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# Acute Appendicitis and Microbiota – Etiology and Effects of Antimicrobial Treatment: Study protocol for the MAPPAC (Microbiology Appendicitis Acuta) Trial

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## ABSTRACT

**Introduction:** Based on epidemiological and clinical data acute appendicitis can present either as uncomplicated or complicated. Recent studies have shown the safety, efficacy and cost-effectiveness of antibiotics for CT-confirmed uncomplicated acute appendicitis. The etiology of these different appendicitis forms remains unknown. Complicated acute appendicitis most often requires emergency appendectomy. Despite appendicitis being one of the most common surgical emergencies, there are very few reports on appendicitis etiology and pathophysiology focusing on the differences between uncomplicated and complicated appendicitis. MAPPAC trial aims to evaluate microbiological etiology of these different forms also assessing both antibiotics non-responders and recurrent appendicitis after successful antibiotic treatment. MAPPAC also aims to determine antibiotic and placebo effects on gut microbiota composition and antimicrobial resistance.

**Methods and analysis:** MAPPAC is a prospective clinical trial with both single- and multicentre arm conducted in close synergy with concurrent trials APPAC II (p.o. vs. i.v+p.o. antibiotics, NCT03236961) and APPAC III (double-blind trial placebo vs. antibiotics, NCT03234296) randomized clinical trials further aiming to optimise the non-operative treatment of uncomplicated acute appendicitis. Based on the enrolment for these trials, patients with CT-confirmed uncomplicated acute appendicitis are recruited also to the MAPPAC study. In addition to conservatively treated uncomplicated acute appendicitis patients, MAPPAC will recruit patients with uncomplicated and complicated appendicitis undergoing appendectomy. Rectal and appendiceal swabs, appendicolith, faecal, and serum samples, appendiceal biopsies, and clinical data are collected during the hospital stay for microbiological and immunological analysis in both study arms with the longitudinal study arm collecting samples also during follow-up up to 12 months after appendicitis treatment.

**Ethics and dissemination:** This study has been approved by the Ethics Committee of the Hospital District of Southwest Finland (Turku University Hospital) and the Finnish Medicines Agency (Fimea). Results of the trial will be published in peer-reviewed journals.

**Trial registration:** Eudra-CT: 2016-003655-29 (2017-01-12), clinicaltrials.gov NCT03257423.

**KEYWORDS**

Appendicitis, appendectomy, appendicitis etiology, appendicolith, microbiota, antimicrobial resistance

## Strengths and limitations of this study

- To our knowledge, MAPPAC (Microbiology APPendicitis ACuta) is the first prospective trial assessing the role of microbiology and immunology in the etiology of acute appendicitis in a large patient cohort consisting of CT-diagnosed uncomplicated and complicated acute appendicitis patients also specifically evaluating microbiological and immunological aspects of appendicoliths and recurrent appendicitis after initial successful conservative treatment.
- The strong synergy between two ongoing randomized clinical trials (APPAC II and APPAC III) enabling a large prospective patient cohort of acute appendicitis patients with associated clinical data to be assessed with the microbiological and immunological findings.
- The application of next generation sequencing combined with traditional culturing methods will provide reliable information about the microbiological factors in the etiology of complicated and uncomplicated acute appendicitis
- The comprehensive approach of the MAPPAC study acquiring a large set of samples in the emergency surgery setting presents a challenge to surgeons on call and some patients may not have all study samples available.

## INTRODUCTION

Acute appendicitis is one of the most common causes of abdominal pain in emergency departments. The lifetime risk of acute appendicitis in males is 8.6% and 6.7% in females [1] with recent meta-analysis showing an increasing trend in appendicitis incidence in the industrialised countries [2]. Appendectomy has unquestionably been the standard treatment for acute appendicitis for over a century with more than 300 000 appendectomies performed annually in the United States [3]. Although appendectomy is generally well tolerated, it is a major surgical intervention potentially associated with postoperative morbidity [4-6]. Based on epidemiological and clinical data, acute appendicitis can present either as uncomplicated or complicated with the majority of cases being uncomplicated. Increasing amount of evidence shows that the majority of patients with uncomplicated acute appendicitis may be treated with antibiotics alone instead of surgery [7-13]. The original APPAC (APPendicitis ACuta) trial reported that at long-term follow-up, the majority of patients with computed tomography (CT) confirmed uncomplicated acute appendicitis were successfully treated with antibiotics, and those patients that required later appendectomy did not have increased or major complications [10, 14]. Antibiotics alone is an efficient and a safe first-line treatment for CT-confirmed uncomplicated acute appendicitis [8, 15-17]. In addition to decreased morbidity, antibiotic therapy was shown to offer significant cost savings compared to surgery [18].

The different epidemiological trends of uncomplicated and complicated acute appendicitis indicate different pathophysiology [3]. Despite the high incidence of acute appendicitis, there are very few reports on appendicitis etiology and pathophysiology especially focusing on the possible differences between uncomplicated and complicated acute appendicitis. Complicated acute appendicitis, defined as a finding of perforation, appendicolith, abscess or a suspicion of tumor, requires emergency appendectomy. Appendicolith is a calcified faecal concretion in the appendix and it is the most common form of complicated acute appendicitis. Even though

the first thorough study on appendicoliths was already reported in 1966 [19], information about appendiceal calculi is scarce. Obstruction of the appendiceal lumen caused by an appendicolith, lymphoid hyperplasia, or swelling has been evaluated to be the primary cause of appendicitis and bacterial overgrowth has been considered a consequence [20]. However, bacterial infection has also been proposed as the primary cause of appendicitis [21, 22]. *Bacteroides* species are reported to be one of most common bacterial findings in appendicitis [23, 24]. Further, certain members of the *Fusobacteria,* especially *F. nucleatum* and *F. necrophorum,* are present in most appendicitis samples [21]. The most common aerobic bacteria organism detected by culturing is *Escherichia coli,* but also *Klebsiella pneumoniae, Streptococcus spp., Enterococcus spp.* and *Pseudomonas aeruginosa* have been reported [25, 26]. To our knowledge, only one study with a very small number of patients has characterised the adult appendiceal microbiota with next generation sequencing (NGS) methods. Appendix seems to have diverse microbiota including both commensal species from gut microbiota (GM) and opportunistic pathogens [27]. Since the interindividual variability in the microbial composition of the appendix samples is high [27], a larger number of appendicitis patients is needed to draw conclusions.

Antimicrobials decrease GM diversity, richness and species variation, and cause a perturbation in its overall balance. Particularly broad-spectrum antibiotics have been shown to have a long-term effect on GM. [31, 32] A prolonged disturbance in GM and the following imbalance with the host and its immune system have been associated with a variety of diseases, such as inflammatory bowel disease [33] and type 2 diabetes [34]. Antibiotic use can further lead to increased prevalence of antibiotic resistant bacteria and antibiotic resistance genes [35-37]. Although the effects of antibiotic treatment on the AMR development is less clear in countries with lower prevalence of resistant bacteria [38, 39], the evaluation of antibiotic treatment effects on GM is essential in the treatment of acute appendicitis.

The MAPPAC (Microbiology APPendicitis ACuta) trial patient enrolment is based on the ongoing APPAC II and APPAC III randomised multicentre clinical trials of our study group. APPAC II trial aims to optimise antibiotic treatment for CT confirmed uncomplicated acute appendicitis in order to both shorten the hospital stay and restrict the antibacterial spectrum. The APPAC III trial aims to assess symptomatic treatment of uncomplicated acute appendicitis and the role of antibiotics in the resolution of uncomplicated appendicitis. To our knowledge, there are so far no similar large microbiological studies focusing on acute appendicitis performed in conjunction with large clinical trials with prospective access to both uncomplicated and complicated appendicitis patients.

MAPPAC trial aims to evaluate the possible role and differences in the microbiological etiology of complicated and uncomplicated appendicitis with a special reference to the presence of an appendicolith. In addition, MAPPAC aims to evaluate the immunological and microbiological factors involved in appendicitis recurrence after successful initial antibiotic therapy. In the longitudinal study arm we also aim to assess the effects of antibiotic and placebo treatment on the GM profile and the effects of hospital stay duration on the AMR reservoir of the GM.

## METHODS AND ANALYSIS

### Study Design

MAPPAC is a prospective clinical trial with a single-centre and a multicentre arm. The single-centre study arm at Turku University Hospital, aims to determine the possible differences in the etiology of complicated and uncomplicated acute appendicitis with a special reference to the role and etiology of appendicolith. In the multi-centre longitudinal arm, MAPPAC trial concentrates on assessment of possible immunological and microbiological factors involved in initial non-responders or appendicitis recurrence after successful initial antibiotic therapy at long-term follow-up. In this longitudinal multicentre study arm we also aim to evaluate the effects of antibiotic and placebo treatment on the GM and the effects of the duration of the hospital stay on the possible AMR reservoir within the GM. MAPPAC protocol was drafted in accordance with the SPIRIT statement [40]. The trial has been registered at both EudraCT (2016-003655-29) and clinicaltrials.gov (NCT03257423).

The three ongoing study entities (APPAC II, APPAC III, and MAPPAC) are strongly interconnected having a common study aim and a patient enrolment population (Figure 1). APPAC II trial is a randomised open-label, non-inferiority multicentre trial to compare intravenous antibiotic therapy followed by per oral (p.o.) antibiotics with p.o. monotherapy in the treatment of uncomplicated acute appendicitis (NCT03236961)[41]. APPAC III trial is a randomised double-blind, placebo-controlled, superiority multicentre study to compare antibiotic therapy with placebo in the treatment of uncomplicated acute appendicitis (NCT03234296) [42]. All incoming patients are informed of all ongoing trials. Enrolment will be based firstly on the time of the day (availability of the hospital pharmacy services for the double-blinding) and secondly on patient preference (patient unwilling to participate in APPAC III, will be informed and invited to participate in APPAC II). During all hours, patients will be invited to participate in APPAC II trial. All patients invited to participate in APPAC II

and III trials will be invited to participate in the MAPPAC trial. Patients recruited for the APPAC II or III trial are asked to sign a separate consent form for the MAPPAC trial allowing for the use of their data and collection of microbiological samples. The study flow is illustrated in Figure 2.

**Patient selection**

Eligible for inclusion are all adult patients 18 – 60 years old admitted to the emergency department with suspected acute appendicitis in whom a CT-confirmed uncomplicated or complicated appendicitis is diagnosed or patients presenting with a suspected recurrent appendicitis after initial successful antibiotic therapy for uncomplicated acute appendicitis. The optimized low-dose and standard CT protocols from our OPTICAP trial [43] are used at all trial hospitals. MAPPAC single-centre arm will enroll patients with both uncomplicated (both APPAC II and III trials and patients declining to participate in these trials undergoing appendectomy) and complicated acute appendicitis (patients excluded from APPAC II and APPAC III trials) at Turku University Hospital. The enrolment of uncomplicated acute appendicitis patients participating in the APPAC III trials will be performed at all APPAC III hospitals (all five Finnish university hospitals: Turku, Helsinki, Tampere, Oulu, and Kuopio). In addition, all of the APPAC II and III trial patients having to undergo appendectomy either for a treatment failure during the primary hospitalisation or for suspected recurrence after a successful initial non-operative treatment will be enrolled in the MAPPAC trial at all ten study hospitals (all five university and five central hospitals in Finland: Jyväskylä, Pori, Mikkeli, Seinäjoki and Rovaniemi).

All adult patients with a clinical suspicion of acute appendicitis will be studied carefully by attending surgeons at the emergency departments of the participating hospitals. Clinical history, physical investigation, and laboratory tests are evaluated and if these indicate acute

appendicitis, the patient will undergo CT imaging with either optimised low-dose (BMI<30 kg/m$^2$) or standard CT (BMI>30kg/m$^2$).

Inclusion criteria

MAPPAC inclusion criteria follow mainly the criteria of APPAC II and APPAC III trials enrolling patients with CT-confirmed uncomplicated acute appendicitis. In addition, MAPPAC includes patients with complicated acute appendicitis and patients under or over the age limit of 18 to 60 years of the APPAC II and APPAC III trials in the single-centre study arm at Turku University Hospital. Inclusion criteria: signed informed consent, CT scan confirmed diagnosis of either uncomplicated acute appendicitis or complicated acute appendicitis (appendicolith, perforation, abscess, suspicion of a tumor), suspected treatment failure of antibiotic therapy for uncomplicated acute appendicitis during the primary hospitalisation of APPAC II or III patients, and suspected recurrent appendicitis after a successful initial antibiotic therapy for uncomplicated acute appendicitis. Patients with uncomplicated acute appendicitis undergoing appendectomy based on the inclusion criteria and declining to participate in the APPAC II or III trials, may also be enrolled in the MAPPAC trial.

Exclusion criteria

MAPPAC exclusion criteria for the patients with uncomplicated acute appendicitis undergoing antibiotic therapy are the same as in APPAC II and III trials: pregnancy or lactating, allergy to contrast media or iodine, allergy or contraindication to antibiotic therapy, renal insufficiency, metformine medication, severe systemic illness such as malignancy, medical condition requiring immunosuppressant medications, inability to co-operate and give informed consent. Diagnostic criteria for uncomplicated acute appendicitis at CT include appendiceal diameter exceeding 6 mm with wall thickening and at least 1 of the following (abnormal contrast enhancement of the appendiceal wall, inflammatory edema or minor fluid collection

around the appendix). CT criteria for complicated acute appendicitis include appendicolith (> 3mm stone within the appendix), abscess (periappendiceal walled of collection with enhancing walls), perforation (appendiceal wall enhancement defect and periappendiceal excess of fluid and/or infectious phlegmon and/or extraluminal air), or tumor suspicion (tumor-like prominence of the appendix).

**Study setting and feasibility**

The study was initiated at Turku University Hospital in April 2017, with the study commencing at all study centres by fall 2017. The majority of MAPPAC patient enrolment is evaluated to be completed in conjunction with the APPAC II and III trials latest by fall 2020. Patient recruitment in the MAPPAC trial will continue for a minimum of five years after APPAC II and III trial enrolment completion based on the planned microbiological assessment of late appendicitis recurrence.

**Interventional groups**

Four interventional groups within MAPPAC are defined as follows:

1. Patients with uncomplicated acute appendicitis participating in the APPAC II trial at Turku University Hospital (MAPPAC single-centre treatment arm) receiving either i.v. antibiotic therapy followed by p.o. antibiotics (i.v. + p.o.) or patients receiving p.o. antibiotic monotherapy (p.o.). *I.v. + p.o.* group receives intravenous ertapenem 1 g once daily for two days followed by p.o. levofloxacin 500 mg once daily and metronidazole 500 mg three times per day for 5 days. *P.o.* group receives *p.o.* moxifloxacin 400 mg once daily for seven days.
2. Patients with uncomplicated acute appendicitis participating in the double-blinded APPAC III trial at all five university hospitals (MAPPAC multicentre treatment arm)

receiving either antibiotic therapy or placebo treatment. *Antibiotic treatment* is intravenous ertapenem 1 g once per day for three days followed by p.o. levofloxacin 500 mg once per day and metronidazole 500 mg three times per day for four days. *Placebo treatment* entails intravenous placebo once per day for three days followed by per oral placebo three times per day for four days.

3. Patients with uncomplicated acute appendicitis or suspected recurrent appendicitis undergoing appendectomy

   The patients in this group will undergo laparoscopic appendectomy either after declining to participate in the APPAC II or APPAC III trials (MAPPAC single-centre treatment arm at Turku University Hospital) or after suspected treatment failure of non-operative treatment or after presenting with suspected appendicitis recurrence after initial successful non-operative treatment (MAPPAC multicentre treatment arm, all APPAC II and APPAC III hospitals)

4. Patients with complicated acute appendicitis undergoing appendectomy

   The patients in this group will undergo laparoscopic appendectomy and are eligible for enrolment only in the MAPPAC single-centre study arm at Turku University Hospital.

**Sample collection**

**Rectal swabs** are collected from all patients in the emergency department prior to antibiotic treatment or surgery (timepoint 0). Further, rectal swabs are collected on the 1st day for APPAC II patients and for APPAC III patients on the 1st day (Turku University Hospital) and on the 3rd day (all five university hospitals). At each time point, two rectal swabs are collected: Amies-Coal transport swab for culturing methods (Sarstedt, Nümbrecht, Germany) and Puritan DNA/RNA swab (Puritan Medical products, Guilford, Maine) with shield fluid (Zymo

Research, Irvine, Canada) for microbial DNA extraction. The shield fluid in this collection tube allows the transportation at room temperature.

**Samples from the appendix** are collected from patients undergoing appendectomy for complicated or uncomplicated acute appendicitis and from patients with suspected disease progression during the primary hospitalisation or appendicitis recurrence after successful initial non-operative therapy. Samples include routine histology as well as specific trial swabs and biopsies. The appendix is opened vertically with a sterile scalpel and two swabs are taken similar to the rectal swab (one for culture and one for DNA extraction). Three biopsies are taken at a distance of approximately 2 cm from the base of the appendix. All biopsies are stored at -75 ◦C, one immediately and the rest two are first embedded in RNAlater solution (Thermo Fisher Scientific, Waltham, MA, USA). Possible appendicoliths are collected into a sterile container, frozen and stored at -75 ◦C. If appendectomy is performed during office hours, appendiceal samples are collected by study personnel and an additional swab for anaerobic culture is then collected and cultured in connection with collection and immediately transferred into an anaerobic jar. During on call hours, the samples are collected by surgeons and the -75 ◦C appendiceal biopsy may not be collected. These appendiceal samples are collected only in the Turku University Hospital and at the other study hospitals only a swab sample (transport tube with DNA shield fluid) from the appendix of non-responders and patients with appendicitis recurrence is collected.

**Additional serum samples** are collected from all the patients recruited in MAPPAC trial at Turku University Hospital and for APPAC III trial at all five study hospitals for future immunological and inflammatory marker analysis and metabolomics approach. The serum samples are divided into six aliquots prior freezing at -75 ◦C.

**Questionnaire**

MAPPAC patients are asked to fill a primary questionnaire during the initial hospitalisation covering topics possibly affecting their GM composition: chronic diseases, special diets, smoking and alcohol consumption, travel history, antibiotic intake, other medications (12 months prior the sampling), consumption of probiotics and other dietary supplements, recent diarrhoea and/or vomiting, Bristol stool form scale estimate [44] measuring stool consistency (1 for very hard stool to 7 for diarrhoea) and the average number of defecations per day.

**Follow-up during the hospitalisation**

During the hospitalisation the following parameters will be recorded every 24 hours: pain assessed by Visual Analogue Scale (VAS), leukocyte count, CRP, temperature and clinical findings at patient reassessment. If the patient is suspected of not responding to the randomized therapy based on clinical deterioration signs combined with laboratory findings (signs of peritonitis, persisting fever, increasing pain, white blood cell count or CRP), the patient will be operated based on the surgeon's decision and the reasons for proceeding to appendectomy will be recorded. For appendectomy, laparoscopic approach is recommended. The operative findings and the histopathology of the appendix will be recorded. After the initial hospitalisation, recurrent acute appendicitis will be diagnosed on a clinical basis and patients with a suspected recurrence of appendicitis will undergo a laparoscopic appendectomy.

**Follow-up**

The specific MAPPAC trial follow-up is conducted for patients also participating in the APPAC II and III trials with collection of three faecal samples at home (at one week, six months and one year). Follow-up samples at home are not collected in MAPPAC interventional groups undergoing appendectomy. The follow-up faecal sample is collected into 10 ml DNA/RNA Shield Faecal Collection Tube (Zymo Research) enabling transportation and storage at room temperature prior to DNA extraction. Patients also deliver a faecal sample into a gel tube for

culture purposes. A follow-up questionnaire is collected at 6 and 12 months consisting of the same questions as the preliminary questionnaire covering the time between hospital discharge and the follow-up time point. Other follow-up for patients in APPAC II trial will include a phone interview at one week after discharge, APPAC III trial patients have their first follow-up call 2-4 days after discharge from the hospital and then at 10 days, both trials at two months and at one, three, five and ten years. The follow-up for APPAC III patients will include laboratory tests (leukocyte count, CRP, additional follow-up serum samples).

**Outcome parameters**

Based on the MAPPAC trial design, no specific primary endpoint can be determined. The following parameters will be recorded for all patients: age, gender, BMI, clinical findings on admission (tympanic temperature, nausea, pain or tenderness in the right lower abdominal quadrant, pain migration, VAS pain scores, white blood cell count, CRP, findings on CT imaging), and data on primary and follow-up questionnaires. Blood cultures will be obtained from patients with complicated acute appendicitis and for APPAC II and III trial patients at Turku University Hospital.

