UNIVERSITA' DEGLI STUDI DI MILANO

Scuola di Dottorato in Scienze Biomediche Cliniche e Sperimentali
Corso di Dottorato in Biotecnologie Applicate alle Scienze Mediche
Ciclo XXIV

Tesi di dottorato di ricerca

DNA METHYLATION AS A PREDISPOSITION FACTOR IN THE PATHOGENESIS OF CONGENITAL HYPOTHYROIDISM IN PRETERM INFANTS

Facoltà di Medicina e Chirurgia
Settore Scientifico Disciplinare Bio13

Coordinatore: Prof. Enrico GINELLI

Tutor: Prof. Luca PERSANI

Dottorando: Federica MARELLI

Matricola n. R08135

Anno Accademico 2011-2012

Table of contests

ABSTRACT

Congenital hypothyroidism (CH) is the most frequent endocrinopathy in newborn. If not promptly treated lead to a severe impairment of psychomotor development. The etiophatogenesis of CH is still poorly understood; although several causative genes are identified, they can explain only a small portion of the pathological phenotypes. This scenario is further complicated if we focused on the incidence of CH in different context. In fact, epidemiological data indicate that children born prematurely have a 3-5 fold higher risk of CH. In addition premature infants born small for gestational age (SGA) have a risk of 12% higher to develop IC compared to prematures with appropriate development (AGA). The mechanisms that justify the increased risk of IC are still unknown. Some studies report that aberrant methylation patterns are associated with prematurity, intrauterine fetal development and the onset of some diseases. This project is focused on the study of DNA methylation, as predisposing factor to permanent thyroid dysfunction with neonatal onset.

Using the *Illumina Infinium-HumanMethylation27* technology we analyzed the global DNA methylation patterns (AVGβ) and selected the differentially methylated genes (DMGs) between 31 CH-cases born premature, AGA or SGA, and 28 term or preterm controls. To better understand the relationship between the DNA methylation and the premature birth, the intrauterine growth and the thyroid defect, the following groups were selected according to the gestational age at birth: 12 CH-with very preterm birth (CH-VPB<32wks) and 19 CH-with preterm birth (CH-PB 32-37wks); Controls: 9-CVPB, 6-CPB, 12-term birth (CTB>37wks). The same subjects were then analyzed according to intrauterine growth (20 CH-SGA, 11 CH-AGA than 6 C-SGA and 20 C-AGA) or the degree of CH: 19 with overt CH (OH, TSH>10 μU/L) and 12 with mild CH (MH, TSH<10 μU/L) than 16 CPB and 12 CTB.

The global methylation analysis showed that infants born prematurely and SGA have a significant hypomethylation than term-controls. These data were confirmed by the gene-specific methylation analysis, through which we selected a large group of differentially methylated genes (DMGs) in CH-cases than term controls. Interestingly, the 95% of the DMGs are hypomethylated and the 70% of them are represented by CpG sites located in DNA non-coding regions. The gene ontology analysis revealed that genes involved in fetal growth and thyroid hormone metabolism were included among DMGs. The analysis of nine maternal genomic DNA for polymorphisms at the *MTHFR* revealed the possible association with folate deficiency during pregnancy and the global hypomethylation status of affected newborns.

This is the first work exploring the role of epigenetic influences in the predisposition to congenital hypothyroidism. Our results suggest that genomic instability caused by global hypomethylation of non-coding regions may be related to premature birth and fetal growth delay. Under these conditions, thyroid defects are more frequent than expected and could result from the increased expression of predisposing genes, rather than from the reduced expression of protective genes. The role of maternal conditions during pregnancy seems to be a key factor to determine a proper DNA methylation pattern on fetus. Based on the Developmental Origin of Health and Disease Theory, we can assume that adverse condition during pregnancy, such as folate deficiency, may produce a fetal epigenetic reprogramming and the adaptation of preterm neonate to the extrauterine life includes among other dysfunctions a thyroid functional impairment. If this data will be confirmed by further experiments, this could represent a new predisposing factor to take into account during pregnancy to prevent and improve the prenatal screening of CH.

INTRODUCTION-CHAPTER I

1. THYROID GLAND DEVELOPMENT AND DEFECTS

1.1 FETAL THYROID DEVELOPMENT: A BRIEF OVERVIEW

The development of fetal thyroid function is dependent on the embryogenesis, differentiation and maturation of the thyroid gland. This is coupled with evolution of the hypothalamic-pituitary-thyroid axis and thyroid hormone metabolism, resulting in the regulation of thyroid hormone action, production and secretion [Fagman and Nilsson, 2011]. Thyroid hormones are critical for development of the fetal and neonatal brain, as well as for many other aspects of fetal growth. Before onset of function of its own thyroid gland, the only source of thyroid hormone for the fetus is the maternal thyroid gland [Panicker, 2011].

1.1.1 Morphological aspects

Thyroid gland is a composite of two different cell types, the follicular cells responsible for the production of thyroid hormones triiodothyronine (T3) and tiroxine (T4), and parafullicular C cells that produce calcitonin. Thyroid follicular cells (TFCs) are required to the thyroid fate from the endodermal epithelium of the foregut. C-cell precursors migrate from the neural crest to the fourth pharyngeal pouch located symmetrically on both sides of the neck (figure 1, panel A) [Fagman and Nilsson, 2010].

Specification of these two cell types marks the beginning of the morphogenesis of the thyroid gland. The development of the thyroid, through anatomic studies in humans and genetic studies in mice, can be described generally in the following steps (figure 1, panel B) [Fagman and Nilsson, 2011]. In human, the first visible manifestation of the gland, the thyroid anlage begins as a thickening of the endodermal epithelium in the midline of the primitive pharynx at embryonic

day 20-22. Cellular proliferation of the TFC results in a thyroid bud that begins to migrate caudally from the pharyngeal floor leaving a remnant of descent known as the thyroglossal duct. The developing thyroid will pass through, or adjacent to, the hyoid bone on its course to the trachea the migration process nears completion by day 45. Under normal conditions the thyroglossal duct, which connects the thyroid to its pharyngeal origin, will disappear. Simultaneously to the thyroid migration process, the C cells within the fourth pharyngeal pouch have localized to a transient embryologic region called the ultimobranchial body (UB). From its lateral origin, the UB bodies will migrate medially from either side of the neck. By day 70, the TFC and C cells that make up the mature and differentiated thyroid gland have now merged anterior to the cricoid cartilage on the trachea. The thyroid gland then begins to expand and the TFC, which vastly outnumber the interspersed C cells, organized into follicles. The final dispersion if C-cells within the thyroid are not uniform as they are concentrated within the middle and upper thirds of the lateral lobes of the gland. Functional differentiation of the TFC, and ultimately hormone production, is the final step in normal organogenesis. The proteins and pathways required for hormone synthesis are expressed once the thyroid reaches its final location on the trachea [Parlato *et al.*, 2004].

Mice models demonstrate that T4 is present shortly after folliculogenesis begins and human fetal serum contains both thyroid-stimulating hormone (TSH) and T4 after 12 weeks. Much of what drivers thyroid morphogenesis, has been determined from a combination of studies on inherited disorders of the thyroid and mice models which are considered an excellent homolog for human thyroid development [Van Vliet, 2003].

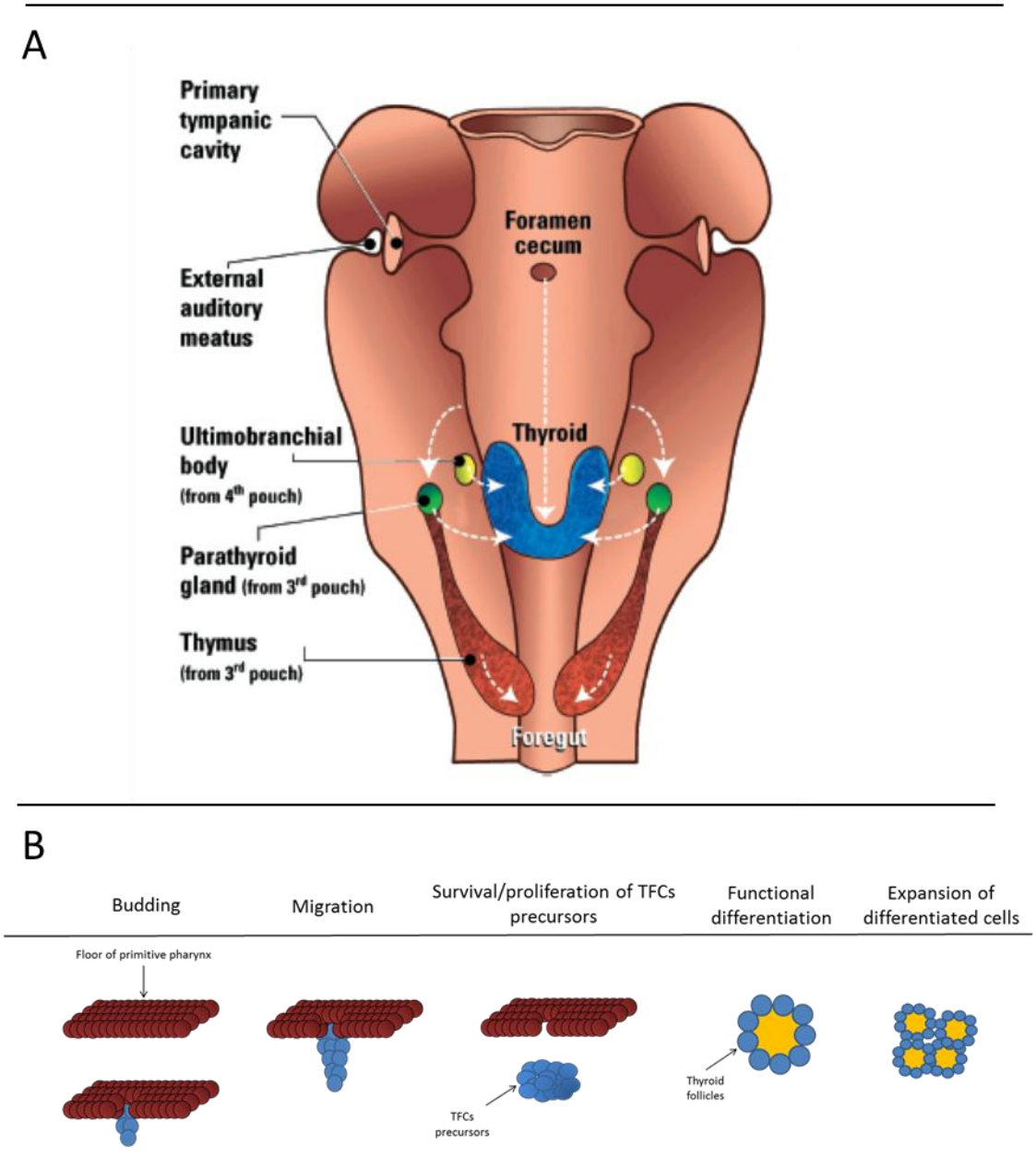


Figure 1: Overview of thyroid morphogenesis. **Panel A**: A frontal view of the migration paths of the lateral and median thyroid anlagen in relationship with other structures of the head and neck [Parlato *et al.*, 2004]. **Panel B**: Schematic representation of the stages of development of the thyroid follicular cells [Van Vliet, 2003].

1.1.2 Genetic component on thyroid development

The molecular basis of thyroid gland development began to be investigated with the discovery that the transcription factor Titf1/Nkx2-1, identified as responsible for the thyroid-specific expression of Tg and TPO, is expressed not only in functioning thyroid cells but also in their precursors [Gillam and Kopp, 2001]. Subsequently, the transcription factors Foxe1, Pax8, and Hhex were also found to be expressed both in mature thyroid cells and in their precursors [Di Lauro, 2003]. The expression of these factors in the thyroid anlage, at the very beginning of thyroid morphogenesis, immediately suggested that these genes might play an important role in the organogenesis of the thyroid gland. These factors are also present in other embryonic tissues, but all four are co-expressed only in the thyroid anlage [Van Vilet, 2003]. Thus, the small number of cells in the primitive pharynx fated to become TFCs already at E24 are univocally characterized by the simultaneous expression of Titf1/Nkx2-1, [De Felice and Di Lauro, 2007], Foxe1 [Kimura, 2011], Pax8 [Trueba *et al.*, 2005], and Hhex [Lacroix *et al.*, 2006]. When the thyroid diverticulum forms and begins its migration, the expression of these factors is restricted to the thyroid primordium as they are never expressed in the thyroglossal duct [Fagman and Nilsson, 2011].

These genes continue to drive thyroid development until the gland has completed migration and begins to enlarge. At this stage other genes are activated and pathways for hormone synthesis begin to develop. In the 10th and 11th week it is the serial expression of genes such as Fgfr2 and Tshr that prompts the production of thyroglobulin (Tg), thyroid peroxidase (TPO), and the TSH receptor (Tshr) [De Felice and Di Lauro, 2007]. By the 12th week the sodium-iodide symporter (NIS) gene is activated and soon after the NIS is found in thyroid cell membranes. This heralds the final step in cellular differentiation as thyroid hormone is detected in fetal circulation shortly after NIS expression (12th week) (Figure 2).

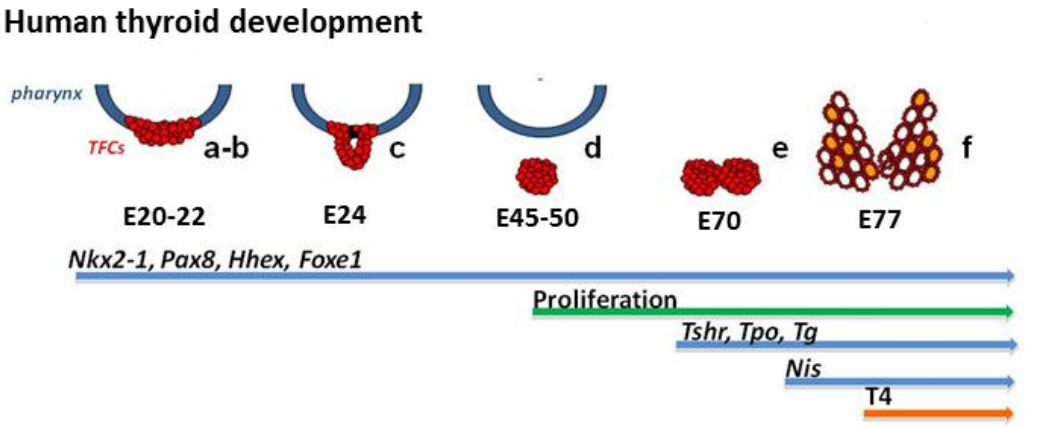


Figure 2: Thyroid development in humans: schematic illustrations. The time of development is E (embryonic day post-fertilization). Red color indicates developing thyroid follicular tissue. (a) early marker gene expression in endoderm; (b) primordium at ventral midline of the pharynx; (c, d) evagination and re-localization; (e) bifurcation of primordium; (f) growth of thyroid follicular cells, surrounded by connective tissue; differentiation into many follicles at the same time; (g) differentiation into one first follicle; (h) growth of follicles. Blue arrows indicate the onset of early and differentiative marker gene expression. Green arrows indicate the onset of thyroid proliferation; orange arrows indicate the onset of thyroid function as judged by T4 production [Fagman and Nilsson, 2010].

1.2 THYROID HORMONE METABOLISM AND ACTION

Thyroid hormone (TH) has long been known to be important for organ function and metabolism in adult vertebrates and for embryo development [Brix *et al.*, 2011]. The most obvious and earliest known abnormalities of human body and behavior associated with TH deficiency are goiter and cretinism [Nishimaki *et al.*, 2009; Ilyés, 2011]. In humans, the most important period of TH action responsible for cretinism appears to be the so-called postembryonic period, a few months before and several months after birth, which is critical for growth and maturation of many organs, including the brain [Yassa *et al.*, 2010].

As previously reported, during human development, TH begins to explain his role very early [Medici *et al.*, 2011]. The thyroid gland is developed within the first 12 weeks of gestation. In vitro organ culture studies of human thyroid tissue suggest that fetal TH production begins as early as 10 weeks of gestation, although significant TH production probably does not occur until 20 weeks or later [De Felice and Di Lauro, 2007]. In the fetal plasma, TH levels, especially 3,5,30-triiodothyronine (T3), remain low until about 4 months prior to birth when endogenous synthesis of TH leads to a rapid increase in plasma TH levels. The TH levels reach a peak at birth and remain high several months after birth. Thus, it may not be surprising that TH deficiency during the neonatal period leads to irreversible, profound neurological deficit and mental retardation [Hubalewska-Dydejczyk *et al.*, 2011]. In addition to fetal TH, maternal TH also plays an important role in early embryogenesis [Mortimer *et al.*, 2012]. On the other hand, excess TH also impairs embryo development, leading to increased miscarriages and lower birth weights [Alvarez-Pedrerol *et al.*, 2008]. Although TH is clearly important for embryogenesis in humans and other mammals, it has been difficult to investigate the underlying mechanisms in mammals. This is in part due to the difficulty to manipulate the uterus-enclosed mammalian embryos and to separate the direct effects of TH on embryos from the indirect maternal effects caused by altering TH levels [Bünger *et al.*, 1998].

The biological effects of TH are mainly mediated through gene regulation by TH receptors (TRs), although non-genomic effects of TH have been well documented [Radenne *et al.*, 2008; Lin *et al.*, 2011]. There are two evolutionary-conserved TR genes, TRa and TRβ, in all vertebrate species and both are members of the nuclear hormone receptor superfamily [Tarim, 2011]. TRs are capable of functioning as monomers, homodimers, and heterodimers with 9-cis-retinoic acid receptors (RXRs), which belong to the same superfamily. For genes induced by TH, TR-RXR heterodimers bind to TH response elements (TREs) in around the promoters of target genes even in the context of chromatin [Jones and Shi, 2003]. In the absence of TH, TR represses transcription from their promoters; when TH is present, TR activates their transcription, accompanied by chromatin

disruption [Wang *et al.*, 2009]. There are also genes that are repressed by the presence of TH. TR accomplishes gene regulation by recruiting corepressor or coactivator complexes to the target promoters, respectively. Many TR-interacting proteins have been isolated and characterized through in vitro and cell culture studies [Ortiga-Carvalho *et al.*, 2005]. Two highly related proteins N-CoR (nuclear corepressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptors) are among the best-studied TR-binding corepressors [Astapova *et al.*, 2011]. Both bind to unliganded TR and form large complexes containing histone deacetylase 3 (HDAC3), transducin beta-like protein 1 (TBL1), TBL1-related protein 1 (TBLR1), and G-protein pathway suppressor 2 (GPS2), suggesting that unliganded TR represses transcription in part through deacetylation of the local histones at target promoters [Jones *et al.*, 2003]. In the presence of TH, TR releases corepressors and recruits coactivators. The known TR coactivators include ATP-dependent chromatin-remodeling proteins, histone acetyltransferases such as CREB-binding protein (CBP) and p300, and TRAP=DRIP=mediator complex that associates with the recruitment and activation of RNA polymerase II [Li *et al.*, 1997].

Among the coactivators, the role of the SRC family (SRC1, 2, 3) in gene regulation by TR has been studied extensively. SRCs bind to TR in the presence of TH and form complexes with other cofactors, including CBP=p300, protein arginine methyltransferase 1 (PRMT1), and coactivator-associated arginine methyltransferase 1 (CARM1 or PRMT4) [34], suggesting that SRCs activate transcription in part through acetylation or methylation of local histones in the promoter region (Figure 3) [Jones *et al.*, 2003].

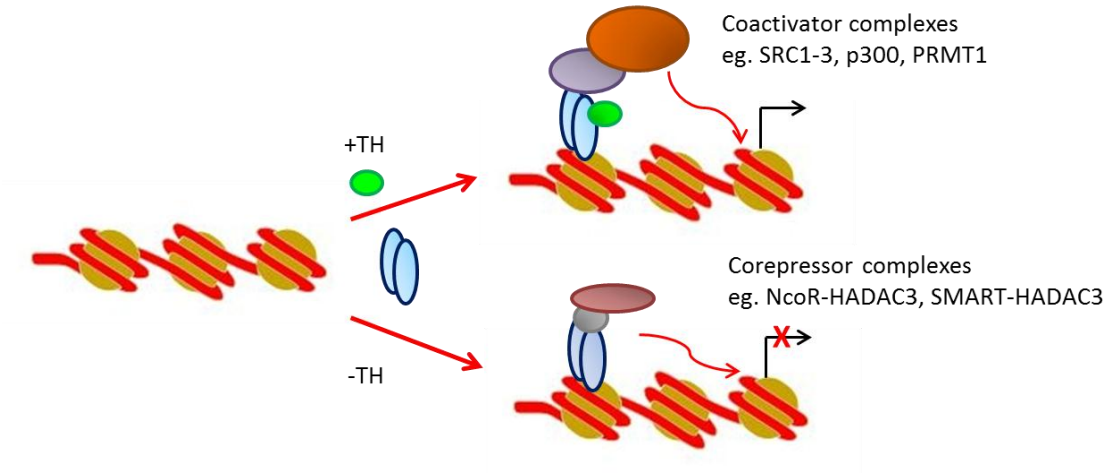


Figure 3: Mechanisms of transcriptional regulation by thyroid hormone (TH) receptor (TR). For TH-inducible genes, TR heterodimerized with 9-cis-retinoic acid receptors (RXR) constitutively binds the TH response elements (TREs) in their promoters or enhancers. In the absence of TH, TR binds corepressor complexes, such as those containing histone deacetylase HDAC3 and the highly related protein N-CoR (nuclear corepressor) or SMRT (silencing mediator of retinoid and thyroid hormone receptors) to inhibit transcription from the promoters through deacetylation of lysine residues of histone H3 and H4 to induce a ‘‘closed’’ chromatin state, as suggested by the folding of histone tails (red beaded structure) on to the DNA helix because of the charge–charge interaction between the positively charged histone tails and negatively charged DNA. The binding by TH induces a conformational change in TR, leading to the binding of coactivator complexes, such as those containing the related coactivators SRC 1, 2, or 3, which in turn binds p300 and PRMT1. SRCs and p300 have histone acetyltransferase (HAT) activity to acetylate histones H3 and H4, and PRMT1 can methylate histone H4. Histone acetylation is believed to facilitate the formation of an ‘‘open’’ chromatin state, as diagramed by the unfolding of histone tails (red beaded structure) away from the DNA helix due to the neutralization of the positive charges on the histone tails by acetylation. There are other corepressors and coactivators that can participate in this process, including those involved in chromatin disruption upon transcriptional activation by TR [Jones *et al.*, 2003].

1.3 CONGENITAL HYPOTHYROIDISM

Congenital hypothyroidism (CH) is the most common congenital endocrine disorder (about one in 3000 newborns). Because the hormone thyroxine (T4), produced by the thyroid gland, is essential for brain development, CH represents the most common preventable cause of mental retardation [Rastologi *et al.*, 2010]. Very rarely, CH is due to pituitary or hypothalamic defects resulting in 'central' hypothyroidism [Nebesio *et al.*, 2010], but it is most often due to a defect at the level of the thyroid gland itself ('primary' hypothyroidism) [Bros *et al.*, 2009]. In the following paragraphs are reported the knowing causes of primary congenital hypothyroidism.

1.3.1 Thyroid Dysgenesis

Unlike in iodine-deficient areas of the world where endemic cretinism continues to be a major health hazard, the majority (85 to 90%) of cases of permanent congenital hypothyroidism are due to thyroid dysgenesis [Thorwarth *et al.*, 2010]. Thyroid dysgenesis may result in the complete absence of thyroid tissue (agenesis) or it may be partial (hypoplasia); the latter often is accompanied by a failure to descend into the neck (ectopy) [Rastologi *et al.*, 2010]. Females are affected twice as often as males. In the United States, thyroid dysgenesis, is less frequent among African Americans and more common among Hispanics and Asians [Miyai *et al.*, 1984]. Babies with CH have an increased incidence of cardiac anomalies, particularly atrial and ventricular septal defects [Devos *et al.*, 1999]. An increased prevalence of renal and urinary tract anomalies has also been reported recently [Trueba *et al.*, 2005].

Most cases of thyroid dysgenesis are sporadic. Although both genetic and environmental factors have been implicated in its etiology, in most cases the cause is unknown [Castanet *et al.*, 2010; Büyükgebiz, 2003]. The occasional familial occurrence, the higher prevalence of thyroid dysgenesis in babies of certain ethnic groups and in female versus male infants as well as the increased incidence in babies with Down syndrome all suggest that genetic factors might play a role in some patients [LaFranchi, 199; Nebesio and Eugster, 2009]. The transcription factors NKX2.1 (TTF1), FOXE1 (TTF2) and PAX8 would appear to be obvious candidate genes in view of their important role in thyroid organogenesis and in thyroid-specific gene expression [Fagman *et al.*, 2011; Di Palma *et al.*, 2010]. To date, however, abnormalities in these genes have been found in only a small proportion of affected patients, usually in association with other developmental abnormalities [Kempers *et al.*, 2009]. For example, the syndrome of congenital hypothyroidism associated with unexplained neonatal respiratory distress, ataxia, and developmental delay has been found in a number of patients with genetic abnormalities of NKX2.1 [Doyle *et al.*, 2004], analogous to the findings of abnormal thyroid, lung, pituitary, and forebrain development in mice with a targeted disruption of this gene [De Felice *et al.*, 1998; Argumedo *et al.*, 2011]. In contrast, no germline mutations in NKX2.1 gene were found in a total of 76 patients with isolated CH [Montanelli and Tonacchera, 2010]. A similar situation has been found with FOXE1 (TTF2), a mutation which has been reported in 2 siblings with the combination of thyroid agenesis, cleft palate, spiky hair and choanal atresia [Tonacchera *et al.*, 2004]. In a different study, germline mutations of PAX8 were found in only 2 of 145 Italian patients with sporadic thyroid dysgenesis studied [Di Palma *et al.*, 2010]. In one of these latter patients, the thyroid gland was hypoplastic and ectopic while in the other patient the thyroid gland was hypoplastic but located in a normal position in the neck [Montanelli and Tonacchera, 2010]. Since PAX8 is also involved in renal development it will be important to determine whether this gene is related to the increased prevalence of renal urinary tract anomalies that has been noted recently [Trueba *et al.*, 2005]. It is possible that thyroid dysgenesis is a polygenic

disease with variable penetrance depending on the genetic background. Alternately, epigenetic modifications, early somatic mutations or stochastic developmental events may play a role [Vassart and Dumont, 2005].

1.3.2 Dysormonogenesis

Inborn errors of thyroid hormonogenesis are responsible for most of the remaining cases (15%) of neonatal hypothyroidism [Topaloglu, 2006]. A number of different defects have been characterized and include: 1) decreased TSH) responsiveness, 2) failure to concentrate iodide, 3) defective organification of iodide due to an abnormality in the peroxidase enzyme or in the H2O2 generating system, 4) defective thyroglobulin synthesis or transport, and 5) abnormal iodotyrosine deiodinase activity [Kota *et al.*, 2011, Moreno and Visser, 2007; Turkkahraman *et al.*, 2009]. The association of an organification defect with sensorineural deafness is known as Pendred syndrome [Coakley *et al.*, 1992]. Though usually included in causes of congenital hypothyroidism because it is caused by a genetic defect, Pendred syndrome rarely presents in the newborn period [Banghová *et al.*, 2008]. Unlike thyroid dysgenesis, a sporadic condition, these inborn errors of thyroid hormonogenesis are commonly associated with an autosomal recessive form of inheritance, consistent with a single gene abnormality [Narumi *et al.*, 2011]. Genetic component involved mutations the genes for the TSH receptor (TSHR), the sodium-iodide symporter (NIS), thyroid peroxidase (TPO), dual oxidase (DUOX) 2, thyroglobulin (Tg), and iodotyrosine deiodinase (DEHAL1) [Grasberger *et al.*, 2011]. All of the inborn errors of thyroid hormonogenesis except decreased TSH responsiveness are associated with a normally placed (‘eutopic’) thyroid gland that may be increased in size at birth and this feature forms the basis for the clinical distinction from thyroid dysgenesis [Park and Chatterjee, 2005]. In contrast, most babies with TSH resistance have a normal or hypoplastic, eutopic gland that may in some cases

mimic an abnormality of thyroid gland development; in rare cases no thyroid gland at all is discernible on thyroid imaging, a picture indistinguishable from thyroid agenesis. Similar to the variability observed in thyroid gland size in this condition, the clinical findings in TSH resistance have varied from subclinical to overt hypothyroidism depending on the severity of the functional defect [Persani *et al.*, 2011]. Some of these patients have been found to have a loss of function mutation of the TSH receptor, analogous to the hyt/hyt mouse [Sprenkle *et al.*, 2001. In a few affected infants, a discrepancy between presumed 'athyreosis' on thyroid scintigraphy and the detection of either a 'normal' serum thyroglobulin concentration or glandular tissue on ultrasound examination has been noted, a feature that may be helpful diagnostically [Alberti *et al.*, 2002].

1.3.3 Incidences of congenital hypothyroidism

Prior to the onset of newborn screening programs, the incidence of congenital hypothyroidism, as diagnosed after clinical manifestations, was in the range of 1;7,000 to 1:10,000 [Dassault *et al.*, 1975]. With the advent of screening of newborn populations, the incidence was initially reported to be in the range of 1:3,000 to 1:4,000 [Harris and Pass, 2007; Olney *et al.*, 2010].

With more experience from state, regional, and national screening programs, it has become apparent that the incidence varies by geographic location. A report from the French newborn screening program summarizing a 20 year period found the incidence of permanent hypothyroidism to be 1:10,000 [Farriaux and Dhondt JL, 1988], whereas a report from the Greek Cypriot population over an 11 year period found the incidence in newborns to be 1:800 [Skordis *et al.*, 2005]. A recent report showed that the incidence in the United States increased from 1:4,094 in 1987 to 1:2,372 in 2002 [Shapira *et al.*, 2010].

Even in Italy, The Italian National Population-Based Registry of Infants with CH (INRCH) was established in Italy in 1987 shown that the incidence of CH is about

1:2400 live births, an incidence slightly higher than that observed in other countries where iodine prophylaxis is carried out [Corbetta *et al.*, 2009].

The reasons for the increased incidence are not clear, but one possible explanation may be a change in testing strategy. With increased sensitivity and accuracy of TSH methods, Italian and other programs around the world have switched from a primary T4-follow- up TSH approach to a primary TSH test. If the TSH cutoff is lowered, more infants with milder congenital hypothyroidism will be detected [Pass and Neto, 2009; Lafranchi, 2010].

Interestingly, there is some variation in the incidence among:

- Different racial and ethnic groups: several U.S. programs have reported a higher incidence in the Asian, Native American, and Hispanic populations and lower in the American Black population as compared to the White population. A summary of the New York State program during the years 2000 to 2003 showed some interesting demographic variations in the incidence of congenital hypothyroidism. As compared to the overall incidence of congenital hypothyroidism, the incidence was somewhat lower in Whites (1:1815) and Blacks (1:1902), somewhat higher in Hispanics (1:1559), and highest in the Asian population (1:1016) [Shapira *et al.*, 2010].

- Multiple deliveries: several reports indicates that the incidence of CH is nearly double in twin births as compared to singletons, and even higher with multiple births [Derom *et al.*, 1995; Olivieri *et al.*, 2007].On this regard, in Italy, the INRICH data have also shown a high frequency of twins in the CH population with a proportion 3-fold higher in the CH population (3.5%) than in the Italian general population (1.1%) [Olivieri *et al*., 2007].It was found 3-fold higher in multiple (10.1 per 10,000 live births) than in single deliveries (3.2 per 10,000 live births) with a relative

risk of CH occurrence in twin deliveries of 3.1 (95% CI, 2.5–3.9) [Corbetta *et al.*, 2009]. Moreover, the analysis of re-evaluated infants with high suspicion of transient hypothyroidism recorded in the INRICH has shown a twin prevalence of 1.9% among infants who were affected by permanent CH and 13.2% in those who resulted affected by transient CH. Taken together these findings have demonstrated an increased risk for both permanent and transient CH in multiple than in single deliveries. This increased CH risk in multiple pregnancies has important implications in terms of public health given the high number of induced pregnancies, in Italy as well as in other Western countries, because of the increasing use of techniques of assisted reproduction and drugs inducing ovulation [Umstad and Gronow, 2003; Olivieri *et al.*, 2010].

- Prematurity and fetal growth: some reports indicate that children born prematurely, independently from their birth weight or length, frequently present with disturbances of the hypothalamus-pituitary-thyroid axis later in life with a incidence 3-fold higher than term infants [Radetti *et al.*, 2007; Srinivasan *et al.*, 2011; Linn *et al.*, 2010]. Instead, other work indicated that intrauterine growth restriction is associated with some endocrine disturbances later on life [Dahlgren *et al.*, 1998; Cianfarani *et al.*, 2003]. Premature babies born small for gestational age (SGA) have an incidence 12% higher than premature children born acceptable for gestational age (AGA) [Radetti *et al.*, 2004]. What causes the alteration in the hypothalamus-pituitary-thyroid-axis is still unknown. Iodine deficiency, adverse maternal conditions such as diabetes and thyroid autoantibodies during pregnancy seems to be play an important role [Simpser *et al.*, 2010]

- Congenital malformations and CH: anomalies of heart, nervous system, eyes (representing precocious structures in the developing embryo) and multiple congenital malformations are significantly associated to CH

[Reddy *et al.*, 2010]. Babies with CH shown a 3-4 fold higher incidence of cardiac disease [Passeri *et al.*, 2011]. These findings have strongly suggested a very early impairment in the first stages of embryo development with a consequent involvement of different organs and structures [Kreisner *et al.*, 2005].

It's clear that the etiophatogenesis of CH is influenced by several factors. It has been demonstrated that CH is a multigenic [Amendola *et al.*, 2005; Amendola *et al.*, 2010] and multifactorial disease [Vassart and Dumont, 2005]. However, the occurrence of mutations in genes known to be involved in the development of the disease has been observed only in a small proportion of the CH patients [Lafranchi, 1999]. These considerations imply that the etiology of CH is still largely unknown and that further efforts to identify new genetic markers and modifiable environmental risk factors are needed to allow an efficient primary prevention of the disease. The multifactorial origin of CH was also supported by results obtained in the above mentioned study on CH twins recorded in the INRICH [Corbetta *et al.*, 2009; Olivieri *et al.*, 2007]. These findings strongly suggested the occurrence of non-inheritable postzygotic events in the etiology of CH and that environmental risk factors may act as a trigger on a susceptible genetic background in the etiology of the disease [Gilbert *et al.*, 2011].

INTRODUCTION-CHAPTER II

2. EPIGENETIC MECHANISMS

2.1 A BRIEF OVERVIEW

The term epigenetics was originally coined in the 1940s to describe the interplay of genes and environmental factors or stimuli that produce a resultant phenotype, long before the mechanisms underscoring such events were known [Waddington, 1942]. While an organism's cells all hold the same genetic code, it is axiomatic that this information is not expressed uniformly across all cell types. Instead, the modification of additional layers of information residing "on top of" the bare genomic sequence helps contribute to cellular phenotypic variance [Wu and Morris, 2001].

It is important to note that epigenetic modification is a normal and ubiquitous process. Initially, certain divergent genes become epigenetically suppressed during embryonic differentiation [Christophersen and Helin, 2010]. This "locks in" the cell's basic expression profile and contributes to the resultant morphological and functional diversity among cell phenotypes. Furthermore, once established, these epigenetic changes can be stable and have the ability to persist through multiple cellular divisions of the differentiated line; in other words, they are heritable. In some circumstances, epigenetic marks may even be persistent across generations, passed meiotically from parent to offspring [De Carvalho *et al.*, 2010].

