Review

Nutritional and environmental contributions to autism spectrum disorders: Focus on nutrigenomics as complementary therapy

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Abstract: The prevalence of autism spectrum disorders (ASD) has risen sharply in the last 30 years, posing a major public health concern and a big emotional and financial challenge for families. While the underlying causes remain to be fully elucidated, evidence shows moderate genetic heritability contribution, but heavy environmental influence. Over the last decades, modern lifestyle has deeply changed our eating, rest, and exercise habits, while exposure to air, water, and food chemical pollution has increased due to indiscriminate use of pesticides, food additives, adjuvants, and antibiotics. The result is a drastic change in the quality of our energy source input, and an overload for antioxidant and detoxification pathways that compromises normal metabolism and homeostasis. Current research shows high prevalence of food selectivity and/or food allergy among children with autism, resulting in essential micronutrient deficits that may trigger or aggravate physical and cognitive symptoms. Nutrigenomics is an emerging discipline that focuses on genotype-micronutrient interaction, and a useful approach to tailor low risk, personalized interventions through diet and micronutrient supplementation. Here, we review available literature addressing the role of micronutrients in the symptomatology of ASD, the metabolic pathways involved, and their therapeutic relevance. Personalized and supervised supplementation according to individual needs is suggested as a complement of traditional therapies to improve outcome both for children with autism and their families.

Keywords: autism spectrum disorder, food selectivity, vitamins, omega-3 fatty acids, supplementation, micronutrient deficit

Abbreviations

5-MTHF	5-methyl-tetrahydrofolate
ASD	autism spectrum disorders
BBB	blood-brain barrier
DHFR	dehydrofolate reductase
FR	folate receptor
FRAA	α-FR autoantibodies
GSH	reduced glutathione
GSSG	oxidized glutathione
MS	methionine synthase
MTHFR	methylene tetrahydrofolate reductase
PCFT	proton coupled folate transporter
PL	pyridoxal
PLP	pyridoxal 5'-phosphate
RA	retinoic acid
RARE	retinoic acid response element
ROS	reactive oxygen species (ROS)
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SNP	small nuclear polymorphism
THF	tetrahydrofolate
VDR	vitamin D receptor
VDRE	vitamin D response element
PUFA	polyunsturated fatty acids
ARA	arachidonic acid (20:4 n-6)
EPA	eicosapentanoic acid (20:5 n-3)
DHA	docosahexanoic acid (22:6 n-3)
eCB	endocannabinoids
PPAR	peroxisome prolifarator-activated receptors

Introduction

Autism spectrum disorders (ASD) are complex neurodevelopmental conditions typically manifested by impaired language and social skills and disruptive behaviors such as self-harm and severe meltdowns [1]. Over the years, many frequent comorbidities have been identified in ASD individuals, including epilepsy, attention deficit and hyperactivity disorder (ADHD), learning disability, allergies, and

sleep, nutritional, and gastrointestinal issues, to mention the most recurrent ones (see [2, 3] for review).

The prevalence of ASD is somewhat controversial since it has showed an exponential increase over the last 3 decades. When ASD numbers began to build-up, so did the arguments about real increase versus improved and earlier diagnosis, as well as changes in diagnosis criteria. Bi-annual reports of the ASD surveillance program, launched by the Centers for Disease Control and Prevention (CDC) in 2000, revealed indeed an alarming increment in ASD frequency in 8-year-old children in the USA, from 1 in 150 in the first report to 1 in 59 in 2014, being 4 times more common in boys than in girls [4]. According to the Summary of ASD Prevalence Studies published in the CDC website, global prevalence is between 1 and 2% considering reports from Asia, Europe and North America [5]. Noteworthy, another study available in this web site indicates that 1 in 6 children in the United States had a developmental disability from 1997 to 2008, and that there has been a 17,1% rate increase according to parental report over the last 12-year period [6]. Since 1994 ASD was diagnosed according to the 4th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria, by the American Psychiatric Association. In May 2013 the 5th edition (DSM-V) established new guidelines for ASD identification, which some authors proposed as a plausible cause for prevalence increase, since disorders that were considered different from autism in DSM-IV (such as pervasive developmental disorder or Asperger's syndrome), fall within the *spectrum* in DSM-V. However, a rate attenuation was observed after DSM-V introduction, between 2013 and 2015 [1, 7, 8]. Furthermore, a population-based cohort study in Denmark showed that changes in diagnosis criteria and report improvement accounted for 60% of ASD prevalence rise, with the remaining 40% reflecting the effective rate increase, prompting intensive research to identify risk factors and prevent exposure [9]. The outcome of these efforts is highly relevant not only for individuals with ASD and their families, which face substantial financial and emotional burdens, but also for public health and education systems [10].

While the underlying causes of autism remain unclear, the original concept of a pure non-genetic neurological disorder has been replaced by one that proposes instead a polygenic and multifactorial origin, emphasizing the role of epigenetic changes [11]. Inherited single-gene and chromosomal defects account for just a minority of ASD cases [12], and studies among identical twins showed that while genetic heritability had a moderate influence, environmental factors (e.g. immune maternal activation, pesticides or heavy metal exposure) may strongly influence the onset and persistence of autistic traits, as evidenced by discordant DNA methylation patterns is monozygotic twins [13–15]. Since genetic variation does not account for ASD prevalence escalation in such a short period of time, the question arises: what else has changed in our lifestyle and/or environment over the last decades that could contribute to increasing rates of autism? Also, while there are still neither definite biochemical diagnosis nor specific medical treatment for autism, what novel strategies have been advanced? On a global scale, we have witnessed significant changes in our nutritional habits over the last few decades. Adoption of Western eating habits led to overconsumption of refined carbohydrates and sugar, protein from processed meat, food additives and preservatives, and saturated fatty acids that accompanied the increase in obesity, diabetes, and cardiovascular disease. Micronutrient malnutrition, a condition where the balance in key micronutrients such as vitamins, minerals and essential fatty acids required for optimal metabolism and health is lost, became more prevalent too with the consumption of processed, rather than raw or minimally processed foods, especially in industrialized societies. Along with these changes, environmental contamination increased as well through continuous release into the air and water of pollutants derived from agriculture, urbanization, and industry [16, 17]. These and other environmental toxicants, including neurotoxins within cosmetic and pharmaceutical adjuvants, as well as the indiscriminate use of antibiotics, were linked to developmental delay and a variety of congenital and acquired health conditions [18–21]. The result is a drastic change in the quality of our energy source input, and an overload for antioxidant and detoxification pathways that compromises normal metabolism and cell and organ development and function. Currently, ASD treatment consists of behavioral interventions (disruptive behavior modification therapies) together with educational and speech therapy for years to come after diagnosis, aimed to improve social integration and provide autonomy in adult life [22, 23]. However, outcome success is generally limited, stressing out the necessity to find novel diagnostic tools and therapeutics to synergistically arrive to earlier, more effective interventions.

Based on current evidence both in humans and in animal models [24–29], we hypothesize that advances in nutrigenomics raise the chance to tailor low risk, personalized interventions through diet and/or micronutrient supplementation according to individual nutritional deficits, to optimize health and complement standard autism treatment. In this review, we summarize data on several promising micronutrients in terms of ASD therapeutic relevance, and list some of the most prevailing disturbances associated with vitamins and omega 3 fatty acids deficiencies in relation to their physiological actions and metabolic pathways.

