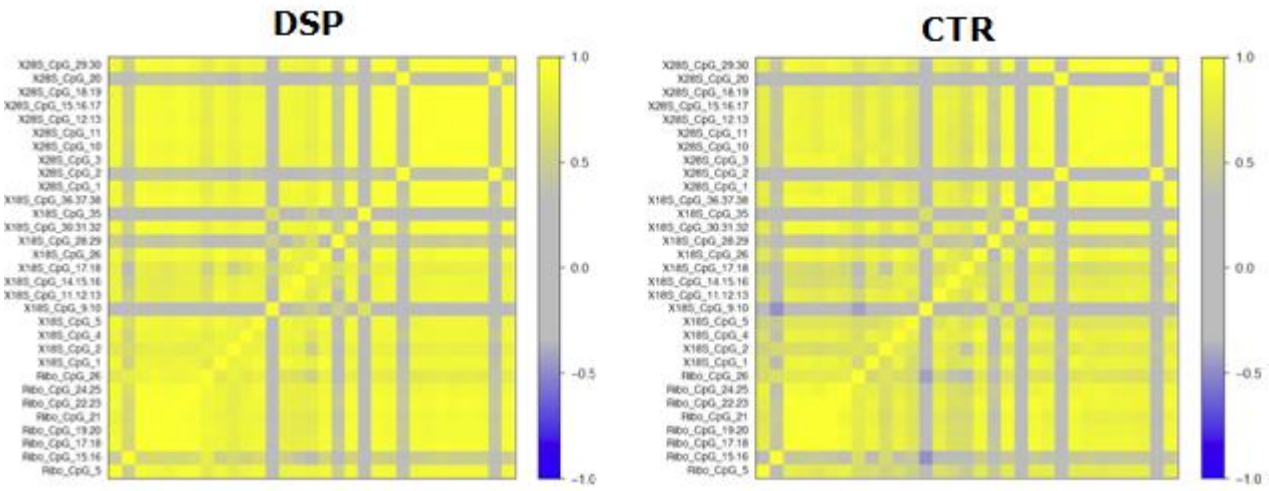

Figure 33. Correlation matrices between CpG units within the sequences *Ribo*, *18S* and *28S*.

EXPRESSION ANALYSIS

On the basis of methylation data, we wondered if DSP hypermethylation of *KDM2B* N Shore and of some CpGs in ribosomal genes could lead to changes in gene expression of *KDM2B* gene and of rRNA 45S. To this aim we conducted a gene expression analysis using Real Time PCR technique. We compared 4 DS samples with age- and sex- balanced 4 controls. Results reported in Figure 34 A and B indicate that the DSP hypermethylation state previously described does not cause alterations in gene expression both for *KDM2B* gene and ribosomal genes.

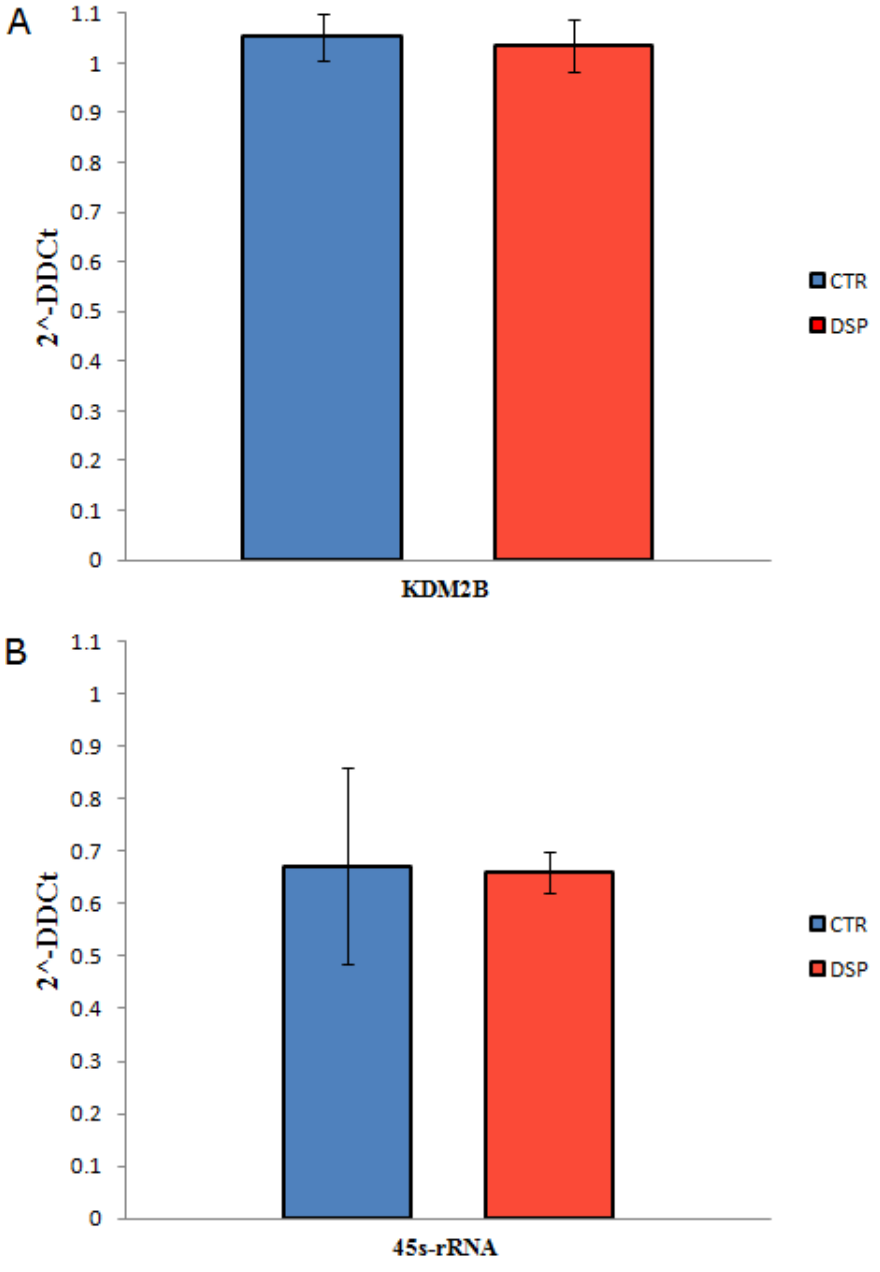

Figure 34. Gene expression levels of *KDM2B* gene **(A)** and 45S-rRNA **(B)** in controls and DSP.

ANALYSIS OF AGE-DEPENDENT EPIGENETIC CHANGES IN DSP

As Down Syndrome is usually considered a segmental progeroid syndrome, we investigated how DSP behave for loci whose methylation status is known to be associated with aging. Recently, Hannum and colleagues used the Infinium 450k to analyse whole blood DNA methylation in 656 individuals from 19 to 101 years and identified an age-associated epigenetic signature including 71 CpG sites (Hannum et al. 2013). Figure 35 A reports the principal component analysis calculated using the methylation values of the 71 CpG probes in the 29 DSP, 29 DSS and 29 DSM included in our cohort. As shown in Figure 35 B, the first principal component (PC1) accounted for around 94% of the variability in the data, while an additional 4% of variability was explained when also the second principal component (PC2) was taken into account. As the methylation status of the 71 CpG probes is age-dependent, we plotted both PC1 and PC2 against the age of the subjects (Figure 35 C and 35 D respectively). PC1 almost perfectly correlated with DSS and DSM age ($\rho = 0.95$, p-value < 0.001) and with DSP age ($\rho = 0.95$). DSP showed a pattern comparable to that of age-matched siblings ($\rho = 0.95$, p-value < 0.001), indicating that the DNA methylation changes in blood cells that occur with aging are not overall replicated in Down Syndrome. Also PC2 correlated with age, both in DSP ($\rho = 0.43$, p-value=0.02) and in DSS and DSM ($\rho = 0.65$, p-value < 0.001). In this case however DSP had PC2 values similar to their mothers, indicating that a minor portion of the variability in DSP methylation patterns could be explained in terms of accelerated aging.

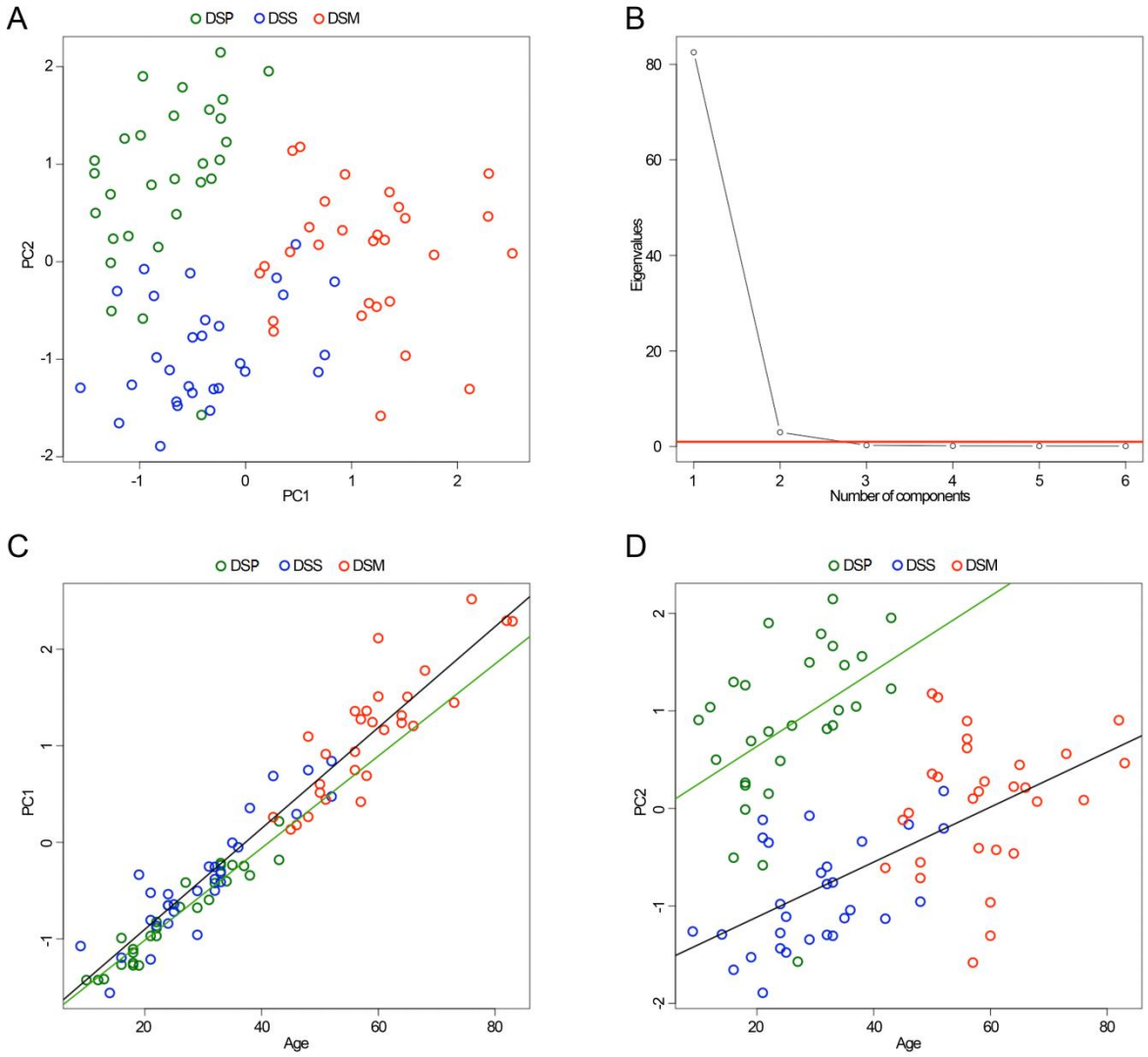

Figure 35. Age-dependent epigenetic changes in DSP. (A) The plot reports the results of principal components analysis calculated using the methylation values of 71 age-dependent CpG probes (Hannum et al. 2013) in our cohort. The first and the second principal components are reported on x and y axes respectively. **(B)** The scree plot reports the fraction of total variance in the data (DNA methylation values of the 71 age-dependent CpGs in 29 DSP, 29 DSS and 29 DSM) as explained by each principal components. **(C)** The first principal component and **(D)** the second principal component are plotted against the age of DSP, DSS and DSM subjects.

IDENTIFICATION OF DMRs BETWEEN DSS AND DSM

Finally, we compared Class A BOPs between DSS and DSM subjects, using sex and cell counts as covariates. We found 4459 BOPs significantly different between DSS and their mothers. Figure 36 shows the MDS calculated on the BOPs that reached statistical significance from this comparison.

