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Review Article

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A comprehensive review of mercury provoked autism

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Emerging evidence supports the theory that some autism spectrum disorders (ASDs) may result from a combination of genetic/biochemical susceptibility, specifically a reduced ability to excrete mercury (Hg), and exposure to Hg at critical developmental periods. Elemental/inorganic Hg is released into the air/water where it becomes methylated and accumulates in animal tissues. The US population is primarily exposed to methyl-Hg by fish consumption. In addition, many pharmaceuticals have been, and some continue to be, a ubiquitous source of danger because they contain mercurials. Mercurials may be found in drugs for the eye, ear, nose, throat, and skin; in bleaching creams; as preservatives in cosmetics, tooth pastes, lens solutions, vaccines, allergy test and immunotherapy solutions; in antiseptics, disinfectants, and contraceptives; in fungicides and herbicides; in dental fillings and thermometers; and many other products. Hg has been found to cause immune, sensory, neurological, motor, and behavioural dysfunctions similar to traits defining/associated with ASDs, and that these similarities extend to neuroanatomy, neurotransmitters, and biochemistry. Furthermore, a review of molecular mechanisms indicates that Hg exposure can induce death, disorganization and/or damage to selected neurons in the brain similar to that seen in recent ASD brain pathology studies, and this alteration may likely produce the symptoms by which ASDs are diagnosed. Finally, a review of treatments suggests that ASD patients who undergo protocols to reduce Hg and/or its effects show significant clinical improvements in some cases. In conclusion, the overwhelming preponderance of the evidence favours acceptance that Hg exposure is capable of causing some ASDs.

Key words Autistic - glutathione - neurodevelopmental disorder - testosterone

Introduction

In order to evaluate the relationship between Hg exposure and autism spectrum disorders (ASDs), this study will review emerging evidence from a variety of scientific disciplines. In considering this relationship, this review will specifically address the science-supported theory that some ASDs may result from a combination of a biochemical/genetic susceptibility, specifically a reduced ability to excrete mercury (Hg), and exposure to toxic levels of Hg at critical times in development.

If this is the case, then the amount of exposure to Hg that produces an ASD in a particular individual is, at low Hg exposure levels, dependent upon that individual's biochemical/genetic susceptibility. With low-level exposure, only those individuals with a high genetic/biochemical susceptibility to Hg intoxication would be affected. Glutathione, sulphation, testosterone, estrogen, and genetic single nucleotide polymorphisms (SNPs) in the body's Hg detoxification pathways are among the identified biochemical/genetic susceptibility

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factors affecting an individual's susceptibility to Hg intoxication. Thus, for low-level Hg exposures, the greater the biochemical/genetic susceptibility to Hg intoxication, the greater the likelihood of developing an ASD.

By contrast, even in cases where the other susceptibility aspects are a factor, as the exposure level to Hg increases, the individual's biochemical/genetic susceptibility becomes increasingly less important as a determining factor in the development of an ASD. Given the preceding realities, individuals exposed to a relatively high level of Hg could be clinically Hg intoxicated, even when, based on the absence of any unusual biochemical/genetic susceptibility indicators, that individual is an efficient eliminator of Hg.

Thus, only when an exposed foetus or infant has biochemical/genetic susceptibility, that makes one less able to remove Hg, would lower levels of Hg exposure lead to the development of an ASD. At higher Hg-exposure levels, as the determining factor is increasingly environmental exposure, rather than biochemical/genetic susceptibility, the excretion system of a foetus or infant with no particular biochemical/genetic susceptibility and a normal ability to withstand Hg exposure may be overcome, resulting in an increasingly high rate of ASD diagnoses in a given population. This overall paradigm is illustrated in Fig. 1.

We evaluate the biochemical indicators for Hg intoxication in ASDs but after assessing these, review effective clinical interventions to treat the child with an ASD based upon these findings.

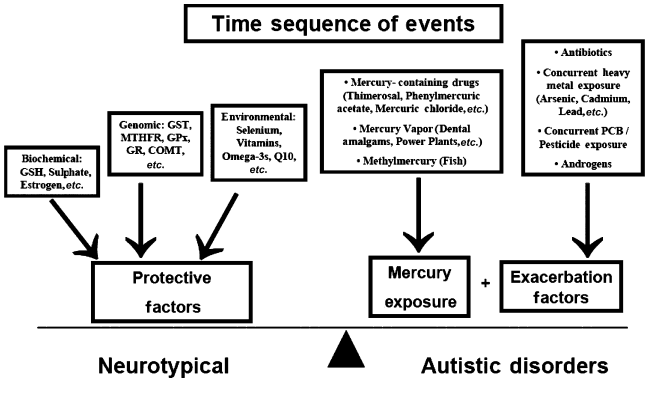

Fig. 1. A summary of the balancing factors involved in the pathogenesis of Hg induced autistic disorders. COMT, catechol-*O*-methyl transferase; GPx, glutathione peroxidase; GSH, glutathione; GST, glutathione *S*-transferase; GR, glutathione reductase; MTHFR, methylenetetrahydrofolate reductase; PCB, polychlorinated biphenyls.

A brief background of Hg exposure

Hg, a heavy metal, is widespread and persistent in the environment. Exposure to hazardous Hg levels can cause permanent neurologic and renal impairment. Humans probably first used the naturally occurring red Hg sulphide, cinnabar, as a pigment in their early art. Aristotle, in the 4th Century B.C., was the first to leave a written account of Hg, which was called liquid silver. Five centuries later Dioscorides recorded that cinnabar was good for eye medicines, in that it healed burnings and the eruption of pustules. He also noted that it was dangerous if swallowed. Roman physicians recognized industrial poisoning with Hg and its derivatives (mercurialism) as the 'disease of the slaves,' because it was common among slaves working in the Spanish cinnabar mines of Almaden. The poisoning was so terrible that only criminal slaves were forced to work in the mines. The first reports of synthetic organic Hg compounds appeared in the mid1800s, and, within a few years, the first cases of organic Hg poisoning were described[1-3].

Elemental Hg or inorganic Hg (mostly as Hg^{2+} salts) released into the air or water becomes methylated in the environment where it accumulates in animal tissues and increases in concentration through the food chain. The US population is primarily exposed to some form of protein bound methyl-Hg by eating fish. The exposure to methyl-Hg is of greater concern to women of childbearing age because foetuses are highly susceptible to Hg's adverse effects[1-3]. In addition to environmental sources of Hg, numerous prescription and over-the-counter drugs have contained or continue to contain mercurials (in both, organic and Hg^{2+} forms). Mercurials have been and/or continue to be a ubiquitous source of danger in drugs for the eye, ear, nose, throat, and skin; in bleaching creams; as preservative in cosmetics, tooth pastes, lens solutions, vaccines, allergy test and immunotherapy solutions; in antiseptics, disinfectants, and contraceptives; in fungicides and herbicides; in dental fillings and thermometers; and many other products[4].

The 1999 National Health and Nutritional Examination Survey (NHANES) conducted by the US Centers for Disease Control and Prevention (CDC) estimated that about 10 per cent of US women of childbearing age (16-49 yr old) had total blood Hg levels in excess of the current US Environmental Protection Agency (EPA)'s blood Hg safety limit (5.8 µg/l) and hair Hg levels in excess of the US EPA's current hair Hg safety limit (1 part-per-million) [5].

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Additionally, the administration of routinely recommended thimerosal-containing vaccines to infants in the US was found to result in increased blood Hg levels with some infants having blood Hg levels in excess of the US EPA's blood Hg limit, as well as the level defined by the US CDC as the threshold for Hg poisoning (10 µg/l) [6-8]. Administration of thimerosal-containing vaccines to infants was reported to result in a substantial increase (446%) in hair Hg levels[9]. This observation is consistent with previously modeled hair Hg concentrations in infants exposed to thimerosal-containing vaccines. Specifically, simple Hg-excretion models have shown that US infants who received Hg exposure from the routinely recommended thimerosal-containing vaccines would have hair Hg levels in excess of the US EPA's hair Hg safety limit for up to 365 days, with several peak concentrations within the first two years of life[10].

Researchers reported on their evaluation of Hg exposures in early childhood from both potential environmental sources of Hg exposure and the most common medicinal sources of Hg exposure, namely routinely recommended thimerosal-containing childhood vaccines[11]. This study found that some US infants born in the 1990s may have been exposed to cumulative Hg doses, from environmental as well as medicinal sources, in excess of 350 µg Hg during the first 6 months of life. In addition, this study reported that about 50 per cent of all the Hg to which some infants were exposed came from routinely recommended thimerosal-containing childhood vaccines. Furthermore, when considering the sum of the estimated environmental Hg exposure and the dose of Hg some children received from the routinely recommended thimerosal-containing vaccines, these researchers concluded that some infants received doses of Hg that were significantly in excess of the Hg exposure limits established the US EPA, US CDC, US Food and Drug Administration (FDA), World Health Organization (WHO), and Health Canada. Finally, the study noted that this Hg exposure occurred across key developmental periods in the first year of life.

Calculations of US foetal/infant exposure to Hg from Thimerosal-containing childhood vaccines/biologics in the mid1980s demonstrate that infants received a cumulative dose of 100 µg Hg during the first 18 months from the 25 µg Hg in each diphtheria-tetanus-pertussis (DTP) vaccine routinely administered at 2, 4, 6, and 18 months of age. Additionally, during this time, infants may have incurred additional Hg exposure through breast milk if they were born to mothers with Hg amalgam fillings and/or Rh-negative mothers, since many Rho(D)-immune globulin formulations, used to prevent isoimmunization in the Rho(D) negative individual exposed to Rho(D) positive foetal blood, contained thimerosal (10.5 to > 50 µg Hg/dose) and were routinely recommended for administration to these mothers within 72 h of birth[12, 13].

Starting in the late 1980s/early 1990s, the cumulative dose of Hg children received from thimerosal-containing childhood vaccines/biologics almost tripled. Specifically, a thimerosal-containing *Haemophilus Influenza* type b (Hib) vaccine (25 µg Hg/dose) was recommended for routine administration at 2, 4, 6 and 18 months of age. Furthermore, a thimerosal-containing hepatitis B vaccine (12.5 µg Hg/dose) was recommended for routine administration at birth, 2 and 6 months. As a result, an infant could have received a cumulative dose of 237.5 µg Hg during the first 18 months of life. Furthermore, since many formulations of Rho(D)-immune globulins were thimerosal-containing (10.5 to > 50 µg Hg/dose) and were recommended for routine administration to all Rh-negative pregnant women at 28 wk gestation starting in the late 1980s/early 1990s (in addition to the recommendation for its routine administration within 72 h of birth), the cumulative dose of Hg received from thimerosal-containing vaccines/biologics was certainly even higher for many US infants[12,13].

By the summer of 1999, the realization that the Hg exposure American infants were incurring through the immunization schedule exceeded some, if not all, safety limits, caused alarm among both private health organizations and public agencies. On July 7, 1999, the US Public Health Service (USPHS) and the American Academy of Pediatrics (AAP) issued a joint statement that urged "all government agencies to work rapidly toward reducing children's exposure to mercury from all sources." The statement recommended that thimerosal be removed from vaccines as soon as possible as part of this overall process [14]. Between 1999 and 2001, many of the thimerosal-preserved vaccines recommended for children <6 yr of age were made available in reduced-thimerosal ("preservative free") formulations in the US[15].

Despite the call, issued by the USPHS and the AAP, to reduce children's exposure to Hg from all sources, thimerosal was re-introduced into the US routinely recommended childhood vaccine schedule in 2002 with

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recommendations to administer two doses of influenza vaccine in the first year of life (starting at 6 and 7 months of age) and to vaccinate all children who were 6 months to 23 months of age. In addition, recommendations were made to vaccinate all pregnant women who would be in their 2nd or 3rd trimester of pregnancy during the US-"flu" season (December to March) as well as those who have medical conditions that might increase their risk for complications of influenza, regardless of the stage of pregnancy[16, 17].

