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RISK FACTORS IN AUTISM SPECTRUM DISORDERS: THE ROLE OF GENETIC, EPIGENETIC, IMMUNE AND ENVIRONMENTAL INTERACTIONS

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Abstract

Autism spectrum disorders (ASD) are a heterogeneous group of neurodevelopmental disorders that affect an individual's ability to communicate and socialize, often associated with repetitive movements or behaviors. Frequently patients associate intellectual disability or digestive problems. Autism is more common in males and affects 1 in 88 children in USA. The mechanism that leads to ASD is very complex, involving genetic, epigenetic, immune and environmental factors that could act in different proportions, at different developmental stages (prenatal, perinatal or postnatal) and on different pathways. The general prototype consists in an initial systemic dysfunction, such as immune dysregulation, inflammation, impaired detoxification or oxidative stress in an individual with genetic predisposition. In this context, ASD may arise due to the harmful action of environmental factors that lead to neuroinflammation and abnormal brain development. Environmental factors involved in autism determinism could be very diverse and include classical extrinsic factors (toxicants, environmental pollutants, medications, food additives, electromagnetic fields and even social influences), maternal disorders or lifestyle factors, as well as intrinsic factors (hormones, inflammatory mediators, microbiota and other biological molecules that make up the microenvironment around the developing fetal or neonatal brain).
The aim of the present review is to discuss actual theories concerning genetic, epigenetic, immunologic and environmental factors interplay in ASD determinism, to present a practical and global approach of this complex problem, as well as to point some of the new directions for ASD prevention and therapy.

Key words: autism spectrum disorders, environmental factors, epigenetic, genetic, microbiota

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1. Introduction

Autism spectrum disorders (ASD) are a heterogeneous group of neurodevelopmental disorders defined by core deficits in social interaction and communication, restrictive interests and repetitive behaviors appearing before age 3 (American Psychiatric Association, 2013). The spectrum encompasses autistic disorder, Asperger syndrome, pervasive developmental disorder not otherwise specified, childhood disintegrative disorder and Rett syndrome. Frequently autistic children associate comorbidities like intellectual disability, seizures, schizophrenia, sleep disorders or gastro-intestinal symptoms (e.g. diarrhea, constipation, bloating and gastro-esophageal reflux) (Rossignol and Frye, 2012).

The mechanism that leads to ASD is very complex, involving genetic, epigenetic, immune and environmental factors that could act in different proportions, at different developmental stages (prenatal, perinatal or postnatal) and on different

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pathways. The general prototype consists in an initial systemic dysfunction, such as immune dysregulation, inflammation, impaired detoxification or oxidative stress (Rossignol and Frye, 2012). In this context, ASD may arise due to the harmful action of environmental factors. Autism is more frequent in males (4 male/ 1 female ratio) (Baron-Cohen et al., 2011) and different genetic, epigenetic, metabolic and social hypotheses tried to explain this finding. In USA, ASD prevalence has increased in time, from 1 in 3,000 individuals in 1966 (Lotter, 1966), to 1 in 150 in 2007 (Kuehn, 2007) and 1 in 88 in 2012, with specific prevalence in boys (1 in 54) comparative to girls (1 in 252) (CDC, 2012). This recent increase pointed to environmental factors as a key issue for ASD determinism and stimulated research in the field. Surprisingly, research showed that ASD could be triggered in a genetically predisposed individual not only by classical external environmental factors (e.g. toxicants, pollutants, pesticides), but also by maternal imbalances or disorders (e.g. hormonal or inflammatory), as well as by disturbances of gut microbiota in the affected child (considered as internal environment).

The aim of the present review is to discuss actual theories concerning genetic, epigenetic, immunologic and environmental factors interplay in ASD determinism, as well as to point some of the new directions for ASD prevention and therapy.

2. Phenotypic approach to ASD studies

To overcome the difficulties of genomic studies (raised by the multitude of factors involved in ASD determinism), some studies in the literature tried to divide ASD cases according to different clinical criteria and to relate each category with a specific genetic defect (Tadevosyan-Leyfer et al., 2003). These studies have identified genes involved in nervous system development and function, as well as genes related to endocrine and immune function (frequently disturbed in ASD) (Hu et al., 2009).

3. Genetic roots

Genetic ASD causes are represented by structural DNA defects that could be either sporadic (the parents of the affected child are normal) or could be inherited in the family.

Early on, ASD concordance in monozygotic twins was considered to be 90%, meaning that nongenetic factors (i.e. the environment) might have a little role in ASD determinism (Steffenburg et al., 1989). However, recent studies have shown that ASD heritability (i.e. contribution of the genetic factors) is only 55% (Hallmayer et al., 2011), environmental influence being much more important than expected before.

DNA investigations have associated ASD with 2,193 genes, 2,806 single nucleotide polymorphisms (SNPs), 4,544 copy number variations (CNVs) and 158 linkage regions (Xu et al., 2012). Single gene disorders with high incidence of ASD, including Angelman, Fragile X, Rett, Smith-Lemli-Opitz and Timothy syndrome, as well as neurofibromatosis and tuberous sclerosis account for less than 20% of autistic patients (Benvenuto et al., 2009). In these disorders genetic counseling and prenatal diagnosis easily allows the prevention of new cases, unlike the rest of the cases where the multitude of factors involved makes the management much more difficult. Copy number variation is the term used when a certain large DNA fragment is either missing (deletion) or present as extra copies (duplication); frequently duplications are associated with ASD, not deletions (deletions produce more severe consequences, being recorded in cases that associate ASD with intellectual disability and multiple congenital anomalies) (Girirajan et al., 2013).

