Adulthood Prenatally Programmed Diseases: Health Relevance and Methods of Study

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1 Introduction

Cell structure and function depends on the synthesis of a number of proteins, such as structural proteins, enzymes, hormone and neurotransmitter receptors, and regulating proteins, among others. These proteins are synthesized following inherited genetic information coded in the genome, and change at the various stages of the differentiation of the cells through the ontogenic development. The above process can be selectively modulated by cell environment, mainly hormonal.

During precise and critical stages of the ontogenic development (which are specific for each cell-type), cells define and program quantity and quality of their hormone receptors, needed for the optimal regulation of their function and their homeostatic adaptation to environmental variations, that lasts for all the host individual life. This process was named imprinting or cell programming. If, during this critical period of cell development, any of these cell-types is exposed to abnormal hormone concentrations or to other chemical agents (i.e., pharmaceuticals, food additives, natural components of some foods, various polluting agents), this program is altered and leads to irreversible qualitative and/or quantitative changes in specific proteins, especially hormone or neurotransmitter receptors. This will originate persistent alterations in the regulation of affected cells function, which usually persist through life, and are the basis for the development of various diseases later in life.

The Hungarian biologist György Csaba and co-workers published the first report showing that experimental animals fetal exposure to various hormones or hormone action-displaying xenobiotics during critical periods of their development induces persistent changes in the action of related hormones (Csaba & Nagy, 1976; Csaba, 1980). These changes, detected later in life, include a modification in the activity of receptors and in the intensity of responses mediated by them (Csaba *et al*., 1980, 1984, 1986; Inczefi-Gonda *et al*., 1982; Csaba & Nagy, 1985; Dobozy *et al*., 1985). This effect of hormones during fetal or neonatal life, permanently modifying the ability of the cells to respond later to hormone stimulation, was named by Csaba "imprinting" (Csaba, 1980; Csaba *et al*. 1986). These results were confirmed in our Laboratory for prenatal (Arriaza *et al*., 1989) or early postnatal (Mena *et al*., 1992) androgens exposure and for prenatal diethylstilbestrol exposure (Tchernitchin & Mena, 2006), which alter responses to estrogen in the uterus later in life.

For humans, the first evidence that synthetic hormone prenatal exposure induces imprinting emerged from clear cell cervicovaginal adenocarcinoma development in young women whose mothers received treatment with diethylstilbestrol during pregnancy (Herbst, 1981; Verheigen *et al*., 1991). The cause-effect relationship was confirmed in experimental animals (Walker, 1983; Newbold *et al*., 1990), suggesting that prenatal or neonatal exposure to this agent induces permanent changes in some cell types. These changes become evident after puberty as enhanced risk for cancer development, probably under the effect of increased estrogen blood levels that normally occur following puberty, in addition to other persistent changes observed in the uterus and other organs (Stillman & Miller, 1984; Haney *et al*., 1986; Wingard & Turiel, 1988; Fenaux *et al*., 2004).

In addition to changes in hormone receptors quantity and quality in affected cell-types once they reach maturity, the imprinting process also involves biochemical, morphological and functional alterations in these cells. We proposed that the process discovered by Csaba *et al*. involves a modification of the routes of normal cell differentiation (Tchernitchin & Tchernitchin, 1992) or programming (Tchernitchin *et al*., 1999). Exposure to environmental agents during critical windows of susceptibility at early stages of development usually remain “silent” for a long period of time without any overt manifestations, but later in life increases the risk for the development of various diseases (Barker, 1999; Barker *et al*., 2002). Alterations to the epigenome are a possible mechanism explaining how early exposures increase

disease risk later in life. These epigenetic changes refer to modifications in gene expression influenced by DNA methylation and/or chromatin structure, histone modification, microRNA (miRNA) expression, RNA editing, and RNA interference without changes in DNA sequences (Baccarelli & Bollati, 2009; Li *et al*., 2012). DNA methylation is the best known epigenetic process regulating gene expression. It is a covalent modification, heritable by somatic cells after cell division. 5-methyl-cytosine represents 2-5% of all cytosines in mammalian genomes and is found primarily on CpG dinucleotides (Millar *et al*., 2003). DNA methylation regulates many cellular processes such as chromatin structure and remodeling, X-chromosome inactivation, genomic imprinting, chromosome stability, and gene transcription (Grewal & Moazed, 2003; Reik *et al*., 2001). Gene promoter hypermethylation is generally associated with decreased gene expression (Orphanides & Reinberg, 2002). Disturbances of DNA methylation process under exposure during the critical periods of life may be involved in the imprinting mechanism (Li *et al*. 2003).

Subsequent studies showed that imprinting is not only induced by prenatal or early postnatal exposure to abnormal hormone levels. It may also be induced by agents and conditions such as pharmaceuticals (Clemens *et al*., 1979; Rodríguez Echandía *et al*., 1988; Cagiano *et al*., 1990; Li *et al*., 2003; Lu *et al*., 2010, 2011a; Gunawardana *et al*., 2011), pollutants (Csaba & Inczefi-Gonda, 1984; Sierra & Uphouse, 1986; Eriksson *et al*., 1990; Dell'Omo *et al*. 1995; Li *et al*., 2003; Suzuki *et al*., 2004; Watanabe, 2005; Adewale *et al*., 2011; Dickerson *et al*., 2011), substances of abuse (Heyser *et al*., 1994; Hickey *et al*., 1995; Rubio *et al*., 1995; Moreno *et al*., 2003; Tchernitchin *et al*., 2009), food additives (Hughes & Beveridge, 1990; Nyakas *et al*., 1990), maternal nutritional characteristics including some natural food components (Csaba & Dobozy, 1977; Karnik *et al*., 1989; Brown *et al*., 1990; Contreras & Ryan 1990; McGrath, 2001; Wu *et al*., 2004), substances present in plants (Tchernitchin & Tchernitchin, 1999), and strong psychological stress (Ward, 1972; Dörner *et al*., 1983; Sobrian *et al*., 1997; Kay *et al*., 1998; Csaba *et al*., 2009; Schöpper *et al*., 2012) – probably through increases in stress-related hormones in maternal blood (Gounaris *et al*., 2007; Soliman *et al*., 2008; Sivamani *et al*., 2009). The imprinting process is induced following interaction of any of the above agents or conditions with the different cell-types at precise stages of their fetal or neonatal development – windows of susceptibility (Tchernitchin & Tchernitchin, 1992; Tchernitchin *et al*., 1999; Tchernitchin, 2005).

Changes in cell differentiation/programming induced by imprinting cause, later in adulthood, the development of diseases, such as cancer, endocrine abnormalities, infertility, immune diseases, psychic alterations, or changes in personality and behavior (Tchernitchin & Tchernitchin, 1992; Signorello & Trichopoulos, 1998; Tchernitchin *et al*., 1999; Holmang, 2001; McGrath, 2001; Miles *et al*., 2005; Tchernitchin, 2005; Tchernitchin & Mena, 2006; Somm *et al*., 2009; Tchernitchin *et al*., 2009; Adewale *et al*., 2011; Dickerson *et al*., 2011; Gunawardana *et al*., 2011). This process takes relevance in the determination of health conditions later on in life considering that it is not only generated by agents that are easy to avoid; it is also induced by agents difficult to detect and avoid, at very low concentrations.

The persistent effects of early exposure to toxic agents is frequently different from the effects caused by acute or chronic exposure to the same agents (vide infra). The most widely known delayed effects of acute or chronic exposure during adulthood are the development of malignancies and mutations affecting germinal cells and originating heritable diseases. Maternal exposure during the first stages of pregnancy may cause fetal malformations by mechanisms independent from imprinting.

The present report describes novel approaches to investigate the adverse effects caused by the imprinting mechanism in complex organs like the uterus, which we take as model in most of our experimental studies. It reviews lead, arsenic and dioxins as the best known imprinting inducing environmental pollutants relevant to human pathology. Finally, it provides a novel hypothesis to the role of imprinting

for species survival under adverse environmental conditions in the phylogenic process of species selection.

2 An Approach to the Study of Imprinting in Complex Organs: The Example of the Uterus

Imprinting is a process defining changes, for all life long, in the intensity and quality of responses of each cell-type to stimulation by a single hormone or neurotransmitter, through affecting their specific receptors. This process occurs at windows of susceptibility, usually at precise critial stages of prenatal or neonatal life, which are different for the different receptors within the same cell-type and for the different cell-types.

Most organs contain a number of different cell types, whose functions are different. Besides, there may exist different kind of receptors for the same hormone in any organ, or even in the same cell-type; therefore, each one can be imprinted at different and precise stages of their development. Therefore, a search for the imprinting effects of any agent in the action of single hormone requires the evaluation of the hormone action separately in every cell-type, considering that not all cell-types should be affected by hormone stimulation. Further, it is necessary to compare the effects of early exposure to the imprinter in the action of the hormone later in life, with the effects of adult acute or chronic exposure to the same agent, since the effects may be mediated by different mechanisms.

2.1 The Uterus is an Excellent Model to Study Imprinting: The dissociation Between Various Responses to Estrogen

The uterus as an excellent model to investigate the delayed effects of early exposure, since it is composed by several cell-types expressing estrogen receptors, and the hormone induces numerous biological responses. If all responses to estrogen were mediated by the same mechanism and all estrogen receptors were identical, as originally proposed (Jensen & DeSombre, 1972), the possibilities of a selective interaction with some but not all estrogenic responses would be scarce and any study of interference with a single estrogenic response would be sufficient to proof the presence or absence of the interaction, at least in the same uterine cell-type.

Up to now, the existence of at least two types of estrogen receptors (α and β) is accepted (Kuiper *et al*., 1996; Wang *et al*., 1999; Damdimopoulos *et al*., 2008). However, various estrogenic responses may be mediated by different kind of receptors. The earliest reports in this direction came from our Laboratories, describing estrogen binding by uterine eosinophil leukocytes (Tchernitchin, 1967, 1973; Tchernitchin & Chandross, 1973) and proposing that the early eosinophil migration to the uterus under estrogen stimulation (Tchernitchin *et al*., 1974a), and several uterine non genomic responses to estrogen (Tchernitchin, 1983; Tchernitchin *et al*., 1985b, 1989), are related to estrogen binding by the eosinophils (Tchernitchin, 1972; Tchernitchin *et al*., 1974b), through a novel mechanism (Tchernitchin, 1979; Galand *et al*., 1985; Tchernitchin *et al*., 1985b, 1989; Grunert *et al*., 1986; López *et al*., 1986). Others reported additional estrogen receptors (or binding proteins) and different mechanisms for hormonal action are: cytoplasmic membrane estrogen receptors (Pietras & Szego, 1980; Nenci *et al*., 1981), type II cytoplasmic and nuclear estrogen receptors (Markaverich *et al*., 1981) and specific antiestrogen receptors (Sutherland *et al*., 1980). Considering that an imprinter may selectively interact with some but not all estrogen receptors, or may act as hormone agonist for some receptors and as antagonist for others, it is

clear that it is necessary to investigate the effects of acute, chronic and prenatal exposure on every response to estrogen in each target organ, to verify the possible effects of exposure.