Methods

A web literature search was conducted in PubMed, Science Direct and Cochrane databases for peer-reviewed English-language articles published from 2000 to the present time. The following keywords and their combinations (made by applying operators AND and OR) were used in the search: autism, nutrition, food selectivity, food allergy, vitamins, supplementation, vitamin A, vitamin B, pyridoxal, folate, folinic acid, cobalamin, vitamin D, vitamin K, omega 3, PUFA, DHA, EPA, PPAR, endocannabinoids, oxidative stress, neurodevelopment, neurotoxicity, methylation, epigenetic, environmental exposure. Reference lists of selected papers were consulted as well. Because randomized, double-blind placebo-controlled studies in large populations including both diagnosed and unrelated neurotypical participants are scarce, results from small studies were carefully analyzed to allow comparisons with larger-scale studies.

Epigenetics: environmental contribution and genetic susceptibility in ASD

Many authors refer an impact of pre- and/or perinatal exposure to different environmental stressors or nutritional deficits on neurodevelopment outcome, according to gene-variant susceptibility [11, 30–33]. Here we discuss metabolic pathways involved in increased neurodevelopmental vulnerability, based on known genetic predisposing factors.

Oxidative stress and methylation deficit

It is well known that reactive oxygen and nitrogen species (ROS and RNS respectively) damage DNA, proteins, and lipids, affecting cell signaling, gene expression, metabolism and ultimately, cell fate and viability [34]. Cellular antioxidant defense status varies according to age, nutritional status and genetics, the latter depending on polymorphisms of key genes coding for enzymes involved in specific pathways, considered next [35]. Several metabolic aspects of ≻l-γ-glutamyl-≻l-cysteinylglycine (glutathione; GSH), the main intracellular antioxidant, have been linked to the pathophysiology of ASD and other disorders. Nearly 90% of the cell and tissue GSH pool is in the reduced form and is crucial for thiol-redox homeostasis [36]. Less than 10% is oxidized, and the molar ratio between the reduced and the oxidized/dimerized (GSSG) forms of GSH is a sign of cell functionality and redox status. Increased plasma levels of homocysteine and GSSG, together with a decrease in cysteine, total GSH, and the GSH/GSSG ratio correlate with the severity of symptoms in autism [37, 38]. Impairment of GSH homeostasis, reflected by lower activities of glutamate cysteine ligase (GCL; the rate limiting enzyme in GSH synthesis), as well as glutathione peroxidase (GPX) and glutathione-S-transferase (GST), was observed in cerebellum samples from ASD individuals [39]. In addition, specific polymorphisms in the genes coding for GST (*rs1695* in *GSTP1*) and GPX (*rs1050450* in *GPX1*) have been linked to diminished antioxidant capacity in ASD [40, 41]. Abnormalities in other antioxidant enzymes, including superoxide-dismutase and/or catalase imbalance, [42, 43] as well as impaired bioenergetics associated to mitochondrial dysfunction and oxidative stress [44–46] have also been described in relation to autism.

DNA methylation and histone acetylation are key features of epigenetic modulation [47]. One-carbon (C1) metabolism (Figure 1) represents a central pathway linked to methylation, *de novo* nucleotide biosynthesis, transsulfuration, GSH metabolism, and redox homeostasis [48, 49]. The onset of autism symptoms was proposed to result from a redox/methylation deficit [50, 51]. Methyl groups for methylation reactions are provided by methionine-synthase (MS), requiring reduced cobalamin (vitamin B_{12}) as coenzyme. Under oxidative stress cobalamin is oxidized, so MS activity drops off and methylation deficit follows. Since MS modulates methyl donor S-adenosylmethionine (SAM) and acceptor S-adenosylhomocysteine (SAH) balance (SAM/SAH ratio), a pro-oxidant status can indirectly lead to hypomethylation, since it would divert homocysteine to GSH synthesis in order to cope with oxidative stress, thus decreasing SAM levels [52, 53]. Other methylation events dependent on the SAM/SAH ratio include protein and RNAs methylation, which in turn contribute to cell function through protein turnover and post-transcriptional gene expression regulation [54, 55]. A specific polymorphism in the MS gene (G allele for *rs1805087*) has been linked to increased autism risk, while significantly decreased levels of MS mRNA have been reported in cerebral cortex from autistic subjects, especially at younger ages [56, 57].

Impaired detoxification mechanisms have been proposed to contribute to ASD etiology [18, 21, 58]. About half of the thousands of chemicals found in consumer products lack developmental toxicity data, and many industrial and environmental chemicals detected on cord blood analyses (including heavy metals, pesticides and endocrine-disruptors) are able to alter chromatin structure, affect DNA methylation, and trigger developmental defects in animal models [59–61]. Thus, the weight of the evidence accumulated this far puts in the spotlight a fine interplay between genetic and epigenetic contributions to ASD

