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AUTISM SPECTRUM (C DISSANAYAKE, SECTION EDITOR)

Environmental Factors in the Onset of Autism Spectrum Disorder

Antonio M. Persico · Sara Merelli

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Abstract Autism spectrum disorder (ASD) is a heterogeneous clinical condition whose prevalence has grown considerably during the last decade. Genetic factors are thought to underpin the disorder, but common genetic variants and epigenetic mechanisms have been increasingly called into question for the majority of ASD cases. Growing prenatal exposure to new environmental toxicants has been shown to potentially affect brain development, leading to altered cognitive, social, attentive, behavioral, and motor performance. Both epidemiological evidence and mechanistic studies assessing oxidative stress, neuroinflammation, epigenetic alterations, and impaired signal transduction, all observed following neurotoxicant exposure, indeed lend biological plausibility to Gene x Environment interactions, whereby environmental toxicants interacting additively or synergistically with genetic liability, can push prenatal neurodevelopmental processes over the threshold for postnatal ASD expression. Research on environmental contributions to ASD and on specific Gene x Environment interaction models ultimately aims at defining targeted preventive strategies.

A. M. Persico (✉)
Unit of Child and Adolescent NeuroPsychiatry, Laboratory of Molecular Psychiatry and Neurogenetics, University "Campus Bio-Medico", Via Alvaro del Portillo 21, 00128 Rome, Italy
e-mail: a.persico@unicampus.it

A. M. Persico
Department of Experimental Neurosciences, I.R.C.C.S. "Fondazione Santa Lucia", Via del Fosso di Fiorano 64, 00143 Rome, Italy

A. M. Persico · S. Merelli
Mafalda Luce Center for Pervasive Developmental Disorders, Via Bernardo Rucellai 36, 20126 Milan, Italy

S. Merelli
e-mail: s.merelli@unicampus.it

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Introduction

Autism spectrum disorder (ASD) is an extended diagnostic category, which includes individuals with impaired social interaction and communication, as well as repetitive stereotyped behaviors, insistence on sameness and sensory abnormalities [1]. Severity ranges from "low-functioning" cases with absence of spoken language and severe intellectual disability, to "high-functioning" individuals with normal to high Intellective Quotient (IQ), subtle social deficits, and some restricted and obsessive interests. Individuals who display few signs of autism without meeting the full diagnostic criteria belong to the "broad autistic phenotype", making ASD the categorical extreme of a quantitative continuum of traits present in the general population [2].

During the last decades, ASD prevalence estimates have risen to as much as 113/10,000 children in the USA [3], and 62/10,000 globally [4], corresponding to 1:88 and 1:161 children, respectively. This increase is so prominent that it appears hardly accountable solely to enhanced awareness, greater service availability, and broader diagnostic categories. While genetic components are considered extremely relevant to ASD etiology, candidate genes and copy-number variants currently explain about 20 % of syndromic and non-syndromic ASD cases [5]. This percentage will indeed rise once whole-genome sequencing becomes routinely implemented in the

clinic, but it will almost certainly never explain all ASD cases. In fact, common genetic variants seemingly account for at least 50 % of ASD liability, leaving ample room for environmental contributions due to their low penetrance and additive effects [6••].

Prenatal exposure to neurotoxic substances has been proven to alter brain maturation and to produce a wide array of neurodevelopmental deficits, in a way that has no counterpart in the adult brain [7]. The developing brain is particularly vulnerable during its "critical periods", time windows of susceptibility when neuronal proliferation, migration, differentiation, maturation (i.e., neurite sprouting and pruning), synaptogenesis, and activity-dependent synaptic remodelling occur. During these paramount processes, exposure to environmental disruptors can affect brain development, leading to functional deficits and behavioral disorders. In general, a positive epidemiological association, if not spuriously due to mere temporal coincidence, can stem from modulatory, additive, permissive, synergistic, and causal effects exerted by the environmental factor under scrutiny (Fig. 1). Patients not carrying rare, disruptive genetic variants may thus be accounted for by a "multiple-hit" pathogenic model based on complex Gene x Environment interactions, whereby multiple combinations of common genetic variants, each conferring a small risk, create a highly individualized spectrum of sensitivity to the detrimental effects of environmental factors [8]. This conceptual framework can provide a better understanding of the increased prevalence and pathogenetic complexities of ASD, as well as of its amazing clinical heterogeneity.

