

C A Shaw<sup>1</sup>, S Sheth<sup>1</sup>, D Li<sup>1</sup>, L Tomljenovic<sup>1</sup>

### Abstract Introduction

Causes & Prevalence

Thus far, most of the research on both neurodevelopmental and

neurodegenerative disorders has been focused on finding the presumed underlying genetic causes, while much less emphasis has been put on potential environmental factors. While some forms of autism are clearly genetic, the fact remains that heritability factors cannot adequately explain all reported cases nor their drastic increase over the last few decades. In particular, studies on twins have now shown that common environmental factors account for 55% of their risk for developing autism while genetic susceptibility explains only 37% of cases. Because the prenatal environment and early postnatal environment are shared between twins and because overt symptoms of autism emerge around the end of the first year of life, it is likely that at least some of the environmental factors contributing to the risk of autism exert their deleterious neurodevelopmental effect during this early period of life. Indeed, evidence has now emerged showing that autism may in part result from early-life immune insults induced by environmental xenobiotics. One of the most common xenobiotic with immuno-stimulating as well as neurotoxic properties to which infants under two years of age are routinely exposed worldwide is the aluminum (Al) vaccine adjuvant. this review we discuss the In

mechanisms by which Al can induce adverse neurological and immunological effects and how these may provide important clues of Al's putative role in autism. Because of the tight connection between the development of the immune and the central nervous system, the possibility that immune-overstimulation in early infancy via vaccinations may play a role in neurobehavioural disorders needs to be carefully considered.

#### Conclusion

There is now sufficient evidence from both human and animal studies showing that cumulative exposure to aluminium adjuvants is not as benign as previously assumed. Given that vaccines are the only medical intervention that we attempt to deliver to every living human on earth and that by far the largest target population for vaccination are healthy children, a better appreciation and understanding of vaccine adjuvant risks appears warranted.

## **Introduction**

The etiologies proposed for the spectrum of neurological disorders falling under the umbrella of autism spectrum disorders (ASD) shows the same range as those proposed for the well-known neurological disorders associated with aging. The latter typically include Alzheimer's disease. Parkinson's disease, and Lou Gerhig's disease (also known as amyotrophic lateral sclerosis or ALS). The widespread, but incorrect, view regarding all of these diseases is that most cases arise from genetic mutations or polymorphisms. In reality, the large majority of these disorders that are not familial have no obvious genetic mutations associated with either the onset or progression of the disorder<sup>1,2</sup> and fall into a category known as "sporadic". Moreover, an apparent increase in both prevalence and incidence of these disorders over a relatively short time span (i.e.,

several decades) rules out a purely genetic origin.

In ALS, for example, much of the literature of the last 20 years has focused sequentially on the search for genetic etiologies leading to motor neuron loss and the list of defective genes has grown larger over this time period. These include a number of variations on the so-called "toxic gain of function" mutation in the gene coding for the antioxidant enzyme superoxide dismutase (SOD)<sup>3</sup>. Additional mutations affect the genes coding for DNA binding protein TDP-43, FUS, or VEGF, or the newest player, C9orf72, and all have added to the complexity of the polygenic picture without necessarily increasing the total percentage of all ALS cases that are clearly gene mutation-derived. Overall, the various mutations may account for about 10% of all ALS and mutant SOD may comprise about 25% of this or 2.5% of the total number of ALS cases<sup>4,5</sup>.

In regard to Alzheimer's disease which remains one of the world's most burdensome and disabling health issues (affecting 24.3 million people with more than 4.5 million new cases/year<sup>6</sup>), only a very small percentage are familial with early onset of symptoms, (<65 years). In contrast, >95% are idiopathic (late onset, >65 years<sup>7</sup>) and most likely due to factors other than genetics<sup>8</sup>.

Thus the literature seems clear that the bulk of neurological disorders arise due to environmental factors, most still not identified (reviewed in<sup>1</sup>). None of this diminishes the putative role of susceptibility genes, also mostly unknown, whose interactions with the various environmental toxicants are highly likely to be crucially involved. Keeping with this, experimentally it has proven possible to develop toxin-based

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<sup>\*</sup>Corresponding author

Email: cashawlab@gmail.com

<sup>&</sup>lt;sup>1</sup> University of British Columbia, Vancouver, British Columbia, Canada



animal models of ALS in outbred mice with no gene mutations and thus model the much higher fraction of ALS which is sporadic<sup>1</sup>. A very similar set of outcomes has been noted in the Parkinson's disease literature. including the ability to generate Parkinsonian features in outbred rats using various toxins<sup>9,10</sup>. The bottom line is that apart from some neurological disorders such as Huntington's disease<sup>11</sup>, most cases of the various disorders do not arise from an obvious genetic mutation or polymorphism.

In spite of these observations, the dominance of a genetic causality perspective for the above disorders remains relative firmly in place. There is insufficient space in the present review to go into the various scientific and sociological reasons why this may be so (see1), but it is important to recognize that much the same perspective dominates the ASD field, a perspective that is likely to be as fundamentally incorrect as it is for ALS and the other age-related neurological disorders. Indeed, the increase in the prevalence of autism has followed an even more dramatic upward curve than that of Alzheimer's and is equally if not more so unlikely to be due to a change in population genetics. The increase in ASD began after the early 1980s. Prior to this, the prevalence of autism was relatively low and relatively stable (<5 in 10,000 children<sup>12,13</sup>).

In 2011 to 2012 the U.S. Department of Health and Human Services and the U.S. Centers for Disease Control and Prevention (CDC), reported that 1 in 50 U.S. children aged 6 to 17 had been diagnosed with autism (200 per 10,000)<sup>14</sup>. This prevalence estimate was significantly higher than the estimate for children in the same age group reported in 2007 (1 in 88)15, representing a 72% increase since 2007 alone. In the United Kingdom, current reported autism prevalence is 1 in 64 children (157 per 10,000)<sup>16</sup>. It should be obvious that the cumulative 3040 to 3900% increase in ASD prevalence in two Western countries

since the 1980s cannot be convincingly explained by genetic factors alone, nor by changes in diagnostic criteria<sup>17,18</sup>, the latter often given as the reason<sup>14</sup>. Consistent with this, in a recent analysis comparing the prevalence of autism with that of other disabilities among successive birth cohorts of U.S. school-aged children, Newschaffer et al.19 showed that autism prevalence has been increasing with time, as evidenced by higher prevalences among younger birth cohorts.

