This supplement contains the following items:

1. Original protocol and statistical analysis plan
2. Final protocol and statistical analysis plan
3. Summary of changes.

Protocol

TITLE: Faecal microbiota transplantation (FMT) for the treatment of active ulcerative colitis (UC)

INVESTIGATORS DETAILS AND QUALIFICATION

Dr Samuel Costello (MBBS, FRACP)
Gastroenterologist
Queen Elizabeth Hospital
Woodville Road
Woodville
South Australia 5011
Email: sam.costello@health.sa.gov.au

Dr Michael Conlon (PhD)
Senior Research Scientist
CSIRO Animal, Food and Health Sciences
Kintore Avenue, Adelaide SA 5000
Email: Michael.Conlon@csiro.au

Dr Patrick Hughes, BSc(Hons)(Pharmacology), PhD
NHMRC Biomedical Research Fellow
Nerve-Gut Research Lab
University of Adelaide
Adelaide SA 5000
Email: patrick.hughes@adelaide.edu.au

Professor Ian Roberts Thomson (MD, FRACP)
Head of Gastroenterology
Queen Elizabeth Hospital
Woodville Road
Woodville SA 5011
Email: ian.roberts-thomson@health.sa.gov.au

Associate Professor Jane Andrews (MBBS, FRACP, PhD)
Head IBD Service and Education
Department of Gastroenterology & Hepatology & School of Medicine
Royal Adelaide Hospital
North Tce, Adelaide
South Australia. 5000
Email: jane.andrews@health.sa.gov.au

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1. OBJECTIVES OF STUDY

Primary

- To determine whether faecal microbiota transplantation (FMT) improves clinical and inflammatory outcomes in patients with active ulcerative colitis (UC)**.**

Secondary

- To determine whether any clinical change is accompanied by an alteration in the faecal and/or mucosal associated microbiome of ulcerative colitis patients prior to and following FMT.
- Assessment of alteration and durability of change in the recipient microbiota after FMT in ulcerative colitis patients
- To examine the mucosal immune changes induced by FMT and to examine whether they are influenced by changes in microbiome and/or disease activity
- To examine the durability of clinical response/improvement after initial response to FMT in subjects with active UC.
- To establish patient satisfaction with FMT as a therapy for UC.

2. BACKGROUND AND SIGNIFICANCE

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) that is characterized by recurring episodes of inflammation primarily involving the mucosal layer and occasionally the submucosa of the colon. Inflammation usually originates in the rectum and progresses in a contiguous fashion proximally. Although the aetiology of UC remains unclear, several factors are believed to play a role in its development and progression, including host genotype, immune disequilibrium, and the composition of microbial communities resident in the gastrointestinal (GI) tract.

There is strong evidence for the involvement of microbes in the development of UC. IBD is associated with changes in the diversity of the gut microbiota, and although alterations in the abundance of specific bacterial species have often been identified there remains no specific organism that is reliably associated with the condition[1]. There also appear to be changes in the functional activity of the microbiome, with changes in gene expression as well as protein production in the microbes of patients with IBD[1]. It is unclear whether the altered microbiota

is a result of, or initiates the inflammatory process in humans. There is some evidence, however, that an altered microbiota develops prior to the onset of colitis in an animal model of interleukin (IL)-10 knockout mice[2].

Intestinal flora and their metabolic products play a critical role in maintaining the health of the colon. Patients who undergo ileostomy and have subsequent diversion of the luminal contents from the colon often develop a "diversion" colitis. The distal colonocytes in this instance are deprived of short chain fatty acids such as butyrate, a product of anaerobic bacterial fermentation of undigested dietary carbohydrates[3]. Yet, animal models of IBD also require bacteria within the colon for inflammation to develop[4]. It has been observed that altering the bacterial and nutrient colonic milieu by diverting the faecal stream using ileostomy reduces the recurrence of Crohn's disease in the colon[5]. Further supporting the notion that bacterial antigens contribute to, or drive the autoimmune injury to the bowel, is evidence that antibiotics have some therapeutic efficacy in UC. In a recent systematic review, antibiotic therapy for UC was significantly associated with remission[6]. Antibiotic therapy gave a statistically significant relative risk reduction for active disease of 0.64 (CI= 0.43-0.96). So, whilst bacteria are necessary to develop IBD, with germ-free animals unable to be induced to develop IBD, bacterial absence (or change in abundance/mix) can also lead to inflammation.

Probiotics have as yet demonstrated only limited therapeutic efficacy in UC[7]. In vitro studies have demonstrated that probiotic bacteria are able to modulate gut immune cells[8,9]. Whilst, in vivo, in a German study, *E.coli* Nissle 1917 was equivalent in efficacy to mesalamine for maintenance of remission of UC[10]. Additionally, a randomized trial of 77 patients with UC found that VSL#3, a cocktail of 8 different bacteria, was more effective than placebo in improving symptoms and inducing remission at 12 weeks[11]. For patients with pouchitis, trials with VSL#3 have shown both therapeutic and prophylactic efficacy[12]. The outcomes in probiotic studies, however, have often been inconsistent and modest. This may be due to the variable actions of the different bacterial species that have been tested as well as the general limitations of most probiotic preparations. These probiotics provide a comparatively low number and diversity of bacterial species in comparison with the vast human gut microbiota. For this reason, some probiotic bacterial strains may not be able to compete effectively against the complex interactions of an established and adapted indigenous gut microbial community[13].

FMT has been described as "the ultimate probiotic" as it provides an entire microbiome to the recipient. This therapy delivers a much greater number and diversity of bacteria than any current commercially available preparation. FMT was first reported in humans by Eiseman *et al* in 1958 in the treatment of 4 patients with pseudomembranous colitis[14]. Three of the four patients were described as terminally or critically ill requiring vasopressor support and all were successfully cured. Over the subsequent years there have been case reports and case series describing FMT predominantly for *Clostridium difficile* colitis but also for treating IBD, irritable bowel syndrome and constipation[13,15,16]. In the past decade, there has been a heightened interest in the use of this therapy, predominantly driven by increasing rates of recurrent *C. difficile* infection. During this time *C. difficile* has become more frequent, more severe and more refractory to standard treatment as well as more likely to relapse[17]. Standard treatment with metronidazole or vancomycin alters the normal gut flora that would usually provide colonization resistance against *C. difficile* infection. For this reason, after successful initial therapy, up to 35% of patients will experience a symptomatic recurrence after ceasing antibiotics[18]. A subset of patients will have multiple recurrences and subsequent relapses occur in 45-65% of patients who have relapsed one or more times[19,20]. For patients with recurrent *C. difficile* colitis, FMT offers the greatest chance of cure of any therapy with success in 87-100% of cases[13, 21-25]. This impressive success rate is presumably due to the ability of the transplanted bacteria to recolonize/occupy the missing components/niches of the normal intestinal microbiota thus removing the microbial niche that *C. difficile* would otherwise exploit.

FMT for UC was first reported in the literature by a gastroenterologist Dr Justin D Bennet, from Kansas City, who described the results of a faecal transplant he received for his own disease[26]. Dr Bennet had continuous active, severe UC for 7 years, confirmed endoscopically and histologically that was refractory to standard therapy. Dr Bennet described receiving antibiotics to "sterilise" his bowel prior to retention stool enemas. At the time of publication in 1989 he had been symptom and medication free for the first time in 11 years, at 6 months post FMT.

Borody *et al* described case reports of six patients (3 men and 3 women aged 25–53 years) with UC for at least 5 years who were treated with FMT[16]. All patients had suffered severe, recurrent symptoms and UC had been confirmed on colonoscopy and histology. Faecal flora

donors were healthy adults who were extensively screened for parasites and bacterial pathogens. Patients were prepared with oral antibiotics and oral polyethylene glycol lavage. Faecal suspensions were administered as retention enemas and the process repeated daily for 5 days. By 1 week post FMT, some symptoms of UC had improved. Complete reversal of symptoms was achieved in all patients by 4 months post-FMT, by which time all other UC medications had been ceased. At 1 to 13 years post FMT, and without any UC medication, there was no clinical, colonoscopic, or histologic evidence of UC in any patient. The authors concluded that colonic infusion of donor human intestinal flora can reverse UC in selected patients and that these results support the concept of abnormal bowel flora or even a specific, albeit unidentified, bacterial pathogen causing UC. However, caution is needed when interpreting their data as this centre is known to have undertaken a large number of these treatments and it is uncertain why only 6 are reported. There is no comment in this paper as to the number of patients at their facility in whom this technique was attempted and if there were any patients in whom the treatment failed, moreover, this is open label treatment, which is now an insufficient standard of proof when evaluating novel therapies. Hence, randomized placebo controlled trials are needed to rigorously examine the efficacy of this proposed "alternative" therapy.

An anticipated concern in the medical community regarding FMT has been patient acceptance. This has been an assumption based on little evidence. To look at this question of patient willingness to undergo FMT, Kahn *et al*, performed a qualitative study to explore the attitudes and concerns of patients and parents of children with UC regarding FMT as a potential treatment[27]. They conducted six focus groups at a clinic in Chicago, Illinois and participants were asked about their perceptions of and interest in FMT as a treatment for UC. Sessions were recorded, transcribed, and reviewed to identify domains, themes, and major concepts. The focus groups included 15 adult patients and seven parents of children with colitis. The study identified five major domains pertaining to FMT; impressions of treatment, benefits, risks, potential mechanisms, and social concerns. All but one participant expressed interest in FMT and several wished it were already available. Participants compared FMT to probiotics, felt it was ''natural,'' easier than current therapies, and with donor screening would be safe. Although initial distaste and the ''yuck factor'' were uniformly mentioned, these concerns were outweighed by perceived benefits. The study concluded that given adequate supporting research, donor selection, and screening, adult patients and parents of children with UC will consider FMT and are eager for it to become available.

FMT for UC is currently undertaken at a private gastroenterology clinic in Sydney and case reports of success from this clinic are reported in the literature[16]. There is also evidence from UC online forums that patients are conducting FMT for UC outside of the healthcare setting[28,29]. This is occurring in an unregulated fashion, with only very limited evidence of efficacy from 7 case reports in the literature. These occurrences underline the need for more robust scientific evidence in this area and a randomised controlled study of efficacy.

2.1 Standard of care for ulcerative colitis

The management of UC involves both maintenance medication and medication used to control flares of the disease. The goal of maintenance therapy in UC is to maintain steroid-free remission, clinically and endoscopically. This requires regular clinical assessment including history, physical examination and at times colonoscopic examination. Other tools of assessment include blood (e.g. CRP, WCC) and stool (calprotectin) testing for inflammatory markers and imaging including MRI, CT or ultrasound.

The choice of maintenance treatment in UC is determined by disease extent, disease course (frequency of flares), failure of previous maintenance treatment, severity of the most recent flare, treatment used for inducing remission during the most recent flare, safety of maintenance treatment, and cancer prevention. The mainstay of maintenance medication are the 5-aminosalicylic acid compounds (5-ASA) such as mesalazine or sulphasalazine[30,31]. These compounds are commonly taken orally in formulations that predominantly deliver the active 5-ASA component to the colon. Alternatively, or in addition, mesalazine preparations can be delivered topically via enema or suppository if the disease only involves the left side of the colon (although it is only PBS funded for topical therapy during a flare and not for maintenance of remission – even though it also works in this setting). The majority of patients can be managed with maintenance 5-ASA compounds most of the time. For patients who have repeated flares of disease on 5-ASA maintenance therapy (1 or more flares in a year needing steroids), thiopurine medication such as azathioprine or 6-mercapropurine should be used[32]. These medications induce systemic immunosuppression, reduce the incidence and severity of flares of colitis but also slightly increase the risk of some infections and malignancy. Anti TNF agents such as infliximab or adalimumab have been shown to have benefit in maintaining remission in UC[33] (and are licensed for this indication by the

TGA), however these agents are very expensive and not funded by the pharmaceutical benefits scheme in Australia and so, are not readily available. The anti TNF agents also give an increased risk of infection, particularly latent TB reactivation.

Mild flares of UC can be managed with higher doses of oral 5-ASA compounds or the addition of topical 5-ASAs given via enema or suppository. More severe flares are usually managed with a course of systemic corticosteroid. These can be given intravenously in acute, severe disease or orally in less severe flares. The steroids should then be tapered over time and discontinued. There is no indication for long term steroid use in UC and prolonged steroid use is associated with a number of complications including infection, osteoporosis, obesity, diabetes, poor wound healing, thinning skin, mood changes and insomnia. Severe flares of UC not responsive to steroids may respond to rescue therapy with the addition of either cyclosporin or anti-TNF therapy.

Patients in whom colonic inflammation cannot be controlled adequately frequently undergo total colectomy. This may be done electively (for refractory disease) or emergently in acute fulminant colitis. Colectomy entails surgical risk that is higher in the emergent setting; this risk includes infection, wound breakdown and a mortality rate. Colectomy is considered "curative" for UC especially if they have an ileostomy stoma created, however, it frequently also leads to complications both short- and long-term. In addition, in patients in whom an ileal-anal pouch is fashioned up to 50% will subsequently develop pouchitis at 4 years post surgery[34].

3. SPECIFIC SAFETY CONSIDERATIONS

A recent review article assessed all cases of FMT in the literature prior to 2011[13]. A total of 239 patients had undergone FMT. The authors did not find any serious adverse events related to the procedure. Some studies reported patient deaths due to the underlying disease, where the patient has not responded to the FMT. In one study, in which donor faeces were instilled via a nasogastric tube, a patient died of peritonitis. This patient was undergoing peritoneal dialysis for end stage renal failure at the time and was septic with severe *C. difficile* colitis. Her condition remained unchanged immediately post transplantation, however on the third

day she developed peritonitis. Although considered more likely the result of peritoneal dialysis, the nasogastric tube insertion could not be discounted to have been contributory[35]. One patient in a study by Silverman *et al* developed irritable bowel symptoms following FMT[25].

Following this literature review in 2011 there have been 4 further cohort studies in the literature of patients who have undergone faecal transplant for *C. difficile* colitis[21,22,36,37]. A total of 216 patients who underwent FMT via colonoscopy were included in these 4 studies with no immediate adverse effects from FMT noted.

There is a potential to transmit infection via contaminated donor stool. The donor stool will therefore undergo microscopy and culture for potential bacterial pathogens, microscopy for ova, cysts and parasites as well as viral studies and *C. difficile* toxin analysis.
Blood testing to exclude HIV, Hepatitis B and C and syphilis will be undertaken.

Changes in faecal microbiota have been found in patients with a number gastrointestinal and extra-intestinal diseases. Changes in the microbiome of patients with IBD and irritable bowel syndrome are well documented in the literature. There have also been associations between various bowel flora and obesity and the metabolic syndrome[38]. The association has not been documented as causal and it appears probably related to the diet consumed by these subjects. It would, however, be prudent to exclude donors with the metabolic syndrome from the study.

In an audit of 16,318 colonoscopies performed in Northern California from 1994 to 2002, Levin *et al.,* found serious complications occurred in 5.0 of 1000 proceedures[39]. The major risk of colonoscopy, bowel perforation, occurred in 0.09% of colonoscopies in this study. Other risks include dehydration from bowel preparation, over-sedation, aspiration, bleeding and splenic laceration. This patient group will however be undergoing regular colonoscopies for their UC and will be familiar with these risks. Risks from standard therapies they would be offered for active disease are also substantial (steroids, immunomodulators, colectomy), thus risks from colonoscopy for FMT are relative.

