A combined treatment with GM-CSF, fosfomycin and metronidazole for pouchitis in ulcerative colitis patients after restorative ileal pouch anal anastomosis surgery

A clinical safety and proof-of-concept study

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Proposed schedule:

Start of trial period Phase I study	01.01.2021
End of trial period Phase I study	31.05.2021
Start of trial period Phase II study	01.06.2021
End of trial period Phase II study	01.03.2022

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Abbreviations

16S rRNA (16S ribosomal ribonucleic acid), 12
AE (adverse event), 21
APC (Antigen-presenting cells, 11
ATC classification (Anatomical Therapeutic Chemical classification), 24
BMI (body mass index), 27
CD (Crohn's disease), 11
cDNA (complementary deoxyribonucleic acid), 24
CLR (C-type lectin receptor), 11
CPR (Civil Person Registry), 33
CRFs (Case report forms), 22
DC (Dendritic cells), 11
ESBL (extended-spectrum β-lactamase-producing *E. coli)*, 14
EUDRACT database (European Union Drug Regulating Authorities Clinical Trials database), 36
FAP (Familiar adenomatous polyposis), 10
FFP (formalin fixed paraffin), 28
GCP unit (Good clinical practice unit), 26
GDPR (General Data Protection Regulation), 32
GM-CSF (granulocyte-macrophage colony stimulating factor), 13
GMP (Good Manufacturing Practice), 24
HE (hematoxylin/eosin), 28
i.m. (intramuscular), 17
i.v. (intravenous), 17
IBD (Inflammatory bowel disease), 11
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IPAA (restorative ileal pouch anal anastomosis), 10
IPEC-J2 cells (Intestinal porcine enterocyte J2 cell line), 15
IUPAC (International Union of Pure and Applied Chemistry), 14
JAK/STAT (Janus kinase/signal transducer and activation of transcription), 13
MAPK (mitogen-activated protein kinase), 13
MRSA (Methicillin-resistant Staphylococcaus aureus), 14
PDAI (Pouchitis Disease Activity Index), 10
PERMANOVA (permutational analysis of variance), 31
PI-3 (phosphatidylinositol 3), 13
PRR (pattern recognition receptors), 11
rhGM-CSF (recombinant human granulocyte-macrophage colony-stimulating factor), 24
s.c. (subcutaneous), 17
SAE (Serious adverse events), 30
SAR (Serious adverse reactions), 30
SUSAR (Suspected unexpected serious adverse reaction), 30
TLR-5 (Toll-like-receptor 5), 13
UC (Ulcerative colitis), 10

1. Summary

A definitive cure for patients with treatment-refractory ulcerative colitis is proctocolectomy with IPAA (restorative ileal pouch anal anastomosis) (1). Up to 50% of all patients develop “pouchitis” within the first five years after surgery, an inflammatory condition that is as yet poorly understood and without official consensus on treatment (2). Treatment modalities include oral antibiotics as well as immunomodulators, steroids, probiotics and biological agents, but up to 20% of these patients develop chronic, treatment-resistant pouchitis, which can result in pouch failure and the need for reoperation with the possible creation of an ileostomy (11,12).

The etiology of pouchitis is thought to be similar to other inflammatory bowel diseases, in that genetic and bacterial factors, a compromised gastrointestinal barrier and immunological components seem to play a role. Its pathogenetic mechanisms seem to mimic Crohn’s disease, in which smaller studies have shown some effect of systemically administered GM-CSF (granulocyte-macrophage colony stimulating factor) on the gut macrophage function in clearing microorganisms and maintaining the mucosal barrier (3–6).

We hypothesize that GM-CSF will have an effect in the treatment of pouchitis because of its similarity to that of Crohn’s disease. In order to maximize effect on the inflamed mucosa and minimize systemic side effects, we intend to administer it locally in the pouch. In a safety and proof-of-concept intervention study we will combine 50 µg GM-CSF with two well-known antibiotics, 400 mg Fosfomycin and 100 mg metronidazole, to target both the suspected immunological as well as the bacterial role in the pathogenesis of pouchitis.

The effect on the pouch will be assessed endoscopically and histologically by taking biopsies that will also be examined for changes in the microbiome. Patients will be clinically examined and have blood samples taken to monitor for adverse reactions. The primary outcome measure will be an assessment of adverse reactions and tolerability of the drug. Secondary outcome measures will be a change in the pouchitis disease activity index (PDAI), a change in the microbial diversity, and a change in inflammatory markers.

This study is based on a non-randomized trial design with an open-label single group assignment.

Phase I

The tolerability of treatment will be tested on 6 patients with pouchitis with a single dose of the combined medication applied endoscopically in the pouch. Endoscopy with the taking of biopsies will be performed before and one week after administration of the medication, as well as blood samples before and after the medication. After the follow-up endoscopy, the patient will receive standard oral metronidazole or ciprofloxacin treatment for 10 days.

Phase II

Depending on effect of the first study, the second study plans for the treatment of 12 patients. Endoscopy with biopsies will be conducted with the first application of the study drug combination in the pouch, and afterward a daily dosage for another 6 days. Clinical and endoscopic control after 14 days with blood samples and biopsies will be done. After the follow-up endoscopy, the patient will receive standard oral metronidazole or ciprofloxacin treatment for 10 days.

Inclusion criteria

Patients

- Of any gender
- Over 18 years of age
- Have a previous diagnosis of ulcerative colitis
- Have had IPAA surgery, and
- Have been diagnosed with pouchitis
- Be able to understand and complete study procedures as determined by the investigator
- Be able to speak either Danish or English
- Be able to comply with study procedures for the length of the study
- Use a highly effective contraception method for the duration of the trial (until day 30 in Phase I and until day 37 in Phase II), such as implants, injectables, oral contraceptives, IUD (intrauterine device), sexual abstinence or vasectomized partner.

Written informed consent must be obtained.

Exclusion criteria

- Patients with a previous allergic reaction to GM-CSF, metronidazole or fosfomycin
- Patients who are currently under antibiotic treatment or have received antibiotic treatment within the past 30 days
- Patients currently pregnant or breastfeeding
- Patients with ASA IV classification (American Society of Anesthesiologists physical status classification)
- Patients with severe pulmonary disease
- Patients with autoimmune thrombocytopenia
- Patients with severe renal impairment (eGFR < 40 ml/min)
- Patients with alcohol use disorder or history of drug abuse
- Patients currently in treatment for any malignant or hematological disease
- Patients with a previous history of cancer will be excluded from the study (except for patients with well-treated and stabile cancer after a control period of more than two years).
- Patients with anticipated compliance problems as determined by the investigator

Patients can voluntarily withdraw from participation in this study at any time as well as withdraw from receiving the study intervention for any reason. The investigator can withdraw patients if the investigator feels the safety of the patient is compromised by participating in the study.

Adverse events and reactions will be monitored and followed up until the end of the study and/or resolution of the adverse event or reaction or until the adverse event or reaction is considered chronic.

In accordance with Danish law on personal data, we have approval for this trial from Region Sjælland (REG-106-2020) through their data application system. Data handling will be in accordance with the act on processing of sensitive personal information, the GDPR (General Data Protection Regulation) and the Data Protection Act. We have received conditional approval for the trial from The Research Ethics Committee (Application no: 75784) and the Danish Medicines Agency (EudraCT: 2020-000609-10).

The study will be conducted in the Surgical Department of Zealand University Hospital in cooperation with the Department of Gastroenterology and Hepatology of Zealand University Hospital. It will contribute to knowledge of the pathogenesis of pouchitis and whether a combination of GM-CSF and antibiotics given locally has an influence on the inflammation of the mucosa.

Following completion of the study, the positive, negative or inconclusive results of this research will be published in an international peer-reviewed scientific journal.

2. Background and scientific rationale

The surgical procedure of choice for patients with therapy-resistant UC (ulcerative colitis) is proctocolectomy with IPAA (ileal pouch anal anastomosis) (1). The intent of the procedure is a definitive cure of the disease and reduction of the risk of developing malignancy while preserving continence.

Pouchitis, inflammation of the mucosa of the ileoanal reservoir, is one of the most common complications after IPAA. (2) Symptoms of pouchitis include hematochezia, increased stool frequency and urgency, abdominal cramping, pain, nausea, incontinence and fever, as well as extraintestinal manifestations. Endoscopy typically shows the mucosa to be erythematous, with friability and loss of vascular patterns. Histology shows signs of inflammation and extravasation of red blood cells in the lamina propria. Interestingly, the incidence of pouchitis is about 10 times lower in patients after IPAA in FAP (Familiar adenomatous polyposis) (7). Various scoring systems have been developed to assess the severity of inflammation in pouchitis, of which the PDAI (Pouchitis Disease Activity Index) is the most common. It assesses clinical, endoscopic and histological features (8) (see Table 1 in Appendix 1).

Pouchitis can be further categorized into acute and chronic pouchitis.

- Acute pouchitis – duration <4 weeks
- Chronic pouchitis – duration >4 weeks despite therapy

The incidence of acute pouchitis has been reported to be up to 50% at 5 years and there is no official consensus on treatment (9,10).

Treatment includes oral antibiotics, most often with metronidazole and/or ciprofloxacin. The standard treatment at Zealand University Hospital for acute pouchitis consists of a 10-day treatment period with oral metronidazole or ciprofloxacin. Metronidazole, while effective, can cause nerve damage after prolonged use in high doses. As patients often experience repeated flare-ups of acute pouchitis or chronic pouchitis, this risk increases due to repeated and lengthy use (11).

Other treatment modalities include probiotics, immunomodulators, steroids and biological agents (12,13). Up to 20% of patients with acute pouchitis can develop chronic pouchitis or chronic refractory pouchitis (14). While these can also be manageable with medical treatment, a small percentage of patients develop therapy-resistant pouchitis. A meta-analysis in 2017 by Segal et al. suggested that, on average, remission can be achieved in up to 59% of patients, but that chronic pouchitis still is challenging because the pathogenesis behind it is not fully understood (15). In therapy-resistant pouchitis, surgery with fecal diversion with the construction of an ileostomy is the treatment of choice and this results in the loss of anal defecation. Pouch failure ranges between 3.5% after 5.5 years to up to 9% after 10 years in various studies (16,17).

Introduction

Pathogenesis of pouchitis and inflammatory bowel disease

The pathophysiology of pouchitis after IPAA is poorly understood. Controversy exists as to whether pouchitis is a remanifestation of ulcerative colitis that mimics CD (Crohn's disease) or a type of Crohn's disease of the pouch. Just as in IBD (Inflammatory bowel disease), its etiology is thought to be multifactorial involving genetic and environmental factors including bacterial, mucosal barrier and immunological components (2,18).

There are several similarities between Crohn's disease and pouchitis, which questions its origin as a simple reactivation of ulcerative colitis in the mucosa of the ileum with colonic metaplasia:

- Pouchitis often responds to antibiotic treatment. Ulcerative colitis generally does not respond to antibiotics, whereas Crohn's disease does (19).
- Fistulas, which can appear in pouchitis, are often associated with Crohn's disease (20).
- Inflammation in ulcerative colitis is limited to the rectum and colon. Both Crohn's disease and pouchitis can also cause inflammation in the small intestine e.g. ileum (21).
- On an immunological level, Crohn's disease is Th1-mediated, while ulcerative colitis is mostly Th2-cytokine driven. Pouchitis seems to be Th1-mediated like Crohn's disease (22,23).

The focus of our study is on the pathogenesis and treatment of pouchitis and the transition from the environmental to the immunological side. This includes bacterial dysbiosis and the compromised intestinal mucosal barrier. In theory, an imbalance of the gut microbiome in the gastrointestinal tract results in an incorrect response of the immune system to bacteria (24). In a healthy individual, macrophages clear away invading microbes without the need to raise a specific immune response (25,26). When the immune system has to be activated, macrophages and dendritic cells act as sensing and APC (Antigen-presenting cells) to create an adequate response (27). DC (Dendritic cells), the connection between the innate and the adaptive immune system, accumulate in the mucosa of patients with IBD (28). When an immature dendritic cell digests a microbe after recognizing it as pathogenic through PRR (pattern recognition receptors), the pathogen is degraded into fragments and presented at the cell surface. The mature cell then migrates to a lymph node where it can activate T-helper cells, T-killer cells and B-cells to create an immune response. Dendritic cells can also induce T-cell tolerance. To determine if an antigen is to be tolerated or whether further activation of the immune system is necessary, a special type of PRR is used, called a CLR (C-type lectin receptor). CLR on the surface of dendritic cells can either induce or repress the production of various inflammatory cytokines or chemokines (29).