With the above as a general introduction, the present article will attempt to address the issue of ASD etiology, putting into perspective the likely roles of genes versus environment in the disorder and the interactions between the two.

### **Discussion**

The authors have referenced some of their own studies in this review. These referenced studies have heen conducted in accordance with the Declaration of Helsinki (1964) and the protocols of these studies have been approved by the relevant ethics committees related to the institution in which they were performed. All human subjects, in these referenced studies, gave informed consent to participate in these studies. Animal care was also in accordance with the institution guidelines.

#### Autism features and central nervous system (CNS) abnormalities

Autism and related disorders of the autism spectrum (i.e., Asperger's syndrome, pervasive developmental disorder not otherwise specified, Rett syndrome etc.) are neurodevelopmental disorders characterized dysfunctional bv function, stereotypic immune behaviour and various degrees of impairments in social skills and verbal communication (i.e., language delays). Other neurological and medical conditions frequently co-occur with autism, including mental retardation (30% of cases score mild to moderate,

and 40% score serious to profound retardation)<sup>20</sup> and epilepsy (40% of cases)<sup>21</sup>. Co-morbid behavioural and psychiatric conditions associated with the core symptoms include aggression, disruption, hyperactivity, self-injury, abnormalities, sensorv anxiety, depression and sleeping disturbances. The most frequent non-neurological comorbidities associated with ASD are gastrointestinal abnormalities and underlying inflammation<sup>22,23,24,25,26,27,28</sup>, difficulties feeding and food sensitivities<sup>29</sup>. Autistic symptoms normally appear before 36 months of age, and regression or loss of skills occurs in 30% of affected children, usually between 18 and 24 months<sup>30</sup>.

Abnormal neural connectivity is one of the key pathological features of the autistic brain. The term connectivity encompasses local connectivity within neural assemblies and long-range connectivity between brain regions. Similarly, there is also physical connectivity ("hard-wiring"), associated with synapses and tracts and functional connectivity ("soft-wiring"), which is Conflict of interests: None declared. associated with neurotransmission<sup>31</sup>. Physically, in the autistic brain, high local connectivity may develop in tandem with low long-range connectivity<sup>32</sup>, potentially as a result of widespread alterations in synapse elimination and/or formation and or changes in inhibitory/excitatory synaptic ratios<sup>31</sup>.

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There is now also abundant evidence supporting the notion that abnormal activity of immune signalling in the brain interferes with the establishment of appropriate neuronal circuitry during development, thus contributing to the emergence of autistic phenotypes <sup>33,34,35</sup>. For example, mice deficient in MHC class I signalling and the classical complement cascade (C1q and C3) exhibit defects in synaptic pruning in specific areas of the brain as well as activity<sup>36,37</sup>. enhanced epileptic Cerebellar Purkinje cells, which are significantly reduced in autism, are a site of prominent MHC class I expression and one hypothesis currently under investigation is that specifically timed changes in neuronal

**Review** 

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class I expression MHC could contribute to autism<sup>31</sup>. Notably. neuropathological examinations on autistic brains by Vargas et al.38 showed evidence of an active neuroinflammatory process in the cerebral cortex and the cerebellum with extensive loss of cerebellar Purkinje cells. In particular, marked reactivity of the Bergmann's astroglia in areas of Purkinje cell loss within the Purkinje cell laver, as well as marked astroglial reactions in the granule cell laver and cerebellar white matter were detected.

In the middle frontal gyrus and the anterior cingulate gyrus, astroglial reactions were prominent in the subcortical white matter, and in some cases panlaminar astrogliosis was observed. Moreover, cvtokine profiling indicated that macrophage chemoattractant protein (MCP)-1 and tumour growth factor-β1, derived from neuroglia, were the most prevalent cytokines in autistic compared to control brain tissues. The cerebrospinal fluid derived from autistic patients likewise showed a unique proinflammatory profile of cytokines, including a marked increase in MCP-1<sup>38</sup>.

Altogether these observations suggest that autistic brain is a result of a disease process that arises from altered activity of immune-related pathways in the brain. Other evidence in support of this notion is the frequent finding of autoimmune particularly those manifestations, affecting the CNS, in autistic individuals which do not appear to be limited to only a few nervous system antigens. For example, Voidani et al.<sup>39</sup> demonstrated elevated levels of immunoglobulins (Ig)G, IgM and IgA against nine different neuron-specific antigens in ASD children. Additionally, significantly more autistic children show serum autoantibodies to human brain (espe-cially the cerebellum and cingulate gyrus), compared to their unaffected control siblings (p < 0.01)<sup>40</sup>. The frequent findings of autoantibodies against neuronal antigens in autistic individuals has led

many researchers to conclude that the blood-brain barrier (BBB) is breached in autism. Indeed, such widespread manifestations of **CNS-related** autoimmunity may have arisen from BBB disruption which would then enabled access have of immunocompetent cells to many different CNS antigens<sup>39</sup>.

Autoantibodies against foetal brain proteins have also been detected in mothers of ASD children, suggesting a disruption of BBB in utero<sup>41</sup>. It is important to emphasize that an in utero initiation of ASD does not imply a genetic etiology. Although it is currently not known how the BBB may become disrupted in autism, perinatal stressors and exposure to BBB-altering environmental stressors including toxic metals (i.e., lead (Pb), mercury (Hg) and aluminum (Al)), polychlorinated biphenyls (PCBs) have been implicated as potential triggering factors<sup>33,39,42,43,44</sup>.