4. ETHICAL CONSIDERATIONS

UC is a chronic, debilitating disease with a near normal life expectancy[40]. Current therapies are inadequate and the disease continues to have an unacceptably high rate of chronic relapsing symptoms. This is underlined by evidence that up to 30 percent of patients will require colectomy after 25 years of disease[41]. For this reason, it is important for the medical community to rigorously examine potential new therapies that may benefit this group of patients.

A small number of case reports of successful treatment of UC with FMT have been reported in the literature[16,26]. However, the findings of these case reports have never been tested in a randomised controlled trial. Despite this very limited evidence there is a clinic in Australia offering UC patients FMT as a therapy[16]. There is also evidence from online forums that patients are undertaking this therapy without medical supervision[28,29]. Despite the minimal evidence in the literature, there is a willingness among sufferers of UC to try this potential therapy[27]. We believe a randomised control trial in this area is necessary to gather evidence for or against the effectiveness of FMT as a treatment for UC. A positive result would avail UC sufferers of a new therapy and a negative one would help discourage the use of an unproven, invasive therapy. Stool analysis of faecal transplant success may also fast track development of tailored probiotic medicines.

Donors will be anonymous and so will not be known to the recipient. This avoids any apportion of blame towards a known donor should a complication or treatment failure arise during the trial.

Colonoscopy will be used to deliver the initial stool transplantation and to assess the colon during follow up. This is an invasive procedure that carries some risk. Most of the recent studies of FMT for *C. difficile* have used colonoscopic delivery[13,22,23] as it allows assessment of the underlying disease and allows the donor bacteria to contact the entire colon. Patients with symptomatic UC would ordinarily undergo examination with colonoscopy as part of the assessment of disease activity to help guide treatment. The initial colonoscopy in this trial will therefore not be an additional procedure. However, the colonoscopy at week 8 and at 12 months may be additional procedures depending on the state of the patient's disease and symptoms.

The colonoscopic examinations will involve biopsy of the mucosa for analysis of microbiota as well as immune function and histopathology. The majority of these biopsies will be additional to that which the patient would have ordinarily received outside of the trial. These biopsies will be critical to detect any changes in the mucosal associated microbiota or immune changes associated with the FMT. The risk of biopsy of the mucosa is small with the major risk being bleeding. Biopsies can be safely performed on a single anti-platelet agent[42]. Patients on duel antiplatelet therapy or anticoagulant medication (i.e. warfarin or heparin) will be excluded from the study.

As FMT has only been performed in large numbers in the past decade, there may be unknown long term risks. However, there have been no reports of major complication of faecal transplant in the literature to date.

Taking blood may cause short-term pain or discomfort and patients will be informed about this before entering the trial. The volume of blood taken is not extreme and will not cause side effects. If patients are of the view that blood sampling is too painful they may withdraw from the study at any time. Blood tests as well as answering questionnaires will involve an increased time burden and patients will be informed about this before the trial begins. It is not anticipated that the FMT procedure will cause any adverse reactions, but participants will be provided with information about supports they can contact should they experience any distress in relation to the study.

Before taking part in the study, informed written consent will be obtained from patients. The researchers will ensure that the patient is given full and adequate verbal and written information about the nature, purpose, possible risk and benefit of the trial. They will be given sufficient time to consider the information, to ask questions and to seek advice prior to being asked whether they wish to participate in the study. Participants will also be assured their participation in the trial is absolutely voluntary. All treatment decisions are at the discretion of the usual treating physician, and will not be altered by the trial. The participation is strictly confidential, and the identity of subjects will not be disclosed to other medical or research staff unless subjects agree.

Once subjects have been enrolled in this study, they will be given a study participant code, and only study investigators will have access to their name and personal details. We intend to

summarise the results in a manuscript and to submit it for publication in a peer reviewed journal. Therefore, all information gathering from this study will be published in a form that does not allow patient identification. We will not provide any feedback with regard to individual microbiota composition or immunologic function.

Our proposed study has the support of the Director of the Royal Adelaide Hospital department of Gastroenterology & Hepatology, Richard Holloway, as well as the head of Endoscopic Services, Mark Schoeman. The Head of IBD Service, Jane Andrews, will be the lead supervisor of the study. The Royal Adelaide Hospital has a large cohort of approximately 800 patients with IBD and a strong record of successful clinical research. The proposed study also has the support of the head of gastroenterology at the Queen Elizabeth Hospital, Ian Roberts-Thomson. The Queen Elizabeth Hospital has a cohort of approximately 300 patients with IBD. The study supervisors all have extensive experience in medical research as well as experience in supervising PhD students.

The Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Adelaide have broad experience in human gut flora and microbial analysis. This is an area of focus for their recent research. The Nerve Gut Research Laboratory at the Royal Adelaide Hospital is a leader in the field of research into the neuro-immunological and neuro-endocrine processes of the human gut.

5. STUDY DESIGN

This study is an 8 week randomised placebo controlled trial with a 44 week open labelled extension.

Randomisation:

- Group 1:
    - Patients receives previously frozen pooled **donor** stool via colonoscopic insertion into right colon
- Group 2
    - Patients receives previously frozen **own** stool via colonoscopic insertion into the right colon

Open label therapy from 8 weeks

Patients who are randomly assigned to the placebo group who do not have a clinically relevant response (achieving remission, having a drop in Mayo score by ≥3 or achieving a endoscopic sub-score of 0-1) by week 8 will then cross over to receive active donor FMT at the 8 week colonoscopy. The FMT will be conducted in an identical manner to Group 1 with FMT followed by 2 enemas on day 3 or 4 and one on day 6 or 7.

5.1 Recruitment

Patients will be recruited from IBD clinics at the Royal Adelaide and Queen Elizabeth Hospitals. Patients on the mailing list for the Royal Adelaide Hospital will be contacted about the trial through the quarterly newsletter. Gastroenterologists in Adelaide will be informed about the trial though a presentation at the South Australian gut club and an email to the South Australian gut club members. The trial will be listed on the Australian and New Zealand clinical trials registry as well as the Gastroenterology Society of Australia website.

We will enrol 70 patients with 35 patients in each arm of the trial.

5.2 Inclusion criteria:

1. *Mild to moderate active UC (Total Mayo score 3 to 10)*
2. *Endoscopic subscore of 2 or greater (to ensure symptoms are due to UC (not post-inflammatory irritable bowel syndrome)*
3. *Patients aged 18 to 75 years with established diagnosis of UC*

5.3 Exclusion criteria:

1. *Severe UC (Mayo score 11-12 or Truelove and Witts criteria)*
2. *More than 25mg of prednisolone per day (or equivalent steroid)*
3. *Previous colonic surgery*

4. *Active gastrointestinal infection*
5. *Pregnancy*
6. *Anticoagulant therapy or duel antiplatelet therapy (i.e. aspirin and clopidogrel)*
7. *Current use of antibiotics*
8. *Anti-TNF therapy*

Activity of disease will be defined by the Mayo score. This scoring system has 3 points each for stool frequency, rectal bleeding, endoscopic findings and physician's global assessment giving a total score out of 12.

A score of

- 0, 1 or 2 would indicate inactive disease and exclusion from the trial.
- 3 to 10 would allow inclusion into the trial. Subjects would need an endoscopic sub-score of at least 2 for inclusion, to prove active disease. (0= normal mucosa and 1= erythema only – most studies start with 2)
- 11-12 would indicate severe disease and these patients would be excluded from the trial.

Similarly any patient who fulfilled Truelove and Witts criteria for severe colitis would be excluded while they met this criteria. Truelove and Witts is defined as >6 bloody bowel motions per day plus one or more of the following: Haemoglobin <10.5g/dL, ESR>30mm/hr, Pulse rate >90 beats per minute or Temperature over 37.5 degrees Celsius.

5.4 Medication prior to enrolment

Stable dosing of UC maintenance therapy is required prior to enrolment.

1. 5-aminosalicylic acid (5-ASA) stable doing for at least 4 weeks
2. Thiopurines and methotrexate stable dosing for at least 6 weeks
3. Biological agents stable dosing for at least 8 weeks
4. Patients can enrol on an oral dose of prednisolone ≤25mg, with a mandatory taper of 5mg per week.

6. OUTCOME MEASURES

6.1 Primary outcome

Steroid-free remission of UC at week 8 defined as

1. Total Mayo score of ≤ 2
   AND
2. Mayo endoscopic score of ≤ 1

6.2 Secondary outcomes

1. Clinical response (≥3 point reduction in total Mayo score at week 8 and 1 year)
2. Clinical remission (Simple clinical colitis activity index (SCCAI) ≤ 2 at week 8 and 1 year)[43]
3. Endoscopic remission (Mayo ≤ 1 at week 8 and 1 year)
4. Safety (assessed at week 8 and 1 year)
5. Changes in mucosal and faecal associated microbiota following FMT assessed by 16s ribosomal RNA sequencing, stratified by:
   a. Change in total Mayo score following FMT
   b. Randomisation
6. Durability of engraftment of donor microbiome following FMT
7. Changes in peripheral blood and colonic lamina propria mononuclear cell populations (assessed by FACS) following FMT
8. Patient perception and palatability

Disease activity measures of symptoms score (SCCAI), endoscopic and histologic grading as well as records of hospitalization, corticosteroid requirement, periods of symptom flares and colectomy rate will be recorded at the 1 year mark as part of the open label observation period from 8 weeks to 1 year.

7 PATIENT PARTICIPATION

7.1 Recruitment

Participants will be recruited from:

Royal Adelaide Hospital (RAH) & The Queen Elizabeth Hospital (TQEH) Gastroenterology IBD databases and newsletters

Gastroenterology in- or out-patient encounter(s) at the RAH and TQEH (and Flinders Medical Centre after relevant approvals) by referral from their clinicians and by searching OPD letters.

Patients on clinical databases who have previously consented to being contacted regarding research studies will receive information about the study in the RAH IBD Service regular newsletter and may also be contacted by telephone, and if no answer is obtained a letter will be sent. All other patients will be contacted via a letter or by their treating clinician in whichever way the clinician feels is most appropriate to the particular patient.

Regarding the use of letters for contact: Subjects who have already consented to be contacted regarding research (on database) will receive a letter signed by A/Prof Andrews (at RAH); Prof. Roberts Thomson (at TQEH) on behalf of the study investigators.

The initial invitation letter will include an opt-out slip for subjects not wishing to be contacted further. Subjects not opting out or responding within 4 weeks after invitation will be contacted up to a further 3 times by 2 different methods (phone, SMS, email or letter) to ascertain whether they wish to participate or not. Demographic details of non-responders will be recorded to enable a full description of the sources of possible bias. All who agree to participate will be subsequently screened to ensure they fulfill inclusion criteria.

Donors will be recruited with a flier advertisement on notice boards at the RAH and TQEH as well as the Adelaide University Medical School and Adelaide University campus.

7.2 Withdrawal criteria

Patients may withdraw from the study at any time. We will ask for their reasons for statistical purposes, however they will not be obliged to provide this. Withdrawal from the study will not affect ongoing standard medical care in any way. Their clinicians will be informed of their participation in the study. We will ask patients to notify us of any changes in their treatment during the course of the study and if necessary, we will seek their permission to verify this with their treating clinician.

8. ULCERATIVE COLITIS PATIENT ASSESSMENT

8.1 The week prior to enrollment

1. The patient should have the opportunity to read the patient information sheet, discuss the trial with family or friends and ask questions of the investigators prior to signing trial consent.
2. Patient questionnaire regarding perception and expectation of faecal transplant prior to procedure
3. Detailed history of UC
    a) Date of diagnosis
    b) Extent of disease
    c) Medication use- current and prior
    d) Previous Surgery
    e) Previous Hospitalisation
    f) Comorbid disease
    g) Current symptoms
    h) Extra-articular manifestations

4. Stool collected for
    a) Infection screen. Microscopy Culture + Sensitivity, *Clostridium difficile* toxin (5g)
    b) Possible re-administration for placebo arm subjects. (50g)
    c) Microbiome analysis. 6 x 0.25g stool in Eppendorf tubes; 2x 5g stool in larger brown stool pots

For collection and processing methods see section 12 (page 31-33)

5. Disease activity assessment
    a. Faecal calprotectin
    b. CRP,ESR, FBC, U+E, LFTs
    c. Symptom severity (SCCAI) at screening and one day prior to FMT
    d. Flexible sigmoidoscopy
        i. Total Mayo score.
        ii. Disease extent (≥10cm of disease required)
        iii. Biopsy for light microscopy and histopathology to exclude CMV inclusions

8.2 Randomisation

Randomisation will be conducted once the patient satisfies the inclusion and exclusion criteria and has consented to enter the study. This should occur within 1-7 days prior to the first faecal transplant that will be delivered via colonoscopy.

Prior to randomisation 3 aliquots of pooled donor stool suspension from a single batch and 3 aliquots of the donor's own stool suspension will each be placed in a clear plastic bags in the -80°C freezer at the endoscopy unit. All stool aliquots will be in identical, yellow topped 250ml cryo-safe containers. These will include 1x 200ml suspension for colonoscopic delivery and 2x 100ml suspensions for enema delivery.

Donor stool pots will be labelled on the lid with:

1. Batch number
2. Date of manufacture of batch

Patient's own stool will be labelled on the lid with:

1. Patient ID consisting of initials and study number ie (AB-1)
2. Date of patient stool donation

Randomisation to be conducted by hospital clinical trial nursing staff using www.random.org

1. Cardboard circular caps with Patient ID and either “Transplant” or “Save” and either “Colon” or “Enema” are then placed on the pots containing donor or patients own stool depending on randomisation.
2. “Transplant” caps are placed on the pots to be given at and in the week following the first colonoscopy.
3. “Save” pots will be saved and the cap removed following the 8 week colonoscopy. If these contain donor stool then they will be given to the patient at the 8 week colonoscopy and in the subsequent week.
4. “Colon” pots will be delivered at colonoscopy and the “Enema” pots then delivered via enema in the following week.
5. The randomisation document for the use by study nursing staff is listed on page 21.