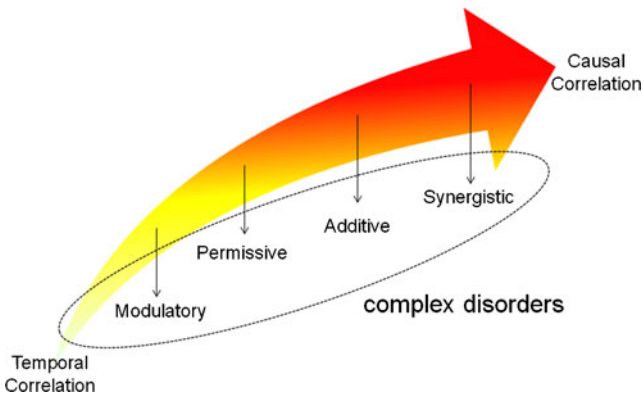

Fig. 1 Environmental factors in human disorders A "none-to-all" upward scaling model, spanning from temporal coincidence without any pathogenic role to full causality. Environmental Factors (EF), in interaction among themselves and with Genetic Factors (GF), can exert: (a) *modulatory* effects: EF influence the phenotypic expression of GF only qualitatively; (b) *permissive* effects: one EF is necessary for another EF to exert damage, or for a GF to become penetrant and to achieve phenotypic expression; (c) *additive* effects: EF and GF act independently and their combined effect equals the sum of each contributing factor; (d) *synergistic* effects: EF and GF potentiate each other, so that the combined effect is greater than the predicted sum of each contributing factor

The Tip of the Iceberg: Known Environmental Causes of ASD

Case reports, patient cohort studies, epidemiological and neuroanatomical investigations, as well as animal models, have proven that, in a limited number of cases, environmental factors can be regarded as the sole causative agent in ASD. This is the case for some teratogenic drugs, namely valproic acid, misoprostol and thalidomide, as well as for prenatal rubella and cytomegalovirus infections (Table 1).

Teratogenic Drugs

Prenatal exposure to antiepileptic drugs (AED) has been linked to "fetal anticonvulsant syndrome" (FAS), characterised by congenital malformations and developmental delay. Autistic symptoms can also be part of FAS, most frequently following prenatal exposure to sodium valproate (VPA), although some ASD cases have been reported after phenytoin, carbamazepine, or polydrug therapy. Several cohort studies have looked at neurodevelopmental outcomes following prenatal AED exposure, and found increased incidence of ASD, ADHD and dyspraxia, as well as decreased adaptive skills and emotional control, with VPA being most frequently involved [9–11].

VPA teratogenicity in ASD has received unequivocal support by epidemiologic and experimental studies, to the point that mice prenatally exposed to VPA are frequently used as a rodent model of autism. Children prenatally exposed to VPA display hazard ratios of 2.9 % and 5.2 % for ASD and childhood autism, respectively, as defined by the ICD-10, after adjusting for parental psychiatric disease and epilepsy [12]. These rates are significantly higher than the 0.6–1.1 % encountered in the general population [3, 4]. Clinical characteristics of VPA-related autism include an M:F ratio close to 1, minor and major malformations (glue ear, joint laxity, hernias, and congenital defects of the neural tube, heart, genitourinary tract, upper airways, eyes, skin and teeth), significant speech delay, and mild motor delay in the absence of severe cognitive impairment or regression (i.e., loss of acquired skills) [13]. VPA teratogenity can be explained by increased oxidative stress, acid folic deficiency, interference with the Ras-ERK and GSK3β intracellular pathways favouring neuronal differentiation over proliferation, and most importantly by inhibition of histone deacetylase, resulting in persistent histone acetylation and cytosine demethylation at promoters controlling the expression of several neurodevelopmentally-relevant genes, such as WNT, FZD-5, GFRA-2, and GATA-3 [14–16]. Animal models of prenatal VPA exposure show interesting neuroanatomical, behavioral and electrophysiological abnormalities, including: (a) enhanced growth and abnormal distribution of serotoninergic terminals in the brainstem, which reflects defective differentiation and migration of