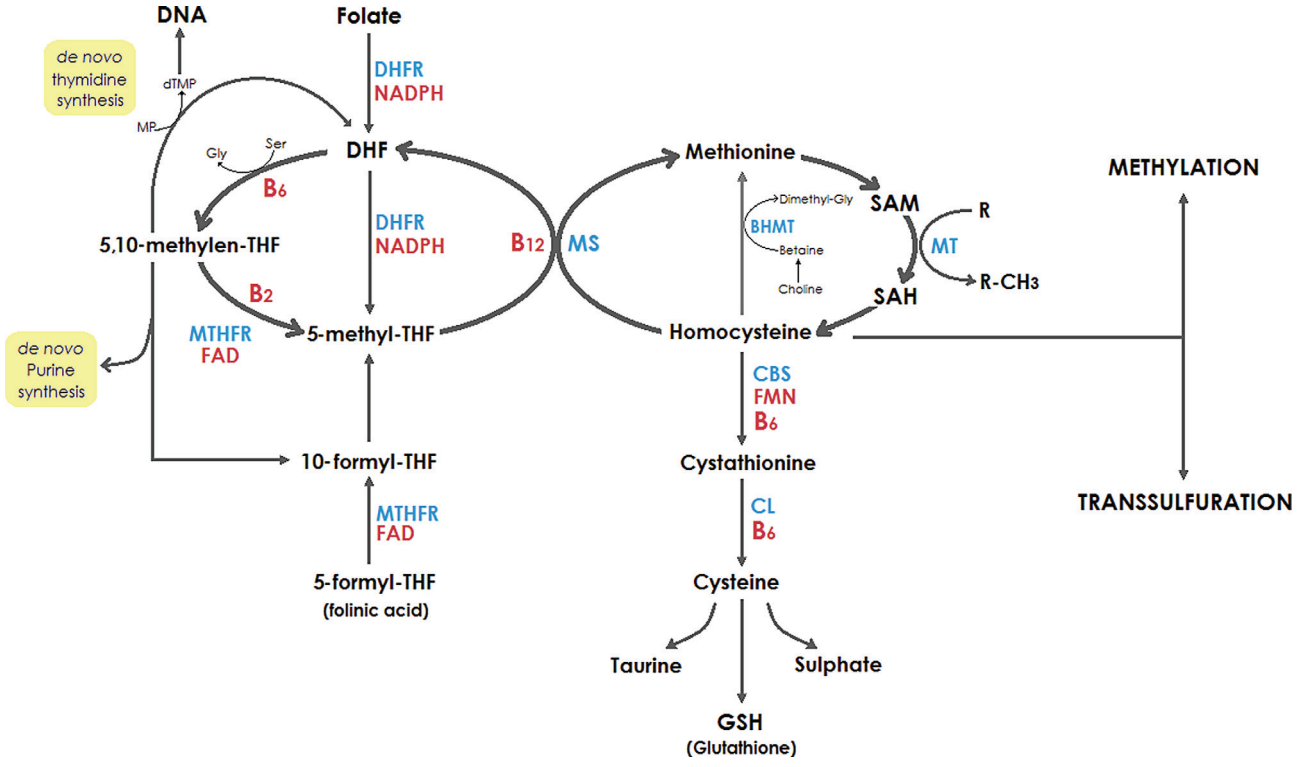

Figure 1. One-carbon (C1) metabolism. DHF (dihydrofolate), THF (tetrahydrofolate), SAM (S-adenosyl methionine), SAH (S-adenosyl homocysteine), DHFR (dihydrofolate reductase), MTHFR (methyl tetrahydrofolate reductase), BHMT (betaine-homocysteine *S*-methyltransferase), MS (methyl synthase); MT (methyl transferase), CBS (cystathionine-β-synthase), CL (cystathionine-γ-lyase), B_2 (riboflavin), B_6 (pyridoxal phosphate), B_{12} (cobalamin), NADPH (reduced nicotinamide adenine dinucleotide phosphate), FAD (flavin adenine dinucleotide), FMN (flavin mononucleotide).

phenotype and severity, emphasizing the need to identify environmental stressors in order to prevent exposure of both gestating mothers and newborns.

Nutritional deficit aspects

Our eating habits changed remarkably in the last decades, with a meaningful decrease in the quality of our energy source input derived from increased consumption of processed, rather than raw or minimally processed foods. The Western diet, enriched in refined carbohydrates, cholesterol, and trans-saturated fats, jeopardizes the supply of key micronutrients such as vitamins, minerals, and essential fatty acids needed for optimal metabolism and health. Worryingly, most children with autism present with food selectivity and/or food allergies [62–64], which compromises their nutritional status and magnifies genetic susceptibility in the presence of gene polymorphisms affecting micronutrient metabolism, or even major metabolic pathways such as glycolysis [65, 66]. Genetic variants related to specific micronutrients and increased susceptibility are considered in the next sections.

Vitamins are essential micronutrients. Although vitamin D can be synthesized by the body upon sunlight exposure, current lifestyle leads to suboptimal endogenous synthesis making it necessary to increase intake, in order to achieve optimal levels (further addressed in section 4.3). All other vitamins or their precursors must be acquired from the diet. An adequate vitamin supply is particularly important since vitamins influence every metabolic pathway, either as coenzymes, transcription factor ligands, or antioxidants. Consequently, several aspects of metabolism may be compromised due to the "picky" eating pattern frequent in children with ASD [67, 68]. In view of recent supplementation studies targeting subjects with autism and pregnant women [24, 26–29, 33, 69–76] (Table 1), in the following sections we will address some of the most relevant micronutrients, in terms of therapeutic relevance for ASD and proper neurodevelopment.

Vitamin deficiencies in ASD

Vitamin B_6, B_9, and B_{12}

These vitamins are water-soluble, have a pivotal role in C1 metabolism and hence in redox status and methylation (Figure 1) [48].

Table 1. Overview of main findings in vitamin supplementation studies in humans, regarding ASD risk or ASD outcome

Reference	Parcitipants	Supplementation type	Intervention lapse	Conclusion/key findings	Study Limitations
Adams JB et al. [24]	65 ASD (3 to 58 y.o.) subjects and 50 non-sibling, neurotypical sex and age matched controls.	Multivitamin and mineral complex, omega3, carnitine, digestive enzymes, antioxidants, healthy gluten-soy-casein-free diet (HGSCF diet), added sequentially.	12 months	Improved nutritional status, non-verbal IQ, autism symptoms, and other symptoms in most individuals with ASD. Parents reported that the vitamin/mineral supplements, essential fatty acids, and HGCSF diet were the most beneficial.	Study of exploratory nature and small sample size. All participants received all treatments. Due to specific diet implementation suggestion, blinding was incomplete.
Mousain-Bosc M et al. [74]	33 Pervasive developmental disorder (PDD) subjects (mean age 4 y.o.) and 36 matched controls.	Magnesium-vitamin B_6 (Mg-B_6) (6 mg/kg/day Mg and 0.6 mg/kg/day vit B_6).	6 months	Supplementation improved PDD symptoms in 23/33 children with no adverse effects: social interactions (23/33), communication (24/33), stereotyped restricted behavior (18/33), and abnormal/delayed functioning (17/33); 15/33 children improved in the first three groups of symptoms. PDD symptoms reappeared few week after treatment was stopped.	Small sample size. Lack of placebo arm, randomization and control subjects supplementation.
Frye RE et al. [28]	48 ASD + language impairment subjects (7,4 y.o.; 82% male).	Randomized. 25 subjects were supplemented with folinic acid 2 mg/kg/day (maximum 50 mcg/day), and 23 subjects with placebo.	3 months	High-dose folinic acid for 12 weeks resulted in improvement in measures of verbal communication as compared with placebo, specially in children positive for folate receptor alpha autoantibodies (FRAA).	Small sample size. Single-site design allows limited generalization of results. Safety of this treatment requires further study to determine the optimal folinic acid dose. Oral folate bioavailability depends on enteric microbiome, altered in children with ASD.
Raghavan R et al. [27]	1257 mother-infant pairs included (86 ASD cases and 1171 children with neurotypical development).	Folate and B_{12} supplementation during pregnancy.	Prospective cohort study. Recruitment in 1998 at Boston Medical Center and follow up until 2013.	Moderate (3-5 times/week) supplementation pregnancy associated with decreased risk of ASD. Low ($\leq$2 times/week) and high (>5 times/week) supplementation associated with increased risk of ASD. Highest ASD risk in children of mothers with both plasma folate and B_{12} elevated values.	Self-reported supplementation data. Lack of maternal dietary intake data previous to conception. Case- and neurotypical development classification not based on research-reliable gold standard diagnostic assessments (based on medical records).
Hendren RL et al. [71]	57 ASD subjects (mean age 5,3 y.o.; 79% male).	Participants randomly assigned to methyl B12 (75 μg/kg) supplementation or saline placebo every 3 days by subcutaneous injection.	2 months	Methyl B_{12} treatment improved ASD symptoms rated by clinician.	Small sample size. Lack of control group.

