Digestive and Liver Disease 51 (2019) 1366–1374

Contents lists available at ScienceDirect

Digestive and Liver Disease

journal homepage: www.elsevier.com/locate/dld

Alimentary tract

Functional analysis of gut microbiota and immunoinflammation in children with autism spectrum disorders

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ARTICLE INFO

Article history:
Received 3 April 2019
Accepted 9 June 2019
Available online 16 July 2019

Keywords:
Autism spectrum disorders
Gut microbiota
Metagenomics
Inflammation
HMGB1

ABSTRACT

Background and Aims: Recent evidence implicates gut microbiota (GM) and immune alterations in autism spectrum disorders (ASD). We assess GM profile and peripheral levels of immunological, neuronal and bacterial molecules in ASD children and controls. Alarmin HMGB1 was explored as a non-invasive biomarker to monitor gastrointestinal (GI) symptoms.
Methods: Thirty ASD children and 14 controls entered into the study. GM metagenomic analysis was performed for 16 ASD patients and 7 controls. GM functional profile was assessed by GO term analysis. Blood levels of IL-1β, TNFα, TGFβ, IL-10, INFγ, IL-8, lipopolysaccharide, Neurotensin, Sortilin1 and GSSG/GSH ratio were analyzed in all subjects by ELISA. Fecal HMGB1 was analyzed by Western blot.
Results: We observed a significant decrease in bacterial diversity. Furthermore, 82 GO terms underrepresented in ASD. Four of them pointed at 3,3 phenylpropionate catabolism and were imputable to *Escherichia coli* (*E. coli*) group. Serum levels of TNFα, TGFβ, NT, and SORT-1 increased in ASD patients. Fecal levels of HMGB1 correlated with GI sign severity in ASD children.
Conclusions: We suggest that a decrease of *E. coli* might affect the propionate catabolism in ASD. We report occurrence of peripheral inflammation in ASD children. We propose fecal HMGB1 as a non-invasive biomarker to detect GI symptoms.

1. Introduction

Autism spectrum disorders (ASD) include complex neurological conditions, characterized by impairment in social interaction and communication as well as restricted, repetitive, and stereotyped patterns of behavior, interests and activities. They affect up to one in 59 births in the United States and are the fastest growing neurodevelopmental disability worldwide [1]. The causes of ASD explaining the great clinical heterogeneity and variable symptomatic course, are largely unknown, except in the rare instance of identifiable genetic abnormalities, such as the Fragile X, Rett Syndrome, tuberous sclerosis complex and *PTEN*-associated ASD [2].

Recent research based on animal models has revealed new insights in the neurobiology alterations associated with autism, highlighting the gut microbiota (GM)-brain interactions in cognitive and emotional brain centers development and expression [3,4]. The routes of connection between GM and brain are not fully elucidated, but a widely agreed view suggests that neural, endocrine and immune pathways, activated at the intestinal epithelium and mucosa, are involved [3,5]. Hence, ASD might be considered not only a brain disorder but rather a whole-body disorder with broad systemic abnormalities in immune and metabolic variables, while GM, interacting with intestinal epithelium and mucosa, as well as with enteric nervous system, plays a critical role in modulating gut-brain axis [6,7].

Autistic neurodevelopmental disorders are frequently associated with GM dysbiosis [8] and gastrointestinal (GI) symptoms [9]. Finally, evidences from animal models suggest that certain gut microbial shifts may produce changes consistent with the clinical picture of autism, with consequent toxin production, aberrations in fermentation processes/products, and immunological

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https://doi.org/10.1016/j.dld.2019.06.006
1590-8658/

and metabolic abnormalities [4,10]. Coherently, in humans, ASD have been linked to altered short-chain fatty acid (SCFA) profiles deriving from GM fermentation [11]. Although findings in human studies are less abundant and the results are heterogeneous and often contradictory [12]; however, some studies have turned to targeting GM for treatment of ASD [10,13,14].

Furthermore, the immune system is emerging as an important regulator of the interaction between GM, brain development and behavior [7,15]. Imbalance of several interleukins has been reported in the peripheral blood of ASD children with increased activation of both Th1 and Th2 pathways [16], therefore, cytokine profiles are currently considered possible immune biomarkers in ASD [17], although results are still controversial due to the high heterogeneity of patients.

The present study aims to: 1) characterize the structural and functional peculiarities of the GM of ASD children as compared to controls through NGS shotgun metagenomics and assess possible relationships between GM composition and psycho-behavioral profiles; 2) analyze the levels of immunological, neuronal and bacterial molecules in the serum of ASD children and controls; 3) test the possible use of the alarmin HMGB1 as a non-invasive biomarker to identify the presence and severity of GI symptoms in ASD patients.

2. Methods

2.1. ASD patient clinical features

44 children were recruited and evaluated at the Department of Human Neurosciences, of the Sapienza University of Rome. Thirty (29 males, 1 female; age: 2–6 years, median: 3) children were affected by ASD, according to the DSM-5 diagnostic criteria (Table 1) while the other 14 (7 males, 7 female; age: 5–16 years, median:10) were controls. All patient and control children underwent immunological analysis, while subgroups of patients (16 subjects) and controls (7 subjects), indicated with the symbol * in Table 1, were selected for the metagenomic analysis. In particular, among ASD patients, 7 were taken that complained of GI symptoms (GIH $\geq$ 7), while 9 patients did not (GIH $\leq$ 3). All children had not been treated with antibiotic and/or functional foods (probiotics and/or prebiotics) for at least two weeks before enrollment. ASD pediatric patients were assessed by clinical observation, neurologic examination and structured diagnostic instruments, such as Griffiths Mental Development Scales (GMDS) and Autistic Diagnostic Observation Schedule (ADOS-2). Families completed in-person assessments, including the CHARGE Gastrointestinal History Questionnaire (GIH), about gastrointestinal symptoms. All patients showed a <-2,326 Griffiths Global Quotient z-score, that means all children had a severe developmental delay. At the ADOS-2 -, 4 patients obtained scores indicating a mild level of ASD symptoms, 16 had a moderate level, and 10 showed severe level of ASD symptoms.

The GIH questionnaire showed high scores (=>10) in 5 patients, medium scores (=>5 < 10) in 10 patients and lower scores (<5) in 15.

2.2. Ethical considerations

All patients entered into the study after informed consent from parents. The study was approved by the Sapienza University of Rome - Policlinico Umberto I Hospital Ethics Committee.