Today, there is indeed growing evidence that external factors such as diet, for example, have the ability to alter phenotypic outcomes via changes in recently-discovered epigenetic machinery [Sebert *et al.*, 2011; Slattery *et al.*, 2010]. Interestingly, some reports suggested that such plasticity under the jurisdiction of environmental cues could constitute an organism's ability to deliberately

integrate environmental information into its genetic function in effect, a new kind of Lamarckism [Kanno *et al.*, 2011; Handel *et al.*, 2010]. In any case, epigenetic processes allow the cell to enhance and augment the information residing in its DNA and to add nuance to the cell's control over gene expression. For example, epigenetic mechanisms are responsible for the dosage compensation phenomena of random X chromosome inactivation and gene imprinting [Tarry-Adkins JL and Ozanne, 2011]. The nature of these processes is discussed below.

2.2 EPIGENETIC COMPONENTS

At a molecular level, epigenetics involves two main constituents, these being histone modification and DNA methylation, although it is increasingly evident that non-coding RNAs are implicated in epigenetic maintenance [Golbabapour *et al.*, 2011]. Collectively, these mechanisms facilitate the expression or silencing of targeted genes that ultimately culminate in the desired cell state, referred to as the epigenome [Dupont *et al.*, 2009]. This section will briefly introduce histone modification and non-coding RNA, before a more thorough review of DNA methylation in section 2.3.

2.2.1 Histones modifications

The histone family (H2A, H2B, H3 and H4) are highly conserved proteins that, when arranged in an octamer, permit 146 bp lengths of genomic DNA to wind around them to form a nucleosome. Millions of nucleosomes together with other scaffold proteins, collectively referred to as chromatin, allow for the efficient packing of DNA into the nucleus [Zweidler, 1984]. The degree to which localized sectors of DNA are packaged also dictates how readily accessible the underlying

genetic information is to transcription machinery responsible for gene expression. Generally, condensed chromatin, or heterochromatin, restricts access to DNA sequence and thus represses gene expression, whereas euchromatin is in an open conformation that allows binding of transcription factors and the RNA polymerase complex [Sierra *et al.*, 1992]. Several covalent modifications of histone N-terminal tails exist which are thought to control the extent of chromatin packing as well as to recruit and regulate attachment of other DNA binding proteins capable of initiating or suppressing transcription. Presently, around thirty acetylation, methylation, phosphorylation and ubiquitination additions are documented, which are facilitated by multiple corresponding acetylases and transferases [Garcia *et al.*, 2008]. Consequently, deciphering this regulation is quite complex and can be seemingly contradictory. For example, methylation of histone H3-Lys4 leads to a open chromatin structure [Hake *et al.*, 2006] whereas methylation of Lys9 of the same histone invokes chromatin condensation [Zhao *et al.*, 2005]. Such residues can be mono-, di- or tri-methylated, further complicating the understanding of such events [Li *et al.*, 2007]. Certainly, the picture describing histone modification and control is far from complete. The exact nature of proteins that bind modified histone residues are still being elucidated. Even the mechanism whereby a histone's modification state is preserved across mitosis is unconfirmed, although division of the octamer in a semi-conservative manner analogous to DNA replication is a possibility [Hajkova *et al.*, 2008].

2.2.2 MicroRNAs

The existence of non-coding microRNAs (miRNAs) as epigenetic regulators is a recent discovery. Non-coding RNAs participate in regulation of gene expression at all stages, including transcription modulation, primary transcript processing and mature mRNA stability, and translation repression [Bartel, 2004]. Notably,

miRNAs have also been implicated in modification of chromatin and associated processes like imprinting. For example, H3-Lys9 methylation in both bacteria and mice has been demonstrated to be dependent upon RNA interference (RNAi) mechanisms [Cowell *et al.*, 2002]. Double stranded RNAs (dsRNAs), transcribed from heterochromatic regions, are processed into small interfering RNAs (siRNAs) and integrated into the RNA-induced transcriptional silencing complex (RITS). This complex directs the histone methyltransferase to further methylate local H3-Lys9 residues, several kilobases around the RNA-targeted site [Bernstein *et al.*, 2007]. Additionally, non-coding RNA can assist genomic DNA methylation, a process that also contributes to heterochromatin formation. RNA-directed DNA methylation (RdDM) functions similarly to the aforementioned procedure, but processed dsRNAs are smaller (21-24 nucleotides) and instead recruit DNA methyltransferases to the promoter region homologous to the dsRNA [Bartel, 2004]. Owing to the fact that only a fraction of non-coding RNAs have been characterised, considerable further investigation is required to determine the extent to which noncoding RNAs control epigenetic events.

In the following paragraphs, we will focus on the DNA methylation, which is the main topic of my PhD thesis.

2.3 THE DNA METHYLATION

2.3.1 Introduction of DNA methylation

DNA methylation is a biochemical process that is important for normal development in higher organisms. It involves the addition of a methyl group to the 5 position of the cytosine pyrimidine ring or the number 6 nitrogen of the adenine purine ring (Figure 4). This modification can be inherited through cell division [Bird, 2002].

DNA methylation stably alters the gene expression pattern in cells such that cells can "remember where they have been" or decrease gene expression [Reik *et al.*, 2009]. DNA methylation is typically removed during zygote formation and re-established through successive cell divisions during development. However, the latest research shows that hydroxylation of methyl group occurs rather than complete removal of methyl groups in zygote [Surani, 2001].

However, these moieties are still able to be added or removed independently of genetic duplication [Santos *et al.*, 2002]. DNA methylation also contributes to the condensation of chromatin and thus gene suppression. Similarly to the mechanisms by which histone modifications are thought to induce changes in chromatin, the modification of cytosines aids the binding of a family of methyl binding domain proteins (MBDs) that facilitate chromatin modification or act as linkers to recruit DNA methyltransferases to further methylate adjacent cytosine [Fulka *et al.*, 2004; Paulsen *et al.*, 2008]. Alternatively, the presence of a methyl group can interfere with trans-factor binding, thus altering the ability of the underlying genetic material to be expressed. This process is depicted in Figure 5.

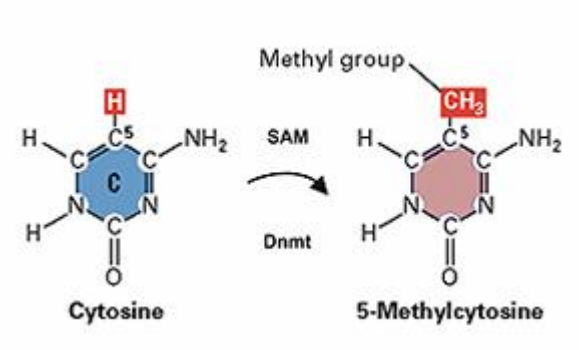


Figure 4: DNA methylation. A covalent addiction of methyl group on carbon in position 5' of cytosine forms a 5Met-Cytosine.

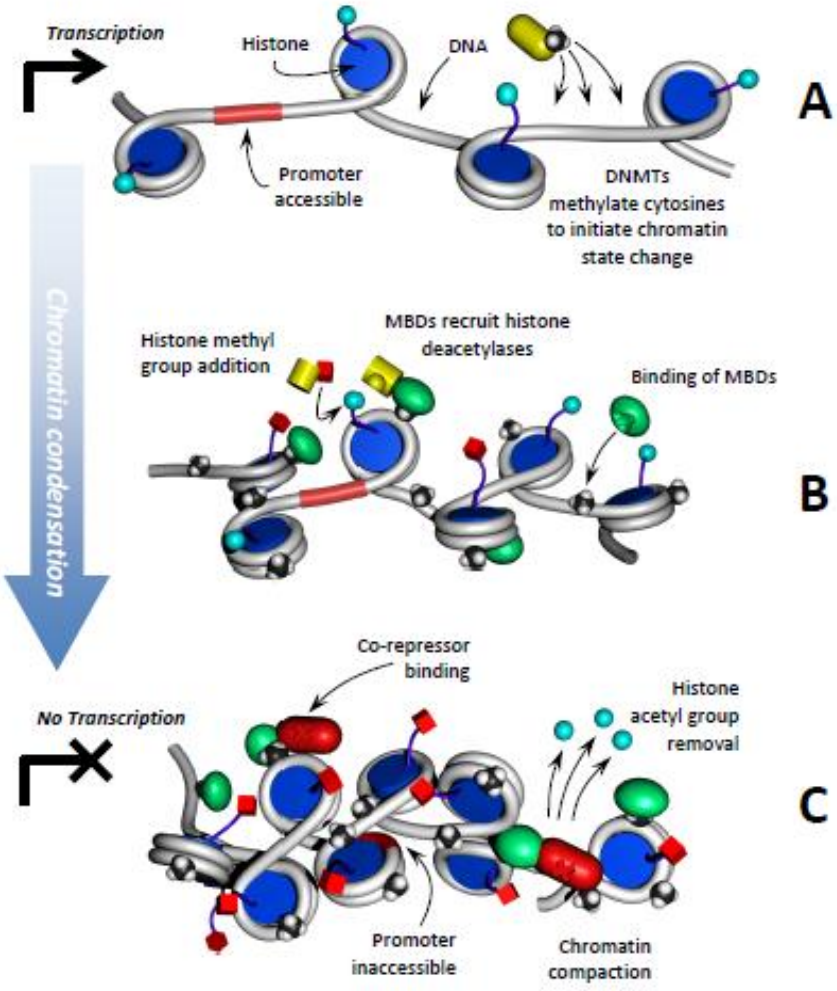


Figure 5: General mechanism of transcriptional silencing involving DNA methylation. Panel A: Active gene promoters are characterized by an open chromatin structure, facilitated by acetylated histones which permit low nucleosome occupancy (acetyl groups denoted by cyan spheres). Cytosine methylation of the DNA locus is performed by DNA methyltransferases (gold cylinder). Panel B: Addition of methyl groups attracts Trans-acting factors with methyl binding domains (MBDs, denoted as green ovals), a family comprised of MeCP2, MBD1, MBD2 and MBD4. These proteins recruit additional factors to interfere with transcriptional machinery (co-repressors shown as red cylinders in Panel C) and condense chromatin by altering histone tags (histone deacetylases and methylated histone tags represented by yellow cylinders and red cubes, respectively), which also occludes DNA sequence from the polymerase complex. Panel C: As a result, transcription is repressed. Note, the temporal sequence of these events varies and in some instances, histone modification precedes DNA methylation [Fulka *et al.*, 2004].

2.3.2 5'-Methylcytosine and the CpG dinucleotide

A methyl moiety covalently bonded to the 5' carbon of the pyrimidine ring of cytosine is the predominant form of DNA methylation. In mammals, cytosine methylation generally only occurs when that residue is adjacent to a 3' guanosine, termed a CpG dinucleotide [Bird, 2002]. The "p" represents the phosphodiester bond between nucleotides. Significant methylation of other cytosine-containing di- and trinucleotides does exist however. For example, plants exhibit more complicated CpNpG and CpNpN patterns, where N is any nucleotide [Zilberman *et al.*, 2007]. Another example is during fetal development, where non CpG methylation is witnessed [Lister *et al.*, 2009].

In essence, each CpG dinucleotide pair is a palindrome and the methyl moiety shares this reciprocity with cytosines of both DNA strands being methylated. The methyl groups of complimentary CpGs both protrude into the major groove of the helix (see Figure 6, panel A). As DNA is replicated semi-conservatively, the newly synthesized daughter strand at S phase is devoid of cytosine methylation; while adenosine, cytosine, guanine and thymine are present in the cell as free

nucleotides, 5'-methylcytosine only exists as a modification of cytosine once the latter has been integrated into the DNA molecule. Therefore, following replication, hemi-methylated CpGs are detected and the remaining dinucleotide is enzymatically methylated [Jeltsch, 2006]. This method allows faithful epigenetic transmission from parent to daughter cell. The process is catalyzed by DNA methyltransferases (DNMTs).

The methyltransferases are coded by three genes, DNMT1, DNMT3A and DNMT3B, all of which contain several conserved motifs [Van Emburgh and Robertson, 2008]. Interestingly, these motifs are present across all studied prokaryotes and eukaryotes [Posfai *et al.*, 1989).]. Multiple splice variants of each gene's transcripts exist in humans. Some isoforms only perform key methylation events during embryogenesis, or are sex specific, while DNMT1, the prime "maintenance" methyltransferase, is responsible for the routine re-methylation of the bulk of DNA following replication [Van Emburgh and Robertson, 2008].

However, in addition to primarily recognising hemi-methylated DNA, DNMT1 can also conduct *de novo* methylation [Pradhan *et al.*, 1999]. Conversely, DNMT3 families are also primarily de novo methyltransferases, but may contribute to maintenance methylation [Liang *et al.*, 2002]. DNMT2, originally presumed to act exclusively on DNA, has recently been re-categorised as a RNA methylase [Goll *et al.*, 2006].

The catalytic mechanism by which DNMTs operate is worth mentioning. Following substrate recognition, one strand of the DNA is flipped out of the helix by the enzyme to expose the acceptor carbon of the unmethylated cytosine. A methyl moiety is then transferred from S-adenosylmethionine to the cytosine [Cheng and Roberts, 2001; Klimasauskas *et al.*, 1994]. Although only established in bacteria, this mechanism is presumed to occur in mammals due to the very close sequence homology between proand eukaryotes (see Figure 6, panel B). Less is known about the process of methylated cytosine removal. Rapid and large scale demethylation is known to occur early during embryonic development, and

adult somatic tissues can display a gradual loss of methylation as part of senescence and cancer [Issa, 2003; Maegawa *et al.*, 2010].

This loss of methylation is generally believed to be a passive event, perhaps as a result of a breakdown in DNMT-facilitated methylation over multiple cell divisions. However, active demethylation mechanisms may exist outside of fetal development. The nuclear protein Gadd45a in conjunction with a DNA repair endonuclease XPG, leaves damaged CpGs unmarked after repair, which could be an attractive mechanism to explain age-related hypomethylation [Barreto *et al.*, 2007]. There is also accumulating evidence that DNA methylation is dynamic in the brain and involved in memory formation [Levenson *et al.*, 2006; Miller and Sweatt, 2007], and recent work in plants has discovered multiple DNA glycosylases capable of actively performing demethylation via excision ' of 5 - methylcytosine [Agius *et al.*, 2006; Morales-Ruiz *et al.*, 2006]. It is presently unclear if this process extends to humans.

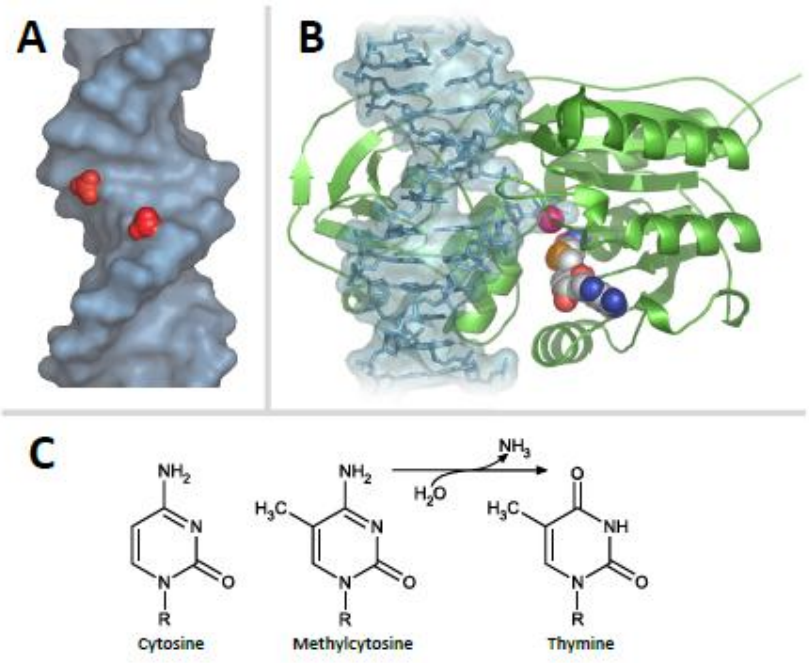


Figure 6: Attributes of 5'-methylcytosine. Panel A: Spatial representation of methyl groups of a CpG dinucleotide pair within the DNA double helix. Methyl moieties protrude into the major groove, which suggests their steric interaction with methylbinding proteins or transcription factors. Methyl groups are depicted in red. Panel B: Cartoon rendering of the 3-dimensional structure of the Haemophilus parahaemolyticus DNMT-DNA complex (PDB ID: 1MHT). The enzyme (green) "flips" one strand of the DNA (light blue) outwards to expose the unmethylated cytosine residue. The methyl moiety (magenta) is then transferred from S-adenosylmethionine to the 5' carbon of cytosine. As there is a very high degree of conservation between bacterial and higher eukaryote DNA methyltransferases, this mechanism is also assumed to occur in mammals. Images in panels A and B rendered using PyMol version 0.94. Panel C: Chemical structures of cytosine, methylcytosine and thymine, highlighting the propensity of methylcytosine to deaminate to thymine. The R-group denotes the deoxyribose common to all three species [Laird, 1999]

2.3.3 Genomic distribution of 5'-methylcytosine

Under conditions of local strand separation, genomic cytosine is prone to spontaneous deamination, becoming uracil [Fryxell and Moon, 2005]. Fortuitously, the cell has the ability to detect, excise and replace uracil in DNA with uracil glycosylase [Lindahl *et al.*, 1997]. However, if the cytosine is methylated, the end product of deamination is thymine (see Figure 1.6, panel C), a legitimate base which is invisible to the aforementioned corrective machinery. Consequently, over time, methylated cytosines have transmogrified to thymine residues and CpGs are statistically underrepresented throughout the human genome [Lander *et al.*, 2001].

Correspondingly, around 1% of nucleotides in adult mammalian genomes are 5'-methylcytosine (and, as it follows, approximately 4.5% of cytosines). In a given human somatic cell, this equates to around 30 million CpG dinucleotides [Costello and Brena, 2008]. The distribution of cytosine methylation throughout the genome is far from even however, with the bulk of methylation occurring in

repetitive elements including transposons and satellite sequences (see Table 1.1). Approximately 75-80% of CpGs are methylated [Dahl and Guldberg, 2003]. Clusters of unmethylated CpGs exist, however, usually within gene promoter CpG islands. CpG islands were originally defined as a region with at least 200 bp and with a GC percentage that is greater than 50% and with an observed/expected CpG ratio that is greater than 0.6 [Gardiner-Garden and Frommer, 1987]. More stringency has since been added to this definition to exclude much of the CpG content of repetitive elements while retaining islands associated with gene regulation [Takai and Jones, 2002]. The revised classification specifies that CpG islands are deemed to possess greater than 60% GC content and be between 0.5 – 2 kb in length. This is often relaxed in circumstances such as algorithmic genome searches [Yamada *et al.*, 2004].

Ultimately, the CpG sites on the genome can be divided into two broad categories: within gene promoters and are called *CpG islands* or within non-coding DNA (such as repetitive sequences) and are called *non-CpG islands* sites (see figure 7). The main difference of this CpG sites resides in the physiological state of methylation and into the mechanism of inhibition of gene expression. As described above, *CpG island sites* are usually hypomethylated and the covalent addition of methyl groups directly prevents the activities of the transcriptional machinery complex. On the contrary, *non-CpG island sites* are normally hypermethylated and the gene silencing occurs in an indirect way, through the recruitment of protein complex of chromatin condensation [Shen *et al.*, 2007]. The details of the mechanisms of gene regulation are described in the following paragraph.

	CpG	GC content (%)	Composition of genome (%)	CpG distribution (%)
Genome	29,848,743	41	100	100
CpG island	1,876,802	62	0.57	5.5
First exon	508,553	56	0.31	1.8
Other exons	1,337,271	48	1.83	4.9
DNA transposons	565,601	29	3.6	2.1
LINE transposons	3,242,225	32	22.3	11.8
SINE transposons	7,479,682	38	16.1	27.2
LTR transposons	1,958,798	37	9.3	7.13
Alpha satellite	~766,000	38	2.07	2.79
Classical satellite	~1,140,000	34	2.1	4.15
Other sequences	8,358,888	42	41.85	32.58

Table 1: Distribution of CpG dinucleotides in the human genome [Rollins, 2006]

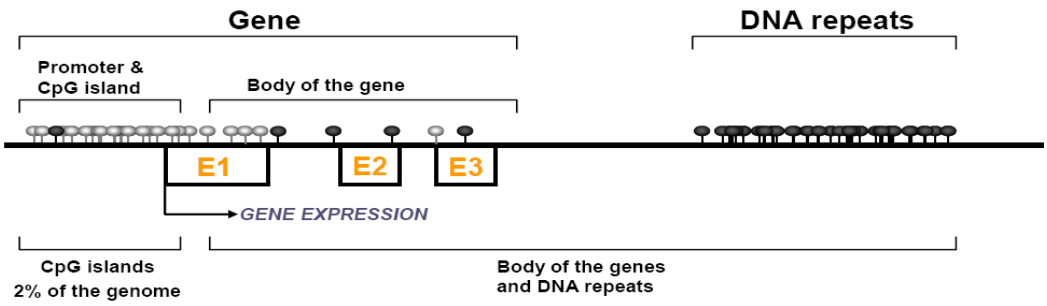


Figure 7: Distribution of CpG sites into the genome. CpG islands are located into gene promoters and are usually hypomethylated. Non-CpG island sites localize into non-coding regions such as the body of gene (introns) and DNA repeats (LINE, Alu repeats) [Shen *et al.*, 2007].

2.3.4 CpG sites help determine gene expression state

The presence of CpG islands around certain gene promoters and first exons, particularly of house-keeping genes, suggests a role in regulation of gene expression. Indeed, the great majority of CpG islands in these regions appear to confer a stable open chromatin conformation and permit ubiquitous expression [Weber *et al.*, 2007]. Conversely, many tissue-specific genes lack CpG islands which may allow them to be shut down more easily upon differentiation. Such genes typically contain a TATA box for a precisely mapped transcription start site and specific transcription factor binding, whereas gene promoters containing CpG islands can have multiple transcription start sites and are frequently devoid of TATA elements [Carninci *et al.*, 2006]. Other factors relating to 5′ CpG islands also contribute to the ultimate expression state of a gene, however. These include the nature of other various regulatory motifs within the island that facilitate transcription factor binding, overall sequence composition of the CpG island and the level of GC content, which can affect the amount of "twist" in the DNA helix and thereby alter the accessibility of associated proteins [Bock *et al.*, 2006]. Incidentally, the methylation status of CpG islands further downstream, outside the transcription start site, generally have little bearing on transcription and do not interfere with elongation [Larsen *et al.*, 1993].

Adding complexity, there is considerable crosstalk between CpG island methylation state, histones, trans-acting factors and non-coding RNA. DNA methyltransferases and histone methyltransferases (HMTs) often interact directly to methylate CpGs and alter histone conformation of both genes and satellite repeat sequences [Lehnertz *et al.*, 2003; Li *et al.*, 2006].

It is important to note too that unmethylated CpG islands are generally, but not always an attribute of positive gene expression. Some strongly unmethylated islands can be devoid of expression [Weber *et al.*, 2007] and conversely, active histone marks have been shown to override methylated CpGs and force transcription [Brinkman *et al.*, 2007]. Additionally, it is not clear if there is a

specific temporal sequence of histone modification and DNA methylation that culminates in the changed chromatin state [Sims *et al.*, 2008]. While the subtleties of CpG island control may not have been completely elucidated, it is clear they play an important part in epigenetic gene regulation.

2.4 ROLES OF DNA METHYLATION

Due to its ability to suppress expression, DNA methylation is utilized by the cell to affect a number of disparate functions that require such repression the sequential switching off of tissue-specific genes during development, accountancy of various dosage imbalances, or the suppression of parasitic elements. Furthermore, the replication of methylated CpG marks across cell divisions allows DNA methylation to act as a faithful epigenetic inheritance mechanism.

2.4.1 Transposon suppression-DNA methylation as a genetic defense

As shown in Table 1, around half of all CpGs reside in repetitive sequences and such sequences constitute a similar proportion of the human genome. The nature of these elements is diverse, including tandem repeats located around centromeres and telomeres and numerous classes of transposable elements. Many of the latter are inactive due to deletions or mutations, but elements such as complete LINE transposons, of which there are almost 100, or LTR retrotransposons (endogenous retroviruses, occupying approximately 10% of the genome) have the potential to become active and invade functional genomic regions [Brouha *et al.*, 2003; Slotkin and Martienssen, 2007]. Doing so can cause genomic recombination or chromosome breaks, or disrupt enhancers,

promoters, polyadenylation and splicing of adjacent genes [Girard and Freeling, 1999].

Not surprisingly, these elements are suppressed by the cell, being heavily methylated and consequently quarantined by heterochromatin. Flagging of these sequences for methylation is presumed to occur by sequence recognition, but also by virtue of their high copy number, experiments increasing the amount of introduced transgene sequence have been shown to increase its methylation by the host, for example [Garrick *et al.*, 1998].

DNA methylation of these "parasitic" elements occurs across animals, plants and fungi. As such, it has been proposed that DNA methylation originally evolved as a host defense mechanism to protect against foreign DNA integration and that utilization of DNA methylation for host gene regulation was a later adoption [Yoder *et al.*, 1997].

2.4.2 DNA methylation and development

Mammalian development is characterized by bimodal DNA methylation reprogramming that occurs initially during germ cell development and then during preimplantation (Figure 8) [Kafri *et al.*, 1992]. Primordial germ cells (PGCs) enter the developing germinal ridge and begin differentiation and expansion. At this time, the highly methylated PGCs undergo rapid genome-wide demethylation such that by day 12.5 most of the methylation is lost [Sandovici *et al.*, 2008]. This reprogramming phase coincides with the erasure and resetting of parent of-origin specific marks that include DNA methylation of imprinted DMRs associated with allele-specific gene expression [Shafa *et al.*, 2010]. The exact timing of de novo methylation has not been firmly established but is initiated in males at 14.5 dpc (days post-coitum) and thereafter in females such that the mature gametes of both sexes will eventually become highly methylated. The

second phase of methylation reprogramming occurs between fertilization and formation of the blastocyst. On fertilization a rapid paternal-specific asymmetric loss of methylation is observed [Shiota, 2004]. This process takes place in the absence of transcription or DNA replication and is termed active demethylation. Thereafter, there is a step-wise decline in methylation until the morula stage. This decline occurs as a result of the absence of the primary DNA methyl transferase, Dnmt1, during DNA replication [Klug *et al.*, 2010]. Thus, the newly replicated strand fails to become methylated and the level of methyl cytosine per nucleus declines. This replication-dependent loss of DNA methylation is referred to as passive demethylation. The initiation of the de novo methylation occurs after the fifth cell cycle and coincides with the time of the first differentiative event. The establishment of the first two cell lineages results in yet another significant asymmetry. The inner cell mass (ICM), which gives rise to all the tissues of the adult, becomes hypermethylated, while the trophectoderm (TE), that forms most of the structure of the placenta, is undermethylated [Jones and Laird, 1999]. This differential is maintained and reflected in highly methylated somatic tissues and the distinctively hypomethylated extra-embryonic tissues of the placenta. Among the somatic tissues that derive from the ICM are the highly methylated PGCs which arise, day 7 in the extraembryonic mesoderm of the developing embryo. Their migration via the allantois to the developing germinal ridges, where they will eventually differentiate into mature gametes, completes the cycle of epigenetic reprogramming [Sarge and Park-Sarge, 2005].

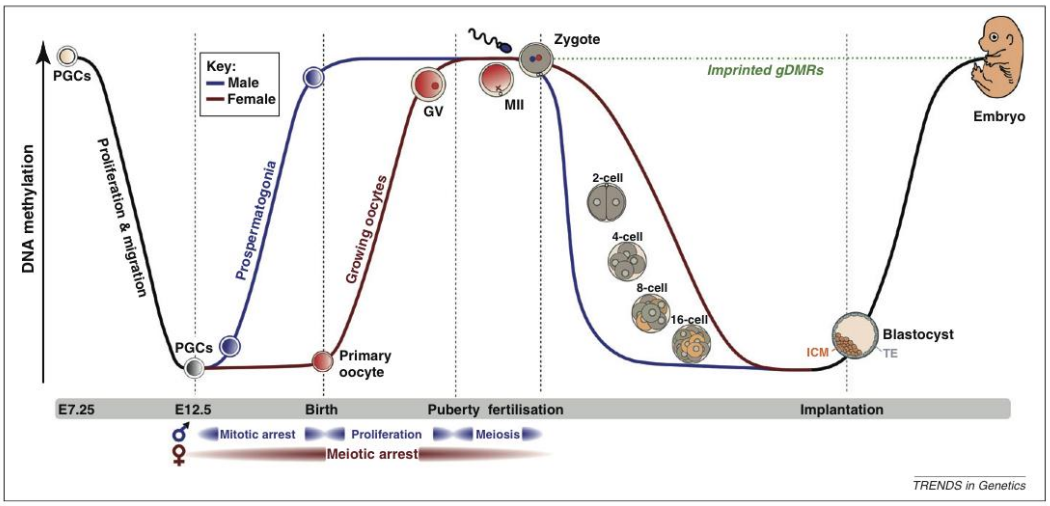


Figure 8: representation of epigenetic reprogramming in mammalian development.

2.4.3 X chromosome inactivation

Another intriguing process in mammalian embryo development is X inactivation. One of the two X chromosomes in females becomes transcriptionally inactive (Xi) to yield a single X chromosome gene dose comparable to a male XY embryo [Boumil and Lee, 2001]. This process is mediated in part by CpG methylation. Specifically, partial inactivation of the paternal X chromosome is observed from the two-cell to the early blastocyst stage. This is retained in the non-embryo-forming cells, which develop into the placenta, but reactivated at the late blastocyst stage in the inner cell mass which later forms the embryo [Sengupta *et al.*, 2008]. One of the two active X chromosomes is then randomly inactivated in these cells so that all future embryonic development is dosage-compensated. The initial paternal X inactivation is carried over from that chromosome's silencing during spermatogenesis. During oogenesis, the maternal X is marked with an imprint to make it resistant to inactivation in extra-embryonic tissues after fertilization [Tada *et al.*, 2000]. Embryonic X inactivation occurs when the

non-translated RNA Xist, transcribed from the chosen Xi chromosome coats itself over the entire chromosome [Clemson *et al.*, 1996]. This induces an ordered progression of histone modification, DNA methylation and heterochromatin formation, transcriptionally silencing the chromosome [Kay *et al.*, 1993; Wutz and Jaenisch, 2000]. Once established, heavy DNA methylation maintains gene promoter silencing and a heterochromatic state over subsequent cell divisions. A small, variable fraction of genes escape this suppression, however, which may contribute to expression heterogeneity among females [Carrel and Willard, 2005]. DNA methylation may also be a determinant of Xist suppression prior to X chromosome inactivation, with DNMT3a recruited to methylate regions flanking the gene, preventing transcription [Panning, 2008].

2.4.4 Imprinting

The phenomenon of imprinting relates to around 50 known genes in the human genome [Morison *et al.*, 2005]. Imprinted genes are characterized by gene methylation (or more specifically, regulatory element patterns) that are specific to each of the parental alleles. Consequently, transcription occurs in a monoallelic, parent-of-origin-specific fashion, i.e. only from the paternally-inherited allele or the maternally-inherited allele [Ferguson-Smith *et al.*, 1993]. Imprints are set up during the formation of parental gametes and escape the resetting of methylation marks in the morula [Reik *et al.*, 2001].

In mammals, imprinting is predominantly associated with genes involved in the regulation of fetal growth and development and postnatal behaviour [Reik and Dean, 2001]. Interestingly, many paternally expressed genes promote prenatal growth whereas many maternally expressed genes limit it. It has been postulated that this pattern has arisen evolutionarily to balance paternal and maternal resource allocation and gene fitness enhanced fetal growth gives a selection

advantage to help continue the paternal DNA lineage, but the associated increased nutritional cost may compromise the total reproductive success of the mother, hence the maternal desire to limit fetal growth [Haig and Graham, 1991]. It should be noted that not all imprinted genes fit this model, however. Probably the best-characterised example of imprinting is that of the paternally-expressed insulin-like growth factor 2 gene (IGF2), which codes for a hormone that promotes growth during gestation, and the reciprocally regulated, maternally expressed H19 gene, which transcribes a putative tumour suppressor non-coding RNA [Barlow *et al.*, 1991; Rachmilewitz *et al.*, 1992; Zhang and Tycko, 1992]. Both genes are co-localized with numerous other imprinted genes at 11p15.5 and are separated by a differentially-methylated, imprinting control region, DMR1. Lack of DMR methylation on the maternal allele allows for expression of the downstream H19 gene, but also facilitates the binding of methylation-sensitive insulator elements (CTCF) to motifs in the DMR which block 3′ enhancers vital for IGF2 expression. Conversely, paternal DMR methylation suppresses H19 expression and prevents CTCF insulators from occluding IGF2 enhancers [Du *et al.*, 2003; Takai *et al.*, 2001; Webber *et al.*, 1998]. Errors in methylation of this region, such as loss of imprinting or hypermethylation of the maternal H19 DMR, result in under or over expression of IGF2 respectively. Overgrowth conditions like Beckwith-Wiedemann Syndrome can often be attributed to this dysregulation, which also have a predisposition towards malignancies such as Wilms tumour [Cui *et al.*, 2001; Weksberg *et al.*, 2003]. In fact, as described in chapter III, numerous perturbations in the wider methylome can have multiple disease outcomes.

INTRODUCTION-CHAPTER III

3. DNA METHYLATION AND HUMAN DISEASES

3.1 EPIGENETIC DYSREGULATION IN HUMAN DISEASES

The role of epigenetic dysregulation in human disease has been reviewed extensively. We therefore summarize only the most salient points here.

3.1.1 DNA Methylation and cancer

Cancer has been traditionally regarded as a cumulative series of genetic alterations resulting in deregulated cell growth. In the early 1980s, hypomethylation of RAS oncogenes in malignant tissue were reported, indicating epigenetic factors may contribute to tumorigenesis [Feinberg and Vogelstein, 1983]. Altered DNA methylation is now regarded as one of the most common molecular changes in human neoplasm development [Baylin *et al.*, 1998; Jones and Baylin, 2002]. Interestingly, aberrant methylation coexists as a non-specific global hypomethylation of the genome and defined regions of hypermethylation. A decrease in global methylation occurs early in tumorigenesis, and its extent mirrors the progression of the disease [Hernandez-Blazquez *et al.*, 2000; Watts *et al.*, 2008]. Hypomethylation can up-regulate proto-oncogenes as well as alter heterochromatin or satellite DNA. The latter perturbations can activate latent transposons and induce chromosomal rearrangement, instability and poor chromosomal segregation during mitosis [Bouzinba-Segard *et al.*, 2006; Howard *et al.*, 2008; Wong *et al.*, 2006]. Cells with this hypomethylated attribute have been described as having a "mutator phenotype", as the propensity for chromosomal deletion or recombination can elevate mutation rates in other genes [Frigola *et al.*, 2006; Novak *et al.*, 2006].

For the most part hypermethylation is confined to discrete promoter CpG islands with neighbouring genes being unaffected, however entire chromosomal bands undergoing methylation-mediated suppression have been reported in colorectal and breast cancer cases [Chen *et al.*, 1998]. Hypermethylation of gene promoters causes their suppression and most aspects of tumorigenesis are affected by such aberrant methylation. Disruption of genes involved in cell cycle [Ferguson *et al.*, 2000; Greger *et al.*, 1989; Herman *et al.*, 1994; Liang *et al.*, 2000; Merlo *et al.*, 1995; Sakai *et al.*, 1991; Stirzaker *et al.*, 1997], DNA repair mechanisms [Esteller *et al.*, 2001; Esteller *et al.*, 2000; Hegi *et al.*, 2005; Herman al., 1998] which can induce chromosomal instability [Ahuja *et al.*, 1997], and apoptosis [Conway *et al.*, 2000; Katzenellenbogen *et al.*, 1999; Teitz *et al.*, 2000] are all affected. Exactly how aberrant methylation manifests during tumorigenesis and what drivers are responsible is an area of active research. Numerous environmental effectors, particularly diet, have been implicated in both cancer and wider aberrant methylation events [Kondo *et al.*, 1995].

