Autism and nutrition: the role of the gut–brain axis

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Abstract

Autism spectrum disorder (ASD) is characterised by deficits in the ability to socialise, communicate and use imagination, and displays of stereotypical behaviour. It is widely accepted that ASD involves a disorder in brain development. However, the real causes of the neurodevelopmental disorders associated with ASD are not clear. In this respect, it has been found that a majority of children with ASD display gastrointestinal symptoms, and an increased intestinal permeability. Moreover, large differences in microbiotic composition between ASD patients and controls have been reported. Therefore, nutrition-related factors have been hypothesised to play a causal role in the aetiology of ASD and its symptoms. Through a review of the literature, it was found that abnormalities in carbohydrate digestion and absorption could explain some of the gastrointestinal problems observed in a subset of ASD patients, although their role in the neurological and behavioural problems remains uncertain. In addition, the relationship between an improved gut health and a reduction of symptoms in some patients was evaluated. Recent trials involving gluten-free diets, casein-free diets, and pre- and probiotic, and multivitamin supplementation show contradictive but promising results. It can be concluded that nutrition and other environmental influences might trigger an unstable base of genetic predisposition, which may lead to the development of autism, at least in a subset of ASD patients. Clear directions for further research to improve diagnosis and treatment for the different subsets of the disorder are provided.

Key words: **Autism spectrum disorder: Treatment: Diagnosis: Gluten-free diets: Gut–brain axis**

Introduction

Autism spectrum disorder (ASD) is a syndrome with various subgroups, boundaries and treatments[1]. Symptoms include a reduced ability to socialise, to communicate and to use imagination, and displays of stereotypical behaviour[2]. The Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) handbook lists five subtypes of ASD: autistic disorder; Asperger's disorder; childhood disintegrative disorder; pervasive developmental disorder not otherwise specified; and Rett's disorder.

Currently, no diagnostically relevant biological tests for ASD exist. Therefore, diagnoses are based on observed deficits in reciprocal social interaction and communication[3]. Among the children diagnosed with autism between 6 and 17 years old, the amount of co-morbidities, such as attention-deficit disorder, attention-deficit/hyperactivity disorder, anxiety problems and behavioural problems are reported as high as 87·3 %[4]. The prevalence of autism is currently estimated to lie between 45 and 110 per 10 000 individuals in the USA. A recently reported increase[5] has been ascribed to increased recognition, changed diagnostic criteria and changing public attitudes towards autism. However, a causal increase by environmental risk cannot be ruled out[6]. Common postulated risk factors for autism include high maternal and paternal age, a low level of parental education, a child of male sex, autism in the family, low birth weight or gestational age, and prenatal virus and drug exposures (for example, rubella infection[7], thalidomide[8] and maternal smoking[9]).

There is scientific consensus that autism involves a disorder in brain function and development[10–13]. The cause of this disorder, however, is not clear. Although several genetic factors are known to influence the aetiology of different types of autism[14], these only apply to a minor part of the autistic population[15,16]. Moreover, several hypotheses point to environmental influences as possibly being causative to autism, including involvement of abnormal gastrointestinal (GI) microbiota composition, autoimmunity, early environmental exposures to viruses and drug compounds[17].

The ambiguity in causation has resulted in a wide variety of proposed treatments[18]. With this, autistic patients are currently subjected to numerous 'alternative interventions' by caregivers and researchers[19,20]. Some of these are specifically aimed at the diet and improving gut health. Described interventions include gluten- and casein-free diets[19,21], supplementation with pre- and probiotics[22] and supplementation with multivitamins[23−25].

In the present review, we evaluate the current theories and hypotheses concerning the aetiology of autism, with a special focus on the gut–brain axis. On this axis, the mechanisms and interactions of sensory cues and biochemical signals that take place between food, the GI tract and the nervous system can best be described. Through this approach, we discuss the possible alternative nutritional interventions and their efficacy on reducing symptoms of ASD.

Discussion

To place the nutritional interventions in context, it is important to study the causation of disorders in brain function and development before developing symptoms related to the different subcategories of ASD. For the purpose of the review, available studies related to the gut–brain axis are described.

Gastrointestinal abnormalities

GI abnormalities in the gut of autistic children compared with healthy or sibling controls have been studied in multiple trials[22,26−36]. In this respect, studies by Valenci-McDermott et al.[37] and Adams et al.[29] have indicated that 70 % of children with ASD report having a history of GI complaints, against 28 % of neurotypical controls, and that GI symptoms are strongly correlated with the severity of autism (r 0·59; $P < 0·001$). The GI abnormalities found in autistic children include malabsorption[38], maldigestion[39,40], microbial overgrowth (fungal, bacterial and viral)[31] and abnormal intestinal permeability[28,41]. These events could cause symptoms including diarrhoea, constipation, gas, belching, probing and visibly undigested foods[42].

In the study of Wakefield et al.[43], twelve children with regressive autism and GI abnormalities were examined. Of these children, ten displayed lymphoid-nodular hyperplasia (LNH) and eight also displayed abnormalities in the mucosa (including granularity, loss of vascular pattern, and patchy erythema). There were no neurological abnormalities detected in the children.

In 2004[44], the same research group conducted a comparable study among 148 children with ASD as well as GI symptoms and thirty developmentally normal controls by ileo-colonoscopy. LNH was found in the ileum of 90 % of autistic children v. 30 % of controls and in the colon of 59 v. 23 % of autistic children and controls, respectively.

The presence and severity of ileal LNH was not influenced by diet or age at colonoscopy.

Another study[27] examined thirty-six children with autism and abnormal gastroenterological symptoms. In this study, reflux oesophagitis in twenty-five of the children, chronic gastritis in fifteen, and chronic duodenitis in twenty-four were observed. Likewise, twenty-one children showed low activity of carbohydrate digestive enzymes in the intestine, and twenty-seven showed increased exocrine secretion of pancreatic−bilary fluid after intravenous administration of the GI hormone secretin (the peptide hormone secretin, released by endocrine cells within the duodenal mucosa, promotes sodium bicarbonate and water secretion by the pancreas[36]).

