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Advanced Paternal Age and Endocrine Disruptors: Two Causes of Psychiatric Disorders in Children, with DNA Methylation Dys-Regulation as a Common Biochemical Mechanism

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INTRODUCTION

Autism is a complex neuro-developmental disorder with a male to female prevalence >4:1. The number of children diagnosed with autism syndrome disorder (**ASD**) has increased at an alarming rate. It's estimated by the Center for Disease Control and Prevention (**CDC**) that 14 out of 1,000 eight-year old children have been identified with ASD during the year 2014. In some North American states, the ratio can reach 1 in 50. The prevalence of the disorder has more than doubled since the year 2000.

Several authors have shown a link between advanced paternal age and increased risk of psychic disorders. This is especially concerning if we consider that, with the development of assisted reproductive technologies (**ARTs**), pregnancies are more and more delayed [1,2].

There is a growing body of evidence showing that exposure to endocrine disruptors (**EDC**) increases neuro-developmental disorders such as a decrease in IQ and poor memory [3,4]. EDCs have been shown to be trans-generational causative agents of endocrine and metabolic pathologies and sperm epi-mutations, linked to DNA methylation, as seen in rodent models. DNA methylation is involved in regulation of important processes such as gene transcription, genomic imprinting and genomic stability but also gene inactivation. DNA methylation is a major effector of epigenetic

regulations a process modifying gene expression without changing the underlying DNA sequence. EDCs are also EDCs are strong inducers of oxidative stress (**OS**) via their interactions with the estrogen receptors (**ER**) and peroxisome proliferator-activated receptors (**PPAR**).

Homocysteine is the epicenter between methylation and oxidative stress [5,6]. Abnormal homocysteine levels have been noted in the body fluids of autistic children, Group B vitamins are mandatory cofactors for the homocysteine recycling and metabolism. In fine, there is a significant association between MTHFR polymorphism causing a decrease in folic acid (vitamin B9) metabolism and autism. All these observations advocate a strong link between methylation errors and oxidative stress during gametogenesis, pregnancy, early life and genesis of psychiatric disorders in children.

ADVANCED PATERNAL AGE AND THE RISKS OF MENTAL DISEASES IN THE CHILDREN

It was common knowledge for decades to think that males had no ticking clock regarding their reproductive capacity. Several studies published in the nineties have clearly established that this old belief is erroneous. Advanced paternal age has a strong impact on the early embryonic developmental capacity with also an increased incidence in early miscarriages [7]. In fact the first scientific data regarding changes in this concept came from who studied in situ sperm DNA alterations in the form of sperm DNA fragmentation (**SDF**) [8]. It was then clearly demonstrated that a strong discrepancy can exist between the classical parameters of semen analysis, concentration, motility, morphology and the quality of transported DNA. From 1980 to 1995 it was thought in ART that the quality of sperm was not important since following the first cleavages the embryos were generally transferred day 2 or 3 after fertilization. Then, following culture to the blastocyst stage, it became clear that a spermatozoon with defective DNA has full capacity to fertilize oocytes and induce first cleavages but fails to promote further proper development.

The origin and causes of sperm DNA insults have now mostly been identified. Oxidative stress (**OS**) is a major effector via a two-step process. First the basic components are oxidized and DNA adducts are formed the most important one is 8-oxo-deoxy-guanosine (**8OHdG**); but more than 15 compounds have already been identified [9]. Moreover the reactive oxygen species (**ROS**) can lead to the formation of adducts after condensation with the by-products of lipid peroxidation [10]. Oxidative process can lead to de-amination of the bases with replication of damaged bases allowing a matching with a wrong opposing base. As a result, ROS induce the formation of apurinic/apyramidic (**AP**) sites. The oocyte is able to repair, to a certain extent, DNA decays borne by sperm but also to repair its own DNA damages. DNA repair activity of the zygote is mandatory in order to avoid mutations. The number of DNA repair operations is estimated at 2 million during the first cycle post fertilization. The oocyte DNA repair capacity is finite and decreases with maternal age. In the man, the situation is rather similar as the capacity to fight ROS decreases with age and the linked decays increase with age. Also DNA damage in sperm is of concern as it's related to paternal

