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THE MICROBIOME IN AUTISM SPECTRUM DISORDER

Approaches to studying and manipulating the enteric microbiome to improve autism symptoms

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There is a growing body of scientific evidence that the health of the microbiome (the trillions of microbes that inhabit the human host) plays an important role in maintaining the health of the host and that disruptions in the microbiome may play a role in certain disease processes. An increasing number of research studies have provided evidence that the composition of the gut (enteric) microbiome (GM) in at least a subset of individuals with autism spectrum disorder (ASD) deviates from what is usually observed in typically developing individuals. There are several lines of research that suggest that specific changes in the GM could be causative or highly associated with driving core and associated ASD symptoms, pathology, and comorbidities which include gastrointestinal symptoms, although it is also a possibility that these changes, in whole or in part, could be a consequence of underlying pathophysiological features associated with ASD. However, if the GM truly plays a causative role in ASD, then the manipulation of the GM could potentially be leveraged as a therapeutic approach to improve ASD symptoms and/or comorbidities, including gastrointestinal symptoms. One approach to investigating this possibility in greater detail includes a highly controlled clinical trial in which the GM is systematically manipulated to determine its significance in individuals with ASD. To outline the important issues that would be required to design such a study, a group of clinicians, research scientists, and parents of children with ASD participated in an interdisciplinary daylong workshop as an extension of the 1st International Symposium on the Microbiome in Health and Disease with a Special Focus on Autism (www.microbiome-autism.com). The group considered several aspects of designing clinical studies, including clinical trial design, treatments that could potentially be used in a clinical trial, appropriate ASD participants for the clinical trial, behavioral and cognitive assessments, important biomarkers, safety concerns, and ethical considerations. Overall, the group not only felt that this was a promising area of research for the ASD population and a promising avenue for potential treatment but also felt that further basic and translational research was needed to clarify the clinical utility of such treatments and to elucidate possible mechanisms responsible for a clinical response, so that new treatments and approaches may be discovered and/or fostered in the future.

Keywords: *autism spectrum disorder*; *clinical trials*; *Clostridia*; *fecal microbiota transplantation (FMT)*; *microbiome*; *mitochondria*; *probiotic*; *short chain fatty acids*; *vancomycin*; *gastrointestinal*

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An increasing number of research studies have provided evidence that the composition of the gut (enteric) microbiome (GM) in autism spectrum disorder (ASD), in at least a subset of individuals, deviates from what is expected in typically developing individuals (1–4). However, there is not a clear consensus regarding possible cause(s) and consequence(s) of these perturbations. There are several lines of research that suggest that specific changes in the GM may be causative in driving core and associated ASD symptoms and gastrointestinal (GI) symptoms, although, at this point in time, this hypothesis has not been satisfactorily and/or systematically evaluated. If such a hypothesis is true, then manipulation of the GM could potentially be leveraged as a possible therapeutic approach to improve core and associated ASD symptoms and also address important comorbid medical factors that may be present in some children with ASD, such as GI symptoms. One approach of investigating this possibility in greater detail includes clinical studies in which the GM is systematically manipulated. Such an approach could answer three important questions: first, whether there is a connection between ASD symptoms and alterations in the GM; second, whether manipulations can be safely used as a therapeutic approach for treating ASD; and third if putative mechanisms responsible for such an effect can be better understood.

To this end, on Friday, June 27, 2014, a GM Workshop at Arkansas Children's Hospital Research Institute was held. The working group included clinicians, research scientists, and parents of children with ASD (see Table 1). This workshop was an extension of the 1st International Symposium on the Microbiome in Health and Disease with a Special Focus on Autism (www.microbiome-autism.com). The aim of this workshop was to consider the design of a clinical trial focused on determining whether children with ASD can respond to therapy aimed at modulating and/or manipulating the GM.

The emerging interest of the microbiome in neurodevelopmental disorders

There is a growing body of clinical and basic science evidence that the GM (the trillions of microbes that inhabit the human digestive tract) affect immune and metabolic function (5–7) as well as modulate gene expression through epigenetic mechanisms (8, 9) that may initiate and exacerbate ASD pathophysiology and/or symptoms (10–12). Emerging research has found that GM and its metabolic byproducts play a major role in normal brain and behavioral development (13), and that these factors are altered in persons with ASD (1–4) [see accompanying papers by Krajmalnik-Brown et al. (14), in this issue]. Thus, alterations and/or perturbations in the GM may contribute to core and associated ASD symptoms by affecting behavior and brain function (12, 15–19) and modulating the expression of ASD-associated genes (20). Although this field is in its infancy, rapidly emerging technological advances have given us an increasing amount of relevant data, thereby increasing the possibilities of creating new prophylactic and therapeutic interventions.

Defining the question

The study of the relationship between ASD and the GM should be conducted very carefully in the manner in which one carefully poses the question and designs the study. The group suggested using a Patient/Populations, Intervention, Comparison, and Outcome (PICO)-like formulation for any question. PICO is a method for formulating a research question, particularly for systematic literature reviews. A PICO like method is essential for creating a well thought out scientific study.