However, in a study of Black et al.[33], it was found that ninety-six children, that at a later stage were diagnosed as 'autistic or 'possibly autistic', did not suffer more from GI inflammation, coeliac disease, food intolerance or recurrent GI symptoms than the 449 controls without subsequent development of autism. In a recent study[34], including 124 children with autism and two matched controls per case, the lifetime incidence of GI symptoms was found to be 77·2 and 72·2 %, respectively. Concentrating on the five formed categories of GI symptoms (constipation; diarrhoea; abdominal bloating, discomfort or irritability; gastro-oesophageal reflux/vomiting; and feeding issues or food selectivity), the autistic children did experience significant higher rates of constipation and 'feeding/food selectivity issues' (specified feeding problems, lactose intolerance, loss of appetite or loss of weight) than did controls. It is suggested, however, that these two categories are primarily influenced by behavioural rather than biological influences[34].

Microbiotic compositions: clostridia and vancomycin

Bolte[45] hypothesised that abnormal gut microbiota may be involved in the aetiology of ASD patients. Finegold et al. found that children diagnosed with late-onset autism differed from non-autistic controls in microbiotic compositions of faecal flora, gastric and small-bowel samples[32]. The authors state that there are several reasons to consider that micro-organisms may be involved in late-onset autism. First, onset of the disease often follows anti-microbial therapy, for example, to treat ear infections that often are present in high frequency and persistency among young ASD patients[46−48]. Second, GI symptoms are common at the onset of ASD and often persist. Finally, other antimicrobials may lead to a clear-cut response and relapse may occur when the antimicrobial is discontinued, which is demonstrated with, for example, the antimicrobials vancomycin[49] and metronidazole[50]. However, in higher doses, over a longer period of time (>6 d of treatment), vancomycin disrupts the anaerobic intestinal microflora and promotes colonisation by pathogens[51].

Indeed, significantly more clostridia and ruminococci were found in the stools of the autistic group (geometric

mean count of 2.1×10^6 colony-forming units) *v.* the control group (1.6×10^5 colony-forming units) using 16S ribosomal RNA gene sequencing. Non-spore-forming anaerobic and micro-aerophilic bacteria were found in four of five children with autism, whereas none of the control children showed these bacteria. Furthermore, both the gastric and small-bowel specimens from children with autism were more likely to have (a higher number of species of) clostridia than was true for the controls. Song *et al.*[26] found significantly higher mean cell counts of *Clostridium* cluster groups I and XI and *Clostridium boltae* in fifteen autistic children compared with eight controls using real-time PCR. Parracho *et al.*[22] used fluorescence *in situ* hybridisation, and reported higher levels of *C. histolyticum* in fifty-two autistic children compared with both healthy unrelated children and healthy siblings ($P<0.01$ and $P<0.05$, respectively). However, no relationship was evident between the levels of any of the bacterial populations examined and age, sex, antibiotic history or diet type. GI problems were associated with high levels of clostridia ($P<0.001$) in patients with ASD, but this is apparent, as 91.4 % of the autistic group and none of the unrelated control group had GI symptoms[22]. Martirosian *et al.*[52] detected higher levels of *C. perfringens*, but equal amounts of *Clostridium* spp. in forty-one autistic children compared with ten non-autistic controls using anaerobic bacterial cultivation.

Concluding from the findings listed above, it was hypothesised that: (1) relapse in autistic children after discontinuation of antibiotic treatment is due to the presence of *Clostridium* spores which then germinate to reproduce the disease; (2) the increased incidence of autism is related to the widespread exposure to *Clostridium* spores in the environment; and (3) the increase in families with multiple cases of autism is also due to contact with spores. However, the studies conducted so far are of low to moderate quality, predominantly due to small sample sizes and inadequate or absent explanation of sources of the sample, timing of the study and potential biases (for an extensive review, see also Cao *et al.*[53]). In addition, the studies used a wide range of different assessment methods, which makes it impossible to make qualitative comparisons. Clearly, carefully designed studies are warranted to verify any cause–effect relationships.

Indolylacryloylglycine

Another aspect in the relationship between micriobiota and (late-onset) ASD involves urinary indolyl-3-acryloylglycine (IAG). IAG is a regular constituent of human urine and is produced by gut microflora[54,55]. It has been speculated that high levels of IAG in urine are an indication of gut dysbiosis[56]. A few studies found increased levels of IAG in autistic individuals compared with asymptomatic controls[54,55].

Normally, tryptophan is catabolised to indole pyruvate and indole acetate and can be detected in the urine of normal subjects[57]. The formation of IAG is possibly the result of another less-investigated pathway. It is likely that intestinal micro-organisms catabolise tryptophan to indole derivatives which are then absorbed and converted to indolylacrylic acid (IAcrA) and after conjugation of IAcrA with glycine in the liver IAG is formed[58]. Shattock *et al.*[59] hypothesised that IAG represents the detoxified version of a acidic precursor that affects membranes throughout the body, particularly those lining the gut wall and the blood–brain barrier, making them permeable to other biologically active products such as peptides. This would occur either by replacing the (flat) long-chain fatty acids that make up the lipid elements of the membrane, or by inserting itself between these layers[60]. However, very little evidence exists to support this suggestion. To the best of our knowledge, only one publication showed that the levels of highly unsaturated fatty acids in erythrocyte membranes are affected by the presence of IAcrA[61].

Abnormal intestinal permeability

Two studies[28,41] found an abnormally high intestinal permeability in autistic children, compared with normal controls. D'Eufemia *et al.*[41] included twenty-one autistic and forty healthy age-matched children, both groups without clinical evidence of any GI disease or allergy. The results highlighted some damage to tight junctions of the gut mucosa, and showed that in some patients with infantile autism damage to these junctions occurs in the absence of established GI disorders. Recent results from a study by de Magistris *et al.*[28] replicated the finding of an abnormal intestinal permeability in 36.7 % of ninety autistic children, as well as in 21.2 % of their 146 siblings. This was significantly higher than in the 146 healthy controls (4.8 %). They also found GI symptoms in 46.7 % of the autistic children, although these symptoms were not related to the intestinal permeability. Furthermore, ASD patients on a reported gluten-free, casein-free (GFCF) diet had significantly lower intestinal permeability values compared with those on an unrestricted diet and controls. Specific causes were not described, although the authors suggest the existence of a genetic GI factor that is involved in the pathology of a subgroup of ASD[28].