Table 1 Summary of the documented environmental factors known to cause ASD, their proposed pathogenetic mechanisms and time-window of maximum teratogenicity in humans

Documented environmental factors able to cause ASD			
Agent	Time-window	Mechanisms Involved	Refs.
Valproic acid	E18-E30	Altered gene expression due to histone hyperacetylation Inhibition of neural progenitor proliferation vie Ras-ERK Oxidative stress Folic acid antimetabolism	9, 12-22
Thalidomide	E20–E30	Altered gene expression ROS-mediated DNA damage Angiogenesis inhibition	16, 17, 24-28
Misoprostol	E18–E42	Altered gene expression Angiogenesis inhibition	26-28
Rubella	Up to E56	Direct viral damage Maternal and fetal immune response	30, 31
CMV	Throughout pregnancy	Direct viral damage Maternal and fetal immune response	30, 32-35

E embryonic day post-fertilization, *CMV* Cytomegalovirus

serotoninergic neurons [17]; (b) reduced cerebellar volume, presumably due to diminished Purkinje cells number [18]; (c) local hyperconnectivity in the neocortex, as well as diminished numbers of putative synaptic contacts between layer-5 pyramidal neurons [19]; (d) abnormal fear conditioning and amygdala processing [20], and (e) enhanced expression of NMDA receptors determining increased long-term potentiation [21]. Importantly, the role of epigenetic alterations in VPA-related autism fits with the broad gene expression dysregulation observed in multiple data sets in idiopathic autism [22]. In particular, this dysregulation may implicate genes encoding chromatin-related proteins involved in the transcriptional regulation of prenatal brain development [23].

Thalidomide was used as a sedative and to relieve morning sickness in pregnant women until 1961, when it was withdrawn from the market because of its teratogenic effects. Thalidomide causes multiple systemic malformations, abnormal cortical development and neuronal hyperexcitability, primarily by inhibiting angiogenesis, altering gene expression, broadly perturbing morphoregulatory processes and producing DNA oxidative damage [24, 25]. Similarly to VPA, thalidomide also disrupts early serotoninergic neurodevelopment [17]. Misoprostol is a methyl ester derivative of prostaglandin E1, used to treat gastric ulcers and to induce abortion due to its stimulatory effect on uterine contractions. Its teratogenicity has been observed in children born after unsuccessful abortion attempts, who show cranial nerve hypoplasia sometimes associated with a Möbius sequence, limb malformation and, in some cases, autistic-like behaviors [26]. Thalidomide and misoprostol show many similarities: (a) both alter vascularisation and gene expression, particularly in genes concerned with balancing proliferation and apoptosis, cell migration, neuronal differentiation, and synaptogenesis; (b) both can induce autism, specifically after prenatal exposure very early during pregnancy (embryonic days 18–30 and 18–42 post-fertilisation for thalidomide and misoprostol, respectively); and (c) both affect limbs, ears, eyes, cranial nerves, and the central nervous system (CNS), yielding mental retardation, language impairment and autism [27, 28]. Thalidomide and misoprostol clearly resemble the prenatal exposure timing and phenotypic manifestations of VPA-induced fetal anticonvulsant syndrome. This evidence, in conjunction with the observation that children with autism frequently display minor physical anomalies established during early organogenesis, support the increased importance of an early prenatal time window of maximum sensitivity over the specific nature of the teratogenic agent [29].