Moreover, the 2002 recommendation has been continually expanded to the point that, in 2008, the US CDC recommended that all pregnant women should receive an influenza vaccine (without regard to the trimester of pregnancy) and that all infants should receive two doses of influenza vaccine in the first year of life, with one influenza vaccine administered on a yearly basis thereafter until 59 months. In addition, the CDC recommends that children under nine who only received one influenza vaccine initially should be given 2 doses of influenza vaccine, at least two weeks apart[18].

Biological plausibility of Hg-induced autistic disorders

Autism is a lifelong neurodevelopmental disorder that disproportionately affects male children (roughly, 5 males per 1 female). Autism is characterized by early onset of impairments in social interaction and communication and unusual, stereotyped behaviours. Unable to learn from the natural environment as most children do, the child with autism generally shows little interest in the world or people around him. Although some children with autism develop normal and even advanced skills in particular areas, most exhibit a wide range of profound behavioural problems and delayed or undeveloped skills. Therefore, in absence of any autism is, in general, a lifelong developmental disability that profoundly affects the way a person comprehends, communicates and relates to others. In the US, autism (*i.e.*, autistic disorder) is often classified with two related, although less severe, developmental disorders: Asperger's Disorder and Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS). Collectively, these three diagnoses constitute the autism spectrum. The diagnosis of an ASD is based exclusively on developmental pattern and behavioural observation[19, 20].

In January 2004, an Autism A.L.A.R.M. was issued by the AAP and the US CDC, stating that 1 in 166 children in the United States suffers from an ASD, and far worse, that 1 in 6 children suffers from a developmental and/or behavioural disorder. Published by the US CDC in 2007, the most recent survey-based US autism prevalence estimates for 8-yr-old children born in the early 1990s suggest that more than 1 in 150 children in the US may have an ASD diagnosis, and that more than 1 per cent of children may have an ASD diagnosis in certain areas in the US (note: no attempt was made to correct the survey results for their inherent undercounting)[19,20]. These epidemic rates for ASD diagnoses in the US have apparently coincided with the previously described several-fold increase in foetal and infant exposure to Hg from medicinal and environmental sources.

As early as 1991, a researcher from the National Institute for Occupational Safety and Health (NIOSH) of the US CDC, reported "organic mercury" was among the "established" "human behavioural teratogens," and according to this researcher, the resulting human behavioural disorders might "…include seizure disorders, autism, childhood schizophrenia, early onset emotional disturbances and attention deficit disorders"[21]. Subsequently, others reported the specific biological effects of Hg exposure on neuronal development as, "…mercury exposure altered cell number and cell division; these impacts have been postulated as modes of action for the observed adverse effects in neuronal development. The potential implications of such observations are evident when evaluated in context with research showing that altered cell proliferation and focal neuropathologic effects have been linked with specific neurobehavioural deficits (*e.g.,* autism)"[22].

In regard to the relationship between methyl-Hg exposure and neurodevelopmental disorders in humans, The National Research Council of the US National Academy of Sciences concluded, "Overall, data from animal studies, including studies on nonhuman primates, indicate that the developing nervous system is a sensitive target organ for low-dose (methyl-Hg) MeHg exposure. Results from animal studies have reported effects on cognitive, motor, and sensory functions… On the basis of the body of evidence from human and animal studies, the committee concludes that neurodevelopmental deficits are the most sensitive, well-documented effects…"[23]. Additionally, researchers reported that exposure to Hg can cause immune, sensory, neurological, motor, and behavioural dysfunctions similar to traits defining or associated with ASDs, and that these similarities extend to neuroanatomy, neurotransmitters, and biochemistry[24-29].

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Historically, developmental regressions with ASD symptoms have been reported following foetal and/or early childhood Hg exposure. For example, a case report of an 11-month-old Swiss boy who was brought to his paediatrician has been described[30]. He had been in good health and had developed normally until then, but his parents mentioned that during a 2 wk period, the child ceased to laugh or play and became more and more restless, and slept only 1 to 2 h a night. He was no longer able to crawl or to stand up, and he had lost weight. Clinical examination revealed swollen hands and feet with skin desquamation, axial hypotonia and brisk reflexes. The child sweated profusely, refused to crawl or stand, showed stereotypic movements of the hands (kneading) and repeatedly bit objects or his own hands. Upon admission to the regional hospital, extensive genetic/biochemical tests were conducted, but their results were all within normal limits. The child was referred for further evaluation due to severe psychomotor regression with autistic features of unknown aetiology.

Thereafter, it was learned that, approximately 4 wk before the onset of symptoms, a thermometer had broken in the child's home and that the Hg spilled onto the living room carpet had only been vacuum cleaned. Based on this newly obtained anamnestic clue and subsequent clinical and laboratory findings, this child was diagnosed with Hg intoxication.

Toxicokinetics of Hg in infant animal model systems

Given that various researchers have suggested an association between Hg exposure and the development of ASDs, and given anecdotal case reports of such exposure leading to ASDs in the published medical literature, it is important to consider the toxicokinetics of Hg, particularly upon the brain in animal models, in order to assess the "biological possibility" of this theory.

Researchers evaluated infant monkeys following oral administration of methyl-Hg hydroxide or injected doses of thimerosal, sodium ethyl-Hg thiosalicylate, comparable to the dosing schedule (weight- and age-adjusted) that US children received during the 1990s[31]. They determined that the maximum Hg content in the brains of the thimerosal-treated infant monkeys averaged about 40-50 parts-per-billion. In addition, they calculated that the half-life for organic Hg in the brain of the infant monkeys examined was about 14 days. By contrast, they determined that maximum Hg content in the brains of methyl-Hg treated infant monkeys averaged about 80-120 parts-per-billion. In this case, they calculated that the half-life for organic Hg in the brain of the infant monkeys examined was about 58 days. Based on these results, it was demonstrated that infant low-dose organic Hg exposure, mimicking the Hg exposure received by US children through the 1990s immunization schedule, was able to induce significant levels of Hg in the monkey brain, and that this organic Hg was present in the brain for several weeks post-dosing.

In addition, post-dosing-schedule testing found the concentration of Hg^{2+} (formed from the ethyl-Hg entering the brain) averaged 16 parts-per-billion in the brains of the thimerosal-treated infant monkeys (all had a measurable level), whereas methyl-Hg treated infant monkeys had significantly less than half as much Hg^{2+} (almost half had a level below the method's detection limit). Moreover, the half-life of Hg^{2+} in the monkeys' brains was too long to estimate from the available data (no significant measurable decline was detectable by 120 days). Additionally, it was previously reported that, as a result of the significant Hg^{2+} fraction of Hg observed in the brain following injection of thimerosal, a longer biological time was observed for the Hg in the brain from ethyl-Hg than for the Hg from methyl-Hg[32].

Other researchers further evaluating the persistence of Hg^{2+} in the brain have reported evidence which supports the finding that Hg^{2+} in the central nervous system (CNS) has a very long biological half-life on the order of several years, in contrast to a biological half-life of days or weeks for organic Hg[33]. Another study, based upon human autopsies, reported that the half-life for Hg^{2+} in the brain was about 20 yr[34].

Another animal study evaluated the distribution pattern of Thimerosal or inorganic Hg administered to rat pups subcutaneously, three times during the suckling period, on days 7, 9, and 11 of life, again imitating the vaccination of American infants in the 1990s.[35] Both groups of test rats were administered equimolar doses of Hg. At 14 days of age, the test and control animals were killed, and the total Hg in their blood and organs (kidney, liver, and brain) was analyzed. Analysis revealed that Hg was present in the blood and organs in both treatment groups, and that blood and organ levels were significantly higher in both treatment groups than in the unexposed controls. The results showed the level of Hg was significantly higher in the liver (1.4-fold) and kidney (4.4-fold) of the inorganic-Hg-exposed group than in the thimerosal-exposed group. However, the brain (1.5-fold) and blood (23-fold) concentration

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of Hg were significantly higher in the thimerosal-exposed group in comparison to the inorganic-Hg-exposed group.

Investigators have also evaluated whether injection of endotoxin (lipopolysaccharide) into mice exerted any effect on Hg content in the cerebrum after injection of low-doses of thimerosal[36]. These researchers showed that administration of endotoxin to mice resulted in significantly increased persistent Hg levels in the cerebrum following low-dose administration of thimerosal in comparison to mice not treated with endoxtoxin. Endotoxin is a cell-wall component of Gram-negative bacteria and induces damage in various types of tissues including brain. Endotoxin accumulates in the brain endothelial cells and, transiently, opens the blood-brain barrier for small molecular weight compounds such as the short-chain alkyl-Hg compounds. Notably, many routinely recommended childhood vaccines such as the whole-cell pertussis vaccines have contained or still contain significant concentrations of endotoxin as well as thimerosal[37-39]. In the US, many infants are inoculated with vaccines when they already have a mild infection that is accompanied by inflammation and fever - conditions likely associated with increased endotoxins levels - which may also increase Hg intoxication in the brain. Given these vaccination co-morbidities, the presence of endotoxin may affect Hg distribution patterns following Hg exposure and result in significantly increased penetration and storage of Hg in the cerebrum.

Toxicological effects of persistent Hg in the brain

Understanding that Hg levels in the brain increase after exposure to this known neurotoxin, one must then evaluate the effects of its presence on neurological structure. The overall importance of persistent Hg^{2+} in the brain stems from the fact that recent studies showed that dealkylation of Hg in the brain is not necessarily a detoxification process[40-44]. After dosing monkeys with organic Hg, researchers showed that the half-life of Hg^{2+} in the brain varied significantly across different regions of the brain, from 227 days to 540 days. In other regions, six months after Hg dosing had ended, the concentrations of Hg^{2+} remained the same (thalamus) or doubled (pituitary)[43,44]. Stereologic and autometallographic studies on the brains of these monkeys indicated that the persistence of the Hg^{2+} in the brain was associated with a significant increase in the number of microglia in the brain, whereas the number of astrocytes declined. Remarkably, "an active neuroinflammatory process" has been demonstrated in brains of ASD patients, including a marked activation of microglia[45]. Notably, these effects were observed in the monkeys 6 months after dosing with the Hg compounds had ended, when Hg^{2+} concentrations were at the highest levels in the animals dosed with organic Hg or solely exposed to inorganic Hg[40-42].

Toxicity of low-dose Hg exposure in animal model systems of ASDs

While the ability of Hg to penetrate the brain, affecting its structure and function, is well demonstrated, only recent studies have examined the effects of low-dose Hg exposure on the developing brain of animal models. These studies are particularly poignant, given the current controversy regarding the effect of these same low levels of Hg upon the developing human brain, where the Hg exposure is from both medicinal and environmental sources during the prenatal and postnatal periods.

Researchers from Columbia University reported that an autoimmune diathesis is described in ASD probands and their first-degree relatives[46]. Major histocompatibility complex (MHC) genes regulate risk of Hg-induced autoimmunity in mice[46]. In order to examine whether immunogenetic factors mediate vulnerability to Hg-related neurodevelopmental damage, these researchers exposed mice of differing MHC (H-2) backgrounds to thimerosal in doses and timing equivalent to the US paediatric immunization schedule of the 1990s[46]. These researchers observed profound behavioural and neuropathological disturbances after post-natal thimerosal exposure in SJL/J ($H\text{-}2^s$) mice, but not in mouse strains without autoimmune sensitivity (BALB/cJ, $H\text{-}2^d$ or C57BL/6J, $H\text{-}2^b$ mice). Many of the symptoms and the altered brain structure observed in the treated SJL/J mice were similar to the symptoms exhibited by children with an ASD diagnosis, including: growth delay; reduced locomotion; exaggerated response to novelty; and, for the brain structure, densely packed, hyperchromic hippocampal neurons with altered glutamate receptors and transporters. Importantly, the SJL/J mouse strain was found to have significantly impaired antioxidant capacity (*i.e.* lower levels of glutathione and superoxide dismutase) in comparison to the other strains of mice used in this study[46].