An essential step in the pathogenesis of Crohn's disease is an inappropriate activation of the immune response likely due to dendritic cells and signaling between PRR and CLR.

Bacteria have been suggested to be involved in the pathogenesis of pouchitis, in that areas of inflammation are found to have a high concentration of certain bacteria (19).

Existing treatment in IBD and pouchitis focuses on either the bacterial side, such as antibiotics and probiotics, or the immunological side with the use of immunomodulators, biologicals and glucocorticoids.

Our study attempts to target the conversion of the bacterial to the immunological side, where dendritic cells are involved while also targeting the bacterial imbalance. These dendritic cells present the broken-down bacteria to the immune system, which reacts incorrectly and creates inflammation.

The microbiome of the pouch

It has previously been shown that up to 60-80% of pouch microbiota cannot be cultured (30), and this has complicated microbiome studies greatly. Culturing bacteria of the gastrointestinal tract greatly favors aerobic bacteria, which can result in misleading findings. The development of 16S rRNA (16S ribosomal ribonucleic acid) sequencing has revolutionized microbial diagnostics. It allows detection and quantification of non-culturable as well as slow-growing bacteria in both a clinical and research setting. However, it does not assess the viability of the detected bacteria.

Studies have shown considerable differences between bacteria found in the pouch lumen and feces, and bacteria adherent to the mucosa (31,32). Especially the bacteria found in the mucosa are thought to be involved in the pathogenesis of IBD and pouchitis because they are in close contact with the host and the immune system (31,33).

Tissue biopsies from the pouch mucosa will be analyzed in our study for change in bacterial composition with 16S rRNA sequencing.

It is still unclear which bacteria are responsible for the development of pouchitis, but several bacteria have been associated with inflammation whereas others usually found in healthy pouches disappear or are reduced in number in pouchitis. In general, it is thought that a lower bacterial diversity leads to inflammation. Enterobacteriaceae, streptococci, Bacteroidetes, and enterococci are thought to be involved in the maintaining of gut homeostasis, and low levels of these bacteria favor the development of pouchitis (34). As opposed to this, a higher incidence of proteobacteria and sulfate-reducing bacteria such as clostridia are found in the inflamed pouch (35–37).

Bacteria increased in pouchitis	Bacteria in healthy pouches or reduced in pouchitis
Proteobacteria Clostridia (especially *C. perfringens*) Bacteriodaceae *Roseburia* *Akkermansia* *Escherichia coli* (AIEC) (38)	Bacteroidetes *Faecalibacterium prausnitzii* *Veillonella* Staphylococci Enterobacteriaceae Streptococci Enterococci Bifidobacteriaceae Eubacteriaceae Bacillaceae Moraxellaceae Burkolderaceae Corynebacteriaceae *Sutterella*
Inconclusive	
Lachnospiraceae *Ruminococcus*	

Fusobacterium

Table 1: Overview of bacteria associated with pouchitis

Roseburia is a genus of anaerobic Gram-positive bacteria, which are found in increased numbers in the inflamed pouch. They are motile, being flagellated. The protein flagellin from the bacterial flagellum induces proinflammatory gene expression by activating TLR-5 (Toll-like-receptor 5) (35). *Akkermansia* is a genus of mucin-degrading bacteria also associated with the development of pouchitis (39).

Butyrate-producing bacteria such as F. prausnitzii are found more often in healthy pouches. The butyrate is an important energy source for colonic epithelial cells, has anti-inflammatory properties and improves the barrier function of the epithelial cells. Pouchitis is associated with a decrease in these bacteria (40).

Targeting dendritic cells in pouchitis and bacterial dysbiosis

GM-CSF (granulocyte-macrophage colony stimulating factor) is a glycoprotein cytokine that stimulates the production, survival, activation and differentiation of white blood cells – granulocytes and monocyte/macrophages. A deficiency of GM-CSF has been shown in previous studies to induce inflammation in animal models with Crohn's disease, as well as being associated with an impaired intestinal barrier function (41–43). When administered intraperitoneally in mice, it has been shown to decrease the number of pro-inflammatory cytokines in the mucosa in experimental colitis and improve healing (44–46). It has recently been shown that GM-CSF might also be involved in a genetic component of Crohn's disease involving decreased signaling in macrophages and monocytes (47,48).

Anti-GM-CSF antibody concentrations also seem to be increased in IBD patients experiencing increased disease activity, especially in Crohn's disease (49).

Several phase 1 studies have shown an ameliorating effect of systemic GM-CSF in Crohn's disease, whereas one study has not (3–6). Moreover, GM-CSF has shown a beneficial effect in some cases of fistulizing Crohn's disease, and with fistulization being a known problem in pouchitis, this holds promise for a possible beneficial effect in patients with this condition. However, systemically administered GM-CSF, e.g. given subcutaneously, may not produce a sufficient local effect in the bowel, while overstimulating the bone marrow and causing side effects such as bone pain (50).

GM-CSF is secreted by various cells in response to proinflammatory stimuli and binds to the GM-CSF receptor on myeloblastic cells. The complex mechanisms behind its multiple functions are not yet fully understood, but it is known to activate at least three different pathways: JAK/STAT (Janus kinase/signal transducer and activation of transcription), MAPK (mitogen-activated protein kinase) and PI-3 (phosphatidylinositol 3) (46).

In the intestine, GM-CSF has been found to have multiple effects on the mucosa:

- It enhances the expression of costimulatory molecules in APCs such as dendritic cells and thereby induces mucosal immunity (51).
- In mice, it has been shown that a GM-CSF deficit was associated with an increased inflammation of the ileum after exposure to NSAID. It is assumed that GM-CSF plays a role in regulation and survival of intestinal epithelial cells and thereby upholds the intestinal barrier (42).

GM-CSF acts physiologically as a cytokine, by paracrine action on neighboring or nearby cells in the same tissue compartment. It does not readily cross tissue barriers, and only when used pharmacologically at

supraphysiological doses does it begin to act at a distance like a classical hormone. This means that systemically administered GM-CSF will act principally on the bone marrow and only to a lesser extent on the granulocytes and macrophages found in peripheral tissues. At the same time, the mucosal macrophage populations of large organs like the lungs (e.g. the pulmonary alveolar macrophages) and gut (gut mucosal macrophages) are semi-independent cell populations which only interchange slowly and incompletely with the monocytes of the circulation. Indeed, a population of self-maintaining gut macrophages has been described that occupies microanatomical niches in the gut submucosa and myenteric plexus and appears to be essential for intestinal homeostasis (52). For GM-CSF to have an effective restorative effect on defective gut macrophage function in clearing microorganisms and maintaining the mucosal barrier, it should be given locally, so that it can reach the local macrophages at an adequate concentration.

Besides treatment with GM-CSF, treatment would also include a combination of the antibiotics metronidazole and fosfomycin. Metronidazole is a common antibiotic used against anaerobic bacteria, where it disrupts DNA-synthesis (53). Fosfomycin is an antibiotic that is used against both Gram-positive and Gram-negative bacteria in that it inhibits bacterial wall synthesis (54).

Fosfomycin is the international non-proprietary name of a broad-spectrum antibiotic isolated and characterized in 1969 from *Streptomyces fradiae* strains under the name phosphomycin or phosphonomycin (55). Its structure was determined to be (-)(IR, 2S)-1,2-epoxypropylphosphonic acid (56) with the systematic IUPAC (International Union of Pure and Applied Chemistry) name [(2*R*,3*S*)-3-methyloxiran-2-yl]phosphonic acid and a formula weight of 138.1 Da. Fosfomycin is bactericidal and inhibits bacterial cell wall biosynthesis by inactivating the enzyme UDP-N-acetylglucosamine-3-enolpyruvyltransferase, also known as MurA (57). This enzyme catalyzes the committed step in peptidoglycan biosynthesis, the ligation of phosphoenolpyruvate to the 3'-hydroxyl group of UDP-N-acetylglucosamine to form N-acetylmuramic acid. Fosfomycin is a phosphoenolpyruvate analogue that inhibits MurA by alkylating an active site cysteine residue. The antibiotic enters the bacterial cell via the glycerophosphate transporter.

Given this mechanism of action, fosfomycin has a broad bactericidal spectrum, being active against aerobic genera such as *Staphylococcus,* including MRSA (Methicillin-resistant *Staphylococcaus aureus*), *Streptococcus, Enterococcus, Neisseria, Escherichia,* including ESBL (extended-spectrum β-lactamase-producing *E. coli), Proteus* (indole-negative), *Serratia, Salmonella, Shigella, Pseudomonas, Haemophilus*, and *Vibrio*, less active against indole-positive *Proteus* spp., *Klebsiella* and *Enterobacter* spp. It is also active against the anaerobic genus *Fusobacterium,* as well as the anaerobic genera *Peptostreptococcus* (including *Peptoniphilus, Finegoldia* and *Anaerococcus*). However, it is inactive against the anaerobic *Bacteroides fragilis* group of bacteria.

There is a low prevalence of bacterial resistance to fosfomycin in the community, and studies of the prevalence of resistant bacteria after the introduction of fosfomycin have shown either no increase or only a modest increase in the prevalence of resistant organisms. However, prolonged exposure to the antibiotic may enable bacteria to develop resistance by selection of mutants that lack the glycerophosphate transporter pathway. Alternative mechanisms of resistance involve the loss of the inducible hexose phosphate transporter, a Cys-Asp mutation in MurA, or acquistion of plasmids coding for the fosfomycin inactivating enzymes *fos*A and *fos*B (in addition to the chromosomal *fos*X in *Listeria monocytogenes*). The mutant strains may, however, also show reduced pathogenicity (58). This may explain why the emergence of bacterial resistance is seen on prolonged exposure *in vitro*, but much less frequently *in vivo*. The appearance of resistant bacterial strains in controlled clinical trials of orally or

intravenously administered fosfomycin has been 3.0% overall, with a maximum of 15% for *Pseudomonas aeruginosa*. In general, fosfomycin is seen to be a valuable addition to the therapeutic armament against multidrug-resistant organisms.

Fosfomycin has been shown to have the capacity to favor phagocytosis and act as an immunomodulator. It is accumulated by polymorphonuclear leukocytes to reach concentrations that are up to twice those of the extracellular fluid, but does not affect their cellular functions, while still exerting a bactericidal effect on *Staphylococcus aureus* (59).

Fosfomycin is also actively taken up by intestinal mucosal cells to reach concentrations that can kill intracellular bacteria such as Fusobacteria. A rapid uptake of fosfomycin has been demonstrated in IPEC-J2 cells (Intestinal porcine enterocyte J2 cell line) exposed to fosfomycin calcium at 580 µg/ml to reach an intracellular concentration approaching 30 µg/ml within 15 minutes (60). This concentration of fosfomycin is readily reached by local administration to the gut mucosa.

Fosfomycin is known to penetrate readily through tissue barriers and into bacterial biofilm (61) so that high, bactericidal concentrations of fosfomycin will result from the direct application of fosfomycin solution to the pouch mucosa. Fosfomycin inhibits the adhesion to epithelial cells of various bacterial species, including those involved in biofilm formation (62).

Fosfomycin shows considerable synergism in bactericidal effect on a large number of strains of organisms from the susceptible genera mentioned, when used in combination with a large number of antibiotics of the penicillin, cephalosporin, aminoglycoside, macrolide and lincosamide types. While early studies showed a synergistic effect on about 70-100% of tested strains for various antibiotic combinations, subsequent more extensive studies showed synergy rates of 36-74%. The remaining strains showed merely additive effects and an inhibitory effect was only seen in one or two individual antibiotic combinations on an individual bacterial strain (62). The fact that fosfomycin shows synergy with many individual antibiotics and indeed abrogates the toxicity of many other antibiotics, including the nephrotoxicity and ototoxicity of the aminoglycosides, favors the use of fosfomycin in combination with other antibiotics to produce a potent bactericidal action and compensate for any development of fosfomycin resistance during more prolonged treatment.

Metronidazole is a semi-synthetic antibiotic which is active against a number of anaerobic bacteria including bacteria of the *B. fragilis* group and *Fusobacterium* spp. Oral metronidazole, alone or in combination with oral ciprofloxacin is one of the standard treatments for pouchitis (63). It has also been used systemically in combination with systemic fosfomycin for prophylaxis against wound infection after abdominal surgery. The intracellular penetration of metronidazole appears to be by rapid passive diffusion, so that intracellular levels approach equilibrium with extracellular levels within 15 minutes (63). It is a part of the study drug combination in the present study, in which it will be locally applied at a low dose to the pouch mucosa to reach a higher concentration than that which can be achieved by conventional oral administration, to reduce the bacterial load of toxin-producing *B. fragilis* group bacteria which may play a role in pouchitis and which would not be susceptible to the locally applied fosfomycin.