Congenital Viral Infections

The two infectious agents most widely acknowledged as conferring ASD risk following congenital infection are rubella and cytomegalovirus (CMV) [30]. Children infected prenatally with these viruses show multisystem impairment and CNS abnormalities ranging from macroscopic neocortical malformations subsequent to migration defects (polymicrogyria, pachygyria, heterotopias), to microscopic alterations in neuronal myelination. Initial evidence linking prenatal rubella infection to ASD came from a longitudinal study involving 448 prenatally infected children [31]. Autism rates were as high as 7.4 % and risk seemed particularly increased when the infection occurred within the first 8 weeks of pregnancy. In addition to autism, deafness, eye defects, cardiopathy and mental retardation were also described. “Late” physical and psychiatric manifestations can appear during adolescence or early adulthood, suggesting that

environmental influences can result in clinical courses less stable than those seen in the majority of children with idiopathic ASD.

Several case reports link prenatal cytomegalovirus (CMV) infection to ASDs [31–34]. To what extent autism stems from direct viral damage, from the nature and location of CMV cerebral lesions, or from the strong immune response driven by the virus, still remains to be established [35].

The Submerged Iceberg: Environmental Factors Potentially Involved in ASD

The number of potential teratogens is rapidly increasing, and developmental neurotoxicity has already been ascertained for over 200 agents [36]. The developing brain is especially sensitive to neurotoxicants [37], and preliminary evidence links neurodevelopmental disorders to early exposure to several neurotoxic agents [36]. Environmental factors potentially involved in autism pathogenesis are listed in Table 2 and discussed below.

Air Pollution

Air pollution is comprised of a diverse mixture of particulate matter (PM), gases (e.g., ground-level ozone, carbon monoxide, sulphur oxide), organic compounds (e.g., polycyclic aromatic hydrocarbons) and metals (e.g., nickel, manganese) present in outdoor and indoor air; many millions of people around the world are chronically exposed to air pollutants above promulgated safety standards [38]. Emissions from mobile and residential fuels, wood combustion, construction and demolition works, and industrial activities (e.g., refineries, metal processing facilities) are some examples of air pollution sources.

Prenatal exposure to air pollution has been found to increase risk for low birth weight, pre-term birth, and postnatal mortality [39, 40]; later in life, low performances on cognitive, attentive and memory tests have been described [41, 42]. Interestingly, genetically susceptible children appear to suffer greater impairment [42], supporting Gene x Environment interactions with synergistic or additive mechanisms. Large-scale epidemiological investigations have shown increased autism risk in children prenatally exposed to air pollutants

Table 2 List of environmental factors putatively involved in ASD, their proposed pathogenetic mechanisms, and time-window of maximum teratogenicity in humans

ENVIRONMENTAL FACTORS POTENTIALLY INVOLVED IN ASD			
Agent	Time-window	Mechanisms involved	Refs.
Air pollution	Throughout pregnancy + Early postnatal life	Oxidative stress CNS and systemic immune activation Cerebral vascular damage Neuronal cell death Altered gene expression through dysregulated DNA methylation	44-54
OPs	Throughout pregnancy	CNS and systemic inflammatio Oxon disruption of neuroglial proliferation & differentiation Decreased Reelin expression and enzymatic activity Decreased BDNF expression Interference with intracellular Ca^{2+} signaling Disruption of GABAergic neurotransmission	5, 6, 59-69
PCBs and PBDEs	Throughout pregnancy	Endocrine disruption Oxidative stress Interference with intracellular Ca^{2+} signaling Altered gene transcription through decreased DNA methylation Immune system activation	62, 69, 70, 74–83, 87-91
Heavy metals	Throughout pregnancy	Neurotoxicity Immune system activation/autoimmunity Altered gene transcription	69, 92, 95-105
Thimerosal	Postnatally	Oxidative stress GSH depletion Interference with intracellular Ca^{2+} signaling Immune system activation/autoimmunity	117-125

OPs Organophosphates, *PCBs* polychlorinated biphenyls, *PCBEs* polybrominated diphenyl ethers, *BDNF* brain-derived neurotrophic factor, *GABA* gamma-amino butyric acid, *ROS* reactive oxygen species, *CNS* central nervous system, *GSH* Glutathione