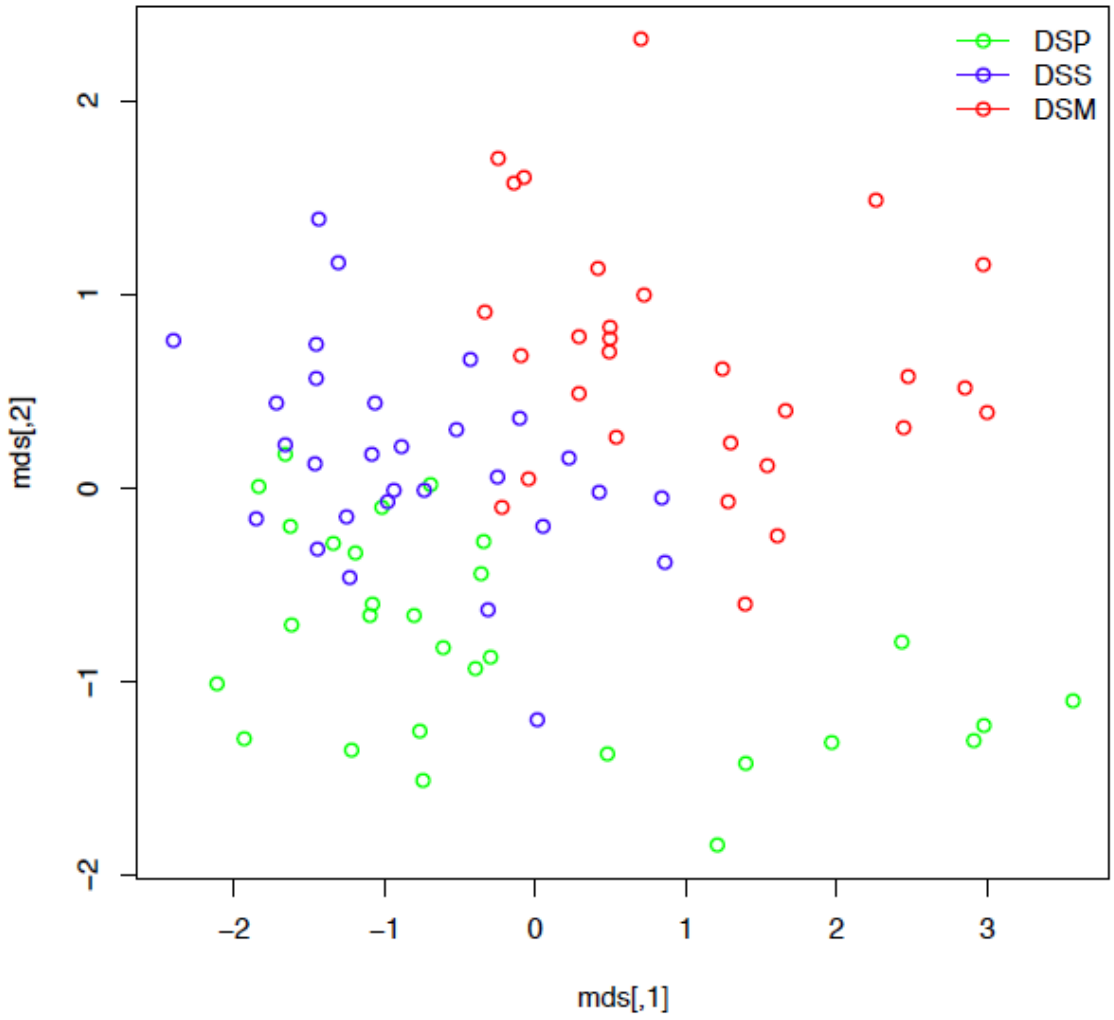

Figure 36. MDS calculated on the BOPs of Class A resulted significantly different between DSS and DSM.

Since both brothers and mothers samples are distributed along the first component of the MDS, we correlated the values of the first component of the MDS (MDS1) with the age of the subjects (Figure 37). The MDS1 correlated very well with the age of DSS and DSM (Pearson correlation coefficient=0.68, p-value=2.8*10-9). Both Figure 36 and Figure 37 evidence that some DSP have a MDS1 comparable with elder subjects, in according to the results described above.

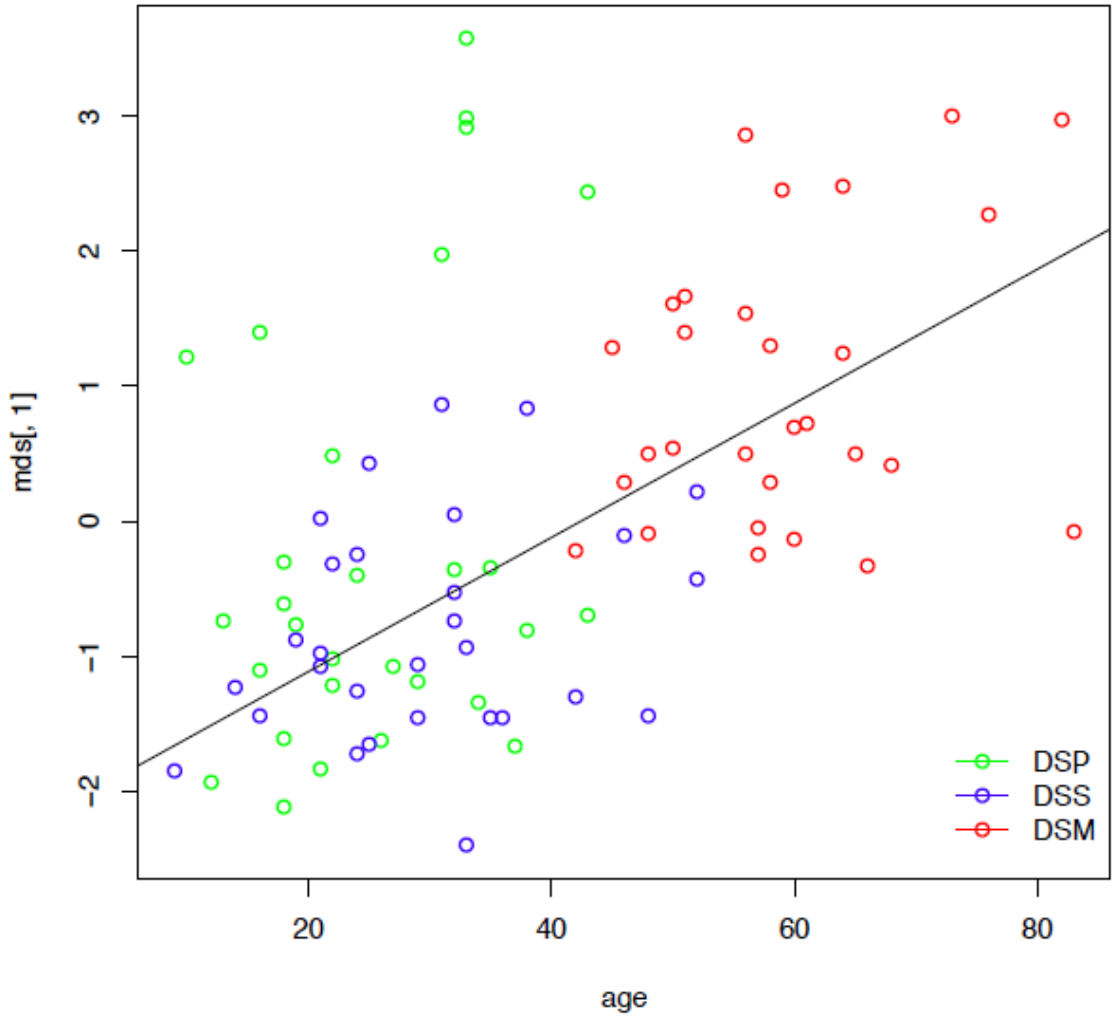

Figure 37. Correlation between MDS-1 of Figure 36 and age.

Among the loci which resulted differentially methylated between DSS and DSM we selected 3 genes in which a bubble of differential methylation was evident: *ELOVL2* (cg16867657, cg21572722, cg24724428), *FHL2* (cg06639320, cg22454769, cg24079702) and *PENK* (cg16419235, cg16219603, cg12877723). All the differentially methylated CpG dinucleotides were located in a CpG island. The three regions resulted hypermethylated in DSM respect to the DSS, while they did not display sex or family-associated differences in methylation levels.

We replicated these results in a larger sex-balanced cohort. Specifically, we analyzed whole blood DNA samples from 494 individuals (245 males and 249 females) aged between 9 and 99 years and 7 DNA samples extracted from cord blood (3 males and 4 females). We divided samples in 5 age classes, whose mean methylation values for each CpG unit are

reported in Figure 38 A. We then calculated Spearman's correlation between age and methylation level for each CpG unit. The highest values of correlation obtained were 0.92 (CpG_11.12.13.14), 0.80 (CpG_9.10 and CpG_19.20) and 0.63 (CpG_23.24) for *ELOVL2*, *FHL2* and *PENK* respectively (Figure 38 B).

In all the cases, the age-correlated sites tended to be hypermethylated with aging (Figure 39 A; for *FHL2* only the CpG_9.10 is shown). *ELOVL2* displayed the widest variation in methylation values, which ranged from 7% to 91% during aging. For *FHL2*, the methylation range was between 12% and 53%, while for *PENK* was compressed between 1% and 27% (Figure 39 A). In each gene, we identified a small subset of CpG units adjacent to the best age-correlated site, which displayed high correlation coefficients with age and whose methylation values were closely correlated with each other (Figure 39 B).

Among the 3 genes, *PENK* showed the lowest level of correlation with age and very small variations in methylation values. These variations are difficult to be assessed quantitatively by the EpiTYPER assay, which can detect at most a difference of 5% in methylation levels (Coolen et al. 2007), thus the evaluation of the correlation with age could be affected by experimental limits. *FHL2* and, to a major extent, *ELOVL2* displayed striking correlations and more consistent variations in DNA methylation with aging. In particular, *ELOVL2* CpG spans from 0% methylation in cord blood to almost 100% methylation in the oldest old, displaying an on-off switch mode between the two extreme regions of lifespan.

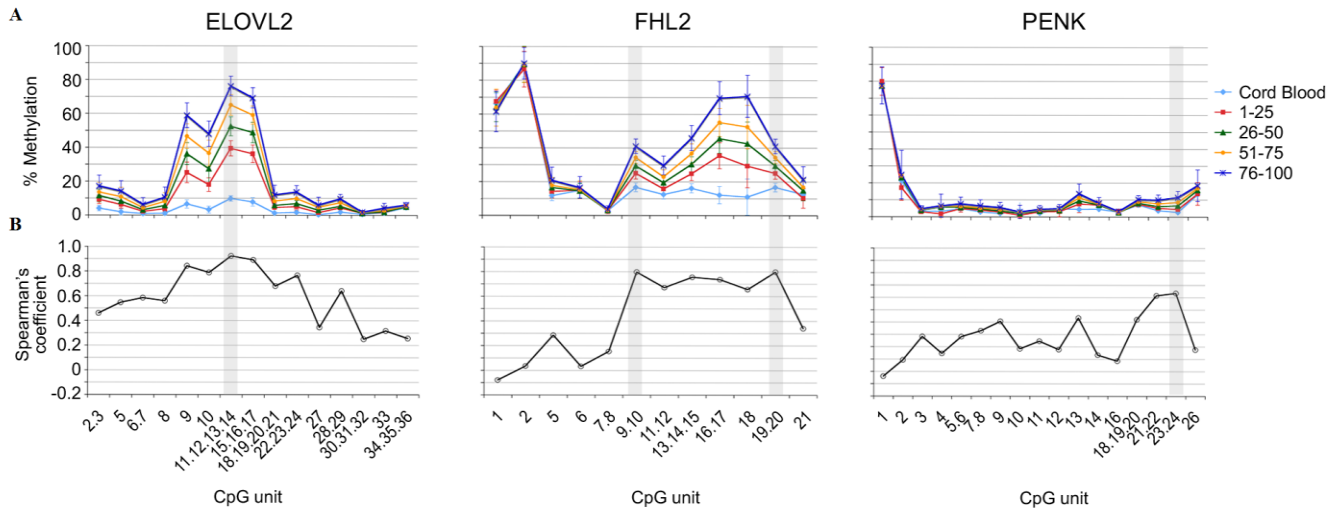

Figure 38. Replication study in Time serie cohort. (A) Mean methylation values ± standard deviation in 5 age classes are reported for each CpG unit. **(B)** Spearman's correlation coefficients for each CpG unit. Highly correlated regions are marked in gray (adapted from Garagnani et al., 2012).

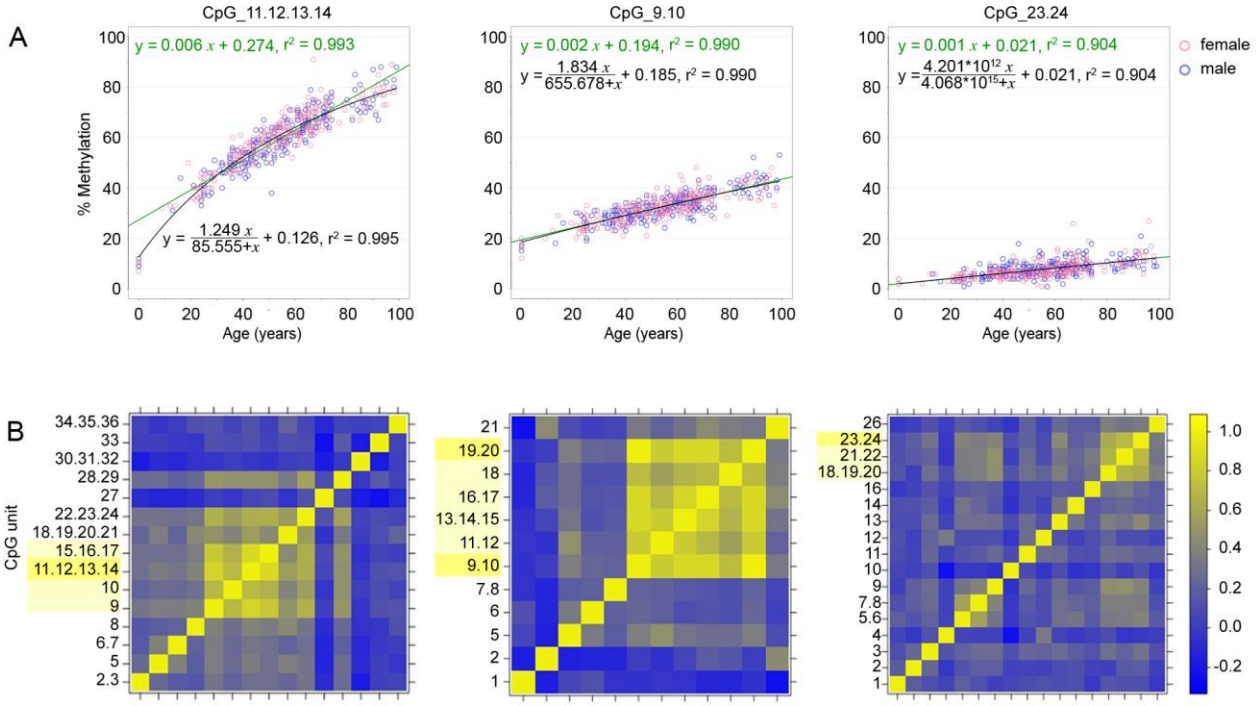

Figure 39. Replication study in Time Serie cohort. (A) Methylation values of the CpG unit that better correlates with age in each gene. **(B)** Correlation between CpG units within each CpG island. The most correlated CpG units are highlighted in yellow (adapted from Garagnani et al., 2012).

Discussion

In this study we investigated the epigenetic status of persons affected by Down Syndrome (DSP). In particular we studied DNA methylation of whole blood samples from DSP, in comparison with their mothers (DSM) and their unaffected sibs (DSS). In this way we obtained a family-based model that is able to minimize confounding genetics and environmental factors.