2.3. DNA extraction and shotgun metagenomic analysis

DNA isolation from stool samples and shotgun metagenomics analysis was performed as previously described [18]. Briefly, stool samples from 16 ASD patients (16 males, indicated in Table 1 with the sign *) and 7 healthy controls (2 males, 5 females) were collected. Microbial DNA was isolated using the QIAamp® DNA stool mini kit (Qiagen, Valencia, CA) following manufacturer's instructions. Samples were processed in parallel to minimize sample variations. DNA was RNAse-treated and then purified by phenol-chloroform and precipitated with isopropanol (0.8v) and sodium acetate (0.1v).

Library preparation and sequencing was performed at Istituto di Genomica Applicata (IGA-Udine, Italy). For shotgun metagenomics analysis, microbial DNA was sequenced by Paired End (PE) sequencing at a depth of at least 20 M reads per sample using Illumina technology.

Raw reads were quality checked (FastQC v0.11.2) and contaminant reads from the human genome (hg38) were removed using Kneaddata (v0.6.1). The reads sequenced and the percentage of reads arising from human DNA contamination are reported in Table 1 supplementary. Remaining reads were processed following the Humann2 pipeline to obtain taxonomic and functional profiles [19]. Multivariate analysis to test for association of specific taxa or GO term with metadata (ASD disease status, ADOS score, GIH, serum cytokines, fecal HMGB1) was performed using Maaslin [20] with default settings (BH p-value adjustments, Grubbs test for outlier detection) with the only exception of dMinAbd = 0.0000001. The number of species identified for each sample (including singletons) is reported in Table 1 supplementary.

Fisher diversity index was computed using the phyloseq R package [21]. To this purpose, metaphlan [22] was run with option -t reads map and the output was processed to count how many raw reads arose from each taxon for each samples (i.e., no sequencing depth normalization was performed, as specified in phyloseq documentation). P-value was computed using Wilcoxon Rank Sum test (one tailed). All figures were generated using R.

Table 1
Clinical and Demographic Characteristics of the 30 Patients with Autism Spectrum Disorder.

N.	Sex	Age (years)	ADOS-2comparison score	GIHscore
1[a]	M	5	Moderate	10
2[a]	M	3	Moderate	19
3[a]	M	5	Severe	0
4	M	2	Severe	4
5	M	2	Severe	6
6	M	4	Severe	6
7[a]	M	3	Mild	7
8	M	2	Moderate	6
9[a]	M	3	Severe	3
10[a]	M	6	Severe	11
11[a]	M	2	Moderate	0
12[a]	M	6	Moderate	10
13[a]	M	2	Severe	1
14[a]	M	3	Moderate	0
15	M	4	Mild	7
16	M	4	Severe	3
17[a]	M	3	Moderate	1
18	M	3	Moderate	4
19[a]	M	3	Moderate	8
20	M	5	Severe	3
21	M	5	Moderate	10
22	M	4	Moderate	6
23	M	4	Moderate	0
24[a]	M	3	Moderate	1
25[a]	M	3	Mild	8
26[a]	M	3	Moderate	2
27	M	5	Moderate	5
28	M	3	Moderate	5
29	F	4	Severe	2
30[a]	M	3	Mild	0

ADOS-2, Autistic Diagnostic Observation Schedule; GIH, Gastrointestinal History.
[a] patients selected for metagenomics analysis.

2.4. Serum samples collection and storage

Peripheral blood samples were collected into tubes without anticoagulant, incubated 2 h at room temperature and then centrifuged at 2000 rpm for 10 min at 4 °C. Clear supernatants were collected and stored at −80 °C.

2.5. Quantification of serum molecules by enzyme-linked immunosorbent assay (ELISA)

Levels of serum molecules were determined by commercial quantitative ELISA kits following the manufacturer's instructions: TNFα (Quantikine HS, R&D Systems, Minneapolis, MN, USA, cat. HSTA00D, detection limit: 0.106 pg/ml); IL-10 (Quantikine HS, R&D Systems, cat. HS100C, detection limit: 0.09 pg/ml); TGFβ (Quantikine, R&D Systems, cat. DB100B, detection limit: 4.61 pg/ml); Neurotensin (Cloud Clone Corp., Katy, TX, USA, cat. CEB203Hu, detection limit: 13.2 pg/ml); Sortilin1 (LifeSpan Bioscience, Seattle, WA, USA; cat. LS-F6828, detection limit: 0.156 ng/ml); IL-1β (Quantikine HS, R&D Systems, cat. DLB50, detection limit: 1 pg/ml); IL-8 (Quantikine HS, R&D Systems, cat. HS800, detection limit 0.02-0.40 pg/mL); LPS (LifeSpan Bioscience, cat. LS-F17912, detection limit: 0.78 ng/ml); INFγ (Quantikine, R&D Systems, cat. DIF50, detection limit: 8 pg/ml). Determination of serum oxidized glutathione (GSSG) and reduced glutathione (GSH) level was performed by the colorimetric assay OxiSelect™ Total glutathione (GSSG/GSH) Kit (Cell Biolabs, San Diego, CA, USA; cat. STA-312).

Standard curves were generated using eight points of concentrations; three replicates of each serum sample were analyzed.

2.6. Protein extraction from stool samples

Stool specimens, stored at −80 °C, were weighed and resuspended in a volume of extraction buffer (ScheBo Biotech AG, Giessen, Germany) to obtain a final concentration of 500 mg/ml. Samples were placed to a vigorous orbital shaking for 1 h at room temperature and then centrifuged twice at 10.000 rpm for 10 min at 4 °C. Clear supernatants were stored at −80 °C. Concentration of total fecal proteins was determined by the Bradford assay (Bio-Rad Laboratories).

2.7. Immunoblot analysis

Twenty micrograms of fecal extracts were fractionated by sodium dodecyl sulfate-(12%) polyacrylamide gel electrophoresis to detect HMGB1. Proteins were transferred in polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA) and blocked with TBS-Tween 20 0.1%, containing 5% non-fat dry milk. Anti-human HMGB1 antibody (1:1.000; R&D, Minneapolis, MN) was diluted in TBS-Tween 20 0.1%, containing 3% non-fat dry milk and incubated overnight at 4 °C. Membranes were washed in TBS-Tween 20 0.1%, incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), washed in TBS-Tween 0.1%, and developed with LiteBlotEXTEND (Euroclone, Milan, Italy). Densitometric analysis of western blot bands was performed using the Software ImageQuant Las500 (GE Healthcare Life Science, Uppsala, Sweden).