Previously, intestinal permeability was found to be involved in the aetiopathogenesis of several autoimmune diseases, including Crohn's disease[62], coeliac disease[63] and type 1 diabetes[64]. Significantly lower levels of the health-promoting bifidobacteria species and the mucolytic bacterium *Akkermansia muciniphila* were found in children with autism[65]. It is suggested that low levels of bifidobacteria are related to unhealthy (more putrefactive) gut microbiome composition and metabolism. The change in *A. muciniphila* suggests mucus barrier changes. *A. muciniphila* is a mucin-degrading bacterium extensively present in the guts of healthy adults, but reduced in patients with Crohn's disease, ulcerative colitis and in

elderly individuals. A thinner GI mucus barrier could represent less substrate for mucin-degrading bacteria and hence lower numbers in the faeces. Thus, although a degraded mucus barrier and less mucus-degrading bacteria seems paradoxical, *A. municiphila* could be a possible marker for altered mucus turnover and a thin, thus possibly more 'leaky', gut barrier[66].

However, another recent study showed no abnormal intestinal permeability in autistic children compared with healthy siblings and non-related controls[67]. The same test (a differential sugar-absorption test) was used as well as measuring glucagon-like peptide-2, an enteroendocrine molecule that is released from the GI tract in response to nutrients, which has been found to reduce intestinal permeability[68,69]. No differences were observed in the fourteen ASD patients compared with eight siblings and seven non-related controls. However, the small size of this pilot study may not have been sufficient to show a statistically significant difference between the two groups. Therefore, further research is warranted to establish whether there is a true higher incidence of enhanced intestinal permeability in ASD.

Metabolic abnormalities

Several metabolic abnormalities have been found in autistic children. These include, amongst others, defects in methylation, oxidative stress[70,71], and disturbed concentration of amino acids in plasma[72]. Also, abnormal sulfur metabolism[72–74], lower concentrations of mammalian–microbial co-metabolites and a more active nicotinic acid metabolism[75], have been found in autistic individuals. Below, we will review this in more detail.

Methylation, sulfation and oxidative stress

Several studies have reported lower baseline plasma concentrations of methionine, *S*-adenosylmethionine, homocysteine, cystathionine, cysteine, and total glutathione and significantly higher concentrations of *S*-adenosyl-homocysteine, adenosine and oxidised glutathione in autistic children[70,71]. These findings implicate impaired methylation and oxidative stress in autistic individuals. Geier *et al.*[73] found that patients with ASD show decreased trans-sulfuration metabolites and reduced sulfate concentrations. The same group of researchers[76] found significantly decreased plasma reduced glutathione, cysteine, taurine, sulfate, and increased oxidised glutathione in the plasma of autistic children relative to age-matched neurotypical controls. Other research also found a decreased ability to form sulfated metabolites[77] and lower plasma sulfation products[78] in autistic individuals. The inability of autistic individuals to properly respond to toxins may be partially due to an undersupply of sulfate substrate for the sulfotransferases, which results in impaired sulfur-dependent detoxification pathways. Sulfate substrate is presumably produced by the sulfoxidation of cysteine[79], which was found to be decreased[73].

Geier *et al.*[73] concluded that the abnormal (oxidised) glutathione levels result in a disturbance of the reduced/oxidised glutathione redox equilibrium. This may subsequently affect processes in which this equilibrium is involved, such as nitrogen and oxygen free radical scavengers, protein redox status and enzyme activity, cell membrane integrity, signal transduction and gene expression. Furthermore, increased levels of oxidised glutathione in plasma were found to be correlated with increased levels of Hg-associated urinary porphyrins. Geier *et al.*[73] suggested that increased levels of oxidised glutathione may contribute to abnormal trans-sulfuration and consistently result, in co-occurrence with low glutathione levels and increased oxidative stress, in a higher risk of infections and inflammation in autistic children[73].

These systemic impairments of sulfation in ASD patients could theoretically threaten the stability of the catecholamine transmitter systems, the integrity of the gut lining, and heighten vulnerability to food-borne or pollutant xenobiotic overload to tissues[77].

Glutamate, glutamine and other amino acids

An altered urinary amino acid excretion has been frequently found in autistic children compared with age-matched controls[80]. In children with ASD, Pangborn & Baker[81] found very low levels of some amino acids on urinalysis, such as taurine (in 62 % of subjects), lysine (in 59 %), phenylalanine (in 54 %) and methionine (in 51 %). The researchers also found, to a lesser extent, below-normal levels of tyrosine, leucine, glutamine, valine and asparagine[81]. In the plasma of autistic children[82], however, increased levels of glutamic acid, phenylalanine, asparagine, tyrosine, alanine and lysine were found. The parents and siblings of these patients had these increased plasma levels as well, compared with non-autistic and non-related controls. The authors suggest that this may reflect a reduced capacity to remove amino acids from the system, or, without supplying evidence, a relative permeable blood-brain barrier. An altered plasma level of amino acids could be related to nutritional intake, but the sampling was done at home, thus under different (nutritional) conditions between the families. Raised plasma levels of phenylalanine and tyrosine levels may theoretically lead to higher concentrations of catecholamines such as dopamine and noradrenaline[82]. It is hypothesised that elevated dopaminergic[83] and (nor)adrenergic[84] functions could be involved in the development of autism. However, the existing research does not provide strong support for these mechanisms due to inconsistent results and lack of methodological quality[83].