Region centric approach in methylation analysis

To identify specific genomic regions whose methylation is altered in DS, we used an approach that simultaneously considers the methylation values of a group of adjacent CpG probes, comparing them between DSP and DSS (region-centric analysis). As previously reported (Wessely and Emes 2012), this approach can have several advantages respect to single-probe analysis. First of all, it better resembles the biological basis of the process, considering that the phenotype is more likely to be affected by changes in a group of adjacent CpG sites, whose methylation status tends to be correlated, than by alterations of single CpG sites. Furthermore this region centric approach tends to provide less spurious results, due for example to the presence of SNPs in the probes included in the array (Wessely and Emes 2012).

Epigenetic Signature of Down Syndrome

In our analysis DS subjects showed a profound rearrangement of genome wide DNA methylation patterns.

Region-centric and site-specific analyses identified a large number of DMRs mapping in all the chromosomes, and Fisher's exact test showed enrichment in HSA21 for the DMRs belonging to Class A and Class C probes. Importantly, similar results were achieved when we considered the overall distributions of DNA methylation values, which resulted significantly different between DSP and healthy subjects for most of the chromosomes, but particularly marked for HSA21.

It is, however, difficult to say for certain if only the extra copy of chromosome 21 is aberrantly methylated or if the modifications of DNA methylation are due to all three copies of HSA21, because the Infinium 450 k provides an estimate of the mean methylation status

of DNA hybridized to the array. This point could be study in deep by analyzing allele-specific DNA methylation.

In our analysis the majority of DMRs previously identified in PBL from DS subjects (Kerkel et al. 2010) was confirmed, but the DMRs enrichment that we found in HSA21 was not observed in the three previous studies on the methylation patterns of individuals with DS. These differences may be due to different experimental procedures, analytical protocols, analyzed tissues and/or age classes of the samples.

Our data show a general hypermethylation of DMRs in DS, in agreement, even if less pronounced, with those reported in the Jin et al. study in placental tissue (Jin et al. 2013). In this study in fact it was observed a global DNA hypermethylation in DS placental tissue. Considering the type of tissue under investigation they proposed that these hypermethylation events may occur early during development, as a consequence of downregulation of Tet enzymes and/or of Rest transcription factor which have an important role in DNA demethylation and histone deacetylation/demethylation respectively (Jin et al. 2013). In our study we found *REST* N-shore slight hypermetilated in DSP, which may be correlated with the *REST* mRNA downregulation in DS suggested by Jin and co-workers. On the contrary, it is counterintuitive the observation that *TET1* S-shore was hypomethylated in blood from DSP compared to healthy controls. It would be interesting to experimentally verify if the methylation status of this region could affect the expression of the enzyme and in which direction.

The gene ontology analysis that we performed on the identified DMRs supports the perspective of DS as a developmental disease (Briggs et al. 2013), since they are involved in morphogenetic and developmental processes. Many differentially methylated genes in our study are involved in the development of the nervous and the immune system. These observations suggest a link between intrinsic defects that occur in the early stages of development (Kusters et al. 2009; Chou et al. 2012; Kusters, Verstegen, and de Vries 2011) and the phenotype of DS, such as premature aging of specific tissues.

Also KEGG analysis provided interesting inputs regarding the molecular basis of DS phenotype. For example PI3K-Akt-mTOR signalling pathway, that regulates fundamental cellular functions and has an important role in aging (Blagosklonny 2009; Blagosklonny

2010), is deregulated in DS in a early step of development (Iyer et al. 2014; Perluigi et al. 2014; Troca-Marín, Alves-Sampaio, and Montesinos 2011). The alteration in methylation that we found in genes involved in this signalling pathway can contribute to DS phenotype, including premature aging.

Starting from the analysis on Class A DMRs, we defined a Down Syndrome epigenetic signature in whole blood. We selected a short list of DMRs that showed methylation differences greater than 0.15 between the two experimental groups and that can be functionally linked to the various phenotypic aspects of the disease.
The signature includes genes involved in hematopoietic process like *RUNX1* and *EBF4*. *RUNX1* gene is located on chromosomal 21 and encodes for a transcription factor that has important functions in hematopoietic development, hematopoietic stem cell homeostasis and various blood malignancies like myeloid leukemia (Lam and Zhang 2012; Nižetić and Groet 2012), whose frequency is high among DS persons (Fonatsch 2010). In this regard *RUNX1* is also known as "acute myeloid leukemia 1" because its sequence was discovered from a patient with acute myeloid leukemia (Miyoshi et al. 1991; Lam and Zhang 2012). Also *EBF4* gene encodes for a transcriptional factor that could be responsible for hematological defects of subjects with DS. In fact *EBF4* product belongs to a family of transcriptional factor implicated in B-cell maturation (Wang, Betz, and Reed 2002). Many studies have identified in DS subjects a significant decrease of B lymphocyte absolute number and percentage (Cossarizza et al. 1990; Cossarizza et al. 1991; de Hingh et al. 2005).

In our epigenetic signature we found also genes involved in morphogenesis and development processes. In vertebrate's embryos the activation of *HOXA* cluster plays an important role in dynamic chromatin modifications suggesting that progressive epigenetic modifications could regulate gene activation and embryonic cell fate (Soshnikova and Duboule 2009).
The other category of genes that we found hypermethylated in DSP, regards genes implicated in neural development like *NCAM1* (also belonging to the previous category), which is located on chromosome 11 and encodes a cell adhesion protein that is a member of the immunoglobulin superfamily. In particular the encoded protein is involved in cell-to-cell interactions as well as cell-matrix interactions during development and differentiation, and

has an important role in development of the nervous system, retinal histogenesis and nerve-muscle interaction. Differences in cell surface distribution of these molecules and extent of glycosilation of *NCAM1* have been suggested as possible mechanisms that regulate cell adhesiveness (Reyes, Small, and Akeson 1991). It has been also demonstrated that in DS children the combined expression of *NCAM1* and *RUNX1* during stress haematopoiesis might be one of the principal causes of overt leukemia (Langebrake et al. 2006).

It is interesting to note that some genes described as DMRs between DSP and DSS have an important role in the development of tissues other than blood, first of all the central nervous system (CNS). Despite DNA methylation profile is highly tissue specific, the differences in DNA methylation observed in whole blood of DSP could be an index of epigenetic defects in other tissues such as the CNS that may contribute to the pathogenesis of DS.

Finally, in our short list are also included genes involved in the regulation of chromatin (Long, Blackledge, and Klose 2013), such as *KDM2B* and *TET1*. In particular *KDM2B* maps on chromosome 12 and its transcript is an histone lysine demethylase involved in the regulation of chromatin structure.

In mouse embryonic stem cells it has been demonstrated that Kdm2b recruits a protein complex known as polycomb repressive complex 1 (Prc1) to CpG islands of early lineage-specific genes (He et al. 2013; Wu, Johansen, and Helin 2013). In addition, the JmjC domain of the protein is required for the demethylation activity of the fourth trimethylated lysine on histone H3, while the zinc finger domain CXXC recognizes and binds to specific regions rich in CpG unmethylated. Human *KDM2B* also encodes for a nucleolar protein that represses the transcription of ribosomal RNA genes, binding the transcribed region of ribosomal DNA (Frescas et al. 2007).

Is important to note that the selected DNA methylation changes were common to all the analysed DS subjects, independently from their genetic and environmental background, and they were reproducible in an independent cohort.

Ribosomal genes methylation and gene expression

Our data show a DNA hypermethylation of *KDM2B* N-Shore in DSP. As Kdm2b activity contributes to repress rDNA expression, the epigenetic silencing of *KDM2B* expression could account for the increase in ribosomal gene activity previously observed in

lymphocytes from DS (Demirtas 2009; Demirtas et al. 2001). Starting from this interesting observation we decided to investigate the methylation status and the expression of the rDNA sequence in DSP, which is not included in the Infinium 450k design.

Most eukaryotic organisms present multiple copies of rRNA coding genes that are found as repeated clusters, usually arranged heat to tail (Reeder 1984). Human diploid cells contain approximately 400 copies of these genes, localized in the fibrillar centres and the dense fibrillar component of the nucleolus. In physiological conditions, about half of these copies are inactivated by epigenetic mechanisms (Bacalini et al. 2014) including the methylation of the promoter. In situ hybridization experiments revealed that the repeated units of rDNA, defined as organization of nucleolar regions (NOR), are localized on the short arms of the five acrocentric human chromosomes (13, 14, 15, 21 and 22) (Henderson, Warburton, and Atwood 1972). Each unit (~43 kb) includes the 47S rRNA encoding sequence, which is ~30 kb long, and a non-transcribed intergenic spacer of about ~430 kb. The ~50 bp region upstream of the initiation site (+1) is the core promoter and it is necessary and sufficient for initiation of basal transcription in most species. RNA pol I transcribes each rRNA copy in a primary immature transcript (rRNA 45S) of 14 kb and it is separated from others repeated units by a long intragenic space (IGS). The entire promoter is localized in the IGS between the transcribed units. Within each transcription unit there are two regions coding for the subunit 18S and 28S rRNA and a smaller region coding for the subunits 5.8S. The sequences for the *18S*, *5.8S* and for the *28S* are separated by internal spacer regions. After transcription the 45S rRNA precursor is processed in the 3 separated ribosomal RNA: 18S rRNA, 5.8S rRNA and 28S rRNA.

Literature shows that levels of Argilophilic Nucleolus Organizer Regions (AgNORs) are greater in lymphocytes and buccal cells of DS babies/infants (Yilmaz and Demirtas 2008; Imamoglu et al. 2005), an increase that is far higher than the expected value due to the extra rRNA genes on the extra-chromosome 21. This means a higher ribosomal expression during the early phase of cell development. On the other hand, the age-associated reduction of ribosome biogenesis seems to be more pronounced in cells of DS patients (Borsatto and Smith 1996). Another study shows that in vivo and in vitro examinations total RNA (80% rRNA) content in young DS patients' peripheral blood mononuclear cells (PBMC) is higher

than controls but it also demonstrated how it decreases with age and finally becomes lower as controls (Hamurcu, Demirtas, and Kumandas 2006).

Based on these considerations, we analysed 3 target regions localized at the promoter of the rDNA (*Ribo)* and at the 5' sequences of *18S* and *28S* regions in our cohort of DSP and healthy controls. As higher rRNA expression was described in DSP, at least in younger subjects, we expected a hypomethylation of rDNA. On the contrary, we found a partial DS hypermethylation in these regions. Although the modification in rDNA methylation was not in the expected direction, it is in any case interesting that DS is associated to an alteration in the epigenetic control of this locus. One intriguing possibility is that rDNA hypermethylation may be a "defense response" against rRNA hyperexpression that occurs in DS during development and the first years of life. Unfortunately, our DSP cohort includes only few young DSP, so it is difficult to unravel the methylation status of rDNA when rRNA is overexpressed. Importantly, a similar behaviour has been described in cancer (Bacalini et al. 2014), where rDNA hypermethylation was observed in several tumors compared to healthy tissues, despite the increase in ribosome biogenesis which characterizes cancer. Further analyses are needed to investigate this complex scenario. Moreover at present it is not possible to know if this alteration is due to the rDNA cluster on chromosome 21 or if a general rearrangement of methylation of several rDNA loci occurs.

Considering our methylation results we wondered whether hypermethylation of *KDM2B* N-shore and of some CpG within ribosomal genes in DSP could be associated to changes in *KDM2B* gene expression and of the 45S rRNA. To answer this question, we conducted an expression analysis by Real Time PCR, comparing 4 SD subjects with 4 sex- and age-matched controls. No expression differences for *KDM2B* and ribosomal genes were observed. Further studies should better investigate the relationship between *KDM2B* and rDNA methylation and expression, as well as the relationship between *KDM2B* and rDNA expression in the context of DS.

Epigenetic Signature of DSP and accelerated aging

Down Syndrome is traditionally classified as a progeroid disease (Martin 1982; Patterson and Cabelof 2012), as affected subjects exhibit precocious appearance of age-associated biomarkers like DNA damage accumulation and chromosomal instability (Jovanovic,

Clements, and MacLeod 1998; Nakamura and Tanaka 1998; Odetti et al. 1998; Praticò et al. 2000). However, accelerated aging in DS is segmental, as it involves some, but not all, organs and tissues, in particular the CNS (Lott and Head 2005; Teipel and Hampel 2006) and the immune system (Cuadrado and Barrena 1996; Cossarizza et al. 1990). To explore this topic, we took advantage of the increasing amount of data on the DNA methylation changes that occur during aging in blood cells (Garagnani et al. 2012; Gentilini et al. 2012; Hannum et al. 2013; Heyn et al. 2012; Rodríguez-Rodero et al. 2010). We selected a small fraction of genes whose methylation levels were age-dependent and verified if their methylation in blood from DSP was more similar to healthy subjects of the same age (DSS) or to healthy older subjects (DSM). The methylation of age-associated genes was substantially comparable between DSP and DSS, and DSP were more similar to DSM only for a minor fraction of the variability in their methylation patterns.

Collectively, our results suggest that from an epigenetic point of view Down Syndrome is not characterized by the early appearance of markers of physiological aging, but rather by intrinsic defects that are established early during development (Chou et al. 2012; Kusters, Verstegen, and de Vries 2011; Kusters et al. 2009) and lead to the precocious impairment in the function of specific tissues.