The following outcome parameters will be assessed based on the sample types collected:

*Parameters from the appendix i.e. patients undergoing appendectomy*

Operative details and histopathology of the appendix, host transcriptomics, proteomics, and immunohistochemical analysis from appendiceal biopsy, possible appendicolith composition, morphology, and classification.

*Microbiological parameters from the rectal and appendiceal swabs, faecal and appendiceal samples*

Microbial profile, metagenome and metatranscriptome, name of different identified bacterial species, number of species identified both by NGS and culture methods, antimicrobial

susceptibility test results, the presence of AMR related genes by molecular analysis methods, bacterial genome data of AMR or virulence genes, all microbial data combined with clinical and additional data.

*Serum samples*

In order to compare possible differences between patients with successful antibiotic therapy to patients with either failed antibiotic therapy or complicated acute appendicitis, the serum samples will be analysed to identify possible inflammatory or immunological markers. Biomarker analysis of numerous different cytokines, chemokines and growth factors as well as serum metabolome will be analysed. Additional analysis include the level and activity of CD73 and soluble vascular adhesion protein-1 (VAP-1).

Laboratory methods used in the trial to analyse the collected samples are described in detail in the online supplementary material.

**Statistical analysis**

Based on the explorative research nature of the MAPPAC study, there is not enough information available about the study aims to enable sample size calculations. Categorical variables of the study will be characterised using frequencies and percentages. For continuous variables means and standard deviations or medians with range and 25th and 75th percentiles will be used. In case of categorical outcomes, groups will be compared using Pearson´s Chi-squared –test and if further analyses will be needed, logistic regression models will be used. Group differences in continuous variables will be evaluated using independent samples t-test, analysis of variance (ANOVA) or non-parametric methods, if needed. Associations between continuous variables will be evaluated using correlation coefficients and linear regression analysis and if adjustments are needed, linear models will be used. Continuous outcomes measured in several time-points will be analysed using linear mixed models. For categorical outcomes with repeated measurements generalised linear mixed

models will be used. The assumptions of the methods will be checked for justification of the analyses and transformations will be used for the variables, if needed. The study site differences will be evaluated in statistical models and if major differences are detected, more complicated statistical models will be used in the analyses. Two-sided p-values will be used and p-values less than 0.05 will be considered statistically significant. The measurements with missing data will automatically be excluded from the analyses of the variables in concern. Statistical analyses will be performed using SAS System for Windows, Version 9.4 and JMP Pro 13 or later versions (SAS Institute, Cary, NC, USA).

**Patients and public involvement**

The MAPPAC research questions and outcome measures were based on the results of original APPAC trial [10] and the study protocol was developed together with the study group surgeons, clinical microbiologists and immunologists. Patients were not directly involved in the design of this study and the burden of study participation was not assessed by patients themselves. Upon recruitment, patients are well informed of all aspects of the trial including antibiotic or symptomatic therapy of CT-confirmed uncomplicated acute appendicitis, difference between complicated and uncomplicated acute appendicitis, treatment success, possible late recurrence, and safety in order to help patients make an informed decision about trial participation. Patients also receive additional instructions in a phone call made prior to follow-up sample collection at 6 and 12 months. After completion of data collection and analysis, the patients will be informed of the study results and they will be provided with an opportunity to ask further questions.

## ETHICS AND DISSEMINATION

### Ethics

This protocol has been accepted by the Ethical Committee of the Hospital district of Southwest Finland (Turku University Hospital) and Finnish Medicines Agency (Fimea). The trial will be conducted in compliance with the Declaration of Helsinki.

### Data collection and confidentiality

All data and samples are handled confidentially and the information in the datasets is non-identifiable. Data are gathered during the emergency room visit, hospitalisation for acute appendicitis, clinical observations, and follow-up phone calls. The main investigators will be in charge of the common database with full access to the data which is, otherwise strictly limited. As the MAPPAC and APPAC II and III trials are based on the same patient population, the interventions partly overlap and the enrolled patients are informed about this overlapping of the trials and the acquired data.

### Withdrawal

Patients are informed of their right to withdraw from the study without explanation at any time. In case of patient withdrawal, they will be asked for permission to use their data.

### Dissemination plan

Results from this trial and reported in articles which will be published in peer-reviewed journals. Results are also presented at national and international conferences to further distribute this research.

## DISCUSSION

As non-operative treatment for uncomplicated acute appendicitis has been shown to be efficient and safe also at long-term [10, 13-17] and cost-effective [18], understanding both the etiology of the different appendicitis forms and potentially predicting the recurrences has become of utmost clinical importance in order to thoroughly evaluate all the optimization of the different treatment options. The MAPPAC trial aims to assess this largely unknown microbiological etiology of the different disease forms of acute appendicitis. In addition, the effects of antibiotic and placebo treatments on the GM composition and the effects of the duration of the hospital stay on the AMR reservoir of the GM will be assessed. With the longitudinal study design, both the immediate and long-term effects of the antibiotic treatment on GM can be analysed. As the hospital stay differs in APPAC II and APPAC III patients, the effects of the duration of the hospital stay on the emergence of resistant bacteria and AMR reservoir can be evaluated. Moreover, the comparison of the effects of antibiotic treatment on GM between two different administration routes (p.o. and i.v. + p.o.) is possible. This study design carried out simultaneously with APPAC II and APPAC III clinical trials allows us to collect a unique set of microbiological samples in a large prospective clinical setting, which may provide clinically relevant new knowledge of appendicitis microbiology and immunology potentially having an impact on the treatment of acute appendicitis patients.

In the 5-year follow up of the original APPAC trial, 39 % (n=100) of the antibiotic group patients underwent appendectomy for either during the primary hospitalization or for suspected late appendicitis recurrence. Out of these hundred patients, 85 were operated on for suspected recurrence and 78 patients had a histopathologically confirmed acute appendicitis [14]. Understanding the pathophysiology and contributing factors in recurrent appendicitis are of vital clinical importance in evaluating the optimal treatment paradigms for uncomplicated acute appendicitis. Therefore, MAPPAC trial is assessing both initial non-responders to

antibiotic therapy and recurrent appendicitis after successful initial antibiotic treatment and these results may provide novel tools to predict the potential recurrence risk and thus help to assess the optimal treatment choice for patients with uncomplicated acute appendicitis.

**Strengths and limitations**

MAPPAC has a strong synergy between APPAC II and III studies enabling the enrolment of both uncomplicated and complicated acute appendicitis patients in a large prospective series with associated clinical data to be assessed in conjunction with the microbiological and immunological findings. In order to thoroughly evaluate the microbiological etiology of appendicitis, MAPPAC has characteristics of an observational study. The determination of one primary outcome is insufficient for the study, as several factors are indispensable for the understanding of etiology and the effects of antibiotics on GM all provided with this unique set of microbiological samples. To our knowledge, only one previous study [27] has characterized the adult appendix microbiota during appendicitis in adult patients using NGS technique and to date no reports on the structure and physicochemical contents of appendicoliths exists. Using these assessments is a strength in our study. Further, to our knowledge this is the first trial aiming to prospectively assess the possible microbiological and/or immunological etiology of appendicitis recurrence after a successful initial conservative treatment with antibiotics or symptomatic therapy and primary non-responders to conservative treatment of uncomplicated acute appendicitis. The third strong element of the study is the accuracy of differential diagnosis between complicated and uncomplicated acute appendicitis as all patients included in the study are imaged with CT protocol. CT scan is the gold standard for acute appendicitis imaging and this near perfect accuracy of CT adds to the reliability of the clinical data and appendicitis severity also in the patients with uncomplicated acute appendicitis without a histopathological confirmation of the appendix.

The study limitations include the difficult challenge of conducting prospective clinical trials in the emergency setting. It is expected that all eligible patients may not be evaluated for enrolment or some patients may not have all study samples available as the recruitment is performed by a large number of surgeons on call. The lack of healthy control group is a limitation in the study when determining the effects of antibiotics on GM, as the results cannot be fully distinguished from the effects of acute appendicitis.

## Authors' contributions

All authors have contributed to the design of this trial protocol. PS is the chief investigator. PS, SV, AJH, EM, SJ, JG, and SS have initiated the project. The protocol was drafted by PS, which was refined by SV, AJH, EM, SS, SJ, JG, HM, EE, and SH. Statistical advice was provided by SH. SV was responsible for drafting the manuscript, which was refined by PS, AJH, and EM. All authors have read and approved the final manuscript.

In addition, The APPAC collaborative study group lead by primary investigator Paulina Salminen includes the following contributors: Sallinen V., Leppäniemi A., Rautio T., Meriläinen S., Nordström P., Laukkarinen J., Rantala T., Savolainen H., Aarnio M., Mattila A., Haijanen J., Sävelä E-L., Imre I., Paajanen H., Rintala J., Pinta T., Sippola T., and Böckerman P. All contributors are local investigators who are responsible for execution of the APPAC II and/or APPAC III trials in addition to execution of the applicable parts of the MAPPAC trial and valid data gathering. They have all read and approved the final manuscript and they will be included in the future MAPPAC trial reports, when applicable. The surgical departments of the following Finnish Hospitals contribute to the execution of this trial: University hospitals of Turku, Helsinki, Oulu, Tampere, and Kuopio, central hospitals of Jyväskylä, Pori, Mikkeli, Seinäjoki, and Rovaniemi.

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## Competing interests

Authors declare they have no competing interests.

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**REFERENCES**

1 Addiss DG, Shaffer N, Fowler BS, et al. The epidemiology of appendicitis and appendectomy in the United States, *Am J Epidemiol* 1990;132:910-25.

2 Ferris M, Quan S, Kaplan B, et al. The Global Incidence of Appendicitis: A Systematic Review of Population-based Studies, *Annals of Surgery* 2017;266:237-41 doi:10.1097/SLA.0000000000002188.

3 Livingston EH, Fomby TB, Woodward WA, et al. Epidemiological Similarities Between Appendicitis and Diverticulitis Suggesting a Common Underlying Pathogenesis, *Archives of Surgery* 2011;146:308-14 doi:10.1001/archsurg.2011.2.

4 Dai L, Shuai J. Laparoscopic versus open appendectomy in adults and children: A meta-analysis of randomized controlled trials, *United European Gastroenterol J* 2017;5:542-53 doi:10.1177/2050640616661931.

5 Leung TTW, Dixon E, Gill M, et al. Bowel obstruction following appendectomy: what is the true incidence? *Ann Surg* 2009;250:51-3 doi:10.1097/SLA.0b013e3181ad64a7.

6 Margenthaler JA, Longo WE, Virgo KS, et al. Risk factors for adverse outcomes after the surgical treatment of appendicitis in adults, *Ann Surg* 2003;238:59-66 doi:10.1097/01.SLA.0000074961.50020.f8.

7 Hansson J, Körner U, Ludwigs K, et al. Antibiotics as first-line therapy for acute appendicitis: evidence for a change in clinical practice, *World J Surg* 2012;36:2028-36 doi:10.1007/s00268-012-1641-x.

8 Harnoss JC, Probst P, Büchler MW, et al. Antibiotics Versus Appendicectomy for the Treatment of Uncomplicated Acute Appendicitis: An Updated Meta-Analysis of Randomised Controlled Trials by Rollins et al, *World J Surg* 2017;41:2411 doi:10.1007/s00268-016-3864-8.

9 Sallinen V, Tikkinen KAO. Antibiotics or Appendectomy for Acute Non-Perforated Appendicitis--How to Interpret the Evidence? *Scand J Surg* 2016;105:3-4 doi:10.1177/1457496916632188.

10 Salminen P, Paajanen H, Rautio T, et al. Antibiotic Therapy vs Appendectomy for Treatment of Uncomplicated Acute Appendicitis: The APPAC Randomized Clinical Trial, *JAMA* 2015;313:2340-8.

11 Styrud J, Eriksson S, Nilsson I, et al. Appendectomy versus Antibiotic Treatment in Acute Appendicitis. A Prospective Multicenter Randomized Controlled Trial, *World J Surg* 2006;30:1033 doi:10.1007/s00268-005-0304-6.

12 Varadhan KK, Humes DJ, Neal KR, et al. Antibiotic therapy versus appendectomy for acute appendicitis: a meta-analysis, *World J Surg* 2010;34:199-209 doi:10.1007/s00268-009-0343-5.

13 Vons C, Barry C, Maitre S, et al. Amoxicillin plus clavulanic acid versus appendicectomy for treatment of acute uncomplicated appendicitis: an open-label, non-inferiority, randomised controlled trial, *Lancet* 2011;377:1573-9 doi:10.1016/S0140-6736(11)60410-8.

14 Salminen P, Tuominen R, Paajanen H, et al. Five-Year Follow-up of Antibiotic Therapy for Uncomplicated Acute Appendicitis in the APPAC Randomized Clinical Trial, *JAMA* 2018;320:1259-65 doi:10.1001/jama.2018.13201 [doi].

15 Rollins KE, Varadhan KK, Neal KR, et al. Antibiotics Versus Appendicectomy for the Treatment of Uncomplicated Acute Appendicitis: An Updated Meta-Analysis of Randomised Controlled Trials, *World J Surg* 2016;40:2305-18 doi:10.1007/s00268-016-3561-7 [doi].

16 Sallinen V, Akl EA, You JJ, et al. Meta-analysis of antibiotics versus appendicectomy for non-perforated acute appendicitis, *Br J Surg* 2016;103:656-67 doi:10.1002/bjs.10147 [doi].

17 Sakran JV, Mylonas KS, Gryparis A, et al. Operation versus antibiotics--The "appendicitis conundrum" continues: A meta-analysis, *J Trauma Acute Care Surg* 2017;82:1129-37 doi:10.1097/TA.0000000000001450 [doi].

18 Sippola S, Grönroos J, Tuominen R, et al. Economic evaluation of antibiotic therapy versus appendicectomy for the treatment of uncomplicated acute appendicitis from the APPAC randomized clinical trial, *British Journal of Surgery* 2017;104:1355-61 doi:10.1002/bjs.10575.

19 Forbes GB, Lloyd-Davies RW. Calculous disease of the vermiform appendix, *Gut* 1966;7:583-92 doi:10.1136/gut.7.6.583.

20 Lamps LW. Infectious Causes of Appendicitis, *Infectious Disease Clinics of North America* 2010;24:995-1018 doi:10.1016/j.idc.2010.07.012.

21 Swidsinski A, Doerffel Y, Loening-Baucke V, et al. Acute appendicitis is characterised by local invasion with Fusobacterium nucleatum/necrophorum, *Gut* 2011;60:34-40 doi:10.1136/gut.2009.191320.

22 Zhong D, Brower-Sinning R, Firek B, et al. Acute appendicitis in children is associated with an abundance of bacteria from the phylum Fusobacteria, *J Pediatr Surg* 2014;49:441-6 doi:10.1016/j.jpedsurg.2013.06.026 [doi].

23 Jindal N, Kaur GD, Arora S, et al. Bacteriology of acute appendicitis with special reference to anaerobes, *Indian J Pathol Microbiol* 1994;37:299-305.

24 Martirosian G, Bulanda M, Wojcik-Stojek B, et al. Acute appendicitis: the role of enterotoxigenic strains of Bacteroides fragilis and Clostridium difficile, *Med Sci Monit* 2001;7:382-6.

25 Chen CY, Chen YC, Pu HN, et al. Bacteriology of acute appendicitis and its implication for the use of prophylactic antibiotics, *Surg Infect (Larchmt)* 2012;13:383-90 doi:10.1089/sur.2011.135 [doi].

26 Reinisch A, Malkomes P, Habbe N, et al. Bad bacteria in acute appendicitis: rare but relevant, *Int J Colorectal Dis* 2017;32:1303-11 doi:10.1007/s00384-017-2862-0 [doi].

27 Guinane CM, Tadrous A, Fouhy F, et al. Microbial composition of human appendices from patients following appendectomy, *MBio* 2013;4:12.

28 Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation, *Proc Natl Acad Sci U S A* 2011;108 Suppl 1:4554-61 doi:10.1073/pnas.1000087107 [doi].

29 Jernberg C, Lofmark S, Edlund C, et al. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota, *ISME J* 2007;1:56-66 doi:ismej20073 [pii].

30 Manichanh C, Rigottier-Gois L, Bonnaud E, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach, *Gut* 2006;55:205-11 doi:gut.2005.073817 [pii].

31 Qin J, Li Y, Cai Z, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes, *Nature* 2012;490:55-60 doi:10.1038/nature11450 [doi].

32 Lindgren M, Lofmark S, Edlund C, et al. Prolonged impact of a one-week course of clindamycin on Enterococcus spp. in human normal microbiota, *Scand J Infect Dis* 2009;41:215-9 doi:10.1080/00365540802651897 [doi].

33 Raymond F, Ouameur AA, Deraspe M, et al. The initial state of the human gut microbiome determines its reshaping by antibiotics, *ISME J* 2016;10:707-20 doi:10.1038/ismej.2015.148 [doi].

34 Jakobsson HE, Jernberg C, Andersson AF, et al. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome, *PLoS One* 2010;5:e9836 doi:10.1371/journal.pone.0009836 [doi].

35 Buelow E, Bello Gonzalez, T D J, Fuentes S, et al. Comparative gut microbiota and resistome profiling of intensive care patients receiving selective digestive tract decontamination and healthy subjects, *Microbiome* 2017;5:z doi:10.1186/s40168-017-0309-z [doi].

36 Card RM, Mafura M, Hunt T, et al. Impact of Ciprofloxacin and Clindamycin Administration on Gram-Negative Bacteria Isolated from Healthy Volunteers and Characterization of the

Resistance Genes They Harbor, *Antimicrob Agents Chemother* 2015;59:4410-6 doi:10.1128/AAC.00068-15 [doi].

37 Chan A, Tetzlaff JM, Altman DG, et al. SPIRIT 2013 statement: defining standard protocol items for clinical trials, *Ann Intern Med* 2013;158:200-7 doi:10.7326/0003-4819-158-3-201302050-00583.

38 Haijanen J, Sippola S, Gronroos J, et al. Optimising the antibiotic treatment of uncomplicated acute appendicitis: a protocol for a multicentre randomised clinical trial (APPAC II trial), *BMC Surg* 2018;18:y doi:10.1186/s12893-018-0451-y [doi].

39 Sippola S, Gronroos J, Sallinen V, et al. A randomised placebo-controlled double-blind multicentre trial comparing antibiotic therapy with placebo in the treatment of uncomplicated acute appendicitis: APPAC III trial study protocol, *BMJ Open* 2018;8:023623 doi:10.1136/bmjopen-2018-023623 [doi].

40 Niiniviita H, Salminen P, Grönroos JM, et al. LOW-DOSE CT PROTOCOL OPTIMIZATION FOR THE ASSESSMENT OF ACUTE APPENDICITIS: THE OPTICAP PHANTOM STUDY, *Radiat Prot Dosimetry* 2017:1-9 doi:10.1093/rpd/ncx070.

41 Lewis SJ, Heaton KW. Stool form scale as a useful guide to intestinal transit time, *Scand J Gastroenterol* 1997;32:920-4 doi:10.3109/00365529709011203 [doi].

