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Study on epigenetic modifications in human placenta and buccal mucosa cells of mothers and newborns

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1.1- The new world of epigenetics

In the early 1940s, Conrad Waddington first coined the term *epigenetics*. The Waddington's neologism means *outside conventional genetics* and integrates the theory of *epigenesis* that assumed the fundamental role of the genotype during development to give the phenotype (Waddington, 1940). Nowadays, the term epigenetics describes the field of study of heritable changes in gene expression, that arise during development and cell proliferation, independently from the DNA sequence itself. An exhaustive definition of epigenetics was reported by Feinberg: "*modifications of DNA or associated factors that have information content, other than the DNA sequence itself, are maintained during cell division, are influenced by the environment, and cause stable changes in gene expression*" (Feinberg, 2018). This modern definition of epigenetics takes its plasticity into account.

Undeniably, every single cell of an organism contains the same genetic information, but the expression of these genes is diversified in a variety of tissues. These different profiles are mediated by epigenetic mechanisms. Epigenetic changes occur in cell differentiation during development, even randomly in adult organisms, and mainly under the influence of environmental stimuli. This suggests the phenotype not to be a direct product of the program encoded in our DNA, but the result of this interaction (Issa *et al.*, 2000). Accordingly, the epigenome can be considered the way the genome acquires a memory of the various experiences resulting in a better adaptation of the individuals to the environment. In this perspective, the period of great importance for its plasticity and vulnerability, at the same time, is the ontogenesis. Perturbations, mediated by the placenta action, can occur and alter the fetal programming of early embryo establishing the basis for the inevitable manifestation of their effects in the postnatal life. The adaptative response at early exposure in the uterine microenvironment can also influence the next generations. The establishment of epigenetic marks in the gametes in response to external conditions sets the basis for transgenerational transmission of the new information that can mean a predisposition to a pathological phenotype.

During the last twenty-five years, scientists and epidemiologists have investigated the connection between this particular sensitivity of the developing organism to distress situations and physiological signals in response to them. Thus, the role of all the chemical and physical factors (e.g. endocrine disruptors and ionizing radiations), biological agents (e.g. viruses) and maternal experiences during gestation period (e.g. stress, depression or nutritional state) was showed to induce potentially adaptive

and predictive epigenetic changes that could be inherited from one generation to another (Burgio, 2015).
In this context takes seed the model of pathogenesis known as the *Developmental Origins of Health and Diseases* (DOHaD). In this lie our ongoing understanding of chronic-degenerative, inflammatory and neoplastic disease onset in response to a past – and very early - exposition. The above-mentioned pathologies showed an increased incidence in latter decades, that can be explained by DOHaD paradigm. Meanwhile, is emerging the necessity to establish a program for monitoring the maternal factors that can lead to a perturbation of intrauterine microenvironment. This would be the key for an early prevention program aiming to reduce the exposure to environmental cues and to protect the physiological programming from the first moment of conception to the whole lifetime: *from the womb to the tomb.*

1.2- The epigenetic mechanisms: an overview

Chromatin is not only the carrier of genetic information, but also of epigenetic ones, including DNA methylation and histone modifications. These mechanisms orchestrate dynamically gene regulation, to perform normal cellular functions and to respond to environmental and physiological changes. These are interacting systems, so that a perturbation at these levels can lead to an alteration resulting in epigenetic diseases linked to both germline and somatic lineage.
DNA methylation is a potent way of transcriptionally silencing genes through chemical modifications that occur directly on DNA sequence. This mechanism will be extensively explored in the next paragraph (see par.1.3).
Instead, at a post-translational level, a variety of modifications occur on tails of histone proteins, modulating chromatin structure. In the nucleus, chromatin exists in two different forms: euchromatin and heterocromatin. The heterochromatin is characterized by a closed structure due to the packaging of DNA into nucleosomes. In this conformation, DNA is poorly accessible by the enzymes that perform replication, transcription, and DNA repair. On the contrary, euchromatin is the open status favourable to the association of transcription machinery and to the subsequent gene expression (Biswas and Rao, 2018). An explosion of literature document the assortment of modifications that can occur within the N-terminal tails of histone proteins and their functions (Tan *et al*., 2011; Li *et al.,*2019; Collins *et al*. 2019). Phosphorylation, acetylation, ubiquitination, methylation, SUMOylation, and GlcNAcylation are the elements that allow to mediate the transition to one chromatin structure to another, events mediated by a wide range of enzyme activities (Wang *et al*., 2004).

Histone acetylation and methylation are the modifications better characterized.

Histone acetylation occurs on ε-amino group of lysine residues in H3 and H4 tails. The addition of the acetyl group and its removal are catalysed by histone acetyltransferase (HATs) and histone deacetylases (HDACs) enzymes respectively. Histone acetylation was the first modification discovered to be associated with activation of transcriptional status, with the cooperation of cofactors such as CREB binding protein (CBP), p300, MYST, and GNAT (Handy *et al*., 2011).

Thus, the addition of a methyl group occurs on the arginine and lysine residues, resulting in mono (me1), di (me2) and trimethylated (me3) states. Because of intricate nature of this system, these modifications can be associated with transcriptional activation or repression based on the location of the lysine residues. Histone methyl transferases enzymes (HMTs) promotes the addition of methyl group, and it is removed by demethylases (HDMs) (Biswas and Rao, 2018).

All these modifications may function, alone or together, as a part of a predictive histone code. Thus, different histone modifications can affect each other or can have interactions with DNA methylations as well (Reyk, 2007).

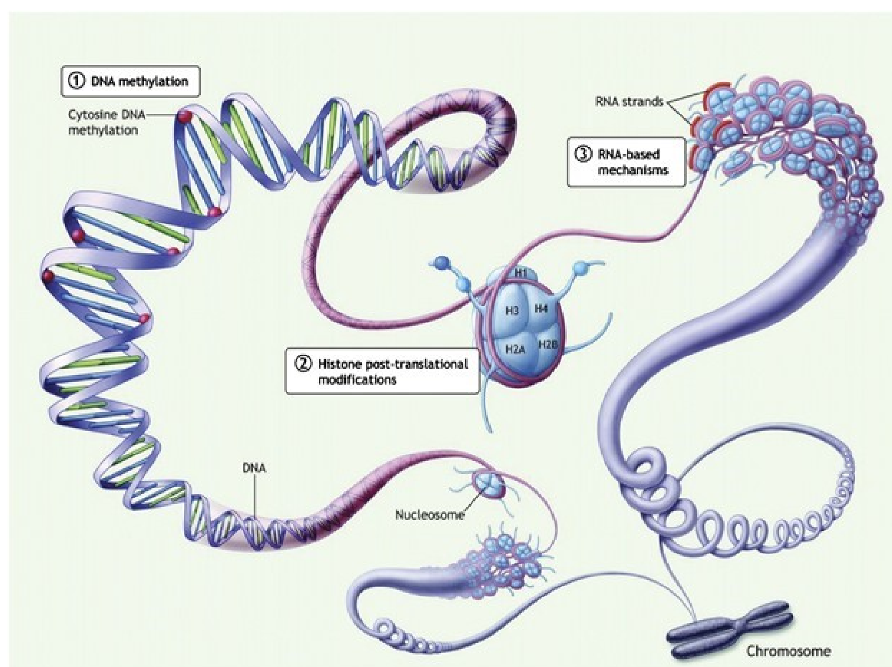


Figure 1 (from Lee *et al*., 2014): *A representation of epigenetic mechanisms.* 1) DNA methylation occurs directly on the DNA sequence at the level of CpG islands. Generally, methylation tags impede the access to DNA strands by transcription machinery. 2) Histone modifications occur at a post-translational level and consist in a variety of modifications on tails of histone proteins. These also reflect the variety of arrangements that can be found to coordinate the chromatin structure. 3) Non-coding RNAs including miRNAs and large non-coding RNAs (lncRNAs)

Among the epigenetic mechanisms, non-coding RNA (ncRNAs) is a regulation that has only been identified relatively recently and is an area of intensive ongoing investigation.
The first discovered ncRNA was *micF* from the bacteria *E. coli*. Masayuki Inouye's teams first characterized the mechanisms through which RNA realize regulation of gene expression by sense-antisense base pairing (Inouye and Delihast, 1988). In the '80s was discovered the phenomenon of genomic imprinting. In the following studies, aimed to investigate these dosage compensation mechanisms, the first eukaryote long non-coding RNA (lncRNA) gene emerged: *H19*. This is an imprinted gene maternally expressed that forms the *H19*/*IGF2* cluster. Inquiringly, *IGF2* paternally expresses protein Igf2 (Insulin Growth Factor 2); even though *H19* presented small open reading frames, it was found the absence of translation. However, the gene showed the features of classic mRNAs: it is transcribed by RNA polymerase II, spliced, 3′polyadenylated, and localized to the cytoplasm (Brannan *et al*., 1990). Since that time, *H19* revealed first to be lethal in prenatal stages, suggesting that the dosage of this lncRNA is tightly controlled and has an important role in embryonic development; then, in the more recent studies, the involvement of *H19* in tumorigenesis, proliferation, apoptosis is emerged, defining it as the prototype of a "multitasking" lncRNA (Jarroux *et al*., 2017; Dai *et al*., 2019).
Nowadays, different classes of ncRNAs have been characterized. These classes do not encode for functional proteins, but have a role in regulating gene expression to control cell differentiation and proliferation. They are characteristically divided in two categories, based on their size: short chain non-coding RNAs (including siRNAs, miRNAs, and piRNAs) and lncRNAs (Wei et al., 2017).
miRNAs are the most studied class of short ncRNAs: they are recognized as one of the main regulators of gene expression. The biogenesis consists of six main steps. Essentially, RNA polymerase II transcribes miRNA genes producing a primary transcript. It is processed first by the ribonuclease DROSHA/DGCR8 complex, and then by the ribonuclease DICER1. Functionally mature miRNAs, approximately 22 nucleotides long, are complexed together with AGO family proteins, the core of the RNAinduced silencing complex (RISC). The miRNA-RISC complex binds target mRNAs and mediates the translational repression or degradation of target (Hrovatin and Kunej, 2018).

1.3- DNA methylation

Among the various forms of epigenetic modifications, DNA methylation is the major epigenetic chemical modification that occurs directly on DNA sequence.

Cytosine methylation was first reported by Wyatt in 1951 (Wyatt, 1951), but it took twenty years to propose its involvement on regulatory maintenance of the 5mC pattern across cell divisions (Riggs, 1975; Chen *et al.,*2016)

The essentiality of mammalian DNA methylation in development or adult homeostasis is well defined (Messerschmidt *et al.,* 2014). DNA methylation is also essential for cellular differentiation, physiological conditions, X-chromosome inactivation (Riggs, 1975), gene imprinting (Henckel *et al.,* 2010), and repression of retrotransposons (Slotkin *et al.,* 2007).

Vertebrate genomes are methylated predominantly within CpG sites that are found throughout the genome in low density and generally in a hight methylated status (>85%) leading gene inactivation (Deichmann, 2016). A minority of CpG dinucleotides is clustered in GpG-rich regions called CpG islands (CGIs), located in the proximity of the transcription start sites (TSSs) of the majority (70%) of human protein-coding genes, that are largely unmethylated (<10%) (Jones and Takai, 2001; Bergman and Cedar, 2013).

The mechanism of methylation consists in the addition of a methyl group to the 5' carbon of a cytosine residue to form 5-methyl cytosine (5-mC). The family enzymes of DNA methyltransferases (DNMTs) play a key role in the transfer of the methyl group from S-adenyl methionine (SAM) to the cytosine ring during cell division (Moore *et al.,* 2013). DNMTs are trans-acting factors targeting DNA sites for methylation using cis-acting signals (Golbabapour *et al*., 2011). In mammals, DNA methylation is performed by DNMT1, DNMT3s (DNMT3A and 3B) and DNMT3L. In the last one lacks a catalytic domain but acts as a crucial cofactor of both DNMT3A and DNMT3B and it is essential also for establishing methylation imprints in the female germ line (Bourc'his *et al*., 2001; Jia *et al*., 2007). They act in S phase, the period that allows enzymes and accessory factors to be load into the newly synthesized DNA strand and to replicate all marks as found in the parental one.

The DNA (cytosine-5) methyltransferase 1 (DNMT1) has a high affinity for hemimethylated DNA substrates *in vitro* (Pradhan *et al*., 1999). His function is to copy the parental methylation pattern on the newly synthesized strand right after the replication. For these reasons, DNMT1 is also called maintenance DNA methyltransferase.

A dysregulation of DNMT1 expression leads to an alteration of embryo phenotype, as was shown in different functional studies (Stancheva & Meehan, 2000; Stancheva *et al.,* 2001; Biniszkiewicz *et al.* 2002). DNA Methyltransferases 3a and 3b are the main engines of *de novo* methylation pattern to unmodified DNA substrates. Expression studies in human and murine models show that *DNMT3A* and *DNMT3B* are highly expressed around the blastocyst stage and during germ cells development, time windows of development in which new DNA methylation patterns are being laid genome-wide (*see paragraph 1.4*) (Li, 2002). However, all three DNMTs are extensively involved in embryo

development until the ultimate cell differentiations are reached and the DNA methylation pattern in postmitotic cells becomes stable (Moore *et al.,* 2013).

However, covalent modifications on DNA are not permanent. These marks can be removed depending on the requirement of the cell, and it occurs through two different processes.

In a first moment, it was discovered that the absence of complex formed by DNMT1 and its cofactor UHRF1 (a key regulator that participates in both DNA methylation and histone modifications) enhances a progressive loss of methylated cytosines pattern following successive DNA replications, defined as passive demethylation. At later time, the second mechanism was shown, subsequentially to the discovery of ten-eleven translocation (TET) family of proteins. The three members of the TET family (TET1, TET2 and TET3) have been identified, which oxidize first 5mC to 5-hydroxymethylcytosine (5hmC), and then produce 5- formylcytosine (5fC) and 5-carboxylcytosine (5caC) in further oxidation steps (Biswas and Rao, 2018).

All the members of TET family are involved in different processes. TET1, TET2 and TET3 remove imprinting marks and paternal DNA methylation marks in the zygote, during the fetal development period (*see paragraph 1.4*). Furtherly, the TET2 role in the modulation of DNA methylation was also detected during haematopoiesis (Solary *et al*., 2014).

As already mentioned, all the epigenetic mechanisms are essential to achieve a correct cellular response. The family of methyl-CpG-binding domain (MBD) proteins coordinate crosstalk between DNA methylation, histone modifications and chromatin organization to orchestrate a coherent transcriptional program. DNA are "read" by these specific translator proteins, that bind methylated CpG dinucleotides, and produce heterochromatic silencing through interactions with histone methylation and chromatin remodelling binding cofactors. MeCP2 and MBD1 are the two main MBD proteins involved in these processes (Du *et al*., 2015).

1.4- Epigenetic events during embryos development: windows of vulnerability

In mammals, gametogenesis and embryo-fetal development are the two major reprogramming periods. Early embryo development can be divided into three steps, preimplantation embryo, organogenesis and fetal growth, and postnatal maturation (fig. 2). These are the windows of maximal epigenetic plasticity, when the epigenetic programming takes place.

Early embryo experiences an erasure of epigenome among the first cellular division of the zygote.

Thus, in preimplantation embryo, all methylation marks are removed as a prelude to resetting totipotency and to subsequently specifying the inner cell mass (ICM) and trophectoderm fates (Monk *et al*., 1987).

The progression of preimplantation development is characterized exclusively by the genome-wide demethylation processes. It starts 10–12 hours after fertilization and continues from the late zygote stage to the four-cell stage. At this point, the genome demethylation is almost complete and continues from the eight-cell stage to the blastocyst stage, when it reaches its lowest level (Zhu *et al*., 2018).

It was shown that during the interval between these stages, two massive events of new methylation occur. The phenomena especially regard particular elements, such as the Alu and LINE1 retroelements, which are transiently active during preimplantation development. The local gain of methylation acts as protection from their transcriptional activity that could affect genome stability. Global DNA methylation reprogramming can be considered a dynamic balance between strong genome-wide demethylation and local re-methylation (Wang *et al*., 2019).

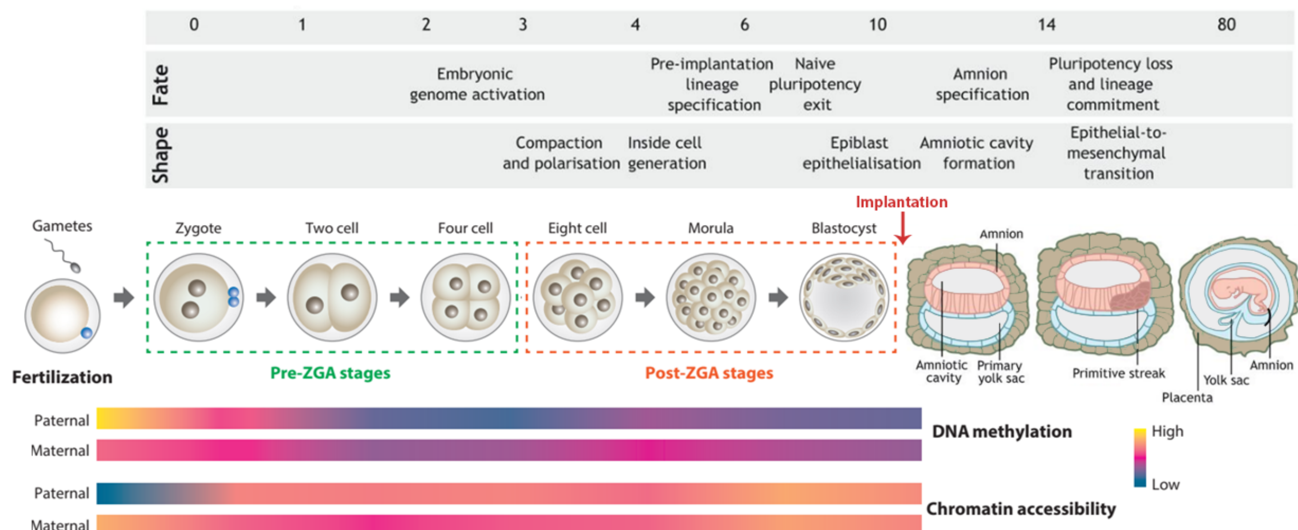
Demethylation starts in the zygote at the level of a specific sequence in the paternal nucleus, followed by genome-wide demethylation due to active DNA-repair processes and passive loss of methylation (Mayer *et al.*, 2000). Paternally imprinted genes, heterochromatin around centromeres and some repetitive element are preserved from demethylation events. On the contrary, the CpG island-like of *Oct3/4* and *Nanog* genes, that are methylated in sperm DNA, are targets for demethylation before implantation: this makes it the key stage for reprogramming the genome in a pluripotent state (Farthing *et al*., 2008).

In fully differentiated gametes, sperm genome methylation is much greater than the oocyte one (82% vs 55%, median values). For these reasons, the paternal genome is faster demethylated to make it accessible like the maternal one. Indeed, after the two-cell stage, paternal genome results less methylated with respect to maternal genome, but the meaning of this parent-specific methylation remains unclear (Guo *et al*., 2014).

At implantation stage, after five cell cycles, a new epigenetic pattern is re-established, and the genome undergoes a *de novo* methylation due to the upregulation of the methylases enzymes DNMT3A, DNMT3B, and DNMT1 (Okano *et al.,* 1999) (fig.2). After this stage, the methylation level is higher in the ICM that gives rise to somatic tissues than in the trophoectoderm, which forms the future placenta (Chen and Zhang, 2011).

Embryo-fetal development represents the phase of life most vulnerable to information from the environment: specifically, the extreme plasticity of the genome increases the sensitivity to maternal-fetal stress, nutritional errors and pollutants. The resulting bimodal pattern is conserved throughout development and aging of the organism unless unexpected alterations (Greenfield *et al*., 2018).

Figure 2 (modified from Shahbazi *et al*., 2020 and Whang *et al*., 2019): *Human developmental time.* After fertilization, a wide erasure of methylation marks of parental genomes occurs. At 4-cell stage, genome wide demethylation is completed, and chromatin reaches its most accessible level in a blastocyst stage. In humans, zygotic genome activation (ZGA) takes place from approximately the 4-cell stage to the 8-cell stage. In pre-ZGA embryos, unique broad open chromatin is highly enriched at oocyte partially methylated domains.