2.8. Statistics

Statistical analysis of ELISA experiments was performed using the Graph Pad InStat Software (La Jolla, CA USA). Data were presented as mean ± Standard Error of the Mean (S.E.M.). Comparison among groups was performed by non-parametric Mann Whitney test. Statistic correlation between serum molecules and clinical scores ADOS-2 and GIH (Gastrointestinal History) was computed by the Spearman's rank correlation. Correlation between fecal HMGB1 and GH score was computed by Spearman's rank correlation. Differences were noted as significant * $p < 0.05$, *** $p < 0.001$.

3. Results

3.1. Taxonomic profiles of GM of ASD patients and controls

GM taxonomic profiles were assessed by NGS shotgun metagenomic analysis of stool samples from 16 ASD patients and 7 neurotypical controls. The number of reads sequenced, the percentage of reads arising from human DNA contamination and the number of species identified for each sample are reported in Table 1 supplementary. With the only exception of sample C2, contamination by human DNA was negligible. Nevertheless, all reads mapping to the human genome (hg38) were removed before analysis.

The taxonomic profiles at phylum (Fig. 1A), class (Fig. 1B), order (Fig. 1C) and family (Fig. 1D) levels are shown for ASD and control groups. Taxonomic profiles for each single sample are reported in Fig. 1Fig. 1 Supplementary. Results did not show any significant association between taxa identified and ASD patients nor between taxa and ADOS or GIH scores.

3.2. Functional profiles of GM differentiate between ASD and control children

To evaluate the GM functional profiles of ASD patients and controls, each DNA fragment was assigned to a gene family and, in turn, each gene was assigned to a Gene Ontology (GO) Term as suggested by the GO classification. Interestingly, upon multivariate analysis with multiple testing corrections, 82 GO terms displayed significant differences between ASD patients and neurotypical controls (Q-value<0.05). In particular, 2 GO terms belonged to the Cell Compartment category, 30 belonged to the Biological Pathway category and 50 belonged to the Molecular Function category (Table 2 supplementary). Notably, 81 out of 82 GO terms were strongly underrepresented in ASD patients (patients A2 and A25 were detected as outliers by Maaslin using Grubbs test and hence excluded from this analysis) as compared to controls, belonging to Biological Processes (Fig. 2A) and Molecular Function (Fig. 2B) categories. This finding highlights that, although the absence of specific correlations between GM taxa and ASD patients or controls, however, there is a strongly reduced biodiversity in ASD patients as compared to controls. Accordingly, the α diversity by Fisher index analysis, measuring the microbial richness, confirmed a statistically significant difference between ASD and control samples (p-value = 0.03261) (Fig. 2C). Notably, no statistically significant difference was observed in the number of reads yielded by NGS for ASD and control samples (Table 1 supplementary), implying that the difference of α diversity value between ASD and controls groups is not attributable to the depth of sequencing, but to a generally higher number of taxa.

3.3. Association between 3,3 phenylpropionate catabolism and Escherichia coli (E. coli) presence

Four GO terms (GO:0019380, GO:0008695, GO:0008688 and GO:0019622) highlighted a loss-of-function in the catabolism of 3,3 phenylpropionate, deriving from propionic acid (PPA), in ASD patients as compared to controls (Fig. 2 Supplementary). When we traced the bacterial DNA sequences related to PPA GO terms, we found that the most of them mapped to *E. coli* genome. Intriguingly, we observed that *E. coli* is more abundant in GM of control than ASD subjects (Fig. 2D). At variance, no difference was found in Clostridia abundance between ASD and control subjects.

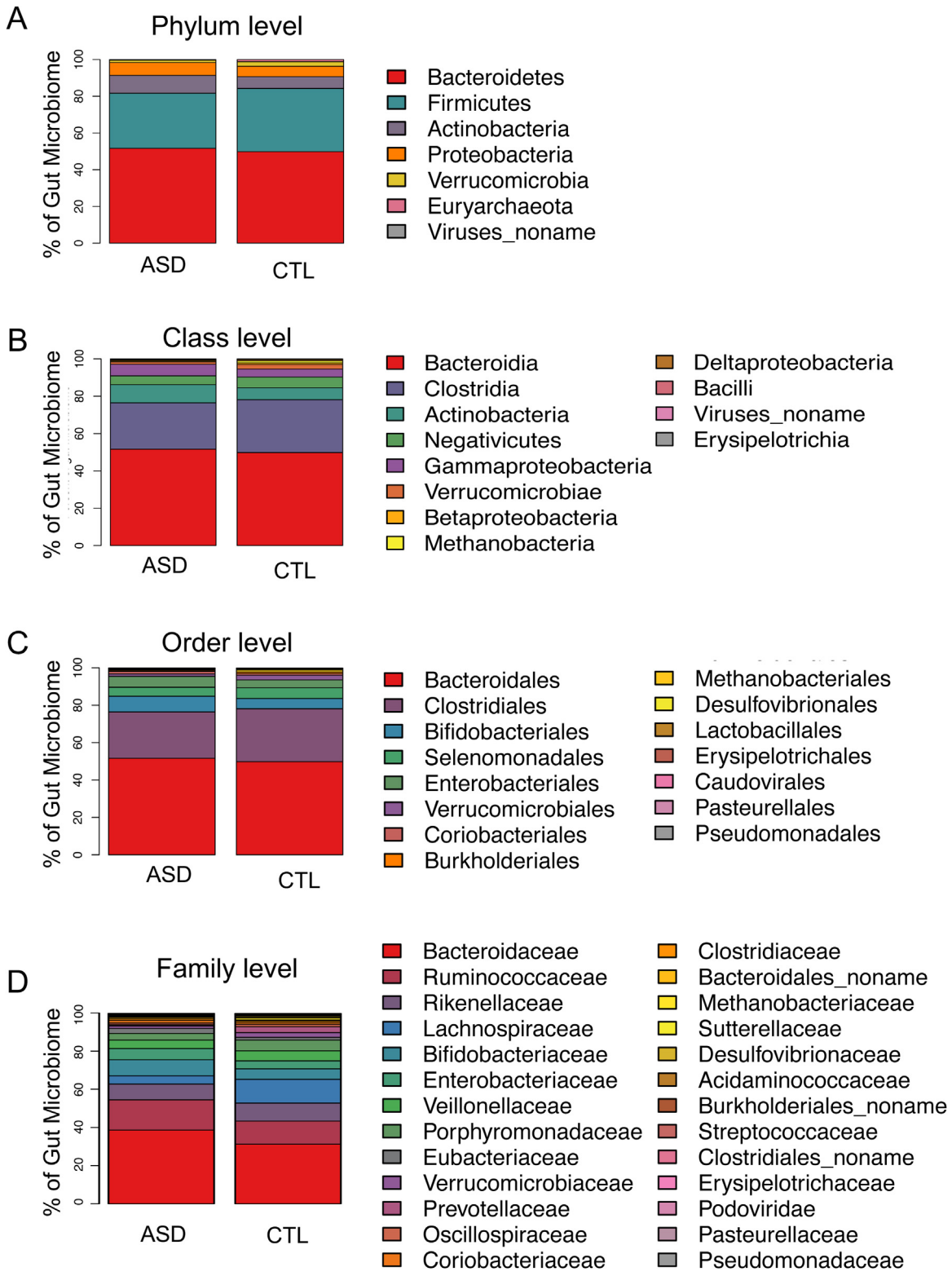

Fig. 1. Taxonomic analysis of gut microbiome from ASD patients and healthy controls.
Taxonomic composition of the gut microbiota is reported at phylum (panel A), class (panel B), order (panel C) and family (D) level. ASD = Autism Spectrum Disorder; CTL = healthy controls.