## Abbreviations

**AMR:** Antimicrobial resistance

**BMI:** Body mass index

**CRP:** C-reactive protein

**CT:** Computed tomography

**GM:** Gut microbiota

**i.v.:** Intravenous

**MALDI-TOF:** Matrix Assisted Laser Desorption/Ionisation time-of-flight mass spectrometry

**MS:** Mass spectrometry

**NGS:** Next generation sequencing

**p.o.:** Per os

**SPIRIT:** Standard Protocol Items: recommendations for Interventional Trials

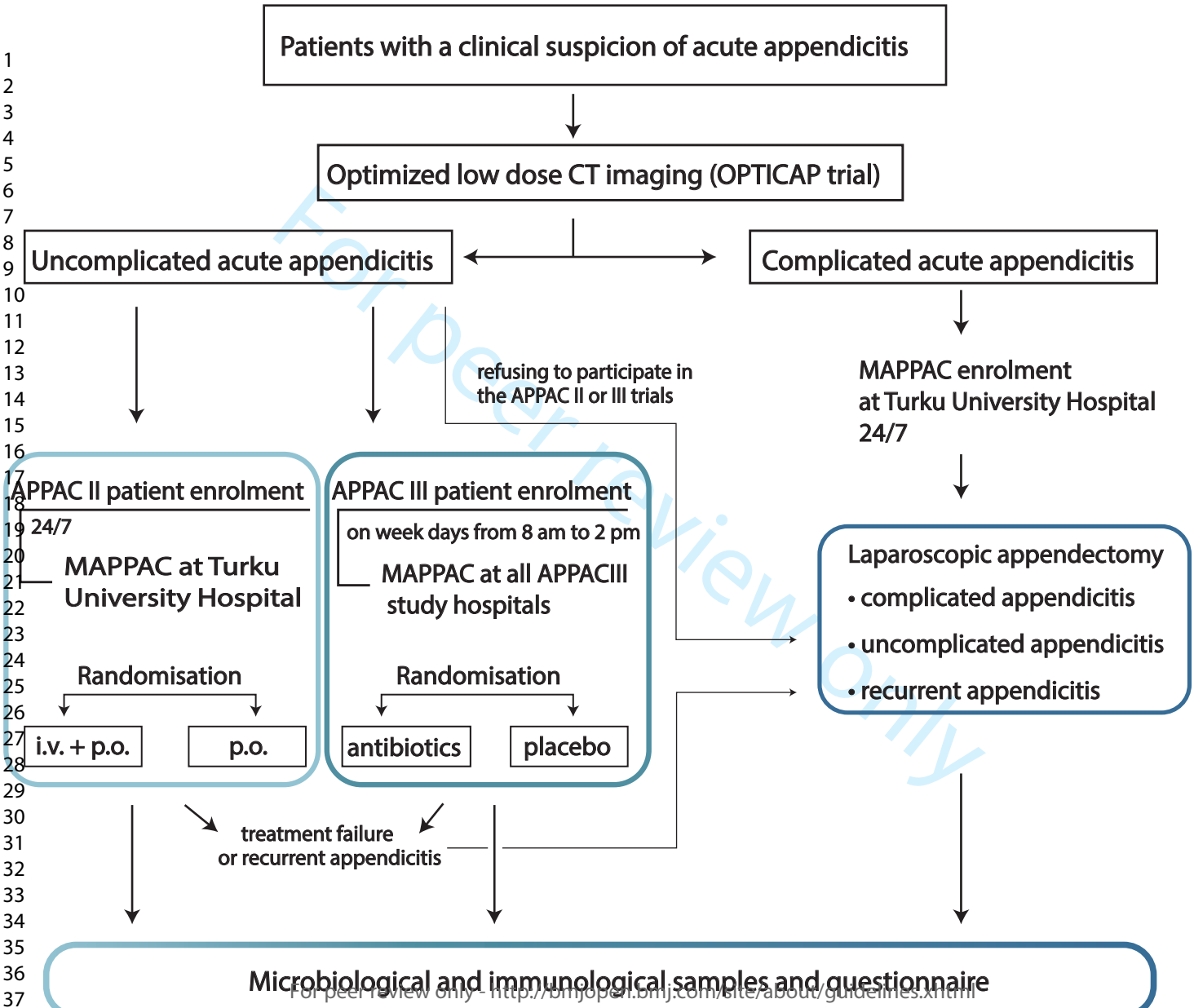
**VAP-1:** Vascular adhesion protein-1

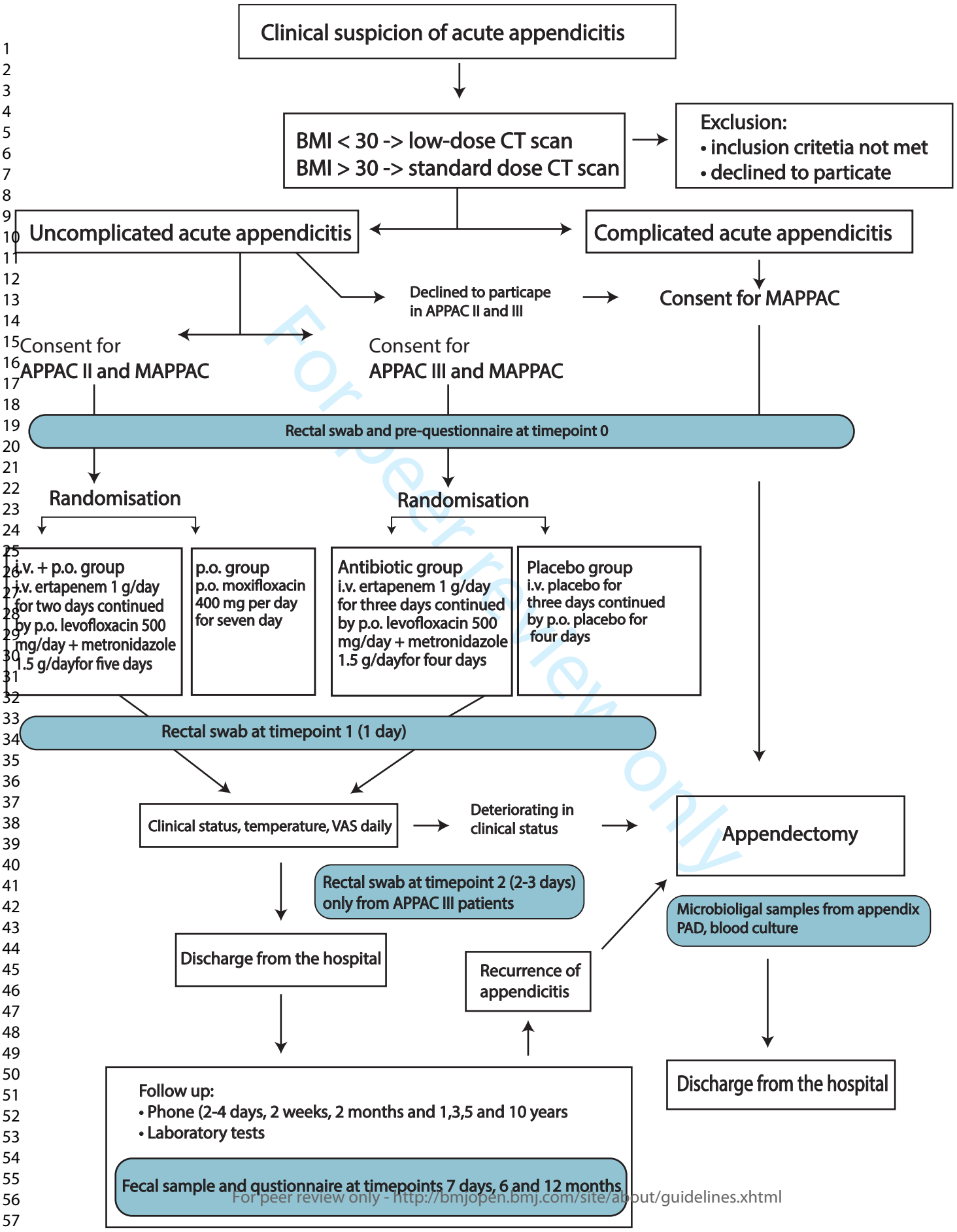
**VAS:** Visual analogue scale

**FIGURE TITLES**

Figure 1.The synergy between MAPPAC, APPAC II and APPAC III studies.

Figure 2. Flow chart of the study protocol





**SUPPLEMENTARY FILE**

**Laboratory methods**

**DNA extraction and analysis**

**Stool samples and rectal and appendiceal swabs**

Bacterial DNA from rectal and appendiceal swabs and faecal samples are extracted with semiautomated DNA extraction kit together with NorDiag Arrow extraction instrument (Diasorin). The following sample preparation is undertaken before the extraction: 500 µl of the sample is added to 700 µl of stool stabilizer in a 1.4 mm Ceramic Powerbead Tube (Qiagen, Hilden, Germany) and homogenized with MO BIO PowerLyzer 24 Bench Top Bead-Based Homogenizer (MO BIO Laboratories, Inc., USA) at 1000 rpm for 3 minutes followed by centrifugation at 5000 x g for 5 minutes and 600 µl of the supernatant is transferred into a new tube, the centrifugation is repeated and 500 µl of the supernatant is transferred into anew tube. This pretreatment is followed by the DNA extraction, which is performed according to the manufacturer´s instructions.

**Appendiceal biopsy:** Microbiological DNA is extracted from appendix biopsy stored in RNAlater with semiautomated DNA extraction kit using NorDiag Arrow extraction instrument. At first RNA later is removed by centrifugation and the pellet is resuspended into ultrapure water. Subsequently, the resuspension containing also the biopsy is treated with proteinase K in thermomixer. From the resulting solution DNA extraction is carried out according to manufacturer's instructions.

The concentration of eluted DNA from all sample types is measured with Qubit dsDNA HS or BR assay kit (Thermo Fisher Scientific) using Qubit fluorometer (Life Technologies, Carlsbad,

California, USA). DNA is divided into two aliquots and stored at -75◦C for later analysis. Microbial profile of samples will be analysed with appropriate methods including NGS approach.

**Analyses of transcriptome and proteome from appendiceal biopsy**

Total RNA and proteins from the appendiceal biopsy are extracted with appropriate methods. Transcriptome is analysed with RNA sequencing using Illumina Hiseq system. In addition, the expression of specific genes is quantified with quantitative real-time PCR. Proteome is characterized using mass spectrometry-based methods with qualitative and quantitative approach.

**Culture methods**

Appendiceal swabs are cultured with both aerobic and anaerobic culture methods. For aerobic culture the following growth media are used: CHROMagar Orientation (Becton Dickinson, Franklin Lakes, New Jersey, USA), Trypticase soy agar with 5% sheep blood (Becton Dickinson), Yersinia selective agar and Streptococcus selective agar (in house production). If the appendectomy is performed during the office hours, an additional anaerobic culture is made in connection with the sample collection. Samples for anaerobic culturing are collected with a sterile cotton swab from the appendix and cultured for fastidious anaerobe agar with blood (FAA) and kanamycin-vancomycin laked blood agar (KVLB) (both in house production), which are immediately transferred into anaerobic jar with anaerobic gas generating sachet (Anaerogen 3,5 l, Thermo Fisher Scientific).

Cultures are incubated at +35 °C for 18-24 hours. Morphologically distinct colonies are subcultured until a pure culture is obtained. Isolated species are frozen in a mixture of skim milk and glycerol at −75 °C.

**MALDI-TOF mass spectrometry**

The identification of isolated bacteria is done with Bruker matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). Isolates are inoculated and cultured for 18-48 hours at +35 °C before the analysis. For the analysis, colony is applied either directly or through ethanol/formic acid extraction on a steel plate and HCCA matrix (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile-2.5% trifluoroacetic acid (Bruker Daltonics, Bremen, Germany)) is added according to the manufacturer´s instructions. The identification is done using Microflex LT instrument and MALDI Biotyper software (Bruker Daltonics). If isolates cannot be identified due to the absence of reference peaks in the database, the isolate is identified with sequencing the 16S rRNA gene.

**Antimicrobial susceptibility testing**

Phenotypic antimicrobial susceptibility testing for the isolated aerobic and anaerobic bacteria is performed by disk diffusion and MIC methods, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. For certain bacteria, the Clinical and Laboratory Standards Institute (CLSI) guidelines can be used.

Further molecular analysis for antimicrobial resistance or virulence genes using e.g., PCR, Sanger sequencing and whole genome sequencing (WGS) are also performed for selected isolates.

**Appendicolith analysis**

Appendicoliths are assessed both on the surface and cross-sectional morphology. Based on the morphological characteristics and a degree of hardness, all appendicoliths will be classified. The composition of selected appendicoliths are analysed with physical and chemical methods.

**Immunological analysis**

The appendiceal biopsies are analysed immunohistochemically by determining the presence of different cell types and blood vessel surface markers. Neutrophils, leukocytes and monocytes will be analysed by cell type in larger groups and special interest will be focused on lymphocyte subtypes (i.e., CD4/CD8 and more detailed subgroup analyses such as regulatory T cells) and monocyte markers (i.e., macrophage M1/M2 / receptor MHCII). Moreover, certain inflammation induced markers on endothelium such as VAP-1, E-selectin and P-selectin will be evaluated. In order to compare possible differences between patients with successful antibiotic therapy to patients with failed antibiotic therapy or complicated acute appendicitis the serum samples will be screened to identify possible inflammatory or immunological markers for identifying the different forms of the disease. For example, cytokines, chemokines and growth factors will be analysed using BioRad Pro multiplex assay system. Moreover, we will use in house analyses to measure soluble VAP-1 (inflammatory) and CD73 (anti-inflammatory). The results obtained from immunological analyses will be correlated to the clinical parameters and to the microbiological findings.

SPIRIT

STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

| Section/item | Item No | Description | Page Number on which item is reported |
|---|---|---|---|
| **Administrative information** | | | |
| Title | 1 | Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym | 1 |
| Trial registration | 2a | Trial identifier and registry name. If not yet registered, name of intended registry | 3 |
| | 2b | All items from the World Health Organization Trial Registration Data Set | n/a |
| Protocol version | 3 | Date and version identifier | n/a |
| Funding | 4 | Sources and types of financial, material, and other support | 22 |
| Roles and responsibilities | 5a | Names, affiliations, and roles of protocol contributors | 1,22 |
| | 5b | Name and contact information for the trial sponsor | 1 |
| | 5c | Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities | 22 |
| | 5d | Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee) | 7-8 |
| **Introduction** | | | |

| | | | |
|---|---|---|---|
| Background and rationale | 6a | Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention | 5-7 |
| | 6b | Explanation for choice of comparators | 6-7 |
| Objectives | 7 | Specific objectives or hypotheses | 7 |
| Trial design | 8 | Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory) | 7 |
| **Methods: Participants, interventions, and outcomes** | | | |
| Study setting | 9 | Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained | 8 |
| Eligibility criteria | 10 | Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists) | 9-11 |
| Interventions | 11a | Interventions for each group with sufficient detail to allow replication, including how and when they will be administered | 11-12 |
| | 11b | Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease) | 14 |
| | 11c | Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests) | 14-15, 17 |
| | 11d | Relevant concomitant care and interventions that are permitted or prohibited during the trial | n/a |
| Outcomes | 12 | Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended | 15-16 |

| | | | |
|---|---|---|---|
| Participant timeline | 13 | Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure) | 12-15, figure 2 |
| Sample size | 14 | Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations | 16, n/a |
| Recruitment | 15 | Strategies for achieving adequate participant enrolment to reach target sample size | 11, 17 |
| **Methods: Assignment of interventions (for controlled trials)** | | | |
| Allocation: | | | n/a |
| Sequence generation | 16a | Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions | n/a |
| Allocation concealment mechanism | 16b | Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned | n/a |
| Implementation | 16c | Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions | n/a |
| Blinding (masking) | 17a | Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how | n/a |
| | 17b | If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial | n/a |
| **Methods: Data collection, management, and analysis** | | | |

| | | | |
|---|---|---|---|
| Data collection methods | 18a | Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol | 12-17, supplementary material |
| | 18b | Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols | 17 |
| Data management | 19 | Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol | 18 |
| Statistical methods | 20a | Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol | 16 |
| | 20b | Methods for any additional analyses (eg, subgroup and adjusted analyses) | 17 |
| | 20c | Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation) | n/a |
| **Methods: Monitoring** | | | |
| Data monitoring | 21a | Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed | 22 |
| | 21b | Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial | n/a |

| | | | |
|---|---|---|---|
| Harms | 22 | Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct | 17, 18 |
| Auditing | 23 | Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor | n/a |
| **Ethics and dissemination** | | | |
| Research ethics approval | 24 | Plans for seeking research ethics committee/institutional review board (REC/IRB) approval | 18 |
| Protocol amendments | 25 | Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators) | n/a |
| Consent or assent | 26a | Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32) | 8-9 |
| | 26b | Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable | 9, 18 |
| Confidentiality | 27 | How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial | 18 |
| Declaration of interests | 28 | Financial and other competing interests for principal investigators for the overall trial and each study site | 22 |
| Access to data | 29 | Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators | 18 |
| Ancillary and post-trial care | 30 | Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation | n/a |
| Dissemination policy | 31a | Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions | 18 |

| | | | |
|---|---|---|---|
| | 31b | Authorship eligibility guidelines and any intended use of professional writers | n/a |
| | 31c | Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code | n/a |
| **Appendices** | | | |
| Informed consent materials | 32 | Model consent form and other related documentation given to participants and authorised surrogates | in finnish only |
| Biological specimens | 33 | Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable | 12-15, supplementary material |

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

BMJ Open

# A Prospective Multicenter Cohort on Acute Appendicitis and Microbiota – Etiology and Effects of Antimicrobial Treatment: Study protocol for the MAPPAC (Microbiology Appendicitis Acuta) Trial



| | |
|---|---|
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| Primary Subject Heading: | Gastroenterology and hepatology |
| Secondary Subject Heading: | Infectious diseases |
| Keywords: | appendicitis, appendectomy, appendicitis etiology, appendicolith, microbiota, antimicrobial resistance |
| | |

# A Prospective Multicenter Cohort on Acute Appendicitis and Microbiota – Etiology and Effects of Antimicrobial Treatment:

# Study protocol for the MAPPAC (Microbiology Appendicitis Acuta) Trial

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## ABSTRACT

**Introduction:** Based on epidemiological and clinical data acute appendicitis can present either as uncomplicated or complicated. The etiology of these different appendicitis forms remains unknown. Antibiotic therapy has been shown to be safe, efficient and cost-effective for computed tomography (CT) confirmed uncomplicated acute appendicitis. Complicated acute appendicitis most often requires emergency appendectomy. Despite appendicitis being one of the most common surgical emergencies, there are very few reports on appendicitis etiology and pathophysiology focusing on the differences between uncomplicated and complicated appendicitis. MAPPAC trial aims to evaluate these microbiological and immunological aspects including immune response in the etiology of these different forms also assessing both antibiotics non-responders and appendicitis recurrence. In addition, MAPPAC also aims to determine antibiotic and placebo effects on gut microbiota composition and antimicrobial resistance.

**Methods and analysis:** MAPPAC is a prospective clinical trial with both single- and multicentre arm conducted in close synergy with concurrent trials APPAC II (p.o. vs. i.v+p.o. antibiotics, NCT03236961) and APPAC III (double-blind trial placebo vs. antibiotics, NCT03234296) randomized clinical trials. Based on the enrolment for these trials, patients with CT-confirmed uncomplicated acute appendicitis are recruited also to the MAPPAC study. In addition to these conservatively treated randomized patients with uncomplicated acute appendicitis, MAPPAC will recruit patients with uncomplicated and complicated appendicitis undergoing appendectomy. Rectal and appendiceal swabs, appendicolith, faecal, and serum samples, appendiceal biopsies, and clinical data are collected during the hospital stay for microbiological and immunological analysis in both study arms with the longitudinal study arm collecting fecal samples also during follow-up up to 12 months after appendicitis treatment.

**Ethics and dissemination:** This study has been approved by the Ethics Committee of the Hospital District of Southwest Finland (Turku University Hospital) and the Finnish Medicines Agency (Fimea). Results of the trial will be published in peer-reviewed journals.

**Trial registration:** Eudra-CT: 2016-003655-29 (2017-01-12), clinicaltrials.gov NCT03257423.

**KEYWORDS**

Appendicitis, appendectomy, appendicitis etiology, appendicolith, microbiota, antimicrobial resistance

## Strengths and limitations of this study

- To our knowledge, MAPPAC (Microbiology APPendicitis ACuta) is the first prospective trial comparing the role of microbiology and immunology including immune response in the etiology of uncomplicated and complicated acute appendicitis in a large patient cohort consisting of CT-diagnosed patients also specifically evaluating appendicoliths and recurrent appendicitis after initial successful conservative treatment.
- The strong synergy between two ongoing randomized clinical trials (APPAC II and APPAC III) enabling a large prospective patient cohort of acute appendicitis patients with associated clinical data to be assessed with the microbiological and immunological findings.
- The application of next generation sequencing combined with traditional culturing methods will provide extensive information about the microbiological factors in the etiology of complicated and uncomplicated acute appendicitis also presenting a challenge in differentiating between etiologic and non-etiologic microbiota in the specimens.
- The comprehensive approach of the MAPPAC study acquiring a large set of samples in the emergency surgery setting presents a challenge to surgeons on call and some patients may not have all study samples available.

## INTRODUCTION

Acute appendicitis is one of the most common causes of abdominal pain in emergency departments. The lifetime risk of acute appendicitis in males is 8.6% and 6.7% in females [1] with recent meta-analysis showing an increasing trend in appendicitis incidence in the industrialised countries [2]. Based on epidemiological and clinical data, acute appendicitis can present either as uncomplicated or complicated with the majority of cases being uncomplicated.