Epigenetic markers of age

We also identified regions differentially methylated between DSS and DSM, identifying 3 regions, the CpG islands of *ELOVL2*, *FHL2* and *PENK* genes, whose methylation status increased with aging (Garagnani et al. 2012). We confirmed this observation by Sequenom MassARRAY in a larger sex-balanced cohort of individuals aged between 0 and 99 years. In particular *ELOVL2* displayed the most consistent variations in DNA methylation with aging, spanning from 0% of methylation in cord blood to almost 100% methylation in the oldest old and displaying therefore an on-off switch mode between the two extreme of lifespan. *ELOVL2* is mainly expressed in the liver while its expression level in human blood cells is still unknown. This gene encodes for a fatty acid elongase that catalyzes the synthesis of polyunsaturated very long chain fatty acid (C20 and C24) as ω3 and ω6 polyunsaturated fatty acids (PUFA). PUFA play a wide range of functions, including regulation of membrane fluidity and function and immune response, where ω3 and ω6

PUFA have respectively anti- and pro-inflammatory roles. It is widely accepted that a correct ω3/ω6 ratio in adult diet positively influences cardiovascular and neurological health. However, it has also been demonstrated that human have a very limited capacity to synthesize long-chain PUFA and that healthy development of infants requires preformed-ω3 intake from milk (Plourde and Cunnane 2007). Genetic variants in *ELOVL2* genes have been associated with serum metabolic profile of human individuals (Illig et al. 2010) and to plasma levels of ω3 PUFA (Lemaitre et al. 2011).We cannot say for certain if age-related hypermethylation of *ELOVL2* CpG island in blood cells could have functional consequences on PUFA metabolism, because the expression and the role of *ELOVL2* in human blood cells have not been specifically addressed. Moreover we cannot exclude that observed variations in methylation levels of *ELOVL2* are caused by alterations in the number of specific subpopulations of blood cells. Considering that PUFAs are involved in crucial biological functions like energy production, modulation of inflammation, and maintenance of cell membrane integrity, *ELOVL2* methylation could play a role in the aging process through the regulation of different biological pathways (Garagnani et al. 2012).

While *PENK* showed the lowest level of methylation variation and correlation with age, for *FHL2* we can advance considerations similar to *ELOVL2*. *FHL2* encodes for a transcriptional cofactor that acts as scaffolding protein in different signalling pathways and that can also localize in cytoplasm, where it regulates cytoskeletal dynamics (Canault et al. 2006). It is involved in cell cycle regulation, differentiation and apoptosis, assembly of extracellular matrix, bone formation, and wound healing (Friedrich et al. 2014). Generally it is more expressed in heart and muscle, while a low expression is detected in blood cells like that we analyzed. *FHL2* can act as both activator and repressor in developmental processes, including osteoblast (Du et al. 2012) and cardiovascular (Chu and Chen 2011) differentiation, and over- and down-regulation of its expression are observed in different types of tumors (Kleiber, Strebhardt, and Martin 2007).

Conclusions

In the last decades two main theories on the molecular pathogenesis of Down Syndrome have been formulated. While the "Reductionist" view (Epstein 1990) also known as "Gene dosage effect" hypothesis, states that the cause of the disease is the presence of a limited number of dosage sensitive genes localized in a critical region (DSCR) of chromosome 21, the "Organicistic" view (Opitz and Gilbert-Barness 1990) or "Amplified developmental instability" hypothesis (Pritchard and Kola 1999) argues that the pathogenesis of Down Syndrome is strictly linked to the development process and suggests that the presence of a trisomic chromosome profoundly alters the genetic homeostasis, leading to a developmental instability.

Current research tends to favour a synthesis of the two theories, sustaining that "a triplicated gene, the solitary effect of which is inconspicuous, could contribute to a trisomic phenotype in combination with other genes, based on the specificity of effects and interactions of these genes" (Olson et al. 2004).

Our results support this new vision. Analysing the epigenome of persons affected by Down Syndrome we found differentially methylation regions that, although enriched on chromosome 21, interested most of the chromosomes. Importantly, several of these DMRs were functionally correlated with developmental defects characteristic of the disease.

As for the aim of the study, initially we posed three question.

Does an epigenetic signature of Down Syndrome exist?

We identified an epigenetic signature of Down Syndrome. In particular, we selected a short list of 68 genomic that were remarkably different in persons affected by Down Syndrome compared to healthy controls.

Does this signature reflect a phenomenon of accelerated aging?

This epigenetic signature reflects, at least in part, a phenomenon of accelerated aging. Our analysis suggests that, from an epigenetic point of view, Down Syndrome is characterized by low but significant early appearance of markers of physiological aging. However, we believe that the precocious functional aging of specific tissues characteristic of the disease is mostly driven by intrinsic defects that are established early during development.

Is there a functional correlate of this signature?

Further studies should better characterize the functional consequences of altered DNA methylation in Down Syndrome. In this thesis we attempted to explore this issue by analysing gene expression of *KDM2B* gene and of rRNA 45S, but we were not able to find significant alterations. However, it should considered that our analyses were performed on a very limited cohort, and that other studies suggest that alterations in ribosomal activity are evident mostly in DSP younger than 20 years. Moreover, it would be interesting to study ribosomal biogenesis using other sensitive and repeatable techniques such as AgNORs staining (Demirtas 2009; Yilmaz and Demirtas 2008).

Finally, it is worth to note that among the genes present on HSA21 there is DNMT3L which encodes for a protein without enzymatic activity, but that helps DNMT3a and DNMT3b in *de novo* methylation of DNA (Gowher et al. 2005). Alteration of DNMT3L expression/function due to trisomy could thus affect the establishment of patterns of DNA methylation during development.

Bibliography

Alvarado, S, Russell D F, Storey K B, and Szyf, M. 2014. "The Dynamic Nature of DNA Methylation: A Role in Response to Social and Seasonal Variation." *Integrative and Comparative Biology* 54 (1): 68–76. doi:10.1093/icb/icu034.

Antonarakis, S E, Lyle, R, Dermitzakis, E T, Reymond, A, and Deutsch, S. 2004. "Chromosome 21 and down Syndrome: From Genomics to Pathophysiology." *Nature Reviews. Genetics* 5 (10): 725–38. doi:10.1038/nrg1448.

Arosio, P, Abbate1, C, Zanetti, ,M, Caputo, L. 2004. "Sindrome Di Down. Alcuni Aspetti Socioambientali." *G Gerontol*. http://www.sigg.it/public/doc/GIORNALEART/182.pdf?r=0,1853187.

Bacalini, M G, Pacilli, A, Giuliani, C, Penzo, M, Treré, D, Pirazzini, C, Salvioli, S, Franceschi, C, Montanaro, L, and Garagnani, P. 2014. "The Nucleolar Size Is Associated to the Methylation Status of Ribosomal DNA in Breast Carcinomas." *BMC Cancer* 14 (January): 361. doi:10.1186/1471-2407-14-361.

Bacalini, M G, Gentilini, D, Boattini, A, Giampieri, E, Pirazzini, C, Giuliani, C, Fontanesi, E, Scurti, M, Remondini, D, Capri, M, Cocchi, G, Ghezzo, A, Collino, S, Del Rio, A, Luiselli, D, Vitale, G, Mari, D, Castellani, G, Fraga, M, Di Blasio, A M, Salvioli, S, Franceschi, C, AND Garagnani, P. 2015a. " Identification of a DNA methylation signature in blood from persons with Down syndrome. " *Aging.* 7 (1). In press.

Bacalini, M G, Boattini, A, Gentilini, D, Gianpieri, E, Pirazzini, C, Giuliani, C, Fontanesi, E, Remondini, D, Capri, M, Del Rio, A, Luiselli, D, Vitale, G, Mari, D, Castellani, G, di Blasio, A M, Salvioli, S, Franceschi, C, and Garagnani, P. 2015b. " A meta-analysis on age-associated changes in blood DNA methylation: results from an original analysis pipeline for Infinium 450k data". *Aging.* 7 (1). In press.

Barrena, M J, Echaniz, P, Garcia-Serrano, C, and Cuadrado, E. 1993. "Imbalance of the CD4+ Subpopulations Expressing CD45RA and CD29 Antigens in the Peripheral Blood of Adults and Children with Down Syndrome." *Scandinavian Journal of Immunology* 38 (4): 323–26. http://www.ncbi.nlm.nih.gov/pubmed/7692590.

Beck, S, Olek, A, and Walter, J. 1999. "From Genomics to Epigenomics: A Loftier View of Life." *Nature Biotechnology* 17 (12). *Nature America Inc.*: 1144. doi:10.1038/70651.

Beyan, H, Down, T A, Ramagopalan, S V, Uvebrant, K, Nilsson, A, Holland, M L, Gemma, C, Giovannoni, G, Boehm, B O, Ebers, G C, Lernmark, A, Cilio, C M, Leslie, R D, Rakyan, V K. 2012. "Guthrie Card Methylomics Identifies Temporally Stable Epialleles That Are Present at Birth in Humans." *Genome Research* 22 (11): 2138–45. doi:10.1101/gr.134304.111.

Biagi, E, Nylund, L, Candela, M, Ostan, R, Bucci, L, Pini, E, Nikkïla, J, Monti, D, Satokari, R, Franceschi, C, Brigidi, P, De Vos, W. 2010. "Through Ageing, and beyond: Gut Microbiota and Inflammatory Status in Seniors and Centenarians." *PloS One* 5 (5): e10667. doi:10.1371/journal.pone.0010667.

Bibikova, M, Barnes, B, Tsan, C, Ho, V, Klotzle, B, M Le, J, Delano, D, Zhang, L, Schroth, G P, Gunderson, K L, Fan, J B, and Shen, R. 2011. "High Density DNA Methylation Array with Single CpG Site Resolution." *Genomics* 98 (4): 288–95. doi:10.1016/j.ygeno.2011.07.007.

Bird, A. 2002. “DNA Methylation Patterns and Epigenetic Memory.” *Genes & Development* 16 (1): 6–21. doi:10.1101/gad.947102.

Biselli, J, Goloni-Bertollo, E, Ruiz, M, and Pavarino-Bertelli, E. 2010. “Cytogenetic Profile of Down Syndrome Cases Seen by a General Genetics Outpatient Service in Brazil.” *Down Syndrome Education International*. doi:10.3104/reports.2010.

Bittles, A H, and Glasson, E J. 2004. “Clinical, Social, and Ethical Implications of Changing Life Expectancy in Down Syndrome.” *Developmental Medicine and Child Neurology* 46 (4): 282–86. http://www.ncbi.nlm.nih.gov/pubmed/15077706.

Bittles, A H, Petterson, B A, Sullivan, S G, Hussain, R, Glasson, E J and Montgomery, P D. 2002. “The Influence of Intellectual Disability on Life Expectancy.” *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences* 57 (7): M470–72. http://www.ncbi.nlm.nih.gov/pubmed/12084811.

Blagosklonny, M V. 2009. “TOR-Driven Aging: Speeding Car without Brakes.” *Cell Cycle (Georgetown, Tex.)* 8 (24): 4055–59. http://www.ncbi.nlm.nih.gov/pubmed/19923900.

Blagosklonny, M V. 2010. “Revisiting the Antagonistic Pleiotropy Theory of Aging: TOR-Driven Program and Quasi-Program.” *Cell Cycle (Georgetown, Tex.)* 9 (16): 3151–56. doi:10.4161/cc.9.16.13120.

Borsatto, B, and Smith, M. 1996. “Reduction of the Activity of Ribosomal Genes with Age in Down’s Syndrome.” *Gerontology* 42 (3): 147–54. http://www.ncbi.nlm.nih.gov/pubmed/8796373.

Braunschweig, C L, Gomez, S, Sheean, P, Tomey, K M, Rimmer, J, and Heller, T. 2004. “Nutritional Status and Risk Factors for Chronic Disease in Urban-Dwelling Adults with Down Syndrome.” *American Journal of Mental Retardation : AJMR* 109 (2): 186–93. doi:10.1352/0895-8017(2004)109<186:NSARFF>2.0.CO;2.

Briggs, J A, Mason, E A, Ovchinnikov, D A, Wells, C A, and Wolvetang, E J. 2013. “Concise Review: New Paradigms for Down Syndrome Research Using Induced Pluripotent Stem Cells: Tackling Complex Human Genetic Disease.” *Stem Cells Translational Medicine* 2 (3): 175–84. doi:10.5966/sctm.2012-0117.

Burgio, G R, Lanzavecchia, A, Maccario, R, Vitiello, A, Plebani, A, and Ugazio, A G. 1978. “Immunodeficiency in Down’s Syndrome: T-Lymphocyte Subset Imbalance in Trisomic Children.” *Clinical and Experimental Immunology* 33 (2): 298–301. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1537565&tool=pmcentrez&rendertype=abstract.

Burgio, G R, Ugazio, A G, Nespoli, L, Marcioni, A F, Bottelli, A M, and Pasquali, F. 1975. “Derangements of Immunoglobulin Levels, Phytohemagglutinin Responsiveness and T and B Cell Markers in Down’s Syndrome at Different Ages.” *European Journal of Immunology* 5 (9): 600–603. doi:10.1002/eji.1830050904.

Bush, A, and Beail, N. 2004. “Risk Factors for Dementia in People with down Syndrome: Issues in Assessment and Diagnosis.” *American Journal of Mental Retardation : AJMR* 109 (2): 83–97. doi:10.1352/0895-8017(2004)109<83:RFFDIP>2.0.CO;2.

Canault, M, Tellier, E, Bonardo, B, Mas, E, Aumailley, M, Juhan-Vague, I, Nalbone, G, and Peiretti, F. 2006. "FHL2 Interacts with Both ADAM-17 and the Cytoskeleton and Regulates ADAM-17 Localization and Activity." *Journal of Cellular Physiology* 208 (2): 363–72. doi:10.1002/jcp.20671.

Capone, G T. 2001. "Down Syndrome: Advances in Molecular Biology and the Neurosciences." *Journal of Developmental and Behavioral Pediatrics : JDBP* 22 (1): 40–59. http://www.ncbi.nlm.nih.gov/pubmed/11265922.

Cevenini, E, Bellavista, E, Tieri, P, Castellani, G, Lescai, F, Francesconi, M, Mishto, M, Santoro, A, Valesin S, Salvioli, S, Capri, M, Zaikin, A, Monti, D, de Magalhães, J P, Franceschi, C. 2010. "Systems Biology and Longevity: An Emerging Approach to Identify Innovative Anti-Aging Targets and Strategies." *Current Pharmaceutical Design* 16 (7): 802–13. http://www.ncbi.nlm.nih.gov/pubmed/20388091.

Cevenini, E, Invidia, L, Lescai, F, Salvioli, S, Tieri, P, Castellani, G, and Franceschi, C. 2008. "Human Models of Aging and Longevity." *Expert Opinion on Biological Therapy* 8 (9). Informa UK Ltd London, UK: 1393–1405. doi:10.1517/14712598.8.9.1393.