Also, the balance between glutamic acid and glutamine, in plasma, was found to be different in ASD patients[85] and

their parents[82] compared with controls. Glutamic acid is an excitatory neurotransmitter crucial to neuronal plasticity and the maintenance of cognitive functioning[85], which is normally removed from the synapse after a receptor has been activated and is carried by transporter proteins to astrocytes[86]. Here, it is stored as glutamine via glutamine synthetase, and later transported back to the presynaptic neurons and reconverted to glutamate, via glutaminase. Glutamine is required for the metabolism of enterocytes, and lower levels may thus have a deleterious effect on gut function, or a disturbed microbiota composition[82]. Glutamine synthase, which converts glutamate into glutamine[87], is down-regulated by activated astrocytes. Glutaminase, which converts glutamine into glutamate[88], is activated by necrotic neurons in microglia. Activated astrocytes and necrotic neurons in microglia characterise gliosis, which is found to be increased in autistic brains[89].

Thus far, most observations made have led to speculations about possible metabolic disturbances. However, it is also suggested that amino acid deficiencies found in children with autism are due to poor protein nutrition and food selectivity[90]. ASD children who were on restricted diets had an increased prevalence of essential amino acid deficiencies and lower plasma levels of essential acids, including tyrosine and tryptophan, than both controls and ASD children on unrestricted diets[91]. Thus, findings of abnormal levels of several amino acids might be attributable to poor nutrition secondary to food selectivity[91], instead of actual metabolic abnormalities. However, the multiple findings indicating metabolic difficulties suggest that food selectivity may not be the only cause of changed amino acid levels in autistic children.

Carbohydrate digestion and absorption

Intestinal bacteria encode the enzymes glycoside hydrolase and polysaccharide lyase, which are absent in humans, necessary for the fermentation of poly- and oligosaccharides. These bacteria produce SCFA as endproducts of polysaccharide fermentation. These SCFA serve as energy substrates for colonocytes, modulate colonic pH, regulate colonic cell proliferation and differentiation, and contribute to hepatic gluconeogenesis and cholesterol synthesis[92,93].

Recently, intestinal biopsies (ileum) from children with autism and GI problems showed a deficiency of ileal transcripts encoding disaccharidases and hexose transporters, indicative of impairment of the primary pathway for carbohydrate digestion and transport in enterocytes. Since the conditions that impact on transcription are not specific for a specific segment but rather for the whole absorptive intestine, it is reasonable to assume that similar effects will also be present in the duodenum and jejunum. Indeed, one other study[27] reported low enzyme activities in 58 % of children with ASD in the duodenum. Reduced carbohydrate digestion and absorption can lead to accumulation of saccharides in the intestinal lumen,

resulting in osmotic diarrhoea, bloating and flatulence[94]. The expression levels of disaccharidases and hexose transporters may be controlled, in part, by the transcription factor CDX2 (caudal type homeobox 2). In autistic children with GI symptoms, 86·7 % had CDX2 levels below the 50th percentile of control children with GI symptoms. Moreover, 46·7 % of autistic children with GI symptoms had at least a two-fold decrease in mean CDX2 expression relative to the control children with GI symptoms[94]. In addition, an altered gut microbiota was observed, including increased levels of caecal *Firmicutes*, especially clostridia, and a higher caecal *Firmicutes*:*Bacteroidetes* ratio[94]. This dysbiosis of the mucoepithelium was associated with the deficiencies in host disaccharidase and hexose transporter messenger RNA expression.

In conclusion, abnormalities in carbohydrate digestion and absorption could possibly explain some of the GI problems observed in a subset of ASD patients, although their role in the neurological and behavioural problems remains uncertain.

Intestinal inflammation

Chronic inflammation in the gut can damage the epithelial cell layer[95]. When present, this may explain the increased intestinal permeability found in ASD patients. In this respect, intestinal biopsies among fifty-two regressive autistic children revealed significantly increased CD3$^+$ and CD3$^+$CD8$^+$ in the epithelium as well as CD3$^+$ in the lamina propria compared with developmentally normal non-inflamed control groups, reaching levels similar to inflamed controls[96]. Up-regulation of pro-inflammatory cytokines in the intestinal mucosa of autistic children with GI symptoms has also been reported[97,98]. Additionally, increasing cytokine levels were associated with more impaired communication and aberrant behaviours[98]. However, in another study[99], pro-inflammatory cytokines IL-6, IL-8 and IL-1β were not found to be elevated in the mucosa. In addition, two independent markers of inflammatory reactions in the gut, i.e. rectal NO and faecal calprotectin, were measured in twenty-four children with autism. As in only two of the children the level of one of these markers was increased, the investigators were unable to disclose evidence of a link between the autistic disorder and active intestinal inflammation[100]. Thus, controversial results exist regarding a causal role of GI inflammation in the aetiology and/or behavioural aspects of autism.

Food allergies

In food allergy, presentation of food antigen leads to a response by T cells and subsequently to an initiation of a food-mediated immune response[101]. Parental reports have shown a significantly greater incidence of food allergy in ASD patients compared with healthy

controls[102]. Lucarelli et al.[103] found in 1995 that an oral challenge with cows' milk protein led to worsening of some of the behavioural symptoms of autistic children, and that these children express significantly higher serum levels of IgA, IgG and IgM for casein and IgA for lactalbumin and β-lactoglobulin compared with healthy controls. Recently, Sabra et al.[104] hypothesised that food allergy is the pivotal causative factor that produces lesions in the ileum that consist of enlarged lymphoid nodules containing large collections of lymphocytes in the GI lymphoid tissues adjacent to Peyer's patches. These GI lesions would allow the entry of food antigens across the inflamed mucosa of the bowel and elicit an inflammatory response in the GI tract[104]. They found LNH, reactive lymphoid follicular hyperplasia and chronic inflammation in twelve children with attention-deficit/hyperactivity disorder, autism, anorexia and/or migraine. Th1-associated cytokines were found to be decreased compared with control values, which, together with a predominance of CD4+ cells, support an immunological basis for non-IgE-mediated food allergy (NFA) in this group[104].

Another research group[105] also supports the hypothesis of an existence of NFA in autistic children. They found more TNF-α and IL-12 in peripheral blood mononuclear cells of children with autism than controls, when stimulated with cows' milk protein, β-lactoglobulin and α-lactoalbumin, irrespective of objective GI symptoms[106]. However, NFA may play a lesser role in GI symptoms in older children with autism, as most children probably outgrow NFA during the first 2 years of life with maturation of the gut immune system and the establishment of oral tolerance[107]. Concluding, NFA could partially explain GI symptoms and the suggested benefits of dietary interventions such as the GFCF diet in some autistic children under the age of 2 years.