- Germ line reprogramming

DNA methylation programming in the germline is of great importance, as methylation marks established in the gametes have the power to regulate gene expression up to the next generation.

At the perigastrulation embryo stage, approximately at embryonic weeks 2-3, occurs the specification of primordial germ cell (PGC), precursors of oocytes and sperm. During weeks 4th, PGCs start their migration from yolk sac wall to the colonization of the developing genital ridge, where rapidly proliferate and proceed their differentiation in a sex-specific manner. Mature parental gametes are programmed differently, because they are functionally non-equivalent (McGrath *et al*., 1984).

After week 9th of gestation, germ cell sexual differentiation produces oogonia and prospermatogonia in female and male gonads, respectively. In humans, before embryonic week 4-7, the first wave of global DNA demethylation in PGCs occurs (von Meyenn and Reik, 2015), and they reach the lowest point, with 6-7% (median level, this is the lowest levels of CpG methylation observed in the human genome to date) of residual methylation approximately at 10–12 week of gestation. This means an extensive erasure of the parental DNA methylation memory.

The demethylated status of proliferating PCG is preserved by the inactivation of methylation maintenance enzymes (e.g., UHRF1) and of *de novo* methylation enzymes (DNMT3A and

DNMT3B), until the embryonic weeks 16th or 19th, in female and male respectively. At this time, the global reestablishment of DNA methylation occurs (White *et al*., 2016).
Tahiliani and colleagues first identified the pivotal role of the TET1 in removing the methylation marks on DNA (Tahiliani *et al*., 2009). TET1 and TET2 convert 5 methylcytosine (5mC) into 5 hydroxymethylcytosine (5hmC), promoting an active demethylation process. They contribute to the global DNA demethylation, especially for the imprinting regions in early PGC development (Hargan-Calvopina *et al*., 2016).
Intriguingly, determined hot spot of transgenerational epigenetic inheritance (such as CpG islands and promoters or gene bodies regions) can escape from this massive global erasure. Among them, several genes were identified with characteristic traits and disease associations, such as obesity-related traits, schizophrenia and multiple sclerosis (Tang *et al*., 2015).

1.5- Physiological responses to environmental stimuli

All the epigenetic marks are involved in the normal regulation of the genome: they are crucial for distinct cell lineages differentiation, during development and in the adult organism. These processes are deeply influenced by the environmental cues - in terms of both exogenous and endogenous influences - that can contribute to an alteration of a phenotype.
The question is: what are the molecular mechanisms leading to specific epigenetic rearrangements that may be involved in DOHaD?
Physiologically speaking, epigenetic responses to metabolism are important for nutrient sensing and environmental adaptation. Accordingly, linking nutrient availability and epigenetic mechanisms is quite intuitive. The pivotal enzymes involved in epigenetic modifications are interrelated with energy metabolism and nutrition, as the major biochemical pathways participate in chromatin remodelling and transcriptional regulation (Lempradl *et al*., 2015). For example, lysine acetylation reactions, promoted by HATs, have as substrate acetyl-Co provided by the tricarboxylic acid (TCA) cycle, β-oxidation and metabolism of aminoacids (Lempradl *et al.,* 2015). In the same way, SAM recycling reactions from S-adenosylhomocysteine (SAH) are mediated by many nutritional factors, such as group-B vitamins (B9, B6 and B12), zinc, choline and methionine. Both histone modification and DNA methylation require SAM as a methyl donor substrate, derived from the one-carbon metabolism cycle. The removal of methylation marks – reactions catalysed by ten-eleven translocation methylcytosine dioxygenase enzyme (TET) – is promoted by Fe (II) and α-ketoglutarate and is inhibited by TCA cycle intermediates (Tahiliani *et al*., 2009; Xiao *et al*., 2012).

Classic signalling, such as the membrane or nuclear receptors (NR), is also affected by the environment and dietary compounds.
NR signalling mediates many physiological processes, such as metabolism, reproduction, inflammation, as well as the circadian rhythm (Weikum *et al*., 2018). Vitamin D, dietary lipids or endocrine disruptors can bind NR mediating specific responses (Safi-Stibler and Gabory, 2020). NR is a family of ligand-activated transcription factors that regulate downstream target genes. Dimerization of NR with ligand molecules can induce the activation of the NRREs (NR-responsive elements) or influence the binding with its cofactors, that can provide transactivation or trans-repression activities. Among all the molecules that act as cofactors, HATs, HDACs, KMTs, KDMs and chromatin remodelling complexes are included. Androgen receptor activation, for example, triggers KDM3A in the same way as ligand binding estrogen receptor (ERα) directs the recruitment of several chromatin modifiers to its target genes (Safi-Stibler and Gabory, 2020).

- Physiological response to stressors

The hypothalamic-pituitary-adrenal (HPA) axis is the central coordinator of neuroendocrine response systems to stressors, both psychological (e.g., anxiety and abuse) and physiological (e.g., hypoxia). Perturbation of physiological homeostasis is restored by primary response mediated by secretion of glucocorticoids. These hormones are directly linked to many metabolic pathways such as glycogenolysis, immune response, and memory (Finsterwald and Alberini, 2014).
Cascade of events leading to release of glucocorticoids (cortisol in humans) starts in the paraventricular nucleus (PVN) of the hypothalamus, with the activation of corticotrophin-releasing hormone (CRH) neurons and the subsequential release of CRH into the hypophyseal portal circulation. These events result in the synthesis, and then, in the release in the peripheral circulation of adrenocorticotrophin (ACTH) from anterior pituitary corticotrophs. ACTH promotes the synthesis and the release of glucocorticoids in the adrenal zona glomerulosa (Matthews and McGowan, 2018).
In the DOHaD context, many studies on both animal models and humans reported that maternal stress or depression, maternal obesity, maternal glucocorticoid exposure, and parental care can affect newborns outcomes (this topic will be discussed in detail in the following paragraphs).
An example is the link between tactile stimulation by the mothers and the serotonin (5-HT) secretion in the hippocampus of the pups. Maternal care promoting the binding of 5-HT on its G protein-coupled receptors (GPCR) activates a signalling cascade that drives the complex (formed by transcription factors and the histone acetyltransferase) on the glucocorticoid receptor (GR) gene promoter promoting its activation (Hellstrom *et al*., 2012). It therefore emerges the role of the DNA

methylation on the regulation of GR or estrogen receptor (ER) transcription levels in response to stimuli, from parental care up to chemical exposure that could be encountered during embryo-fetal development or early postnatal stage (Mirbahai and Chipman, 2014).

1.6- The importance of placental health

Placenta is a transitory unique organ that guarantees fetal health and survival *in utero*. It emerges from blastocyst trophectoderm, after its implantation into the maternal endometrium and is the result of a mixture of maternal (decidua) and fetal (chorion) cells. Placenta is the vascular interface between mother and developing conceptus. Placental villous tree represents the transport unit of the organ. Transfer of substances between the intervillous space and fetal capillaries takes place across a multilayered structure called placental barrier, which is composed of trophoblasts, connective tissue, basal lamina, and the fetal endothelium. Extravillous trophoblast cells migrate from the placenta into the uterine wall, in which they interact with cells of the maternal immune system. These interactions have a physiological, rather than a classical immunological, outcome and probably mediate remodelling of the uterine spiral arteries that supply the placenta (Burton and Jauniaux, 2015).
Oxygen, nutrients and hormones are delivered to the fetus by the placenta, while it removes waste products. Moreover, it has a wide range of basic roles including the immune protection from the mother response, nourishment and support of the developing embryo. This occurs through complex signalling networks that include cytokines, growth factors and hormones. Nevertheless, placenta also represents the vehicle of maternal stress, dysregulated pathways signalling, or environmental toxicants that can reach the womb (Aplin *et al*., 2020).
Accordingly, the placenta is the crucial organ for a successful pregnancy, and alterations in its biomolecular functions may contribute to both adverse birth and later-life health outcomes.
Both genetic and epigenetic profiles of human placental genome have been meticulously investigated, revealing their unique nature.
As it has been previously described (*see paragraph 1.4*), the zygote experiences wide methylation of the genome. Thus, at the blastocyst stage, it results almost hypomethylated (except for imprinted epigenetic marks). Following lineages differentiation, ICM lineage acquires *de novo* DNA methylation pattern, while hypomethylation is a feature of extraembryonic tissue (derived from trophoblast). Accordingly, chorionic villi show a general hypomethylation compared to somatic tissues at birth – as assessed by total 5-methylcytosine content – reflecting its derivation and their faculty to invade and remodel the maternal decidua. This is an epigenomic peculiarity of the *pseudomalignant* nature of placentation (Novakovic1 and Richard Saffery, 2013). Furthermore,

placenta undergoes many changes throughout gestation and several studies have examined genome wide expression differences comparing at first, second and third trimester of placental methylation (Robinson and Price, 2015).

The downregulation of *DNMT1* gene in placenta has been reported. The absence of the maintenance DNMT1 enzyme may contribute to placental DNA hypomethylation. Intriguingly, *DNMT1* gene has been found to show placental-specific imprinting, with maternal allele-specific methylation and paternal expression. This highlights the peculiar placental expression of the most imprinted genes, with spatial and temporal specific patterns relative to somatic tissues (Monk, 2015).

Monoallelic imprinted genes expression creates an equilibrium between giving and taking resources, depending on paternal or maternal imprints respectively. This balance is known as a parental *tug of war*, that guarantee an appropriate fetal growth and a normal placental function. Therefore, aberrations in genomic imprinting have been linked to several human diseases, adverse pregnancy outcomes and gestational exposures to environmental factors (Monk *et al*., 2019).

Almost 50 human genes have been recently identified as imprinted genes, the large majority of which has been characterised in placental tissue and related to birth outcomes (Vincenz *et al*., 2020; Pilvar *et al*., 2019). *H19*, imprinted gene implicated in the Silver-Russel syndrome, is hypomethylated (loss of methylation) in the placenta of growth-restricted infants. On the other hand, gain in methylation at *H19* DMR has been associated with syndromes of fetal overgrowth, such as Beckwith-Wiedemann syndrome (BWS) (Ounap, 2016; Yamaguchi *et al*., 2019).

H19 hypomethylation and its higher expression was found to relate with intrauterine growth restriction (IURG); *IGF2-DMR2* hypomethylation with decreased expression was detected in the same condition, also associated with small for gestational age (SGA, smaller than the 10th percentile) infants (Koukoura *et al*., 2011; O'Callaghan *et al*., 2020).

Moreover, a comprehensive placental profiling of imprinted genes in a large birth cohort – 677 term human pregnancies – found 2-fold increased expression of 9 imprinted genes (*BLCAP*, *DLK1*, *H19*, *IGF2*, *MEG3*, *MEST*, *NNAT*, *NDN*, and *PLAGL1*) in placenta to be positively correlated with large for gestational age (LGA, larger than the 90th percentile) status of fetus (Kappil *et al*., 2015).

Global DNA methylation level can also reflect the fetal size for gestational age in placenta. Several studies (most of them measured LINE-1 methylation as a surrogate for global DNA methylation) didn't find differences between SGA and normal controls (Bourque *et al*., 2010; Tzschoppe *et al*., 2013; Mukhopadhyay *et al*., 2016), but different global placental DNA methylation levels in LGA were reported by Dwi Putra and co-workers (2020). They have investigated global DNA methylation in a very large cohort of 1023 mothers revealing a higher level of global placental DNA methylation

compared to SGA births and controls with any association between pre-pregnancy BMI or maternal diabetes (Dwi Putra *et al*., 2020).
However, common birth complications result in abnormal fetal growth. Both SGA and LGA births are related to maternal disease such as hypertension, preeclampsia (PE), and preexisting or gestational diabetes mellitus (GDM) (Januar *et al*., 2015).
Interestingly, a monoallelic expression was reported also in absence of imprinting. For example, it occurs in leptin gene (*LEP*), that shows a variable degree of nonimprinted monoallelic DNA methylation. *LEP* hypomethylation has been observed in pregnancy complicated by early onset pre-eclampsia (EOPE) associated with more biallelic *LEP* expression rather than skewed allelic expression of the control placentae (Hogg *et al*., 2013). Instead, a 535 mother-infant cohort study investigation has revelated higher methylation of placental *LEP* promoter in GDM compared to non-GDM (Lesseur *et al*., 2014).
These findings suggest that the growth-related genes deregulation may contribute to metabolic programming of obesity and linked conditions. Additionally, altered methylation status in the placental genome was detected in pregnancies complicated by abnormal infant's growth, in particular in multiple genes involved in inflammatory (e. g., *IL10*, *CD28*) or cardiovascular (e.g., *ACE*, *NO53*, *CASZI*) pathways, as well as preterm birth (O'Callaghan *et al*.,2020).
Thus, placenta can be considered as the "diary" of the pregnancy and, as easily available tissue at birth, placental tissue has been and will continue to be used as non-invasive tracking investigated from several birth cohort studies.

1.7- The Developmental Origins of Health and Disease

In the paragraphs above, the events that occurred after fertilization were described. During these events, specific epigenetic patterns are established in embryo-fetal developing cells shaped on the information derived from the mother and, through it, from the outside world.
An enormous variety of stimuli or insults could define a specific uterine microenvironment (through placenta mediation) and affect first the cellular differentiation processes, and then tissues and systemic programming. Early exposure leads to permanent effects on structure, physiology and metabolism of the organisms potentially causing a profound impact on public health.
The *Developmental Origins of Health and Disease* (DOHaD) hypothesis has been developed from the notion of foetal programming, initially proposed by David Barker in the 1990s, and could represents the strategy for the prevention of major illnesses.

Barker was an English epidemiologist, and his work on the *fetal origins of the disease* is recognized as a milestone in this field of research, modified in 2003 to the *developmental origins of health and disease* to better reflect both the gestational and postnatal periods. In his works, Barker first examined a retrospective cohort of men born between the 1911 and 1930, on which had been good preserved measurements of size at birth and growth in the first years of life. Consequentially, he realised that the deaths from the cardiovascular or metabolic syndrome in adults were common in men who were small at birth and at 1 year, highlighting the relation between the neonatal death and the geographical distribution of ischemic heart disease in adult survivors (Barker *et al.*, 1993, 1989; Barker and Osmond, 1986). He also investigated the abdominal fatness in adult men as indicator of increased risk of cardiovascular disease and diabetes independently of body mass in association with retarded fetal growth, revealing a persisting response to adverse conditions in fetal life (Law et al., 1992).
Thus, Barker pioneered the idea that the 20th century epidemic of coronary heart disease in the United Kingdom might have originated in fetal life – directly influenced by the nutritional, hormonal and metabolic environment provided by the mother – rather than to adult standard of living (Barker, 2007).
Following studies conducted on different animal models and observational human studies confirmed the Barker's insight and elucidated the sensitive physiological mechanisms through which the early stimuli are translated and their memory are established, such as through the epigenetic processes (Ledo Husby Phillips and Roth, 2019).
Moreover, a growing range of pathologies have been linked to developmental origins, in particular a wide range of non-communicable diseases (NCD), such as cardiovascular disease, diabetes, chronic lung disease, the ageing-related diseases and the cognitive decline. More recently mental illnesses including depression, anxiety or newborns' neurobehavior became a focus of this research field (Cao-Lei *et al*., 2016). At the same time, the investigations also concerned the factors that can insult first the developing life in the womb – mediated by the placenta action – and then that can be strongly related to early life outcomes as well in the lifespan. For example, the development of functional organs is influenced by the quantity and the quality of food intake during the gestational period. Thus, nourishment deficiency can lead to IURG that are associated with a higher risk of type 2 diabetes and coronary disease among the offspring. However, UGR-related adult morbidities also include a high risk of neurodevelopmental disorders due, for example, to the low supply of some micronutrients (e.g. Fe). On the other hand, maternal obesity can also be a vehicle for neurocognitive and behavioral outcomes in the offspring. An obesity-related potential cause of this impairment could be the mother's increased susceptibility to severe infections – due to the already altered maternal immune and inflammatory status associated to adiposity – and the drugs used for the treatments of infections

(Godfrey et al., 2016). Impaired fetal development has also been largely associated with exposure to environmental toxic substances, such as smoke, pollutants or bisphenol A (BPA) that could insult developmental fetus at different levels, from the growth to brain development *(all these factors will be explored in the next paragraphs*).
Thus, parental lifestyle, nutrition, endocrine disruptors, environmental toxicants are all modulators in the early life programming.
These signals are mediated by epigenome response and subsequently are reflecting the fetal transcription profiles (Mirbahai and Chipman, 2014).

1.7.1- The Nutritional Programming

Barker's work revealed that nutritional exposure during early life is particularly important, as the plasticity of developing organs that shape the way in which the body will respond to the future challenges.
Several famine and longitudinal cohort studies followed, revealing that poor nutrition and/or growth during the first 1000 days of life – from the conception to the 2nd year of life – are important risk factors that contribute to many chronic diseases later in life.
A seminal contribution to this research field was given by the Dutch famine cohort, which included men and women born before, during, and after the Nazi-imposed famine of 1944–1945. This period is known as the Hunger Winter, during which the daily caloric intake was less than 25% of the normal. However, extra rations were provided to pregnant and lactating women, although they were not sufficient and did not help to avoid severe dietary restriction. The Dutch famine study suggests the impact of starvation on the onset of several pathologies, also concerning the timing of the exposure during the pregnancy period. Indeed, the offspring that suffered an early gestational exposure was inclined to develop coronary heart disease, hyperlipidemia, and obesity, while obstructive airways disease and microalbuminuria were associated with a mid-gestation exposure (Ravelli *et al*., 1998; Roseboom *et al*., 2001).
Schizophrenia and spina bifida at birth were phenotypic outcomes also associated to famine exposure in humans (Susser et al., 1996; Brown et al.,1997). A diet lacking micronutrients such as folate and vitamin B12 led to downregulation genes such as *BDNF*, *CREB*, *NGF*, and TrkB, essential for offspring normal brain development and functions. Indeed, nowadays folic acid supplementation prior to conception and during pregnancy is a validated recommendation to significantly reduce the risk of neural tube defects. However, despite the indisputable advantages of this diet supplementation, studies on animal models showed unexpected effects on dams following in utero exposure to maternal

methyl donors diet supplementation: increased susceptibility to allergic airway disease, resulting from high methylation of the runt-related transcription factor 3 (*Runx3*) (Hollingsworth *et al*., 2008).

Moreover, not only the time-windows of development are fundamental to define the intensity of exposure outcomes, but other factors including type of nutrient, species and sex are important in the establishment of the log-term consequences of a nutritional dysregulation (Tobi *et al*., 2009; Hsu and Tain, 2019).

Impaired DNA methylation pattern was observed in the key genes of glucose metabolism as well as in fetal and early growth. Demethylation of the *IGF2* gene was found in 50 years old adults of the Dutch famine cohort related to the fetal protein-energy restrictive environment. On the other hand, higher methylation levels of *LEP*, as well for *ABCA1* and *GNAS* genes were observed (De Luca, 2017).

However, complications afflicting the placenta functions, such as preeclampsia or hypertension (*see paragraph 1.6*), do not guarantee nutrients and hormones correct influx. In response to intrauterine undernutrition, the fetus readjusts its energy demand by reducing plasma concentrations of insulin and insulin-like growth factor 1 (Igf-1), two promoters of fetal growth. Further adaptation processes are activated, including a redistribution of blood flow in order to favour the use of the few nutrients available to critical organs, such as the fetal brain or heart, in order to protect them. Consequently, the development and functionality of the remaining organs are disadvantaged (Salam *et al*., 2014).

However, also obesity during pregnancy and increased gestational weight gain (GWG) has been linked to obstetric pathology and long life offspring outcomes (Oken, 2009; Poston et al., 2011), as well as SGA or LGA infants have an increased risk of obesity and other chronic diseases.

Altered methylation at multiple imprint regulatory regions was found in children born to obese parents, compared with children born to non-obese parents, supposing this imprint instability may be passed to the next generation, increasing the risk for chronic diseases in adulthood (Soubry *et al*.,2015)

Lesseur and colleagues (2013) also defined the *LEP* gene promoter patterns in couples mother newbons and placenta. They have observed that lower blood methylation was associated with pre-pregnancy obesity; while in cord blood *LEP* methylation was negatively associated with pre-pregnancy obesity but was positively related whit excessive maternal GWG and pregnancy smoking. In placental tissue, *LEP* methylation was associated with infant gender (Lesseur *et al*., 2013). The same group has also demonstrated that the methylation of the *LEP* gene was negatively correlated with gene expression in placentas from male infants, associated with an increased risk of neurobehavioral responses characterized as lethargic with poor arousal and non-optimal reflexive response (Lesseur *et al*., 2014). Indeed, a growing body of literature reported a poorer cognitive

outcome in offspring related to an excessive pre-pregnancy weight as well as behavioural and emotional problems, including autism or schizophrenia (Godfrey *et al*., 2016).