3.1.2 DNA methylation and reproduction

Abnormal DNA methylation patterns have also been linked to several reproductive problems and associated diseases. During normal spermatogenesis, it is known that substantial epigenetic programming occurs in two waves [Rousseaux *et al.*, 2005; Li, 2002; Saitou *et al.*, 2002; Santos and Dean, 2004; Biermann and Steger, 2007]. Extensive erasure of DNA methylation marks characteristic of somatic cells is followed by the establishment of sex-specific patterns by de novo methylation. In one study, Houshdaran *et al.* used an extensive panel of DNA methylation assays to characterize the DNA methylation profile of abnormal sperm and reported elevated methylation at numerous DNA sequences indicating that improper erasure of somatic DNA methylation marks is implicated in poor quality sperm and male infertility [Houshdaran *et al.*, 2007].

Furthermore, assisted reproductive technologies that involve the manipulation of embryos are associated with an increased risk of children with Angelman syndrome and Beckwith-Wiedemann syndrome [Gicquel *et al.*, 2003]. Molecular analysis of both syndromes revealed a loss of maternally imprinted methylation at specific gene loci on chromosome 15 and 11 respectively, implicating epigenetic abnormalities in the diseases [Gicquel *et al.*, 2003]. In addition, epigenetic abnormalities are responsible for a large percentage of embryonic losses and placental hyperplasia observed in animal cloning models [Alberio and Campbell, 2003]. However, there is some emerging evidence that underlying infertility problems may play a role rather than the assisted reproductive technologies themselves [Doornbos *et al.*, 2007].

3.1.3 DNA methylation and cardiovascular disease

Cardiovascular Disease CVD, in particular CHD, is now the leading cause of death worldwide, accounting for 27% of deaths in industrialized countries and 21% of deaths in developing countries [Botto *et al*., 2001]. With genetics explaining less than 5% of CHD, adult lifestyle factors and epigenetics likely explain most of the variation [Botto and Correa, 2003]. The most obvious link between epigenetics and cardiovascular disease is hyperhomocysteinemia. The basis for the association of hyperhomocysteinemia with cardiovascular disease is unknown, but since elevated homocysteine concentrations can impair one carbon metabolism and DNA methylation, epigenetic mechanisms have been postulated [Kapusta *et al*., 1999].

Aberrant DNA methylation (both hypo and hypermethylation) secondary to nutritional factors has been implicated as an early step in atherogenesis [Hobbs *et al*., 2005; van Driel *et al*., 2008a].

There are multiple reported instances of DNA hypomethylation occurring in atherosclerosis, specifically in the immediate atherosclerotic lesion (Hiltunen *et al.*, 2002) as well as in peripheral white blood cells (Castro *et al.*, 2003) and smooth muscle (Hiltunen and Yla-Herttuala, 2003; Yideng *et al.*, 2007). Being a multifactorial disease, the hypothesis that hypomethylation could be a contributor to atherosclerosis was raised about a decade ago, although the aberration could also be a secondary event. As already mentioned, elevated homocysteine is a risk factor for the disease. Homocysteine and S adenosyl homocysteine are efficient inhibitors of DNA methyltransferases which could manifest as genomic hypomethylation, implying a secondary development. However hypomethylation is often observed early in atherosclerotic development (Lund *et al.*, 2004) and 5-lipoxygenase and 15-lipoxygenase genes, key enzymes implicated in atherosclerosis development (Zhao and Funk, 2004), are upregulated under promoter hypomethylation (Liu *et al.*, 2004; Uhl *et al.*, 2002).

3.1.4 DNA methylation and Type 2 diabetes

An article proposing DNA methylation profiling in diabetes reviewed indirect evidence that epigenetic dysregulation contributes to type 2 diabetes [Goh and Sum, 2010]. A recent data-mining analysis of more than 12 million Medline records identified epigenetic factors among the strongest statistical associations to type 2 diabetes. The most direct evidence implicating epigenetic dysregulation in human diabetes is from studies of transient neonatal diabetes (TND), a rare form of diabetes that presents within the first few days after birth and, although normally resolving within one year, often recurs later in life. Two studies recently showed that infants with sporadic TND show aberrant methylation at several imprinted genes in peripheral blood leukocytes [Zhao *et al*., 2012; Kantlehner *et al*, 2011].

Effects of parental and grandparental nutrition on diabetes risk in humans have been reported, suggesting transgenerational inheritance of epigenetic alterations that affect diabetes susceptibility [Chaudhary *et al*., 2012; Frantz *et al*., 2012]. Several animal models showing persistent effects of prenatal and early postnatal nutrition on endocrine pancreas function and gene expression suggest an epigenetic basis [Canani *et al*., 2011; Godfrey *et al*., 2011].

3.1.5 DNA methylation and obesity

Prader-Willi syndrome is a developmental syndrome that causes hyperphagic obesity, hypogonadism, and characteristic facial features [Smith *et al*., 2011]. Whereas the disease most commonly results from genetic abnormalities in an imprinted region of chromosome 15, some sporadic cases result from aberrant epigenetic silencing of that region, providing a clear example of epigenetic dysregulation causing human obesity. Genome-wide parent-of-origin linkage analyses suggest that maternally imprinted loci in chromosome regions 10p12 [Alegría-Torres *et al*., 2011] and 2q37 [Boyle *et al*., 2011] also influence human obesity. Moreover, imprinted genes affect the development of the hypothalamus, which plays a central role in regulating energy homeostasis [Herrera *et al*., 2011]. Animal models provide further illustrations that epigenetic dysregulation can cause obesity [Seky *et al*., 2012]. When mice are cloned, they have normal birth weights but often develop adult-onset obesity. A similar phenomenon, termed “large offspring syndrome,” appears to be related to epigenetic dysregulation in cloned sheep [Zhang *et al*., 2011]. Avy mice provide another animal model of epigenetically based obesity. Agouti protein binds antagonistically to the melanocortin 4 receptor in the hypothalamus. Avy/a mice with Avy hypomethylation therefore develop not only yellow coats but also hyperphagic obesity [Morgan *et al*., 1999].

3.2. DEVELOPMENTAL ORIGIN OF HEALTH AND DISEASE HYPOTHESIS (DOHaD)

Subsequent to the highly regulated changes during development, it has been generally assumed that the methylome is fairly immutable and uniform in adult cells. There is, however, the potential for the epigenome to be modulated by external factors, under certain circumstances. In the case of DNA methylation, there is an accumulation of evidence to suggest that exogenous effectors, including diet, have the ability to alter methylation either of the individual or that individual's progeny. The developmental origins hypothesis [Barker *et al*., 1989] proposes Barker and colleagues that during critical periods of prenatal and postnatal mammalian development, nutrition and other environmental stimuli influence developmental pathways and thereby induce permanent changes in metabolism and chronic disease susceptibility. Although extensive human epidemiologic and animal model data support this thesis [Waterland, 2009], the underlying biologic mechanisms are poorly understood.

3.2.1 Fetal programming

Traditionally, the intrauterine environment has been regarded as critical only for prenatal development of the fetus. However, there is accumulating evidence showing that adverse influences during early development can increase the risk of developing disease in adult life. It was first observed by Barker and co-workers that the weights at birth were correlated with the risk of developing coronary artery diseases in adults [Barker and Osmond, 1986; Barker *et al*., 1989]. Subsequently, it has also been found that birth weights were associated with chronic diseases, such as hypertension [Curhan *et al*., 1996a; Curhan *et al*., 1996b] and Type 2 diabetes [Hales *et al*., 1991; Ravelli *et al*., 1998].

Based largely on the epidemiological data, Barker suggested an hypothesis that the alterations of fetal nutrition and endocrine status may result in developmental adaptations that permanently change the structure, physiology, and metabolism which then predispose individuals to cardiovascular, metabolic and endocrine disease in adult life [Barker, 1992; Barker, 2004]. This paradigm is referred to as “fetal programming”. The term “programming” refers to the permanent or long term effects of a stimulus or insult at a critical or sensitive period [Lucas, 1991].

Further studies in experimental animals have provided proof of principle for fetal programming, suggesting that intrauterine environment is important for long term postnatal development [Armitage *et al*., 2004; Gluckman and Hanson, 2004b; Hoet and Hanson, 1999]. The most commonly used approach to study the effect of intrauterine environment has been to alter maternal nutrition during pregnancy, for example, by subjecting the pregnant animals to protein malnutrition. It has been shown that the mice with maternal protein malnutrition result in various degrees of disturbed glucose metabolism [Dahri *et al*., 1991] and cardiovascular function [Langley and Jackson, 1994] in the offspring. It has also been shown that other perturbations of maternal physiology, such as administration of corticosteroids [Dahlgren *et al*., 2001], cytokines [Nyirenda *et al*., 1998] or experimental reduction of uterine blood flow can lead to fetal programming of obesity [Jansson and Lambert, 1999; Simmons *et al*., 2001] or diabetes [Nyirenda *et al*., 1998; Simmons et al. 2001]. These phenomena were referred to as developmental plasticity of the fetus during pregnancy, which conveys the ability to change the structure and function of the fetus in an irreversible fashion during a critical time window in response to the environmental cue [Gluckman and Hanson, 2004a; Gluckman and Hanson, 2004b]. The concept of fetal programming has more broadly been defined as developmental and evolutionary strategies, termed “predictive adaptive response”. This theory proposed „the developmental plasticity as adaptive responses to environmental cues acting early in the life cycle, but where the advantage of the induced phenotype is primarily manifest in a later phase of the

life cycle" [Gluckman and Hanson, 2004a; Gluckman and Hanson, 2004b]. Therefore, instead of causing developmental disruption immediately, the plasticity allows the fetus to respond to the environmental influences by following a developmental trajectory that may be associated with an adaptive advantage *in utero* [Gluckman and Hanson, 2008]. The resulting phenotype is likely to be advantageous in an anticipated future environment. The cue thus acts as a predictor of the nature of this environment. For instance, if the fetal metabolism and growth are adapted to the predicated postnatal environment by the nutrient supply during fetal life as the primary cue, intrauterine nutrient restriction will cause inappropriate fetal predictive response for subsequent abundance supply of nutrients in postnatal stage. Such mismatch of anticipation will then result in susceptibility for chronic diseases in adulthood [Gluckman and Hanson, 2004b] (Figure9).

These observations highlight the importance of investigating the relationship between maternal-fetal interface and the fetal development.

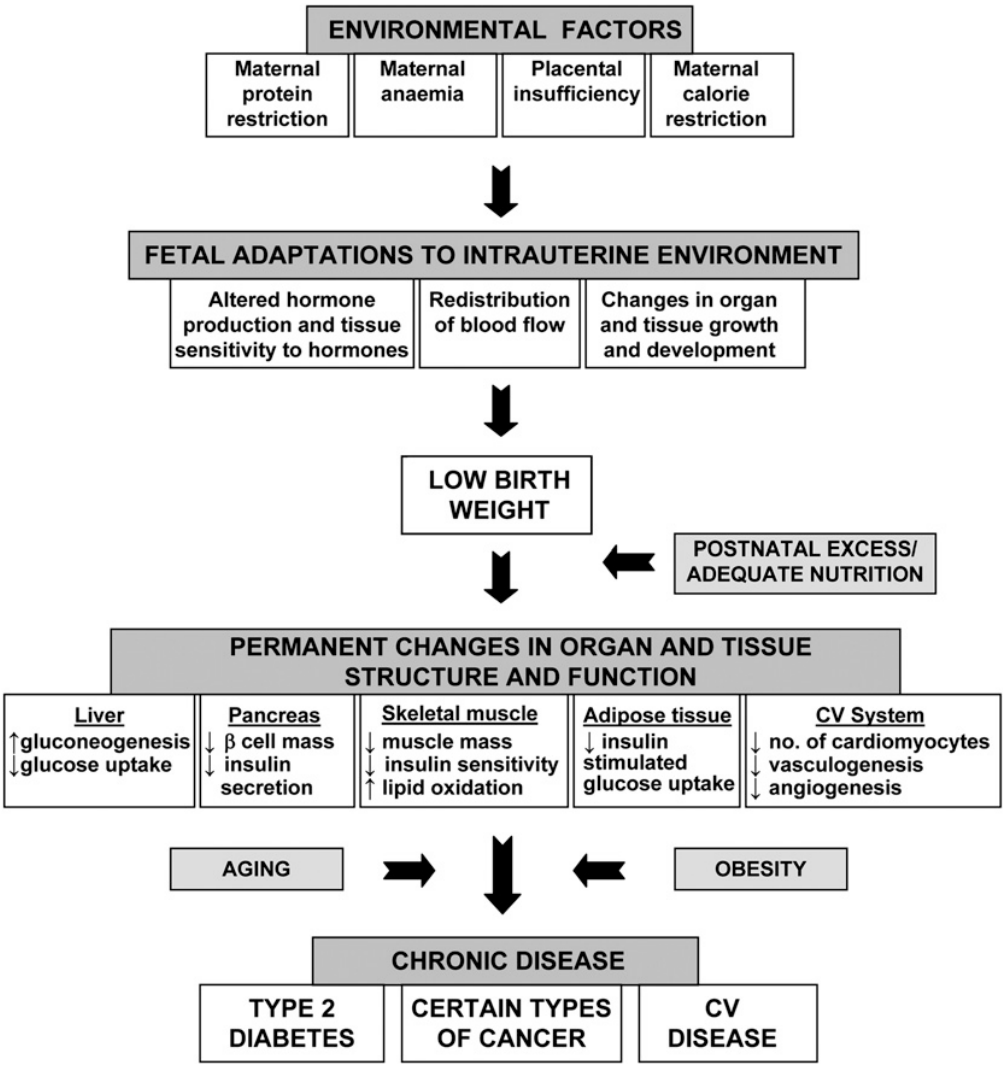


Figure: DOHaD Theory: This suggested that the relationships between birth weight and metabolic disease arose because of the response of a growing fetus to a suboptimal nutritional environment. Central to this hypothesis was the suggestion that during times of nutritional deprivation, the growing fetus adopts a number of strategies to maximize its chances of survival postnatally in similar conditions of poor nutrition. Such adaptations include the preservation of brain growth at the expense of other tissues such as skeletal muscle and the endocrine pancreas, and the programming of metabolism in a manner that would encourage storage of nutrients when they were available. This has no detrimental effect and is in fact beneficial to survival if the fetus is born into conditions of poor nutrition. Thus, in populations where there is chronic malnutrition, these adaptations are beneficial and prevalence of metabolic disease is low. However, detrimental consequences of developmental programming were proposed to arise if the fetus was born into conditions that differed from those experienced in utero. The imbalance between the early and postnatal environments

may then conflict with the programming that occurred during fetal life and predispose the offspring to the subsequent development of metabolic diseases in adulthood [Gluckman and Hanson 2004].

3.2.2 Retrospective studies

There are a limited number of epidemiological studies where associations between nutrition and disease outcomes have been attributed to epigenetics, albeit tenuously. For example, epigenetic profiling of 80 monozygotic twins found that variation in global and locus-specific DNA methylation and histone acetylation accumulated between twins with age, mirroring age-dependent morphological differences, or disease susceptibility discordance. A difference in diet between twins was given as one possible explanation [Fraga *et al.*, 2005]. Also, a recent Swedish study reported a link between men starting smoking at an early age and a higher body mass index in their sons, which they tentatively attributed to epigenetics [Pembrey *et al.*, 2006].The same laboratory assessed other cohorts that were exposed to poor nutrition at discrete time periods early in life. Participants' grandchildren subsequently displayed a decreased risk of heart disease, but only if the grandchild was of the same sex. Conversely, an excess of food in the grandfather produced a four-fold increase in risk of death from diabetes in the grandchild [Kaati *et al.*, 2002; Pembrey *et al.*, 2006].The sex-specific results in particular, suggest an epigenetic influence, although changes in DNA methylation were not investigated.

More robust examples are found in the Hunger Winter Families Studies. In 1944, the occupied Western territories of the Netherlands underwent severe famine, due to a particularly harsh winter and a Nazi-imposed food embargo. Throughout this period however, birth registries and healthcare continued and existing food rations were well documented. This allowed the famine to be clearly defined and exposed individuals to be traced. Various studies have investigated the

epigenetic effect of the famine on those who were in utero at that time. Interestingly, such individuals later displayed aberrant IGF2 imprinting, six decades after the event, compared to their siblings who were not exposed to such periconceptual shock [Heijmans *et al.*, 2008]. Further methylated loci have since been shown to be differentially methylated in these individuals, often in a sex-specific manner [Tobi *et al.*, 2009]. Interestingly, colorectal cancer incidence was decreased for individuals who were adolescents during the famine, despite hypermethylation of discrete loci associated with a neoplastic phenotype [Dirx *et al.*, 2003; Hughes *et al.*, 2009].

3.2.3 Experimental studies

The epidemiological nature of the above studies makes delineating specific contributors to disease difficult, whether they be epigenetic or otherwise. More success has been achieved in experimental animal model systems. Agouti mice are characterised by a variable coat colour that is epigenetically mediated. Mice with the Avy (agouti, viable yellow) allele together with a wildtype allele (a) display a range of coats: yellow, where the agouti gene is overexpressed, through increasingly mottled shades due to partial agouti overexpression, to brown, which is referred to as psuedoagouti and which has minimal aberrant expression [Wolff *et al.*, 1998]. Furthermore, ectopic expression of the agouti allele interferes with metabolic and endocrine systems in multiple tissues resulting in increased morbidity later in life; yellow and mottled mice are prone to obesity, cancer and diabetes, while brown psuedoagoutis are not [Wolff *et al.*, 1999].

The variation in agouti gene expression is dependent upon the methylation of a neighbouring upstream LTR promoter, termed an intracesternal. A particle, or IAP [Cooney *et al.*, 2002]. The degree of methylation in this element produces the coat colour spectrum [Michaud *et al.*, 1994; Morgan *et al.*, 1999].

Intriguingly, experiments that altered maternal nutritional supplementation shifted the epigenetic spectrum of the mother's litter, methyl donors such as methionine fed to the mother increased methylation of the IAP, suppressed agouti gene expression and pushed the colour range of her offspring towards the brown end of the spectrum. This also improved morbidity in the pups when fully grown [Cooney *et al.*, 2002; Waterland and Jirtle, 2003]. Furthermore, such modified agouti phenotypes as a result of supplementation persisted for multiple generations [Cropley *et al.*, 2006].

The agouti model has also been used to probe other environmental exposures, such as the effect of maternal alcohol consumption on developing embryos. In this case, early gestational exposure to ethanol produced hypermethylation of the Avy allele and produced more offspring with a pseudo-agouti-coloured coat [Kaminen-Ahola *et al.*, 2010].

More nebulous effectors like behaviour have also been shown to influence DNA methylation and produce different phenotypic trajectories. Rats that show high levels of care and attention towards their pups in the first week of life, in the form of licking and grooming, produce lower stress responses in the offspring as adults [Francis *et al.*, 1999; Liu *et al.*, 1997].

Cross-fostering of pups to mothers that did not groom as much created higher-stressed pups in adulthood. This suggested an epigenetic rather than genetic mechanism. High rates of maternal licking and grooming altered the expression of multiple genes in the hippocampus of the young, including increased hippocampal glucocorticoid receptor (GR) expression, a protein implicated in short and long term response to stressors [Weaver *et al.*, 2004; Weaver *et al.*, 2005]. Differences in the DNA methylation and histone acetylation of the GR promoter were apparent by day eight in the pups between high care and low care mothers and these differences persisted through adulthood. Additionally, infusing trichostatin, a histone deacetylase inhibitor, into the brains of adult offspring of low care mothers increased histone acetylation, decreased cytosine methylation in the GR promoter and increased expression of the gene. Response

to stress became comparable to rats who received high levels of licking and grooming as pups [Weaver *et al.*, 2004]. Conversely, injecting methionine into the brains of offspring from high-care mothers increased the methylation state of the GR promoter, decreased GR expression and increased their anxiety [Weaver *et al.*, 2005; Weaver *et al.*, 2006]. Methionine treatment altered methylation patterns of a further 300 hippocampal genes, suggesting that such a phenomenon was not limited to the GR locus [Weaver *et al.*, 2006].

4. AIMS OF PhD PROJECT

In the light of all the literature reports shown in previous chapters is evident that the etiology of the CH is still largely unclear. In this scenario, epigenetic mechanisms and in particular an aberrant DNA methylation may explain the increased risk of CH in premature infants and/or with low birth weight and intrauterine growth retardation. We suggested that adverse maternal conditions such as diet or others adverse lifestyle factors influence the establishment of a proper methylation program on fetus during pregnancy. This leads to an early fetal adaptation to extrauterine life, in which changes in pathways involved in the development and in metabolism play an important role. This adjustment could be associated with complications during pregnancy and defects in growth and thyroid development. For that reasons we analyze the global and the gene-specific DNA methylation profile of affected children with congenital hypothyroidism with preterm birth and intrauterine growth delay, against term and preterm healthy controls.

CHAPTER V

5. MATERIALS AND METHODS

In briefly, after the collection of blood sample of cases and controls (5.1.1), the genomic DNA was extracted (5.1.2) and quantified (5.1.3) to test the quality and concentrations for the DNA methylation analysis.

The DNA was than treated with bisulfite to converts the unmethylated cytosine to uracil, allowing to discriminate between methylated cytosine (M) and unmethylated (U) (5.2.1).

We than performed the DNA methylation analysis by the Illumina HumanMethylation27 BeadChip Array (5.2.2).

The raw data of methylation were exported and validated by the *GenomeStudio* software to normalizing and selects the samples than not reached the standards of quality (5.3.1 and 5.3.2).

The clean data were also analyzed by several statistical methods to study the global and the gene-specific methylation patterns (5.3.3).

Finally the selected genes that are differentially methylated between cases and control were studied by their biological functions and pathways involved (5.3.4).

Ultimately, we studied the role of maternal folate deficiency by the analysis the DNA of nine mothers for *MTHFR* polymorphisms (5.4.1).

5.1 SAMPLES COLLECTION AND DNA EXTRACTION

5.1.1 Recruitment of patients

Sixty-eight peripheral-blood samples from CH-subjects, healthy children and parents were collected between 2008 and 2010 thanks to the collaborations of the Pediatric Units of San Raffaele Hospital of Milan and Bolzano Hospital. All participants were informed and agreed to participate at this projects. The details about the clinical features of patients and the design of the DNA methylation study are reported in the section of results.

5.1.2 DNA isolation from peripheral blood samples

Fasting blood samples were collected in EDTA-Vacutainer tubes and immediately chilled on ice before they were centrifuged at 4000 x g for 10 min at 4ºC. DNA was isolated from the stored frozen blood samples according the manufacturer's protocol for the PureGene DNA isolation kit (Gentra Systems, Minneapolis, MN):

Cell Lysis

1. Add 300 µl whole blood (or bone marrow) to a 1.5 ml microfuge tube containing 900 µl RBC Lysis Solution. Incubate 1 minute at room temperature and invert gently 10 times during the incubation.

2. Centrifuge for 20 seconds at 13,000-16,000 x g. Remove as much supernatant as possible with a pipette leaving behind the visible pellet and about 10-20 µl of the residual liquid.

3. Vortex the tube vigorously for 10 seconds to resuspend the white cells in the residual liquid;

4. Add 300 µl Cell Lysis Solution and 3uL 10ng/uL proteinase K (not included in kit) to the resuspended cells and pipet up and down to lyse the cells. Incubate at 55°C over night.

RNase Treatment

1. Add 1.50 µl RNase A Solution to the cell lysate.

2. Mix the sample by inverting the tube 25 times and incubate at 37°C for 15'

Protein Precipitation

1. Cool sample to room temperature.

2. Add 100 µl Protein Precipitation Solution to the cell lysate.

3. Vortex vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution uniformly with the cell lysate.

4. Centrifuge at 13,000-16,000 x g for 1 minute. The precipitated proteins will form a tight, dark brown pellet.

DNA Precipitation

1. Pour the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a clean 1.5 ml microfuge tube containing 300 µl 100% Isopropanol (2-propanol).

2. Mix the sample by inverting gently 50 times.

3. Centrifuge at 13,000-16,000 x g for 1 minute; the DNA will be visible as a small white pellet.

4. Pour off supernatant and add 300 µl 70% Ethanol and invert the tube several times to wash the DNA pellet.

5. Centrifuge at 13,000-16,000 x g for 1 minute. Carefully pour off the ethanol.

6. Invert and drain the tube on clean absorbent paper for 5 seconds.

DNA Hydration

1. Add 50 µl DNA Hydration Solution and vortex 5 seconds to mix.

2. Incubate sample at 65°C for 5 minutes to accelerate rehydration. Note: samples may be incubated at 65ºC for up to 1 hour.

3. Vortex 5 seconds and spin to collect sample at the bottom of the tube.

4. Store DNA at 4°C. For long-term storage, store at –20° or –80° C.

5.1.3 DNA quality and quantity

A NanoDrop® ND1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) was used to measure the DNA concentration and the absorbance ratio (A260/A280). When DNA is extracted from biological samples, protein frequently remains in the DNA solution. Protein is tightly bound to DNA, and complete removal of protein is not always possible. In general, the peak of UV absorption is at 260 nm for DNA and at 280 nm for protein. Thus, when a solution contains both DNA and protein, absorbance at 260 nm is mainly due to the DNA present, and absorbance at 280 nm is due to protein. A pure sample of DNA has the ratio at 1.8 and is relatively free from protein contamination. Generally, the expected ratios for extracted DNA samples should range from 1.7–2.0 [Hebron *et al*., 2009].

To visualize DNA quality, 1 µl of each DNA was loaded on a 1% agarose gel. The extracted DNA sizes were estimated using the DNA marker of GeneRuler™1 kb Plus (Fermentas, Glen Burnie, MD, USA). A digital image was taken under UV light in a Universal Hood II (Bio-Rad, Hercules, CA, USA).

5.2 DNA METHYLATION ANALYSIS

5.2.1 DNA bisulfite treatment

The process of bisulfite conversion of DNA exploits the different sensitivities of cytosine and 5-methylcytosine (5-MeC) to deamination by bisulphite under acidic conditions, in which cytosine undergoes conversion to uracil, whereas methylated cytosine is non-reactive. The efficiency of the reaction is well documented with approximately 99% of unmethylated CpGs undergoing conversion and 99% of methylated CpGs being protected from conversion [Patterson *et al.*, 2011]. Bisulfite treatment of 500ng to 1µg DNA per sample was done using the EZ DNA Methylation-Gold Kit (Zymo Research) according to the manufacturer's instructions prior to the Illumina Infinium DNA Methylation array:

1. Add 130 µl of CT Conversion Reagent solution to each 20 µl sample in the

Conversion Plate. Mix the samples by pipetting up and down.

2. Seal the Conversion Plate with Cover Film. Place the plate in a thermal cycler and perform the following steps:

1. 98°C for 8 minutes

2. 64°C for 3.5 hours

3. 4°C storage for up to 20 hours

3. Add 400 µl of M-Binding Buffer to the wells of a Silicon-A™ Binding Plate on a provided Collection Plate.

4. Load the samples (from Step 2) into the wells of the Silicon-A™ Binding Plate containing the M-Binding Buffer. Mix by pipetting up and down.

5. Centrifuge at ≥ 3,000 x g for 5 minutes. Discard the flow-through and reuse the Collection Plate for the following wash steps.

6. Add 400 µl of M-Wash Buffer to each well of the plate. Centrifuge at ≥ 3,000 x g for 5 minutes.

7. Add 200 µl of M-Desulphonation Buffer to each well and let the plate stand at room temperature (20°C - 30°C) for 15 - 20 minutes, then centrifuge at ≥ 3,000 x g for 5 minutes. Discard the flow-through.

8. Add 400 µl of M-Wash Buffer to each well of the plate. Centrifuge at ≥ 3,000 x g for 5 minutes. Add another 400 µl of M-Wash Buffer and centrifuge for an additional 5 minutes.

9. Place the Silicon-A™ Binding Plate onto a provided Elution Plate. Add 30 µl of MElution Buffer directly to each well. Wait 5 minutes, then centrifuge for 3 minutes at ≥ 3,000 x g to elute the DNA.

The converted DNA was resuspended in 20 µl of TE buffer and stored at −80°C until the samples were ready for analysis.

5.2.2 Infinium Assay for Methylation

DNA methylation analysis was conducted using the Illumina® Infinium Human Methylation27 BeadChip. Each HumanMethylation27 BeadChip consists of 12 arrays, and up to 4 bead chips were processed simultaneously. The assay allows the interrogation of over 27,000 CpG sites located within the proximal promoter regions of over 14,000 consensus coding sequences (CCDS) genes throughout the genome [Bibikova *et al*., 2009]. In addition, the assay includes 110 miRNA promoters and also includes imprinted genes (see table 2). The standard protocol provided by Illumina® was used for DNA methylation analysis.

Briefly, 4 µl of bisulfite converted DNA was isothermally amplified at 37ºC overnight. The amplified DNA product was fragmented by an endpoint enzymatic process. Fragmented DNA was precipitated, resuspended, and applied to an Infinium Human Methylation27 BeadChip and hybridized overnight. During hybridization, the amplified and fragmented DNA samples anneal to specific oligomers that are covalently linked to over 27,000 different bead types.

Each bead type corresponds to the nucleotide identity and thus the methylation status of a bisulfite-converted cytosine in a specific CpG site. The bead chips were then subjected to a single-base extension reaction using the hybridized DNA as a template incorporating fluorescently labeled nucleotides of two different colors, each corresponding to the cytosine (methylated) or uracil (unmethylated) identity of the bisulfite-converted nucleotide at a specific CpG site (figure 10).

The fluorescently stained chip was imaged by the Illumina® BeadArray Reader. The Illumina® Genome Studio program was used to analyze BeadArray data to assign site-specific DNA methylation β-values to each CpG site.

The proportion of methylation (β) for each subject at each CpG site was computed by first subtracting the background signal intensity of negative controls from both the methylated and unmethylated signals and then taking the ratio of the methylated signal intensity to the sum of both methylated and unmethylated signals. Thus, the β-value is a continuous variable ranging between 0 and 1.

Table 1: Markers of the HumanMethylation27 BeadChip.[3]		
Type of target	**CpG sites present**	**Avg # of CpG sites per target**
RefSeq Genes	14,475	1.9 sites
Well-annotated genes described in the NCBI CCDS database (Genome build 36)	12,833	1.9 sites
Methylation hotspots in cancer genes	144	7.6 sites
Cancer-related targets	982	1.9 sites
miRNA promoters	110	2.3 sites

Table 2: CpG sites represented on the Illumina HumanMethylation27 BeadChip.

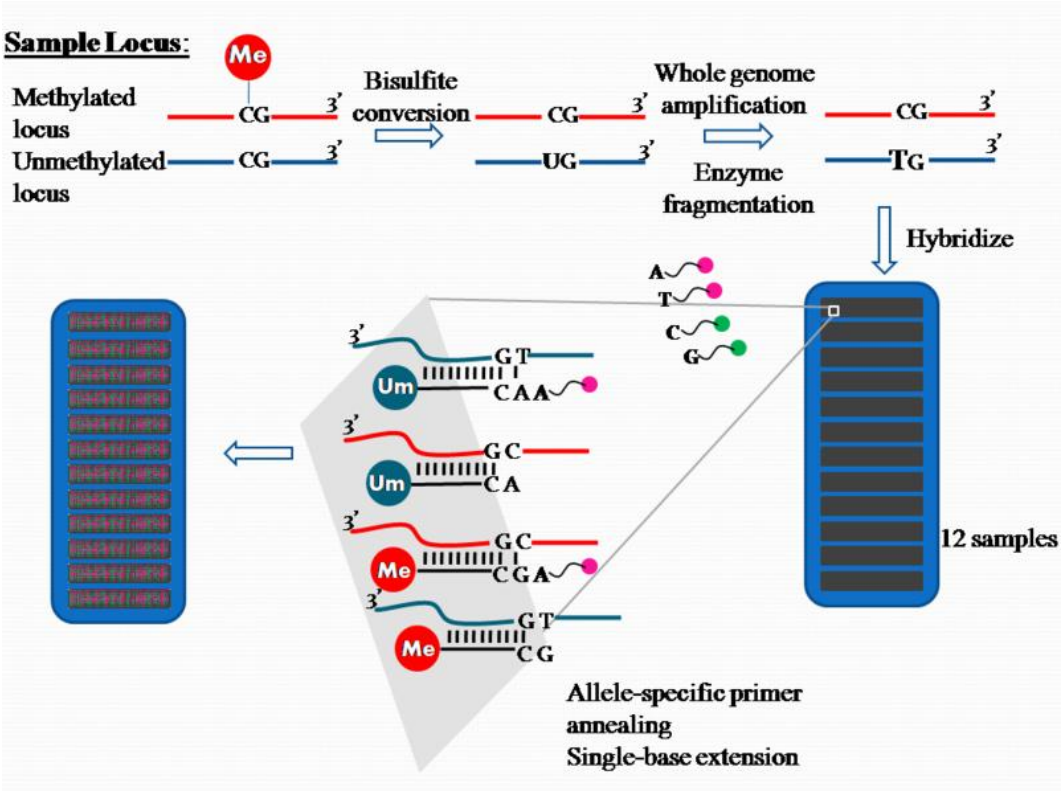


Fig 10: Workflow of the Illumina Infinium technology.

5.3 ANALYSIS OF DNA METHYLATION DATA

5.3.1 Experimental design and quality control

A total of one batch of five bead chips (60 samples) was assayed. Cases and controls were randomly placed within the bead chips. Replicate samples were included in each batch for quality control and normalization measures. All replicate samples achieved a correlation coefficient of greater than 0.985 (data not shown).

Using the Illumina® Genome Studio, background adjusted β-values and assay control probe information was used for initial quality control. Subjects were required to pass three initial quality control criteria in order to be included in further analysis:

1) Subjects achieved a 95% CpG site call rate, which equals 26,250 sites called at an $\alpha<0.05$ significance level;

2) Background signal for each subject was under 1000 units, determined from the controls dashboard in Genome Studio;

3) Clear separation was observed in the non-polymorphic controls, also determined from the controls dashboard.

One sample did not meet these criteria and was removed from the analysis. Individual CpG sites were examined using the detection pvalue metric provided by Illumina® in which the signal generated from each CpG site is compared to negative controls. A threshold of $p<0.05$ was used as a cutoff.

CpG sites that did not reach this threshold were eliminated from the analysis. Using this criterion, 329 of 27,578 CpG sites (1.2%) had missing or invalid β-

values in ten or more samples; these sites were dropped from subsequent analysis, leaving 27,249 CpG sites among 58 subjects.

5.3.2 Principal Component Analysis

Principal component analysis (PCA) was performed on the β-value data matrix of 58 subjects (rows) and 27,249 CpG sites (columns). Data were log it transformed prior to PCA and subsequent association testing. As described above, 329 CpG sites with missing or invalid β-values in ten or more samples were removed from the data; before performing PCA, any remaining missing β- values were imputed using k nearest neighbor averaging (Troyanskaya *et al.*, 2001). The first 20 principal components (PCs) explain 62.8% of overall variance in the logit-transformed β-value matrix, and no single PC among the remaining 347 PCs explains more than 0.34%.

5.3.3 Determination of differentially methylated CpG sites

After initial quality control and PCA of the β value data, a sample of 58 subjects (31 cases and 27 controls) was tested for association between disease status (i.e. case/control) and maternal gene-specific methylation. In this analysis, each CpG site was tested for association by regressing its logittransformed β value on case/control status using multiple linear regression models. Additional covariates included in these models were experimental batch (as a factor), bisulfite-conversion (BSC) efficiency, age, gestational age, birth weight and TSH levels. BSC efficiency for each subject has two channels, red and green. The red and green channels are highly correlated but are not sufficiently collinear, thus both channels were included in the models.