Nutritional interventions

As introduced, some treatment strategies of autism are specifically aimed at dietary measures to improve gut health. The most important are described below.

Gluten- and milk protein-free diets

A regularly proposed treatment to reduce food-related effects in autism is a GFCF diet. Many non-peer-reviewed articles, books[108] and websites support and encourage the application of this diet. Previous research among 284 autistic children indicates that approximately 15·5 % of autistic children in the USA use this diet as a complementary treatment[109]. Consequently, there have been many reports on the role of a GFCF diet on alleviating several symptoms of autistic individuals[19,110–116] (however, not all of sufficient methodological quality; see below). Significant improvements have been noted within psychological and behavioural categories in vocal and non-vocal communication, attention and concentration, episodes of aggressiveness, affection, motor skills, sleeping patterns, displaying of routines and rituals, anxiety, empathy and responses to learning[19,111,112,115,116]. Moreover, reintroduction of dietary gluten elicited a worsening of behaviours in areas of hyperactivity and impulsivity, stereotyped behaviours, aggression and language and communication skills[19]. A slight initial worsening in behaviour after introduction of the GFCF diet was also noted, which was suggested to be comparable with the withdrawal behaviours exhibited by opioid addicts on the removal of opioids[19]. Changes in physical and physiological areas were measured in some studies as well. One patient showed abnormal peptides not found in controls, including β-casomorphin (BCM), α-gliadin, dermorphin, deltorphin I and II, and morphine-modulating neuropeptide[117]. Some of these have also been observed in other studies[57,112,118].

Nevertheless, when critically reviewing these studies it appears that most of these lack sufficient methodological quality[119,120], and a short overview of the trials that have been conducted, to our knowledge, is shown in Table 1.

All the studies except for one lack a control group, and therefore have a high placebo response rate, due to high levels of parental and/or clinician expectancy, a presumed lack of side effects and the degree of parental effort and resources that are invested in the treatment. The outcomes of the three studies with the highest experimental validity are negative, and showed no statistically significant difference between the control and intervention groups after implementing the GFCF diet[110,113,114]. Also, other studies showed no changes in urinary peptide levels of gluten, casein and IAG[22,121]. Moreover, exclusion of casein from the diet was found to decrease intake of dairy foods as well as micronutrients associated with dairy foods. Although supplementation improved many of the inadequacies seen in autistic children on a GFCF diet, nutrients important for bone health such as Ca and P were still inadequate[122].

The introduction of the GFCF diet is derived from the opioid excess theory of autism. Opioids are chemical substances that have a morphine-like activity in the body, and act by binding to opioid μ-, δ- and κ-receptors. These receptors are located principally in the central nervous system as well as the GI tract and activation elicits adenylatecyclase inhibition, K+ channel activation, or Ca2+ inactivation[123]. The opioid excess theory states that children with autism are symptomatic due to excess of peptides, derived endogenously, as well as exogenously from incomplete breakdown of certain foods, both possibly due to peptidase deficiencies. In particular, gluten-derived peptides from wheat and some other cereals and casein from milk and dairy products are suspected to be involved in autism[19,124,125]. Opioid-like molecules include, for example, α-gliadin, dermorphin, novel autism peptide I

Table 1. Studies assessing the effect of gluten and casein related dietary interventions on autism spectrum disorder (ASD) symptoms

Author	Participants	Intervention	Dependent variable	Outcome	Remarks
Adams & Conn (1997)[163]	1 m, 1 f, age 3 years, autism	GFCF diet for 24 months	Anecdotal parent report of overall behaviour	Positive; authors report improvements after 2 weeks of intervention, but no quantifiable data are displayed	No experimental design Not enough detail to replicate No operational definitions No treatment fidelity No inter-observer agreement No attempt to control alternative explanations
Bird et al. (1977)[110]	1 m, age 9 years, autism	GFCF diet for approximately 9 d	Direct observation of pica, inappropriate vocalisations, cooperation and motor activity	Neutral; PND = 3 % (averaged across dependent variables)	No attempt to control alternative explanations and diet Implemented for a brief time
Cade et al. (2000)[112]	28 m, 22 f, age 3·5−16 years, autism	GFCF diet for 12 months	UPL, blood tests of antibodies to gluten and casein, parent, physician, and teacher ratings of social isolation, eye contact, speech, learning skills, hyperactivity, stereotypical activity, hygiene, panic attacks and self-mutilation	Positive; baseline levels of antibodies and UPL were higher in the group with autism than in the neurotypical control group. Significant changes from baseline on ratings of social isolation, eye contact, speech, learning skills, hyperactivity, stereotypical activity, panic attacks and self-mutilation	Analysis of antibodies and UPL was conducted at pre-intervention only No operational definitions of parent- and teacher-rated behaviours No treatment fidelity No inter-observer agreement No attempt to control alternative explanations Not blinded No control group
Harris & Card (2012)[164]	9 m, 5 f, mean age 9 (SD 1·9) years, austism (including Asperger's syndrome)	Seven of thirteen children were on a GFCF diet as assessed by FFQ	GSRS and CARS	Neutral; GSRS and CARS scores did not differ significantly according to GFCF diet	Small sample size Subjective nature of the questionnaires Uncertain ability of parents to accurately quantify the severity of their children's symptoms
Herbert & Buckley (2013)[165]	1 f, followed from age 4 to13 years, severe autism	Multiple interventions including a GFCF ketogenic 1·5:1 ratio diet (14 months at 12 years)	Among others, seizures, brain activity (measured through an electroencephalogram), weight and cholesterol	Positive; reported symptoms decreased (although not quantified). Weight loss of 60 pounds (27 kg) in 1 year. Cholesterol increased slightly	Case study including multiple interventions Causality remains unproven
Knivsberg et al. (2002)[115]	20 m, age 7·5 years, autism	GFCF diet for 12 months	UPL, Leiter Nonverbal Intelligence Test, linguistic abilities using the ITPA and the Reynells språktest, Movement Assessment Battery for Children, parent and teacher behaviour ratings using the DIPAB	Positive; pre−post test showed improvements in the DIPAB, and statistically significant changes in the other standardised assessments	Categorising groups according to gains after intervention increased the likelihood of finding statistical significance No treatment fidelity No attempt to control alternative explanations Single blind
Knivsberg et al. (1990, 1995)[166,167]	8 m, 7 f, age 6−14 years, autism	Three diets for 48 months used: GF, milk reduced; milk free, gluten reduced; and milk free, GF; number of subjects assigned to each diet not reported	UPL, C-Raven, Tajford Observation Scheme, parent and teacher behaviour ratings using the DIPAB	Positive; decreased bizarre behaviour and isolation, statistically significant improvement on C-Raven and Tafjord scores; and improvement in multiple areas on teacher evaluations. Three subjects who stopped the diet had a reversal of gains made in C-Raven scores	No experimental design Not enough information to replicate No treatment fidelity No inter-observer agreement No attempt to control alternative explanations Results for the different diets not reported separately No control group