Table 1. List of attendees

Attendee	Institution
Richard E. Frye	Arkansas Children's Hospital Research Institute, Little Rock, AR, USA
John Slattery	Arkansas Children's Hospital Research Institute, Little Rock, AR, USA
John Rodakis[a]	N of One: Autism Research Foundation, Dallas, TX, USA
Stephen Kahler	Arkansas Children's Hospital Research Institute, Little Rock, AR, USA
Jana Jennings[a]	Private Practice, Benton, AR, USA
Ellen Bolte[a]	N of One: Autism Research Foundation, Dallas, TX, USA
Carl E. Cerniglia	National Center for Toxicological Research, Jefferson, AR, USA
Tore Midtvedt	Karolinska Institutet, Solna, Stockholm, Sweden
Emma Allen-Vercoe	University of Guelph, Guelph, ON, Canada
William Parker	Duke University, Durham, NC, USA
James Adams[a]	Arizona State University, Tempe, AZ, USA
Rosa Krajmalnik-Brown	Arizona State University, Tempe, AZ, USA
Derrick MacFabe	Western University, London, ON, Canada
Susan Swedo	National Institutes of Mental Health, Bethesda, MD, USA
Jill James	Arkansas Children's Hospital Research Institute, Little Rock, AR, USA

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Trial design

Subgroups

Clinical trials in children with ASD have been extremely challenging for many reasons, including the fact that there is significant heterogeneity in the ASD phenotype without much agreement on how different endophenotypic subgroups should be defined. Thus, the working group believed that selection of specific ASD subgroups would be best based upon the goal of the trial. This is discussed in more detail in the Selection of Participants section below.

Concurrent treatments

Children with ASD have a wide range of medical abnormalities, which require various behavioral, psychiatric, and biomedical treatments that can be difficult to equate across subjects and challenging to keep constant throughout a clinical trial. The working group felt that it was important to carefully control concurrent treatments and keep such treatments stable. The working group felt that one of the most difficult factors to control might be educational and behavioral therapy and schooling. Factors such as summer and winter breaks, changing of therapists, and changing of the content of therapeutic programs, especially as symptoms worsen or improve, all have the potential to be confounding factors. Although it would be optimal to tightly control the behavioral and educational therapy, it would be very difficult and expensive. Although approaches such as timing study entry to the start of school is possible, this could significantly limit the recruitment window and could pose a challenge to completing a study within a reasonable time frame. The working group felt that another critical factor was diet, especially considering that many children with ASD are on special diets or have very limited diets which can influence the GM.

Trial length

Length of any clinical trial depends on the treatment selected and its effect on the putative underlying biological mechanism(s). Since the mechanisms involved in altering the GM likely involve a cascade of biological events, a number of factors need to be considered. The working group felt that one of the most important considerations is the timeframe in which the composition and functions of GM will change by the selected treatment. Whereas treatments that change the GM composition may take significant time, other treatments that change the GM's influence on metabolic, immune, and/or epigenetic factors may occur more rapidly. The timeline for changes in symptoms associated with ASD also needs to be considered. ASD is currently considered to be a life-long disorder with a minority of individuals with ASD attaining optimal outcomes. Even those that demonstrate optimal outcomes still exhibit subtle difficulties in language and executive function (21–23). Thus, changes in ASD symptoms can occur over days, months, or years. While some core or associated ASD symptoms may change or improve in the short-term, other important symptoms that involve complex cognitive constructs, such as socialization, may take significant time to change. However, timing of any trial also depends on the aim of the study. For example, the working group felt that it might be adequate to conduct short-term trials if the question focused on biological mechanisms using biomarkers as the outcome variables, whereas trials that assess behavior or cognition may require much longer time periods and long-term follow-up.

Efficacy versus effectiveness versus mechanistic trials

Efficacy trials that demonstrate the significance of a treatment in a carefully controlled setting are the gold standard for proving that a treatment can work. However, the working group felt that other types of trials, such as an effectiveness trial that measures the benefit of a treatment under 'real world' settings, are equally important. The fact that the practical aspect of treating a child with ASD is very complex coupled with the high degree of heterogeneity within the ASD spectrum highlights the possibility that effectiveness trials could be significantly advantageous. Alternatively, given the complicated nature of ASD, the working group felt that understanding the underlying biological mechanisms behind any treatment could be of upmost importance, especially if high-quality biomarkers were used.

Blinded versus open-label

Although it was recognized that blinded, particularly double-blind placebo-controlled (DBPC) studies are the gold standard for proving efficacy of a treatment, the working group also felt that given that the exact mechanism of disease and action of the treatment are still unclear for many GM treatments, open-label exploratory trials that examine biological mechanisms of response and provide information to define biomarkers and characterize responders could be equally important and could help guide future DBPC trials.