Of note, neuronal accumulation of immunoglobulins (IgGs) and CNS autoantibodies have also been detected in Alzheimer's and disease45,46,47. Parkinson's Additionally, most recent studies show that the relevance of circulating anti-NMDA receptor autoantibodies in neuropsychiatric disease patients depends on BBB integrity<sup>48</sup>. In particular, seropositive schizophrenic with patients history а of neurotrauma or birth complications (indicating at least temporarily compromised BBB), had more neurological abnormalities than patients with seronegative comparable history<sup>48</sup>. The above observations further suggest that a common immune-mediated mechanism underlies hoth neurodevelopmetal and neurodegenerative disorders.

Immune abnormalities in ASD are not confined to the nervous system. Indeed, a large body of data points to a role of systemic immune system dysregulation in the pathophysiology of ASD which is likely to precede the inflammatory and autoimmune

manifestations in the brain<sup>44,49,50,51</sup>. Concurrent with aberrant cytokine profiles, various studies have shown abnormal levels of blood lymphocytes. incomplete or partial activation of Tcells following stimulation, as well as lower levels and decreased activity of circulating natural killer (NK) cells in ASD22,52,53.

Given the above, it has been proposed that the widespread manifestations of immune abnormalities in ASD stems from deleterious effects of immune insults that occur during a narrow window of postnatal development which is characterized by extensive shaping of both the CNS and the immune system<sup>33,49,50</sup>.

### Evaluating the evidence for a genetic etiology in autism

Efforts to understand the etiology of ASD as a genetically-based disorder have been largely centred on three approaches: 1) whole genome scanning chromosomal predicting the localization of the disease by scanning families with more than one affected member, especially twin studies<sup>54</sup>; 2) cytogenetic and molecular studies which have sought de novo chromosomal anomalies and inherited mutations, including gene copy number variations; and 3) candidate genes studies which examine the relationship between those genes known to be associated with abnormal brain development and phenotype of the disease. Using these approaches, chromosomes 2q21-33, 3q25-27, 3p25, 4q32, 6q14-21, 7q22, 7q31-36, 11p12-13, 17q11-21 have shown to some linkage ASD<sup>55</sup>. have to Duplications, inherited maternally, on the 15q11-q13 region of chromosome declared 15 have been particularly associated<sup>56</sup>. Deletions on chromosome 16p11 have also shown to be associated with ASD. retardation mental and other developmental disabilities<sup>57</sup>. Replicated copy number variations from genome wide studies are located on the following chromosomes: 1q21, 2p16.3 (NRXN1), 3p25-26 (CNTN4), 7q36.2 (DPP6), 15q11-13 (UBE3A, OR4M2, OR4N4); 16p11.2 (MAPK3, MAZ. DOC2A, SEZ6L2, HIRIP3, IL6);

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22q11.2<sup>55</sup>. However, some of these are also shown to be more frequently present in patients with schizophrenia and mental retardation than controls. thus raising questions about specificity to ASD<sup>55</sup>. Several studies have demonstrated that ASD reflects disorders heterogeneous whose etiology is linked with several rare monogenetic disorders such as fragile X syndrome, mutations in TSC1/TSC2, LAMB1, CNTNAP2, PTEN, DHCR7, SHANK3, NLGN3/4, or RPL1055. Synaptic cell adhesion and associated molecules, including neurexin 1, neuroligin 3 and 4 and SHANK3, glutamatergic indicate which abnormalities in ASD have also been cited<sup>58</sup>.

А kev problem with genetic investigations into ASD origins has been the lack of reproducibility and overlap in genetic linkage studies. Although to date 10 full genome screens have been reported<sup>59,60,61</sup> and have indeed identified numerous regions of suggestive linkage, only a small subset of these overlap across studies. Similarly, although more than 100 candidate ASD genes have been studied<sup>61</sup>, there is no consistent replication of positive results.

What we can conclude from the above is that while there are some genetic associations, it is quite clear that the mode of inheritance of autism is not Mendelian but rather must reflect a polygenic, multifactorial etiology with multiple gene-gene and/or geneenvironment interactions<sup>62,63</sup>. Indeed, rapidly accumulating evidence appears to support a model of autism as a multisystem disorder with genetic influence. environmental contributors, and a distinct immune component38,42,44,64. Studies of animal models have suggested that genetic variations in ASD, rather than being causal to the disorder, instead confer an altered vulnerability to exposure to environmental stressors<sup>65</sup>. In this regard, an epidemiological study on ASD that included a comparison amongst siblings suggested that individuals with ASD may react with

less tolerance to the same environmental stressors<sup>66</sup>.

One of the reasons why autism is considered to be a clear example of a neurodevelopmental heritable disorder, is the large difference in rates concordance between monozygotic and dizygotic twins. In particular, three studies67,68,69 of twins ascertained from clinical samples with a total of 36 monozygotic pairs (concordance rate of 72%) and 30 dizygotic pairs (concordance rate of 0%) have estimated the heritability of autism, or proportion of liability attributable to genetic factors, at about 90%. However, a recent study on identical and fraternal twin pairs with autism by Hallmayer et al.70 showed that genetic susceptibility to ASD was lower than estimates from prior twin studies. In particular, environmental factors common to twins accounted for 55% of their risk for developing autism, while genetic heritability explained only 37% of the risk of ASD.

The findings by Hallmayer et al.<sup>70</sup> indicate that the rate of concordance in dizygotic twins may have been seriously underestimated in previous studies and the influence of genetic factors on the susceptibility to develop autism, overestimated. The authors noted that because of the reported high heritability of autism, a major focus of research in autism has been on finding the underlying genetic causes, with less emphasis on environmental potential triggers. Because the prenatal environment and early postnatal environment are shared between twin individuals and because evidence is accumulating that overt symptoms of autism emerge around the end of the first year of life, Hallmayer et al.<sup>70</sup> have concluded that at least some of the environmental factors impacting susceptibility to autism exert their effect during this critical period of life.