Table 1. *Continued*

Author	Participants	Intervention	Dependent variable	Outcome	Remarks
Lucarelli *et al.* (1995)[103]	30 m, 6 f, age 8–13 years, autism	Free of allergens identified for individual participants and restriction of cows' milk for 2 months	BSE, and a battery of Ig antibody tests	Mixed; statistically significant reductions in Ig antibody levels, and improvements in five of the seven behaviours measured by the BSE	Results for chemical testing of allergens are conclusive; however, results regarding behaviour are suggestive due to: No treatment fidelity No inter-observer agreement No attempt to control alternative explanations Not blinded Adaptation of BSE No control group
O'Banion *et al.* (1987)[168]	1 m, age 8 years, autism	Alternating 4 d periods of fasting (only water allowed) and 4 d periods in which only one type of food was allowed per d	Direct observation of challenging behaviour, movement and laughing	Unclear; wheat, maize, tomatoes, sugar, mushrooms and dairy products were suggested by the authors to be associated with increases in behaviour	No experimental design Cessation of experiment during key phase of study Extremely high likelihood for carry-over effects from extreme food deprivation, stress and fear Confounded data
Patel & Curtis (2007)[111]	9 m, 1 f, age 5–8 years, five with autism + ADHD, and five with Asperger's syndrome + ADHD	Eight components for 3–6 months: 1. Avoidance of mites, moisture, mould, smoke, pesticides and toxic cosmetics/cleaners 2. Organic GCFC diet 3. Oral administration of berbine, artemisinin, citrus abstract and walnut hulls 4. Injections of antigens 5. Administration of common multivitamins and cocktail of over-the-counter herbs, oils and extracts 6. Intravenous chelation 7. Injections up to three times weekly of vitamin B$_{12}$ 8. Special education, behaviour modification, speech language pathology, occupational therapy and physical therapy	Urinary metal concentrations and parent report of behaviour change	Positive; statistically significant ($P < 0.001$) decrease in urinary Pb concentrations, reported behavioural improvements from parents	Pilot study No experimental design No treatment fidelity No inter-observer agreement Potential behaviour improvement from components other than diet (for example, behaviour modification)
Reichelt *et al.* (1990)[169]	10 m, 5 f, age 3–17 years, autism	Prescribed participants specific diets based on children's UPL pattern. Diet variations included: GFCF, gluten-restricted, casein-free, and GF, casein-restricted. Each diet was implemented for 12 months	UPL, blood tests of antibodies, and behaviour questionnaire	Positive; statistically significant decrease in UPL and improvements in antibodies and behaviour	No operational definitions No treatment fidelity No inter-observer agreement No attempt to control alternative explanations Results for the different diets not reported separately No statistical analysis reported No control group

Table 1. *Continued*

Author	Participants	Intervention	Dependent variable	Outcome	Remarks
Seung *et al.* (2007)[170]	10 m, 3 f, age 2–16 years, autism	GFCF diet for 3 months	Direct observation of verbal responses to questions, verbal imitations, different words produced, and total utterances	Neutral; no statistical significance	No control group
Whiteley *et al.* (1999)[19]	15 m 3 f, mean age 5·5 years, fourteen with autism, and four with Asperger's syndrome	GF diet for 5 months	UPL, parental/teacher interview concerning autistic behaviours, and K-ABC	Mixed; some statistically significant behavioural improvements, no statistically significant reduction of UPL. Of the parents, 67 % rated their children improved; 94 % would continue the diet	No experimental design; No treatment fidelity; No inter-observer agreement; No attempt to control alternative explanations; Unblinded comparison on outcome measure K-ABC
Whiteley *et al.* (2010)[171]	72 children, age 4–10 years, ASD	GFCF diet for 24 months	ADOS-G, parental rating of VABS and GARS, symptom severity by ADHD-IV	Slightly positive; while the majority of statistical tests yielded insignificant results, at least one test associated with each subdomain of all instruments produced *P* values lower than 0·05	Different Bonferroni adjustments to α levels for statistical tests; Significant level of participant attrition; Discrepancy across comparable measures; Parents not blinded

m, Male; f, female; GFCF, gluten-free, casein-free; PND, percent non-overlapping data; UPL, urinary peptide level; GSRS, Gastrointestinal Symptoms Rating Scale; CARS, Childhood Autism Rating Scale; ITPA, Illinois Test of Psycholinguistic Ability; DIPAB, Diagnose of Psykotisk Adfœrd hos Børn (Diagnosis of Psychotic Behaviour in Children); GF, gluten-free; C-Raven, C-Raven Progressive Matrices; BSE, Behaviour Summarized Evaluation; ADHD, attention-deficit/hyperactivity disorder; K-ABC, Kaufmann Assessment Battery for Children; ADOS-G, Autism Diagnostic Observation Schedule-Generic; VABS, Vineland Adaptive Behavior Scale; GARS, Gilliam Autism Rating Scale.

and III[117] and BCM[40,126]. The resulting peptides may have direct opioid action or may act as ligands to the enzymes that would break down the endogenous opioids[125]. A few studies have reported indirect supportive results for this hypothesis, namely an abnormal intestinal permeability in autistic subjects, observed using a differential sugar-absorption test[41,28] and an abnormal (high) peptide content in the urine of autistic children[57,112,117,118,127]. Through the immune system, an alternative account of this issue is proposed. It was recently discussed that microglial in the brain can be activated by infection and inflammation, causing behavioural changes[128].