Single nucleotide polymorphisms are very common DNA defects consisting in very small altered DNA sequences that can affect the function of a gene. More than 100 SNPs in genes involved in the detoxification of environmental pollutants have been involved in ASD, because they increase susceptibility to the adverse effects of environmental toxicants (Livingston et al., 2004). Environmental agents may act as mutagens in at least two ways: either by contributing to oxidative stress (leading to DNA damage by free radicals) (Valko et al., 2005), or by inhibiting DNA repair systems (thereby leading to the accumulation of mutations) (Filipic and Hei, 2004). To maintain intracellular balance and protect against oxidative stress, cells produce glutathione. Mercury, cadmium and nickel are toxic because they reduce intracellular glutathione and bind to proteins used for DNA packing (Valko et al., 2005), leaving the DNA vulnerable to mutagenic effects of reactive oxygen species (Kinney et al., 2010).

The defects mentioned above are related to two key elements for ASD determinism:

a) Synaptic development and functioning and intracellular Ca^{2+} signaling (Levitt and Campbell, 2009);
b) Epigenetic regulation of gene functioning.

4. Epigenetic mechanisms

Waddington introduced the term “epigenetics” to refer to causal mechanisms by which the genes of the genotype bring about phenotypic traits (Waddington, 1942). He has anticipated interactions between genes and between genes and the environment as an important foundation to understand development (Millan, 2013). Thus, epigenetic mechanisms act on the genome to regulate gene expression, affecting the phenotype. This includes regulation by DNA methylation, histone modification, chromatin remodeling and microRNA expression, all processes being important for the development and function of the nervous system (Hu, 2013). The DNA encodes the information for all human traits. It is tightly packed with specific

proteins called histones. Chromatin represents the DNA and associated proteins within the nucleus. The tightness of wrapping (and thus the activity of the genetic material in the area) depends on the variant histone proteins used, as well as on the addition or removal of methyl groups to histone proteins (Posavec et al., 2013).

Mutations of the genes involved in this process have been found in ASD (Lasalle, 2013). Some environmental toxins act by reducing levels of DNA methylation (Baccarelli 2009). Fortunately, nutritional factors (e.g. folate and B vitamins) may counteract the harmful effect of chemical pollutants on DNA methylation levels. The typical pathway is the one carbon metabolism cycle that supplies methyl donors from the diet for methylation reactions to both DNA and histones (LaSalle, 2011).

Before implantation the embryo undergoes extensive DNA demethylation (methyl groups removed) followed by specific remethylation (methyl groups inserted) after implantation (Reik and Dean, 2001). Children with ASD and their mothers have prejudiced the capacity of methylation (James et al., 2010). In the absence of periconceptional vitamin supplementation, maternal gene variants give less efficient one carbon metabolism and higher homocysteine levels. MTHFR is the key enzyme that regulates folate metabolism. MTHFR 677TT genotype decreases enzyme activity leading to inefficient folate metabolism, decreased blood folate, elevated plasma homocysteine and reduced methylation, mainly in individuals with low B vitamins levels (Hustad et al., 2007). Mutations in other genes involved in folate metabolism also increase maternal and fetal homocysteine with subsequent increase in ASD risk in the absence of maternal periconceptional vitamin intake (Boyles et al., 2006).

5. Oxidative stress and mitochondrial dysfunction

Mitochondria are complex, biochemically active and dynamic cellular components with a major role in energy production and management of reactive oxygen species (Shetty et al., 2012). They are providing energy for maintaining ion gradients, key elements in synaptic transmission (Toescu, 2000). The brain is extremely vulnerable to oxidative stress because of both high energy request and poor equippment with antioxidant enzyme defenses (Natelson, 2013), mitochondrial dysfunction increasing ASD risk. Mitochondrial dysfunction leads to three main consequences: a) decreased energy production; b) increased production of reactive oxygen species (ROS) and oxidative damage; c) induction of apoptosis (cell death) (Rossignol and Frye, 2012).

All these changes have been recorded in autism, but may be also induced by pesticides (Franco et al., 2009). Most pesticides induce dysregulation of Ca^{2+}-mediated signaling and production of mitochondrial ROS (Mariussen and Fonnum, 2006).

6. Immune dysfunction

Recent reviews of the immune dysregulation in autism have revealed different defects, the most important being altered cytokine profiles (Ashwood et al., 2003), neuro-inflammation and auto-antibodies directed at nuclear brain proteins (Goines and Van de Water, 2010).

Cytokines are proteins that control the nature, duration and intensity of an immune response. They are produced by different immune cells, as well as by non-immune cells like neurons (that produce them and also respond to them) (Goines and Ashwood, 2013). Cytokines are involved in normal neurodevelopment, including progenitor cell differentiation, cellular localization/migration within the nervous system and synaptic network formation (Deverman and Patterson, 2009). Levels of pro-inflammatory cytokines are elevated in ASD patients in a dose - response fashion (Ashwood et al., 2011), demonstrating an active neuro-inflammatory process (El-Ansary et al., 2013).

Many autistic individuals have food allergies (Jyonouchi, 2010) and allergic-like symptoms (Angelidou et al., 2011), but often with no positive test results, suggesting mast cell activation by non-allergic triggers (Theoharides et al., 2012). Mast cells are the “immune gate to the brain” (Theoharides, 1990). They could be stimulated by allergic, environmental (mercury), infectious (viruses), mitochondrial, stress and toxic (propionic acid) triggers and release inflammatory and neurotoxic molecules that trigger focal brain allergies. They also increase blood brain barrier permeability, allowing circulating lymphocytes to enter the brain leading to focal encephalitis (Theoharides, 2013).