Evidence for an environmental etiology in autism

Extensive research has underscored the tight connection between development of the immune system and that of the CNS, thus substantiating the notion that disruption of critical events in immune development may play a role in neurobehavioral disorders including those of the autism spectrum<sup>49,50</sup>. Indeed, early-life immune insults (both peri- and post-natal) have been shown to produce long-lasting, highly abnormal cognitive and behavioural responses, including increased fear and anxiety, impaired social interactions, deficits in object recognition memory and sensorimotor gating deficits<sup>33,71,72,73,74,75,76,77</sup>.

These symptoms are typical of ASD and results from the heightened vulnerability of the developing immune system to disruption by immuno-modulating environmental pollutants<sup>49</sup>.

Neuroinflammatory processes and immune dysfunction associated with autism can result following early-life exposure to various xenobiotics (i.e., bisphenol A, PCBs, Pb, Hg and Al<sup>33,49,50,75,78</sup>). Of the later, although Hg and Al in particular can come from various sources, the one common source to which infants and pregnant women are universally exposed is through vaccinations. The burden of paediatric vaccines received in the first two years of life (i.e., currently in the U.S. 27 doses of vaccines; Table 1) has been blamed the most by some proponents of environmental etiologies as being the key factor driving the upward trend of autism prevalence worldwide. Hg has historically been used in some vaccines in the form of an ethyl Hg compound, trade marked as Thimerosal, a bacteriostatic agent. It is no longer in widespread use in most Western countries, although it is still routinely in use in the Third World with older vaccine stocks78.

The evidence that Thimerosal may be involved in ASD remains controversial<sup>79</sup>. Certainly, compelling data exist on the capacity of low-dose Thimerosal (in vaccine-relevant exposures) to harm the

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Table 1: 2013 vaccination schedule for preschool children recommended by the U.S. Centers for Disease Control and Prevention<sup>167</sup>. Shaded boxes indicate the age range the vaccine can be given. Asterisks denote Al-adjuvanted vaccines. Hep A is given in 2 doses spaced at least 6 months apart. According to this schedule, by the time a child is two years of age, they would have received 27 vaccinations (3x HepB, 3x Rota, 4x DTaP, 4x Hib, 4x PCV, 3x IPV, 2x Influenza, 1x MMR, 1x Varicella and 2x HepA).

Birth	1 month	2	4	6	12	15	18	19-23	2-3 years	4-6
		months	months	months	months	months	months	months		years
HepB*	HepB*				HepB*					
		Rota	Rota	Rota						
		DTaP*	DTaP*	DTaP*	DT		aP*			DTaP*
		Hib*	Hib*	Hib*	Hib*					
		PCV*	PCV*	PCV*	PCV*					
		IPV	IPV		IPV					IPV
				Influenza (yearly)						
					MI	MR				MMR
					Vari	cella				Varicella
					HepA*					

Abbreviations: Hep A, hepatitis A; Hep B, hepatitis B; Rota, rotavirus; DTaP, diphtheria-pertussis-tetanus; Hib, *Haemophilus influenzae* type b; PCV, pneumococcal; IPV, inactivated polio; MMR, measles-mumps-rubella.

developing nervous system in animal models in a manner consistent with the pathology of autism<sup>72,75,78,80,81</sup>, and because of this, the safety of TCVs appears to stand on uncertain grounds. However, the fact that a significant reduction of Thimerosal from vaccines in use in the Western world implemented in 200182 was not accompanied by a correspondingly dramatic reduction in the reported rate of autism suggests that Hg alone cannot be the main culprit behind the increased autism rates. Nonetheless, it should be noted that Thimerosal was subsequently re-introduced to vaccines administered to pregnant women as well infants of 6 months of age (and then yearly throughout childhood) in the form of multi-dose flu vaccines<sup>83</sup>.

This recommendation to reintroduce Thimerosal at the same time when the U.S. medical authorities recommended its removal from routine childhood vaccines has created a false overall impression that the impact of Thimerosal has been reduced, when in actuality, the administration during the gestational period has increased the potential to damage the developing CNS. In contrast to Hg, Al continues to be used in most paediatric and adult vaccines as an adjuvant, as it has been for almost 90 years (since 1926)<sup>84</sup>. The highly effective and currently indispensable adjuvant properties of Al, make its removal from vaccines problematic since without the various Al salts most vaccines would fail to stimulate the immune system to a sufficient extent to produce acceptable antibody titres<sup>85,86</sup>.

adjuvant-mediated immune-Al's enhancing effect is accomplished via mechanisms that impinge on both the innate and adaptive immune systems<sup>87</sup>. While the potency and toxicity of Al adjuvants should be adequately balanced so that the necessary immune stimulation is achieved with minimal side effects, such balance can be difficult to accomplish in practice. This is because the same mechanisms that drive the immune-stimulatory effects of adjuvants have the capacity to provoke a variety of autoimmune and/or inflammatory adverse reactions<sup>86,88,89,90,91,92,93</sup>. Moreover, as we will demonstrate below, the evidence for a role, direct or indirect, of Al in ASD, perhaps in concert to certain susceptibility genes as cited above, is increasing.

We note that for many in the medical community, the notion that any compound in vaccines might be involved in the changing rates of ASD is sometimes taken as a form of apostasy. It is not our intention in the following to incite this concern, merely to show that Al has the neurotoxic capability and ubiquity to contribute to ASD in humans and that some of the features of the disorder can be demonstrated in animals treated with Al by injection.

# Aluminum as a neurotoxicant and its impact in the CNS

There is now an abundant literature on Al and its ubiquity in the modern "age of aluminum" and widespread use in a variety of materials, as well as in food, water. and various medicinal products<sup>8,17,94,95</sup>. Much of this was suspected as early as 100 years ago and repeatedly confirmed<sup>96</sup>. Indeed, in spite of rather ill-informed views that Al is non-toxic and inert and may even be beneficial for a developing human foetus97, since the seminal work of William Gies' 191198. in epidemiological, clinical and experimental data have clearly identified the CNS as the most sensitive target of Al's toxic effects regardless of mode of exposure (i.e., oral, injectable as adjuvant in vaccines, etc.)<sup>92,94,99,100</sup>. The neurotoxicity of Al typically manifests in learning, memory, concentration and speech deficits, impaired psychomotor control, increased seizure activity, and altered

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behaviour (i.e., confusion, anxiety, repetitive behaviours, and sleep disturbances)8. One hundred years of general ignorance of Gies' concerns and recommendations (that Al should excluded from he human consumption)<sup>98</sup>, has brought us to the need of re-evaluating our increased intake of Al from whatever source. This need is highlighted in burgeoning evidence that links Al to the spectrum of neurological diseases which plague the 21<sup>st</sup> century including Alzheimer's<sup>8</sup>. ALS/Parkinsonism dementia<sup>101</sup>, multiple sclerosis<sup>102,103</sup> and autism spectrum and neurological impairments in children<sup>99,104,105,106</sup>.

