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High-Efficacy Treatment Protocols for Autism Spectrum Disorders with Novel Targeted Therapeutic Modalities of Regenerative Medicine

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ABSTRACT

The article examines high-efficacy treatment protocols for Autism Spectrum Disorders (ASD), focusing on novel targeted therapeutic modalities within the realm of regenerative medicine. We reviewed and analyzed the implementation of advanced regenerative medicine techniques in treating ASD, emphasizing the integration of novel therapeutic modalities with biologicals targeting main pathological pathwaysof the autism. Authors discuss cellular therapies, neuropeptide interventions, therapy with precursors of neuromediators et cetera. Clinical outcomes were assessed based on improvements in behavioral, cognitive, and social functions. Patients on autism spectrum that receivedholistic biological therapy demonstrated rapid gains in self-regulation and appropriate behavior across various settings within the first weeks of the treatment. After 2-3 months, notable advancements were observed in eye contact, attention span, verbal responsiveness, and speech development. Six-month follow-ups revealed enhanced communication fluency and greater self-management. The subsequent addition of neuropeptides during the maintenance phase led to significant reductions in anxiety, aggression, and improved social skills and communication abilities. Long-term therapy showed sustained improvements in conversational ability, reduced repetitive behaviors, and enhanced social and emotional reciprocity. The integration of novel therapies with biological and neutraceuticals offers a promising approach to managing ASD, showing significant improvements in behavioral and cognitive outcomes. These findings support the potential of regenerative medicine modalities in enhancing therapeutic efficacy for ASD and warrant further investigation to optimize treatment protocols and validate long-term benefits.

Keywords

Autism, Autism spectrum disorders, Stem cells, Regenerative medicine, Neurodevelopmental disorders, Neuropeptides, Behavioral therapy, Cognitive development, Social skills.

Introduction

Autism spectrum disorders (ASD) are a diverse group of neurodevelopmental disorders, characterized by some degree of difficulty in social interaction and communication, atypical patterns of activities and behaviours, such as difficulty with transition from one activity to another, and focus on details and unusual reactions to sensations. Often diagnosed in early childhood, ASD often produces a negative impact on education and employment opportunities, and demands families and caretakers to provideadequate necessary care and support to the autistic individual. Generally characterized by failure of a various degree of the affected person to communicate and interact socially with others, ASD individuals usually demonstrate restricted, repetitive, and stereotyped behavioural patterns [1].

According to the WHO the prevalence of ASD worldwide is 1 in 100 children [2]. Most of the studies show a dramatic increase in ASD prevalence since the late 1900s and early 2000s. According to data from the Autism and Developmental Disabilities Monitoring (ADDM) Network, established by the Centers for Disease Control and Prevention (CDC) in USA, the prevalence of ASD among 8-year-old children in the United States has increased from 67 per 10,000 in 2000 to 90 per 10,000 in 2006 and 145 per 10,000 in 2012 [3-5]. The latest report from 2018 indicates that approximately 230 per 10,000 children are affected by ASD, reflecting a more than

200% increase in prevalence since the initial ADDM Network study in 2000 [6]. A systematic review that analyzed 71 studies and 99 prevalence estimates from 34 countries, covering publications from 2012 to 2021, indicated a median prevalence of 100 per 10,000 children [7]. In contrast, the meta-analysis by Salari et al., which included 74 studies published between 2008 and 2021, reported a pooled prevalence of 60 per 10,000 [8].

Persistent deficits in social communication and social interaction:
Making little or inconsistent eye contact
Tending not to look at or listen to people
Rarely sharing enjoyment of objects or activities by pointing or showing things to others
Failing to, or being slow to, respond to someone calling their name or to other verbal attempts to gain attention
Having difficulties with the back and forth of conversation
Often talking at length about a favorite subject without noticing that others are not interested or without giving others a chance to respond
Having facial expressions, movements, and gestures that do not match what is being said
Having an unusual tone of voice that may sound sing-song or flat and robot-like
Having trouble understanding another person's point of view or being unable to predict or understand other people's actions

Table 1: Changes in social communication and social interaction in ASD.

Restricted, repetitive patterns of behavior, interests, or activities:
Repeating certain behaviors or having unusual behaviors. For example, repeating words or phrases, a behavior called *echolalia*
Having a lasting intense interest in certain topics, such as numbers, details, or facts
Having overly focused interests, such as with moving objects or parts of objects
Getting upset by slight changes in a routine
Being more or less sensitive than other people to sensory input, such as light, noise, clothing, or temperature

Table 2: Behavioral changes, restricted and repetitive behaviors in ASD.

In spite of the high prevalence of the ASD, there are no standardized and success-guaranteed treatment strategies for this conditions. According to the CDC, the current treatment options include behavioural, developmental, educational, psychological, social-relational, pharmacological, and complementary and alternative modalities. Developmental approaches aim to enhance specific developmental skills, such as language or motor skills, or a broader spectrum of interconnected developmental abilities. These approaches are frequently integrated with behavioral strategies. Among developmental therapies for individuals with ASD, speech and language therapy is the most prevalent. This therapy focuses on improving communication abilities, including understanding and using speech and language. While some individuals with ASD communicate verbally, others may use signs, gestures, pictures, or electronic communication devices. Occupational therapy is designed to promote independence by teaching essential daily living skills, such as dressing, eating, bathing, and social interactions. It may also include sensory integration therapy to address challenges with sensory input that can be overwhelming or restrictive. Additionally, physical therapy targets the enhancement of physical skills, including fine motor movements of the fingers and larger body movements.

Pharmaceutical approach meant address to co-occurring symptoms that happen along with ASD and can help people with ASD function better. For instance, medication may help manage inability to focus or self-harming behavior, such as head banging or hand biting. Medication can also help manage co-occurring anxiety or depression, in addition to medical conditions such as seizures, sleep problems, or stomach or other gastrointestinal problems.

Complementary and alternative treatments are frequently employed alongside conventional therapies to enhance overall care. These treatments may encompass specialized diets, herbal supplements, chiropractic adjustments, animal-assisted therapy, art therapy, mindfulness practices, or relaxation techniques. It is essential for individuals and families to consult with their healthcare provider before initiating any complementary or alternative treatments. However, in spite of the increasing occurrence of the ASD and high attention to the topic, there are no conventional commonly accepted treatment strategies that could yield any notable beneficial outcomes. In our previous works we have discussed some aspects of etiology, pathogenesis, symptomatology and treatment options in ASD [9,10].

The main objectives of the current work are to conduct an in-depth analysis of neuroanatomy of the ASD, discuss its pathogenesis and metabolic disruptions occurring in the ASD brain and provide the high-efficacy pathogenetically substantiated treatment protocols for the spectrum disorders.

Neuroanatomy of ASD

Let us look into the anatomical differences described between ASD and neurotypical non-ASD brains. Numerous instances of ASD are linked to an increased size of the frontal cortex during early childhood. Additional studies indicate variations in the size of various other brain areas and possible epigenetic changes that could suggest differences in brain function. At the cellular level, ASD has been associated with cortical dysgenesis, including alterations in cortical thickness, neuronal and minicolumn density, and localized disorganization. These observations of atypical brain structure have sparked a renewed focus on the core mechanisms that govern brain development [11].

Difficulties with social interaction, reward prediction, emotional memory, and facial and emotional recognition in ASD may reflect dysfunction in the amygdala and its connected regions [12,13]. Recent research has investigated how abnormal growth patterns of the amygdala affect children and adolescents with ASD [14].

Kemper & Bauman were among the first to identify abnormalities in the amygdala in individuals with autism, noting increased cell densities and smaller neuronal cell sizes [15]. However, their findings were based on a limited sample of only six cases, including four with comorbid seizure conditions, which may have influenced the observed anatomical changes.