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Table 1. Overview of main findings in vitamin supplementation studies in humans, regarding ASD risk or ASD outcome (Continued)

Reference	Parcitipants	Supplementation type	Intervention lapse	Conclusion/key findings	Study Limitations
Feng J et al. [26]	215 ASD subjects (mean age 4,76 y.o.; 173 male, 42 female = 4:1) and 285 Control (mean age 5,12 y.o.).	Vit D_3 150.000 UI/month (intramuscular injection) + 400 UI/day (oral).	3 months	Vitamin D_3 supplementation improved clinical outcome, especially in the younger children with ASD.	Sunlight exposure was not assessed. Lack of placebo arm, blindness, randomization and control group supplementation.
Saad K et al. [72]	122 ASD subjects (mean age 5,09 y.o.) and 100 matched controls (mean age 4,88; 75% male).	Open label trial. 106 ASD subjects with low serum 25-OH-vitaminD levels (<30 ng/ml) supplemented with vitamin D_3 300 IU/kg/day.	3 months	81% of subjects with improved outcome. Improvement in irritability, lethargy/social withdrawal, hyperactivity, and stereotypic behavior.	Small sample size. Lack of placebo arm, randomization and control subjects supplementation.
Stubbs G et al. [73]	19 pregnant women with a previously ASD diagnosed child, and their newborns.	Open label trial. Mothers supplemented with vitamin D_3 5000 IU/day during pregnancy, newborns supplemented with 1000 IU/day for 3 years (oral).	3 years	Only 1 out of 19 (5%) newborns developed autism in contrast to the recurrence rate of approximately 20% reported in the literature.	Small sample size. Lack of control group, placebo, blinding and randomization.

Vitamin B_6 or pyridoxal (PL)

Pyridoxal (PL) is required for over 160 different catalytic reactions spanning amino acid and neurotransmitter synthesis and degradation, tryptophan catabolism (involved in melatonin and serotonin synthesis), and nicotinamide adenine dinucleotide (NAD^+) synthesis [77]. The active form of vitamin B_6 is pyridoxal 5'-phosphate (PLP). Absorption kinetics for PLP and other phosphorylated B_6 vitamers proceeds by a series of tissue-specific, tightly regulated dephosphorylation and rephosphorylation reactions [78]. PLP is also required as coenzyme for glutamic acid decarboxylase (GAD) responsible for gamma aminobutyric acid (GABA) synthesis from glutamate (Glu). Glu and GABA are, respectively, the major excitatory (E) and inhibitory (I) neurotransmitters in the adult brain. An imbalance in E/I neurotransmission has been observed in ASD and has been linked to epilepsy, a frequent ASD comorbidity. Vitamin B_6-dependent seizures, triggered by PLP deficiency and reduced GAD activity, contribute to increased Glu and decreased GABA signaling and may underlie epileptic episodes in children with autism [79–81].

The role of B_6 metabolism on ASD remains controversial, since either low or high plasma total B_6 levels have been reported in patients with autism [82–84]. Increased B_6 levels could result from polymorphisms involving pyridoxal kinase [84] or tissue nonspecific alkaline phosphatase, another enzyme related to B_6 metabolism [85]. Despite this uncertainty, vitamin B_6 plus magnesium supplementation was reported to ameliorate ASD core symptoms; strikingly, autistic traits reappeared a few weeks after supplement discontinuation [74]. However, B_6 supplementation studies in autism are scarce, and methodological limitations such as small number of participants or lack of placebo arm are common [86, 87]. Still, considering the potential benefits and low cost of B_6-Mg supplementation, future research should address this matter in order to safely recommend it as a therapeutic complement upon individual assessment.

Vitamin B_9 or folate

The term vitamin B_9 refers to a family of molecules derived from folic acid. Folic acid does not exist in nature but can result from folate oxidation and is also the synthetic form found in most dietary supplements and fortified foods. Tetrahydrofolate (THF) and 5-methyl-tetrahydrofolate (5-MTHF) are the biologically active molecules, so other members of the family need to undergo chemical transformation by dihydrofolate reductase (DHFR), in the case of folic acid, or by methylene tetrahydrofolate reductase (MTHFR), for folinic acid and other reduced forms of folate (Figure 1). The folate cycle is the key motor of C1 metabolism. 5-MTHF is the donor of reactive one-carbon units used by MS to methylate homocysteine to methionine, which in turn is converted into *S*-adenosyl-methionine (SAM), the

universal methyl donor species for most methylation reactions in the cell. Dietary folate is absorbed by enterocytes by two carriers, the proton-coupled folate transporter (PCFT) and the reduced folate carrier (RFC). In most tissues, folate uptake involves mainly four folate receptors (α-FR, *β*-FR, *γ*-FR, δ-FR) with differential expression among tissues [88, 89]. Given folate's central role in C1 metabolism, many aspects of its chemistry and genetics have been an active topic in autism research. To this extent, different polymorphisms in *MTHFR* (C677T and 1298AC) [90], *DHFR* (19 bp deletion) [91], and the maternal *RFC1* gene (maternal G allele in A80G) for the reduced folate carrier were associated with increased ASD risk [92]. Evidence suggests that folate bioavailability may also be compromised in ASD. 5-MTHF accounts for over 90% of circulating folate and binding to the aforementioned carrier proteins allows it to cross the blood-brain barrier (BBB). While α-FR shows high affinity for folate and is the main transport mechanism across the BBB, RFC has lower affinity and is the main carrier in neurons within the central nervous system (CNS). Several studies showed a high prevalence of serum autoantibodies against α-FR (FRAA) not only in ASD cases, but also in their parents and typically-developing (TD) siblings [93, 94]. Furthermore, in double-blind placebo controlled trials, supplementation with *folinic* acid (which is transported by RFC across the BBB) improved core ASD symptoms, especially verbal communication [28]. Current research in this field will hopefully provide a new treatment option to complement speech and behavior therapies.

Vitamin B_{12} or cobalamin

Cobalamin is a coenzyme for MS in C1 metabolism, and also for mitochondrial methylmalonyl-CoA mutase, the enzyme that converts methylmalonyl-CoA into succinyl-CoA, which connects vitamin B_{12} to bioenergetics as well. Clinical B_{12} deficiency presents with classic hematological (e.g. pernicious anemia) and neurological manifestations. Dietary cobalamin is absorbed and transported through a series of carrier proteins and receptors that may be involved in vitamin B_{12} deficits. For example, mutations in the protein amnionless, part of the cubam B_{12} receptor, can lead to Imerslund-Gräsbeck syndrome and impair vitamin B_{12} transport into the CNS [95]. Under oxidative stress, the cobalt atom in cobalamin is oxidized, inactivating B_{12} in an irreversible way. In most cell types, oxidized cobalamin is remethylated by a methyltransferase, with SAM as the methyl donor. In neurons, however, oxidized cobalamin dissociates from MS and must be replaced by another reduced cobalamin molecule [50, 96]. In fact, B_{12} was shown to be an efficient superoxide anion scavenger, which could contribute to B_{12} depletion upon inactivation by oxidation [97]. Concerning ASD, significantly lower cobalamin levels, similar to those of elderly subjects, were found in postmortem frontal cortex from children with autism compared to age-matched controls [53]. Plasma levels of cobalamin in ASD subjects were reported to be normal, so lower cortex level could imply deficient transport across the BBB. In this regard, a lower affinity binding polymorphism for B_{12} plasma carrier transcobalamin II was identified [98], the frequency of the homozygous genotype for this variant was found to be 10% higher in ASD versus TD children, increasing autism risk 1.7-fold [37]. Furthermore, polymorphisms in the genes coding for cubilin, also part of the cubam receptor [99], and the renal receptor LRP2 [100] showed that cobalamin homeostasis is crucial for normal neurodevelopment. Interestingly, LRP2 and cubilin are also involved in α-FR-folate complex endocytosis, confirming the importance of folate- and B_{12}-dependent DNA methylation for normal development [101]. Although multivitamin supplementation is now commonly recommended during pregnancy, and folate supply has been shown to decrease neural tube defects, appropriate vitamin intake limits should be considered: whereas extremely high maternal plasma folate and B_{12} levels at birth were linked to increased autism risk, moderate concentration in maternal blood was shown to reduce autism risk in offspring [27]. Finally, B_{12} supplementation trials on ASD population are scarce, and only one randomized trial of methyl-B_{12} showed improvement [71]. The study in question indicated better methylation capacity associated to increased SAM/SAH ratio, although no significant behavioral changes were observed in parent-rated ABC (aberrant behavior checklist) and SRS (social responsiveness scale) scores.