Parallel versus cross-over trials

Cross-over trials can be very useful for controlling individual differences when significant individual heterogeneity exists. However, cross-over studies require significant assumptions that may not be wise to accept in the ASD population for several reasons. First, it is assumed that the placebo will not change the outcome measure. This assumption provides power to a cross-over study because the participant serves as their own control, reducing the need to match an individual in a control group with an individual in the treatment group on particular characteristics. However, many children with ASD, especially those that are medically complex, demonstrate wide variability in ASD symptoms over time with waxing and waning in the

severity of their symptoms. The reasons for these behavioral fluctuations are multifactorial with little known evidence regarding the ability to predict these variations. These factors complicate the ability of an individual patient being used as their own control unless such variability is taken into account. Second, if the active treatment is the first arm of a cross-over study, it is assumed that after a relatively short washout period, the effect of the treatment will be lost and the participants in the trial will return to their own respective baseline. However, if the treatment results in a major change in GM composition, a short washout period may not be sufficient time to recover to the baseline GM (if ever). Thus, given the fact that most treatments will influence the composition and functions of GM, the working group recognized that in most instances it is probably best to use a parallel design.

Treatments that may alter the GM

Many treatments for children with ASD that are already in use have the potential to change the GM. The specific effect on the GM of many of these treatments, such as special diets, may not be clear, while other treatments, such as antibiotic therapy, may have predictable effects on specific bacteria and/or their metabolites, but unknown downstream long-term effects. Other treatments, such as fecal microbiota transplantation (FMT), have been well established for other conditions such as *Clostridium Difficile* overgrowth but are still considered experimental for ASD. Below is a summary of treatments that may manipulate the GM and may be appropriate for study.

Treatments that reduce specific gut microbiome components

Antibiotics

Several studies have suggested that children with ASD have a history of increased antibiotic use for recurrent infections prior to their diagnosis (24) and other studies have suggested that antibiotic use during pregnancy is linked to the development of ASD (25). Some groups have even hypothesized that use of specific antibiotics early in life could be causative for ASD (26) and others have suggested that antibiotic use early in life facilitates a vicious cycle between immune system impairment and dysbiosis (27).

Vancomycin and metronidazole are two antibiotics used to treat ASD symptoms both within clinical and research studies that are believed to target specific GM bacteria (28). Vancomycin broadly targets Gram-positive bacteria, including Gram-positive anaerobic bacteria such as those that are members of the *Clostridium* genus, and is believed to have a favorable safety profile when administered orally since, under normal physiological circumstances, vancomycin is not absorbed from the GI tract into the circulation to any significant extent (29, 30). Metronidazole also targets similar GM bacteria, but since it is systematically absorbed, it is felt to have a less favorable safety profile because of the possibility of systemic adverse effects (28).

In a small, partially blinded 8-week clinical trial of vancomycin conducted in the United States on children with ASD, significant GI symptoms and high irritability, temporary effectiveness was found for treating ASD symptoms in 8 of 11 participants (31). Approximately half of participants had an initial burst of hyperactivity lasting 1–4 days. Vancomycin levels were measured in the patients with the highest levels of intestinal permeability to assure that there was no absorption of vancomycin, although the data remain unpublished.

The therapeutic response to vancomycin is supported by the propionic acid rodent model of ASD (12, 15–20) and may in part or in total be related to mitochondrial dysfunction resulting from the overproduction of short-chain fatty acid fermentation products such as propionic acid from GM bacteria [see accompanying paper by Frye et al. (32) in this issue]. At the same time, it is possible that an aberrant immune response toward particular components of the GM or aberrant gut epithelial barrier function is responsible for the therapeutic response to vancomycin [see accompanying paper by Bilbo et al. (33) in this issue].

When discussing the use of vancomycin, even in a clinical research setting, it is of the utmost importance to stress that vancomycin-resistant enterococcus (VRE) is a very serious health concern. If a future clinical trial confirms a clinically meaningful response to vancomycin in children with ASD, then the findings can be applied to other treatments in order to prevent vancomycin overuse. Indeed, the use of vancomycin in a clinical trial could be very useful for mechanistically demonstrating the behavioral, biochemical, and microbial effects of manipulating the GM, and to learn and produce new hypotheses, but may not be a viable long-term solution. Other less problematic treatments may be developed based on the knowledge gained from such a trial.

Antibiotics which do not specifically target *Clostridium* genus are used to treat ASD symptoms under the diagnostic umbrella of Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcal infections (PANDAS), Pediatric Acute-onset Neuropsychiatric Syndrome (PANS), or chronic Lyme disease (34, 35). These antibiotics have well-known effects on pathogenic organisms such as *Streptococcus* and *Borrelia* but can disrupt healthy gut bacteria, resulting in dysbiosis. Antibiotics can also have direct effects on human host cells. Some have speculated that common antibiotics, such as aminoglycosides, may either be triggering or improving ASD symptoms by disrupting polymerase function (36). Likewise, chronic administration of beta-lactam antibiotics for routine pediatric infections may impair gut and renal carnitine transport, possibly worsening a relative

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carnitine deficiency and causing mitochondrial dysfunction (12, 15, 16).

