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RATIONALE FOR INVESTIGATING STOOL METABOLITES AND MICROBIOTA IN WOMEN WITH FECAL INCONTINENCE

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Introduction

Childbirth and its associated neuromuscular injury to the anal sphincter muscles have traditionally been considered as a common cause of fecal incontinence (FI) in women.[1] However, the onset of FI is usually after age 50 which is remote from delivery.[2] Recent large epidemiologic studies report that bowel symptoms related to gut motility and sensation, such as diarrhea and fecal urgency, are significant contributing factors that are more important than obstetric factors in the pathogenesis of FI.[1–3] In these studies examining the association of multiple demographic, obstetric, and gastrointestinal risk factors for FI, the number of fecal urgency episodes per month and diarrhea emerged as risk factors that were most closely associated with FI.[1–3] Therefore, investigating mechanisms that worsen fecal urgency and diarrhea could advance our understanding of factors that modulate the severity of FI and its response to treatment.

Stool metabolites, produced by the interaction of gut microbiota with the host, can modulate neuro-hormonal mechanisms implicated in gut motility and gut sensation. Several studies show that stool metabolites enhance contractile responses of intestinal smooth muscle and neuronal excitability of the enteric nervous system resulting in symptoms such as diarrhea, fecal urgency, and sense of incomplete emptying that are common in women with FI.[4–5] Given that stool metabolites and microbiota are potentially modifiable, understanding the role of these factors in the pathogenesis of FI could provide a paradigm change to its management.

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Stool Microbiota and Microbiome-Controlling Anal Incontinence by Performing Anal Exercises with Biofeedback or Loperamide (SMM-CAPABLe) Study

The Eunice Kennedy Shriver NICHD sponsored Pelvic Floor Disorders Network, a multi-center network of eight sites, is currently performing a study investigating stool metabolites and microbiota in women with FI. Our overall hypothesis is that the stool metabolome and microbiome has a role in mechanisms underlying FI and may modulate response to treatment.

SMM-CAPABLe is a prospective case control study that is supplementary to the CAPABLe randomized controlled trial investigating the efficacy of non-surgical treatments for FI (NCT02008565). In the CAPABLe RCT, subjects are being randomized using a factorial design to one of four treatments, loperamide, anorectal biofeedback, combined loperamide and anorectal biofeedback or no treatment. In the supplementary SMM-CAPABLe study, cases are women with FI and who are participating in CAPABLe. Similarly aged controls without FI are being recruited from each of the eight participating sites. Women with stools at the extremes of spectrum of consistency, watery stool (type 7) and hard lumpy stools (type 1) are being excluded. Conditions known to be associated with abnormal stool metabolites and microbiota such as inflammatory bowel disease and recent antibiotic use are also being excluded.

The primary aim of SMM-CAPABLe is to compare baseline levels of metabolites in stool samples of women with FI from CAPABLE to unaffected similarly aged controls. The secondary aim of SMM-CAPABLe is to compare baseline proportional abundance of Clostridiales and other microbiota in stool samples of women with FI to unaffected similarly aged controls. The primary outcome for this study is levels of butyrate in stool, and important secondary outcomes are the levels of bile acids and tryptamine and microbiota in stool. SMM-CAPABLe will also explore if levels of stool metabolites or microbiota can predict response to treatments being tested in CAPABLe, such as loperamide and anorectal biofeedback.

All women (cases and controls) participating in SMM-CAPABLe contributed a stool specimen at baseline and at the end of 24 weeks of treatment. Stool samples are being analyzed for metabolites using targeted metabolite profiling of fecal water. Stool microbiota are being analyzed using 16S rRNA gene sequencing. The planned sample size, based on reported levels of butyrate in stool of human controls, is 82 cases and 41 unaffected controls.

In sections below, we present our rationale for measuring specific metabolites and microbiota in the stool of women with FI.