Schumann observed changes in the amygdala's relative size specifically in young children. An MRI study of 89 children aged 1 to 5 years revealed that toddlers with a confirmed ASD diagnosis had larger right and left amygdalae compared to controls, when adjusted for total brain volume [16]. Interestingly, the extent of amygdala enlargement in these toddlers was positively associated with the severity of social interaction and communication difficulties at age 5. Other research has also reported increased amygdala volume in autistic individuals during early childhood (under 5 years) [17,18]. However, Schumann and colleagues found no evidence of amygdala enlargement in adolescents with ASD, suggesting that changes in amygdala size and cell count may be age-dependent [19]. The prevailing view is that while the amygdala may enlarge early in life for those with ASD, this enlargement typically slows and may even reverse, resulting in a smaller amygdala with fewer neurons in adults with ASD compared to controls.

The cerebellum is well-known for its role in proprioception and fine motor control. Recent research, however, has increasingly highlighted its involvement in higher cognitive functions, including language, cognitive processing, and emotional regulation [20-22]. Early studies identified hypoplasia of the central cerebellar vermis lobules (VI+VII) as the first neuroanatomical alteration in individuals with ASD [23]. Since then, numerous studies have documented cerebellar abnormalities in ASD [22]. A recent meta-analysis by Stoodley, which examined 17 voxel-based morphometry studies of grey matter volume, compared the cerebella of male ASD patients to age-matched neurotypical controls [24]. This analysis revealed consistent reductions in the inferior cerebellar vermis (lobule XI), right crus I, and left lobule VIIIB. However, these findings are not universally observed across all studies or cohorts. For instance, some research has reported no changes in cerebellar volume [25], and Courchesne's group has noted cases of cerebellar hyperplasia [26].

Social Impairment	Communication deficits	Repetitive behaviours
OFC – Orbitofrontal cortex ACC – Anterior cingulate cortex FG – Fusiform gyrus STS – Superior temporal sulcus A – Amygdala mirror neurons IFG – Inferior frontal gyrus PPC – Posterior parietal cortex	IFG – Inferior frontal gyrus (Broca's area) STS – Superior temporal sulcus SMA – Supplementary motor area BG – Basal ganglia SN – Substantianigra Th – Thalamus PNC – Pontine nuclei cerebellum	OFC – Orbitofrontal cortex ACC – Anterior cingulate cortex BG – Basal ganglia Th – Thalamus

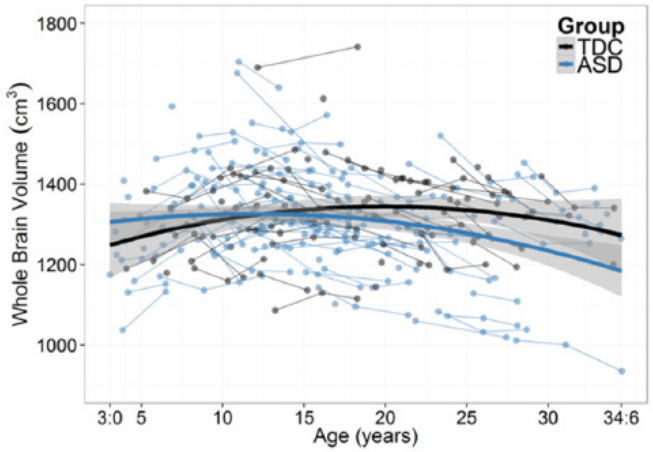
Table 3: Neuroanatomy of ASD.

One of the most consistently observed neuroanatomical abnormalities in postmortem ASD brains is a marked decrease in the size and number of Purkinje cells, particularly in the posterolateralneocerebellar and archicerebellar cortices [27]. Since Purkinje cells are the sole output neurons of the cerebellum, their reduction may have significant functional implications. Data from 24 postmortem studies revealed a notable 79% incidence of reduced Purkinje cell numbers in the cerebellar hemispheres of ASD brains [28]. A recent study provided an stereological assessment of Purkinje cell size and morphology in ASD, showing a 25% decrease in total Purkinje cell count and a 24% reduction in cell density in the cerebella of 14 ASD subjects compared to 14 age-matched controls (ages 4–60) [29]. Another study reported significant reductions in Purkinje cell volume, approximately 31% in 4- to 8-year-olds and around 23% in individuals aged 29 to 60 [30].

The frontal cortex is crucial for various executive functions, including complex cognitive tasks such as decision-making, planning, working memory, emotion regulation, social behavior, learning, and communication. Given the social and emotional challenges associated with ASD, this brain region has become a focal point of recent research. Key findings in ASD include atypical cortical growth patterns, irregularities in cortical thickness, and disorganization of neurons across cortical layers and their connections to other brain areas.

Earlier MRI studies had indicated that children with ASD experienced atypical growth patterns, including early overgrowth of the frontal cortex [31]. The analysisof MRI scans of 41 toddlers with diagnosed ASD and 44 typically developing controls across various time points revealed a notable 7% increase in overall cerebrum size in autistic toddlers compared to controls up to 2.5 years of age, with a 10% rise in white matter and a 5% increase in grey matter [32]. These findings reinforced previous observations of abnormal cortical growth patterns in ASD toddlers, starting before the age of 2. The results emphasized the importance of including younger children in studies to better understand the developmental disruptions linked to ASD. More recent findings from a large scale study are aligned with previous research, demonstrating increased cortical thickness in early childhood, which was followed by a slowdown in growth and a halt in development during later childhood, continuing into adolescence [33]. They observed a normalization of cortical thickness during mid- to late childhood (ages 8–18), but noted that this normalization might not reflect broader structural or functional improvements.

Neuronal cell bodies in the neocortex are arranged one above the other forming the minicolumns. The analysis of the microstructure of layer III in the dorsolateral prefrontal cortex and Brodmann area 9 (BA9) using 14 postmortem ASD samples, finding a reduction in the spacing between minicolumns [34-36]. The study of McKavanagh et al. observed wider minicolumns in the primary auditory, auditory association, orbital frontal, and parietal cortices of ASD brains, especially in younger individuals [37]. These findings further support the idea of an atypical developmental trajectory in ASD.



Graph 1: Dynamics of the whole brain volume in ASD individuals over the years of development. A comparison with the typically developing brain (from: Lange et al., Autism Research, 2015).

The analysis of postmortem tissue from the prefrontal, temporal, and occipital neocortices of 11 children with ASD and 11 typically developing controls, ranging in age from 2 to 15 years. Using in situ hybridization on tissue sections, the study identified patches of altered or reduced gene expression, measuring between 5 and 7 mm, in 10 out of 11 ASD patients, compared to only 1 out of 11 controls [38]. Such alterations can occur due to abnormal neuronal proliferation, differentiation, migration and/or survival, ultimately leading to mis-positioning of neurons within the wrong layers.

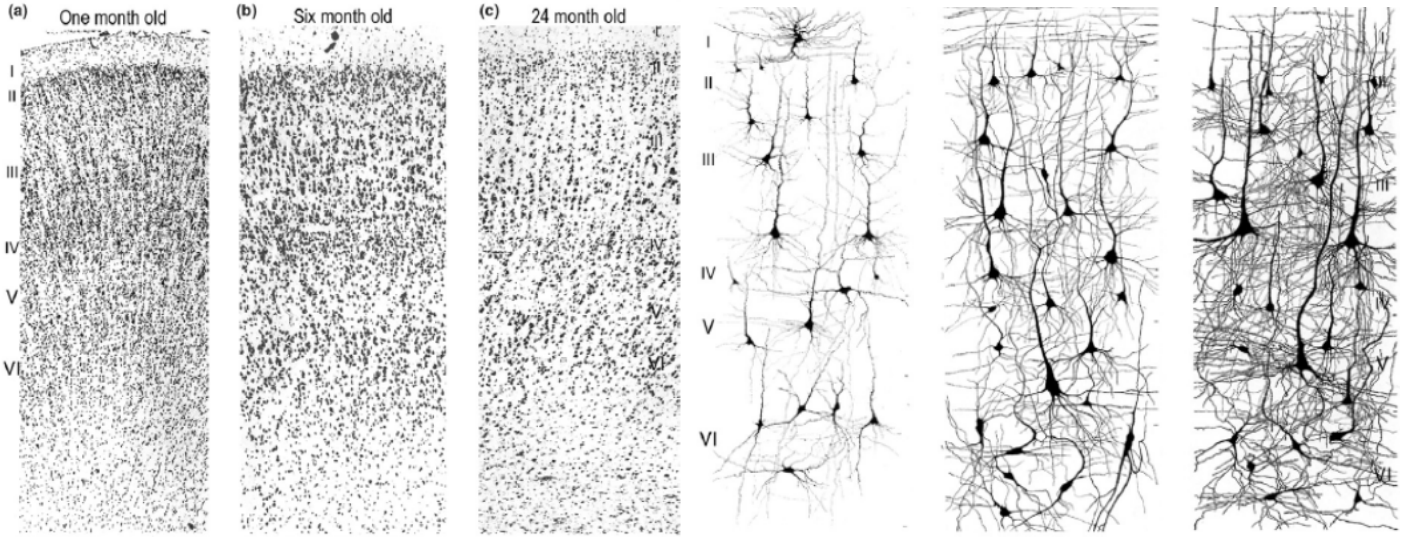
Hyperconnectivity and misfiring in ASD brain