The different epidemiological trends of uncomplicated and complicated acute appendicitis indicate different pathophysiology [3]. Despite the high incidence of acute appendicitis, there are very few reports on appendicitis etiology and pathophysiology especially focusing on the possible differences between uncomplicated and complicated acute appendicitis. Complicated acute appendicitis, defined as a finding of perforation, appendicolith, abscess or a suspicion of tumor, requires emergency appendectomy. Appendicolith is a calcified faecal concretion in the appendix and it is the most common form of complicated acute appendicitis. Even though the first thorough study on appendicoliths was already reported in 1966 [4], information about appendiceal calculi is scarce. Obstruction of the appendiceal lumen caused by an appendicolith, lymphoid hyperplasia, or swelling has been evaluated to be the primary cause of appendicitis and bacterial overgrowth has been considered a consequence [5]. However, bacterial infection has also been proposed as the primary cause of appendicitis [6, 7]. *Bacteroides* species are reported to be one of most common bacterial findings in appendicitis [8, 9]. Further, certain members of the *Fusobacteria,* especially *F. nucleatum* and *F. necrophorum,* are present in most appendicitis samples [6]. The most common aerobic bacteria organism detected by culturing is *Escherichia coli,* but also *Klebsiella pneumoniae, Streptococcus spp., Enterococcus spp.* and *Pseudomonas aeruginosa* have been reported [10, 11]. To our knowledge, only one study with a very small number of patients has characterised

the adult appendiceal microbiota profile with next generation sequencing (NGS) methods. Appendix seems to have diverse microbiota including both commensal species from gut microbiota (GM) and opportunistic pathogens [12, 13]. Since the interindividual variability in the microbial composition of the appendix samples is high [12], a larger number of appendicitis patients is needed to draw the microbiological conclusions. Since most of the species identified from the appendix with both culturing and NGS methods can also be part of normal gut microbiota it is challenging to determine their role in the infection [14]. In addition, the immune response and predisposition for infection by specific bacteria varies between individuals. Consequently, innate immunity is considered to be a contributing factor in the development of complicated appendicitis [15]. In conclusion, several factors have been proposed to take part in the development of appendicitis, most importantly obstruction of the lumen and bacterial infection with this research largely focusing on complicated acute appendicitis [5-7, 16, 17].

Antimicrobials decrease GM diversity, richness and species variation, and cause a perturbation in its overall balance. Particularly broad-spectrum antibiotics have been shown to have a long-term effect on GM profile. [18, 19] A prolonged disturbance in GM and the following imbalance with the host and its immune system have been associated with a variety of diseases, such as inflammatory bowel disease [20] and type 2 diabetes [21]. Antibiotic use can further lead to increased prevalence of antibiotic resistant bacteria and antibiotic resistance genes [22-24]. Although the effects of antibiotic treatment on the development of antimicrobial resistance (AMR) development is less clear in countries with lower prevalence of resistant bacteria [25, 26], the evaluation of antibiotic treatment effects on GM is essential in the treatment of acute appendicitis.

Appendectomy has unquestionably been the standard treatment for acute appendicitis for over a century with more than 300 000 appendectomies performed annually in the United States [3]. The original APPAC (APPendicitis ACuta) trial reported that at long-term follow-up, the majority of patients with computed tomography (CT) confirmed uncomplicated acute appendicitis were successfully treated with antibiotics, and those patients that required later appendectomy did not have increased or major complications [27, 28]. Antibiotic therapy for CT-confirmed uncomplicated acute appendicitis has been shown to be safe, efficient and cost-effective both in adult and paediatric patients [29-33]. APPAC II trial aims to optimise antibiotic treatment for CT confirmed uncomplicated acute appendicitis in order to both shorten the hospital stay and restrict the antibacterial spectrum. The APPAC III trial aims to assess symptomatic treatment of uncomplicated acute appendicitis and the role of antibiotics in the resolution of uncomplicated appendicitis. The MAPPAC (Microbiology APPendicitis ACuta) trial patient enrolment is based on the ongoing APPAC II and APPAC III randomised multicentre clinical trials of our study group. To our knowledge, there are so far no similar large microbiological studies focusing on acute appendicitis performed in conjunction with large clinical trials with prospective access to both uncomplicated and complicated appendicitis patients. MAPPAC trial aims to evaluate the possible role and differences in the microbiological etiology of complicated and uncomplicated appendicitis with a special reference to the presence of an appendicolith. In addition, MAPPAC aims to evaluate the immunological and microbiological factors involved in appendicitis recurrence after successful initial antibiotic therapy. In the longitudinal study arm we also aim to assess the effects of antibiotic and placebo treatment on the GM profile and the effects of hospital stay duration on the AMR reservoir of the GM.

## METHODS AND ANALYSIS

### Study Design

MAPPAC is a prospective clinical trial with a single-centre and a multicentre arm. The single-centre study arm at Turku University Hospital, aims to determine the possible differences in the etiology of complicated and uncomplicated acute appendicitis with a special reference to the role and etiology of appendicolith. In the multi-centre longitudinal arm, MAPPAC trial concentrates on assessment of possible immunological and microbiological factors involved in initial non-responders or appendicitis recurrence after successful initial antibiotic therapy at long-term follow-up. In this longitudinal multicentre study arm we also aim to evaluate the effects of antibiotic and placebo treatment on the GM and the effects of the duration of the hospital stay on the possible AMR reservoir within the GM. MAPPAC protocol was drafted in accordance with the SPIRIT statement [34]. The trial has been registered at both EudraCT (2016-003655-29) and clinicaltrials.gov (NCT03257423).

The three ongoing study entities (APPAC II, APPAC III, and MAPPAC) are strongly interconnected having a common study aim and a patient enrolment population (Figure 1). APPAC II trial is a randomised open-label, non-inferiority multicentre trial to compare intravenous antibiotic therapy followed by per oral (p.o.) antibiotics with p.o. monotherapy in the treatment of uncomplicated acute appendicitis (NCT03236961)[35]. APPAC III trial is a randomised double-blind, placebo-controlled, superiority multicentre study to compare antibiotic therapy with placebo in the treatment of uncomplicated acute appendicitis (NCT03234296) [36]. All incoming patients are informed of all ongoing trials. All patients invited to participate in APPAC II and III trials will be invited to participate in the MAPPAC trial. Patients recruited for the APPAC II or III trial are asked to sign a separate consent form for the MAPPAC trial allowing for the use of their data and collection of microbiological samples. The study flow is illustrated in Figure 2.

**Patient selection**

Eligible for inclusion are all adult patients 18 – 60 years old presenting with either a CT-confirmed uncomplicated or complicated appendicitis or patients with a suspected recurrent appendicitis after initial successful antibiotic therapy for uncomplicated acute appendicitis. MAPPAC single-centre arm will enroll patients with both uncomplicated (both APPAC II and III trials and patients declining to participate in these trials undergoing appendectomy) and complicated acute appendicitis (patients excluded from APPAC II and APPAC III trials) at Turku University Hospital. The enrolment of uncomplicated acute appendicitis patients participating in the APPAC III trials will be performed at all APPAC III hospitals (all five Finnish university hospitals: Turku, Helsinki, Tampere, Oulu, and Kuopio). In addition, all of the APPAC II and III trial patients having to undergo appendectomy either for a treatment failure during the primary hospitalisation or for suspected recurrence after a successful initial non-operative treatment will be enrolled in the MAPPAC trial at all ten study hospitals (all five university and five central hospitals in Finland: Jyväskylä, Pori, Mikkeli, Seinäjoki and Rovaniemi).

**Study setting and feasibility**

The study was initiated at Turku University Hospital in April 2017, with the study commencing at all study centres by fall 2017. The majority of MAPPAC patient enrolment is evaluated to be completed in conjunction with the APPAC II and III trials latest by fall 2020. Patient recruitment in the MAPPAC trial will continue for a minimum of five years after APPAC II and III trial enrolment completion based on the planned microbiological assessment of late appendicitis recurrence.

**Interventional groups**

Four interventional groups within MAPPAC are defined as follows:

1. Patients with uncomplicated acute appendicitis participating in the APPAC II trial at Turku University Hospital (MAPPAC single-centre treatment arm) receiving either i.v. antibiotic therapy followed by p.o. antibiotics (i.v. + p.o.) or patients receiving p.o. antibiotic monotherapy (p.o.). *I.v. + p.o*. group receives intravenous ertapenem 1 g once daily for two days followed by p.o. levofloxacin 500 mg once daily and metronidazole 500 mg three times per day for 5 days. *P.o.* group receives *p.o.* moxifloxacin 400 mg once daily for seven days.
2. Patients with uncomplicated acute appendicitis participating in the double-blinded APPAC III trial at all five university hospitals (MAPPAC multicentre treatment arm) receiving either antibiotic therapy or placebo treatment. *Antibiotic treatment* is intravenous ertapenem 1 g once per day for three days followed by p.o. levofloxacin 500 mg once per day and metronidazole 500 mg three times per day for four days. *Placebo treatment* entails intravenous placebo once per day for three days followed by per oral placebo three times per day for four days.
3. Patients with uncomplicated acute appendicitis or suspected recurrent appendicitis undergoing appendectomy

   The patients in this group will undergo laparoscopic appendectomy either after declining to participate in the APPAC II or APPAC III trials (MAPPAC single-centre treatment arm at Turku University Hospital) or after suspected treatment failure of non-operative treatment or after presenting with suspected appendicitis recurrence after initial successful non-operative treatment (MAPPAC multicentre treatment arm, all APPAC II and APPAC III hospitals)
4. Patients with complicated acute appendicitis undergoing appendectomy

The patients in this group will undergo laparoscopic appendectomy and are eligible for enrolment only in the MAPPAC single-centre study arm at Turku University Hospital.

**Sample collection**

**Rectal swabs** are collected from all patients in the emergency department prior to antibiotic treatment or surgery (timepoint 0). Further, rectal swabs are collected on the 1st day for APPAC II patients and for APPAC III patients on the 1st day (Turku University Hospital) and on the 3rd day (all five university hospitals). At each time point, two rectal swabs are collected: Amies-Coal transport swab for culturing methods (Sarstedt, Nümbrecht, Germany) and Puritan DNA/RNA swab (Puritan Medical products, Guilford, Maine) with shield fluid (Zymo Research, Irvine, Canada) for microbial DNA extraction. The shield fluid in this collection tube allows the transportation at room temperature.

**Samples from the appendix** are collected from patients undergoing appendectomy for complicated or uncomplicated acute appendicitis and from patients with suspected disease progression during the primary hospitalisation or appendicitis recurrence after successful initial non-operative therapy. Samples include routine histology as well as specific trial swabs and biopsies. The appendix is opened vertically with a sterile scalpel and two swabs are taken similar to the rectal swab (one for culture and one for DNA extraction). Three biopsies are taken at a distance of approximately 2 cm from the base of the appendix. All biopsies are stored at -75 ◦C, one immediately and the rest two are first embedded in RNAlater solution (Thermo Fisher Scientific, Waltham, MA, USA). Possible appendicoliths are collected into a sterile container, frozen and stored at -75 ◦C. If appendectomy is performed during office hours, appendiceal samples are collected by study personnel and an additional swab for anaerobic culture is then collected and cultured in connection with collection and immediately transferred into an anaerobic jar. During on call hours, the samples are collected

by surgeons and the -75 ◦C appendiceal biopsy may not be collected. These appendiceal samples are collected only in the Turku University Hospital and at the other study hospitals only a swab sample (transport tube with DNA shield fluid) from the appendix of non-responders and patients with appendicitis recurrence is collected.

**Additional serum samples** are collected from all the patients recruited in MAPPAC trial at Turku University Hospital and for APPAC III trial at all five study hospitals for the identification of possible disease-form specific inflammatory biomarkers in serum. The serum samples are divided into six aliquots prior freezing at -75 ◦C.

**Questionnaire**

MAPPAC patients are asked to fill a primary questionnaire during the initial hospitalisation covering topics possibly affecting their GM profile: chronic diseases, special diets, smoking and alcohol consumption, travel history, antibiotic intake, other medications (12 months prior the sampling), consumption of probiotics and other dietary supplements, recent diarrhoea and/or vomiting, Bristol stool form scale estimate [37] measuring stool consistency (1 for very hard stool to 7 for diarrhoea) and the average number of defecations per day.

**Follow-up during the hospitalisation**

During the hospitalisation the following parameters will be recorded every 24 hours: pain assessed by Visual Analogue Scale (VAS), leukocyte count, C-reactive protein (CRP), temperature and clinical findings at patient reassessment. If the patient is suspected of not responding to the randomized therapy based on clinical deterioration signs combined with laboratory findings (signs of peritonitis, persisting fever, increasing pain, white blood cell count or CRP), the patient will be operated based on the surgeon's decision and the reasons for proceeding to appendectomy will be recorded. For appendectomy, laparoscopic approach is recommended. The operative findings and the histopathology of the appendix will be recorded. After the initial hospitalisation, recurrent acute appendicitis will be diagnosed on a

clinical basis and patients with a suspected recurrence of appendicitis will undergo a laparoscopic appendectomy.

**Follow-up**

The specific MAPPAC trial follow-up is conducted for patients also participating in the APPAC II and III trials with collection of three faecal samples at home (at one week, six months and one year). Follow-up samples at home are not collected in MAPPAC interventional groups undergoing appendectomy. The follow-up faecal sample is collected into 10 ml DNA/RNA Shield Faecal Collection Tube (Zymo Research) enabling transportation and storage at room temperature prior to DNA extraction. Patients also deliver a faecal sample into a gel tube for culture purposes. A follow-up questionnaire is collected at 6 and 12 months consisting of the same questions as the preliminary questionnaire covering the time between hospital discharge and the follow-up time point. Other follow-up for patients in APPAC II trial will include a phone interview at one week after discharge, APPAC III trial patients have their first follow-up call 2-4 days after discharge from the hospital and then at 10 days, both trials at two months and at one, three, five and ten years. The follow-up for APPAC III patients will include laboratory tests (leukocyte count, CRP, additional follow-up serum samples).

**Outcome parameters**

Based on the MAPPAC trial design, no specific primary endpoint can be determined. The following parameters will be recorded for all patients: age, gender, Body mass index, clinical findings on admission (tympanic temperature, nausea, pain or tenderness in the right lower abdominal quadrant, pain migration, VAS pain scores, white blood cell count, CRP, findings on CT imaging), and data on primary and follow-up questionnaires. Blood cultures will be obtained from patients with complicated acute appendicitis and for APPAC II and III trial patients at Turku University Hospital.

The following outcome parameters will be assessed based on the sample types collected:

*Parameters from the appendix i.e. patients undergoing appendectomy*

Operative details and histopathology of the appendix, host transcriptomics, proteomics, and immunohistochemical analysis from appendiceal biopsy, possible appendicolith composition, morphology, and classification.

*Microbiological parameters from the rectal and appendiceal swabs, faecal and appendiceal samples*

Microbial profile, metagenome and metatranscriptome, name of different identified bacterial species, number of species identified both by NGS and culture methods, antimicrobial susceptibility test results, the presence of AMR related genes by molecular analysis methods, bacterial genome data of AMR or virulence genes, all microbial data combined with clinical and additional data.

*Serum samples*

In order to compare possible differences between patients with successful antibiotic therapy to patients with either failed antibiotic therapy or complicated acute appendicitis, the serum samples will be analysed to identify possible inflammatory or immunological markers. Different cytokines, chemokines and growth factors as well as serum metabolome will be included in these analyses. Additional analysis include the level and activity of CD73 and soluble vascular adhesion protein-1 (VAP-1). List for analytes that will be screened is provided in the supplementary methods.

Laboratory methods used in the trial to analyse the collected samples are described in detail in the online supplementary material.

## Statistical analysis

Based on the explorative research nature of the MAPPAC study, there is not enough information available about the study aims to enable sample size calculations. Categorical variables of the study will be characterised using frequencies and percentages. For continuous variables means and standard deviations or medians with range and 25th and 75th percentiles will be used. In case of categorical outcomes, groups will be compared using Pearson´s Chi-squared –test and if further analyses will be needed, logistic regression models will be used. Group differences in continuous variables will be evaluated using independent samples t-test, analysis of variance (ANOVA) or non-parametric methods, if needed. Associations between continuous variables will be evaluated using correlation coefficients and linear regression analysis and if adjustments are needed, linear models will be used. Continuous outcomes measured in several time-points will be analysed using linear mixed models. For categorical outcomes with repeated measurements generalised linear mixed models will be used. The assumptions of the methods will be checked for justification of the analyses and transformations will be used for the variables, if needed. The study site differences will be evaluated in statistical models and if major differences are detected, more complicated statistical models will be used in the analyses. Two-sided p-values will be used and p-values less than 0.05 will be considered statistically significant. The measurements with missing data will automatically be excluded from the analyses of the variables in concern. Statistical analyses will be performed using SAS System for Windows, Version 9.4 and JMP Pro 13 or later versions (SAS Institute, Cary, NC, USA).

## Patients and public involvement

The MAPPAC research questions and outcome measures were based on the results of original APPAC trial [27] and the study protocol was developed together with the study group

surgeons, clinical microbiologists and immunologists. Patients were not directly involved in the design of this study and the burden of study participation was not assessed by patients themselves. Upon recruitment, patients are well informed of all aspects of the trial including antibiotic or symptomatic therapy of CT-confirmed uncomplicated acute appendicitis, difference between complicated and uncomplicated acute appendicitis, treatment success, possible late recurrence, and safety in order to help patients make an informed decision about trial participation. Patients also receive additional instructions in a phone call made prior to follow-up sample collection at 6 and 12 months. After completion of data collection and analysis, the patients will be informed of the study results and they will be provided with an opportunity to ask further questions.

## ETHICS AND DISSEMINATION

### Ethics

This protocol has been accepted by the Ethical Committee of the Hospital district of Southwest Finland (Turku University Hospital) and Finnish Medicines Agency (Fimea). The trial will be conducted in compliance with the Declaration of Helsinki.

### Data collection and confidentiality

All data and samples are handled confidentially and the information in the datasets is non-identifiable. Data are gathered during the emergency room visit, hospitalisation for acute appendicitis, clinical observations, and follow-up phone calls. The main investigators will be in charge of the common database with full access to the data which is, otherwise strictly limited. As the MAPPAC and APPAC II and III trials are based on the same patient population, the interventions partly overlap and the enrolled patients are informed about this overlapping of the trials and the acquired data.

## Withdrawal

Patients are informed of their right to withdraw from the study without explanation at any time. In case of patient withdrawal, they will be asked for permission to use their data.

## Dissemination plan

Results from this trial and reported in articles which will be published in peer-reviewed journals. Results are also presented at national and international conferences to further distribute this research.

## DISCUSSION

As non-operative treatment for uncomplicated acute appendicitis has been shown to be efficient and safe also at long-term [27-30, 33, 38] and cost-effective [39], understanding both the etiology of the different appendicitis forms and potentially predicting the recurrences has become of utmost clinical importance in order to thoroughly evaluate all the optimization of the different treatment options. The MAPPAC trial aims to assess this largely unknown microbiological etiology of the different disease forms of acute appendicitis. Further, the whole microbial entity i.e. profile both in the appendix and in gut is studied, and their role in the disease severity and form is assessed. In addition, the effects of antibiotic and placebo treatments on the GM profile and the effects of the duration of the hospital stay on the AMR reservoir of the GM will be assessed. With the longitudinal study design, both the immediate and long-term effects of the antibiotic treatment on GM can be analysed. As the hospital stay differs in APPAC II and APPAC III patients, the effects of the duration of the hospital stay on the emergence of resistant bacteria and AMR reservoir can be evaluated. Moreover, the comparison of the effects of antibiotic treatment on GM between two different administration routes (p.o. and i.v. + p.o.) is possible.

In the 5-year follow up of the original APPAC trial, 39 % (n=100) of the antibiotic group patients underwent appendectomy for either during the primary hospitalization or for suspected late appendicitis recurrence. Out of these hundred patients, 85 were operated on for suspected recurrence and 78 patients had a histopathologically confirmed acute appendicitis [28]. Understanding the pathophysiology and contributing factors in recurrent appendicitis are of vital clinical importance in evaluating the optimal treatment paradigms for uncomplicated acute appendicitis. Therefore, MAPPAC trial is assessing both initial non-responders to antibiotic therapy and recurrent appendicitis after successful initial antibiotic treatment and

these results may provide novel tools to predict the potential recurrence risk and thus help to assess the optimal treatment choice for patients with uncomplicated acute appendicitis.