Chen, T, Ueda, Y, Xie, S, and Li, E. 2002. "A Novel Dnmt3a Isoform Produced from an Alternative Promoter Localizes to Euchromatin and Its Expression Correlates with Active de Novo Methylation." *The Journal of Biological Chemistry* 277 (41): 38746–54. doi:10.1074/jbc.M205312200.

Chou, S T, Byrska-Bishop, M, Tober, J M, Yao, Y, Vandorn, D, Opalinska, J B, Mills, J A, Choi, J K, Speck, N A, Gadue, P, Hardison, R C, Nemiroff, R L, French, D L, Weiss, M J. 2012. "Trisomy 21-Associated Defects in Human Primitive Hematopoiesis Revealed through Induced Pluripotent Stem Cells." *Proceedings of the National Academy of Sciences of the United States of America* 109 (43): 17573–78. doi:10.1073/pnas.1211175109.

Chu, P H, and Chen, J. 2011. "The Novel Roles of Four and a Half LIM Proteins 1 and 2 in the Cardiovascular System." *Chang Gung Medical Journal* 34 (2): 127–34. http://www.ncbi.nlm.nih.gov/pubmed/21539754.

Contestabile, A, Benfenati, F, and Gasparini, L. 2010. "Communication Breaks-Down: From Neurodevelopment Defects to Cognitive Disabilities in Down Syndrome." *Progress in Neurobiology* 91 (1). Elsevier Ltd: 1–22. doi:10.1016/j.pneurobio.2010.01.003.

Coolen, M W, Statham, A L, Gardiner-Garden, M, and Clark, S J. 2007. "Genomic Profiling of CpG Methylation and Allelic Specificity Using Quantitative High-Throughput Mass Spectrometry: Critical Evaluation and Improvements." *Nucleic Acids Research* 35 (18): e119. doi:10.1093/nar/gkm662.

Corder, E H, Saunders, A M, Strittmatter, W J, Schmechel, D E, Gaskell, P C, Small, G W, Roses, A D, Haines, J L, and Pericak-Vance, M A. 1993. "Gene Dose of Apolipoprotein E Type 4 Allele and the Risk of Alzheimer's Disease in Late Onset Families." *Science (New York, N.Y.)* 261 (5123): 921–23. http://www.ncbi.nlm.nih.gov/pubmed/8346443.

Cossarizza, A, Monti, D, Montagnani, G, Ortolani, C, Masi, M, Zannotti, M and Franceschi, C. 1990. "Precocious Aging of the Immune System in Down Syndrome: Alteration of B Lymphocytes, T-Lymphocyte Subsets, and Cells with Natural Killer Markers." *American*

Journal of Medical Genetics. Supplement 7 (January): 213–18. http://www.ncbi.nlm.nih.gov/pubmed/2149950.

Cossarizza, A, Ortolani, C, Forti, E, Montagnani, G, Paganelli, R, Zannotti, M, Marini, M, Monti, D, and Franceschi, C. "Age-Related Expansion of Functionally Inefficient Cells with Markers of Natural Killer Activity in Down's Syndrome." *Blood* 77 (6): 1263–70.

Cossarizza, A. 1991. "Age-Related Expansion of Functionally Inefficient Cells with Markers of Natural Killer Activity in Down's Syndrome." *Blood* 77 (6): 1263–70.

Cuadrado, E, and Barrena, M J. 1996. "Immune Dysfunction in Down's Syndrome: Primary Immune Deficiency or Early Senescence of the Immune System?" *Clinical Immunology and Immunopathology* 78 (3): 209–14.

Cummings, M. 2010. *Human Heredity: Principles and Issues*. http://books.google.com/books?hl=it&lr=&id=1bw8AAAAQBAJ&pgis=1.

De Hingh, Y C M, van der Vossen, P W, Gemen, E F A, Mulder, A B, Hop, W C J, Brus, F, and de Vries, E. 2005. "Intrinsic Abnormalities of Lymphocyte Counts in Children with down Syndrome." *The Journal of Pediatrics* 147 (6): 744–47. doi:10.1016/j.jpeds.2005.07.022.

Deb, S, Braganza, J, Norton, N, Williams, H, Kehoe, P G, Williams, J, and Owen, M J. 2000. "APOE Epsilon 4 Influences the Manifestation of Alzheimer's Disease in Adults with Down's Syndrome." *The British Journal of Psychiatry : The Journal of Mental Science* 176 (May): 468–72. http://www.ncbi.nlm.nih.gov/pubmed/10912224.

Dekker, A D, De Deyn, P P, and Rots, M G. 2014. "Epigenetics: The Neglected Key to Minimize Learning and Memory Deficits in Down Syndrome." *Neuroscience and Biobehavioral Reviews* 45C (May). Elsevier Ltd: 72–84. doi:10.1016/j.neubiorev.2014.05.004.

Demirtas, H, Imamoglu, N, Dönmez, H, Cücer, N, Yilmaz, A, and Candemir, Z. 2001. "Condensed Chromatin Surface and NORs Surface Enhancement in Mitogen-Stimulated Lymphocytes of Down Syndrome Patients." *Annales de Génétique* 44 (2): 77–82.

Demirtas, H. 2009. "AgNOR Status in Down's Syndrome Infants and a Plausible Phenotype Formation Hypothesis." *Micron (Oxford, England: 1993)* 40 (5-6): 511–18. doi:10.1016/j.micron.2009.02.014.

Doi, A, Park, I-H, Wen, B, Murakami, P, Aryee, M J, Irizarry, R, Herb, B, Ladd-Acosta, C, Rho, J, Loewer, S, Miller, J, Schlaeger, T, Daley, GQ, and Feinberg, A P. 2009. "Differential Methylation of Tissue- and Cancer-Specific CpG Island Shores Distinguishes Human Induced Pluripotent Stem Cells, Embryonic Stem Cells and Fibroblasts." *Nature Genetics* 41 (12): 1350–53. doi:10.1038/ng.471.

Down, J L. 1995. "Observations on an Ethnic Classification of Idiots. 1866." *Mental Retardation* 33 (1): 54–56. http://www.ncbi.nlm.nih.gov/pubmed/7707939.

Du, J, Wang, Q, Wang, L, Wang, X, and Yang, P. 2012. "The Expression Pattern of FHL2 during Mouse Molar Development." *Journal of Molecular Histology* 43 (3): 289–95. doi:10.1007/s10735-012-9409-z.

Du, P, Zhang, X, Huang, C-C, Jafari, N, Kibbe, W A, Hou, L, and Lin, S M. 2010. "Comparison of Beta-Value and M-Value Methods for Quantifying Methylation Levels by Microarray Analysis." *BMC Bioinformatics* 11 (January): 587. doi:10.1186/1471-2105-11-587.

Dussault, A-A, and Pouliot, M. 2006. "Rapid and Simple Comparison of Messenger RNA Levels Using Real-Time PCR." *Biological Procedures Online* 8 (January): 1–10. doi:10.1251/bpo114.

Earl, C. J. C. 1934. "Mental Defect: By L. S. PENROSE, M.A., M.D. London: Sidgwick & Jackson, Ltd., 1933. Pp. 175. Price 8s. 6d." *The British Journal of Psychiatry* 80 (328): 114–15. doi:10.1192/bjp.80.328.114.

Eckmann-Scholz, C, Bens, S, Kolarova, J, Schneppenheim, S, Caliebe, A, Heidemann, S, von Kaisenberg, C, Kautza, M, Jonat, W, Siebert, R, and Ammerpohl, O. "DNA-Methylation Profiling of Fetal Tissues Reveals Marked Epigenetic Differences between Chorionic and Amniotic Samples." *PLoS ONE* 7 (6): e39014. doi:10.1371/journal.pone.0039014.

Eden, E, Navon, R, Steinfeld, I, Lipson, D, and Yakhini, Z. 2009. "GOrilla: A Tool for Discovery and Visualization of Enriched GO Terms in Ranked Gene Lists." *BMC Bioinformatics* 10: 48. doi:10.1186/1471-2105-10-48.

Epstein, C J. 1990. "The Consequences of Chromosome Imbalance." *American Journal of Medical Genetics. Supplement* 7 (January): 31–37. http://www.ncbi.nlm.nih.gov/pubmed/2149968.

Esteller, M. 2007. "Epigenetic Gene Silencing in Cancer: The DNA Hypermethylome." *Human Molecular Genetics* 16 Spec No (April): R50–59. doi:10.1093/hmg/ddm018.

Fazzari, M J, and Greally, J M. 2004. "Epigenomics: Beyond CpG Islands." *Nature Reviews. Genetics* 5 (6): 446–55. doi:10.1038/nrg1349.

Filion, G J P, Zhenilo, S, Salozhin, S, Yamada, D, Prokhortchouk, E, and Defossez, P-A. 2006. "A Family of Human Zinc Finger Proteins That Bind Methylated DNA and Repress Transcription." *Molecular and Cellular Biology* 26 (1): 169–81. doi:10.1128/MCB.26.1.169-181.2006.

Fonatsch, C. 2010. "The Role of Chromosome 21 in Hematology and Oncology." *Genes, Chromosomes & Cancer* 49 (6): 497–508. doi:10.1002/gcc.20764.

Franceschi, C, Monti, D, Cossarizza, A, Fagnoni, F, Passeri, G, and Sansoni, P. 1991. "Aging, Longevity, and Cancer: Studies in Down's Syndrome and Centenarians." *Annals of the New York Academy of Sciences* 621 (January): 428–40. http://www.ncbi.nlm.nih.gov/pubmed/1830464.

Franceschi, C, Monti, D, Cossarizza, A, Tomasi, A, Sola, P, and Zannotti, M. 1990. "Oxidative Stress, poly(ADP)ribosylation and Aging: In Vitro Studies on Lymphocytes from Normal and Down's Syndrome Subjects of Different Age and from Patients with Alzheimer's Dementia." *Advances in Experimental Medicine and Biology* 264 (January): 499–502. http://www.ncbi.nlm.nih.gov/pubmed/2173880.

Franceschi, C, Monti, D, Sansoni, P, and Cossarizza, A. 1995. "The Immunology of Exceptional Individuals: The Lesson of Centenarians." *Immunology Today* 16 (1): 12–16. http://www.ncbi.nlm.nih.gov/pubmed/7880382.

Franceschi, C, Monti, D, Scarfí, M R, Zeni, O, Temperani, P, Emilia, G, Sansoni, P, Lioi, M B, Troiano, L, Agnesini, C, Salvioli, S, Cossarizza A. 1992. "Genomic Instability and Aging." *Annals of the New York Academy of Sciences* 663 (1 Aging and Cel): 4–16. doi:10.1111/j.1749-6632.1992.tb38643.x.

Francesconi, M, Remondini, D, Neretti, N, Sedivy, J M, Cooper, L N, Verondini, E, Milanesi, L, and Castellani, G. 2008. "Reconstructing Networks of Pathways via Significance Analysis of Their Intersections." *BMC Bioinformatics* 9 Suppl 4: S9. doi:10.1186/1471-2105-9-S4-S9.

Frescas, D, Guardavaccaro, D, Bassermann, F, Koyama-Nasu, R, and Pagano, M. 2007. "JHDM1B/FBXL10 Is a Nucleolar Protein That Represses Transcription of Ribosomal RNA Genes." *Nature* 450 (7167): 309–13. doi:10.1038/nature06255.

Friedrich, Felix W, Silke Reischmann, Aileen Schwalm, Andreas Unger, Deepak Ramanujam, Julia Münch, Oliver J Müller, et al. 2014. "FHL2 Expression and Variants in Hypertrophic Cardiomyopathy." *Basic Research in Cardiology* 109 (6): 451. doi:10.1007/s00395-014-0451-8.

Frisoni, G B, J Louhija, C Geroldi, and M Trabucchi. 2001. "Longevity and the epsilon2 Allele of Apolipoprotein E: The Finnish Centenarians Study." *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences* 56 (2): M75–78. http://www.ncbi.nlm.nih.gov/pubmed/11213279.

Garagnani, P, Bacalini, M G, Pirazzini, C, Gori, D, Giuliani, C, Mari, M, Di Blasio, A M, Gentilini, D, Viatle, G, Collino, S, Rezzi, S,Castellani, G, Capri, M, Salvioli, S, and Franceschi, C. 2012. "Methylation of ELOVL2 Gene as a New Epigenetic Marker of Age." *Aging Cell* 11 (6): 1132–34. doi:10.1111/acel.12005.

Gardiner-Garden, M, and Frommer, M. 1987. "CpG Islands in Vertebrate Genomes." *Journal of Molecular Biology* 196 (2): 261–82. http://www.ncbi.nlm.nih.gov/pubmed/3656447.

Gentilini, D, Mari, D, Castaldi, D, Remondini, D, Ogliari, G, Ostan, R, Bucci, L, Sirchia, S M, Tabano, S, Cavagnini, F, Monti, D, Franceschi, C, Di Blasio, A M, and Vitale, G. 2012. "Role of Epigenetics in Human Aging and Longevity: Genome-Wide DNA Methylation Profile in Centenarians and Centenarians' Offspring." *Age (Dordrecht, Netherlands)*, August. doi:10.1007/s11357-012-9463-1.

Ghezzo, A, Salvioli, S, Solimando, M C, Palmieri, A, Chiostergi, C, Scurti, M, Lomartire, L, Bedetti, F, Cocchi, G, Follo, D, Pipitone, E, Rovatti, P, Zamberletti, J, Gomiero, T, Castellani, G, and Franceschi, C. 2014. "Age-Related Changes of Adaptive and Neuropsychological Features in Persons with down Syndrome." *PloS One* 9 (11): e113111. doi:10.1371/journal.pone.0113111.

Giannone, S, Strippoli, P, Vitale, L, Casadei, R, Canaider, S, Lenzi, L, D'Addabbo, P, Frabetti, F, Facchin, F, Farina, A, Carinci, P, and Zannotti, M. 2004. "Gene Expression Profile Analysis in Human T Lymphocytes from Patients with Down Syndrome." *Annals of Human Genetics* 68 (Pt 6): 546–54. doi:10.1046/j.1529-8817.2003.00123.x.

Glasson, EJ, Sullivan, S G, Hussain, R, Petterson, B A, Montgomery, P D, and Bittles, A H. 2002. "The Changing Survival Profile of People with Down's Syndrome: Implications for Genetic Counselling." *Clinical Genetics* 62 (5): 390–93. doi:10.1034/j.1399-0004.2002.620506.x.