Potential risks of the study include the relatively low risk of endoscopy of the pouch, as well as any potential analgesic medicine given during the procedure. The low dosage and local application of the study drug combination is also deemed to be of low risk to the patients. Previous studies have tested higher dosages of the same combination with intraperitoneal application of the drug, without serious adverse reactions. The safety of application of a lower dosage in the pouch will be the subject of Phase I of this study. A more thorough description of all side effects of GM-CSF, Fosfomycin and metronidazole is given in chapter three.

Potential benefits of the study include a potential new treatment for pouchitis, as well as a better understanding of the role of bacteria in pouchitis. Moreover, administering a combination drug locally in the pouch instead of systemically will potentially enable lower dosage and lessen side effects.

3. Study objectives

The hypothesis of the study is that both systemically administered GM-CSF (given subcutaneously) and systemically administered antibiotics (given orally) respectively act relatively weakly, when given at tolerable doses, on the immunocompetent cells of the bowel mucosa and the bacterial dysbiosis that contributes to the pathogenesis of pouchitis. It is hypothesized that the beneficial action of these agents will be potentiated if they are administered locally into the pouch at higher concentrations than can be achieved with systemic administration at acceptable doses. At the same time, the total daily local doses of these agents will be considerably lower than the conventionally used systemic doses, and their limited absorption from the pouch will significantly reduce the systemic side effects of conventional pouchitis treatment.

We aim to investigate whether GM-CSF in combination with metronidazole and fosfomycin, applied topically in the pouch, can be used in patients with pouchitis. As pouchitis seems to mimic Crohn's disease, and responds to therapy used in Crohn's disease, we suspect that this combination therapy can target the bacterial dysbiosis and inadequate immune response in pouchitis.

The therapy will be applied topically, both under endoscopic supervision in an initial safety study and depending on safety data, later by daily administration for one week using an enema in the pouch in a proof of concept study. We will investigate if this therapy has an effect on pouchitis by assessing if the patient has a lowering in the Pouchitis Disease Activity Index, by clinical history and examination, taking biopsies from the pouch mucosa and taking blood samples.

Phase I – Safety study

Primary outcome measure

- Safety: Determine serious adverse reactions or adverse reactions from the application of GM-CSF, metronidazole and fosfomycin in the pouch as seen in Appendix 8. The category of the reactions will be determined by the principal investigator according to the safety assessment described in chapter 7.
  A summary of the product characteristics of GM-CSF, fosfomycin and metronidazole will function as a reference document in evaluation of adverse effects. We will monitor adverse reactions from the day of the intervention in Phase I and II until respectively day 30 and 37. The safety of the intervention will be measured by adverse reactions.

Secondary outcome measures

- Change in the pouchitis disease activity index (PDAI) – A decrease of 3 points or more will be determined as an improvement in PDAI from before application of the study drug to 7 days after application of the study drug
- Change in median WBC, CRP, creatinine and liver enzymes from before application of the study drug to 7 days after application of the study drug
- Change in microbial diversity in the pouch using 16S rRNA sequencing from before application of the study drug to 7 days after application of the study drug

Phase II – Proof of concept study

Primary outcome measure

- Change in the pouchitis disease activity index (PDAI) – A decrease of 3 points or more will be determined as an improvement in PDAI from before application of the study drug to 7 days after application of the study drug

Secondary outcome measures

- Change in the clinical, endoscopic or histological PDAI – A decrease of 3 points or more will be determined as an improvement in PDAI from before application of the study drug to 7 days after application of the study drug
- Change in median WBC, CRP, creatinine and liver enzymes from before application of the study drug to 7 days after application of the study drug
- Change in microbial diversity in the pouch using 16S rRNA sequencing from before application of the study drug to 7 days after application of the study drug
- Safety: Determine serious adverse reactions or adverse reactions from the application of GM-CSF, metronidazole and fosfomycin in the pouch as seen in Appendix 8.

Potential benefits of the study

Pouchitis affects a considerable amount of patients after IPAA surgery, and can result in either loss of anal defecation due to removal of the pouch and creation of a permanent ileostomy, or in certain centers the construction of a new pouch. Both are associated with risks of additional surgeries for the patient. In addition, the current treatment with oral metronidazole can result in serious side effects due to repeated, high doses, and it is important to determine whether an alternative or lower dosage can be as effective. We hypothesize that GM-CSF with Fosfomycin and metronidazole will have an effect on the PDAI and bacterial dysbiosis, and potentially give rise to fewer side effects than conventional medical therapy because of local instead of systemic application.

Potential risks of the study

The risk of endoscopy of the pouch (pouchoscopy) will be relatively low, compared to colonoscopy, and includes bleeding, perforation, infection and adverse reaction to sedatives and analgesia given. There are no data on perforation risk of pouchoscopy, but in colonoscopy overall, the pooled prevalence is described as 0.5/1000 (95% CI 0.4-0.7), including both diagnostic and interventional colonoscopy. Bleeding rate for colonoscopy occurs in 2.6/1000 (95% CI 1.7-3.7) and mortality at 2,9/100,000 (95% CI 1.1-5.5).

Because endoscopy of the pouch is not technically complicated and only takes a short time, most patients will not require sedation or analgesia, but is possible, depending on the patient's wishes. The endoscopy of the pouch will be performed by a trained specialist surgeon in the outpatient endoscopy clinic.

GM-CSF

Knowledge about adverse reactions after GM-CSF is mainly based on i.v. (intravenous), i.m. (intramuscular) and s.c. (subcutaneous) administration. We suspect that application of a low dose of 50 µg to the mucosa of the pouch will result in less frequent systemic adverse reactions.

Fever is the most common adverse effect, often accompanied by myalgia and malaise, and occurs in more than 20% of recipients of the conventional systemic dose of 250 µg. For GM-CSF application, the following known and usual side effects (1-10%) and known and non-usual side effects (0.1-1%) will not be registered as adverse reactions: fever, malaise, myalgia, abdominal pain, diarrhea, rash, chest pain, weight loss, nausea and vomiting, peripheral edema, dyspnea, hypoxemia, hypotension, tachycardia, flushing, metabolic disease, hyperbilirubinemia, cardiac dysrhythmia and pharyngitis (65).

Effects are more common when GM-CSF is administered intravenously and with doses greater than 3 µg/kg. High doses (20 µg/kg/day) of GM-CSF have been reported to cause a generalized capillary leak syndrome (4,66). In our study, only 50 µg are applied locally in the pouch.

Metronidazole

Treatment with metronidazole is known to create Antabuse-like reactions in patients who continue to drink alcohol. Patients who are currently heavy drinkers are excluded from study participation. Patients will be orally instructed not to drink alcohol from the day before the therapeutic endoscopy during the enrollment meeting until one week after the last intake of metronidazole.

For metronidazole application, the following known and usual side effects (1-10%) and known and less usual side effects (0.1-1%) will not be registered as adverse reactions: nausea, glossitis, dry mouth, dysgeusia, stomatitis, myalgia, superinfection with candida, dark coloration of the urine.

Fosfomycin

Based on the literature on the effects of fosfomycin, the following known and usual side effects (1-10%) and known and less usual adverse reactions (0.1-1%) will not be registered as adverse reactions: phlebitis, abdominal pain, decreased appetite, dysgeusia, vertigo, light headache, nausea, vomiting, diarrhea, rash, fatigue (54).

The chief adverse effects of fosfomycin are gastric irritation from orally administered fosfomycin disodium, evidence of allergy in the form of transient rashes (0.3% of cases) and eosinophilia (0.2%), as well as transiently raised liver enzymes (0.3% of cases) from systemically administered fosfomycin (62). In general, however, fosfomycin displays remarkably low toxicity, so that when high doses of fosfomycin disodium are given systemically (up to 28 or 32 g per day), it is the sodium load that is the dose-limiting factor (54). It is recommended that the dosage of Fosfomycin is lowered in patients with a GFR < 40 ml/min. Our study only administrates 400 mg Fosfomycin daily, substantially lower than normal doses of Fosfomycin (8-12 grams daily). Therefore, we do not expect there to be a need for lowering the dosage in patients with renal impairment. For ensuring optimal safety, however, we will exclude patients with an eGFR < 40 ml/min in this study.

Drug combination risks

One recent study tested intraperitoneal administration of GM-CSF for peritonitis on patients undergoing laparoscopic appendectomy for uncomplicated appendicitis in 14 otherwise healthy male individuals. In this Phase II trial, 50 µg of molgramostim was administered into the peritoneal cavity directly after appendectomy, along with 4 g of fosfomycin and 1 g of metronidazole.

No serious adverse events or complications occurred. Adverse events included dizziness, diarrhea, hypotension, no flatus and discomfort when breathing deeply. However, it was not possible to determine if these occurred due to the study drug combination, or were complications related to the anesthesia,

surgery or appendicitis. It is also unclear if these adverse events, if related to the study drug, were a reaction to the GM-CSF, fosfomycin or metronidazole (67).

We hypothesize therefore, that application of the study drug combination is safe in the pouch. We will also be using a lower dosage of fosfomycin and metronidazole.

Bias

This study is an open-label proof-of-concept study with a single treatment arm. There is no blinding of patients or the investigator. The only blinded examination is the pathology examination of mucosal biopsies of the pouch and 16S rRNA sequencing. The pathologist is unaware of whether samples are taken before the application of the study drug or after. Therefore, the histology of the PDAI should not be influenced by bias.

4. Study design

This study is a clinical proof-of-concept intervention study. It is based on a non-randomized trial design with an open-label single group assignment. See chapter 7 for a flow chart of the trial phases and study visits.

Phase I

Tolerability of the treatment is tested on 6 patients with a single dose of the combined medication applied endoscopically. See also appendix 14 for a flow-chart.

Patients will be identified by the treating gastroenterologist through the outpatient clinic when presenting with pouchitis and then informed in detail and screened according to the inclusion and exclusion criteria. The patient then gets at least 24 hours to reflect before signing the informed consent form to enter the trial.

- Day 1:
    - Clinical examination is performed and pre-treatment blood samples taken
    - Endoscopy with biopsies is performed and GM-CSF, metronidazole and fosfomycin is sprayed into the pouch as a single dose
- Day 7:
    - Clinical examination, post-treatment blood samples,
    - Endoscopy with biopsies
    - Recording of any adverse reactions or other adverse events (can be any day between day 6-9)
- Afterwards, the patient receives the standard treatment at Zealand University Hospital for acute pouchitis, consisting of a 10-day course of oral metronidazole 500 mg x 3 or ciprofloxacin 500 mg x 2.
- Day 30:
    - Telephone call to the patient to ask about symptoms of pouchitis and any side effects. (Can be any day between day 30 and 37).

After the last patient in phase I has had their 30 day follow-up, the safety and feasibility of the study drug application will be assessed by the sponsor and investigators of the study. If no serious adverse events of reactions occurred, the Phase II study will be initiated.

Phase II:

Depending on effect of the first study, this phase will comprise the treatment of 12 patients with a daily dose of the combined medication. The study drug will be applied in the hospital, to ensure the correct dosage and concentration. See also appendix 15 for a flow-chart.

Clinical and endoscopic control after 14 days with blood samples and biopsies. Patients who have been a part of the Phase I study, can be included if they fulfill inclusion criteria at time of inclusion for Phase II.

Inclusion will follow the procedures from the Phase I trial. Patients will be identified by the treating gastroenterologist through the outpatient clinic when presenting with pouchitis and then informed in detail and screened according to the inclusion and exclusion criteria. The patient then gets at least 24 hours of reflection time before signing the informed consent form to enter the trial.

- Day 1:
  - Clinical examination, blood samples
  - Endoscopy with biopsies and GM-CSF, fosfomycin and metronidazole applied in the pouch as a spray
- Day 2-7:
  - Enema with GM-CSF and metronidazole/fosfomycin applied in the hospital once daily
- Day 14:
  - Clinical examination, blood samples
  - Endoscopy with biopsies
  - Recording of any adverse reactions or other adverse events (Can be any day between day 13-16)
- Afterwards, the patient gets the standard treatment at Zealand University Hospital for acute pouchitis, which consisting of oral metronidazole 500 mg x 3 for 10 days or ciprofloxacin 500 mg x 2 for 10 days.
- Day 37:
  - Telephone call to the patient to ask for symptoms of pouchitis, any side effects. (Can be any day between day 37 and 44).