Apart from the macro and microanatomical changes, the autistic brain shows a greater functional hyperconnectivity compared to the typically developing brain. Supekar et al. examined data from 110 children gathered across three separate sites, making this the largest pediatric brain imaging dataset to date [39]. The results provided strong and consistent evidence of widespread functional brain hyper-connectivity in children with ASD. This functional hyper-connectivity is supported not only by the exceptional triple-replication but also by its correlation with clinical ASD symptoms. Specifically, children with more severe social impairments showed increased functional connectivity. This study is the first to establish a significant relationship between atypical whole-brain functional connectivity and a core symptom of ASD.

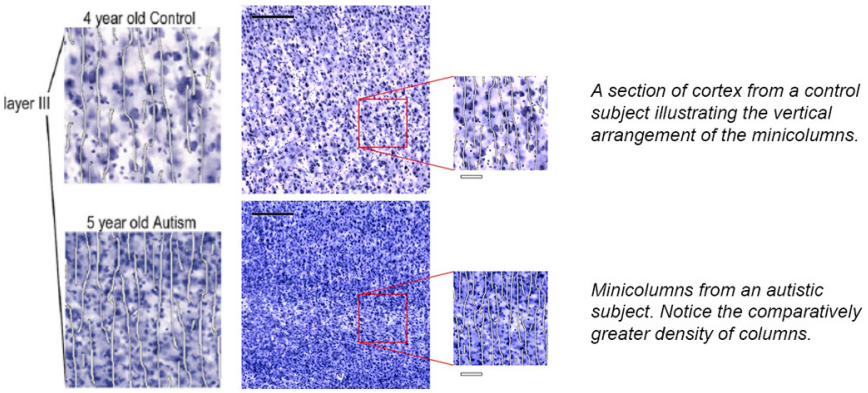
Abnormal age-related cortical thickness trajectories in ASD and brain volume changes through the cause of neurodevelopment
Accelerated total brain volume growth around 2~4 years of age predominately due to enlarged brain volume of the frontal and temporal lobesAccelerated expansion of cortical surface area (grey matter) but not cortical thickness
Impaired maturation of the cortical white matter
Followed by arrest of growth and possible decline in brain volume after around 10~15 years of age

Table 4: Dynamics in brain volume and cortical thickness during the development of the autistic brain.

A study involving children aged 7 to 13 found increased connectivity within striatal systems in those with ASD compared to typically developing peers [40]. This observation of "ectopic" hyper-connectivity in a specific brain system aligns with our current findings and extends them to a whole-brain perspective for the first time. The analysis of functional subsystems revealed hyper-connectivity in various areas, including sensory and association cortices. These results highlight abnormal connectivity patterns in brain systems involved in cognitive, social, and emotional processes [41]. The examining of connectivity patterns in relation to anatomical distance has revealed hyper-connectivity in ASD between both nearby and distant brain regions, indicating atypical integration and segregation within both short- and long-range circuits [39]. Collectively, these findings offer new insights into widespread functional brain hyper-connectivity in childhood



Graph 2: Cortical microcolumns in ASD (from Amaral et al. Trends in Neurosciences, 2003).



Graph 3: Cortical Micro columns changes in ASD (from Casanova, M.F. et al. (2006) Minicolumnar abnormalities in autism).

ASD, affecting both whole-brain connectivity and major functional subsystems across various anatomical distances.

Neurotoxicity in ASD

Genetic and environmental factors are believed to play an important role in pathogenesis of ASD. Recently, there has been increased focus on the impact of exposure to neurotoxic substances. These substances, whether naturally occurring or synthetic, affect the functioning of the nervous system. Numerous everyday products contain chemicals that are suspected to have detrimental effects on neurodevelopment. Therefore, there has been growing concern about the effects of exposure to neurotoxic substances. These compounds, which can be either natural or man-made, impact the nervous system's function. Many common products include chemicals believed to negatively influence neurodevelopment.

For instance, recent research has linked acetaminophen use in children to an increased risk of developing autism. A parents' survey found an association between acetaminophen use following measles-mumps-rubella (MMR) vaccination and autistic behaviors in children up to 5 years old [42]. Animal studies have shown that acetaminophen exposure can affect social behavior in mice [43]. It was suggested that acetaminophen might influence the endocannabinoid system, potentially leading to neuromodulatory effects during development that could trigger autism [44]. Hence, cannabinoid receptor type 2 has been found to be upregulated in the blood cells of children with ASD [45].

Neurotoxic substances can cross the placental barrier, meaning that exposure can begin during the prenatal period. During this time, the embryo and fetus undergo rapid processes such as cell division, differentiation, and organ formation [46]. In the first nine months of life, the human brain evolves from a simple cell layer into a sophisticated organ with billions of specialized, intricately connected cells [47]. Consequently, exposure to neurotoxic compounds can be especially detrimental to brain development during this critical phase. However, brain development does not stop at birth. The growth of glial cells and the myelination of axons continue for several years, while synaptic development persists through childhood and adolescence, extending the period of susceptibility to neurotoxic agents [47,48]. Disruptions in neurotransmitter function could be a significant factor, as studies have linked neurotoxic compounds to alterations in neurotransmitter activity, which in turn are associated with ASD. Several neurotransmitters, including γ-aminobutyric acid (GABA), glutamate (Glu), serotonin (5-HT), and dopamine (DA), as being related to ASD [27]. Changes in both the levels of these neurotransmitters and their associated proteins - such as receptors and transporters - have been connected to the disorder. Disruptions in acetylcholine neurotransmission caused by organophosphate exposure may also affect other neurotransmitter systems [49]. Additionally, organochlorine pesticides have been associated with the inhibition of $GABA_A$ receptors [50]. Specifically, the organochlorine pesticide endosulfan interferes with the binding of GABA to its receptor site, resulting in excessive neuronal excitation.

There has been much speculations regarding the role of mercury in neurotoxicity and development of ASD. Earlier studies had found that hair mercury levels were significantly higher in children with autism compared to age- and sex-matched healthy controls [51]. This finding aligns with the another study results, which also observed elevated hair mercury levels in autistic children compared to their non-autistic peers [52]. Additionally, Adams et al. noted that pathways for excreting toxic metals might differ significantly between individuals with moderate to severe ASD and those with milder forms of the disorder [53]. Children are more vulnerable to environmental toxins due to their higher rates of absorption and lower detoxification abilities compared to adults. Mercury is particularly noted for its significant impact on neurodevelopment because of its neurotoxic effects. Additionally, exposure to multiple toxic elements simultaneously may have compounded effects, worsening neurotoxicity and making it challenging to isolate the impact of individual elements on ASD. Thiomersal, a mercury-based compound used as a preservative in vaccines like the Measles-Mumps-Rubella (MMR) vaccine and as an antiseptic and antifungal agent, has historically been suspected of contributing to autism cases. In children with autism, issues related to toxic elements are believed to be linked to oxidative stress, reduced methylation and transsulfuration capacities, and mitochondrial dysfunction.