**Strengths and limitations**

MAPPAC has a strong synergy between APPAC II and III studies enabling the enrolment of both uncomplicated and complicated acute appendicitis patients in a large prospective series with associated clinical data to be assessed in conjunction with the microbiological and immunological findings. In many aspects, MAPPAC is an exploratory study of possible associations of whole microbial community and host immune characteristics with uncomplicated vs. complicated appendicitis and antibiotic response among patients in clinical trials treated with and without antibiotics. MAPPAC trial aims to generate hypotheses to better understand the role of disease progression and host susceptibility for future studies; i.e. determination of one primary outcome is insufficient for the study, as several factors are indispensable for the understanding of etiology and the effects of antibiotics on GM all provided with this unique set of microbiological samples. To our knowledge, only one previous study [12] has characterized the adult appendix microbiota during appendicitis in adult patients using NGS technique and to date no reports on the structure and physicochemical contents of appendicoliths exists. Using these assessments is a strength in our study. Further, to our knowledge this is the first trial aiming to prospectively assess the possible microbiological and/or immunological etiology of appendicitis recurrence after a successful initial conservative treatment with antibiotics or symptomatic therapy and primary non-responders to conservative treatment of uncomplicated acute appendicitis. One of the main hypothesis of the MAPPAC study is that the microbial composition of appendix differs between CT differentiated complicated and uncomplicated appendicitis. Therefore, strong element of the study is that all patients included in the study are imaged with CT protocol. CT scan is the gold standard for

acute appendicitis imaging and this near perfect accuracy of CT adds to the reliability of the clinical data and appendicitis severity also in the patients with uncomplicated acute appendicitis without a histopathological confirmation of the appendix.

The study limitations include the difficult challenge of conducting prospective clinical trials in the emergency setting. It is expected that all eligible patients may not be evaluated for enrolment or some patients may not have all study samples available as the recruitment is performed by a large number of surgeons on call. The lack of healthy control group is a limitation in the study regarding both the etiology and in determining the effects of antibiotics on GM, as the results cannot be fully distinguished from the effects of acute appendicitis.

## Authors' contributions

All authors have contributed to the design of this trial protocol. PS is the chief investigator. PS, SV, AJH, EM, SJ, JG, and SS have initiated the project. The protocol was drafted by PS, which was refined by SV, AJH, EM, SS, SJ, JG, HM, EE, and SH. Statistical advice was provided by SH. SV was responsible for drafting the manuscript, which was refined by PS, AJH, and EM. All authors have read and approved the final manuscript.

In addition, The APPAC collaborative study group lead by primary investigator Paulina Salminen includes the following contributors: Sallinen V., Leppäniemi A., Rautio T., Meriläinen S., Nordström P., Laukkarinen J., Rantala T., Savolainen H., Aarnio M., Mattila A., Haijanen J., Sävelä E-L., Imre I., Paajanen H., Rintala J., Pinta T., Sippola T., and Böckerman P. All contributors are local investigators who are responsible for execution of the APPAC II and/or APPAC III trials in addition to execution of the applicable parts of the MAPPAC trial and valid data gathering. They have all read and approved the final manuscript and they will be included in the future MAPPAC trial reports, when applicable. The surgical departments of the following Finnish Hospitals contribute to the execution of this trial: University hospitals of Turku, Helsinki, Oulu, Tampere, and Kuopio, central hospitals of Jyväskylä, Pori, Mikkeli, Seinäjoki, and Rovaniemi.

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## Competing interests

Authors declare they have no competing interests.

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**REFERENCES**

1 Addiss DG, Shaffer N, Fowler BS, et al. The epidemiology of appendicitis and appendectomy in the United States, *Am J Epidemiol* 1990;132:910-25.

2 Ferris M, Quan S, Kaplan B, et al. The Global Incidence of Appendicitis: A Systematic Review of Population-based Studies, *Annals of Surgery* 2017;266:237-41 doi:10.1097/SLA.0000000000002188.

3 Livingston EH, Fomby TB, Woodward WA, et al. Epidemiological Similarities Between Appendicitis and Diverticulitis Suggesting a Common Underlying Pathogenesis, *Archives of Surgery* 2011;146:308-14 doi:10.1001/archsurg.2011.2.

4 Forbes GB, Lloyd-Davies RW. Calculous disease of the vermiform appendix, *Gut* 1966;7:583-92 doi:10.1136/gut.7.6.583.

5 Lamps LW. Infectious Causes of Appendicitis, *Infectious Disease Clinics of North America* 2010;24:995-1018 doi:10.1016/j.idc.2010.07.012.

6 Swidsinski A, Doerffel Y, Loening-Baucke V, et al. Acute appendicitis is characterised by local invasion with Fusobacterium nucleatum/necrophorum, *Gut* 2011;60:34-40 doi:10.1136/gut.2009.191320.

7 Zhong D, Brower-Sinning R, Firek B, et al. Acute appendicitis in children is associated with an abundance of bacteria from the phylum Fusobacteria, *J Pediatr Surg* 2014;49:441-6 doi:10.1016/j.jpedsurg.2013.06.026 [doi].

8 Jindal N, Kaur GD, Arora S, et al. Bacteriology of acute appendicitis with special reference to anaerobes, *Indian J Pathol Microbiol* 1994;37:299-305.

9 Martirosian G, Bulanda M, Wojcik-Stojek B, et al. Acute appendicitis: the role of enterotoxigenic strains of Bacteroides fragilis and Clostridium difficile, *Med Sci Monit* 2001;7:382-6.

10 Chen CY, Chen YC, Pu HN, et al. Bacteriology of acute appendicitis and its implication for the use of prophylactic antibiotics, *Surg Infect (Larchmt)* 2012;13:383-90 doi:10.1089/sur.2011.135 [doi].

11 Reinisch A, Malkomes P, Habbe N, et al. Bad bacteria in acute appendicitis: rare but relevant, *Int J Colorectal Dis* 2017;32:1303-11 doi:10.1007/s00384-017-2862-0 [doi].

12 Guinane CM, Tadrous A, Fouhy F, et al. Microbial composition of human appendices from patients following appendectomy, *MBio* 2013;4:12.

13 Peeters T, Penders J, Smeekens SP, et al. The fecal and mucosal microbiome in acute appendicitis patients: an observational study, *Future Microbiol* 2019;14:111-27 doi:10.2217/fmb-2018-0203 [doi].

14 Bennion RS, Baron EJ, Thompson JE,Jr, et al. The bacteriology of gangrenous and perforated appendicitis--revisited, *Ann Surg* 1990;211:165-71 doi:10.1097/00000658-199002000-00008 [doi].

15 Rivera-Chavez FA, Peters-Hybki DL, Barber RC, et al. Innate immunity genes influence the severity of acute appendicitis, *Ann Surg* 2004;240:269-77 doi:00000658-200408000-00012 [pii].

16 Reinisch A, Malkomes P, Habbe N, et al. Bad bacteria in acute appendicitis: rare but relevant, *Int J Colorectal Dis* 2017;32:1303-11 doi:10.1007/s00384-017-2862-0.

17 Martirosian G, Bulanda M, Wojcik-Stojek B, et al. Acute appendicitis: the role of enterotoxigenic strains of Bacteroides fragilis and Clostridium difficile, *Med Sci Monit* 2001;7:382-6.

18 Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation, *Proc Natl Acad Sci U S A* 2011;108 Suppl 1:4554-61 doi:10.1073/pnas.1000087107 [doi].

19 Jernberg C, Lofmark S, Edlund C, et al. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota, *ISME J* 2007;1:56-66 doi:ismej20073 [pii].

20 Manichanh C, Rigottier-Gois L, Bonnaud E, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach, *Gut* 2006;55:205-11 doi:gut.2005.073817 [pii].

21 Qin J, Li Y, Cai Z, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes, *Nature* 2012;490:55-60 doi:10.1038/nature11450 [doi].

22 Lindgren M, Lofmark S, Edlund C, et al. Prolonged impact of a one-week course of clindamycin on Enterococcus spp. in human normal microbiota, *Scand J Infect Dis* 2009;41:215-9 doi:10.1080/00365540802651897 [doi].

23 Raymond F, Ouameur AA, Deraspe M, et al. The initial state of the human gut microbiome determines its reshaping by antibiotics, *ISME J* 2016;10:707-20 doi:10.1038/ismej.2015.148 [doi].

24 Jakobsson HE, Jernberg C, Andersson AF, et al. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome, *PLoS One* 2010;5:e9836 doi:10.1371/journal.pone.0009836 [doi].

25 Buelow E, Bello Gonzalez, T D J, Fuentes S, et al. Comparative gut microbiota and resistome profiling of intensive care patients receiving selective digestive tract decontamination and healthy subjects, *Microbiome* 2017;5:z doi:10.1186/s40168-017-0309-z [doi].

26 Card RM, Mafura M, Hunt T, et al. Impact of Ciprofloxacin and Clindamycin Administration on Gram-Negative Bacteria Isolated from Healthy Volunteers and Characterization of the Resistance Genes They Harbor, *Antimicrob Agents Chemother* 2015;59:4410-6 doi:10.1128/AAC.00068-15 [doi].

27 Salminen P, Paajanen H, Rautio T, et al. Antibiotic Therapy vs Appendectomy for Treatment of Uncomplicated Acute Appendicitis: The APPAC Randomized Clinical Trial, *JAMA* 2015;313:2340-8.

28 Salminen P, Tuominen R, Paajanen H, et al. Five-Year Follow-up of Antibiotic Therapy for Uncomplicated Acute Appendicitis in the APPAC Randomized Clinical Trial, *JAMA* 2018;320:1259-65 doi:10.1001/jama.2018.13201 [doi].

29 Sakran JV, Mylonas KS, Gryparis A, et al. Operation versus antibiotics--The "appendicitis conundrum" continues: A meta-analysis, *J Trauma Acute Care Surg* 2017;82:1129-37 doi:10.1097/TA.0000000000001450 [doi].

30 Sallinen V, Akl EA, You JJ, et al. Meta-analysis of antibiotics versus appendicectomy for non-perforated acute appendicitis, *Br J Surg* 2016;103:656-67 doi:10.1002/bjs.10147 [doi].

31 Podda M, Gerardi C, Cillara N, et al. Antibiotic Treatment and Appendectomy for Uncomplicated Acute Appendicitis in Adults and Children: A Systematic Review and Meta-analysis, *Ann Surg* 2019 doi:10.1097/SLA.0000000000003225 [doi].

32 Harnoss JC, Probst P, Büchler MW, et al. Antibiotics Versus Appendicectomy for the Treatment of Uncomplicated Acute Appendicitis: An Updated Meta-Analysis of Randomised Controlled Trials by Rollins et al, *World J Surg* 2017;41:2411 doi:10.1007/s00268-016-3864-8.

33 Rollins KE, Varadhan KK, Neal KR, et al. Antibiotics Versus Appendicectomy for the Treatment of Uncomplicated Acute Appendicitis: An Updated Meta-Analysis of Randomised Controlled Trials, *World J Surg* 2016;40:2305-18 doi:10.1007/s00268-016-3561-7 [doi].

34 Chan A, Tetzlaff JM, Altman DG, et al. SPIRIT 2013 statement: defining standard protocol items for clinical trials, *Ann Intern Med* 2013;158:200-7 doi:10.7326/0003-4819-158-3-201302050-00583.

35 Haijanen J, Sippola S, Gronroos J, et al. Optimising the antibiotic treatment of uncomplicated acute appendicitis: a protocol for a multicentre randomised clinical trial (APPAC II trial), *BMC Surg* 2018;18:y doi:10.1186/s12893-018-0451-y [doi].

36 Sippola S, Gronroos J, Sallinen V, et al. A randomised placebo-controlled double-blind multicentre trial comparing antibiotic therapy with placebo in the treatment of uncomplicated acute appendicitis: APPAC III trial study protocol, *BMJ Open* 2018;8:023623 doi:10.1136/bmjopen-2018-023623 [doi].

37 Lewis SJ, Heaton KW. Stool form scale as a useful guide to intestinal transit time, *Scand J Gastroenterol* 1997;32:920-4 doi:10.3109/00365529709011203 [doi].

38 Vons C, Barry C, Maitre S, et al. Amoxicillin plus clavulanic acid versus appendicectomy for treatment of acute uncomplicated appendicitis: an open-label, non-inferiority, randomised controlled trial, *Lancet* 2011;377:1573-9 doi:10.1016/S0140-6736(11)60410-8.

39 Sippola S, Grönroos J, Tuominen R, et al. Economic evaluation of antibiotic therapy versus appendicectomy for the treatment of uncomplicated acute appendicitis from the APPAC randomized clinical trial, *British Journal of Surgery* 2017;104:1355-61 doi:10.1002/bjs.10575.

## Abbreviations

**AMR:** Antimicrobial resistance

**CRP:** C-reactive protein

**CT:** Computed tomography

**GM:** Gut microbiota

**i.v.:** Intravenous

**MALDI-TOF:** Matrix Assisted Laser Desorption/Ionisation time-of-flight mass spectrometry

**MS:** Mass spectrometry

**NGS:** Next generation sequencing

**p.o.:** Per os

**SPIRIT:** Standard Protocol Items: recommendations for Interventional Trials

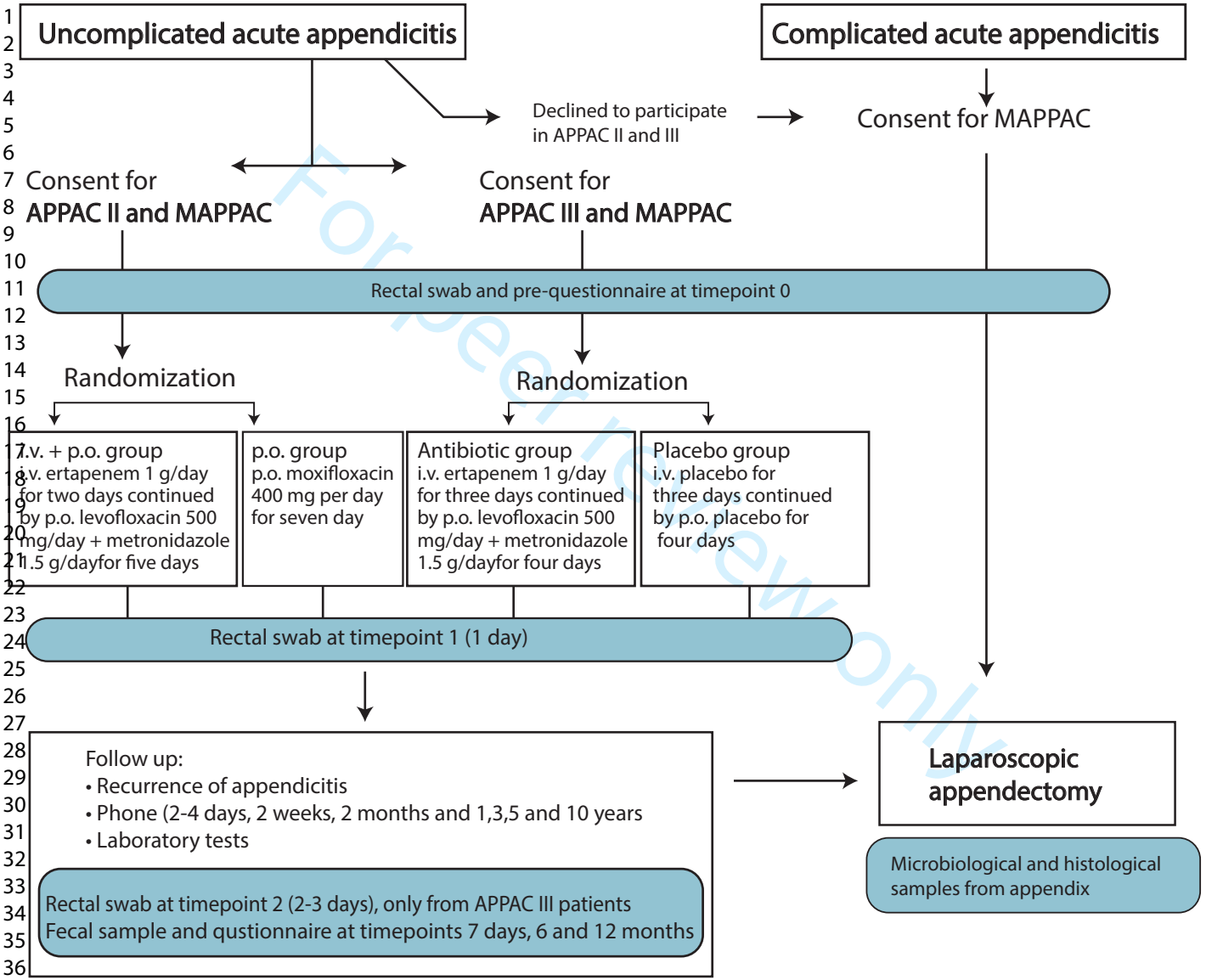
**VAP-1:** Vascular adhesion protein-1

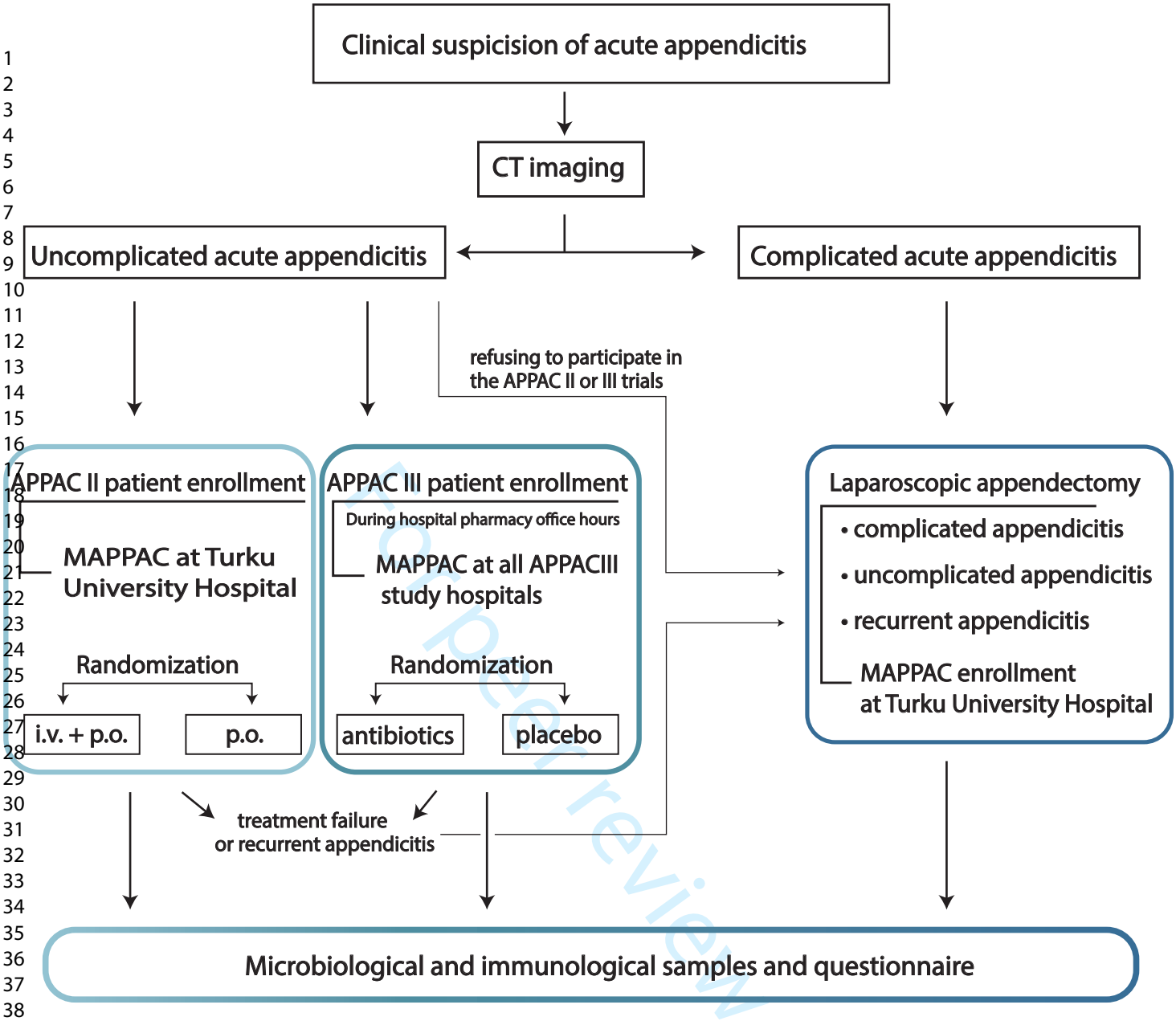
**VAS:** Visual analogue scale

## FIGURE LEGENDS

Figure 1.The synergy between MAPPAC, APPAC II and APPAC III studies.