The “leaky gut” theory considers that increased gut permeability allows different substances to enter the blood stream and damage the central nervous system leading to ASD, (De Theije et al., 2011). Autoimmune diseases like type 1 diabetes, Hashimoto’s thyroiditis or psoriasis were frequently recorded in families with autistic children, especially in the mother (Comi et al., 1999); maternal allergies, asthma, rheumatoid arthritis or celiac disease have also been related to ASD risk in children (Atladottir et al., 2009). Major consequences of the prenatal disruption of the immune development consist in atopy, allergy and autoimmunity in early childhood (Hertz-Picciotto et al., 2008). Exposure to several types of pesticides may decrease immune competence and enhance autoimmunity (Corsini et al., 2008). Organo-phosphorus pesticides are particularly immunotoxic (Li, 2007), as well as pyrethroid pesticides (Gabbianelli et al., 2009). The chemical structure of the compound influences proinflammatory and immunosuppressive properties of the pesticide (Rooney et al., 2003).

7. Environmental causes

7.1. General context

The term "environment" used here means any factor that is not part of the genome (i.e. genetic material) and may have a role in ASD determinism. It includes intrinsic factors (hormones, inflammatory mediators, microbiota that make up the microenvironment around the developing fetal or neonatal brain), as well as extrinsic factors (toxicants, environmental pollutants, medications, food additives, maternal disorders and even social influences that may have an impact on maternal, fetal or neonatal tissues). Environmental agents that have been involved in ASD determinism may act before, during or after birth because even if the generation of new neurons is completed well before birth, their migration and differentiation, as well as the patterning of neural circuits continue after birth (Buss et al., 2006).

Advanced parental age is an important preconceptional factor related to ASD risk. Sperm from older men contain more DNA mutations (Kong et al., 2012), whereas older women are at higher risk of having ASD children due to disturbed epigenetic regulation. The season of conception was also correlated to ASD risk. Children conceived in winter months have an increased risk of autism (Zerbo et al., 2011). This risk is correlated with maternal allergies before pregnancy (Croen et al., 2005), viral infections in the first months of pregnancy, nutritional factors (vitamin/mineral deficiency during winter time), with the precipitation rates (no sun exposure with subsequent lack of vitamin D) and with the use of pesticides in summertime (second and third trimester of pregnancy) (Lyall et al., 2013).

Results of the studies referring to toxicant environmental exposure should be interpreted with caution because genetic factors could influence the threshold for susceptibility to certain toxicants. Moreover, toxicant tissue levels may be very dynamic, depending on factors like caloric state, exercise, fever etc. For all these reasons, emerging testing methods incorporate tissue mobilization of toxicant using techniques such as caloric restriction (Gavrilescu et al., 2015; Rossignol et al., 2014).

A few recent meta-analyses identified significant ASD pregnancy-related risk factors: maternal disorders (diabetes, infections, bleeding during pregnancy and HTA), maternal medication (e.g. valproate), maternal exposure to organo-phosphate insecticides, first-born children (compared to the next ones) and mother born abroad (pregnancy close to migration moment; ASD risk probably related to maternal stress and pregnancy-induced low immunity for common infections). Potential risk factors in the perinatal and neonatal period could be: abnormal fetal presentation, umbilical cord complications, fetal distress, birth trauma, multiple birth (twin pregnancy), maternal hemorrhage, summer birth, small for gestational age, meconium aspiration, neonatal anemia, ABO/Rh incompatibility and hyperbilirubinemia (Gentile et al., 2013). Risk factors are associated with hypoxia that leads to oxidative stress and neurotoxic effects (Gardener et al., 2009).

7.2. Exogenous medically related factors

Congenital rubella infection or an impaired immune response to rubella vaccination has been observed in some ASD children (Stubbs, 1976). Other viral infections (herpes simplex virus, cytomegalovirus, varicella zoster virus, mumps virus, influenza virus and polyomaviruses) have been associated with ASD (Ciaranello and Ciaranello, 1995; Lintas et al., 2010). The most significant association was reported when the viral infection was recorded in the first trimester of pregnancy (Atladottir et al., 2010).

There are two possible mechanisms linking infections to ASD: either direct neurotoxic effect or neurotoxic effect mediated by the immune system. The direct neurotoxic theory appreciates that the pregnancy is an immune-suppressive condition and women could easily contract infections that damage the incompletely developed fetal brain (Sells et al., 1975). For the immune mediated effects of viral infections there are two possible hypotheses: "molecular mimicry" (an immune cell recognizes a viral protein that resembles a human protein and triggers an immune response against both structures) and "bystander activation" (expansion of an immune response directed at tissues altered by inflammation induced by a viral infection) (Munz et al., 2009); the viral infection could produce a transient increase of pro-inflammatory cytokines without viral persistence ("hit-and-go" mechanism) or could produce chronically elevated pro-inflammatory cytokines due to viral persistence (Libbey et al., 2005); cytokines may be produced directly in the brain or get access through the immature blood-brain barrier; these events could occur also in the maternal immune system, with subsequent attack of the fetus and abnormal neurological development (Libbey et al., 2005).