One of the attendees indicated that parents of children with ASD seem to support the idea that some children with ASD may be positive responders to antibiotic treatments when prescribed for PANDAS/PANS or infections in an informal social media discussion [see accompanying paper by Rodakis in this issue]. Other attendees were aware of at least one unpublished small, open-label trial that demonstrated positive effects of antibiotics on ASD symptoms. However, published evidence supporting these observations are lacking, underlying the need for well-controlled, objective clinical studies to document any effect on ASD symptoms and/or to understand underlying mechanisms. Furthermore, given the public health concerns of antibiotic resistance and health risks associated with antibiotic misuse and overuse, it is critical to determine if this practice is able to provide clinical benefits.

Antifungals

Children with ASD are treated in clinical practice with antifungal agents despite the lack of scientific evidence of fungal overgrowth. One study that specifically examined yeast in the gut demonstrated no difference between ASD and typically developing children (37). However, it should be noted that detection of yeast strains is technically challenging, potentially resulting in a high false negative rate. A national survey by the Autism Research Institute (Table 2) found that parents reported that antifungal therapy with Diflucan or Nystatin was often beneficial and rarely worsened symptoms (38). It should be noted that antifungal treatments have known toxicity and may require monitoring of liver transaminases for safety.

Caveats

The working group noted that the treatments that could change specific components and diversity of the GM are commonly used in the treatment and management of children with ASD. The evidence for the effectiveness of these treatments for improving ASD symptoms is very limited. This highlights the need for well-controlled clinical trials. Several challenges were noted in this area of ASD treatment research. First, there may be subgroups of children with ASD who respond positively or negatively to these treatments and the treatments themselves may have a significant carry-over effect meaning that long-term observation may be needed. Furthermore, antibiotic resistance is a major health concern for the United States and other industrialized nations, making the safety of long-term and repeated treatments with these agents problematic, both to the patient and to the hospital, clinics, and the general population.

Treatments that replace gut microbiome components

Fecal microbiota transplantation

FMT is an intervention in which the fecal microbiota from a donor is delivered to a recipient in an attempt to replace a dysbiotic GM with a healthy one. It is highly efficacious (approximately 92%) in curing recurrent *C. difficile* infections (39). There is interest in using it in a number of disease states which appear to be associated with disruption of the GM. These disorders include GI diseases such as inflammatory bowel disease, irritable bowel syndrome, and chronic constipation as well as non-GI diseases such as autoimmune diseases, chronic fatigue syndrome, and obesity (40). Further safety and efficacy studies in clinically controlled trials are necessary to evaluate the use of FMT. For example, recently, new-onset obesity was observed in a FMT recipient for recurrent *C. difficile* infection after transplantation from an obese donor (41). Thus, current FMT studies are very careful regarding the selection of donors. Given the growing evidence for imbalance in the GM in ASD, there is an increasing interest in using FMT in ASD. Several attendees are currently involved in FMT treatment research for ASD and other disorders. However, the attendees felt that this approach is still in need of refinement, particularly in understanding the type of transplant, the delivery system to use, dosage, and duration of treatment as well as the need for pretreatment use of antibiotics and bowel-cleansing regimes [see accompanying papers by Krajmalnik-Brown et al. (14), and Toh and Allen-Vercoe (42) in this issue].

Helminths

There is a growing body of literature indicating that changes in the environment have resulted in a rise in immune disease over the past century [see accompanying paper by Bilbo et al. (33) in this issue]. One significant change that is underappreciated is the loss of exposure to eukaryotic, multicellular organisms living in the gut (e.g. worms called helminths) as a result of the widespread use of technological advances such as indoor plumbing, water treatment facilities, and modern food processing facilities (43). Our immune system has evolutionarily adapted over

Table 2. Autism Research Institute survey of parent ratings of treatment efficacy

	% Worse	% No change	% Better	Number of reports
Antifungals: Diflucan	5	34	62	1,214
Antifungals: Nystatin	5	43	52	1,969
Candida diet	3	39	58	1,141

millennia to protect our bodies from these gut dwelling organisms and it is believed by some that the sudden loss of these complex multicellular organisms may have destabilized the immune system, leading to an increased reactivity to environmental stimuli and, in some cases, to the body itself. This view, originally labeled the 'hygiene hypothesis', is more appropriately known as the 'biome depletion theory' (44). To combat this destabilization of the immune system, researchers have investigated treatments with helminths such as the pig whipworm, *Trichuris suis*, which can stimulate the enteric immune system but cannot maintain prolonged residence in the bowel. The idea of the use of this treatment in ASD has recently been introduced (45) and clinical trials are currently underway.