Figure 2. Flow chart of the study protocol





**SUPPLEMENTARY FILE**

**Laboratory methods**

**DNA extraction and analysis**

**Stool samples and rectal and appendiceal swabs**

Bacterial DNA from rectal and appendiceal swabs and faecal samples are extracted with semiautomated DNA extraction kit together with NorDiag Arrow extraction instrument (Diasorin). The following sample preparation is undertaken before the extraction: 500 µl of the sample is added to 700 µl of stool stabilizer in a 1.4 mm Ceramic Powerbead Tube (Qiagen, Hilden, Germany) and homogenized with MO BIO PowerLyzer 24 Bench Top Bead-Based Homogenizer (MO BIO Laboratories, Inc., USA) at 1000 rpm for 3 minutes followed by centrifugation at 5000 x g for 5 minutes and 600 µl of the supernatant is transferred into a new tube, the centrifugation is repeated and 500 µl of the supernatant is transferred into anew tube. This pretreatment is followed by the DNA extraction, which is performed according to the manufacturer´s instructions.

**Appendiceal biopsy:** Microbiological DNA is extracted from appendix biopsy stored in RNAlater with semiautomated DNA extraction kit using NorDiag Arrow extraction instrument. At first RNA later is removed by centrifugation and the pellet is resuspended into ultrapure water. Subsequently, the resuspension containing also the biopsy is treated with proteinase K in thermomixer. From the resulting solution DNA extraction is carried out according to manufacturer's instructions.

The concentration of eluted DNA from all sample types is measured with Qubit dsDNA HS or BR assay kit (Thermo Fisher Scientific) using Qubit fluorometer (Life Technologies, Carlsbad, California, USA). DNA is divided into two aliquots and stored at -75◦C for later analysis. Microbial

profile of samples will be analysed with NGS approach using appropriate methods and Illumina Miseq system.

**Analyses of metagenome, transcriptome and proteome from appendiceal biopsy**

Total RNA and proteins from the appendiceal biopsy are extracted with appropriate methods. Transcriptome and metagenome are analysed with Illumina Hiseq system. In addition, the expression of specific genes is quantified with quantitative real-time PCR. Proteome is characterized using mass spectrometry-based methods with qualitative and quantitative approach.

**Culture methods**

Appendiceal swabs are cultured with both aerobic and anaerobic culture methods. For aerobic culture the following growth media are used: CHROMagar Orientation (Becton Dickinson, Franklin Lakes, New Jersey, USA), Trypticase soy agar with 5% sheep blood (Becton Dickinson), Yersinia selective agar and Streptococcus selective agar (in house production). If the appendectomy is performed during the office hours, an additional anaerobic culture is made in connection with the sample collection. Samples for anaerobic culturing are collected with a sterile cotton swab from the appendix and cultured for fastidious anaerobe agar with blood (FAA) and kanamycin-vancomycin laked blood agar (KVLB) (both in house production), which are immediately transferred into anaerobic jar with anaerobic gas generating sachet (Anaerogen 3,5 l, Thermo Fisher Scientific).

Cultures are incubated at +35 °C for 18-24 hours. Morphologically distinct colonies are subcultured until a pure culture is obtained. Isolated species are frozen in a mixture of skim milk and glycerol at −75 °C.

**MALDI-TOF mass spectrometry**

The identification of isolated bacteria is done with Bruker matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). Isolates are inoculated and cultured for 18-48 hours at +35 °C before the analysis. For the analysis, colony is applied either directly or through ethanol/formic acid extraction on a steel plate and HCCA matrix (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile-2.5% trifluoroacetic acid (Bruker Daltonics, Bremen, Germany)) is added according to the manufacturer´s instructions. The identification is done using Microflex LT instrument and MALDI Biotyper software (Bruker Daltonics). If isolates cannot be identified due to the absence of reference peaks in the database, the isolate is identified with sequencing the 16S rRNA gene.

**Antimicrobial susceptibility testing**

Phenotypic antimicrobial susceptibility testing for the isolated aerobic and anaerobic bacteria is performed by disk diffusion and MIC methods, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. For certain bacteria, the Clinical and Laboratory Standards Institute (CLSI) guidelines can be used.

Further molecular analysis for antimicrobial resistance or virulence genes using e.g., PCR, Sanger sequencing and whole genome sequencing (WGS) are also performed for selected isolates.

**Appendicolith analysis**

Appendicoliths are assessed both on the surface and cross-sectional morphology. Based on the morphological characteristics and a degree of hardness, all appendicoliths will be classified. The composition of selected appendicoliths are analysed with physical and chemical methods.

**Immunological analysis**

The appendiceal biopsies are analysed immunohistochemically by determining the presence of different cell types and blood vessel surface markers. Neutrophils, leukocytes and monocytes will be analysed and special interest will be focused on lymphocyte subtypes (i.e., CD4/CD8 and more detailed subgroup analyses such as regulatory T cells) and monocyte markers (i.e., macrophage M1/M2 / receptor MHCII). Moreover, certain inflammation induced markers on endothelium such as VAP-1, E-selectin and P-selectin will be evaluated. In order to compare possible differences between patients with successful antibiotic therapy to patients with failed antibiotic therapy or complicated acute appendicitis the serum samples will be screened to identify possible inflammatory or immunological markers for identifying the different forms of the disease. Cytokines, chemokines and growth factors will be tested using Bio-Plex Pro Human Cytokine 48-Plex Screening Panel (BIO-RAD) containing the following analytes: Basic FGF, CTACK, eotaxin, G-CSF, GM-CSF, GRO-a, HGF, ICAM-1, IFN-a2, IFN-g, IL-1a, IL-1b, IL-1ra, IL-2, IL-2Ra, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-16, IL-17A, IL-18, IP-10, LIF, MCP-1 (MCAF), MCP-3, M-CSF, MIF, MIG, MIP-1a, MIP-1b, b-NGF, PDGF-BB, RANTES, SCF, SCGF-b, SDF-1a, TNF-a, TNF-b, TRAIL, VCAM-1 and VEGF-A. Moreover, we will use in house analyses to measure soluble VAP-1 (inflammatory) and CD73 (anti-inflammatory). In addition, metabolomics approach using nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS) metabolomics platforms will be used for biomarker analysis. The results obtained from immunological analyses will be correlated to the clinical parameters and to the microbiological findings.

SPIRIT

STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

| Section/item | Item No | Description | Page Number on which item is reported |
|---|---|---|---|
| **Administrative information** | | | |
| Title | 1 | Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym | 1 |
| Trial registration | 2a | Trial identifier and registry name. If not yet registered, name of intended registry | 3 |
| | 2b | All items from the World Health Organization Trial Registration Data Set | n/a |
| Protocol version | 3 | Date and version identifier | n/a |
| Funding | 4 | Sources and types of financial, material, and other support | 22 |
| Roles and responsibilities | 5a | Names, affiliations, and roles of protocol contributors | 1,22 |
| | 5b | Name and contact information for the trial sponsor | 1 |
| | 5c | Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities | 22 |
| | 5d | Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee) | 7-8 |
| **Introduction** | | | |

| | | | |
|---|---|---|---|
| Background and rationale | 6a | Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention | 5-7 |
| | 6b | Explanation for choice of comparators | 6-7 |
| Objectives | 7 | Specific objectives or hypotheses | 7 |
| Trial design | 8 | Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory) | 7 |
| **Methods: Participants, interventions, and outcomes** | | | |
| Study setting | 9 | Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained | 8 |
| Eligibility criteria | 10 | Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists) | 9-11 |
| Interventions | 11a | Interventions for each group with sufficient detail to allow replication, including how and when they will be administered | 11-12 |
| | 11b | Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease) | 14 |
| | 11c | Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests) | 14-15, 17 |
| | 11d | Relevant concomitant care and interventions that are permitted or prohibited during the trial | n/a |
| Outcomes | 12 | Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended | 15-16 |

| | | | |
|---|---|---|---|
| Participant timeline | 13 | Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure) | 12-15, figure 2 |
| Sample size | 14 | Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations | 16, n/a |
| Recruitment | 15 | Strategies for achieving adequate participant enrolment to reach target sample size | 11, 17 |
| **Methods: Assignment of interventions (for controlled trials)** | | | |
| Allocation: | | | n/a |
| Sequence generation | 16a | Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions | n/a |
| Allocation concealment mechanism | 16b | Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned | n/a |
| Implementation | 16c | Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions | n/a |
| Blinding (masking) | 17a | Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how | n/a |
| | 17b | If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial | n/a |
| **Methods: Data collection, management, and analysis** | | | |

| | | | |
|---|---|---|---|
| Data collection methods | 18a | Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol | 12-17, supplementary material |
| | 18b | Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols | 17 |
| Data management | 19 | Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol | 18 |
| Statistical methods | 20a | Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol | 16 |
| | 20b | Methods for any additional analyses (eg, subgroup and adjusted analyses) | 17 |
| | 20c | Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation) | n/a |
| **Methods: Monitoring** | | | |
| Data monitoring | 21a | Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed | 22 |
| | 21b | Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial | n/a |

| | | | |
|---|---|---|---|
| Harms | 22 | Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct | 17, 18 |
| Auditing | 23 | Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor | n/a |
| **Ethics and dissemination** | | | |
| Research ethics approval | 24 | Plans for seeking research ethics committee/institutional review board (REC/IRB) approval | 18 |
| Protocol amendments | 25 | Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators) | n/a |
| Consent or assent | 26a | Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32) | 8-9 |
| | 26b | Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable | 9, 18 |
| Confidentiality | 27 | How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial | 18 |
| Declaration of interests | 28 | Financial and other competing interests for principal investigators for the overall trial and each study site | 22 |
| Access to data | 29 | Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators | 18 |
| Ancillary and post-trial care | 30 | Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation | n/a |
| Dissemination policy | 31a | Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions | 18 |

| | | | |
|---|---|---|---|
| | 31b | Authorship eligibility guidelines and any intended use of professional writers | n/a |
| | 31c | Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code | n/a |
| **Appendices** | | | |
| Informed consent materials | 32 | Model consent form and other related documentation given to participants and authorised surrogates | in finnish only |
| Biological specimens | 33 | Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable | 12-15, supplementary material |

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

BMJ Open

# A Prospective Multicenter Cohort Trial on Acute Appendicitis and Microbiota – Etiology and Effects of Antimicrobial Treatment: Study protocol for the MAPPAC (Microbiology Appendicitis Acuta) Trial



| | |
|---|---|
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| Primary Subject Heading: | Gastroenterology and hepatology |
| Secondary Subject Heading: | Infectious diseases |
| Keywords: | appendicitis, appendectomy, appendicitis etiology, appendicolith, microbiota, antimicrobial resistance |
| | |

# A Prospective Multicenter Cohort Trial on Acute Appendicitis and Microbiota – Etiology and Effects of Antimicrobial Treatment: Study protocol for the MAPPAC (Microbiology Appendicitis Acuta) Trial

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Word count: 4143

## ABSTRACT

**Introduction:** Based on epidemiological and clinical data acute appendicitis can present either as uncomplicated or complicated. The etiology of these different appendicitis forms remains unknown. Antibiotic therapy has been shown to be safe, efficient and cost-effective for computed tomography (CT) confirmed uncomplicated acute appendicitis. Despite appendicitis being one of the most common surgical emergencies, there are very few reports on appendicitis etiology and pathophysiology focusing on the differences between uncomplicated and complicated appendicitis. MAPPAC (Microbiology APPendicitis ACuta) trial aims to evaluate these microbiological and immunological aspects including immune response in the etiology of these different forms also assessing both antibiotics non-responders and appendicitis recurrence. In addition, MAPPAC aims to determine antibiotic and placebo effects on gut microbiota composition and antimicrobial resistance.

**Methods and analysis:** MAPPAC is a prospective clinical trial with both single- and multicentre arm conducted in close synergy with concurrent trials APPAC II (p.o. vs. i.v+p.o. antibiotics, NCT03236961) and APPAC III (double-blind trial placebo vs. antibiotics, NCT03234296) randomized clinical trials. Based on the enrolment for these trials, patients with CT-confirmed uncomplicated acute appendicitis are recruited also to the MAPPAC study. In addition to these conservatively treated randomized patients with uncomplicated acute appendicitis, MAPPAC will recruit patients with uncomplicated and complicated appendicitis undergoing appendectomy. Rectal and appendiceal swabs, appendicolith, faecal, and serum samples, appendiceal biopsies, and clinical data are collected during the hospital stay for microbiological and immunological analysis in both study arms with the longitudinal study arm collecting fecal samples also during follow-up up to 12 months after appendicitis treatment.

**Ethics and dissemination:** This study has been approved by the Ethics Committee of the Hospital District of Southwest Finland (Turku University Hospital, approval number

ATMK:142/1800/2016) and the Finnish Medicines Agency (Fimea). Results of the trial will be published in peer-reviewed journals.

**Trial registration:** Eudra-CT: 2016-003655-29 (2017-01-12), clinicaltrials.gov NCT03257423.

**KEYWORDS**

Appendicitis, appendectomy, appendicitis etiology, appendicolith, microbiota, antimicrobial resistance

## Strengths and limitations of this study

- To our knowledge, MAPPAC (Microbiology APPendicitis ACuta) is the first prospective trial comparing the role of microbiology and immunology including immune response in the etiology of uncomplicated and complicated acute appendicitis in a large patient cohort consisting of CT-diagnosed patients also specifically evaluating appendicoliths and recurrent appendicitis after initial successful conservative treatment.
- The strong synergy between two ongoing randomized clinical trials (APPAC II and APPAC III) enabling a large prospective patient cohort of acute appendicitis patients with associated clinical data to be assessed with the microbiological and immunological findings.
- The application of next generation sequencing combined with traditional culturing methods will provide extensive information about the microbiological factors in the etiology of complicated and uncomplicated acute appendicitis also presenting a challenge in differentiating between etiologic and non-etiologic microbiota in the specimens.
- The comprehensive approach of the MAPPAC study acquiring a large set of samples in the emergency surgery setting presents a challenge to surgeons on call and some patients may not have all study samples available.

## INTRODUCTION

Acute appendicitis is one of the most common causes of abdominal pain in emergency departments. The lifetime risk of acute appendicitis in males is 8.6% and 6.7% in females [1] with recent meta-analysis showing an increasing trend in appendicitis incidence in the industrialised countries [2]. Based on epidemiological and clinical data, acute appendicitis can present either as uncomplicated or complicated with the majority of cases being uncomplicated.

The different epidemiological trends of uncomplicated and complicated acute appendicitis indicate different pathophysiology [3]. Despite the high incidence of acute appendicitis, there are very few reports on appendicitis etiology and pathophysiology especially focusing on the possible differences between uncomplicated and complicated acute appendicitis. Complicated acute appendicitis in this trial is defined as a finding of perforation, appendicolith, abscess or a suspicion of tumor. Appendicolith is a calcified faecal concretion in the appendix and it is the most common form of complicated acute appendicitis. Even though the first thorough study on appendicoliths was already reported in 1966 [4], information about appendiceal calculi is scarce. Obstruction of the appendiceal lumen caused by an appendicolith, lymphoid hyperplasia, or swelling has been evaluated to be the primary cause of appendicitis and bacterial overgrowth has been considered a consequence [5]. However, bacterial infection has also been proposed as the primary cause of appendicitis [6, 7]. *Bacteroides* species are reported to be one of most common bacterial findings in appendicitis [8, 9]. Further, certain members of the *Fusobacteria,* especially *F. nucleatum* and *F. necrophorum,* are present in most appendicitis samples [6]. The most common aerobic bacteria organism detected by culturing is *Escherichia coli,* but also *Klebsiella pneumoniae, Streptococcus spp., Enterococcus spp.* and *Pseudomonas aeruginosa* have been reported [10, 11]. To our knowledge, only one study with a very small number of patients has characterised the adult appendiceal microbiota profile

with next generation sequencing (NGS) methods. Appendix seems to have diverse microbiota including both commensal species from gut microbiota (GM) and opportunistic pathogens [12, 13]. Since the interindividual variability in the microbial composition of the appendix samples is high [12], a larger number of appendicitis patients is needed to draw the microbiological conclusions. Since most of the species identified from the appendix with both culturing and NGS methods can also be part of normal gut microbiota it is challenging to determine their role in the infection [14]. In addition, the immune response and predisposition for infection by specific bacteria varies between individuals. Consequently, innate immunity is considered to be a contributing factor in the development of complicated appendicitis [15]. In conclusion, several factors have been proposed to take part in the development of appendicitis, most importantly obstruction of the lumen and bacterial infection with this research largely focusing on complicated acute appendicitis [5-7, 16, 17].

Antimicrobials decrease GM diversity, richness and species variation, and cause a perturbation in its overall balance. Particularly broad-spectrum antibiotics have been shown to have a long-term effect on GM profile. [18, 19] A prolonged disturbance in GM and the following imbalance with the host and its immune system have been associated with a variety of diseases, such as inflammatory bowel disease [20] and type 2 diabetes [21]. Antibiotic use can further lead to increased prevalence of antibiotic resistant bacteria and antibiotic resistance genes [22-24]. Although the effects of antibiotic treatment on the development of antimicrobial resistance (AMR) development is less clear in countries with lower prevalence of resistant bacteria [25, 26], the evaluation of antibiotic treatment effects on GM is essential in the treatment of acute appendicitis.

Appendectomy has unquestionably been the standard treatment for acute appendicitis for over a century with more than 300 000 appendectomies performed annually in the United States

[3]. The original APPAC (APPendicitis ACuta) trial reported that at long-term follow-up, the majority of patients with computed tomography (CT) confirmed uncomplicated acute appendicitis were successfully treated with antibiotics, and those patients that required later appendectomy did not have increased or major complications [27, 28]. Antibiotic therapy for CT-confirmed uncomplicated acute appendicitis has been shown to be safe, efficient and cost-effective both in adult and paediatric patients [29-33]. APPAC II trial aims to optimise antibiotic treatment for CT confirmed uncomplicated acute appendicitis in order to both shorten the hospital stay and restrict the antibacterial spectrum. The APPAC III trial aims to assess symptomatic treatment of uncomplicated acute appendicitis and the role of antibiotics in the resolution of uncomplicated appendicitis. The MAPPAC (Microbiology APPendicitis ACuta) trial patient enrolment is based on the ongoing APPAC II and APPAC III randomised multicentre clinical trials of our study group. To our knowledge, there are so far no similar large microbiological studies focusing on acute appendicitis performed in conjunction with large clinical trials with prospective access to both uncomplicated and complicated appendicitis patients. MAPPAC trial aims to evaluate the possible role and differences in the microbiological etiology of complicated and uncomplicated appendicitis with a special reference to the presence of an appendicolith. In addition, MAPPAC aims to evaluate the immunological and microbiological factors involved in appendicitis recurrence after successful initial antibiotic therapy. In the longitudinal study arm we also aim to assess the effects of antibiotic and placebo treatment on the GM profile and the effects of hospital stay duration on the AMR reservoir of the GM.

## METHODS AND ANALYSIS

### Study Design

MAPPAC is a prospective clinical trial with a single-centre and a multicentre arm. The single-centre study arm at Turku University Hospital, aims to determine the possible differences in the etiology of complicated and uncomplicated acute appendicitis with a special reference to the role and etiology of appendicolith. In the multi-centre longitudinal arm, MAPPAC trial concentrates on assessment of possible immunological and microbiological factors involved in initial non-responders or appendicitis recurrence after successful initial antibiotic therapy at long-term follow-up. In this longitudinal multicentre study arm we also aim to evaluate the effects of antibiotic and placebo treatment on the GM and the effects of the duration of the hospital stay on the possible AMR reservoir within the GM. MAPPAC protocol was drafted in accordance with the SPIRIT statement [34]. The trial has been registered at both EudraCT (2016-003655-29) and clinicaltrials.gov (NCT03257423).