Faecal transplant for active ulcerative colitis trial

Protocol for randomisation of FMT

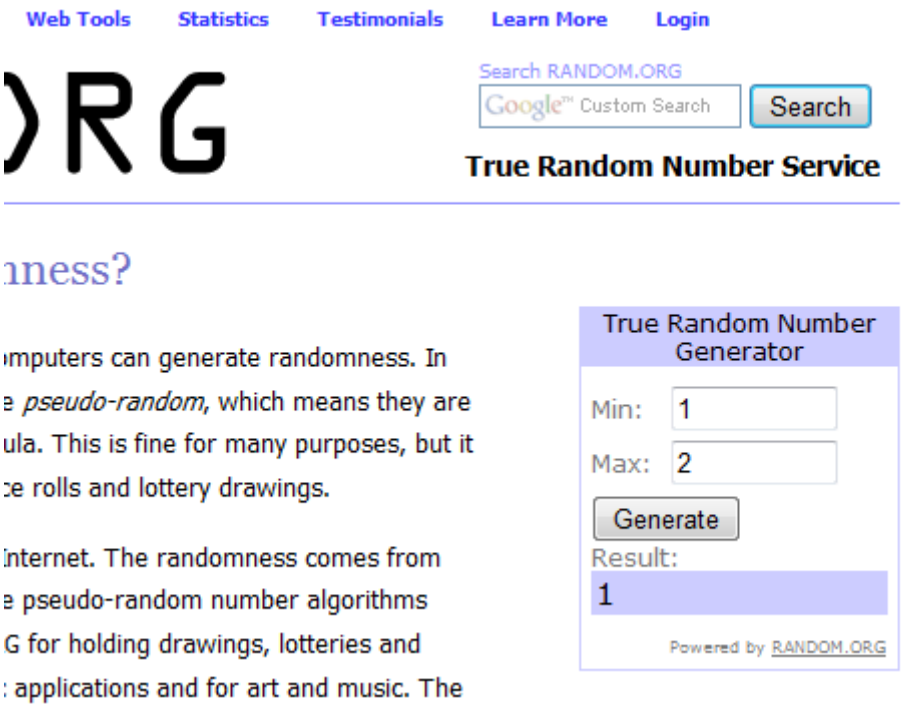
1-7 days prior to faecal transplant use the random number generator http://www.random.org/

Into the "true random number generator" box on the right of the screen set the minimum to 1 and maximum to 2

Select: Generate

1 = Donor faecal transplant

2= Placebo faecal transplant (patient's own stool)



Go to the -80°C Freezer.

1. For Donor Faecal transplant stick the "Transplant" disks on the top of the donor stool yellow pots and the "Save" disks on the placebo pots. Record the batch number in the transplant record book.
2. For Placebo Faecal transplant stick the "Save" disks on the donor stool yellow pots and the "Save" disks on the donor pots. The donor stool will then be saved to transplant at 8 weeks at the open label cross over.

The **donor stool** pots are labelled with a Batch number and date. (ie Batch 3, 2/7/13)

The **patient's own stool** (placebo) is labelled with patient study number

8.3 Week 1 of trial

8.3.1 Day prior to Colonoscopy

1. Patient to take a light breakfast and then to fast from solids
2. Maintain high fluid intake throughout the day
3. Take 3 sachets of Colonlyetly bowel preparation (polyethylene glycol) in 3 L of water

8.3.2 Morning of Colonoscopy

1. Randomised faecal aliquot labeled “Transplant” and “Colon” to be removed from the -80°C freezer and thawed at room temperature for 3.5 hours prior to delivery
2. Patient to receive Loperamide 2mg orally prior to colonoscopy
3. SCCAI score diary to be collected
4. Mayo symptom scores to be taken (endoscopic score to be added at colonoscopy)
5. Biopsy posts should be pre-labelled with the site and number of biopsies required
6. Consent should be obtained for this procedure on a standard CALHN consent form in addition to the study consent form that has previously been signed.
7. While inserting canula, take 60mls of blood
    a. 50 mls into 6x heparin tubes (green and black top) for Peripheral Blood Mononuclear Cell flow cytometry (to be taken to Dr Hughes at nerve gut laboratory).
    b. 5mls into a small EDTA (purple tube) (to be sent to clinical laboratory)
    c. 5mls into a GEL (white top) Electrolytes and liver function, C-reactive protein (to be sent to SA pathology laboratory)

8.3.3 At Colonoscopy

1. Assess and disease severity (using endoscopic Mayo score at point of maximum inflammation) and disease extent.
2. Biopsies should be taken on colonoscope insertion*

a. Left sided biopsies- 11 total; 6 in RPMI media, 2 into RNA later (microbiome), 1 RNA later (PCR), 1 formalin (histopath), 1 PFA (IHC)
b. Right sided biopsies- 9 biopsies; 6 in RPMI media, 2 into RNA later (microbiome), 1 formalin (histopath).

3. An attempt should be made to remove any residual fluid or faecal material during colonoscope insertion with suction and washing if required.
4. Once at caecum patient should be rolled onto the right lateral position and randomized faecal suspension delivered into the right colon. If caecum cannot be reached then delivery of faecal suspension into the right colon beyond the hepatic flexure is acceptable.
5. Patient should then remain on their right side for 1 hour following procedure.
6. Following 1 hour the patient should be assessed for any adverse effects and if well sat up and offered food and drink prior to discharge.

*Biopsies- At each colonoscopy in more detail

- 2 biopsies will be taken from both the recto-sigmoid region and ascending colon-caecal region of the colon for microbiota analysis. Each in 2.5ml RNAlater.
- 1 biopsy from each region will be taken for histopathology analysis into formalin.
- 1 biopsy from left colon for immunohistochemistry (formalin) and
- 1 from the left colon for PCR (cytokines, transcription factors)- (RNA later)
- 4 biopsies will be taken from the left and 4 from the right colon for flow cytometry (FACS) analysis. (RPMI complete media). Processed the same day as colonoscopy.
- 2 biopsies from the left colon and 2 from the right for supernatant release for cytokines / mast cell mediators. (RPMI complete media)
- This would amount to 9 biopsies in the right colon and 11 in the left colon.

8.3.4 Enemas

Two enemas of 100ml faecal suspension will be delivered by a Gastroenterologist at the clinic in the week following colonoscopy Days (2-4) Days (5-7).

Patient should:

1. Take 2mg of loperamide prior to enema
2. Lay on left lateral position for enema insertion.
3. Roll from the left lateral to prone position then right lateral and then back to left lateral position following enema insertion. This is to encourage proximal distribution of the enema.
4. Attempt to hold the enema for 1 hour

8.4 Week 4 assessment

1. Stool collection for faecal calprotectin level and microbiome analysis
2. Stool to be collected in sterile blue bags that are placed over the toilet.
3. Patient to zip tie blue bag closed and place blue bag in clear zip lock bag
4. Deliver to CSIRO laboratory within 1 hour
5. Bag to be opened and stool processed under anaerobic conditions in anaerobic chamber
    a) Microbiome analysis. 6 x 0.25g stool in Eppendorf tubes; 2x 5g stool in larger brown stool pots

8.5 Week 8 assessment

8.5.1 Two days prior to Colonoscopy

1. Stool collection for faecal calprotectin level and microbiome analysis
2. Stool to be collected in sterile blue bags that are placed over the toilet.
3. Patient to zip tie blue bag closed and place blue bag in clear zip lock bag
4. Deliver to CSIRO laboratory within 1 hour
5. Bag to be opened and stool processed under anaerobic conditions in anaerobic chamber
    a) Microbiome analysis. 6 x 0.25g stool in Eppendorf tubes; 2x 5g stool in larger brown stool pots

8.5.2 One day prior to Colonoscopy

1. Patient to take a light breakfast and then to fast from solids
2. Maintain high fluid intake throughout the day
3. Take 3 sachets of Colonlyetly bowel preparation (polyethylene glycol) in 3 L of water

8.5.3 Morning prior to Colonoscopy

1) Randomised faecal aliquot labeled “Save” and “Colon” to be removed from the -80°C freezer and thawed at room temperature for 3.5 hours prior to delivery
2) Patient to receive loperamide 2mg orally prior to colonoscopy
3) SCCAI score diary to be collected
4) Mayo symptom scores to be taken (endoscopic score to be added at colonoscopy)
5) Adverse events since randomization recorded
6) Biopsy posts should be pre-labelled with the site and number of biopsies required
7) Consent should be obtained for this procedure on a standard CALHN consent form in addition to the study consent form that has previously been signed.
8) While inserting canula, take 60mls of blood
    a) 50 mls into 6x heparin tubes (green and black top) for Peripheral Blood Mononuclear Cell flow cytometry (to be taken to Dr Hughes at nerve gut laboratory).
    b) 5mls into a small EDTA (purple tube) (to be sent to clinical laboratory)
    c) 5mls into a GEL (white top) Electrolytes and liver function, C-reactive protein (to be sent to clinical laboratory)

8.5.4 At Colonoscopy

1) Assess disease severity using endoscopic Mayo score at point of maximum inflammation and disease extent.
2) Biopsies should be taken on colonoscope insertion*
    a) Left sided biopsies- 11 total; 6 in RPMI media, 2 into RNA later (microbiome), 1 RNA later (PCR), 1 formalin (histopath), 1 PFA (IHC)

b) Right sided biopsies- 9 biopsies; 6 in RPMI media, 2 into RNA later (microbiome), 1 formalin (histopath)

3) An attempt should be made to remove any residual fluid or faecal material during colonoscope insertion with suction and washing if required.
4) Once at caecum (and disease severity has been assessed and recorded) the cardboard
5) "Save" cap should be removed from the pot to reveal the contents of the faecal pot.
   a) If this is labeled as the patient's own stool it should be discarded and the colonoscope withdrawn.
   b) If this is labeled as donor stool then the patient should be rolled onto the right lateral position and the un-blinded faecal suspension delivered into the right colon.
6) If caecum cannot be reached then delivery of faecal suspension into the right colon beyond the hepatic flexure is acceptable.
7) Patient should then remain on their right side for 1 hour following procedure.
8) Following 1 hour the patient should be assessed for any adverse effects offered food and drink and if prior to discharge.
9) Patient to be informed about randomization. If they were initially randomized to placebo/ autologous FMT then they will require 2 further donor FMTs via enema.

*Biopsies- At each colonoscopy in more detail

- 2 biopsies will be taken from both the recto-sigmoid region and ascending colon-caecal region of the colon for microbiota analysis. Each in 2.5ml RNAlater.
- 1 biopsy from each region will be taken for histopathology analysis into formalin.
- 1 biopsy from left colon for immunohistochemistry (formalin) and
- 1 from the left colon for PCR (cytokines, transcription factors)- (RNA later)
- 4 biopsies will be taken from the left and 4 from the right colon for flow cytometry (FACS) analysis. (RPMI complete media). Processed the day of colonoscopy.
- 2 biopsies from the left colon and 2 from the right for supernatant release for cytokines / mast cell mediators. (RPMI complete media)
- This would amount to 9 biopsies in the right colon and 11 in the left colon.

8.5.5 Enemas (Patients randomized to placebo/ autologous FMT)

Two enemas of 100ml faecal suspension will be delivered by a medical practitioner at the clinic in the week following colonoscopy Days (2-4) Days (5-7).

1) Patient to take 2mg of loperamide prior to enema
2) Lay on left lateral position for enema insertion.
3) Roll into prone positions, right lateral and then back to left lateral position following enema insertion.
4) Patient should attempt to hold the enema for 1 hour

8.6 1 year assessment

Patient will be posted or emailed

1) SCCAI symptoms score
2) Patient questionnaire regarding experience of faecal transplant prior to procedure and adverse events
3) Invitation to undergo disease activity assessment

Patients who do not return forms within 2 weeks will be contacted via telephone

8.6.1 Two days prior to Flexible Sigmoidoscopy

1) Stool collection for faecal calprotectin level and microbiome analysis
    a) Stool to be collected in sterile blue bags that are placed over the toilet.
    b) Patient to zip tie blue bag closed and place blue bag in clear zip lock bag
    c) Deliver to CSIRO laboratory within 1 hour
    d) Bag to be opened and stool processed under anaerobic conditions in anaerobic chamber
        i) Microbiome analysis. 6 x 0.25g stool in Eppindorf tubes; 2x 5g stool in larger brown stool pots
        ii) Faecal calprotectin

7.6.2 Flexible Sigmoidoscopy

1) Mayo symptom scores to be taken (endoscopic score to be added at colonoscopy)
2) Adverse events since randomization recorded
3) Biopsy posts should be pre-labelled with the site and number of biopsies required
4) Consent should be obtained for this procedure on a standard CALHN consent form in addition to the study consent form that has previously been singed.
5) While inserting cannula, take 60mls of blood
    a) 50 mls into 6x heparin tubes (green and black top) for Peripheral Blood Mononuclear Cell flow cytometry (to be taken to Dr Hughes at nerve gut laboratory).
    b) 5mls into a small EDTA (purple tube) (to be sent to clinical laboratory)
    c) 5mls into a GEL (white top) Electrolytes and liver function, C-reactive protein (to be sent to clinical laboratory)
6) Assess disease severity using endoscopic mayo score at point of maximum inflammation
7) Biopsies should be taken on the left side only
    a) Left sided biopsies- 11 total; 6 in RPMI media, 2 into RNA later (microbiome), 1 RNA later (PCR), 1 formalin (histopath), 1 PFA (IHC)
8) Following 1 hour the patient should be assessed for any adverse effects offered food and drink and if prior to discharge.

8.6.3 Care during the follow up period

During the trial subjects will be treated to the standard of care for UC. This involves a fixed maintenance medication as prescribed/advised by their own physician. Patients will enter this trial due to a flare, and all therapy they are on at entry will be continued except for the steroid taper as described.

A subject who experiences a flare of their disease during the study will be treated with standard therapy as they would if they were not in the study. This will include increasing their oral 5-ASA and/or adding a topical enema or suppository therapy. Systemic steroid therapy may also be used. Steroid use will be quantified during the study and steroid

requirement over the 12 month period will be another secondary end point. Once patients are commenced on steroid it will be tapered as explained above.

If a subject deteriorates on steroid therapy they may require escalation of their medical therapy or surgery. Escalation of medical therapy may involve increasing the steroid dose temporarily. Patients who are naive to thiopurine therapy may benefit from the addition of a thiopurine. Thiopurines can take up to 12 weeks to reach their therapeutic effect and so "rescue therapy" may be needed in the intervening period. Rescue therapy involves the addition of cyclosporine or an anti-TNF agent such as infliximab (if available through compassionate access) in the short term. Rescue therapy would be continued for 6 to 12 weeks to allow the thiopurine medication to reach its full effect.

Patients who have a severe flare of UC that does not respond to intravenous steroid medication within 3 to 5 days are unlikely to improve and should be assessed for surgical colectomy[44], as would be the case in routine care.

9. STOOL DONOR RECRUITMENT AND SCREENING

9.1 Donor recruitment

Posters will be placed on noticeboards on the University of Adelaide Campus. These will detail that we are recruiting stool donors and the posters will have the contact details of Dr Costello and Dr Andrews.

9.2 Donor screening

Potential donors would be sent the donor information sheet via email or post.

Donors who consent will undergo a four stage screening process with medical history, physical examination, blood testing and stool testing with the aim of reducing the risk of disease transmission from donor to recipient.