Vitamin D deficiency has increased in the last time due to life style modification (work inside, longer time spent inside watching television, use of solar filters and sun avoidance during pregnancy) (Cannell et al., 2008). Apart of the classic regulatory pathway of calcium metabolism, vitamin D is also involved in controlling the immune response by modulating immune cells (Baeke et al., 2010). It also promotes DNA synthesis and repair (Ellison et al., 2008). Vitamin D level correlates with the latitude (Holick, 2005), the month of conception (Cannell et al., 2008), urban populations (Williams et al., 2006), air pollution (Windham et al., 2006), regions with high levels of precipitation and no sunny weather (Waldman et al., 2008), mother's metabolic status (obese individuals have a higher risk of vitamin D deficiency) and darker skin (Cannell et al., 2008), as

well as increased access to cable television with longer time spent indoors (Waldman et al., 2008). In females, estrogen increases neural vitamin D and protects against vitamin D deficiency, unlike testosterone that makes male brains susceptible to vitamin D deficiency, providing one reason why ASD is more frequent in males (Cannell et al., 2008).

Iron deficiency during the critical period of neurodevelopment could also trigger epigenetic mechanisms and increase ASD risk (Georgieff, 2008). Pre-pregnancy obesity (≥ 90 kg) and excessive weight gain (≥ 18 kg) during the pregnancy have been significantly associated to ASD (Krakowiak et al., 2012). In women with non-insulindependent diabetes, increased fetal metabolism induces chronic intrauterine tissue hypoxia (Eidelman and Samueloff, 2002) and fetal iron deficiency (Georgieff, 2008), affecting neurodevelopment and neuronal connectivity (Georgieff, 2006). Moreover, increased cytokine levels disrupt normal fetal brain development by crossing the placental barrier (Krakowiak et al., 2012).

Mothers of ASD children frequently have auto-immune disorders, including rheumatoid arthritis, celiac disease and insulindependent diabetes (Atladottir et al., 2010), maternal autoimmune reaction having negative effects on fetal brain development (Careaga et al., 2010).

Valproic acid (frequently used as an anticonvulsivant) has been associated with a substantial increase in autism risk (Rasalam et al., 2005). It enhances DNA demethylation and interferes with the methylation processes necessary for normal brain development (Gadad et al., 2013). In humans, it seems that assisted reproductive technologies are not associated with an increased risk of autism, except ovulation-inducing drugs use (Hvidtjorn et al., 2011).

Pregnant women who receive paracetamol in the third trimester of pregnancy have an increased risk of HTA and subsequent ASD in the child (Rebordosa et al., 2010). Their ability to sulfate paracetamol is reduced, with subsequent activation of an immune response and pro-inflammatory cytokine signaling (Jetten et al., 2012). There has been a lot of debate about the initial suggestion that MMR (measles – mumps – rubella) vaccine, through its thimerosal content, may contribute to autism (Wakefield et al., 1998). Thimerosal contains ethyl mercury and has been widely used as a preservative in many drug products and vaccines. The scientific consensus based on multiple epidemiologic studies rejected the relationship between thimerosal-containing vaccines and autism (Parker et al., 2004).

7.3. Maternal lifestyle factors

Short inter-pregnancy interval was associated with increased risk for ASD (Cheslack-Postava et al., 2011). A possible explanation of this finding could be maternal nutrient depletion, especially folate (van Eijsden et al., 2008). Essential nutrients (including folate) are preferentially distributed to the fetus, resulting in depleted stores in the mother, a state that remains for at least 12 months after the delivery (Milman et al., 2006). Folic acid supplements have a protective role, especially in the presence of an inefficient folate metabolism (either in the mother or in the child) (Schmidt et al., 2011). No association has been found between average alcohol consumption and autism (Eliasen et al., 2010). However, high alcohol consumption facilitates folate deficiency and may increase ASD risk.

Maternal smoking has been associated with autism. A possible mechanism could be reduced blood flow to the fetal brain due to placental insufficiency produced by smoking and oxygen deprivation (Albuquerque et al., 2004). Maternal smoking is also associated with stress and this triggers epigenetic ASD risk. Heavy metals from cigarette smoke (especially cadmium and lead) accumulate in maternal bones and are co-transferred with calcium to the fetus and infant during pregnancy and lactation (Sanders et al., 2012). In the fetus they induce epigenetic alterations that predispose to autism. Maternal diet poor in leafy vegetables, meat and eggs could lead to folate deficiency with subsequent increase of ASD risk. For this reason in most of the countries worldwide peri-conceptional vitamins (including high doses of folic acid) are recommended and some countries decided to fortify bread with folic acid.

Some studies (McCanlies et al., 2012) have associated ASD in children with occupational workplace exposure of the parents (especially mother) during the three months preceding pregnancy through birth or weaning. Moreover, a home environment with polyvinyl chloride flooring, cable TV (with subsequent vitamin D deficiency due to less time spent outside), microwave, wireless technology and pesticides for pets, as well as home close to highways or fields with pesticides could also increase ASD risk.

7.4. Environmental chemicals

The effect of various toxicants in ASD determinism could be potentiated by the concomitant action of electromagnetic frequency and radiofrequency exposures (Juutilainen et al., 2006).

7.4.1. Endocrine-disrupting chemicals

Adequate levels of in utero thyroid hormones are critical for brain development. Maternal thyroid impairment has been associated with exposures to environmental chemicals (Caliman and Gavrilescu, 2009; Winneke, 2011). Pesticides interfere with thyroid function by preventing iodine uptake and thyroid hormones synthesis (Colborn, 2004; Gavrilescu et al., 2015), with neurodevelopmental consequences in the child (Zimmermann, 2007). The human fetus does not start producing sufficient thyroid hormones until gestational week 18 (Burrow et al., 1994), meaning that adequate maternal thyroid hormones are critical to neurodevelopment in early

fetal life (Pathak et al., 2011). Polychlorinated biphenyls (PCB) were used as coolant fluids, plasticizers, adhezives, industrial oils, lubricants and pesticides, banned 40 years ago due to their toxicity, but they are still released into the environment due to their persistence or from building materials (Jamshidi et al., 2007). Breast milk could be a source of exposure due to bioaccumulation of PCBs through the food chain (Hertz-Picciotto et al., 2008). In the prenatal brain, PCBs act on signaling pathways that regulate neuronal connectivity (Kodavanti, 2005). So, children with mutations in genes involved in these processes are more sensitive to PCBs and prone to ASD (Stamou et al., 2013).