Other researchers reported that the developing brain is highly sensitive to methyl-Hg[47]. These researchers undertook a study to determine whether an

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environmental toxicant could also impact brain development with rapid (6-7 h) effects on DNA synthesis and cell cycle machinery in neuronal precursors. *In vivo* studies in newborn rat hippocampus and cerebellum, two regions of postnatal neurogenesis, were followed by an *in vitro* analysis of cortical and cerebellar cells used as surrogates for the brain's developmental status. The *in vitro* analyses performed focused on the proteins that regulate the G1/S transition. In post-natal day 7 pups, a single subcutaneous injection of methyl-Hg (3 μg/g) acutely (7 h) decreased DNA synthesis in the hippocampus by 40 per cent and produced long term (2 wk) reductions in total cell number, estimated by DNA quantification. These authors estimated that the rat brain Hg levels which induced these significant adverse effects, were about 200-300 parts-per-billion[47].

Surprisingly, cerebellar granule cells were resistant to methyl-Hg effects *in vivo* at comparable tissue concentrations, suggesting region-specific differences in precursor populations. *In vitro*, methyl-Hg altered proliferation and cell viability, with DNA synthesis selectively inhibited at an early time-point (6 h) corresponding to these researchers' *in vivo* observations. Considering that G1/S regulators are targets of exogenous signals, these researchers used a well-defined cortical cell model to examine methyl-Hg effects on relevant cyclin-dependent kinases (CDK) and CDK inhibitors. At 6 h, methyl-Hg decreased the levels of cyclin E, a cell cycle regulator with roles in proliferation and apoptosis, by 75 per cent without altering p57, p27 or CDK2, or the levels of activated caspase 3. In aggregate, these researchers concluded that their observations identified the G1/S transition as an early target of methyl-Hg toxicity and raised the possibility that cyclin E degradation contributes to both decreased proliferation and eventual cell death.

Another study reported that normal brain development requires co-ordinated regulation of several processes including proliferation, differentiation, and cell death[48]. Multiple factors from endogenous and exogenous sources interact to elicit positive as well as negative regulation of these processes. In particular, the perinatal rat brain is highly vulnerable to specific developmental insults that produce later cognitive abnormalities. Therefore, these researchers used a rat model system to examine the developmental effects of methyl-Hg.

Seven day old rats received a single injection of methyl-Hg, 5 μg/g body weight. This resulted in an estimated rat brain Hg concentration of about 500 parts-per-billion. Methyl-Hg inhibited DNA synthesis by 44 per cent and reduced levels of cyclins D1, D3, and E at 24 h in the hippocampus, but not in the cerebellum. Toxicity was highly associated with caspase-dependent programmed cell death. The methyl-Hg treatments resulted in reductions in hippocampal size (21per cent) and cell numbers 2 weeks later, especially in the granule cell layer (16per cent) and hilus (50per cent) of the dentate gyrus defined stereologically. Collectively, these findings suggest that neurons may be particularly vulnerable to Hg exposure. Consistent with this, perinatal exposure led to profound deficits in juvenile hippocampal-dependent learning during training on spatial navigation tasks. It was also postulated that the methyl-Hg exposure in the rats induced effects on cell migration as well as axon and synapse formation. In addition, these researchers described how, in preliminary studies, they found that a greater than 15-fold lower dose of methyl-Hg (0.3 μg/g body weight) elicited a 3-fold increase in the number of caspase 3-positive cells in the treated animals as compared to the controls. Overall, their results indicated that exposure to one dose of methyl-Hg during the perinatal period acutely induced apoptotic cell death, which, in turn, led to later deficits in hippocampal structure and function[48].

Epidemiological studies of environmental Hg and autistic disorders

While epidemiology is not intended to provide an absolute demonstration of drug safety or harm to an individual, use of this academic discipline as a surveillance tool is appropriate in determining whether low levels of Hg exposure may have contributed to an increased risk for a child developing an ASD.

One study indicating an association between low-dose Hg exposure through vaccines administered to infants and ASDs was a new meta-analysis study, performed on data from the Vaccine Adverse Events Reporting System (VAERS) database[49]. The VAERS is an epidemiological database that has been maintained by the US CDC since 1990 as a surveillance tool to evaluate vaccine safety. The VAERS Working Group of the US CDC and the US FDA analyze and publish epidemiologic studies based upon VAERS. The VAERS Working Group notes that VAERS is simple to use, flexible by design, and the data are available in a timely fashion, but it also warns that the potential limitations may include systematic error due to under-reporting,

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erroneous reporting, frequent multiple exposures, multiple outcomes, and the lack of precise denominators[50].

In order to examine the VAERS database appropriately, the meta-analysis study used the general epidemiological technique developed by researchers at the US CDC'S the National Immunization Program (NIP). This technique involves comparing two different types of vaccines that were administered to age-matched populations, and using the net number of doses distributed from the Biological Surveillance Summaries of the US CDC to estimate the number of doses administered[50].

In this meta-analysis study, the VAERS database was examined for the incidence rate of adverse events reported following DTP vaccines in comparison to diphtheria-tetanus-pertussis-*H. influenza* type b (DTPH) vaccines (administered: 1994-1997) and, for the period 1997-2000, the incidence rate of adverse events reported following thimerosal-containing diphtheria-tetanus-acellular pertussis (DTaP) vaccines in comparison to thimerosal-free DTaP vaccines. In both cases, there should have been, maximally, an additional exposure to approximately 100 μg Hg.

When, during 1994 to 1997, the DPT and Hib vaccines were combined in the DTPH vaccine, children receiving it were nominally exposed to 25 μg Hg per vaccine administration. By contrast, children receiving the individual DTP and Hib vaccines were nominally exposed to the 25 μg Hg from the DTP and 25 μg Hg from the Hib. Thus, with respect to the possible combinations/alternatives of vaccine administrated in accord with the immunization schedule, examining specifically DTP and Hib versus DTPH, children receiving separate DTP and Hib vaccines potentially received a nominal maximum of 200 μg Hg from these vaccines, whereas children receiving DTPH in the same schedule potentially received a nominal maximum of 100 μg Hg from these vaccines during the first 18 months of life. These vaccines were administered in the U.S. during the 1994-1997 study period under a childhood vaccination schedule that specified inoculation for each vaccine selected at 2, 4, 6, and 18 months.

Similarly, the VAERS database was examined for the incidence rate of adverse events reported following thimerosal-containing DTaP vaccines in comparison to thimerosal-free DTaP vaccines (administered: 1997-2000). Maximally, there should have been an additional exposure to approximately a nominal 100 μg Hg, among those children receiving thimerosal-containing DTaP vaccines in comparison those receiving thimerosal-free DTaP vaccines. This is the case because children receiving thimerosal-containing vaccines were nominally administered an additional 25 μg Hg with each dose of vaccine. Thus, among the vaccines under study, children receiving thimerosal-containing DTaP vaccines potentially received a nominal maximum of 100 μg Hg from these vaccines, whereas children receiving thimerosal-free DTaP vaccines in the same schedule potentially received a nominal maximum of 0 μg Hg from these vaccines during the first 18 months of life. This is the case because these vaccines were administered in the U.S. during the 1997-2000 study period under a childhood vaccination schedule that specified inoculation with one of the selected two vaccines at 2, 4, 6, and 18 months.

In this study, unique VAERS reports that stated a DTP or thimerosal-containing DTaP was administered were assigned to the exposed group, and the unique VAERS reports that stated that DTPH or thimerosal-free DTaP was given were assigned to the unexposed group. The Biological Surveillance Summary reports from the CDC, sorted by vaccine manufacturer, indicated that there were a total of 57,151,417 vaccine doses administered to children in the exposed group, those receiving additional doses of Hg from thimerosal-containing vaccines (*i.e.*, the DTP or thimerosal-containing DTaP vaccines), and 47,985,230 vaccine doses administered to children in the unexposed group, those receiving lower doses of Hg from vaccines (*i.e.*, the DTPH or thimerosal-free DTaP vaccines).

The following numbers of study-associated neurodevelopmental disorder adverse events were identified in VAERS: autism (133 reports), speech disorders (115 reports), mental retardation (143 reports), personality disorders (124 reports), thinking abnormalities (41 reports), ataxia (41 reports), and neurodevelopmental disorders in general (374 reports). In addition, significantly increased adjusted risk ratios for neurodevelopmental disorder adverse events reported to VAERS were apparent in the exposed group, when compared to the unexposed group, for the outcomes of autism, speech disorders, mental retardation, personality disorders, thinking abnormalities, ataxia, and neurodevelopmental disorders in general. By contrast, none of the control adverse events (note: these were selected on an *a priori* basis as not "biologically plausibly linked" to an

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increased risk following additional doses of Hg from thimerosal-containing vaccines) of conjunctivitis, febrile seizures or lymphandeonopathy reported to VAERS had a significantly increased risk ratio in the exposed group when compared to the unexposed group[49].

A previous epidemiological study examined the consistency of the results observed in VAERS with those in the Vaccine Safety Datalink (VSD) database.[51] The VSD database was created in 1991 by the US CDC's NIP. The VSD links medical event information, vaccine history, and selected demographic information from the computerized clinical databases of four health maintenance organizations (HMOs). Analysis of the VSD database showed significant associations between increasing cumulative exposures to thimerosal and increased risk ratios for the following types of neurodevelopmental disorders: unspecified developmental delay, tics, attention deficit disorder (ADD), language delay, speech delay, and neurodevelopmental delays in general. This study[51] showed that exposure to Hg from Thimerosal-containing vaccines administered in the US was a consistent significant risk factor for the development of neurodevelopmental disorders.

A prior VSD-based epidemiological study conducted by Verstraeten *et al*[52] on US children also found significant increasing dose-response effects between Hg exposure from thimerosal-containing childhood vaccines and some types of neurodevelopmental disorders in the first phase of the study, but the effects observed were not consistent in the second phase of the study. The lead author concluded that the study was neutral with respect to thimerosal causality for neurodevelopment impairment[53].

Three ecological studies conducted on children in the US[54-56] found significant correlations between increasing cumulative doses of vaccine Hg and ASDs. The first two examined children born from the mid-1980s through the mid-1990s and found a significant statistical correlation between the birth cohort prevalence of autistic disorders from the US Department of Education and the estimated Hg dose children received from thimerosal-containing vaccines[54, 55.] The third study correlated increasing cumulative doses of Hg exposure from thimerosal-containing childhood vaccines with the increasing population prevalence of children diagnosed with autism-like disorders seeking special education services for autism in the California Department of Developmental Services (CDDS) from 1987 to 1998 by birth-year cohort[56].

In contradiction to these three studies, a fourth ecological study assessed time trends in the prevalence by age and birth cohort of children who were active status clients of the CDDS from January 1, 1995 through March 31, 2007. It concluded that the estimated prevalence of autism for children at each year of age, from 3 to 12 yr, increased throughout the study period, in spite of the removal of thimerosal from some of the vaccines administered to US children[57]. This study, however, suffered from an incomplete assessment of potential Hg exposure incurred by children from all Thimerosal-containing vaccines and biologics as well as from potential exposures to other environmental sources of Hg. Furthermore, this study did not have any means to evaluate the potential effects of the introduction of influenza vaccine to the US vaccine schedule for pregnant women and infants starting in 2002.

Several epidemiological studies conducted outside the US have also examined the relationship between vaccine Hg exposure and neurodevelopmental disorders and come to conflicting conclusions. Two studies in the United Kingdom showed significant increasing dose-response effects for some neurodevelopmental problems such as tics or behavioural problems and increasing vaccine Hg exposure, but the results for other conditions showed no significant association[58,59]. The researchers concluded that they could not find convincing evidence that early exposure to Hg doses from thimerosal-containing vaccines had deleterious effects on neurodevelopmental outcomes. Other studies in Canada, Sweden, and Denmark evaluated the relationship between Hg exposure from thimerosal-containing vaccines and autism[56,60,61]. These studies found no significant relationship. In considering these later studies, however, concerns have been raised regarding their applicability to the US experience with thimerosal-containing vaccines.