[43–46]. In the large cohort of the CHARGE study, a dispersion model of traffic-related pollutants, combined with measurements of nitrogen dioxide (NO_2), ozone (O_3), and particulate matter (PM10 and PM25) air concentrations, estimated a two-fold increase in ASD risk among children prenatally exposed to traffic-related air pollution and a three-fold increase when exposure occurred also during their first year of postnatal life [45]. Even when considered individually, exposure to PM25, PM10, or NO_2 was associated with a two-fold increase in ASD risk [45]. A different cohort of 7,603 children with autism and 10 matched controls per case, all born between 1995 and 2006 to mothers residing in Los Angeles county at the time of child birth, yielded relative increases in the odds of an ASD diagnosis per interquartile range (IQR) of increased exposure to NO/NO_2, PM25 and O_3 at 3–9 %, 5–15 % and 6–12 %, respectively, confirming previous results but with smaller effect sizes [46]. These air pollutants mostly derive from traffic, and their association with autism is strongest in the offspring of mothers with low educational level and poor socio-economic status [46].

Neurodevelopmental damage following early exposure to air pollution may ensue from several cellular and molecular mechanisms, depending on the chemical and physical characteristics of each pollutant. In general, oxidative stress, microglial activation, neuroinflammation, cerebral vascular damage and neurodegeneration have all been found after air pollution exposure [47, 48]. Inflammatory damage may follow direct exposure to air pollutants, reaching the CNS through either the olfactory mucosa or systemic circulation, or indirectly by the action of proinflammatory cytokines, released in the respiratory system [48]. Damage to the blood–brain barrier can then enhance pollutant access to the CNS and generate further neural damage in a vicious cycle [47, 48].Animal and cellular models have shown that: (a) dopaminergic neurons are particularly sensitive to the effect of acute and chronic exposure to O_3 and to diesel exhaust particles (DEP), which activate microglial cells, causing CNS oxidative damage and cell death in the striatum and substantia nigra [49, 50]; (b) behaviorally, prenatal DEP exposure increases locomotor activity, rearing behaviors, and self-grooming in the presence of an unfamiliar mouse, rodent analogues of the restricted and repetitive patterns of behavior seen in human autism [51]; (c) prenatal or early postnatal exposure during lactation to polycyclic aromatic hydrocarbons (PAH) like benzo(a)pyrene alters DNA methylation and the postnatal expression of ASD-relevant genes, like MET and the 5HT1A receptor gene, producing persistent behavioral abnormalities [52••, 53]. Interestingly, altered DNA methylation has been found also in human cord blood after prenatal exposure to benzo(a)pyrene [54].

Collectively, these data suggest that prenatal and early postnatal exposure to air pollutants can trigger oxidative, inflammatory, and epigenetic alterations in the CNS. In turn, these effects enhance autism risk, provided the exposure occurs during neurodevelopment, is sufficiently prolonged, and likely acts upon genetically susceptible feto-maternal units. The major current limitation of the studies to date is the difficulty to disentangle the effect of air pollution from those of other neurotoxicants potentially more present in California among pregnant women of low education and socioeconomic status.

Insecticides and Pesticides

Organophosphates (OPs) are the most widely used pesticides in agriculture, as well as insecticides in residential, commercial and industrial settings, after organochloride banning. Children may be exposed to OPs via the placenta or through breast milk, food, and inhalation, and appear particularly vulnerable to OPs and to oxidative stress compared to adults, because of their lower activity levels of the enzyme paraoxonase, involved in OP inactivation and lipid peroxide degradation [55]. Prenatal exposure to OPs has been linked to early neurodevelopmental deficits, which appear to be maintained during childhood, including deficits in cognitive abilities, working memory and perceptual reasoning [56–58]. Increased cortical surface in brain regions involved in attention, receptive language, social cognition, reward and behavioral inhibition has been correlated with lower IQ scores in humans prenatally exposed to chlorpyrifos [59•]. Increased risk of developing ASD following prenatal OP exposure has also been reported [60].