Vitamin A

Vitamin A is liposoluble and has three active isoforms, retinol, retinal, and retinoic acid (RA), the latter being an important transcriptional regulator. Vitamin A actions include antioxidant defense as a free radical scavenger, cell growth and differentiation, and immune function [102, 103]. Vitamin A is stored as retinyl esters mainly in the liver, and hydrolyzed to retinol according to physiological needs (Figure 2). Diet-related vitamin A deficiency is prevalent in children with autism [67]. In addition, a small number of ASD cases present E3 ubiquitin ligase (UBE3A) hyperactivity, leading to excessive ubiquitylation of retinaldehyde dehydrogenase (ALDH1A; the rate limiting enzyme in RA synthesis), reduced RA signaling, and impaired RA-mediated synaptic plasticity. Accordingly, ASD-like symptoms were observed in mice overexpressing UBE3A in the prefrontal cortex or administered an ALDH1A antagonist, while RA supplementation alleviated the autistic phenotype [104]. RA nuclear receptor alpha (RARα) plays a crucial role in nervous system development,

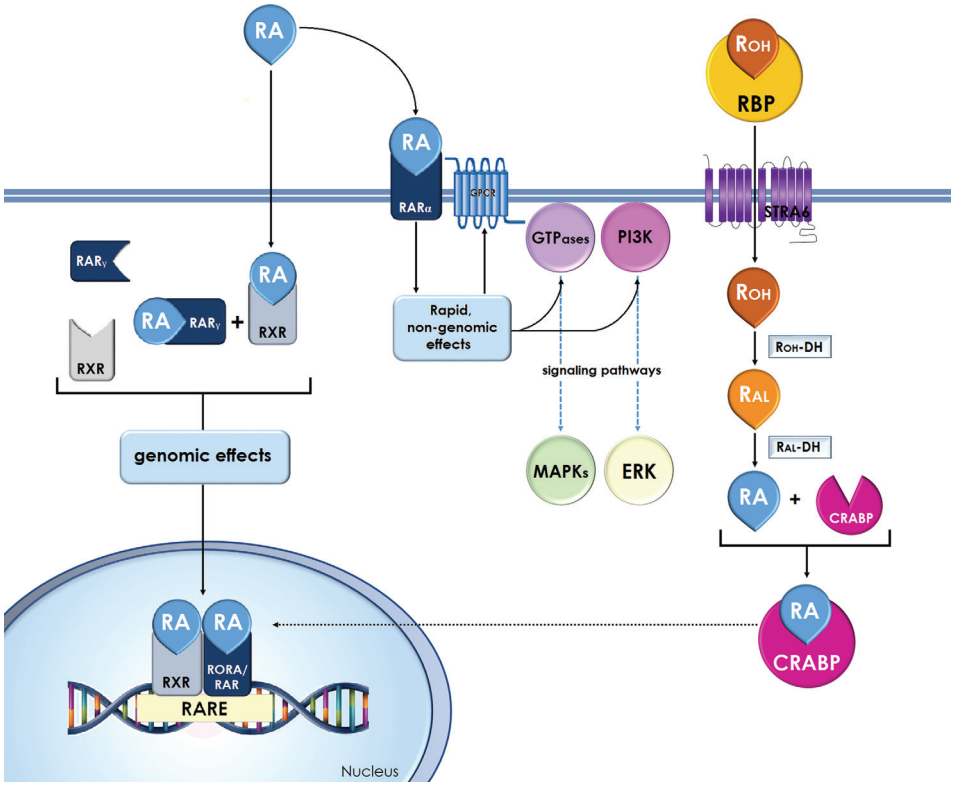

Figure 2. Vitamin A genomic and non-genomic actions. CRABP (cellular retinoic acid binding protein), GPCR (G-protein coupled receptor), MAPKs (mitogen-activated protein kinases pathway), ERK (extracellular signal-regulated kinase pathway), PI3K (phosphatidylinositol 3-kinase pathway), RA (retinoic acid), R_{AL} (retinaldehyde), R_{AL}-DH (retinaldehyde dehydrogenase), RARE (retinoic acid response element), RAR (retinoic acid receptor alpha), RARγ (retinoic acid receptor gamma), RBP (retinol binding protein), R_{OL} (retinol), R_{OL}-DH (retinol dehydrogenase), RORA (retinoic acid related orphan receptor), RXR (retinoid X receptor), STRA6 [stimulated by retinoic acid gene 6 homolog (mouse)] [103, 189].

learning, and memory, through epigenetic modifications, particularly by modulating histone acetylation in the hippocampus. In vitamin A-deficient rats, significant lower histone acetylation was correlated with impaired learning and memory, pinpointing the relevance of vitamin A intake during pregnancy and childhood to prevent learning and memory decline in adulthood [105].

ASD prevalence is 4 times higher in males than females, a bias suggested to be related to high fetal testosterone levels in boys [106]. Decreased levels of aromatase, the enzyme that converts androgens to estrogens, were detected in the frontal cortex of individuals diagnosed with ASD. Studies suggested that this finding may be secondary to alterations in nuclear RA-related orphan receptor alpha (RORA) expression, which modulates aromatase activity and is in turn modulated by vitamin A levels [107]. Since *RORA* activity is regulated in an opposite fashion by sex hormones, with dihydrotestosterone (DHT) suppressing and estradiol promoting its expression [108], this model provides a plausible answer for increased testosterone levels in the ASD male brain tissue, while higher estrogen levels in females might buffer RORA suppressing agent [107] or else vitamin A deficiency. RORA has also been associated to circadian rhythm control (frequently altered in ASD) and neuroprotection against oxidative stress and inflammation [109, 110]. Noteworthy, DNA methylation and immunohistochemical analyses demonstrated prevalence of RORA hypermethylation in lymphoblasts from children with autism, while low expression of *RORA* (and other regulators of the circadian rhythm) in ASD brain tissue was linked to severe language impairment [111]. Collectively, these findings suggest that addressing vitamin A deficiency may prevent or alleviate ASD symptoms.