Cows' milk-derived BCM, which is thought to have bioactive properties, is extensively studied for its potential role in several human diseases. It is found in bovine as well as mother's milk[129] and acts as a substrate for dipeptidyl peptidase 4 (DPP-IV), which is a cell-surface protease present on the brush border of the intestine, kidney and liver[130]. A defect in DPP-IV, a brush-border peptidase which normally digests dietary opioids[131], could cause the presence of opioid peptides such as BCM in the urine of children with autism[117]. In a study by Kost *et al.*[132], ninety infants, of which thirty-seven were fed with mother's milk and fifty-three were fed with formula containing cows' milk, were investigated on levels of human and bovine BCM. Elevated basal bovine BCM was found in blood plasma of cows' milk-fed infants who showed delay in psychomotor development and elevated muscle tone. The authors therefore suggested the deterioration of bovine BCM elimination as a risk factor for delay in psychomotor development and autism. However, other studies, reviewed by Hunter *et al.*[133], could not find defects or abnormalities in DPP-IV in autistic subjects. Lacking direct evidence, however, this theory remains speculative.

Dietary pre- and probiotic supplementation

The symptoms of inflammatory bowel disease and irritable bowel syndrome are quite similar to those of the GI symptoms in ASD patients, namely abdominal pain or discomfort (which sometimes lessens upon defecation), and are associated with changing stool frequency and/or changing stool consistency[134]. Due to these similarities and the established role of probiotics in the treatment of inflammatory bowel disease (see below), a possible treatment of autism with probiotics has been proposed. Since autistic children also frequently suffer from other GI disorders, studies have been undertaken to understand the composition of gut microbiota in autistic children and to modify the gut microbiota and its metabolism favourably when required. In this respect, some bacteria are believed to be harmful through the production of harmful metabolites, and some bacteria are thought to be health-promoting, such as the bifidobacteria and lactobacilli[135]. The latter

are believed to provide protection against infection in the gut and to help in maintaining an efficient barrier function and a healthy immune function[135].

Probiotics are live micro-organisms, which when administered in adequate amounts confer a health benefit on the host[136]. They are supposed to give relief from lactose maldigestion[137,138], reducing the related episodes of diarrhoea[139,140]. Additionally, probiotics are suggested to help reduce risk factors associated with inflammatory bowel disease[141,142], colorectal cancer[143] and impaired gut-associated immune responses[144–146].

In a double-blind, placebo-controlled study by Parracho *et al.*[147], *Lactobacillus plantarum* feeding of children with autism resulted in significant increased levels of the beneficial bacteria lactobacilli and enterococci, and a significant reduction of a cluster of *Clostridium*, compared with the placebo group. Through a 12-week study, the probiotic feeding resulted in reduced GI problems and, more importantly, in improved behaviour scores compared with baseline. In this respect, it is noteworthy that, during another double-blind, cross-over study, addressing the effects of the probiotic *L. plantarum* on autism failed during the changing of treatments in the cross-over period, because parents (who were blinded for the intervention) of children treated with the actual probiotics refused to make the switch, as they wanted their autistic children to continue their improvement[148]. Noted improvements were decreased levels of clostridia bacteria in the stools and a positive effect on mood and general behaviour, as described by parents. Since this can only be considered as anecdotal evidence, further well-controlled studies are warranted.

Another probiotic trial in autistic children was recently conducted by Kałużna-Czaplińska & Błaszczyk[149]. Probiotic supplementation with *L. acidophilus* over 2 months led to a significant decrease in D-arabinitol and to a significant improvement in the ability to concentrate and carry out orders. D-Arabinitol is a metabolite of most pathogenic *Candida* species and its excretion in urine is elevated in autistic patients[149]. Candida infections have been associated with autism previously[35]. In a trial regarding the oral supplementation of vancomycin, followed by supplementation of a probiotic mixture of *L. acidophilus*, *L. bulgaricus* and *Bifidobacterium bifidum* (40×10^9 colony-forming units/ml), positive results were found in communication and behaviour[150]. However, the effects were attributed to vancomycin, the subject of study, and no attention was given to the possible contribution of the probiotic mixture.

Unfortunately, these studies were not of sufficient methodological quality due to the absence of control groups, multiple treatments at once and/or small sample sizes, as shown in an overview in Table 2.

Prebiotics are non-digestible food ingredients that have a beneficial effect through their selective metabolism in the intestinal tract. A reason for a potential influence of prebiotics on the treatment of symptomatology of autism concerns especially the selective stimulation of growth of lactobacilli and bifidobacteria and the production of SCFA that have an influence on gut energy metabolism, gut barrier function, water fluxes and motility[151]. However, no systematic studies have been conducted so far.

Vitamin supplementation

Autistic children often experience significant eating difficulties, specific food selectivity[91,152,153], poor digestion[94], inflammatory conditions in the gut[154,155] and reduced levels of vitamin-producing microbiota in the intestines[65,156]. These factors could lead to a poor nutritional status[157]. A survey among physicians showed that multivitamin supplements are among the most widely recommended medical interventions for autism, and are recommended for children with autism by almost half of the interviewed physicians[158]. As such, a plethora of research has been published in respect of which vitamins, combined with a range of minerals, have been supplemented with various outcomes.

The available studies involving vitamin/mineral supplementation of autistic individuals generally lead to slightly promising results. Nutritional high-dose supplementation with ascorbic acid resulted in a statistically significant reduction in autism severity[159]. There have been many double-blind, placebo-controlled studies of very high doses of vitamin B_6 with Mg, and all but one showed positive behavioural improvements[25]. However, the studies were limited by lack of sufficient methodological quality due to small sample size and the use of assessment tools of limited validity[25].