3.4. *Immuno-inflammatory profiles of ASD and control subjects*

A chronic state of low grade inflammation and alterations in the immune response has been reported in ASD patients [15]. Therefore, we analyzed the presence of molecules related to inflammation or neuroinflammation in serum samples of ASD and control subjects. Peripheral blood levels of IL-1β, TNFα, TGFβ, IL-10, INFγ, IL-8 and the bacterial endotoxin lipopolysaccharide (LPS) were analyzed in 30 ASD and 14 control subjects by ELISA. Moreover, proteins of neuronal signaling, Neurotensin peptide (NT) and Sor-

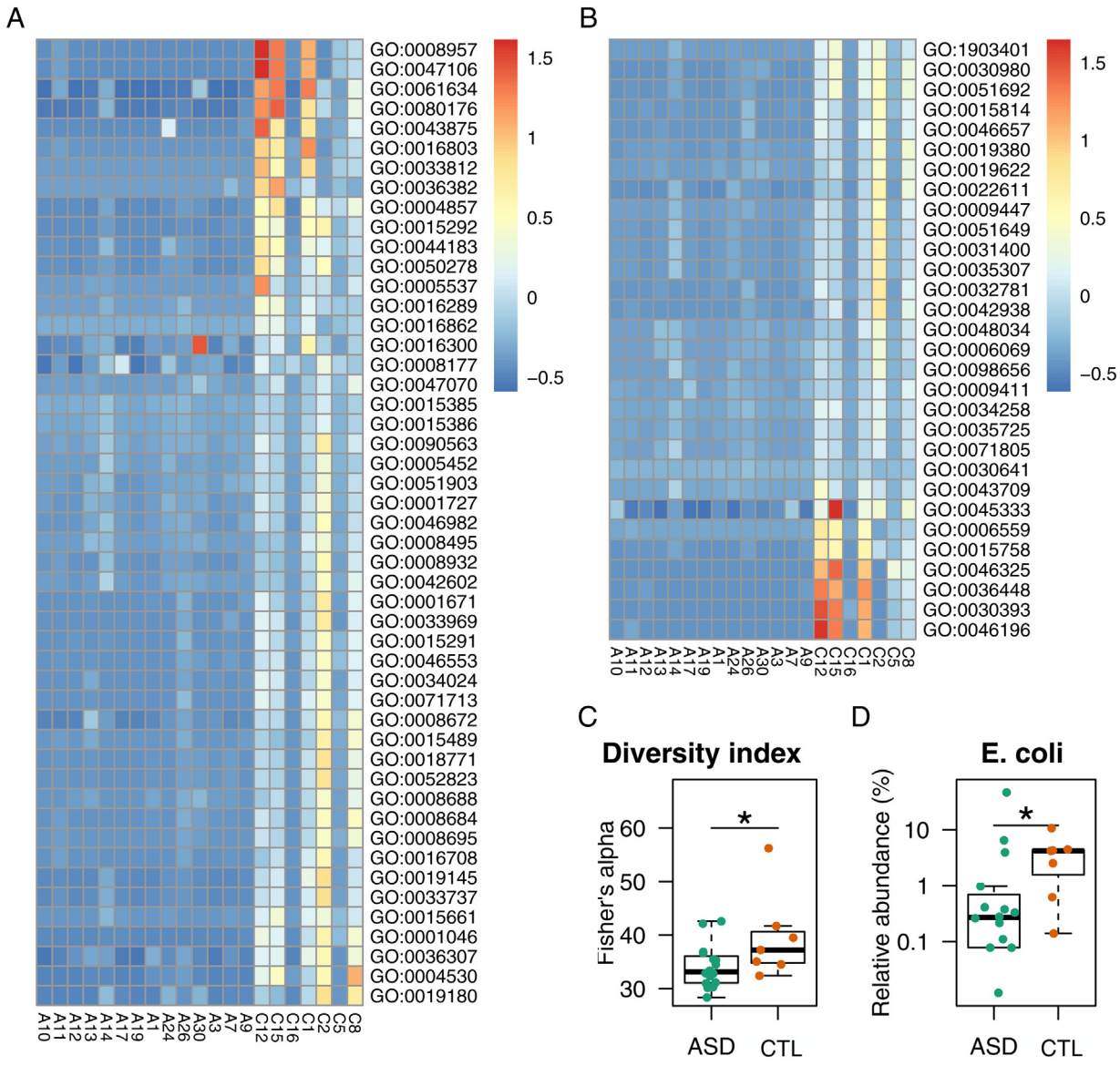

Fig. 2. Differences between GM of ASD patients and healthy controls.
Molecular Function (panel A) and Biological Process (panel B) GO terms significantly under-represented in the gut microbiota of ASD as compared to healthy controls (CTL). A1-30: ASD patients; C1-16 healthy controls. Fisher diversity index for ASD and Control samples (panel C). Proportion of reads mapping to *E. coli* in ASD and Control samples (panel D). $*p < 0.05$ (Wilcoxon Rank Sum test, one sided).

tilin1 (SORT-1) as well as the oxidative stress marker, GSSG/GSH ratio, all involved in neuroinflammation, were also investigated.

Although no significant difference was found in serum levels of IL-1β, IL-8, INFγ, LPS and GSSG/GSH ratio between groups, results showed significantly higher serum levels of TNFα, IL-10 ($p < 0.05$), TGFβ ($p < 0.001$), NT ($p < 0.001$) and SORT-1 ($p < 0.001$) in ASD patients as compared to controls (Fig. 3).

No significant correlation was found between levels of cytokines, neuropeptides or oxidative stress markers and the severity of symptoms of ASD patients, as assessed by the ADOS-2 score, GM composition and GI symptoms (as indicated by GIH score).