The dissociation of responses to estrogen in the uterus and in other target organs confirms the need to consider all responses to hormone stimulation in each target organ. This dissociation was reported under the action of the following agents or conditions: (a) route of estrogen administration, allowing physiological hormone levels locally or both locally and systemically (Tchernitchin & Galand, 1983); (b) the following estrogenic compounds: estriol (Tchernitchin *et al*., 1975b), estradiol-17α (Tchernitchin *et al*., 1989), diethylstilbestrol (Grunert *et al*., 1986), clomiphene (Grunert *et al*., 1987), nafoxidine (Galand *et al*., 1984, 1985), 2(OH)-estradiol-17β or 4(OH)estradiol-17β (Baumann *et al*., 1986); (c) interaction with glucocorticoids (Tchernitchin *et al*., 1975a), progesterone (Grunert *et al*., 1984b), insulin (Steinsapir *et al*., 1982a), or thyroid hormones (Steinsapir *et al*., 1982b)); (d) the following pharmaceuticals or biological reagents: teophylline (Steinsapir *et al*., 1982c), bromocriptine (Unda *et al*., 1999); actinomycin D (Tchernitchin & Galand, 1982) or colloidal carbon (López *et al*., 1986); (e) exposure to following environmental pollutants: lead (Tchernitchin *et al*., 2003) or, cadmium (Tchernitchin *et al*., 2008); and (f) phytoestrogens present in food (Gaete *et al*., 2010, 2011).

In experimental animals, delayed effects of prenatal (15^{th} or 18^{th} day of pregnancy) or neonatal exposures to imprinting-inducing agents may be investigated on uterine estrogenic responses induced in 21-day-old pre-pubertal or adult ovariectomized rats, and compared to effects of acute or chronic exposure at pre-pubertal or adult ages. Below we describe the methods we used in our Laboratory to evaluate these estrogenic responses.

2.2 Histological, Histochemical and Morphometrical Procedure for the Study of Responses to Estrogen in the Uterus

Each uterine formalin fixed and dehydrated horn was paraffin-embedded to obtain 5 μm thick uterine cross sections. One group of hydrated sections was stained 1-3 min in hematoxylin, washed in several changes of tap water, and transferred for 1 min to a saturated lithium carbonate solution. Subsequently, they were stained in 1% eosin y aqueous solution, washed quickly in distilled water, and dehydrated in a graded series of ethyl alcohols, absolute ethanol and xylene (Grunert *et al*., 1984a). This procedure was used for eosinophil quantification (Tchernitchin *et al*., 1974a, 1985a), various uterine cell types mitoses counting (Grunert *et al*., 1986, 1987), morphometry (Grunert *et al*., 1984a), including computer assisted image analysis of luminal epithelial cell volume (Tchernitchin *et al*., 2003). Another group of hydrated uterine cross sections was stained 5 min with phosphate-buffered 0.01% acridine orange (pH 7.4) in distilled water, differentiated in 0.1% (wt/v) calcium chloride and covered with phosphate buffer (pH 7.4) to evaluate RNA and DNA densitometry by epifluorescence microscopy under excitation light λ=380-420 nm (Konarev, 1966).

2.3 Quantification of Estrogenic Responses in the Uterus

The following estrogenic responses were quantified in the uterus: uterine eosinophilia, percentage of eosinophils according to the distribution in different uterine histological layers and to their degree of degranulation; endometrial stroma edema; luminal epithelial cell RNA content, luminal epithelial and myometrium cell hypertrophy, and number of mitotic figures in luminal and glandular epithelia, endometrial stroma and myometrium. Each effect of estrogen action was evaluated at times it reaches its maximum (Tchernitchin *et al*., 1974a, 2003; Grunert *et al*., 1984a, 1986). Uterine eosinophilia reaches maximal response at 6 or 24 h, endometrial edema at 6 h, luminal epithelial RNA content, luminal epithelial

and myometrial cell hypertrophy, and luminal and glandular epithelial, stroma and myometrial cell proliferation reach maximal responses at 24 h after treatment. For each imprinting inducing agent, to compare the alterations of the different parameters of estrogen stimulation, all responses were expressed as % of maximal response, i.e., the value of vehicle-treated animals was considered as 0% response, and the value of maximal response to estradiol – 100% response.

Uterine eosinophilia (Tchernitchin *et al*., 1974a) was assessed in 30 uterine sections, distributed along the uterus (proximal, medial and caudal); eosinophils were classified according to their location within the uterine histological layers and to their degree of degranulation (Tchernitchin *et al*., 1985a; Grunert *et al*., 1986). The distribution of the eosinophils within the uterus reflect their migration mesometrium blood vessels towards the endometrium through uterine extravascular space (Soto & Tchernitchin, 1979). Edema in deep endometrial stroma (Grunert *et al*., 1984a) was evaluated by counting the number of nuclei a number of 1054 μm^2 areas delimited by a standard circle located in the ocular piece of the microscope. An increase in the reciprocal value of cell density (a decrease in cell counts within a standard area) in a location containing few cells and large extracellular space, reflects edema, since an increase in extracellular space, but not in the cell volume, decreases cell density (Grunert *et al*., 1984a). Myometrial cell hypertrophy (Grunert *et al*., 1984a) was evaluated by counting the number of nuclei in a number of standard areas. Increases in cell density reciprocal values in areas containing cell bodies with negligible extracellular space reflects increase in cell size (Grunert *et al*., 1984a). Luminal epithelial cell hypertrophy (increases in cell volume) was evaluated through computer assisted image analysis from digital micrographs, to calculate luminal epithelial cell volume (Tchernitchin *et al*., 2003). Luminal epithelial RNA content was evaluated from acridine orange stained uterine sections digital micrographs, through epifluorescence microscopy under excitation light, λ 380-420 nm, using computer assisted morphometry and cell densitometry (Tchernitchin *et al*., 2003). Estrogen-induced mitotic response was evaluated as an increase in the number of mitotic figures in luminal epithelium, glandular epithelium, endometrial stroma and myometrium, and quantified for each animal in a number of uterine cross sections considering all three uterine pieces (Grunert *et al*., 1986, 1987).

2.4 Other Estrogenic Responses that Needs to Investigate in the Uterus

Additional estrogenic responses should be investigated since they may be mediated by different estrogen receptors. Estrogen-induced myometrial contractility, nitric oxide release from uterine eosinophils (Suburo *et al*., 1995; Feder *et al*., 1997) or other uterine cells (involved in myometrial and in blood vessel relaxation), uterine hyperemia (Clewell *et al*., 1977), cAMP (Singhal, 1973; Kvinnsland, 1976; Flandroy & Galand, 1978) and cGMP (Flandroy & Galand, 1978) release, glycogen synthesis (Tchernitchin *et al*., 1975b) and concentration in uterine epithelial and myometrial cells; apoptoses in the various uterine cell-types (Stewart *et al*., 1999), and progesterone receptor expression (Reel *et al*., 1975; Aldad *et al*., 2011), among other responses.

Estrogen receptor expression in the different uterine cell-types may also be selectively modified in some uterine cell-types but not others, by perinatal, acute or chronic exposure. This effect may selectively affect some responses to hormone stimulation but not others. Estrogen receptor expression may be evaluated in the different uterine cell-types by radioautographic techniques (Tchernitchin *et al*., 1985b), and complementarily by immune cytochemistry, the latter may evaluate the number of hormone receptors but not their functionality, considering that hypothetical changes in the structure of a receptor may affect the response but not binding.

Additionally, some estrogenic responses in the uterus may be modulated by extrauterine conditions. For instance, taking into consideration the proposed role of eosinophils in some non-genomic re-

sponses to estrogen in the uterus (Tchernitchin, 1979; Tchernitchin & Galand, 1982; Tchernitchin *et al*., 1985b, 1989), the number of eosinophils in the blood is a required condition for their migration to the uterus under estrogen stimulation, and for the induction of eosinophil-mediated responses (Tchernitchin & Galand, 1983). Any condition decreasing blood eosinophils numbers, such as a treatment with cortisol (Tchernitchin *et al*., 1975a) or other eosinopenic hormones (Steinsapir, 1982a, 1982b), inhibits eosinophil-mediated responses in the uterus, such as endometrial edema (water imbibition) and histamine release. Eosinophil degranulation in the blood (Tchernitchin *et al*., 1985a; Grunert *et al*., 1986, 1987) may interfere with the estrogen-induced recognition of uterine vascular endothelial lining by the eosinophils, or with the release of the eosinophil enzymes into the uterus, required for the development of eosinophil-mediated responses. Estrogen-induced eosinophil migration to the uterus decreases following intravenous administration of colloidal carbon, which is known to be uptaken through phagocytosis by eosinophils and/or uterine vascular endothelial cells (López *et al*., 1986); these cells may have lost their surface estrogen receptors following the process of phagocytosis (López *et al*., 1986).

3 From Lead-Induced Infertility to a Hypothesis on the Relevance of Imprinting for Species Evolution

Several environmental agents cause reproductive alterations. Among them, lead affects male and female reproductive systems in humans (Winder, 1993) and experimental animals (Ronis *et al*., 1996). In women cause infertility, increase in time to achieve pregnancy, miscarriage, preeclampsia, pregnancy hypertension, premature delivery (Winder, 1993; Guerra-Tamayo *et al*., 2003; Al-Saleh *et al*., 2008), abnormal menstruations, hypermenorrhea, polymenorrhea, and spontaneous abortions (Tang & Zhu, 2003). In 1965, Gilfillan (1965) suggested that the declining birth rate in Rome's ruling class, which may have been at the root of the empire's dissolution, resulted from exposure to lead in food and wine. In monkeys, chronic lead exposure inhibits menstruation, ovulation, and follicular growth (Eck & Meigs, 1960). It delays vaginal opening in prepubertal rats (Kimmel *et al*., 1980) and decreases ova implantation and pregnancies in mice (Odenbro & Kihlstrom, 1977).

Several mechanisms for lead-induced inhibition of fertility were studied in laboratory animals. Changes occur at the enzyme levels (Wiebe *et al*., 1988; Kempinas *et al*., 1994) or in the action of sex hormones, mainly estrogens, in the uterus (Tchernitchin *et al*., 1998a, 1998b, 2003). The interaction of lead with hormone action may be direct, via changes in hormone receptors (Wide & Wide, 1980), or levels of other hormones modifying sex steroids action: glucocorticoids (Tchernitchin *et al*., 1975a) and prolactin (Unda *et al*., 1989), which increase under lead exposure (Vyskocil *et al*., 1991). Lead decreases pituitary response to GHRF (Camoratto *et al*., 1993), affecting levels of gonadotropin-releasing hormone, somatostatin (Sierra & Tiffany-Castiglioni, 1992), FSH and LH (McGivern *et al*., 1991). In agreement with the existence of independent mechanisms of estrogen action in the uterus, involved in separate groups of estrogenic responses, differences in the regulation of estrogen action in each uterine cell-type (Tchernitchin, 1979, 1983; Tchernitchin *et al*., 1985b, 1989; Gaete *et al*., 2010, 2011), exposure to lead dissociates estrogenic responses in the uterus: it selectively enhances some of them, inhibits others while a third group remains unaffected (Tchernitchin *et al*., 1998a, 1998b, 2003).

The heterogeneity of biochemical fertility related processes affected by lead, and the existence of multiple and independent mechanisms of estrogen action (*vide supra*), explain reported time-dependent differences between the different effects of lead on reproductive changes (Kempinas *et al*., 1994). We previously reported selective changes in some parameters of estrogen action following acute (Tcher-

nitchin *et al*., 1998b), subacute (Tchernitchin *et al*., 1998a), or chronic (Tchernitchin *et al*., 2003) exposure to lead of prepubertal rats, which can be additionally explained, by hematologic lead induced changes (Tchernitchin *et al*., 1997) which also affect estrogen action in the uterus (Tchernitchin *et al*., 1974a, 1985a, b, 1989).