transmission of mutations and potential cancer [11]. OStargets the complexes containing DNA methyltransferases, polycomb members to CpGislands [12]. In murine, sperm submitted to OS impairs embryo development and further on alters offspring metabolism [13]. This is especially disconcerting as most of the natural barriers to fertilization are overcome by intra-cytoplasmic sperm injection (**ICSI**). Embryos conceived by older men have a higher probability to lead to early miscarriages. The abortion rate at age 45 is more than double that of paternal age 30, going from 15% to 32.4% [3]. Definitely a link with paternal aging and significant increase in psychiatric problems such as autism or schizophrenia is well established [14]. It meant that the embryos escaping miscarriages might be more subject to an altered neurological development. The above findings are somewhat troubling knowing that with the advance in ART, pregnancies are more and more delayed.

THE ENDOCRINE DISRUPTORS (EDCS) (FIGURE 1)

Recent decades of modern life have had an impact on many aspects of life but also on fertility, through the use of pesticides, xenoestrogens, and endocrine disrupting chemicals involved in plastic technology such as polychlorinated bisphenyls (**PCB**), bisphenol A (**BPA**), dibutyl phthalates (**DBP**) and alkyl phenols, Di-(2-ethylhexyl) phthalate (**DEHP**) and other cosmetic additives (Figure 1) [15]. Many substances in the domestic or professional environment have not been tested before being launched in the market. Plastic Derived Endocrine Disruptors, are common in domestic use. DHEP and BPA are currently found in urine of men and they negatively impact sperm quality [16]. DEHP, parabens and BPA are regularly found in the urine and amniotic fluid of pregnant patients [17-20].