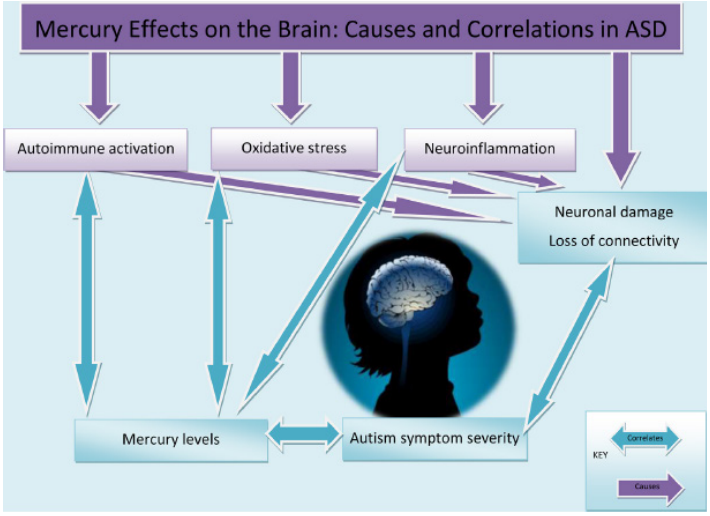
The recent Spanish study examined mercury concentrations in ASD children's hair and urine and had findings that mercury concentrations were no different to the concentrations found in the group of normally-developing children. More than half of urine Hg determinations in the ASD and control groups were below the detection capacity. In hair, mercury concentrations in both groups were even much lower than the percentage of limits of detection [54].

A detailed meta-analysis has examined 18 studies on aluminum (Al), 18 on cadmium (Cd), and 23 on mercury (Hg) in ASD individuals. Albeit the meta-analysis revealing significant associations between all three metals and ASD, the nature of these associations varied. Specifically, elevated levels of Hg in hair, urine, and blood were consistently linked to ASD. Conversely, while Al levels in hair and urine showed a positive correlation with ASD, Al levels in blood were negatively associated. For Cd, higher levels in hair and urine were inversely related to ASD. These findings suggest that, although these metals are all neurotoxic, their effects on ASD and their mechanisms of action may differ. Further research is needed to explore the long-term impacts of these toxic metals on ASD risk, identify critical exposure periods, and investigate factors that might modify their effects. Overall, the results support policies aimed at reducing exposure to neurotoxic metals, especially for pregnant women and young children, to help mitigate the increasing prevalence of ASD [55].

Another study involving one hundred children with ASD in comparison to a hundred of control non-ASD individuals showed the mean levels of mercury, lead, and aluminum in hair of the autistic children were significantly higher than controls [56]. These findings allowed researchers to conclude that environmental exposure to these toxic heavy metals, at key times in development, may play a causal role in autism. Authors also suggested that mercury, lead, and aluminum levels were positively correlated with maternal fish consumptions, living nearby gasoline stations, and the usage of aluminum pans, respectively.

Excitotoxicity in the Pathogenesis of ASD

Excitotoxicity refers to a pathological condition marked by excessive neuronal stimulation due to overactivation of excitatory amino acid receptors, such as those for glutamate and aspartate [57]. Morphologically, excitotoxicity is characterized by neuronal swelling, vacuolation, and cell death [58]. Normally, excitatory receptors allow the movement of sodium, calcium, and potassium ions, which leads to neuronal activation [59]. However, elevated levels of glutamate and other excitatory substances can lead to excessive activation of ionotropic glutamatergic receptors, such as N-methyl-d-aspartate (NMDA) and 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionate (AMPA). This excessive activation results in elevated intracellular calcium levels. The increase in calcium triggers the activation of inducible nitric oxide synthase (iNOS) and protein kinase C, which further amplifies nitric oxide (NO) production and activates phospholipase A2. This process generates pro-inflammatory molecules [60,61]. The resulting free radicals can impair oxidative phosphorylation and damage mitochondrial enzymes involved in the electron transport chain, reducing energy production [62].



Graph 4: Mercury effects on the brain and its associations and correlations with ASD (J.K. Kern et al. Journal of Trace Elements in Medicine and Biology (2016)).

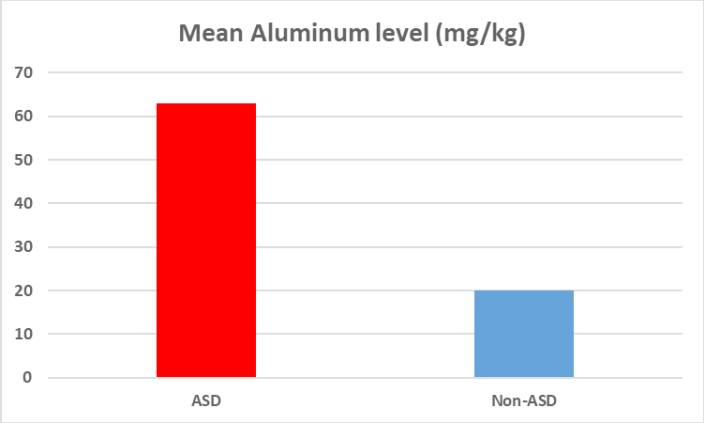


Chart 1: Mean Aluminum level in the hair of ASD vs non-ASD individuals (from: Mohamed Fel B. et al. Assessment of Hair Aluminum, Lead, and Mercury in a Sample of Autistic Egyptian Children: Environmental Risk Factors of Heavy Metals in Autism. Behav Neurol. 2015).

In children with autism, excitotoxicity is also associated with the onset of seizures. Experimental evidence supports a link between seizures and excitotoxicity, with elevated brain glutamate levels being a potential trigger for seizures [63,64]. Additionally, immune responses triggered by microglial activation and cytokine release contribute to excitotoxicity [65].

Biochemical assessments have revealed higher serum levels of glutamate in individuals with autism compared to controls [66]. Research has also linked disruptions in immune system regulation with autism [67]. Additionally, increased levels of tumor necrosis factor-alpha (TNF-alpha) receptor II have been noted in the blood of children with autism spectrum disorders [68]. During immune responses, microglial activation leads to the release

of TNF-alpha, which exacerbates excitotoxicity by boosting reactive oxygen and nitrogen species and further impeding the reuptake of glutamate [69]. Furthermore, activated microglia can release potent excitotoxins, including glutamate and quinolinic acid [70]. Research has indicated a connection between the development of seizures and excitotoxicity, particularly involving glutamate accumulation [71]. Seizures can lead to the production of excitotoxic amino acids by increasing the generation of free radicals. Importantly, the developing brain in infants is especially susceptible to excitotoxic damage due to a higher density of synaptic glutamate receptors compared to newborns, with this receptor density gradually decreasing with age [72].

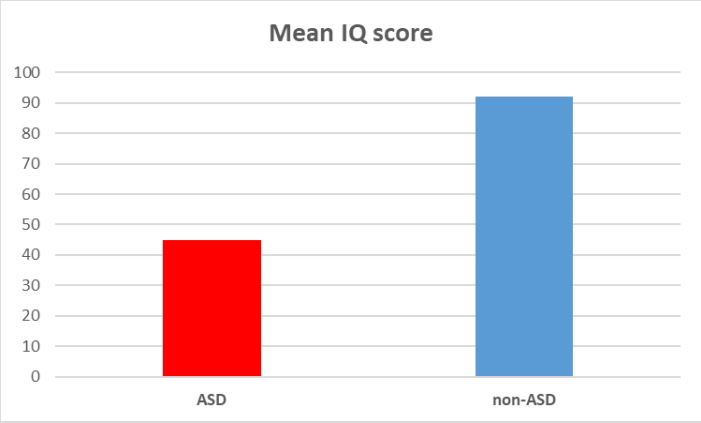


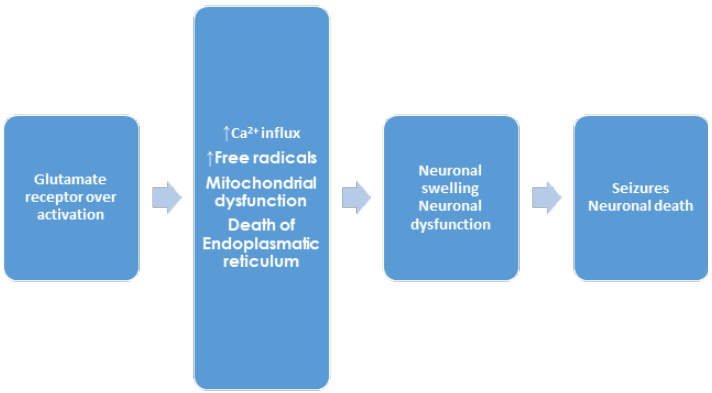
Chart 2: Mean IQ score in ASD vs non-ASD individuals in the same cohorts corresponding to the Aluminum levels in hair (from Mohamed Fel B. et al. Assessment of Hair Aluminum, Lead, and Mercury in a Sample of Autistic Egyptian Children: Environmental Risk Factors of Heavy Metals in Autism. Behav Neurol. 2015).