The three ongoing study entities (APPAC II, APPAC III, and MAPPAC) are strongly interconnected having a common study aim and a patient enrolment population (Figure 1). APPAC II trial is a randomised open-label, non-inferiority multicentre trial to compare intravenous antibiotic therapy followed by per oral (p.o.) antibiotics with p.o. monotherapy in the treatment of uncomplicated acute appendicitis (NCT03236961)[35]. APPAC III trial is a randomised double-blind, placebo-controlled, superiority multicentre study to compare antibiotic therapy with placebo in the treatment of uncomplicated acute appendicitis (NCT03234296) [36]. All incoming patients are informed of all ongoing trials. All patients invited to participate in APPAC II and III trials will be invited to participate in the MAPPAC trial. Patients recruited for the APPAC II or III trial are asked to sign a separate consent form for the MAPPAC trial allowing for the use of their data and collection of microbiological samples. The study flow is illustrated in Figure 2.

**Patient selection**

Eligible for inclusion are all adult patients 18 – 60 years old presenting with either a CT-confirmed uncomplicated or complicated appendicitis or patients with a suspected recurrent appendicitis after initial successful antibiotic therapy for uncomplicated acute appendicitis. MAPPAC single-centre arm will enroll patients with both uncomplicated (both APPAC II and III trials and patients declining to participate in these trials undergoing appendectomy) and complicated acute appendicitis (patients excluded from APPAC II and APPAC III trials) at Turku University Hospital. The enrolment of uncomplicated acute appendicitis patients participating in the APPAC III trials will be performed at all APPAC III hospitals (all five Finnish university hospitals: Turku, Helsinki, Tampere, Oulu, and Kuopio). In addition, all of the APPAC II and III trial patients having to undergo appendectomy either for a treatment failure during the primary hospitalisation or for suspected recurrence after a successful initial non-operative treatment will be enrolled in the MAPPAC trial at all ten study hospitals (all five university and five central hospitals in Finland: Jyväskylä, Pori, Mikkeli, Seinäjoki and Rovaniemi).

**Study setting and feasibility**

The study was initiated at Turku University Hospital in April 2017, with the study commencing at all study centres by fall 2017. The majority of MAPPAC patient enrolment is evaluated to be completed in conjunction with the APPAC II and III trials latest by fall 2020. Patient recruitment in the MAPPAC trial will continue for a minimum of five years after APPAC II and III trial enrolment completion based on the planned microbiological assessment of late appendicitis recurrence.

**Interventional groups**

Four interventional groups within MAPPAC are defined as follows:

1. Patients with uncomplicated acute appendicitis participating in the APPAC II trial at Turku University Hospital (MAPPAC single-centre treatment arm) receiving either i.v. antibiotic therapy followed by p.o. antibiotics (i.v. + p.o.) or patients receiving p.o. antibiotic monotherapy (p.o.). *I.v. + p.o*. group receives intravenous ertapenem 1 g once daily for two days followed by p.o. levofloxacin 500 mg once daily and metronidazole 500 mg three times per day for 5 days. *P.o.* group receives *p.o.* moxifloxacin 400 mg once daily for seven days.
2. Patients with uncomplicated acute appendicitis participating in the double-blinded APPAC III trial at all five university hospitals (MAPPAC multicentre treatment arm) receiving either antibiotic therapy or placebo treatment. *Antibiotic treatment* is intravenous ertapenem 1 g once per day for three days followed by p.o. levofloxacin 500 mg once per day and metronidazole 500 mg three times per day for four days. *Placebo treatment* entails intravenous placebo once per day for three days followed by per oral placebo three times per day for four days.
3. Patients with uncomplicated acute appendicitis or suspected recurrent appendicitis undergoing appendectomy

   The patients in this group will undergo laparoscopic appendectomy either after declining to participate in the APPAC II or APPAC III trials (MAPPAC single-centre treatment arm at Turku University Hospital) or after suspected treatment failure of non-operative treatment or after presenting with suspected appendicitis recurrence after initial successful non-operative treatment (MAPPAC multicentre treatment arm, all APPAC II and APPAC III hospitals)
4. Patients with complicated acute appendicitis undergoing appendectomy

The patients in this group will undergo laparoscopic appendectomy and are eligible for enrolment only in the MAPPAC single-centre study arm at Turku University Hospital.

**Sample collection**

**Rectal swabs** are collected from all patients in the emergency department prior to antibiotic treatment or surgery (timepoint 0). Further, rectal swabs are collected on the 1st day for APPAC II patients and for APPAC III patients on the 1st day (Turku University Hospital) and on the 3rd day (all five university hospitals). At each time point, two rectal swabs are collected: Amies-Coal transport swab for culturing methods (Sarstedt, Nümbrecht, Germany) and Puritan DNA/RNA swab (Puritan Medical products, Guilford, Maine) with shield fluid (Zymo Research, Irvine, Canada) for microbial DNA extraction. The shield fluid in this collection tube allows the transportation at room temperature.

**Samples from the appendix** are collected from patients undergoing appendectomy for complicated or uncomplicated acute appendicitis and from patients with suspected disease progression during the primary hospitalisation or appendicitis recurrence after successful initial non-operative therapy. Samples include routine histology as well as specific trial swabs and biopsies. The appendix is opened vertically with a sterile scalpel and two swabs are taken similar to the rectal swab (one for culture and one for DNA extraction). Three biopsies are taken at a distance of approximately 2 cm from the base of the appendix. All biopsies are stored at -75 ◦C, one immediately and the rest two are first embedded in RNAlater solution (Thermo Fisher Scientific, Waltham, MA, USA). Possible appendicoliths are collected into a sterile container, frozen and stored at -75 ◦C. If appendectomy is performed during office hours, appendiceal samples are collected by study personnel and an additional swab for anaerobic culture is then collected and cultured in connection with collection and immediately transferred into an anaerobic jar. During on call hours, the samples are collected

by surgeons and the -75 ◦C appendiceal biopsy may not be collected. These appendiceal samples are collected only in the Turku University Hospital and at the other study hospitals only a swab sample (transport tube with DNA shield fluid) from the appendix of non-responders and patients with appendicitis recurrence is collected.

**Additional serum samples** are collected from all the patients recruited in MAPPAC trial at Turku University Hospital and for APPAC III trial at all five study hospitals for the identification of possible disease-form specific inflammatory biomarkers in serum. The serum samples are divided into six aliquots prior freezing at -75 ◦C.

**Questionnaire**

MAPPAC patients are asked to fill a primary questionnaire during the initial hospitalisation covering topics possibly affecting their GM profile: chronic diseases, special diets, smoking and alcohol consumption, travel history, antibiotic intake, other medications (12 months prior the sampling), consumption of probiotics and other dietary supplements, recent diarrhoea and/or vomiting, Bristol stool form scale estimate [37] measuring stool consistency (1 for very hard stool to 7 for diarrhoea) and the average number of defecations per day.

**Follow-up during the hospitalisation**

During the hospitalisation the following parameters will be recorded every 24 hours: pain assessed by Visual Analogue Scale (VAS), leukocyte count, C-reactive protein (CRP), temperature and clinical findings at patient reassessment. If the patient is suspected of not responding to the randomized therapy based on clinical deterioration signs combined with laboratory findings (signs of peritonitis, persisting fever, increasing pain, white blood cell count or CRP), the patient will be operated based on the surgeon's decision and the reasons for proceeding to appendectomy will be recorded. For appendectomy, laparoscopic approach is recommended. The operative findings and the histopathology of the appendix will be recorded. After the initial hospitalisation, recurrent acute appendicitis will be diagnosed on a

clinical basis and patients with a suspected recurrence of appendicitis will undergo a laparoscopic appendectomy.

**Follow-up**

The specific MAPPAC trial follow-up is conducted for patients also participating in the APPAC II and III trials with collection of three faecal samples at home (at one week, six months and one year). Follow-up samples at home are not collected in MAPPAC interventional groups undergoing appendectomy. The follow-up faecal sample is collected into 10 ml DNA/RNA Shield Faecal Collection Tube (Zymo Research) enabling transportation and storage at room temperature prior to DNA extraction. Patients also deliver a faecal sample into a gel tube for culture purposes. A follow-up questionnaire is collected at 6 and 12 months consisting of the same questions as the preliminary questionnaire covering the time between hospital discharge and the follow-up time point. Other follow-up for patients in APPAC II trial will include a phone interview at one week after discharge, APPAC III trial patients have their first follow-up call 2-4 days after discharge from the hospital and then at 10 days, both trials at two months and at one, three, five and ten years. The follow-up for APPAC III patients will include laboratory tests (leukocyte count, CRP, additional follow-up serum samples).

**Outcome parameters**

Based on the MAPPAC trial design, no specific primary endpoint can be determined. The following parameters will be recorded for all patients: age, gender, Body mass index, clinical findings on admission (tympanic temperature, nausea, pain or tenderness in the right lower abdominal quadrant, pain migration, VAS pain scores, white blood cell count, CRP, findings on CT imaging), and data on primary and follow-up questionnaires. Blood cultures will be obtained from patients with complicated acute appendicitis and for APPAC II and III trial patients at Turku University Hospital.

The following outcome parameters will be assessed based on the sample types collected:

*Parameters from the appendix i.e. patients undergoing appendectomy*

Operative details and histopathology of the appendix, host transcriptomics, proteomics, and immunohistochemical analysis from appendiceal biopsy, possible appendicolith composition, morphology, and classification.

*Microbiological parameters from the rectal and appendiceal swabs, faecal and appendiceal samples*

Microbial profile, metagenome and metatranscriptome, name of different identified bacterial species, number of species identified both by NGS and culture methods, antimicrobial susceptibility test results, the presence of AMR related genes by molecular analysis methods, bacterial genome data of AMR or virulence genes, all microbial data combined with clinical and additional data.

*Serum samples*

In order to compare possible differences between patients with successful antibiotic therapy to patients with either failed antibiotic therapy or complicated acute appendicitis, the serum samples will be analysed to identify possible inflammatory or immunological markers. Different cytokines, chemokines and growth factors as well as serum metabolome will be included in these analyses. Additional analysis include the level and activity of CD73 and soluble vascular adhesion protein-1 (VAP-1). List for analytes that will be screened is provided in the supplementary methods.

Laboratory methods used in the trial to analyse the collected samples are described in detail in the online supplementary material.

## Statistical analysis

Based on the explorative research nature of the MAPPAC study, there is not enough information available about the study aims to enable sample size calculations. Categorical variables of the study will be characterised using frequencies and percentages. For continuous variables means and standard deviations or medians with range and 25th and 75th percentiles will be used. In case of categorical outcomes, groups will be compared using Pearson´s Chi-squared –test and if further analyses will be needed, logistic regression models will be used. Group differences in continuous variables will be evaluated using independent samples t-test, analysis of variance (ANOVA) or non-parametric methods, if needed. Associations between continuous variables will be evaluated using correlation coefficients and linear regression analysis and if adjustments are needed, linear models will be used. Continuous outcomes measured in several time-points will be analysed using linear mixed models. For categorical outcomes with repeated measurements generalised linear mixed models will be used. The assumptions of the methods will be checked for justification of the analyses and transformations will be used for the variables, if needed. The study site differences will be evaluated in statistical models and if major differences are detected, more complicated statistical models will be used in the analyses. Two-sided p-values will be used and p-values less than 0.05 will be considered statistically significant. The measurements with missing data will automatically be excluded from the analyses of the variables in concern. Statistical analyses will be performed using SAS System for Windows, Version 9.4 and JMP Pro 13 or later versions (SAS Institute, Cary, NC, USA).

## Patients and public involvement

The MAPPAC research questions and outcome measures were based on the results of original APPAC trial [27] and the study protocol was developed together with the study group

surgeons, clinical microbiologists and immunologists. Patients were not directly involved in the design of this study and the burden of study participation was not assessed by patients themselves. Upon recruitment, patients are well informed of all aspects of the trial including antibiotic or symptomatic therapy of CT-confirmed uncomplicated acute appendicitis, difference between complicated and uncomplicated acute appendicitis, treatment success, possible late recurrence, and safety in order to help patients make an informed decision about trial participation. Patients also receive additional instructions in a phone call made prior to follow-up sample collection at 6 and 12 months. After completion of data collection and analysis, the patients will be informed of the study results and they will be provided with an opportunity to ask further questions.

## ETHICS AND DISSEMINATION

### Ethics

This protocol has been accepted by the Ethical Committee of the Hospital district of Southwest Finland (Turku University Hospital) and Finnish Medicines Agency (Fimea). The trial will be conducted in compliance with the Declaration of Helsinki.

### Data collection and confidentiality

All data and samples are handled confidentially and the information in the datasets is non-identifiable. Data are gathered during the emergency room visit, hospitalisation for acute appendicitis, clinical observations, and follow-up phone calls. The main investigators will be in charge of the common database with full access to the data which is, otherwise strictly limited. As the MAPPAC and APPAC II and III trials are based on the same patient population, the interventions partly overlap and the enrolled patients are informed about this overlapping of the trials and the acquired data.

**Withdrawal**

Patients are informed of their right to withdraw from the study without explanation at any time. In case of patient withdrawal, they will be asked for permission to use their data.

**Dissemination plan**

Results from this trial and reported in articles which will be published in peer-reviewed journals. Results are also presented at national and international conferences to further distribute this research.

## DISCUSSION

As non-operative treatment for uncomplicated acute appendicitis has been shown to be efficient and safe also at long-term [27-30, 33, 38] and cost-effective [39], understanding both the etiology of the different appendicitis forms and potentially predicting the recurrences has become of utmost clinical importance in order to thoroughly evaluate all the optimization of the different treatment options. The MAPPAC trial aims to assess this largely unknown microbiological etiology of the different disease forms of acute appendicitis. Further, the whole microbial entity i.e. profile both in the appendix and in gut is studied, and their role in the disease severity and form is assessed. In addition, the effects of antibiotic and placebo treatments on the GM profile and the effects of the duration of the hospital stay on the AMR reservoir of the GM will be assessed. With the longitudinal study design, both the immediate and long-term effects of the antibiotic treatment on GM can be analysed. As the hospital stay differs in APPAC II and APPAC III patients, the effects of the duration of the hospital stay on the emergence of resistant bacteria and AMR reservoir can be evaluated. Moreover, the comparison of the effects of antibiotic treatment on GM between two different administration routes (p.o. and i.v. + p.o.) is possible.

In the 5-year follow up of the original APPAC trial, 39 % (n=100) of the antibiotic group patients underwent appendectomy for either during the primary hospitalization or for suspected late appendicitis recurrence. Out of these hundred patients, 85 were operated on for suspected recurrence and 78 patients had a histopathologically confirmed acute appendicitis [28]. Understanding the pathophysiology and contributing factors in recurrent appendicitis are of vital clinical importance in evaluating the optimal treatment paradigms for uncomplicated acute appendicitis. Therefore, MAPPAC trial is assessing both initial non-responders to antibiotic therapy and recurrent appendicitis after successful initial antibiotic treatment and

these results may provide novel tools to predict the potential recurrence risk and thus help to assess the optimal treatment choice for patients with uncomplicated acute appendicitis.

**Strengths and limitations**

MAPPAC has a strong synergy between APPAC II and III studies enabling the enrolment of both uncomplicated and complicated acute appendicitis patients in a large prospective series with associated clinical data to be assessed in conjunction with the microbiological and immunological findings. In many aspects, MAPPAC is an exploratory study of possible associations of whole microbial community and host immune characteristics with uncomplicated vs. complicated appendicitis and antibiotic response among patients in clinical trials treated with and without antibiotics. MAPPAC trial aims to generate hypotheses to better understand the role of disease progression and host susceptibility for future studies; i.e. determination of one primary outcome is insufficient for the study, as several factors are indispensable for the understanding of etiology and the effects of antibiotics on GM all provided with this unique set of microbiological samples. To our knowledge, only one previous study [12] has characterized the adult appendix microbiota during appendicitis in adult patients using NGS technique and to date no reports on the structure and physicochemical contents of appendicoliths exists. Using these assessments is a strength in our study. Further, to our knowledge this is the first trial aiming to prospectively assess the possible microbiological and/or immunological etiology of appendicitis recurrence after a successful initial conservative treatment with antibiotics or symptomatic therapy and primary non-responders to conservative treatment of uncomplicated acute appendicitis. One of the main hypothesis of the MAPPAC study is that the microbial composition of appendix differs between CT differentiated complicated and uncomplicated appendicitis. Therefore, strong element of the study is that all patients included in the study are imaged with CT protocol. CT scan is the gold standard for

acute appendicitis imaging and this near perfect accuracy of CT adds to the reliability of the clinical data and appendicitis severity also in the patients with uncomplicated acute appendicitis without a histopathological confirmation of the appendix.

The study limitations include the difficult challenge of conducting prospective clinical trials in the emergency setting. It is expected that all eligible patients may not be evaluated for enrolment or some patients may not have all study samples available as the recruitment is performed by a large number of surgeons on call. The lack of healthy control group is a limitation in the study regarding both the etiology and in determining the effects of antibiotics on GM, as the results cannot be fully distinguished from the effects of acute appendicitis.

## Authors' contributions

All authors have contributed to the design of this trial protocol. PS is the chief investigator. PS, SV, AJH, EM, SJ, JG, and SS have initiated the project. The protocol was drafted by PS, which was refined by SV, AJH, EM, SS, SJ, JG, HM, EE, and SH. Statistical advice was provided by SH. SV was responsible for drafting the manuscript, which was refined by PS, AJH, and EM. All authors have read and approved the final manuscript.

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## Competing interests

Authors declare they have no competing interests.

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## REFERENCES

1 Addiss DG, Shaffer N, Fowler BS, et al. The epidemiology of appendicitis and appendectomy in the United States, *Am J Epidemiol* 1990;132:910-25.

2 Ferris M, Quan S, Kaplan B, et al. The Global Incidence of Appendicitis: A Systematic Review of Population-based Studies, *Annals of Surgery* 2017;266:237-41 doi:10.1097/SLA.0000000000002188.

3 Livingston EH, Fomby TB, Woodward WA, et al. Epidemiological Similarities Between Appendicitis and Diverticulitis Suggesting a Common Underlying Pathogenesis, *Archives of Surgery* 2011;146:308-14 doi:10.1001/archsurg.2011.2.

4 Forbes GB, Lloyd-Davies RW. Calculous disease of the vermiform appendix, *Gut* 1966;7:583-92 doi:10.1136/gut.7.6.583.

5 Lamps LW. Infectious Causes of Appendicitis, *Infectious Disease Clinics of North America* 2010;24:995-1018 doi:10.1016/j.idc.2010.07.012.

6 Swidsinski A, Doerffel Y, Loening-Baucke V, et al. Acute appendicitis is characterised by local invasion with Fusobacterium nucleatum/necrophorum, *Gut* 2011;60:34-40 doi:10.1136/gut.2009.191320.

7 Zhong D, Brower-Sinning R, Firek B, et al. Acute appendicitis in children is associated with an abundance of bacteria from the phylum Fusobacteria, *J Pediatr Surg* 2014;49:441-6 doi:10.1016/j.jpedsurg.2013.06.026 [doi].

8 Jindal N, Kaur GD, Arora S, et al. Bacteriology of acute appendicitis with special reference to anaerobes, *Indian J Pathol Microbiol* 1994;37:299-305.

9 Martirosian G, Bulanda M, Wojcik-Stojek B, et al. Acute appendicitis: the role of enterotoxigenic strains of Bacteroides fragilis and Clostridium difficile, *Med Sci Monit* 2001;7:382-6.

10 Chen CY, Chen YC, Pu HN, et al. Bacteriology of acute appendicitis and its implication for the use of prophylactic antibiotics, *Surg Infect (Larchmt)* 2012;13:383-90 doi:10.1089/sur.2011.135 [doi].

11 Reinisch A, Malkomes P, Habbe N, et al. Bad bacteria in acute appendicitis: rare but relevant, *Int J Colorectal Dis* 2017;32:1303-11 doi:10.1007/s00384-017-2862-0 [doi].

12 Guinane CM, Tadrous A, Fouhy F, et al. Microbial composition of human appendices from patients following appendectomy, *MBio* 2013;4:12.

13 Peeters T, Penders J, Smeekens SP, et al. The fecal and mucosal microbiome in acute appendicitis patients: an observational study, *Future Microbiol* 2019;14:111-27 doi:10.2217/fmb-2018-0203 [doi].

14 Bennion RS, Baron EJ, Thompson JE,Jr, et al. The bacteriology of gangrenous and perforated appendicitis--revisited, *Ann Surg* 1990;211:165-71 doi:10.1097/00000658-199002000-00008 [doi].