Chronic exposure to lead and prenatal or early postnatal exposure are common conditions affecting human population (Tchernitchin *et al*., 2005). Studies of delayed effects of lead prenatal or early postnatal exposure in the brain, ovary, and uterus of experimental animals suggested the mechanism of imprinting. We investigated the delayed effects of prenatal exposure to lead on responses to estrogen in the prepubertal rat uterus (Tchernitchin *et al*., 2011), and compared them to those previously reported effects of chronic exposure (Tchernitchin *et al*., 2003). Additionally, we investigated hematologic changes in prenatally lead exposed rats, considering the role of eosinophils in estrogen action (Tchernitchin *et al*., 1989), and compared them to effects of chronic lead exposure. The experimental conditions intended to investigate, in an animal model, conditions frequently occurring in the human species: pregnant women displaying high lead blood levels as a cause of their child's prenatal exposure to lead; subsequent breast feeding as the source of further exposure after birth.

3.1 Effects of Lead Exposure on Uterine Responses to Estrogen and on Hematologic Parameters in the Rat: Comparison between Prenatal Exposure and Chronic Exposure.

Blood lead levels. In non lead-exposed adult pregnant rats, levels were under 2 µg/dL. In pregnant rats 1 to 7 days after lead exposure, they increased to 35.1 µg/dL; no differences were detected within the 7 days following exposure. In the offspring of lead-exposed mothers levels were 20.6 µg/dL at the time of blood and uterus sample collection; in the offspring of no exposed mothers levels were 4.4 µg/dL (Tchernitchin *et al*., 2011).

Uterine eosinophilia. Figure 1 shows that prenatal lead exposure strongly enhances estrogen-induced increase in endometrial eosinophilia at 6 h of hormone treatment (Tchernitchin *et al*., 2011) and that this effect is the opposite to that caused by chronic exposure, which inhibits endometrial eosinophilia (Tchernitchin *et al*., 2003). Subacute lead exposure also strongly inhibits estrogen-induced uterine eosinophilia (Tchernitchin *et al*., 1998a). No significant changes occur in estrogen-induced mesometrial eosinophilia under prenatal exposure to lead (data not shown), while it is slightly increased under the effect of chronic lead exposure (Tchernitchin *et al*., 2003). Prenatal exposure to lead decreases the proportion of eosinophils located in the mesometrium at 6 h of hormone stimulation (from 36.8% to 20.1%; $p<0.001$, χ^2 test). On the contrary, chronic lead exposure increases the proportion of eosinophils located in the mesometrium (from 38.1% to 51.9%; $p<0.01$, χ^2 test). From these data, it can be concluded that eosinophil migration from mesometrium towards myometrium and endometrium is enhanced in prenatally lead exposed animals while it is inhibited in chronically lead exposed rats.

Endometrial edema. Figure 2 shows that prenatal exposure to lead strongly enhances estrogen-induced edema in deep endometrial stroma at 6 h of hormone stimulation (Tchernitchin *et al*., 2011). Figure 2 also shows that this response is different from the previously reported (Tchernitchin *et al*., 2003) inhibition of estrogen-induced endometrial edema following chronic exposure to lead.

Uterine luminal epithelial cell hypertrophy. Figure 3 shows that prenatal exposure to lead strongly enhances estrogen-induced increase in luminal epithelial cell volume 24 h after hormone stimulation (Tchernitchin *et al*., 2011) and that this effect is similar to the previously reported enhancement of estrogen-induced luminal epithelial hypertrophy under the effect of chronic exposure to lead (Tchernitchin *et al*., 2003).

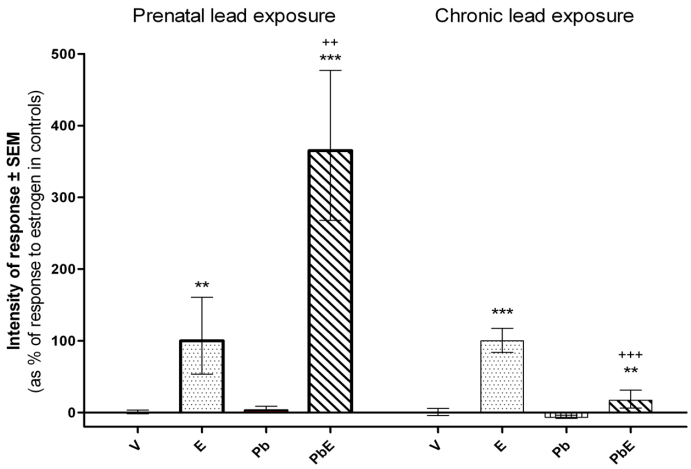

Figure 1: Compares the effects of prenatal lead exposure and chronic lead exposure on estrogen-induced uterine eosinophilia in the endometrium. Pregnant mothers of prenatally exposed rats were s.c. injected with lead acetate (P) or saline physiological solution at 14th day of pregnancy. From birth on, the pups were breastfed by their mothers. At the age of 21 days they were treated with estradiol-17ß (E) or vehicle (V), and the uteri were obtained 6 h thereafter. Previously reported data from chronically exposed rats are shown for comparison purposes (Tchernitchin *et al*., 2003). Bars indicate means (expressed as % of maximal response to estradiol) ± standard error of the mean. Statistics: LSD test. ** or $^{++}$, P<0.01; *** or $^{+++}$, P<0.001; *, comparisons to the homologous condition without estrogen; $^{+}$, comparisons to the homologous condition without lead (reproduced from: Tchernitchin *et al*., 2011; an open access journal).

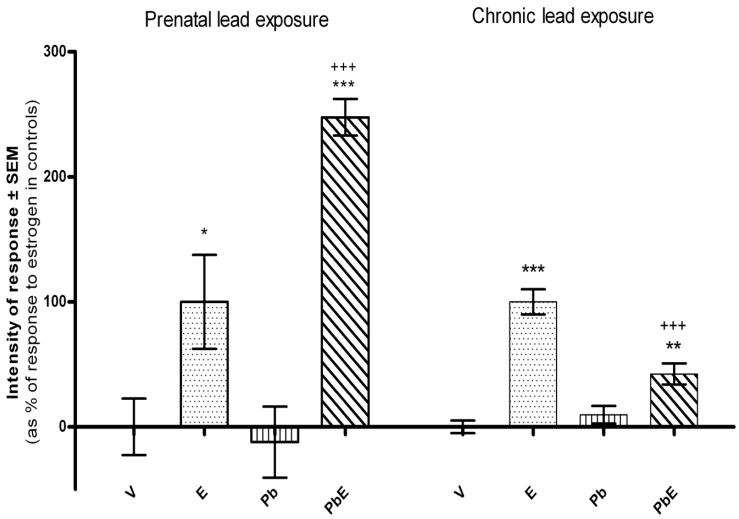

Figure 2: Compares the effects of prenatal exposure to lead and chronic exposure to lead on estrogen-induced edema in deep endometrial stroma, measured as increases in reciprocal value of cell density. Experimental conditions as in Figure 1. Statistics: LSD test. *, P<0.05; **, P<0.01; *** or $^{+++}$, P<0.001; *, comparisons to the homologous condition without estrogen; $^{+}$, comparisons to the homologous condition without lead (reproduced from: Tchernitchin *et al*., 2011; an open access journal).

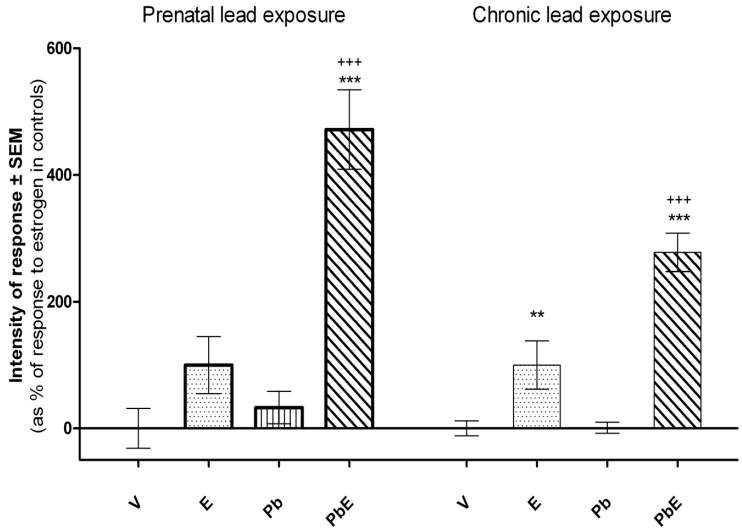

Figure 3: Compares the effects of prenatal exposure to lead and chronic exposure to lead on estrogen-induced hypertrophy of luminal epithelial cells, measured as increase in their cellular volume. Prenatally exposed rats were subjected to s.c. injection of their pregnant mothers with a lead acetate (P) or saline physiological solution at their 14th day of pregnancy. From birth on, the pups were breastfed by their mothers. At the age of 21 days the animals were treated with estradiol-17ß (E) or vehicle (V). The uteri were obtained 24 h after hormone or vehicle administration. Previously reported data from chronically exposed rats are shown for comparison purposes (Tchernitchin *et al*., 2003). Bars indicate means (expressed as % of maximal response to estradiol) ± standard error of the mean. Statistics: LSD test. **, P<0.01; *** or $^{+++}$, P<0.001; *, comparisons to the homologous condition without estrogen; $^{+}$, comparisons to the homologous condition without lead (reproduced from: Tchernitchin *et al*., 2011; an open access journal).

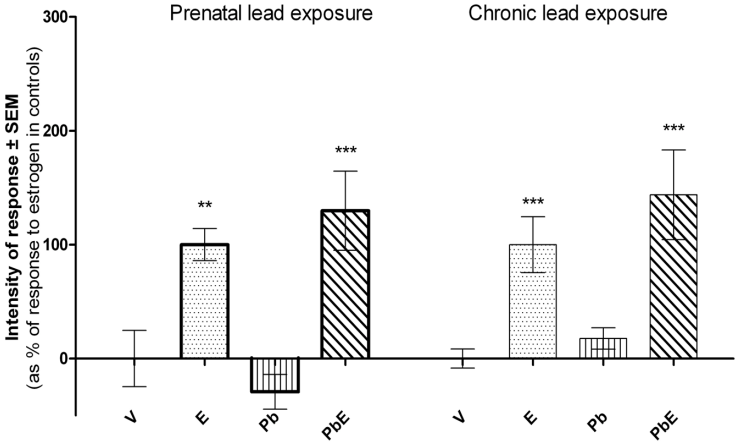

Figure 4: Compares the effects of prenatal exposure to lead and chronic exposure to lead on estrogen-induced myometrial hypertrophy, measured as increase in cell volume of circular myometrium. Experimental conditions as in Figure 3. Statistics: LSD test. **, P<0.01; ***, P<0.001; *, comparisons to the homologous condition without estrogen (reproduced from: Tchernitchin *et al*., 2011; an open access journal).

Circular myometrial cell hypertrophy. Figure 4 shows that prenatal exposure to lead does not induce significant changes in estrogen-induced cell hypertrophy in this histological layer, 24 h after hormone stimulation (Tchernitchin *et al*., 2011). The figure compares this effect to previously reported (Tchernitchin *et al*., 2003) lack of any significant effect of chronic exposure to lead on this response to estrogen

Hematologic parameters. Prenatal exposure to lead exposure does not cause any significant change in the count of total leukocytes, eosinophils, lymphocytes, monocytes, and neutrophils in the blood, as compared to control animals non-exposed to lead (results not shown). Estrogen treatment also does not cause any significant change in the number of these blood cells in either prenatally lead exposed or non-exposed rats (results not shown). This lack of significant effect of prenatal lead exposure is quite different from the previously reported increases in total leukocytes, eosinophils, lymphocytes, monocytes, and neutrophils in the blood under the effect of subacute (Villagra *et al*., 1997) or chronic (Tchernitchin *et al*., 1997) exposure to lead.