In addition, an epidemiological study was conducted on a cohort of 82 infants in Brazil that assessed variance in pre- and post-natal variables associated with Gesell neurodevelopment scores at 180 days, including birth weight and thimerosal exposure from vaccination at birth, 1, 2, 4, and 6 months[62]. These researchers used principal component analysis to identify sets of interrelated variables. The principal component analysis yielded a two-factor solution,

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explaining 92 per cent of variance. From the two composite factors identified, it was clear that there was a significant inverse relationship between the normalized Hg doses (Hg dose per kilogram of birth weight) from the thimerosal-containing childhood vaccines administered at specific times during the first six months of life and Gesell neurodevelopment scores measured at 6 months-old infants in the areas of motor development, language development, adaptive development, and general development. By contrast, the variability in the other pre- and post-natal variables measured was not significantly associated with neurodevelopment at 180 days.

While much attention has been paid to Hg exposure from vaccines, only very recently have studies been designed to evaluate the effects of Hg exposure from thimerosal-containing Rho(D)-immune globulins. Now, several epidemiological studies have evaluated the relationship between prenatal Hg exposure from thimerosal-containing Rho(D)-immune globulins and the risk of a child being diagnosed with an ASD in the US.

Rho(D)-immune globulin is an immune globulin preparation containing antibodies to Rho(D) which is intended for intramuscular injection. Rho(D)-immune globulin is used to prevent isoimmunization in the Rho(D) negative individual exposed to Rho(D) positive blood. Historically, starting in the 1970s, Rho(D)-immune globulin was administered within 72 h of a full-term delivery of a Rho(D) positive infant by a Rho(D) negative mother or following known potential exposure between maternal and foetal blood. Subsequently, in the late 1980s/early 1990s, the American College of Obstetricians and Gynecologists (ACOG) adopted the recommendation that, in addition to birth and times of potential mixing of foetal and maternal blood, Rho(D)-immune globulin preparations should be routinely administered to all Rh-negative mothers at 28 wk of gestation[13].

Until 2001, when the last doses of thimerosal-containing Rho(D)-immune globulin preparations were manufactured, many formulations of Rho(D)-immune globulin contained thimerosal in the US. Thimerosal was added to Rho(D)-immune globulin preparations at the preservative level of 0.003 to 0.01 per cent (from 10.5 μg Hg to >50 μg Hg/dose)[13].

In evaluating the relationship between thimerosal-containing Rho(D)-immune globulin administration and ASDs, one study[13] examined a total of 53 consecutive non-Jewish Caucasian patients with an ASD diagnosis who were born between 1987 and 2001, and brought to the Genetic Centers of America for outpatient genetic/developmental evaluations[13]. Data from these patients were prospectively collected from June 1, 2005 through March 31, 2006. To rule out other causal factors for a diagnosis of an ASD, imaging and laboratory testing were conducted on each patient. Using appropriate matched controls, the researchers determined the frequency of Rh-negativity from 926 non-Jewish Caucasian pregnant women who had presented for outpatient prenatal genetics care to the Genetic Centers of America between 1980 and 1989. The children with an ASD were significantly more likely to have Rh-negative mothers than controls. Each ASD patient's mother was determined to have been administered at least one thimerosal-containing Rho(D)-immune globulin during her pregnancy.

A second study, conducted in an autism treatment clinic, examined the frequency of Rh-negative mothers and Rho(D)-immune globulin exposure among 94 patients with ASDs born between 1985 and 1999, in comparison to 45 matched normal controls born between 1990 and 1999[63]. The researchers found that number of Rho(D)-immune globulin injections received by mothers in the ASD group was significantly higher than the number of those injections received by mothers of controls.

By contrast, other researchers surveyed families of children with an ASD ascertained through a University-based autism clinic[64]. Between 2004 and 2006, 305 mothers of 321 children with an ASD agreed to participate in a telephone interview. Analysis of their complete medical records, including the blood group status and Rho(D) immune globulin exposure of 214 families, showed that Rh-negative status was no higher in mothers of children with autism than in the general population and that exposure to antepartum Rho(D) immune globulin, preserved with thimerosal, was not higher for children with ASDs.

Recently, however, this study was criticized for several significant limitations[65] First, there was no neurotypical children control group to evaluate the general population frequency of maternal Rh-negativity and Rho(D)-immune globulin administration (controls included blood bank estimates of the frequency of Rh-negativity and children with chromosomal abnormalities). Second, more than 50 per cent of families with children diagnosed with ASDs were lost to followup according to the researchers. Third, information on the racial

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demographics of several of the control groups employed was not provided. Finally, it is difficult to reconcile the results reported with their previous findings.

In 2002, researchers from Harvard Medical School conducted an epidemiological study of low-dose Hg exposure and the prevalence of neuro-otological symptoms among 114 primarily school children from gold-mining areas of Ecuador[66]. In the study areas, the sources of Hg exposure included: inhalation of Hg vapours during the burning of Hg, a process used to separate gold particulates from alluvial sediment and rock soil in the mountain, mines and rivers, and probably, consumption of methyl-Hg contaminated fish from local rivers and of domestic chickens and pigs that ate plants/products from Hg-contaminated ground soil. These researchers observed prevalent learning disabilities, attention deficits, and autism among the school children examined. Subsequently, these researchers reported, "…investigations of Hg exposure and the general neurologic status of Andean children living in gold mining settlements where Hg is used extensively in the gold amalgamation process, we have found elevated levels of Hg in blood (HgB), urine (HgU), and hair (HgH) in the children, particularly in some of the indigenous AmerIndian families who are most active in the gold mining operations. Some children had neurosensory disorders, including brainstem impairment and auditory dysfunction. Others had epileptic seizures, motoric involvement, and autism"[67].

Finally, a series of epidemiological studies conducted in the US (in California, Louisiana, and Texas) have all also found significant associations between Hg exposure and ASDs[68-70]. In the study published from California (supported by the US CDC), 283 children with ASDs and 657 controls, born in 1994 in the San Francisco Bay area, were examined[70]. These researchers assigned exposure level by census tract of birth residence for 19 chemicals. Among these 19 chemicals to which children were exposed, Hg was found to be the single largest risk factor associated with ASDs. When comparing high Hg exposure relative to low Hg exposure, there was a significant increase of the risk, which was about double, for being diagnosed with an ASD.

Clinical Hg toxicity/increased Hg body-burden in autistic disorders

With animal models demonstrating the toxic effect of Hg upon the developing brain, and epidemiological studies suggesting a correlation between early pre- and post-natal Hg exposure and the rate of ASDs, one must assess whether this association is theoretical or actual. This requires an assessment of Hg body burden and/or detoxification between children with ASDs and neurotypical controls.

In 2003, researchers reported that differential rates of post-natal Hg elimination may explain why similar gestational and infant exposures to Hg produce variable neurological effects[63]. First baby haircut samples were obtained from children diagnosed with autism and matched controls. Information on diet, maternal dental amalgam fillings, vaccine history, Rho(D) immune globulin administration, and autism symptom severity were collected through a maternal survey questionnaire and clinical observation. The mothers in the autistic group had significantly higher levels of Hg exposure through Rho(D) immune globulin injections, fish consumption, and amalgam fillings than control mothers. In addition, autistic children had significantly lower levels of Hg in first baby haircut samples than matched controls. Within the autistic group, hair Hg levels varied significantly across mildly, moderately, and severely autistic children.

Subsequently, other researchers from Arizona State University (ASU) and the Massachusetts Institute of Technology conducted a study (supported by the National Institutes of Health) to replicate the previous study by Holmes *et al*[63] regarding a correlation between the level of Hg in baby hair among children diagnosed with ASDs in comparison to matched controls[71]. Logistic regression analysis showed that, compared to children with higher levels of Hg, children with lower levels of Hg in their hair were 2.5-fold significantly more likely to be diagnosed with ASDs. Children with ASDs and controls had similar Hg exposure. These researchers stated that the lower level of Hg in the baby hair of children with ASDs indicates an altered metabolism of Hg, and may be due to a decreased ability to excrete Hg. Furthermore, these researchers stated that their data are consistent with reports of high Hg body burden in children with ASDs during foetal/infant development, and thus early exposure to Hg appears to be involved in the aetiology of ASDs.

Consistent with these previous reviewed observations that implicated foetal/early postnatal Hg exposures with ASDs, measures of post-first baby haircut sampling have revealed minimal differences in hair Hg levels among patients with ASDs and controls (*i.e.* the older the patients examined, the smaller the differences in hair Hg

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levels between ASD patients and controls)[72-74]. This observation can apparently be explained by the fact that significant lower exposure to Hg occurred in children following the foetal and early infant periods, and thus potential variations in Hg excretion rates would tend to be minimized.

Consistent with the hypothesis that individuals exposed to relatively high Hg could be affected even if their bodies were efficient eliminators, researchers from the University of Kuwait identified a patient population diagnosed with ASDs that showed very elevated levels of Hg (median = 4.50 parts per million), which were found to be significantly higher than matched controls[75]. In support of the observation that the autism diagnosed population examined did not have identifiable susceptibility factors to Hg toxicity, these researchers evaluated the ratios of essential element to Hg levels (selenium/Hg, zinc/Hg, and sulphur/Hg), and all were found to be within normal reference ranges. As a result, this study supports the reality that, at high Hg-dose exposures, even individuals who are capable of good Hg excretion may potentially become Hg toxic and subsequently be diagnosed with an ASD.

Other researchers, including from ASU, examined urinary heavy metal concentrations following therapy for 3 days with *meso*-2,3-dimercaptosuccinic acid (DMSA), a US FDA-approved chelating agent, in children diagnosed with an ASD in comparison to matched controls[76]. Overall, urinary Hg concentrations were significantly higher in children with an ASD than in neurotypical controls. In contrast, similar urinary cadmium and lead concentration levels were observed for the children diagnosed with an ASD and for control children. This study concluded that the DMSA treatment described might be useful in estimating and tracking the burden of Hg in ASD patients.

An examination of a case-series of nine patients diagnosed with ASDs revealed that eight of nine patients had normal development for the first year of life[77]. Subsequently, following significant exposure to medicinal sources of Hg, they suffered Hg toxic encephalopathies with clinical symptoms of a regressive ASD that manifested between their first and second birthdays. In this study, the researchers found that, following chelation therapy to remove heavy metals from the body, eight of the nine patients examined presented with significantly elevated concentrations of Hg in their urine, faecal, and/or hair samples.

ASU and University of Texas researchers have also evaluated baby teeth as a measure of cumulative exposure to toxic metals during foetal development and early infancy in ASD children relative to matched controls[78]. They observed that mean Hg levels in baby teeth from ASD children were significantly higher than the levels in the controls, whereas the lead and zinc levels in both groups were similar. These researchers concluded that ASD children had a higher body burden of Hg during foetal/infant development than neurotypical children.

Investigators from the University of Northern Iowa performed a re-analysis of data from a cross-sectional cohort study over a 5-month period in 2000 comparing hair and blood Hg levels of children with an ASD and a matched control group of normal children[79]. Logistic regression was performed using blood Hg level as the predictor and the ASD/control group as the criterion. Results of their re-analysis indicated that blood Hg level can be used to predict autism diagnosis. These researchers concluded that their finding indicates a significant relationship between Hg levels in the blood and diagnosis of an ASD. In addition, they found, when re-analyzing the data, that the correlation between blood levels of Hg and Hg excreted in the hair is lower for those with an ASD compared with normal controls. Furthermore, this relationship difference between a child with an ASD and the normal controls was most pronounced at high blood levels of Hg.