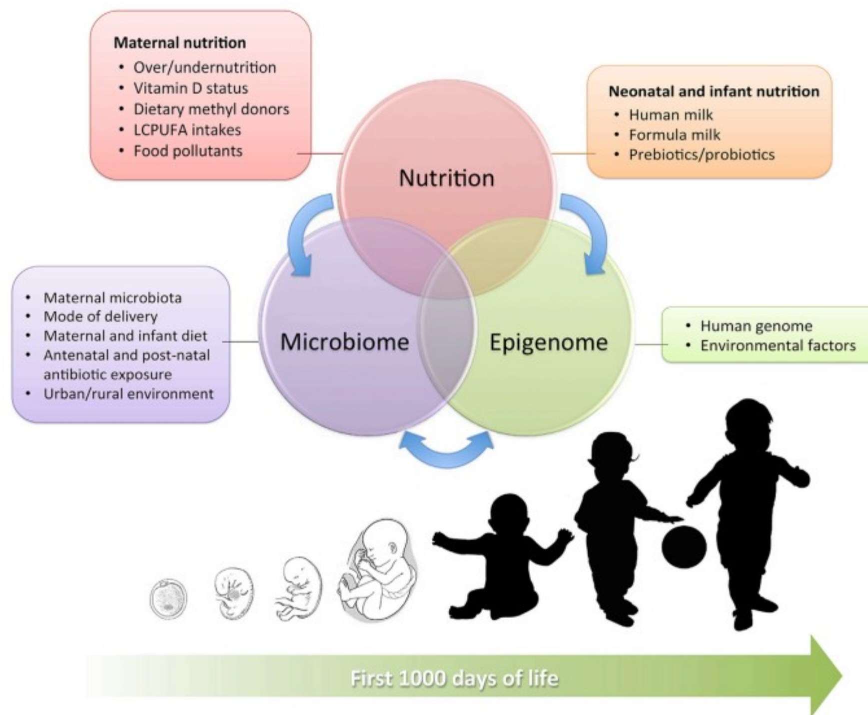


Figure 3: *Interrelation between maternal and neonatal factors*. Nutrition, gut microbiota, and epigenetics during the first 1,000 days of life. The main influencing factors are detailed in the boxes. From Indrio *et al.,* 2017.

In contrast to the traditional "sterile womb" idea, it was shown that also maternal diet could impact the early establishment of the fetal and neonatal microbiome (through breastfeeding), promoting specific epigenetic signatures that may predispose to the onset of late-life obesity. These findings support the concept that microbiota exists in the placenta, umbilical cord blood, amniotic fluid and meconium in healthy pregnancy supporting the *in utero* colonization hypothesis (Li, 2018).
In conclusion, maternal starvation or overnutrition, as well as diabetes and other metabolic conditions, have been shown to insult offspring's epigenome also in periconceptional environment, including the features of the father (fig. 3).

1.7.2- The environmental stressors

The World Health Organization (WHO) estimated that 93% circa of the children in the world breathe polluted air daily, causing an increasing number of deaths from acute respiratory infections. Environmental risk factors, such as air, water and soil pollution, chemical exposures or radiation are

the leading causes of the onset of more than 100 diseases and injuries. Thus, these expositions are being the focus of epidemiological studies principally in early life period, in which metabolic and physiological changes in the fetus have been detected through altered epigenetic profiles (Li *et al.*, 2019).

Methylation changes of genes related to oxidative stress, immunity and inflammatory responses have been suggested to be an important mechanism in response to air pollution exposure that lead to adverse pregnancy outcomes.

Despite the transport of particles across the human placenta is still debated because of the rather limited evidence (often limited to *in vitro* cell cultures, *ex vivo* and animal models), their harmful power on placental function and fetal growth is evident (Wick *et al.*, 2009; Grafmuller *et al.,* 2013; Bové *et al.*, 2019; Mi Yung *et al.*, 2020).

Particular matter (PM) <2.5 µm exposure result in a decrease of global DNA methylation in placenta tissue, as well as in placental mitochondria numerosity and mtDNA methylation (Jansen *et al.*,2012). Birth weight and gestational age result compromised as well as cognitive development and behaviour also in a sex specific manner (Cowell *et al.*, 2015; Li *et al.*, 2017). Male birth weight was most susceptible to these kinds of exposures particularly among those with obese mothers, as well as neurodevelopmental outcomes with preschool aged females reduced memory ability of preschool aged females (Lakshmanan *et al.*, 2015; Chiu *et al.*, 2016)

A perturbation of epigenetic pattern has been found in pregnant women living close to high-traffic roads, which showed lower levels of placental DNA methylation in LINE-1 but not in AluYb8 (Kingsley *et al.*, 2016). Exposure during the first trimester to PM2.5 fine inhalable particles was also found to be inversely associated with placental global methylation, as well as with PM10 exposure (Janssen *et al.,* 2013; Cai *et al.*, 2017). PM2.5 and PM10 exposures during the first two trimesters of pregnancy can influence gene specific methylation of *LEP*, *NR3C1* and *HSD11B2* placental promoters, and the differences in methylation are much more evident in the fetal growth-restricted newborn (Saenen *et al.*, 2019).

Moreover, important is the exposure to a wide range of heavy metals, that can cross the placenta barrier (as PM) and reach the fetus. Arsenic, cadmium, mercury, manganese and lead have been investigated as modulators of methylation status, promoting neurodevelopment impairment. Indeed, numerous neurobehavioral, neurodevelopmental and neurodegenerative diseases have been known to develop due to one or a combination of the metals, promoting by epigenetic mechanisms (Ijomone *et al.*, 2020). However, human observational studies reveal also a positive association between low birth weight causing by lead or cadmium exposure and obesity, cardiovascular disease or metabolic syndrome. Indeed, a rapid adiposity gain in response to low birth weight is a known risk of long-life

metabolic impairment. These observations were also confirmed in animal models of exposure to lead and cadmium. Lead exposure shows increased fat mass, body weight or food intake in adulthood, while cadmium exposure increases the fat mass in male mice (Park *et al*., 2016).

Furthermore, altered placental global DNA methylation patterns have been associated with exposures to folic acid, BPA, and phthalates (Nahar *et al*., 2015; Zhao *et al*., 2015). BPA, polybrominated diphenyl ethers (PBDEs), phthalates and phenols are commonly used in consumer products as either in plasticizers or flame retardants, and they are known as endocrine disruptor chemicals (EDC) for their faculty to alter the function of the endocrine system.

The effects on brain development induced by BPA exposure was largely demonstrated in several animal studies, supporting the data from human cohorts (Perera *et al*., 2012; Kundakovic and Jaric 2017). It was shown that prenatal exposure to low, environmentally relevant doses of BPA induces epigenetic alterations in the brain that influences the behavioural and learning abilities. A study conducted on children at 3–5 years of age, showed that high maternal BPA exposure is associated with a sex specific disturbed emotional regulation and increased aggressive behavior in boys (Perera *et al*., 2012).

Impairment of DNMTs methylation and expression could be a possible mechanism through which the functionality of many genes is affected, contributing to BPA-induced neurobehavioral consequences. For example, both methylation and expression of *Bdnf* gene are significantly changed in the hippocampus and blood of BALB/c mice, in according with *BDNF* changes in the cord blood of humans exposed to high maternal BPA levels in utero (Kundakovic et al., 2015).

There are also several evidences that particular environmental exposures are able to modulate the methylation of mitochondrial DNA (Linqing *et al*., 2016; Xu *et al*., 2017) which can also undergo modifications at the placental level in relation to maternal hormonal variations (Janssen *et al.*, 2017) and in the umbilical cord following alterations of the placenta (Novielli *et al.,* 2017).

These findings suggest that cells respond to various environmental stressors with oxidative stress, inflammation and changes in energy production, especially during the critical period of implantation.

-Smoke exposure

Pregnant women often smoke during the gestational period - while a minority stops temporarily and start again after giving birth - heedless of the risk that this habit creates for themselves and their babies. Indeed, cigarette smoke is composed of almost 4000 toxic chemicals, that can insult directly placental or membrane physiology, and increase the risk of premature birth and perinatal mortality. For instance, nicotine easily passes the placental barrier and reaches the fetus with concentrations 15% higher than in the mother (Pintican *et al*., 2019). Nicotine modulation on placenta structure and

function was demonstrated in both in vivo and in vitro studies. It exerts a harmful effect by causing an alteration of trophoblast invasion and the poor development of placental vascularity. These actions are mediated by placental nAChR α4-subunit (nicotinic acetylcholine receptors), causing the increase of placental hypoxia as well as the unbalance between proangiogenic and antiangiogenic factors (Holloway *et al*., 2014).

Recent studies corroborate the existence of an epigenetic memory of the *in utero* smoking exposition, often associated with a transcriptome alteration, in different placental loci also in a sex specific manner (Morales *et al*., 2016; Martin *et al*., 2017; Cardenas *et al*., 2019; Rousseaux *et al*., 2020).

The placental methylation modulation of specific genes, such as *RUNX3* and *NR3C1* (glucocorticoid receptor gene) has also been linked to adverse infant outcomes, e.g., preterm birth and low birth weight, respectively. Many candidate genes, including *CYP1A1, AHHR* and *HSD11β2* (11β–hydroxysteroid dehydrogenase type 2) exhibit altered gene methylation and expression linked to maternal smoking in both placenta and cord blood (Joubert *et al*.,2012). All these genes code for enzymes involved in basic pathways that protect the fetus from exogenous factors and maternal context. For example, the HSD11β2 enzyme regulates the availability of glucocorticoids to their receptor, promoting the inactive state of the cortisone (normally overproduced during pregnancy and afterwards to stressful events), while CYP is a xenobiotic-metabolizing enzyme family acting also in placental tissue.

Maternal tobacco use during pregnancy has also been associated with global methylation changes – measured as LINE-1 and AluYb8 - and CpG-specific in cord blood and buccal cells from children. For example, *IGF2* gene has also been associated with pre pregnancy smoke, displaying higher DMR methylation levels and a lower expression in cord blood (Murphy *et al*. 2012; Nielsen *et al.,* 2016). Moreover, maternal smoking during pregnancy was associated with epigenetic modifications within the *BDNF* gene in adolescent offspring, further confirming that the smoking induced epigenetic changes can be long-lasting (Toledo-Rodriguez *et al*., 2010).

1.7.3-Psychological influence on neurodevelopmental programming

Maternal adverse psychosocial experiences during pregnancy are considered a risk factors for poor birth outcome. Placenta mediates the stimuli concerning the maternal psychological sphere that could influence intrauterine environment and can lead to physical and mental injuries in the offspring (fig.4).

An alteration of HPA axis is often found in psychiatric disorders, particularly in patients suffering from depression and anxiety disorders.

Specific neonatal movements have been reported in association to maternal distress. In particular, reduced fetal heart rate variability and increased movement have been observed related to maternal stress, as well as higher levels of cortisol amount are directly related to time spent moving. On the other hand, fetus spents more time in quiet and sleep and showed poor body movements in depressed mothers (Monk *et al.,* 2012).

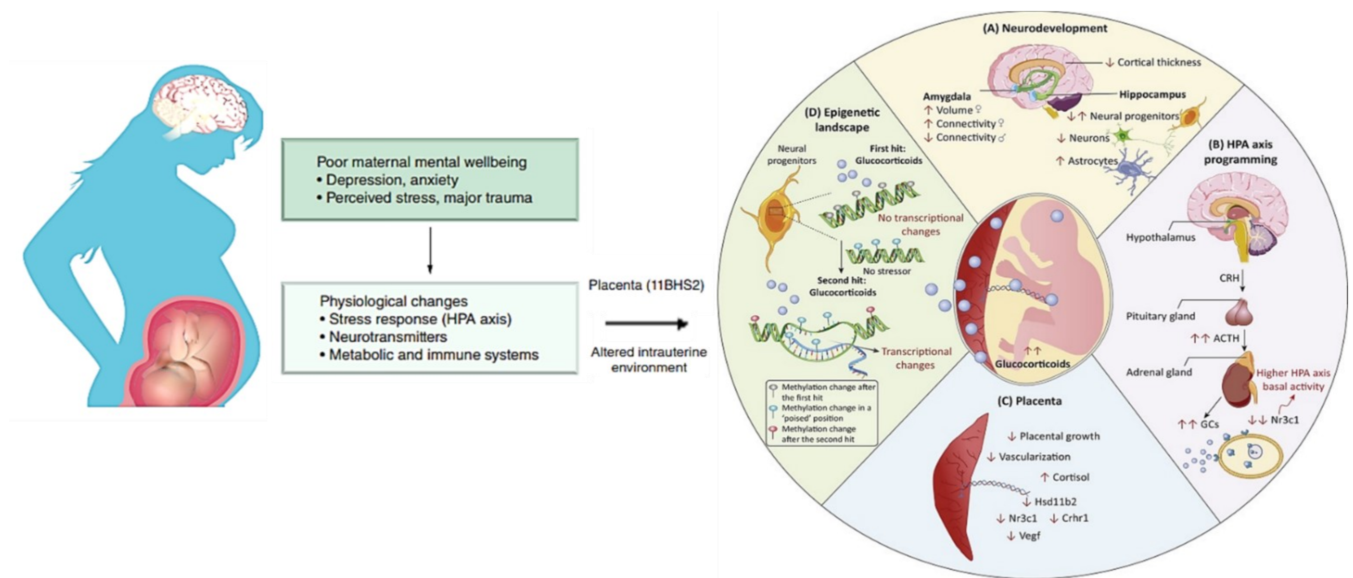
Different studies aimed to investigate how distress may affect children's outcomes via epigenetics (as reviewed by Palma-Gudiel *et al*., 2018). These papers focused on regulation and activation of HPA axis functioning, as well as on *BDNF* gene regulation (that codes for a neurotrophic factor critically involved in neurodevelopment). High levels of *HSD11B2* normally characterize the second and third trimesters of pregnancy, regulating the passage of cortisol from the maternal to the fetal circulation (Glover *et al*., 2018). Higher anxiety maternal mood downregulates placental *HSD11B2* mRNA, and an increase of its DNA methylation has been observed also linked to lower muscle tone in newborns (Lester *et al*., 2014; Monk *et al*., 2016). Moreover, in an independent study of 181 infants, birth weight was positively associated with the degree of placental DNA methylation levels of *IGF2* and *AHRR* (Aryl hydrocarbon receptor repressor) and inversely associated with *HSD11B2* methylation (Xiao *et al*., 2016).

Similarly, birthweight was found affected in babies of women that experienced war-related stress, in association with 3 placental CpG sites of HPA axis genes, including *NR3C1* (that seems to be an upstream regulator of placental *HSD11B2* gene expression; Kertes *et al*., 2016). Furthermore, greater reflex asymmetry was found in infants characterised by low placental *HSD11B2* methylation but high *NR3C1* methylation levels, while the opposite combination leads to lower excitability outcome. Impairments in stimulus habituation have been associated with high methylation levels in both genes (Nugent and Bale, 2015).

On the other hand, BDNF was found in amniotic fluid in both human and animal pregnancy, and the placenta may be the main source of these neurotrophin, suggesting its action on the fetus (Deuschle *et al*., 2018). Indeed, decreased in *BDNF IV* DNA methylation in both male and female infants was detected in infants' buccal cells as well as an increased *NR3C1* methylation, associated to maternal depressive symptoms during pregnancy (Braithwaite *et al*., 2015).

Oxytocin signalling also seems to be impaired in relation to anxious mood of the mothers. To this regard, decreased *OXTR* (oxitocine receptor gene) DNA methylation could be considered an adaptive molecular mechanism in children to facilitate the expression of the gene potentially afflicted by restricted maternal cares (Unternaehrer *et al.,* 2016).

Figure 4: A simplified diagram showing how poor maternal mental wellbeing during pregnancy could influence the early development (modified from Ryan *et al*., 2017 and Krontira *et al*., 2020)



Aim

The aim of this work is to investigate the possible correlations among the maternal environmental exposures in the prenatal period and peripheral biomarkers (alterations in global DNA methylation as well as at specific genes level).

This study represents a part of a longer-term prospect study aiming at investigating the predisposition to the development of neurodevelopmental diseases in children during the first 1000 days of life, as a result of environmental exposures in the womb, with the ultimate goal to identify potential early biomarkers useful for primary prevention strategies.

The whole study is funded by the CCM, (National Center for Disease Prevention and Control, Ministry of Health), and is entitled "Environment, fetal epigenetic programming and chronic disease prevention".

The different Operative Units are involved both in the recruitment of mother-newborn couples, and in carrying out specific analyses on the biological material collected (cells of the buccal mucosa and urine of the mother and of the newborn, placenta). The Pisa operational unit was involved in the recruitment of 20 mother-child pairs and in methylation analyses.

In this frame the methylation profiles of 13 candidate genes involved in different metabolic pathways – also known to be sensitive to maternal conditions and exposures during pregnancy –, and potentially able to interfere with growth and neurodevelopment have been considered.

Gene-specific and global methylation analyses were performed on DNA obtained from oral swabs carried out on mother-child pairs and from placental tissue samples.

Since from the placenta tissue principal heavy metals and dioxin compounds were in parallel measured by the Operative Unit of Bologna (ISZLER), and data were already available, it has been established to use them, in order to correlate them with our methylation data, searching for predictors of maternal and fetal toxicant exposure.

2.1- Study design

This work is part of a study entitled "Environment, fetal epigenetic programming and prevention of chronic diseases", funded by the Italian Ministry of Health.

This project aims to improve our knowledge on the maternal-fetal exposure to environment and pollutants, as well as on the main biological-molecular mechanisms of damage (with regard to epigenetic mechanisms) during the first 1000 days of life. The ultimate goal is to create a study model of the relationship between environment, pregnancy and the peri-post-natal period for an advanced environment-health surveillance system.

A multidisciplinary approach is adopted to identify the presence of early biomarkers of exposure at the fetal level and, in particular, at placental ones, that could be used for the development of a surveillance system and primary prevention of the main emerging chronic diseases and disorders.

Thus, pre- and perinatal risk factors for early molecular, epigenetic and metabolomic biomarkers were researched. A 12-month follow-up of the babies will allow establishing the correlations with the features of babies 'growth and cognitive development, with a probable embryo-fetal origin.

Furthermore, 20 pregnant healthy women and their babies were recruited in four Italian regions (Sardegna, Toscana, Lombardia and Puglia). From the mothers, urine, placenta and buccal cells samples were collected the day of delivery; from the newborns, buccal cells and urine samples were collected at birth and after the 12-month follow-up.

On the buccal cells and placenta samples of each couple, the epigenetic profile has been defined in our lab. The methylation of mitochondrial DNA was investigated to establish their activity in placenta tissue. Additionally, the main heavy metal concentrations (such as Pb, Cr, As, Hg, Cd and others), IPA and PFAS were measured on the whole placenta using ICP-MS (Inductively Coupled Plasma-Mass Spectrometry) technique. The 1H NMR spectroscopy was applying on both placenta and urine samples to establish the metabolomic profile of the couple recruited.

All the data that will be obtained from epigenetic, morphological and metabolomic profiles and the detection of the epigenotoxic compounds will be compared, allowing to:

a) correlate the significant placental and fetal alterations with upstream specific biomarkers, with downstream signs and symptoms of perinatal and postnatal distress;
b) establish the early biomarkers and signs that can be used for primary prevention strategies, early diagnosis, individualized counselling and targeted intervention for chronic disorders and diseases with possible epigenetic/fetal origin.

2.2- Population

Between February 2019 and February 2020, 26 pregnant women were recruited at Department of Obstetrics and Gynaecology, Santa Chiara Hospital in Pisa. The clinical characteristics and anthropometric measurements of each couple newborn/mother were shown in table 1.