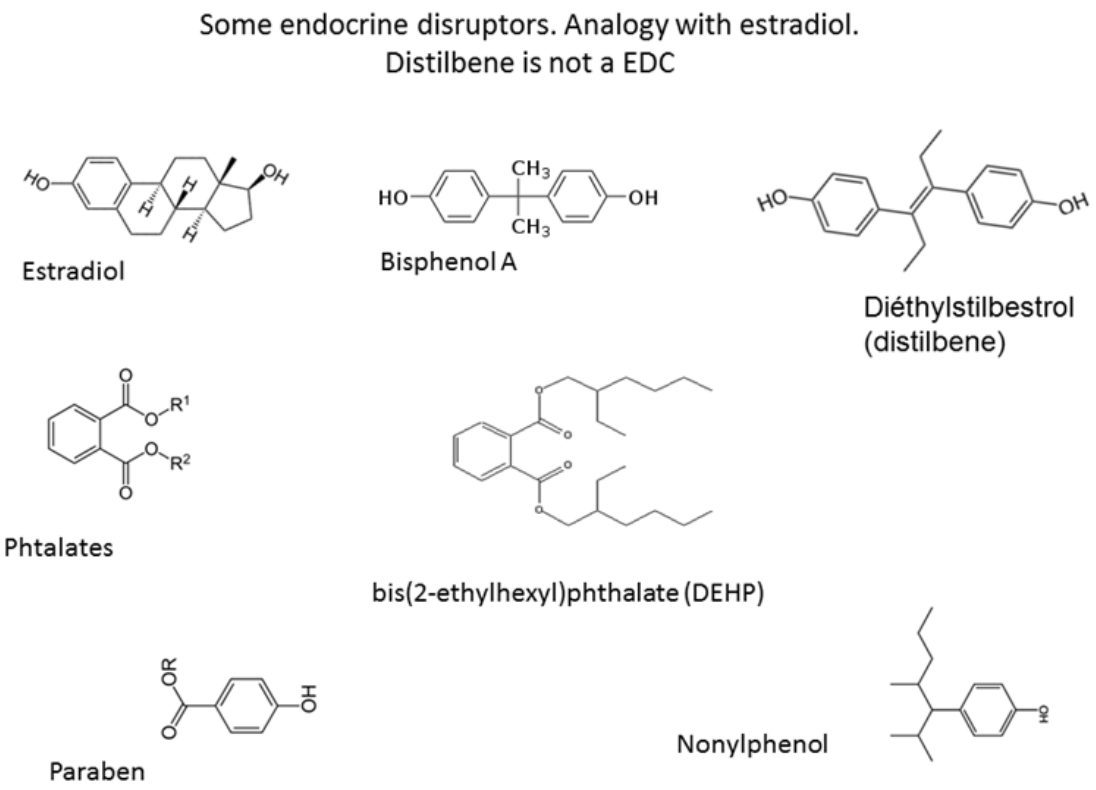

Figure 1: Main EDCs.

DEHPs are peroxisome proliferators and they impair mitochondrial function according to Iso T et al [21]. BPA can induce DNA damage independently through its estrogenic activity on receptors. In some cases, metabolites may be even more toxic than the native compounds. They also induce DNA strand breaks and chromosomal aberrations, as measured by several *in vitro* and *in vivo* tests (Ames test, unscheduled DNA synthesis (**UDS**), comet assay, etc.). Paraben basic compounds, a class of widely used preservatives in cosmetic or pharmaceutical products, or found in US foodstuffs [22] cause DNA damage and thus are potent inducers of oxidative stress [23]. One of the most important areas of concern is potential interactions/synergy. i.e. "cocktail effect" that may exist between these molecules, all now permanently present in the environment. Most of the EDCs, especially DHEPs and BPS are inhibitors of DNA methylation. This has to be considered on the female side: oocyte and then embryo.

`CpG islands are regions of DNA where Cytosine nucleotide (**C**) is followed by Guanine nucleotide (**G**) in linear sequences. The fact that methylation occurs mainly on CpG islands is an important feature: G is the base the most sensitive to oxidation and CpG cannot be performed correctly if G is oxidized. Then if methyl cytosine is oxidized, leading to the formation of 5-hydroxymethyl cytosine (**5HmC**) at a high level, an aberrant DNA de-methylation process is induced. The aberrant

methylation process due to the EDCs is concerning for gamete quality and embryos development. We have seen in the preceding paragraph that gametogenesis is impaired leading to a risk in the next generation. The female gamete is not protected during its periods of quiescence and then during follicular growth [9,24]. The oocyte needs a high content of reduced glutathione for counteracting the ROS and avoid apoptosis, but also in order to allow sperm swelling immediately post fertilization. During its growth, the oocyte stores mRNAS coding for the protection against ROS [25], but also the amino acids precursor for the synthesis of glutathione, necessary for the so called "oocyte competence". This permits a harmonious embryo development post genomic activation and additionally, it is important to note here that the oocyte and preimplantation embryos have a poor capacity to recycle Homocysteine the by-product formed post DNA methylation, after the loss of the methyl group by S Adenosyl methionine (**SAM**).

Two pathways the cystathionine beta synthase (**CBS**) pathway and the betaine homocysteine methyl transferase (**BHMT**) pathway are poorly expressed. So the preimplantation embryo has to rely mainly on the methionine synthase pathway. Both male and female gametes are sensitive to the negative impact of EDCs. Experiments in rodents have shown that plastic-derived endocrine disruptors (BPA, DEHP and DBP) affect strong gene methylation [26-28]. They induce severe pathologies in the pups that can be reversed by a supplementation with methyl donors (i.e. support of the one carbon cycle: 1-CC, figure 2) which positively affect gene expression and counteract the hypo-methylation effects of BPA. This means that the negative impact is especially related to DNA methylation. We can consider that EDCs after affecting negatively the gametes methylation process perturb methylation maintenance during the very early stage and then methylation resetting in fetal germ cells (Figure 3).