5. Study enrollment and withdrawal

Patients

- Of any gender
- Over 18 years of age
- Have a previous diagnosis of ulcerative colitis
- Have had IPAA surgery, and
- Have been diagnosed with pouchitis
- Be able to understand and complete study procedures as determined by the investigator
- Be able to speak either Danish or English
- Be able to comply with study procedures for the length of the study
- Use a highly effective contraception method for the duration of the trial as mentioned below (until day 30 in Phase I and until day 37 in Phase II). In men, there is no evidence of GM-CSF or

the administered antibiotics increasing risk for genetic damage in the spermatozoa that can result in embryopathies.

Written informed consent must be obtained.

Exclusion criteria

- Patients with a previous allergic reaction to GM-CSF, metronidazole or fosfomycin
- Patients who are currently under antibiotic treatment or have received antibiotic treatment within the past 30 days
- Patients currently pregnant or breastfeeding. A pregnancy test (serum Hcg) must be negative before inclusion into the trial.
- Patients with ASA IV classification (American Society of Anesthesiologists physical status classification)
- Patients with severe pulmonary disease
- Patients with autoimmune thrombocytopenia
- Patients with severe renal impairment (eGFR < 40 ml/min)
- Patients with alcohol use disorder or history of drug abuse
- Patients currently in treatment for any malignant or hematological disease
- Patients with a previous history of cancer will be excluded from the study (except for patients with well-treated and stabile cancer after a control period of more than two years).
- Patients with anticipated compliance problems as determined by the investigator

A completed test person

A completed test person is defined as:
Phase I: a patient who received the therapeutic endoscopy, clinical examination and had pre-treatment blood samples drawn, and then came for a clinical appointment on day 7 with new blood samples drawn, clinical examination, and control endoscopy
Phase II: a patient who received the initial endoscopy, clinical examination and had pre-treatment blood samples drawn, and then received the enema daily for 7 days and came for a clinical appointment on day 14, when new blood samples were drawn, and clinical examination and control endoscopy performed

Reasons for withdrawal

A patient will be discontinued from participation in the study

- if any clinical AE or AR (adverse event or reaction), laboratory abnormality, intercurrent illness, or other medical condition or situation occurs which suggests that continued participation in the study would not be in the best interest of the patient. This also concerns the necessity of initiating a particular drug treatment, e.g. antibiotics for infection or taking a 'rescue drug' for a medical condition, that interacts with the study drug
- when developing any of the exclusion criteria

Patients can voluntarily withdraw from participation in this study at any time as well as withdraw from receiving the study intervention for any reason. The patients will be informed of this right in accordance with the Helsinki Declaration.

The investigator can withdraw a patient if the investigator considers that the safety of the patient is compromised by the participation in the study. In that case, the investigator must inform the patient about the reason for withdrawal.

When patients withdraw from the study, reasons for withdrawal, if given, will be documented in the CRFs (Case report forms). The number of dropouts and withdrawals will be reported. No further data will be collected from the patients withdrawing from the study; however, the previously collected data will be part of the final data analysis unless the patient specifically requests all data to be deleted. Patients that have received the study medicine will be contacted by telephone two weeks after receiving the study medicine, to ensure patient wellbeing. This data after withdrawal from the study, will not be collected and is meant solely to ensure the safety of the patient.

In case of withdrawal due to AE or AR, safety data will be collected and the patient will be followed until given appropriate care under medical supervision or until symptoms of any AE or AR resolve or become stable.

Participants who discontinue early will be replaced. This concerns all patients that have not undergone the second endoscopy. In case of withdrawal due to a serious adverse event (SAE) or serious adverse reaction (SAR), continuity of inclusion will be discussed with the sponsor.

Contraception

During the enrollment visit, a serum HCG test is conducted on female patients of fertile age. Women of fertile age must use safe contraceptive products, as recommended by the Clinical Trials Facilitation Group (CTFG).

Contraceptive products considered safe are: intrauterine devices or hormonal contraception (oral contraceptive pills, implants, transdermal patches, vaginal rings or long-acting injections) or if the patient practices sexual abstinence. They must be used during the whole duration of the trial (until day 30 in Phase I of the trial, and day 37 in Phase II of the trial). Subjects that are sterilized or infertile, are exempt from contraceptive use. This includes: patients that have undergone surgical sterilization (vasectomy, bilateral salpingectomy, hysterectomy, bilateral oophorectomy), or patients postmenopausal defined as no menstruation for 12 months or more prior to inclusion in the trial.

Unless a female patient is suspected of having become pregnant during the trial, no further pregnancy testing is conducted.

In men, there is no evidence of GM-CSF or the administered antibiotics increasing risk for human teratogenicity or fetotoxicity in early pregnancy. There are no data on the concentration of GM-CSF in the seminal fluid after GM-CSF administration in the pouch. There are also no data on potential subsequent exposure to a sexual partner through seminal fluid, but it is theoretically possible that a certain systemic concentration of GM-CSF can be achieved. Therefore, we recommend that male patients use a condom during the duration of the trial (until day 30 in Phase I and day 37 in Phase II).

There are no populations exempt from using the above-mentioned methods.

Subsequent studies

Patients that leave or terminate the trial can participate in subsequent studies and or treatment with the study medicine, if they still meet inclusion and exclusion criteria at time of enrollment.

6. Study intervention

Composition of the combined medication

A single dose of the medication will consist of 50 µg of molgramostim (non-glycosylated recombinant human GM-CSF produced in *E. coli*), 400 mg of fosfomycin disodium neutralized with succinic acid and 100 mg of metronidazole.

In this study, patients are administered the study drugs as a solution containing 50 µg of molgramostim, 400 mg of fosfomycin and 100 mg of metronidazole, first sprayed endoscopically in the pouch in the Phase I study, and then given as an enema in the Phase II study.

The medication is combined on-site by the principal investigator with 0.2 ml molgramostim intraintestinal solution (from 1.2 ml molgramostim 250 µg/ml) in low acyl Kelcogel CG-LA 0.25% (from CPKelco) and 100 mg metronidazole (from B. Braun i.v. solution 5 mg/ml solution in 100 ml) and 400 mg fosfomycin (from i.v. Infectofos 2 g) in sterile water with 0.25% calcium chloride (see appendix 10). Components A (molgramostim) and B (fosfomycin and metronidazole) are given subsequently into the pouch, they are therefore only combined *in situ*, not prior to administration. The 0.25% calcium chloride solution and Kelcogel 0.25% (gellan gum) are both inactive substances that, when combined, become an adhesive gel, that will possibly lower the risk of the patient immediately defecating (and thereby keeping the medicine in the pouch for a longer time to ensure exposure of the inflamed mucosa to the study medication).

The administration of fosfomycin and metronidazole with Kelcogel 0.25% and calcium chloride has previously been approved by the Danish Medicines Association (Reg.-no.: 2019-000131-51) in the MeFo-trial for application in the colon. Here, the investigator combined the two antibiotics with calcium chloride 0.25% immediately before application and this was given with kelcogel 0.25% through an endoscope to become an adhesive gel.

The general dosage guideline for adults treated with fosfomycin infusions is 12-24 g intravenously per day, divided onto 2 to 3 dosages. In those with impaired renal function and a glomerular filtration rate under 40 ml/minute dosages should be lowered. As we are only administering 400 mg of fosfomycin daily, we do not expect the need for lowering the dosage in patients with renal impairment. However, to ensure optimum safety of our trial participants, we will exclude patients with a eGFR < 40 ml/min from participating in this trial.

The general dosage guideline for treatment with metronidazole is 1500 mg intravenously per day.
We are using much lower dosages in this study, 100 mg of metronidazole, applied topically in the pouch.

The general dosage guideline for treatment with GM-CSF in chemotherapy-induced neutropenia is a maximum of 10 µg/kg/day for 7-10 days, which is a much higher dosage than we will be using in this study.

Formulation, packaging and labeling, and acquisition

Molgramostim, which is a water-soluble, non-glycosylated rhGM-CSF (recombinant human granulocyte-macrophage colony-stimulating factor) produced under GMP (Good Manufacturing Practice) rules in a strain of *E. coli* bearing a genetically engineered plasmid which contains a human GM-CSF cDNA (complementary deoxyribonucleic acid) coding sequence. The product is purified through a series of tested methods to remove process-related impurities. According to the ATC classification (Anatomical Therapeutic Chemical classification), GM-CSF belongs to the class L Antineoplastic and immunomodulating agents; L03 Immunostimulants; L03a Immunostimulants; and L03AA Colony stimulating factors. This results in the ATC code L03AA03 for molgramostim.

Repomol, for intraintestinal administration, contains molgramostim formulated in a solution at pH 7.0-7.4. The drug product must be stored at 2-8 ^{o}C. Repomol used in this trial is supplied by Reponex Pharmaceuticals A/S, Hørsholm, Denmark. The manufacturer is responsible for the study drug, including the packaging.

Fosfomycin (Infectofos, from Infectopharm) is a marketed medicine, used routinely in the surgical department of the study site. 1 vial contains 2 grams of fosfomycin in powder form, designed to be dissolved in either sterile water or isotonic glucose. Every box contains 10 vials of Infectofos. When dissolved, Infectofos has a pH 7-8. The drug is supplied by Unimedic Pharma (Sundbybergsvagen 1, Solna, Stockholm, Sweden).

Metronidazole (Metronidazole 'B. Braun'), is a marketed medicine, used routinely in the surgical department of the study site. 1 container consists of 500 milligrams of metronidazole in a soluble form, that can be diluted in either isotonic saline or isotonic glucose. The drug is supplied by B. Braun Medical (Dirch Passers Allé 27, 3. Sal, 2000 Frederiksberg).

The application of the medicine and the dose is recorded in the CRF (see appendix 4).

Product storage and stability

Repomol is delivered by Reponex pharmaceuticals A/S, and received at the study site by the principal investigator according to the Good Clinical Practice guidelines for extended recipient control.

Repomol is provided in a 1.2-ml vial. Repomol must be stored in a refrigerator at 2-8^{o}C in the original package, and must be protected from light. The temperature will be monitored by a temperature log.

The solution must not be frozen. Each vial contains 1.2 ml of Repomol containing a total of 300 µg molgramostim, the concentration of which is 250 µg/ml. The expiry date is indicated on on each vial. The 1.2 ml vials each bear a label with relevant information e.g. identification name, batch number, expiry date and will be clearly marked as a trial drug. Each vial will only be used for application of the study drug for one patient. Administration and maintenance procedures will be outlined in detailed instructions before the administration of the first dose of Repomol.

Fosfomycin (Infectofos) should be kept at room temperature (between 15-30^{o}C) away from excess heat.

Metronidazol (Metronidazol 'B. Braun') should be kept at room temperature (between 15-30^{o}C) in a container in the original box to protect it from light.

Dosage, preparation and administration of the study drug

The drug combination will be applied endoscopically as a spray and in the form of an enema, in Phase I and II respectively. The drug combination will be administered at the hospital by the investigator to ensure the correct concentration and dosage of the study drug.

Only designated staff will be allowed to handle and administer the study drug in the hospital. The study drug Repomol will be taken out of refrigerated storage approximately 10-30 minutes before use. An information sheet with clear instructions will be provided to guide the study staff.

Repomol is in a vial of 1.2 ml containing 300 micrograms of molgramostim. Of this, 0.2 ml (containing 50 micrograms of molgramostim) are administered to the patient.

One vial with 2g fosfomycin powder is dissolved in 100 ml metronidazole (5 mg/ml) and 25 ml calcium chloride giving a total volume of 125 ml of which 25 ml are administrated to the patient (400 mg fosfomycin and 100 mg metronidazole in 25 ml calcium chloride 0,25%)

Assessment of patient compliance with study drug

During Phase I of the study, the application of the drug is done endoscopically as a spray. Therefore, correct application is confirmed visually. During Phase II of the study, the patient gets the first dosage of the drug in the outpatient clinic, with the investigator ensuring that application is done correctly. The daily application of the study drug on day 2-7 will also be in the hospital to ensure the correct dosage and compliance.

Therapeutic endoscopy Phase I and II

In this study, endoscopy of the pouch only is needed, which does not require a full bowel preparation. Instead, the patient can self-administer an enema (e.g. Microlax, containing sodium citrate and sodium laurylsulfoacetate) 1 hour before the examination. Visual assessment of the pouch mucosa will be performed, and biopsies of the pouch will be taken to assess the mucosa according to the PDAI, as well as to examine the microbial diversity. Biopsy size will be 2 mm with regular biopsy forceps, and 2 biopsies will be taken for histology and 2 of these for microbiology assessment.