3.2 Dissociation of Responses to Estrogen in Lead Exposed Animals

Prenatal lead exposure enhancemenet of estrogen-induced uterine eosinophilia 6 h after hormone treatment, mainly in the endometrium but not in the mesometrium suggests increased eosinophil migration from mesometrium towards myometrium and endometrium (Soto & Tchernitchin, 1979). This effect parallels the potentiation of estrogen-induced edema in endometrial stroma in prenatally exposed animals, the edema is caused by an increase in endometrial osmotic pressure following macromolecule depolymerization by nzymes released from the eosinophils (Tchernitchin *et al*., 1985b, 1989).

Several explanations were suggested for the enhancement of estrogen-induced eosinophil migration towards endometrium in prenatally exposed rats. Eosinophils may have increased intrinsic ability for migration through connective tissue, they may contain an increased amount of enzymes required to increase ground substance fluidity due to macromolecule depolymerisation (Tchernitchin *et al*., 1985b, 1989), or display increased sensitivity to chemotactic agents; the secretion of a putative eosinophil chemotactic substance by endometrial tissue (Lee *et al*., 1989) may be increased as well. These alterations may be mediated by changes in estrogen receptors, reported following prenatal lead exposure (Wiebe & Barr, 1988), or by alterations in regulatory mechanisms. The latter may occur in the affected uterine cell types and include heat shock proteins, such as hsp90, known to interact with estrogen receptors and modify their activity (Knoblauch & Garabedian, 1999), or hsp70, which binds estrogen receptors and may protect them against a number of adverse conditions (Klinge *et al*., 1997; Ciocca *et al*., 1998). It may also involve systemic endocrine changes, such as glucocorticoids, catecholamines, prolactin or growth hormone (Vyskocil *et al*., 1991; Shouman & El-Safty, 2000), which are known to modify responses to estrogen (Tchernitchin *et al*., 1985b; Unda *et al*., 1989).

We describe an important enhancement of estrogen-induced uterine luminal epithelium hypertrophy under the effect of prenatal exposure to lead. It is similar to that reported following acute (Tchernitchin *et al*., 1998b), subacute (Tchernitchin *et al*., 1998a), or chronic (Tchernitchin *et al*., 2003) exposure to lead. In contraposition to findings in uterine luminal epithelium, prenatal exposure does not cause a significant change in estrogen-induced hypertrophy in circular myometrium (Tchernitchin *et al*., 2011), an effect that does not occur either following chronic exposure either (Tchernitchin *et al*., 2003). The difference between both cell types may be explained by the accumulation of lead in luminal epithelium (Nilsson *et al*., 1991), or to differences in sensitivity of these cell-types to lead. This finding confirms our previous suggestions that toxic substances may interact in a different way with the different uterine cell-

types (Tchernitchin *et al*., 1998a, b, 2003, 2008), and points to the need for separate consideration of all cell types in every study of toxicity in reproductive organs.

With regard to hematologic parameters reported to be affected following subacute (Villagra *et al*., 1997) or chronic (Tchernitchin *et al*., 1997) exposure to lead (increase in total leukocyte counts, eosinophils, lymphocytes, monocytes, and neutrophils in the blood), prenatal exposure to lead does not reveal any delayed effects on them. Considering the role of eosinophils in estrogen action in the uterus, the lack of effect of prenatal exposure on blood eosinophils may contribute to explain differences in uterine responses to estrogen following the different times of exposure to lead.

3.3 Hypothesis Explaining Success for Species Survival in Toxic Environments: Role of Imprinting in Species Evolution

It is striking that three responses to estrogen stimulation in the uterus (estrogen-induced endometrial eosinophilia, eosinophil migration from mesometrium towards myometrium and endometrium, and endometrial edema), but not the remaining responses (luminal epithelial and myometrial cell hypertrophy), display just the opposite behavior when comparing prenatal exposure and chronic exposure. Estrogen-induced increase in eosinophils in the endometrium, endometrial edema and eosinophil migration from mesometrium towards myometrium and endometrium appear enhanced in prenatally exposed rats while are inhibited following chronic exposure. The differences appears even more conspicuously, considering that lead blood levels in prenatally exposed rats at the age of estrogen stimulation are much higher than those in no exposed controls, although lower than following chronic exposure, that is, mimicking a less severe chronic exposure.

It was reasonable to expect in prenatally exposed rats an inhibition of endometrial eosinophilia and edema of smaller magnitude than in chronically exposed animals, considering that blood lead levels were lower (20.6 μg/dL) in prenatally exposed than in chronically exposed rats (35.1 μg/dL). Therefore, our finding of enhancement of the estrogenic responses in prenatally exposed animals was completely unexpected. Considering that in prenatally exposed animals at the time of estrogen treatment blood lead levels remain increased and should inhibit these estrogenic responses, we conclude that prenatal exposure antagonize the effects of chronic exposure to lead on these estrogenic responses, thus providing a partial protection of cell function against adverse effects of chronic exposure to lead.

We have previously proposed that estrogen-induced endometrial edema and destruction of endometrial extracellular matrix by plasmin formed by eosinophil plasminogen activators are required to facilitate blastocyst implantation (Tchernitchin, 1985b). Eosinophils were also proposed to suppress some immune reaction sequelae that could affect the development of the product of conception (Tchernitchin, 1985b). Therefore, if the inhibition of estrogen-induced endometrial eosinophilia and edema by chronic lead exposure contribute to the infertile condition, we suggest that prenatal exposure may neutralize these effects and provide a partial protection against these effects of chronic exposure.

Nevertheless, at this point, it is not possible to ascertain whether the remaining uterine effects of prenatal lead exposure may interfere with blastocyst implantation and its development. Further work is needed to evaluate this possibility as well as effects in other reproductive organs.

Imprinting can be considered a general biological epigenetic mechanism incorporated at early stages of the phylogenic species development (Csaba *et al*., 1982; Csaba, 2008). Hormonal imprinting is present already at the unicellular level causing the development of specific receptors for surrounding molecules (Csaba *et al*., 1982); this process may be an important factor in the survival of the species, since the information needed for the recognition of foreign molecules is transmitted to the progeny cell generations (Csaba, 2008).

In multicellular organisms, and particularly in mammals, it is accepted that imprinting causes changes in the differentiation or programming in various cell-types under the effect of perinatal exposure to various agents. We now propose that this process may have persisted through evolution as a non-genetic adaptive mechanism to provide protection against long-term environmental variations that otherwise may cause the extinction of species not displaying this kind of adaptation. In the particular case of lead, it may protect several reproductive functions and their mechanisms against damage by this pollutant, allowing offspring of prenatally or perinatally exposed individuals to survive and further reproduce in a newly polluted toxic environment.

We conclude from this study that prenatal exposure to lead causes persistent changes in several responses to estrogen in the rat uterus, detected at postnatal age of 21 days, and suggest that prenatal exposure may constitute a non-genetic adaptive mechanism to antagonize some of the adverse effects of chronic exposure to lead in the uterus, thus protecting it against reproductive impairment caused by chronic exposure to lead.

4 Lead Exposure Effects

4.1 Reproductive Effects Not Involving the Imprinting Mechanism

The effects not mediated by imprinting may recover following a decrease in blood lead levels. Among them, fertility depression and reproductive dysfunctions in both males are females (Winder, 1993) following lead exposure at adolescent or adult ages. In women, infertility, miscarriage, preeclampsia, pregnancy hypertension, polymenorrhea, hypermenorrhea, premature delivery and spontaneous abortions (Winder, 1993; Guerra-Tamayo *et al*., 2003; Tang & Zhu, 2003; Al-Saleh *et al*., 2008) are some of these dysfunctions.

4.2 Reproductive Effects Mediated by the Imprinting Mechanism

The infertility that is mediated by the imprinting mechanism following prenatal or early postnatal exposures to lead usually persists through life (Tchernitchin & Tchernitchin, 1992; Tchernitchin *et al*., 1999). In the experimental animal model, prenatal lead exposure causes persistent changes in uterine estrogen receptors expression and characteristics (Wiebe and Barr, 1988), decreases ovary luteinizing hormone (LH) receptors, and alters steroidogenesis (Wiebe *et al*., 1988). These changes may explain the delay in puberty that occurs with very low blood lead level increases from dietary origin in mice. In fact, while modest increases in blood lead levels from a normal background of 2-3 to 13.2 µg/dL delayed the onset of puberty by 15-20% to about 40-43 days, reducing blood lead from 2-3 to 0.7 µg/dL was associated with an acceleration of puberty to 21 days, an enhancement by over 30% (Iavicoli *et al*., 2004). This dose-response relationship represents relevant findings of possible ecological as well as public health significance indicating that lead is able to induce biologically significant changes at blood lead levels previously thought to be without effect.

It was suggested that lead-induced oxidative stress was one of the biochemical mechanisms responsible for altered ovarian steroidogenic transcriptional machinery, on reaching adulthood after early developmental exposure to lead (Pillai *et al*., 2010), that may additionally contribute to fertility inhibition in lead exposed experimental animals and humans (Rom, 1976; Needleman & Landrigan, 1981; Winder, 1993; Ronis *et al*., 1996).

4.3 Non-Reproductive Effects Not Involving the Imprinting Mechanism

In the rat, chronic lead exposure induces brain astroglial changes, reduces dopaminergic neurons in the substantia nigra (Sansar *et al*., 2011), increases brain serotonin and dorsal raphe nucleus immunoreactive serotoninergic cell bodies density, and increases anxiety (Sansar *et al*., 2012).The main effects caused by chronic lead exposure of adult human population are: progressive damage to the central and peripheral nervous systems (Banks *et al*. 1997; Zhang *et al*., 2012), a moderate increase in blood pressure (Fiorim *et al*., 2011), hematopoietic system damage (Graziano *et al*. 1991), thyroid function depression (Bledsoe *et al*., 2011; Wu *et al*., 2011), nephropathy (Cardenas *et al*. 1993), intestinal colic and gastrointestinal symptoms (Pagliuca *et al*. 1990). Lead exposure immune system damage was reported in humans and in laboratory animals (Koller, 1990; Tchernitchin *et al*., 1997; Villagra *et al*., 1997; García-Lestón *et al*., 2012). In humans, it causes effects on chromosomes (Al-Hakkak *et al*., 1986), increases mortality rate (Cooper, 1988) and decreases life expectancy.

4.4 Non-Reproductive Effects Mediated by the Imprinting Mechanism

The most relevant delayed effects following of prenatal or early postnatal lead exposure affect the central nervous system. Several neurobehavioral changes attributed to lead exposure were reported in high lead pollution level countries (Tchernitchin *et al*., 2005; Laidlaw *et al*., 2008; Donaldson *et al*., 2010; Mielke *et al*., 2011).