Table 1: *Characteristics of the birth cohort and mothers.*

a) Women			***b) Newborns***	
Mothers	n= 26		Male	n=16
Age	34,12 ± 4,6		Female	n=10
Pregravid BMI (Kg/m^2)	21,91 ± 2,69		Gestational age (days)	38,91 ± 0,47
Previously pregnancies	92%		Weight (kg)	3278,19 ± 375,17
Number of miscarriages	19%		Length (cm)	50,27 ± 1,76
			Head circumference(cm)	35,19 ± 1,42
			Apgar score	>7

To standardize the deliveries features, exclusively elective caesarean delivery without labor or general anaesthesia were included.

In order to exclude confounding factors, known to affect methylation patterns, a wide range of exclusion criteria were established. They included obese, pre-existing diabetes, autoimmune diseases (e.g., thyroiditis), current multiple pregnancy, fetal chromosomopaties, alcohol or drugs abuse and other characteristics summarized in table 2.

Table 2: *Inclusion and exclusion criteria*

Inclusion criteria		***Exclusion criteria***
Italian nationality		Family history without metabolic disorders (e.g., gestational diabetes, autoimmune diseases)
Age (from 18 to 40 years)		Preeclampsia
Pregravid BMI 18,5-25 kg/m2		Twin pregnancy
Same residence of WG		Assisted reproductive pregnancy
Ability to understand the protocol and willingness to accept		Chromosomopaties
Propensity to breastfeed		Maternal infections
Singleton birth		Farmacological treatments
Natural pregnancy		Drugs and alcohol abuse
Elective cesarean delivery		Labor
Gestational age ≥ 34 weeks		General anesthesia

The eligibility of the subjects was established after the administration of a questionnaire, during the medical exam scheduled to plan the delivery date. The aim of the questionnaire was to explore the periconceptional and gestational periods, in order to establish the influences occurred, in term of professional or environmental exposures (e.g., chemicals, solvents, dioxin or air pollutants) and maternal characteristics and stressors. Maternal stress factors that could insult the neurodevelopment were previously investigated by our group, by using the same questionnaire (Grossi *et al*., 2018). Furthermore, data on parental lifestyle and newborns measurement at birth were collected (see supplementary material 1- Questionnaire).

Clinical variables of the infants included sex, birth weight, body length, head circumference, placenta weight, gestational age, and eventually neonatal complications were collected at birth.

Informed written consent was signed by parents who accepted to contribute to the project, and the sampling and experimental processes were performed with the approval of Area Vasta Nord Ovest Ethic Committee (CEAVNO) (study protocol number 40896; approved on 19/07/2018).

2.3- Samples collection

For each couple mother-newborn samples of buccal cells and urine were collected. Placenta samples were also set up within an hour from the delivery.

Buccal cells and placenta samples were used for the epigenetic investigations.

-Buccal cells collection and DNA extraction

Buccal cells from both mothers and newborns were obtained during the days of hospitalization. Cells were collected easily and quickly using DNA Buccal Swabs kit (Isohelix) by gently swabbing the swab on the inner walls of the cheeks, following the instructions of the company. Addition of Isohelix Dri-Capsules preserved, at room temperature, genomic DNA from degradation after collection (fig. 5). Maternal (matDNA) and neonatal (nwbDNA) genomic DNAs were extracted using NEW Buccal-Prep Plus DNA Isolation Kit (Isohelix) and quantified with Nanodrop 2000C (Thermo Scientific). DNA was stored at -20°C until use.

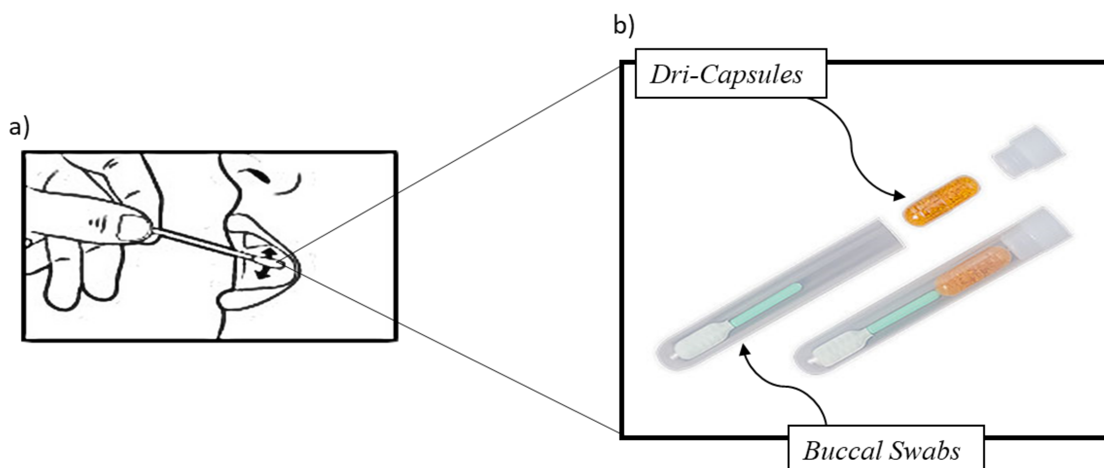


Figure 5: *Buccal cells collection*. a) Mucosa cells are collected swabbing the cheeks for one minute for side. b) Buccal swab provides by Isohelix: both cotton swab and capsule are shown.

-Placenta samples and DNA extraction

The placenta samples were collected after childbirth in operating room following standard obstetrical practice (fig. 6). Placenta was delivered and immediately passed in a sterile container and weighed. First, a macroscopic anatomopathological examination to verify the health of the organ was done. To avoid the placenta being contaminated by maternal skin or surrounding atmosphere, the maternal decidua was discarded, with only the inner part of the placenta retained. Subsequentially, four 1-2 cm^3 sections were excised from the maternal side of placenta and washed several times in Phosphate Buffered Saline, PBS (Gibco – ThermoFisher) to remove the presence of maternal blood. Placenta and umbilical cord samples for each working group were also collected, in order to send samples to other collaborating Units, to test mitochondria activity, to evaluate the histopathological

characteristics and allow the toxic compounds measurement. Finally, the tissue samples were stored at -80°C for further analysis.

Placental genomic DNA (plcDNA) was extracted using QIAamp®DNA Mini and Blood Mini Handbook kit (QIAGEN, Milan, Italy), following the manufactory protocol. pDNA was stored at -20°C until use.

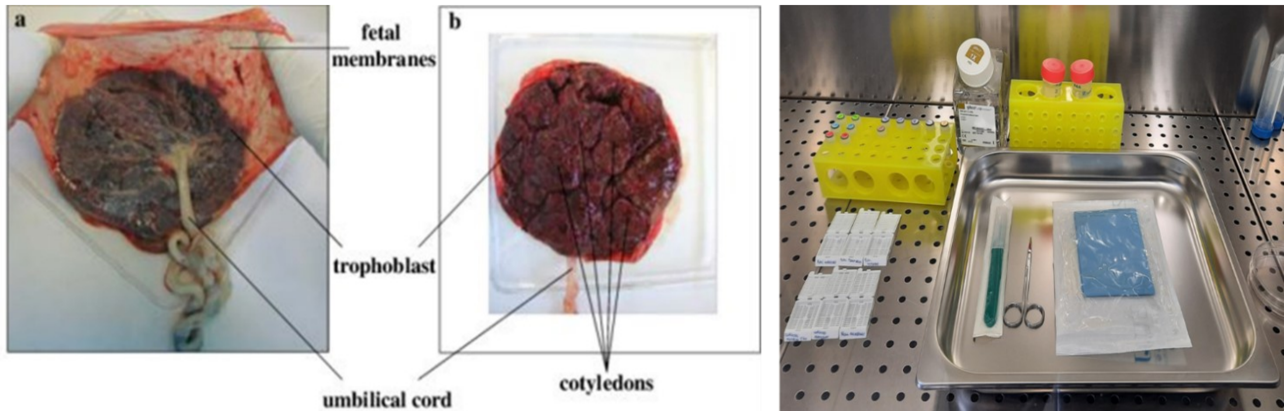


Figure 6: *The human placenta.* **a)** Fetal side. It is possible to distinguish the fetal membranes, trophoblast, and umbilical cord. **b)** Maternal side. The placenta is characterized by irregular lobed structures, termed cotyledons. (Evangelista *et a*l.,2008) **c)** Preparation of biological laminal flow for placenta sampling

2.4 Gene specific methylation assay

- Bisulfite modification

Preliminarily, 200 ng of DNA derived from each tissue were bisulfite treated using "EpiTect® Bisulfite Handbook" (QIAGEN, Milan, Italy). Reaction with sodium bisulfite promotes cytosine deamination and its conversion in uracil; 5-methylcytosine (5-mC) - methylated form of the cytosine - reacts poorly with bisulfite and is resistant to deamination by bisulfite treatment, leaving methylated cytosines unchanged.

- Methylation Sensitive High Resolution Melting (MS-HRM)

Gene methylation was assessed by means of methylation sensitive-high resolution melting (MS-HRM) analysis in a CFX96 Real-Time PCR detection system (Bio-Rad, Milan, Italy).

For MS-HRM analysis, we developed in-house protocols, using methylation independent primers designed by MethPrimer software, or we used primers taken from previous literature when it is

possible (table 3). Each couple of primers was first tested by a Gradient PCR in order to identify the annealing temperature (Ta) that allows to properly separate the standard DNA curves.
Specifically, standard DNA was prepared by mixing the fully methylated DNA and the unmethylated one (EpiTectH methylated and unmethylated human control DNA, bisulfite converted, Qiagen). Six growing ratios of methylation were thus obtained: 0, 12.5, 25, 50, 75 and 100%. Standard DNA samples with known methylation ratios were included in each assay in order to generate standard curves that were used to deduce the methylation levels of each sample, using an interpolation method (Fig. 7).

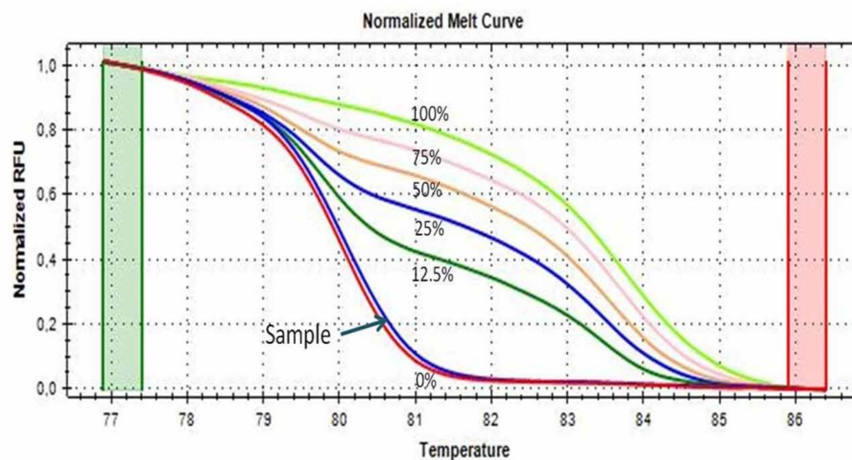


Figure 7: *Standard curves and a sample.*
Primer sequences and Ta used during MS-HRM analysis, amplicon length, number of investigated CpG sites of the analyzed amplicons are reported in Table 3.

Table 3: Primer sequences and Ta used during MS-HRM analysis, amplicon length, number of investigated CpG sites of the analyzed amplicons.

Gene	Primer sequence	Ta	Amplification length	CpG site	Accession Number and Nucleotide Position
LEP	F 5′- GGGTGGGATTTTAGAATTTTTAATT-3′ R 5′- AAACCAACCCCTTAAAAAAATACTT-3′	55°	259 bp	28	NG_007450. From 14444 to 4702 bp
MECP2	F 5′-AATTAAGGTTTTTTAGTTGGGGTAA-3′ R 5′-TTAACCCTCTATCCACAAATACACC-3′	62°	145 bp	5	NC_000023.11 From 154097160 to 154097350 bp
IGF2- IV [a]	F 5′- GGAGGGGGTTTATTTTTTTAGGAAG -3′ R 5′- AACCCCAACAAAAACCACTAAACAC-3′	60°	93 bp	3	NG_008849.1 From 6281 to 6374 bp
MTHFR	F 5′-TTTTAATTTTTGTTTGGAGGGTAGT-3′ R 5′-AAAAAAACCACTTATCACCAAATTC-3′	54°	155 bp	7	NM_005957.4 From 30 to 184 bp
DNMT3b	F 5′-TGGTGTTGTGTGATTATAGTGG-3′ R 5′-TCACCCTAAAAAATCAAAAACC-3′	55°	174 bp	6	NG_007290.1 from −397 to −223 bp

OXTR	F 5′-AATTATTGTAAAATAAATTTATTTGTTAAG-3′ R 5′-AACTAAAATCTCTCACTAAAACCTC-3′	53°	274 bp	26	NC_000003.11 From – 8812437 to -8812711 bp
H19-ICR1	F 5′-TGGGTATTTTTGGAGGTTTTTTT-3′ R 5′-ATAAATATCCTATTCCCAAATAA-3′	56,5°	216 bp	17	NG_041945.1 From 3549 to 3764 bp
HSD11B2	F 5′-TAGGTTTAAGTTTGGAAGGAAAG-3′ R 5′-ACCACAAAACCTACCTAAAACAAAA-3′	59°	107 bp	5	NG_016549.1 From 4302 to 4409 bp
BDNF-I	F 5′-GGGTTGTTAATTTATATTTGGGAAGT-3′ R 5′-ACCACTAATTACCCACAAAACC-3′	58°	119 bp	4	CM000673.2 From 46058 to 46177 bp
CYP1A1 [b]	F 5′-TGTTATAGGGTTTTTAGGAAAAA-3′ R 5′-AAATTATTTTCTAACCTAAACCAAC-3′	54,8°	147 bp	4	GRCh37/hg19 From 75013061 to 75017877 bp
ERα	F 5′-GGGAGATTAGTATTTAAAGTTGGAGGT-3′ R 5′-CAAAACAAAAAACTCAAAAACC-3′	55,4°	233 bp	22	NG_008493.2 From 155.951 to 156.184 bp
MGMT	F 5′-GCGTTTCGGATATGTTGGGATAAGT -3′ R 5′-AACGACCCAAACACTCACCAAA -3′	58°	110	12	NC_000010.11 From 129467205 to 129467315 bp

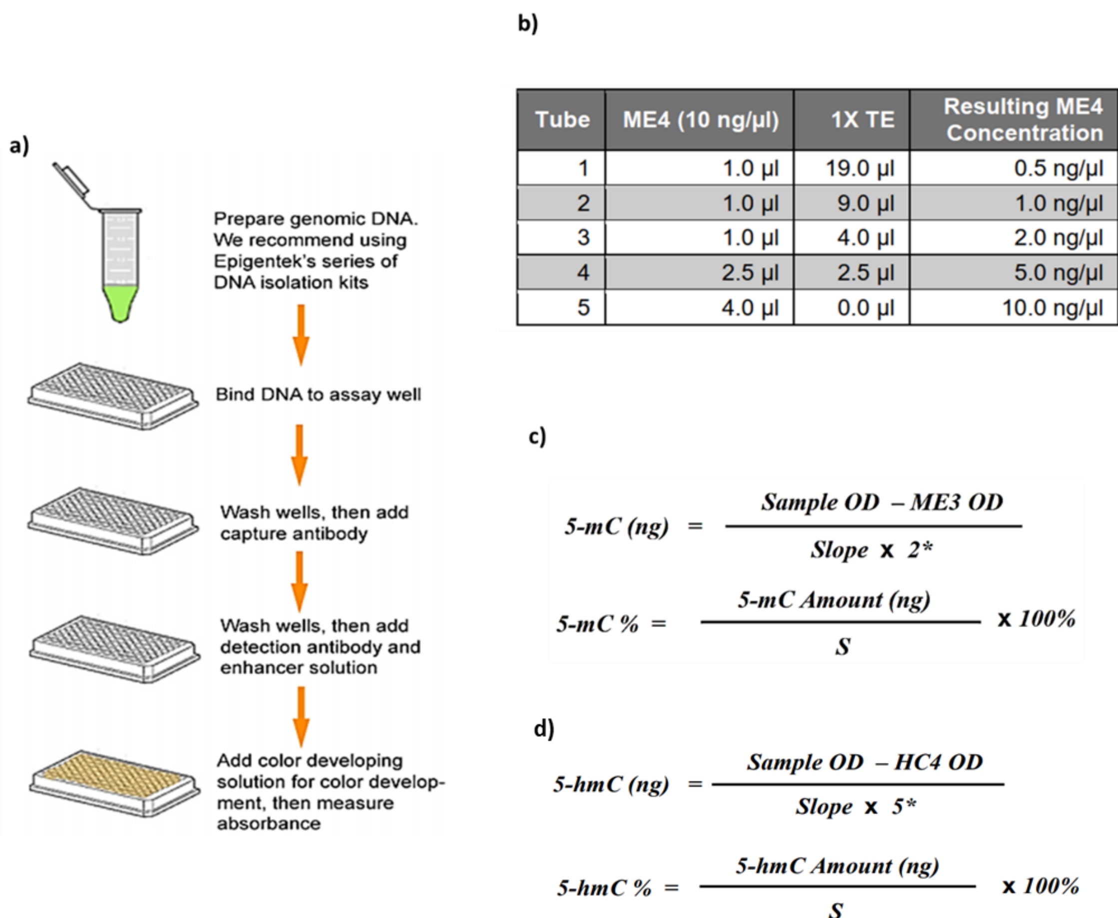
a) Murphy *et al*., 2012; b) Janssen *et al.,* 2017

All the analyses were run according to the following conditions: 1 cycle of 95°C for 12 min, 60 cycles of 95°C for 30 s, Ta for 30 s and 72°C for 15 s; followed by an HRM step of 95◦C for 10 s and 50°C for 1 min, 65°C for 15 s, and continuous acquisition to 95°C at one acquisition per 0.2°C.
PCR was performed in a final volume of 10 µl, containing 5 µl of master mix (Qiagen), 10 pmol of each primer and 10 ng of bisulfite modified DNA template. Each reaction was performed in duplicate and the samples were randomly repeated.

2.5 Global DNA methylation assay

The MethylFlashTM Methylated DNA Quantification Kit (Colorimetric) (Epigentek) and the MethylFlashTM Hydroxymethylated DNA Quantification Kit (Colorimetric) were used for detecting global DNA methylation status of DNA isolated from all the tissues samples.
These methods allow to detect and quantify 5-mC and 5-hmC methylated DNA, using optimized antibodies and enhancer solutions with high specificity to them, with no cross-reactivity to unmethylated cytosine and no or negligible cross-reactivity to hydroxymethylcytosine within the indicated concentration range of the sample DNA. Thus, 50 ng of DNA were used, and each reaction was performed in duplicate. The experiments were conducted according to the manufacture protocols.

Briefly, in these assays, DNA is bound to strip wells that are specifically treated to have a high DNA affinity. The methylated fraction of DNA is detected using capture and detection antibodies and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer (BioRad). The amount of methylated DNA is proportional to the OD 450 nm intensity (fig. 8).



Tube	ME4 (10 ng/µl)	1X TE	Resulting ME4 Concentration
1	1.0 µl	19.0 µl	0.5 ng/µl
2	1.0 µl	9.0 µl	1.0 ng/µl
3	1.0 µl	4.0 µl	2.0 ng/µl
4	2.5 µl	2.5 µl	5.0 ng/µl
5	4.0 µl	0.0 µl	10.0 ng/µl

Figure 8*: Figure from Epigentek guide*. a) The main steps of the experiment are shown. b) example of standard curved preparation from the positive control (ME4) dilutions in 5mC protocols. All the resulting concentration are shown in the last column of the table. c) Formula used to extrapolate the percent of 5-mC. Sample OD: 450 nm; ME3: negative control; S: amount of input sample DNA in ng; * 2 is a factor to normalize 5-mC in the positive control to 100%, as the positive control contains only 50% of 5-mC. d) Formula used to extrapolate the percent of 5-hmC. Sample OD: 450 nm; HC4: negative control; S: amount of input sample DNA in ng; * 5 is a factor to normalize 5-hmC in the positive control to 100%, as the positive control contains only 20% of 5-hmC.