Under standard theory a t-statistic, derived from the least-squares estimate of the case/control parameter and its standard error, was compared with the theoretical t-distribution to test the null hypothesis of no association between disease status and site-specific methylation, H0 : c j= 0, where j=1,…,27,249 indicates the CpG site. Because of the potential for spurious association due to chip bias, however, a randomization testing approach was used to evaluate statistical significance. For each pseudo-dataset, disease status was randomized in a two-stage process for each chip. First, disease status was randomly permuted among the subjects on that chip, and then with 50% probability, disease status was swapped between case and control for all subjects on the chip. In each pseudo-dataset, therefore, each subject was equally likely to be assigned case or control status, which means that, H0 : c j= 0 is known to be (synthetically) true. However, because the randomization is performed separately within chips, the structure of the experimental design, and therefore the chance for spurious disease association due to chip effect, is retained. The empirical null distributions resulting from this randomization approach had heavier tails than the theoretical null distribution, leading to less significant p-values. The randomization approach is therefore a conservative approach and properly controls type-I error in the presence of chip-to-chip variation of β-values. To properly account for the large number of statistical tests being performed, false discovery rate (FDR) q-values [Teschendorff *et al*., 2009] were computed for each CpG site. In our context, FDR is the proportion of detected disease associations (under a given hypothesis testing procedure) that are false, while the q-value is defined as the minimum FDR at which a given test can be considered significant. The q-value is therefore the FDR equivalent of the p-value and was computed using the qvalue package in the R statistical programming environment, under default settings [R Development Core Team, 2008].

5.4 Biological Processes and Pathway analysis

5.4.1 Functional annotation of gene lists

Based on the results from our association testing, a list of differentially methylated CpG sites with p-values less than 0.005 was generated. The CpG sites of interest were mapped to their corresponding genes and the list of genes were then tested for potential overlaps with biological processes and pathways using the freely available Database for Annotation, Visualization and Integrated Discovery (DAVID) database and KEEG pathway database were used for analyzing large lists of genes to mine for functionally related groups of genes and genes within pathways [Subramanian *et al*., 2005]. The software is designed to evaluate microarrays at the level of gene sets. Gene sets are defined as groups of genes that share common biological function, chromosomal location or regulation. The gene set database used in the analysis is established based on prior biological knowledge.

5.4.2 Individual gene information

The National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) and GeneCards (http://www.genecards.org/) were used to gather information on the functions, published work and general information for individual genes.

5.5 ANALYSIS OF *MTHFR* POLYMORPHISMS

The SNP was typed by direct DNA sequencing using primers flanking the A1298C and T1317C polymorphism region. We used the (CAAGGAG-GAGCTGCTGAAGA) forward and the (CCACTCCAG-CATCACTCACT) reverse primers, which result in a 128 base-pair (bp) fragment. The amplification conditions for 200 ng of DNA were: 10 mM of Tris-HCl (pH 8.4); 50 mM of potassium chloride (KCl); 2.0 mM of magnesium chloride (MgCl2); 0.2 mM each of deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP); 0.2 pmol/µL of each primer, and 1.0 U of Taq DNA polymerase. The initial denaturation cycle at 94°C for 4 minutes was followed by 30 amplification cycles at 94°C for 60 seconds for DNA denaturation, 60°C for 50 seconds for annealing of the primers, and 72°C for 60 seconds for chain extension. A last cycle of 10 minutes at 72°C was performed for final extension of the chains. For sequencing by ABI PRISMA 3100 Genetic Analyzer (Applied Biosystems), we used a forward primer (ForwardC) according to Ranjith *et al.* (2003). The amplification product was purified using a QIAquick PCR Purification Kit (Qiagen, Germany), according to the manufacturer's instructions. The sequencing reaction was performed with a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, UK), according to the manufacturer's instructions. Analysis was based on the chromatograms reproduced by the automated sequencer.

CHAPTER VI

6. RESULTS

6.1 DESIGN OF DNA METHYLATION STUDY

6.1.1 Statistical methods

Unsupervised hierarchical clustering of samples and analysis of DNA methylation were done using the *Illumina GenomeStudio®* software. Differentially methylated probes in the Illumina array from each comparison were identified using Siggenes package from R with a cut-off of false discovery rate (FDR) <0.1% and Differential Score (DS)> 13. FDRs were generated after comparison of 1000 random permutations between samples. DS of 13, corresponding to a p value of 0.05, takes into account background noise and sample variability. Pearson linear correlation was used to determine the similarity of DNA methylation profiles between samples. The Database for Annotation, Visualization and Integrated Discovery (DAVID) and Kyoto Encyclopedia of Genes and Genomes (KEGG), were used for gene ontology and pathway analysis using total number of genes presented in the array as a background for comparison (Dennis *et al.* 2003; Huang da *et al.* 2009). The results are presented as mean (SEM) or proportions. Student's t-test, chi-squared test, analysis of variance and rank-sum test were used to test group differences. A p-value<0.05 was considered significant. The association between covariates or potential confounders and outcome were tested using simple, linear regression or ANOVA.

6.1.2 Collections of CH and control subjects

Thanks to the partnership of the Pediatric Units of Hospital of Bolzano and San Raffaele Hospital of Milan, we collected a total of 31 children born prematurely with CH (*cases group*) and 28 term/preterm healthy subjects (*controls group*). As reported in detail in table 3, cases and controls have comparable ages at sample collection and gender proportions.

Most of them were born from spontaneous conception while the remaining 20% by *in vitro* fertilization techniques. The percentages about only child/twins per reproductive event are 29-71 for CH-cases and 43-57 for controls.

Focused on twin pairs data, the fraction of monozygotic and dizygotic twins (MZ/DZ) are 41/59 for CH-cases and 19/81 for ctrls groups. Based on thyroid phenotype, the 64% of affected subjects are concordant while the others are discordant for CH.

According to the clinical data at birth, all of CH-cases were born preterm while about half of healthy controls were premature and half born at term. The 65% of affected children was born small for gestational age whereas the majority of controls had an acceptable intrauterine growth. Concerning the thyroid phenotype, the 42% of the cases showed a subclinical hypothyroidism and the 58% exhibit an overt hypothyroidism. All of them had an *in situ* gland with volume and free-T4 values within normal limits.

GENERAL FEATURES			CH-CASES	CONTROLS	p-VALUE
Number of subjects					
		N	31	28	
Age at sample's collection					
		years	5.1 (2.6)	5.7 (2.8)	0.91
Gender					
	Male	%	58	47	
	Female	%	42	53	
Conception					
	Spontaneus	%	87	81	
	FIVET/IUI	%	13	19	
Number of child/reproductive event					
	Only child	%	29	43	
	Twins	%	71	57	
Twin pairs data					
	MZ/DZ	%	41/59	19/81	
	discordance/concordance for CH	%	36/64	50/-	
CLINICAL DATA AT BIRTH					
Gestational Age					
	Preterm birth	%	100	57	
	At term birth	%	-	43	
Percentile of fetal growth					
	Small for Gestational Age (SGA)	%	65	29	
	Acceptable for Gestational Age (AGA)	%	35	71	
Serum TSH					
	Subclinical Hypothyroidism (SH)	%	42	-	
	Overt Hypothyroidism (OH)	%	58	-	
Free T4					
		ng/L	1.04 (0.43)	-	

Table 3: General characteristics and clinical data of subjects enrolled in this study. The features are reported as numbers or percentages. To show a significant difference of the age at sample collection between cases and controls, a p-value are reported.

To understand the relationship between DNA methylation and prematurity and/or the intrauterine growth delay associated with the onset of CH, we divided the subjects about their a) gestational age, b) intrauterine growth and c) thyroid function, and performed three independent DNA methylation studies.

These approaches can let to find which factors that have undergone aberrant methylation, affects the state related to prematurity or intrauterine fetal growth or are direct linked to thyroid development of at thyroid hormone action at peripheral level (Table 4 a, b, c).

The details of the DNA methylation analysis and subjects' categorization are reported in the following chapter (6.1.4).

DNA METHYLATION APPROACHES		CASES GROUP	CONTROLS GROUP	DNA METHYLATION INVOLVED IN
a	**Gestational Age**	*Very Preterm Birth (VPB)* *Preterm Birth (PB)*	*Very Preterm Birth (VPB)* *Preterm Birth (PB)* *Term Birth (TB)*	**Preterm birth delivery associated with CH**
b	**Intrauterine Growth**	*Small for gestational age (SGA)* *Acceptable for gestational age (AGA)*	*Small for gestational age (SGA)* *Acceptable for gestational age (AGA)*	**Intrauterine growth delay associated with CH**
c	**Thyroid Function**	*Subclinical hypothyroidism (SH)* *Overt Hypothyrpidism (OH)*	*Preterm Birth (PB)* *Term Birth (TB)*	**Thyroid organogenesis or thyroid hormone actions at peripheral level**

Table 4: Categorization of cases and controls based on their clinical data at birth and thyroid function. a) Gestational Age approach: CH-VPB, CH-PB vs C-VPB, C-PB and C-TB; b) Intrauterine growth approach: CH-SGA, CH-AGA vs C-SGA and C-AGA; c) Thyroid function approach: SH and OH cases vs C-PB and C-TB.

6.1.3 Design of DNA methylation studies

The DNA methylation analysis by Illumina's array platform was the same for each approach. This method allows quantifying the DNA methylation levels of 27,252 CpG sites belonging to 14,495 genes. The majorities of these sites (73%) is localized into "*CpG islands*" of promoter of target genes and are characterized by the direct action of methylation on gene expression. The remained 27% are called "*non-CpG island*" sites and are localized into non-promoter regions also called non-coded DNA such as repetitive elements (*Line* and *Alu* repeats) and intronic sequences, whereas the DNA methylation acts indirectly on gene expression by recruiting the complexes responsible of chromatin condensation [Shen et al, 2009].

The results regarded *CpG island* sites and *non-CpG islands* site as well as the autosomes and the X-linked CpG sites were reported separately, since these regions have different DNA methylation levels (figure 11).

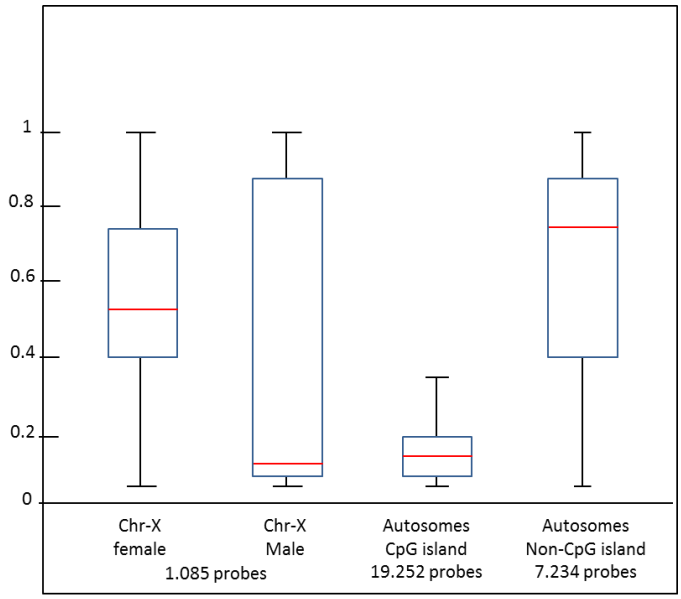


Figure 11: DNA methylation levels of ChX from female and male and of autosomes from CpG island and Non-CpG island sites. Boxes represent the global methylation levels (0 to 100% of AVG Beta) and the red line shows the mean values.

In briefly, we firstly applied an ANOVA statistical method of standard deviations of AVG Beta values from cases and controls groups. As previously described, the presence of potential confounders, such as the ratio of only child/twin pairs or the ages at sample collection, can influence the DNA methylation patterns and the internal variability of groups [Hwang and Park, 2009]. In fact, the genetic similarities of twins as well as genetic differences of single child can influence the internal variability of groups and subsequently the DNA methylation analysis. This method represents a good index of the variability between and within groups, where non-significant differences between cases and controls indicate a good homogeneity of samples (6.2.1).

Subsequently we performed an analysis of clustering to display the Euclidian distances of cases and controls. This analysis can allow us to see the closeness or discrepancy between subjects based on their methylome (6.2.2).

Then, we analyzed the global DNA methylation profile of *"CpG island sites"* and *"non-CpG island sites"* of autosomes and sexual chromosome.

We compared the DNA methylation trends by correlating the AVG beta values with the 19.252 *CpG island* sites and the 7.234 *non-CpG island* sites. The graphical representation indicates roughly how many CpG sites and how much are differentially methylated between cases and controls (6.3.1).

A more accurate estimate is obtained with the comparison of the mean AVG beta values of groups and a t-test method allows us to find which subjects have a statistically significant difference of methylation (6.3.2).

Afterwards, we performed a gene-specific methylation analysis to select the sets of differentially methylated genes (DMGs) that are hypo- or hypermethylated in CH-cases groups than controls (6.4.1). Through a differential score plot analysis we exported a sets of genes with FDR<0.01, Diff Score <0.05 and AVGdeltaBeta> 5% (for details of statistical analysis see Materials and Methods).

Subsequently, to find which factors are associated to epigenetic changes we selected a set of genes in common between the three approaches and clustered CH-patients for these DMGs (6.4.2)

The peculiar characteristics of DMGs were investigated by their CpG sites locations, chromosomes distribution and imprinted status with *GeneImprint Software* (6.4.3).

In the 6.4.4 paragraph we described the genetic analysis of *MTHFR* polymorphism because these variants are reported to be involved in folate deficiency, pregnancy complications and birth defects.

In the last section (chapter 6.5.1), by the ontology enrichment analysis, the DMGs were categorized by their biological processes with *David Gene Functional Database* and *KEEG Pathway* software. Finally, we selected a list of "top genes" which are particularly interesting for further studies (6.5.2).

These analysis let to identify the biological pathway affected on aberrant DNA methylation, and can potentially explains the higher predisposition of the onset of congenital hypothyroidism associated with premature birth and/or intrauterine fetal growth impairment.

6.1.4 Categorization of subjects according to their clinical data

a) Gestational age approach: we used the last menstrual period to estimate gestational age (GA) of infants. We divided cases and controls into two and three groups respectively, depending on the severity of prematurity: *Very Preterm Birth* with 32 completed weeks or less (VPB), *Preterm Birth* with 33 to 37 weeks (PB) and *Term Birth* with 38 to 40 weeks of gestation (TB). As reported in table 5, there's no significant difference about the ages at sample collection of subgroups. Interestingly, the TSH-levels of CH-PB are significant higher then CH-VPB. No linear correlation was found about GAs and AVG Beta levels in affected infants both for *CpG island* and *Non-CpG island* sites (figure 12).

		GENDER (m/f)	AGE AT SAMPLE COLLECTION (years)	GESTATIONAL AGE (weeks)	TSH (μg/ml)
CASES GROUP	**VPB**	8/4	6.5 (2.57)	30.6 (2.12)	9.16 (5.65)
	PB	10/9	5.3 (2.29)	35.5 (1.60)	50.4 (78.63)*
CONTROLS GROUPS	**VPB**	3/6	6.4 (2.69)	30.3 (2.07)	n
	PB	3/3	6.6 (3.6)	34.5 (1.23)	n
	TB	7/5	5.2 (2.22)	39.2 (1.56)	

Table 5. Clinical data of Gestational Age approach. The details about the age at sample collection, GA and TSH values are reported as a mean and in parentheses are shown the standard deviations. CH-PB subjects have TSH levels significant higher than CH-VPB (*$p<0.05$).

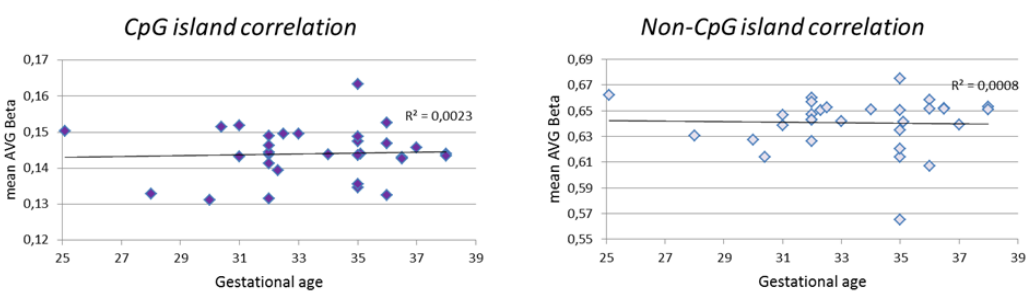
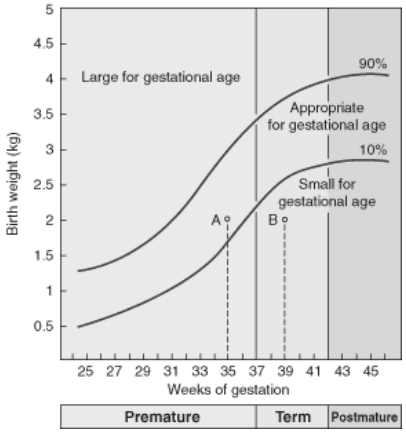


Figure 12. Distribution of GAs and AVG Beta values of CH children. No linear correlation was found both for CpG island and Non-CpG island sites.

b) Intrauterine growth: birth weight (BW) and gestational age (GA) of these children are compared with reference standards for the normal spread of "gestation and weight-for-gestational age at birth.



Infants are considered *Small for Gestational Age* or SGA when the gestational age/birth weight ratio (GA/BW ratio) was below of 10th percentile than standard curve; while, a normal intrauterine development or *Appropriate for Gestational Age* or AGA has a GA/BW ratio between 10th and 90th percentile [Milde *et al.*, 2006].

According to the percentile of intrauterine growth we categorized both cases and controls as SGA and AGA. As shown in table 6, no significant differences are found in the age of sample collection or at the TSH levels. No linear correlation was found about percentiles of intrauterine growth and AVG Beta levels of affected subjects, both for *CpG island* and *Non-CpG island* sites (figure 13).

		GENDER (m/f)	AGE AT SAMPLE COLLECTION (years)	GESTATIONAL AGE (weeks)	BIRTH WEIGHT (g)	PERCENTILE (th)	TSH (µg/ml)
CASES GROUP	SGA	11/9	5.53 (2.51)	33.8 (2.4)	1427 (466.3)	3.5 (2.3)	32.6 (57.7)
	AGA	8/3	5.5 (2.83)	32.9 (3.6)	1964 (644.7)	23 (19.8)	42.3 (83.6)
CONTROLS GROUPS	SGA	3/3	6.2 (2.26)	32.6 (1.94)	1452.5 (405.5)	4.25 (3.8)	n
	AGA	12/8	5.6 (2.76)	36.5 (4.4)	2720 (850.3)	57.8 (36.6)	n

Table 6: Clinical data of Intrauterine Growth approach. The details about the age at sample collection, GA, BW, percentile and TSH values are reported as a mean and in parentheses are shown the standard deviations.

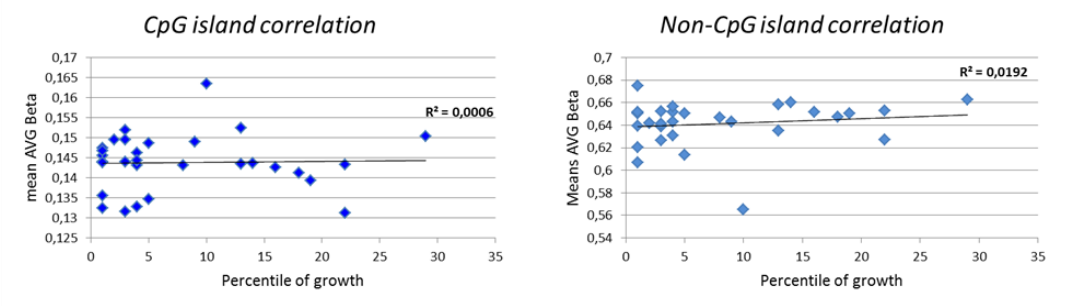


Figure 13: Distribution of percentiles of intrauterine growth and AVG Beta values of CH children. No linear correlation was found both for *CpG island* and *Non-CpG island* sites.

c) Thyroid function: it was assessed through the measurement of thyrotropin (TSH) and the free thyroxin (fT4) levels. Serum TSH level is generally considered the best screening test for thyroid disease; this test has proved to be both sensitive and specific. In this study, the Pediatric Units of Bolzano and Milan had routinely performed thyroid function test using radioimmunoassay for TSH and fT4 measured within the first 10 days of life and repeated every 2 to 4 weeks after the first test [Gruters and Krude, 2011].

The newborn thyroid capabilities was considered abnormal when the serum-TSH is higher than 4 µU/ml (infant normal range, 0.5 < TSH < 4 µU/ml) and fT4 was normal or lower than 0.8 ng/L (infant normal range, 0.8 < fT4 < 1.8). Considering

this TSH cut-off, we categorized the affected infants of cases group as *Subclinical Hypothyroidism* (CH-SH) with a TSH value between 4 to 10 μU/ml and *Overt Hypothyroidism* (CH-OH) with a TSH value greater than 10 μU/ml, where as the controls group was divided as *preterm birth* (C-PB, GA< 37 weeks) and *term birth* (C-TB, GA>37). Unfortunately, we were not able to recall a complete data concerning the presence or absence of autoantibodies against thyroglobulin and tireoperoxidase and how many subjects were treated with L-thyroxine. As reported in Table 7, significant differences are found when compared the GA and BW of CH-SH and the others groups. Also in this case, CH children don't show a direct link between TSH levels and AVG Beta values for *CpG island* and *Non-CpG island* sites (figure 14).

		GENDER (m/f)	AGE AT SAMPLE COLL. (years)	GESTATIONAL AGE (weeks)	BIRTH WEIGHT (g)	TSH (μU/ml)
CASES GROUP	**SH**	9/3	6,6 (2,47)	32,1 (2,71)*	1299 (417,7)**	6,02 (1,47)
	OH	9/10	5,2 (2,89)	34,6 (3,07)	1793 (596,5)	60,4 (78,83)
CONTROLS GROUP	**PB**	6/10	6,1 (2,29)	32,4 (2,76)	1625 (487,9)	n
	TB	7/5	5,3 (3,8)	39,3 (2,1)	2652 (679,5)	n

Table 7: Clinical data of Thyroid Function approach. The details about the age at sample collection, GA, BW, percentile and TSH values are reported as a mean and in parentheses are shown the standard deviations. Significant differences (*p<0.05 and **p<0.001) about GA and BW of -SH than OH, C-PB and C-TB.

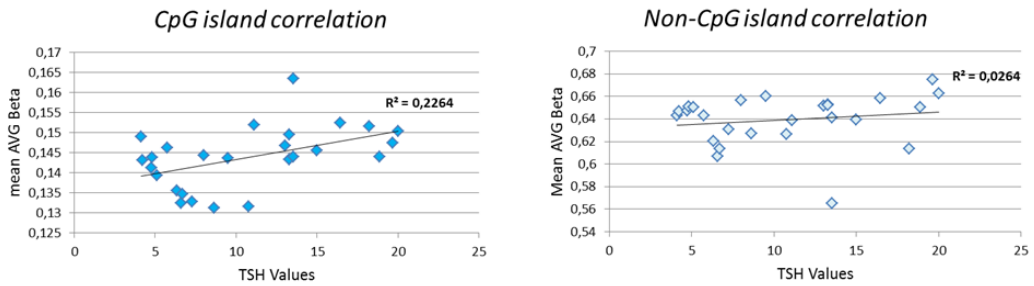


Figure 14: Distribution of TSH and AVG Beta values of CH children. No linear correlation was found both for *CpG island* and *Non-CpG island* sites.

6.2 ANOVA STATISTICAL ANALYSIS

The ANOVA analysis of autosomes between cases and controls groups of gestational age and thyroid function approaches don't shown statistically significant differences of standard deviations, for both *CpG island* and a *non-CpG island* sites. The intrauterine growth approach analysis has revealed that affected infants with AGA have a significant internal variance (*$p<0.05$) when compared with the C-SGA group about *CpG island* sites.

When we compare separately the Chr-X values of standard deviations, we found some differences between cases and ctrls:

- *CpG island* sites about *female* and *male* CH-VPB vs C-PB and C-TB
- *CpG island* sites about *female* and *male* CH-SGA vs C-SGA and at *Non-CpG island* sites of *female* CH-SGA versus C-AGA
- *CpG island* sites of *female* CH-SH vs C-PB and C-TB, and at *Non-CpG island* sites of *male* CH-OH than C-TB

These results may be due to the small numbers of subjects in CH-AGA and the different fractions of females and males. This means that the following studies of global and gene-specific methylation status between these subgroups may be influenced (figure 15).

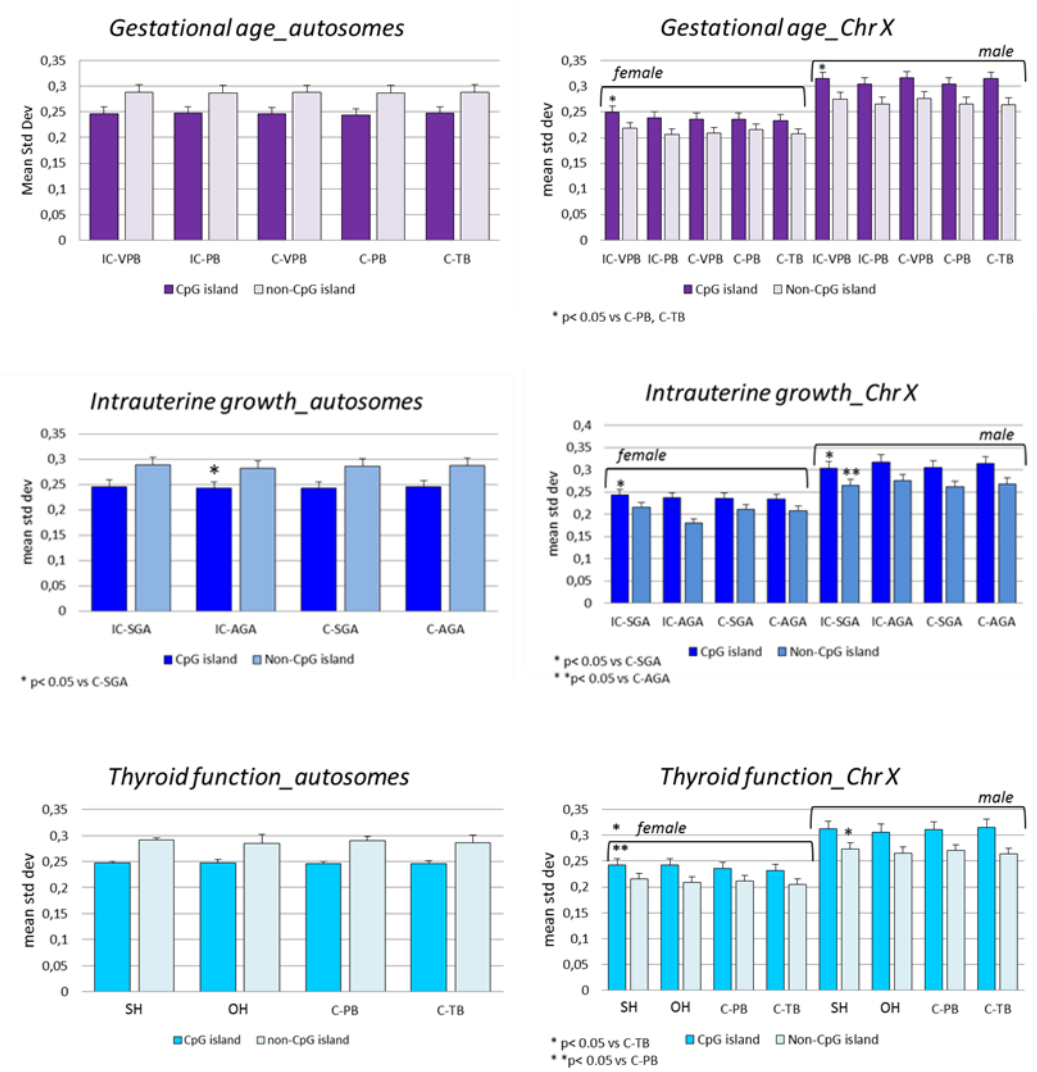
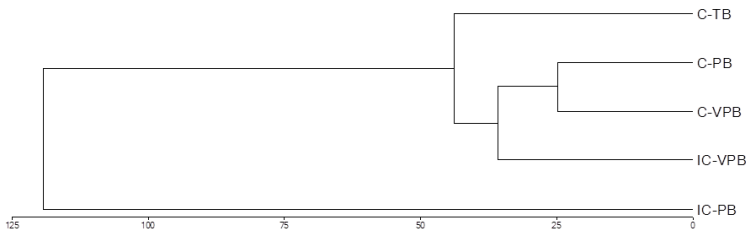


Figure 15: ANOVA analysis of internal variance. Gestational age approach: significant differences of mean standard deviation about *females* and *males* Chr X of CH-VPB versus C-PB and C-TB for *CpG island* sites. Intrauterine growth approach: p<0.05 of *CpG island* sites of autosomes between CH-AGA and C-SGA; for *female* and *male* Chr X between CH-SGA and C-SGA; p<0.05 of *Non-CpG island* sites of *male* Chr X about IC-SGA and C-AGA. Thyroid function approach: significant differences of *CpG island* sites of female Chr X between CH-SH and C-PB and C-TB and about *Non-CpG island* sites of *male* Chr X between CH-SH and C-TB.

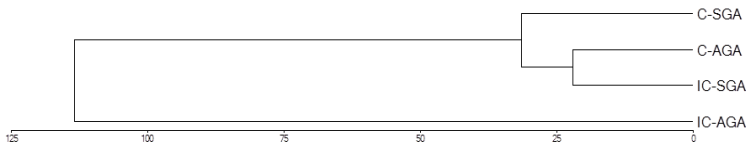
6.3 CLUSTERING OF SUBJECTS

After background adjustment and normalization, we performed unsupervised hierarchical clustering with all the groups based on a distance measure of 1-r, where r is the Pearson correlation coefficient between different samples. As display in figure 15, for all of the three approaches, cases and controls are distributed according to their features at birth. In particular we have seen that affected children with preterm birth, acceptable intrauterine growth and overt hypothyroidism clustered alone than other groups. CH-children with very preterm birth and intrauterine growth delay are distributed between controls and other cases, while SH cases are localized with preterm and term controls.

Gestational age approach



Intrauterine growth approach



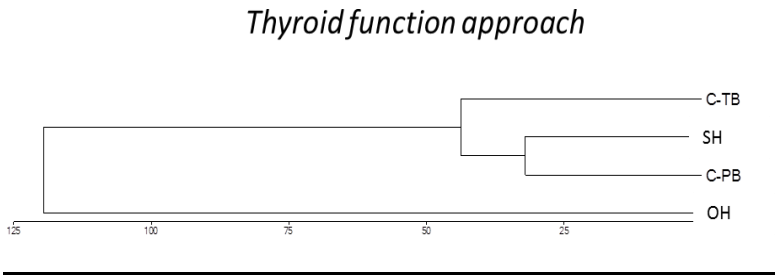


Figure 16: Clustering on Euclidian distances of cases and controls for the three study approaches.

Interestingly, when categorized separately the 31 cases and 28 ctrls, we found that 9/12 TB controls clustered together while PB controls are distributed randomly (data not shown). Focused affected patients, the only child with percentile of growth <10th and comparable levels of TSH are localized closely. On the contrary, CH subjects with a normal intrauterine development are relatively casually distributed. Concordant twin pairs from cases and controls clustered together while for discordant twin pairs, affected twin with TSH<10 μU/ml clustered with his brother, whereas CH-twin with TSH>10 μU/ml is localized far from healthy twin.

6.4 GLOBAL DNA METHYLATION ANALYSIS

6.4.1 Representation of DNA methylation trends

As expected, the correlation of probes methylation values between different sample groups is consistent with that observed in the cluster analysis. The analysis of global methylation data between cases and controls shows that both *CpG island* and *non-CpG island* sites have methylation profiles not fully overlapping (figure 17):

- *CpG island methylation trends:* in all three studies, it appears that CpG sites that show the largest differences in methylation are those which are physiologically hypomethylated. In fact, approximately 3.000 of 19.252 CpG sites that have a different methylation status between cases and controls ranges from 10 to 20% of AVG Beta.

- *Non-CpG island methylation trends:* on the contrary in this case, the major differences are found in CpG sites that are hypermethylated. About 3.500 of 7.354 CpG sites which shown different AVG Beta levels ranging from 50 to 80% of DNA methylation.

Generally, in all of studies, controls with terminal birth and adequate intrauterine growth display a high methylation status followed by preterm and SGA controls and lastly by affected subjects.

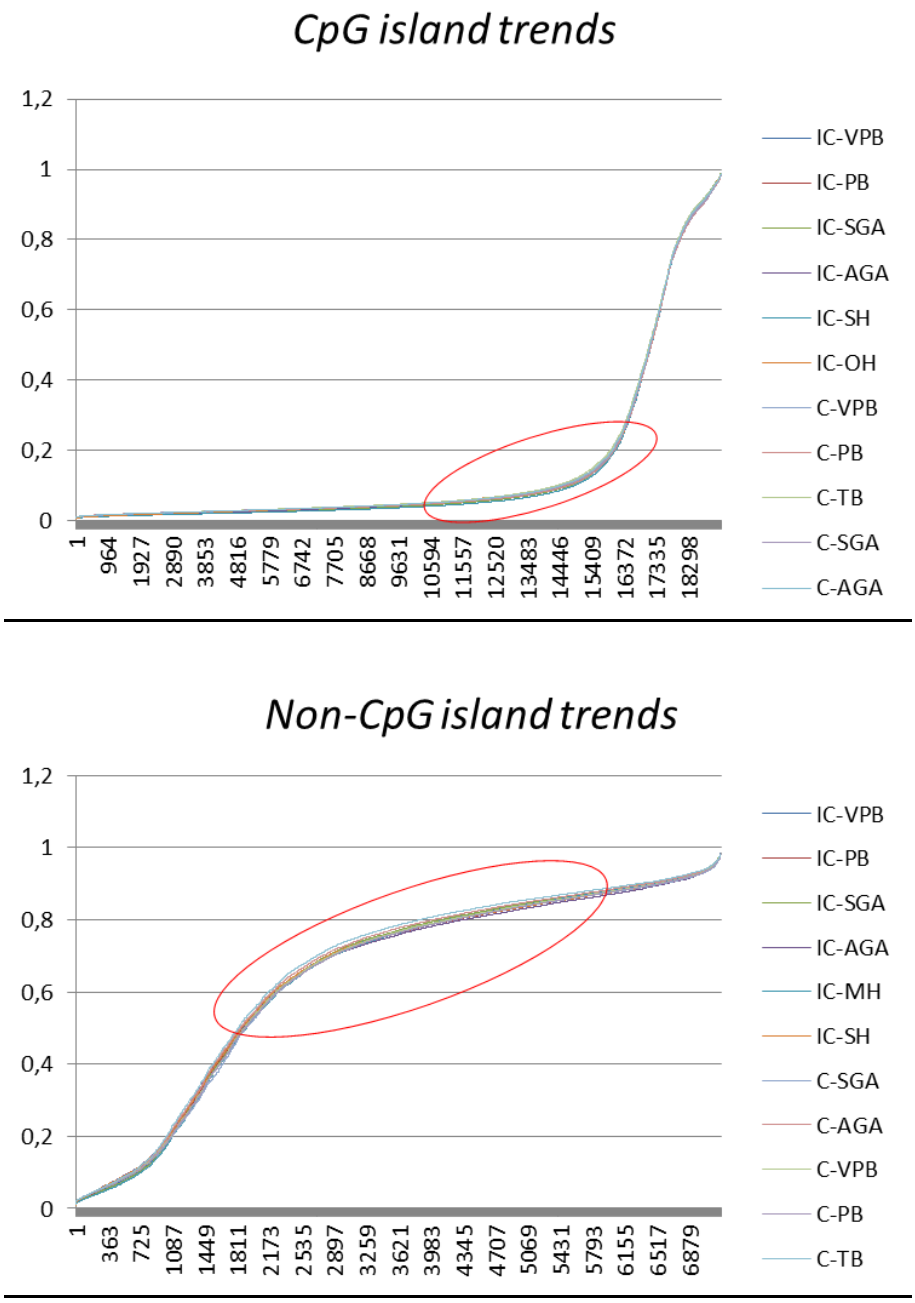


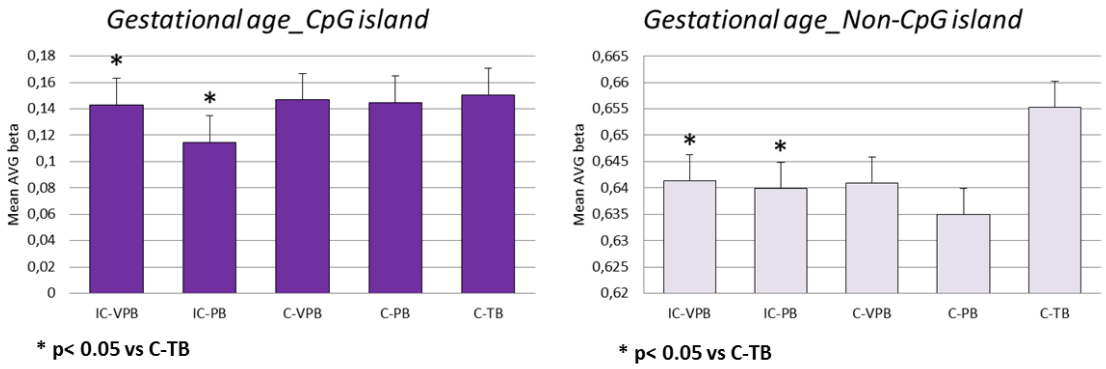
Figure 17: DNA methylation trends of cases and ctrls categorized by *gestational age*, *intrauterine growth* and *thyroid function*. About 4.000 of 19.252 *CpG island sites*, which ranging from 5 to 20% of AVG Beta values shown different methylation levels. Approximately 3.500 of 7.354 *Non-CpG island sites* that range from 50 to 80% of AVG Beta values shown different have different methylation status.