3.5. *Fecal HMGB1 correlates with the presence and severity of GI symptom in ASD patients*

ASD is frequently associated with GI symptoms [9], but the identification of a GI disorder in ASD children, who often have difficulty in reporting symptoms, might be difficult. Therefore, the identification of biomarkers for intestinal inflammation related to GI symptoms in ASD children is crucial. Our data showed that nor GM composition neither immuno-inflammatory profile correlate with GI symptoms.

Since the alarmin HMGB1 is detected in the stools of patients affected by gut inflammation [23,24], we assessed the presence of fecal HMGB1 in our cohort of patients and controls. Interestingly, the amount of HMGB1 in the stools of ASD patients analyzed by western blot significantly correlated with the occurrence and severity of GI symptoms ($r = 0.69$, $p < 0.001$) (Fig. 4A and B).

4. Discussion

There is a growing body of evidence indicating the GM influences neurodevelopment and behavior [8]. Previous studies, mostly performed by 16S rDNA amplicon sequencing, report conflicting results on the association between GM dysbiosis and ASD [4].

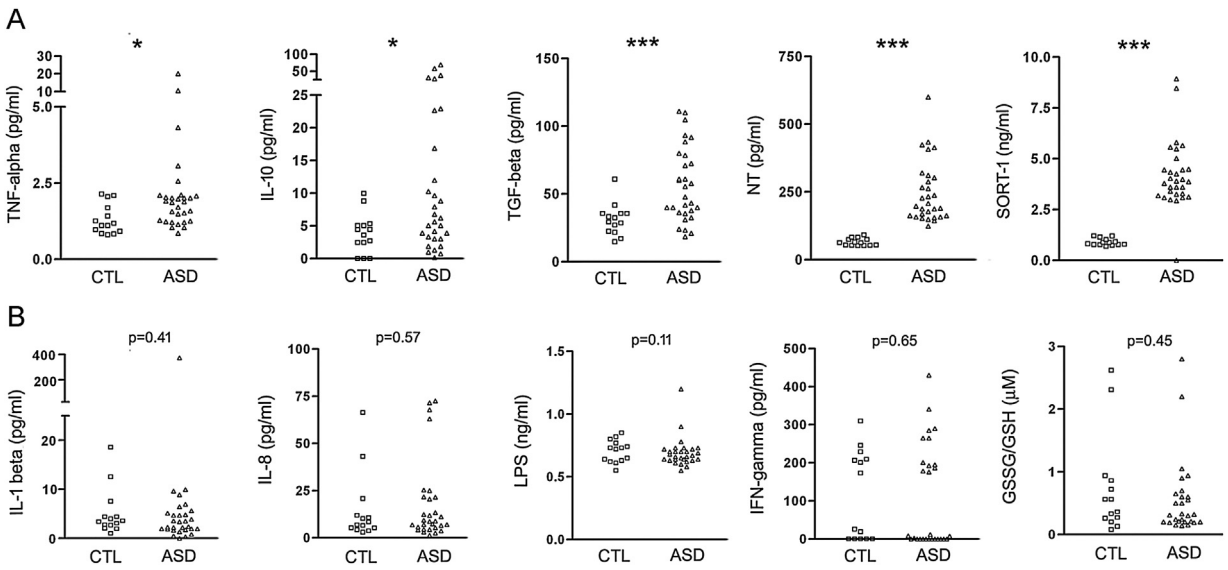

Fig. 3. Immuno-inflammatory profiles in ASD and control subjects.
Elisa assay of serum molecules in ASD patients (n = 30) and controls (n = 14). Mann-Whitney t-test: *p < 0,05, ***p < 0,001.

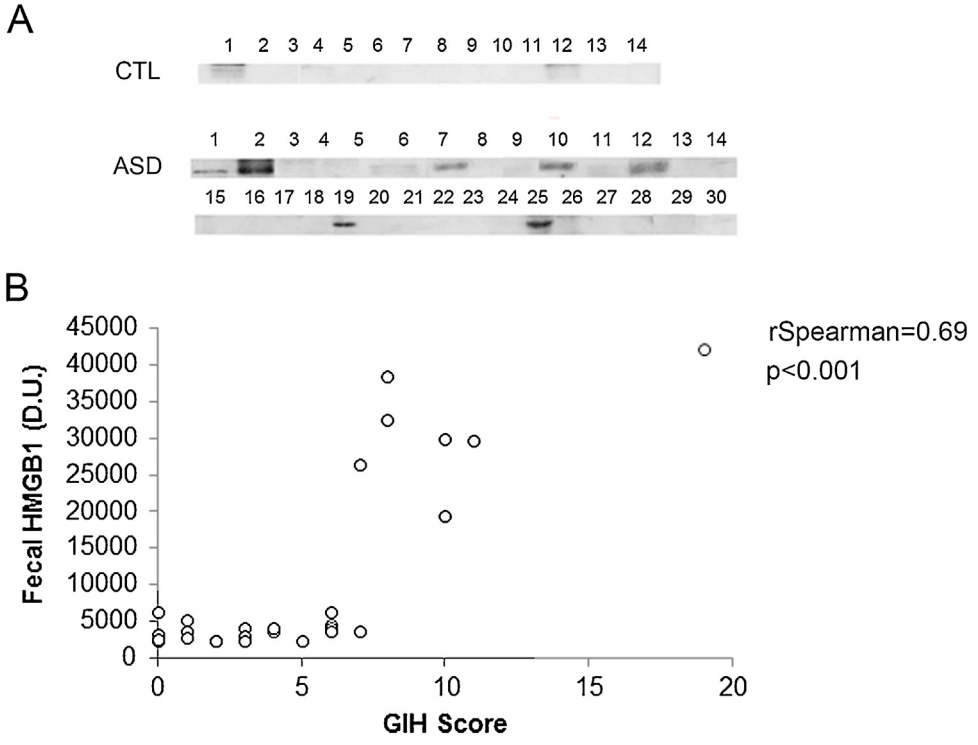

Fig. 4. Fecal HMGB1 indicates the presence and severity of GI symptoms in ASD patients. Western Blotting of fecal HMGB1 in Autism Spectrum Disorders (ASD) patients (n = 30) and controls (n = 14) (panel A); Correlation between fecal HMGB1 and GIH score (panel B) (two-tailed P < 0.0001).

Here, we investigate the GM composition in the stools of a cohort of 16 ASD patients with comparable developmental level and 7 neurotypical children, by using NGS shotgun sequencing. The application of NGS to the study of GM metagenome is more relevant as compared to the 16S approach since it may provide a functional other than taxonomic view of the bacterial community allowing a wider comprehension of the role of bacteria in the physiological as well as pathological state of the host. Indeed, in this study, we used the Gene Ontology classification to assign each gene to a GO term, depending on its functional characteristics, instead of calculating for each gene a relative abundance.