Among these changes, lead exposure impairs learning in experimental animals (Massaro *et al*., 1986) and humans (Needleman *et al*., 1990; Mielke *et al*., 2011). In humans, it causes deficits in central nervous system functioning that persists into adulthood, including learning impairment, psychometric intelligence scores deficit, lower IQ scores, poorer school performance, increased school failure, reading disabilities and poorer eye-hand coordination (Rothenberg *et al*., 1989; Needleman *et al*., 1990). A deficit in the IQ scores can already be detected in children with lead blood levels as low as 9 μg/dL (Royce, 1990); IQ declined by 7.4 points as lifetime average blood lead concentrations increased from 1 to 10 μg/dL (Canfield *et al*., 2003). Brain protein kinase C inhibition by prenatal lead exposure probably explains the persistent memory damage (Xu *et al*., 2005). Figure 5 illustrates learning impairment by lead: Blood lead levels from 5 to 9 μg/dL increased the percentage of students requiring special education in public schools in Detroit, Michigan, USA, as compared to students with levels between 1 to 4 μg/dL; that further increases in blood lead levels caused a greater increase in percentage of students requiring special education (Tarr *et al*., 2009; Raymond *et al*., 2012). Figure 6 shows that the Michigan Education Assessment Program scores for 3rd, 5th and 8th graders students revealed impairment in mathematics, reading and science from 5 to 8 μg/dL lead in blood (Raymond *et al*., 2012). Impairment in school performance was detected at even lower blood lead levels: A blood lead level of 5 μg/dL at early ages (1-2 years old) is associated with a decline in end of grade (4th grade students) reading, mathematics and science scores, as compared to early childhood blood lead levels as low as 2 μg/dL (Miranda *et al*., 2007).

Maternal lead exposure causes permanently elevated offspring corticosterone blood levels, effect potentiated by maternal stress. It suggests a mechanism by which lead exposure enhance susceptibility to diseases and dysfunctions and induce cognitive deficits (Cory-Slechta *et al*., 2004). A report from our Laboratory describes neurological damage with blood lead levels 10 to 19 μg/dL or higher in children 1-10 years of age, living near a lead mineral storage site in Antofagasta, Chile (Tchernitchin *et al*., 2005). A delay in response to auditory stimulus in children 8 or 9 years old living in a lead polluted area in Arica, Chile, occurs with lead blood levels ≥ 10 μg/dL; this delay occurs at much lower levels than those

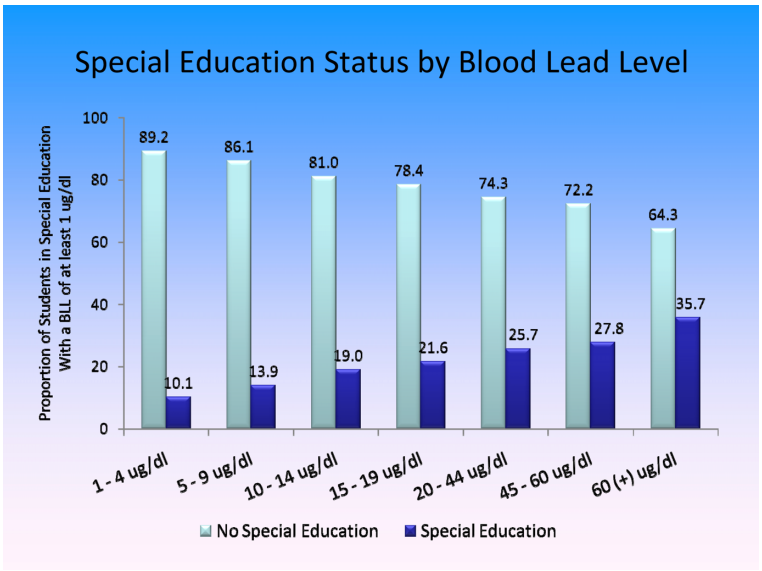

Figure 5: Effect of lead exposure on school outcome among children living and attending public schools in Detroit, Michigan, USA. The figure shows the proportion of students in special education in relation to blood lead levels (Tarr *et al*., 2009; Raymond *et al*., 2012; reproduced from Raymond *et al*., 2012, with kind permission of Dr. Randall E. Raymond, geographic information specialist Detroit Public Schools Office of Research, Evaluation, and Accountability, Department of Health and Wellness Promotion, City of Detroit, Mi, USA).

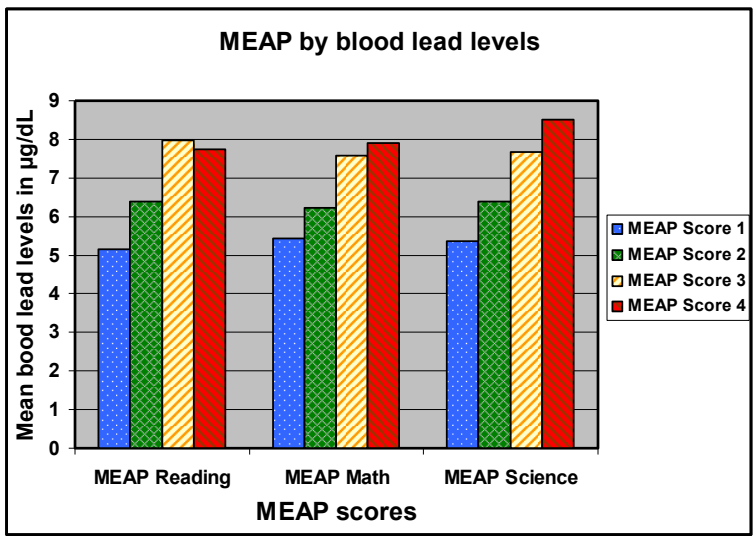

Figure 6: Effect of lead exposure on school outcome among children living and attending public schools in Detroit, Michigan, USA. The Michigan Education Assessment Program scores October 2099 3rd, 5th and 8th graders in mathematics, reading and science dramatically impair when blood lead levels increase from 5 to 8 µg/dL (Redrawn with data from Raymond *et al*., 2012).

reported to cause a decrease in the velocity of nerve conductance through motor myelin nerve (Tchernitchin *et al*., 2005).

Evidence suggests that Pb exposure can be a risk factor promoting the pathogenesis of Alzheimer's disease (Basha *et al*.,2005; Wu *et al*., 2008, Zawia *et al*., 2009). Population-based studies reported that adult occupational exposure to Pb is related to Alzheimer's disease increased risk (cited by Li *et al*., 2012). In a study on 23-year-old Cynomolgus monkeys that were exposed to lead as infants, the activity of tissue DNA-methyltransferase decreases, suggesting that developmental exposure to lead is probably a risk factor for Alzheimer's disease pathogenesis (Wu *et al*., 2008). Postnatal rodent exposure to lead results in a transient increase in amyloid precursor protein mRNA expression in neonates followed by a return to basal levels by 1 year and a delayed overexpression at 20 month after lead exposure ceased (Basha *et al*.,2005). The upregulation in amyloid precursor protein mRNA levels occurred concurrently with lead exposure early in life. Data from Cynomolgus monkeys exposed to lead as infants showed that the amyloid precursor protein mRNA, amyloid precursor protein, and amyloid β are elevated in old age. The demethylation of amyloid precursor protein gene was permanent, suggesting that lead exerts its neurotoxic effects through mechanisms altering the global and promoter methylation patterns of amyloid precursor protein gene, and that lead interference with epigenetic program of amyloid precursor protein gene promotes the pathogenesis of Alzheimer's disease (Li *et al*., 2012).

Similarly to the findings in the rat (De Marco *et al*., 2005), perinatal or infant human exposure to lead causes the development of a hyperactive and aggressive behavior (Tchernitchin *et al*., 2005). Early Pb exposure (K x-ray tibia fluorescence spectroscopy at 12 years age), associated to increased risk for antisocial and delinquent behavior and to anxious/depressed behavior (Needleman, 1996, 2002). Similar correlation was shown between dental enamel lead levels and antisocial behavior in Brazilian adolescents (Olympio *et al*., 2010).

Soils are the main reservoir of Pb dust; lead is mainly originated from leaded gasoline and in a smaller proportion, from paint sources (Mielke *et al*., 2011); its amount in a city or a country can be inferred from amounts of leaded gasoline used (Mielke *et al*., 2010). Lead in soil correlates with blood lead levels in urban children population (Zahran *et al*., 2011). Under the hypothesis that early lead exposure causes neurobehavioral alterations in children increasing delinquent behavior at later ages, single nation regressions of data showed a very strong association between preschool blood lead and subsequent crime rate trends over several decades in the USA, Britain, Canada, France, Australia, Finland, Italy, West Germany, and New Zealand. Despite divergent international crime and blood lead trends, regression R^2 (and blood lead t-level) is near its peak in each nation at time lags consistent with peak offending ages for each crime category; for instance, for index crime broadly defined was 19 years (Nevin, 2007). An analysis lead tons per thousand population, for leaded paint and gasoline from 1876 - 1979 in the USA, correlated with murders with a lag of 21 years (1897 - 2000) (Nevin 2000). A correlation between lead content in air particulate matter and homicide rates was also reported in a cross-sectional ecological study in all counties in 48 states of the USA (Stretesky & Lynch, 2001).

It was suggested that the hyperactive or aggressive behavior induced by early lead exposure, is an irreversible risk behavior for antisocial or delinquent behavior development later in life (Tchernitchin *et al*., 2005). If this aggressivity is channeled, by a psychological treatment, to socially-positive aggression-like activities (such as sports, dance, or other activities), then it should be possible to avoid the transformation of these children into a delinquent population (Tchernitchin *et al*., 2005).

Prenatal exposure altered at the postnatal age energy status of neuronal mitochondria, changing neuronal function in such a way that it could play a role in neurodegeneration (Baranowska-Bosiacka *et al*., 2011).

Following prenatal exposure, a permanent increase in the affinity of δ- (McDowell & Kitchen, 1988) and μ-opioid, but not κ–opioid receptors (Kitchen, 1993) was reported in the rat brain. This change parallels the impairment of opioid but not non-opioid stress-induced antinociception in developing rats (Jackson & Kitchen, 1989), and a depression-like behavior (de Souza Lisboa *et al*., 2005). It has not been investigated whether these changes also occur in humans; if they do, they may explain behavior changes that occur in exposed population (Rothenberg *et al*., 1989; Needleman *et al*., 1990), and increased frequency of opioid or other drugs or abuse addictions high lead contaminated environments (Tchernitchin & Tchernitchin, 1992). The dopamine and 5-hydroxyindoleacetic acid response to amphetamine enhancement in lead-exposed animals (Lasley *et al*., 1985), also suggested that the responses to other stimulant substances of abuse may be enhanced as well (Tchernitchin & Tchernitchin, 1992).

The report of changes in the opiate deprivation syndrome in prenatally lead exposed animals and the suggestion of a possible link between lead and opioid addiction (Kitchen & Kelly, 1993) support our former hypothesis on the role of early lead exposure on abuse drug addiction (Tchernitchin & Tchernitchin, 1992). Developmental lead exposure throughout gestation and lactation alters sensitivity to various psychoactive drugs when adult animals are tested much later. Perinatal lead exposure enhanced the rate of acquisition of cocaine self-administration (Rocha *et al*., 2005), produced a shift to the left in the dose effect curve (Nation *et al*., 2004), and increased the stimulatory properties of cocaine when animals were tested in postnatal days 30 or 90 (Nation *et al*., 2000). Perinatal lead exposure increased the vulnerability to reinstatement, so that perinatally exposed adult rats were more vulnerable than controls to drug reinstatement (relapse) (Nation *et al*., 2003). Perinatally lead-exposed animals self administered cocaine at doses too low to sustain responding in non lead exposed animals (Nation *et al*, 2004; Valles *et al*, 2005). By contrast, developmental exposure to lead attenuates the psychoactive heroin effects (Rocha *et al*., 2004) and the methamphetamine dose-effect self-administration performance (Rocha *et al*., 2008), suggesting a decreased rewarding sensitivity to opiates (Miller *et al*., 2001).