Several researchers have examined urinary porphyrins among large ASD cohorts in comparison to controls[80-82]. Urinary porphyrins are those heme precursors in the multi-step heme synthesis pathway that are excreted by the kidneys. For certain substances, disturbances in the urinary porphyrin profile affords a measure of environmental exposures. The steps in the heme synthesis pathway that are most vulnerable to heavy metal inhibition are those that involve the enzymes uroporphyrin decarboxylase (UROD) and coproporphyrinogen oxidase (CPOX). Inhibition of these enzymes by Hg poisoning results in specific elevations of coproporphyrin and pentacarboxyporphyrin in the urine. A causal relationship between heavy metal inhibition and porphyrinuria was demonstrated both in rats and humans exposed to Hg. Precoproporphyrin (also known as keto-isocoproporphyrin) is produced by *in vivo* conversion of pentacarboxyporphyrinogen under pressure of heavy metal interference, providing, in particular, a specific porphyrin marker for Hg exposure. Additionally, significant correlations have been found between certain urinary porphyrin profiles and Hg body burden as well

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as specific neurobehavioural deficits associated with low-level Hg exposure. Overall, certain urinary porphyrin profiles have been reported to be a useful biomarker for Hg exposure and potential adverse health effects in human subjects[83-86].

In general, researchers have found that there were significantly increased concentrations and ratios to uroporphyrin for the urinary porphyrins specifically associated with Hg (*i.e.* precoproporphyrin, pentacarboxyporphyrin, and coproporphyrin) among ASD individuals in comparison with controls, with >50 per cent of ASD children having urinary coproporphyrin levels more than 2 standard deviations above the control mean level of urinary coproporphyrin. Also, increasing clinical severity of ASDs correlated with increasing levels of the affected urinary porphyrins. Finally, post-chelation studies on some children with an ASD have found that chelation significantly reduced the levels of the Hg-affected urinary porphyrins in the treated ASD individuals relative to their pre-treatment levels[80-82].

Other researchers have observed significantly increased peripheral oxidative stress/inflammation markers among patients with ASDs in comparison to controls[87-90]. These observations are consistent with significant increases in oxidative stress and/or inflammation markers reported in Hg poisoning[91-96].

An excess accumulation of advanced glycation end products (AGEs) has been reported in the brains of those with an ASD diagnosis[87]. Through their interaction with their putative receptor for advanced glycation end products (RAGE), AGEs can promote neuroinflammation, oxidative stress and neuronal degeneration. To shed more light on the possible alterations of the AGEs-RAGE axis in autism, these researchers measured plasma levels of endogenous secretory RAGE (esRAGE) and its proinflammatory ligand S100A9 in 18 young adults with an ASD diagnosis and 18 age- and gender-matched healthy comparison subjects. The Childhood Autism Rating Scale (CARS) was used to assess the severity of ASD symptoms. Significantly reduced levels of esRAGE and elevated concentrations of S100A9 were found in the patients with an ASD as compared to controls. In ASD patients, there was a statistically significant positive correlation between CARS scores and S100A9 levels, but no significant correlation was seen between esRAGE and S100A9 values. These researchers concluded that their results of a significantly reduced peripheral level of esRAGE coupled with elevated S100A9, point to a subtle but definite dysfunction of the AGEs/RAGE axis in autism that could play a role in the pathophysiology of this disorder[87].

Other researchers from the New York State Institute for Basic Research in Developmental Disabilities compared lipid peroxidation status in the plasma of children with ASDs and their developmentally normal non-ASD siblings by quantifying the levels of malonyldialdehyde, an end product of fatty acid oxidation[88]. Lipid peroxidation was found to be elevated in autism indicating that oxidative stress is increased in this disorder. Levels of major antioxidant proteins, namely, transferrin (iron-binding protein) and ceruloplasmin (copper-binding protein) in the serum, were significantly reduced in ASD children as compared to their developmentally normal non-ASD siblings. A striking correlation was observed between reduced levels of these proteins and the loss of previously acquired language skills in children with ASDs. These researchers concluded that their results indicate altered regulation of transferrin and ceruloplasmin in ASD children who lose acquired language skills. They also suggested that such changes may lead to abnormal iron and copper metabolism in ASDs, and that increased oxidative stress may have pathological role in ASDs.

Robert Wood Johnson Medical School investigators evaluated children diagnosed with autism for the presence of two oxidative stress biomarkers[89]. Urinary excretion of 8-hydroxy-2-deoxyguanosine (8-OHdG) and 8-isoprostane-F2alpha (8-iso-PGF2alpha) was determined in 33 children with autism and 29 healthy controls. 8-iso-PGF2alpha levels were significantly higher in children with autism. The isoprostane levels in ASD subjects were variable with a bimodal distribution. The majority of ASD subjects showed a moderate increase in isoprostane levels while a smaller group of ASD children showed dramatic increases in their isoprostane levels. There was a trend of an increase in 8-OHdG levels in children with autism but it did not reach statistical significance. There was no significant correlation between the levels of the biomarkers and vitamin intake, dietary supplements, medicine, medical disorders, or history of regression. These researchers concluded that their results suggest that the lipid peroxidation biomarker is increased in this cohort of children diagnosed with an ASD.

Researchers from the University of Pennsylvania evaluated urinary levels of isoprostane F(2alpha)-VI, a marker of lipid peroxidation; 2,3-dinor-thromboxane B(2), which reflects platelet activation; and 6-keto-

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prostaglandin F(1alpha), a marker of endothelium activation, in subjects diagnosed with autism and healthy in control subjects[90]. Compared with controls, children with autism had significantly higher urinary levels of isoprostane F(2alpha)-VI, 2,3-dinor-thromboxane B(2), and 6-keto-prostaglandin F(1alpha). Lipid peroxidation levels directly correlated with both vascular biomarker ratios.

The most recent research[97-99], extending the aforementioned studies of peripheral samples showing increased Hg levels and increased potentially Hg-associated oxidative stress/inflammation in patients diagnosed with an ASD, directly implicates significant increases in Hg and Hg-related oxidative stress/ inflammation in pathological studies of the brain in patients diagnosed with an ASD in comparison with controls.

Harvard University researchers reported that oxidative stress and/or Hg compounds play an important role in the pathophysiology of ASDs[97]. For the first time, this study compared the cerebellar levels of the oxidative stress marker 3-nitrotyrosine (3-NT), Hg and the antioxidant selenium levels between control and ASD subjects. Tissue homogenates were prepared in the presence of protease inhibitors from the frozen cerebellar tissue of ASD and matched control subjects. Mean cerebellar 3-NT was elevated in those with an autism diagnosis by 68.9 per cent and the increase was significant. Cerebellar Hg in the ASD cases was also increased by 68.2 per cent in comparison to controls. There was a positive correlation between cerebellar 3-NT and Hg levels. Furthermore, a small decrease was observed in cerebellar Se levels in ASDs, and this small decrease in Se was accompanied by a statistically significant 42.9 per cent reduction in the molar ratio of Se to Hg in the ASD cerebellum. These researchers concluded that their results add elevated oxidative stress markers in the brain to the growing body of data reflecting greater oxidative stress in ASDs.

Other researchers, including from the University of Maryland, the University of Texas, and Case Western Reserve University, described that oxidative damage has been documented in the peripheral tissues of autism patients[98]. In this study, the researchers sought evidence of oxidative injury in the ASD brain. Carboxyethyl pyrrole (CEP) and iso[4]levuglandin (iso[4]LG)E2-protein adducts, which are uniquely generated through peroxidation of docosahexaenoate and arachidonate-containing lipids respectively, and heme oxygenase 1 were detected immunocytochemically in cortical brain tissues and by ELISA in blood plasma. In every autism case examined, the study found significant immunoreactivity in regard to all three of these markers of oxidative damage in the white matter and, based on these damage markers, the immunoreactivity often extended well into the grey matter of axons. This resulting threadlike pattern appears to be a distinguishing hallmark of the ASD brain because this pattern was not seen in any control brain for the oxidative assays. Western blot and immunoprecipitation analysis confirmed neurofilament heavy chain to be a major target of CEP-modification. In contrast, when examining the plasma from patients with an ASD in comparison to age-matched healthy controls, the study found similar levels of plasma CEP, iso[4]LGE2 protein adducts, anti-CEP and anti-iso[4]LGE2 autoantibody titre, as well as no differences between the ratio of NO2Tyr/Tyr. These findings provide the first direct evidence of increased oxidative stress in the brains of those with an ASD. Therefore, the oxidative injury of proteins in the brain seems to be associated with neurological abnormalities and to provide a cellular basis at the root of ASDs.

Finally, investigators studied brain samples for evidence of impaired speech/language function, one of the key criteria for the diagnosis of ASDs[99]. These researchers examined brain samples from age-matched ASD and control subjects and compared brain regions associated with the production and processing of speech. The Wernicke's area (Brodmann 22, speech recognition), Broca's area (Brodmann 44, speech production) and the gyrus angularis (Brodmann 39, reading) from ASD subjects and matched-control subjects were examined microscopically. Striking differences in the density of glial cells, the density of neurons and the number of lipofuscin-containing neurons were observed in the ASD group when compared with controls. The mean density of glial cells was significantly greater in the ASD cohort than controls in area 22, area 39, and area 44. Also, the density of the neurons was significantly less in autism in area 22 and area 39. In addition, the ASD group exhibited significantly greater numbers of lipofuscin-containing cells in area 22 and area 39. These results are consistent with accelerated neuronal death in association with gliosis and lipofuscin accumulation in autism.

These researchers[99] hypothesized that environmental exposure in sensitive subjects might underlie glial proliferation and neuronal death in the

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pathogenesis of autism. In support of this hypothesis, these researchers described that toxins, including metals such as Hg, specifically induce glial proliferation, degeneration and decreased cellular function in some regions of the brain. Neurotoxicity of metals is primarily mediated by increased oxidative stress and both increased metals and increased oxidative stress are reported in autism. They also reported that lipofuscin is an intralysosomal polymeric material that originates from autophagocytosed cellular components which cannot be degraded or exocytosed. Biochemical analysis of lipofuscin reveals a complex aggregated byproduct composed primarily of oxidatively-modified proteins and lipids. In addition, lipofuscin is a depot for heavy metals such as Hg[100]. Lipofuscin accumulation in cells is accelerated under conditions of oxidative stress, and experimentally, lipofuscin itself induces neurotoxicity via generation of free radicals.

Clinical studies implicating biochemical/genomic Hg susceptibility in autistic disorders

Researchers from Northeastern University have reported that methylation events play a critical role in the ability of growth factors to promote normal development[101]. They observed folate-dependent, phospholipid methylation in the lymphoblasts of ASD children was, in a dose-response manner, significantly more sensitive to thimerosal exposure than in their unaffected siblings.

Wake Forest University researchers postulated that thimerosal may be a potential triggering mechanism contributing to ASDs in susceptible individuals, and that one potential risk factor in these individuals may be an inability to adequately upregulate metallothionein (MT) biosynthesis in response to heavy metals (MTs may help to modulate Hg neurotoxicity)[102]. Cultured lymphocytes from ASDs challenged with zinc responded with an impressive upregulation of MT transcripts (at least nine different MTs were over-expressed), whereas these same cells, when challenged with thimerosal responded by upregulating numerous heat shock protein transcripts, but not MTs.

In heavy metal toxicity, Hg binds to cysteine thiol (-SH) groups on intracellular proteins and inactivates their function. The cysteine-SH group of glutathione binds Hg and protects essential proteins from functional inactivation. The synthesis of glutathione has been directly linked to the rate of Hg excretion and cellular protection from Hg induced damage. Individuals with genetic deficiencies in glutathione synthesis will be less able to excrete Hg and will be more sensitive to its adverse effects[103]. Several recent studies have examined blood markers in the transsulphuration pathway in ASDs. These studies have demonstrated that ASD patients have significant reductions in cysteine, sulphate, total glutathione, and free glutathione (*i.e.* active glutathione that can bind Hg) and significant increases in oxidized glutathione (*i.e.* inactive glutathione that cannot bind Hg) in comparison to controls[103-108].