Probiotics

Probiotics are living microorganisms, usually lactic-acid-producing bacteria, which are believed to be beneficial to health. Probiotics are widely used for children with ASD and there is growing excitement as a result of recent studies on animal models of ASD (46). Specifically, animal research suggests that treating the maternal immune activation mouse model, which is an animal model with behavioral and metabolic characteristics similar to ASD and alterations in the enteric microbiome, with a humanly derived strain of *Bacteroides fragilis* improves metabolic, enteric microbiome, and behavioral abnormalities, suggesting that this might be a promising treatment for children with ASD. The theorized mechanisms by which *B. fragilis* produces a treatment effect, at least in a mouse model, is through ameliorating enteric microbiome imbalances (47) including upstream and downstream by-products which modulate behavior. Other studies not related to ASD have demonstrated that some probiotic strains can influence the immune system (48, 49), at least for a certain period of time, and metabolic abnormalities such as redox imbalances (50).

Although the working group is supportive of the use of probiotics, it should be highlighted that commercial probiotics vary considerably in their quality, composition, and efficacy, and clinical studies are lacking in children with ASD regarding their effectiveness. There are several limitations of commercial probiotics that should be noted. First, they contain only a tiny fraction of the total bacteria in the gut and the bacteria they do contain are limited in diversity compared to the broad number and types of bacteria in the human gut. For example, the human gut contains more than 1,000 different strains of bacteria while commercial probiotics usually contain less than 10 strains of bacteria. Second, they rarely contain obligate anaerobes that constitute a meaningful percentage of the typical GM. Third, since they are derived from milk, the human GM is not a natural environment for these organisms.

Unprocessed foods

Certain natural, unprocessed foods have become popular as treatments for children with ASD. Fermented foods and raw milk such as camel's milk have natural bacteria and prebiotics that may, at least theoretically, influence the GM in a variety of ways. Some interesting preliminary studies suggest some benefit of camel's milk in children with ASD. In one case study, the author claims that the consumption of raw camel's milk heralded the start of a 6-year improvement in ASD symptoms for his son (51). One DBPC study demonstrated that 2 weeks of either raw or boiled camel's milk consumption resulted in significant improvement in biochemical measures of oxidative stress, including glutathione, superoxide dismutase, and myeloperoxidase as well as ASD symptoms as indexed by the Childhood Autism Rating Scale (CARS), while cow's milk did not result in such improvements (52). The fact that the boiled camel's milk had a similar effect as compared to the raw camel's milk suggests that the effect of camel's milk was not due to live bacteria, as some believe. A second DBPC study found that both raw and boiled camel's milk resulted in improvement in thymus and activation-regulated chemokine serum levels, but that only raw camel's milk resulted in improvement in the CARS as compared to cow's milk (53). The use of cow's milk as a control could be a significant factor in these studies as cow's milk is believed to stimulate immune function and potentially increase oxidative stress. Thus, using cow's milk as a comparison may not be optimal to answer the question whether the effects seen in these studies are due to camel's milk specifically or due to the lack of cow's milk. Indeed, milk-free diets, including casein-free diets, are believed to be beneficial in children with ASD. Bovine milk products can stimulate the production of the folate receptor α autoantibodies (54). This autoantibody can bind to the folate receptor α and block the transport of folate into the brain resulting in depressed folate concentration in the brain (55). Unpublished laboratory studies suggest that the folate receptor α exists at the same level in camel's milk as compared to cow's milk (Dr Edward Quadros, personal communication).

Fermented foods

Information about the use of fermented foods, such as keffirs, to treat ASD is purely anecdotal, so scientific evidence for these treatments are currently lacking. Thus, although there are reasons to believe that these treatments could be useful, at least in some children with ASD, further research is needed to determine whether there is any true benefit to these treatments, and, if so, whether such benefits are due to changes in the microbiome and/or related to other biological mechanisms.

Treatments that may change the gut environment

Dietary treatments

It is not uncommon in ASD for several specific dietary interventions to be prescribed by medical professionals.

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Furthermore, it is not uncommon for parents to independently implement specific dietary changes independent of medical recommendations. Such diets (i.e. gluten-free/casein-free, specific carbohydrate diet, elemental diet) contain various types and qualities of carbohydrates while other diets, such as the modified Atkin's diet, limit carbohydrates. Other diets empirically attempt to limit refined carbohydrates, foods containing additives, such as the food preservative propionic acid, or artificial sweeteners, which have recently been shown to alter the GM, short-chain fatty acid production, and lipid metabolism (12, 15, 56, 57). In contrast, other approaches attempt to normalize an otherwise self-limited diet. There is preliminary evidence for effectiveness of some of these diets in ASD. Small studies provided inconsistent evidence for the effectiveness of the gluten-free, casein-free diet (58, 59), while a larger single-blind study provides more promising results (60). There is enthusiasm for the modified Atkin's diet (61) for children with ASD, especially for those with concurrent epilepsy (62). Some of these diets have been used for individuals with inflammatory bowel disease, but studies are inconsistent (63). Diet can have a profound effect on the composition and metabolic products of the GM (64) and on the immune system (65), but little is known about the correlations between these special diets and the GM in ASD. Clearly this is an interesting area of clinical research that is still very preliminary. Well-designed clinical trials focusing on diets in ASD may be particularly fruitful for understanding the mechanism of action of these diets, especially if they involve carefully characterization of the GM. Given the likely heterogeneity of ASD, a helpful approach may be to focus attention on dietary responders in larger controlled clinical trials.