Vitamin D

This liposoluble vitamin is traditionally known for its role in calcium and phosphorus homeostasis and skeletal health. However, interest in its role influencing gene expression has grown over the last two decades, after ~2000 genes were found to encompass vitamin D response elements (VDREs) within their regulatory regions. Vitamin D is an active neurosteroid that plays crucial neuroprotective roles in the developing brain, participating in cell proliferation and differentiation, immunomodulation, regulation of

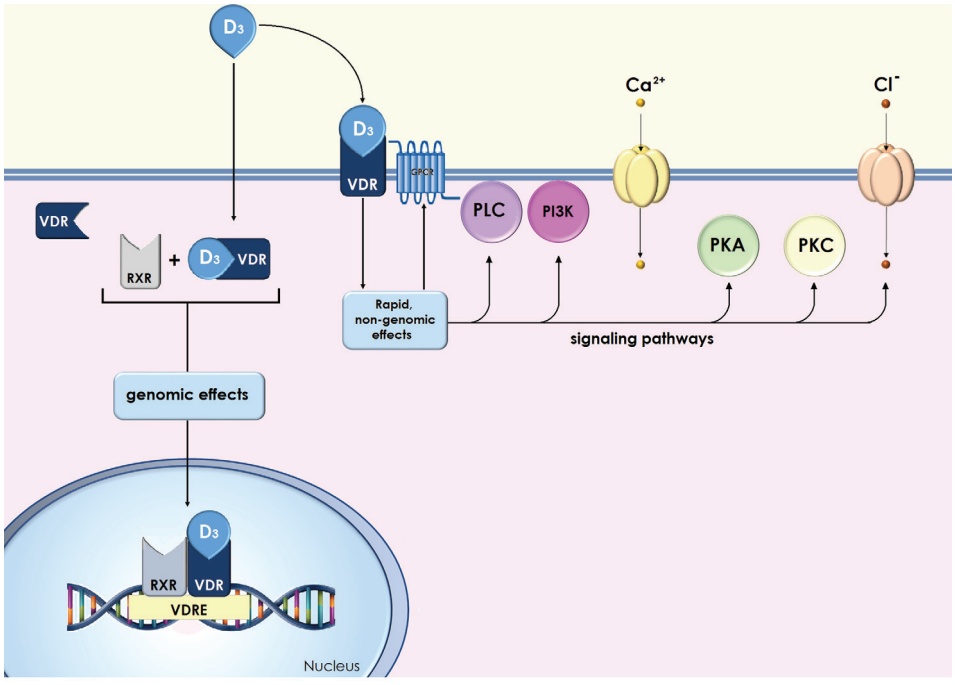

Figure 3. Vitamin D genomic and non-genomic actions. D3 (calcitriol), GPCR (G-protein coupled receptor), PI3K (phosphatidylinositol 3-kinase pathway), PKA (protein kinase A), PKC (protein kinase C), PLC (phospholipase C), RXR (retinoid X receptor), VDR (vitamin D receptor), VDRE (vitamin D response element) [190, 191].

neurotransmission, and steroidogenesis [112, 113]. Vitamin D is the only vitamin that can be synthesized *de novo* by humans. Nutritional vitamin D intake represents roughly 10% of daily needs, so most of our vitamin D requirement must come from endogenous sources. In the skin, 7-dehydrocholesterol renders pre-vitamin D_3 upon ultraviolet irradiation exposure, which is converted into vitamin D_3 or cholecalciferol [114]. The dietary vitamin D from vegetable origin is D_2 or ergosterol. Both D_2 and previtamin-D_3 reach the liver through the bloodstream bound to the vitamin D transporter protein DBP, to be hydroxylated by hepatic D-25-hydroxylase (CYP2R1) to 25-hydroxy-vitamin D (25-OH-D) or calcidiol, the main circulating form of vitamin D. Next, calcidiol is further hydroxylated to 1,25-dihydroxy-vitamin D (1,25-diOH-D) or calcitriol by renal 25-OH-D-1 -hydroxylase (CYP27B1). Although CYP27B1 was believed to be expressed only in the kidney, it was later found in many other cells and tissues including brain, immune cells such as macrophages and T-lymphocytes, and placenta, highlighting a more general role for vitamin D as an autocrine or paracrine modulator of gene expression, versus the endocrine role of renal calcitriol [115]. Calcitriol exerts its actions by binding to cellular vitamin D Receptor (VDR), with both genomic and non-genomic effects (Figure 3). In line with current scientific evidence, normal range minimun level for serum 25-OH-D has recently changed to 30 ng/ml (75 nmol/l) and values from 40 to 60 ng/ml (100 to 150 nmol/l) are considered optimal for health benefits. Serum levels between 20 and 29 ng/ml are considered as vitamin D insufficiency, while scores under 20 ng/ml indicate vitamin D deficiency [116]. Remarkably, it seems likely that the prevailing medical advice against sunlight exposure (tempered by recommendation of preventive measures, i.e. sunscreen protection) to reduce skin cancer risk has actually contributed to widespread D hypovitaminosis. Achieving optimal vitamin D serum levels from supplements alone would demand 1000-4000 IU/day for adults, while most "over-the-counter" supplements provide in average 500 IU/dose. Cannell [117] was the first to propose a link between increased autism rates and gestational/early childhood vitamin D deficiency caused by medically-recommended sun avoidance. Supporting this theory, several studies from different latitudes suggest that ASD prevalence rates tend to be the lowest in countries near the Equator and to increase moving poleward (reviewed by [118]). Vitamin D is crucial not only for proper neurodevelopment but also for brain function, and several neuropsychiatric conditions have been related to vitamin D status [119–123]. Vitamin D contributes to antioxidant defense mechanisms by increasing GSH synthesis, thus improving redox status [124, 125]. Concerning calcitriol homeostasis, functional polymorphism analyses revealed an increased ASD risk for the AA genotype of the *GC* gene encoding DBP [126], a variant previously associated with lower plasma 25-OH-D levels [127]. Similarly, the GG genotype for the *CYP2R1* gene coding