The endoscopy of the pouch will be performed by a trained specialist surgeon or gastroenterologist in the outpatient endoscopy clinic.

7. Study schedule

See Appendix 2 for schedule of events

Screening

Recruitment of patients will be done consecutively in the outpatient clinic. All patients with a flare up of acute pouchitis will receive information about the trial in the outpatient clinic and referred to the principal investigator. The treating physician, an experienced gastroenterologist on pouchitis, will only inform the patient that a clinical trial is ongoing, and give written information on the trial to the patient. If the patient is interested in hearing more about the trial, he or she will be offered a consultation with the principal investigator. Initial information and a consultation with the principal investigator therefore takes place before informed written consent. The identity of the patient and basic demographic information is given to the investigator after verbal consent from the patient in the outpatient clinic.

Enrollment/Baseline

Patients eligible for the study are offered a consultation with the principal investigator, and are informed about their right to bring a bystander along to this consultation. During this consultation, the patient will receive information about the trial. Afterwards, the patient will be offered the therapeutic endoscopy if they meet inclusion criteria. They will be asked to sign the informed consent form if they accept entering into the study.

Enrollment meeting

Patients who are eligible for inclusion will receive both oral and written information about the study. The oral information will be delivered in a room only used for that purpose to avoid disturbances. The patient can be offered to have the enrollment meeting at his/her home. The investigator will have enough time to explain the content, extent, purpose, expected risks and advantages of the study in plain Danish or English. The patient will have enough time to read the written information and to ask questions. The following will be covered during the interview:

- The purpose and organization of the study
- The procedures during the therapeutic endoscopy
- The permission to access the patient's files to register any adverse reaction, medication use, blood tests and other clinical parameters perioperatively, and that this permission will also give the sponsor, the investigators, the Danish Medicines Agency, the GCP unit (Good clinical practice unit), and the Danish Data Protection Agency access to patient files during monitoring
- The methods used in the study
- The right to ask for additional information at all times
- The right of the patient to withdraw at any time during trial without explanation
- The insurance
- The contact person

At the end of the enrolment meeting the patient may sign the informed consent if they wish, and may also be given the time needed for consideration (at least 24 hours). The informed consent will be written in accordance with Danish law (see Annex III). Patients will be informed if any changes in the study occur, as this might influence the patient's willingness to participate or the safety of the participating patients. The approved document will be updated accordingly.

Intervention

The intervention of the study will be according to the phase of the trial.

Phase I: Day 1, medical history, clinical examination and laboratory testing of the patient, as well as the pouchoscopy with biopsies to determine PDAI. During the same endoscopy, the study drug will be applied.

Phase II: Day 1, medical history, clinical examination and laboratory testing of the patient, as well as the pouchoscopy with biopsies to determine PDAI.
Day 1-7, patient is given the study drug combination as an enema daily for one week in the hospital, the first dose during endoscopy, to ensure correct administration and compliance.

Follow-up

Follow-up will be:

- Phase I: Day 7 after the therapeutic endoscopy (Can be day 6-9)
- Phase II: Day 14 after the first diagnostic endoscopy (Can be day 13-16)

Follow-up will be a medical history, clinical examination of the patient, laboratory testing and a diagnostic endoscopy. Patients' pouchitis will be assessed according to the PDAI.

After the follow-up endoscopy, the patient will receive the standard treatment at Zealand University Hospital for acute pouchitis, which consists of oral metronidazole 500 mg x 3 for 10 days or ciprofloxacin 500 mg x 2 for 10 days. The type of treatment is decided on by the patient's gastroenterologist.

Final study visit

30 days after the follow-up, the patient will receive a telephone consultation. Patients will be asked about symptoms of pouchitis and if they had any adverse reactions or side effects to the treatment. See Appendix 8.

Study procedure - evaluation

Clinical evaluations

At the first baseline visit, a thorough medical history will be taken both by using a patient interview as well as by using past medical records from the electronic health records. A list of current medications is made, where all currently taken medications should be included, both prescription and over-the-counter medicine, as well as medicine taken within the last 30 days. The patients PDAI score must be recorded.

The physical examination will include a measurement of vital signs (blood pressure, pulse, temperature, respiratory frequency), as well as cardiopulmonary, gastrointestinal and gross neurological examination. Current weight, height, BMI (body mass index), ASA score, performance status.

Subsequent visits will include a short update of the medical history, an update of the PDAI as well recording any adverse reactions during the study period. A clinical symptom-directed examination is done if the investigator deems this necessary.

All data is recorded in Easytrial by direct entry, using autoquery to minimize faulty data entry.

Laboratory evaluations

The following blood sample analyses will be taken to monitor signs of inflammation as well as adverse reactions to the medication.

Type of vial:	Clinical biochemistry test:	Amount of samples
EDTA	Hemoglobin; B Leukocytes; B Thrombocytes; B Leukocyte type;	2 blood samples per patient taken at baseline (Day 1) and 7 days after end of treatment
Heparin	Creatinine; P Potassium; P HCG (in females); P Alkaline phosphatase; P	2 blood samples per patient taken at baseline (Day 1) and 7 days after end of treatment
Li-Heparin	Sodium; P Bilirubin; P ALAT; P Amylase; P Lactate dehydrogenase; P	2 blood samples per patient taken at baseline (Day 1) and 7 days after end of treatment

	CRP; P	

Blood samples will be analyzed by the clinical biochemistry department at the Sjælland University Hospital Køge. Per vial between 7-10 ml of blood are drawn, with 3 vials drawn both at baseline (Day 0) and 7 days after treatment. In total 6 vials of blood are drawn, with a maximum of 60 ml of blood.

Control endoscopy Phase I and II

After seven days, endoscopy of the pouch will be performed again after application of a cleansing enema by the patient 1 hour before the examination. The pouch mucosa will be visually assessed and biopsies of the pouch mucosa taken to assess change in the mucosa according to the PDAI, as well as to examine change in the microbial diversity.
The endoscopy of the pouch will be performed by a trained specialist surgeon in the outpatient endoscopy clinic.

Special assays or procedures

Histology

Two biopsies of 2 mm in size each will be taken for assessment of inflammation of the mucosa, according to the PDAI. Biopsies will be fixed in a 4% neutral buffered formaldehyde ('formalin') solution. Biopsy samples will be prepared in sections of FFP (formalin fixed paraffin) wax-embedded tissue, for histology with routine HE (hematoxylin/eosin)-staining. A trained pathologist will review the samples, without prior knowledge of the patient's clinical or treatment status.

Biopsies are reported descriptively and using the PDAI. They will assess the quantity of acute and chronic inflammation, presence of crypt abscesses, erosions and ulcers as well as loss of villous height, granulomas and dysplasia. Chronic inflammatory changes will be distinguished from true pouchitis, as the mucosa of the healthy pouch undergoes adaptive changes after surgery. These changes, which are also frequently described as 'colonic metaplasia' include villous atrophy, crypt hyperplasia, infiltration of the lamina propria of mononuclear cells, eosinophils and histiocytes (68).

Microbial diversity

Two biopsies will be taken for assessment of the microbial diversity of the mucosa. It has previously been shown that up to 60-80% of pouch microbiota cannot be cultured (30). Furthermore, there is a difference in analyzing the luminal microbiota (fecal samples) or mucosa-adherent microbiota (mucosal samples). It is assumed that the mucosal microbiota is more relevant in the pathogenesis of IBD, and we will focus on these in our study (31). Therefore, we will take mucosal biopsies, which will be analyzed by 16S rRNA sequencing.

Safety assessments and adverse events or reactions

An adverse event (AE) is any untoward medical occurrence in a patient or clinical investigation subject that has been given a pharmaceutical product, regardless of its causal relationship to the study treatment. This can be an abnormal laboratory finding, a symptom or disease coincident in time with the use of the investigational medicinal product.

During and after administration of GM-CSF, fosfomycin and metronidazole, any adverse events or intercurrent illnesses will be reported. Patients who have known allergy to GM-CSF, fosfomycin and or metronidazole are excluded from study participation. The administration of the study drug combination will be in the presence of a medical doctor, the principal investigator. Patients will be observed in the endoscopy unit for at least 1 hour after the first administration of the drug. In case of the occurrence of

an adverse event or serious adverse event, monitoring and further treatment steps will be taken according to the type and severity of reaction.

The severity of the AE will be assessed using the following grading system:

- Mild: requires minimal or no treatment, does not interfere with the patient's daily activities
- Moderate: low level of inconvenience or concern with therapeutic measures, some interference with the patient's daily activities
- Severe: interrupts a patient's daily activities, may require systemic drug therapy or other treatment, and is usually incapacitating
- Life threatening: places the patient at immediate risk of death from the reaction as it occurs

Changes in the severity of the AE will be documented.

All AEs, including both local and systemic eventsnot meeting the criteria for 'serious adverse events' will be captured on the appropriate form for recording AEs. We will collect information on

- Description of the event
- Time of onset
- Clinician's assessment of the severity
- Relationship to the study product
- Time of resolution/stabilization of the event

Any medical condition that the patient has at baseline (time of inclusion in the study), will not be considered or reported as an AE. If an existing medical condition worsens during the study, it will be recorded as an AE.

Adverse reactions

An adverse reaction is an adverse event where there is a reasonable possibility of a causal relationship between the investigative medicinal product (IMP) and an adverse event. The assessment of causality can be done using the WHO-UMC Case Causality Assessment, which uses certain criteria to determine if causality is plausible. Causality can hereafter be

- Certain
- Probable/likely
- Possible
- Unlikely
- Conditional/Unclassified
- Unassessable/Unclassifiable

We will attempt to assess the relationship of the AE to the study drug. All AEs will be reported, using the following guidelines:

- Related: reasonable possibility that the study drug caused the AE or evidence to suggest a causal relationship between study drug and the AE. The adverse event can thereafter be classified as an adverse reaction (AR).
- Not related: no reasonable possibility that the administration of the study drug caused the event

Serious adverse events or reactions

An AE or AR is considered serious when resulting in death, is life-threatening , results in hospitalization or prolongation of existing hospitalization, causes persistent or significant incapacity or substantial disruption of the patient's daily activity or causes a congenital anomaly or birth defect, and other adverse events that according to the investigator's judgement are medically significant. A medical event that

requires surgery or medical intervention to prevent one of these outcomes is also considered a serious AE or AR. All SAE (Serious adverse events) and SAR (Serious adverse reactions) will be recorded on the appropriate form, and followed through resolution by the investigator as well as reviewed and evaluated.

All SAE and SAR will be registered and reported to the sponsor by the investigator within 24 hours after the event has been recorded by e-mail. All adverse reactions will be reported to the Danish Medicines Agency and the local Ethics Committee in an annual report with a report on patient safety of the trial. All SAR will be reported in an annual report together with a report on patient safety to the Danish Medicines Agency. All SAE and SAR will be evaluated by the investigator according to the known possible adverse reactions reported in the product summary (see below) if they are a possible SUSAR (Suspected unexpected serious adverse reaction).

As there are no previous data on the administration of GM-CSF, fosfomycin and metronidazole in the pouch, there are no data on expected serious adverse events or reactions in the reference safety information of the Investigator's Brochure of GM-CSF. Therefore, all SAE and possible SAR are considered to be related to the investigational medicinal product and will be reported as SUSAR.

The investigators are responsible for reporting all lethal or life-threatening SUSAR to the Danish Medicines Agency and the regional Ethical Committee as soon as possible and no later than 7 days after knowledge of such reaction has been received. No later than 8 days after the reporting, the Danish Medicines Agency will be informed of the follow-up. All other SUSAR will be reported to the Danish Medicines Agency and the regional Ethics Committee no later than 15 days after knowledge of the reaction has been received by the sponsor and the investigators. In these situations, the patient will be followed until the reaction has terminated either via the outpatient clinic at the hospital or via contact with the sponsor or the investigators.

Life-threatening adverse events

An adverse event is considered as life threatening if, in the investigator's opinion, the occurrence places the patient at immediate risk of death.

Unexpected adverse event

If an adverse event is not listed in the investigator brochure, it is considered 'unexpected'.

We will immediately report to the sponsor any unexpected serious adverse events ,drug-related or not, including an assessment of whether we suspect the drug as the causative agent.

Adverse events and reactions will be monitored and followed up until end of the study and/or resolution of the adverse event or reaction, or until the adverse event or reaction is considered chronic.