Digestive enzymes

Given that children with ASD appear to have defects in certain intestinal digestive enzymes, particularly enzymes responsible for carbohydrate digestion (1), it is logical that supplementation of digestive enzymes may be useful, especially since carbohydrates that go undigested in the small bowel will be transported to the colon where they can stimulate bacterial fermentation. However, clinical treatment studies using digestive enzymes report mixed results. In an open-label study of 46 patients, a digestive enzyme containing Caso-Glutenase 10,000 AU, Bromelain 230 BTU, Acid Fast Protease 100 SAPU, Lactase 330 LacU, Phytase 125 U, and Galactose 100 mg was given with meals (66). The 22 patients who completed the study showed significant gains in several ASD-related symptoms. However, a DBPC cross-over study of 43 children treated with a digestive enzyme containing peptidase and proteases only during one meal per day failed to demonstrate improvements in ASD symptoms (67). Although a DBPC design is the gold standard as compared to the open-label study, clearly the two studies used different approaches with the open-label study using a mixture of enzymes that digests both proteins and carbohydrates with every meal and the DPBC using a digestive enzyme that only digests proteins and only with one meal. Unfortunately, the open-label study provided very biased results as only the patients that continued the treatment were analyzed. Clearly this is an interesting area of treatment research that deserves greater attention.

Vitamins

Vitamins can be consumed or made more bioavailable by the GM. Deficiencies in vitamins can change the GM composition and the GM composition can change the requirement for specific vitamins and minerals. For example, *Lactococcus spp* have been associated with folate biosynthesis in animal studies (68) and human omnivores metabolize carnitine into a proatherogenic compound, trimethylamine-N-oxide, through a microbiota-dependent mechanism whereas vegetarians or vegans do not metabolize carnitine in this way (69). Likewise biotin and B12 are essential cofactors for propionic acid breakdown, and dietary deficiencies of these vitamins may further impair propionic acid and carnitine metabolism and contribute to ASD-associated mitochondrial dysfunction (12). One study measured levels of all vitamins in children with ASD and found that they were significantly lower in biotin compared to controls (70). Biotin is a vitamin which is produced by GM bacteria in amounts that are comparable to dietary intake, and a follow-up treatment study with a vitamin/mineral supplement found that initial low levels of biotin or vitamin K (also produced in substantial amounts by GM bacteria) was highly correlated with degree of improvement resulting from the treatment (71). Collectively, this suggests that children with ASD may have low amounts of the GM bacteria that produce biotin and may benefit from biotin supplements. Clearly, the interactions between the GM and vitamin requirements and bioavailability are complex and require further study.

Toilet training

Many children with ASD have difficulty with toilet training and may be withholding bowel movements leading to functional constipation. An abnormal frequency in bowel movements can result in a change in the GM and in the production of microbial metabolites (12, 15). Conversely, changes in the GM can result in constipation and difficulty in toilet training.

Participants: defining subgroups

Properly screening and selecting participants is of upmost importance in clinical studies.

First, it is important to carefully define the inclusion criteria so that the selected study cohort is optimal. Children with ASD should be diagnosed carefully,

preferably with a gold-standard tool such as the Autism Diagnostic Observation Scheduled (ADOS) and/or the Autism Diagnostic Interview – Revised (ADI-R) by a certified research reliable rater.

Second, it is important to exclude those children with complicated medical conditions or medications that could interfere with the study. Examples of possible exclusion criteria include

- Inborn errors of metabolism
- Failure to thrive
- Epilepsy
- Inflammatory bowel disease
- PANDAS/PANS
- Chronic infections
- Recent antifungal, antiviral, and/or antibacterial medications
- Treatments that alter the GM such as pre/probiotic usage or special diets
- Immunosuppressant medications
- High-dose vitamins since these treatments may alter both the GM and metabolism
- Puberty
- An inability to maintain other therapies constant throughout the study
- A caretaker who is not able to comply with protocol
- Specific genetic abnormalities, such as Fragile X syndrome

Finally, it is important to either select a group of children with ASD who are hypothesized to respond most significantly to a treatment or to stratify the participants into two or more subgroups to understand whether specific factors are important in predicting treatment response. For example, Finegold et al. suggest that children with regressive ASD were more likely to have a wide variety of non-spore-forming anaerobes and microaerophilic bacteria in their feces (72), so children with regressive ASD may be an optimal group for study. Other possibilities include children with a history of positive response to specified classes of antibiotics, those with significant GI symptoms, or those with high irritability scores. For children with waxing and waning symptoms, it might be best to follow them longitudinally to determine the optimal time to start the intervention.