Statistical analyses

Data obtained from both methylation analyses and toxicant concentrations were tested for normality using the Kolmogorov-Smirnov test. Since methylation levels obtained from both the genes specific analyses and 5hmc toxicant determination were not all normally distributed, natural logarithm transformation was done before the analyses. Data are presented ad mean ± SD (standard deviations). One-way multifactorial ANOVA was used to compare epigenetic data (gene specific and global DNA levels) among the exposition factors obtained from the questionnaire and clinical features of the couple mother-newborn. Maternal analyses were corrected for maternal age and pre-pregnancy BMI; gestational age, gender and birthweight were used as covariates in newborn analyses; parental age, gender and birthweight were used as covariates in placenta analyses. Bonferroni adjustment of the *p value* was applied in multiple comparisons performed; with the alpha level being divided by the number of the genes considered.

Linear regression analysis was performed to search for correlations among methylation levels obtained, clinical women and newborn data and toxicants concentrations in placenta tissues. Statistical analyses were performed whit STATGRAPHICS 5.1 plus software package for Windows.

3.1- Data collection from questionnaire

Questionnaire data processing allowed to characterize the women and to obtain information on the presence of any previous exposure factors that occurred during the pregnancy period (Tab. 4).
Most of women recruited lived in an urban context; about half of them conducted an active lifestyle (and had a regular sun exposure.
27% of them also claimed to smoke before pregnancy and 23% was exposed to passive smoke. Just a few of the interviewees declared an environmental or occupational exposition to toxicants.
Furthermore, 30% had influenza and/or febrile symptoms during the pregnancy period, while 46% reported familial pathologies affecting the parents.
The questionnaire was also aimed to detect stressful events that eventually occurred during their pregnancy: 30% of the women interviewed lived a family bereavement, job loss, relocation and legal problems.

Table 4: *Questionnaire data processing.*

Factors investigated	***Number (%) of exposed***
Miscarriages	19
Ex-smokers	27
Passive exposure to cigarette smoke	23
Influenza and/or febrile symptoms	30
Stressful events during pregnancy	30
Urban environment	65

3.2- Gene specific methylaytion analysis

Methylation percentage obtained from all genes using MS-HRM technique is reported in table 5. The values reported refer to the mean and standard deviation.

Table 5: *Methylation levels of the candidate genes analysed expressed as mean and standard deviation (SD)*

GENE	***Placental methylation (%)***	***Maternal buccal cells methylation (%)***	***Newborn buccal cells methylation (%)***
BDNF	7,45 ± 8,00	10,85 ± 10,39	7,33 ± 8,42
CYP1A1	19,99 ± 10,20	26,99 ± 6,92	14,89 ± 7,45
DNMT-3B	5,63 ± 5,61	7,72 ± 11,33	7,08 ± 8,14
ERα	0,33 ± 0,75	6,02 ± 7,13	6,26 ± 7,28
H19	58,32 ± 10,57	71,57 ± 9,50	69,75 ± 12,05
HSD11β2	4,92 ± 3,85	0,55 ± 1,00	1,20 ± 2,12
IGF2	51,30 ± 7,48	40,54 ± 11,27	32,52 ± 11,26
LEP	64,87 ± 18,19	11,98 ± 5,99	5,29 ± 5,21
MECP2	7,49 ± 6,02	35,67 ± 13,30	11,91 ± 10,13
MGMT	0,50 ± 0,57	0,16 ± 0,31	0,34 ± 0,60
MTHFR	11,05 ± 3,08	17,78 ± 8,10	9,51 ± 8,48
OXTR	1,17 ± 1,68	1,20 ± 1,11	0,91 ± 0,71
REL	2,06 ± 2,77	2,93 ± 3,80	3,78 ± 3,73

3.3- Data from questionnaire, mother-newborn parameters and gene specific methylation

Methylation levels of all the candidate genes were investigated in all the tissues collected. Data obtained from the comparisons between DNA methylation and exposure factors or couples' parameters were divided considering the source of the tissues.

First, each relation was evaluated individually; for what concern all the comparisons obtained, the *p*-value was adjusted for Bonferroni correction based on the number of genes included in the analysis. Only the relations that survived after Bonferroni adjustment were considered statistically significant.

- Maternal buccal cells methylation

Maternal methylation levels of all genes were compared with data collected: all the findings are summarized in Table 7.

A statistically significant difference in *matBDNF* gene promoter methylation levels was found related to stressful events occurred during pregnancy period (*p* = 0,0416) (fig.9, a). In detail, women who experienced stressful events showed high levels of *matBDNF* gene methylation compared with women who did not live them. The same gene showed a positive correlation with infants gestational age in maternal mucosa cells (*p* = 0,0105; r = 0,5) (fig.9, f).
Additionally, low *matHSD11B2* gene methylation levels were found in women exposed to passive exposure to cigarette smoke (*p*=0,0455) (fig.9, b).
For what concerns the features of the couple mother-newborn, a correlation with maternal age was found with *matH19* and *matLEP* gene methylation levels. The first one showed a negative correlation with age (*p* = 0,0038; r = -0,5) (fig.9, d), while a positive correlation with maternal age was detected in the second one (*p* = 0,0114; r = 0,5) (fig.9, c).
Lastly, *matMTHFR* (*p* = 0,0316; r = 0,5) (fig.9, g) and *matCYP1A1* (*p* = 0,0407; r = 0,4) (fig.9, d) gene methylation levels showed a positive correlation with head circumference.

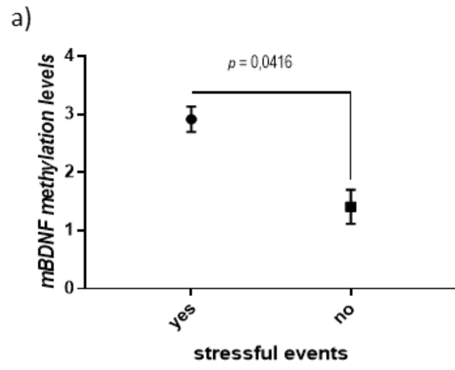
Table 7: Summary of all data obtained from statistical analyses on the maternal buccal cells derived DNA. Maternal age and pre-pregnancy BMI were used as covariates in Multifactorial ANOVA test. Data that were still statistically significant after Bonferroni adjustment are in bold.

Maternal buccal cells methylation				
Exposure factors	***Gene***	***findings***	***p value***	***Bonferroni adjusted p value***
Multifactorial ANOVA (Fig.8 a and b)				
Stressful Events (n of exposed =8)	*BDNF*	High levels of *BDNF* methylation in women who experience stressful events during pregnancy	**0,0032**	**0,0416**
Miscarriages (n of exposed =5)	*LEP*	Low genes methylation levels in women who experience previous miscarriages events	0,0347	0,4511
	MECP2		0,0053	0,0689
	REL		0,0073	0,0949
	IGF2	High genes methylation levels in women who experience miscarriages events	0,0316	0,4108
	CYP1A1		0,0147	0,1911
Passive smoke exposure (n of exposed =6)	*MECP2*	Low genes methylation levels in women exposed to passive smoke	0,0163	0,2132
	HSD11B2		**0,0035**	**0,0455**
Linear Regression (Fig.8 c,d,e,f,g)				
Maternal age	*H19*	The gene methylation levels show a negative correlation with age	0,0038; r= -0,5	
	LEP	The gene methylation levels shown a positive correlation with age	0,0114; r= 0,5	
Neonatal cranic circumference	*MTHFR*	The gene methylation levels show a positive correlation with cranic circumference	0,0316; r= 0,5	
	CYP1A1		0,0407; r= 0,4	

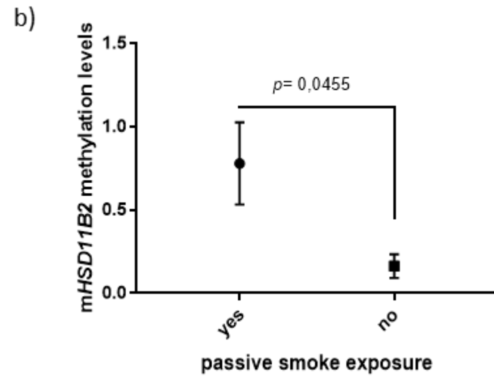
Gestational age	*BDNF*	The gene methylation levels show a positive correlation with gestational age	0,0105; r= 0,5

Figure 8: The influence of exposure factors on maternal DNA methylation. Graphs show methylation values transformed with natural log. The mean and SD of the gene methylation (%) for each group were:
a) ***yes (n=8)***= 20,04 ± 9,60 ***no (n=18)*** 6,76±7,96; **b) yes (n=6)**= 1,51±1,39 ***no (n=20)***= 2,52± 10,24.

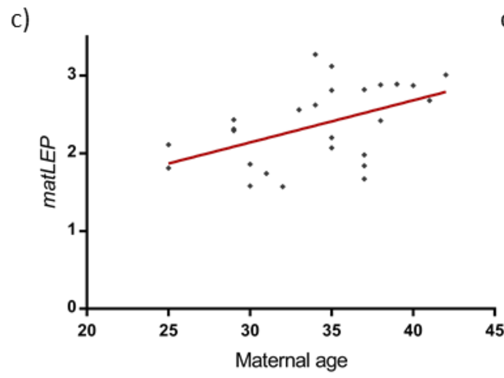
a)



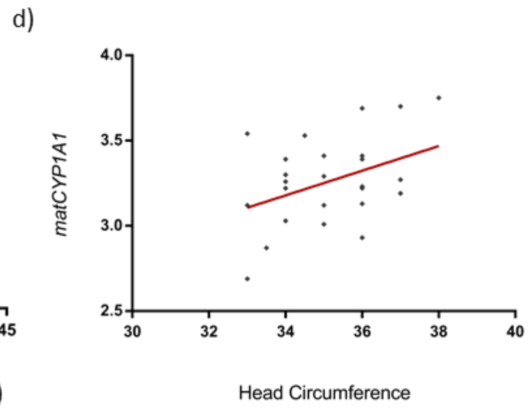
b)



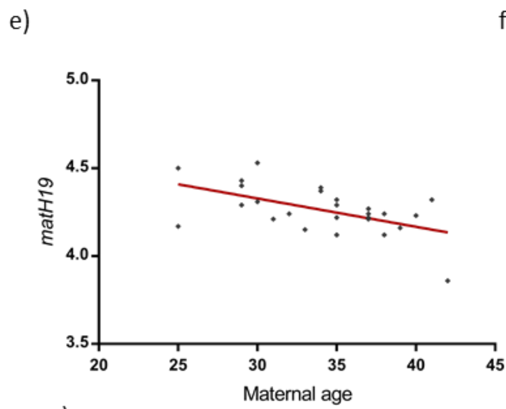
c)



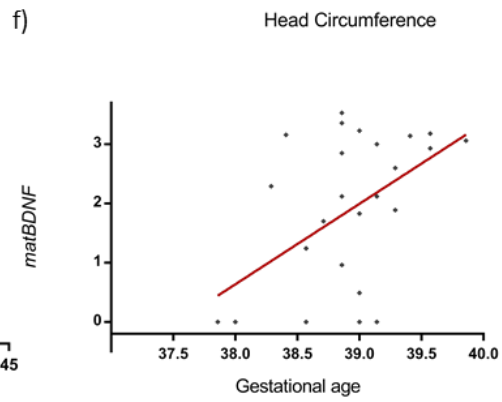
d)



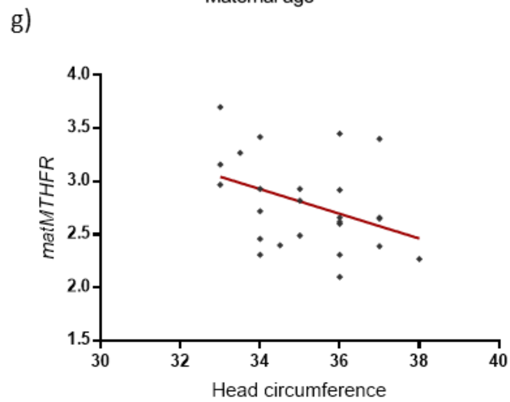
e)



f)



g)



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- *Newborns buccal cells methylation*

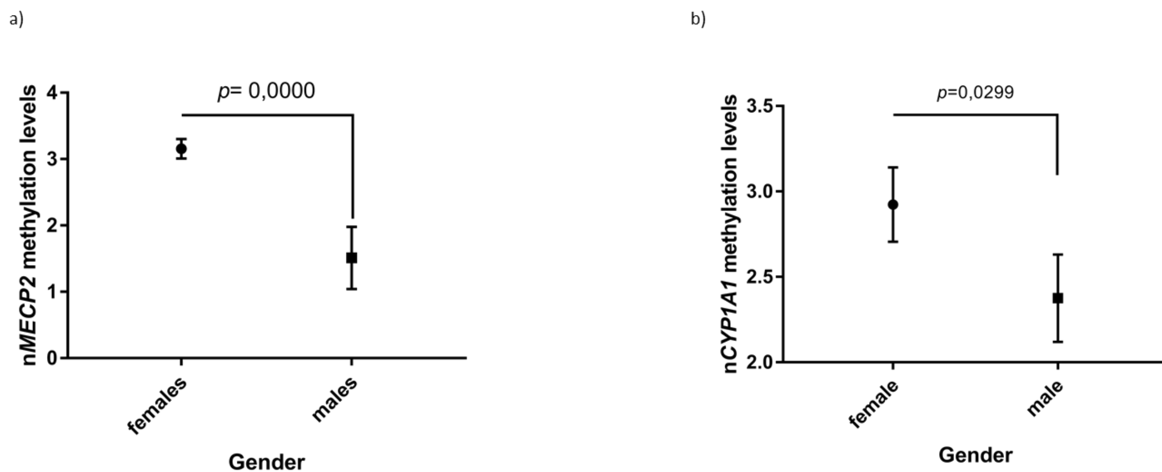
Neonatal methylation levels of all genes were compared with data collected: all the findings are summarized in Table 8.

Among all data collected, statistically significant differences were detected regarding neonatal gender only. In detail, a higher methylation of *nwbMECP2* (*p* = 0,0000) (fig.10, a) and *nwbCYP1A1*(fig.10, b) (*p* = 0,0299) was found in female infants.

Table 8: Summary of data obtained from statistical analyses on the neonatal buccal cells derived DNA. Gestational age, birthweight and gender were used as covariates in Multifactorial ANOVA test. Data that were still statistically significant after Bonferroni adjustment are in bold.

Neonatal buccal cells methylation				
Exposure factors	***Genes***	***findings***	***p value***	***Bonferroni adjusted p value***
Multifactorial ANOVA (Figure 10)				
Gender (*M=16; F=10)*	***IGF2***	Boys shows a higher methylation than girls	0,0419	0,5447
	MECP2	Girls shows a higher methylation than boys	**0,0000**	**0,0000**
	CYP1A1		**0,0023**	**0,0299**
Ex-smokers (*n of exposed* =7)	***DNMT3B***	Babies born from mothers who smoke before pregnancy show a low methylation of *DNMT3B* gene	0,0077	0,1001
Passive smoke exposure (*n of exposed* =6)	***HSD11B2***	Babies exposed to passive smoke during pregnancy period show a higher methylation of *HSD11B2* gene	0,0265	0,3445

Figure 10: The influence of gender on neonatal DNA methylation Graphs show methylation values transformed with natural log. The mean and SD of the gene methylation (%) for each group were: **a)** ***F (n=10)*** **=** 22,926±4,6906, ***M (n=16)*** **=** 5,02±3,88.



- ***Placenta cells methylation***

Placental methylation levels of all candidate genes were compared with data collected: all the findings are summarized in Table 9.

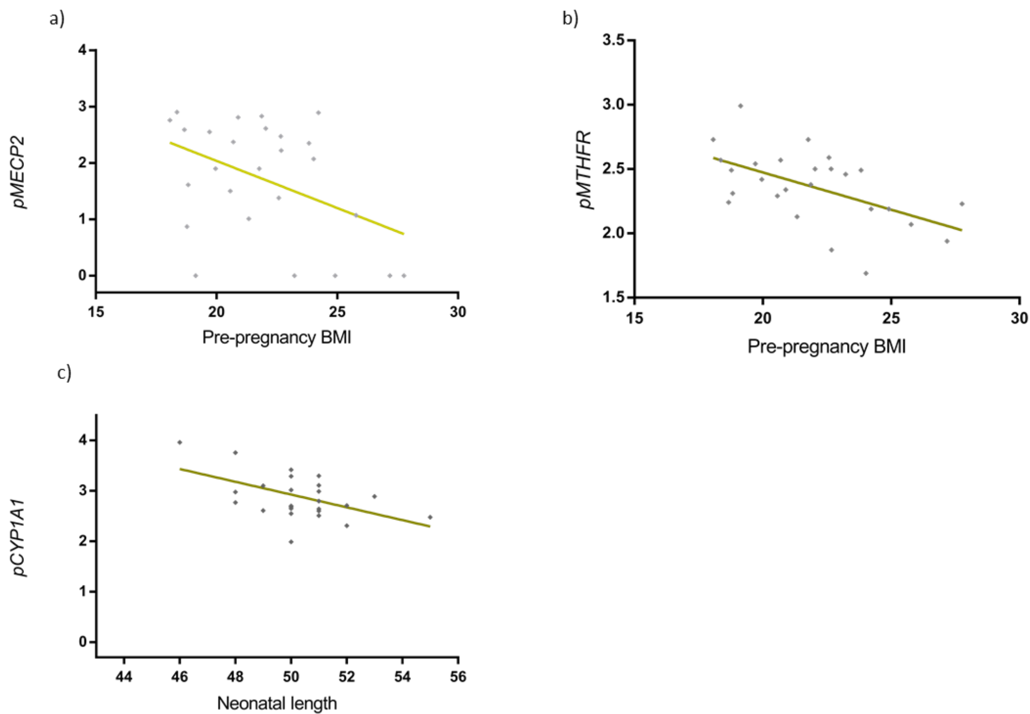
None of the genes analysed showed a pattern specifically related with the questionnaire data. Indeed, statistically significant results were related only to newborn parameters.

In detail, placental *MTHFR* (*p* = 0,0038; r = -0,5)(fig.11, b) and *plcMECP2* (*p* = 0,0269; r = -0,4) (fig.11, a) genes methylation levels showed a negative correlation with maternal pre-pregnancy BMI, and *plcCYP1A1* gene methylation showed the same trend related to newborn length (*p* = 0,009; r = -0,5) (fig.11, c) .

Table 9: Summary of data obtained from statistical analyses on the placenta cells derived DNA. Parental age, birthweight and gender were used as covariates in Multifactorial ANOVA test. Data that were still statistically significant after Bonferroni adjustment are in bold.

Placenta cells methylation				
Exposure factors	***Gene***	***Findings***	***p value***	***Bonferroni adjusted p value***
Multifactorial ANOVA				
Ex-smokers *(n of exposed =7)*	***IGF2***	Low *IGF2* methylation levels were found in placenta cells of ex-smoker women	0,0294	0,3822
Linear regression (figure 11)				
Newborn length	***CYP1A1***	The placental gene methylation levels show a negative correlation with babies' length	0,009; r= -0,5	
Pre-pregnancy BMI	***MTHFR***	The placental genes methylation levels show a negative correlation with maternal pre-pregnancy BMI	0,0038; r= -0,5	
	MECP2		0,0269; r=-0,4	

Figure 11: The influence of clinical data on placental DNA methylation Graphs show methylation values transformed with natural log.



3.4- Global DNA methylation and hydroxymethylation

Global DNA methylation and hydroxymethylation levels were investigated using ELISA assays in order to establish the global 5mC and 5hmC content in all the tissues collected.

The values are reported in Table 10 and are expressed as mean ± standard deviation. Statistically significant differences were not found among the groups.