Butyrate

Several animal and human studies show that butyrate has an important role in gut motility and gut sensation and are discussed below. The short chain fatty acids (SCFAs), butyrate, propionate, and acetate, are microbial metabolites produced in the intestinal lumen by

fermentation of dietary fiber by SCFA-producing bacteria (primarily *Clostridiales* class). More than 95% of the butyrate produced in the gut is absorbed and butyrate levels in stool represent the level of butyrate in the distal colon. Normally, fecal butyrate levels are low and fairly constant with acetate > propionate > butyrate in molar concentrations of 65: 20: 15 respectively.[6]

The effect of butyrate on gut motility has been well documented. Experimental intraluminal administration of butyrate stimulates high amplitude propulsive contractions as well as colonic transit and in both rats and humans.[6–9] In patients with irritable bowel syndrome (IBS), higher levels of butyrate have been associated with increased motility and diarrhea while lower levels are associated with reduced motility and constipation.[10–11] The mechanism through which butyrate stimulates gut motility has also been elucidated. In rats, butyrate stimulates colonic motility through the release of a well known neurotransmitter in the host gut, 5-hydroxytryptamine (5-HT) or serotonin.[6] In fact, a butyrate-inducible transcription factor, ZBP-89, regulates the expression of Tph1, the rate limiting enzyme in 5-HT synthesis.[12] The effect of butyrate on colonic transit is abolished by pretreatment with 5-HT3 receptor antagonist and can be stimulated by probiotics containing *Propionibacterium*.[6,13]

Butyrate has also been shown to have a role in gut sensation. In a classic animal model of noninflammatory colonic hypersensitivity, Bourdu et al demonstrated that rectal instillation of butyrate induces rectal hypersensitivity and the effect is abolished by CGRP receptor antagonists.[7] In humans, high concentrations of luminal SCFAs have been implicated in fecal urgency, disordered defecation, and liquid stool.[9–10, 14] In healthy human volunteers, instillation of SCFA was associated with symptoms of cramps and an urge to defecate even at small volumes.[9] However, the motility stimulated by the SCFAs was not associated with systemic release of gastrointestinal regulatory peptides suggesting that the motor effect of SCFAs is through the coloileal reflex of the enteric nervous system in humans. Repetitive instillation of larger doses of butyrate enemas is associated with a decrease in visceral perception, likely due to overstimulation and subsequent desensitization of TRPV1 receptors.[14] These findings suggest a ‘threshold effect’ for butyrate on gut sensation where levels above a certain ‘threshold’ cause fecal urgency but high levels of rectal butyrate reduce rectal sensation. Since both rectal hypersensitivity and lack of rectal sensation can cause FI, investigating stool butyrate levels could identify a new factor that contributes to FI. Furthermore, since the effect of butyrate can be modulated by probiotics and drugs, elucidating the role of butyrate in FI could help identify new treatments that modulate the severity of FI and/or its response to treatment.

Bile Acids

The primary bile acids (BAs), cholic acid and chenodeoxycholic acid, are synthesized and conjugated with taurine and glycerine in the liver and secreted into the bile. The majority of the primary BAs are reabsorbed into the enterohepatic circulation in the distal ileum and only 5% escapes into the colon, where they undergo deconjugation by gut microbiota to form the secondary bile acids, deoxycholic acid and lithocholic acid. The predominant BAs

in stool are the secondary BAs, deoxycholic acid and lithocholic acid, and only small amounts of the primary BAs are found in the stool.[15]