We are aware that in our metagenomics analysis control patients are mostly females whereas ASD are principally males. Several studies have provided some evidence regarding differences in the GM community structure between sexes at taxonomic level, with no changes in α diversity [25,26]. However sexual maturation and sexual hormones were considered as major determinants [25,27]. Thus, since our data are collected in pre-pubertal age and show differences in α diversity between control and ASD patients,

we believe that the effects of gender differences can be considered negligible.

Interestingly, although multivariate analysis did not show any significant association between specific bacterial taxa and ASD patients, however, we found 82 GO terms underrepresented in ASD GM, suggesting a decreased biodiversity. Accordingly, we found a lower α-diversity in the GM of ASD children as compared to controls. Currently, microbial diversity is considered to play a key role in the maintenance of intestinal homeostasis, in the development of the immune system of the gut mucosa and, in general, in the preservation of the human wellness. Accordingly, there are many evidences that most of the human diseases affecting westernized countries are associated with loss of microbial diversity in the GM [28–30].

Furthermore, we observed that 4 of the 82 GO terms shared a role in the catabolic processing of the 3,3 phenylpropionate, a monocarboxylic acid anion that is the conjugate base of *3*-phenylpropionic acid deriving from propionic acid (PPA). PPA is a ubiquitous short-chain fatty acid (SCFA), a major class of signaling molecules produced during bacterial fermentation of dietary carbohydrates. Whereas PPA is an energy substrate and has many proposed beneficial effects, it is also associated with human neurodevelopmental disorders, including ASD [31–33]. Indeed, increased mean levels of PPA in stools of ASD children have been shown [34]. Moreover, the 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA) was found in higher concentrations in urinary samples of autistic children than in the controls and its measurement was suggested to have potential clinical utility for identifying subgroups of ASD subjects [35–37].

The main source of 3-phenylpropionic acid and HPHPA appear to be multiple species of anaerobic bacteria of the *Clostridium* genus [37,36,38,39] whose colonization in the intestinal tract has been related to the probability of developing and/or aggravating autism among children [12,40]. Remarkably, we do not find any significant difference between the Clostridia group in ASD and controls, but we instead observed a lower prevalence of *E. coli* in the former. Moreover, tracing the bacterial DNA sequences linked to PPA metabolism- GO terms, we found that the most mapped to *E. coli* group. It is worth noting that the involvement of *E. coli* in the degradation of the phenylpropionate has been well documented by several authors [41–43]. On the basis of our findings, we suggest that the elevated concentration of propionate metabolites described in ASD children and traditionally related to an enhanced production by Clostridia, could be alternatively due to their reduced degradation as a consequence of the *E. coli* drop.

In addition to intestinal microbial community structure and functions, strong inflammation states are associated with ASD. This inflammatory condition is often linked to immune system dysfunction. Neuro-inflammation and neuro-immune abnormalities have now been established in ASD as key factors in its development and maintenance [44]. However, results are still controversial. Hence, we analyzed the expression levels of the cytokines IL-1β, TNFα, TGFβ, IL-10, INFγ and IL-8 in the peripheral blood of ASD patients and controls. We also analyzed the concentrations of neuronal signaling molecules (NT and SORT-1), bacterial endotoxins (LPS) and oxidative stress molecules (GSSG/GSH ratio) that have been involved in neuroinflammation and brain damage [45,46]. We found increased serum levels of TNFα and TGFβ, indicating a discrete immune dysfunction in ASD of pro-inflammatory character. High levels of TNFα have been already shown by several authors [47,48]. Interestingly, TNFα is supposed to cross the blood-brain barrier and trigger neurodegeneration [49,50]. On the contrary, the behavior of TGFβ is less clear and results are more contradictory [51], with papers describing both elevated [52,53] and reduced TGFβ amounts in the serum/plasma of ASD children [54,53]. However, our results are consistent with the higher level of TGF-β1 found in post-mortem brain tissue from ASD patients [55]. Differently from previous literature showing decreased IL-10 in ASD patients [56] or levels similar to controls [57], we found the counterregulatory cytokine IL-10 increased in ASD patients. Further investigation into this immune regulatory mechanism in ASD is needed.

Overall, this finding confirms that a chronic state of low-grade inflammation is detected in children with ASD.

We also showed increased concentrations of NT and SORT-1 in ASD children. NT, a neuropeptide thought to modulate dopaminergic and other neurotransmitter systems, is involved in the pathophysiology of various mental disorders, including autism. Elevated NT levels in the periphery have been associated with immune reactivity and GI problems in ASD [58]. Moreover, NT stimulates the release of cellular mitochondrial DNA, which is elevated in serum of children with ASD [59] and seems to cause elevation of cytokines [60]. Recently, SORT-1 has been also found increased in children with ASD and a link between NT and SORT-1 has been suggested [45].

Clinical manifestation of digestive tract problems in children with ASD may differ as compared to children with typical development, and the diagnosis of a GI disorder in children may be more difficult and delayed in time. Alarmins are a multifunctional heterogeneous group of proteins that are released in the surrounding tissues as a consequence of cell damage or inflammation. Their functions are multiple as they could activate innate immunity or recruit and activate antigen-presenting cells stimulating an adaptive response. Alarmins are interesting both for understanding the inflammatory process and for diagnostic purposes as biomarkers [61].

HMGB1, an alarmin prototype, normally resides in the nucleus, where it functions as a structural co-factor critical for proper transcriptional regulation in somatic cells. However, under appropriate signal stimulation, it is released into the extracellular milieu and activates the immune system promoting inflammation. In previous studies, we found that HMGB1 is abundantly found in the stools of subjects with gut inflammation, such as those with Inflammatory Bowel Disease (IBD), and correlates with the severity of symptoms, so it can be considered a robust noninvasive biomarker of clinically overt and subclinical gut inflammation [23,24]. Thus, we tested the usefulness of the alarmin HMGB1 to identify the presence and severity of GI problems in children with ASD who often have difficulty in reporting symptoms. Interestingly, we found a strong correlation between the presence of HMGB1 in the stools of ASD children and the presence and severity of GI signs. Accordingly, we believe that fecal HMGB1 should be further investigated as a tool to verify the occurrence of GI impairment, and eventually its resolution after therapy, also in children with extra-intestinal diseases, such as ASD.