Goll, M G, Kirpekar, F, Maggert, K A, Yoder, J A, Hsieh, C-L, Zhang, X, Golic,, K G, Jacobsen, S E, and Bestor, T H. 2006. "Methylation of tRNAAsp by the DNA Methyltransferase Homolog Dnmt2." *Science (New York, N.Y.)* 311 (5759): 395–98. doi:10.1126/science.1120976.

Gowher, H, Liebert, K, Hermann, A, Xu, G, and Jeltsch, A. 2005. "Mechanism of Stimulation of Catalytic Activity of Dnmt3A and Dnmt3B DNA-(cytosine-C5)-Methyltransferases by Dnmt3L." *The Journal of Biological Chemistry* 280 (14): 13341–48. doi:10.1074/jbc.M413412200.

Hamurcu, Z, Demirtas, H, and Kumandas, S. 2006. "Flow Cytometric Comparison of RNA Content in Peripheral Blood Mononuclear Cells of Down Syndrome Patients and Control Individuals." *Cytometry. Part B, Clinical Cytometry* 70 (1): 24–28. doi:10.1002/cyto.b.20077.

Hannum, G, Guinney, J, Zhao, L, Zhang, L, Hughes, G, Vas Sadda, S, Klotzle, B, Bibikova, M, Fan, J B, Gao, Y, Deconde, R, Chen, M, Rajapakse, I, Friend, S, Ideker, T, and Zhang, K. 2013. "Genome-Wide Methylation Profiles Reveal Quantitative Views of Human Aging Rates." *Molecular Cell* 49 (2): 359–67. doi:10.1016/j.molcel.2012.10.016.

Hasle, H, Clemmensen, I H, and Mikkelsen, M. 2000. "Risks of Leukaemia and Solid Tumours in Individuals with Down's Syndrome." *Lancet* 355 (9199): 165–69. doi:10.1016/S0140-6736(99)05264-2.

Hata, K, Okano, M, Lei, H, and Li, E. 2002. "Dnmt3L Cooperates with the Dnmt3 Family of de Novo DNA Methyltransferases to Establish Maternal Imprints in Mice." *Development (Cambridge, England)* 129 (8): 1983–93. http://www.ncbi.nlm.nih.gov/pubmed/11934864.

He, J, Shen, L, Wan, M, Taranova, O, Wu, H, and Zhang, Y. 2013. "Kdm2b Maintains Murine Embryonic Stem Cell Status by Recruiting PRC1 Complex to CpG Islands of Developmental Genes." *Nature Cell Biology* 15 (4): 373–84. doi:10.1038/ncb2702.

Head, E, Garzon-Rodriguez, W, Johnson, J K, Lott, I T, Cotman, C W, and Glabe, C. 2001. "Oxidation of Abeta and Plaque Biogenesis in Alzheimer's Disease and Down Syndrome." *Neurobiology of Disease* 8 (5): 792–806. doi:10.1006/nbdi.2001.0431.

Heijmans, B T, Kremer, D, Tobi, E W, Boomsma, D I, and Slagboom, P E. 2007. "Heritable rather than Age-Related Environmental and Stochastic Factors Dominate Variation in DNA Methylation of the Human IGF2/H19 Locus." *Human Molecular Genetics* 16 (5): 547–54. doi:10.1093/hmg/ddm010.

Hellman, A, and Chess, A. 2007. "Gene Body-Specific Methylation on the Active X Chromosome." *Science (New York, N.Y.)* 315 (5815): 1141–43. doi:10.1126/science.1136352.

Henderson, A S, Warburton, D, and Atwood, K C. 1972. "Location of Ribosomal DNA in the Human Chromosome Complement." *Proceedings of the National Academy of Sciences of the United States of America* 69 (11): 3394–98. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=389778&tool=pmcentrez&rendertype=abstract.

Hermann, A, Goyal, R, and Jeltsch, A. 2004. "The Dnmt1 DNA-(cytosine-C5)-Methyltransferase Methylates DNA Processively with High Preference for Hemimethylated Target Sites." *The Journal of Biological Chemistry* 279 (46): 48350–59. doi:10.1074/jbc.M403427200.

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, Savols, S, Pujana, M A, Serra-Musach, J, Iglesias-Platas, I, Formiga, F, Fernandez, A F, Fraga, M F, Health, S C, Valencia, A, Gut, I G, Wang, J, and Esteller, M. 2012. "Distinct DNA Methylomes of Newborns and Centenarians." *Proceedings of the National Academy of Sciences of the United States of America* 109 (26): 10522–27. doi:10.1073/pnas.1120658109.

Hook, E B, Cross, P K, and Schreinemachers, D M. 1983. "Chromosomal Abnormality Rates at Amniocentesis and in Live-Born Infants." *JAMA* 249 (15). *American Medical Association*: 2034. doi:10.1001/jama.1983.03330390038028.

Houseman, E A, Accomando, W P, Koestler, D C, Christensen, B C, Marsit, C J, Nelson, H H, Wiencke, J K and Kelsey, KT. 2012. "DNA Methylation Arrays as Surrogate Measures of Cell Mixture Distribution." *BMC Bioinformatics* 13 (January): 86. doi:10.1186/1471-2105-13-86.

Illig, T, Gieger, C, Zhai, G, Römisch-Margl, W, Wang-Sattler, R, Prehn, C, Altmaier, E, Kastenmüller, G, Kato, B S, Mewes, H W, Meitinger, T, de Angelis, M H, Kronenberg, F, Soranzo, N, Wichmann, H E, Spector, T D, Adamski, J, and Suhre, K. 2010. "A Genome-Wide Perspective of Genetic Variation in Human Metabolism." *Nature Genetics* 42 (2): 137–41. doi:10.1038/ng.507.

Imamoglu, N, Demirtas, H, Donmez-Altuntas, H, and Ilten, A. 2005. "Higher NORs-Expression in Lymphocyte of Trisomy 21 Babies/children: In Vivo Evaluation." *Micron (Oxford, England : 1993)* 36 (6): 503–7. doi:10.1016/j.micron.2005.05.002.

Irizarry, R A, Ladd-Acosta, C, Wen, B, Wu, Z, Montano, C, Onyango, P, Cui, H, Gabo, K, Rongione, M, Webster, M, Ji, H, Potash, J B, Sabunciyan, S, and Feinberg, A P. 2009. "The Human Colon Cancer Methylome Shows Similar Hypo- and Hypermethylation at Conserved Tissue-Specific CpG Island Shores." *Nature Genetics* 41 (2): 178–86. doi:10.1038/ng.298.

Isbir, T, Agaçhan, B, Yilmaz, H, Aydin, M, Kara, I, Eker, D, and Eker, E. 2001a. "Interaction between Apolipoprotein-E and Angiotensin-Converting Enzyme Genotype in Alzheimer's Disease." *American Journal of Alzheimer's Disease and Other Dementias* 16 (4): 205–10. http://www.ncbi.nlm.nih.gov/pubmed/11501342.

Isbir, T, Agaçhan, B, Yilmaz,H, Aydin, M, Kara, I, Eker, E, and Eker, D. 2001b. "Apolipoprotein-E Gene Polymorphism and Lipid Profiles in Alzheimer's Disease." *American Journal of Alzheimer's Disease and Other Dementias* 16 (2): 77–81. http://www.ncbi.nlm.nih.gov/pubmed/11302074.

Iyer, A M, van Scheppingen, J, Milenkovic, I, Anink, J J, Adle-Biassette, H, Kovacs, G G, and Aronica, E. 2014. "mTOR Hyperactivation in down Syndrome Hippocampus Appears Early during Development." *Journal of Neuropathology and Experimental Neurology* 73 (7): 671–83. doi:10.1097/NEN.0000000000000083.

Jaenisch, R, and Bird, A. 2003. "Epigenetic Regulation of Gene Expression: How the Genome Integrates Intrinsic and Environmental Signals." *Nature Genetics* 33 Suppl (March): 245–54. doi:10.1038/ng1089.

Ji, H, Ehrlich, L I R, Seita, J, Murakami, P, Doi, A, Lindau, P, Lee, H, Aryee, M J, Irizarry, R A, Kim, K, Rossi, D J, Inlay, M A, Serwold, T, Karsunky, H, Ho, L, Daley, G Q, Weissman, I L,

and Feinberg, A P. 2010. “Comprehensive Methylome Map of Lineage Commitment from Haematopoietic Progenitors.” *Nature* 467 (7313): 338–42. doi:10.1038/nature09367.

Jia, D, Jurkowska, R Z, Zhang, X, Jeltsch, A, and Cheng, X. 2007. “Structure of Dnmt3a Bound to Dnmt3L Suggests a Model for de Novo DNA Methylation.” *Nature* 449 (7159): 248–51. doi:10.1038/nature06146.

Jin, S, Lee, Y K, Lim, Y C, Zheng, Z, Lin, X M, Ng, D P Y, Holbrook, J D, Law, H Y, Kwek, K Y, Yeo, G S, and Ding, Cet. 2013. “Global DNA Hypermethylation in down Syndrome Placenta.” *PLoS Genetics* 9 (6): e1003515. doi:10.1371/journal.pgen.1003515.

Jones, M J, Farré, P, McEwen, L M, Macisaac, J L, Watt, K, Neumann, S M, Emberly, E, Cynader, M S, Virji-Babul, N, and Kobor, M S. 2013. “Distinct DNA Methylation Patterns of Cognitive Impairment and Trisomy 21 in Down Syndrome.” *BMC Medical Genomics* 6 (January): 58. doi:10.1186/1755-8794-6-58.

Jones, P L, Veenstra, G J, Wade, P A, Vermaak, D, Kass, S U, Landsberger, N, Strouboulis, J, and Wolffe, A P. 1998. “Methylated DNA and MeCP2 Recruit Histone Deacetylase to Repress Transcription.” *Nature Genetics* 19 (2): 187–91. doi:10.1038/561.

Jones, P A. 2012. “Functions of DNA Methylation: Islands, Start Sites, Gene Bodies and Beyond.” *Nature Reviews. Genetics* 13 (7). Nature Publishing Group: 484–92. doi:10.1038/nrg3230.

Jones, P A, and Baylin, S B. 2002. “The Fundamental Role of Epigenetic Events in Cancer.” *Nature Reviews. Genetics* 3 (6): 415–28. doi:10.1038/nrg816.

Jovanovic, S V, Clements, D, and MacLeod, K. 1998. “Biomarkers of Oxidative Stress Are Significantly Elevated in Down Syndrome.” *Free Radical Biology & Medicine* 25 (9): 1044–48.

Kerkel, K, Schupf, N, Hatta, K, Pang, D, Salas, M, Kratz, A, Minden, M, Murty, V, Zigman, W B, Mayeux, R P, Jenkins, E C, Torkamani, A, Schork, N J, Silverman, W, Croy, B A, Tycko, B. 2010. “Altered DNA Methylation in Leukocytes with Trisomy 21.” *PLoS Genetics* 6 (11): e1001212. doi:10.1371/journal.pgen.1001212.

Kleiber, K, Strebhardt, K, and Martin, B T. 2007. “The Biological Relevance of FHL2 in Tumour Cells and Its Role as a Putative Cancer Target.” *Anticancer Research* 27 (1A): 55–61. http://www.ncbi.nlm.nih.gov/pubmed/17352216.

Korenberg, J R, Chen, X N, Schipper, R, Sun, Z, Gonsky, R, Gerwehr, S, Carpenter, N, Daumer, C, Dignan, P, and Disteche, C. 1994. “Down Syndrome Phenotypes: The Consequences of Chromosomal Imbalance.” *Proceedings of the National Academy of Sciences of the United States of America* 91 (11): 4997–5001. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=43917&tool=pmcentrez&rendertype=abstract.

Korenberg, J R, Kawashima, H, Pulst, S M, Ikeuchi, T, Ogasawara, N, Yamamoto, K, Schonberg, S A, West, R, Allen, L, and Magenis, E. 1990. “Molecular Definition of a Region of Chromosome 21 That Causes Features of the Down Syndrome Phenotype.” *American Journal of Human Genetics* 47 (2): 236–46.

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1683719&tool=pmcentrez&rendertype=abstract.

Krasuski, J S, Alexander, G E, Horwitz, B, Rapoport, S I, and Schapiro, M B. 2002. "Relation of Medial Temporal Lobe Volumes to Age and Memory Function in Nondemented Adults with Down's Syndrome: Implications for the Prodromal Phase of Alzheimer's Disease." *The American Journal of Psychiatry* 159 (1): 74–81. http://www.ncbi.nlm.nih.gov/pubmed/11772693.

Kuroda, A, Rauch, T A, Todorov, I, Ku, H T, Al-Abdullah, I H, Kandeel, F, Mullen, Y, Pfeifer, G P, and Ferreri, K. 2009. "Insulin Gene Expression Is Regulated by DNA Methylation." *PloS One* 4 (9): e6953. doi:10.1371/journal.pone.0006953.

Kusters, M A A, Verstegen, R H J, Gemen, E F A, and de Vries, E. 2009. "Intrinsic Defect of the Immune System in Children with Down Syndrome: A Review." *Clinical and Experimental Immunology* 156 (2): 189–93. doi:10.1111/j.1365-2249.2009.03890.x.

Kusters, M A A, Verstegen, R H J, and de Vries, E. 2011. "Down Syndrome: Is It Really Characterized by Precocious Immunosenescence?" *Aging and Disease* 2 (6): 538–45. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3295065&tool=pmcentrez&rendertype=abstract.

Lai, F, Kammann, E, Rebeck, G W, Anderson, A, Chen, Y, and Nixon, R A. 1999. "APOE Genotype and Gender Effects on Alzheimer Disease in 100 Adults with Down Syndrome." *Neurology* 53 (2): 331–36. http://www.ncbi.nlm.nih.gov/pubmed/10430422.

Lam, K, and Zhang, D-E. 2012. "RUNX1 and RUNX1-ETO: Roles in Hematopoiesis and Leukemogenesis." *Frontiers in Bioscience: A Journal and Virtual Library* 17: 1120–39.

Langebrake, C, Klusmann, J H, Wortmann, K, Kolar, M, Puhlmann, U, and Reinhardt, D. 2006. "Concomitant Aberrant Overexpression of RUNX1 and NCAM in Regenerating Bone Marrow of Myeloid Leukemia of Down's Syndrome." *Haematologica* 91 (11): 1473–80. http://www.haematologica.org/content/91/11/1473.full-text.pdf+html.