The extent of the Al's neurotoxic impacts may depend in large part on the form(s) of Al, the route of administration, and the concentration and duration of exposure. Included in this latter category is the issue of dietary versus injected Al. It should be obvious that the route of exposure bypasses the protective which barriers of the gastrointestinal tract and/or the skin will likely require a much lower dose to produce a toxic outcome. In the case of Al, only ~0.25% of dietary Al is absorbed into systemic circulation<sup>107</sup> and then rapidly filtered by the kidneys in those with mature and patent kidney function. In contrast, Al hydroxide (the most common adjuvant form) injected intramuscularly may be absorbed at nearly 100% efficiency over time<sup>108</sup> and follow a completely different route in the body. Namely, intramuscularly or subcutaneously injected Al (mimicking vaccine exposure) may accumulate in other organs including the spleen and the brain where it is still detected up to 1 post-injection<sup>109</sup>. Notably, year research on human subject suffering from post-vaccination syndromes showed retention of Al adjuvants up to 8 to 10 years following exposure<sup>110,111,112</sup>.

The prolonged hyperactivation of the system and chronic immune inflammation triggered by repeated exposure and unexpectedly long persistence of Al adjuvants in the human body are thought to be the principal factors underlying the toxicity of these compounds. One of the reasons for this long retention of Al adjuvants in bodily compartments including systemic circulation is most likely due to its tight association with the vaccine antigens or other vaccine excipients (i.e., DNA residuals)113,114 which makes such Al complexes resistant to both kidney excretion and enzymatic degradation. Even dietary Al has been shown to accumulate in the CNS over time, producing Alzheimer outcomes in type experimental animals feed equivalent amounts of Al to what humans consume through a typical Western diet<sup>115,116</sup>.

With respect to Al in the vaccine adjuvant form, in the last decade, studies on animal models and humans have indicated that Al adjuvants have an intrinsic ability to inflict adverse neurological and immunoinflammatorv

manifestations<sup>91,92,117,118</sup>. This research culminated in delineation of ASIA-"autoimmune/inflammatory

syndrome induced by adjuvants", which encompasses the wide spectrum of adjuvant-triggered medical conditions characterized by a misregulated immune response<sup>89,119</sup>. Notably, a large portion of adverse experimentallv manifestations triggered by Al in animal models<sup>92,120</sup>, those associated and with administration of adjuvanted vaccines in humans are neurological and neuropsychiatric<sup>121,122</sup>. The ability of Al adjuvants to cross the blood-brain blood-cerebrospinal and fluid barriers<sup>92,109,122,123</sup> may in part explain the reason the adverse manifestations following vaccinations tend to be neurological with an underlying immuno-inflammatory component<sup>121,124,125</sup>.

Thus it appears that Al impacts on the CNS and immune system are not disparate actions but rather are reciprocally linked<sup>17,33,93,105</sup>.

CNS damage has been directly linked to the immunostimulatory properties of Al adjuvants in mice92,126 and

sheep122 where in both cases damage to the motor system was noted at both behavioral and cellular levels. In particular, the "sheep ASIA syndrome" mimics in many aspects human neurological diseases linked to Al adjuvants<sup>122</sup>. The adverse chronic phase of this syndrome affects 50 to 70% of flocks and up to 100% of animals within a flock. It is characterized by severe neurobehavioral outcomes all of which are consistent with Al toxicity (restlessness, compulsive wool biting, generalized weakness, muscle tremors. loss of response to stimuli, ataxia, tetraplegia, stupor, coma and death), inflammatory lesions in the brain and the presence of Al in central nervous svstem tissues122. The main histopathologic change of the chronic phase of the "sheep ASIA syndrome" is located at the spinal cord and consists in multifocal neuronal necrosis and neuron loss in both dorsal and ventral column of the grey matter.

In humans, the best studied condition to adjuvant Al linked is the neuromuscular disorder macrophagic myofasciitis (MMF) syndrome and associated cognitive impairment. MMF is a condition characterized by highly specific myopathological alterations at deltoid muscle biopsy due to long-term persistence of vaccine-derived Al hydroxide nanoparticles within macrophages at the site of previous vaccine injections<sup>110,117,118</sup>. Patients diagnosed with MMF tend to be female Conflict (70%) and middle-aged at time of biopsy (median age 45 years), having received 1 to 17 intramuscular (i.m.) Alcontaining vaccines (mean 5.3) in the 10 years before MMF detection<sup>127</sup>. Clinical manifestations in MMF patients include diffuse myalgia, arthralgia, Competing interests: declared chronic fatigue, muscle weakness and cognitive dysfunction. Overt cognitive alterations affecting memory and attention are manifested in 51% of cases<sup>127</sup>. In addition to chronic fatigue syndrome, 15–20% of patients with MMF concurrently develop an autoimmune disease, the most frequent multiple sclerosis-like being demyelinating disorders, Hashimoto's thyroiditis, and diffuse dysimmune neuromuscular diseases, such as

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dermatomyositis, necrotizing autoimmune myopathy, myasthenia gravis, and inclusion body myositis<sup>127</sup>. Even in the absence of overt autoimmune disease. low titres of various autoantibodies. increased inflammatory biomarkers, and abnormal iron status are commonly detected<sup>128</sup>.