9.2.1 Medical History

Inclusion of patients who

1. Are 18 to 65 years of age
2. Have not received antibiotic therapy for the past 6 months
3. Have not had unprotected sexual intercourse in the last 1 month outside of a long term monogamous relationship?
4. Have not travelled outside of Australia for past 1 month

Have no active medical problems or a history of

1. Inflammatory bowel disease
2. Irritable bowel syndrome
3. Colonic polyps
4. Bowel cancer
5. Any other gastrointestinal disorder
6. Obesity
7. High blood pressure
8. Diabetes
9. Heart disease
10. Stroke
11. Major depression
12. Infection with Hepatitis B or C, HIV or syphilis
13. Autoimmune disease (ie rheumatoid arthritis, SLE)

9.2.2 Physical Examination

Cardiovascular and gastrointestinal examination
Height and Weight. Obesity (BMI <18 and >30) is an exclusion

9.2.3 Blood testing

1. Full blood count (Anaemia, WCC>12.5 are exclusions)
2. Electrolytes, Urea and Creatinine (renal impairment eGFR<60 is an exclusion)
3. Liver function tests (abnormal LFTs are exclusions)
4. Human T-cell lymphotropic virus 1 and 2 serology (positive serology is an exclusion)
5. Epstein Barr Virus IgM and IgG (positive IgM is exclusion)
6. Cytomegalovirus IgM and IgG (positive IgM is exclusion)
7. Syphilis (positive rapid plasma regain is an exclusion)
8. *Strongyloides stercoralis*, *Entamoeba histolytica (positive serology is an exclusion)*
9. Toxoplasma serology (positive serology is an exclusion)
10. Hepatitis A virus IgM (positive serology is an exclusion)
11. Hepatitis B PCR (positive PCR is an exclusion)
12. Hepatitis C PCR (positive PCR is an exclusion)
13. HIV PCR (positive PCR is an exclusion)
14. Fasting lipids and Blood sugar level (Total Cholesterol > 4.0 mmol/L, LDL >2.5 mmol/L, Triglycerides >2.0 mmol/L, HDL <1.0 mmol/L are exclusions)
15. C-Reactive Protein (>8 exclusion)

9.2.4 Stool testing

1. Microscopy and Culture
2. *Clostridium difficle* toxin PCR
3. Egg, cysts and parasites (including Cryptosporidium spp., Giardia spp., and *Entamoeba histolytica* PCR)

10. STOOL COLLECTION AND PROCESSING

Once donors have passed all the screening requirements they are eligible to donate for 1 month. To donate stool beyond this time will require repeat screening.

10.1 Stool collection

1. Stool collected in sterile blue bags that are placed over the toilet.
2. Stool donor to zip tie blue bag closed and place blue bag in clear zip lock bag
3. Stool donor to produce stool at CSIRO or deliver to CSIRO laboratory in Esky within 1 hour of defecation
4. 4-6 stool donors will be asked to provide stool on each collection day

10.2 Stool processing

10.2.1 Donor stool processing

Setting up

Ensure anaerobic chamber is primed with gas and is anaerobic.
See instructions on setting up anaerobic chamber

Set up

Blender case as well as spatulas, glass beaker and glass measuring cylinder to be autoclaved within 24 hours of commencing stool processing (ideally the night prior)

1. Weigh stool (Empty clear and blue bag weight = 47g)
2. Saline (mls) = 2.6 x total stool weight (g)
3. Glycerol (mls) = 0.4 x total stool weight (g)
4. Sterile 200ml yellow pots (number) = total stool weight/ 50 (rounded up)
5. Transfer these minimum amounts into the anaerobic chamber

Equipment

Blender (cylinder and base)
Stainless steel spatulas (autoclaved)
Glass beaker (autoclaved)
Glass measuring cylinder (autoclaved)

8x Eppendorf tubes labelled

- Donor number
- Date
- Tube number
- F= fresh. G= Glycerol

Note pad, pen and scissors

Scientific weigh scales

Prior to blending

1. Add 0.25g of stool to each of 6 labelled capped Eppendorf tubes
2. Add 5g of stool to 2x larger brown pots
3. Record weight of stool in note pad

Blending process

1. Stool from four donors will be pooled and blended with normal saline and sterile pharmaceutical grade glycerol (in the ratio 25% stool, 65% saline, 10% glycerol).
    - The number of donors to be pooled will be limited to four so as to reduce the risk of transmissible disease from a single donor.
2. Blend on low power for 20 seconds and then high power for a further 20 seconds.
3. Aliquot the stool suspension into the sterile yellow pots (Colonoscopy -200mls or Enema- 100mls) and label with batch number and date
    - Each batch consists of 1x 200ml pot and 2x 100ml pot.
    - Each recipient will receive the same batch (same blend of donor stool from single day donation) for each of their three faecal transplants.
    - Multiple such batches can be produced from each donor stool blend.
4. Half fill a further 2 Eppendorf tubes with blended stool mix
5. Transfer the stool suspensions and tubes directly into -80 degree freezer

10.2.2 Documentation and tracing of donors

1) Each stool donor will be recorded in the secure and confidential study "stool donor register" document. This will include
   a) donors name
   b) date of birth
   c) address and contact details
   d) result of screening history, physical examination and blood and stool tests.
2) Each stool donor will be assigned a donor number.
3) Each stool aliquot will be numbered and recorded in the secure and confidential faecal transplant aliquot document that will list the four stool donors who contributed to each aliquot. In this way any possible transmission of infection could be traced.
4) A small amount of each individual donation will be set aside and frozen individually. This will allow repeat testing and tracing of each individual donation in the future in the event of possible transmission of infection.

10.2.3 UC patient stool processing

1) Each subject potentially suitable for the study, will also be asked to donate a stool sample of their own.
2) A small portion of the stool will be set aside to undergo faecal associated microbiota analysis.
3) 50g of the remainder will be mixed with 20mls sterile pharmaceutical grade glycerol and 130mls of saline and placed into frozen storage at -80 degrees C. This stool will then be used to transplant those subjects randomized to receive "placebo" with their own stool. In this way the FT will remain blinded to both the subject and colonoscopist.

10.2.4 Cleaning equipment

Blender case, stainless steel implements and glassware should all be cleaned following stool processing in the order listed below.

1. Rinsed with water in the sink
2. Washed with detergent and water
3. Rinsed with water
4. Washed with enzymatic wash
5. Rinsed with water
6. Autoclaved

11. ANALYSIS AND REPORTING OF RESULTS

All of the outlined techniques are well established and have been used in previous studies

Analysis of stool microbiota and microbiota metabolites will mainly be conducted at CSIRO Animal, Food and Health research laboratories in Adelaide under the guidance of Dr. Michael Conlon. Some analyses may be outsourced to other laboratories, but under the broad direction of Dr. Conlon in consultation with Dr Costello and other collaborators. The abundance and/or activities of faecal and mucosal (biopsy)-associated microbes will be analysed using molecular methods. This is expected to include the use of QPCR for a range of bacterial targets but may also include deep sequencing of microbial DNA for an in-depth analysis of microbial population changes. Isolation (culture) of bacteria from stool samples may be considered to further understanding of metabolic changes occurring in bacteria of IBD patients when compared to healthy controls. Stool will be analysed for short chain fatty acids, ammonia, phenols, cresols and bile acids using a range of methods established at CSIRO where sufficient material is available. Other metabolites may also be measured.

Gut mucosal immunological analysis with be performed with Dr Patrick Hughes at the Nerve Gut Research Laboratory.

Blood sampling
A total of 60ml will be taken at each time point which will be used for further experiments outlined below:

Isolation of PBMC and LPMC cells

Peripheral Blood Mononuclear Cells (PBMC) are isolated from whole blood via density gradient centrifugation. Lamina Propria Mononuclear Cells (LPMC) are isolated from colonic biopsies via collagenase digestion and density gradient centrifugation. Cells will be stored under liquid nitrogen until further analysed.

PBMC cells and biopsy tissue will be used for Flow cytometry and cell sorting.

PBMC and LPMC are surface stained using monoclonal antibodies against specific immune cell subsets (e.g. T memory cells CD45(RO), T helper cells (CD4), cytoxic T-cells (CD8), B-cells (CD19), 6B11 (natural killer cells), monocytes (CD14) and the integrins **a**4, **b**7 and CCR9). PBMC and LPMC will be surface stained, permeabilised and stained with anti-cytokine or opioid antibodies to detect intracellular cytokine content / opioid content (e.g TNF-**a**, IL-1**b**, **b**-endorphin), and also transcription factor content (e.g. FOXP3).

12. Statistical analysis

Patient information will be de-identified and the results of microbiota, immune analysis as well as clinical scores will be recorded in an excel spread sheet. This data will then be imported into the R program for statistical analysis. Statistical analysis will be conducted in collaboration with the University of Adelaide department of statistics.

12.1 Primary outcome power analysis

The study is powered to detect a significant difference in the primary outcome of inducing remission at 8 weeks post FT with 32 patients in each arm. This was calculated using a Z test with pooled variance for the difference of two independent proportions. The significance level was set at 5% and the power at 80%. The estimated remission rate in the placebo group was 26.4% and the minimally clinically relevant remission rate we are powered to detect is 60%.

Comparisons between treatment groups of the primary and secondary dichotomous outcomes will be assessed using Fisher's exact tests with an intention to treat analysis.

The placebo remission rate is difficult to predict based on the heterogeneous nature of previous studies that investigated induction of remission in UC. Our placebo remission rate was derived from the active ulcerative colitis trials 1 and 2[45] (ACT-1 and ACT- 2). The ACT-1 and 2 trials were randomized, double-blind, placebo controlled studies that evaluated the efficacy of IV infliximab 5- or 10-mg/kg IV infusion for induction and maintenance treatment in adults with UC. The clinical response rate in those patients in the ACT -2 trial who were not steroid dependent was 26.4%. These patients had moderate to severe colitis with a MAYO score of 6 to 12 on enrolment and so had more severe disease on average than our patients. Response was defined as at least a 3 point reduction and 30% reduction in the MAYO score to determine clinical response at week 8. Another trial of patients with mild to moderate ulcerative colitis[46] found a remission rate at 8 weeks with oral mesalamine 2.4g daily of 22%. Many of our patients will be taking an oral aminosalicilate compound and some a concomitant steroid. The remission rate in this case would be expected to be higher than 22%.

12.2. Safety

The analysis of serious adverse effect at week 8 will be by Fischer's exact test. Assessment of treatment on the change in serum creatinine, ALT, ALP, bilirubin and haemoglobin will be assessed using linear mixed effects regression with week 8 values as the outcome. Adverse effects at 1 year will be recorded, however there will not be a comparator group.

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Final protocol

TITLE: Faecal microbiota transplantation (FMT) for the treatment of active ulcerative colitis (UC)

INVESTIGATORS DETAILS AND QUALIFICATIONS

Dr Samuel Costello (MBBS, FRACP)
Gastroenterologist
Queen Elizabeth Hospital
Woodville Road
Woodville
South Australia 5011
Email: sam.costello@health.sa.gov.au

Dr Michael Conlon (PhD)
Senior Research Scientist
CSIRO Animal, Food and Health Sciences
Kintore Avenue, Adelaide SA 5000
Email: Michael.Conlon@csiro.au

Dr Patrick Hughes, BSc(Hons)(Pharmacology), PhD
NHMRC Biomedical Research Fellow
Nerve-Gut Research Lab,
South Australian Health and Medical Research Institute
North Tce, Adelaide SA 5000
Email: patrick.hughes@adelaide.edu.au

Professor Ian Roberts Thomson (MD, FRACP)
Head of Gastroenterology
Queen Elizabeth Hospital
Woodville Road
Woodville SA 5011
Email: ian.roberts-thomson@health.sa.gov.au

Associate Professor Jane Andrews (MBBS, FRACP, PhD)
Head IBD Service and Education
Department of Gastroenterology & Hepatology & School of Medicine
Royal Adelaide Hospital
North Tce, Adelaide
South Australia. 5000
Email: jane.andrews@health.sa.gov.au

Dr Oliver Waters (MBBS, FRACP)
Gastroenterologist
Fiona Stanley Hospital
Murdoch WA 6150
Email: Oliver.Waters2@health.wa.gov.au

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1. OBJECTIVES OF STUDY

Primary

- To determine whether faecal microbiota transplantation (FMT) improves clinical and inflammatory outcomes in patients with active ulcerative colitis (UC)**.**

Secondary

- To determine whether any clinical change is accompanied by an alteration in the faecal and/or mucosal associated microbiome of ulcerative colitis patients prior to and following FMT.
- Assessment of alteration and durability of change in the recipient microbiota after FMT in ulcerative colitis patients.
- To examine the mucosal immune changes induced by FMT and to examine whether they are influenced by changes in microbiome and/or disease activity
- To examine the durability of clinical response/improvement after initial response to FMT in subjects with active UC.
- To establish patient satisfaction with FMT as a therapy for UC.

2. BACKGROUND AND SIGNIFICANCE

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) that is characterized by recurring episodes of inflammation primarily involving the mucosal layer and occasionally the submucosa of the colon. Inflammation usually originates in the rectum and progresses in a contiguous fashion proximally. Although the aetiology of UC remains unclear, several factors are believed to play a role in its development and progression, including host genotype, immune disequilibrium, and the composition of microbial communities resident in the gastrointestinal (GI) tract.

There is strong evidence for the involvement of microbes in the development of UC. IBD is associated with changes in the diversity of the gut microbiota, and although alterations in the abundance of specific bacterial species have often been identified there remains no specific organism that is reliably associated with the condition[1]. There also appear to be changes in the functional activity of the microbiome, with changes in gene expression as well as protein production in the microbes of patients with IBD[1]. It is unclear whether the altered microbiota is a result of, or initiates the inflammatory process in humans. There is some evidence,

however, that an altered microbiota develops prior to the onset of colitis in an animal model of interleukin (IL)-10 knockout mice[2].

Intestinal flora and their metabolic products play a critical role in maintaining the health of the colon. Patients who undergo ileostomy and have subsequent diversion of the luminal contents from the colon often develop a "diversion" colitis. The distal colonocytes in this instance are deprived of short chain fatty acids such as butyrate, a product of anaerobic bacterial fermentation of undigested dietary carbohydrates[3]. Yet, animal models of IBD also require bacteria within the colon for inflammation to develop[4]. It has been observed that altering the bacterial and nutrient colonic milieu by diverting the faecal stream using ileostomy reduces the recurrence of Crohn's disease in the colon[5]. Further supporting the notion that bacterial antigens contribute to, or drive the autoimmune injury to the bowel, is evidence that antibiotics have some therapeutic efficacy in UC. In a recent systematic review, antibiotic therapy for UC was significantly associated with remission[6]. Antibiotic therapy gave a statistically significant relative risk reduction for active disease of 0.64 (CI= 0.43-0.96). So, whilst bacteria are necessary to develop IBD, with germ-free animals unable to be induced to develop IBD, bacterial absence (or change in abundance/mix) can also lead to inflammation.

Probiotics have as yet demonstrated only limited therapeutic efficacy in UC[7]. In vitro studies have demonstrated that probiotic bacteria are able to modulate gut immune cells[8,9]. Whilst, in vivo, in a German study, *E.coli* Nissle 1917 was equivalent in efficacy to mesalamine for maintenance of remission of UC[10]. Additionally, a randomized trial of 77 patients with UC found that VSL#3, a cocktail of 8 different bacteria, was more effective than placebo in improving symptoms and inducing remission at 12 weeks[11]. For patients with pouchitis, trials with VSL#3 have shown both therapeutic and prophylactic efficacy[12]. The outcomes in probiotic studies, however, have often been inconsistent and modest. This may be due to the variable actions of the different bacterial species that have been tested as well as the general limitations of most probiotic preparations. These probiotics provide a comparatively low number and diversity of bacterial species in comparison with the vast human gut microbiota. For this reason, some probiotic bacterial strains may not be able to compete effectively against the complex interactions of an established and adapted indigenous gut microbial community[13].