Polybrominated diphenyl ethers (PBDE) are another class of endocrine-disrupting chemicals. They are used for flame retardants, foam in furniture, children's clothing and household materials. In spite of their banned production, PBDE levels in humans are high, as they bioaccumulate (Frederiksen et al., 2009). Children react by robust inflammation to PBDE exposure during the critical developmental windows, with long term neurologic consequences and subsequent ASD (Goines and Ashwood, 2013). Both PCB and PBDE interact with specific receptors (Gu et al., 2012), disrupt endocrine systems (Lema et al., 2008) and interfere with calcium homeostasis (Langeveld et al., 2012), all of them leading to neurologic and immunologic consequences (Herbstman et al., 2010).

Bisphenol A (BPA) and phthalates have also been reported as endocrine disruptors. BPA is a plasticizer found in plastic drink containers, the lining of food cans and plastic food wrappings, dental resins and baby bottles, as well as thermal paper. BPA has estrogenic properties. Phthalates are used in cosmetics, lotions, fragrances, vinyl flooring and a variety of plastics and have anti-androgenic effects. In-house flooring material made of polyvinyl chloride (a source of airborne phthalates), unlike wood flooring, has been associated with an increased risk of ASD (Larsson et al., 2009). Vinyl chloride is also a mutagenic agent, being metabolized to products that act directly on DNA, leading to chromosomal deletions (Chiang et al., 1997).

One possible mechanism by which endocrine-disrupting chemicals influence brain development and increase ASD risk is through disruption of thyroid homeostasis during pregnancy that affects nervous cells (Hertz-Picciotto et al., 2008). Alternative mechanisms are either by altering molecular signaling, including calcium (Shafer et al., 2005) or direct effects on neural development or the placental or blood-brain barriers. Endocrine-disrupting chemicals could also contribute to the male preponderence of ASD due to their effects on steroid hormones.

7.4.2. Pesticides

Pesticides are composed of a parent product, inert ingredients and agonists that enhance the functionality of the parent compound. All these compounds could be degraded to metabolites that distribute throughout the body, meaning that the mechanism by which pesticides determine ASD could be either direct (pesticide action) or indirect (metabolite action) (Shelton et al., 2012). Pregnant women could be exposed to pesticides from very variable sources. They may be applying pesticides in or around their homes or to their pets, consuming food with residues of pesticides or pesticide metabolites or inhaling air from agricultural or urban spraying near their home or workplace (Gavrilescu, 2005; Shelton et al., 2012).

Exposure to organo-chlorine (OC) pesticides (e.g. hexachlorobenzene, DDT, dicofol and endosulfan – many of them banned – (Crinnion, 2009)) in early pregnancy has been associated with an ASD risk in children of women that lived within 500 m of fields treated with high doses of pesticides (Roberts et al., 2007). Another study (Roberts et al., 2013) identified two critical periods of developmental vulnerability to ASD: one extended from one month before pregnancy till the end of the fifth month of pregnancy and the second one (postnatal) extending from 2 to 8 months of age. OCs interfere with calcium signaling, voltage sensitive sodium channels and GABA receptors, being neuro- and immuno-toxic (Heusinkveld and Westerink, 2012). The characteristic immune profile following OC exposure predisposes to allergic and asthmatic disorders, as well as autism (Gupta et al., 1998).

Organo-phosphorus pesticides (OP) (e.g. chlorpyrifos, diazinon) are the most commonly used pesticides in the world (Zaim and Jambulingam, 2007). ASD susceptibility is influenced by functional polymorphisms (SNPs) in paraoxonase PON-1, the enzyme involved in OP detoxification (D'Amelio et al., 2005). OP interfere with synapse formation in mammalian brain by interfering with specific signaling pathways and Ca^{2+} signaling, conferring an increased ASD risk (Forster et al., 2010). OPs also induce persistent inflammatory states (Li, 2007). More recently, as OPs have been banned for residential use, pyrethroid sales have increased rapidly (Williams et al., 2008).

Pyrethroids (e.g. cyfluthrin) are a group of insecticides and repellants derived from natural compounds of Chrysantemum genus. They disrupt calcium signaling, interfere with voltage sensitive sodium channels and induce oxidative stress (Soderlund, 2012). Pyrethroids stimulate the expression of genes involved in cytokines production and signaling (Mense et al., 2006).

Imidacloprid is used in the agriculture, but it is also an active ingredient of flea and tick treatments for household pets (Cox, 2001). Its primary action is similar to that of OP (Pessah and Lein, 2008). Dermal absorbtion of imidacloprid can result from petting recently treated animals, but the dose that reaches and harms the fetus is unknown. Depending on the chemical structure, pesticides could increase ASD risk through a variety of mechanisms, including: target voltage-gated sodium channels,

acetyl-cholinesterase or GABA receptors, interference with the development of the serotonergic nervous system, changes in activity of monoamine oxidase, endocrine disruption, immune dysregulation, altered lipid metabolism and calcium signaling (Casida, 2009; Heusinkveld and Westerink, 2012; Malaviya et al., 1993; Pessah et al., 2010; Preda et al., 2012; Soderlund, 2012).