6.4.2 Comparison of mean-AVGbeta values

The study of the global methylation status about their AVG Beta levels confirmed these different trends.

Analysis of autosomes: are shown in histograms reported below (figure 18), we found that the mean of AVG Beta values was statistically significant different when we compared:

- CH-VPB and CH-PB with TB controls, both for *CpG island* and *non-CpG island* sites.
- CH-SGA with AGA controls for *CpG island* sites.
- SH than OH cases, PB and TB controls for *CpG island* sites and SH with TB controls for *non-CpG island* sites.



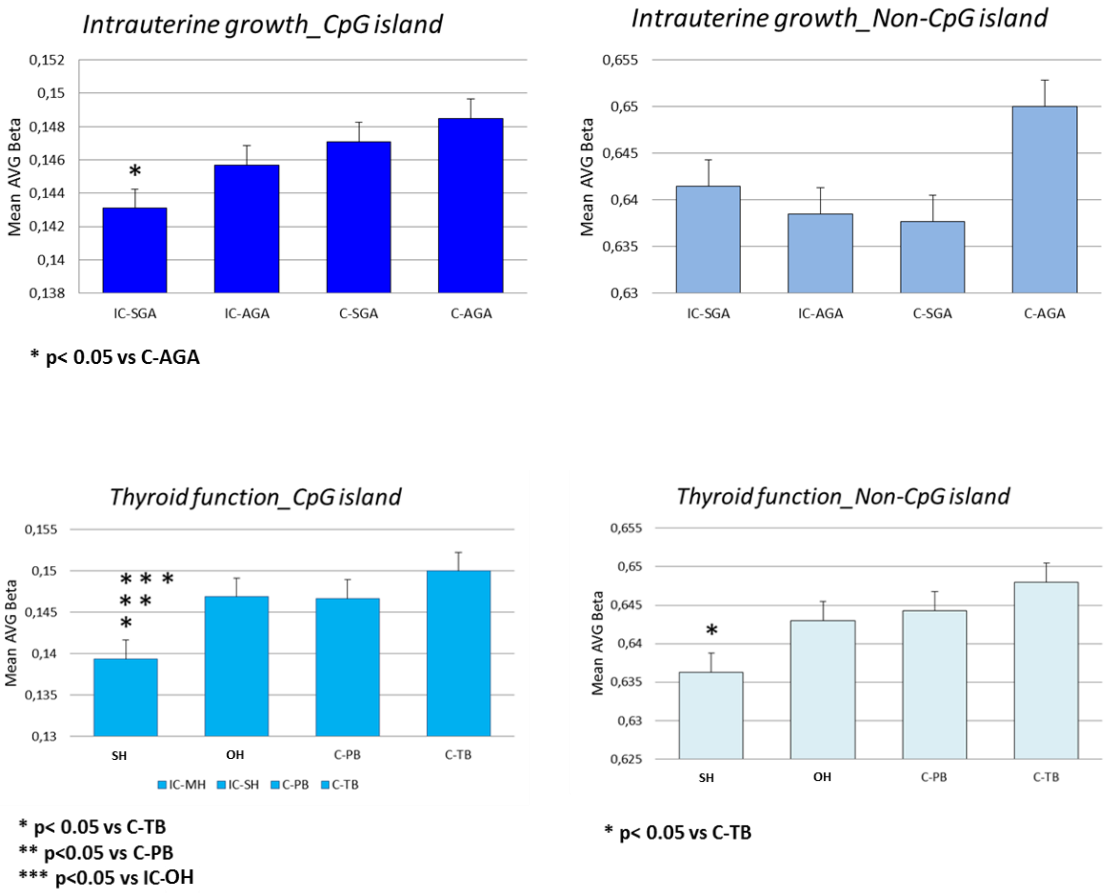


Figure 18: Comparison of autosome mean-AVG Beta values. Gestational age approach: significant differences of global methylation were found between CH-VPB and C-TB both for CpG island and Non-CpG island sites. Intrauterine growth approach: p<0.05 of CpG island sites between CH-SGA and C-AGA. Thyroid function approach: significant differences of CpG island sites between SH and OH, C-PB and C-TB and about Non-CpG island among SH and C-TB.

Sexual chromosome: the analysis of Chr-X methylation (figure 19), revealed significant differences between:

- VPB *female* and *male* with TB ctrls for *CpG island* and VPB *female* with TB ctrls.
- SGA and AGA *female* with AGA ctrls for *CpG island* and AGA female with TB ctrls for non-CpG island sites.

- SH *female* and *male* with TB ctrls for *CpG island* and VPB *female* with TB ctrls.

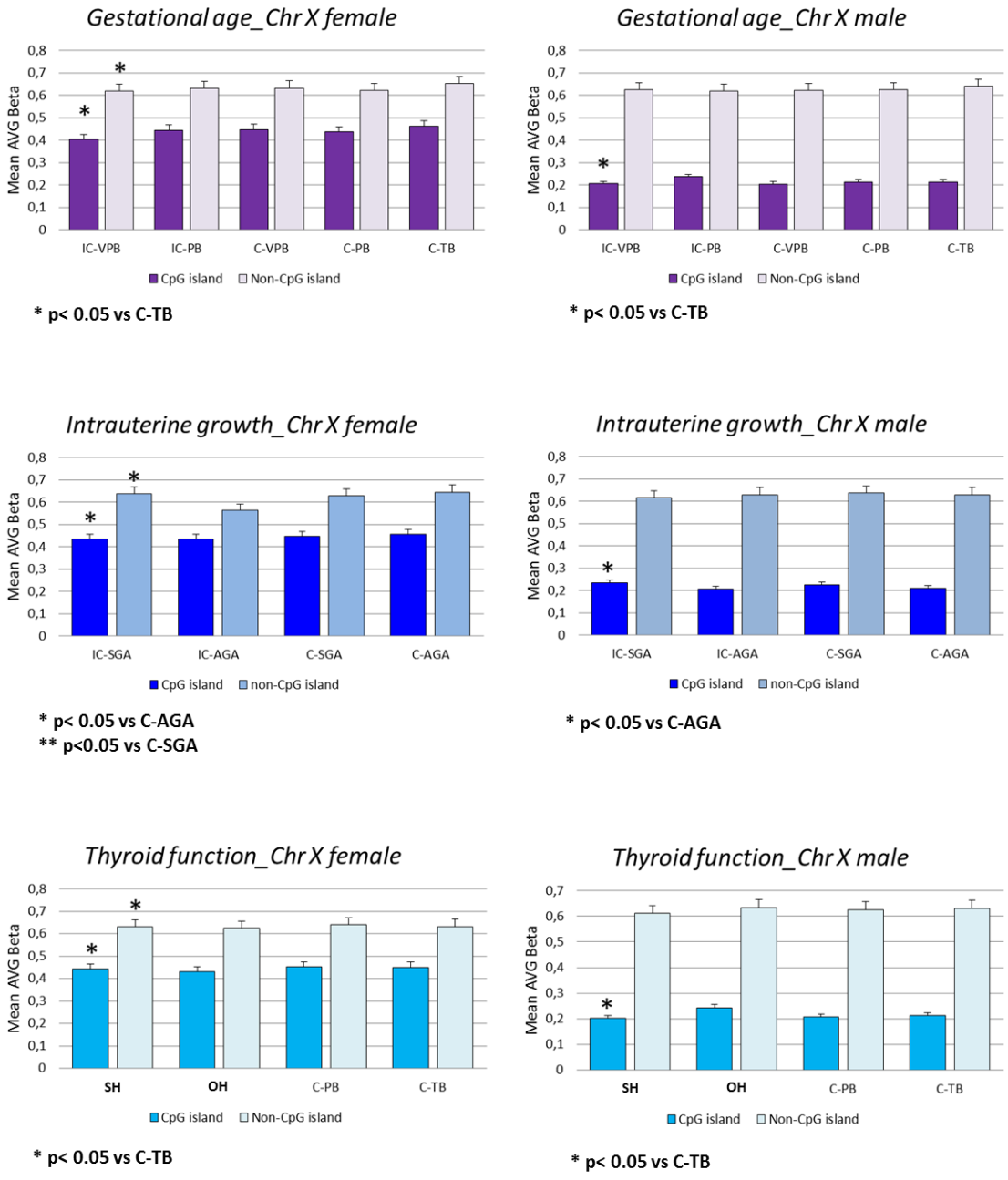


Figure 19: Comparison of Chr X mean-AVG Beta values. Gestational age approach: significant differences of global methylation were found between CH-VPB *female* and *male* and C-TB for CpG island sites and CH-VPB *female* vs C-TB for Non-CpG island sites. Intrauterine growth approach: p<0.05 of CpG island sites between both CH-SGA *female* and *male* and C-AGA. p<0.05 of Non-CpG island sites among CH-SGA *female* vs C-AGA.

Thyroid function approach: significant differences of CpG island sites between *female* and *male* SH and C-TB and about Non-CpG island among SH *female* and C-TB.

6.4.3 Summary of global methylation analysis results

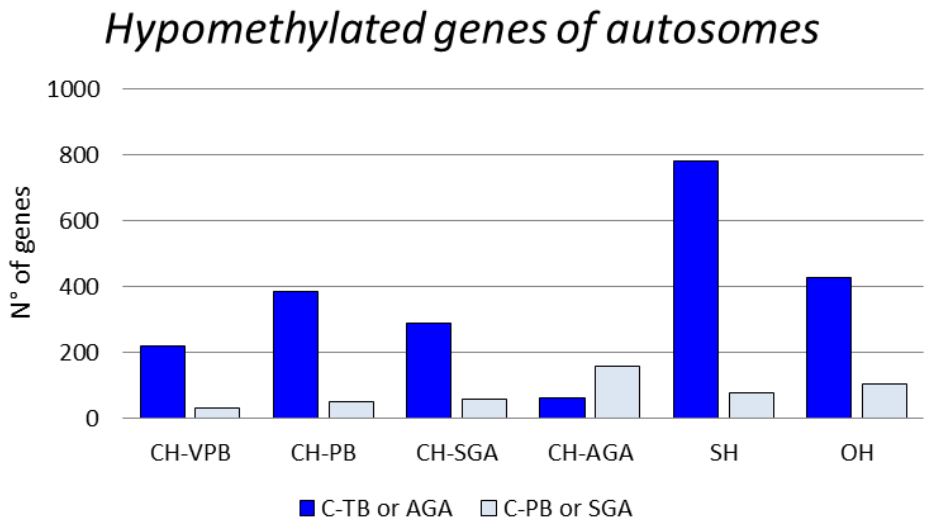
The results of the global methylation analysis have shown that affected subjects with prematurity associated with CH have a different methylation status than healthy controls. In facts, the clustering of subjects revealed that only child with SGA and comparable levels of TSH localized vey close. Also, twin pairs discordant for CH clustered together in presence of SH while twin with OH are localized far form his brother. These results suggested that TSH levels may be correlated with methylome status even in the presence of high genetic similarity. The comparison of the DNA methylation trends and the mean values of AVG Beta highlighted that cases are hypomethylated than controls with term birth. Generally, these differences are statistically significant when compared cases with severe or not prematurity and intrauterine growth delay than C-TB for both *CpG island* and *Non-CpG island* for autosomes and X-chromosome. The analysis of thyroid function approach indicates that CH with subclinical hypothyroidism has a significant hypomethylation status than controls. This finding could be explained by clinical data of these patients. As listed in table, the SH group has average levels of gestational age and birth weight significantly lower than others. This suggests that such factors are associated and justifying the low levels of DNA methylation. Based on these early results, it seems that the premature birth associated with CH, especially in the presence of intrauterine growth restriction is related with global hypomethylation. The direct link between TSH levels and hypomethylation remain unclear but the analysis of clustering and the low but not significant levels of methylation of OH-cases suggest its possible involvement.

6.5 GENE-SPECIFIC DNA METHYLATION ANALYSIS

6.5.1 Selection of Differentially Methylated Genes (DMGs)

Through the gene-specific DNA methylation analysis by FDR<0.01, Differential Score< 0.05 and AVG delta Beta> 0.05 we selected the sets of DMGs that are hypomethylated in affected children than term and AGA controls.

As shown in the histograms and table below, approximately 90-96% of DMGs are hypomethylated in cases with CH (figure 20). Interestingly, about 70% of these genes are represented by *Non-CpG island* sites. On the contrary the comparison with preterm controls doesn't show a significant number of DMGs.

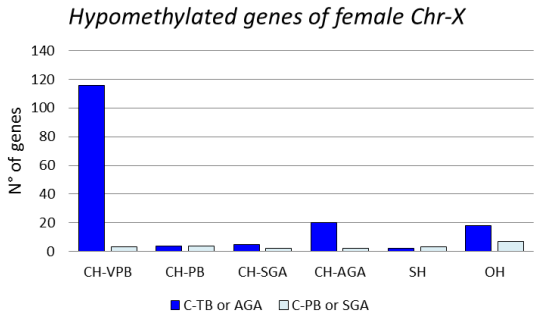


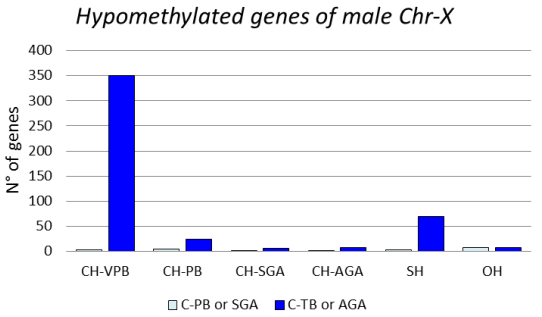
Tot of DMGs	CH-VPB	CH-PB	CH-SGA	CH-AGA	SH	OH
	225	405	335	89	814	473
Hypomethylated	218 (97)	386 (95)	289 (86)	62 (69)	783 (96)	428 (90)
CpG island	51 (23)	103 (35)	99 (34)	41 (66)	308 (39)	142 (33)
Non-CpG island	168 (77)	283 (75)	190 (66)	21 (34)	275 (61)	286 (67)

Figure 20: Graphical representation of the number of DMGs in cases versus controls of autosomes. In dark blue are display hypomethylated genes in cases than C-TB and C-AGA, and in a light blue the hypomethylated genes in cases than C-PB and C-SGA. In the table are reported the total of DMGs selected, the number of hypomethylated genes and how many of them are members of CpG island or Non-CpG island. In parentheses are reported the percentages of these data.

Regarding on the analysis of sexual chromosome we selected (figure 21):

- *Female*: a total of 116 DMGs, that are almost totally hypomethylated in CH-VPB than term controls. The majorities of these genes are represented by *CpG island* sites.
- *Male*: we found a total of 373 DMGs, which the 94-98% is hypomethylated in CH-VPB then term controls.





female DMGs	CH-VPB	CH-PB	CH-SGA	CH-AGA	SH	OH
	116	10	5	24	7	21
Hypomethylated	116 (100)	10 (100)	2 (40)	20 (86)	7 (100)	18 (85)
CpG island	102 (88)	2 (20)	2 (100)	10 (50)	7 (100)	13 (72)
Non-CpG island	14 (12)	8 (80)		10 (50)		5 (28)

male DMGs	CH-VPB	CH-PB	CH-SGA	CH-AGA	SH	OH
	373	25	26	14	70	14
Hypomethylated	351 (94)	25 (100)	6 (23)	10 (86)	70 (100)	7 (50)
CpG island	330 (94)	6 (24)	4 (66)	5 (50)	24 (34)	5 (72)
Non-CpG island	21 (6)	19 (76)	2 (34)	5 (50)	46 (66)	2 (28)

Figure 21: Graphical representation of the number of DMGs in cases versus controls of Chr-X. In dark blue are display hypomethylated genes in cases than C-TB and C-AGA, and in a light blue the hypomethylated genes in cases than C-PB and C-SGA both for *female* and *male*. In the table are reported the total of DMGs selected, the number of hypomethylated genes and how many of them are members of CpG island or Non-CpG island. In parentheses are reported the percentages of these data.

6.5.2 Selection of "common genes"

Through a differential score plot comparison of all DMGs resulting from the three studies, we selected a total of 154 of autosomes. In addition we found 79 DMGs in common between male and female VPB cases (figure 22). We then clustered all CH-patients according to the 154 DMGs to find which factors are related to the gene-specific hypomethylation.

As reported in the figure 23, we find that CH-children with comparable gestational age and low weight at birth clustered together. On the contrary, affected infants with low gestational age and proper weight at birth are spread far away. The most significant results are those concerning the analysis of concordant and discordant twin pairs for CH. In fact, both of twin pairs with low birth weight clustered together, unlike the siblings with normal birth weight. These results confirm that gene-specific hypomethylation is associated to prematurity with low birth weight. Thyroid defects seem to be, therefore, a secondary effect from this condition.

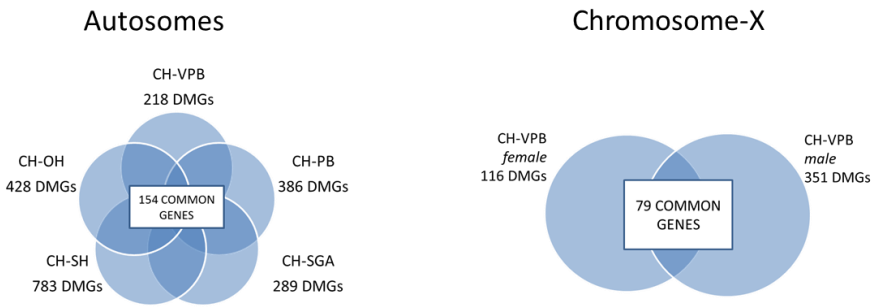


Figure 22: Venn diagram having the number of DMGs of autosomes and of Chr-X that are in common between CH subgroups.

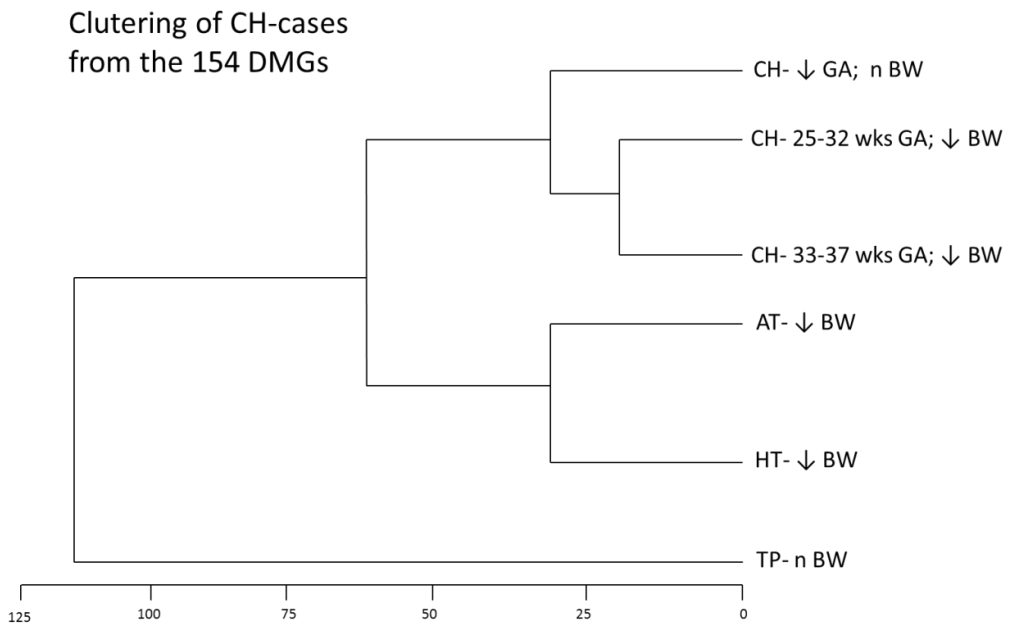


Figure 23: schematic representation of cluster of CH-patients according to the 154 DMGs.

6.5.3 Features of differentially methylated loci

In table 8 are reported the full list and the details of the 154 DMGs resulted by the gene-specific methylation analysis. Reflecting the global DNA methylation analysis, the 75% of these genes were represented by CpG sites located into non-coding regions, while the remained 25% was characterized by CpG island sites situated into promoters of target genes. Several works reported the association of DNA hypomethylation and genomic instability. This condition is known to be related to cancer predisposition, adverse pregnancy outcomes and birth defects. Then we conducted a topological analysis to study the distribution of chromosomal epigenetic changes related to CH onset and fetal development. In this regard, using the list of sites previously identified as CpG significant variables in both the preterm than in intrauterine growth restriction associated with CH it

was calculated distribution at the chromosomal level. In particular, for each chromosome, the results for the frequencies have been reported as the percentage ratio between the numbers of CpG sites differently methylated and the number of the CpG sites represented by Illumina BeadChip. The epigenetic changes in Non-CpG island sites are found to have a significantly greater rate in chromosomes 1, 2, 3, 6, 7 and 17 compared to that expected for randomly selected genes (figure 24). Instead, CpG island sites seem to be distributed homogeneously within the genome.

Finally, through *GeneImprint* public database we search which DMGs are imprinted or not (http://igc.otago.ac.nz and http://www.geneimprint.com). Three genes are known to be dependent of parent-of-origin (*DLK1*, *GNAS* and *KCNQ1*) and two are predicted to be imprinted (*HOXB2* and *SLC38A4*) (Table 8).

The interpretation of DNA methylation of imprinted control regions (ICRs) of these genes is more difficult that non-imprinted genes. For example, this complexity can be illustrated by the data of *GNAS* genes for which 30 probes across 3 promoter regions of 3 alternative transcripts (*NESP55*, *GNASXL* and exon 1A of *GNAS*), were present on the Infinium array (figure 25). 8/30 probes are differentially methylated and have AVGΔβ values between 10 to 20% (figure 26).

The interpretation of the methylation data for these genes depends on the number of probes that show significant differences and the percentage of loss or acquisition of DNA methylation. For these reasons it is necessary to validate these findings with other methods.

Symbol	Product	Chormosome	CpG island	AVGΔβ	Imprinted	Allele
A2M	alpha-2-macroglobulin precursor	12	N	-7%		
ABCB1	ATP-binding cassette sub-family B member 1	7	N	-5%		
ACF	apobec-1 complementation factor isoform 1	10	N	-6%		
ADAMTSL5	thrombospondin; type I; domain containing 6	19	N	-8%		
ADH1C	class I alcohol dehydrogenase; gamma subunit	4	N	-7%		
ADH7	class IV alcohol dehydrogenase 7 mu or sigma subunit	4	N	-7%		
ADIPOQ	adiponectin precursor	3	N	-6%		
ALDOB	aldolase B, fructose-bisphosphate	9	N	12%		
APOBEC1	apolipoprotein B mRNA editing enzyme isoform 2	12	N	-5%		
APOBEC4	apolipoprotein B mRNA editing enzyme	1	N	-8%		
APOD	apolipoprotein D precursor	3	N	-6%		
ATP5A1	ATP synthase; H+ transporting; mitochondrial F1 complex	18	N	-6%		
BMP10	bone morphogenetic protein 10 preproprotein	2	N	-6%		
BMP4	bone morphogenetic protein 4 preproprotein	14	N	-6%		
CALCA	calcitonin isoform CALCA preproprotein	11	Y	-6%		
CCL5	small inducible cytokine A5 precursor	17	N	-5%		
CER1	cerberus 1 homolog	21	N	-5%		
CHIA	eosinophil chemotactic cytokine	1	N	-8%		
CIR	CBF1 interacting corepressor isoform 2	2	N	-5%		
CLEC12A	myeloid inhibitory C-type lectin-like receptor isoform alpha	12	N	-7%		
CPA5	carboxypeptidase A5	7	N	-6%		
CRYM	crystallin; mu isoform 1	16	N	-6%		
CYP1A2	cytochrome P450, family 1, subfamily A, polypeptide 2	15	N	-7%		
CYP3A5	cytochrome P450, family 3, subfamily A, polypeptide 5	7	N	-7%		
DHRS4	dehydrogenase/reductase (SDR family) member 4	14	Y	-21%		
DHRS4L2	dehydrogenase/reductase (SDR family) member 4 like 2	14	N	-5%		
DLK1	delta-like 1 homolog isoform 2	14	Y	-6%	Y	paternal
EPS8L1	epidermal growth factor receptor 8-like protein	19	N	-19%		
ESR1	estrogen receptor 1	6	Y	-12%		
FABP1	fatty acid binding protein 1, liver	2	N	-5%		
FABP2	fatty acid binding protein 2, liver	4	N	-6%		
FABP4	fatty acid binding protein 4, liver	8	N	-5%		
FCRL4	Fc receptor-like 4	1	N	-6%		
FGF7	fibroblast growth factor 7 precursor	15	N	-6%		
FORL2	folate receptor 2	17	N	-7%		
GCG	glucagon preproprotein	2	N	-7%		
GDF15	growth differentiation factor 15	19	Y	-7%		
GLI2	GLI-Kruppel family member GLI2 isoform delta	2	N	-8%		
GNA11	guanine nucleotide binding protein; alpha 11 (Gq class)	19	Y	-15%		
GNAS	guanine nucleotide binding protein; alpha stimulating activity	20	Y	-25%	Y	isoform-dip
GRM5	glutamate receptor; metabotropic 5 precursor	11	N	-7%		
GRM8	glutamate receptor, metabotropic 8	7	N	-5%		
GSTA5	glutathione transferase A5	6	N	-6%		
GSTM1	glutathione S-transferase M1 isoform 1	1	Y	-10%		
GSTM5	glutathione S-transferase M5	1	Y	-7%		
GSTT1	glutathione S-transferase theta 1	22	Y	-6%		
GUCA1C	guanylate cyclase activator 1C	3	N	-9%		
GUCY2C	guanylate cyclase 2C (heat stable enterotoxin receptor)	12	N	-5%		
HLA-C	major histocompatibility complex, class I, C	6	N	-7%		
HLA-DQB1	major histocompatibility complex; class II; DQ beta 1 precursor	6	N	-7%		
HLA-DQB2	major histocompatibility complex; class II; DQ beta 2	6	N	-12%		
HOXB2	homeo box B2	17	N	-5%	P	maternal
HOXB5	homeo box B5	17	N	-5%		
HOXB6	homeo box B6 isoform 1	17	N	-7%		
HRH1	histamine receptor H1	3	N	-8%		
HTR5A	5-hydroxytryptamine (serotonin) receptor 5A	7	N	-8%		
HTR1F	5-hydroxytryptamine (serotonin) receptor 1F	3	N	-6%		
IFNA16	interferon, alpha 16	9	Y	-7%		
IL10	interleukin 10	1	N	-6%		
IL5	interleukin 5	11	N	-8%		
KCNAB3	potassium voltage-gated channel; shaker-related subfamily	17	Y	-12%		
KCNE2	potassium voltage-gated channel; Isk-related family	21	Y	-7%		
KCNJ16	potassium inwardly-rectifying channel J16	17	N	-6%		
KCNQ1	potassium voltage-gated channel; KQT-like subfamily	11	Y	-9%	Y	maternal
KCNRG	potassium channel regulator isoform 1	13	Y	-7%		
KCTD1	potassium channel tetramerisation domain containing 1	18	N	-6%		
KCTD2	potassium channel tetramerisation domain containing 2	17	N	-7%		
KRTAP19-2	keratin associated protein 19-2	21	N	-6%		
KRTAP21-1	keratin associated protein 21-1	21	N	-5%		
KRTAP21-2	keratin associated protein 21-2	21	N	-7%		
KRTAP4-4	keratin associated protein 4.4	17	N	-9%		
KRTAP9-4	keratin associated protein 9-4	17	N	-5%		
LCE2B	late cornified envelope 2B	1	N	-5%		
LDHC	lactate dehydrogenase C	11	N	-11%		
LTB4R	leukotriene B4 receptor	14	N	-9%		
MAPK9	mitogen-activated protein kinase 9 isoform 1	5	N	-6%		

MASP1	mannan-binding lectin serine protease 1 isoform 1 precursor	3	N	-6%		
MBNL2	muscleblind-like 2 isoform 1	13	N	-5%		
MGMT	O-6-methylguanine-DNA methyltransferase	10	N	-6%		
MGP	matrix Gla protein	12	N	-7%		
MMP10	matrix metalloproteinase 10 preproprotein	11	Y	-6%		
MMP14	matrix metalloproteinase 14 preproprotein	14	Y	-5%		
MMP21	matrix metalloproteinase 21 preproprotein	10	N	-6%		
MSX1	msh homeo box homolog 1	5	Y	-6%		
MTHFD2	methylene tetrahydrofolate dehydrogenase 2 precursor	11	Y	-10%		
MYH3	myosin; heavy polypeptide 3; skeletal muscle; embryonic	17	N	-7%		
NALP12	PYRIN-containing APAF1-like protein 7 isoform 1	19	N	-7%		
NOS3	nitric oxide synthase 3 (endothelial cell)	7	N	-6%		
NUDT12	nudix -type motif 12	5	N	-6%		
PAN3	PABP1-dependent poly A-specific ribonuclease subunit PAN3	13	N	-5%		
PARG	poly (ADP-ribose) glycohydrolase	10	N	-5%		
PARVB	parvin; beta isoform b	22	Y	-7%		
PDE1A	phosphodiesterase 1A; calmodulin-dependent isoform 2	2	N	-7%		
PDE3B	phosphodiesterase 3B; cGMP-inhibited	11	Y	-6%		
PDE4D	cAMP-specific phosphodiesterase 4D	5	Y	-6%		
PEBP1	phosphatidylethanolamine binding protein 1	12	N	-6%		
PHC2	polyhomeotic 2-like isoform a	1	N	-6%		
PLD4	phospholipase D family; member 4	14	Y	-5%		
PLG	plasminogen	6	N	-7%		
PRL	prolactin	6	N	-8%		
PSCA	prostate stem cell antigen	8	Y	-17%		
PSMD5	proteasome 26S non-ATPase subunit 5	9	Y	-7%		
PTH	parathyroid hormone preproprotein	11	N	-6%		
PTMA	prothymosin; alpha (gene sequence 28)	2	N	-6%		
PTPN3	protein tyrosine phosphatase; non-receptor type 3	9	N	-6%		
RALBP1	ralA binding protein 1	18	Y	-5%		
RARRES2	retinoic acid receptor responder (tazarotene induced) 2	7	Y	-9%		
RDH12	retinol dehydrogenase 12 (all-trans and 9-cis)	14	Y	-5%		
REG1B	regenerating islet-derived 1 beta precursor	2	N	-5%		
RPL4	ribosomal protein L4	15	N	-6%		
RRH	peropsin	4	N	-6%		
RUNX2	runt-related transcription factor 2 isoform b	6	N	-6%		
SELE	selectin E precursor	1	Y	-6%		
SLC10A2	solute carrier family 10; member 2	13	Y	-6%		
SLC13A1	solute carrier family 13; member 1	7	Y	-5%		
SLC16A4	solute carrier family 16; member 4	1	N	-7%		
SLC17A2	solute carrier family 17 (sodium phosphate); member 2	6	N	-6%		
SLC17A4	solute carrier family 17 (sodium phosphate); member 4	6	N	-5%		
SLC25A15	solute carrier family 25; member 15	13	N	-5%		
SLC25A20	carnitine/acylcarnitine translocase	3	N	-6%		
SLC2A9	solute carrier family 2; member 9 protein isoform 1	4	Y	-5%		
SLC30A8	solute carrier family 30 member 8	8	Y	-5%		
SLC38A4	solute carrier family 38; member 4	15	Y	-8%	P	UN
SLC39A12	solute carrier family 39 (zinc transporter); member 12	10	Y	-6%		
SLC4A5	sodium bicarbonate transporter 4 isoform a	2	N	-7%		
SLC4A7	solute carrier family 4; sodium bicarbonate cotransporter	3	N	-6%		
SLC5A10	solute carrier family 5; member 10	17	N	-5%		
SLC5A4	solute carrier family 5 (low affinity glucose cotransporter)	22	N	-5%		
SMURF	SMAD specific E3 ubiquitin protein ligase 1	7	N	-6%		
SOX6	SRY (sex determining region Y)-box 6	11	N	-6%		
SRD5A2	3-oxo-5 alpha-steroid 4-dehydrogenase 2	2	Y	-7%		
STK38	serine/threonine kinase 38	6	N	-6%		
SULT1A1	sulfotransferase family, cytosolic, 1A, phenol-preferring	11	N	-9%		
SULT1A2	sulfotransferase family, cytosolic, 2A, phenol-preferring	16	N	-12%		
SUMO2	small ubiquitin-like modifier 2 isoform a precursor	17	N	-7%		
SUMO3	small ubiquitin-like modifier protein 3	17	N	-5%		
SUSD1	sushi domain containing 1	9	Y	-6%		
TAS2R1	taste receptor T2R1	5	N	-7%		
TAS2R10	taste receptor; type 2; member 10	12	N	-6%		
TDG	thymine-DNA glycosylase isoform 2	12	Y	-8%		
TFDP2	transcription factor Dp-2 (E2F dimerization partner 2)	3	N	-5%		
TG	thyroglobulin	8	Y	-6%		
TGIF	TG-interacting factor isoform c	18	N	-6%		
TGM3	transglutaminase 3 precursor	20	N	-10%		
THOP1	thimet oligopeptidase 1	19	N	-5%		
THRSP	thyroid hormone-responsive protein	11	N	-6%		
THSD4	thrombospondin, type I, domain containing 4	15	N	-5%		
TIN	tubulointerstitial nephritis antigen-like 1	1	N	-6%		
TMPRSS11D	transmembrane protease; serine 11D	4	N	-9%		
TREML2	triggering receptor expressed on myeloid cells-like 2	6	N	-6%		
TRIM16	tripartite motif-containing 16	17	N	-8%		
TRIP6	thyroid hormone receptor interactor 6	7	N	-5%		
UCN2	urocortin 2	3	N	-6%		
UGT2A1	UDP glycosyltransferase 2 family; polypeptide A1	4	N	-6%		

Table 8: general features of the 154 DMGs: symbols, products, chromosomes and CpG sites location are reported. The hypomethylated status of each gene is display as a percentage of the differences between cases and controls (AVGΔβ). In the last two columns are the genes subjected to imprinting (Y) or predicted (P), and the type of inheritance as maternal, paternal, isoform-dependent or unknown (UN).

