THE IMPORTANCE OF GUT- BRAIN AXIS IN THE PATHOGENESIS OF AUTISM SPECTRUM DISORDERS

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ABSTRACT

In recent years, there has been increasing interest in the study of the intestinal microbiota in autism spectrum disorders (ASD) children, and the possible implication of gut-brain axis in the etiology of ASD. Current literature comes with impressive data regarding the possible relationship between intestinal flora abnormalities and how these changes impact brain development through increased neuroinflammation and oxidative stress.

The aim of this article is to present the current knowledge on the link between intestinal flora and the brain as a bidirectional communication pathway and how intestinal microbiota changes could be involved in the pathogenesis of autism.

1. AUTISM SPECTRUM DISORDERS (ASD)

ASD is a neurodevelopmental disorder characterized by social interaction impairments, repetitive behavior, cognitive and language delays. The autism spectrum includes - according to ICD-10 and DSM V - different forms of severity of the condition, from more severe forms like Infantile Autism to milder forms like Asperger syndrome.

According to ICD-11, Autism spectrum disorder is characterized by persistent deficits in the ability to initiate and to sustain reciprocal social interaction and social communication, and by a range of restricted, repetitive, and inflexible patterns of behavior and interests. The onset of the disorder occurs during the developmental period, typically in early childhood, but symptoms may not become fully manifest until later, when social demands exceed limited capabilities. Deficits are sufficiently severe to cause impairment in personal, family, social, educational, occupational or other important areas of functioning and are usually a pervasive feature of the individual's functioning, and observable in all settings, although they may vary according to social, educational, or other context. Individuals along the spectrum, exhibit a full range of intellectual functioning and language disabilities.

Besides the core symptoms of autism, symptoms like sleep disturbances, gastrointestinal disturbances, food selectivity, irritability and aggressive behavior are found in a great number of cases.

There is currently no unique cause to pinpoint as being responsible for the development of ASD. The number of studies trying to find the etiology and pathogenesis have increased in the last years. A numerous number of hypotheses have been studied in accordance to autism etiology, from genetic polymorphism and epigenetic influences, to the importance of the immune system, intestinal microbiota and a whole range of environmental factors. The currently available

scientific information is consistent with the idea that ASD has a complex etiopathogenic mechanism, that implies both genetic traits and a range of gene-environment interactions.

Neither has there been major progress regarding the treatment of the main symptoms of autism, at the moment, no treatment being approved for the core symptomatology. The currently approved pharmacological molecules are being used for aggression, irritability or associated comorbidities.

The need to discover both the etiological factors and an efficient treatment of ASD, is an important aim at the moment, as ASD has become a public health problem, both in terms of prevalence of the disorder and persistent disabilities during adolescence and adulthood, but also in terms of financial outcomes. The prevalence of ASD has been rising rapidly in the last years, according to the data released by Centers of Disease Control and Prevention (CDC) in 2014, 1:59 children has been identified with autism [17].

The total yearly cost for children with ASD in the United States was estimated to be between $11.5 billion – $60.9 billion (2011 US dollars). This significant economic burden represents a sum of direct and indirect costs: from medical care, to special education, to lost parental productivity [5, 22].

2. HUMAN MICROBIOME

The human microbiome represents the totality of bacteria, archaea, viruses, fungi and protists which reside in our tissues or fluids, including the skin, oral mucosa, cavities and gastrointestinal tract [30].

The human gastrointestinal tract is populated with approximately 1000 species of bacteria and the number of bacterial genes that are in the gut is about 9.9 million.[28] The gastrointestinal microbiota is important for many physiological processes like protecting our bodies from pathogenic microorganisms, maintaining the integrity of the intestinal lining through mucus production, producing vitamins, metabolizing non-digestible carbohydrates and, nevertheless, interacting with the immune system. All these processes are essential, many of them being disrupted when dysbioses occur. The gut bacterial imbalance has been linked in several studies with the occurrence of certain intestinal or extra-intestinal diseases, like irritable bowel syndrome, coeliac disease, allergies, asthma but also psychiatric and neurological disorders like anxiety, depression, Alzheimer's disease, multiple sclerosis and ASD [6,7]. The intestinal microbiota has been linked with shaping the immune response, while dysbiosis has been linked to anomalies in the host's immune response [23].