7.4.3. Air pollution and proximity to freeways

Different studies reported the following air pollutants as being associated with ASD risk: quinolone and styrene (Kalkbrenner et al., 2010), ozone (Becerra et al., 2013), nitrogen dioxide (Volk et al., 2013), pooled metals, mercury, lead, nickel, manganese, diesel particulate and methylene chloride (stronger association for boys compared to girls) (Roberts et al., 2013). Collectively, significant association with ASD risk seems to be for traffic-related air pollution, big particulate matter and nitrogen dioxide, whereas small particulate matter and ozone do not show consistent association (Rossignol et al., 2014). Endocrine disruption may also be a pathway for air pollutants like diesel particulates, mercury and other metals, as they have been shown to influence levels of thyroid hormone (Takser et al., 2005).

7.4.4. Heavy metals

The most prominent heavy metals involved in ASD determinism are mercury, cadmium and nickel (Kinney et al., 2010). They act as mutagens in two ways: either by contributing to oxidative stress (DNA damage by free radicals) (Valko et al., 2005), or by inhibiting DNA repair systems (accumulation of mutations) (Filipic and Hei, 2004; Pavel et al., 2013). Heavy metals are also associated with lower IQ, behavioral disturbances, endocrine disruptions (Winneke, 2011), immunotoxic properties leading to autoantibody production (Rowley and Monestier, 2005) and abnormal cytokine profiles. Some studies (Tian et al., 2011) suggest that individuals with ASD might have a particular immunologic susceptibility to heavy metals.

Glutathione S-transferases are the enzymes that catalyze the detoxification of heavy metals. Polymorphisms affecting their genes have been associated with an increased ASD risk (Rossignol et al., 2014). Notably, SNPs in genes that impair toxicant elimination might not become functionally relevant in individuals with ASD until toxicant exposure levels reach a certain threshold and defense mechanisms are overwhelmed (Grandjean, 1995).

Mutagenicity of mercury has been proven (Agency for Toxic Substances and Disease Registry accessed 2009 – cited in (Kinney et al., 2010), the most dangerous being mercury acetate, that has a dose-dependent effect on the type of mutation (Silva-Pereira et al., 2005). Apart of the genotoxic effect, mercury may bind groups like thiols, hydroxyls and carboxyls (Bridges and Zalups, 2010), increase dramatically intracellular calcium levels (Limke et al., 2003) and alter cytokine profile with subsequent development of autoimmunity (Kempuraj et al., 2010). Ethyl mercury is a component of thimerosal, a widely used vaccine preservative. In vitro it has neurotoxic properties, may alter calcium signaling and cytokine production (Goth et al., 2006). However, many independent epidemiological studies showed no association between thimerosal and ASD in humans (Price et al., 2010). Testosterone may increase toxicity of mercury (Muraoka and Itoh, 1980), whereas estrogen is protective, providing a possible explanation for increased ASD frequency in males (Oliveira et al., 2006).

Lead is neurotoxic and highly immuno-toxic (Mishra, 2009). At high levels, lead is immuno-suppressive, with increased production of regulatory cytokines and increased risk of infection (Valentino et al., 2007). At low levels, lead is immuno-stimulatory (Flohe et al., 2002). Pro-inflammatory status (Goebel et al., 2000) is frequently found in ASD (Li et al., 2009). Lead toxicity may have unusual clinical presentation in some ASD individuals, with flu-like syndrome, weight loss, abdominal pain, diarrhea and vomiting (Newton et al., 2005), reason why periodic screening for lead exposure in children with ASD has been recommended (Filipek et al., 1999). Nickel produces reactive oxygen species (Galaris and Evangelou, 2002), inhibits DNA repair (Wozniak and Blasiak, 2004), but also potentiates the effect of other mutagens (Deng et al., 2006).

7.5. Electromagnetic frequency and radiofrequency exposures

Electromagnetic fields and radiofrequency radiations (EMF/RFR) are very diverse types of environmental radiations provided by different sources including X-rays used for diagnostic purposes, cell phones, wireless connections, microwaves etc. Electromagnetic fields enhance free radical activity, having a cumulative effect (De Iuliis et al., 2009). Free radicals destroy cells by damaging macromolecules (DNA, proteins and membrane components). Moreover, it was discovered that in ASD individuals very low intensity EMF and RFR modulate glutathione, affecting mitochondrial metabolism (Choudhury et al., 2012). Their damaging effects may be reduced by supplementation with antioxidants and radical scavengers like vitamins E and C (Guney et al., 2007) and gingko biloba (Ilhan et al., 2004).

EMF/RFR also act on the physico-chemical characteristics of membranes (Beneduci et al., 2012), membrane potential (Linz et al., 1999) and calcium signaling (Nesin et al., 2012). EMF/RFR may also compromise barrier structures that separate blood flow from organs like brain (blood – brain barrier), gut, eye or placenta (Salford et al., 2012). When these barriers become pathologically leaky, albumin, toxins, pro-inflammatory cytokines and infectious agents may cross the barriers, trigger immune

responses and affect the developing fetus, finally producing ASD (Somosy et al., 1993). This mechanism has been associated with non-thermal exposures comparable with normal cell phone radiation exposure (Nittby et al., 2008).

Some studies have documented the genotoxic effect of EMF/RFR (Sage and Carpenter, 2009). Many of the genetic defects predisposing to ASD are de novo mutations produced in sperm DNA by cell phone radiation (O'Roak et al., 2012). The proper mechanism of genotoxic action of EMF/RFR in ASD consists in oxidative stress and DNA damage by free radicals, challenge to DNA repair mechanisms or chromatin condensation (Herbert and Sage, 2013).