DNA methylation is of major importance in game to genesis, because primordial germ cells undergo a process of DNA de-methylation when they enter the developing gonads (imprint erasure). This process is subsequently reversed during prenatal life in males, and during post-natal follicle development in females, but the negative impact can continue during adult life. A cumulative effect seems the rule. EDCs cause incomplete methylation of specific gene regions in the young brain and impair neural development and brain functions across generations. This last negative impairment will lead to trans generational endocrine and possible psychiatric disorders via the gametes. A sex related sensibility towards the brain has been claimed but this parameter is controversial; it could be explained by the time difference observed for the epigenetic resetting between male and female. Considering that the brain inhibition of methylation/epigenesist/ methylation by EDCs can occur all along fetal life, during "brain growth and maturation", it can continue during early life [29]. Low-dose prenatal BPA exposure induces lasting epigenetic disruption in the brain that possibly under lie enduring effects on brain function and behavior [30].

Prenatal exposure to DDT has been shown to induce global DNA hypomethylation and leads to a depression like effect in the mouse, affecting serotonin signaling [31]. Exposure to EDCs could be

associated with depression and other psychiatric disorders such as schizophrenia. Incomplete or disorganized methylation can impair neural development and brain functions across generations [32]. These methylation errors can also lead to carcinogenesis in the next generation [33]. Finally the negative epigenetic reprogramming related to BPA may affect brain disorders on a cumulative way and through a cocktail effect, from the gamete and embryo development to adult life.

IMPORTANCE OF THE METHYLATION PROCESS: ONE CARBON CYCLE (1-CC, FIGURE 2) INEFFICIENCY MAY CAUSE MENTAL DISORDERS

The 1-CC also known as the methionine cycle (Figure 2) or the methylation cycle is a network of inter-related biochemical reactions allowing the transfer of one methyl group to molecules. The main compound is methionine. To be active methionine (**Met**) has to be transformed to S-Adenosyl Methionine (**SAM**) by the Methionine Adenosyl Transferases (**MATs**). Then SAM adds a methyl group on DNA (mainly on the cytosine at CpG site) or on histones. After loss of the methyl group, S-Adenosyl Homocysteine (**SAH**) is formed and hydrolyzed to Homocysteine (**Hcy**) by the SAH hydrolase, with liberation of Adenosine.

Hcy is a neurotoxic compound; it is an inhibitor of methylation and it competes for the same amino acid transporter with Met thus inhibiting Met regular entry in any cell [34]. So Hcy has to be recycled to Methionine by the addition of a methyl group. This is normally possible via two different pathways: one normally highly active and efficient, the Methionine synthase (**MS**) pathway and one generally less active: the betaine homocysteine methyl transferase (**BHMT**) pathway. A third pathway, the cystathionine beta synthase (**CBS**) pathway allows the removal of Hcy, with formation of cysteine, which can be, later on, used for the synthesis of 2 very interesting antioxidant molecules, glutathione and hypotaurine (highly present in the surrounding of the preimplantation embryo).

To be active the Methionine synthase (**MS**) pathway relies on the folate pathway (Figure 2). This pathway forms 5- Methyl Tetrahydrofolate (**5MTHF**), which interacts with Hcy to form Met under the control of two enzymes: MS and Methionine synthase reductase (MSR also called MTRR). Tetrahydrofolate (**THF**) is released and will be recycled in 5 MTHF by the folic acid cycle. Vitamins B3, B6, B9, B12 and Zinc are mandatory cofactor for a harmonious activity of the 1-CC. Most of the steps, if negatively affected by genetics, contribute more or less to psychiatric disorders. Abnormal levels of Hcy are found in the biological fluids of autistic children [35]. Elevated Hcy is also a risk factor for vascular dementia and Alzheimer's disease and schizophrenia [36,37].