The development of gut microbiota is influenced by a series of factors like: the mother's microbiota, genetic factors, the type of diet, various associated disorders and exposure to antibiotics. The common knowledge regarding the human microbiota has been expanding over the years, thus resulting in changes to many theories and beliefs. One example is represented by the fact that previously, the newborn gut was considered sterile, but nowadays there are several studies showing that during delivery the baby's intestine is colonized with bacteria from the skin, vagina and the anus, or just with skin bacteria in caesarean deliveries [24,35]. Moreover, there is increasing evidence that not even the amniotic fluid is sterile and, in animal studies, the meconium was proven not to be sterile, suggesting the hypothesis that intestinal colonization begins before birth [19].

The environmental factors also play an important role in producing changes of the intestinal microbiota. For example, the exposure to antibiotic treatment during the first years of life or the exposure to antibiotics during pregnancy have been associated with

important changes in the gut microbiota composition [4].

The first bacteria that are found in a healthy newborn intestinal tract are Enterobacteriaceae, *Staphylococcus* and *Streptococcus*, which are facultative anaerobes. They consume the oxygen in the gut creating an anaerobic environment. Afterwards, anaerobic species such as Bacteroides, *Bifidobacterium* and *Clostridium* appear [10]. This dynamic and diverse process stabilizes around the age of 2 or 3, being influenced by many environmental factors [4]. It is an interesting overlap between the window of gut microbiota development and the window of one of the most active periods of brain development [9]. Between the age of 2-3 months and the age of 2 there are numerous processes of neuroplasticity. During this period, the brain of the baby almost triples its volume. It is in this period of time that the brain is sensitive to many internal and external factors which could influence this highly dynamic neurodevelopment process, and we should consider that altered internal factors - like an intestinal dysbiosis - could disrupt the underlying growth mechanisms.

The intestinal microbiota development is a multivariable process, but the extent of the heterogeneity of transformations appearing during this process have not been completely understood.

3. GASTROINTESTINAL SYMPTOMS AND FOOD SELECTIVITY IN ASD

There are several studies outlining the high prevalence of gastrointestinal symptoms in autistic children compared to healthy controls. The most common symptom is represented by constipation, but also diarrhea, bloating, abdominal pain, reflux, vomiting, gaseousness, foul smelling stools, may be present [15,29]. A study by Gorrindo et al. identified constipation as the most common symptom (85%) in children with ASD, according to parental reports and evaluations by pediatric gastroenterologists. There are studies that found a correlation between the severity of gastrointestinal symptoms and the severity of main ASD traits [18]. Moreover, auto-aggressive behaviors and irritability have been more frequent in children with ASD and more severe gastrointestinal disturbances. It was even stipulated the fact that the aggressive behaviors could be a consequence of abdominal pain or similar intestinal complains [11].

The associated gastrointestinal symptoms in ASD must be taken into consideration for the importance of gut microbiota in the physiopathology of this medical condition, even though there is still insufficient evidence to draw a cause-effect conclusion.

Food selectivity in ASD is another important trait, as the affected children, when compared with healthy controls, have a reduced consumption of vegetables, fruits and meat, which is translated into a reduced intake of minerals, vitamins and proteins that play a key role in the normal brain and gut microbiota development. Moreover, usually ASD children have restrictive diets that contain high caloric products, with high quantities of carbohydrates which could disrupt normal gut microbiota.

Furthermore, a series of studies have shown that the development of intestinal microbiota is influenced by the period of breastfeeding, also revealing that using only formula feeding could affect normal gut bacterial growth. In addition to this, in some studies ASD children have been breastfed for shorter periods of time comparing to typical children, being exposed to formula feeding earlier in their development [20]. It is considered that vaginal delivery and breastfeeding are two important aspects that help the development of a normal healthy intestinal flora, with more beneficial bacteria than pathogenic ones [3].

Besides food selectivity, there is an entire debate on food allergies in children with ASD, and various studies have tried to examine the importance of this atopic trait in the etiology of ASD. Even though, there is no unitary conclusion at this time, the higher prevalence of food allergies and casein or gluten intolerance in ASD children have led scientists to believe that they should further study how these could impact the gut bacterial development and the brain-gut interactions.

4. THE GUT-BRAIN AXIS

The gut-brain axis is a pathway of bidirectional communication between the gastrointestinal system and the cerebral system, through neuroendocrine, neuroimmune and autonomic nervous signaling. There is an increasing number of evidence regarding the importance of intestinal microbiota in the pathogenesis of ASD through a series of abnormal processes [16].