Researchers from the University of Arkansas further extended the role of glutathione in ASDs by reporting on evaluations of lymphoblastoid cell lines from children with ASD diagnoses who have at least one affected sibling[109]. Samples of ASD-derived and sibling-control cells were cultured under identical conditions. Rates of free radical generation and free glutathione/oxidized glutathione levels were measured at baseline and after 3 and 24 h exposures to thimerosal. The experiments provided experimental evidence that, following low dose thimerosal exposure, the ASD-derived lymphoblastoid cells were, in a dose- and time-dependent manner, significantly more susceptible to generating more free radicals and had lower free glutathione and higher oxidized glutathione levels than control cells. Since both cell lines were cultured at the same time under identical conditions with identical media, the differences at baseline and after exposure to thimerosal must reflect inherent genetic differences, and thus, suggest that children with an ASD diagnosis are more genetically sensitive to pro-oxidant environmental exposures.

A series of clinical studies have shown significant altered functionality of key antioxidant enzymes among patients with ASDs in comparison to controls[110-113]. These abnormalities were associated with dysregulation of oxidative stress in ASDs, and were demonstrated to be significantly altered by Hg toxicity[94].

In addition to biochemical susceptibilities to heavy metal toxicity in ASDs, several recent studies have assessed genomic susceptibilities to heavy metal toxicity in ASDs. The results showed that there were significant correlations between genomic changes associated with reduced functioning in heavy metal detoxification enzymes (*i.e.* gene deletions/polymorphisms) and ASDs, including reduced folate carrier (RFC); catechol-*O*-methyltransferase (COMT); transcobalamin II (TCN2); glutathione *S*-transferase M1 (GSTM1); glutathione *S*-transferase P1 (GSTP1); 5, 10-methylenetetrathydrofolate reductase (MTHFR); metal-

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regulatory transcription factor 1 (MTF1); and divalent metal ion transporter SLC11A3[106, 114-117].

Hormones as a mediating factor in mercury toxicity

In animal models and in human Hg poisonings, males were found to be significantly more susceptible to Hg toxicity (including neurotoxicity) than females[118-120]. Additionally, in a series of tissue culture experiments with neurons, testosterone was able to potentiate the neuronal toxicity of Hg, whereas estradiol significantly lessened the neuronal toxicity of Hg[121, 122]. Also of interest was the fact that estradiol exposure possibly contributed to maintaining normal neuronal cell glutathione levels, and thus, helped to significantly lessen neuronal Hg toxicity[122]. It was also observed that estradiol administration in rats significantly protected the hypothalamus-pituitary axis from methyl-Hg induced damage and significantly reduced Hg content of the anterior pituitary gland and medial hypothalamus[123]. Similarly, other researchers observed greater Hg excretion in female rats in comparison to male rats following methyl-Hg exposure[124]. Increased testosterone and other androgen levels were also observed to occur in tissue culture, in animals, and in humans (including paediatric patients) following low-dose Hg exposure[125-128], whereas aromatase activity, a key steroidogenic enzyme that catalyses the conversion of androgens to estrogens, was inhibited by Hg exposure[129]. In addition, *in vitro* studies showed that Hg exposure significantly inhibited hydroxysteroid sulphotransferase (HST) activity, thus reducing the conversion of dehydroepiandrosterone (DHEA) to dehydroepiandrosterone-sulphate (DHEA-S)[130, 131].

Several studies have examined androgen and estradiol levels in those with an ASD. In these studies, individuals with ASDs (and other neurodevelopmental disorders) had evidence of significantly increased pre- and post-natal levels of testosterone and other androgen metabolites, and significantly decreased pre- and post-natal levels of estradiol[104, 132-139]. Patients with an ASD diagnosis were also found to have significantly elevated DHEA levels in comparison to controls, yet these patients had significantly decreased DHEA-S levels in comparison with controls. These findings suggest that those most vulnerable to Hg toxicity are characterized by high testosterone and other androgens, and that Hg may also increase already high levels of androgen metabolites in the testosterone pathway in ASD patients.

Molecular mechanisms by which mercury causes autistic disorders

In examining the biomolecular mechanisms by which mercury causes ASDs, the following studies have documented phenomena of neuronal death, disorganization and/or damage in the brain as a result of Hg exposure. The resulting Hg-induced alterations in brain structure, signaling, function, *etc.* appear to produce the symptoms used to diagnose ASD.

In considering the biomolecular mechanisms by which Hg can cause ASDs, it is important to note that Hg can cross blood-brain barrier and that this transport mechanism can lead to significant brain concentrations of Hg^{2+}, which have been shown to persist there for prolonged periods of time[31]. Researchers have taken this discovery further by summarizing the overall pathogenesis of ASD as a condition resulting from environmental factors (*e.g.,* Hg) coupled with decreased levels of key body antioxidants (*i.e.* low glutathione, antioxidant enzymes, *etc.*), leading to the increased production of free radicals[140]. These researchers reported that the resultant increased free radical production can cause an increase in lipid peroxidation, protein oxidation, and DNA oxidation, all leading to increased oxidative stress. In considering the effects of oxidative stress on the mechanisms that mediate neuronal dysfunction and clinical symptoms in autism, increased oxidative stress was shown to result in impaired neuronal development, increased inflammatory response, impaired energy production, cell death, decreased synaptic efficiency, *etc.* These combined effects lead directly to the pathogenesis and clinical presentation of ASDs[140].

Furthermore, it is known that Hg can cause neuronal degeneration (*i.e.* the neuronal circuitry does not form appropriately and brain connectivity disorders develop – this phenomena has been directly observed in the brains of ASD patients), neuronal cell death (*i.e.* decreased numbers of specific cells types that are crucial for normal brain development follows – this phenomenon has also been directly observed in the brains of ASD patients), and inhibit mechanisms of normal neuronal development (*i.e.* proliferation/ migration of specific cell types that are a part of normal brain development does not occur – this phenomenon has also been directly observed in the brains of ASD patients). Such phenomenon result in the alteration of crucial neurodevelopmental steps in brain development, and hence, neurodevelopment is directly hindered at the cellular level, leading to neurodevelopmental disorders, including the neurodevelopmental disorder of autism.

Investigators from the University of Calgary demonstrated that exposure to Hg ions markedly disrupts the membrane structural integrity of neurites

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and the growth cones in identified neurons[141]. These investigators reported that the phenomenon observed appeared specific for Hg at low parts-per-billion concentrations utilized, since exposure to four other heavy metals at the same molar concentrations had no observable effect on either growth cone morphology or individual neurites. The biomolecular basis for the investigators' findings was that Hg inhibits guanosine-5'-triphosphate (GTP) nucleotide binding to β-tubulin, a requisite step for tubulin polymerization in the formation of microtubules. Thus, these investigators thought the Hg-induced disassembly of the neurite membrane was a physical manifestation of a disrupted microtubule-tubulin polymerization cycle.

Investigators from Northeastern University, the University of Nebraska, and Johns Hopkins University reported that *S*-adenosylmethionine (SAM) provides methyl groups to numerous acceptors, including phospholipids and DNA, and that methionine synthase (MS) can promote methylation[142]. Growth factors [*e.g.* nerve growth factor, brain derived neurotrophic factor and insulin-like growth factor 1 (IGF-1)] promote development of neuronal phenotype and support the function and survival of differentiated nerves. The capacity to activate simultaneously both phosphoinositide 3 (PI3)-kinase and mitogen-activated protein-kinase (MAPK) pathways is a feature of many growth factors. Blocking the methionine cycle [*e.g.* with inhibitors of *S*-adenosylhomocysteine (SAH) hydrolase] interferes with neurotrophic responses, indicating an essential role for methylation in growth factor action. Since differences in cellular phenotype reflect varied patterns of methylation-dependent gene silencing, growth factors directly or indirectly modulate genomic methylation status during development. IGF-1 exerts trophic and anti-apopotic effects on a wide variety of cell types, and its involvement in brain development is well documented. In addition to its neurotrophic action, IGF-1 promotes differentiation and survival of myelin-producing oligodendrocytes, an action in which divalent copper plays a role. Thus the chelation of copper causes demyelination and upregulation of IGF-1. Vitamin B12 deficiency and chronic nitrous oxide exposure, both of which impair MS, also cause demyelination. It was observed that copper promotes MS activity and protects against the inhibitory effects of other metals, while copper chelation has an opposite effect. Thus oligodendrocytes provide a specific example of how IGF-1, metal ions and methylation can combine to affect cellular differentiation and brain development. During post-natal development, myelination is critical for the specification of fixed connections between brain regions (*i.e.* hard-wiring), and there have been a number of reports of abnormal white matter (*i.e.* myelination) in autism.

Neurodevelopmental insults affecting myelination could lead to abnormal neuronal connections, resulting in the enhancement of certain relationships, but deficiencies in others, as is frequently observed in autism. Reduced IGF-1 levels have been reported in autism, which may also contribute to impaired myelination. These investigators concluded that the discovery of the PI3-kinase/MAPK/MS pathway and its potent inhibiting by developmental neurotoxins such as Hg provides a molecular explanation for how Hg could promote the development of autism[142,143].

Investigators from Virginia Tech (supported by the US National Institutes of Health) reported that thimerosal is an organic Hg compound that consists of an organic moiety, ethyl-Hg, which is bound to the sulphur atom of the thiol group of salicylic acid[144]. Under physiological conditions, thimerosal is rapidly converted into ethyl-Hg chloride and hydroxide. Ethyl-Hg (as hydroxide or chloride) and its decomposition product, Hg^{2+}, rapidly accumulate in the tissues – preferentially in the kidneys and brain. Following *in vivo* administration, ethyl-Hg compounds pass through cellular membranes and concentrate in cells of vital organs, including the brain, where metabolic processes operate to de-ethylate these ethyl-Hg compounds and release Hg^{2+}, significantly raising its concentration higher than equimolar doses of its admittedly highly toxic methyl-Hg relatives, whose toxicity and harmfulness are widely accepted.

Some recent *in vitro* studies show that certain concentrations of thimerosal have decreased cellular viability in human neurons and fibroblasts. For example, Baskin *et al*[145] noted an increase in membrane permeability to DAPI dye as early as two hours after incubation of human cortical neurons and fibroblasts with 250 μM thimerosal. A six-hour incubation resulted in membrane damage (loss of DAPI dye exclusion), DNA breaks, and apoptosis as indicated by morphology and caspase-3 activation. The studies cited above identified a number of molecular targets for thimerosal, including micronuclei induction, disturbances of intracellular calcium, and inhibition of glutathione content, but the unique dependence of the developing nervous system on growth factors suggests that the

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neurotrophins and their receptors represent possible targets for thimerosal. There are several studies suggesting that thimerosal may alter neurotrophin signaling, including binding of secondary messengers; microtubule assembly; and intracellular calcium concentrations. Concentrations of thimerosal showing an effect on the development and viability of undifferentiated human neuroblastoma cells and neurotrophin cell signaling in terms of protein phosphorylation and cell viability are of interest. Neurotrophins, especially nerve growth factor (NGF), but also the related neurotrophins, brain-derived growth factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5), are known to affect the biological behaviour of neuronal cells during development, survival, and differentiation of the central and peripheral nervous systems.

In the study by Parran *et al*[144], the SH-SY5Y human neuroblastoma cell line (SY5Y cells) was used as an *in vitro* model for neurotrophin (NGF)-induced differentiation. SY5Y cells represent a well characterized *in vitro* model system used to study the actions of neurotrophins on developing neurons. In addition, continuously dividing cell lines such as SY5Y cells have been widely used as a model to study neurodegeneration. SY5Y cells differentiate into cells that are biochemically, ultrastructurally, and electrophysiologically similar to neurons. In this cell line, activation of the TrkA receptor by its ligand NGF leads to differentiation. These actions of NGF on SY5Y cells are dependent on the high affinity interaction with the TrkA receptor. Binding of NGF to the TrkA receptor induces receptor dimerization and tyrosine kinase activity that results in autophosphorylation of several tyrosine residues on the cytoplasmic domain of the receptor. These phosphorylated tyrosines serve as anchors for binding and activating downstream signaling elements such as phospholipase C (PLC), Shc, SNT and phosphatidyl inositide 3-kinase. These proteins couple TrkA to several intracellular pathways such as the MAPK cascade and protein kinase C (PKC). Activation of the MAPK cascade by NGF leads to activation of transcription factors and induction of immediate-early genes, ultimately leading to differentiation (*i.e.,* neurite outgrowth). Specific isoforms of PKC have been associated with NGF-induced differentiation, in particular PKC-d and PKC-e. PKC-d differs in that it is translocated to the membrane in response to NGF. In addition, PKC-d is required for NGF-induced activation of the MAPK cascade, and contributes to MEK-induced neurite outgrowth (differentiation). Parran *et al*[144] examined the NGF signal transduction cascade in human neuroblastoma SY5Y cells that were stimulated with NGF in the presence of various concentrations of thimerosal. The effects of thimerosal on NGF induced TrkA autophosphorylation, MAPK activation, and PKC-d phosphorylation were determined.