7.6. Endogenous factors

7.6.1. Microbiota

The vast collection of microbes that live on or inside us (microbiota) and their collective genes (microbiome) have been recently proved as being involved in ASD due to the extensive use of genomic techniques (germ identification by DNA tests, not by germ culture) (Gonzalez et al., 2011).

The development of the human microbiome is a complex process, starting in pregnancy, when maternal bacteria are transported to the placenta via bloodstream, umbilical cord and amniotic fluid (Valles et al., 2012). After birth, gut microbiota changes continuously, but in general gut microbial community is established in the first 3 years of life (Koenig et al., 2011). Gut bacteria have a set of digestive enzymes that are missing in the human host and complete the host set (Flint et al., 2008). Children with ASD frequently associate intestinal dysbiosis that determines abnormal digestion with additional growth substrates for bacteria that trigger dysbiosis (Williams et al., 2011).

Most of the microbiota germs are located in our gut and consists of approximately 10^{14} bacteria that balance the immune system, help digestion, produce vitamins and promote gastro-intestinal motility (Berg, 1996). The human microbiome represents the interface between our genes and our history of environmental exposures. Early environmental exposures include physical contact with family members (that explains why family members share a core microbiome) and the diet (maternal milk). Some studies in the literature suggest that milk formulas could be involved in ASD determinism due to the reduced content of water and high molecular weight (Hahr, 2013).

The most important factors that influence gut microbiota are: mode of delivery (Dominguez-Bello et al., 2010), geographic origin (De Filippo et al., 2010), host genotype (Li et al., 2012), diet (Walker et al., 2011), antibiotics (Willing et al., 2011), probiotics (Rauch and Lynch, 2012), age (Tiihonen et al., 2010) and stress (Konturek et al., 2011). Alterations of indigenous microbiota have been associated with many diseases, including obesity, metabolic syndrome, nonalcoholic steato-hepatitis, inflammatory bowel disease, irritable bowel syndrome, atherosclerosis, type I diabetes, autism, allergy, asthma and celiac disease (Backhed et al., 2012). Gut microbiota could be involved in different ways in ASD pathogeny: alteration in sulfur metabolism, production of organic acids (propionic acid) and presence of bioactive peptides in urine (phenols produced by specific bacteria are transformed at liver level and released in urine) (Midtvedt, 2012).

Different microbiota unbalances have been identified in ASD, including increased levels of Clostridia (Parracho et al., 2005), Bacteroidetes (Finegold et al., 2010), Ruminococcus torques (Wang et al., 2013), Desulfovibrio (Finegold, 2011) and Sutterella spp. (Williams et al., 2012), as well as decreased levels of Firmicutes (Finegold et al., 2010) and Verrucomicrobia (Wang et al., 2011).

7.6.2. Mineral imbalances

Mineral imbalances associated with ASD are zinc and magnesium/calcium deficiency (frequent), iron, chromium, manganese, copper and cobalt deficiency (rare) and toxic metal burdens (aluminium, cadmium, lead, arsenic and mercury). An inverse relationship was found between zinc and lead, aluminium and cadmium concentrations, suggesting that these toxic metal burdens associate with infantile zinc deficiency. Three types of metallomic profiles have been identified in ASD children: zinc and magnesium deficiency associated with burdens of cadmium and lead; burden of aluminium, mercury or arsenic; high sodium and potassium (Yasuda and Tsutsui, 2013). Most of the ASD cases are diagnosed clinically until 3 years of age and in these cases a mineral check is indicated for treatment/prevention purposes.

Zinc is a component of many enzymes and it is also involved in gene functioning. It plays important roles in nucleic acid/protein synthesis, cell division, as well as in tissue growth and repair, especially in pregnant women and infants. In brain it plays an important role in synaptic transmission. Zinc deficiency observed in autistic children induces epigenetic mechanisms and by gene/environment interaction interferes with neuronal maturation during early development (Grabrucker, 2012). Cadmium and arsenic also induce epigenetic alterations (Jakovcevski and Akbarian, 2012). Recently, it was shown that dietary restriction-induced zinc deficiency up-regulates intestinal zinc-importer, increasing the risk of high-uptake of toxic metals such as cadmium and lead (Goyer, 1997).

Similarly, deficiency in magnesium/calcium seems to enhance toxic effects of lead (Mahaffey et al., 1986). The most common lead exposure pathway in children is ingestion or inhalation of road dust, fossil fuel, asphalt and paints (lead chromate or lead carbonate) (Dixon et al., 2009), as well as maternal cigarette smoking (Razagui and Ghribi, 2005). Cadmium and lead from cigarette smoke accumulate in maternal bones and are co-transferred with

calcium to the fetus and infant during pregnancy and lactation (Sanders et al., 2012).

7.6.3. Medication metabolism

Autistic children have a decreased ability to sulfate paracetamol (primary metabolic pathway for children) (Alberti et al., 1999). When paracetamol is metabolized through alternative routes it induces oxidative stress and immune dysregulation (Bauer and Kriebel, 2013).