OPs inhibit the enzyme acetylcholinesterase (AChE), determining excessive cholinergic transmission; however, OPs main neurotoxic actions are seemingly exerted by their oxon metabolites [61]. At toxicologically relevant doses, these compounds disrupt neuronal proliferation, differentiation, gliogenesis and apoptosis by interfering directly with cell signaling molecules, or indirectly with AchE morphogenetic activities, which are distinct from its enzymatic activity [61]. At the molecular level, OPs and oxons impair neuroligin-neurexin-SHANK signalling, decrease the expression of BDNF and other neurotrophins, interfere with Ca^{2+}-dependent signaling and with the PI3K/mTOR pathway, and disrupt GABAergic neurotransmission, ultimately resulting in altered neuronal connectivity [62, 63]. These effects are predicted to be most functionally significant in genetically vulnerable individuals. For example, OPs inhibit Reelin's proteolytic activity, crucial for neuronal migration, dendritic spine maturation, and synaptic function [64, 65]. This effect, in conjunction with differences in OP clearance due to interindividual variation in paraoxonase (PON1) activity [66, 67], modulate neurodevelopmental abnormalities, albeit in ways more complex than initially suggested [68]. Also, inflammatory imbalances have been observed after prenatal OP exposure, with

upregulation of TH1 and TH2 cytokines in the periphery, and increased IL-6 and IL-1β levels in the CNS [69].

Halogenated Aromatic Hydrocarbons

High hydrophobicity and long half-lives are key features of halogenated aromatic hydrocarbons, a large family of chemicals broadly spread in the environment. Until their banning in 1977, polychlorinated biphenyls (PCBs) were used as coolants, lubricants, and in building materials, whereas polybrominated diphenyl ethers (PBDEs) are flame retardants, still largely employed on infant products, electronic items, and furniture. Early exposure may occur through the placenta and breast milk [70, 71], while postnatal exposure may result from hand-to-mouth behaviors, since these compounds are semivolatile, and accumulate on household surfaces and dust. Impairment in cognition, attention, and motor development has been recorded following prenatal exposure to PBDEs and PCBs [72–74]. Moreover PCBs and PBDEs can alter endocrine and immune functions, as observed in animal models and human studies [75–78]. Multiple neuronal populations (e.g., cerebellar granule cells, hippocampal neurons, nigrostriatal dopaminergic cells) are affected by PBDEs neurotoxic proprieties, apparently due to oxidative stress [79, 80]. On the other hand PCBs produce neurotoxic effects by opening ryanodine receptors (RyR), resulting in enhanced and/or prolonged cytosolic Ca^{2+} spikes [81]. Excessive and/or prolonged cytosolic Ca^{2+} spikes can also account for the excessive, spontaneous and the blunted, experience-dependent dendritic growth observed in developing neuronal cells of rats prenatally exposed to PCBs [82, 83•]. Moreover, Gene x Environment interactions involving autism-related genes relevant to Ca^{2+} management, such as ATP2B2 and SLC25A12 [84, 85•, 86], and PCB exposure could result in altered neuronal connectivity [62]. Interestingly, Gene x Environment interactions converging onto epigenetic alterations relevant to ASD have been found in animal models. Mice carrying a truncated form of the *MECP2* gene (i.e., $Mecp2^{308}$ with a premature stop codon after codon 308), which causes Rett syndrome in humans, after prenatal exposure to PBDEs display globally reduced DNA methylation and enhanced deficits in social behaviour and learning abilities [87].

A limited number of human studies have directly addressed the potential role of PCBs and PBDEs in autism, yielding inconclusive results. No difference in PBDE plasma concentrations have been found in children with autism contrasted with typical or delayed development [88]. Similarly, no difference in PCB levels have been recorded in archived serum samples of Finnish mothers pregnant with 75 children that were later diagnosed with ASD compared to 75 matched controls, although the adjusted model yielded a promising O.R. = 1.92 for autism (P=0.29), which deserves an independent replication and extension [89]. Curiously, higher amounts of PCB95 were found in postmortem brains of children with maternal 15q11-q13 duplication or deletion compared to brains of children with non-syndromic autism or typical development (TD) [90]. These same brains exhibited lower levels of repetitive DNA methylation, suggesting that exposure to PCB95 might contribute to the generation of specific CNVs [90]. Finally, altered immune responses following LPS stimulation have been observed in BDE-47-pre-treated mononuclear cells of autistic children as compared to control children [91], suggesting that the former may respond differently to PBDEs.