5 Arsenic Exposure Effects

5.1 Effects Not Involving the Imprinting Mechanism

Arsenic is an environmental agent affecting several broad areas of our planet. The effects of chronic exposure to arsenic in humans are well known, and they are not related to the imprinting mechanism. Chronic exposure to arsenic favors the development of several malignancies, specially broncogenic lung cancer and bladder, kidney, liver and skin non-melanoma cancer (Rivara & Corey, 1995; Steinmaus *et al*., 2000; Mostafa *et al*., 2008). It also increases mortalities from cardiovascular and cerebrovascular diseases (Navas-Acien *et al*., 2005), and is teratogenic mainly for the kidney and the nervous system. Inorganic arsenic is enzymatically methylated for detoxication, using up S-adenosyl-methionine in the process. The observation that DNA methyltransferases also require S-adenosyl-methionine as their methyl donor suggested a role for DNA methylation in arsenic carcinogenesis and other arsenic-related effects (Zhao *et al*., 1997). In rat-liver epithelial cell lines treated with chronic low arsenic doses, malignant transformation was associated with depressed S-adenosyl-methionine levels, global DNA hypomethylation, and decreased DNA methyltrasferase activity (Zhao *et al*., 1997). Following this findings, several studies reported that arsenic is associated with gene-specific hypermethylation (Zhong & Mass, 2001; Chanda *et al*., 2006; Pilsner *et al*., 2007; Zhang *et al*., 2007; Terry *et al*., 2011), as well as global DNA hypomethylation (Chen *et al*., 2004; Sciandrello *et al*., 2004; Reichard *et al*., 2007). In a human study from India, significant DNA hypermethylation of p53 and p16 promoter regions was

observed in blood DNA of subjects exposed to toxic level of arsenic compared to controls (Chanda *et al*., 2006); hypermethylation showed a dose-response relationship with arsenic measured in drinking water. Arsenic toxicity has been related to changes in miRNA expression. Alterations were reported in miRNA profiles of human lymphoblastic cells grown under sodium arsenite treatment; alterations affected expression of specific miRNAs that were involved in one-carbon metabolism (Marsit *et al*., 2006). The lack of carcinogenicity in most animal models is supposed to be due to the absence of arsenic methylating enzyme.

5.2 Effects Mediated by the Imprinting Mechanism

The first report on human prenatal arsenic exposure delayed effects was from an epidemiological study in Antofagasta, Chile (Smith *et al*., 2006). This city had a distinct period of time of very high arsenic exposure from drinking water, that began in 1958 and lasted until 1971, when an arsenic removal plant was installed (Figure 7). The mortality rates in Antofagasta in the period 1989-2000 were compared with the rest of Chile, focusing on subjects who were born during or just before the peak exposure period and who were 30-49 years of age at the time of death (prenatally exposed); they were compared to the cohort born before the high-exposure period (1950-1957) and exposed to high arsenic levels in childhood or later but not prenatally. Standard mortality ratio for bronchiectasis in prenatally exposed was four times higher than in non-exposed prenatally. Bronchiestasis standard mortality rates for those born in 1958–1970 (prenatally exposed) was 46.2; for those born in 1950–1957 before the very high exposures started (non-prenatally exposed) standard mortality ratio was 12.4. The Poisson regression test revealed that the difference between prenatally exposed and non-exposed population was p=0.02 (Smith *et al*., 2006) (Figure 8). Mortality for chronic obstructive pulmonary disease was higher in prenatally exposed population than in non-exposed population, but mortality rates for lung cancer did not significantly differ between prenatally exposed and non-exposed population (Smith *et al*., 2006) (Figure 8).

In Bangladesh, arsenic exposure in pregnancy was associated with increased morbidity in infectious diseases during childhood; it mainly increased the risk of lower respiratory tract infection and diarrhoea during infancy in Bangladesh (Rahman *et al*., 2011). A possible causal pathway for an effect of arsenic exposure on incidence of lower respiratory tract infection and diarrhea is through immunosupression, as suggested by several studies: Arsenic exposure suppressed theimmunoglobulin (Ig) M and IgG antibody–forming cell response (Selgrade, 2007), decreased interleukin-2 mRNA expression (Conde *et al*., 2007), inhibited antigen-driven T-cell proliferation and macrophage activity, and suppressed contact hypersensitivity responses (Patterson *et al*., 2004). Inorganic arsenic impaired the function of human macrophages both *in vivo* and *in vitro* (Lemarie *et al*., 2006; Banerjee *et al*., 2009). Arsenic exposures through drinking water during pregnancy in rural Bangladesh reduced placental T cells ($CD3^+$ cells) and altered cord blood cytokines, indicating effects of arsenic on immune function (Ahmed *et al*., 2011). It was reported that arsenic induces reactive oxygen species in mothers of the investigated cohort (Ahmed *et al*., 2011), finding of relevance under the consideration that oxidative stress is known to affect the immune system (Knight, 2000). In another study of exposed women, an association between prenatal arsenic exposure and decreased thymus size in the offspring was observed (Moore *et al*. 2009), as well as secretion of trophic factors such as lactoferrin and interleukin-7 in breast milk (Raqib *et al*., 2009). The immunosupression induced by prenatal exposure to arsenic in drinking water may explain, at least in part, the increases in mortality by bronchiestasis, chronic obstructive pulmonary disease (Smith *et al*., 2006) and the high prevalence of respiratory complications like breathing alterations such as chest sound, asthma, bronchitis and cough, together with other morbidities associated with drinking water arsenic toxicity

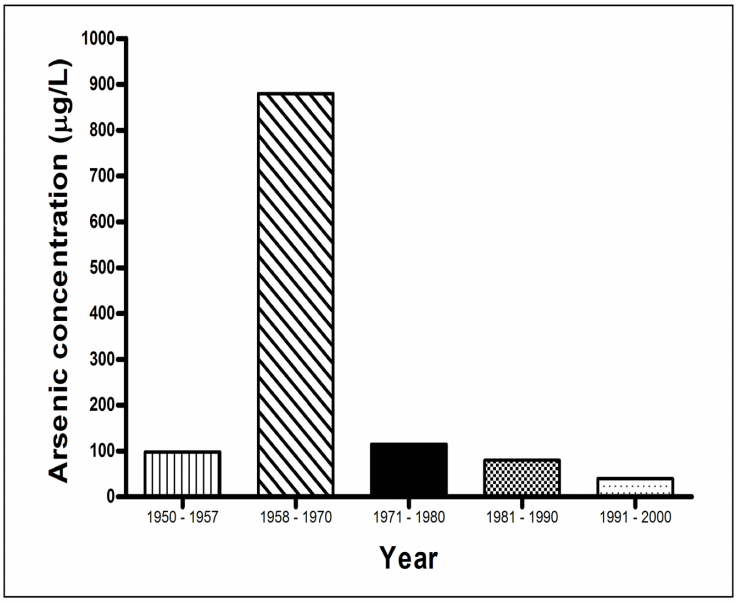

Figure 7: Arsenic concentration in Antofagasta/Mejillones water by year. An arsenic removal plant was installed in 1971 (modified from Smith *et al*., 2006)

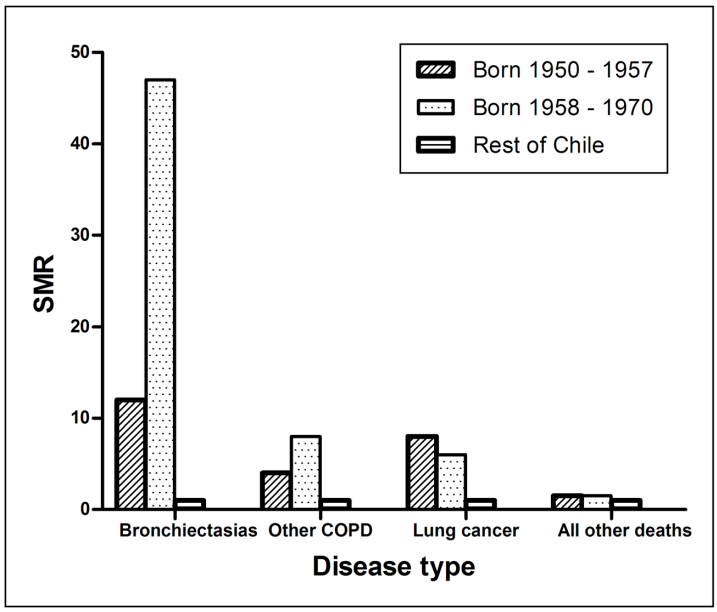

Figure 8: Chronic obstructive pulmonary disease standard mortality rates for Antofagasta/Mejillones for individuals 30-49 years of age (modified from Smith *et al*., 2006).

(Islam *et al*., 2007). All these alterations can be consequences of *in utero* exposure to arsenic, possibly in combination with maternal toxicity during pregnancy, with indirect effects on the fetus.

Gestational exposure of experimental animals to inorganic arsenic plus post-weaning exposure to dimethylarsinic acid increase renal tumour incidence, while no renal tumours occurred in controls or in dimethylarsinic acid treated animal that were not prenatally exposed to inorganic arsenic (Tokar *et al*., 2012). The finding suggested that its inorganic forms are early life carcinogens in humans and mice that can initiate tumours as well as other lesions or alterations promotable by other agents in later life (Tokar *et al*., 2012).

Adult mice prenatally exposed to arsenic showed a 2-fold increase in lesion formation in the aortic roots and aortic arch, compared to non-exposed animals. The exposed mice had a 20-40% decrease in total triglycerides, no change in total cholesterol and phospholipids, and abundance of VLDL or HDL particles. Arsenic-exposed mice also showed a vascular relaxation defect in response to acetylcholine, suggesting disturbance of endothelial cell signalling (Srivastava *et al*., 2007). These results show an early atherosclerosis onset without a hyperlipidemic diet and alerts that *in utero* arsenic may be atherogenic in humans (Srivastava *et al*., 2007).

Current arsenic concentrations in urine, which reflect all sources of recent exposure, were associated with small decrements in intellectual testing (vocabulary test, object assembly test, and picture completion test) in school-aged children in West Bengal; no associations were detected, however, between long-term water arsenic concentrations and intellectual function (von Ehrenstein *et al*., 2007). A more recent study, however, reported adverse effects of arsenic exposure on intelligence quotient (IQ) in girls (verbal IQ and full scale IQ), but not boys, at 5 years of age (Hamadani *et al*., 2011).

6 Dioxins and Related Compounds Exposure Effects

Dioxin is the common name for dibenzo-p-dioxins and dibenzofurans, contaminants nearly ubiquitous in the environment and highly resistant to chemical and biological degradation. These compounds can survive for decades in the environment and accumulate in the human and animal food chains. Polychlorinated biphenyls (PCB) display toxicological effects that are nearly similar to those from dioxins. Usually contaminated environments contain a mixture of dibenzo-p-dioxins, dibenzofurans and polychlorinated biphenyls. As compared to polychlorinated dioxins or polychlorinated biphenyls (World Health Organization, 1989), the polybrominated compounds (World Health Organization, 1998) or those containing both chlorine and bromide atoms in their molecules display grossly equivalent toxicity.