The hypothesis regarding the involvement of gut-brain axis in the etiology of ASD is much more complex than the main modified processes showed in Figure 1. The present article is supposed to present a brief overview of this concept, but have in mind that more profound changes in many key mechanisms like abnormal mitochondrial functioning, DNA methylation and microglial activation have significant importance in having a complete understanding of the process.

Having the above-mentioned limitations in mind, we will try to draw a picture of how the gut and brain interact in the ASD gut-brain theory.

First of all, the increased permeability of the gut lining is linked with the modified gut flora in ASD children as many bacterial species have the role of maintaining the integrity of the intestinal wall through various mechanisms. Some of the bacterial species that are supposed to be involved in the mechanisms of maintaining the gut epithelium integrity are scarce in the feces of ASD children.

Decreased Lactobacillus could be linked with the changes in the intestinal permeability, because of the important role it has in the maintenance of tight junctions in the epithelial barrier [18].

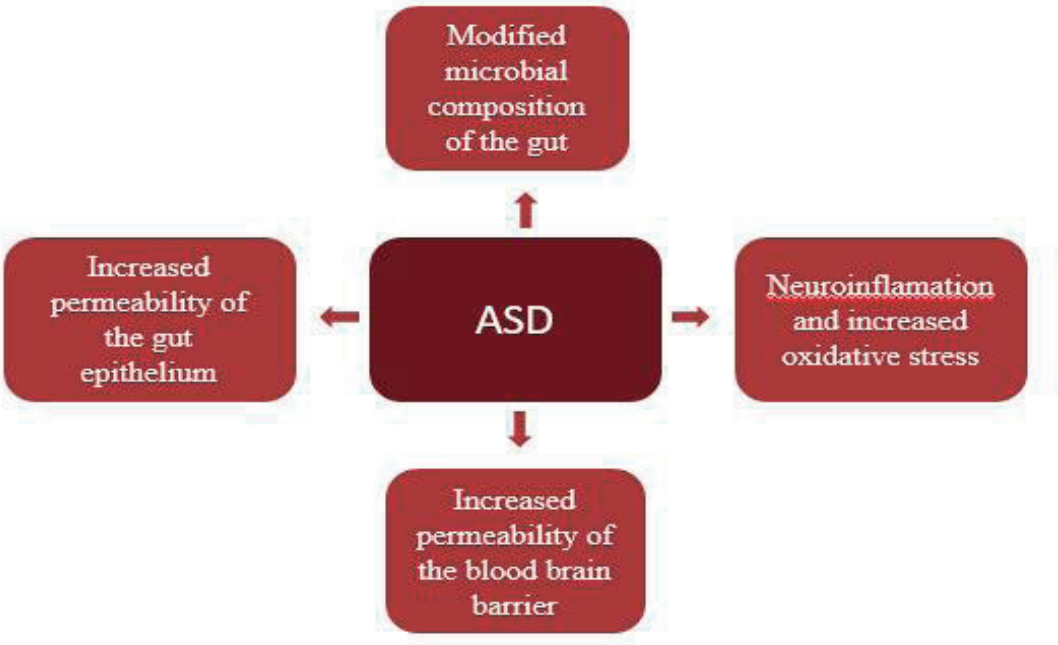

Figure 1 - Showing the main disturbed processes regarding gut-brain communication in ASD

Elevated intestinal permeability, or the - so called - "leaky gut" of ASD children, could lead to increased passage of bacterial metabolites like LPS (lipopolysaccharides), exotoxins and cells from the gut to the blood flow. This movement can be more intense in episodes of intestinal photogenic infections and during the recovery, but can be also a continuous baseline trans-passing associated with high inflammatory processes in the body. The systemic immune response is associated with elevated cytokine levels.

The increased permeability of blood-brain barrier observed in post-mortem brains of ASD children may be a consequence of many disturbed processes. An important factor is represented by the increased level of cytokines in the blood that can damage the tight junctions of microvascular endothelial cells forming the blood-brain barrier (BBB). Moreover, some cytokines can pass the BBB getting directly to the brain causing considerable damage.

Besides the mechanisms already described, the increased permeability of both the gut and the BBB allows the passage of LPS, exotoxins, and sometimes of pathogen cells directly into the brain, inducing the process of microglial activation, and thus increasing the oxidative stress in the cerebrum. The increased neuroinflamation contributes to the malfunction of synapsis and could interfere with the normal processes of brain development.