FMT has been described as "the ultimate probiotic" as it provides an entire microbiome to the recipient. This therapy delivers a much greater number and diversity of bacteria than any current commercially available preparation. FMT was first reported in humans by Eiseman *et al* in 1958 in the treatment of 4 patients with pseudomembranous colitis[14]. Three of the four patients were described as terminally or critically ill requiring vasopressor support and all were successfully cured. Over the subsequent years there have been case reports and case series describing FMT predominantly for *Clostridium difficile* colitis but also for treating IBD, irritable bowel syndrome and constipation[13,15,16]. In the past decade, there has been a heightened interest in the use of this therapy, predominantly driven by increasing rates of recurrent *C. difficile* infection. During this time *C. difficile* has become more frequent, more severe and more refractory to standard treatment as well as more likely to relapse[17]. Standard treatment with metronidazole or vancomycin alters the normal gut flora that would usually provide colonization resistance against *C. difficile* infection. For this reason, after successful initial therapy, up to 35% of patients will experience a symptomatic recurrence after ceasing antibiotics[18]. A subset of patients will have multiple recurrences and subsequent relapses occur in 45-65% of patients who have relapsed one or more times[19,20]. For patients with recurrent *C. difficile* colitis, FMT offers the greatest chance of cure of any therapy with success in 87-100% of cases[13, 21-25]. This impressive success rate is presumably due to the ability of the transplanted bacteria to recolonize/occupy the missing components/niches of the normal intestinal microbiota thus removing the microbial niche that *C. difficile* would otherwise exploit.

FMT for UC was first reported in the literature by a gastroenterologist Dr Justin D Bennet, from Kansas City, who described the results of a faecal transplant he received for his own disease[26]. Dr Bennet had continuous active, severe UC for 7 years, confirmed endoscopically and histologically that was refractory to standard therapy. Dr Bennet described receiving antibiotics to "sterilise" his bowel prior to retention stool enemas. At the time of publication in 1989 he had been symptom and medication free for the first time in 11 years, at 6 months post FMT.

Borody *et al* described case reports of six patients (3 men and 3 women aged 25–53 years) with UC for at least 5 years who were treated with FMT[16]. All patients had suffered severe, recurrent symptoms and UC had been confirmed on colonoscopy and histology. Faecal flora

donors were healthy adults who were extensively screened for parasites and bacterial pathogens. Patients were prepared with oral antibiotics and oral polyethylene glycol lavage. Faecal suspensions were administered as retention enemas and the process repeated daily for 5 days. By 1 week post FMT, some symptoms of UC had improved. Complete reversal of symptoms was achieved in all patients by 4 months post-FMT, by which time all other UC medications had been ceased. At 1 to 13 years post FMT, and without any UC medication, there was no clinical, colonoscopic, or histologic evidence of UC in any patient. The authors concluded that colonic infusion of donor human intestinal flora can reverse UC in selected patients and that these results support the concept of abnormal bowel flora or even a specific, albeit unidentified, bacterial pathogen causing UC. However, caution is needed when interpreting their data as this centre is known to have undertaken a large number of these treatments and it is uncertain why only 6 are reported. There is no comment in this paper as to the number of patients at their facility in whom this technique was attempted and if there were any patients in whom the treatment failed, moreover, this is open label treatment, which is now an insufficient standard of proof when evaluating novel therapies. Hence, randomized placebo controlled trials are needed to rigorously examine the efficacy of this proposed "alternative" therapy.

An anticipated concern in the medical community regarding FMT has been patient acceptance. This has been an assumption based on little evidence. To look at this question of patient willingness to undergo FMT, Kahn *et al*, performed a qualitative study to explore the attitudes and concerns of patients and parents of children with UC regarding FMT as a potential treatment[27]. They conducted six focus groups at a clinic in Chicago, Illinois and participants were asked about their perceptions of and interest in FMT as a treatment for UC. Sessions were recorded, transcribed, and reviewed to identify domains, themes, and major concepts. The focus groups included 15 adult patients and seven parents of children with colitis. The study identified five major domains pertaining to FMT; impressions of treatment, benefits, risks, potential mechanisms, and social concerns. All but one participant expressed interest in FMT and several wished it were already available. Participants compared FMT to probiotics, felt it was ''natural,'' easier than current therapies, and with donor screening would be safe. Although initial distaste and the ''yuck factor'' were uniformly mentioned, these concerns were outweighed by perceived benefits. The study concluded that given adequate supporting research, donor selection, and screening, adult patients and parents of children with UC will consider FMT and are eager for it to become available.

FMT for UC is currently undertaken at a private gastroenterology clinic in Sydney and case reports of success from this clinic are reported in the literature[16]. There is also evidence from UC online forums that patients are conducting FMT for UC outside of the healthcare setting[28,29]. This is occurring in an unregulated fashion, with only very limited evidence of efficacy from 7 case reports in the literature. These occurrences underline the need for more robust scientific evidence in this area and a randomised controlled study of efficacy.

2.1 Standard of care for ulcerative colitis

The management of UC involves both maintenance medication and medication used to control flares of the disease. The goal of maintenance therapy in UC is to maintain steroid-free remission, clinically and endoscopically. This requires regular clinical assessment including history, physical examination and at times colonoscopic examination. Other tools of assessment include blood (e.g. CRP, WCC) and stool (calprotectin) testing for inflammatory markers and imaging including MRI, CT or ultrasound.

The choice of maintenance treatment in UC is determined by disease extent, disease course (frequency of flares), failure of previous maintenance treatment, severity of the most recent flare, treatment used for inducing remission during the most recent flare, safety of maintenance treatment, and cancer prevention. The mainstay of maintenance medication are the 5-aminosalicylic acid compounds (5-ASA) such as mesalazine or sulphasalazine[30,31]. These compounds are commonly taken orally in formulations that predominantly deliver the active 5-ASA component to the colon. Alternatively, or in addition, mesalazine preparations can be delivered topically via enema or suppository if the disease only involves the left side of the colon (although it is only PBS funded for topical therapy during a flare and not for maintenance of remission – even though it also works in this setting). The majority of patients can be managed with maintenance 5-ASA compounds most of the time. For patients who have repeated flares of disease on 5-ASA maintenance therapy (1 or more flares in a year needing steroids), thiopurine medication such as azathioprine or 6-mercapropurine should be used[32]. These medications induce systemic immunosuppression, reduce the incidence and severity of flares of colitis but also slightly increase the risk of some infections and malignancy. Anti TNF agents such as infliximab or adalimumab have been shown to have benefit in maintaining remission in UC[33] (and are licensed for this indication by the

TGA), however these agents are very expensive. The anti TNF agents also give an increased risk of infection, particularly latent TB reactivation.

Mild flares of UC can be managed with higher doses of oral 5-ASA compounds or the addition of topical 5-ASAs given via enema or suppository. More severe flares are usually managed with a course of systemic corticosteroid. These can be given intravenously in acute, severe disease or orally in less severe flares. The steroids should then be tapered over time and discontinued. There is no indication for long term steroid use in UC and prolonged steroid use is associated with a number of complications including infection, osteoporosis, obesity, diabetes, poor wound healing, thinning skin, mood changes and insomnia. Severe flares of UC not responsive to steroids may respond to rescue therapy with the addition of either cyclosporin or anti-TNF therapy.

Patients in whom colonic inflammation cannot be controlled adequately frequently undergo total colectomy. This may be done electively (for refractory disease) or emergently in acute fulminant colitis. Colectomy entails surgical risk that is higher in the emergent setting; this risk includes infection, wound breakdown and a mortality rate. Colectomy is considered "curative" for UC especially if they have an ileostomy stoma created, however, it frequently also leads to complications both short- and long-term. In addition, in patients in whom an ileal-anal pouch is fashioned up to 50% will subsequently develop pouchitis at 4 years post surgery[34].

3. SPECIFIC SAFETY CONSIDERATIONS

A recent review article assessed all cases of FMT in the literature prior to 2011[13]. A total of 239 patients had undergone FMT. The authors did not find any serious adverse events related to the procedure. Some studies reported patient deaths due to the underlying disease, where the patient has not responded to the FMT. In one study, in which donor faeces were instilled via a nasogastric tube, a patient died of peritonitis. This patient was undergoing peritoneal dialysis for end stage renal failure at the time and was septic with severe *C. difficile* colitis. Her condition remained unchanged immediately post transplantation, however on the third day she developed peritonitis. Although considered more likely the result of peritoneal dialysis, the nasogastric tube insertion could not be discounted to have been contributory[35].

One patient in a study by Silverman *et al* developed irritable bowel symptoms following FMT[25].

Following this literature review in 2011 there have been 4 further cohort studies in the literature of patients who have undergone faecal transplant for *C. difficile* colitis[21,22,36,37]. A total of 216 patients who underwent FMT via colonoscopy were included in these 4 studies with no immediate adverse effects from FMT noted.

There is a potential to transmit infection via contaminated donor stool. The donor stool will therefore undergo microscopy and culture for potential bacterial pathogens, microscopy for ova, cysts and parasites as well as viral studies and *C. difficile* toxin analysis.
Blood testing to exclude HIV, Hepatitis B and C and syphilis will be undertaken.

Changes in faecal microbiota have been found in patients with a number gastrointestinal and extra-intestinal diseases. Changes in the microbiome of patients with IBD and irritable bowel syndrome are well documented in the literature. There have also been associations between various bowel flora and obesity and the metabolic syndrome[38]. The association has not been documented as causal and it appears probably related to the diet consumed by these subjects. It would, however, be prudent to exclude donors with the metabolic syndrome from the study.

In an audit of 16,318 colonoscopies performed in Northern California from 1994 to 2002, Levin *et al.,* found serious complications occurred in 5.0 of 1000 proceedures[39]. The major risk of colonoscopy, bowel perforation, occurred in 0.09% of colonoscopies in this study. Other risks include dehydration from bowel preparation, over-sedation, aspiration, bleeding and splenic laceration. This patient group will however be undergoing regular colonoscopies for their UC and will be familiar with these risks. Risks from standard therapies they would be offered for active disease are also substantial (steroids, immunomodulators, colectomy), thus risks from colonoscopy for FMT are relative.

4. ETHICAL CONSIDERATIONS

UC is a chronic, debilitating disease with a near normal life expectancy[40]. Current therapies are inadequate and the disease continues to have an unacceptably high rate of chronic

relapsing symptoms. This is underlined by evidence that up to 30 percent of patients will require colectomy after 25 years of disease[41]. For this reason, it is important for the medical community to rigorously examine potential new therapies that may benefit this group of patients.

A small number of case reports of successful treatment of UC with FMT have been reported in the literature[16,26]. However, the findings of these case reports have never been tested in a randomised controlled trial. Despite this very limited evidence there is a clinic in Australia offering UC patients FMT as a therapy[16]. There is also evidence from online forums that patients are undertaking this therapy without medical supervision[28,29]. Despite the minimal evidence in the literature, there is a willingness among sufferers of UC to try this potential therapy[27]. We believe a randomised control trial in this area is necessary to gather evidence for or against the effectiveness of FMT as a treatment for UC. A positive result would avail UC sufferers of a new therapy and a negative one would help discourage the use of an unproven, invasive therapy. Stool analysis of faecal transplant success may also fast track development of tailored probiotic medicines.

Donors will be anonymous and so will not be known to the recipient. This avoids any apportion of blame towards a known donor should a complication or treatment failure arise during the trial.

Colonoscopy will be used to deliver the initial stool transplantation and to assess the colon during follow up. This is an invasive procedure that carries some risk. Most of the recent studies of FMT for *C. difficile* have used colonoscopic delivery[13,22,23] as it allows assessment of the underlying disease and allows the donor bacteria to contact the entire colon. Patients with symptomatic UC would ordinarily undergo examination with colonoscopy as part of the assessment of disease activity to help guide treatment. The initial colonoscopy in this trial will therefore not be an additional procedure. However, the colonoscopy at week 8 and at 12 months may be additional procedures depending on the state of the patient's disease and symptoms.

The colonoscopic examinations will involve biopsy of the mucosa for analysis of microbiota as well as immune function and histopathology. The majority of these biopsies will be additional to that which the patient would have ordinarily received outside of the trial. These

biopsies will be critical to detect any changes in the mucosal associated microbiota or immune changes associated with the FMT. The risk of biopsy of the mucosa is small with the major risk being bleeding. Biopsies can be safely performed on a single anti-platelet agent[42]. Patients on duel antiplatelet therapy or anticoagulant medication (i.e. warfarin or heparin) will be excluded from the study.

As FMT has only been performed in large numbers in the past decade, there may be unknown long term risks. However, there have been no reports of major complication of faecal transplant in the literature to date.

Taking blood may cause short-term pain or discomfort and patients will be informed about this before entering the trial. The volume of blood taken is not extreme and will not cause side effects. If patients are of the view that blood sampling is too painful they may withdraw from the study at any time. Blood tests as well as answering questionnaires will involve an increased time burden and patients will be informed about this before the trial begins. It is not anticipated that the FMT procedure will cause any adverse reactions, but participants will be provided with information about supports they can contact should they experience any distress in relation to the study.

Before taking part in the study, informed written consent will be obtained from patients. The researchers will ensure that the patient is given full and adequate verbal and written information about the nature, purpose, possible risk and benefit of the trial. They will be given sufficient time to consider the information, to ask questions and to seek advice prior to being asked whether they wish to participate in the study. Participants will also be assured their participation in the trial is absolutely voluntary. All treatment decisions are at the discretion of the usual treating physician, and will not be altered by the trial. The participation is strictly confidential, and the identity of subjects will not be disclosed to other medical or research staff unless subjects agree.

Once subjects have been enrolled in this study, they will be given a study participant code, and only study investigators will have access to their name and personal details. We intend to summarise the results in a manuscript and to submit it for publication in a peer reviewed journal. Therefore, all information gathering from this study will be published in a form that does not allow patient identification. We will not provide any feedback with regard to

individual microbiota composition or immunologic function.

Our proposed study has the support of the Director of the Royal Adelaide Hospital department of Gastroenterology & Hepatology, Richard Holloway, as well as the head of Endoscopic Services, Mark Schoeman. The Head of IBD Service, Jane Andrews, will be the lead supervisor of the study. The Royal Adelaide Hospital has a large cohort of approximately 800 patients with IBD and a strong record of successful clinical research. The proposed study also has the support of the head of gastroenterology at the Queen Elizabeth Hospital, Ian Roberts-Thomson. The Queen Elizabeth Hospital has a cohort of approximately 300 patients with IBD. The study supervisors all have extensive experience in medical research as well as experience in supervising PhD students.

The Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Adelaide have broad experience in human gut flora and microbial analysis. This is an area of focus for their recent research. The Nerve Gut Research Laboratory at the Royal Adelaide Hospital is a leader in the field of research into the neuro-immunological and neuro-endocrine processes of the human gut.

5. STUDY DESIGN

This study is an 8 week randomised placebo controlled trial with a 44 week open labelled extension.

Randomisation:

- Group 1:
    - Patients receives previously frozen pooled **donor** stool via colonoscopic insertion into right colon
- Group 2
    - Patients receives previously frozen **own** stool via colonoscopic insertion into the right colon

Open label therapy from 8 weeks

Patients who are randomly assigned to the placebo group by week 8 will then be offered active donor FMT at the 8 week colonoscopy. The FMT will be conducted in an identical manner to Group 1 with FMT followed by 2 enemas on day 3 or 4 and one on day 6 or 7.