Behavioral and cognitive measures

Baseline characteristics

It is of the upmost importance to carefully characterize participants at baseline. The following baseline measures were proposed:

- ASD diagnosis using gold-standard instruments such as the ADOS and/or ADI-R.
- Assessment of social function using an instrument such as the Social Responsiveness Scale.
- Measurement of intellectual and/or developmental functioning. Many intelligence tests have been used with children with ASD, but the limitations in language commonly associated with ASD can cause a downward bias in intellectual quotient scores leading some to suggest that non-verbal intellectual testing should be used in children with ASD to account for this potential confounding factor.
- An index of development such as the Adaptive Behavioral Composite from the Vineland Adaptive Behavioral Scale.
- Dietary history with a comprehensive assessment due to dietary effects on the GM.
- Psychiatric history to look for important comorbidities.
- Comprehensive medical history looking for important medical factors such as GI symptoms pre- and post-ASD diagnosis, as well as other important factors such as oral antibiotic usage during the pre- and postnatal periods, probiotic usage, delivery method (vaginal or C-section), and neonatal complications and hospitalization duration.
- Developmental profile must be examined in detail with a clear and detailed understanding of the timing of the development of ASD symptoms and whether regression in any skills occurred. Any trigger that may have caused regression should be examined in detail. Regression can be investigated using a standardized method (73).
- Executive function is an important deficit in children with ASD, so specific age-appropriate instruments such as the Delis-Kaplan Executive Function System should be considered.

Outcome measures

Important quantitative outcomes measures should be employed in any study of the microbiome in ASD. Measures that were felt to be important included:

- *Autonomic Tone:* The autonomic nervous system has been shown to be disrupted in children with ASD and is closely linked to GI function (74). Measures such as pupillometry (75) and skin conductance (76), among other autonomic nervous system measures, have been used to document abnormal autonomic nervous system reactivity in individuals with ASD.
- *Behavior:* Behavioral dysregulation is a key symptom of functional impairment in children with ASD and it is measured in various ways including caregiver questionnaires such as the Aberrant Behavior Checklist (ABC) which has been used in multiple clinical trials and the Children's Yale-Brown Obsessive Compulsive Scale (Y-BOCS) (77). Other studies

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have used a visual analog scale for rating of parental selected target behaviors (78). Clinician rated Clinical Global Impression (CGI) Scale is common, but a new scale specific for ASD behaviors known as the Ohio Autism Clinical Impression Scale (OACIS) may be more specific. The OACIS (79), previously known as the OSU Autism Rating Scale (80), has been shown to be reliable across cultures (81) and has been successfully used in several ASD clinical trials (82–84). Other studies have carefully coded behaviors based on video recordings using rating scales adapted from animal studies (85). Actigraphy can also be used to objectively monitor behavior and activity level during the daytime. Finally, eye-tracking paradigms can be used to detect changes in visual attention (86).

- *Fine Motor:* Fine motor function is often disrupted in children with ASD. Examining changes in handwriting or in the ability to copy a picture can demonstrate fine motor changes.
- *Sleep:* Sleep is commonly disrupted in children with ASD. Studies have used several methods for measuring sleep. One of the most objective means for measuring sleep quality is actigraphy (87) that appears to be superior, especially for measuring nighttime wakening, as compared to parental sleep diaries (88). Two questionnaires have been used to measure sleep symptoms in ASD: the Modified Simonds and Parraga Sleep Questionnaire and the Children's Sleep Habits Questionnaire (89).
- *Gastrointestinal Symptoms:* Most studies create their own unique GI symptoms scales, although a few standardized scales have been developed. One study used a simple nine question Gastrointestinal Severity Index (GSI) (90) that has been shortened to six key questions (GSI-6) in another study (37). Others have developed very detailed questionnaire such as the Questionnaire on Pediatric Gastrointestinal Symptoms (91) while others have used the Gastrointestinal Symptom Rating Scale (92). Scales that measures stool characteristics such as the Bristol stool scale could also be useful to use on a daily basis during a study.

Biomarkers

Biological sample collection

The working group felt that it was critical to standardize any stool collection protocol such as obtaining the first morning stool or obtaining a 24 h collection. Timing of biomarker collection may be critical as research studies have demonstrated diurnal oscillations in the composition and function of the GM (93). It is also important to ensure that specimens are immediately frozen at -20 to -80°C. Of course, GI issues which are common in ASD, such as chronic constipation and chronic diarrhea, can complicate any collection, standardization, and comparison across individual samples.

Microbiota identification

The various conventional and culture-independent methods to determine the microbiota content of the GM have their individual strengths and weaknesses. Rapid improvements and cost reductions in molecular microbiome identification methods make such investigations attractive for clinical studies. Such techniques are essential since anaerobic bacteria are difficult to culture in the lab. This has led to the popularity of techniques that identify the genetic fingerprint of bacteria, for example, variable regions of 16S ribosomal RNA gene sequences. Techniques range from semiquantitative methods such as temperature gradient gel electrophoresis, denaturing gradient gel electrophoresis, and terminal restriction fragment length polymorphisms as well as quantitative genetic techniques such as microarrays and direct sequencing. 16S ribosomal RNA genes are the most popular targets because they are specific to bacteria (and archaea), and not found in human cells. Shotgun metagenome sequencing methods sequence all genetic material in the entire microbiome, allowing more detailed classification of the bacterial taxonomy and the genetic potential of a given microbiome community. Next generation sequencing methods have lowered sequencing costs and enable megagenomics to become cost-effective, although a bottleneck remains in the time required for post-sequencing analysis. Bioinformatics methods that can predict metagenomes using 16S ribosomal RNA gene information such as Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (94) are also available and when used correctly could save a significant amount of time and money.