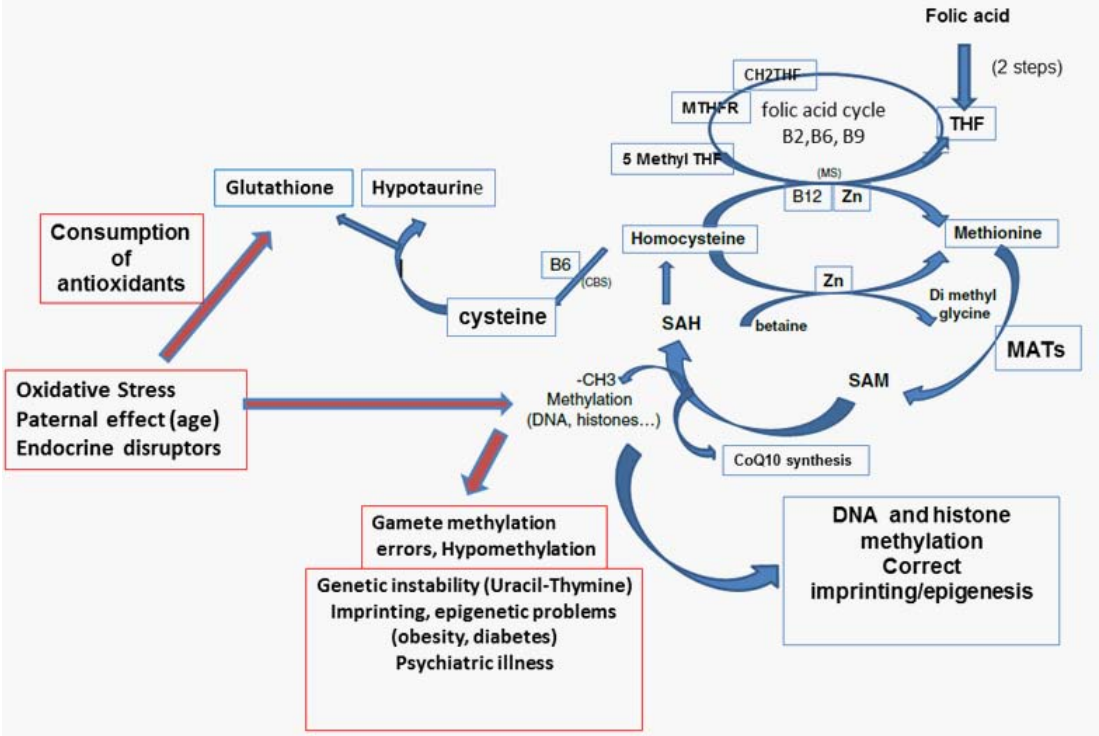

Figure 2: The one carbon cycle.

CBS: cystathionine betasynthase pathway, **CH2THF** : Methylenetetrahydrofolate, **5 MethylTHF**: 5 Methyltetrahydrofolate, downstream product formed by MTHFR activity, **MAT**: Methionine adenosyl transferase,

MTHFR: MethylenetetraHydrofolate Reductase, **SAH**: S Adenosyl Homocysteine, **SAM**: S Adenosyl Methionine

THF: tetrahydrofolate.

The most common genetic variants linked to the 1-CC is the MethyleneTetraHydrofolate reductase (**MTHFR**) an important piece of the folate cycle. The most common are the C677T and the A1298C, their prevalence can reach easily 50% of the population in some areas. These variant are a lower activity and so limit the supply of 5MTHF into the 1-CC (and especially methionine synthase). An increase level of Hcy is often observed in the homozygotes (especially for C677T). An association is usually observe between the C677T isoform and the risk of Autism spectrum disorders [38,39] but also schizophrenia [40].

Another enzyme of the folic acid cycle, the Serine Hydroxymethyl transferase (SHMT, allowing the synthesis of Methylene THF) has a C1420T variant form leading to hyperhomocysteiemia. There are several other enzymes affected by genetic polymorphism with important functional consequence; The MTR variant P1173L causes megaloblastic anemia and development delay.

The A2756G generates hyperhomocysteiemia especially when in a homozygous form. The defective variant A66G of MTRR is clinically linked to hyperhomocysteiemia; its prevalence is nearly as important as MTHFR. Mutations in the BHMT pathway induce neural tube defect and/or hyperhomocysteiemia. As the vitamins B are mandatory cofactors of the 1-CC, all types of deficiencies, transport and metabolism will lead to brain problems such as depression and Alzheimer for review [41,42]. Any dysfunction or slowing down of the 1-CC may affects brain development and functionality with Hcy at its epicenter.