Researchers have observed that concurrent exposure to NGF and thimerosal decreased TrkA autophosphorylation[144]. The inhibition of TrkA autophosphorylation was concentration-dependent and was evident at thimerosal concentrations as low as 50 nM. Inhibition of TrkA activity is likely to have consequences on downstream signaling. TrkA activation leads to the initiation of several effects including Rac1, the MAPK cascade, PLCc, and PKC. The time course for activation of these proteins follows closely to that of TrkA. Studies have demonstrated that another neural cell line (PC12 cells) can either differentiate or proliferate in response to growth factor stimulation according to the strength (threshold) or duration (or both) of the stimulus. It has been shown that both the strength and duration of the signal generated by a receptor with tyrosine kinase activity can influence the downstream signaling pathway, leading to cell differentiation instead of cell proliferation.

Therefore, thimerosal-induced inhibition of the early peak of neurotrophin signaling may reduce TrkA activity below a threshold, leading to effects on downstream signaling and differentiation in SY5Y cells. Phosphorylation of tyrosine 490 and 785 within the TrkA receptor leads to activation of the MAPK cascade and PKC, respectively. The MAPK cascade has been shown to be important for NGF signaling. In the absence of Thimerosal, phosphorylation of MAPK in SY5Y occurred rapidly upon exposure to NGF and peak activity was observed at 5 min. MAPK phosphorylation then decreased but remained above the initial (0 min) level of phosphorylation for the next 60 min. Concurrent exposure to NGF and thimerosal decreased MAPK phosphorylation. A more detailed examination revealed a concentration-dependent inhibition with effects observed at concentrations of thimerosal as low as 100 nM. The inhibition of MAPK phosphorylation by thimerosal may be a consequence of its actions upstream on TrkA, or due to a direct effect on enzymes in the MAPK cascade. The results suggest that TrkA signaling through MAPK is a sensitive target for thimerosal[144].

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The results indicate that the thimerosal cytotoxicty to SY5Y cells depended on both the presence of NGF and the time of exposure to thimerosal. Following a 24 h exposure in the absence of NGF, thimerosal toxicity was approximately 15 times higher when compared to thimerosal exposure in the presence of NGF. This trend was also observed following a 48 h exposure where thimerosal's effects on cell viability was approximately 24 times higher in the absence of NGF. This may be attributed to NGF's ability to promote survival as well as regulate and modulate differentiation and some investigators have suggested that NGF acts to help stabilize the reorganization of cytoskeletal elements of cells[144].

When comparing the EC_{50}s of cytotoxicity, thimerosal toxicity was also time dependent. In the presence and absence of NGF, thimerosal cytotoxicity was six and nine times higher, respectively, following a 48 h exposure compared to a 24 h exposure[144]. It was shown that thimerosal-induced cytotoxicity in SY5Y cells ranged from low nanomolar to the micromolar concentrations. Similar to the results observed by Parran *et al*[144], high cellular toxicity of thimerosal in low micromolar concentrations was reported using other cell culture models including human neurons and fibroblasts and Jurkat cells. These studies also concluded that thimerosal exposure resulted in caspase-3 activation, which is induced during apoptotic cell death[144]. Following 24 h of exposure to thimerosal, DNA fragmentation first increased and then decreased, with fragmentation highest at 0.01 μM and significantly lower at >1 μM. The elevated DNA fragmentation could suggest that thimerosal was inducing apoptotic cell death in a concentration-dependent fashion, perhaps through a mechanism similar to that induced by serum/neurotrophin withdrawal. Apoptosis plays an important role during neuronal development and defects in apoptosis may underlie various neurodegenerative disorders. The process of DNA fragmentation into specific oligonucleosomal fragments has been found to accompany apoptosis in many cell types and has become a biochemical hallmark of classic apoptosis. At concentrations above 1 μM of thimerosal, there was a steep decline in DNA fragmentation. This steep decline in DNA fragmentation was observed in both the presence and absence of NGF. This would suggest that at these higher concentrations, cell death was produced through non-apoptotic pathways[144].

Hg species, including thimerosal, are reported to have effects on the antioxidant status of various cells types including astrocytes and neurons, lymphocytes, and thymoctes. Previous mechanistic studies[144] of methyl-Hg toxicity have implicated reactive oxygen species and depletion of intracellular glutathione as a contributor to Hg-induced cytotoxicity. Reduced glutathione provides the major intracellular defense against reactive oxygen species, oxidative stress-induced cell damage and apoptosis. Its depletion was shown to precede the increase in reactive oxygen species associated with loss of viability and apoptosis. Previous studies[144] have demonstrated that thimerosal neurotoxicity is associated with glutathione depletion and this action is likely to be related to changes in cellular redox status modulating channel and receptor activities as well as cell growth and death related to cellular redox state.

Parran *et al*[144] observed significant inhibition of TrkA autophosphorylation and MAPK activation at 0.1 μM thimerosal. These effective concentrations are lower concentrations than those that were observed in other proposed modes of action for the developmental neurotoxicity of methyl-Hg, including microtubule disruption, decreased expression of NCAM, and inhibition of the cell cycle and the induction of Gadd45/153.

Parran *et al*[144] concluded that their data do not exclude the possibility that thimerosal can act at other sites, either directly or indirectly, to inhibit NGF-induced signaling. In response to NGF stimulation, a significant fraction of MAPK is redistributed to the nuclei and is retained there for several hours. This enables transmission of neurotrophin signaling to the nucleus, where an important end result is transcriptional control. Nuclear uptake is strongly correlated with MAPK-dependent regulation of DNA synthesis in differentiating PC12 cells and appears to require positive signaling through the MAPK cascade and phosphorylation of MAPK. Several proteins have been identified as putative substrates for MAPK, including pp90rsk, PLA2, p62TCF, c-Fos, and c-Jun. Thimerosal could affect these pathways indirectly by inhibiting MAPK activation or directly by inhibiting one of the components of the cascade. In light of the proclivity of thimerosal to bind any protein containing sulphydryl groups, it is likely that the effects of thimerosal on differentiation are the result of multiple sites of action[144].

Researchers from the University of Torino, funded by the Italian Ministry of University and Scientific Research, have reported that during the development of the nervous system, many neurons and glial cells

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migrate to reach their final location[146]. Distinct and elaborate modes of migration are required to generate the complexity of the brain structures and the peripheral innervation. Diverse roles of glial cells in this process have been recognized, in both the peripheral nervous system (PNS) and the CNS. For example, in the cerebral and cerebellar cortex of vertebrates, glial cells act as a scaffold for migrating neurons; in insects and vertebrates, glial cells function as guideposts or intermediate targets in axon guidance; and, in the developing insect antennal lobe, glial cell migration is a crucial step for glomerulus formation and axon sorting.

The researchers described the ability of embryonic chick ciliary ganglion (CG) cells to migrate and reaggregate when uniformly dispersed in a culture dish[146]. CG neurons, when dissociated from E7 ganglia and cultured in the presence of neurotrophic factors such as bFGF and GDNF, migrate in association with nonneuronal cells to form cellular aggregates connected by bundles of fibers. CG nonneuronal cells are midbrain/hindbrain neural crest derivatives mainly composed of satellite cells and undifferentiated glial precursors. These researchers also showed that neuronal migration in the absence of nonneuronal cells is strongly inhibited: only a small percentage of neurons move by means of a mechanism of soma translocation along the neurite. These studies point to a key role of nonneuronal cells in the formation of neural cells networks; thus, embryonic CG provides a good model to study migration mechanisms and neuron–glia interactions during this process.

On the other hand, calcium signals are involved in several ways in the regulation of migratory processes, including cell migration cycle co-ordination, cell guidance by chemorepulsive and chemoattractive cues, axon outgrowth, and growth cone turning and deadhesion. The subcellular localization and the specific frequency patterns of these signals are key factors in determining the extent, the directionality and the completion of migration.

By tracking the soma of neurons that migrate in association with glial cells and by monitoring intracellular calcium in both cells types Ariano *et al*[146] showed that (*i*) migration of CG cells is a calcium dependent process; (*ii*) both neurons and nonneuronal cells generate spontaneous calcium signals; (*iii*) the amplitude of nonneuronal calcium oscillations can be directly correlated with the rate of migration of the complex. They determined that their data suggest that Ca2+-signaling in nonneuronal cells plays a key role in the migration of neurons. Furthermore, they found that application of thimerosal, a compound that stimulates calcium mobilization from internal stores, increased the amplitude of spontaneous nonneuronal oscillations, the area of migrating nonneuronal cells and the velocity of the neuronal–nonneuronal cell complex[146].

Another common pathological lesion observed in specific regions of the brains of ASD patients is characterized by significant neuronal loss, accompanied by a significant increase in glial cells in specific regions of the brain[99]. Researchers have evaluated mitochondrial viability and apoptosis induced by mercuric Hg and methyl-Hg in cell lines of neuronal origin (including neuroblastoma, glioblastoma, and retinal pigment epithelial cells). Mitochondrial dysfunction and cell death assays showed a clear dose-response and exposure time-response to mercuric Hg and methyl-Hg. They also found that mitochondrial dysfunction and cell death assays following Hg exposures showed clear cell-line specificity, with the following order of decreasing sensitivity to cellular damage: neuroblastoma > glioblastoma > retinal pigment epithelial cells[147]. Other researchers have observed similar sensitivities of neuronal cell lines to thimerosal induced cell death[148].

Additionally, it was reported, that in microscopic photographs of co-cultures of glioblastoma and neuroblastoma cells with Hg exposure, low-dose exposure to $HgCl_2$ or methyl-Hg, induced significant cellular damage/death in neuroblastoma cells, whereas glioblastoma cells fared significantly better and, in some cases, cellular proliferation continued. By contrast, at high-dose exposure to $HgCl_2$ or methyl-Hg, Hg induced significant cellular damage and/or death in both neuroblastoma and glioblastoma cells[147].

Moreover, various and significant underlying molecular mechanisms by which Hg, at levels shown to be present from environmental sources, including drugs, produce such neuronal damage have been well investigated and documented by a wide variety of investigators.