There are insufficient arguments for the moment to make the assumption that the abnormal gut microbiota is the primary change in ASD, which triggers the whole cascade of mechanisms described in the summary above, but its important role in ASD pathogenesis should not be mitigated

The main changes identified in ASD - presented in Figure 1, will be discussed in detail, as it follows:

a) Modified microbial composition of the gut in ASD

b) Increased permeability of the gut epithelium

c) Increased permeability of the blood brain barrier

d) Neuroinflamation and increased oxidative stress

a) Modified microbial composition of the gut in ASD

A very commonly observed phenomenon in the intestinal flora of ASD children, is a decreased ratio of between the phyla Bacteroidetes to Firmicutes, translated as increased concentration of Firmicutes spp [31].

Table 1 – Common identified changes of gut flora in ASD children [8]

Increased concentrations of bacterial spp./genera in ASD gut microbiota	Decreased concentrations of bacterial spp./genera in ASD gut microbiota
Akkermansia muciniphila Anaerofilum Barnesiella intestinihominis Clostridium spp, Dorea spp The family Enterobacteriaceae Faecalibacterium spp (especially Faecalibacterium prausnitzii) Roseburia spp, Parasutterella excrementihominis, Prevotella copri Prevotella oris,Turicibacter spp Aeromonas, Odirobacter splanchnicus, Parabacteroides, Porphyromonas, Pseudomonas, and Turicibacter sanguinis.	Escherichia coli Bifidobacterium, Fusobacterium, Oscillospira, Sporobacter, Streptococcus and Subdoligranulum Enterococcus spp, Lactobacillus, Lactococcus and Staphylococcus Collinsella spp except Collinsella aerofaciens

The changes of bacterial flora presented in Table 1 – are changes that have been identified in the feces of ASD children. It should be taken into account that other studies that have analyzed the bacterial flora through intestinal biopsies and have shown different results. There is also a high heterogeneity of the current studies regarding the control groups and the type of technique used for microbiota analysis. Some of the studies comparing ASD children microbiota with a sibling control group do not identify significant changes in gut microbiota. In the future these should be considered for further analysis as the abnormal microbiota changes could be a phenotype, also present in ASD children siblings. There is a need for more extensive studies that could integrate all the information and maybe determine a specific microbiota pattern of ASD children.

In the present paper we will outline in summary how the modified bacterial flora in ASD could affect the body and brain integrity and function.

First of all, we should highlight the fact that in normal gut microbiota there is an equilibrium between bacterial species that have pro-inflammatory and anti-inflammatory activity. As stipulated above, the *Bifidobacterium* and *Lactobacillus* concentrations are low in ASD children microbiota, these changes being associated with decreased anti-inflammatory activity. On the other hand, increased pro-inflamatory activity in the gut is due to high levels of *Clostridium* and *Ruminococcus* [13].

There are other bacterial species that influence different mechanisms through their metabolites. An example from this class is represented by *Desulfovibrio* which produces hydrogen sulfide as a metabolic product, hydrogen sulfide being toxic for humans. An important role is also played by *Clostridium* spp. through the exotoxins these species produce, that pass through the gut lining and reach the blood flow and eventually the brain, causing high oxidative stress to the nervous cells [1].

Some bacterial metabolites were tracked in order to become possible biomarkers in ASD. This is the case of p-cresol which is produced only in the gut by *Clostridium* spp., and has been identified in several studies as being increased in the urine and feces of ASD children [34].

P-cresol is a compound that has an inhibitory effect on dopamine-beta-hydroxylase, regulating the level of dopamine in the brain. Moreover, some studies have identified a directly proportional relation between p-cresol and the severity of the autistic behavior [32].

The last aspect discussed will be the increased role of short-chain fatty acids (SCFAs) and free amino acids (FAA) that were found in high levels in feces of ASD groups compared to typical controls. [33] SFCAs are mainly produced by *Clostridium*, Bacteriodetes and *Desulfovibrio* by fermentation of non-digestible carbohydrates. The production of SCFAs includes acetic acids (AA), propionic acids (PPA), butyrate, valeric acid isovaleric acids and others [2]. SCFAs and FAA have multiple effects in our body and brain, such as regulating the expression of genes encoding tyrosine hydroxylase which has an important role in dopamine, norepinephrine and epinephrine synthesis [25].

There is a diversity of research papers studying the impact of microbial flora changes and the effects of their metabolites, but also still a need to undergo further research in order to have a complete and homogeneous view of these complex differences.

b) Increased permeability of the gut epithelium

The increased permeability of the intestinal wall has been demonstrated in ASD children by measuring blood lactulose after oral administration. Increased permeability of the gut lining was also identified in ASD sib-

lings, raising the possibility that this change could be part of a broad autistic phenotype.