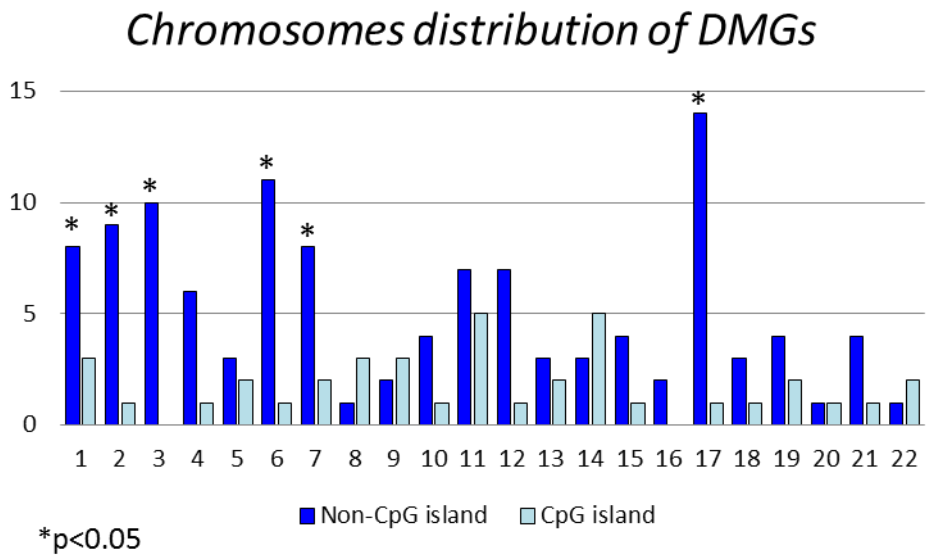


Figure 24: Chromosomes localization of the 154 DMGs. Data are reported as a number of DMGs for each chromosome and then we compared the % of these genes with total genes represented by the Illumina array. Chromosomes 1, 2, 3, 6, 7 and 17 shown a higher frequency of CpG sites then expected (*).

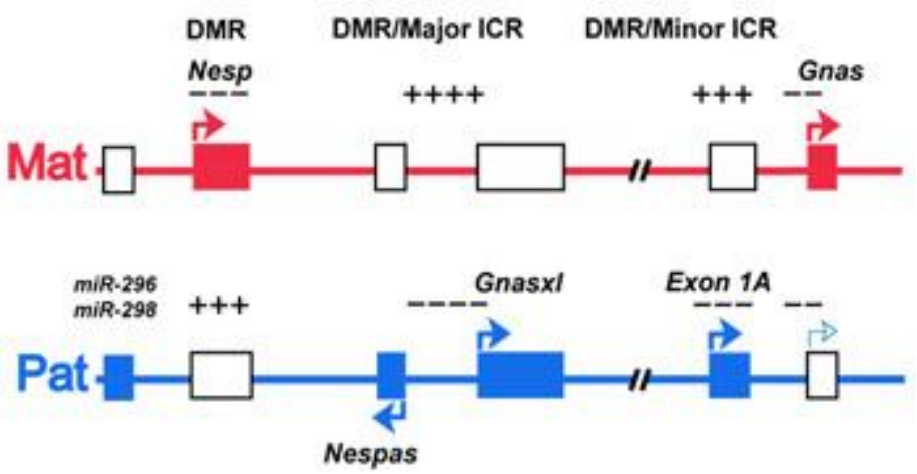


Figure 25: GNAS locus has a highly complex imprinted expression pattern. It gives rise to maternally, paternally, and biallelically expressed transcripts that are derived from four alternative promoters and 5' exons. The GNAS locus is imprinted and encodes 5 main transcripts: Gs-α, XLAS, NESP55, A/B transcript, antisense GNAS transcript. Some transcripts contain a differentially methylated region (DMR) at their 5' exons, and this DMR is commonly found in imprinted genes and correlates with transcript expression. An antisense transcript exists, and this antisense transcript and one of the transcripts are paternally expressed, produce noncoding RNAs, and may regulate imprinting in this region. In addition, one of the transcripts contains a second overlapping ORF, which encodes a structurally unrelated protein.

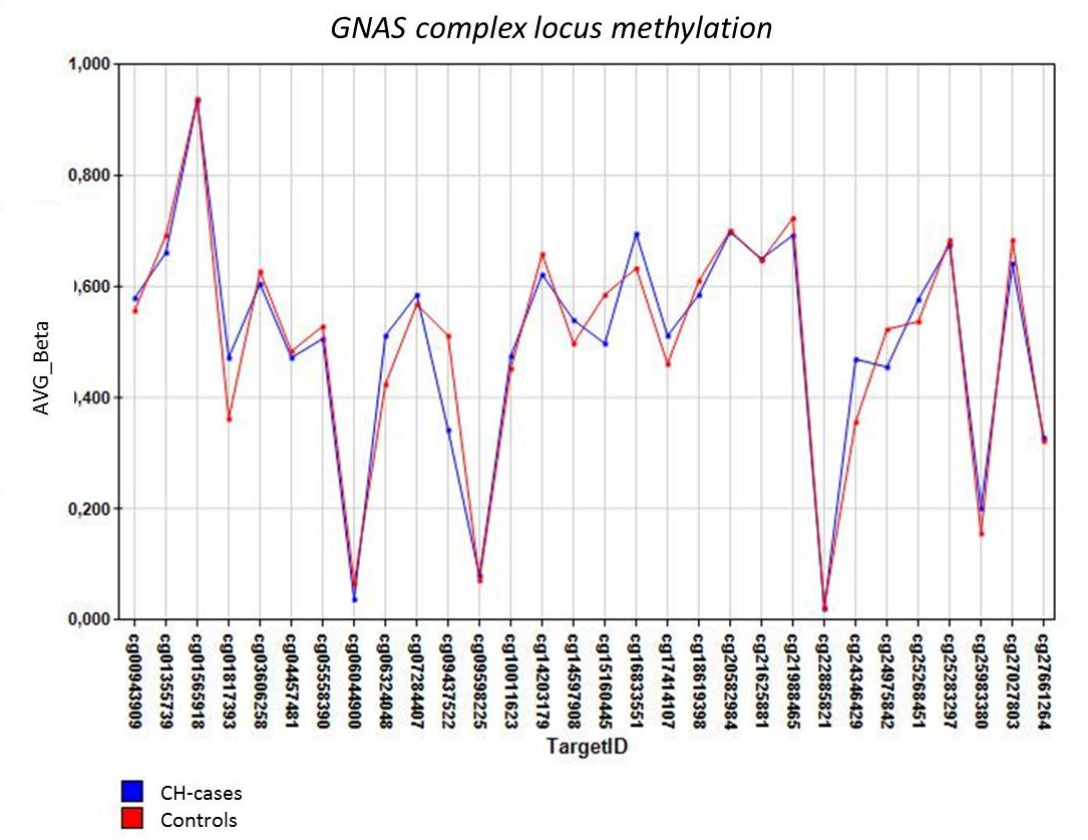


Figure 26: GNAS complex on Illumina's array. 30 probes cover the differentially methylated regions (DMRs) of the five alternative transcripts. 8 to 30 probes are differentially methylated and have AVGΔβ values between 10 to 20%.

6.6 CATEGORIZATION OF DIFFERENTIALLY METHYLATED GENES

6.6.1 Gene Ontology Enrichment analysis

The analysis with DAVID conducted on this restricted list of 154 DMGs has allowed characterizing with higher resolution the biological processes involved in cellular functions during fetal and thyroid development. Thus, we tested whether specific GO terms of the genes associated with one or more DMGs were enriched when compared to the GO distribution of all the 154 autosomal genes associated with CpG sites present on the array that we analyzed.

Biological processes such as regulation and response to hormone stimulus, post-embryonic development, regulation of retinoic metabolism, metabolism of vitamins and sulfur metabolism are found to be regulated at the epigenetic level (figure 27).

The inclusion of these specific processes within the top ten most significant gene set overlaps provided evidence of strong biological relevance of the differentially methylated genes to fetal development and thyroid function.

We then analyzed the DMGs with *KEEG pathway search database* to find which signal pathways are involved in these epigenetics changes. Interestingly, pathways implicated in the autoimmune thyroid disease, metabolism of xenobiotic and in metabolism of retinol are found to be significantly enriched (table 9).

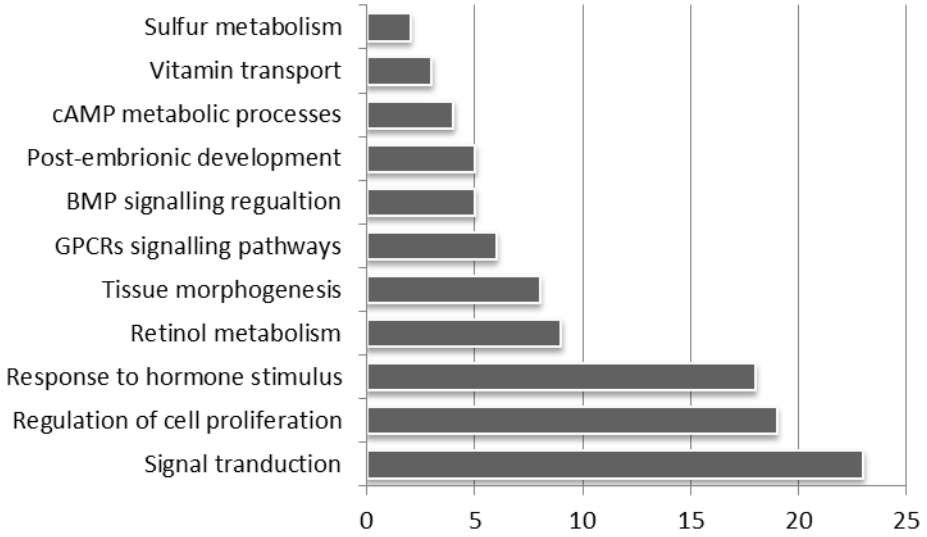


Figure 27: Gene Ontology Enrichment analysis of 154 DMGs by DAVID database. On the horizontal axis are reported the number of genes and in the vertical axes the biological processes that are enriched.

KEGG-Pathway Database		
PATHWAY	**P VALUE**	**GENES**
Metabolism of xenobiotic P450	9.4 E-4	UGT2A1, ADH7, ALDH3B2, GSTA5, GSTM1, GSTM5, GSTT1
Autoimmune thyroid disease	6.3 E-3	IFNA16, IL10, IL5, HLA-C, HLA-DQBA, TG
Neuroactive ligand-receptor interactior	7.3 E-3	HTRA1F, HTR5A, MAS1, GRM5, GRM8, GHRHR, HRH2, LTB4R, PLG, PTAFR, PRSS3
Glycolysis/gluconeogenesis	1.2 E-3	ADH7, ALDH3B2, ALDOB, GALB, LDHC
PPAR signalling pathway	5.7 E-2	ACSL5, ADIPOQ, FABP1, FABP2, FABP4
Calcium signalling pathway	8.2 E-2	HTR5A, GNAS, GRM5, GNA11, HRH2, NOS3, PDE1A, PTAFR
Retinol metabolism	8.8 E-2	UGT2A1, ADH7, DHRS4L2, RDH12

Table 9: Pathway search analysis of DMGs by KEEG Database. In this table are display the biological pathway enriched, the p-value and the lists of genes involved.

6.6.2 Selection of the "top genes"

Finally, we selected a short list of genes of particular interests for further studies since it's involved in fetal development and action of thyroid hormones at peripheral level:

BMP10, BMP4 and SMURF: BMPs proteins belong to the TGF-β pathway family and are multifunctional growth factors involved in many aspects of tissue development and morphogenesis [Mandel *et al.*, 2010]. The important functioning of BMP signals in physiology is emphasized by the multitude of roles for dysregulated BMP signalling in pathological processes. Especially cancerous disease often involves alterations of the BMP signalling system. Absence of BMP signalling is, for instance, an important factor in the progression of colon cancer [Samavarchi-Tehrani *et al.*, 2010] and conversely overactivation of BMP signalling following reflux-induced esophagitis provokes Barrett's esophagus and is thus instrumental in the development of adenocarcinoma in the proximal portion of the gastrointestinal tract [Barros *et al.*, 2008]. Interestingly, thyroid hormones induce the local BMP expression to stimulate the cell metabolism and growth [Milano *et al.*, 2007].

DLK1, CIR1: Dlk1 has evoked considerable interest because in mammals it is a paternally expressed imprinted gene that is epigenetically regulated, and defective imprinting of Dlk1 results in developmental abnormalities [Begemann *et al.*, 2012]. The developmental mechanisms leading to these abnormalities are unknown and in particular it is unclear the extent to which this protein has the capability of regulating Notch signalling in vivo, because it lacks an essential extracellular DSL domain common to all known Notch ligands. Both its lack of a DSL-domain and its short intracellular domain suggest therefore that DLK1 would not be able to function as a Notch ligand. However, as an inverse correlation

between Dlk1 levels and Notch activity have been observed in cultured cells, it is possible that DLK1 could be a receptor antagonist [Sánchez-Solana *et al*., 2011].

Corepressor interacting with RBPJ (CIR1) may modulate splice site selection during alternative splicing of pre-mRNAs. CIR1 regulates transcription and acts as a corepressor for RBPJ and play a central role in Notch signaling. Recruits RBPJ to the Sin3-histone deacetylase complex (HDAC) and is required for RBPJ-mediated repression of transcription [Hsieh *et al*., 1999].

The notch signaling pathway is important for cell-cell communication, which involves gene regulation mechanisms that control multiple cell differentiation processes during embryonic and adult life. Notch signaling is dysregulated in many cancers, and faulty notch signaling is implicated in many diseases including T-ALL (T-cell acute lymphoblastic leukemia), CADASIL (Cerebral Autosomal Dominant Arteriopathy with Sub-cortical Infarcts and Leukoencephalopathy), MS (Multiple Sclerosis), Tetralogy of Fallot, Alagille syndrome, and many other disease states [Mead *et al*., 2012]. Recently a possible role for Notch signal in thyroid development has been proposed [Ferretti *et al*, 2008; Geers *et al*., 2011].

GSTA5, GSTM1, GSTT1, and GSTM5: Genes encoded for glutathione-S-transferases (GSTs) are strongly hypomethylated in affected infants. It's well known that oxidative stress is closely related to fetal stress and adverse pregnancy outcomes [257]. Level of expression of GSTs is a crucial factor in determining the sensitivity of cells to a broad spectrum of carcinogens, antitumor drugs, environmental pollutants, and products of oxidative stress. Some reports demonstrated the association between oxidative stress and hypothyroidism [258]. Hypothyroid rats shown elevated plasma levels of homocysteine, nitric oxygen (NO) metabolites and oxidized glutathione/reduced glutathione (GSSG/GSH) ratio due to a selenium deficiency and GSTs expression.

Folic acid supplementation combined with L-tiroxine therapy ameliorating not only the thyroid state but also the antioxidant status. It's also reported that thyroid hormone (THs) have a strong impact on the antioxidant system. Physiological levels of THs repress the expression of these genes and protect cells to the adverse effects of oxidative stress [259].

ADH7, DHRS4L2, and DHR12: these hydrolytic enzymes are involved in the homeostasis of retinoic acid. The biosynthesis of active forms of retinoids are critical for many physiological processes, including embryonic development, reproduction, postnatal growth, differentiation and maintenance of various epithelia, immune responses, and vision and could activate gene expression through a thyroid hormone response element [260].

Retinol is converted by alcohol dehydrogenase to retinaldehyde, which is subsequently converted by retinaldehyde dehydrogenase to all-trans retinoic acid (ATRA). ATRA undergoes isomerization in hepatic microsomes to 13-cisretinoic acid (RA) and 9-cis RA, depending on the levels of converting enzymes and cellular retinol binding protein. These retinoids can influence expression of many genes through nuclear receptors [retinoic acid receptor (RAR) and retinoid X receptor (RXR)]. It was reported that that a synthetic RXR-selective retinoid, LG1069, can cause central hypothyroidism in patients through a profound suppression of serum TSH levels [261].

PDE4D, PDE3B: Cyclic nucleotide phosphodiesterases (PDEs) catalyze the hydrolytic inactivation of the common intracellular second messenger's cyclic adenosine (cAMP). Thus these enzymes play a critical role in the regulation of a wide range of physiological processes such as the TSH regulation and the negative feedback of thyroid hormone actions [262]. PDE4D is expressed in the thyroid and its overexpression is believed to counteract the constitutive

activation of cAMP pathway induced by TSHR or Gsa gain-of-function mutations in thyroid adenomas (Persani *et al.*, JCEM 2000).

SULT1A1, SULT1A2: Sulfation, catalyzed by sulfotransferase enzymes (SULTs), is an important pathway of thyroid hormone metabolism by which T(4) is irreversibly converted to inactive reverse T(3) rather than active T(3) [263]. Impairment in SULT1A1 and SULT1A2 activity have a mild effects and thyroid status but have a strong effect in liver, brain and kidneys in animal model [264].

IFNA16, HLA-C and HLA-DQBA: Autoimmune thyroid disease (AITD) causes cellular damage and alters thyroid gland function by humoral and cell-mediated mechanisms. Cellular damage occurs when sensitized T-lymphocytes and/or autoantibodies bind to thyroid cell membranes causing cell lysis and inflammatory reactions. Anti-thyroid antibodies have been implicated in adverse pregnancy outcomes [265] and the onset of congenital hypothyroidism [266].

6.7 Analysis of *MTHFR* polymorphisms

In this paragraph the genetic analysis of *MTHFR* is reported. Although several factors are known to be related to global and gene-specific hypomethylation, polymorphisms in this gene are responsible to maternal folate deficiency and fetal hypomethylation.

Methylenetetrahydrofolate reductase (MTHFR) catalyzes the conversion of 5,10-methylenetetrahydrofolate (5,10-CH2-THF) to 5-methyltetrahydrofolate (5-MeTHF), a co-substrate for homocysteine remethylation to methionine. Genetic variations in this gene are associated with *MTHFR* are positively correlated with lower serum and red blood cell folate concentrations [267].

Several studies reported that maternal folate deficiency during pregnancy influences the susceptibility to occlusive vascular disease, neural tube defects, colon cancer and acute leukemia. Exactly how *MTHFR* mutations can cause pregnancy complications is unknown; a logical hypothesis is that endothelial damage from hyperhomocysteinemia leads to venous thromboembolism and placental insufficiency [268].

Several non-synonymous single nucleotide polymorphisms (SNPs) are present in the coding region of the gene including positions 677 and 1298. The most studied SNP is the 677 C>T substitution that results in an amino acid change from alanine to valine at codon. The MTHFR 1298A>C polymorphism results in a substitution of glutamate with alanine. MTHFR enzyme activity is reduced by 35% among the 677C/T carriers and by 50% to 70% among 677T/T carriers. The function of polymorphism 1298A>C has not been consistently demonstrated; however carriers of the heterozygous genotypes (677CT/1298AC) show MTHFR activity similar to that in $MTHFR^{677T/T}$ carriers. The reduction in MTHFR activity increases homocysteine levels and in turn reduces the availability of the DNA methyl groups [270].

To date, nine maternal blood samples were analysed; six of them are parents of concordant and discordant twin pairs and three are mothers of affected only child. Genomic DNA from mothers was extracted and DNA amplification and genotyping of *MTHFR* 677 C>T and 1298 A>C were performed by polymerase chain reaction (PCR-RFLP) according to the protocol suggested by Chen and Weisberg [271].

As shown in table 10, two of four mothers of discordant twin pairs carry the homozygous variant *MTHFR*$^{677T/T}$, while the others have the heterozygous polymorphisms *MTHFR*$^{677C/T}$ and *MTHFR*$^{1298A/C}$, respectively.

These single heterozygous genotypes are also found in four mothers of concordant twins and only child. One parents of CH-only child bring a double heterozygous polymorphisms (*MTHFR*$^{677C/T,\ 1298A/C}$).

Mothers with the *MTHFR*$^{677T/T}$ genotypes are those whose affected twins are born small for gestational age. Single *MTHFR*$^{677C/T}$ polymorphisms are found in mothers of one AGA discordant twin pairs and two SGA only children. *MTHFR*$^{1298A/C}$ variants are found in mothers of AGA discordant twins, SGA/AGA concordant twins and in SGA only child. The *MTHFR*$^{677C/T,\ 1298A/C}$ genotype is carried by parents of SGA only-child.

Although these data have to be extended and validated, the presence of these genetic variants appears invariably associated with intrauterine growth delay. Maternal folate deficiency caused by MTHFR deficiency leads to a shortage of methyl-donors and as a consequence to the hypomethylation status. The genomic instability and the altered regulation of certain factors may have potentially affected the pregnancy outcome and fetal development.

Mothers	CH-infants	GA (wks)	BW (g)	TSH (μU/ml)	SGA	MTHFR 677C/T	MTHFR 1298A/C
M1	Discordant-TP	35.1	1830/2043	13.55/ 1.13	y/n	T/T**	A/A
M2	Discordant-TP	25.1	680/843	20/1.92	n/n	C/T*	A/A
M3	Discordant-TP	30.4	2300/2630	18.23/2.24	n/n	C/C	A/C*
M4	Discordant-TP	31	930/1122	11.1/1.35	y/n	T/T**	A/A
M5	Concordant-TP	35	1330/2150	19.6/13.5	y/n	C/C	A/C*
M6	Concordant-TP	36.5	2650/2300	262.8/150	n/n	C/C	A/A
M7	Only child	32,5	1330	13,27	y	C/T*	A/A
M8	Only child	33	1262	125	y	C/T*	A/C*
M9	Only child	37	2030	15	y	C/C	A/C*

Table 10: MTHFR genotyping. Nine mothers are collected and analyzed for the presence of 677 C/T and 1298 A/C polymorphisms. Kinship and clinical data of sons are reported: gestational age (GA), birth weight (BW), TSH values and the presence (y) or absence (n) of intrauterine growth delay (SGA). (*) marks the heterozygous state while (**) the homozygous variant.

6.6.3 Summary of results of gene-specific methylation analysis

The gene-specific methylation analysis confirmed the scenario of the global methylation study. Almost all of DMGs of autosomes are less methylated in CH patients than in term and AGA controls, and about 70% of DMGs are located into *Non-CpG island* sites. To find which factors are directly related to the hypomethylation, through a scatter plot and association analyses we selected a subsets of 154 DMGs that are in common between the subgroups of cases. By a sample clustering we find that the gestational age and the birth weight are the principal indicators of this methylome aberration. In fact, affected SGA infants with low BW are clustered together while the others are distributed randomly. No direct association on TSH levels was found. These data are confirmed to the fact that twins concordant and discordant for IC are distributed according to birth weight; twin pairs with comparable weight are grouped together; on the contrary, twins with different weight are not clustered together. This finding suggests that the degree of prematurity and intrauterine growth is directly linked to the DNA hypomethylation and the increased predisposition to CH onset could represent a secondary event or a strongly associated co-variate. Interestingly, the 75% of the hypo-DMGs are localized into the *Non-CpG island* regions and for

the 25% into a promoter *CpG* island. About the chromosomes distribution of the 154 DMGs, non-CpG island sites showed an overestimation than the expected frequencies in chromosomes 1, 2, 3, 6, 7 and 17. By *GeneImprint* software, we found 5 genes known or predicted to be imprinted but the interpretation of their methylation status and the biological significance has to be further validated. Then we analyzed the presence of *MTHFR* polymorphisms in nine mothers and we found that two of them are homozygous and three are heterozygous for 677C/T variants. Two mothers are heterozygous for the 1298A/C variants and one of them have a double heterozygous genotype. In the end, by the Gene Ontology and KEEG Pathway database we have found that several genes belong to the biological processes involved in fetal development and maintenance the homeostasis of hormone levels. We then selected a set a top genes that are particularly interesting for further studies.

CHAPTER VII

6. DISCUSSION

The etiophatogenesis of congenital hypothyroidism (CH) is still poorly understood. Studies in animal model and human screening programs have been demonstrated that CH is a multigenic [Amendola *et al.* 2004; Trueba *et al.*, 2005] and a multifactorial [Olivieri *et al.*, 1999; Medda *et al.*, 2005] disease. Although the genetic component is still widely studied, the occurrences of mutations in genes known to be involved in the development of the disease have been observed only in a small proportion (15-20%) of the CH patients [Olivieri *et al.*, 2009].

During pregnancy, the fetus is exposed to a plethora of endocrine and metabolic stimuli that enable it to a proper growth [Mitskevich *et al.*, 1990]. It has been hypothesized that environment affects maternal and fetal metabolism which results in an increased risk in birth thyroid defects [Liu *et al.*, 2009]. However, the etiological role of specific environmental risk factors is far to be understood. Recent works published by Olivieri *et al.*, indicates that the risk of CH onset depends on maternal and gestational factors. Iodine deficiency and maternal diseases as diabetes play a role in the development of CH [Olivieri *et al.*, 2007]. The multifactorial origin of CH was also supported by results obtained in the study on CH twins recorded in the INRICH between 1989 and 2000. This study showed that, despite a low concordance rate (4.3%) for permanent CH observed among twins at birth, a high recurrence risk for the disease was present among siblings of CH cases. Moreover, given the high discordance rate for CH their data suggest the importance of environmental risk factors in the etiology of CH and a possible role of competitive conditions regarding metabolic factors *in utero* [Medda *et al.*, 2005].

Works from Radetti and colleagues, conducted in groups of children with term and preterm birth reported interestingly but non-conclusive results. They examined a group of children of the same gestational age, they found an increased prevalence of thyroid dysfunction in those born SGA and identified in the shorter length at birth the cause for the thyroid alteration [Radetti *et al.*, 2004].

In a further work, conducted in a large group of patients born either preterm or at term, children born prematurely, independently from their birth weight or length frequently presented mild dysfunctions of the hypothalamus-pituitary-thyroid axis later in life [Radetti *et al.*, 2007].

These data are in agreement with the results of Cianfarani *et al.*, who showed that SGA children born at term, while having higher TSH values, compared with controls, all had TSH levels less than 10 μU/ml [Cianfarani *et al.*, 2003]. Even if the association between intrauterine environment, premature birth and the onset of CH is not well known, these data highlighted the occurrence of non-inheritable post-zygotic events in the etiology of CH and that environmental risk factors may act as a trigger on a susceptible genetic background in the etiology of the disease. Supporting this hypothesis, it has been demonstrated that some congenital extra-thyroidal malformations affects heart, nervous system, eyes (representing precocious structures in the developing embryo) and multiple congenital malformations are significantly associated to CH [Passeri *et al.*, JCEM, 2011; Monroy-Santoyo, 2012]. These findings have strongly suggested a very early impairment in the first stages of embryo development with a consequent involvement of different organs and structures.

In this scenario, epigenetic alterations appear to play an important role. Over the past decade, there has been increasing evidence supporting the concept that adverse factors in the perinatal environment predispose an individual to disease later in life [Waterland *et al.*, 2006]. Central to this concept, and driven by the initial epidemiological studies of David Barker and colleagues [Barker *et al.* ,1993], is the well substantiated link between birth weight and the development of a number of adult diseases, including obesity (Herrera *et al*., 2011),

hypertension (Curhan *et al*., 1996), coronary artery disease (Hiltunen *et al*., 2006) and insulin resistance (Zhao *et al*., 2012). In other words, adverse intrauterine environmental factors, often associated with low birth weight, increase disease risk postnatally (Barker *et al*., 1994; Canani *et al*., 2011; Lucas, 1991). Traditionally, cardiovascular and metabolic disorders including coronary artery disease, hypertension and type II diabetes have been thought to be caused by lifestyle risk factors acting upon a fixed genetic background.

However, based on multiple epidemiological studies, the fetal origin of adult diseases hypothesis suggests that environmental factors acting early in life play an important role in the pathogenesis of these diseases in adulthood (Armitage *et al*., 2004). Exposure to an inappropriate diet or toxins during the developmental period is related to aberrant DNA methylation and adverse pregnancy outcomes [Robins *et al.*, 2011] and in exacerbating the risk of disease onset [Bloomfield *et al.*, 2011]. The intrauterine environment can affect the intake of methyl donors and the establishment of a correct pattern of DNA methylation during both in the early phase of fetal development [Rogers *et al.*, 2011] and the latter stages of pregnancy [Reynolds *et al.*, 2011]. Changes in the fetal physiological, neuroendocrine or metabolic environment may result in permanent reprogramming of the developmental pattern of cellular proliferation and differentiation, resulting in alterations of organ physiology, morphology and/or metabolism in adult life. It's reported that these changes affect the development of the kidneys [Woroniecki *et al.*, 2011], pancreatic b-cells [Thornburg, 2010], heart [Szostak-Węgierek *et al.*, 2011] muscle [Alexander *et al.*, 2011], liver [Hogg *et al.*, 2011], adipose tissue [Lillycrop, 2011] or the hypothalamic-pituitary-adrenal (HPA) axis [Seckl *et al.*, 2006].

To our knowledge, we have performed the first case-control study to provide evidences of association between DNA methylation and CH onset in premature children. We collected a total of 31 infants born premature with normal *in situ* gland and 28 healthy term and preterm controls.

The DNA methylation patterns were measured in the genomic DNA isolated from peripheral blood lymphocytes. The interpretation of results from methylation studies must always be evaluated with caution as epigenetic marks may vary depending on cell type and can be influenced by various factors.

However, some published works have used Illumina Infinium technology to assess DNA methylation in peripheral blood lymphocytes and have identified CpG sites associated with diseases [Idaghdour *et al*., 2008; Pedersen *et al.*, 2011]. The accumulation of studies that have assessed DNA methylation patterns in lymphocytes in multiple diseases and exposures provides evidence that methylation patterns in peripheral blood cells may be useful in identifying biomarkers of various diseases.

As previously described in details, to understand the relationship between epigenetic changes, prematurity, intrauterine growth and CH onset we categorized cases and controls according to 1) gestational age, 2) intrauterine growth percentile and 3) TSH level and performed three independent DNA methylation studies.

The median global methylation level measured as the %5mC (5-methyl-cytosine) in leukocyte-derived DNA, was significantly lower in: 1) CH patients with very preterm birth (GA<32 weeks) and preterm birth (32<GA<37 weeks) than term healthy controls (37<GA<40 weeks); 2) CH children were born small for gestational age (SGA<10^{th} percentile) than AGA controls (10^{th}<AGA<90^{th} percentile) and 3) affected subjects with subclinical hypothyroidism (SH, 4μU/ml<TSH<10μU/ml) than children with overt hypothyroidism (OH, 10μU/ml<TSH<300μU/ml) and preterm and term controls. Based on these early results, it seems that the premature birth associated with CH, especially in the presence of small intrauterine development is related to global hypomethylation.

The findings about the thyroid function approach appear to open novel perspectives on the cause of the three to five folds increased risk for mild CH with GIS in premature babies. Firstly, if we focus on the clinical data for patients with subclinical hypothyroidism, we see that the mean values of gestational age and birth weight were significantly lower than other groups. This would explain and confirm the hypothesis that the prematurity and the intrauterine growth delay, but not the TSH levels, are associated with global hypomethylation. Thyroid defects seems to be a secondary event or a co-variate and epigenetic modifications may be restricted to only patients with subclinical hypothyroidism or mild thyroid dysfunction. So, it's possible that changes in the DNA methylation display a noticeable effect on fetal growth and metabolism, and may be sufficient to produce a mild thyroid dysfunction or a resetting of HPT axis regulation that can be detected by using low TSH cutoffs for neonatal CH screening. Obviously, severe CH would then require additional (genetic?) mechanisms to occur.

Nevertheless, if cases and controls are clustered according to their epigenetic profile, infants with overt hypothyroidism showed the most significant differences. This suggests that epigenetic changes may directly affect thyroid function, but the small number of patients and the influence of potential confounders indicate the need for a validation of this interpretation.

These results are confirmed by the gene-specific methylation analysis, where approximately 90-96% of DMGs are hypomethylated in affected subjects than controls in all the three approaches. To find which factors are related to these gene-specific changes, we selected a total of 154 common DMGs and clustered all of patients about such genes. We find that affected infants with comparable gestational age and low birth weight clusters together unlike those with a proper weight. These data are strongly supported by the study of the twin pairs discordant or concordant for CH. Interstingly, both concordant and discordant

twins cluster together according to their birth weight unlike their genetic similarity and the thyroid disease.

This allows us to speculate the presence of a direct relationship between the gestational age and birth weight with the gene-specific DNA hypomethylation, and the increased CH risk could represent an associated co-variate. Interestingly, about 75% of these genes are represented by probes located into the DNA non-coding regions, suggesting that the hypomethylation affects the chromosome stability. About the chromosomes distribution of the 154 DMGs, *non-CpG island* sites showed an overestimation than the expected frequencies in chromosomes 1, 2,3,6,7 and 17, but the reasons and consequences of this stratification remain to be clarified.

In literature, DNA hypomethylation is strongly associated with genomic instability and the relationship with developmental growth retardation [Koukoura *et al.*, 2011], premature birth [Hofman *et al.*, 2006] and the onset of some pathologies in later life such as cancer [Watanabe *et al.*, 2010], dementia [Miller *et al.*, 2003], neural tube defects [Chang *et al.*, 2011], congenital heart defects [Huang *et al.*, 2007] and diabetes [Toperoff *et al.*, 2012] are reported.

It acts through the deregulation of transposable elements, pericentromeric regions, or activation of endoparasitic sequences. To a lesser extent, DNA hypomethylation also impacts growth regulatory genes, imprinted genes, developmentally critical genes, genes regulated by transposable elements and tissue specific genes, such as germline-specific tumor antigen genes [Raptis *et al.*, 2006]. DNA hypomethylation patterns also represent footprints of transcription factor activities, and therefore are indicative of active cellular pathways. In fact, it can thus be used as prognostic indicators of diseases or as predictive markers of sensitivity or resistance to particular therapies or environmental exposures [Davis *et al.*, 2004].

Alterations in both global and gene-specific DNA methylation have been linked to exposure to metals, peroxisome proliferators, air pollutants, endocrine-

disrupting/reproductive toxicants, diet, maternal behavior, viruses, bacteria, simulated-microgravity exposure, metabolic imprinting and stress [LaSalle *et al.*, 2011; Madrigano *et al.*, 2011; Stouder *et al.*, 2011; Pilsner *et al.*, 2011]. More closely associated with the early onset of disease hypothesis, global DNA hypomethylation in cord blood DNA has been linked to in utero exposure to cigarette smoke, polycyclic aromatic hydrocarbons, lead and polyfluoroalkyl compounds [Waterland *et al.*, 2008].

Global DNA methylation changes may also be evolution-conserved adaptive responses that maintain homeostasis and assure cell survival in the face of threatening and noxious environmental stimuli. Adaptive changes in global patterns of differential DNA methylation variability at specific genetic loci can also result from inherited genetic variants that predispose demethylating phenotypes with selective advantages [Feinberg *et al.*, 2010]. Global DNA methylation may be a phenotype of the stochastic adaptation to repeated exposures to environmental stressors, which would select for epigenetic heterogeneity, and thus the ability of cells to grow outside of their normal milieu [Feinberg *et al.*, 2010]. Therefore, changes in global DNA methylation content may explain the phenotypic variation described by both Darwinian selection forces and Lamarckian evolution development [Handel *et al.*, 2010].