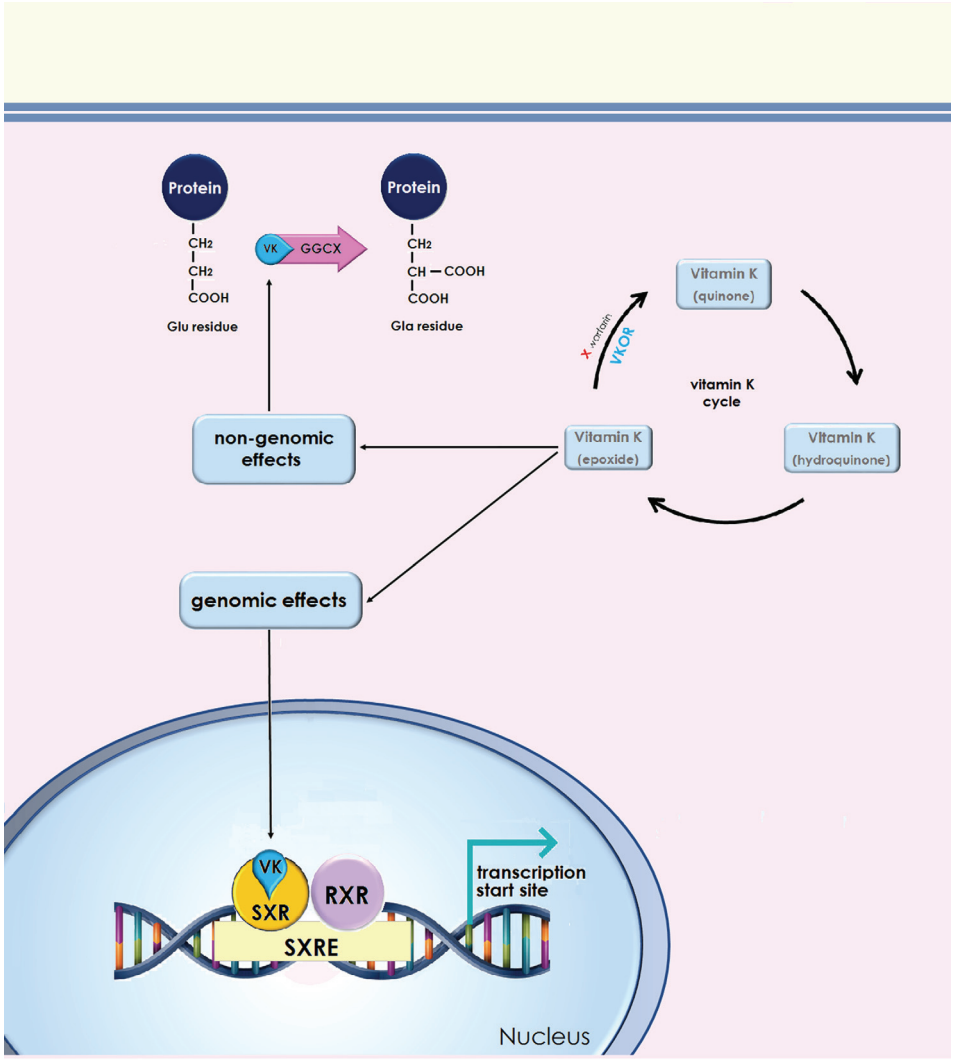

Figure 4. Vitamin K genomic and non-genomic actions. VK (vitamin K), GGCX (gamma-glutamyl carboxylase), Glu (glutamic acid), Gla (γ-carboxyglutamic acid), RXR (retinoid X receptor), SXR (steroid and xenobiotic nuclear receptor), SXRE (steroid and xenobiotic response element) [192].

25-D-hydroxylase was also linked to higher ASD risk after being associated to reduced 25-OH-D plasma concentrations [128]. Two paternal and child variants in the *VDR* gene (*TaqI* and *BsmI*) were also linked to increased risk for ASD [126, 129, 130]. Meanwhile, lower 25-OH-D plasma levels in ASD versus TD [131, 132], as well as a relationship between decreased 25-OH-D level and ASD severity [133, 134] have been demonstrated. Thus, as evidence of higher risk of autism due to maternal vitamin D deficit keeps growing [135, 136], several randomized trials reported significant improvement in core symptoms of autism upon vitamin D_3 supplementation [26, 72, 137].

The recurrence prevalence for ASD is nearly 20% in siblings [138], but according to a small study, vitamin D supplementation in both expecting mothers who already had a child diagnosed with autism (5000 IU/day) and their newborns (1000 IU/day until the age of 3), decreased the expected incidence to 5% [73]. In agreement with these findings, earlier research associated maternal 25-OH-D insufficiency during pregnancy with offspring language impairment and suggested that gestational vitamin D supplementation may reduce this outcome [139]. Given the lack of negative result reports upon supervised vitamin D supplementation, and documented high cost-effectiveness, larger randomized trials are warranted.

Vitamin K

Although recent research has focused on investigating associations between vitamin K status and several health outcomes, the therapeutic potential of vitamin K in ASD has remained so far unexplored.

Vitamins K_1 (phylloquinone) and K_2 (menaquinone) are liposoluble molecules with a wide action repertoire including epigenetic function, calcium metabolism regulation, coagulation, oxidative stress, inflammation, and cell growth and proliferation. There is also a water-soluble synthetic form of vitamin K known as K_3 or menadione, which is turned into K_2 upon isoprenylation in the liver by UbiA prenyltransferase domain containing protein 1 (UBIAD1). UBIAD1 is involved in the biosynthesis of both vitamin K_2 and coenzyme Q10, and exhibits multiple subcellular localizations including mitochondria, endoplasmic reticulum, and Golgi [140]. The metabolism of dietary vitamin K is represented in Figure 4. Compared to other liposoluble vitamins, the vitamin K reserve is low; given its central role in coagulation, a vitamin K cycle is at work to prevent depletion in case of insufficient dietary intake. Vitamin K is a coenzyme for microsomal γ-glutamyl carboxylase, which carboxylates glutamate residues of target proteins to enable calcium binding (Figure 4). Because of this post-translational modification of Glu to γ-carboxyglutamic acid (Gla) residues, these proteins are collectively known as Gla-proteins or vitamin K dependent proteins (VKDP). About 14 VKDP have been identified; half of them are produced in the liver and participate in blood coagulation, and the rest contribute to bone remodeling (including cell proliferation and differentiation) and tissue calcification [141, 142]. Vitamin K_1 draw attention and was first described because of its antihemorrhagic role, but lately K_2 gained interest as a plausible aid in bone health and cardiovascular disease given its role not only in regulating bone remodeling but also in the calcification of soft tissue such as blood vessels [143]. Bone health in young ASD individuals has been overlooked compared to behavioral, cognitive or social issues. Lower bone mineral density (BMD) was observed in peripubertal boys with ASD, while a higher fracture rate was described in both children and adults (male and female) with autism. Additionally, compared to TD children, boys with ASD showed lower protein, calcium, and phosphorus intake in association with lower BMD [144–147]. Vitamin K_2 has been proposed as a therapeutic agent for osteoporosis treatment. Evidence accumulating over the last few years suggested a synergistic effect of vitamin D_3 and K_2 co-supplementation on bone metabolism, calcium bioavailability, and cardiovascular health [143, 148, 149]. On the other hand, a study showed that vitamin K_2 improved anxiety and depression in an animal model of metabolic syndrome [150]. Furthermore, vitamin K_2 was shown to provide neuroprotection after neurotoxin exposure [151] and to potentially prevent neurodegenerative diseases such as Alzheimer's and Parkinson's through anti-apoptotic and antioxidant effects [152, 153]. Along these lines, synthetic K_3 was found to modulate amyloid plaque formation kinetics by inhibiting protein aggregation, conferring neuroprotection against amyloid-induced cytotoxicity [154].

Two Gla-proteins (Gas6 and Protein S) were shown to critically influence neurite growth, redox status, and anti-inflammatory responses in the developing nervous system. Gas6 is also involved in the synthesis of sphingolipids, and vitamin K deficiency was shown to decrease ceramide and sphingomyelin synthesis and increase gangliosides content in the rat brain [155, 156].

As mentioned above, mitochondrial dysfunction and bioenergetics failure are common events in ASD. Ubiquinone, a vitamin K-related molecule, is a key component of the electron transport chain. In fact, vitamin K_2 was proposed to act as a mitochondrial electron carrier between electron-donating and electron-accepting enzyme complexes, contributing to normal ATP synthesis and restoring mitochondrial function [157, 158].

No vitamin K supplementation trials have been conducted in subjects diagnosed with ASD, thus future research should address this matter, considering the potential metabolic gain.