6.1 Effects Not Involving the Imprinting Mechanism

Dioxin chronic exposure affects the skin (Pasarini *et al*., 2010), liver (Bock & Köhle, 2009), the immune system (Lu *et al*., 2011b; Yoshida *et al*., 2012), reproductive system (Brunnberg *et al*., 2011) and many other organs and systems. Although there is some controversy (Boffetta *et al*., 2011), it is generally accepted that it induces the development of various cancers (Peng *et al*., 2009; Apostoli *et al*., 2011a, 2011b; Kogevinas, 2011; Warner *et al*., 2011). The toxicological effects of polychlorinated dibenzo-paradioxins and dibenzofurans, and of polybrominated dibenzo-p-dioxins and dibenzofurans are extensively reviewed elsewhere (World Health Organization, 1989, 1998). Dioxin toxicity is mediated by the arylhydrocarbon receptor (AhR) (Bunger *et al*., 2003; Baccarelli *et al*., 2003; Okey. 2007) and requires altered transcription of target genes (Bunger *et al*., 2003). Although miRNAs might be responsible for this

mRNA downregulation in dioxin/AhR-related pathways, few changes in miRNA levels suggested its limited role in dioxin toxicity (Moffat *et al*., 2007).

6.2 Effects Mediated by the Imprinting Mechanism

Prenatal exposure to dioxins and related agents was reported to cause persistent changes in the immune, respiratory, and reproductive systems, in mammary glands, to determine persistent neurobehavioral alterations and irreversibly increases the susceptibility of various organs for the development of cancer under the action of several carcinogenic agents. These effects are for life and are mediated by the mechanism of imprinting.

Perinatal Exposure Delayed Effects in the Immune System of Experimental Animals. Cellular immunity suppression occurs in rats and mice following maternal treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin (Vos and Moore, 1974). In mice, prenatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin causes persistent effects in the immune system: thymus gland atrophy and immune suppression that were explained by an alteration lymphocyte stem cells differentiation (Fine *et al*., 1989). Persistent suppression of contact hypersensitivity and altered T-cell parameters (increase in CD4+) was reported in F344 rats perinatally exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (Walker *et al*., 2004).

Perinatal dioxin exposure interferes with responses that participate in allergic reactions: for instance, it decreases allergic sensitisation (Tarkovski *et al*., 2010), probably caused by prenatal dioxin exposure induced changes on T regulatory cells, which have the potential to suppress immune reactions thus protecting from allergy development (ten Tusscher *et al*., 2003). However, the decrease in allergic sensitisation may be related to inhibition of IL-4 synthesis rather than suppression mediated by T regulatory cells (Tarkovski *et al*., 2010).

Exposure to tetrachlorodibenzodioxin during immune system development causes persistent humoral immune dysregulations and altered cell-mediated responses, with a profile of changes suggesting increased risk for autoimmune disease (Mustafa *et al*., 2008). In autoimmune disease-prone mice treated with TCDD during gestation, postnatal autoimmunity was exacerbated, suggesting that prenatal dioxin may interfere with normal thymus development of central tolerance and supporting the hypothesis of increased risk of postnatal autoimmune responses as result of dioxin exposure during time of immune system establishment (Gogal & Holladay, 2008). Prenatal tetrachlorodibenzo-p-dioxin augmented the hallmark indicators of systemic lupus eritematosus progression in the lupus-prone SNF(1) mice: renal immune complex deposition, glomerulonephritis, and mesangial proliferation, suggesting that it causes persistent modulation of the postnatal immune response and exacerbate inflammatory disease in these mice (Mustafa *et al*., 2011).

Perinatal Exposure Delayed Effects in the Human Immune System. Early studies suggested that background levels of polychlorinated biphenyls/dioxin exposure influences the human fetal and neonatal immune system. Higher prenatal polychlorinated biphenyls/dioxin exposure caused an increase in the number of TcR$\gamma\delta^+$ T cells at birth and an increase in the total number of T cells and number of $CD8^+$ (cytotoxic), TcR$\alpha\beta^+$, and TcR$\gamma\delta^+$ T cells at 18 months of infant age; no relationship was found between prenatal polychlorinated biphenyls/dioxin exposure and humoral antibody production (Weisglas-Kuperus *et al*., 1995). Further studies confirmed that perinatal exposure to dioxins and to polychlorinated biphenyls cause immune depression (increase in lymphocytes and T cells, changes in markers and decrease in antibodies) that persists through childhood. This may explain the increased susceptibility to infectious diseases, and a higher prevalence of coughing, chest congestion and phlegm detected in Dutch prenatally

exposed children (Weisglas-Kuperus *et al*., 2000). It is also related to a higher prevalence of recurrent middle ear infections (Weisglas-Kuperus *et al*., 2004).

Hematological and immunological alterations were detected in 8-year-old Dutch children that were prenatally exposed to dioxin (ten Tusscher *et al*., 2003). A decrease in the number of polymorphic neutrophils in adolescents with higher serum polychlorinated biphenyls was also reported (Leijs *et al*., 2009).

The immunodepression caused by perinatal exposure to dioxin causes an increase in infectious diseases. In the Hokkaido Study in Japan, relatively higher polychlorinated dibenzofuran levels were associated with a significantly increased risk of otitis media at 18 months age; relatively higher levels of 2,3,4,7,8-pentachlorodibenzofuran were also associated with an increased risk of otitis media (Miyashita *et al*., 2011). In the Taiwan Yucheng accident, children born to mothers who had accidentally ingested high levels of contaminated rice oil had higher frequencies of bronchitis, reduced serum levels of immunoglobulin (Ig) A, IgG, and IgM at 6 months (Yu *et al*., 1998), and a higher incidence of influenza and otitis media at 6 years of age than unexposed controls (Rogan *et al*., 1988; Chao *et al*., 1997). In Japan, infants born to mothers occupationally exposed to high levels of polychlorinated biphenyls have a higher frequency of colds and gastrointestinal complaints (Hara, 1985). In Inuit infants born to mothers who had ingested high levels of contaminated marine mammals, higher prenatal PCB exposure led to a significantly elevated incidence of infections (acute otitis and respiratory problems) (Dallaire *et al*., 2004, 2006). In the Rotterdam study, polychlorinated biphenyls in maternal blood and dioxins in breast milk were associated with a higher prevalence of otitis media and chicken pox, related to a reduction in measles, mumps, and rubella reactivity after primary vaccination and an increased number of T lymphocytes at 42 months of age (Weisglas-Kuperus *et al*., 2000, 2004).

Similarly to what was found in the experimental animals (Tarkovski *et al*., 2010), in 8 years old Dutch children it was found that prenatal or postnatal dioxin exposure causes a significant decrease in allergy, with no change in blood eosinophil granulocytes, persistently decreased thrombocytes, increased thrombopoietin, increased CD4+ T-helper, and increased CD45RA+ cell counts (ten Tusscher *et al*., 2003). The decrease in allergy in relation to perinatal exposure to polychlorinated biphenyls was also reported (Weisglas-Kuperus *et al*. 2000). The decrease in allergy could be caused by a relative deficient immune memory, an altered antigen receptor, or an insufficient immune cells mobilization; prenatal dioxin may "protect" against allergies by means of interference in the Th1:Th2 ratio (ten Tusscher *et al*., 2003). The allergy "protecting" effects of prenatal dioxin may occur by its effects on T regulatory cells; although others suggest that allergic sensitisation decrease is due to IL-4 synthesis inhibition and not suppression mediated by T regulatory cells (Tarkovski *et al*., 2010).

Perinatal Exposure Delayed Effects in the Human Respiratory System. A decrease in lung function, assessed by spirometry in 7 to 12 year-old children, was associated with perinatal exposure to Dutch background levels of dioxins; a clinical association between chest congestion and perinatal dioxin exposure was also established (ten Tusscher *et al*., 2001).

Perinatal Exposure Delayed Reproductive Effects in Male Laboratory Animals. Exposure to tetrachlorodibenzodioxins during development permanently alters reproductive function in male rats and hamsters. It reduces ejaculated and epididymal sperm numbers and sex accessory gland weights in offspring with normal testosterone blood and androgen receptor levels (Gray *et al*., 1995). It causes in males a morphologic and behavioral demasculinization and feminization, and a decrease in fertility (Gray *et al*., 1995). In prenatally exposed rats it inhibitions further development of male genitalia, decreases the urogenital complex weight and the anogenital distance, with no change in serum testosterone or luteinizing hormone (Ohsako *et al*., 2001). Tetrachlorodibenzodioxin exposure resulted in both a dose-dependent

increase in 5alpha-reductase type 2 (5alphaR-II) mRNA level and a dose-dependent decrease in androgen receptor mRNA level. Ohsako *et al*. (2001) suggested that low-dose dioxin exposure had a greater effect on external genital organs and ventral prostate development than on testis and other internal genital organs development, and that the decrease in ventral prostate size by maternal dioxin exposure might be due to decreased prostate responsiveness to androgen due to insufficient androgen receptor expression level during puberty. Further reports from Ohsako (2002) confirmed the decreases in the urogenital complex and ventral prostate weights, in urogenital-glans-penis length of male rat offspring at postnatal day 70, from rats exposed at gestational day 15 but not in rats exposed on gestational day 18 or postnatal day 2. Testicular and epididymal weights were also lower than control group in dioxin-exposed animals at gestational day 15 only. Anogenital distance was significantly reduced in the animals exposed at gestational days 15 and 18 but not in the postnatal day 2 group. PCR analysis showed that androgen receptor mRNA levels were decreased in the dioxin-exposed gestational day 15 group only, suggesting the presence of a critical window during development with regard to impairments of male reproductive organs by *in utero* and lactational exposure to a low dose of dioxin (Ohsako *et al*. 2002).

Prenatal dioxin exposure determines impaired growth of the rat seminal vesicles, associated with a decrease in the epithelium development. Seminal vesicle weights were not significantly decreased until postnatal day 32, and androgen receptor mRNA expression in postnatal day 25 seminal vesicles was not different from control. Dioxin exposure, however, decreased seminal vesicle epithelial branching and differentiation. Proliferating nuclear antigen was immunolocalized in controls in undifferentiated basal epithelial cells only; in dioxin-exposed animals was found in both basal and luminal seminal vesicle cells (Hamm *et al*., 2000).

In further studies of the inhibition of prostate development in prenatally dioxin-exposed mice Lin *et al*. (2003) found that exposure causes an aryl hydrocarbon receptor-dependent inhibition of prostatic epithelial bud formation commensurate with its inhibitory effects on ventral and dorsolateral prostate development, and that the inhibition of budding is not due to insufficient dehydrotestosterone. They suggested that inhibited bud formation is the primary cause of abnormal prostate development. In this context, Moriguchi *et al*. (2003) investigated the effect of dioxin exposure in homozygous mice for human type aryl hydrocarbon receptor gene and reported that these animals were less sensitive to dioxin-damage; in fact, human-type gene exposed animals developed hydronephrosis but not cleft palate, while mouse-type gene developed both abnormalities.

Hurst *et al*. (2000) reported that prenatal dioxin exposure of male fetuses at gestational day 15 caused a delay in puberty and decreased epididymal sperm counts, and warned that low-level dioxin exposure during the perinatal stage of life can produce adverse effects within the developing pups.

Perinatal Exposure Delayed Male Reproductive Effects in Humans. Prenatal exposure to polychlorinated biphenyls and dibenzofuranes determine, in adolescents at young adult age, persistent alterations in sperm quality and inhibits spermatozoid capacity to penetrate hamster oocytes; therefore, it may cause male infertility (Guo *et al*., 2000). Prenatal exposure to polychlorinated biphenyls also showed a significant delay in puberty in boys (Roy *et al*., 2009). Changes in children gender-related play behavior following perinatal exposure to dioxins, furans or polychlorinated biphenyls are reported below, *vide infra*.