Heavy Metals

Heavy metals, particularly lead (Pb) and mercury (Hg), are widespread environmental toxins that can produce multisystem damage. Fetal exposure through the placenta can result in impaired cognitive development and behavioral disturbances, such as low IQ, ADHD and impaired motor skills [92–94]. The relationship between heavy metal exposure and ASD is highly controversial. Contrasting results have been found with regard to serum levels of Pb and Hg in ASD children [95–98]. The debate on the possible role of the vaccine preservative thimerosal (composed by ethyl-mercury) is outlined in the next section. However, sources other than thimerosal could potentially determine chronic Hg exposure resulting in increased ASD risk, as suggested by ecological studies in areas with higher levels of Hg in ambient air [99, 100]. Furthermore, the descendants of a cohort of individuals with Hg hypersensitivity showed ASD prevalence above general population rates [101], again supporting the role of Gene x Environment interactions. Moreover, differences in gene transcription profiles correlated with Hg and Pb plasma levels were detected between blood samples of ASD and TD children, suggesting that some ASD children may display a unique susceptibility to heavy metals, presumably due to their peculiar genetic profile [102, 103]. Finally, the concomitant administration of Pb and proinflammatory cytokines altered the expression of metalloproteinase genes in glial cells and acted on neuronal tissue remodelling, while the administration of only one of the two agents did not, unveiling possible synergistic or permissive interactions between heavy metals and proinflammatory factors [104].

In summary, exposure to heavy metals from sources other than vaccines, combined with genetic variants promoting immune dysfunction or impairing neurodevelopmental processes, could potentially exert small additive effects in a limited number of ASD children. However, it should be considered that the elevated blood levels of heavy metals found in many autoimmune disorders, such as rheumatoid arthritis [105], stem from active dysimmunity rather than from environmental contamination, and that similarly active dysimmunity may well be present in a consistent subset of

ASD children, for which metal chelation therapy appears absolutely unjustified.

Vaccines

In the late 1990s, the MMR (measles mumps and rubella) vaccine and thimerosal, an ethyl mercury preservative, began to be regarded as potential causal factors for ASD. Public concern rose following Andrew Wakefield's paper, which connected MMR and autism [106], and the almost coincidental recommendation to remove thimerosal-containing vaccines from the market, made in 1999 by the American Academy of Pediatrics (AAP), jointly with Public Health Services. Huge research efforts have been made to clarify whether the suggested causal link is scientifically valid. Large national databases have been analyzed to take a deeper look at the effects of changes in vaccine policies on autism prevalence rates. Collectively, the majority of epidemiological studies have not found a relationship between autism prevalence and MMR or thimerosal exposure. For example, in the UK, autism prevalence increased between 1979 and 1992, but no "step-up" effect, or change in trend, was seen after the introduction of MMR in 1988 [107]. Moreover, autism incidence continued to rise after steady MMR coverage was obtained, indicating no correlation between the two [108]. A large retrospective cohort study of all Danish children born from January 1991 through December 1998 showed no increase in the risk of ASD for MMR vaccinated children [109]. No differences were found in rates of developmental regression and gastrointestinal symptoms before and after the introduction of the MMR vaccine, and between MMR-exposed and -unexposed children with autism [110]. Similar results were obtained in epidemiological studies addressing the role of thimerosal-containing vaccines (TCVs). A large Danish study investigated ASD incidence between 1971 and 2000: no increase was seen until 1990, although thimerosal had been in use for several years. Conversely, the rising trend of autism incidence, recorded starting in 1991, continued unchanged in spite of the removal of TCVs in 1992 [111]. Another population-based cohort study of all children born in Denmark between 1990 and 1996 also provided negative findings, showing a lack of dose–response association between thimerosal and autism severity [112]. Similarly, results from studies conducted in the UK and USA did not show increased autism risk in children exposed to TCVs, nor significant associations between TCVs and neurodevelopmental adverse outcomes [113–115]. Consequently, in 2002 the AAP withdrew its 1999 recommendation, and in 2004, the Institute of Medicine concluded that evidence favored the rejection of a link between the MMR vaccine or thimerosal and autism [116].