THERAPEUTIC APPLICATIONS

There is no debate anymore. The methylation process is strongly involved in the transgenerational transmission of mental, but also of endocrine and reproductive disorders. The link with oxidative is not less obvious; tis can appear less evident for the genetic impact on the 1-CC, but one must not forget that the recycling of homocysteine allows the synthesis of cysteine. Cysteine is a precursor of glutathione and hypotaurine synthetized via the cysteine sulfonate pathway (**CSD**). Hypotaurine is the most important antioxidant in the surrounding of the preimplantation embryo [43]. Moreover the synthesis of CoQ10 requires 3 methylation steps. All the steps, from gametes to newborn (and after) can be affected by methylation/epigenetics errors.

A very interesting information was recently provided by a Japanese team, indicating that there is a strong methylation maintenance activity during preimplantation development up to genomic activation and immediately after day 3 to 4 of the human embryonic development [44]. It was confirming our work indicating a strong expression of DNA methyl transferase 1 (**DMT1**) and folate receptor 1 and folate transporter member1 in the oocyte and early embryo [45]. It was thought that early preimplantation was characterized by a global demethylation. This DNA maintenance is probably of major importance for further embryo "normality/quality" and the embryo, as early as conception is sensitive to methylation "aggressions" (Figure 3). Due to the small quantity of material in a preimplantation embryo (70-80 ng of dry mass), an extreme sensitivity to external insults can be expected. This is the reason why current *In vitro* fertilization (**IVF**) culture media generate methylation anomalies in the babies [46-48].