Lemaitre, R N, Tanaka, T, Tang, W, Manichaikul, A, Foy, M, Kabagambe, E K, Nettleton, J A, King, I B, Weng, L C, Bhattacharya, S, Bandinelli, S, Bis, J C, Rich, S S, Jacobs, D R Jr, Cherubini, A, McKnight, B, Liang, S, Gu, X, Rice, K, Laurie, C C, Lumley, T, Browning, B L, Psaty, B M, Chen, Y D, Friedlander, Y, Djousse, L, Wu, J H, Siscovick, D S, Uitterlinden, A G, Arnett, D K, Ferrucci, L, Fornage, M, Tsai, M Y, Mozaffarian, D, and Steffen, L M. 2011. "Genetic Loci Associated with Plasma Phospholipid N-3 Fatty Acids: A Meta-Analysis of Genome-Wide Association Studies from the CHARGE Consortium." *PLoS Genetics* 7 (7): e1002193. doi:10.1371/journal.pgen.1002193.

Letourneau, A, Santoni, F A, Bonilla, X, Sailani, M R, Gonzalez, D, Kind, J, Chevalier, C, Thurman, R, Sandstrom, R S, Hibaoui, Y, Garieri, M, Popadin, K, Falconnet, E, Gagnebin, M, Gehrig, C, Vannier, A, Guipponi, M, Farinelli, L, Robyr, D, Migliavacca, E, Borel, C, Deutsch, S, Feki, A, Stamatoyannopoulos, J A, Herault, Y, van Steensel, B, Guigo, R, and Antonarakis, S E. 2014. "Domains of Genome-Wide Gene Expression Dysregulation in Down's Syndrome." *Nature* 508 (7496): 345–50. doi:10.1038/nature13200.

Levin, S, Schlesinger, M, Handzel, Z, Hahn, T, Altman, Y, Czernobilsky, B, and Boss, J. 1979. "Thymic Deficiency in Down's Syndrome." *Pediatrics* 63 (1): 80–87. http://www.ncbi.nlm.nih.gov/pubmed/155804.

Li, C-M, Guo, M, Salas, M, Schupf, N, Silverman, W, Zigman, W B, Husain, S, Warburton, D, Thaker, H, and Tycko, B. 2006. "Cell Type-Specific over-Expression of Chromosome 21 Genes in Fibroblasts and Fetal Hearts with Trisomy 21." *BMC Medical Genetics* 7 (January): 24. doi:10.1186/1471-2350-7-24.

Li, Y, Zhu, J, Tian, G, Li, N, Li, Q, Ye, M, Zhang, H, Chen, Q, Luo, R, Chen, M, He, Y, Jin, X, Zhang, Q, Yu, C, Zhou, G, Sun, J, Huang, Y, Zheng ,H, Cao, H, Zhou, X, Guo, S, Hu, X, Li, X, Kristiansen, K, Bolund, L, Xu, J, Wang, W, Yang, H, Wang, J, Li, R, Beck, S, Wang, J, and Zhang, X. 2010. "The DNA Methylome of Human Peripheral Blood Mononuclear Cells." *PLoS Biology* 8 (11): e1000533. doi:10.1371/journal.pbio.1000533.

Lockitch, G, Singh, V K, Puterman, M L, Godolphin, W J, Sheps, S, Tingle, A J, Wong, F, and Quigley, G. 1987. "Age-Related Changes in Humoral and Cell-Mediated Immunity in Down Syndrome Children Living at Home." *Pediatric Research* 22 (5): 536–40. doi:10.1203/00006450-198711000-00013.

Long, H K, Blackledge, N P, and Klose, R J. 2013. "ZF-CxxC Domain-Containing Proteins, CpG Islands and the Chromatin Connection." *Biochemical Society Transactions* 41 (3): 727–40. doi:10.1042/BST20130028.

Lopez-Serra, L, and Esteller, M. 2008. "Proteins That Bind Methylated DNA and Human Cancer: Reading the Wrong Words." *British Journal of Cancer* 98 (12): 1881–85. doi:10.1038/sj.bjc.6604374.

Lott, I T, and Head, E. 2005. "Alzheimer Disease and Down Syndrome: Factors in Pathogenesis." *Neurobiology of Aging* 26 (3): 383–89. doi:10.1016/j.neurobiolaging.2004.08.005.

Lubec, G, and Engidawork, E. 2002. "The Brain in Down Syndrome (TRISOMY 21)." *Journal of Neurology* 249 (10): 1347–56. doi:10.1007/s00415-002-0799-9.

Lyle, R, Béna, F, Gagos, S, Gehrig, C, Lopez, G, Schinzel, A, Lespinasse, J, Bottani, A, Dahoun, S, Taine, L, Doco-Fenzy, M, Cornillet-Lefèbvre, P, Pelet, A, Lyonnet, S, Toutain, A, Colleaux, L, Horst, J, Kennerknecht, I, Wakamatsu, N, Descartes, M, Franklin, J C, Florentin-Arar, L, Kitsiou, S, Aït Yahya-Graison, E, Costantine, M, Sinet, PM, Delabar, JM, and Antonarakis, S E. 2009. "Genotype-Phenotype Correlations in Down Syndrome Identified by Array CGH in 30 Cases of Partial Trisomy and Partial Monosomy Chromosome 21." *European Journal of Human Genetics : EJHG* 17 (4): 454–66. doi:10.1038/ejhg.2008.214.

Mao, R, Wang, X, Spitznagel, E L, Frelin, L P, Ting, J C, Ding, H, Kim, J-W, Ruczinski, I, Downey, T J, and Pevsner, J. 2005. "Primary and Secondary Transcriptional Effects in the Developing Human Down Syndrome Brain and Heart." *Genome Biology* 6 (13): R107. doi:10.1186/gb-2005-6-13-r107.

Martin, G M. 1982. "Syndromes of Accelerated Aging." *National Cancer Institute Monograph* 60: 241–47.

Mayeux, R, Stern, Y, Ottman, R, Tatemichi, T K, Tang, M X, Maestre, G, Ngai, C, Tycko, B, and Ginsberg, H. 1993. "The Apolipoprotein Epsilon 4 Allele in Patients with Alzheimer's Disease." *Annals of Neurology* 34 (5): 752–54. doi:10.1002/ana.410340527.

Miyoshi, H, Shimizu, K, Kozu, T, Maseki, N, Kaneko, Y, and Ohki, M. 1991. "t(8;21) Breakpoints on Chromosome 21 in Acute Myeloid Leukemia Are Clustered within a Limited Region of a Single Gene, AML1." *Proceedings of the National Academy of Sciences of the United States of America* 88 (23): 10431–34. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=52942&tool=pmcentrez&rendertype=abstract.

Moarefi, A H, and Chédin, F. 2011. "ICF Syndrome Mutations Cause a Broad Spectrum of Biochemical Defects in DNMT3B-Mediated de Novo DNA Methylation." *Journal of Molecular Biology* 409 (5): 758–72. doi:10.1016/j.jmb.2011.04.050.

Montanaro, L, Calienni, M, Bertoni, S, Rocchi, L, Sansone, P, Storci, G, Santini, D, Ceccarelli, C, Taffurelli, M, Carnicelli, D, Brigotti, M, Bonafè, M, Treré, D, and Derenzini, M. 2010. "Novel Dyskerin-Mediated Mechanism of p53 Inactivation through Defective mRNA Translation." *Cancer Research* 70 (11): 4767–77. doi:10.1158/0008-5472.CAN-09-4024.

Nakamura, E, and Tanaka, S. 1998. "Biological Ages of Adult Men and Women with Down's Syndrome and Its Changes with Aging." *Mechanisms of Ageing and Development* 105 (1-2): 89–103.

Nasi, M, Troiano, L, Lugli, E, Pinti, M, Ferraresi, R, Monterastelli, E, Mussi, C, Salvioli, G, Franceschi, C, and Cossarizza, A. 2006. "Thymic Output and Functionality of the IL-7/IL-7 Receptor System in Centenarians: Implications for the Neolymphogenesis at the Limit of Human Life." *Aging Cell* 5 (2): 167–75. doi:10.1111/j.1474-9726.2006.00204.x.

Neri, G, and Opitz, J M. 2009. "Down Syndrome: Comments and Reflections on the 50th Anniversary of Lejeune's Discovery." *American Journal of Medical Genetics. Part A* 149A (12): 2647–54. doi:10.1002/ajmg.a.33138.

Newberger, D S. 2000. "Down Syndrome: Prenatal Risk Assessment and Diagnosis." *American Family Physician* 62 (4): 825–32, 837–38. http://www.ncbi.nlm.nih.gov/pubmed/10969860.

Nimura, K, Ishida, C, Koriyama, H, Hata, K, Yamanaka, S, Li, E, Ura, K, and Kaneda, Y. 2006. "Dnmt3a2 Targets Endogenous Dnmt3L to ES Cell Chromatin and Induces Regional DNA Methylation." *Genes to Cells : Devoted to Molecular & Cellular Mechanisms* 11 (10): 1225–37. doi:10.1111/j.1365-2443.2006.01012.x.

Nižetić, D, and Groet, J. 2012. "Tumorigenesis in Down's Syndrome: Big Lessons from a Small Chromosome." *Nature Reviews. Cancer* 12 (10): 721–32. doi:10.1038/nrc3355.

Odetti, P, Angelini, G, Dapino, D, Zaccheo, D, Garibaldi, S, Dagna-Bricarelli, F, Piombo, G, Perry, G, Smith, M, Traverso, N, and Tabaton, M. 1998. "Early Glycoxidation Damage in Brains from Down's Syndrome." *Biochemical and Biophysical Research Communications* 243 (3): 849–51. doi:10.1006/bbrc.1998.8186.

Okano, M, Bell, D W, Haber, D A, and Li, E. 1999. "DNA Methyltransferases Dnmt3a and Dnmt3b Are Essential for de Novo Methylation and Mammalian Development." *Cell* 99 (3): 247–57. http://www.ncbi.nlm.nih.gov/pubmed/10555141.

Okano, M, Xie, S, and Li, E. 1998. "Cloning and Characterization of a Family of Novel Mammalian DNA (cytosine-5) Methyltransferases." *Nature Genetics* 19 (3): 219–20. doi:10.1038/890.

Olson, L E, Richtsmeier, J T, Leszl, J, and Reeves, R H. 2004. "A Chromosome 21 Critical Region Does Not Cause Specific Down Syndrome Phenotypes." *Science (New York, N.Y.)* 306 (5696): 687–90. doi:10.1126/science.1098992.

Opitz, J M, and Gilbert-Barness, E F. 1990. "Reflections on the Pathogenesis of Down Syndrome." *American Journal of Medical Genetics. Supplement* 7 (January): 38–51. http://www.ncbi.nlm.nih.gov/pubmed/2149972.

Oster, J, Mikkelsen, M and Nielsen, A. 1975. "Mortality and Life-Table in Down's Syndrome." *Acta Paediatrica Scandinavica* 64 (2): 322–26. http://www.ncbi.nlm.nih.gov/pubmed/124122.

Patterson, D. 2007. "Genetic Mechanisms Involved in the Phenotype of Down Syndrome." *Mental Retardation and Developmental Disabilities Research Reviews* 13 (3): 199–206. doi:10.1002/mrdd.20162.

Patterson, D, and Cabelof, D C. 2012. "Down Syndrome as a Model of DNA Polymerase Beta Haploinsufficiency and Accelerated Aging." *Mechanisms of Ageing and Development* 133 (4): 133–37. doi:10.1016/j.mad.2011.10.001.

Penrose, L S. 1949. "The Incidence of Mongolism in the General Population." *The British Journal of Psychiatry* 95 (400): 685–88. doi:10.1192/bjp.95.400.685.

Penzo, M, Casoli, L, Pollutri, D, Sicuro, L, Ceccarelli, C, Santini, D, Taffurelli, M, Govoni, M, Brina, D, Trerè, D, and Montanaro, L. 2014. "JHDM1B Expression Regulates Ribosome Biogenesis and Cancer Cell Growth in a p53 Dependent Manner." *International Journal of Cancer. Journal International Du Cancer*, October. doi:10.1002/ijc.29240.

Perluigi, M, Pupo, G, Tramutola, A, Cini, C, Coccia, R, Barone, E, Head, E, Butterfield, D A, and Di Domenico, F. 2014. "Neuropathological Role of PI3K/Akt/mTOR Axis in Down Syndrome Brain." *Biochimica et Biophysica Acta* 1842 (7): 1144–53. doi:10.1016/j.bbadis.2014.04.007.

Pike, C J, Carroll, J C, Rosario, E R, and Barron, A M. 2009. "Protective Actions of Sex Steroid Hormones in Alzheimer's Disease." *Frontiers in Neuroendocrinology* 30 (2): 239–58. doi:10.1016/j.yfrne.2009.04.015.

Plourde, M, and Cunnane, S C. 2007. "Extremely Limited Synthesis of Long Chain Polyunsaturates in Adults: Implications for Their Dietary Essentiality and Use as Supplements." *Applied Physiology, Nutrition, and Metabolism = Physiologie Appliquée, Nutrition et Métabolisme* 32 (4): 619–34. doi:10.1139/H07-034.

Portela, A, and Esteller, M. 2010. "Epigenetic Modifications and Human Disease." *Nature Biotechnology* 28 (10): 1057–68. doi:10.1038/nbt.1685.

Prandini, P, Deutsch, S, Lyle, R, Gagnebin, M, Delucinge Vivier, C, Delorenzi, M, Gehrig, C, Descombes, P, Sherman, S, Dagna Bricarelli, F, Baldo, C, Novelli, A, Dallapiccola, B, Antonarakis, S E. 2007. "Natural Gene-Expression Variation in Down Syndrome Modulates the Outcome of Gene-Dosage Imbalance." *American Journal of Human Genetics* 81 (2): 252–63. doi:10.1086/519248.

Prasher, V P, Chowdhury, T A, Rowe, B R, and Bain, S C. 1997. "ApoE Genotype and Alzheimer's Disease in Adults with Down Syndrome: Meta-Analysis." *American Journal of Mental Retardation : AJMR* 102 (2): 103–10. doi:10.1352/08958017(1997)102<0103:AGAADI>2.0.CO;2.