Despite the epidemiological evidence outlined above, the controversy on vaccine safety continues. Some have argued that thimerosal exposure has continued through influenza vaccines, recommended to pregnant women and infants, thus undermining epidemiological studies attempting to monitor the consequences of its removal after 1999. Animal models have shown that thimerosal may affect the CNS, especially during development: persistent abnormalities in brain monoaminergic system after prenatal exposure to thimerosal at E9 have been reported in mice [117, 118]; increased oxidative stress in cerebellar cells, as well as impaired motor learning and delayed startle response have been observed in rats prenatally and postnatally exposed to Et-Hg [119]. In vitro studies have found that Et-Hg increases oxidative stress, reduces GSH availability, and alters Ca^{2+} signaling [120, 121]. Even more interestingly, both in mice and rats, males and strains prone to develop autoimmunity appear more sensitive to thimerosal than females and strains resistant to autoimmunity [122–125]. A recent study on 11 families with an autistic child showed unaffected twins and non-twin siblings having thimerosal hypersensitivity in B lymphocytes in 4 of the 11 families, as indicated by elevated oxidative stress targeting mitochondria [126].

Collectively, these results indicate that vaccines and thimerosal per se do not cause autism in the vast majority of patients. Changes in ASD incidence trends should have reflected changes in vaccination policies, as promptly and faithfully as lung cancer incidence has followed trends in smoking habits. Ironically, by preventing substantial numbers of infectious diseases like rubella, vaccinations actually prevent many cases of autism [127]. Nonetheless, some genetically vulnerable individuals could conceivably suffer from the consequences of vaccination and thimerosal, as well as of several other incidental conditions activating the immune system and enhancing oxidative stress during early infancy (a common example being the recurrent ear infections so frequently affecting children later diagnosed with autism during the first 12–18 months of life). By hindering energy metabolism, vaccines, thimerosal, and infections could each provide small, additive contributions toward the precipitation of a "regressive" form of autism, which would have otherwise evolved spontaneously at a slower and more progressive pace. However, many purely genetic forms of autism, such as those due to NLGN3 mutations, also typically display a regressive onset. This clearly speaks against regression as necessarily stemming from an environmental factor acting postnatally at the time when behavioral alterations are first noticed. Rather, this could be the time when defective neural networks fail to come "on-line" and to support the acquisition of novel cognitive functions.

Conclusions

An increasing body of evidence is showing that environmental factors can variably increase autism risk through multiple

mechanisms, namely deregulating gene expression, altering signal transduction or cytosolic calcium homeostasis, and inhibiting enzymatic activities critical to brain development and neural function, causing oxidative stress or neuroinflammation. The array of environmental agents potentially involved in ASD is broad, including several not discussed here such as prenatal and perinatal stressors [128, 129], prenatal infections boosting IL6 production [130••], and neuroactive compounds produced by gut bacteria [131–133]. Meanwhile, the well-established causal role demonstrated for pathogenic mutations and CNVs in many cases of autism imposes a balanced view. This multiplicity of environmental agents and pathways, paired with the multiplicity of common and rare genetic variants causing autism in some and merely conferring susceptibility in others, should help us abandon the unrealistic hope of finding the "one cause" of autism in every patient and to tolerate complexity, as it occurs in nature and in human disease.

Compliance with Ethics Guidelines

Conflict of Interest Antonio M. Persico has grants/grants pending with the Autism Research Institute.

Sara Merelli declares that she has no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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