Omega-3 polyunsaturated fatty acid (n-3 PUFA) deficit and ASD

Restricted food range and nutritional habits in people with autism also compromise essential fatty acids proper intake. Lipids from diet, particularly n-3 and n-6 polyunsaturated fatty acids (PUFA) are known to influence plasma membrane lipid composition and lateral domain organization, affecting membrane proteins partition and thus, cell signaling and cell metabolism [159–161]. Our ancestry's diet involved an n-6/n-3 ratio fairly close to 1:1, but in recent decades modern lifestyle changed the odds up to 20:1 in favor of n-6, resulting in a plethora of health consequences [162]. N-3 PUFA are enriched in oily marine fish and sea food, which are a rare food choice among individuals with autism. Today we know PUFA modulate physiology in many ways. For instance, eicosanoids derived from 20:4 n-6 arachidonic acid (ARA) (e.g. prostaglandin E2 and leukotriene B4) are stronger pro-thrombotic and pro-inflammatory agents than eicosanoids derived from 20:5 n-3 eicosapentenoic acid (EPA) (prostaglandin E3 and leukotriene B5), actually considered anti-inflammatory. In fact, n-3 PUFA give rise to lipoxins, resolvins, maresins and protectins, collectively known as specialized pro-resolving mediators or SPMs that return the system to homeostasis after an inflammatory event. It is interesting that failure of this final stage of resolution of the

inflammatory response could lead to chronic (neuro)inflammation, a condition often linked to ASD [163–166]. Moreover, 22:6 n-3 docosahexanoic acid (DHA) bioaccumulates in brain tissue since the third trimester during pregnancy and on through the lactation period, accounting for about 30% of brain fatty acids, while EPA level is kept low and tightly regulated by beta-oxidation denoting precise functions in the CNS [167]. A study in an animal model showed that maternal deprivation of n-3 PUFA during pregnancy and lactation affected neurogenesis and apoptosis in adult offspring, involving increased DNA methylation of the BDNF coding gene. This outcome did not revert upon restoring n-3 PUFA supply after weaning, suggesting a role of n-3 PUFA in long-term imprinting [168]. By modulating membrane biophysical properties, PUFA also influence neurotransmission by regulating vesicular release from presynaptic cells and receptor partition in the postsynaptic membrane as well, due to lipid domain organization and membrane fluidity [169, 170]. Endocannabinoids (eCB) constitute another neurobiological relevant PUFA-derived group of molecules, which perform their action by binding to cannabinoid receptors (CBR) types 1 and 2; CBR1 is highly expressed in the nervous system, while CBR2 is mainly (but not exclusively) found outside the CNS. The endocannabinoid system plays an important role in learning and memory by restraining neurotransmitter release in the presynaptic neuron [171]. The eCB system is also a key modulator of the immune system through CBR2, highly expressed in macrophages and microglia, and is noteworthy that CBR2 signaling was found to be upregulated in mononuclear cells derived from autistic children's blood samples [172, 173]. In addition, n-3PUFA bind to certain neurotransmitters (e.g. DHA- or EPA-serotonin, DHA- or EPA-dopamine) resulting in eCB-like molecules with novel physiological roles that merit further investigation in the near future, but have already been linked to inflammation, cancer and pain [174]. PUFA also modulate gene expression as ligands of a nuclear transcription factors family, the peroxisome proliferator-activated receptors (PPAR) with three members, PPARα, PPARβ/δ and PPARγ, expressed in different organs and tissues including adipose, liver, muscle and brain. These transcription factors are involved in lipids homeostasis, energy metabolism [175, 176], inflammatory response [177], and neurologic functions such as memory, learning and behavior [178], all of which have been previously related to autism. Like liposoluble vitamins intracellular receptors, PPAR require heterodimerization (e.g. with RXR) in order to bind to response elements in DNA and modulate gene expression. Interestingly, recent research suggests that PPAR agonists should be further explored as complementary treatment, since animal and small sized studies in humans showed improvement in cognitive function, behavior and biochemical indicators of oxidative stress and inflammation [76, 179–183].

Decreased n-3 PUFA plasma levels have been correlated with several neuropsychiatric disorders, including ASD [184], proving their crucial role in neurodevelopment and proper neurologic function. The role of EPA and DHA in neurodevelopment and their epigenetic contribution to autism (and ADHD), as well as a summary of the available supplementation trials has been recently and extensively reviewed by Martins et al. [75]. They highlight that the Food and Drug Administration (FDA) of the United States has recently increased the suggested amount of fish consumption for women considering getting pregnant, pregnant or nursing, and they conclude that n-3 PUFA supplementation is an option for mothers with inadequate dietary intake as well as fortification of formula, considering the benefits and the lack of serious adverse effect reports (mainly mild gastrointestinal symptoms). Supporting supplementation in autistic subjects, certain polymorphism for the rate limiting enzymes responsible for the synthesis of n-3 and n-6 PUFA from essential fatty acids (fatty acid desaturases 1 and 2, and elongase 2; FADS1, FADS2 and ELOVL2 respectively) were analyzed and related to autism risk. A study showed that the A/A genotype of *rs10498676* in *ELOVL2* and the G allele for *rs526126 in FADS2* actually correlated with a decline in the Autism Diagnostic Interview-Revised communication (verbal and nonverbal) domain, in Chinese children, suggesting lower risk for carriers [185]. Moreover, the G allele for *rs526126 in FADS2* was linked to lower levels of ARA [186]. Considering different variants for FADS and/or ELOV could modulate PUFA levels and hence their derivatives (in spite of adequate essential fatty acids intake), n-3 PUFA level should be assessed in order to guarantee metabolic needs and avoid adverse downstream consequences.

Concluding remarks and future guidelines

Autism is a very complex, highly heterogeneous and multifactorial disorder with symptoms varying in occurrence and severity. Variation depends on individual genotype *x* environment interactions, resulting in an aptly called *spectrum* of phenotypes. As mentioned, there is a large number of genes and variants linked to autism and yet, most cases are idiopathic, highlighting the impact of both environment and interindividual genetic differences. Exposure to certain risk factors is in many cases inevitable (e.g. air and water chemical contamination derived from industrialization).

However, one major risk factor that we can handle is micronutrient deficiency. Eating disorders, food selectivity and food allergies are common in ASD, making it difficult to introduce diet modifications towards improved nutrition, and nutrigenomics is certainly a powerful tool in this regard. Tackling nutritional deficits in ASD is a worthy effort that entails little risk and many potential benefits. As knowledge in this field continues to grow, it becomes clear that both genetic risks and nutritional status should be addressed in order to characterize and attend individual deficiencies through supervised, tailored supplementation plans suited to specific needs, since food choice range varies among subjects and hence, interindividual deficiencies as well. Additionally, scientific evidence on the link between gastrointestinal health, the gut microbiome, and their impact on nervous system function in autism is growing (see [187, 188] for review), although its analysis exceeds the scope of this manuscript. Nevertheless, gastrointestinal issues including dysbiosis, should be assessed before initiation of macro- or micronutrient supplementation regimens.

This work summarized current evidence on selected vitamins and omega-3 fatty acids regarding potential impact on ASD, therapeutic uses, and the status of supplementation trials. Some systematic reviews and meta-analyses question the relevance of most supplementation and/or environmental risk factor studies due to methodological limitations such as small sampling size, or lack of placebo arm or neurotypical control group. However, a growing number of investigations suggest the strong association of environmental factors with both autistic traits and the global increase in ASD prevalence, meriting therefore our full attention.

Today, autism treatment predominantly focuses on lifetime behavior modification therapies, aimed to lower symptoms severity and improve communication and social skills to promote autonomy, independence, and social inclusion. Although further research and thoughtful experimental design is needed, we feel nutritional intervention will help complement conventional interdisciplinary treatment to improve physical and cognitive results and increase life quality for people with autism and their families.

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