Over activation of the glutamate receptors NMDA and AMPA leads to the release of additional excitotoxins and an increase in glutamate levels [73]. High concentrations of glutamate cause elevated calcium levels within the cytosol. This occurs because excessive glutamate prolongs the opening of calcium channels, resulting in an increased influx of calcium into cells. The rise in calcium levels activates inducible nitric oxide synthase and protein kinase C, which in turn generate free radicals, ROS, and arachidonic acid. The production of these oxidants can impair mitochondrial function, lead to the buildup of pro-inflammatory molecules, and ultimately result in cell death.

GABA, being the principal inhibitory neurotransmitter, and its reduced inhibitory function is one of the many abnormalities seen in ASD. When GABAergic inhibition is diminished, it can lead to an overabundance of glutamate activity in neurons and impaired sensory gating. This suppression may occur through the direct dysfunction of GABA receptors or through the antagonism of GABAergic neurons that have receptors sensitive to the glutamate analog NMDA [74]. Additionally, excessive activation of non-NMDA glutamatergic receptors can lead to a reduction in the number of synapses and limit dendritic growth in pyramidal neurons within the hippocampus.

Calcium ions (Ca^{2+}) can play a crucial role in mediating excitotoxic damage within cells. Calcium is essential for various cellular functions, including membrane excitability, cell growth, exocytosis, and synaptic activity. Neurons typically have specialized mechanisms to regulate cytosolic Ca^{2+} levels and maintain low calcium concentrations under normal conditions. However, excessive glutamate release from synapses and overstimulation of glutamate receptors, such as NMDA, AMPA, and kainate receptors, can lead to excessive receptor activation. This overstimulation causes associated ion channels to open, resulting in an increased influx of calcium and sodium ions. This disruption in calcium regulation can ultimately lead to cell death [75].

Genetic factors also contribute significantly to excitotoxicity in ASD. Mutations in genes that encode voltage-gated calcium channels can be linked to AS. These channels, present in dendrites and cell bodies, play a key role in regulating neuronal excitability and calcium-dependent signaling pathways [76,77]. Mutations can cause these channels to remain open longer than normal, leading to excessive calcium influx [78]. Additionally, mutations in the gene for the Ca2+-activated K+ channel (BKCa), specifically KCNMA1, can also lead to abnormal calcium influx. BKCachannels are primarily located in the presynaptic active zones of the brain and are involved in regulating synaptic transmission and neuronal excitability [79]. Normally, BKCa channels help hyperpolarize the membrane in response to depolarization or increased Ca2+ levels, thereby reducing calcium influx through voltage-gated Ca2+ channels. Reduced activity of BKCa channels, combined with impaired inactivation of voltage-gated Ca2+ channels, results in abnormal calcium influx in individuals with autism [79].

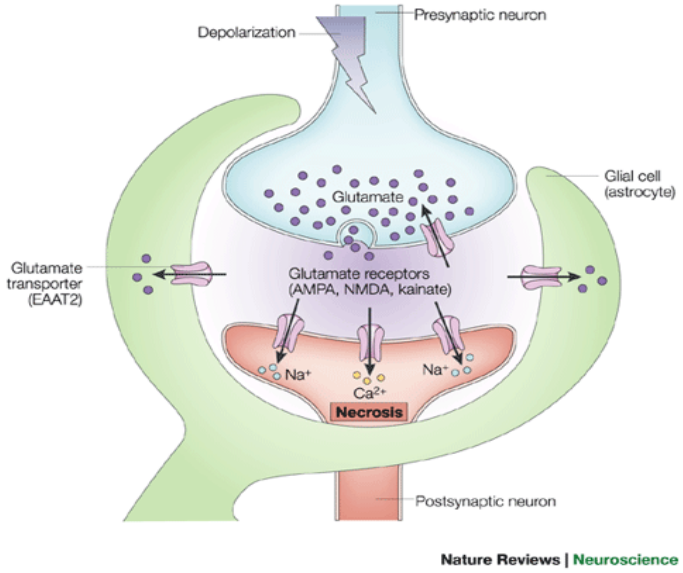


Graph 5: Mechanisms of excitotoxicity and seizures in ASD.

Metabolic disruptions and mitochondrial dysfunction in the ASD brain

The brain has exceptionally high energy requirements, utilizing about 20% of the body's total caloric intake despite representing just 2% of body weight. It requires substantial amounts of adenosine triphosphate (ATP) to sustain ionic gradients crucial for neurotransmission and neural plasticity [80]. Mitochondria play a crucial role in several aspects of neural development and function. They are essential for the proliferation, differentiation, and maturation of neural stem cells. Additionally, mitochondria

are involved in the growth of dendritic processes, as well as in developmental and synaptic plasticity. They also influence cell survival and apoptosis [81-83].



Graph 6: The mechanisms of neuronal necrosis caused by glutamate excitotoxicity in ASD (from Syntichaki, P., Tavernarakis, N. The biochemistry of neuronal necrosis: rogue biology?. Nat Rev Neurosci,2003, 4, 672–684).).

The rate of mitochondrial disorders among individuals with ASD is estimated to be around 5.0%, which is approximately 500 times greater than the incidence in the general population, where it is about 0.01%. Moreover, the prevalence of abnormal metabolic markers is even higher, indicating that up to 30% of children with ASD might have metabolic issues. Nearly one-third of autistic children show increased levels of plasma lactate and/or an elevated lactate-to-pyruvate ratio. Additionally, other mitochondrial biomarkers, such as pyruvate, carnitine, and ubiquinone, also exhibit significant differences between children with ASD and typically developing controls [84].

ASD has been associated with disruptions in folate metabolism. Variations in folate-related genes may contribute to an increased risk of developing ASD through complex polygenic mechanisms. Additionally, autoantibodies that inhibit folate transport into the brain have been linked to ASD, and high doses of folinic acid can help counteract these antibodies. Similar metabolic issues are observed in mothers of children with ASD, and folate supplementation during preconception and pregnancy has been shown to lower the risk of ASD in offspring. These findings suggest that abnormalities in the folate pathway could be a significant metabolic factor in ASD and could be used to develop biomarkers for improving symptoms and potentially preventing the disorder [85].

ASD is also known to be associated with myriads of various metabolic disturbances. Abnormal amino acid levels in plasma, urine, platelets, or cerebrospinal fluid have been documented since the 1970s. Research has shown that children with phenylketonuria often exhibit characteristics similar to those of ASD. Similarly, individuals with purine metabolism disorders have been identified with ASD. Elevated levels of certain organic acids have been observed in children with autism, including increased Krebs cycle analogues and arabinose in two siblings with autistic traits. There have also been reports of altered metabolism in neurotransmitters and hormones such as serotonin, catecholamines, melatonin, oxytocin, GABA, and endorphins in individuals with ASD.

Over time, there has been growing evidence of mitochondrial dysfunction in ASD, with abnormal lactate and pyruvate levels frequently reported. Additionally, oxidative stress has been consistently documented in children with ASD through various experimental methods. Research has also highlighted biomarkers of vitamin deficiency, impaired energy transport, reduced sulfation, and detoxification in these individuals. Abnormal fatty acid metabolism has been noted in some cases of ASD.