Perinatal Exposure Delayed Reproductive Effects in Female Laboratory Animals. In addition to congenital genital malformations reported following prenatal exposure to dioxins (Hurst *et al*., 2000), *in utero* and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin and 2,3,4,7,8-pentachlorodibenzofuran reduces growth and affects reproductive parameters in female rats (Salisbury & Marcinkiewicz, 2002). Prenatal exposure disrupts regular estrous cycles, inhibits ovulation rate, and de-

creases serum estradiol following chorionic gonadotrophin stimulation. In mice, prenatal exposure to dioxins reduces fertility and increases incidence of premature birth (Bruner-Tran & Osteen, 2011).

Endometriosis develops in rhesus monkeys following prenatal and postnatal chronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (Rier *et al*., 1993). In the mouse, perinatal plus adult exposure to tetrachlorodibenzodioxin increases the size of surgically induced endometriotic lesions (Cummings *et al*., 1999). Dioxin-induced progesterone-resistance may play a role in the development of endometriosis (Nayyar *et al*., 2007; Bruner-Tran *et al*., 2010). In fact, exposure *in utero* of female mouse to tetrachlorodibenzo-p-dioxin causes a dose-dependent reduction in uterine sensitivity to progesterone during adult age (Nayyar *et al*., 2007; Bruner-Tran *et al*., 2010).

Perinatal Exposure Delayed Female Reproductive Effects in Humans. Developmental exposure to polybrominated biphenyls causes earlier menarche, thelarche and earlier pubic hair stage in pubertal girls (Roy *et al*., 2009). Changes in children gender-related play behavior following perinatal exposure to dioxins, furans or polychlorinated biphenyls are reported below, *vide infra*.

Perinatal Exposure Delayed Effects in the Mammary Gland of Laboratory Animals. Prenatal exposure to dioxins irreversibly inhibits the development of the mammary gland (reduced primary branches, decreased epithelial elongation, and fewer alveolar buds and lateral branches). This phenomenon persisted through postnatal day 68 when, unlike fully developed glands of controls, dioxin-exposed rats retained undifferentiated terminal structures. Gestational day 15 was a critical period for consistent inhibition of epithelial development (Fenton *et al*., 2002). Experiments using mammary epithelial transplantation between control and dioxin-exposed females suggested that the stroma plays a major role in the retarded development of the mammary gland following dioxin exposure (Fenton *et al*., 2002). This may be explained by an earlier proposition that stromal tissue regulates hormone effects of epithelial tissue via stimulating in the epithelium synthesis of hormone receptors which in turn respond to hormone stimulation; these mesenchymal-epithelial interactions may explain a weak epithelial response to hormone stimulation, or on the contrary a strong response or even carcinogenesis of epithelial tissues, depending on the development degree of its stroma (Cunha *et al*., 1983). This way, dioxin exposure prior to migration of the mammary bud into the fat pad may permanently alter the mammary epithelial development (Fenton *et al*., 2002). The glands, however, retain their ability to respond to estrogen stimulation with progesterone receptor synthesis and tissue differentiation (Lewis *et al*., 2001).

Perinatal Exposure Delayed Effects in the Human Mammary Gland. It was reported that prenatal or perinatal exposure to dioxin causes abnormal breast development in pre-pubertal girls (Roy *et al*., 2009).

Perinatal Exposure Delayed Neurobehavioral Effects in Laboratory Animals. Prenatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin determines demasculinization and feminization of sex behavior in male rats (Mably *et al*., 1992; Bjerke *et al*., 1994), which is not associated with alterations in estrogen receptor binding or volumes of sexually differentiated brain nuclei (Bjerke *et al*., 1994). Prenatal exposure to dioxin causes permanent morphologic and behavior male demasculinization and feminization, and decreases in fertility in rats and hamsters (Gray *et al*., 1995). *In utero* exposure to dioxin caused changes in sexual dimorphisms of saccharin preference and in the volume of the sexually dimorphic nucleus in the preoptic area in adult offspring; the demasculinization of male offspring was proposed to be caused by inhibition of brain aromatase activity in the hypothalamus preoptic area during central nervous system development (Ikeda *et al*., 2005). Prenatal exposure to a mixture of polychlorinated or polybrominated biphenyls causes feminization of sex-dependent sweet preference behavior and reductions of serum testosterone and testes weights in adult male offspring (Kaya *et al*., 2002, Lilienthal *et al*., 2006).

The dioxin-like polychlorinated biphenyl congener 3,4,3',4'-tetrachlorobiphenyl (PCB 77) has estrogenic and anti-estrogenic properties, and affects brain chemistry and behavior of developing rats when administered during gestation, especially affecting offspring maternal behavior (Simmons *et al*., 2005).

In the rat, prenatal exposure to dioxin inhibits the development of attentional processes (Markowski *et al*. 2002). Some of behavior effects evoked by prenatal exposure of laboratory animals are sexually dimorphic (Hojo *et al*., 2002). It was suggested that the anatomical and biochemical substrate for these alterations are a decrease in serotonergic neurons in prenatally exposed animals in all raphe nuclei as well as an immunostaining intensity decrease (Kuchiiwa *et al*., 2002). Approximately a quarter to half of immunoreactive neurons were detected in dioxin-exposed offspring raphe nuclei, as compared to control offspring. Perinatal exposure to dioxin also affects the expression of both subunits of the N-methyl-D-aspartate in the neocortex and hippocampus at postnatal day 49, without changes at postnatal day 5 (Kakeyama *et al*., 2001). The alteration consists in an enhancement of the nuclear receptor 2A subunit mRNA expression in the neocortex and hippocampus, and an inhibition of the 2B subunit mRNA expression. Similar alterations were found in the first postnatal months (Nayyar *et al*., 2003), supporting the finding of Kakeyama *et al*. (2001) who provided evidence that the perinatal exposure to dioxin can alter the molecular basis of the offspring brain in adulthood.

Perinatal exposure to polychlorinated biphenyls also causes persistent behavioral changes in rats (Pantaleoni *et al*., 1988). Dosage-dependent differences in the evaluated behaviors were found in the offspring of the polychlorinated biphenyls fenclor-42 -exposed females when compared to controls. Differences in the development of cliff avoidance reflexive behavior, swimming ability, and open field activity were particularly evident. Polychlorinated biphenyls exposure of female rats during gestation and lactation resulted in offspring impaired acquisition of the active avoidance behavior while preconceptional polychlorinated biphenyls exposure significantly affected active avoidance performance (Pantaleoni *et al*., 1988). Learning and memory is negatively affected by gestational or lactational exposure to dioxin (Seo *et al*., 1999; Haijima *et al*., 2010).

Perinatal Exposure Delayed Neurobehavioral Effects in Humans. Changes in 7-8 year-old children gender-related play behavior were reported following prenatal exposure to dioxins or furans: it determines feminization of male children gender-related play behavior and causes further feminization of female children play behavior (Vreugdenhil *et al*., 2002b). Prenatal exposure to polychlorinated biphenyls also determines feminization of in 7-8 year-old male children gender-related play behavior, but, in contraposition to the action of dioxins, prenatal exposure to polychlorinated biphenyls causes its masculinization in female children (Vreugdenhil *et al*., 2002b).

Various epidemiological studies revealed predictive relationships between prenatal or neonatal exposure to polychlorinated biphenyls, a delay in cognitive development (Lai *et al*., 1994) and subtle cognitive deficits in infancy through the preschool or school years (Patandin *et al*., 1999; Stewart *et al*., 2000; Vreugdenhil *et al*., 2002a, 2004). In 9-year-old children of the Rotterdam PCB-dioxin cohort, higher prenatal polychlorinated biphenyls levels correlated with longer response times, more variation in response times, and lower scores on the "Tower of London" (Vreugdenhil *et al*., 2004).

Perinatal Exposure Delayed Increase in Sensitivity to Carcinogenic Agents. Prenatal exposure to dioxins increases their sensitivity to estrogens to induce mammary cancer development in adult females (Birnbaum & Fenton, 2003). Dioxin effects on the developing breast are delayed proliferation and differentiation of the mammary gland, as well as an elongation of the window of sensitivity to potential carcinogens. It is suggested that the causes of endocrine-related cancers or susceptibility to cancer is a result of developmental exposures rather than exposures existing near the time of tumor detection. Prenatal dioxin

also increases mammary gland sensitivity to the carcinogen metilnitrosourea to induce breast cancer development (Desaulniers *et al*., 2001).

The carcinogens potentiation following dioxin exposure (perinatal or subsequent exposure) may reflect dioxin-induced aryl-hydrocarbon receptor suppression of expression of genes associated with cell cycle progression, DNA replication, spindle assembly checkpoint or DNA repair activation, as shown with a regulator of prostate cancer progression that contribute to enhanced carcinoma cell plasticity (Hrubá *et al*., 2011).

Other Perinatal Exposure Delayed Effects in Experimental Animals. Perinatal exposure to a low dose of the mono-ortho pentachlorobiphenyl PCB 118 permanently disrupted the hypothalamus-pituitary-thyroid axis causing a significant increase in thyroxin levels in offspring, as a "thyroid resistance syndrome" (Kuriyama *et al*., 2003 The mechanism of thyroid homeostasis disruption seems to be arylhydrocarbon receptor-independent, suggesting a different mechanism of toxicity from that of dioxins (Kuriyama *et al*., 2003).

Other Perinatal Exposure Delayed Effects in Humans. An association between polychlorinated biphenyls exposure and diabetes was reported for population younger than 55 years but not for older population in Anniston, Alabama, USA, where these chemical agents were manufactured from 1929 to 1971, causing significant environmental contamination (Silverstone *et al*., 2012). The age difference may reflect a window of susceptibility to exposure and suggest a role of the imprinting mechanism in the development of the disease. In this context, it was proposed that dioxin-like substances contribute to mitochondrial dysfunction (Lee, 2011) and that mitochondrial DNA, that may be imprinted in early life, may play a key role in development of insulin resistance and type 2 diabetes as it also occurs with poor fetal growth (Lee *et al*., 2005). Alterations in nail development were reported in prenatally exposed human population (Hsu *et al*., 1995).

7 Conclusions

For the success of species evolution, the protection of the individual's reproductive function from adverse environmental events is required. One of the mechanisms allowing this protection, according to our hypothesis, is the mechanism of imprinting which determines persistent hormone receptor changes resulting from prenatal exposure to toxic environmental agents, aimed to reproductive function conservation. For human beings, however, this process usually causes a myriad of delayed adverse effects which persist through individual's life. Limited reports have shown so far that several environmental pollutants, pharmaceuticals, food additives, and some food constituents, many of them considered as non-dangerous, cause adverse effects through the imprinting process. Of particular interest are the well documented effects of lead, arsenic and dioxin-like compounds. These effects should constitute a warning alerting that a large percentage of adulthood diseases may have been programmed before or around birth. Further research is needed to investigate the delayed effects caused by early human exposure to agents people may be exposed. Efforts to promote research will not be sufficient to obtain important improvements of health conditions and quality of life for the future generations unless research outcomes are followed by changes in public health policies and environmental standards, public information through formal education and mass communication media, and protection programs for population at sensitive stages of development, i.e., pregnant women, late fetal and early neonatal ages.

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