5.1 Recruitment

Patients will be recruited from IBD clinics at the Royal Adelaide and Queen Elizabeth Hospitals. Patients on the mailing list for the Royal Adelaide Hospital will be contacted about the trial through the quarterly newsletter. Gastroenterologists in Adelaide will be informed about the trial though a presentation at the South Australian gut club and an email to the South Australian gut club members. The trial will be listed on the Australian and New Zealand clinical trials registry as well as the Gastroenterology Society of Australia website.

We will enrol 70 patients with 35 patients in each arm of the trial.

5.2 Inclusion criteria:

1. *Mild to moderate active UC (Total Mayo score 3 to 10)*
2. *Endoscopic subscore of 2 or greater (to ensure symptoms are due to UC (not post-inflammatory irritable bowel syndrome)*
3. *Patients aged 18 to 75 years with established diagnosis of UC*

5.3 Exclusion criteria:

1. *Severe UC (Mayo score 11-12 or Truelove and Witts criteria)*
2. *More than 25mg of prednisolone per day (or equivalent steroid)*
3. *Previous colonic surgery*
4. *Active gastrointestinal infection*
5. *Pregnancy*
6. *Anticoagulant therapy or duel antiplatelet therapy (i.e. aspirin and clopidogrel)*
7. *Current use of antibiotics*

Activity of disease will be defined by the Mayo score. This scoring system has 3 points each for stool frequency, rectal bleeding, endoscopic findings and physician's global assessment giving a total score out of 12.

A score of

- 0, 1 or 2 would indicate inactive disease and exclusion from the trial.
- 3 to 10 would allow inclusion into the trial. Subjects would need an endoscopic sub-score of at least 2 for inclusion, to prove active disease. (0= normal mucosa and 1= erythema only – most studies start with 2)
- 11-12 would indicate severe disease and these patients would be excluded from the trial.

Similarly, any patient who fulfilled Truelove and Witts criteria for severe colitis would be excluded while they met this criteria. Truelove and Witts is defined as >6 bloody bowel motions per day plus one or more of the following: Haemoglobin <10.5g/dL, ESR>30mm/hr, Pulse rate >90 beats per minute or Temperature over 37.5 degrees Celsius.

5.4 Medication prior to enrolment

Stable dosing of UC maintenance therapy is required prior to enrolment.

1. 5-aminosalicylic acid (5-ASA) stable doing for at least 4 weeks
2. Thiopurines and methotrexate stable dosing for at least 6 weeks
3. Biological agents stable dosing for at least 8 weeks
4. Patients can enrol on an oral dose of prednisolone ≤25mg, with a mandatory taper of 5mg per week.

6. OUTCOME MEASURES

6.1 Primary outcome

Steroid-free remission of UC at week 8 defined as

1. Total Mayo score of ≤ 2

   AND

2. Mayo endoscopic score of ≤ 1

6.2 Secondary outcomes

1. Clinical response (≥3 point reduction in total Mayo score at week 8 and 1 year)
2. Clinical remission (Simple clinical colitis activity index (SCCAI) ≤ 2 at week 8 and 1 year)[43]
3. Endoscopic remission (Mayo < 1 at week 8 and 1 year)
4. Safety (assessed at week 8 and 1 year)
5. Changes in mucosal and faecal associated microbiota following FMT assessed by 16s ribosomal RNA sequencing, stratified by:
   a. Change in total Mayo score following FMT
   b. Randomisation
6. Durability of engraftment of donor microbiome following FMT
7. Changes in peripheral blood and colonic lamina propria mononuclear cell populations (assessed by FACS) following FMT
8. Patient perception and palatability

Disease activity measures of symptoms score (SCCAI), endoscopic and histologic grading as well as records of hospitalization, corticosteroid requirement, periods of symptom flares and colectomy rate will be recorded at the 1 year mark as part of the open label observation period from 8 weeks to 1 year.

7. PATIENT PARTICIPATION

7.1 Recruitment

Participants will be recruited from:

Royal Adelaide Hospital (RAH), The Queen Elizabeth Hospital (TQEH) and Fiona Stanley Hosptial (FSH) Gastroenterology IBD databases and newsletters

Gastroenterology in- or out-patient encounter(s) at the RAH, TQEH and FSH by referral from their clinicians and by searching OPD letters.

Patients on clinical databases who have previously consented to being contacted regarding research studies will receive information about the study in the RAH IBD Service regular newsletter and may also be contacted by telephone, and if no answer is obtained a letter will be sent. All other patients will be contacted via a letter or by their treating clinician in whichever way the clinician feels is most appropriate to the particular patient.

Regarding the use of letters for contact: Subjects who have already consented to be contacted regarding research (on database) will receive a letter signed by A/Prof Andrews (at RAH); Prof. Roberts Thomson (at TQEH) or Dr Waters (at FSH) on behalf of the study investigators.

The initial invitation letter will include an opt-out slip for subjects not wishing to be contacted further. Subjects not opting out or responding within 4 weeks after invitation will be contacted up to a further 3 times by 2 different methods (phone, SMS, email or letter) to ascertain whether they wish to participate or not. Demographic details of non-responders will be recorded to enable a full description of the sources of possible bias. All who agree to participate will be subsequently screened to ensure they fulfill inclusion criteria.

Donors will be recruited with a flier advertisement on notice boards at Adelaide University Medical School and Adelaide University campus.

7.2 Withdrawal criteria

Patients may withdraw from the study at any time. We will ask for their reasons for statistical purposes, however they will not be obliged to provide this. Withdrawal from the study will not affect ongoing standard medical care in any way. Their clinicians will be informed of their participation in the study. We will ask patients to notify us of any changes in their treatment during the course of the study and if necessary, we will seek their permission to verify this with their treating clinician.

8. ULCERATIVE COLITIS PATIENT ASSESSMENT

8.1 The week prior to enrollment

1. The patient should have the opportunity to read the patient information sheet, discuss the trial with family or friends and ask questions of the investigators prior to signing trial consent.
2. Patient questionnaire regarding perception and expectation of faecal transplant prior to procedure
3. Detailed history of UC
    a) Date of diagnosis
    b) Extent of disease
    c) Medication use- current and prior
    d) Previous Surgery
    e) Previous Hospitalisation
    f) Comorbid disease
    g) Current symptoms
    h) Extra-articular manifestations

4. Stool collected for
    a) Infection screen. Microscopy Culture + Sensitivity, *Clostridium difficile* toxin (5g)
    b) Possible re-administration for placebo arm subjects. (50g)
    c) Microbiome analysis. 6 x 0.25g stool in Eppendorf tubes; 2x 5g stool in larger brown stool pots

For collection and processing methods see section 12 (pages 32-33)

5. Disease activity assessment
    a. Faecal calprotectin
    b. CRP, ESR, FBC, U+E, LFTs
    c. Symptom severity (SCCAI) at screening and one day prior to FMT
    d. Flexible sigmoidoscopy
        i. Total Mayo score.

ii. Disease extent (≥10cm of disease required)
iii. Biopsy for light microscopy and histopathology to exclude CMV inclusions

8.2 Randomisation

Randomisation will be conducted once the patient satisfies the inclusion and exclusion criteria and has consented to enter the study. This should occur within 1-7 days prior to the first faecal transplant that will be delivered via colonoscopy.

Prior to randomisation 3 aliquots of pooled donor stool suspension from a single batch and 3 aliquots of the donor's own stool suspension will each be placed in a clear plastic bags in the -80°C freezer at the endoscopy unit. All stool aliquots will be in identical, yellow topped 250ml cryo-safe containers. These will include 1x 200ml suspension for colonoscopic delivery and 2x 100ml suspensions for enema delivery.

Donor stool pots will be labelled on the lid with:

1. Batch number
2. Date of manufacture of batch

Patient's own stool will be labelled on the lid with:

1. Patient ID consisting of initials and study number ie (AB-1)
2. Date of patient stool donation

Randomisation to be conducted by hospital clinical trial nursing staff using www.random.org

1. Cardboard circular caps with Patient ID and either "Transplant" or "Save" and either "Colon" or "Enema" are then placed on the pots containing donor or patients own stool depending on randomisation.
2. "Transplant" caps are placed on the pots to be given at and in the week following the first colonoscopy.

3. “Save” pots will be saved and the cap removed following the 8 week colonoscopy. If these contain donor stool then they will be given to the patient at the 8 week colonoscopy and in the subsequent week.
4. “Colon” pots will be delivered at colonoscopy and the “Enema” pots then delivered via enema in the following week.
5. The randomisation document for the use by study nursing staff is listed on page 22.

Faecal transplant for active ulcerative colitis trial

Protocol for randomisation of FMT

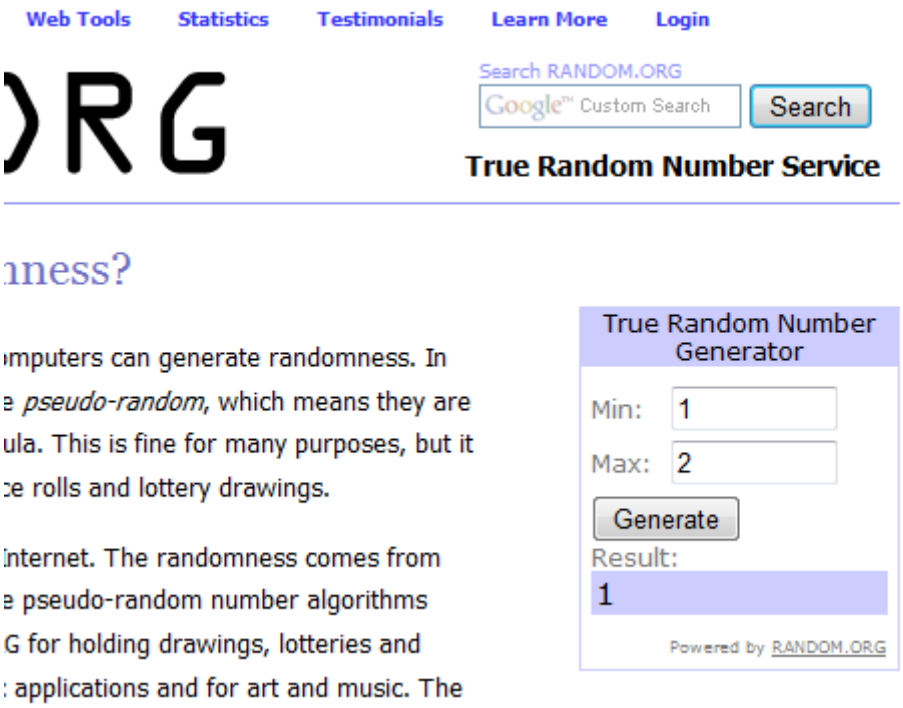
1-7 days prior to faecal transplant use the random number generator http://www.random.org/

Into the "true random number generator" box on the right of the screen set the minimum to 1 and maximum to 2

Select: Generate

1 = Donor faecal transplant

2= Placebo faecal transplant (patient's own stool)



Go to the -80°C Freezer.

1. For Donor Faecal transplant stick the "Transplant" disks on the top of the donor stool yellow pots and the "Save" disks on the placebo pots. Record the batch number in the transplant record book.
2. For Placebo Faecal transplant stick the "Save" disks on the donor stool yellow pots and the "Save" disks on the donor pots. The donor stool will then be saved to transplant at 8 weeks at the open label cross over.

The **donor stool** pots are labelled with a Batch number and date. (ie Batch 3, 2/7/13)

The **patient's own stool** (placebo) is labelled with patient study number

8.3 Week 1 of trial

8.3.1 Day prior to Colonoscopy

1. Patient to take a light breakfast and then to fast from solids
2. Maintain high fluid intake throughout the day
3. Take 3 sachets of Colonlyetly bowel preparation (polyethylene glycol) in 3 L of water

8.3.2 Morning of Colonoscopy

1. Randomised faecal aliquot labeled “Transplant” and “Colon” to be removed from the -80°C freezer and thawed at room temperature for 3.5 hours prior to delivery
2. Patient to receive Loperamide 2mg orally prior to colonoscopy
3. SCCAI score diary to be collected
4. Mayo symptom scores to be taken (endoscopic score to be added at colonoscopy)
5. Biopsy posts should be pre-labelled with the site and number of biopsies required
6. Consent should be obtained for this procedure on a standard CALHN consent form in addition to the study consent form that has previously been signed.
7. While inserting cannula, take 60mls of blood
    a. 50 mls into 6x heparin tubes (green and black top) for Peripheral Blood Mononuclear Cell flow cytometry (to be taken to Dr Hughes at nerve gut laboratory).
    b. 5mls into a small EDTA (purple tube) (to be sent to clinical laboratory)
    c. 5mls into a GEL (white top) Electrolytes and liver function, C-reactive protein (to be sent to SA pathology laboratory)

8.3.3 At Colonoscopy

1. Assess and disease severity (using endoscopic Mayo score at point of maximum inflammation) and disease extent.
2. Biopsies should be taken on colonoscope insertion*

    a. Left sided biopsies- 11 total; 6 in RPMI media, 2 into RNA later (microbiome), 1 RNA later (PCR), 1 formalin (histopath), 1 PFA (IHC)
    b. Right sided biopsies- 9 biopsies; 6 in RPMI media, 2 into RNA later (microbiome), 1 formalin (histopath).
3. An attempt should be made to remove any residual fluid or faecal material during colonoscope insertion with suction and washing if required.
4. Once at caecum patient should be rolled onto the right lateral position and randomized faecal suspension delivered into the right colon. If caecum cannot be reached then delivery of faecal suspension into the right colon beyond the hepatic flexure is acceptable.
5. Patient should then remain on their right side for 1 hour following procedure.
6. Following 1 hour the patient should be assessed for any adverse effects and if well sat up and offered food and drink prior to discharge.

*Biopsies- At each colonoscopy in more detail

- 2 biopsies will be taken from both the recto-sigmoid region and ascending colon-caecal region of the colon for microbiota analysis. Each in 2.5ml RNA later.
- 1 biopsy from each region will be taken for histopathology analysis into formalin.
- 1 biopsy from left colon for immunohistochemistry (formalin) and
- 1 from the left colon for PCR (cytokines, transcription factors)- (RNA later)
- 4 biopsies will be taken from the left and 4 from the right colon for flow cytometry (FACS) analysis. (RPMI complete media). Processed the same day as colonoscopy.
- 2 biopsies from the left colon and 2 from the right for supernatant release for cytokines / mast cell mediators. (RPMI complete media)
- This would amount to 9 biopsies in the right colon and 11 in the left colon.

8.3.4 Enemas

Two enemas of 100ml faecal suspension will be delivered by a Gastroenterologist at the clinic in the week following colonoscopy Days (2-4) Days (5-7).