The pathological significance of the MMF lesion has long been illunderstood because of the lack of an obvious link between persistence of Al agglomerates in macrophages at sites of previous vaccination and delayed onset of systemic and neurological manifestations. However, recent studies from these same investigators have demonstrated in experimental animals a clear pathway for injected Al hydroxide from muscle in which the Al particles are transported via the draining lymph nodes by circulating macrophages into the brain<sup>109</sup>. Once there, Al's unusual physical and biophysical properties and its ability to bind to and disrupt normal biochemical reactions render it capable of altering normal signalling at every level of the CNS and also able trigger autoimmune reactions<sup>8,17,33,89,90</sup>.

In spite of the observation that longterm cumulative exposure from Al adjuvants in adults can result in adverse autoimmune and neurological children worldwide outcomes. continue to be exposed to a much greater Al burden from vaccines. While an adult MMF patient may have received up to 17 vaccines in 10 years prior to diagnosis<sup>127</sup>, an average U.S. child would have received the same number of Al-adjuvanted vaccines in their first 18 months of life according to the latest U.S. CDC vaccination schedule (Table 1). Of note, in humans, important aspects of brain development (i.e., synaptogenesis) occur during the first 2 years after birth<sup>129,130</sup>, a period in which the immature brain extremely is vulnerable to neurotoxic and immunotoxic insults49,50,129, and in which children receive the majority of their paediatric vaccinations. Yet another factor that is universally overlooked in the design of routine vaccination schedules is that simultaneous administration of as little as two to three immune adjuvants, or repeated stimulation of the immune system by the same overcome genetic antigen. can resistance to autoimmunity<sup>131,132</sup>. The lax view about the potential toxicity of Al is perhaps best exemplified by its historical and routine use as a placebo in vaccine safety trials<sup>133,134</sup>, a practice that puts in doubt all widely held assumptions about Al safety in vaccines.

### Aluminum as a candidate risk factor in autism

The ability of Al to adversely affect both the immune and the nervous system as described above make it a plausible candidate risk factor for triggering disorders of the autism spectrum. Indeed, the two principal component of ASD are neurological malfunction and immune system dysfunction. Al is also a known BBB toxin<sup>135,136</sup> and there is increasing widespread evidence that the manifestation of **CNS-related** autoimmunity in autistic patients is partially due to disruption of the BBB<sup>33,39,42,43,44</sup>.

The mechanism by which peripheral (systemic) immune stimulation affects responses in the brain is critical to understanding of the potential role of Al adjuvants in neurodevelopmental disorders of the autism spectrum. An important advance in understanding of the function of the normal and the diseased brain was the recognition that there is an extensive communication between the immune system and cells in the CNS137,138. As a result of this neuro-immune crosstalk. neural activity be can dramatically altered in response to a variety of immune stimuli<sup>139,140,141</sup>. Such peripheral immune stimuli lead to de novo production of proinflammatory cytokines within the brain by the activated microglia, the brain's resident immune cells<sup>140,142</sup>. It should be emphasized that repeated activation of once resting microglia

sometimes induces an irreversible shift of these cells to a neurodestructive proinflammatory and excitotoxic phenotype<sup>140,143,144</sup>. That adjuvant Al can induce proinflammatory responses in the brain including a dramatic activation of glial cells has been repeatedly shown in the literature<sup>91,92</sup>. Moreover, the studies by Vargas et al.<sup>38,64</sup> on autistic brain samples suggests that neuroglial activation and neuroinflammation could be critical in initiating and maintaining some of the CNS abnormalities present in autism.

Proinflammatory responses arising from peripheral immune stimuli early in the postnatal period are further detrimental because they result in accumulation proinflammatory of cytokines and excitotoxic levels of the neurotransmitter glutamate within the brain, thus promoting inflammation and disrupting neural development<sup>71,145</sup>. Moreover. such immune stimuli can increase CNS vulnerability to subsequent immune insults and the latter can then permanently impair CNS function<sup>146,147,148,149</sup>. For example, in rodents, peripheral immune stimuli with either bacterial antigens or viral mimetics within the first two postnatal weeks are sufficient to cause deficits in Conflict of interests: social interactions, altered responses to novel situations, anxiety-like behaviours, impairments in memory, long-lasting increase in seizure susceptibility, abnormal immune cytokine profiles and increase glutamate the extracellular in declared in the article. hippocampus<sup>71,73,74,149,150</sup>. All of these abnormalities are to various degrees observed in autistic children<sup>21,29,38,151</sup>.

Repeated administration of bacterial and viral antigens (most of which are adsorbed to Al adjuvants) through present vaccination schedules (Table 1) is clearly analogous both in nature and peripheral timing to immune stimulation with microbial mimetics in experimental animals during early periods of developmental vulnerability of the CNS. If administered during these periods (including early postnatal), such potentiated immune stimuli have the intrinsic capacity to produce

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adverse neurodevelopmental outcomes, and also to permanently immune responses impair subsequent immune challenges later in life<sup>150,152,153</sup>. In spite of these clear paediatric vaccinations analogies, have been historically dismissed as a plausible cause for the growing burden of neurodevelopmental and immune abnormalities in children<sup>154</sup>.

With this background in mind, we undertook an ecological study of ASD incidence in 7 Western countries, including the U.S., in relation to Aladjuvanted vaccine use in each country' recommended paediatric vaccination schedule<sup>105</sup>. The results showed a clear, and statistically significant correlation between the number of Al adjuvants administered (and the estimated total Al body burden) and the rate of ASD over the period examined. Further, the rate, which varied by country, changed in synchrony to the number of Aladjuvanted vaccine in any country's schedule. Recognizing that correlation does not demonstrate causality, we applied Hill's criteria<sup>155</sup> and found that 8 of the 9 criteria could reasonably be satisfied.