There are many aspects that influence gut permeability. Intestinal inflammation should be considered as the main cause, because it can result from various processes such as toxins, like those produced by *Clostridium*, *Desulfovibrio* and LPS (endotoxins) that are part of the Gram-negative bacterial cell wall(e.g. *Bacteroides*). Moreover, with the increased levels of Clostridia and decreased levels of *Bifidobacterium*, pro-inflammatory cytokines will be high resulting in an increased permeability of the intercellular junctions [21].

The intestinal dysbiosis has a critical role to play in the elevated gut permeability in ASD, as the absence of an equilibrium between pro-inflammatory and anti-inflammatory metabolites can trigger a chronic inflammatory process in the gut, but also creates a favorable environment for opportunistic and pathogenic infections.

c) Increased permeability of the blood brain barrier

The study of postmortem cerebral tissue has offered significant information about the structure of the BBB.

Studies have shown that the integrity of the BBB in ASD is affected by a low expression of some claudins (), proteins that are essential for the structure of tight junctions of the cells. There were two main changes observed in ASD cortex and cerebellum compared to typical brain tissue, both claudin (CLDN5 and CLDN12) expression was reduced in the brain tissue and their genes were overexpressed. [14] Both proteins play an essential role in tight junctions of endothelial cells in the brain. The most plausible cause for these changes is that the overexpression of the CLDN5 and CLDN12 is due to low integrity of BBB through a destruction of these two proteins.

Similar to gut permeability the tight junctions between endothelial cells loosens when there is a systemic inflammatory response. Exotoxins and endotoxins from the microbial flora impact both gut and blood brain barrier permeability.

d) Neuroinflammation and increased oxidative stress

Neuroinflammation represents the biochemical and cellular response of the brain to various factors like injury, infections and toxins. The innate immune response of the brain is provided by microglial cells, that maintain the homeostasis of the brain under normal conditions. In some cases, when there is an increased inflammatory systemic response or associated mitochondrial dysfunctions, the process of microglial activation becomes aberrant and toxic to the brain tissue.

Under normal condition of microglial activation there is a balance between cytokines and chemokines involved in the inflammation mechanism and the factors involved in tissue repair like resolvins, IL-10 and neuroprotectins, thus ensuring a low oxidative stress level. The oxidative stress appears when there is an imbalance between the production of reactive oxygen species and the ability to detoxify and repair the damage.

The main natural antioxidant of cells is glutathione (GSH) and there are studies that show decreased plasma levels of GSH can be a marker of increased oxidative stress [12].

Besides the normal functioning of the BBB and the normal microglial activation process, an important role for maintaining brain homeostasis is played by normal mitochondrial activation. Mitochondria are important for generating adenosinetriphosphate (ATP) for the normal metabolic activity of cells. When mitochondrial dysfunction occurs and there is no sufficient energy provided for cellular metabolism, the phenomenon of apoptosis appears, leading to inflammation and oxidative stress.

There are studies showing an underlying mitochondrial dysfunction in children with

ASD, as the mitochondria in the granulocytes of these children consume less oxygen than the mitochondria of typical children, this change being translated as mitochondrial abnormality [27]. Moreover, other studies revealed in the post-mortem brains of individuals with autism higher levels of Th1 cytokines than the control group [26] and also deviations in the levels of cells of the immune system such as natural killer cells and macrophages were observed in children with ASD.

All this data, including the abnormal permeability of BBB with increased inflammatory systemic response, the decreased levels of anti-inflammatory factors like GSH and increased inflammatory status is suggestive for immune system abnormalities in ASD children.

CONCLUSIONS

The intestinal microbiota has essential functions in our body and the dysbiosis identified in children with autism could impact the associated clinical manifestation of ASD and also may play a critical role in the pathogenesis of the disorder. There are many things to be further studied in order to draw a complete and convincing hypothesis regarding the gut-brain axis role in the etiology of autism.

On one hand, there is a need for an increased homogeneity of the research in this area, from defining what GI symptoms mean, because GI symptoms vary in the current literature between 9-91% [15], to the use of similar methodological approach.

On the other hand, further data will help define a stable pattern of gut microbiota in ASD children and maybe establish a metabolomics profile, that could be interrelated with functional pathways from our body.

This field sounds promising in making a further step towards understanding ASD and maybe in finding novel pathways in the management, screening but also in developing new treatment approaches.

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