Patient should:

1. Take 2mg of loperamide prior to enema
2. Lay on left lateral position for enema insertion.
3. Roll from the left lateral to prone position then right lateral and then back to left lateral position following enema insertion. This is to encourage proximal distribution of the enema.
4. Attempt to hold the enema for 1 hour

8.4 Week 4 assessment

1. Stool collection for faecal calprotectin level and microbiome analysis
2. Stool to be collected in sterile blue bags that are placed over the toilet.
3. Patient to zip tie blue bag closed and place blue bag in clear zip lock bag
4. Deliver to CSIRO laboratory within 1 hour
5. Bag to be opened and stool processed under anaerobic conditions in anaerobic chamber
    a) Microbiome analysis. 6 x 0.25g stool in Eppendorf tubes; 2x 5g stool in larger brown stool pots

8.5 Week 8 assessment

8.5.1 Two days prior to Colonoscopy

1. Stool collection for faecal calprotectin level and microbiome analysis
2. Stool to be collected in sterile blue bags that are placed over the toilet.
3. Patient to zip tie blue bag closed and place blue bag in clear zip lock bag
4. Deliver to CSIRO laboratory within 1 hour
5. Bag to be opened and stool processed under anaerobic conditions in anaerobic chamber
    a) Microbiome analysis. 6 x 0.25g stool in Eppendorf tubes; 2x 5g stool in larger brown stool pots

8.5.2 One day prior to Colonoscopy

1. Patient to take a light breakfast and then to fast from solids
2. Maintain high fluid intake throughout the day
3. Take 3 sachets of Colonlyetly bowel preparation (polyethylene glycol) in 3 L of water

8.5.3 Morning of Colonoscopy

1) Randomised faecal aliquot labeled “Save” and “Colon” to be removed from the -80°C freezer and thawed at room temperature for 3.5 hours prior to delivery
2) Patient to receive loperamide 2mg orally prior to colonoscopy
3) SCCAI score diary to be collected
4) Mayo symptom scores to be taken (endoscopic score to be added at colonoscopy)
5) Adverse events since randomization recorded
6) Biopsy posts should be pre-labelled with the site and number of biopsies required
7) Consent should be obtained for this procedure on a standard CALHN consent form in addition to the study consent form that has previously been signed.
8) While inserting cannula, take 60mls of blood
    a) 50 mls into 6x heparin tubes (green and black top) for Peripheral Blood Mononuclear Cell flow cytometry (to be taken to Dr Hughes at nerve gut laboratory).
    b) 5mls into a small EDTA (purple tube) (to be sent to clinical laboratory)
    c) 5mls into a GEL (white top) Electrolytes and liver function, C-reactive protein (to be sent to clinical laboratory)

8.5.4 At Colonoscopy

1) Assess disease severity using endoscopic Mayo score at point of maximum inflammation and disease extent.
2) Biopsies should be taken on colonoscope insertion*

a) Left sided biopsies- 11 total; 6 in RPMI media, 2 into RNA later (microbiome), 1 RNA later (PCR), 1 formalin (histopath), 1 PFA (IHC)
b) Right sided biopsies- 9 biopsies; 6 in RPMI media, 2 into RNA later (microbiome), 1 formalin (histopath)

3) An attempt should be made to remove any residual fluid or faecal material during colonoscope insertion with suction and washing if required.
4) Once at caecum (and disease severity has been assessed and recorded) the cardboard
5) "Save" cap should be removed from the pot to reveal the contents of the faecal pot.
   a) If this is labeled as the patient's own stool it should be discarded and the colonoscope withdrawn.
   b) If this is labeled as donor stool then the patient should be rolled onto the right lateral position and the un-blinded faecal suspension delivered into the right colon.
6) If caecum cannot be reached then delivery of faecal suspension into the right colon beyond the hepatic flexure is acceptable.
7) Patient should then remain on their right side for 1 hour following procedure.
8) Following 1 hour the patient should be assessed for any adverse effects offered food and drink and if prior to discharge.
9) Patient to be informed about randomization. If they were initially randomized to placebo/ autologous FMT then they will require 2 further donor FMTs via enema.

*Biopsies- At each colonoscopy in more detail

- 2 biopsies will be taken from both the recto-sigmoid region and ascending colon-caecal region of the colon for microbiota analysis. Each in 2.5ml RNA later.
- 1 biopsy from each region will be taken for histopathology analysis into formalin.
- 1 biopsy from left colon for immunohistochemistry (formalin) and
- 1 from the left colon for PCR (cytokines, transcription factors)- (RNA later)
- 4 biopsies will be taken from the left and 4 from the right colon for flow cytometry (FACS) analysis. (RPMI complete media). Processed the day of colonoscopy.
- 2 biopsies from the left colon and 2 from the right for supernatant release for cytokines / mast cell mediators. (RPMI complete media)
- This would amount to 9 biopsies in the right colon and 11 in the left colon.

8.5.5 Enemas (Patients randomized to placebo/ autologous FMT)

Two enemas of 100ml faecal suspension will be delivered by a medical practitioner at the clinic in the week following colonoscopy Days (2-4) Days (5-7).

1) Patient to take 2mg of loperamide prior to enema
2) Lay on left lateral position for enema insertion.
3) Roll into prone positions, right lateral and then back to left lateral position following enema insertion.
4) Patient should attempt to hold the enema for 1 hour

8.6 1 year assessment

Patient will be posted or emailed

1) SCCAI symptoms score
2) Patient questionnaire regarding experience of faecal transplant prior to procedure and adverse events
3) Invitation to undergo disease activity assessment

Patients who do not return forms within 2 weeks will be contacted via telephone

8.6.1 Two days prior to Flexible Sigmoidoscopy

1) Stool collection for faecal calprotectin level and microbiome analysis
    a) Stool to be collected in sterile blue bags that are placed over the toilet.
    b) Patient to zip tie blue bag closed and place blue bag in clear zip lock bag
    c) Deliver to CSIRO laboratory within 1 hour
    d) Bag to be opened and stool processed under anaerobic conditions in anaerobic chamber
        i) Microbiome analysis. 6 x 0.25g stool in Eppindorf tubes; 2x 5g stool in larger brown stool pots

ii) Faecal calprotectin

8.6.2 Flexible Sigmoidoscopy

1) Mayo symptom scores to be taken (endoscopic score to be added at colonoscopy)
2) Adverse events since randomization recorded
3) Biopsy posts should be pre-labelled with the site and number of biopsies required
4) Consent should be obtained for this procedure on a standard consent form in addition to the study consent form that has previously been signed.
5) While inserting cannula, take 60mls of blood
    a) 50 mls into 6x heparin tubes (green and black top) for Peripheral Blood Mononuclear Cell flow cytometry (to be taken to Dr Hughes at nerve gut laboratory).
    b) 5mls into a small EDTA (purple tube) (to be sent to clinical laboratory)
    c) 5mls into a GEL (white top) Electrolytes and liver function, C-reactive protein (to be sent to clinical laboratory)
6) Assess disease severity using endoscopic mayo score at point of maximum inflammation
7) Biopsies should be taken on the left side only
    a) Left sided biopsies- 11 total; 6 in RPMI media, 2 into RNA later (microbiome), 1 RNA later (PCR), 1 formalin (histopath), 1 PFA (IHC)
8) Following 1 hour the patient should be assessed for any adverse effects offered food and drink and if prior to discharge.

8.6.3 Care during the follow up period

During the trial subjects will be treated to the standard of care for UC. This involves a fixed maintenance medication as prescribed/advised by their own physician. Patients will enter this trial due to a flare, and all therapy they are on at entry will be continued except for the steroid taper as described.

A subject who experiences a flare of their disease during the study will be treated with standard therapy as they would if they were not in the study. This will include increasing

their oral 5-ASA and/or adding a topical enema or suppository therapy. Systemic steroid therapy may also be used. Steroid use will be quantified during the study and steroid requirement over the 12 month period will be another secondary end point. Once patients are commenced on steroid it will be tapered as explained above.

If a subject deteriorates on steroid therapy they may require escalation of their medical therapy or surgery. Escalation of medical therapy may involve increasing the steroid dose temporarily. Patients who are naive to thiopurine therapy may benefit from the addition of a thiopurine. Thiopurines can take up to 12 weeks to reach their therapeutic effect and so "rescue therapy" may be needed in the intervening period. Rescue therapy involves the addition of cyclosporine or an anti-TNF agent such as infliximab (if available through compassionate access) in the short term. Rescue therapy would be continued for 6 to 12 weeks to allow the thiopurine medication to reach its full effect.

Patients who have a severe flare of UC that does not respond to intravenous steroid medication within 3 to 5 days are unlikely to improve and should be assessed for surgical colectomy[44], as would be the case in routine care.

9. STOOL DONOR RECRUITMENT AND SCREENING

9.1 Donor recruitment

Posters will be placed on noticeboards on the University of Adelaide Campus. These will detail that we are recruiting stool donors and the posters will have the contact details of Dr Costello and Dr Andrews.

9.2 Donor screening

Potential donors would be sent the donor information sheet via email or post.

Donors who consent will undergo a four stage screening process with medical history, physical examination, blood testing and stool testing with the aim of reducing the risk of disease transmission from donor to recipient.

9.2.1 Medical History

Exclusion of patients who

- Age: <18 or >65
- Antimicrobial therapy or probiotics in the past 3 months
- Active medical illness or symptoms
- Any medications (other than oral contraceptive pill)
- International travel in last 6 months to areas at high risk of travellers' diarrhoea
- High risk sexual activity (unprotected sex in last 1 month outside of a monogamous relationship, men who have sex with men, sex for drugs or money)
- Illicit drug use
- Tattoo or body piercing within 6 months
- Known HIV or viral hepatitis exposure in the last 12 months
- Incarceration or a history of incarceration.
- Family history of colorectal carcinoma involving 2 or more first degree relatives
- Household members with active GI infection

Have no active medical problems or a history of

1. Inflammatory bowel disease
2. Irritable bowel syndrome
3. Colonic polyps
4. Bowel cancer
5. Any other gastrointestinal disorder
6. Obesity
7. High blood pressure
8. Diabetes
9. Heart disease
10. Stroke
11. Major depression
12. Infection with Hepatitis B or C, HIV or syphilis

13. Autoimmune disease (ie rheumatoid arthritis, SLE)

9.2.2 Physical Examination

Cardiovascular and gastrointestinal examination
Height and Weight. BMI <18 and >30 is an exclusion

9.2.3 Blood testing

1. Full blood count (Anaemia, WCC>12.5 are exclusions)
2. Electrolytes, Urea and Creatinine (renal impairment eGFR<60 is an exclusion)
3. Liver function tests (abnormal LFTs are exclusions)
4. Human T-cell lymphotropic virus 1 and 2 serology (positive serology is an exclusion)
5. Epstein Barr Virus IgM and IgG (positive IgM is exclusion)
6. Cytomegalovirus IgM and IgG (positive IgM is exclusion)
7. Syphilis (positive rapid plasma regain is an exclusion)
8. *Strongyloides stercoralis*, *Entamoeba histolytica* (positive serology is an exclusion)
9. Toxoplasma serology (positive serology is an exclusion)
10. Hepatitis A virus IgM (positive serology is an exclusion)
11. Hepatitis B PCR (positive PCR is an exclusion)
12. Hepatitis C PCR (positive PCR is an exclusion)
13. HIV PCR (positive PCR is an exclusion)
14. Fasting lipids and Blood sugar level (Total Cholesterol > 4.0 mmol/L, LDL >2.5 mmol/L, Triglycerides >2.0 mmol/L, HDL <1.0 mmol/L are exclusions)
15. C-Reactive Protein (>8 exclusion)
16. ANA (>1/160 is an exclusion)
17. Helicobacter serology (positive serology is an exclusion)

9.2.4 Stool testing

1. Microscopy and Culture
2. *Clostridium difficle* toxin PCR
3. Egg, cysts and parasites (including Cryptosporidium spp., Giardia spp., and *Entamoeba histolytica* PCR)

4. Rotavirus, Norovirus and Adenovirus PCR

10. STOOL COLLECTION AND PROCESSING

Once donors have passed all the screening requirements they are eligible to donate for 1 month. To donate stool beyond this time will require repeat screening.

10.1 Stool collection

1. Stool collected in sterile blue bags that are placed over the toilet.
2. Stool donor to zip tie blue bag closed and place blue bag in clear zip lock bag
3. Stool donor to produce stool at CSIRO or deliver to CSIRO laboratory in Esky within 1 hour of defecation
4. 4-6 stool donors will be asked to provide stool on each collection day

10.2 Stool processing

10.2.1 Donor stool processing

Setting up

Ensure anaerobic chamber is primed with gas and is anaerobic.
See instructions on setting up anaerobic chamber

Set up

Blender case as well as spatulas, glass beaker and glass measuring cylinder to be autoclaved within 24 hours of commencing stool processing (ideally the night prior).

1. Weigh stool (Empty clear and blue bag weight = 47g)
2. Saline (mls) = 2.6 x total stool weight (g)
3. Glycerol (mls) = 0.4 x total stool weight (g)
4. Sterile 200ml yellow pots (number) = total stool weight/ 50 (rounded up)

5. Transfer these minimum amounts into the anaerobic chamber

Equipment

Blender (cylinder and base)
Stainless steel spatulas (autoclaved)
Glass beaker (autoclaved)
Glass measuring cylinder (autoclaved)

8x Eppendorf tubes labelled

- Donor number
- Date
- Tube number
- F= fresh. G= Glycerol

Note pad, pen and scissors

Scientific weigh scales

Prior to blending

1. Add 0.25g of stool to each of 6 labelled capped Eppendorf tubes
2. Add 5g of stool to 2x larger brown pots
3. Record weight of stool in note pad

Blending process

1. Stool from four donors will be pooled and blended with normal saline and sterile pharmaceutical grade glycerol (in the ratio 25% stool, 65% saline, 10% glycerol).

- The number of donors to be pooled will be limited to four so as to reduce the risk of transmissible disease from a single donor.

2. Blend on low power for 20 seconds and then high power for a further 20 seconds.
3. Aliquot the stool suspension into the sterile yellow pots (Colonoscopy -200mls or Enema- 100mls) and label with batch number and date
    - Each batch consists of 1x 200ml pot and 2x 100ml pot.
    - Each recipient will receive the same batch (same blend of donor stool from single day donation) for each of their three faecal transplants.
    - Multiple such batches can be produced from each donor stool blend.
4. Half fill a further 2 Eppendorf tubes with blended stool mix
5. Transfer the stool suspensions and tubes directly into -80 degree freezer

10.2.2 Documentation and tracing of donors

1) Each stool donor will be recorded in the secure and confidential study "stool donor register" document. This will include
    a) donors name
    b) date of birth
    c) address and contact details
    d) result of screening history, physical examination and blood and stool tests.
2) Each stool donor will be assigned a donor number.
3) Each stool aliquot will be numbered and recorded in the secure and confidential faecal transplant aliquot document that will list the four stool donors who contributed to each aliquot. In this way any possible transmission of infection could be traced.
4) A small amount of each individual donation will be set aside and frozen individually. This will allow repeat testing and tracing of each individual donation in the future in the event of possible transmission of infection.

10.2.3 Ulcerative colitis patient stool processing

1) Each subject potentially suitable for the study, will also be asked to donate a stool sample of their own.

2) A small portion of the stool will be set aside to undergo faecal associated microbiota analysis.
3) 50g of the remainder will be mixed with 20mls sterile pharmaceutical grade glycerol and 130mls of saline and placed into frozen storage at -80 degrees C. This stool will then be used to transplant those subjects randomized to receive "placebo" with their own stool. In this way the FT will remain blinded to both the subject and colonoscopist.

10.2.4 Cleaning equipment

Blender case, stainless steel implements and glassware should all be cleaned following stool processing in the order listed below.