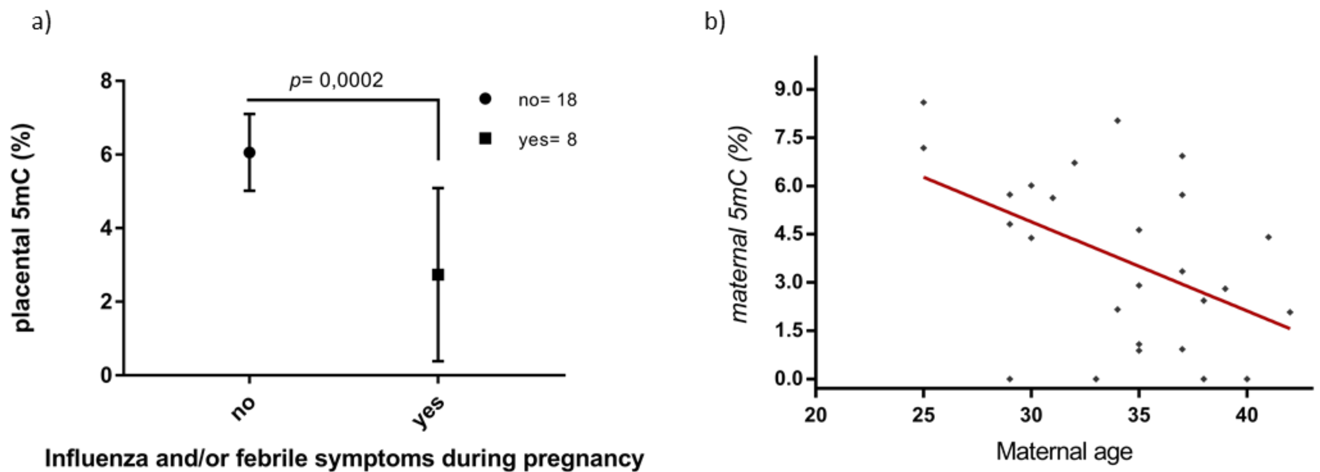
Table 10: Global DNA methylation and hydroxymethylation levels, expressed as mean and standard deviations (SD).

	Placental tissue	***Maternal buccal cells***	***Newborns buccal cells***
5mC- global methylation (%)	5,04 ± 2,77	3,74 ± 2,68	3,77 ± 2,53
5hmC- global methylation (%)	0,41 ± 0,50	0,19 ± 0,25	0,26 ± 0,32

- ***Correlations among data from questionnaire, mother-newborn parameters and global methylation***

Global DNA methylation data were analysed to search for possible relations with survey data and couple's parameters. All *p*-values obtained were adjusted for Bonferroni correction.
For what concerns data from the questionnaire, a statistically significant difference was found in women who contracted influenza or presented fever during gestation (p = 0,0002) (Fig. 12 a).
The analyses also highlighted a negative correlation of 5mC % obtained from maternal tissues with maternal age (p = 0,0326; r = -0,420223) (Fig. 12 b).

Figure 12: The influence of exposure factors on global DNA methylation.
The mean and SD of the gene methylation (%) for each group were: ***yes (n=8)*** = 2,73± 2,81 ***no (n=18)*** = 6,06±2,10.



3.5- Heavy metals, PCB dioxin-like and dioxin exposure

Concentration of dioxins, PCB (Polychlorinated biphenyls; ng/g, wet weight) and the main heavy metals (Mn, Pb, Cr, As, Hg, Cd and Ni; mg/Kg, wet weight) were measured on the whole placenta using ICP-MS (Inductively Coupled Plasma-Mass Spectrometry) technique by the IZSLER Institute, Bologna.
Concentrations of each toxicant were compared with both gene-specific and global methylation in all the three tissue samples collected. Among the 26 analysed, only one placenta showed As presence. For this reason, it was excluded from the analyses.

- ***Maternal buccal cells methylation and heavy metals exposure***

Epigenetic data obtained from buccal cells of mothers were compared with Mn, Pb, Cr, As, Hg, Cd and Ni as well as with dioxin and PCB dioxin-like compound concentrations.

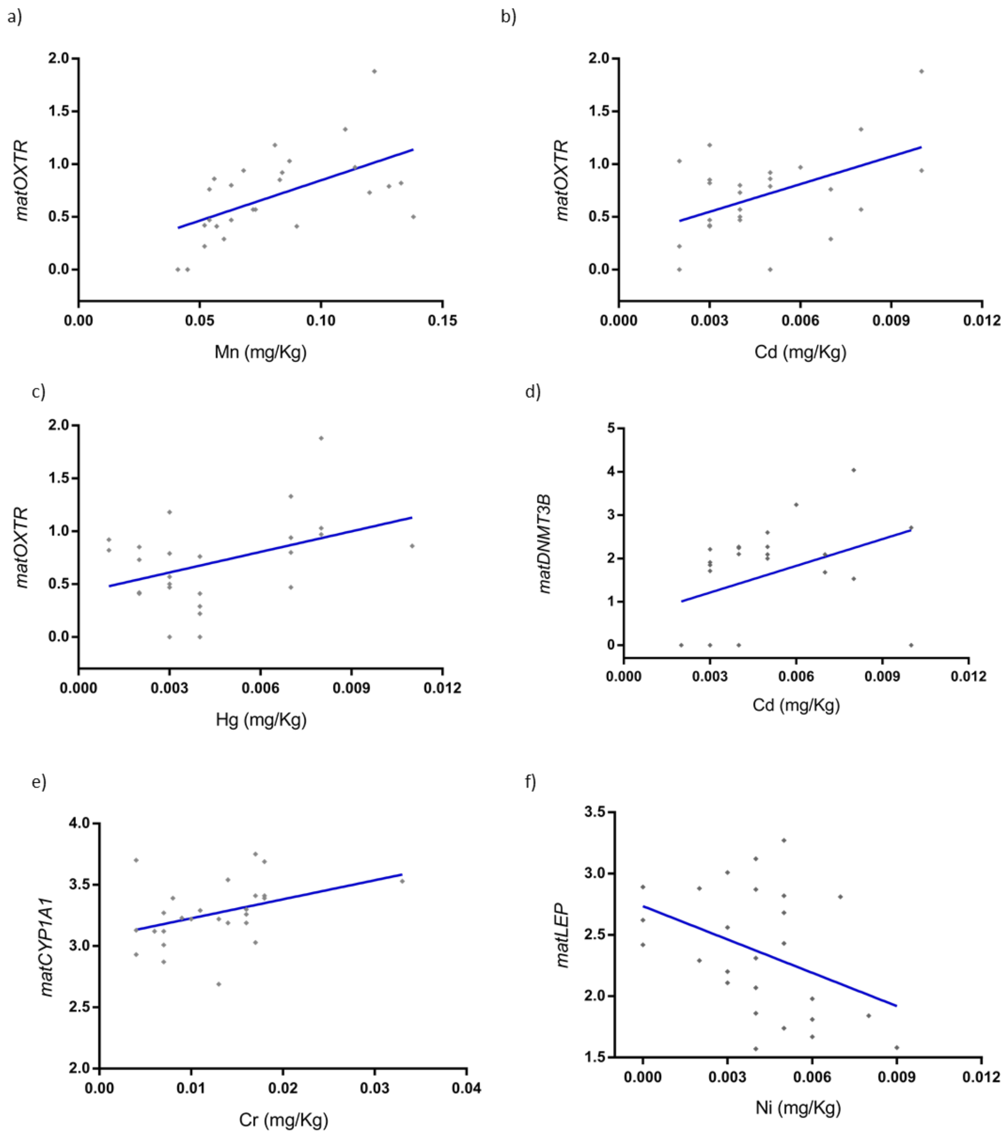
In detail*, matOXTR* gene promoter methylation was found to positive correlate with Mn (p = 0,0032; r = 0,5) (Fig. 13, a) and Hg (p = 0,0359; r = 0,4) (Fig. 13, c) concentration in placenta tissue, as well as with Cd exposures (p = 0,0099; r = 0,5) (Fig. 13, b): these results reflect a significant increase of gene methylation in response to these toxicants (Fig.13).

Additionally, a positive correlation was also found between promoter methylation of both *matDNMT3B* (p= 0,030; r = 0,4) (Fig. 13, d) and *matCYP1A1* (p = 0,0472; r = 0,4) (Fig. 13, e) genes and Cd and Cr detection respectively.

Indeed, low methylation levels were found in *matLEP* (p = 0,0431; r = -0,4) (Fig. 13, f) gene in response to the increased Ni concentration in placenta.

No correlations were found between mothers' DNA and Pb.

Figure 13: The influence of heavy metals exposure on maternal buccal cells methylation. Graphs show methylation values transformed with natural log.

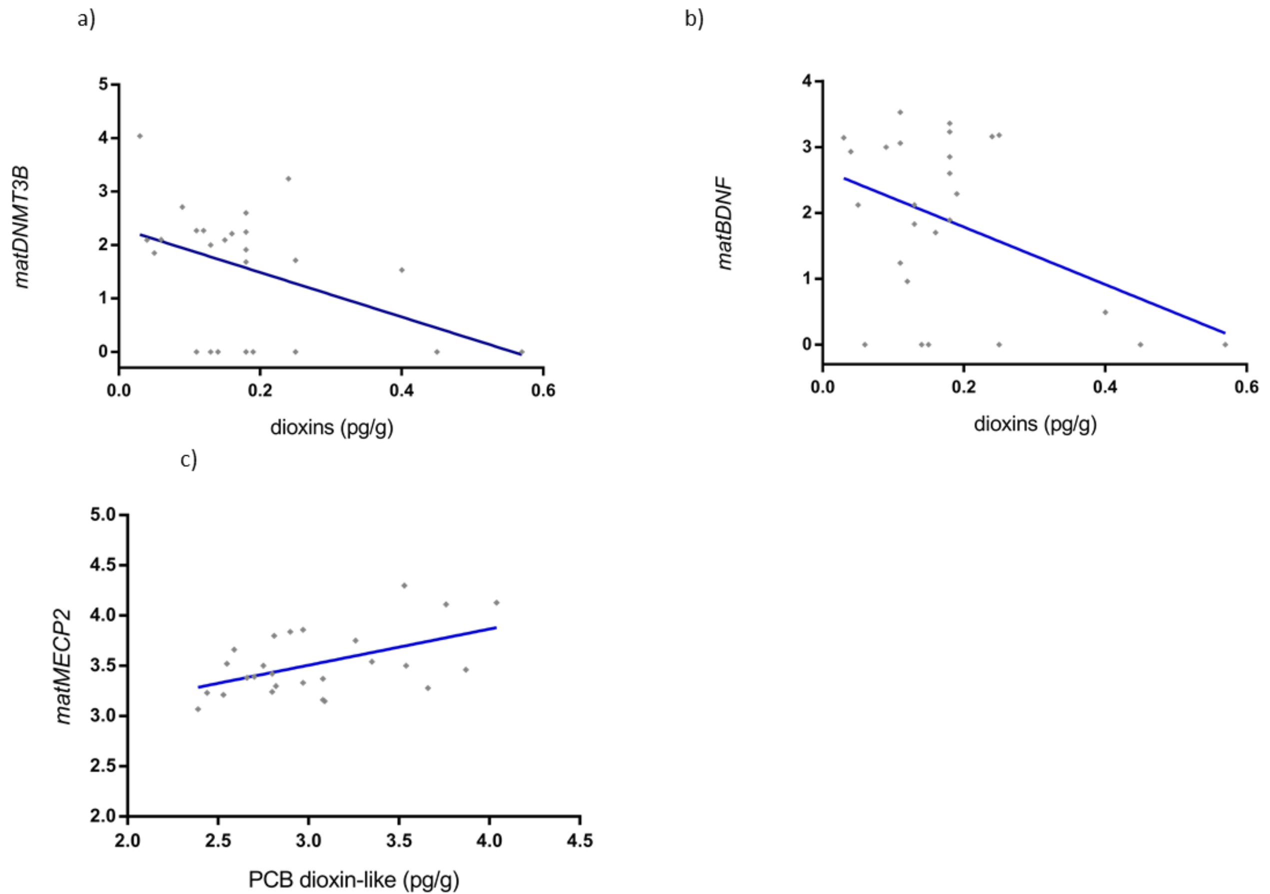


- Maternal buccal cells methylation compared to dioxin and PCB dioxin-like exposure

Total ng/g of detected dioxin in placenta samples were compared with data obtained from epigenetic analyses conducted on maternal DNA (Fig.14).

Statistical analyses revealed a negative correlation between maternal *BDNF* (p = 0,0305; r = -0,4) (Fig.14, b) and *DNMT3B* (p = 0,0226; r = -0,4) (Fig.14, a) gene methylation levels and total amount of dioxins detected, while *MECP2* gene showed an opposite trend (p=0,0112; r=0,5) (Fig.14, c) in response to PCB dioxin-like exposure.

Figure 14: The influence of dioxins exposure on maternal buccal cells. Graphs show methylation values transformed with natural log.



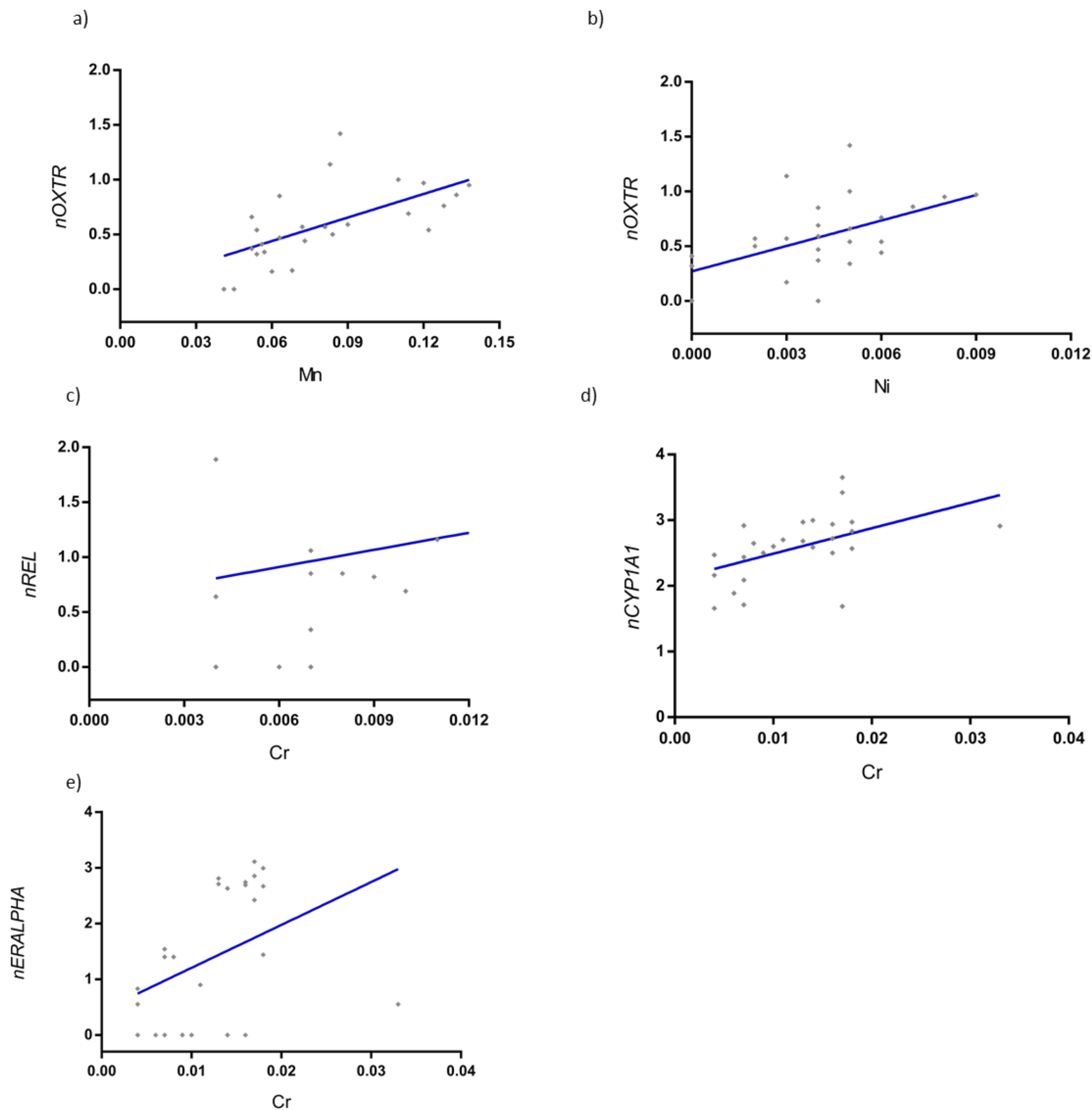
- ***Newborns***

Epigenetic data obtained from buccal cells of newborns were compared with Mn, Pb, Cr, As, Hg, Cd and Ni as well as with dioxin and PCB dioxin-like compound concentrations.

In detail, *nOXTR* gene methylation levels was found to positive correlate with both Mn (p = 0,0007; r = 0,6) (Fig. 15, a): and Ni (p = 0,0081; r = 0,5) (Fig. 15, b) in buccal cells of newborns. Additionally, the increase of *nwbCYP1A1* (p = 0,0087; r = 0,5) (Fig. 15, d), *nwbERα* (p = 0,038; r = 0,4) (Fig. 15, e) and *nwbREL* (p = 0,0434; r = 0,4) (Fig. 15, c) gene methylation levels was found related to a growing presence of Cr (fig.15).

No correlations were found between infants' DNA and Pb, Hg and Cd exposure.

Figure 15: The influence of heavy metals exposure on neonatal buccal cells. Graphs show methylation values transformed with natural log.

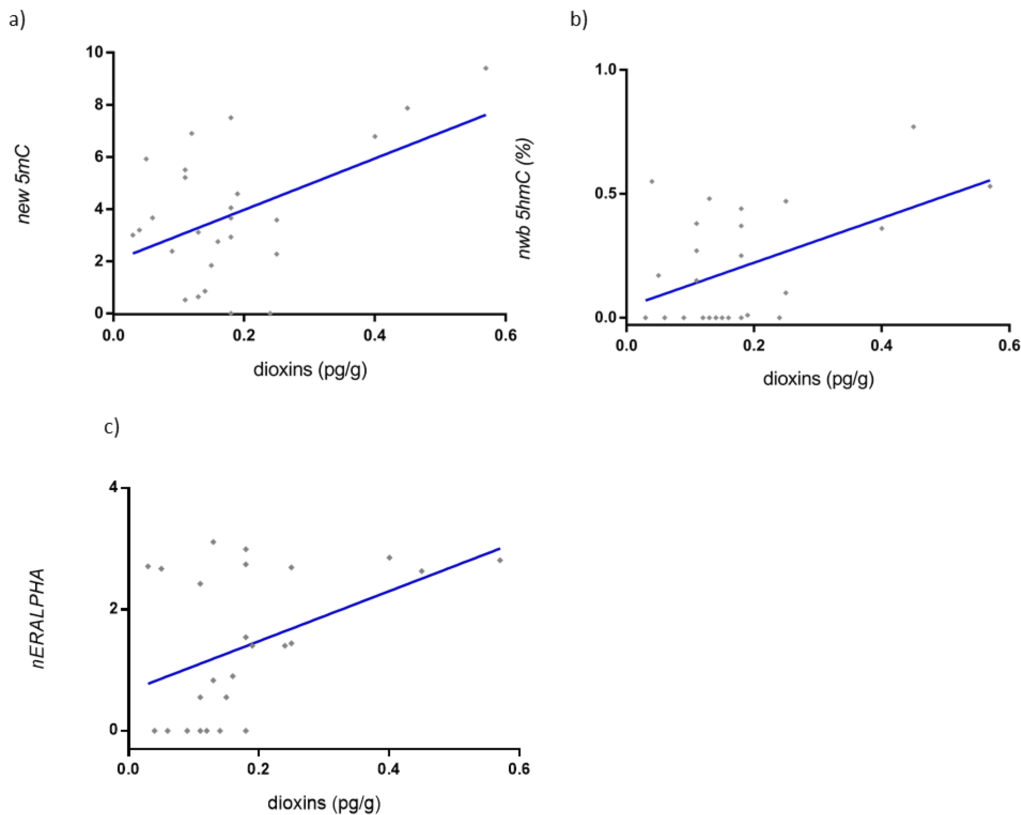


- Neonatal buccal cells methylation compared to dioxin and PCB dioxin-like exposure

Total ng/g of dioxin detected in placenta samples were compared with data obtained from epigenetic analyses conducted on neonatal DNA (Fig. 16).

Data obtained from statistical analyses showed a growing *ERα* gene promoter methylation corresponding to high ng/g of dioxin detected in placenta tissue (p = 0,0291; r = 0,4) (Fig. 16, d). Additionally, both 5mC and 5hmC levels were found to positively correlate with total amount of dioxins (Fig. 16, a-b). No correlation was found with PCB dioxin-like exposure.

Figure 16: The influence of dioxins exposure on maternal buccal cells



- ***Placenta***

Epigenetic data obtained from placenta samples were compared with Mn, Pb, Cr, As, Hg, Cd and Ni as well as with dioxin and PCB dioxin-like compound concentrations.

Among all the comparisons done, a positive correlation was found between *plcBDNF* (p = 0,008; r = 0,508149), *plcH19*(p = 0,0001; r = 0,677818) and *plcOXTR* (p = 0,002; r = 0,577667) genes and Mn concentration (Fig 17,a, c, d).