It is clear that a myriad of factors can affect the global hypomethylation but in this study we focused our attention on maternal folate deficiency. Folate-dependent one-carbon metabolism is a highly polymorphic metabolic pathway that regulates the distribution of one-carbon derivatives between DNA synthesis (proliferation), and DNA methylation (cell-specific gene expression and differentiation). As such, normal functioning of this pathway is essential to support the rapid fire shifts between proliferation, differentiation and cell death that are essential for normal fetal programming and organogenesis. Inadequate enzyme activities and imbalances of substrates and cofactors in one-carbon

metabolism, together referred to as the 'methyldietary' constituents, may cause homocysteine and S-adenosylhomocysteine accumulation.

Several studies in both animal models [Ibrahim *et al.*, 2011] and humans [Orzechowska-Pawilojc *et al.*, 2009] showed the association between increase in plasma total homocysteine and risk of subclinical and overt hypothyroidism.

In a recent work, a group of hypothyroid rats treated with a folate diet during and after receiving PTU showed a better and more rapid response of oxidative stress and hypothalamic parameters during restoration of the euthyroid state than control animals treated with PTU alone which in contrast had higher levels homocysteine during the hypothyroid state (Ibrahim *et al.*, 2011). In humans, the treatment of hypothyroid woman with Levotiroxine, display a reduction of total plasma homocysteine levels and an improvement in the indicators of cellular stress (Cakal *et al.*, 2007). In addition, several experimental studies have shown that hypothyroidism affects folate metabolism and the enzymes involved in the remetylation pathway of homocysteine. In hypothyroid condition the hepatic activity of flavoenzyme - MTHFR is decreased. Thyroid hormone may affect the availability of FMN and FAD - necessary for stabilizing MTHFR. An impairment of enzyme involved in trans-sulfuration pathway is than suggested. The increased serum creatinine level in hypothyroidism probably reflects a reduced glomerular filtration rate, which is linked to impaired renal homocysteine clearance and hyperhomocysteinemia.

Maternal polymorphic variants, nutritional deficiencies and/or environmental exposures that negatively affect availability of one-carbon precursors have been associated with increased risk of structural birth defects, chromosomal anomalies, schizophrenia, and prematurity [Doolin *et al.* 2002; Van der Linden *et al.* 2006; James *et al.* 1999; Johnson 1999; Bukowski *et al.* 2009; Scholl and Johnson 2000; Picker and Coyle 2005]. Defects in Methylenetetrahydrofolate

reductase (MTHFR) activity is strongly related to maternal folate deficiency during pregnancy [La Merrill *et al.*, 2011].

MTHFR is highly polymorphic, and the variant genotypes result in decreased MTHFR enzyme activity and lower plasma folate level. Several non-synonymous single nucleotide polymorphisms (SNPs) are present in the coding region of the gene including positions 677 and 1298. MTHFR enzyme activity is reduced by 35% among the 677C/T carriers and by 50% to 70% among 677T/T carriers. The function of polymorphism 1298A>C has not been consistently demonstrated; however carriers of the heterozygous genotypes (677CT/1298AC) show MTHFR activity similar to that in $MTHFR^{677T/T}$ carriers. The genetic analysis of nine mothers of discordant and concordant twin pairs and CH-only children revealed that mothers with the $MTHFR^{677T/T}$ genotypes are those whose affected twins are born small for gestational age. Single $MTHFR^{677C/T}$ polymorphisms are found in mothers of one AGA discordant twin pairs and two SGA only children. $MTHFR^{1298A/C}$ variants are found in mothers of AGA discordant twins, SGA/AGA concordant twins and in SGA only child. The $MTHFR^{677C/T,\ 1298A/C}$ genotype is carried by parents of SGA only-child. Although these data have to be extended and validated, intrauterine growth delay appears invariably associated with the presence of these genetic variants. Maternal folate deficiency caused by MTHFR deficiency leads to a shortage of methyl-donors and as a consequence to the hypomethylation status. The genomic instability and the altered regulation of certain factors potentially affected pregnancy outcomes and fetal growth.

By Gene Ontology and KEEG Pathway databases we have found that several genes belong to the biological processes involved in fetal development and maintenance the homeostasis of hormone levels. Biological processes such as regulation and response to hormone stimulus, post-embryonic development, regulation of retinoic metabolism, metabolism of vitamins and sulfur metabolism are found to be regulated at the epigenetic level. The most significant signal pathways are involved development, in autoimmune thyroid disease and in

retinol and glutathione metabolism. The inclusion of these specific processes within the top ten most significant gene set overlaps provided evidence of strong biological relevance of the differentially methylated genes to fetal development and thyroid function.

If the methylation status of these genes in thyroid gland or TH target tissues would be similar to that seen in leukocytes, the altered expression of several of these genes (eg, PDEs affecting the sensitivity of thyroid tissue to TSH stimulation or retinoic acid signal cooperating at the hypothalamic-pituitary level with that of TH in the negative feedback mechanism) may affect the setting of the HPT axis function.

CHAPTER VIII

7. CONCLUSIONS AND FUTURE PERSPECTIVES

This project sheds light for the first time on the possible involvement of environmental factors in predisposition to congenital hypothyroidism. The results obtained, although they are preliminary, indicate a direct relationship between hypomethylation and low gestational age and birth weight in hypothyroid infants. The findings about the involvement of DMGs in development, metabolism and hormone action, are in agreement with the DOHaD theory (fig 27).

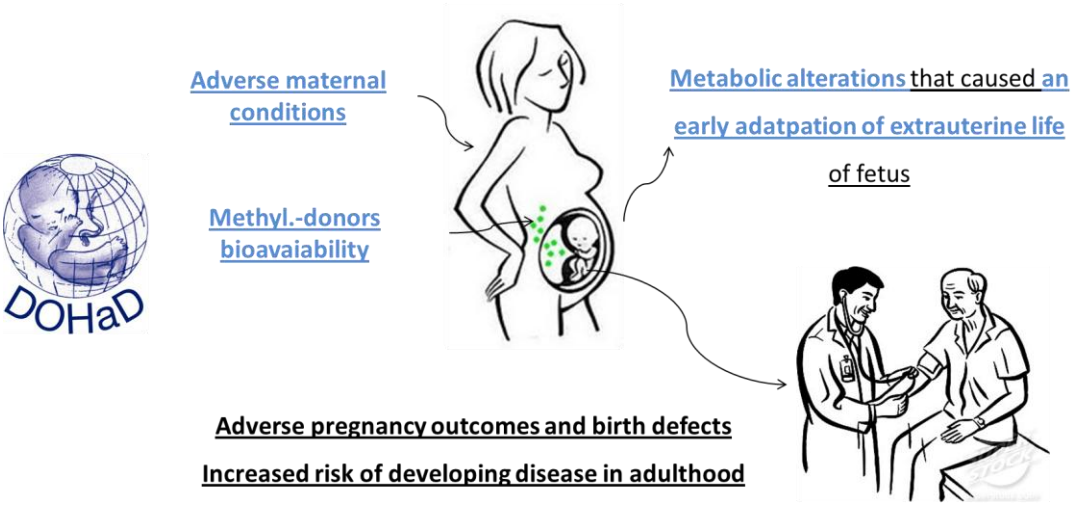


Figure 28: representation of DOHaD theory.

We suggest that several environmental factors, such as maternal nutrients deficiency or toxins exposures caused a global hypomethylation of fetus. Low methylation levels of non-coding regions affect chromosomes stability that is strongly associated to prematurity and low birth weight. Rather, stochastic hypomethylation of promoters of specific genes can explains the early adaptation of fetus of the extrauterine life. In fact, it's demonstrated that the fetus when exposed to metabolic, nutritional or hormonal deficiencies, adapts by increasing the expression of specific genes, such as hormone receptors or growth factors to overcome this condition even during the postnatal period. This adaptation is a double edged sword since although it is an advantage during fetal development, this leads to permanent alterations in sensitivity to internal and external stimuli and this kind cell memory predisposes newborns of further diseases in adulthood. In light of this evidences, congenital hypothyroidism may be caused not only by an abnormal development due to prematurity and low birth weight but also to the an altered sensitivity of cells of thyroid hormones to adapts on the intrauterine deficiency of nutrients and on the reducing maternal metabolism . This stable and altered cellular response to hormonal stimuli may explain the higher risk of premature infants of CH and elucidated why these patients do not have increased levels of methylation after treatment with L-thyroxine.

To validate this hypothesis we aim to:

- Increase the number of CH patients and make a new methylation analysis to confirm the association with hypomethylation and GA and BW;
- Analysis of global and the gene-specific methylation on the DNA extracted from hair follicles in the patients included in this study to confirm that PBMCs are a good model for studying thyroid diseases.
- Assess the levels of expression by Real Time PCR of selected genes list to view if they correlate with the DNA methylation levels.

If confirmed by further experiments, our results could propose that folic acid can be used as an adjuvant therapy in neonatal mild thyroid dysfunction in association with or in alternative to thyroxine replacement therapy.

References

Ahuja, N., Mohan, A.L., Li, Q., Stolker, J.M., Herman, J.G., Hamilton, S.R., Baylin, S.B., and Issa, J.P. (1997). Association between CpG island methylation and microsatellite instability in colorectal cancer. Cancer Res 57, 3370-3374.

Alberio R, Campbell KH (2003). Epigenetics and nuclear transfer. Lancet. 361(9365):1239-40.

Alberti L, Proverbio MC, Costagliola S, Romoli R, Boldrighini B, Vigone MC, Weber G, Chiumello G, Beck-Peccoz P, Persani L (2002). Germline mutations of TSH receptor gene as cause of nonautoimmune subclinical hypothyroidism. J Clin Endocrinol Metab. 87(6):2549-55.

Alegría-Torres JA, Baccarelli A, Bollati V (2011). Epigenetics and lifestyle. Epigenomics. 3(3):267-77.

Alvarez-Pedrerol M, Guxens M, Mendez M, Canet Y, Martorell R, Espada M, Plana E, Rebagliato M, Sunyer J (2008). Iodine levels and thyroid hormones in healthy pregnant women and birth weight of their offspring. Eur J Endocrinol. 160(3):423-9.

Amendola E, De Luca P, Macchia PE, Terracciano D, Rosica A, Chiappetta G, Kimura S, Mansouri A, Affuso A, Arra C, Macchia V, Di Lauro R, De Felice M (2005). A mouse model demonstrates a multigenic origin of congenital hypothyroidism. Endocrinology. 146(12):5038-47.

Amendola E, Sanges R, Galvan A, Dathan N, Manenti G, Ferrandino G, Alvino FM, Di Palma T, Scarfò M, Zannini M, Dragani TA, De Felice M, Di Lauro R (2010). A locus on mouse chromosome 2 is involved in susceptibility to congenital hypothyroidism and contains an essential gene expressed in thyroid. Endocrinology. 151(4):1948-58. Epub 2010 Feb 16.

Argumedo GS, Sanz CR, Olguín HJ (2011). Experimental Models of Developmental Hypothyroidism. Horm Metab Res.

Armitage, J.A., I.Y. Khan, P.D. Taylor, P.W. Nathanielsz, and L. Poston (2004). Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? J Physiol 561: 355-377.

Astapova I, Vella KR, Ramadoss P, Holtz KA, Rodwin BA, Liao XH, Weiss RE, Rosenberg MA, Rosenzweig A, Hollenberg AN (2011). The nuclear receptor

corepressor (NCoR) controls thyroid hormone sensitivity and the set point of the hypothalamic-pituitary-thyroid axis. Mol Endocrinol. 25(2):212-24.

Banghová K, Al TE, Novotná D, Zapletalová J, Hníková O, Cáp J, Klabochová J, Kúseková M, Lebl J (2008). Pendred syndrome among patients with hypothyroidism: genetic diagnosis, phenotypic variability and occurrence of phenocopies. Cas Lek Cesk. 147(12):616-22. Czech.

Barker, D.J. (1992). Fetal growth and adult disease. Br J Obstet Gynaecol 99: 275-276.

Barker, D.J. (1997). The long-term outcome of retarded fetal growth. Clin Obstet Gynecol 40: 853-863.

Barker, D.J. (2004). The developmental origins of adult disease. J Am Coll Nutr 23: 588S-595S.

Barker, D.J. and C. Osmond. (1986). Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet 1: 1077-1081.

Barker, D.J., C. Osmond, J. Golding, D. Kuh, and M.E. Wadsworth (1989). Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. Bmj 298: 564-567.

Barros R, Pereira B, Duluc I, Azevedo M, Mendes N, Camilo V, Jacobs RJ, Paulo P, Santos-Silva F, van Seuningen I, van den Brink GR, David L, Freund JN, Almeida R (2008). Key elements of the BMP/SMAD pathway co-localize with CDX2 in intestinal metaplasia and regulate CDX2 expression in human gastric cell lines. J Pathol. 215(4):411-20.

Bartel D.P (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 116:281–297.

Baylin, S.B., Herman, J.G., Graff, J.R., Vertino, P.M., and Issa, J.P. (1998). Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 72, 141-196.

Begemann M, Spengler S, Kordaß U, Schröder C, Eggermann T (2012). Segmental maternal uniparental disomy 7q associated with DLK1/GTL2 (14q32) hypomethylation. Am J Med Genet A. 158A(2):423-428.

Bernstein B.E., Meissner A., Lander E.S. (2007). The mammalian epigenome. Cell. 128:669–681. doi: 10.1016/j.cell.2007.01.033.

Biermann K, Steger K (2007). Epigenetics in male germ cells. J Androl. 28(4):466-80.

Bird, A. (2002). DNA methylation patterns and epigenetic memory. Genes Dev., 16, 6–21.

Bloomfield FH (2011). Epigenetic modifications may play a role in the developmental consequences of early life events. J Neurodev Disord. 3(4):348-55. Epub 2011 Oct 8.

Bock, C., Paulsen, M., Tierling, S., Mikeska, T., Lengauer, T., and Walter, J. (2006). CpG island methylation in human lymphocytes is highly correlated with DNA sequence, repeats, and predicted DNA structure. PLoS Genet 2, e26.

Botto, L. D. and Correa, A. (2003). Decreasing the burden of congenital heart anomalies: an epidemiologic evaluation of risk factors and survival. Prog Pediatr Cardiol. 18, 111-21.

Botto, L.D., Correa, A., and Erickson, J.D. (2001). Racial and temporal variations in the prevalence of heart defects. Pediatrics 107, E32.

Boumil, R.M., and Lee, J.T. (2001). Forty years of decoding the silence in X chromosome inactivation. Hum Mol Genet 10, 2225-2232.

Bouzinba-Segard, H., Guais, A., and Francastel, C. (2006). Accumulation of small murine minor satellite transcripts leads to impaired centromeric architecture and function. Proc Natl Acad Sci U S A 103, 8709-8714.

Boyle J, Hawkins M, Barton DE, Meaney K, Guitart M, O'Grady A, Tobi S, Ramsden SC, Elles R, Gray E, Metcalfe P, Hawkins JR (2011). Establishment of the first WHO international genetic reference panel for Prader Willi and Angelman syndromes. Eur J Hum Genet. 19(8):857-64

Brinkman, A.B., Pennings, S.W., Braliou, G.G., Rietveld, L.E., and Stunnenberg, H.G. (2007). DNA methylation immediately adjacent to active histone marking does not silence transcription. Nucleic Acids Res 35, 801-811.

Brix K, Führer D, Biebermann H (2011). Molecules important for thyroid hormone synthesis and action - known facts and future perspectives. Thyroid Res. 3;4 Suppl 1:S9.

Bros JA, Lluís PG, Cabot GL, Pedragós AC (2009). Primary hypothyroidism: Considerations for a rational use of levothyroxine therapy. Med Clin. 26;136(5):207-14.

Brouha, B., Schustak, J., Badge, R.M., Lutz-Prigge, S., Farley, A.H., Moran, J.V., and Kazazian, H.H., Jr. (2003). Hot L1s account for the bulk of retrotransposition in the human population. Proc Natl Acad Sci U S A 100, 5280-5285.

Bünger L, Wallace H, Bishop JO, Hastings IM, Hill WG (1998). Effects of thyroid hormone deficiency on mice selected for increased and decreased body weight and fatness. Genet Res. 72(1):39-53.

Büyükgebiz A (2003). Congenital hypothyroidism clinical aspects and late consequences. Pediatr Endocrinol Rev. 2003 Dec;1 Suppl 2:185-90; discussion 190. Review.

Canani RB, Di Costanzo M, Leone L, Bedogni G, Brambilla P, Cianfarani S, Nobili V, Pietrobelli A, Agostoni C (2011). Epigenetic mechanisms elicited by nutrition in early life. Nutr Res Rev. 18:1-8

Carninci, P., Sandelin, A., Lenhard, B., Katayama, S., Shimokawa, K., Ponjavic, J., Semple, C.A., Taylor, M.S., Engstrom, P.G., Frith, M.C., et al. (2006). Genome-wide analysis of mammalian promoter architecture and evolution. Nat Genet 38, 626-635.

Carrel, L., and Willard, H.F. (2005). X-inactivation profile reveals extensive variability in X-linked gene expression in females. Nature 434, 400-404.

Castanet M, Marinovic D, Polak M, Léger J (2010). Epidemiology of thyroid dysgenesis: the familial component. Horm Res Paediatr. 73(4):231-7.

Chaudhary N, Nakka KK, Maulik N, Chattopadhyay S (2012). Epigenetic Manifestation of Metabolic Syndrome and Dietary Management. Antioxid Redox Signal.

Chen, R.Z., Pettersson, U., Beard, C., Jackson-Grusby, L., and Jaenisch, R. (1998). DNA hypomethylation leads to elevated mutation rates. Nature 395, 89-93.

Cheng, X., and Roberts, R.J. (2001). AdoMet-dependent methylation, DNA methyltransferases and base flipping. Nucleic Acids Res 29, 3784-3795.

Christophersen NS, Helin K (2010). Epigenetic control of embryonic stem cell fate. J Exp Med. 207(11):2287-95.

Cianfarani S, Maiorana A, Geremia C, Scirè G, Spadoni GL, Germani D (2003). Blood glucose concentrations are reduced in children born small for gestational age (SGA), and thyroid-stimulating hormone levels are increased in SGA with blunted postnatal catch-up growth. J Clin Endocrinol Metab. 88(6):2699-705.

Clemson, C.M., McNeil, J.A., Willard, H.F., and Lawrence, J.B. (1996). XIST RNA paints the inactive X chromosome at interphase: evidence for a novel RNA involved in nuclear/chromosome structure. J Cell Biol 132, 259-275.

Coakley JC, Keir EH, Connelly JF (1999). The association of thyroid dyshormonogenesis and deafness (Pendred syndrome): experience of the Victorian Neonatal Thyroid Screening Programme. J Paediatr Child Health. 28(5):398-401.

Conway, K.E., McConnell, B.B., Bowring, C.E., Donald, C.D., Warren, S.T., and Vertino, P.M. (2000). TMS1, a novel proapoptotic caspase recruitment domain protein, is a target of methylation-induced gene silencing in human breast cancers. Cancer Res 60, 6236-6242.

Corbetta C, Weber G, Cortinovis F, Calebiro D, Passoni A, Vigone MC, Beck-Peccoz P, Chiumello G, Persani L (2009). A 7-year experience with low blood TSH cutoff levels for neonatal screening reveals an unsuspected frequency of congenital hypothyroidism (CH). Clin Endocrinol (Oxf). 71(5):739-45. Epub 2009 Mar 28.

Costello, J.F., and Brena, R.M. (2008). Cancer Epigenetics. In Epigenetics, J. Tost, ed. (Norfolk, Caister Academic Press), pp. 303-330.

Cowell, I.G., Aucott, R., Mahadevaiah, S.K., Burgoyne, P.S., Huskisson, N., Bongiorni, S., Prantera, G., Fanti, L., Pimpinelli, S., Wu, R. et al. (2002) Heterochromatin, HP1 and methylation at lysine 9 of histone H3 in animals. Chromosoma, 111, 22–36.

Cropley, J.E., Suter, C.M., Beckman, K.B., and Martin, D.I. (2006). Germ-line epigenetic modification of the murine A vy allele by nutritional supplementation. Proc Natl Acad Sci U S A 103, 17308-17312.

Curhan, G.C., G.M. Chertow, W.C. Willett, D. Spiegelman, G.A. Colditz, J.E. Manson, F.E. Speizer, and M.J. Stampfer (1996a). Birth weight and adult hypertension and obesity in women. Circulation 94: 1310-1315.

Curhan, G.C., W.C. Willett, E.B. Rimm, D. Spiegelman, A.L. Ascherio, and M.J. Stampfer (1996b). Birth weight and adult hypertension, diabetes mellitus, and obesity in US men. Circulation 94: 3246-3250.

Dahl, C., and Guldberg, P. (2003). DNA methylation analysis techniques. Biogerontology 4, 233-250.

Dahlgren J, Boguszewski M, Rosberg S, Albertsson-Wikland K (1998). Adrenal steroid hormones in short children born small for gestational age . Clin Endocrinol (Oxf). 49(3):353-61.

Dahlgren, J., C. Nilsson, E. Jennische, H.P. Ho, E. Eriksson, A. Niklasson, P. Bjorntorp, K. Albertsson Wikland, and A. Holmang (2001). Prenatal cytokine exposure results in obesity and gender-specific programming. Am J Physiol Endocrinol Metab 281: E326-334.

De Carvalho DD, You JS, Jones PA (2010). DNA methylation and cellular reprogramming. Trends Cell Biol. 20(10):609-17.

De Felice M, Di Lauro R (2007). Murine models for the study of thyroid gland development. Endocr Dev. 10:1-14.

De Felice M, Ovitt C, Biffali E, Rodriguez-Mallon A, Arra C, Anastassiadis K, Macchia PE, Mattei MG, Mariano A, Schöler H, Macchia V, Di Lauro R (1998). A mouse model for hereditary thyroid dysgenesis and cleft palate. Nat Genet. 19(4):395-8.

Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA (2003). DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol. 4(5):P3

Derom R, Orlebeke J, Eriksson A, Thiery M (1995). The epidemiology of multiple births in Europe. New York: Parthenon Publishing Group; 145–162

Devos H, Rodd C, Gagné N, Laframboise R, Van Vliet G (1999). A search for the possible molecular mechanisms of thyroid dysgenesis: sex ratios and associated malformations. J Clin Endocrinol Metab. 84(7):2502-6.

Di Lauro R (2003). Molecular abnormalities of organogenesis and differentiation of the thyroid gland. Ann Endocrinol (Paris). 64(1):53.

Di Palma T, Zampella E, Filippone MG, Macchia PE, Ris-Stalpers C, de Vroede M, Zannini M (2010). Characterization of a novel loss-of-function mutation of PAX8 associated with congenital hypothyroidism. Clin Endocrinol (Oxf). 73(6):808-14.

Di Palma T, Zampella E, Filippone MG, Macchia PE, Ris-Stalpers C, de Vroede M, Zannini M (2010). Characterization of a novel loss-of-function mutation of PAX8 associated with congenital hypothyroidism. Clin Endocrinol (Oxf). 73(6):808-14.

Dirx, M.J., van den Brandt, P.A., Goldbohm, R.A., and Lumey, L.H. (2003). Energy restriction early in life and colon carcinoma risk: results of The Netherlands Cohort Study after 7.3 years of follow-up. Cancer 97, 46-55.

Doornbos ME, Maas SM, McDonnell J, Vermeiden JP, Hennekam RC (2007). Infertility, assisted reproduction technologies and imprinting disturbances: a Dutch study. Hum Reprod. 22(9):2476-80.

Doyle DA, Gonzalez I, Thomas B, Scavina M (2004). Autosomal dominant transmission of congenital hypothyroidism, neonatal respiratory distress, and ataxia caused by a mutation of NKX2-1. J Pediatr. 145(2):190-3.

Dupont C, Armant DR, Brenner CA (2009). Epigenetics: definition, mechanisms and clinical perspective. Semin Reprod Med. 27(5):351-7.

Dussault JH, Coulombe P, Laberge C, Letarte J, Guyda H, Khoury K (1975). Preliminary report on a mass screening program for neonatal hypothyroidism. J Pediatr. 86:670–674

Esteller, M., Risques, R.A., Toyota, M., Capella, G., Moreno, V., Peinado, M.A., Baylin, S.B., and Herman, J.G. (2001). Promoter hypermethylation of the DNA repair gene O(6)-methylguanine-DNA methyltransferase is associated with the presence of G:C to A:T transition mutations in p53 in human colorectal tumorigenesis. Cancer Res 61, 4689-4692.

Esteller, M., Silva, J.M., Dominguez, G., Bonilla, F., Matias-Guiu, X., Lerma, E., Bussaglia, E., Prat, J., Harkes, I.C., Repasky, E.A., et al. (2000). Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. J Natl Cancer Inst 92, 564-569.

Fagman H, Nilsson M (2010). Morphogenesis of the thyroid gland. Mol Cell Endocrinol. 323(1):35-54.

Fagman H, Nilsson M (2011). Morphogenetics of early thyroid development. .J Mol Endocrinol. 46(1):R33-42. Review.

Farriaux JP, Dhondt JL (1988). French screening programs for congenital hypothyroidism. Am J Dis Child. 142(11):1137-8.

Feinberg, A.P., and Vogelstein, B. (1983). Hypomethylation of ras oncogenes in primary human cancers. Biochem Biophys Res Commun 111, 47-54.

Ferguson, A.T., Evron, E., Umbricht, C.B., Pandita, T.K., Chan, T.A., Hermeking, H., Marks, J.R., Lambers, A.R., Futreal, P.A., Stampfer, M.R., et al. (2000). High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. Proc Natl Acad Sci U S A 97, 6049-6054.

Ferguson, A.T., Evron, E., Umbricht, C.B., Pandita, T.K., Chan, T.A., Hermeking, H., Marks, J.R., Lambers, A.R., Futreal, P.A., Stampfer, M.R., et al. (2000). High

frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. Proc Natl Acad Sci U S A 97, 6049-6054.

Ferretti E, Tosi E, Po A, Scipioni A, Morisi R, Espinola MS, Russo D, Durante C, Schlumberger M, Screpanti I, Filetti S, Gulino A (2008). Notch signaling is involved in expression of thyrocyte differentiation markers and is down-regulated in thyroid tumors. J Clin Endocrinol Metab. 93(10):4080-7. Epub 2008 Jul 29.

Fraga, M.F., Ballestar, E., Paz, M.F., Ropero, S., Setien, F., Ballestar, M.L., Heine-Suner, D., Cigudosa, J.C., Urioste, M., Benitez, J., Boix-Chornet, M., Sanchez-Aguilera, A., Ling, C., Carlsson, E., Poulsen, P., Vaag, A., Stephan, Z., Spector, T. D.,Wu, Y. Z., Plass, C., Esteller, M., (2005). Epigenetic differences arise during the lifetime of monozygotic twins. Proc Natl Acad Sci U S A 102, 10604-10609.

Francis, D., Diorio, J., Liu, D., and Meaney, M.J. (1999). Nongenomic transmission across generations of maternal behavior and stress responses in the rat. Science 286, 1155-1158.

Frantz ED, Peixoto-Silva N, Pinheiro-Mulder A (2012). Endocrine pancreas development: effects of metabolic and intergenerational programming caused by a protein-restricted diet. Pancreas. 41(1):1-9.

Frigola, J., Song, J., Stirzaker, C., Hinshelwood, R.A., Peinado, M.A., and Clark, S.J. (2006). Epigenetic remodeling in colorectal cancer results in coordinate gene suppression across an entire chromosome band. Nat Genet 38, 540-549.

Fryxell, K.J., and Moon, W.J. (2005). CpG mutation rates in the human genome are highly dependent on local GC content. Mol Biol Evol 22, 650-658.

Fulka, H., Mrazek, M., Tepla, O. and Fulka, J., Jr (2004). DNA methylation pattern in human zygotes and developing embryos. Reproduction, 128, 703–708

Garcia BA, Thomas CE, Kelleher NL, Mizzen CA (2008). Tissue-specific expression and posttranslational modification of histone H3 variants. J Proteom Res 7:4225-36

Gardiner-Garden, M., and Frommer, M. (1987). CpG islands in vertebrate genomes. J Mol Biol 196, 261-282.

Garrick, D., Fiering, S., Martin, D.I., and Whitelaw, E. (1998). Repeat-induced gene silencing in mammals. Nat Genet 18, 56-59.

Geers C, Colin IM, Gérard AC (2011). Delta-like 4/Notch pathway is differentially regulated in benign and malignant thyroid tissues. Thyroid. 21(12):1323-30.

Gicquel C, Gaston V, Mandelbaum J, Siffroi JP, Flahault A, Le Bouc Y (2003). In vitro fertilization may increase the risk of Beckwith-Wiedemann syndrome related to the abnormal imprinting of the KCN1OT gene. Am J Hum Genet. 72(5):1338-41.

Gilbert ME, Rovet J, Chen Z, Koibuchi N (2011). Developmental thyroid hormone disruption: Prevalence, environmental contaminants and neurodevelopmental consequences. Neurotoxicology.

Gillam MP, Kopp P (2001). Genetic regulation of thyroid development. Current Opinion of Pediatrics. 13(4):358-63.

Girard, L., and Freeling, M. (1999). Regulatory changes as a consequence of transposon insertion. Dev Genet 25, 291-296.

Gluckman, P.D. and M.A. Hanson (2004a). The developmental origins of the metabolic syndrome. Trends Endocrinol Metab 15: 183-187.

Gluckman, P.D. and M.A. Hanson (2004b). Living with the past: evolution, development, and patterns of disease. Science 305: 1733-1736.

Gluckman, P.D., M.A. Hanson, C. Cooper, and K.L. Thornburg (2008). Effect of in utero and early-life conditions on adult health and disease. N Engl J Med 359: 61-73.

Godfrey KM, Sheppard A, Gluckman PD, Lillycrop KA, Burdge GC, McLean C, Rodford J, Slater-Jefferies JL, Garratt E, Crozier SR, Emerald BS, Gale CR, Inskip HM, Cooper C, Hanson MA (2011). Epigenetic gene promoter methylation at birth is associated with child's later adiposity. Diabetes. 2011 May;60(5):1528-34.

Godfrey KM, Sheppard A, Gluckman PD, Lillycrop KA, Burdge GC, McLean C, Rodford J, Slater-Jefferies JL, Garratt E, Crozier SR, Emerald BS, Gale CR, Inskip HM, Cooper C, Hanson MA (2011). Epigenetic gene promoter methylation at birth is associated with child's later adiposity. Diabetes. 60(5):1528-34.

Goh KP, Sum CF (2010). Connecting the dots: molecular and epigenetic mechanisms in type 2 diabetes. Curr Diabetes Rev. 6(4):255-65.

Golbabapour S, Abdulla MA, Hajrezaei M (2011). A concise review on epigenetic regulation: insight into molecular mechanisms. Int J Mol Sci. 12(12):8661-94.

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 311, 395-398.

Grasberger H, Refetoff S (2011). Genetic causes of congenital hypothyroidism due to dyshormonogenesis. Curr Opin Pediatr. 23(4):421-8. Review.

Greger, V., Passarge, E., Hopping, W., Messmer, E., and Horsthemke, B. (1989). Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. Hum Genet 83, 155-158.

Grüters A, Krude H (2011). Detection and treatment of congenital hypothyroidism. Nat Rev Endocrinol. 8(2):104-13.

Haig, D., and Graham, C. (1991). Genomic imprinting and the strange case of the insulin-like growth factor II receptor. Cell 64, 1045-1046.

Hajkova P, Ancelin K, Waldmann T, Lacoste N, Lange UC, Cesari F (2008). Chromatin dynamics during epigenetic reprogramming in the mouse germ line. Nature. 452:877-81

Hake SB, Garcia BA, Duncan EM, Kauer M, Dellaire G, Shabanowitz J (2006). Expression patterns and post-translational modifications associated with mammalian histone H3 variants. J Biol Chem 281:559-68

Hales, C.N., D.J. Barker, P.M. Clark, L.J. Cox, C. Fall, C. Osmond, and P.D. Winter (1991). Fetal and infant growth and impaired glucose tolerance at age 64. Bmj 303: 1019-1022.

Handel AE, Ramagopalan SV (2010). Is Lamarckian evolution relevant to medicine? BMC Med Genet. 11:73. Review.

Harris KB, Pass KA (2007). Increase in congenital hypothyroidism in New York State and in the United States. Mol Genet Metab. 91:268–277.

Hebron HR, Yang Y, Hang J (2009). Purification of genomic DNA with minimal contamination of proteins. J Biomol Tech. 20(5):278-81.

Hegi, M.E., Diserens, A.C., Gorlia, T., Hamou, M.F., de Tribolet, N., Weller, M., Kros, J.M., Hainfellner, J.A., Mason, W., Mariani, L., et al. (2005). MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 352, 997-1003.

Heijmans, B.T., Tobi, E.W., Stein, A.D., Putter, H., Blauw, G.J., Susser, E.S., Slagboom, P.E., and Lumey, L.H. (2008). Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci U S A 105, 17046-17049.

Herman, J.G., Latif, F., Weng, Y., Lerman, M.I., Zbar, B., Liu, S., Samid, D., Duan, D.S., Gnarra, J.R., Linehan, W.M., et al. (1994). Silencing of the VHL tumorsuppressor gene by DNA methylation in renal carcinoma. Proc Natl Acad Sci U S A 91, 9700-9704.

Herman, J.G., Umar, A., Polyak, K., Graff, J.R., Ahuja, N., Issa, J.P., Markowitz, S., Willson, J.K., Hamilton, S.R., Kinzler, K.W., et al. (1998). Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci U S A 95, 6870-6875.

Hernandez-Blazquez, F.J., Habib, M., Dumollard, J.M., Barthelemy, C., Benchaib, M., de Capoa, A., and Niveleau, A. (2000). Evaluation of global DNA hypomethylation in human colon cancer tissues by immunohistochemistry and image analysis. Gut 47, 689- 693.

Herrera BM, Keildson S, Lindgren CM (2011). Genetics and epigenetics of obesity. Maturitas. 69(1):41-9. Review.

Hiltunen MO, Tuomisto TT, Niemi M, Bräsen JH, Rissanen TT, Törönen P, Vajanto I, Ylä-Herttuala S (2002). Changes in gene expression in atherosclerotic plaques analyzed using DNA array. Atherosclerosis. 165(1):23-32

Hiltunen MO, Ylä-Herttuala S (2003). DNA methylation, smooth muscle cells, and atherogenesis. Arterioscler Thromb Vasc Biol. 23(10):1750-3. Epub 2003 Aug 28. Review.

Hobbs, C.A., Cleves, M.A., Melnyk, S., Zhao, W., and James,S.J. (2005). Congenital heart defects and abnormal maternal biomarkers of methionine and homocysteine metabolism. Am. J. Clin. Nutr. 81, 147-153.

Hogg K, Wood C, McNeilly AS, Duncan WC (2011). The in utero programming effect of increased maternal androgens and a direct fetal intervention on liver and metabolic function in adult sheep. PLoS One. 6(9):e24877.

Houshdaran S, Cortessis VK, Siegmund K, Yang A, Laird PW, Sokol RZ (2007). Widespread epigenetic abnormalities suggest a broad DNA methylation erasure defect in abnormal human sperm. PLoS One. 2(12):e1289.

Howard, G., Eiges, R., Gaudet, F., Jaenisch, R., and Eden, A. (2008). Activation and transposition of endogenous retroviral elements in hypomethylation induced tumors in mice. Oncogene 27, 404-408.

Hsieh JJ, Zhou S, Chen L, Young DB, Hayward SD (1999). CIR, a corepressor linking the DNA binding factor CBF1 to the histone deacetylase complex. Proc Natl Acad Sci U S A.;96(1):23-8.