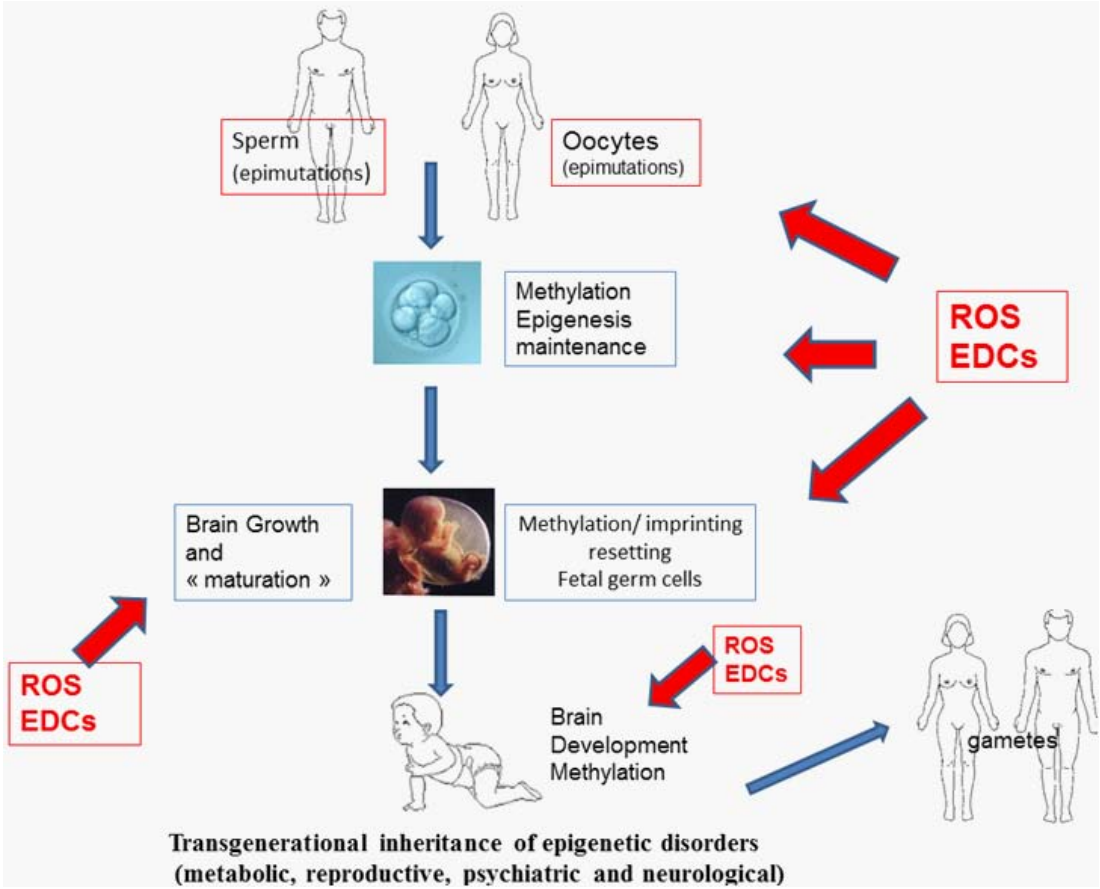

Figure 3: Embryo development and sensitivity to EDCs and Oxidative stress.

Presently, the available simple and complex culture media are often lacking certain essential amino acids such as methionine and cysteine, as proposed and expressed by several authors [49]. Actually, in order to complete full methylation maintenance the preimplantation embryo needs methionine, cysteine and methyl donors; Patients carrying genetic problems linked to the 1-CC have a more important handicap in this regulatory process. This is why these patients'embryos have a decreased developmental potential [50]. There is a link between reproductive, endocrine and psychiatric disorders [51,29]. As in rodent models methyl donors can improve, to a certain extent the methylation process [26]. We have tried to find out if support of the 1-CC cycle can partly alleviate metabolic derangements induced by environment, and improve reproductive capacity in infertile couples [52,53]. The treatments, Procrelia women R and CondensylR, (Nurilia, Lyon France), led to a real improvement when compared to a control group with no treatment. The formulation contains vitamins B2, 3, 6, 12, folic acid, Zinc (on a chelated form) and N acetyl cysteine. One of the most striking effect was the occurrence of spontaneous pregnancies in women having failed several ART attempts. In addition, the circulating Anti Mullerian Hormone (**AMH**), a

marker of the ovarian quality, increased significantly. Eight out of 49 patients, who were advised to enter an oocyte donation program became spontaneously pregnant and delivered [54].

In men, a significant decrease was observed in the sperm DNA fragmentation and decondensation. In the same way of thinking, we proposed to couples carrying the MTHFR C677T isoform, either heterozygous (**HTZ**) or homozygous (**HMZ**), having a severe history of miscarriages (up to 9) and/or infertility, a treatment with 5 MTHF as they metabolize folic acid with difficulty. In fact, 5MTHF is replacing folic acid in our previous treatment in TetrafolicR (Nurilia , Lyon, France) and ImprylR (Parthenogen, Lugano, Switzerland). From the 31 couples having fulfilled treatment, 17 started a pregnancy, but still 4 miscarried. From these pregnant 13 couples (>9 weeks of gestation including 3 deliveries)), one was HMZXHMZ and 8 were HMZXHTZ and 4 were HTZXHTZ [55]. Further studies involving more patients are ongoing.

Our results are highly encouraging when they refer to the reproductive process. Two points must be highlighted 1- We will eventually need to confirm these results in regard to prevention of psychiatric disorders. 2- Prevention means treatment of the parents before conception, of the mother during gestation, but also of the children after birth. Breast-feeding by the mother during treatment must be recommended. Further on, for the children all doses and length of treatment have to be carefully defined

GENERAL CONCLUSIONS

We can generally agree that the average lifetime expectancy has increased. There is also no doubt that modern life has negative effects on some aspects of reproduction, endocrinology and mental health. There is a common trend in western countries in delaying pregnancies. Assisted reproductive technologies have allowed us to prolong that delay. If oocyte early freezing and donation have solved some of the problems on the female side, paternal age may still have a noxious effect on children's health. The endocrine disruptors impact on the gametes epigenetic processes have added more problems. Finally, the MTHFR genetic component must not be overlooked. A support of the one-carbon-cycle may alleviate some of the risks. However, a definition of guidelines must be established for prophylaxis and treatment of the children.

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