The summary of the product characteristics and or investigator's brochure of GM-CSF, fosfomycin and metronidazole will function as a reference document for the evaluation of adverse effects. We will monitor adverse events and reactions from the day of the intervention in Phase I and II until respectively day 30 and 37. Monitoring will depend on asking patients if they have developed known adverse events or reactions as seen in Appendix 8, and self-reporting by the patient. Patients will have the possibility to contact the investigator by telephone during the duration of the trial, to report any adverse event or reaction. Patients will be encouraged to contact the relevant authority and initiate relevant help in case of serious acute adverse events or reactions.

8. Statistical considerations

We plan to include 6 patients in the Phase I study to test tolerability, and 12 patients in the Phase II study to test effectiveness of the treatment. The trial will end with the last visit of the last patient (Day 37 of the last patient). This is a safety and clinical proof-of concept intervention study, and we are, to the best of our knowledge, the first group to look into local treatment with GM-CSF and antibiotics of biofilm in patients with pouchitis after IPAA for ulcerative colitis. We expect this number of patients to be sufficient to provide the necessary experience in implementing the technique and form a basis for future studies, including future sample size calculation. This is based on calculations on number of patients required to achieve statistical significance of paired before-and-after comparisons by non-parametric tests. To achieve statistical significance at 1% level in Phase II, 9 patients are needed. As there is a risk of patients withdrawing from the trial prematurely or not receiving all interventions, we aim to include 3 additional patients.

Our primary objective is to test for adverse events or reactions in Phase I and a significant change in PDAI in Phase II, defined by a decrease of 3 points. Differences pre- and post-treatment will be described by means and standard deviations, or median and range when appropriate, and will be analyzed by the appropriate statistical tests. All patients described in chapter 5 as 'Completed test persons' will be included in the statistical analysis.

Microbial diversity changes will be analyzed using PERMANOVA (permutational analysis of variance). Other secondary objectives like change in WBC and CRP will be analyzed using the appropriate statistical tests.

Data will be analyzed using non-parametric or parametric statistics depending on the distribution of the data. P-values ≤0.05 are considered significant. Correction for multiple comparisons will be performed if needed. Descriptive statistics will be used for baseline characteristics.

Missing data will be handled depending on the type of data missing. If missing data results in a patient not being able to be defined as a 'Completed test person' (Chapter 5), the patient is excluded from the trial and a new patient will be recruited. If individual results are missing (e.g. laboratory values), the last known values will be used for analysis.

Due to the small number of patients enrolled and only one open treatment arm, there are no scheduled interim statistical analyses. Instead, a continuous evaluation of adverse reactions and serious adverse reactions is made according to the guidelines by the Danish Medicines Agency and Ethics Committee. The sponsor will be involved promptly in case of a SAR or SUSAR, and the trial may be halted or terminated at the least suspicion of impacted patient safety.

All deviations from the original statistical analysis plan will be provided in the final clinical study report.

We will use the program R statistics 3.6.2. to analyze all statistical data.

9. Data collection, handling and quality assessment

In accordance with Danish law on personal data, we have approval for this trial from Region Sjælland through their data application system (REG-106-2020). Data handling will be in accordance with legislation on the processing of sensitive personal information, the GDPR (General Data Protection

Regulation) and the Data Protection Act. We will also seek approval for the trial from The National Committee of Health Research Ethics and the Danish Medicines Agency.

Data from each patient will be recorded continuously and written in forms prepared before the trial begins. Appendix 3-6 and 8 are considered CRF on which source data is directly recorded. During the recruitment meeting, we will use paper work sheets for baseline data and inclusion/exclusion criteria. During the therapeutic endoscopy, we will use paper work sheets for data collection; afterwards data will be entered into an electronic database using the Easytrial platform. All other data will be recorded directly to the electronic Easytrial database. The paper work sheets will be stored in an identifiable manner in a locked cabinet at the respective research sites. All collected data will have source documentation.

Data will be stored as personally identifiable for 10 years, after which they are deleted on December 31, 2030. Data will be stored in compliance with GDPR and at EasyTrial (secure data storage). According to the Danish Medicines Agency, data from a clinical trial involving study medicine, must be stored for a minimum of 5 years after conclusion of the trial. This is necessary in case a potential reexamination of the data is needed.

If a patient withdraws from the study, the collected data will be anonymized. If the patient requests the collected data to be deleted, this will be done.

Data will be handled by Viviane Lin and Ismail Gögenur and access to EasyTrial will be restricted solely to them. The investigators will be allowed direct access to data and documents, among these the patients' medical records.

The trial will be monitored by the Good Clinical Practice Unit of the University of Copenhagen. During the duration of this trial, they will also have access to the data we collect. Similarly, the Danish Medicines Agency, according to section 90(2)-5 of the Danish Medicines Act, and The Danish Data protection Agency can conduct inspection visits and get access to the data.

When the handling of data has been completed, the sponsor role and responsibilities will be assigned to Reponex Pharmaceuticals A/S (Slotsmarken 12, 1.th.DK-2970 Hørsholm). The completion of data handling is defined as either the completion of the PhD of Viviane Lin or the submission of an article to a journal. The date of this assignment will be communicated to the authorities (Research Ethics Committee, Danish Medicines Agency) when it is known. The data they receive will be pseudo-anonymized. The key-files to the pseudo-anonymization will be kept on separate secure data sites according to GDPR in their region. During and after the assignment, the data handling will be in accordance with legislation on processing of sensitive personal information, the GDPR and the Data Protection Act.

Data from patients' files

The data collected from patients- files consists of demographic data (age, gender, BMI, comorbidities, WHO performance score, ASA class, history of smoking, medication, date of diagnosis with ulcerative colitis, date of pouch surgery, previous pouchitis treatment. We will collect data on the clinical findings, biochemical findings and PDAI index pre- and post-intervention as well as histology and biofilm results. Patient files will be accessible for reading by authorized personnel until 12 months after the intervention. Before informed consent is obtained from each patient, the following data are necessary for screening of patients by the researchers, which are readily available in a clinical setting:

- Demographic data: age, gender, CPR-number, ASA score, diagnosis of ulcerative colitis, surgical history, diagnosis of pouchitis
- Previous endoscopy data

All other data will be collected after informed consent has been given.

- Data from the therapeutic endoscopy and control endoscopy

Informed written consent by the subject will give the responsible researchers, the sponsor and the supervisory authority access to the patient's electronic health records. This is necessary to assess the subjects' health when conducting the research project, as well as to ensure quality control and monitoring of the project itself.

Storage of biological material

During the trial, biological material will be stored in a research biobank especially set up for this study. The use of a research biobank is to ensure that differences in analyses from patient to patient will be minimal. The research biobank will be reported to the Region Sjælland, and will be maintained using their regulations.

The biopsies are each 2 mm in diameter. The biological material will be stored in an identifiable fashion through a patient code (pseudo-anonymous, as the CPR (Civil Person Registry) number will be stored in a key-file at EasyTrial). Frozen samples will be stored according to the local protocol.

After the cutting of the FFPE blocks containing the biopsy specimens collected from the pouch, these blocks will be returned to the respective pathological archives. Both the biological material (stained glass slides and FFPE blocks of biopsies) and the key-file will be stored until conclusion of the clinical trial, after which both the key-file and the biopsy samples are destroyed.

Good Clinical Practice and quality assessment

This trial is performed in accordance with the trial protocol and ordinary procedures for quality control and assurance are complied with according to ICH-GCP guidelines. This trial is also in compliance with current laws by health authorities including Danish Medicines Act, §88, section 2, and section 3 and 4 of the Danish executive order on Good Clinical Practice number 744 of June 29, 2006. The trial will be monitored by a Good Clinical Practice unit, Capital Region of Denmark. The existing procedures for quality assurance and control in intervention studies will be followed through a scheduled monitoring agreement at the before the start of phase I, between phase I and II and at the conclusion of phase II. At auditing, monitoring and/or inspection from the Danish Medicines Agency, GCP-unit or from other relevant authorities, these will all be allowed relevant access to the data and information stored. The sponsor is informed in writing by the principal investigator when changes from the approved protocol are made.

Data controllers

Viviane Lin, MD, Department of Surgery, Zealand University Hospital

Project manager

Ismail Gögenur, professor, MD, Department of Surgery, Zealand University Hospital

10. Ethical considerations

Informed consent

Participation in this trial is entirely voluntary, and the patient will only receive preliminary information from the treating gastroenterologist. The treating gastroenterologist will not be directly involved in the study procedures and interventions, in order to minimize any risk of coercion or undue influence of participants. There will be no waiving of the process of informed consent. In the opinion of the investigator, participants must be capable of understanding the purpose of the trial, as well as the potential risks and benefits.

Study patients are included after they have received oral and written information and after written consent has been obtained. At any time, the patients can withdraw their consent without impairing their relationship to the investigator or the doctors involved and without prejudicing their treatment at the Department. All patients can require further information about the project from the responsible clinician. The process of informed consent is described in more detail in chapter 7.

Potential harms and benefits

A large part of the trial procedures are part of regular examinations and treatment of patients seen in the outpatient clinic for pouchitis. The minimal risk of endoscopy of the pouch, blood samples and antibiotic treatment with metronidazole are part of the standard approach in this patient population. This trial adds an additional endoscopy of the pouch after one week, as well as the local application of GM-CSF and fosfomycin. As the medication has previously been tested in more invasive settings (intravenous, subcutaneous, intramuscular and intraperitoneal) without serious adverse reactions, we consider this additional risk permissible. Phase I of the study will test safety and tolerability of the study drug combination, and only if these results are favorable in terms of risk and safety, will a Phase II trial be initiated.

The potential therapeutic benefit of the study would be identifying a new treatment for pouchitis, as well as a better understanding of the disease itself. As of yet, no gold standard of pouchitis treatment exists. Current treatment consists of repeated high doses of oral metronidazole or ciprofloxacin, of which especially metronidazole can have lasting side effects. Loss of effect can result in the need for reoperation in patients with all its potential risks, and creation of an ostomy.

Previous studies have shown some promise in administering GM-CSF in patients with inflammatory bowel disease, and this study will examine the application with a combination with antibiotics locally in the pouch. If effective, this drug combination could potentially lower side effects both short-term and long-term for patients with pouchitis. If no effect can be shown, results from the study will still contribute to our knowledge of the pathophysiology of pouchitis.

We consider the potential harms of the study both in themselves and in relation to the potential benefits of the trial justifiable, and believe that the gain in knowledge and potential new treatment for future patients warrants this trial. It will contribute to the knowledge on the pathogenesis of pouchitis and whether a combination of GM-CSF and antibiotics given locally has an influence on the inflammation of the mucosa.

Standard of care

Patients will not be experiencing any delay in traditional treatment of pouchitis when participating in this trial. Current waiting times on outpatient clinic visits and endoscopy of the pouch, result in start of antibiotic treatment after a few weeks. If included in the trial, patients receive endoscopy and the trial medicine within two weeks, after which standard treatment of oral metronidazole or ciprofloxacin is

administered. Patients participating in the trial will therefore not be suffering symptoms of pouchitis longer than patients not participating in the trial, and no established effective intervention is withheld.

Post-trial access

Post-trial access to the study drug combination will be dependent on the outcome of this study, as well as further clinical studies to assess efficacy and safety in a larger, potentially, randomized placebo-controlled trial.

Choice of study design

The study design is aimed at being a proof-of-concept study, in which it was decided by the sponsor and investigators, that a Phase I of the trial will include 6 patients, and a potential Phase II of the trial 12 patients. Any results from the study will not be able to definitively answer questions of efficacy of the treatment, but will support development of further trials and research into pouchitis. The number of patient visits was chosen to minimize the burden on the participants, while at the same time ensuring patient safety. Follow-up by telephone calls was decided, whenever this was possible.

Choice of study population

The participants studied in this trial all suffer from pouchitis, as diagnosed by a gastroenterologist with experience in the field. Participants are representative of the population that most likely will benefit from results of the study. No healthy volunteers will be studied in this trial, as this is not possible. The study drug combination has previously been tested in healthy males with appendicitis, but not in patients with inflammatory bowel disease or pouchitis. GM-CSF on its own, has been previously tested in invididuals with Crohn's disease, while metronidazole has been used extensively in pouchitis.

The patients will be informed of any additional health information found in the study.