A summary of the overall biochemistry found in autistic disorders

Overall, it is apparent that the biochemical basis for abnormalities in the androgen synthesis pathway in those with an ASD may involve the regulating metabolite DHEA. DHEA can either be converted into

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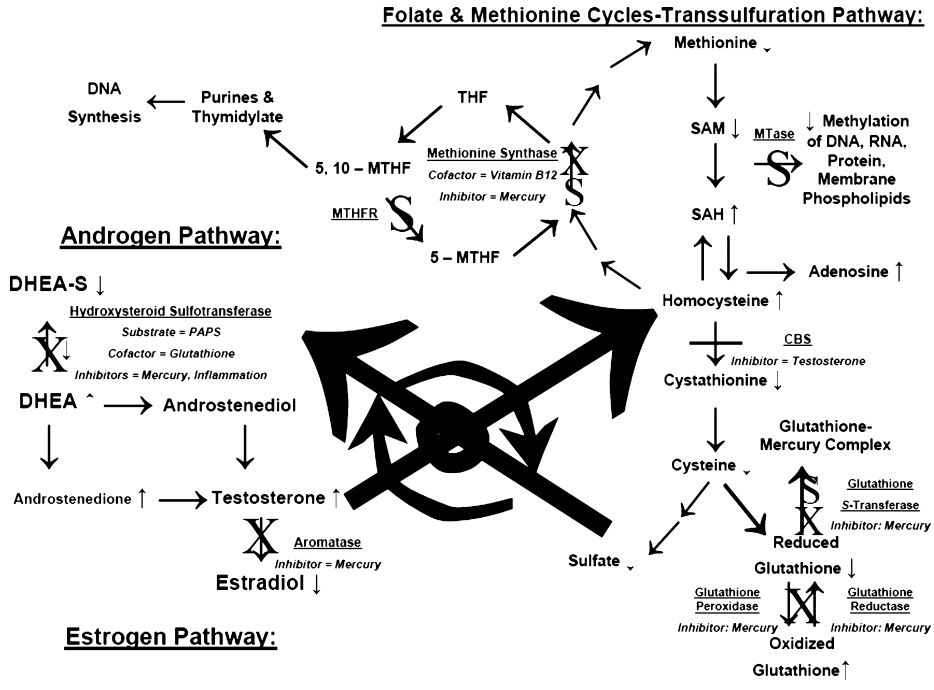

Fig. 2. A summary of the biochemical abnormalities found in autistic disorders and how the biochemical pathways interact with one another. CBS, cystathionine β-synthase; DHEA-S, dehydroepiandrosterone-sulphate; DHEA, dehydroepiandrosterone; PAPS, 3'-phosphoadenylyl sulphate; MTase, methyl transferases; MTHFR, methylenetetrahydrofolate reductase; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylmethionine; THF, tetrohydrofolate; 5-MTHF, 5-methyltetrahydrofolate; 5, 10-MTHF, 5; 10-methyltetrahydrofolate; SAHH, SAH hydrolase; X, Hg inhibition; S, genetic susceptibility sites associated with autistic disorders; _____ , androgen inhibition.

the normally favoured storage molecule, (DHEA-S, or, further) down the androgen pathway toward testosterone, it can be converted into androstenedione or androstenediol. The conversion of DHEA to DHEA-S by the enzyme HST is dependent upon sulphation, its functional activity is enhanced by glutathione, and its activity is inhibited by inflammation and Hg[130,131,149].

Since, evaluations of the transsulfuration pathway in those with an ASD diagnosis has revealed significant decreases in transsulfuration metabolites including cysteine[103-107], glutathione[103-107], and sulphate[108], impaired sulphation[150], as well as significant increases in common polymorphic variants known to modulate the methionine cycle and transsulfuration pathways[106, 114-116], there may be a marked shift toward DHEA, and subsequent metabolites in the androgen synthesis pathway. In addition, the presence of significant oxidative stress in those with an ASD diagnosis[87-90,97-99,105,106] (including pathologically confirmed inflammation[45]) and the apparent significant increase in Hg body-burden and toxicity in those with an ASD diagnosis[63,71,75-82] (Hg is a known inducer of pathologically confirmed inflammation[40]) may also lead to a significant overproduction of androgens in those with an ASD diagnosis. Previous studies in testicular tissue culture[127], animal models[128], and humans[125,126] have shown that low-dose exposure to Hg can induce significant increases in androgen levels. The apparent result, as demonstrated in ASD cases, is a significantly increased DHEA level[104,139] and a significantly lowered DHEA-S level[151], relative to controls.

It has been shown that testosterone, and possibly other androgen metabolites, may have a negative impact on the transsulfuration pathway. A series of transsexual human clinical studies demonstrated that testosterone administration at least partially blocked the conversation of homocysteine to cystathionine by cystathionine β-synthase (CBS), whereas estrogen administration had the opposite affect[152, 153]. Additionally, researchers have shown significant positive correlations between homocysteine and androstenedione levels and glutathione and DHEA-S levels in humans[154]. It has been recently demonstrated in human tissue culture cells that CBS is significant inhibited by testosterone and that this inhibition results in significantly lower levels of glutathione[155]. Thus, high levels of androgens are expected to block the transsulfuration

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pathway. The apparent result, as demonstrated in those with an ASD diagnosis, is significantly increased homocysteine[110], SAH[105, 106], and/or adenosine[105, 106] levels, in comparison to controls.

Exposure to Hg compounds was previously shown to significantly reduce the enzymatic activitiy of aromatase[129], a key steroidogenic enzyme that catalyses the conversion of androgens to estrogens. Hence, this reduction may contribute to the significant elevations observed in androgens, particularly testosterone and androstenedione, and the significant decreases observed in estrogens, particularly estradiol, among patients with an ASD in comparison to controls[104, 133-139].

In synthesizing this information, Hg exposure can adversely effect HST, and the transsulfuration pathway can cause a cyclical biochemical interaction pattern to develop between the transsulfuration and androgen pathways that directly correlates with the biochemistry observed in those having an ASD diagnosis. As expected, this interaction pattern and androgen elevations are consistent with the behavioural/physical traits associated with or defining those who have an ASD diagnosis[156-160]. Fig. 2 summarizes the overall potential interactions between the androgen and transsulfuration pathways in those having an ASD, as well as in the estrogen and methionine cycle pathways.

Additionally, HST was shown to be necessary for appropriate function of bile salts[161]. As a result, given the aforementioned abnormalities observed in patients diagnosed with an ASD, this interference with HST production may contribute to malabsorption and the high prevalence of gastrointestinal disease found in ASD cases[162]. Furthermore, impaired sulphation may also play an important role in other common biochemical abnormalities found in ASD cases, which involve neurotransmitters, peptides, glycosaminoglycans, amines, and/or phenols[108,150].

Notably, the aforementioned understanding of the biochemical interactions and regulation between the transsulfuration and androgen pathways represents a potentially new understanding of control mechanisms in living systems. The understanding that the transsulfuration and androgen pathways interact so as to help to regulate one another may have significant importance in a number of other important chronic diseases with biomarkers similar to those who have an ASD diagnosis[163], and it may also be of significant importance in understanding how the human body undergoes maturational transitions[139].

Suggested therapies

The new identification of abnormalities/imbalances in the androgen pathways offers new possibilities for successful biochemical intervention in ASDs. Based upon this finding, it has been suggested that therapies which address the steroid hormone pathways in ASD, seeking to correct abnormally high levels of metabolites in these pathways, may help to improve clinical outcomes[163]. Recently, a review of several studies showed that drugs with known anti-androgen effects including leuprolide acetate, cyproterone acetate, spironolactone, risperidone, haloperidol, and pioglitazone produce, according to reports, beneficial effects in ASDs[139]. Each of the studies examined noted that the therapies utilizing these drugs resulted in significant clinical ameliorations in hyperactivity/impulsivity, stereotypy, aggression, self-injury, abnormal sexual behaviours, and/or irritability behaviours that frequently occur in those with an ASD diagnosis. It has been reported that leuprolide acetate (LUPRON®) administration to a cohort of nearly 200 patients diagnosed with ASDs, significantly lowered androgen levels and has resulted in very significant overall clinical improvements in socialization, sensory/cognitive awareness, and health/physical/behaviour skills, with few non-responders and minimal adverse clinical effects to the therapy[139].

In addition, researchers have described the results of data compiled by the Autism Research Institute[164]. The Autism Research Institute collected data from over 22,300 parents of children with autism on the behavioural effects of biomedical interventions. The survey includes a list of 45 medications, 23 nondrug supplements or biomedical treatments, and 9 special diets. The parents were asked to rate the treatment on a 6-point scale. Of these 77 choices, parents rated chelation therapy (or the removal of heavy metals) as the most effective. Seventy six per cent of parents said that their child "got better" on this treatment. Other researchers have also reported that significant improvements in ASD symptoms were observed following small clinical trials using chelation therapy to remove heavy metals[165-167].

Overall analysis of causation: Can mercury cause autistic disorders?

In considering the scientific method of investigating the causes of a disease, first a scientific hypothesis is put forward, which leads to a model of the cause of the disease. The model is then scientifically tested to see if the data known about the disease fit within the model and its predictions.

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Table. Summary of evidences supporting that Hg exposure during foetal and/or early childhood periods significantly contributes to the development of ASDs

Category	Support
↑*Hg body-burden*	* ↑ Hg in ASD baby teeth. * ↑ Hg-associated urinary porphyrins in ASDs. * ↑ or ↓ Hg in ASD hair samples. * ↑ Hg in urine & faecal samples in ASDs. * ↑ Hg in the brain of ASDs. * ↑ Hg in the blood of ASDs.
Biochemical susceptibility factors	* Abnormalities in transsulfuration metabolites among ASDs associated with ↑ Hg toxicity. (*e.g.* ↓ reduced glutathione, ↑ oxidized glutathione, ↓ cysteine, ↓ sulphate, *etc.*) * ↓ Abnormalities in antioxidant enzymes associated with ↑ Hg toxicity. (e.g. ↑ or ↓ GPx, ↑ or ↓ SOD, etc.)
Pre-existing genomic susceptibility factors	* Increased genomic susceptibility factors in Hg-excretion pathways among ASDs. (*e.g.* MTHFR, GSTM1, GSTP1, *etc.*)
Similarity of ASD & Hg poisoning symptoms/markers	* Hg-associated oxidative stress markers among ASDs. (↑ Lipid peroxidation, ↑ Nitrotyrosine, ↑ Lipofuscin, ↑ Isoprostane, *etc.*)
Male/female ratio	* Testosterone co-exposure ↑ Hg poisoning, whereas estradiol co-exposure ↓ Hg poisoning. * More males than females diagnosed with ASDs, and have ↑ testosterone & ↓ estradiol.
Epidemiological studies	* Thimerosal-containing vaccines & biologics linked to ASDs. * Environmental Hg linked to ASDs.
Animal models	* Low-dose Hg exposure (organic or inorganic) results in long-term bound Hg^{2+} in the infant monkey brain & is associated with neuroinflammatory pathology found in ASD brains. * Low-dose thimerosal exposure linked to ASD symptoms & brain pathology in a susceptible (*i.e.* ↑ autoimmunity & ↓ antioxidant capacity) mouse model. * Low-dose methyl-Hg exposure linked to ASD symptoms & brain pathology in rat models.
Cellular susceptibility to Hg toxicity	* Cultured cells of ASDs have show significant dose- and time-dependent greater susceptibility to Hg-induced cellular toxicity than unaffected controls. * Cultured neuronal cells exposed to low-dose Hg have similar pathology as ASD brains.
Temporal association	* Symptoms of ASDs emerge within the first several years of life, Hg exposure (*i.e.* foetal & early infancy) proceeds development of ASD symptoms.

ASDs, autism spectrum disorders; GPx, glutathione peroxidase; MTHFR, methylenetetrahydrofolate reductase; GSTM1, glutathione *S*-transferase M1; GSTP1, glutathione *S*-transferase P1; NA, not available; SOD, superoxide dismutase

The present review was undertaken to evaluate the evidence supporting the hypothesis that at least some ASDs are caused or significantly exacerbated by Hg exposure from medicinal (including vaccinal), environmental, dental and/or dietary Hg, during the foetal and/or the early childhood period. This model further posits that the Hg toxicity is far more likely to result in autism in individuals who have a pre-existing susceptibility to Hg intoxication. The Table summarizes the evidence reviewed in the present study which supports the theory that Hg exposure during foetal and/or early childhood periods significantly contributes to the development of ASDs. The overwhelming preponderance of the evidence from the peer-reviewed scientific and medical literature favours acceptance that medicinal, dental and environmental Hg exposures are capable of causing the set of symptoms commonly used to diagnose autism, particularly in children who are biochemically and/or genomically susceptible to Hg intoxication.

Potential conflicts of interest: David A. Geier has been a consultant in vaccine/biologic cases before the no-fault National Vaccine Injury Compensation Program (NVICP) and in civil litigation. Mark R. Geier has been a consultant and expert witness in vaccine/biologic cases before the no-fault NVICP and in civil litigation. Mark and David Geier have a patent pending for the treatment of autistic disorders. Paul G. King has no conflicts of interest. Lisa K. Sykes has been involved in vaccine/biologic cases in civil litigation.

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