Further recognizing that even this was not sufficient, we undertook detailed behavioural studies on newborn male and female outbred mice given an "equivalent" to high and low exposure to Al from vaccines (according to the U.S. and Scandinavian vaccination schedules respectively)<sup>77</sup>. The results of the light/dark box test showed that Al injections in the neonatal period significantly increased anxiety-like behaviours and reduced exploratory activities in mice when they were tested as adults approximately 4 months later. These adverse behavioural outcomes were longlasting and persisted throughout the two month period of testing. In particular, mice of both sexes injected according to the "high Al" schedule showed a highly significant increase in anxiety (p=0.0001 males; p<0.0001 females) and a highly significant reduction in exploratory activities

(p<0.0001 males; p<0.0001 females) compared to saline controls.

Females however were more severely affected, showing significant increase in anxiety even at "low Al" exposure (p<0.034). In addition, males but not females receiving "high Al" were significantly more lethargic and less active in the open field test than control males or those on the "low Al" schedule (p<0.0001). In particular, the young male CD-1 mice exposed to high doses of Al adjuvant travelled shorter distances (p<0.0001), spent significantly less time moving (p<0.0001) and moved more slowly (p<0.0001) than the control animals. These mice also showed reduced rearing frequency compared to controls (p<0.0004). Overall, the adverse effects of high Al adjuvant exposure on locomotor activities in male mice were also long-lasting and persisted throughout the period of testing.

The various behavioural outcomes noted, and the differences between male and female mice treated with Al point to sex difference in sensitivity to neurotoxic/neurodisruptive action of Al. For example, while locomotor activity seemed to be disrupted in males treated with "high Al", in females under same treatment no impairments were observed. Of note, Olczak et al.<sup>72</sup> while investigating the neurotoxic potential of Thimerosal in vaccine relevant exposures in young adult Wistar rats, reported similar outcomes in locomotor activity. Namely, male rats were more sensitive to Thimerosal disruption in the locomotor parameters measured in the open field while the anxiety parameters were altered in both sexes even at the lowest doses of Thimerosal, a result which may reflect the higher intrinsic acute neurotoxic potential of Hg when compared to Al<sup>156</sup>.

At least two other studies in the current literature support the role of neurological Al in and neurodevelopmental disorders. Seneff al.'s<sup>106</sup> recent comprehensive et

analysis of the data from the U.S. Vaccine Adverse Event Reporting System (VAERS) showed that reports of autism in VAERS increased steadily at the end of the last century during a period when Hg was being phased out of U.S. vaccines, while Al adjuvant burden was being increased.

Using standard log-likelihood ratio techniques, Seneff et al.<sup>106</sup> also identified several signs and symptoms that were significantly more prevalent in vaccine reports after 2000, including cellulitis, seizure, depression, fatigue, pain and death, which were also associated with significantly A1containing vaccines. Finally, Melendez et al.<sup>104</sup> found that autistic patients have higher than normal blood serum levels of Al and other toxic metals (Cr and As) while having lower levels of essential metals including Zn, Cu and Mg. Al is well known to displace essential metals from bio-enzymes, especially Mg and this property of Al is linked to its role in triggering neurodegenerative disorders 8. In the study by Melendez et al.<sup>104</sup> several important factors regarding exposure to toxic metals were identified: in 80% of the cases the autistic children had used or made use of controlled drugs and 90% of them had taken all recommended vaccines. In addition, 70% of mothers of ASD children received vaccines and 80% of them ate canned food and fish during pregnancy.

## Aluminum alters gene expression

Conflict of interests: None declared. The ability of Al to bind to DNA and article. change gene expression patterns has been established by Lukiw et al.<sup>157,158</sup>. At nanomolar concentrations, Al Competing interests: declared in the inhibits brain-specific gene transcription from selected AT-rich promoters of human neocortical genes<sup>158</sup>. Al's repressive action on gene transcription is linked to its ability to 1) decrease the access of transcriptional machinery to initiation sites on DNA template by enhancing chromatin condensation<sup>157,159</sup>; or 2) interfere with ATP-hydrolysis-powered separation of DNA strands either indirectly (by binding to phosphonucleotides and increasing the stability and melting temperature of DNA)158,160 or directly

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(by inhibiting the ATPase-dependent action of RNA polymerase)<sup>158</sup>. These effects were experimentally physiologicallydemonstrated at relevant Al concentrations (10-100 nm)<sup>158,161</sup> and at levels that have been in Alzheimer disease reported chromatin fractions<sup>162</sup>. patients' Furthermore, Al content expressed per gram of DNA was found to be significantly increased in nuclear and heterochromatin fractions in presenile Alzheimer's disease patients when compared to age-matched controls<sup>163</sup>.

It is particularly interesting to note that in spite of its overall repressive action on some gene expression, Al can also promote transcription. Al promotes lipid peroxidation and oxidative stress and in this way the **ROS-sensitive** activates transcription factors, hypoxia inducible factor-1 (HIF-1) and nuclear factor (NF)-kB and augments specific neuroinflammatory and pro-apoptotic signalling cascades by driving the expression from a subset of HIF-1 and NF- $\kappa$ B - inducible promoters<sup>164,165</sup>.

Out of eight induced genes upregulated in cultured human neurons by 100 nm Al sulphate (the same compound that is used as a flocculant in water), seven showed expression patterns similar to those observed in Alzheimer's, including HIF-1/NF-ĸBresponsive A<sub>β</sub>PP, interleukin-1<sub>β</sub> (IL-1β) precursor, NF-κB subunits, cytosolic phospholipase A2 (cPLA2), cyclooxygenase (COX)-2 and DAXX, a regulatory protein known to induce and apoptosis repress transcription<sup>165</sup>. Both HIF-1 and NFκB are up-regulated in Alzheimer's disease where thev fuel the proinflammatory cycle which leads to further exacerbation of oxidative stress and inflammation, culminating in neuronal death<sup>2</sup>.