- **N-*Acetylaspartate*** – energy production in the mitochondria correlates with the synthesis of NAA in neurons and axons. Decrements of the concentration of NAA represent the loss of neuronal integrity and represent markers for neuronal dysfunction
- ***Creatine*** - reductions in the concentration of Cr indicate deficient cellular energy metabolism
- ***Choline*** - component of the manufacture and the breakdown of cellular membranes. Increments in Cho likely represent a greater cell growth and gliosis. Thus, Cho is a marker of cell membrane turnover and an increase in its level means gliosis or increased cellular proliferation
- ASD patients have a reduced signal intensity indicating a reduced concentration of **NAA** and corresponding neuronal loss or hypofunction in the left amygdala–hippocampal region and the left cerebellar hemisphere. Reduced concentrations of NAA suggesting general neuronal damage in all brain regions and particularly in the left frontal cortex
- ASD symptoms may result from abnormally low concentrations of gamma-aminobutyric acid (**GABA**), the primary inhibitory neurotransmitter
- Decreased synaptic neuronal density consistent with the lower concentrations of **NAA** and **Choline**
- 60% of ASD individuals with seizures have a biochemically-confirmed mitochondrial disease
- The prevalence of mitochondrial disease in the ASD population is 500 times higher than that found in the general population
- The prevalence of abnormal metabolic biomarkers is 30% of children with ASD may experience metabolic abnormalities

Table 5: Summary of metabolic disruptions in ASD.

Moreover, imbalances in gut microbiota and their potential link to gastrointestinal issues in ASD have gained attention. Overgrowth of specific gut bacteria has been reported, with studies showing

increased levels of certain Clostridium species in the feces of autistic children compared to controls [86]. For instance, a study comparing 13 children with autism and 8 controls found that nine Clostridium species were present in the feces of the autistic children but not in the controls. Additionally, certain anaerobic bacteria were found in the gastric and duodenal samples of autistic children but absent in the controls. Subsequent analyses confirmed significantly higher levels of Clostridium clusters in the fecal samples of autistic children. Furthermore, increased prevalence of Clostridium histolyticum and associations between high levels of Sutterella species and gastrointestinal disturbances in children with autism have been reported.

Neuroinflammation in ASD

Neuroinflammation involves the interaction of neurons, microglia, and macroglia within the CNS [87,88]. This inflammatory response is a common feature in various neurodegenerative disorders, including multiple sclerosis (MS), Alzheimer's disease (AD), Parkinson's disease (PD), and autism spectrum disorders [88,89]. Individuals with ASD frequently exhibit altered inflammatory responses and disruptions in the neuro-immune system throughout their lives, suggesting inflammation might play a role in ASD's development. Increasing clinical and experimental evidence supports this, linking immune and inflammatory abnormalities to ASD pathogenesis [90]. Additionally, post-mortem analyses have revealed significant neuroinflammation in various brain regions of individuals with ASD [91].

Evidence of chronic low-grade inflammation in Central Nervous System in ASD:
Microglial and astrocytic activation
Pro-inflammatory profile of cytokines
Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation

Table 6: Neuroinflammatory changes in ASD brain.

Microglia, the brain's resident immune cells, are essential for mediating neuroinflammation and maintaining brain development and homeostasis. They play a crucial role in defense and tissue repair, with their activation being one of the earliest indicators of neuroinflammation. Abnormalities in microglia have been linked to autism [88,89]. Upon activation, microglia can contribute to neuronal dysfunction and cell death, exhibiting neurodegenerative effects. Activated microglia demonstrate several biological responses, including cell rounding, proliferation, migration, phagocytosis, antigen presentation to T-cells, and the release of reactive oxygen species. They also activate genes and proteins such as inducible nitric oxide synthase (iNOS), cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), and proinflammatory cytokines like interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α). These responses are notably observed in autism. Chronic or excessive neuroinflammation has been identified in ASD, with persistent glial activation and inflammatory dysfunction potentially contributing to the behavioral symptoms of autism [92]. Chronic peripheral inflammation and abnormal brain inflammatory responses may lead to cognitive impairments [90].

Algorithms of therapeutic approach to ASD

Research and development of safe and effective holistic protocols for ASD has been our task for many decades. As we have highlighted in our previous works, conventional approach focusing on education, behavioral strategies and facilitative therapies that target communication, social skills, promotes academic potential, and maladaptive behaviour, as well as generally accepted medications are not been able to correct core deficits of ASD [9]. Notable benefits have been achieved through integration of novel therapeutic modalities with biological agents targeting the intrinsic subcellular biochemical pathways leading to ASD. The holistic paradigms, which can be referred to as the *roadmap of ASD*, include multidimensional therapeutic modalities ranging from nutritional corrections, nutraceuticals, orthomolecular therapy, application of energy devices stimulating neurodevelopment and neuroplasticity, brain engagement, neuropeptides, and cell therapy. Thus, precursor stem cells (PSC) are found capable of bringing improvements not only in early childhood but also in adults with autism, who were not treated timely in the past. During neurogenesis neural progenitor cells express regulatory genes that subdivide each area of the brain into compartments and regulate their size. Progenitor cells generate neurons during early to mid-embryogenesis. Neurons increasingly migrate to their target sites sharing the transcription factors expressed by neuronal progenitors [93,94]. The *roadmap* of ASD begins with holistic profound diagnostic measures, which include but not limited to biochemical laboratory tests, allergy & food intolerance tests, functional assessment, ASD scoring. It is crucial in our opinion to conduct long-term regular reassessments of the main biochemical, metabolic, and functional parameters of the ASD individual to keep the track of the progress. Various scoring systems are used, including but not limited to such as Childhood Autism Rating Scale (CARS), Gilliam Autism Rating Scale (GARS), the Autism Treatment Evaluation Checklist (ATEC), the Vineland Adaptive Behavior Scales (VABS), the Clinical Global Impression Scales for the Severity of Illness (CGI-S) and Global Improvement (CGI-I), Pervasive Developmental Disorder Behavior Inventory (PDDBI), European Wellness Functional Assessment Score*et cetera*.

Nutrition with supplementation of macro and micronutrients deficiency, which is typically associated with ASD, and avoiding the nutrients and toxins that can possibly exacerbate the symptoms. Therefore, sugars, MSG, casein or gluten cause intolerance and/or contribute to worsening of ASD symptoms, hence are advisable to be excluded from the diet. Vitamin D3 and Omega-3 fatty acids are beneficial in reducing hyperactivity and irritability. Folic acid supplementation yields progresses in social, behavioral, and cognitive functions. In addition, maternal supplementation with folic acid is known to reduce the risk of developing ASD in child [95]. Such clinical symptoms as irritability, aggression, as well as some of the metabolic dysfunctions can be effectively addressed with supplementation of vitamin B12. Vitamin B6 and Magnesium are supplemented in adequate doses according to the child's age and bodyweight and are essential micronutrients involved in speech development and relaxation of the CNS.

Prebiotics are stimulating the growth of beneficial gut microbiota, and upon degradation by the gut microbiota, produce increased level of circulating short-chain fatty acids [96]. Probiotics *Bifidobacteriuminfantis* and bovine colostrum improved overall aberrant behaviors and gastrointestinal disorders, and decreased levels of circulating inflammatory cytokines IL-13 and TNF-α [97].

Gamma-aminobutyric acid (GABA) serves as the main inhibitory neurotransmitter in the central nervous system. Due to its main function to "diminish neuronal excitability across the nervous system", by lowering overall neural activity, GABA helps to mitigate responses associated with the fight-or-flight reaction, including fear, anxiety, aggression, stress, and agitation.

Few studies have reported a correlation between abnormal melatonin concentrations and the severity of ASD symptoms [98]. Dozens of clinical trials have reported improvements in sleep parameters with exogenous melatonin supplementation in ASD, including longer sleep duration, less night-time awakenings and quicker sleep onset [99]. In our observations the melatonin supplementation in ASD brings significant improvements in sleep duration, sleep onset latency, and promotes positive changes in daytime behaviors. Phosphatidylcholine plays a crucial role in maintaining healthy brain function in individuals on the autism spectrum. Autism is associated with reduced levels of phosphatidylcholine in the brain tissue, which lead to various cognitive and behavioral symptoms. In particular, it is the choline crucially needed for fetal brain development, and its supplementation is potentially lowering the risk of neural tube defects and occurrence of autism.