General considerations

The study is performed in accordance with the Helsinki Declaration and Danish law 593 of June 14, 2011, on the Research Ethics Committee's practices regarding health research trials. The project has been approved by the departmental management, Region Sjællands data application system (REG-106-2020), the Regional Research Ethics Committee (conditional approval, application no: 75784), and the Danish Medicines Agency (conditional approval). The study is registered at the EudraCT (2020-000609-10) and will be registered at www.clinicaltrials.gov. The trial will be monitored by the local GCP Unit. Any modification of the protocol which may impact the conduct of the study, potential benefit of the patient or may affect patient safety, including changes of study objectives, study design, patient population, sample sizes, study procedures, or significant administrative aspects will require a formal amendment to the protocol. An amendment will be agreed upon by the study group and will need approval by the Ethics Committee and other authorities as appropriate, prior to implementation.

The study will be conducted in the Department of Surgery of Zealand University Hospital.

11. Funding and insurance

Ismail Gögenur DMSc (sponsor) and Viviane Lin MD (principal investigator) initiated this project. The investigators and the sponsor have no financial interests or conflicts of interest in this study. Reponex Pharmaceuticals A/S (Slotsmarken 12, 1. th. DK-2970 Hørsholm) will distribute the trial drugs to the participating department as well as cover salary expenses for Viviane Lin and the running costs of the trial. The funding source had no role in the design of this study and will not have any role during its

execution, analyses, interpretation of the data, or decision to submit results. The funding is an unrestricted grant to the research unit of 2.3 million DKK. After completion of the trial Reponex Pharmaceuticals A/S will be assigned the sponsor role and its responsibilities.

Information about external funding is included in the written information for the patients. Any external funding will be reported to the Research Ethics Committee with information of the amount and the contributors' names. The written information for the patients will also be updated. There will be no fees for trial participation. Participants will not receive payment for participation, but will get their transportation to treatment refunded, including a taxi from or to work or their home. Also, oral metronidazole or ciprofloxacin for 10 days, the current treatment for pouchitis, will be provided to the patient for free after the second endoscopy.

Patients experiencing injury or harm due to study treatment are covered by the already existing insurance for hospitalized patients in the Department of Surgery, Zealand University Hospital (Patient Insurance Scheme). This is according to the 'Danish Act on the Right to Complain and Receive Compensation' chapter 3, paragraph 19, clause 3: *'Individuals who take part in health research, including clinical trials with drugs, that is not part of the diagnosis or treatment of the individual's disease or illness, are considered equal to patients. This also applies to patients from whom tissue and other biological material is taken.'*

12. Publication of research findings

Following completion of the study, the positive, negative or inconclusive results of this research will be published in an international peer-reviewed scientific journal. Authorship eligibility will be based on the guidelines of the ICMJE (International Committee of Medical Journal Editors).

The Research Ethics Committee and the Danish Medicines Agency will be informed within 90 days when the last patient has had their last visit, and a final report will be submitted within 12 months to the EUDRACT database (European Union Drug Regulating Authorities Clinical Trials database).

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Appendices

Appendix 1 – PDAI

Criteria	Score
Clinical	
Stool frequency	
Usual postoperative stool frequency	0
1–2 stool/day >postoperative usual	1
⩾3 stools/day >postoperative usual	2
Rectal bleeding	
None or rare	0
Present daily	1
Faecal urgency or abdominal cramps	
None	0
Occasional	1
Usual	2
Fever (temperature >37.8°C)	
Absent	0
Present	1
Endoscopic inflammation	
Oedema	1
Granularity	1
Friability	1
Loss of vascular pattern	1
Mucous exudate	1
Ulceration	1
Acute histological inflammation	
Polymorphonuclear leucocyte infiltration	
Mild	1
Moderate+crypt abscess	2
Severe+crypt abscess	3
Ulceration per low power field (mean)	
<25%	1
25–50%	2
>50%	3

Table 1: Pouchitis Disease Activity Index (PDAI). A score of 7 or higher is diagnostic of pouchitis.

Appendix 2 – Study schedules

Phase I Study Schedule

					Follow-Up Schedule
Procedures		Screening Day 0	Baseline Day 1	Follow-up Day 7	Follow-up Day 30
Signed Consent Form		X	X		
Assessment of Eligibility Criteria		X	X		
Review of Medical History		X	X		
Review of Concomitant Medications		X	X	X	X
Application of study drug			X		
Physical Examination	Complete	X		X	
	Symptom-Directed		X		
	Vital Signs		X	X	
Assessment of Adverse reactions			X	X	X
Clinical Laboratory	Chemistry	X		X	
	Hematology	X		X	
Histology	Endoscopy with Biopsies		X	X	
Microbiology			X	X	

Phase II Study Schedule

					Follow-Up Schedule	
Procedures		Screening Day 0	Baseline Day 1	Hospital administrationDay 2-7	Follow-up Day 14	Follow-Up Day 37 (1 month after treatment
Signed Consent Form		X	X			
Assessment of Eligibility Criteria		X	X			
Review of Medical History		X	X			
Review of Concomitant Medications		X	X		X	X
Application of study drug as enema			X	X		
Physical Examination	Complete	X			X	
	Symptom-Directed		X			
	Vital Signs		X		X	
Assessment of Adverse reactions			X	X	X	X
Clinical Laboratory	Chemistry	X			X	
	Hematology	X			X	
Histology	Endoscopy with Biopsies		X		X	
Microbiology			X		X	

Appendix 3 – Enrollment form

Name:

Address:

Telephone no:

Date of birth
Gender
Year diagnosed with ulcerative colitis
Year of IPAA surgery
Allergies
Past medical history □ COPD □ Others □ Diabetes □ Asthma □ Hypertension □ Ischemic heart disease □ Liver disease □ Neurological diseases
Medicine
Past medicine used for pouchitis □ Metronidazole □ Ciprofloxacin □ Ampicillin □ Piperacillin □ Probiotics □ Others
Diets used for pouchitis

Clinical PDAI to be filled in at enrollment:

Criteria	Score	Patient score
Clinical		
Stool frequency Usual postoperative stool frequency 1-2 stools/day > than postoperative usual >3 stools/day > postoperative usual	 0 1 2	
Rectal bleeding None or rare Present daily	 0 1	
Fecal urgency or abdominal cramps None Occasional Usual	 0 1 2	
Fever (temperature > 37.8) Absent Present	 0 1	
Total	**6**	

Clinical examination

Height: Weight: ASA: PS:

Vitals BP Pulse Temperature Saturation RF

Pulmonary stethoscopy

Cardiac stethoscopy

Abdomen

Rectal examination:

Gross neurological examination:

Date Place

Clinical examiner:

Appendix 4 – Endoscopy 1, Phase I and II

Patient name:

Date:

Endoscopic PDAI to be filled in:

Criteria	Score	Patient score
Endoscopy		
Endoscopic inflammation		
Edema	1	
Granularity	1	
Friability	1	
Loss of vascular pattern	1	
Mucous Exudate	1	
Ulceration	1	
Total	**6**	

Endoscopy complications:_____

Did the patient receive analgesia/sedation

□ No

□ Yes, which: _____

Biopsies taken for histology: _____

Biopsies taken for microbiology: _____

GM-CSF Metronidazole/Fosfomycin applied:_____

Medical complications:_____

Date Place

Endoscopy physician:

Appendix 5 – Endoscopy 2, Phase I and II

Patient name:

Date:

Date of application of GM-CSF metronidazole/Fosfomycin:

Endoscopic PDAI to be filled in:

Criteria	Score	Patient score
Endoscopy		
Endoscopic inflammation		
Edema	1	
Granularity	1	
Friability	1	
Loss of vascular pattern	1	
Mucous Exudate	1	
Ulceration	1	
Total	**6**	

Endoscopy complications:_____

Did the patient receive analgesia/sedation

□ No

□ Yes, which: _____

Biopsies taken for histology: _____

Biopsies taken for microbiology: _____

Date Place

Endoscopy physician:

Appendix 6 – Histology

Patient name:

Date:

Histological PDAI to be filled in:

Criteria	Score	Patient score
Histology		
Acute histological inflammation		
Polymorphonuclear leucocyte infiltration		
Mild	1	
Moderate + crypt abscess	2	
Severe + crypt abscess	3	
Ulceration per low power field (mean)		
<25%	1	
25-50%	2	
>50%	3	
Total	**6**	

Date Place

Pathologist:

Appendix 7 – Patient PDAI overview

Patient name:

Criteria	Score	Patient score before treatment Date: __/___/____	Patient score after treatment Date: __/___/____
Clinical			
Stool frequency			
Usual postoperative stool frequency	0		
1-3 stools/day > than postoperative usual	1		
>3 stools/day > postoperative usual	2		
Rectal bleeding			
None or rare	0		
Present daily	1		
Fecal urgency or abdominal cramps			
None	0		
Occasional	1		
Usual	2		
Fever (temperature > 37.8)			
Absent	0		
Present	1		
Endoscopic inflammation			
Edema	1		
Granularity	1		
Friability	1		
Loss of vascular pattern	1		
Mucous Exudate	1		
Ulceration	1		
Acute histological inflammation			
Polymorphonuclear leucocyte infiltration			
Mild	1		
Moderate + crypt abscess	2		
Severe + crypt abscess	3		
Ulceration per low power field (mean)			
<25%	1		
25-50%	2		
>50%	3		
Total	**18**		

Date Place

Examiner:

Appendix 8 – Follow-up Day 7/14/(30/37)

Name:

<table>
<tr><td colspan="3">Side effects:</td></tr>
<tr><td>GM-CSF
□ Fever
□ Myalgia
□ Malaise
□ Abdominal pain
□ Diarrhea
□ Rash
□ Chest pain
□ Weight loss
□ Nausea
□ Vomiting
□ Peripheral edema
□ Dyspnea
□ Hypoxemia
□ Hypotension
□ Tachycardia
□ Flushing □ Metabolic disease
□ Hyperbilirubinemia
□ Cardiac dysrhythmia
□ Pharyngitis</td><td>Metronidazole
□ Nausea
□ Glossitis
□ Dry mouth
□ Dysgeusia
□ Stomatitis
□ Myalgia
□ Candida-infection
□ dark urine</td><td>Fosfomycin
□ Phlebitis
□ Vomiting
□ Abdominal pain
□ Diarrhea
□ Rash
□ Fatigue
□ Dysgeusia
□ Decreased appetite
□ Vertigo
□ Headache</td></tr>
<tr><td colspan="3">Adverse reactions</td></tr>
<tr><td colspan="3">Serious adverse reactions / Life-threatening adverse reactions
□ Capillary leak syndrome</td></tr>
</table>

Clinical PDAI to be filled in at follow-up:

Criteria	Score	Patient score
Clinical		
Stool frequency Usual postoperative stool frequency 1-4 stools/day > than postoperative usual >3 stools/day > postoperative usual	 0 1 2	
Rectal bleeding None or rare Present daily	 0 1	
Fecal urgency or abdominal cramps None Occasional Usual	 0 1 2	
Fever (temperature > 37.8) Absent Present	 0 1	
Total	**6**	

Clinical examination

Height: Weight: ASA: PS:

Vitals BP Pulse Temperature Saturation RF

Pulmonary stethoscopy

Cardiac stethoscopy

Abdomen

Rectal examination:

Gross neurological examination:

Date Place

Clinical examiner:

Appendix 9 – Addendum NVK protocol in Danish

Klausuler i kontrakten mellem sponsor og forsøgsstedet om publikation, honorar til forsker, samt forskers adgang til data på dansk: (jvf. National Videnskabsetisk Komités Krav til protokol)

Publikation

Efter studiets afslutning vil forsøgsresultaterne, såvel positive, negative eller inkonklusive, blive publiceret i et international peer-reviewed videnskabelig tidsskrift. National Videnskabsetisk Komité og Lægemiddelstyrelsen vil blive informeret indenfor 90 dage efter sidste patientkontakt og et afsluttende rapport vil blive indsendt til EUDRACT database.

Honorar til forsker

Dette projekt er initieret af Prof. Ismail Gögenur, forskningsansvarlig overlæge på Kirurgisk Afdeling Sjælland's Universitetshospital og forskningsleder på Center for Surgical Science og læge og PhD-studerende Viviane Lin. Reponex Pharmaceuticals A/S har finansieret projektet og betaler PhD-studerende Viviane Lins løn. Reponex Pharmaceuticals A/S er ikke involveret i hverken planlægning, gennemførelse, analyserne eller interpretation af data samlet af studiet. Støttebeløbet er en ubegrænset bevilling til kirurgisk forskningsenhed 'Center for Surgical Science'.