Methylation of *plcH19* and *plcOXTR* genes was found to correlate with a concentration of Ni (p=0,0176; r= 0,461657) and Pb *pH19*(p=0,035; r= 0,415059); *plcOXTR* (p=0,0029; r= 0,560251) respectively (Fig. 17, b. e)).

However, it should to be noted that only one placenta sample showed an elevated Pb concentration (0,043 mg/Kg) that strongly affected the final results. Indeed, after the removal of this outlier from the analyses, the positive association between *plcOXTR* gene and Pb concentration was lost (p=0,0558), while the *H19* one was strengthened (p= 0,004; r=0,6) (fig.18).

No correlations were found between methylation and Hg and Cr toxicant concentrations.

Figure 17: The influence of heavy metals exposure on placental cells methylation Graphs show methylation values transformed with natural log.

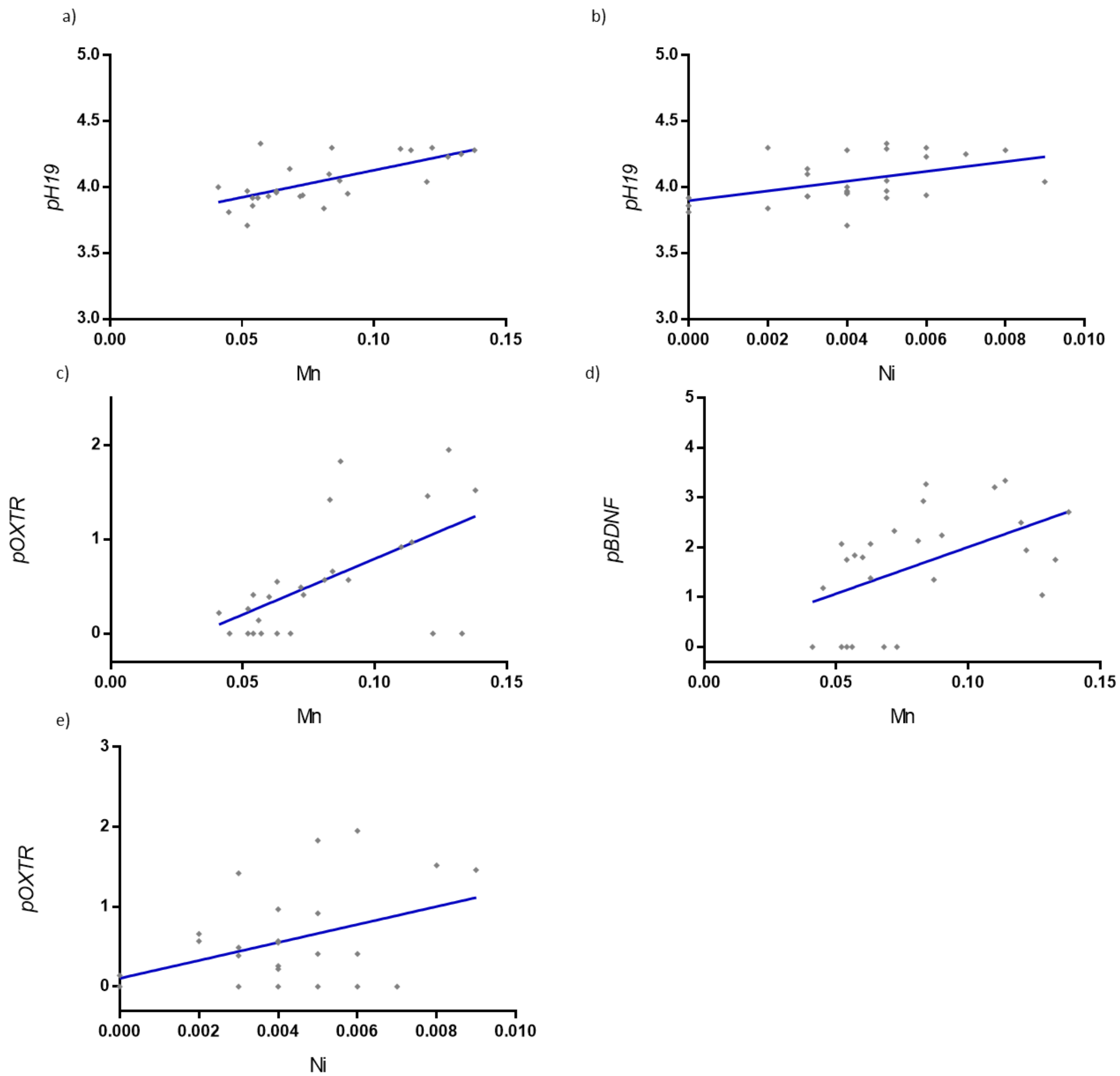
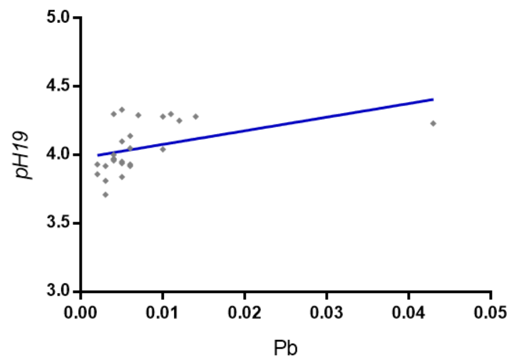
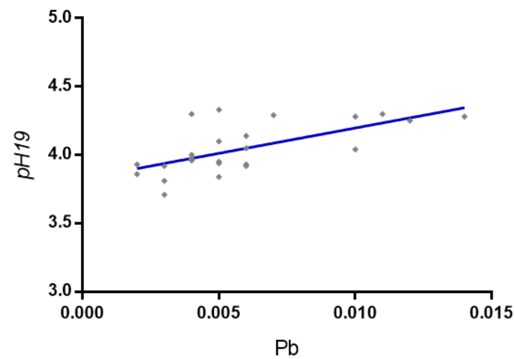


Figure 18: On the left, the correlation between H19 gene and Pi are showed including the outlier. On the right, the same analysis is showed without the outlier. Graphs show methylation values transformed with natural log.





Discussion and Conclusions

This work was inspired by the hypothesis of Barker, who first presupposed the potential role of early life environmental exposure on the risk of developing later in life complex diseases.
About 20 years later, his considerations are now a widely validated theory known as DOHaD theory. Indeed, this intriguing and novel research field has been explored in a plethora of different scientific works aimed at investigating the variety of exposure factors and of environmental cues that can influence mother health and consequently the life in the womb.
Undoubtedly, placenta reveals its central role in receiving and mediating the adaptative responses to these external stimuli. Thus, placental physiology allows the correct fetal programming based on the adverse (or not) conditions in which the new life will grow. These influences can mark DNA also at the level of reproductive cells, guaranteeing a memory of the exposure in the next generations.
Coherently with this perspective, the present thesis aimed at investigating how methylation - at both global and gene specific levels - can be influenced by the environment during gestation.
In detail, gene-specific methylation levels of 13 candidate genes involved in different cellular pathways were established using MS- HRM technique, while the total percentage of global 5mC and hmC contents were investigated using ELISA assays.
The epigenetic analyses were performed on a study cohort of 26 pregnant women recruited in Tuscany, one of the Italian regions involved in a larger project funded by the Italian Ministry of Health. Two different tissues from the cohort were collected: buccal epithelial cells both from the mothers and their neonates, as well as placental tissues, allowing to obtain information from both the peripheral tissues and from the organ tissue directly involved in fetal development.
Moreover, buccal cell tissue is widely used in peripheral biomarker research, due to its easier accessibility and storage (at room temperature until one year) with respect to the peripheral blood, even without specialistic competence (Lehmann *et al.,* 2011).

Indeed, this tissue was chosen for its low sampling invasiveness, which also made easier to obtain the consent of the mothers to participate to the project.
Global epigenetic analyses conducted on mucosa cells of the mother-newborn couples and on placental tissue reveal the specific tissue global methylation patterns.
Our data reveal a percentage of placenta global methylation (5mc: 5,04 ± 2,77 and 5hmC: 0,41 ± 0,50) greater than the percentage detected in mucosa cells of both mother (5mC: 3,74 ± 2,68 and 5hmC: 0,19 ± 0,25) and newborns (5mC: 3,77 ± 2,53 and 5hmC: 0,26 ± 0,32): however, such differences do not reach statistical significance. In literature, global hypomethylation of both mucosa cells and the placenta tissue was reported (Schroeder *et al*., 2013; He *et al.,* 2014 Godderis *et al*., 2015). Lowe and colleagues (2013) identified several buccal cells specific DMR, that are significantly hypomethylated relative to blood.
On the other hand, placental hypomethylation pattern could be explained because of the presence of large blocks named Partially Methylated Domains (PMDs), accounting for about 37% of the placental genome, together with LINE1 hypomethylation observed in the third trimester placenta.
Statistical analyses considered all factors singularly, revealing several correlations with maternal and neonatal features at both peripheral and placental levels.
Data concerning relevant exposures occurred during pregnancy - such as living context, stressful events, occupational exposures etc. - and maternal habits were collected after the administration of a questionnaire, as well as maternal and neonatal clinical data.
Furthermore, heavy metals, dioxin and PCB were detected in placenta samples by the IZSLER Institute of Bologna.

I. Comparisons among epigenetic results, questionnaire and clinical data

According to inclusion criteria, all the women recruited were characterized by a normal pre-pregnancy BMI (21,91 ± 2,69 kg/m^2) and did not present metabolic or immune diseases (such as diabetes, gestational diabetes, thyroiditis, heart failure), nor placenta anomalies (such as placenta previa, accreta, percreta etc.) that can affect fetal development (Apicella *et al*., 2019).
Moreover, the women were not addicted to any substance – smoke, drugs or alcohol – during the pregnancy period.
However, despite some comparisons were lost after Bonferroni correction – probably because of the sample size that we have collected –, most of them remained. The impact of gender, stressful events

and passive smoke exposure on the epigenetic patterns were emphasized, as well as the correlations with maternal/neonate age and clinical parameters (pre-pregnancy BMI, length and head circumference) (Tab.7,8,9).

The contribution of parents in the correct development of the embryo is intuitively important. Our data suggest that maternal age influences both gene-specific methylation levels – *matLEP* positively and *matH19* negatively – and global 5methyl cytosine content in maternal buccal cells, that decreases with maternal age. Generally, age-dependent changes in DNA methylation include global hypomethylation and locus specific hypermethylation (Xiao *et al.*, 2016). These changes have been the focus of several studies that investigated the role of maternal age on both pregnancy complications and newborns outcomes. Indeed, GD, diabetes, gestational hypertension, cesarean delivery rates or preterm birth were found more common in the very advanced maternal age (usually considered women aged > 40 years) (Odibo *et al*., 2006; Carolan *et al*., 2013; Başer *et al.*,2013; Mutz-Dehbalaie *et al*.,2014; Kahveci *et al*., 2018).

Moreover, an increased risk in developing several pathologies, such as type 1 diabetes and neurodevelopmental diseases, was found associated with parental age (Adkins *et al*., 2011).

Interestingly, in our study methylation changes of genes involved in these pregnancy complications have been identified, and, for some of them, these relations can be observed also in newborns. For example, a previous study showed a lower *H19* gene methylation also in cord blood of neonates related to maternal age as well as to serum folate concentrations, serum homocysteine concentrations (Miyaso *et al*.,2017).

Moreover, maternal BMI also emerges in this work as a negative modulator of *plcMTHFR* methylation. Literature reports a raised *MTHFR* gene expression in the placentas of obese women with GD. The increase in the synthesis of 5-MTHF could play a role in inhibiting the release of intracellular homocysteine. The catabolism of folate in the placenta would limit the accumulation of homocysteine within the trophoblast, thus avoiding fetal complications (Martino *et al*., 2018). However, more recently an epigenome-wide association study investigated placental DNA methylation changes associated with maternal pre-gravid BMI and the weight gain during pregnancy, revealing several CpGs previously implicated in obesity traits in children and adults (Shrestha *et al*., 2020). Coherently, elevated BMI before pregnancy and excessive gestational weight gain have been linked to an increased risk of gestational diabetes mellitus, gestational hypertension and macrosomia and LGA newborns (Nucci *et al*., 2018; Sun *et al*., 2020).

Among our data, gestational age was found positively related with *matBDNF* promoter methylation. Other studies investigated this gene, revealing higher maternal blood but lower cord blood and

placental BDNF levels. These findings were associated with prematurity and lower gestational age: furtherly, cord blood levels could also predict birth complications such as abnormal fetal growth and brain development. These may also increase the risk for cardiovascular disease, metabolic syndromes and neurodevelopmental disorders in children born preterm (Dhobale *et al*., 2014; Kertes *et al*.,2017).
In this work, we also investigated the contribution of gender in methylation pattern establishment.
In detail, the neonatal promoters of *CYP1A1* and *MECP2* genes were significantly more methylated in females. The expression of several genes is known to be characterized by sexual dimorphism, which depends on several mechanisms including sex chromosomes, sex hormones, and even epigenetic mechanisms. Moreover, the incidence of several human diseases, phenotypic outcome, response to treatment or exposure are deeply influenced by gender.
MECP2 gene was also found more methylated in females, according with its location on X chromosome. Sex specific patterns of this gene were widely studied for their involvement in neurodevelopmental disorder, mainly in males (for example, ASD showed a male-to-female ratio of 4:1). Indeed, mild reductions in MeCP2 protein expression have typically been associated with social and neurodevelopmental disorders, predominantly in males, with specific timing. Some authors have identified a critical time windows for brain development in the postnatal period. They consider both hormonal and chromosomal features at the basis of the differences in expression in the two sexes.
A first observation concerns the different exposure to steroid hormones produced by the gonads that follow a different temporal development in the two sexes. The second proposed mechanism concerns the inactivation of the X chromosome, which could help in the sex stratification of disorders associated with *MECP2* (Kurian *et al*., 2007).
Functional studies in rodents and cells have highlighted also the differential regulation of the CYP family between the sexes. Penaloza and colleagues (2014) thoroughly investigated CYP family promoters, including *Cyp1a1* gene. Interestingly, its promoter region showed both the ERE (estrogen response element) and GRE (glucocorticoid response element) regions, suggesting a specific hormonal response. Furthermore, the promoter contains 3 specific CpG sites for females, while in males there are 2 (1 of them is common) also defining a sex-specific regulation at the epigenetic level. Indeed, two of the three CpG sites that are more methylated in females show the same pattern in the cord blood of infants with prenatal tobacco exposure (Joubert *et al.,* 2012).
In the context of this study, these differences between neonatal *CYP1A1* methylation and smoke exposure did not arise, but a higher methylation level in *matHSD11B2* was found in women exposed to second-hand smoke. At the best of our knowledge, this is the first study in which this result has reported in maternal buccal cells genome associated with passive smoke exposure, that could mean a reduction of enzyme activity (according to gene features reported by Alikhani-Koopaei *et al*., 2004).

The adverse consequences of cigarette smoke – as well as its passive exposure – on health are well known as powerful cardiovascular and hypertension risk factors (Kim *et al.*, 2019). Regulation of 11βHSD2 enzyme expression plays a relevant role in blood pressure control and hypertension. Indeed, Friso and colleagues (2008) found higher *HSD11B2* promoter methylation in blood of patients with elevated urinary THFs/THE ratio – tetrahydrocortisol/tetrahydrocortisone metabolites were used as a biochemical indicator of enzyme activity – associated with hypertension.
Despite the different experimental design and the tissue considered for the epigenetic analyses, their results could support our findings.
However, *HSD11B2* has been extensively studied in placental tissues for its action – cells protection from the growth inhibitory and proapoptotic effects of cortisone – during fetal development. Higher methylation of this gene was found related to a different kind of exposure, from environmental pollutants to maternal anxiety and distress.
Indeed, stressful events that occur during gestation increase the circulating glucocorticoids that can interfere with *BDNF* signalling pathways – which is a crucial element for embryo neurodevelopment and synaptic plasticity – and co-regulate activation of the HPA axis (Chiba *et al*., 2012; Jeanneteau *et al.,*2012).
Among our data, *matBDNF* promoter was found with higher levels of methylation in the 8 women that experienced stressful events during pregnancy, such as family bereavement, job loss, relocation and legal problems.
The expression of BDNF has been associated with learning, memory formation and consolidation, as well as with behaviour and fear. It has been extensively studied in animal models, revealing a spatial, temporal and gender specific pattern (Bekinschteine *et al*., 2007, Miao et al. 2020).
A similar pattern emerges in humans, where altered *BDNF* regulation was detected in a variety of neurodevelopmental and mental disorders (Hauck *et al*., 2010; Suliman *et al*., 2013).
In the adults, *BDNF* methylation levels were investigated in exons I and IV in blood or saliva samples, related to anxiety, depression, post-traumatic stress disorder, and a history of childhood maltreatment or domestic violence (Perround *et al*., 2013; Moser *et al.,* 2015). Maternal experiences of war trauma and chronic stress reflect specific DNA methylation levels across the *BDNF* gene in maternal blood as well as in cord blood and placental tissue (Kertes et al., 2017).
Therefore, maternal mood can affect the offspring through perturbation of maternal HPA axis during pregnancy, in which *BDNF regulation is implicated as well as main genes that regulate the activity of HPA axis response, including NR3C1, HSD11B2 and FK506 binding protein (FKBP5) (Kunugi et al., 2010;* Conradt *et al*., 2013; Monk *et al.,* 2016)*. It results an important mechanism in prenatal programming and was investigated by several studies aimed to elucidated early life consequences*

(Freson et al., 2013; Hompes *et al., 2013;* Togher *et al*., 2017*). For example,* Braithwaite and colleagues (2015) have investigated the effect of maternal prenatal depressive symptoms of both *NR3C1 1F and BDNF* promoter IV in buccal cells of 2-month-old infant. Their study revealed changes in DNA methylation within the *NR3C1* gene, predominantly in male infants, and a decrease of DNA *BDNF* IV methylation in association with maternal depression.

Intriguingly, Vangeel and colleagues (2015) have investigated maternal emotional stress and cortisol levels related with DNA methylation of *IGF2* and *GNAS* genes of infants. Thus, the contribution of maternal anxiety can be integrated with many other exposure factors – such as prenatally famine or toxicant exposure – that are known to affected methylation of these genes.

In this work, correlation with maternal stress and imprinting genes methylation were not found. However, only the I promoter region of *BDNF* gene was included in the epigenetic analyses. Further investigation including the IV exon of the gene could be relevant in the detection of newborn and placenta response to maternal stress during gestation. Additionally, will be of great interest to integrate *NR3C1* gene in our investigation, because of its known involvement in maternal mood or stress response.

In this thesis, also prenatal exposure to infections was investigated. A relevant decrease of 5mC global placental content was found in mothers who had influenza or fever during pregnancy. Our result is of great actuality considering the historical period that we are living. An explosion of recent works are aimed to define the consequence of the transmission of novel coronavirus (SARS-CoV-2) in pregnancy (Alberca *et al*., 2020; Forestieri *et al*., 2020; Chen *et al*.,2020)

However, at the best of our knowledge, this is the first study that shows this difference in placental 5mC percentage consequent to febrile manifestation or influenza. In the literature, epidemiological studies – supported by translational work in animal models – associate prenatal exposure to infection or inflammatory manifestations as risk factors for neurodevelopmental disorders onset, including schizophrenia and ASD. Animal strong immunological stimulation during gestation – using polyinosinic-polycytidylic acid (Poly (I:C)) and lipopolysaccharide (LPS) which mimic viral and bacterial maternal infections and activate the Toll-like receptor 3 and 4 pathways respectively – resulted in a general epigenetic alteration involving acetylation, DNA methylation, and microRNA expression (Brown, 2012; Zerbo *et al*., 2015; Bergdolt and Dunaevsky, 2019). Physiologically, immune molecules have regulatory roles throughout neurodevelopment, contributing to the correct formation of neural circuits (Dunaevsky, 2019). Thus, maternal infection dysregulates the immune equilibrium between the maternal and fetal environments, resulting in an altered immune profile in the developing brain.