The European wellness road map of ASD
Know your child
Diagnosis, tests (genetic, metabolic panel, macro nutrients, Ig, food allergies and intolerance, porphyrins, oxidative stress, mitochondrial function, methylation, heavy metals, trace minerals, neurological assessment, hearing assessment, digestive system, immune system, scoring systems, ASD assessment)
Modify the diet (remove gluten, casein, soy, sugars, MSG, fast food and processed food; eliminate toxins, dyes, preservatives, phenols, allergens; proper macronutrients – fats, protein, grains, vegetables; make sure essential micronutrients are in – Omega-3, MCT, Mg, Zn, Vitamins B6 and B12, Vit D, Iodine, flavonoids, etc)
Proceed with treatment: behavioural and speech therapy, special educational programs, social skills, manage immune and digestive systems (probiotics, antifungal), support of the CNS with neurotransmitters (melatonin, GABA etc), treat seizures et cetera
Special treatment: transcranial stimulation, brain engagement, hyperbaric oxygenation, electromagnetic therapy, neuropeptides and cell therapy
BE VERY VERY PATIENT!

Table 6: Summary of the treatment algorithms in ASD.

Cellular based therapies, specifically with neural progenitor cells, exhibit significant improvements in several neurological conditions including ASD. Multifactorial beneficial therapeutic effects of cell-based therapies with neural progenitor (precursor) cells are achieved via the homing effects with further proliferation and differentiation potential of the stem cells, as well as through multiple paracrine effects – the massive production of cytokines, chemokines, tissue repair-related growth factors, and neurotrophic factors [9,10,100].

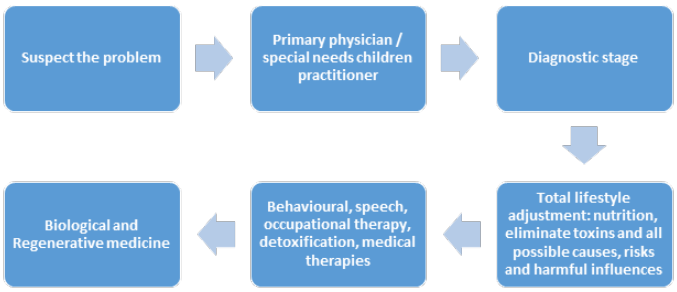


Chart 3: Algorithms of the European wellness *roadmap* of ASD.

Multicentric multinational cohort description and the retrospective data of the observed ASD cases treatment outcomes

Over the last 2 decades we had provided holistic therapeutic solutions to 590 ASD patients from 16 countries, among which were 413 boysand177 girls (chart).

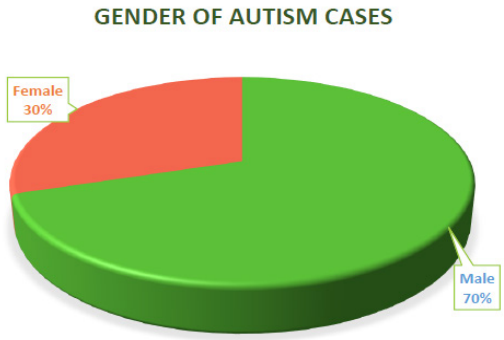


Chart 4: Observed and treated ASD individuals by gender.

The total amount of cell-based therapies done in the entire cohort was 910: 242 patients received only 1 stem cell therapy session, while the rest 348 patients received multiple engrafts of precursor stem cells. As we have mentioned in our previous work, "precursor stem cells are specialized organ-specific stem cells thatstructurally and functionally belong to exact area of the central nervoussystem." [9]. Thetechnicalities of the precursor stem cell protocols were discussed in our previous publications and revisiting this topic is not the objective of the current paper [9,10,100]. The clinical approaches to design of a specific cellular therapy protocols in each particular case were also discussed in details in previous works [9,10,101-103]. The age distribution in the observed cohortis given in the chart below.

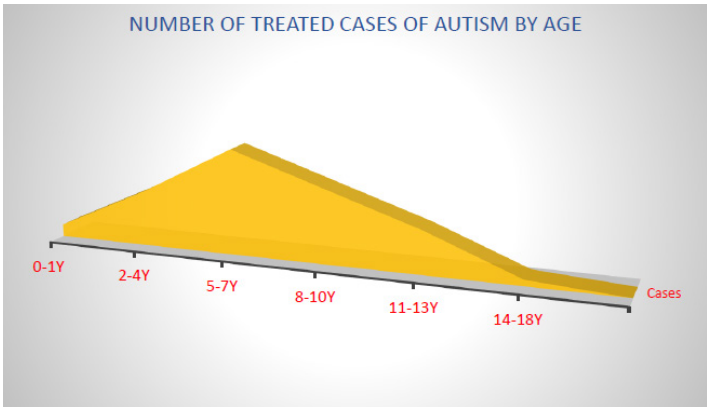


Chart 5: The trend of ASD cases presentation by the families and caretakers for treatment according to the age category in the observed cohort.

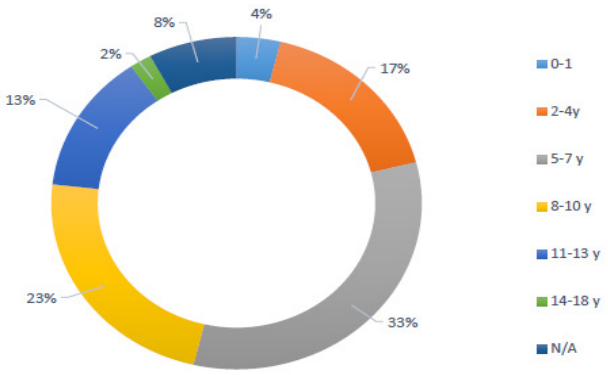


Chart 6: The distribution of ASD individuals by age in the observed cohort.

The amount of cellular therapies per patient among the 348 patients receiving multiple cell-therapy sessions varied from 2 to 7 implantations (chart).

Among the 348 ASD individuals received multiple engraftments of stem cells, 165 (28%) received 2 sessions, 41 (7%) – 3 sessions, 53 (9%) - 4 sessions, and 89 (15%) – 7 sessions. The intervals between sessions of cellular therapies varied from 4 months to 18 months depending on the severity of ASD symptoms and the clinical response of the patients.

As outcomes of the holistic pathogenetically-substantiated therapeutic protocols of ASD, observed patients quickly learn to independently manage their behaviors and act appropriately in various settings, including at home, school, with family, and within the community. Most patients begin to show positive changes in social and language development within the first two weeks. After 2-3 months, ASD children start making eye contact, improving their attention span, responding to verbal instructions, and developing meaningful speech. They become more active, focused, and engaged with their environment. By six months, children typically show increased fluency in communication and greater independence in self-management. With continued treatment, patients are better able to handle anxiety, mood issues, attention deficits, and depression, while also enhancing their social skills. The provided therapies also helped managing seizures, gastrointestinal disorders, dietary imbalances, and disrupted sleep patterns.

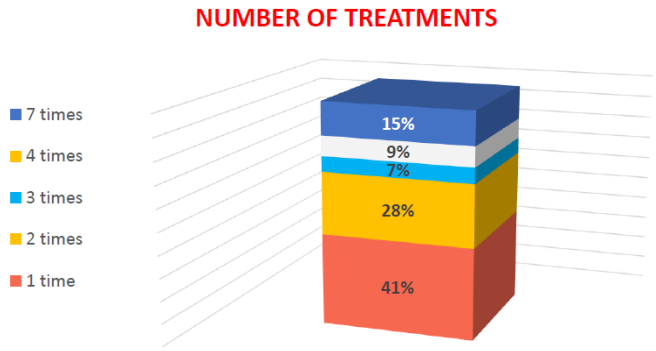


Chart 7: The number of cell therapy sessions with precursor stem cells in the observed cohort of ASD individuals.