In conclusion, our study highlights several novel aspects of ASD pathophysiology: 1) decreased GM biodiversity in ASD children as compared to healthy controls; 2) under-representation in GM of ASD subjects of several functions, such as catabolism of 3,3 phenylpropionate; 3) the loss of *E. coli* strains known to regulate the propionate catabolism [41–43]in the GM of ASD children; 4) the occurrence of peripheral immune dysfunctions of pro-inflammatory character in ASD children; 5) relationship between fecal HMGB1 levels and presence and severity of GI symptoms in ASD children, suggesting its use as non-invasive tool to early diagnose and monitor the occurrence of intestinal inflammation.

We believe that overall our findings could be a catalyst for extensive research into the mechanisms through which the GM and immune system influence the brain and behavior opening new

perspectives into the knowledge and management of this complex disorder.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by a grant MIUR Progetti di Ateneo Sapienza Universita'di Roma to Dr. Salvatore Cucchiara.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.dld.2019.06.006.

References

[1] Dadalko OI, Travers BG. Evidence for brainstem contributions to autism Spectrum disorders. Front Integr Neurosci 2018;12:47.
[2] Fernandez BA, Scherer SW. Syndromic autism spectrum disorders: moving from a clinically defined to a molecularly defined approach. Dialogues Clin Neurosci 2017;19(4):353–71.
[3] Roman P, Rueda-Ruzafa L, Cardona D, Cortes-Rodríguez A. Gut-brain axis in the executive function of austism spectrum disorder. Behav Pharmacol 2018;29(7):654–63.
[4] Hughes HK, Rose D, Ashwood P. The gut microbiota and dysbiosis in autism Spectrum disorders. Curr Neurol Neurosci Rep 2018;18(11):81.
[5] Martin CR, Osadchiy V, Kalani A, Mayer EA. The brain-gut-microbiome axis. Cell Mol Gastroenterol Hepatol 2018;6(2):133–48.
[6] Li Q, Han Y, Dy ABC, Hagerman RJ. The gut microbiota and autism Spectrum disorders. Front Cell Neurosci 2017;11:120.
[7] Azhari A, Azizan F, Esposito G. A systematic review of gut-immune-brain mechanisms in Autism Spectrum disorder. Dev Psychobiol 2018.
[8] Vuong HE, Hsiao EY. Emerging roles for the gut microbiome in autism Spectrum disorder. Biol Psychiatry 2017;81(5):411–23.
[9] Holingue C, Newill C, Lee L-C, Pasricha PJ, Daniele Fallin M. Gastrointestinal symptoms in autism spectrum disorder: a review of the literature on ascertainment and prevalence. Autism Res 2018;11(1):24–36.
[10] Yang Y, Tian J, Yang B. Targeting gut microbiome: a novel and potential therapy for autism. Life Sci 2018;194:111–9.
[11] Morris G, Berk M, Carvalho A, et al. The role of the microbial metabolites including tryptophan catabolites and short chain fatty acids in the pathophysiology of immune-inflammatory and neuroimmune disease. Mol Neurobiol 2017;54(6):4432–51.
[12] Ding HT, Taur Y, Walkup JT. Gut microbiota and autism: key concepts and findings. J Autism Dev Disord 2017;47(2):480–9.
[13] Fowlie G, Cohen N, Ming X. The perturbance of microbiome and gut-brain Axis in autism Spectrum disorders. Int J Mol Sci 2018;19(8).
[14] Frye RE, Rossignol DA. Identification and treatment of pathophysiological comorbidities of autism Spectrum disorder to achieve optimal outcomes. Clin Med Insights Pediatr 2016;10:43–56.
[15] Meltzer A, Van de Water J. The role of the immune system in autism Spectrum disorder. Neuropsychopharmacology 2017;42(1):284–98.
[16] Jiang NM, Cowan M, Moonah SN, Petri WA. The impact of systemic inflammation on neurodevelopment. Trends Mol Med 2018;24(9):794–804.
[17] Masi A, Glozier N, Dale R, Guastella AJ. The immune system, cytokines, and biomarkers in autism Spectrum disorder. Neurosci Bull 2017;33(2):194–204.
[18] Laudadio I, Fulci V, Palone F, Stronati L, Cucchiara S, Carissimi C. Quantitative assessment of shotgun metagenomics and 16S rDNA amplicon sequencing in the study of human gut microbiome. OMICS 2018;22(4):248–54.
[19] Franzosa EA, McIver LJ, Rahnavard G, et al. Species-level functional profiling of metagenomes and metatranscriptomes. Nat Methods 2018;15(11):962.
[20] Morgan XC, Tickle TL, Sokol H, et al. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biol 2012;13(9):R79.
[21] McMurdie PJ, Holmes S. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 2013;8(4):e61217.
[22] Segata N, Waldron L, Ballarini A, Narasimhan V, Jousson O, Huttenhower C. Metagenomic microbial community profiling using unique clade-specific marker genes. Nat Methods 2012;9(8):811–4.
[23] Vitali R, Stronati L, Negroni A, et al. Fecal HMGB1 is a novel marker of intestinal mucosal inflammation in pediatric inflammatory bowel disease. Am J Gastroenterol 2011;106(11):2029–40.
[24] Palone F, Vitali R, Cucchiara S, et al. Fecal HMGB1 reveals microscopic inflammation in adult and pediatric patients with inflammatory bowel disease in clinical and endoscopic remission. Inflamm Bowel Dis 2016;22(12):2886–93.
[25] Takagi T, Naito Y, Inoue R, et al. Differences in gut microbiota associated with age, sex, and stool consistency in healthy Japanese subjects. J Gastroenterol 2019;54(1):53–63.
[26] Haro C, Rangel-Zúñiga OA, Alcalá-Díaz JF, et al. Intestinal microbiota is influenced by gender and body mass index. PLoS One 2016;11(5):e0154090.
[27] Org E, Mehrabian M, Parks BW, et al. Sex differences and hormonal effects on gut microbiota composition in mice. Gut Microbes 2016;7(4):313–22.
[28] Mosca A, Leclerc M, Hugot JP. Gut microbiota diversity and human diseases: should we reintroduce key predators in our ecosystem? Front Microbiol 2016;7:455.
[29] Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature 2012;489(7415):220–30.
[30] Prehn-Kristensen A, Zimmermann A, Tittmann L, et al. Reduced microbiome alpha diversity in young patients with ADHD. PLoS One 2018;13(7):e0200728.
[31] Frye RE, Rose S, Chacko J, et al. Modulation of mitochondrial function by the microbiome metabolite propionic acid in autism and control cell lines. Transl Psychiatry 2016;6(10):e927.
[32] MacFabe DF. Enteric short-chain fatty acids: microbial messengers of metabolism, mitochondria, and mind: implications in autism spectrum disorders. Microb Ecol Health Dis 2015;26:28177.
[33] Nankova BB, Agarwal R, MacFabe DF, La Gamma EF. Enteric bacterial metabolites propionic and butyric acid modulate gene expression, including CREB-dependent catecholaminergic neurotransmission, in PC12 cells–possible relevance to autism spectrum disorders. PLoS One 2014;9(8):e103740.
[34] Wang L, Christophersen CT, Sorich MJ, Gerber JP, Angley MT, Conlon MA. Elevated fecal short chain fatty acid and ammonia concentrations in children with autism spectrum disorder. Dig Dis Sci 2012;57(8):2096–102.
[35] Xiong X, Liu D, Wang Y, Zeng T, Peng Y. Urinary 3-(3-Hydroxyphenyl)-3-hydroxypropionic acid, 3-Hydroxyphenylacetic acid, and 3-Hydroxyhippuric acid are elevated in children with autism Spectrum disorders. Biomed Res Int 2016;2016:9485412.
[36] Shaw W. Increased urinary excretion of a 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA), an abnormal phenylalanine metabolite of Clostridia spp. In the gastrointestinal tract, in urine samples from patients with autism and schizophrenia. Nutr Neurosci 2010;13(3):135–43.
[37] Keşli R, Gökçen C, Buluğ U, Terzi Y. Investigation of the relation between anaerobic bacteria genus clostridium and late-onset autism etiology in children. J Immunoassay Immunochem 2014;35(1):101–9.
[38] Moss CW, Lambert MA, Goldsmith DJ. Production of hydrocinnamic acid by clostridia. Appl Microbiol 1970;19(2):375–8.
[39] Finegold SM. State of the art; microbiology in health and disease. Intestinal bacterial flora in autism. Anaerobe 2011;17(6):367–8.
[40] Argou-Cardozo I, Zeidán-Chuliá F. Clostridium Bacteria and autism Spectrum conditions: a systematic review and hypothetical contribution of environmental glyphosate levels. Med Sci (Basel) 2018;6(2).
[41] Manso I, Torres B, Andreu JM, et al. 3-Hydroxyphenylpropionate and phenylpropionate are synergistic activators of the MhpR transcriptional regulator from *Escherichia coli*. J Biol Chem 2009;284(32):21218–28.
[42] Turlin E, Perrotte-piquemal M, Danchin A, Biville F. Regulation of the early steps of 3-phenylpropionate catabolism in *Escherichia coli*. J Mol Microbiol Biotechnol 2001;3(1):127–33.
[43] Turlin E, Sismeiro O, Le Caer JP, Labas V, Danchin A, Biville F. 3-phenylpropionate catabolism and the *Escherichia coli* oxidative stress response. Res Microbiol 2005;156(3):312–21.
[44] Siniscalco D, Schultz S, Brigida AL, Antonucci N. Inflammation and neuroimmune dysregulations in autism spectrum disorders. Pharmaceuticals (Basel) 2018;11(2).
[45] Patel AB, Tsilioni I, Leeman SE, Theoharides TC. Neurotensin stimulates sortilin and mTOR in human microglia inhibitable by methoxyluteolin, a potential therapeutic target for autism. Proc Natl Acad Sci USA 2016;113(45):E7049–58.
[46] Rudzki L, Szulc A. "Immune gate" of psychopathology-the role of gut derived immune activation in major psychiatric disorders. Front Psychiatry 2018;9:205.
[47] Xie J, Huang L, Li X, et al. Immunological cytokine profiling identifies TNF-α as a key molecule dysregulated in autistic children. Oncotarget 2017;8(47):82390–8.
[48] Ferguson BJ, Marler S, Altstein LL, et al. Associations between cytokines, endocrine stress response, and gastrointestinal symptoms in autism spectrum disorder. Brain Behav Immun 2016;58:57–62.
[49] Kalkan Z, Durasi İM, Sezerman U, Atasever-Arslan B. Potential of GRID2 receptor gene for preventing TNF-induced neurodegeneration in autism. Neurosci Lett 2016;620:62–9.
[50] Hong H, Kim BS, Im H-I. Pathophysiological role of neuroinflammation in neurodegenerative diseases and psychiatric disorders. Int Neurourol J 2016;20(Suppl 1):S2–7.
[51] Xu N, Li X, Zhong Y. Inflammatory cytokines: potential biomarkers of immunologic dysfunction in autism spectrum disorders. Mediators Inflamm 2015;2015:531518.
[52] Hu C-C, Xu X, Xiong G-L, et al. Alterations in plasma cytokine levels in chinese children with autism spectrum disorder. Autism Res 2018;11(7):989–99.
[53] Ashwood P, Enstrom A, Krakowiak P, et al. Decreased transforming growth factor beta1 in autism: a potential link between immune dysregulation and impairment in clinical behavioral outcomes. J Neuroimmunol 2008;204(1–2):149–53.
[54] Okada K, Hashimoto K, Iwata Y, et al. Decreased serum levels of transforming growth factor-beta1 in patients with autism. Prog Neuropsychopharmacol Biol Psychiatry 2007;31(1):187–90.
[55] Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA. Neuroglial activation and neuroinflammation in the brain of patients with autism. Ann Neurol 2005;57(1):67–81.