Metabolic biomarkers

Stool, urine, and blood biomarkers of metabolic products of enteric bacteria have been described. Although there is, as yet, only preliminary data to support their use as GM biomarkers, further studies could validate these approaches. Both targeted methods which identify specific compounds and untargeted metabolomics methods have been used.

Urinary HPHPA

3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA) has been shown to be increased both in children with ASD and in adults with recurrent *C. difficile* infections, and was found to decrease in a patient with acute schizophrenia after treatment with oral vancomycin leading the authors to suggest that this is a valid biomarker for the *Clostridium* genus (95). Although this preliminary data are interesting, clearly further studies are needed to validate this biomarker as specific to *Clostridium* spp. and determine its role in

ASD pathophysiology. Complicating these findings is the fact that the taxonomy of the *Clostridium* genus is currently in a state of flux and not clearly defined.

Urine p-cresol

p-cresol (4-methylphenol) is an organic aromatic compound that is not produced by the body but is a relatively common compound that can be absorbed through the skin, GI, and respiratory systems. It is also produced by enteric bacteria including *C. difficile, C. scatologenes*, *Lactobacillus* spp., *Pseudomonas stutzeri*, and *Pseudomonas mendocina* (96). Studies have found that urinary p-cresol is elevated in children with ASD younger than 8 years of age (particularly females), those who are more severely affected, and those with regressive ASD (97). Further analysis found that fractions of p-cresol, p-cresylsulfate, and p-cresylglucuronate were also elevated in children with ASD (96).

Acyl-carnitines

Studies have linked a pattern of abnormal elevations in acyl-carnitines, biomarkers of fatty-acid metabolism, to metabolic changes associated with excess exposure to propionic acid in animal models of ASD and have demonstrated that this same pattern is found in children with ASD and mitochondrial dysfunction (12, 15, 16).

Untargeted metabolomics

New metabolomics techniques can detect many metabolites produced by the microbiota, which can influence host physiology such as choline metabolites, secondary bile acids, phenols, and short-chain fatty acids (98).

Immune function

The microbiome is closely associated with immunological abnormalities and diseases. Several studies have demonstrated that changes in the GM modulate cytokines and immune cells (99, 100). Such immune measures have the potential to be used as biomarkers of microbiome activity and *vice versa.*

Metabolic/cellular function

Changes in the GM are linked to changes in redox metabolism (50, 101–103) and metabolites produced by the microbiota could significantly influence mitochondrial function (15, 16). Thus, measurements of redox metabolism and mitochondrial function may be important to consider when studying the human microbiome. GM metabolites, particularly short-chain fatty acids, are histone deacetylase inhibitors (104) and have been shown to modulate gene expression (105), including ASD-related genes and pathways (20), so the measurement of gene expression may be very important in microbiome studies.

Stool characteristics

Other stool physiochemical characteristics may also be important including, stool pH, viscosity, short-chain fatty acids, water versus solid content, and water holding capacity. The relative volatility of many microbiome-derived metabolites should be considered in sample storage and measurement. One study found major differences in short-chain fatty acids in children with ASD compared to controls (37).

Safety

In any clinical trial, it is important to ensure that the treatment is safe. Given that changes in the GM have been linked to disease and that overgrowth and infection with particular bacteria such as *C. difficile* can be harmful to individuals, a plan needs to be in place for assuring safety of any treatment. Safety parameters discussed include *C. difficile* antigen in stool, complete blood count, and liver function tests. Furthermore, collection of biospecimens and safety requirements need to be carefully considered to ensure that the spread of potentially infectious waste or material is not transmitted or transferred to personnel involved in the study or caretakers.

Ethics

Because of the novelty of this approach, patients enrolled in studies which manipulate the GM should be informed that many of the treatments are based more on theory rather than strong empirical evidence. Some of the treatments, such as vancomycin, may only provide short-term gain and any antibiotic treatment could, theoretically, alter and even destabilize the GM (106–110) in unpredictable ways that could be harmful to the host but may not be immediately apparent. One approach toward studies of treatments might be to enroll children with ASD who have comorbid medical conditions, such as *C. difficile* infection, into a microbiome treatment study such as a FMT study and to determine if ASD symptoms and putative biomarkers (i.e. acyl-carnitines) improve in parallel with the primary outcome.

Discussion

Overall, the working group concluded that the GM was important to study in ASD and that there were many promising avenues in which treatments that may influence the GM very well could be beneficial to some children with ASD. The group highlighted and noted the very early stages of this research and reiterated that much more basic and translational research will be needed before a clear treatment(s) for ASD involving manipulation of the GM will be clinically applicable.

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