Summary of treatment outcomes in ASD

Improvements in the domains of **social relationships** and reciprocity (improved eye contact, social smile, and reaching out to others)

Improvements in **cognitive** aspects (attention, concentration, and time of response)

Improvements in **speech and language patterns** (reduction in echolalic speech, engaging in stereotyped repetitive use of language, production of infantile squeals or unusual noises, inability to initiate or sustain conversation with others, inability to grasp the pragmatics of the conversation, and speech regression)

Decrease in inappropriate **emotional responses**, exaggerated emotions, engaging in self-stimulating emotions

Functional neuroimaging (PET) scan showed increased 18F-FDG uptake in the areas of frontal lobe, cerebellum, amygdala, hippocampus, parahippocampus, and mesial temporal lobe after 6 months of cellular therapy

Within and after the 6 months of cellular therapy, **improvements in concentration, sitting tolerance, attention, sleep, eye contact, social interactions, and memory**

ATEC scores at follow-up times of 6 and 12 months showed improvements in **sociability, cognitive ability, and behaviours**

Improved cell-mediated **immunity**, due to a significant increase of CD3+ T lymphocytes and CD4+ T helpers, and a decrease in CD19+ B lymphocytes counts after the cellular treatment (pretreatment values were abnormal)

Procedures are well tolerated with **minimal adverse reactions** (transitory low-grade fever)

The first theory on mechanism of action of stem cells was that cell therapy could act by a "cell replacement" mechanism; nowadays, large emerging evidences have shown that cell therapy works by providing trophic or "chaperone" support to the injured tissue and brain Only 5 Clinical Trials performed so far, all show safety of Cell therapy and 4 out of 5 clinical trialsdemonstrate significant efficacy.

Table 7: Summary of treatment outcomes in ASD.

The dynamics of the ATEC score before and after the treatment are provided in the chart below. The response rate to the treatment was essentially 100%, as practically all subjects in the observed cohort had improvements of a various degree in at least some of the developmental aspects, i.e. either cognitive, speech or behavioural, if not in all of them (chart).

To enhance the effectiveness of our integrative holistic treatment protocols for ASD, we have incorporated novel neuropeptides, growth factors andtissue repair-related growth factors, naturally-occurring and procured from the culture of fetal neural precursor stem cells. These neuropeptides play a crucial role in regulating higher nervous functions, such as conditioned and unconditioned reflexes, and complex mental processes that ensure appropriate behavior in varying natural and social environments. CNS peptides act as vital mediators of emotional and social behavior, as well as speech and intellectual development.

The use of mitochondrial peptides and neuropeptides during the post-cellular engraftment of neuronal precursors has led to a significant reduction in anxiety and notable improvements in behavioral aspects for patients with autism. These novel CNS peptides enhance confidence, reduce aggressive behaviors, and improve communication abilities and social adaptation skills. Additionally, incorporating pineal gland cell extracts into the treatment protocol helps address sleep disorders in ASD patients.

It is important to note that after 4-6 months of therapy, patients find it easier to engage in conversations, show fewer repetitive motor behaviors, and exhibit more flexible adherence to routines. Furthermore, improvements in higher mental functions are evident, including enhanced peer relationships, increased social or emotional reciprocity, and progress in speech development, verbal communication, and learning. Concurrently with the mentioned above novel therapeutic modalities of regenerative biological medicine, children with autism benefit from Hyperbaric Oxygen Therapy (HBOT) due to its potential to enhance cerebral perfusion. The inhalation of oxygen at higher-than-atmospheric pressures may increase the arterial partial pressure of oxygen, thereby boosting oxygen delivery to the brain. Additionally, HBOT possesses anti-inflammatory effects by reducing levels of pro-inflammatory cytokines, such as tumor necrosis factor-α, interferon-γ, and interleukins 1 and 6. Moreover, HBOT additionally improves mitochondrial function and stimulate the production of antioxidant enzymes. To enhance the clinical effects from the conducted treatment and to influence thecortical mechanisms of excitability, connectivity, and plasticity, which are abnormal in ASD, we incorporate transcranial electric and electromagnetic stimulation therapy, which has the capacity to modulate these mechanisms. The transcranial stimulation acts through rapid pulse of electrical current that induces a rapidly fluctuating magnetic field, which

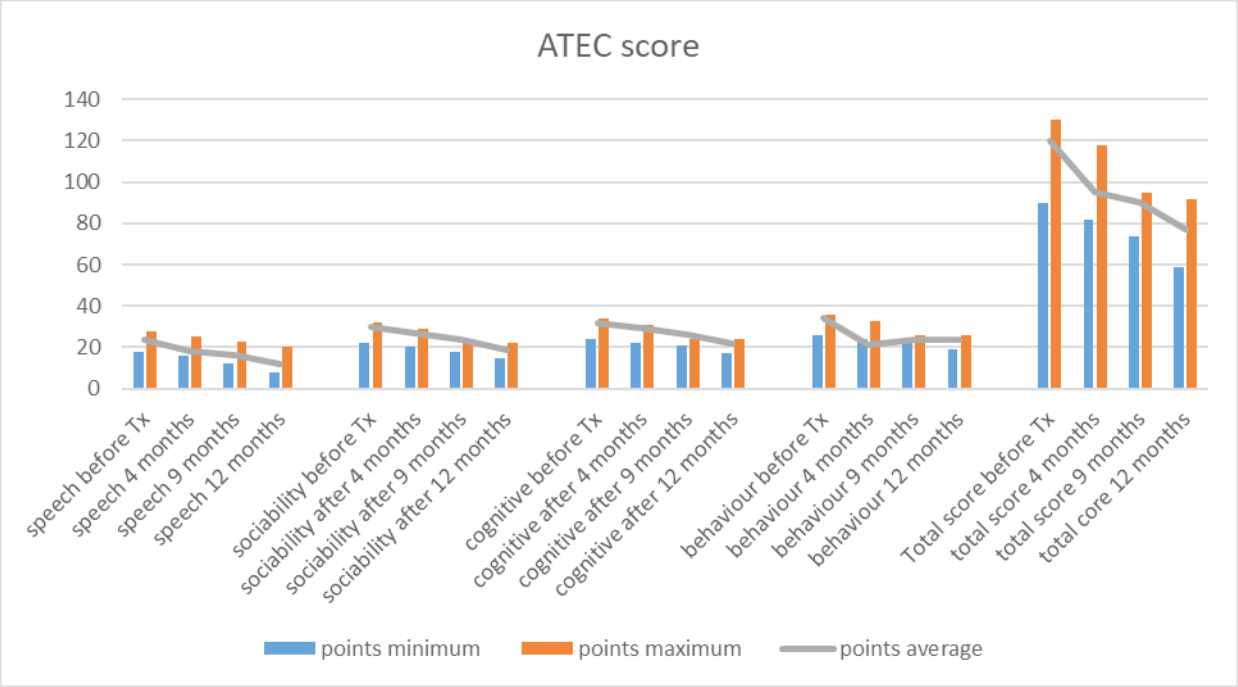


Chart 8: Dynamics of the ATEC score in the treated ASD individuals before the treatment, and after – 4, 9, and 12 months later. Minimum, maximum and mean score in observed ASD cohort (N=590).

in turn induces an electrical current in the underlying brain tissue [104]. The behavioral effects of transcranial stimulation extend beyond the specific cortical regions directly beneath the electrode - they also influence brain areas connected through network interactions with the stimulated regions. Consequently, applying transcranial stimulation therapy to a single cortical area can impact an entire network or system. As a clinical outcome, transcranial stimulation protocols produce a profound impact on the behavioral impairments in ASD individuals.

Conclusions

The integration of regenerative medicine techniques into the treatment of Autism Spectrum Disorders (ASD) represents a groundbreaking advancement in therapeutic approaches. The analysis presented highlights how novel modalities, including cellular therapies with neural progenitor (precursor) cells, neuropeptide interventions, neuromediator precursors, and neutraceuticals have demonstrated promising efficacy in improving behavioral, cognitive, and social functions in individuals with ASD. The rapid gains observed in self-regulation and behavior, as well as the sustained improvements in communication and social skills, underscore the potential of these advanced treatments. The data from clinical outcomes suggest that a holistic approach incorporating biologicals and nutraceuticals can significantly enhance therapeutic efficacy. These findings not only provide hope for more effective management of ASD but also underscore the need for continued research to refine these protocols and validate their long-term benefits. As the field evolves, ongoing investigations will be crucial in optimizing these innovative treatments and ensuring they deliver enduring, meaningful improvements for individuals on the autism spectrum.

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