8. Global mechanism

The most important and recent hypotheses concerning autism pathogeny are listed below:

- In individuals with vulnerable genetic background, advanced parental age and low birth-weight, the risk of ASD may be aggravated by environmental factors: infections provoking an immune activation response, maternal diabetes, obesity or poor nutrition, fetal distress and birth trauma, faulty fetus presentation, hyper-bilirubinaemia and maternal bleeding. The environmental factors dysregulate epigenetic mechanisms resulting in an initial brain overgrowth and aberrant patterns of cerebral connectivity, clinically expressed as ASD (Gentile et al., 2013);
- Allergic, environmental, infectious, mitochondrial, stress or toxic triggers stimulate mast cells that release inflammatory and neurotoxic molecules (resulting in brain allergy) and increase blood-brain barrier permeability, leading to focal encephalitis (Theoharides, 2013);
- Impaired gastro-intestinal absorption of cysteine (main glutathione precursor) leads to local and systemic oxidative stress (because reactive oxygen species produced by metabolism cannot be neutralized anymore by glutathione), leading to disruption of normal epigenetic regulation of gene expression. If cysteine absorption is severely affected the individual will associate overt gastro-intestinal inflammation, whereas if cysteine absorption is mildly decreased, only immune and/or neurological development and function will be affected (Waly et al., 2012);
- Increased gut permeability facilitates the entrance of gluten, casein or lipo-polysaccharides into the blood stream and triggers peripheral inflammatory responses that lead to de novo production of cytokines in the brain with subsequent neuroinflammation (Critchfield et al., 2011);
- The individual lacks appropriate genetic machinery to excrete toxins and they accumulate, with toxic effects on the brain and the immune system. An environmental challenge during a critical window of development may have severe consequences, including abnormal neurodevelopment, altered immune phenotype and autism (Goines and Ashwood, 2013).

Probably the real mechanism is a mixture of all these theories, meaning that in a genetically predisposed individual different (and frequently combined) environmental factors trigger an immune reaction that leads to neuroinflammation with neurodevelopmental consequences (Fig. 1).

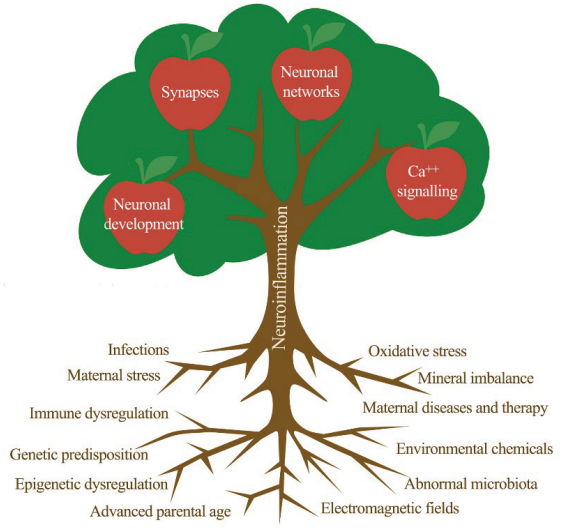

Fig. 1. Complex mechanism of autism spectrum disorders

9. Management and therapy directions

As presented in detail above, autism cases are rarely produced by a single cause. In most of the instances the disorder is produced by many different factors that activate each other and finally lead to neurodevelopmental consequences. For the moment there is no curative therapy for autism. However, because relatively often ASD is produced by a chain of reactions, simply by breaking one chain link we can stop the complex mechanism that leads to autism. Some of the most promising actions include:

- Vaccination of would-be mothers against viruses known to increase ASD risk (Millan, 2013);
- In mothers with less efficient folate metabolism (carrying at least one MTHFR 677T allele) folic acid administration should be increased and extended to three months before pregnancy (Schmidt et al., 2012);
- Strategies to create a beneficial shift in the microbiome include: antibiotics, probiotics (live microorganisms that are not part of the host microbiome, but confer a health benefit to the host) (Rauch and Lynch, 2012), prebiotics (nondigestible food components that are selectively fermented by beneficial members of the gut microbial community (Brownawell et al., 2012) and immune modulators (Backhed et al., 2012). Lactobacilli and Bifidobacteria from probiotics are also capable of transforming toxic mercury compounds into excretable metabolites (Brudnak, 2002);
- The nutritional approach supplements deficient nutrients (e.g. zinc) or vitamins (e.g. vitamin D) and detoxifies accumulated toxic metals (Yasuda and Tsutsui, 2013);
- Nutraceuticals are defined as “any substance that is food or a part of food and provides medical or health benefits, including the prevention and

treatment of disease" (Alissa and Ferns, 2012). They are generally dietary supplements and promote healthy gut, lower body burdens of toxins, improve antioxidant capacity, enhance immune-modulatory systems and minimize stress and environmental contamination (Alanazi, 2013). Nutraceuticals could be multivitamins with high folate content for periconceptional supplementing (Schmidt et al., 2011), vitamin B_{12} and glutathione combined with low fructose and food additive/color organic diet (Patel and Curtis, 2007), essential fatty acids (especially omega-3, but results are controversial) (Vancassel et al., 2001), tetrahydrobiopterin (Frye et al., 2010), casein-free (milk protein) and gluten-free (wheat protein) diets (Elder et al., 2006).

10. Conclusions

Autism is a very complex developmental disorder and genetic, epigenetic, immune and environmental factors should be considered in a comprehensive approach when investigating a case. In most of the situations these factors act together in a sequence of events that leads to neuro-inflammation and abnormal brain development. The knowledge of the factors involved provides ways for medical intervention and complication prevention. The recent increase in ASD prevalence underlines the growing importance of environmental factors in autism determinism.

Environmental factors involved in ASD do not refer only to classic extrinsic agents (like environmental pollutants, electromagnetic fields etc.), but also involve maternal disorders or lifestyle factors, as well as intrinsic factors (hormones, inflammatory mediators and gut bacteria), that may influence the developing fetal or neonatal brain.

Early diagnosis is a key issue because it allows a multimodal intervention plan, including education, medical intervention aiming to prevent complications and genetic counseling.

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