Two small pilot studies involving multivitamin/mineral supplementation, although of limited quality, showed positive preliminary results[159,160]. Recently, 141 children and adults with autism enrolled in a randomised, double-blind, placebo-controlled vitamin/mineral supplementation study[23]. The autistic subjects improved in levels of vitamins, minerals, and biomarkers of sulfation (higher levels of free and total sulfate), methylation (higher levels of *S*-adenosylmethionine and lower levels of uridine), glutathione (higher levels of reduced glutathione) and oxidative stress (higher reduced:oxidised glutathione ratio and lower levels of nitrotyrosine), compared with a control group. Significant improvements were also noted in behavioural problems, including hyperactivity, tantrumming (display of disruption, non-compliance, instigation and/or interruption frequently accompanied by aggression[161]) and receptive language. In addition, plasma and whole-blood levels of several vitamins and minerals, plasma levels of ATP and coenzyme Q10, and erythrocyte levels of NADH and NADPH increased from below normal to normal levels after supplementation[23]. The combined effects of vitamin B_6 and Mg were systematically reviewed[162], and it was concluded that the data were insufficient to use such supplementation as a treatment for ASD.

Table 2. Studies assessing the effect of probiotic interventions on autism spectrum disorder (ASD) symptoms

Author	Participants	Intervention	Dependent variable	Outcome	Remarks
Kałużna-Czaplińska & Błaszczyk (2012)[149]	20 m 2 f, mean age 5·6 years, ASD	Sugar free diet Oral administration of L. acidophilus (Rosell-11, 5 × 10⁹ CFU/g) twice daily for 2 months	DA and LA were identified by capillary GC/MS. Creatine determined by HPLC	Positive; decreased level of DA and DA:LA ratio, improvement in important behaviours such as ability to concentrate and carry out orders	No control group No standardised tests for autistic behaviour No treatment fidelity No statement of blinding No attempt to control alternative explanations
Parracho et al. (2010)[147]	Children with ASD	Probiotic Lactobacillus plantarum WCFS1 treatment in autistic subjects	Faecal microbiota, gut function. Secondary: behavioural scores	Positive; significant improvement in stool consistency, and behavioural scores	High between-subjects variability High dropout rate among the participants
Sandler et al. (1999)[150]	10 m, 1 f, age 43–84 months (mean 59·4 (SD 12·7) months), regressive autism	500 mg oral vancomycin/d divided into three times/d for 8 weeks. Followed by 4 weeks oral probiotic mixture of L. acidophilus, L bulgaricus and Bifidobacterium bifidum (40 × 10⁹ CFU/ml)	Observer rating analogue assessment scales for behaviour, communication and social skills; quantitative faecal flora	Positive; improvement for the group as a whole in communication (P=0·003) and behaviour (P=0·003). Parental-noted deterioration in behaviour within 2 weeks after discontinuation of treatment	All subjects had diarrhoea before enrolment Small sample size No control group Results attributed to vancomycin, no wording regarding possible contributions of probiotics Subjective rating of scores No treatment fidelity

m, Male; f, female; CFU, colony-forming unit; DA, D-arabinitol; LA, L-arabinitol.

Summarising considerations

In conclusion, the exact possible biological causes and symptomatology of the autistic spectrum are still poorly understood. A tremendous amount of theories and hypotheses exist regarding this subject, even when only looking at metabolic aspects. It might well be that not all possible circulating hypotheses are described in the present paper. It can at least be stated firmly that there is considerable evidence that the gut–brain axis is involved in the aetiology of autism.

There is convincing evidence that a genetic predisposition, strengthened by early exposure to environmental agents in a vulnerable period, provides an unstable base allowing a possible development of autism. Factors such as nutrition, infections and the use of antibiotics might trigger this base and lead to the development of autism. However, there are many possible mechanisms, not all completely understood, by which this is possible and many contradicting results from an extensive amount of studies make it even harder for parents and children to find sufficient help in dealing with this disease. There is need for further research to improve diagnosing and treatment for different subsets of autism. Maybe not the severity of symptoms are diagnostic criteria for the different groups (classic autism, Asperger's syndrome, regressive autism, etc.), but the origin of symptoms (GI problems, presence of vitamin deficiencies, brain abnormalities, etc.) will determine the classification in the next Diagnostic and Statistical Manual of Mental Disorders (DSM), which would aid in finding the right treatment for the right individual.

Data supporting the presence of GI problems in ASD patients are convincing, at least for some kind of subset of children who experience a worsening of autistic symptoms along with GI symptoms. Furthermore, the increased incidence of autism might be related to exposure to Clostridium spores in the environment. In addition, cases of increased intestinal permeability were observed, although the evidence so far has been inconclusive. Multiple findings indicating metabolic abnormalities suggest that these are present in ASD patients, and that these abnormalities may partially cause the observed change in amino acid levels and levels of inflammation in these patients. Abnormalities in carbohydrate digestion and absorption could possibly explain some of the GI distress observed in a subset of ASD patients, although their role in the neurological and behavioural problems remains uncertain. Thus, a subset of ASD patients displays measurable changes in GI symptoms, composition of gut microbiota, excretion of metabolites, intestinal permeability and metabolism of several compounds. Feeding selectivity problems frequently detected in ASD patients can be a major cause of these abnormalities, and should certainly be taken into account when investigating other causes. However, this does not change the importance of investigating the effect of these abnormalities on autistic symptoms, and methods to alleviate both GI and autistic symptoms.

Moreover, we feel that additional trials involving gluten-free diets and casein-free diets should be conducted. Current research on the hypothesised relationship between an improved gut health and a reduction of symptoms in some patients is mostly lacking sufficient methodological quality. The few trials involving pre- and probiotic, and multivitamin supplementation have been conducted with contradictive but partly promising results in reducing symptomatology. Results from these trials show the importance of conducting further research in this area. It is clear, however, that children with autism need personal support and advice, because every child experiences slightly different symptoms.

Although there is substantial evidence that the GI tract and the gut–brain axis have a central role in autism, further studies are required to understand the aetiology and the mechanisms by which these factors act, and to understand the possible cause–effect relationship between food choice and autism.

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