Praticò, D, Iuliano, L, Amerio, G, Tang, L X, Rokach, J, Sabatino, G, and Violi, F. 2000. "Down's Syndrome Is Associated with Increased 8,12-Iso-iPF2alpha-VI Levels: Evidence for Enhanced Lipid Peroxidation in Vivo." *Annals of Neurology* 48 (5): 795–98.

Price, M E, Cotton, A M, Lam, L L, Farré, P, Emberly, E, Brown, C J, Robinson, W P, and Kobor, M S. 2013. "Additional Annotation Enhances Potential for Biologically-Relevant Analysis of the Illumina Infinium HumanMethylation450 BeadChip Array." *Epigenetics & Chromatin* 6 (1): 4. doi:10.1186/1756-8935-6-4.

Pritchard, M A, and Kola, I. 1999. "The 'Gene Dosage Effect' Hypothesis versus the 'Amplified Developmental Instability' Hypothesis in Down Syndrome." *Journal of Neural Transmission. Supplementum* 57 (January): 293–303. http://www.ncbi.nlm.nih.gov/pubmed/10666684.

Pueschel, S M. 1990. "Clinical Aspects of Down Syndrome from Infancy to Adulthood." *American Journal of Medical Genetics. Supplement* 7 (January): 52–56. http://www.ncbi.nlm.nih.gov/pubmed/2149974.

Rachidi, M, and Lopes, C. 2007. "Mental Retardation in Down Syndrome: From Gene Dosage Imbalance to Molecular and Cellular Mechanisms." *Neuroscience Research* 59 (4): 349–69. doi:10.1016/j.neures.2007.08.007.

Rachidi, M, and Lopes, C. 2008. "Mental Retardation and Associated Neurological Dysfunctions in Down Syndrome: A Consequence of Dysregulation in Critical Chromosome 21 Genes and Associated Molecular Pathways." *European Journal of Paediatric Neurology : EJPN : Official Journal of the European Paediatric Neurology Society* 12 (3): 168–82. doi:10.1016/j.ejpn.2007.08.010.

Reeder, R H. 1984. "Enhancers and Ribosomal Gene Spacers." *Cell* 38 (2): 349–51. http://www.ncbi.nlm.nih.gov/pubmed/6467370.

Reyes, A A, Small, S J, and Akeson, R. 1991. "At Least 27 Alternatively Spliced Forms of the Neural Cell Adhesion Molecule mRNA Are Expressed during Rat Heart Development." *Molecular and Cellular Biology* 11 (3): 1654–61. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=369464&tool=pmcentrez&rendertype=abstract.

Roat, E, Prada, N, Lugli, E, Nasi, M, Ferraresi, R, Troiano, L, Giovenzana, C, Pinti, M, Biagioni, O, Mariotti, M, Di Iorio, A, Consolo, U, Balli, F, and Cossarizza, A. 2008. "Homeostatic Cytokines and Expansion of Regulatory T Cells Accompany Thymic Impairment in Children with Down Syndrome." *Rejuvenation Research* 11 (3): 573–83. doi:10.1089/rej.2007.0648.

Robakis, N K, Wisniewski, H M, Jenkins, E C, Devine-Gage, E A, Houck, G E, Yao, X L, Ramakrishna, N, Wolfe, G, Silverman, W P, and Brown, W T. 1987. "Chromosome 21q21 Sublocalisation of Gene Encoding Beta-Amyloid Peptide in Cerebral Vessels and Neuritic (senile) Plaques of People with Alzheimer Disease and Down Syndrome." *Lancet* 1 (8529): 384–85. http://www.ncbi.nlm.nih.gov/pubmed/2880184.

Rodríguez-Rodero, S, Fernández-Morera, J L, Fernandez, A F, Menéndez-Torre, E, and Fraga, M F. 2010. "Epigenetic Regulation of Aging." *Discovery Medicine* 10 (52): 225–33.

Roizen, N J, and Patterson, D. 2003. "Down's Syndrome." *The Lancet* 361 (9365): 1281–89. doi:10.1016/S0140-6736(03)12987-X.

Royston, M C, Mann, D, Pickering-Brown, S, Owen, F, Perry, R, Ragbavan, R, Khin-Nu, C, Tyner, S, Day, K, Crook, R, Hardy, J, and Roberts, G W. 1996. "ApoE2 Allele, Down's Syndrome, and Dementia." *Annals of the New York Academy of Sciences* 777 (January): 255–59. http://www.ncbi.nlm.nih.gov/pubmed/8624094.

Royston, M C, Mann,D, Pickering-Brown, S, Owen, F, Perry, R, Raghavan, R, Khin-Nu, C, Tyrer, S, Day, K, and Crook, R. 1994. "Apolipoprotein E Epsilon 2 Allele Promotes Longevity and Protects Patients with Down's Syndrome from Dementia." *Neuroreport* 5 (18): 2583–85. http://www.ncbi.nlm.nih.gov/pubmed/7696609.

Rozovski, U, Jonish-Grossman, A, Bar-Shira, A, Ochshorn, Y, Goldstein, M, and Yaron, Y. 2007. "Genome-Wide Expression Analysis of Cultured Trophoblast with Trisomy 21 Karyotype." *Human Reproduction (Oxford, England)* 22 (9): 2538–45. doi:10.1093/humrep/dem214.

Rubinsztein, D C, Hon, J, Stevens, F, Pyrah, I, Tysoe, C, Huppert, F A, Easton, D F, and Holland, A J. 1999. "Apo E Genotypes and Risk of Dementia in Down Syndrome." *American Journal of Medical Genetics* 88 (4): 344–47. http://www.ncbi.nlm.nih.gov/pubmed/10402500.

Schupf, N, Kapell, D, Lee, J H, Zigman, W, Canto, B, Tycko, B, and Mayeux, R. 1996. "Onset of Dementia Is Associated with Apolipoprotein E epsilon4 in Down's Syndrome." *Annals of Neurology* 40 (5): 799–801. doi:10.1002/ana.410400518.

Schupf, N, Kapell, D, Nightingale, B, Rodriguez, A, Tycko, B, and Mayeux, R. 1998. "Earlier Onset of Alzheimer's Disease in Men with Down Syndrome." *Neurology* 50 (4): 991–95. http://www.ncbi.nlm.nih.gov/pubmed/9566384.

Schupf, N, and Sergievsky, G H. 2002. "Genetic and Host Factors for Dementia in Down's Syndrome." *The British Journal of Psychiatry : The Journal of Mental Science* 180 (May): 405–10. http://www.ncbi.nlm.nih.gov/pubmed/11983636.

Scriver, C R. 2001. "The Metabolic & Molecular Bases of Inherited Disease". McGraw-Hill,.

Singer, J, Roberts-Ems, J, and Riggs, A. 1979. "Methylation of Mouse Liver DNA Studied by Means of the Restriction Enzymes Msp I and Hpa II." *Science* 203 (4384): 1019–21. doi:10.1126/science.424726.

Soshnikova, N, and Duboule, D. 2009. "Epigenetic Regulation of Vertebrate Hox Genes: A Dynamic Equilibrium." *Epigenetics: Official Journal of the DNA Methylation Society* 4 (8): 537–40.

Stazi, M A, Nisticò, L, and Serino, L. 2012. "Al Di Là Della Genetica." *Epidemiologia & Prevenzione*. http://www.epiprev.it/rubrica/al-di-là-della-genetica.

Straussman, R, Nejman, D, Roberts, D, Steinfeld, I, Blum, B, Benvenisty, N, Simon, I, Yakhini, Z, and Cedar, H. 2009. "Developmental Programming of CpG Island Methylation Profiles in the Human Genome." *Nature Structural & Molecular Biology* 16 (5): 564–71. doi:10.1038/nsmb.1594.

Suetake, I, Shinozaki, F, Miyagawa, J, Takeshima, H, and Tajima, S. 2004. "DNMT3L Stimulates the DNA Methylation Activity of Dnmt3a and Dnmt3b through a Direct Interaction." *The Journal of Biological Chemistry* 279 (26): 27816–23. doi:10.1074/jbc.M400181200.

Talens, R P, Christensen, K, Putter, H, Willemsen, G, Christiansen, L, Kremer, D, Suchiman, H E D, Slagboom, P E, Boomsma, D I, and Heijmans, B T. 2012. "Epigenetic Variation during the Adult Lifespan: Cross-Sectional and Longitudinal Data on Monozygotic Twin Pairs." *Aging Cell* 11 (4): 694–703. doi:10.1111/j.1474-9726.2012.00835.x.

Teipel, S J, and Hampel, H. 2006. "Neuroanatomy of Down Syndrome in Vivo: A Model of Preclinical Alzheimer's Disease." *Behavior Genetics* 36 (3): 405–15. doi:10.1007/s10519-006-9047-x.

Thompson, R F, Fazzari, M J, and Greally, J M. 2010. "Experimental Approaches to the Study of Epigenomic Dysregulation in Ageing." *Experimental Gerontology* 45 (4): 255–68. doi:10.1016/j.exger.2009.12.013.

Thompson, R F., Suzuki, M, Lau, K W, and Greally, J M. 2009. "A Pipeline for the Quantitative Analysis of CG Dinucleotide Methylation Using Mass Spectrometry." *Bioinformatics* 25 (17): 2164–70. doi:10.1093/bioinformatics/btp382.

Thomson, J P, Skene, P J, Selfridge, J, Clouaire, T, Guy, J, Webb, S, Kerr, A R W, Deaton, A, Andrews, R, James, K D, Turner, D J, Illingworth, R, and Bird, A. 2010. "CpG Islands Influence Chromatin Structure via the CpG-Binding Protein Cfp1." *Nature* 464 (7291): 1082–86. doi:10.1038/nature08924.

Troca-Marín, J A, Alves-Sampaio, A and Montesinos, M L. 2011. "An Increase in Basal BDNF Provokes Hyperactivation of the Akt-Mammalian Target of Rapamycin Pathway and Deregulation of Local Dendritic Translation in a Mouse Model of Down's Syndrome." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 31 (26): 9445–55. doi:10.1523/JNEUROSCI.0011-11.2011.

Ugazio, A G, Maccario, R, Notarangelo, L D, and Burgio, G R. 1990. "Immunology of Down Syndrome: A Review." *American Journal of Medical Genetics. Supplement* 7 (January): 204–12. http://www.ncbi.nlm.nih.gov/pubmed/2149949.

Valle, D. 2006. "The Online Metabolic and Molecular Bases of Inherited Disease." http://ommbid.mhmedical.com/book.aspx?bookid=474.

Visser, F E, Aldenkamp, A P, van Huffelen, A C, Kuilman, M, Overweg, J, and van Wijk, J. 1997. "Prospective Study of the Prevalence of Alzheimer-Type Dementia in Institutionalized Individuals with Down Syndrome." *American Journal of Mental Retardation : AJMR* 101 (4): 400–412. http://www.ncbi.nlm.nih.gov/pubmed/9017086.

Wang, D, Yan, L, Hu, Q, Sucheston, L E, Higgins, M J, Ambrosone, C B, Johnson, C S, Smiraglia, D J, and Liu, S. 2012. "IMA: An R Package for High-Throughput Analysis of Illumina's 450K Infinium Methylation Data." *Bioinformatics (Oxford, England)* 28 (5): 729–30. doi:10.1093/bioinformatics/bts013.

Wang, S S, Betz, A G, and Reed, R R. 2002. "Cloning of a Novel Olf-1/EBF-like Gene, O/E-4, by Degenerate Oligo-Based Direct Selection." *Molecular and Cellular Neurosciences* 20 (3): 404–14.

Wessely, F, and Emes, R D. 2012. "Identification of DNA Methylation Biomarkers from Infinium Arrays." *Frontiers in Genetics* 3 (January): 161. doi:10.3389/fgene.2012.00161.

Wisniewski, K E, Wisniewski, H M, and Wen. G Y. 1985. "Occurrence of Neuropathological Changes and Dementia of Alzheimer's Disease in Down's Syndrome." *Annals of Neurology* 17 (3): 278–82. doi:10.1002/ana.410170310.

Wu, X, Johansen, J V, and Helin, K. 2013. "Fbxl10/Kdm2b Recruits Polycomb Repressive Complex 1 to CpG Islands and Regulates H2A Ubiquitylation." *Molecular Cell* 49 (6): 1134–46. doi:10.1016/j.molcel.2013.01.016.

Yilmaz, S I, and Demirtas, H. 2008. "AgNOR Increase in Buccal Epithelial Cells of Trisomy 21 Infants." *Micron (Oxford, England : 1993)* 39 (8): 1262–65. doi:10.1016/j.micron.2008.03.014.

Zana, M, Janka, Z, and Kálmán, J. 2007. "Oxidative Stress: A Bridge between Down's Syndrome and Alzheimer's Disease." *Neurobiology of Aging* 28 (5): 648–76. doi:10.1016/j.neurobiolaging.2006.03.008.

Zigman, W B, Schupf, N, Jenkins, E C, Urv, T K, Tycko, B, and Silverman, W. 2007. "Cholesterol Level, Statin Use and Alzheimer's Disease in Adults with Down Syndrome." *Neuroscience Letters* 416 (3): 279–84. doi:10.1016/j.neulet.2007.02.023.

Zigman, W B. 2013. "Atypical Aging in down Syndrome." *Developmental Disabilities Research Reviews* 18 (1): 51–67. doi:10.1002/ddrr.1128.

Zipursky, A. 2003. "Transient Leukaemia--a Benign Form of Leukaemia in Newborn Infants with Trisomy 21." *British Journal of Haematology* 120 (6): 930–38. http://www.ncbi.nlm.nih.gov/pubmed/12648061.

Zizka, Z, Calda, P, Fait, T, Haakova, L, Kvasnicka, J, and Viskova, H. 2006. "Prenatally Diagnosable Differences in the Cellular Immunity of Fetuses with Down's and Edwards' Syndrome." *Fetal Diagnosis and Therapy* 21 (6): 510–14. doi:10.1159/000095663.

Zweiger, G, and Scott, R W. 1997. "From Expressed Sequence Tags to 'Epigenomics': An Understanding of Disease Processes." *Current Opinion in Biotechnology* 8 (6): 684–87. http://www.ncbi.nlm.nih.gov/pubmed/9425656.