In light of the above data, we selected 18 candidate genes which have implied functions in both ASD and macrophage-initiated innate immune response<sup>166</sup>. We measured the expression levels of these 18 genes

using semi-quantitative RT-PCR in brain samples from 3 male control and 3 Al-injected mice from the study cited above<sup>77</sup>. In total 7 genes showed changes in expression. Some of the activators and effectors of immunoinflammatory response were significantly up-regulated, including interferon gamma (IFNG), tumour necrosis factor (TNF), chemokine CCL2 and lymphotoxin beta (LTB). while the inhibitors of immune reaction NF-κBIB (inhibitor of NF-κB), complement component C2 and a gene controlling the regulation of the degradative enzyme for the neurotransmitter acetylcholine (acetylcholinesterase, ACHE), were significantly down-regulated (Figure 1A & Figure 1B). In 5 out of these 7 genes, the analysis of the corresponding protein levels showed significant changes in expression: IFNG, TNF and CCL2 were upregulated while NF-kBIB and ACHE were down-regulated (Figure 1C & Figure 1D).

Although it is still premature to make definitive conclusions given the still small sample size (the data are currently being collected on other samples), these results suggest that an immuno-inflammatory response was activated and the neural activity decreased by Al injection. Moreover, our results are in agreement with Lukiw et al.<sup>165</sup> who demonstrated upregulation of NF-κB responsive and proinflammatory genes by nanomolar Al.

Altogether, the gene expression studies following Al treatment point to a greater complexity than perhaps previously anticipated: not only can Al evoke direct neural damage and trigger activation of adverse immunemediated signals, but it can also directly influences gene expression, thus triggering more complex interactions between genes and toxins. Insofar as the latter may be correct, it will be highly important in the future to determine where in the lifespan can Al impact gene expression and how long such changes might last.

## **Conclusion**

It should be clear by now that the etiology of ASD is not a simple process involving only genetic factors, but rather involves a multiple "hit" etiology. This is not particularly surprising given the existing literature on neurodegenerative disorders associated with aging, e.g., Alzheimer's disease, Parkinson disease, and ALS have reached much the same conclusions. Based on the likelihood of gene-environment interaction, we propose that some combination of genetic predispositions can sensitize the developing CNS of some individuals to a secondary toxic insult. That second insult could conceivably be that from Al from various sources including paediatric vaccines, although clearly other toxicants may also be involved.

Al salts are the most widely used adjuvants in current use. The fact that thev can trigger pathological immunological responses and a cascade of adverse health effects is now well documented, albeit still not widely recognized in the medical community. As detailed in this article, the risks associated vaccine-derived Al are fourfold. First, Al can persist in the body; second, Al can trigger pathological immunological responses; third, Al can Conflict of interests: make its way into the CNS where it can activate deleterious immunoinflammatory and excitotoxic fourth, processes; Al can alter expression of numerous genes involved in the immuno-inflammatory responses and cell-to-cell signaling.

Further, given that a strong adjuvant effect can overcome even genetic resistance to autoimmunity, it is likely that an increasing number of individuals, regardless of their genetic background, will react adversely if exposures to compounds with immune Competing interests: adjuvant properties exceed a certain threshold. Prior genetic susceptibilities may in such cases only determine the degree of severity of manifestations of the disease spectrum, rather than being the principal driving factor in their increase. The fact that as many as 2% of 6 to17 year olds in the U.S. currently

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show some form of autism (representing over a 70% increase since 2007), argues in favour of this hypothesis. No genetic susceptibility can account for such a dramatic increase in such a short time span as genes in a population are highly unlikely to change that rapidly. Notably, studies on twins have now

shown that common environmental factors account for 55% of their risk for developing autism while genetic susceptibility explains only 37% of cases. Because the prenatal environment and early postnatal environment are shared between twins and because overt symptoms of autism emerge around the end of the

first year of life, it is reasonable that at least some of the environmental factors contributing to the risk of autism exert their deleterious neurodevelopmental effect during this early period of life. Indeed, important aspects of human brain development take place during the first two postnatal years, when the immature brain is extremely vulnerable



**Figure 1:** A: The effect of Al on the expression of 18 autism-related genes in the brains of Al-injected (n=3) and control (Con; n=3) male CD-1 mice.  $\beta$ -actin was used as the internal standard. B: Quantification of the expression change shown in A. The expression levels of 7 autism- and innate immunity-related genes were significantly altered in Al-injected mouse brains compared to saline control (Con) as measured by semi-quantitative RT-PCR analyses. Data are presented as fold difference as compared to controls. Histograms report the mean ±SEM of three independent experiments determined by densitometry. \*\*p < 0.01, \*p < 0.05. C: The protein levels of the 7 genes with altered expression levels after aluminum injection were verified by western blots.  $\beta$ -actin was used as internal standard. D: Quantification of the protein level change shown in C. Data are shown as mean signal intensity ± SEM of three independent experiments. \*\*p < 0.01, \*p < 0.05. Abbreviations: ACHE, acetylcholinesterase; C2, complement component 2; CCL2, chemokine (C-C motif) ligand 2; CEBPC, CCAAT/enhancer binding protein beta; CRP, C-reactive protein; IFNG, interferon gamma, KLK1, kallikrein; LTB, lymphotoxin B; MMP9, matrix metalloproteinase 9; NFKBIB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor beta; NFKBIE, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor epsilon; PPARG, peroxisome proliferator-activated receptor gamma; SELE, selectin E; SERPINE1, serpin peptidase inhibitor, clade E member 1; SFTPB, surfactant protein B; STAT4, signal transducer and activator of transcription 4; TNF (TNF-alpha), tumor necrosis factor.

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to neurotoxic an d immunotoxic insults. This is also a period during children worldwide which are routinely exposed to the majority of Al-adjuvanted vaccines. Al is both a neurotoxin and an immunotoxin and there is now sufficient evidence from both human and animal studies that cumulative exposure to this adjuvant is not as benign as previously assumed. Because infants represent the most vulnerable population that is universally and routinely exposed to Al adjuvants, a more rigorous evaluation of its potentially adverse neurodevelopmental impacts is needed.

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None declared.

#### **Competing interests**

C.A. Shaw is a founder and shareholder of Neurodyn Corporation, Inc. The company investigates early-state neurological disease mechanisms and biomarkers. This work and any views expressed within it are solely those of the authors and not of any affil.

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