1. Rinsed with water in the sink
2. Washed with detergent and water
3. Rinsed with water
4. Washed with enzymatic wash
5. Rinsed with water
6. Autoclaved

11. ANALYSIS AND REPORTING OF RESULTS

All of the outlined techniques are well established and have been used in previous studies

Analysis of stool microbiota and microbiota metabolites will mainly be conducted at CSIRO Animal, Food and Health research laboratories in Adelaide under the guidance of Dr. Michael Conlon. Some analyses may be outsourced to other laboratories, but under the broad direction of Dr. Conlon in consultation with Dr Costello and other collaborators. The abundance and/or activities of faecal and mucosal (biopsy)-associated microbes will be analysed using molecular methods. This will include the use of 16s ribosomal RNA sequencing. Isolation (culture) of bacteria from stool samples may be considered to further understanding of metabolic changes occurring in bacteria of IBD patients when compared to healthy controls. Stool will be analysed for short chain fatty acids using a range of methods established at CSIRO where sufficient material is available. Other metabolites may also be measured.

11.1 Bacterial analysis

Bacterial DNA will be extracted from the samples using the MoBio PowerMag Microbial DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. All stool samples will be extracted and processed in duplicate. Amplicon library preparation will be performed using a modified dual-index PCR approach. The V4-V5 hypervariable region of the 16S rRNA gene will be amplified using first-step primers (515F, 806R), modified by the inclusion of a phaser, and the indexed barcodes added to the second set (i5, i7) enable multiplexing of the large number of samples[45]. The library will be pooled at equi-molar concentrations and run on an Illumina HiSeq2500 Rapid instrument using 2 x 250 base pair paired end chemistry (Ramaciotti Centre for Genomics, University of New South Wales).

11.2 Bioinformatics

Raw sequencing data will be processed using a combination of both in-house and open source software. The bioinformatic pipeline will utilise USEARCH algorithms[46] which include merging, quality-filtering, partitioning/de-replicating and clustering into operational taxonomic units (OTUs) at 97% similarity. Representative sequences from each OTU will be classified in two ways: via the RDP Naïve Bayesian Classifier and by finding the closest match in a set of curated reference sequences (RDP 16S Training Set + RefSeq 16S)[47]. The use of two independent classification techniques improves confidence in the taxonomic assignments.

11.3 Immunological analysis via flow cytometry

Gut mucosal immunological analysis with be performed with Dr Patrick Hughes at the Nerve Gut Research Laboratory.

Blood sampling
A total of 60ml will be taken at each time point which will be used for further experiments outlined below:

Isolation of PBMC and LPMC cells

Peripheral Blood Mononuclear Cells (PBMC) are isolated from whole blood via density gradient centrifugation. Lamina Propria Mononuclear Cells (LPMC) are isolated from colonic biopsies via collagenase digestion and density gradient centrifugation. Cells will be stored under liquid nitrogen until further analysed.

PBMC cells and biopsy tissue will be used for the following:

LPMC isolation: Colonic mucosal biopsies will be incubated twice in Hepes buffered HBSS supplemented with 1mM EDTA and 1mM DTT (Sigma) for 10 min. at 37°C under slow rotation, with the suspension strained (100µM) between incubations. Residual tissue will be incubated in Hepes buffered Ca^{2+}/Mg^{2+} free HBSS for 10 min at 37° C under slow rotation and strained (100µM). Residual tissue will be minced and incubated in complete media ((RPMI 1640 (Gibco, Germany) supplemented with fetal calf serum, glutamax and penicillin

/ streptomycin, Collagenase D (1mg/ml, Roche), DNAse1 (0.5mg/ml, Sigma) and Dispase (3mg/ml, Roche)). Collagenase D (Roche, NSW, Australia), 0.5mg/ml DNAse1 (Sigma) and 3mg/mL Dispase (Roche) for 20 min. twice with supernatant removal from centrifugation (300*g*, 5min.) after each incubation. Residual suspensions will be sequentially strained (100μM followed by 40μM), with the supernatant centrifuged (300*g*, 5min), resuspended, stained with trypan blue to determine viability and cell number as previously described[48-50].

Cell staining:

0.5 x 10^6 F_c blocked (BD Biosciences, NSW, Australia) cells will be stained for viability (FVD eFlour450, eBioscience) and the following anti-human monoclonal antibody panels (BD Bioscience unless otherwise stated): a) HLADR-APC, CD11C-FITC, Lin (CD3, CD14, CD16, CD19, CD34, CD56 all APC-Cy7, CD33-PerCP Cy5.5), b) CD3-APC, CD45RO-PerCP Cy5.5, CD19-APC Cy7, CD20-APC Cy7, CD16-PE, CD56-PE, Vα24jα-FITC (eBioscience), c) CD3-APC, CD8-FITC, CD45RO-PerCP Cy5.5, γδT-PE (eBioscience). For T_{REG}, cells will be stained with CD4-APC Cy7, CD8-PE, CD45RO PerCP Cy5.5, CD25 PE Cy7, β7-FITC, followed by fixation and permeabilization (Transcription buffer staining set, eBioscience) and staining with FOXP3-APC (eBioscience). The following gating strategy will be used to identify cell populations: Macrophages (lin-ve/HLADR/CD33+ve), dendritic cells (lin –ve HLADR+/CD33+/CD11c+), T_{HELPER} (CD4+ CD8-), $T_{CYTOTOXIC}$ (CD8+ CD4-), $T_{REGULATORY}$ (CD4+/CD8-/CD25+/FOXP3+), B (CD3-, CD19+ CD20+), Natural Killer (CD3-/CD16+/CD56+/CD45RO-), Natural Killer T (CD3+/NKT+), γδ T (CD3+/γδT+) in LPMC, and gut homing T_{HELPER} (CD4+/CD8-/CD45RO+/β_7+) and gut homing $T_{REGULATORY}$ (CD4+/CD8-/CD45RO+/β_7+/CD25+/FOXP3+) will be determined in PBMC.

12. STATISTICAL ANALYSIS

Patient information will be de-identified and the results of microbiota, immune analysis as well as clinical scores will be recorded in an excel spread sheet. This data will then be imported into the R program for statistical analysis. Statistical analysis will be conducted in collaboration with the University of Adelaide department of statistics.

12.1 Primary outcome power analysis

The study is powered to detect a significant difference in the primary outcome of inducing remission at 8 weeks post FT with 32 patients in each arm. This was calculated using a Z test with pooled variance for the difference of two independent proportions. The significance level was set at 5% and the power at 80%. The estimated remission rate in the placebo group was 26.4% and the minimally clinically relevant remission rate we are powered to detect is 60%.

The placebo remission rate is difficult to predict based on the heterogeneous nature of previous studies that investigated induction of remission in UC. Our placebo remission rate was derived from the active ulcerative colitis trials 1 and 2[51] (ACT-1 and ACT- 2). The ACT-1 and 2 trials were randomized, double-blind, placebo controlled studies that evaluated the efficacy of IV infliximab 5- or 10-mg/kg IV infusion for induction and maintenance treatment in adults with UC. The clinical response rate in those patients in the ACT -2 trial who were not steroid dependent was 26.4%. These patients had moderate to severe colitis with a MAYO score of 6 to 12 on enrolment and so had more severe disease on average than our patients. Response was defined as at least a 3 point reduction and 30% reduction in the MAYO score to determine clinical response at week 8. Another trial of patients with mild to moderate ulcerative colitis[52] found a remission rate at 8 weeks with oral mesalamine 2.4g daily of 22%. Many of our patients will be taking an oral aminosalicilate compound and some a concomitant steroid. The remission rate in this case would be expected to be higher than 22%.

12.2 Clinical outcomes

Comparisons between treatment groups of the primary and secondary dichotomous outcomes will be assessed using Fisher's exact tests with individuals analysed in the group to which they are allocated (intention to treat). Assessment of treatment on the change in total Mayo score will be assessed using linear mixed effects regression with week 8 total Mayo score as outcome and adjusting for baseline total Mayo score and steroid use at either time point. A random intercept will be included for each group of individuals receiving the same donor mix. Associations between baseline factors and change in total Mayo score will be assessed in a similar manner, with treatment group also adjusted for as a fixed effect covariate. To

assess the effect of oral steroid use at either time point a mixed effects regression will be constructed with total Mayo score (at either assessment) as outcome with oral steroid use, assessment time and the treatment-assessment time pairwise interaction as fixed effects. Two non-nested random intercepts will be included one for correlations due to treatment batch effects, the other to account for observations within the same patient. The random effects will be non-nested as the treatment batch effects are only present at week 8.

12.3 Safety

As with the clinical outcomes the comparison between treatment groups and occurrence of SAEs will be assessed using a Fischer's exact test. Assessment of treatment on the change in serum creatinine, ALT, ALP, bilirubin and haemoglobin will be assessed using linear mixed effects regressions with week 8 values as outcome. Fixed effects covariates included treatment group and baseline values with a random intercept to account for within batch correlations.

12.4. Inflammatory markers

The models used to assess the differences due to treatment in white blood cell count, neutrophil count and C-reactive protein will be the same as those used to assess the safety blood markers (see above). The exception being Calprotectin, which has an extra assessment at week 4. This model extends the mixed effects regressions with assessment time (week 4 v week 8) and the pairwise interaction with treatment as additional fixed effects. As before random effect intercepts will be included for each individual and each treatment batch, with individual effects nested with batch. After inspection of the residual distribution these analyses will be performed on the change in log transformed Calprotectin, with results being converted back to the original scale.

12.5 Microbiome - Diversity

Diversity will be defined as the fraction of unique species present at an assessment out of all species present at any analysis in any sample. As such logistic mixed effects regressions will be used to compare between treatment groups with donor stool and stool mix samples. Outcome will be the presence of a species in a particular sample. Fixed effects will include sample origin (donor v mix v treated patient v untreated patient) and total sample count (log-

transformed). Three non-nested random effects will be included; patient identifier, donor batch and the microbiome species identifier. To assess the effect of treatment a separate model will be contrasted with only post baseline samples included as outcome. This model will be identical to the previous, except that the fixed effects will be baseline prevalence (logit transformed), treatment allocation, assessment time (week 4 v week 8), the pairwise treatment-assessment time interaction and total sample count (log-transformed). Associations between both baseline diversity and change in diversity, and change in total Mayo score will be assessed as before (re Clinical outcomes). A two-stage approach will be taken, first the mean diversity will be estimated using the logistic mixed effects models previously described in this section. These diversity estimates will then be included in the models of total Mayo score as fixed effects.

12.6 Microbiome – Abundance v Total mayo score

Associations between changes in biome species abundance with change in total Mayo score will be modelled in a similar manner. For each sample the mean proportion of total counts will be calculated, and subsequently for individuals with samples at both week 4 and 8 averaged to estimate baseline and post randomization prevalence estimates. The change in prevalence will then be included in linear mixed effects models of total Mayo score (re Clinical outcomes). A false discovery rate (FDR) analysis will be performed to provide evidence of associations beyond what would be expected due to multiple testing, with the FDR being compared with the same analysis repeated, but with outcome (total Mayo score) permuted between individuals.

12.7 Microbiome – Abundance v Treatment

The change in prevalence by treatment group and assessment time will be assessed using a negative binomial mixed effects regression for each microbiome species. Fixed effects included treatment allocation, assessment time (baseline, week 4, week 8 and 12 months) and their pairwise interaction. Nested random intercepts per patient and assessment will be included in the model, with total sample count (log transformed) included as an offset.

12.8 Metabolome

Baseline levels of butyrate and dietary fibre will be compared between donors and UC patients using non-parametric Mann-Whitney-Wilcox tests. The effect of treatment on these and other SCFA will be assessed using linear mixed effects regressions. Fixed effects including assessment time (week 4 v 8), treatment group and baseline SCFA abundance, with two nested random intercepts at the donor batch and patient levels. After examination of residual distributions all SFCA variables will be log transformed and results reported as percentages of baseline scores. Associations between baseline total Mayo scores and SCFA will be assessed using linear regressions adjusting for oral steroid use, with baseline SCFA levels log transformed. Associations between change in total Mayo scores and change in SCFA will be performed in the same two stage approach. SCFA change levels will be estimated per individual using linear mixed effects regressions adjusting for baseline levels and treatment, with individual random effects nested within batch. Patient level estimates of SCFA change will be entered into linear mixed effects regressions of total Mayo score as a fixed effect using the same methodology described above (re Clinical Outcomes).

12.9 Immune

The models used to assess associations between immunological measures and total Mayo score both at baseline and post treatment change will be the same as those used for SCFA (see Metabolome above). With baseline and week 8 assessments for the immunological data, the difference in log transformed values will be included in the mixed effects regression of total Mayo score.

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Summary of Changes

Page numbers apply to the final protocol

1. Title page. Oliver Waters added as investigator at Fiona Stanley Hospital in Western Australia. Fiona Stanley Hospital was added as a third site for the trial in 2015.
2. Page 8-9. Under "Background and significance" Deleted "Anti TNF agents not funded by the pharmaceutical benefits scheme in Australia and so, are not readily available". Funding became available for anti-TNF agents in 2014 (after commencement of the study)
3. Page 14 Under "Open label therapy". Changed such that all who are randomly assigned to the placebo group by week 8 will then be offered active donor FMT at the 8 week colonoscopy. Previously only those who do not have a clinically relevant response (achieving remission, having a drop in Mayo score by ≥3 or achieving a endoscopic subscore of 0-1) would be offered donor FMT. This change was made as patient 4 was enrolled and as such all patients in the placebo arm were offered donor FMT at week 8.
4. Page 14 Under "Exclusion criteria". Anti-TNF therapy removed as exclusion criteria. This therapy because funded for ulcerative colitis in 2014 and at this time we allowed patients on this medication to enter the trial.
5. Page 15 Under "Medication prior to enrolment". Biological agents dosing stable for at least 8 weeks. This change was made when biological agents were no longer an exclusion
6. Page 16. Under "Recruitment". Fiona Stanley Hospital in Western Australia added as a study site in 2015 and Dr Oliver Waters added as an investigator at that site.
7. Page 17. Under "Recruitment". Donor recruitment flyers were not placed on the hospital grounds and only the University. The University population were considered to be more suitable to be stool donors.
8. Page 30 Under "11.2.2 Medical history". Exclusion criteria broadened in 2015 to keep up with latest screening practices internationally and on advice from local experts.
9. Page 31 Under "11.2.4 Blood testing". ANA and helicobacter serology added in 2015
10. Page 31 Under "11.2.5 Stool testing" Viral studies and added in 2015.
11. Page 36. Under "11 Analysis and reporting of results". More detailed plan for bacterial and immunological analysis added.
12. Page 39. Under 12.2 Statistical analysis. The statistical plan for clinical outcomes is expanded on beyond using Fischer's exact test alone to test the primary and secondary clinical end points. Factors affecting remission will be analysed using linear mixed effect regression.
13. Page 40 Under "12.4 Inflammatory markers" Statistical plan for analysis of inflammatory markers included with linear mixed effects regression
14. Page 40 Under "12.5-12.7 Microbiome. Statistical plan for the analysis of diversity, abundance vs total Mayo score and abundance vs treatment added.
15. Page 42 Under "12.8 Metabolome". Statistical analysis of stool short chain fatty acid and dietary fibre intake. Again linear mixed effects regression analysis was proposed.

16. Page 42 Under “12.9 Immune”. Statistical analysis of immune cell populations was added. Again linear mixed effects regression analysis was proposed to compare immune populations to total Mayo score.