It has long been known that BA metabolites, produced by intestinal microbiota, have a profound effect on intestinal motility. Excessive loss of BAs in the stool is a well known cause of chronic diarrhea, such as in bile acid malabsorption and as a sequel to ileal resection or inflammatory bowel disease.[16] The BA sequestering drugs, cholestyramine and colesevelam, have been shown to be useful adjunctive treatment to anorectal biofeedback for reducing the severity of bowel symptoms in subjects with FI and cancer, respectively.[16–17] The well known stimulatory effect on BAs on gut motility has been confirmed in recent translational studies. The introduction of BAs directly into the sigmoid colon and rectum has been shown to cause diarrhea by a variety of mechanisms including stimulating colonic motility, activating intracellular secretory mechanisms, and increasing mucosal permeability.[18] Shin et al reported higher levels of individual primary and secondary but not total BAs in the stool of subjects with IBS-Diarrhea and IBS-Constipation respectively, thus demonstrating the utility of measuring BA profile in subjects with unexplained functional diarrhea or constipation.[15] These studies suggest that similar measurements of BA profile in the stool of women with FI could advance our understanding of factors that contribute to the severity of FI and potentially identify subgroups of women who may benefit from BA sequestering treatments.

Tryptamine

Tryptamine is a powerful neuroactive metabolite that is derived from the decarboxylation of dietary tryptophan by decarboxylase enzymes contained in gut bacteria.[19] Tryptamine mimics the effects of serotonin on gut motility and gut neurons through the endogenous release of physiologically active serotonin or 5-HT. An analysis of the Human Microbiome Data shows that individuals have varying ability to sequester dietary tryptophan to generate tryptamine in the gut.[19] At least 10% of the population harbors *C. sporogenes* and *Ruminococcus gnavus*, two gut bacteria that contain enzymes that decarboxylate tryptophan to tryptamine. The gut microbiome of patients with IBS patients is often dominated by Firmicutes, the phylum from which the above decaroboxylase containing bacteria are derived.[10] Since tryptamine levels could potentially be modulated by probiotics or dietary changes, measurement of tryptamine levels or of bacteria that generate tryptamine in the stools of women with FI could identify subgroups of patients who benefit from such treatments.

Stool Microbiota

Since the metabolites of many microbiota have not been identified, direct measurement of stool microbiota could also advance our understanding of bowel disturbances in women with FI. In healthy adults, 80% of the identified fecal microbiota can be classified into three dominant phyla: Firmicutes, Bacteroidetes, and Actinobacteria, but there is substantial variation in species among individuals.[10] In humans, butyrate producing bacteria are elevated in functional disorders associated with fecal urgency and increased motility. In studies using 16S rRNA gene sequencing of stool microbial DNA, gut microbiota of patients

with irritable bowel syndrome (IBS) were dominated by the Clostridiales class, Clostridium IVa clusters and Ruminococcaceae, and showed less diversity (alpha diversity) and different microbial composition (beta diversity or dissimilarity) compared to controls.[10] At the highest taxonomic level (phylum), these IBS groups were defined by an increase in Firmicutes and decrease in Bacteroides. The Firmicutes phylum includes the Clostridiales class, which can be further grouped into the Ruminococcaceae and Clostrdiium IVa clusters, known to be enriched producers of butyrate. Similarly, BA producing bacteria, such as the *Leptum* group, have been noted to be elevated and correlated with stool consistency and primary bile acid levels in subjects with IBS-Diarrhea.[20] Analysis of subgroups in patients with IBS has shown microbial associations with colonic transit time, rectal sensation, bloating, and depression, suggesting that certain bacterial phylotypes were clinical markers of IBS.[10] Given the potential role of gut motility and rectal sensation in the pathogenesis of FI, analysis of microbiota in women with FI compared to healthy controls may allow identification of subgroups of patients who are characterized by abnormal microbiota and/or metabolites.

Though the above discussion provides biologically plausible evidence that stool metabolites modulate gut motility and sensation, direct evidence on the role of stool metabolites and microbiota in women with FI is lacking. The SMM-CAPABLe study will measure both stool metabolome (metabolites resulting from host-microbiota interaction) and stool microbiome (gut bacterial environment) in women with FI compared to similarly aged controls. Analysis of metabolites could advance our understanding of the mechanisms that modulate severity of FI and response to treatment. Analysis of the microbiome could demonstrate the source of altered metabolites. Specific metabolites and/or microbiota could serve as clinical biomarkers for FI subgroups and identify potential new targeted treatments for women with this debilitating condition.

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