[56] Jyonouchi H, Geng L, Davidow AL. Cytokine profiles by peripheral blood monocytes are associated with changes in behavioral symptoms following immune insults in a subset of ASD subjects: an inflammatory subtype? J Neuroinflammation 2014;11:187.
[57] Molloy CA, Morrow AL, Meinzen-Derr J, et al. Elevated cytokine levels in children with autism spectrum disorder. J Neuroimmunol 2006;172(1–2):198–205.
[58] Boules MM, Fredrickson P, Muehlmann AM, Richelson E. Elucidating the role of neurotensin in the pathophysiology and management of major mental disorders. Behav Sci (Basel) 2014;4(2):125–53.
[59] Zhang B, Angelidou A, Alysandratos K-D, et al. Mitochondrial DNA and anti-mitochondrial antibodies in serum of autistic children. J Neuroinflammation 2010;7:80.
[60] Koon H-W, Kim YS, Xu H, et al. Neurotensin induces IL-6 secretion in mouse preadipocytes and adipose tissues during 2,4,6,-trinitrobenzensulphonic acid-induced colitis. Proc Natl Acad Sci USA 2009;106(21):8766–71.
[61] Di Salvo E, Casciaro M, Quartuccio S, Genovese L, Gangemi S. Do alarmins have a potential role in autism spectrum disorders pathogenesis and progression? Biomolecules 2018;9(1).