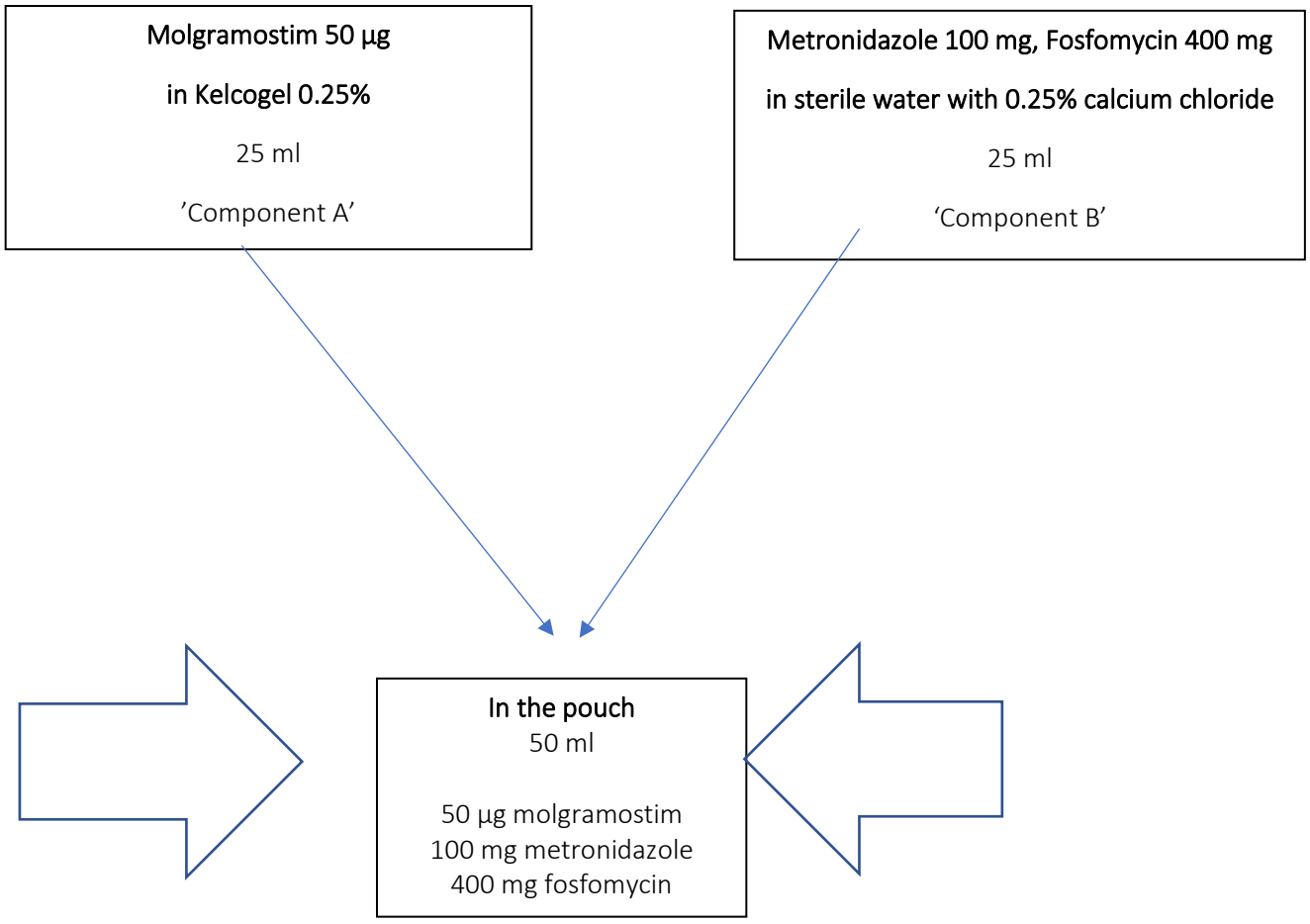
Forskers adgang til data

I overensstemmelse med dansk lovgivning omkring personlige data, søger vi for vores studie godkendelse fra Datatilsynet. Håndtering af data vil ske i overensstemmelse med den generelle databeskyttelsesforordning (GDPR) og loven om databeskyttelse. Vi har fået betinget godkendelse for forsøget fra Videnskabsetisk Komité og Lægemiddelstyrelsen.

Data bliver gemt sikkert i overensstemmelse med GDPR på EasyTrial (sikker datalagring). Hvis en patient frasiger deltagelse i studiet, vil samlet data blive anonymiseret. Hvis patienten ønsker at deres samlet data slettes, gøres dette. Data samles og håndteres af Viviane Lin og Ismail Gögenur (investigatorer) og adgang til Teamsite er begrænset til dem.

Data fra Region Sjælland bliver opbevaret indenfor regionen. Filerne for pseudoanonymisering vil blive opbevaret på en separat data site jvf. GDPR i Region Sjælland.

Appendix 10 – Combination of medicine



Appendix 11 – Suspect adverse reaction report

SUSPECT ADVERSE REACTION REPORT		

I. REACTION INFORMATION

1. PATIENT INITIALS	1a. COUNTRY	2. DATE OF BIRTH			2a. AGE	3. SEX	4-6 REACTION ONSET			8-12 CHECK ALL
(first, last)		Day	Month	Year	Years		Day	Month	Year	APPROPRIATE TO ADVERSE REACTION
7 + 13 DESCRIBE REACTION(S) (including relevant tests/lab data)										☐ PATIENT DIED ☐ INVOLVED OR PROLONGED INPATIENT HOSPITALISATION ☐ INVOLVED PERSISTENT OR SIGNIFICANT DISABILITY OR INCAPACITY ☐ LIFE THREATENING ☐ CONGENITAL ANOMALY ☐ OTHER MEDICALLY IMPORTANT CONDITION

II. SUSPECT DRUG(S) INFORMATION

14. SUSPECT DRUG(S) (include generic name)		20. DID REACTION ABATE AFTER STOPPING DRUG? ☐ YES ☐ NO ☐ NA
15. DAILY DOSE(S)	16. ROUTE(S) OF ADMINISTRATION	21. DID REACTION REAPPEAR AFTER REINTRO-
17. INDICATION(S) FOR USE		DUCTION? ☐ YES ☐ NO ☐ NA
18. THERAPY DATES (from/to)	19. THERAPY DURATION	

III. CONCOMITANT DRUG(S) AND HISTORY

22. CONCOMITANT DRUG(S) AND DATES OF ADMINISTRATION (exclude those used to treat reaction)
23. OTHER RELEVANT HISTORY (e.g. diagnoses, allergies, pregnancy with last menstrual period, etc.)

IV. MANUFACTURER INFORMATION

24a. NAME AND ADDRESS OF MANUFACTURER		26-26a. NAME AND ADRESS OF REPORTER (INCLUDE ZIP CODE)
ORIGINAL REPORT NO.	24b. MFR CONTROL NO.	
24c. DATE RECEIVED BY MANUFACTURER	24d. REPORT SOURCE ☐ STUDY ☐ LITERATURE ☐ HEALTH PROFESSIONAL ☐ REGULATORY AUTHORITY ☐ OTHER	
DATE OF THIS REPORT	25a. REPORT TYPE ☐ INITIAL ☐ FOLLOW-UP	

(Source: cioms.ch)
Council for International Organizations of Medical Sciences (CIOMS)
Case postale 2100
CH-1211 Geneva
info@cioms.ch

Appendix 12 – Procedure for afgivelse af mundtlig deltagerinformation

Hvem afgiver den mundtlige information
Den mundtlige information gives af hovedinvestigator Viviane Lin eller en person bemyndiget hertil, der har de faglige kompetencer til at kunne formidle indholdet.

Hvornår kontaktes forsøgspersonen
Den potentielle forsøgsdeltager med pouchitis gøres opmærksom på at der er et klinisk studie omkring sygdommen, i medicinsk gastroenterologisk ambulatoriet. Hvis patienten er interesseret i at høre mere om forsøget, henvises patienten til hovedinvestigator Vivane Lin. Patienten kan allerede inden et informationsmøde med hovedinvestigatoren, få udleveret skriftlig information omkring forsøget.

Afgivelse af mundtlig og skriftlig information
Den skriftlige information afgives før den mundtlige information. Den mundtlige information vil blive tilpasset modtagerens individuelle forudsætninger, og uden brug af tekniske eller værdiladede vendinger Der vil være tilstrækkelig tid til at lytte til informationen og til at stille spørgsmål til eller få uddybet indholdet.

Forstyrrelser
Informationssamtalen kan finde sted på et i samtalerum tilknyttet afdelingen. På denne måde sikres det, at samtalen kan foregå uforstyrret.

Bisidder
Forsøgspersonen informeres om muligheden for bisidder. Ønskes dette, udskydes eller gentages informationssamtalen.

Betænkningstid
Behovet for betænkningstid mellem den mundtlige/skriftlige information og underskrift på samtykkeerklæringen afgøres af den informerede part, alternativt af den person, der giver informationerne, hvis denne skønner at der er behov for dette. Der skal være mulighed for mindst 24 timers betænkningstid mellem informationsmødet og underskrift på samtykkeerklæringen.
Såfremt der er behov for betænkningstid, vil samtykket forsøges indhentet den efterfølgende dag.

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Appendix 14: Flow-chart of the clinical trial – Phase I

Screening gastroenterology outpatient clinic

Patients with pouchitis

Enrollment visit

- Information meeting with the investigator
- Inclusion & Exclusion criteria

Informed Consent

Day 1 – The Intervention

- Physical examination
- Blood samples
- Endoscopy with biopsies and **study drug administration**
- Adverse reactions

Day 7 – Follow-up visit

- Physical examination
- Blood samples
- Endoscopy with biopsies
- Adverse reactions

10 days oral metronidazole or ciprofloxacin

Day 30 – Follow up

- Telephone call with the investigator to ask about symptoms of pouchitis and ask for adverse reactions

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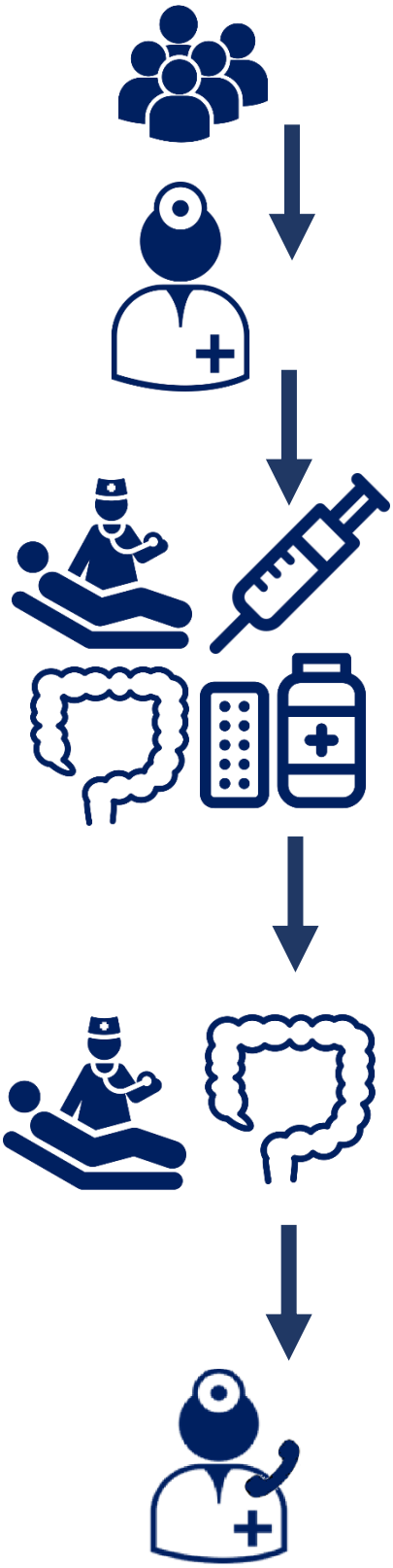
Appendix 15: Flow-chart of the clinical trial – Phase II



Screening gastroenterology outpatient clinic

Patients with pouchitis

Enrollment visit

- Information meeting with the investigator
- Inclusion & Exclusion criteria

Informed Consent

Day 1 – The Intervention

- Physical examination
- Blood samples
- Endoscopy with biopsies and **study drug administration**
- Adverse reactions

6 further days with daily study drug application at the hospital

Day 14 – Follow-up visit

- Physical examination
- Blood samples
- Endoscopy with biopsies
- Adverse reactions

10 days oral metronidazole or ciprofloxacin

Day 37 – Follow up

- Telephone call with the investigator to ask about symptoms of pouchitis and ask for adverse reactions