II. Comparisons among epigenetic data, heavy metal and dioxin levels

In the second part of this work, we focused on methylation changes (at gene-specific and global levels) that occur in response to both heavy metals – including Mn, Pb, Cr, Hg, Ni and Cd –, dioxin and PCB dioxin like. These compounds were measured in placenta tissue by IZSLER Institute of Bologna, and their concentration on wet weight was correlated with our epigenetic data.
According to European community As, Cd, Co, Cr, Cu, Hg, Mn, Ni, and Pb are the chemical elements of highest concern for human health (WHO report).
Some heavy metals have essential roles for physiological processes – such as copper for what concern the activity of several enzymes including ferro-oxidase or cytochrome C oxidase –, while others are essential nutrients in human diets (e.g., Ni or Cr). However, most of them have an intrinsic toxicity that affect the central nervous system (Hg, Pb, As), as well as the kidney, the liver (Hg, Pb, Cd), and even skin, bones, or teeth (Ni, Cd, Cu, Cr) (Bitto *et al*., 2014).
Thus, several studies investigated the role of this toxicants on the human health, particularly related to prenatal exposure and child outcomes. For example, influence of heavy metals on birth weight has been related to Cd level concentration in both maternal and fetal serum of the small foetuses (Sabra *et al*., 2017)
In this study, we first related methylation changes in maternal/neonatal mucosa cells and placental tissue with Pb, Mn, Cr, Hg, Ni Cd and both dioxin and PCB concentrations.
Intriguingly, among the data obtained, a direct evidence was that *OXTR* gene was more methylated in all the three tissue samples considered in response to higher Mn concentration, as well as placental *BDNF* methylation level. Moreover, the same trend can be observed in neonatal and placental *OXTR* gene methylation in response to Ni concentration. *OXTR* gene methylation was also responsive to Cd and Hg, particularly in maternal mucosa cells. Additionally, *REL* methylation detected in newborn buccal cells increase with growing Cr concentration.
Higher methylation of *OXTR* gene has been related to a wide range of mental impairments, social cognitive deficits in ASD, affects regulation problems or problems with facial and emotional recognition. On the other hand, low methylation of this gene has been associated with autism, perinatal stress, postnatal depression and social distress (Maud *et al*., 2018). Similarly, *BDNF* and *REL* methylation changes were investigated in response to mater/newborn stress transmission, neurodevelopmental impairment and neuropsychiatric disorders including autism, schizophrenia, bipolar disorder and others (Folsom and Fatemi, 2013).

Currently, it has been well documented that Pb, Cd, Hg, Ni, Mg and As exposures *in utero* produce several adverse birth outcomes in humans, due mainly to the permeable nature of the placenta.
The exposure results in DNA damage, oxidative cell stress, nervous system affection, glucose metabolism impairment, and endocrine disruption (McDermot *et al*., 2015).
For example, Mn is common in the environment, and it is also present in the whole food chain and drinking water (as a main toxicant analyzed in this context). It induces oxidative stress in the brain, that is most vulnerable due to its great metabolic activity and low levels of antioxidants (Emam *et al*., 2020). Indeed, Mn was further associated with an increased risk of behavioural disorders and affected the Full-Scale IQ and verbal IQ of school-aged children with Pb co-exposure (Gorini *et al*., 2014).
Additionally, a recent review has reported associations between: maternal exposures to Pb and low birth weight, preterm birth, stillbirths, spontaneous abortions and hypertension; Cd and low birth weight, neurological impairment and low Apgar score; and Hg and spontaneous abortions and neurotoxic effects (Rahman *et al*., 2016).
Shah-Kulkarni and colleagues (2020) showed data on prenatal exposures to metal mixtures (measured in women venous blood) at different time points of pregnancy, and neurodevelopment in 6 months children early exposed to lead, mercury and cadmium. Their results revealed that the exposure to lead in late pregnancy showed the most neuro-toxic effects.
All these considerations are coherent with our findings, that show a response of the gene analysed that are involved in neurodevelopment in presence of higher levels of these neurotoxicant compounds. Additionally, some studies corroborated our data showing a lower BDNF level in blood serum in response to Mn and other toxicants co-exposure (Zou et al., 2014; Zhou *et al*., 2019).
Among our results, of great interest are the correlations between placental *H19* gene methylation, that positively increase with Pb, Ni as well as Mn concentration. Abnormal *IGF2/H19* methylation in placenta suggests the fact that the developing fetus may be exposed to an adverse intrauterine environment. At the best or our knowledge, the current literature does not report associations with heavy metals considered singularly.
However, exposures to phthalates or phenols were associated with decreased methylation at the imprinting control region *H19*, *IGF2DMR0* and *IGF2DMR2* in the placenta. They also found sexually dimorphic alterations of methylation following prenatal phthalate and phenol exposure (LaRocca et al., 2014). Moreover, Li and colleagues (2016) showed that the early infant Pb exposure (detected in infants' blood) survives as a sex-specific methylation mark in different DMRs, including the female-specific decrease of *IGF2/H19* DMRs, during the growth.
Additionally, prenatal $PM_{2.5}$ exposure was found to have a genetic region-specific significant association with *IGF2* and *H19* in cord blood during specific gestational weeks, also in a sex-specific

manner (Wang et al., 2020). Furthermore, maternal exposure to $PM_{2.5}$ and black carbon was found associated with changes in placental imprinted gene expression, which suggests that air pollution can affect fetal growth and development (Kingsley et al.,2017).
Moreover, our analyses reveal also the exclusive maternal response to Cr and Ni exposure, resulting in an alteration of *LEP* and *CYP1A1* methylation respectively. In detail, *LEP* gene promoter methylation decreases, while the *CYP1A1* one shows an opposite trend.
Cr, as well as Ni, is wildly applicated in industrial processes (e.g., chrome plating and stainless-steel welding). Thus, it could be included among the occupational exposure compounds, and it is also be related to lung cancer. An in vitro exposure of murine hepatoma cells to Cr showed transcriptional repression of Cyp1a1 by local cross-linking of Hdac and Dnmt1 and altered histone marks. This may hinder the access of histone-modifying complexes, resulting in *CYP1a1* reduced expression (Schnekenburger et al., 2007; Christensen and Marsit, 2011).
Although we did not register any pathological manifestation in the women recruited, our finding could reflect these mechanisms.
On the other hand, similarly to our findings, Saenen and colleagues (2017) reported a negative association with *LEP* promoter methylation in placenta tissue following PM2.5 exposure during the second trimester, as well as with placental 3-NTp, a marker of oxidative/nitrosative stress.
Nowadays, different pathways affected by chemical exposure are showed - that could increase the risk of metabolic diseases later in life -, in which the role of aberrant *LEP* promoter methylation is known (Lesseur *et al.,* 2013). Indeed, significant shreds of evidence highlight the effect of these exposures on fetal and postnatal growth, organ development, and hormonal axes impairment.
For example, the increased concentrations of urinary Ni in early pregnancy have been associated with a greater risk of GDM, either evaluated individually or as a metal mixture (Wang *et al*., 2020).
Thus, several extensive evidences associate these early outcomes in an increased incidence of adult-onset diseases, including obesity, type 2 diabetes, hypertension, and others (De Long *et al.,* 2017).

The second class of toxicant that we considered was dioxin and PCB dioxin-like compounds.
Dioxin is a common designation for a large group of heterocyclic polychlorinated compounds.

Human exposure to both dioxin and dioxin-like compounds was documented in several historical accidents, such as a chemical factory in Seveso (Italy), the production of phenoxy herbicides (Russia, New Zealand), or the use of Agent Orange (AO) defoliant during the Vietnam War.
Following these accidental exposures, the genotoxic effects on health induced by these compounds

was revelated: malignant neoplasms, peripheral neuropathy, Parkinson's disease, coronary heart disease, immunodeficiency disorders, congenital malformations end many others.

In our study, we explored the methylation changes that could mediate the response to dioxin and dioxin-like compounds during pregnancy.

Our results reveal a positive correlation in both 5mC and 5hmC content in newborn buccal cells, that increased in response to dioxins concentrations.
Animal studies support our results: it was shown that maternal exposure to TCDD leads to an increase in global DNA methylation in fetal C57BL/6J mice (Yuan et al., 2016).
On the other hand, several human studies have been reported. Recently, Xu and co-workers (2019) showed that DNA damage and global DNA hypomethylation (as % of 5mC and 5hmC) affect child living near municipal waste incinerator (that was negatively correlated with blood PCDD/Fs).
Other studies found an inverse correlation between serum concentrations of persistent organic pollutants and global DNA methylations (mainly measured as LINE1 and Alu-repeat methylation levels in leucocytes) in adults (Rusiecki *et al*., 2008; Kim *et al*., 2010; Itoh *et al*., 2014). Instead, global DNA hypermethylation levels were associated with the increasing concentrations of PCB126, TCDD dioxin, and DDE pesticide in elderly representatives of the Swedish population (Lind *et al*., 2013). Moreover, Alu hypomethylation was detected in cord blood cells at birth associated with prenatal exposure to DDT/E (Kuzmina and Rubanovich, 2020).
All the differences that can be found among these studies and our results, could be explained first by the difference in tissues analysed. Indeed, the above-mentioned studies principally used blood as peripherical tissue, and the analyses were conducted on children, while we detected these changes in newborns. Finally, most of them used Line and Alu elements to establish the global methylation pattern of their cohort.
In addition to the global methylation level, our data suggest also a specific response to dioxins and PCB dioxin-like exclusively in maternal cells.
In details, dioxins exposure affects *BDNF* and *DNMT3D* methylation levels, resulting in a decrease in their methylation patterns. On the other hand, PCB dioxin-like was found positively related to *MECP2* promoter methylation.
Studies on animal models show the impact of BPA exposure on increased expression of Mecp2 and MECP2 binding in rat primary neuron cultures (Yeo *et al*., 2013), while more investigation need in human studies. This could be a first evidence of MECP2 promoter pattern in response to PCB dioxin-like expositions, that may cause adverse neurodevelopmental outcomes (Locke and Lein, 2020).

Animal models also gave rise to transcriptomic data that confirmed TCDD-induced downregulation of DNA methyltransferase genes, including dnmt3b.
We know from the literature data that both early and postnatal exposure to PCBs was associated with adverse neurodevelopmental outcomes and behaviour. New evidences about the link between POPs, especially PCBs, and autism have been highlighted, but the studies are still limited (Lyall et al., 2014; Rossignol et al., 2014). In a systematic review of Gascon and colleagues (2013) the association between prenatal exposure to DDE, PCBs and dioxins and risk of respiratory infections was also reported, as well as the reduced immune response after vaccination in child postnatally exposed to PCBs.

Conclusions

Critical periods of human development, including perinatal period and early life, are sensitive to the environmental cues. Indeed, embryo genome is set by these environmental influences (that are translated by epigenetic mechanisms), in a way that is either adaptive or maladaptive.
Indeed, these adaptations can prepare the individual to better survive in its environment, but could also predispose to several metabolic, cardiovascular, neurodevelopmental even affective disorders.
In this thesis, preliminary data of a broader project funded by the Italian Ministry of Health are showed and discussed.
This is a multicentric study that proposes to improve our knowledge on the maternal-fetal exposure to environmental pollutants, and on the main biological-molecular mechanisms of damage (particularly epigenetic) that occur during the first 1000 days of life.
It is a structured and multidisciplinary approach aimed at identifying early biomarkers of exposure and damage at the fetal level. These data will be useful in the neonatology and pediatric field for a surveillance system development for primary prevention and early diagnosis of the main emerging chronic disorders and diseases. The long-term goal of the project is to develop a study model that relates maternal-fetal exposure to heavy metals and potentially epi-genotoxic compounds with the increase of endocrine-metabolic pathologies, neurodevelopmental disorders and immune-mediated pathologies, through a multidisciplinary approach.
Within the project, this thesis had as its objective the research and evaluation of epigenetic biomarkers: gene-specific methylation in biological samples (placenta and buccal mucosal cells of mothers and children) from one of the four cohorts identified: the Pisa cohort.

The epigenetic profiles obtained from our cohort in a first step have been integrated with data concerning the estimation of the maternal-fetal pollution rate by analyzing the content of heavy metals, PAHs and PFAS in the placenta, using mass spectrometry by Working Group of Bologna.
In parallel, data concerning the mitochondrial regulation and activity in the placenta, metabolic profile in the urine of the couples mother-newborn and histological analysis of the placenta tissue have being collected for each cohort.
Finally, fundamental is the follow up of the babies, expected at one year of age, that started in these last months. The follow up will allow to collect information about the babies' growth during their first year of life. Additionally, data concerning the onset of pathologies that could be related to our findings (e.g. specific methylation patterns, exposure to toxicants.) during the babies' examination will be collected. Moreover, buccal swab and urine samples from the babies were being collected, and the epigenetic and metabolomic profile will be further analysed.
The first appreciable data within this project reveal the role of genes involved in cellular pathways capable of perceiving and responding to different stressors.
The gene expression remodelling is critical in the early stages of neuronal development. This can contribute to the onset of pathologies related to neurodevelopment - and often characterized by a different incidence between the two sexes - or predisposing the unborn child to them.
The epigenetic data - obtained from the Unit of Pisa - regarding gene-specific methylation analyses of 13 candidate gene and global % of 5mC and 5hmC content are shown.
Our results suggest interesting responses of the methylation patterns to the maternal and uterine environment, as well as to the exposure to toxic compounds.
Indeed, we found stress-specific response in maternal *BDNF* gene related to maternal stress, gender-specific epigenetic pattern and correlation with newborn and maternal clinical characteristics.
It is known that birth outcomes, for example birth weight or head circumference, are early markers of impairment that could be consolidated with growing (neurodevelopmental disorders, or more later in life, metabolism disorders).
Instead, for what concerns the toxicant analyses we found a massive response of a major gene involved in neurodevelopment pathways in response to heavy metals that could have a role in neurocognitive impairments. A global change in neonatal epigenome also emerged from our data.
Undoubtedly, the limitation of our study concerns the sample size obtained so far. However the grouping of the different cohorts, which will be done at the end of the various recruitments will be fundamental and will allow corroborating the findings obtained until now. Moreover, data concerning the analyses from the other Units will better contextualize all the exposures observed. It will allow also to have an overview of the pathways involved, that are responsive to different cues and exposure

observed. Additionally, the follow-up of the babies likely will give us the first information on how these early exposures can affected the early fetal programming.

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Supplementary material 1- Questionnaire

Gentili Signori,
stiamo conducendo uno studio, finanziato dal Ministero della Salute, il cui obiettivo principale è quello di individuare le relazioni tra inquinamento ambientale, gravidanza e periodo peri-post-natale (primi 1000 giorni di vita) per un sistema avanzato di sorveglianza ambiente-salute. Per svolgere questa ricerca avremmo bisogno della vostra collaborazione e disponibilità nel rispondere ad alcune domande specifiche sul vostro stato di salute e sul decorso della gravidanza. La vostra partecipazione è volontaria e in ogni momento avrete la facoltà di recedere dallo studio senza alcun obbligo nei confronti della nostra Struttura e senza che questo comporti qualsiasi problema nella futura assistenza e collaborazione fornita a vostro figlio. Essendo uno degli obiettivi principali dello studio quello di poter fornire una miglior assistenza sanitaria ai bambini, i dati da noi raccolti potranno anzi essere di notevole aiuto al vostro pediatra.

Codice identificativo del padre	
Codice identificativo della madre	

Età del padre	
Età della madre	

<u>Per il padre</u>

Luogo di nascita	

Che studi ha fatto? (barrare con una X)	Scuola elementare
	Scuola media inferiore
	2 o 3 anni di scuola media superiore
	Scuola superiore fino al diploma
	Qualche anno di università
	Università
	Corsi o specializzazioni post - laurea

Che lavoro svolge? (es. operaio, insegnante,…)	

Nella sua famiglia ci sono soggetti affetti o deceduti per: patologie cardiovascolari, neurologiche, psichiatriche, obesità, patologie metaboliche, malattie genetiche o malformazioni? (indicare patologia e grado di parentela; es. Nonno paterno, infarto)	

Lei è affetto da alcune delle patologie sopra elencate? Quali?	

Negli ultimi anni ha avuto esposizione prolungata e consistente a: solventi, insetticidi, metalli (in particolare arsenico, cadmio, piombo, nichel e mercurio), idrocarburi policiclici aromatici (IPA), plastificanti (Bisfenolo A, ftalati), bifenili policlorurati (PCB), diossine, ritardanti di fiamma a base di bromo?	

<u>**Per la madre**</u>

Luogo di nascita	

Che studi ha fatto? (barrare con una X)	Scuola elementare
	Scuola media inferiore
	2 o 3 anni di scuola media superiore
	Scuola superiore fino al diploma
	Qualche anno di università
	Università
	Corsi o specializzazioni post - laurea

Che lavoro svolge? (es. operaio, insegnante,…)	

Nella sua famiglia ci sono soggetti affetti o deceduti per: patologie cardiovascolari, neurologiche, psichiatriche, obesità/patologie metaboliche, malattie genetiche o malformazioni? (indicare patologia e grado di parentela; es. Nonno paterno, infarto)	

Lei è affetta da alcune delle patologie sopra elencate? Quali? *	

Negli ultimi anni ha avuto esposizione prolungata e consistente a: solventi, insetticidi, metalli (in particolare arsenico, cadmio, piombo, nichel e mercurio), idrocarburi policiclici aromatici (IPA), plastificanti (Bisfenolo A, ftalati), bifenili policlorurati (PCB), diossine, ritardanti di fiamma a base di bromo?	

In quale settimana della gravidanza si trova attualmente?	
Quante gravidanze a termine ha avuto?	
Quanti aborti spontanei ha avuto?	

Dove abita attualmente (durante la gravidanza): in un contesto urbano o rurale? (indirizzo completo e dettagli sul traffico veicolare o sulla vicinanza a impianti industriali o altre fonti particolari di inquinamento)	

Che lavoro svolgeva all'epoca del concepimento e della gravidanza? Fino a che mese ha lavorato?	

Era solita bere acqua del rubinetto o in bottiglia nel medesimo periodo?	

Sono state utilizzate tecniche di procreazione medicalmente assistita (FIVET, ICSI, ecc.)?*	

Che numero di gravidanza è quella di suo/a figlio/a?	

Assume alcol o droghe?*	

Fumava al momento del concepimento?	

Fuma in gravidanza?*	

Ci sono fumatori in casa o nell'ambiente lavorativo (fumo passivo)?	

Ha assunto continuativamente farmaci in gravidanza?	
Quali? In quale trimestre? Con quale posologia?	

Ha assunto folati, integratori vitaminici o probiotici in gravidanza?	
Quali? In quale trimestre? Con quale posologia?	

Ha avuto episodi di febbre o di influenza in gravidanza?	

Qual era il suo peso prima della gravidanza?	
E la sua altezza?	

Ha una dieta o esigenze nutrizionali specifiche? (i.e. vegetariana, vegana, allergie)?	
Se sì, specificare	

Mangia carne 2-3 volte a settimana?	
Mangia regolarmente più di 2-3 porzioni di frutta o verdura al giorno?	
Mangia pesce almeno 1-2 volte alla settimana?	
Consuma latticini (-latte, formaggi, yogurt) tutti i giorni?	
Mangia cibi integrali (pane, pasta, riso integrale) almeno 1 volta al giorno?	
Consuma snack confezionati, torte, dolciumi o bevande zuccherate meno di 5 volte a settimana?	
Utilizza sale iodato?	

Si espone regolarmente al sole (viso, braccia e mani per almeno 10-15 minuti al giorno)?	

Ha fatto un esame del sangue con emocromo e dosaggio dell'emoglobina?	
Se sì, il valore dell'emoglobina è superiore a 110 g/l? Inserire il valore	

Quanti caffè/tè beve al giorno?	

Quante volte alla settimana cammina più di 30 minuti consecutivi? (barrare con una X)	nessuna meno di 2 volte 2 o più volte

Durante la gravidanza si sono verificati eventi stressanti? (barrare con una X)	
Morte di un parente	
Morte di un amico	
Perdita del lavoro proprio o del partner	
Sfratto o perdita della casa	
Abusi o violenze	
Trasloco o un cambio di residenza	
Divorzio o separazione dal partner	
Terremoti, inondazioni, uragani o altre catastrofi naturali	
Problemi legali	
Problemi con i superiori al lavoro	
Diminuzione del reddito familiare	
Indebitamento del nucleo familiare	

La gravidanza è stata complicata da particolari problemi? Quali? (es. infezioni, diabete, gestosi)	

Intende allattare al seno il bambino?	

* Risposte positive rappresentano criteri di esclusione