Huang da W, Sherman BT, Lempicki RA (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 4(1):44-57.

Hubalewska-Dydejczyk A, Lewiński A, Milewicz A, Radowicki S, Poręba R, Karbownik-Lewińska M, Kostecka-Matyja M, Trofimiuk-Müldner M, Pach D, Zygmunt A, Bandurska-Stankiewicz E, Bar-Andziak E, Bednarczuk T, Buziak-Bereza M, Drews K, Gietka-Czernel M, Górska M, Jastrzębska H, Junik R, Nauman J, Niedziela M, Reroń A, Sworczak K, Syrenicz A, Zgliczyński W (2011). Management of thyroid diseases during pregnancy. Endokrynol Pol. 62(4):362-81.

Hughes, L.A., van den Brandt, P.A., de Bruine, A.P., Wouters, K.A., Hulsmans, S., Spiertz, A., Goldbohm, R.A., de Goeij, A.F., Herman, J.G., Weijenberg, M.P., et al. (2009). Early life exposure to famine and colorectal cancer risk: a role for epigenetic mechanisms. PLoS One 4, e7951.

Hwang T, Park T (2009). Identification of differentially expressed subnetworks based on multivariate ANOVA. BMC Bioinformatics. 2009 Apr 30;10:128.

Idaghdour Y, Storey JD, Jadallah SJ, Gibson G (2008). A genome-wide gene expression signature of environmental geography in leukocytes of Moroccan Amazighs. PLoS Genet. 4(4)

Ilyés I (2011). Current questions of thyroid diseases in childhood. Orv Hetil. 152(16):617-27. Review. Hungarian.

Issa, J.P. (2003). Age-related epigenetic changes and the immune system. Clin Immunol 109, 103-108.

Jeltsch, A. (2006). On the enzymatic properties of Dnmt1: specificity, processivity, mechanism of linear diffusion and allosteric regulation of the enzyme. Epigenetics 1, 63-66.

Jones PL, Shi YB (2003). N-CoR-HDAC corepressor complexes: roles in transcriptional regulation by nuclear hormone receptors. Curr Top Microbiol Immunol. 274:237-68.

Jones, P.A., and Baylin, S.B. (2002). The fundamental role of epigenetic events in cancer. Nat Rev Genet 3, 415-428.

Jones, P.A., and Laird, P.W. (1999). Cancer epigenetics comes of age. Nat Genet 21, 163-167.

Kaati, G., Bygren, L.O., and Edvinsson, S. (2002). Cardiovascular and diabetes mortality determined by nutrition during parents' and grandparents' slow growth period. Eur J Hum Genet 10, 682-688.

Kafri, T., Ariel, M., Brandeis, M., Shemer, R., Urven, L., McCarrey, J., Cedar, H., and Razin, A. (1992). Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line. Genes Dev 6, 705-714.

Kaminen-Ahola, N., Ahola, A., Maga, M., Mallitt, K.A., Fahey, P., Cox, T.C., Whitelaw, E., and Chong, S. (2010). Maternal ethanol consumption alters the epigenotype and the phenotype of offspring in a mouse model. PLoS Genet 6, e1000811.

Kanno Y, Vahedi G, Hirahara K, Singleton K, O'Shea JJ (2011). Transcriptional and Epigenetic Control of T Helper Cell Specification: Molecular Mechanisms Underlying Commitment and Plasticity. Annu Rev Immunol.

Kantlehner M, Kirchner R, Hartmann P, Ellwart JW, Alunni-Fabbroni M, Schumacher A (2011). A high-throughput DNA methylation analysis of a single cell. Nucleic Acids Res. 39(7):e44.

Kapusta, L., Haagmans, M.L., Steegers, E.A., Cuypers, M.H., Blom, H.J., and Eskes, T.K. (1999). Congenital heart defects and maternal derangement of homocysteine metabolism. J. Pediatr 135, 773-774.

Katzenellenbogen, R.A., Baylin, S.B., and Herman, J.G. (1999). Hypermethylation of the DAP-kinase CpG island is a common alteration in B-cell malignancies. Blood 93, 4347-4353.

Kay, G.F., Penny, G.D., Patel, D., Ashworth, A., Brockdorff, N., and Rastan, S. (1993). Expression of Xist during mouse development suggests a role in the initiation of X chromosome inactivation. Cell 72, 171-182.

Kempers MJ, Ozgen HM, Vulsma T, Merks JH, Zwinderman KH, de Vijlder JJ, Hennekam RC (2009). Morphological abnormalities in children with thyroidal congenital hypothyroidism. Am J Med Genet A. 149A(5):943-51.

Kimura S (2011). Thyroid-specific transcription factors and their roles in thyroid cancer. J Thyroid Res. 2011:710213.

Klimasauskas, S., Kumar, S., Roberts, R.J., and Cheng, X. (1994). HhaI methyltransferase flips its target base out of the DNA helix. Cell 76, 357-369.

Klug, M., Heinz, S., Gebhard, C., Schwarzfischer, L., Krause, S.W., Andreesen, R., and Rehli, M. (2010). Active DNA demethylation in human postmitotic cells correlates with activating histone modifications, but not transcription levels. Genome Biol 11, R63.

Kondo, M., Suzuki, H., Ueda, R., Osada, H., Takagi, K., and Takahashi, T. (1995). Frequent loss of imprinting of the H19 gene is often associated with its overexpression in human lung cancers. Oncogene 10, 1193-1198.

Kota SK, Modi K, Kumaresan K (2011). Elevated thyroid stimulating hormone in a neonate: Drug induced or disease? Indian J Endocrinol Metab. 15(Suppl 2):S138-40.

Kreisner E, Neto EC, Gross JL (2005). High prevalence of extrathyroid malformations in a cohort of Brazilian patients with permanent primary congenital hypothyroidism. Thyroid. 15(2):165-9.

Lacroix L, Michiels S, Mian C, Arturi F, Caillou B, Filetti S, Schlumberger M, Bidart JM (2006). HEX, PAX-8 and TTF-1 gene expression in human thyroid tissues: a comparative analysis with other genes involved in iodide metabolism. Clin Endocrinol (Oxf). 64(4):398-404.

LaFranchi S (1999). Congenital hypothyroidism: etiologies, diagnosis, and management. Thyroid.9(7):735-40. Review.

Lafranchi SH (2010). Newborn screening strategies for congenital hypothyroidism: an update. J Inherit Metab Dis.33(Suppl 2):S225–33.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., et al. (2001). Initial sequencing and analysis of the human genome. Nature 409, 860-921.

Larsen, F., Solheim, J., and Prydz, H. (1993). A methylated CpG island 3' in the apolipoprotein-E gene does not repress its transcription. Hum Mol Genet 2, 775-780.

Lehnertz, B., Ueda, Y., Derijck, A.A., Braunschweig, U., Perez-Burgos, L., Kubicek, S., Chen, T., Li, E., Jenuwein, T., and Peters, A.H. (2003). Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin. Curr Biol 13, 1192-1200.

Li B, Carey M, Workman JL (2007). The role of chromatin during transcription. Cell 128:707-19.

Li E (2002). Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet. 3(9):662-73.

Li H, Leo C, Schroen DJ, Chen JD (1997). Characterization of receptor interaction and transcriptional repression by the corepressor SMRT. Mol Endocrinol. 11(13):2025-37.

Li, H., Rauch, T., Chen, Z.X., Szabo, P.E., Riggs, A.D., and Pfeifer, G.P. (2006). The histone methyltransferase SETDB1 and the DNA methyltransferase DNMT3A interact directly and localize to promoters silenced in cancer cells. J Biol Chem 281, 19489-19500.

Liang, G., Chan, M.F., Tomigahara, Y., Tsai, Y.C., Gonzales, F.A., Li, E., Laird, P.W., and Jones, P.A. (2002). Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. Mol Cell Biol 22, 480-491.

Liang, G., Robertson, K.D., Talmadge, C., Sumegi, J., and Jones, P.A. (2000). The gene for a novel transmembrane protein containing epidermal growth factor and follistatin domains is frequently hypermethylated in human tumor cells. Cancer Res 60, 4907-4912.

Lin HY, Davis FB, Luidens MK, Mousa SA, Cao JH, Zhou M, Davis PJ (2011). Molecular basis for certain neuroprotective effects of thyroid hormone. Front Mol Neurosci. 4:29.

Lindahl, T., Karran, P., and Wood, R.D. (1997). DNA excision repair pathways. Curr Opin Genet Dev 7, 158-169.

Linn M, Yoder BA, Clark RH (2010). Increasing supplemental thyroid hormone use among premature infants born at 23 to 32 weeks' gestation. Am J Perinatol. 27(9):731-5.

Lister, R., Pelizzola, M., Dowen, R.H., Hawkins, R.D., Hon, G., Tonti-Filippini, J.,Nery, J.R., Lee, L., Ye, Z., Ngo, Q.M., et al. (2009). Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462, 315-322.

Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., Sharma, S., Pearson, D., Plotsky, P.M., and Meaney, M.J. (1997). Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science 277, 1659-1662.

Lucas, A (1991). Programming by early nutrition in man. Ciba Found Symp 156: 38-50; discussion 50-35.

Lund KC, Wallace KB (2004). Direct effects of nucleoside reverse transcriptase inhibitors on rat cardiac mitochondrial bioenergetics. Mitochondrion. 4(2-3):193-202.

Maegawa, S., Hinkal, G., Kim, H.S., Shen, L., Zhang, L., Zhang, J., Zhang, N., Liang, S., Donehower, L.A., and Issa, J.P. (2010). Widespread and tissue specific age-related DNA methylation changes in mice. Genome Res 20, 332-340.

Mandel EM, Kaltenbrun E, Callis TE, Zeng XX, Marques SR, Yelon D, Wang DZ, Conlon FL (2010). The BMP pathway acts to directly regulate Tbx20 in the developing heart. Development. 137(11):1919-29.

Mead TJ, Yutzey KE (2012). Notch pathway regulation of neural crest cell development in vivo. Dev Dyn. 241(2):376-8.

Medici M, de Rijke YB, Peeters RP, Visser W, de Muinck Keizer-Schrama SM, Jaddoe VV, Hofman A, Hooijkaas H, Steegers EA, Tiemeier H, Bongers-Schokking JJ, Visser TJ (2011). Maternal Early Pregnancy and Newborn Thyroid Hormone Parameters: The Generation R Study. J Clin Endocrinol Metab.

Merlo, A., Herman, J.G., Mao, L., Lee, D.J., Gabrielson, E., Burger, P.C., Baylin, S.B., and Sidransky, D. (1995). 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat Med 1, 686-692.

Michaud, E.J., van Vugt, M.J., Bultman, S.J., Sweet, H.O., Davisson, M.T., and Woychik, R.P. (1994). Differential expression of a new dominant agouti allele (Aiapy) is correlated with methylation state and is influenced by parental lineage. Genes Dev 8, 1463-1472.

Milano F, van Baal JW, Buttar NS, Rygiel AM, de Kort F, DeMars CJ, Rosmolen WD, Bergman JJ, VAn Marle J, Wang KK, Peppelenbosch MP, Krishnadath KK (2007). Bone morphogenetic protein 4 expressed in esophagitis induces a columnar phenotype in esophageal squamous cells. Gastroenterology. 132(7):2412-21.

Milde K, Tomaszewski P, Sienkiewicz-Dianzenza E, Nowicki D, Wiśniewski A, Stupnicki R (2006). Physical fitness of short-statured boys as related to percentile norms for calendar or growth age. Endokrynol Diabetol Chor Przemiany Materii Wieku Rozw. 12(2):127-30.

Miyai K, Connelly JF, Foley TP Jr, Irie M, Illig R, Lie SO, Morissette J, Nakajima H, Rochiccioli P, Walfish PG (1984). An analysis of the variation of incidence of

congenital dysgenetic hypothyroidism in various countries. Endocrinol Jpn. 31(1):77-81.

Montanelli L, Tonacchera M (2010). Genetics and phenomics of hypothyroidism and thyroid dys- and agenesis due to PAX8 and TTF1 mutations. Mol Cell Endocrinol. 322(1-2):64-71. Epub 2010 Mar 17. Review.

Moreno JC, Visser TJ (2007). New phenotypes in thyroid dyshormonogenesis: hypothyroidism due to DUOX2 mutations. Endocr Dev. 10:99-117. Review.

Morgan HD, Sutherland HG, Martin DI, Whitelaw E (1999). Epigenetic inheritance at the agouti locus in the mouse. Nat Genet. 23(3):314-8.

Morgan, H.D., Sutherland, H.G., Martin, D.I., and Whitelaw, E. (1999). Epigenetic inheritance at the agouti locus in the mouse. Nat Genet 23, 314-318.

Morison, I.M., Ramsay, J.P., and Spencer, H.G. (2005). A census of mammalian imprinting. Trends Genet 21, 457-465.

Mortimer RH, Landers KA, Balakrishnan B, Li H, Mitchell MD, Patel J, Richard K (2012). Secretion and transfer of the thyroid hormone binding protein transthyretin by human placenta. Placenta.

Narumi S, Muroya K, Asakura Y, Aachi M, Hasegawa T (2011). Molecular basis of thyroid dyshormonogenesis: genetic screening in population-based Japanese patients. J Clin Endocrinol Metab. 96(11):E1838-42. Epub 2011 Sep 7.

Nebesio TD, Eugster EA (2009). Unusual thyroid constellation in Down syndrome: congenital hypothyroidism, Graves' disease, and hemiagenesis in the same child. J Pediatr Endocrinol Metab. 22(3):263-8.

Nebesio TD, McKenna MP, Nabhan ZM, Eugster EA (2010). Newborn screening results in children with central hypothyroidism. J Pediatr. 156(6):990-3.

Nishimaki M, Isozaki O, Yoshihara A, Okubo Y, Takano K (2209). Clinical characteristics of frequently recurring painless thyroiditis: contributions of higher thyroid hormone levels, younger onset, male gender, presence of thyroid autoantibody and absence of goiter to repeated recurrence. Endocr J. 56(3):391-7. Epub 2009 Feb 18.

Novak, P., Jensen, T., Oshiro, M.M., Wozniak, R.J., Nouzova, M., Watts, G.S., Klimecki, W.T., Kim, C., and Futscher, B.W. (2006). Epigenetic inactivation of the HOXA gene cluster in breast cancer. Cancer Res 66, 10664-10670.

Nyirenda, M.J., R.S. Lindsay, C.J. Kenyon, A. Burchell, and J.R. Seckl (1998). Glucocorticoid exposure in late gestation permanently programs rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. J Clin Invest 101: 2174-2181.

Olivieri A, Medda E, De Angelis S, Valensise H, De Felice M, Fazzini C, Cascino I, Cordeddu V, Sorcini M, Stazi MA (2007). High risk of congenital hypothyroidism in multiple pregnancies. J Clin Endocrinol Metab. 92(8):3141-7.

Olney RS, Grosse SD, Vogt RF Jr (2010). Prevalence of congenital hypothyroidism--current trends and future directions: workshop summary. Pediatrics. 125 Suppl 2:S31-6

Ortiga-Carvalho TM, Shibusawa N, Nikrodhanond A, Oliveira KJ, Machado DS, Liao XH, Cohen RN, Refetoff S, Wondisford FE (2005). Negative regulation by thyroid hormone receptor requires an intact coactivator-binding surface. J Clin Invest. 115(9):2517-23.

Panicker V (2011). Genetics of thyroid function and disease. Clin Biochem Rev. 32(4):165-75.

Panning, B. (2008). X-chromosome inactivation: the molecular basis of silencing. J Biol 7, 30.

Park SM, Chatterjee VK (2005). Genetics of congenital hypothyroidism. J Med Genet. 42(5):379-89. Review.

Parlato R, Rosica A, Rodriguez-Mallon A, Affuso A, Postiglione MP, Arra C, Mansouri A, Kimura S, Di Lauro R, De Felice M (2004). An integrated regulatory network controlling survival and migration in thyroid organogenesis. Dev Biol. 276(2):464-75.

Pass KA, Neto EC (2009). Update: newborn screening for endocrinopathies. Endocrinol Metab Clin North Am. 38:827–837.

Passeri E, Frigerio M, De Filippis T, Valaperta R, Capelli P, Costa E, Fugazzola L, Marelli F, Porazzi P, Arcidiacono C, Carminati M, Ambrosi B, Persani L, Corbetta S (2011). Increased risk for non-autoimmune hypothyroidism in young patients with congenital heart defects. J Clin Endocrinol Metab. 96(7):E1115-9.

Patterson K, Molloy L, Qu W, Clark S (2011). DNA methylation: bisulphite modification and analysis. J Vis Exp. 56). pii: 3170. doi: 10.3791/3170.

Paulsen M, Tierling, S., Walter, J (2008). DNA methylation in the mammalian genome. Trost Je, editor. Norfolk, UK: Caister Academic Press.

Pedersen KS, Bamlet WR, Oberg AL, de Andrade M, Matsumoto ME, Tang H, Thibodeau SN, Petersen GM, Wang L (2011). Leukocyte DNA methylation signature differentiates pancreatic cancer patients from healthy controls. PLoS One. 6(3):e18223.

Pembrey, M.E., Bygren, L.O., Kaati, G., Edvinsson, S., Northstone, K., Sjostrom, M., and Golding, J. (2006). Sex-specific, male-line transgenerational responses in humans. Eur J Hum Genet 14, 159-166.

Persani L, Gelmini G, Marelli F, Beck-Peccoz P, Bonomi M (2011). Syndromes of resistance to TSH. Ann Endocrinol (Paris). 72(2):60-3. Review.

Pradhan, S., Bacolla, A., Wells, R.D., and Roberts, R.J. (1999). Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation. J Biol Chem 274, 33002-33010.

Radenne A, Akpa M, Martel C, Sawadogo S, Mauvoisin D, Mounier C (2008). Hepatic regulation of fatty acid synthase by insulin and T3: evidence for T3 genomic and nongenomic actions. Am J Physiol Endocrinol Metab. 295(4):E884-94.

Radetti G, Fanolla A, Pappalardo L, Gottardi E (2007). Prematurity may be a risk factor for thyroid dysfunction in childhood. J Clin Endocrinol Metab. 92(1):155-9. Epub 2006 Oct 10.

Radetti G, Renzullo L, Gottardi E, D'Addato G, Messner H (2004). Altered thyroid and adrenal function in children born at term and preterm, small for gestational age. J Clin Endocrinol Metab. 89(12):6320-4.

Rastogi MV, LaFranchi SH (2010). Congenital hypothyroidism. Orphanet J Rare Dis. 10;5:17.

Ravelli, A.C., J.H. van der Meulen, R.P. Michels, C. Osmond, D.J. Barker, C.N. Hales, and O.P. Bleker (1998). Glucose tolerance in adults after prenatal exposure to famine. Lancet 351: 173-177.

Reddy PA, Rajagopal G, Harinarayan CV, Vanaja V, Rajasekhar D, Suresh V, Sachan A (2010). High prevalence of associated birth defects in congenital hypothyroidism. Int J Pediatr Endocrinol. 2010:940980.

Reik, W., and Dean, W. (2001). DNA methylation and mammalian epigenetics. Electrophoresis 22, 2838-2843.

Reik, W., Dean, W. and Walter, J (2009). Epigenetic reprogramming in mammalian development. Science, 293, 1089–1093.

Reik, W., Dean, W., and Walter, J. (2001). Epigenetic reprogramming in mammalian development. Science 293, 1089-1093.

Reynolds LP, Caton JS (2011). Role of the pre- and post-natal environment in developmental programming of health and productivity. Mol Cell Endocrinol.

Robins JC, Marsit CJ, Padbury JF, Sharma SS (2011). Endocrine disruptors, environmental oxygen, epigenetics and pregnancy. Front Biosci. 3:690-700. Review.

Rousseaux S, Caron C, Govin J, Lestrat C, Faure AK, Khochbin S (2005). Establishment of male-specific epigenetic information. Gene. 345(2):139-53.

Saitou M, Barton SC, Surani MA (2002). A molecular programme for the specification of germ cell fate in mice. Nature.418(6895):293-300.

Sakai, T., Toguchida, J., Ohtani, N., Yandell, D.W., Rapaport, J.M., and Dryja, T.P. (1991). Allele-specific hypermethylation of the retinoblastoma tumor-suppressor gene. Am J Hum Genet 48, 880-888.

Salvatore D (2011). Deiodinases: keeping the thyroid hormone supply in balance. J Endocrinol. 209(3):259-60.

Samavarchi-Tehrani P, Golipour A, David L, Sung HK, Beyer TA, Datti A, Woltjen K, Nagy A, Wrana JL (2010). Functional genomics reveals a BMP-driven mesenchymal-to-epithelial transition in the initiation of somatic cell reprogramming. Cell Stem Cell. 7(1):64-77. Epub 2010 Jun 17.

Sánchez-Solana B, Nueda ML, Ruvira MD, Ruiz-Hidalgo MJ, Monsalve EM, Rivero S, García-Ramírez JJ, Díaz-Guerra MJ, Baladrón V, Laborda J (2011). The EGF-like proteins DLK1 and DLK2 function as inhibitory non-canonical ligands of NOTCH1 receptor that modulate each other's activities. Biochim Biophys Acta. 1813(6):1153-64.

Sandovici, I., Smith, N.H., Ozanne, S.E., and Constancia, M. (2008). The dynamic epigenome: the impact of the environment on epigenetic regulation of gene expression and developmental programming. In Epigenetics, J. Tost, ed. (Norfolk, Caister Academic Press), pp. 343-370.

Santos F, Dean W (2004). Epigenetic reprogramming during early development in mammals. Reproduction. 127(6):643-51.

Santos, F., Hendrich, B., Reik, W. and Dean, W. (2002). Dynamic reprogramming of DNA methylation in the early mouse embryo. Dev. Biol., 241, 172–182.

Sarge, K.D., and Park-Sarge, O.K. (2005). Gene bookmarking: keeping the pages open. Trends Biochem Sci 30, 605-610.

Sebert S, Sharkey D, Budge H, Symonds ME (2011). The early programming of metabolic health: is epigenetic setting the missing link? Am J Clin Nutr. 94(6 Suppl):1953S-1958S.

Seckl JR, Meaney MJ (2006). Glucocorticoid "programming" and PTSD risk. Ann N Y Acad Sci. 1071:351-78. Review.

Seki Y, Williams L, Vuguin PM, Charron MJ (2012). Minireview: Epigenetic Programming of Diabetes and Obesity: Animal Models. Endocrinology. 2012 Jan 17.

Sengupta, A.K., Ohhata, T., and Wutz, A. (2008). X chromosome inactivation. In Epigenetics, J. Tost, ed. (Norfolk, Caister Academic Press), pp. 273-301.

Shafa, M., Krawetz, R., and Rancourt, D.E. (2010). Returning to the stem state: Epigenetics of recapitulating pre-differentiation chromatin structure. Bioessays.

Shapira SK, Lloyd-Puryear MA, Boyle C (2010). Future research directions to identify causes of the increasing incidence rate of congenital hypothyroidism in the United States. Pediatrics. 125 Suppl 2:S64-8.

Shen L, Kondo Y, Guo Y, Zhang J, Zhang L, Ahmed S, Shu J, Chen X, Waterland RA, Issa JP (2007). Genome-wide profiling of DNA methylation reveals a class of normally methylated CpG island promoters. PLoS Genet. 3(10):2023-36.

Shiota, K. (2004). DNA methylation profiles of CpG islands for cellular differentiation and development in mammals. Cytogenet Genome Res 105, 325-334.

Sierra F, Lichtler A, Marashi F, Rickles R, Van Dyke T, Clark T (1982). Organization of human histone genes. Proc Natl Acad Sci USA. 79:1795-9.

Simmons, R.A., L.J. Templeton, and S.J. Gertz (2001). Intrauterine growth retardation leads to the development of type 2 diabetes in the rat. Diabetes 50: 2279-2286.

Sims, J.K., Magazinnik, T., Houston, S.I., Wu, S., and Rice, J.C. (2008). Histone Modification and Epigenetics. In Epigenetics, J. Tost, ed. (Norfolk, Caister Academic Press), pp. 105-126.

Skordis N, Toumba M, Savva SC, Erakleous E, Topouzi M, Vogazianos M, Argyriou (2005). A. High prevalence of congenital hypothyroidism in the Greek Cypriot population: results of the neonatal screening program 1990-2000. J Pediatr Endocrinol Metab. 18(5):453-61.

Slattery ML, Curtin K, Wolff RK, Herrick JS, Caan BJ, Samowitz W (2010). Diet, physical activity, and body size associations with rectal tumor mutations and epigenetic changes. Cancer Causes Control. 21(8):1237-45.

Slotkin, R.K., and Martienssen, R. (2007). Transposable elements and the epigenetic regulation of the genome. Nat Rev Genet 8, 272-285.

Smith EY, Futtner CR, Chamberlain SJ, Johnstone KA, Resnick JL (2011). Transcription is required to establish maternal imprinting at the prader-willi syndrome and angelman syndrome locus. PLoS Genet. 7(12):e1002422.

Sprenkle PM, McGee J, Bertoni JM, Walsh EJ (2001). Consequences of hypothyroidism on auditory system function in Tshr mutant (hyt) mice. J Assoc Res Otolaryngol. 2(4):312-29.

Srinivasan R, Harigopal S, Turner S, Cheetham T (2011). Permanent and transient congenital hypothyroidism in preterm infants. Acta Paediatr. 1651-2227.

Stirzaker, C., Millar, D.S., Paul, C.L., Warnecke, P.M., Harrison, J., Vincent, P.C., Frommer, M., and Clark, S.J. (1997). Extensive DNA methylation spanning the Rb promoter in retinoblastoma tumors. Cancer Res 57, 2229-2237.

Surani, M.A. (2001). Reprogramming of genome function through epigenetic inheritance. Nature, 414, 122–128.

Szostak-Węgierek D, Szamotulska K (2011). Fetal development and risk of cardiovascular diseases and diabetes type 2 in adult life. Med Wieku Rozwoj. 15(3 Pt 1):203-15.

Tada, T., Obata, Y., Tada, M., Goto, Y., Nakatsuji, N., Tan, S., Kono, T., and Takagi, N. (2000). Imprint switching for non-random X-chromosome inactivation during mouse oocyte growth. Development 127, 3101-3105.

Takai, D., and Jones, P.A. (2002). Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci U S A 99, 3740-3745.

Tarım Ö (2011). Thyroid hormones and growth in health and disease. J Clin Res Pediatr Endocrinol. 3(2):51-5.

Tarry-Adkins JL, Ozanne SE (2011). Mechanisms of early life programming: current knowledge and future directions. Am J Clin Nutr. 94(6 Suppl):1765S-1771S.

Teitz, T., Wei, T., Valentine, M.B., Vanin, E.F., Grenet, J., Valentine, V.A., Behm, F.G., Look, A.T., Lahti, J.M., and Kidd, V.J. (2000). Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. Nat Med 6, 529-535.

Thornburg KL, Shannon J, Thuillier P, Turker MS (2010). In utero life and epigenetic predisposition for disease. Adv Genet. 71:57-78.

Thorwarth A, Mueller I, Biebermann H, Ropers HH, Grueters A, Krude H, Ullmann R (2010). Screening chromosomal aberrations by array comparative genomic hybridization in 80 patients with congenital hypothyroidism and thyroid dysgenesis. J Clin Endocrinol Metab. 95(7):3446-52.

Tobi, E.W., Lumey, L.H., Talens, R.P., Kremer, D., Putter, H., Stein, A.D., Slagboom, P.E., and Heijmans, B.T. (2009). DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. Hum Mol Genet 18, 4046-4053.

Tonacchera M, Banco M, Lapi P, Di Cosmo C, Perri A, Montanelli L, Moschini L, Gatti G, Gandini D, Massei A, Agretti P, De Marco G, Vitti P, Chiovato L, Pinchera A (2004). Genetic analysis of TTF-2 gene in children with congenital hypothyroidism and cleft palate, congenital hypothyroidism, or isolated cleft palate. Thyroid. 14(8):584-8.

Topaloglu AK (2006). Athyreosis, dysgenesis, and dyshormonogenesis in congenital hypothyroidism. Pediatr Endocrinol Rev. 3 Suppl 3:498-502.

Trueba SS, Augé J, Mattei G, Etchevers H, Martinovic J, Czernichow P, Vekemans M, Polak M, Attié-Bitach T (2005). PAX8, TITF1, and FOXE1 gene expression patterns during human development: new insights into human thyroid development and thyroid dysgenesis-associated malformations. J Clin Endocrinol Metab. 90(1):455-62.

Trueba SS, Augé J, Mattei G, Etchevers H, Martinovic J, Czernichow P, Vekemans M, Polak M, Attié-Bitach T (2005). PAX8, TITF1, and FOXE1 gene expression patterns during human development: new insights into human thyroid development and thyroid dysgenesis-associated malformations. J Clin Endocrinol Metab. 90(1):455-62.

Turkkahraman D, Alper OM, Aydin F, Yildiz A, Pehlivanoglu S, Luleci G, Akcurin S, Bircan I (2009). Final diagnosis in children with subclinical hypothyroidism and mutation analysis of the thyroid peroxidase gene (TPO). J Pediatr Endocrinol Metab. ;22(9):845-51.

Umstad MP, Gronow MJ (2003). Multiple pregnancy: a modern epidemic?. Med J Aust 178:613– 61.

van Driel, L.M., Smedts ,H.P., Helbing, W.A., Isaacs, A., Lindemans, J., Uitterlinden, A.G., van Duijn, C.M., de Vries, J.H., Steegers, E.A., and Steegers-Theunissen, R.P. (2008b). Eight-fold increased risk for congenital heart defects in children carrying the nicotinamide Nmethyltransferase polymorphism and exposed to medicines and low nicotinamide. Eur Heart J 29, 1424-1431.

Van Emburgh, B.O., and Robertson, K.D. (2008). DNA methyltransferases and methyl-CpG binding proteins as multifunctional regulators of chromatin structure and development in mammalian cells. In Epigenetics, J. Tost, ed. (Norfolk, Caister Academic Press), pp. 23-61.

Van Vliet G (2003). Development of the thyroid gland: lessons from congenitally hypothyroid mice and men. Clin Genet. 63(6):445-55.

Vassart G, Dumont JE (2005). Thyroid dysgenesis: multigenic or epigenetic ... or both? Endocrinology. 146(12):5035-7. Review.

Waddington CH (1942). The epigenotype. Endeavour. 1:18–20.

Wang D, Xia X, Liu Y, Oetting A, Walker RL, Zhu Y, Meltzer P, Cole PA, Shi YB, Yen PM (2009). Negative regulation of TSHalpha target gene by thyroid hormone involves histone acetylation and corepressor complex dissociation. Mol Endocrinol. 23(5):600-9.

Waterland RA (2009). Is epigenetics an important link between early life events and adult disease? Horm Res. 71 Suppl 1:13-6

Waterland, R.A., and Jirtle, R.L. (2003). Transposable elements: targets for early nutritional effects on epigenetic gene regulation. Mol Cell Biol 23, 5293-5300.

Watts, G.S., Futscher, B.W., Holtan, N., Degeest, K., Domann, F.E., and Rose, S.L. (2008). DNA methylation changes in ovarian cancer are cumulative with disease progression and identify tumor stage. BMC Med Genomics 1, 47.

Weaver, I.C., Cervoni, N., Champagne, F.A., D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M., and Meaney, M.J. (2004). Epigenetic programming by maternal behavior. Nat Neurosci 7, 847-854.

Weaver, I.C., Champagne, F.A., Brown, S.E., Dymov, S., Sharma, S., Meaney, M.J., and Szyf, M. (2005). Reversal of maternal programming of stress responses in adult offspring through methyl supplementation: altering epigenetic marking later in life. J Neurosci 25, 11045-11054.

Weaver, I.C., Meaney, M.J., and Szyf, M. (2006). Maternal care effects on the hippocampal transcriptome and anxiety-mediated behaviors in the offspring that are reversible in adulthood. Proc Natl Acad Sci U S A 103, 3480-3485.

Weber, M., Hellmann, I., Stadler, M.B., Ramos, L., Paabo, S., Rebhan, M., and Schubeler, D. (2007). Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet 39, 457-466.

Weber, M., Hellmann, I., Stadler, M.B., Ramos, L., Paabo, S., Rebhan, M., and Schubeler, D. (2007). Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet 39, 457-466.

Wolff, G.L., Kodell, R.L., Moore, S.R., and Cooney, C.A. (1998). Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice. FASEB J 12, 949- 957.

Wolff, G.L., Roberts, D.W., and Mountjoy, K.G. (1999). Physiological consequences of ectopic agouti gene expression: the yellow obese mouse syndrome. Physiol Genomics 1, 151-163.

Wong, N.C., Wong, L.H., Quach, J.M., Canham, P., Craig, J.M., Song, J.Z., Clark, S.J., and Choo, K.H. (2006). Permissive transcriptional activity at the centromere through pockets of DNA hypomethylation. PLoS Genet 2, e17.

Woroniecki R, Gaikwad AB, Susztak K (2011). Fetal environment, epigenetics, and pediatric renal disease. Pediatr Nephrol. 26(5):705-11.

Wu Ct, Morris JR (2001). Genes, genetics, and epigenetics: a correspondence. Science.293(5532):1103–1105.

Wutz, A., and Jaenisch, R. (2000). A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. Mol Cell 5, 695-705.

Yamada, Y., Watanabe, H., Miura, F., Soejima, H., Uchiyama, M., Iwasaka, T., Mukai, T., Sakaki, Y., and Ito, T. (2004). A comprehensive analysis of allelic methylation status of CpG islands on human chromosome 21q. Genome Res 14, 247-266.

Yassa L, Marqusee E, Fawcett R, Alexander EK (2010). Thyroid hormone early adjustment in pregnancy (the THERAPY) trial. J Clin Endocrinol Metab. 95(7):3234-41.

Yideng J, Jianzhong Z, Ying H, Juan S, Jinge Z, Shenglan W, Xiaoqun H, Shuren W (2007). Homocysteine-mediated expression of SAHH, DNMTs, MBD2, and DNA hypomethylation potential pathogenic mechanism in VSMCs. DNA Cell Biol. 26(8):603-11.

Yoder, J.A., Walsh, C.P., and Bestor, T.H. (1997). Cytosine methylation and the ecology of intragenomic parasites. Trends Genet 13, 335-340.

Zhang S, Rattanatray L, McMillen IC, Suter CM, Morrison JL (2011). Periconceptional nutrition and the early programming of a life of obesity or adversity. Prog Biophys Mol Biol. 106(1):307-14.

Zhao J, Goldberg J, Bremner JD, Vaccarino V (2012). Global DNA methylation is associated with insulin resistance: a monozygotic twin study. Diabetes. 61(2):542-6.

Zhao L, Funk CD (2004). Lipoxygenase pathways in atherogenesis. Trends Cardiovasc Med. 14(5):191-5. Review.

Zhao W, Soejima H, Higashimoto K, Nakagawachi T, Urano T, Kudo S, Matsukura S, Matsuo S, Joh K, Mukai T (2005). The essential role of histone H3 Lys9 di-methylation and MeCP2 binding in MGMT silencing with poor DNA methylation of the promoter CpG island. J Biochem. 137(3):431-40.

Zilberman, D., and Henikoff, S. (2007). Genome-wide analysis of DNA methylation patterns. Development 134, 3959-3965.

Zilberman, D., Gehring, M., Tran, R.K., Ballinger, T., and Henikoff, S. (2007). Genome-wide analysis of Arabidopsis thaliana DNA methylation uncovers an interdependence between methylation and transcription. Nat Genet 39, 61-69.

Zweidler A (1984). Core histone variants of the mouse: primary structure and differential expression. Wiley

Zwicker JI, Uhl L, Huang WY, Shaz BH, Bauer KA (2004). Thrombosis and ELISA optical density values in hospitalized patients with heparin-induced thrombocytopenia. J Thromb Haemost. 2(12):2133-7.