15 Rivera-Chavez FA, Peters-Hybki DL, Barber RC, et al. Innate immunity genes influence the severity of acute appendicitis, *Ann Surg* 2004;240:269-77 doi:00000658-200408000-00012 [pii].

16 Reinisch A, Malkomes P, Habbe N, et al. Bad bacteria in acute appendicitis: rare but relevant, *Int J Colorectal Dis* 2017;32:1303-11 doi:10.1007/s00384-017-2862-0.

17 Martirosian G, Bulanda M, Wojcik-Stojek B, et al. Acute appendicitis: the role of enterotoxigenic strains of Bacteroides fragilis and Clostridium difficile, *Med Sci Monit* 2001;7:382-6.

18 Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation, *Proc Natl Acad Sci U S A* 2011;108 Suppl 1:4554-61 doi:10.1073/pnas.1000087107 [doi].

19 Jernberg C, Lofmark S, Edlund C, et al. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota, *ISME J* 2007;1:56-66 doi:ismej20073 [pii].

20 Manichanh C, Rigottier-Gois L, Bonnaud E, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach, *Gut* 2006;55:205-11 doi:gut.2005.073817 [pii].

21 Qin J, Li Y, Cai Z, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes, *Nature* 2012;490:55-60 doi:10.1038/nature11450 [doi].

22 Lindgren M, Lofmark S, Edlund C, et al. Prolonged impact of a one-week course of clindamycin on Enterococcus spp. in human normal microbiota, *Scand J Infect Dis* 2009;41:215-9 doi:10.1080/00365540802651897 [doi].

23 Raymond F, Ouameur AA, Deraspe M, et al. The initial state of the human gut microbiome determines its reshaping by antibiotics, *ISME J* 2016;10:707-20 doi:10.1038/ismej.2015.148 [doi].

24 Jakobsson HE, Jernberg C, Andersson AF, et al. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome, *PLoS One* 2010;5:e9836 doi:10.1371/journal.pone.0009836 [doi].

25 Buelow E, Bello Gonzalez, T D J, Fuentes S, et al. Comparative gut microbiota and resistome profiling of intensive care patients receiving selective digestive tract decontamination and healthy subjects, *Microbiome* 2017;5:z doi:10.1186/s40168-017-0309-z [doi].

26 Card RM, Mafura M, Hunt T, et al. Impact of Ciprofloxacin and Clindamycin Administration on Gram-Negative Bacteria Isolated from Healthy Volunteers and Characterization of the Resistance Genes They Harbor, *Antimicrob Agents Chemother* 2015;59:4410-6 doi:10.1128/AAC.00068-15 [doi].

27 Salminen P, Paajanen H, Rautio T, et al. Antibiotic Therapy vs Appendectomy for Treatment of Uncomplicated Acute Appendicitis: The APPAC Randomized Clinical Trial, *JAMA* 2015;313:2340-8.

28 Salminen P, Tuominen R, Paajanen H, et al. Five-Year Follow-up of Antibiotic Therapy for Uncomplicated Acute Appendicitis in the APPAC Randomized Clinical Trial, *JAMA* 2018;320:1259-65 doi:10.1001/jama.2018.13201 [doi].

29 Sakran JV, Mylonas KS, Gryparis A, et al. Operation versus antibiotics--The "appendicitis conundrum" continues: A meta-analysis, *J Trauma Acute Care Surg* 2017;82:1129-37 doi:10.1097/TA.0000000000001450 [doi].

30 Sallinen V, Akl EA, You JJ, et al. Meta-analysis of antibiotics versus appendicectomy for non-perforated acute appendicitis, *Br J Surg* 2016;103:656-67 doi:10.1002/bjs.10147 [doi].

31 Podda M, Gerardi C, Cillara N, et al. Antibiotic Treatment and Appendectomy for Uncomplicated Acute Appendicitis in Adults and Children: A Systematic Review and Meta-analysis, *Ann Surg* 2019 doi:10.1097/SLA.0000000000003225 [doi].

32 Harnoss JC, Probst P, Büchler MW, et al. Antibiotics Versus Appendicectomy for the Treatment of Uncomplicated Acute Appendicitis: An Updated Meta-Analysis of Randomised Controlled Trials by Rollins et al, *World J Surg* 2017;41:2411 doi:10.1007/s00268-016-3864-8.

33 Rollins KE, Varadhan KK, Neal KR, et al. Antibiotics Versus Appendicectomy for the Treatment of Uncomplicated Acute Appendicitis: An Updated Meta-Analysis of Randomised Controlled Trials, *World J Surg* 2016;40:2305-18 doi:10.1007/s00268-016-3561-7 [doi].

34 Chan A, Tetzlaff JM, Altman DG, et al. SPIRIT 2013 statement: defining standard protocol items for clinical trials, *Ann Intern Med* 2013;158:200-7 doi:10.7326/0003-4819-158-3-201302050-00583.

35 Haijanen J, Sippola S, Gronroos J, et al. Optimising the antibiotic treatment of uncomplicated acute appendicitis: a protocol for a multicentre randomised clinical trial (APPAC II trial), *BMC Surg* 2018;18:y doi:10.1186/s12893-018-0451-y [doi].

36 Sippola S, Gronroos J, Sallinen V, et al. A randomised placebo-controlled double-blind multicentre trial comparing antibiotic therapy with placebo in the treatment of uncomplicated acute appendicitis: APPAC III trial study protocol, *BMJ Open* 2018;8:023623 doi:10.1136/bmjopen-2018-023623 [doi].

37 Lewis SJ, Heaton KW. Stool form scale as a useful guide to intestinal transit time, *Scand J Gastroenterol* 1997;32:920-4 doi:10.3109/00365529709011203 [doi].

38 Vons C, Barry C, Maitre S, et al. Amoxicillin plus clavulanic acid versus appendicectomy for treatment of acute uncomplicated appendicitis: an open-label, non-inferiority, randomised controlled trial, *Lancet* 2011;377:1573-9 doi:10.1016/S0140-6736(11)60410-8.

39 Sippola S, Grönroos J, Tuominen R, et al. Economic evaluation of antibiotic therapy versus appendicectomy for the treatment of uncomplicated acute appendicitis from the APPAC randomized clinical trial, *British Journal of Surgery* 2017;104:1355-61 doi:10.1002/bjs.10575.

## Abbreviations

**AMR:** Antimicrobial resistance

**CRP:** C-reactive protein

**CT:** Computed tomography

**GM:** Gut microbiota

**i.v.:** Intravenous

**MALDI-TOF:** Matrix Assisted Laser Desorption/Ionisation time-of-flight mass spectrometry

**MS:** Mass spectrometry

**NGS:** Next generation sequencing

**p.o.:** Per os

**SPIRIT:** Standard Protocol Items: recommendations for Interventional Trials

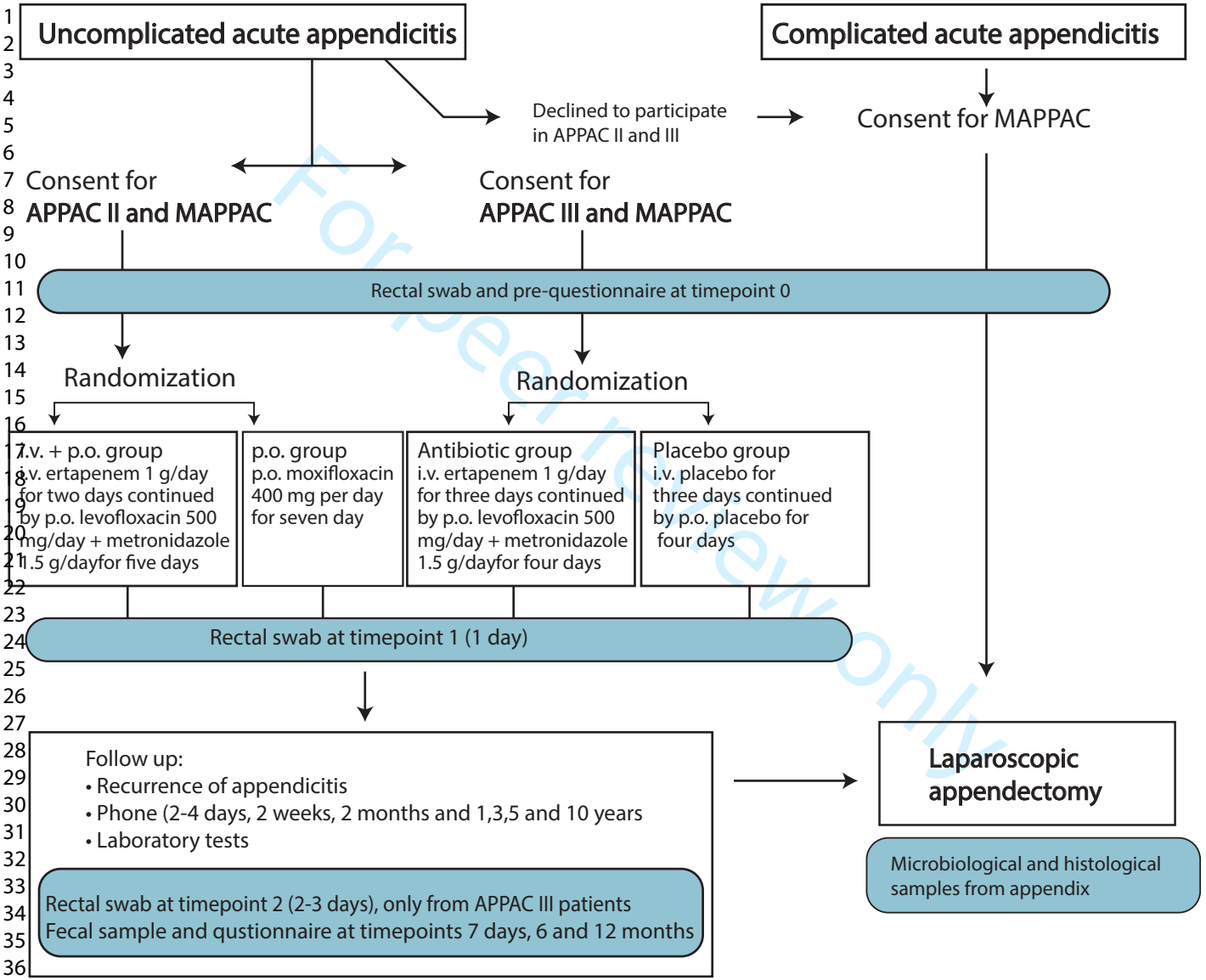
**VAP-1:** Vascular adhesion protein-1

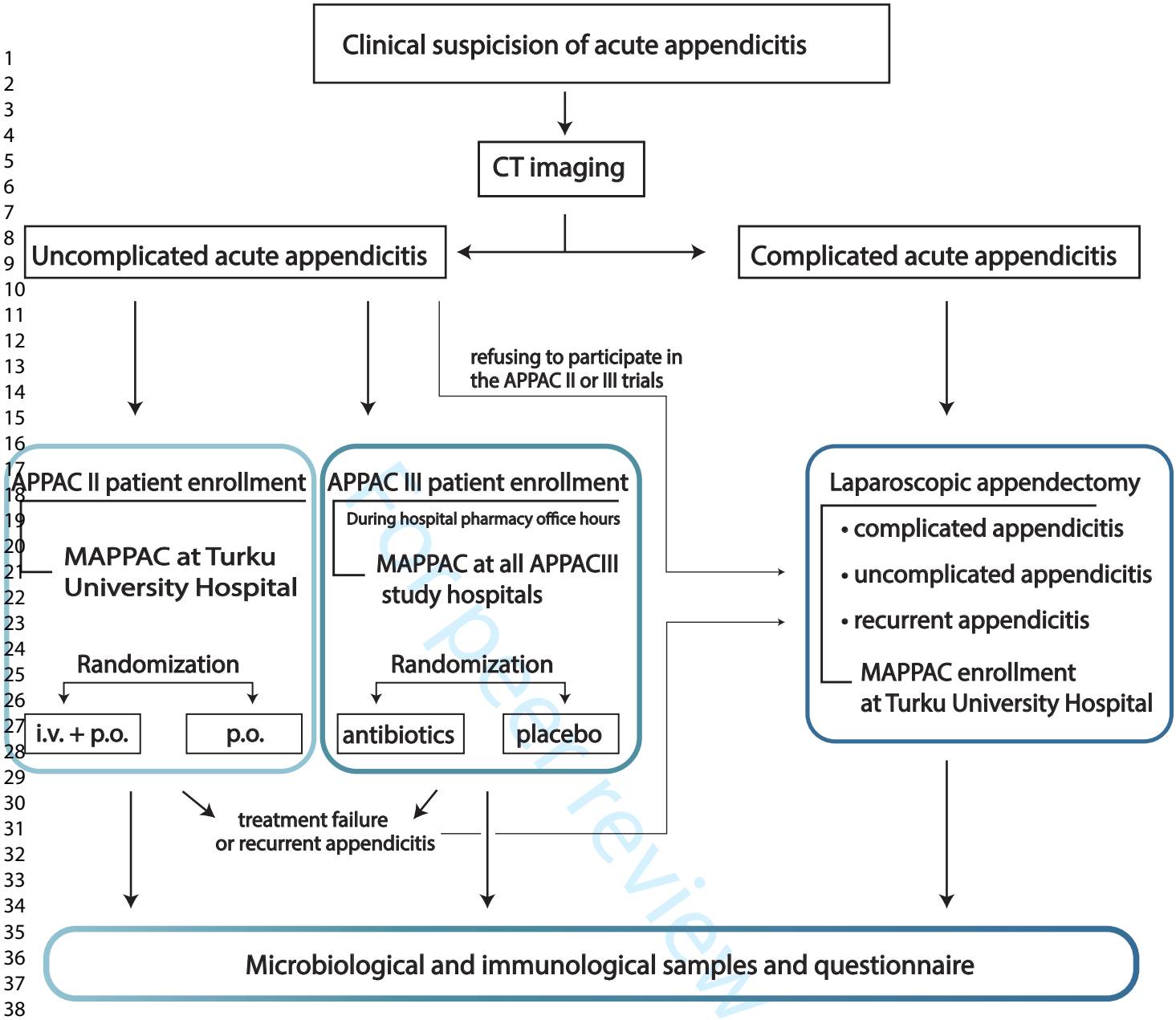
**VAS:** Visual analogue scale

## FIGURE LEGENDS

Figure 1.The synergy between MAPPAC, APPAC II and APPAC III studies.

Figure 2. Flow chart of the study protocol





**SUPPLEMENTARY FILE**

**Laboratory methods**

**DNA extraction and analysis**

**Stool samples and rectal and appendiceal swabs**

Bacterial DNA from rectal and appendiceal swabs and faecal samples are extracted with semiautomated DNA extraction kit together with NorDiag Arrow extraction instrument (Diasorin). The following sample preparation is undertaken before the extraction: 500 µl of the sample is added to 700 µl of stool stabilizer in a 1.4 mm Ceramic Powerbead Tube (Qiagen, Hilden, Germany) and homogenized with MO BIO PowerLyzer 24 Bench Top Bead-Based Homogenizer (MO BIO Laboratories, Inc., USA) at 1000 rpm for 3 minutes followed by centrifugation at 5000 x g for 5 minutes and 600 µl of the supernatant is transferred into a new tube, the centrifugation is repeated and 500 µl of the supernatant is transferred into anew tube. This pretreatment is followed by the DNA extraction, which is performed according to the manufacturer´s instructions.

**Appendiceal biopsy:** Microbiological DNA is extracted from appendix biopsy stored in RNAlater with semiautomated DNA extraction kit using NorDiag Arrow extraction instrument. At first RNA later is removed by centrifugation and the pellet is resuspended into ultrapure water. Subsequently, the resuspension containing also the biopsy is treated with proteinase K in thermomixer. From the resulting solution DNA extraction is carried out according to manufacturer's instructions.

The concentration of eluted DNA from all sample types is measured with Qubit dsDNA HS or BR assay kit (Thermo Fisher Scientific) using Qubit fluorometer (Life Technologies, Carlsbad, California, USA). DNA is divided into two aliquots and stored at -75◦C for later analysis. Microbial

profile of samples will be analysed with NGS approach using appropriate methods and Illumina Miseq system.

**Analyses of metagenome, transcriptome and proteome from appendiceal biopsy**

Total RNA and proteins from the appendiceal biopsy are extracted with appropriate methods. Transcriptome and metagenome are analysed with Illumina Hiseq system. In addition, the expression of specific genes is quantified with quantitative real-time PCR. Proteome is characterized using mass spectrometry-based methods with qualitative and quantitative approach.

**Culture methods**

Appendiceal swabs are cultured with both aerobic and anaerobic culture methods. For aerobic culture the following growth media are used: CHROMagar Orientation (Becton Dickinson, Franklin Lakes, New Jersey, USA), Trypticase soy agar with 5% sheep blood (Becton Dickinson), Yersinia selective agar and Streptococcus selective agar (in house production). If the appendectomy is performed during the office hours, an additional anaerobic culture is made in connection with the sample collection. Samples for anaerobic culturing are collected with a sterile cotton swab from the appendix and cultured for fastidious anaerobe agar with blood (FAA) and kanamycin-vancomycin laked blood agar (KVLB) (both in house production), which are immediately transferred into anaerobic jar with anaerobic gas generating sachet (Anaerogen 3,5 l, Thermo Fisher Scientific).

Cultures are incubated at +35 °C for 18-24 hours. Morphologically distinct colonies are subcultured until a pure culture is obtained. Isolated species are frozen in a mixture of skim milk and glycerol at −75 °C.

**MALDI-TOF mass spectrometry**

The identification of isolated bacteria is done with Bruker matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). Isolates are inoculated and cultured for 18-48 hours at +35 °C before the analysis. For the analysis, colony is applied either directly or through ethanol/formic acid extraction on a steel plate and HCCA matrix (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile-2.5% trifluoroacetic acid (Bruker Daltonics, Bremen, Germany)) is added according to the manufacturer´s instructions. The identification is done using Microflex LT instrument and MALDI Biotyper software (Bruker Daltonics). If isolates cannot be identified due to the absence of reference peaks in the database, the isolate is identified with sequencing the 16S rRNA gene.

**Antimicrobial susceptibility testing**

Phenotypic antimicrobial susceptibility testing for the isolated aerobic and anaerobic bacteria is performed by disk diffusion and MIC methods, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. For certain bacteria, the Clinical and Laboratory Standards Institute (CLSI) guidelines can be used.

Further molecular analysis for antimicrobial resistance or virulence genes using e.g., PCR, Sanger sequencing and whole genome sequencing (WGS) are also performed for selected isolates.

**Appendicolith analysis**

Appendicoliths are assessed both on the surface and cross-sectional morphology. Based on the morphological characteristics and a degree of hardness, all appendicoliths will be classified. The composition of selected appendicoliths are analysed with physical and chemical methods.

**Immunological analysis**

The appendiceal biopsies are analysed immunohistochemically by determining the presence of different cell types and blood vessel surface markers. Neutrophils, leukocytes and monocytes will be analysed and special interest will be focused on lymphocyte subtypes (i.e., CD4/CD8 and more detailed subgroup analyses such as regulatory T cells) and monocyte markers (i.e., macrophage M1/M2 / receptor MHCII). Moreover, certain inflammation induced markers on endothelium such as VAP-1, E-selectin and P-selectin will be evaluated. In order to compare possible differences between patients with successful antibiotic therapy to patients with failed antibiotic therapy or complicated acute appendicitis the serum samples will be screened to identify possible inflammatory or immunological markers for identifying the different forms of the disease. Cytokines, chemokines and growth factors will be tested using Bio-Plex Pro Human Cytokine 48-Plex Screening Panel (BIO-RAD) containing the following analytes: Basic FGF, CTACK, eotaxin, G-CSF, GM-CSF, GRO-a, HGF, ICAM-1, IFN-a2, IFN-g, IL-1a, IL-1b, IL-1ra, IL-2, IL-2Ra, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-16, IL-17A, IL-18, IP-10, LIF, MCP-1 (MCAF), MCP-3, M-CSF, MIF, MIG, MIP-1a, MIP-1b, b-NGF, PDGF-BB, RANTES, SCF, SCGF-b, SDF-1a, TNF-a, TNF-b, TRAIL, VCAM-1 and VEGF-A. Moreover, we will use in house analyses to measure soluble VAP-1 (inflammatory) and CD73 (anti-inflammatory). In addition, metabolomics approach using nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS) metabolomics platforms will be used for biomarker analysis. The results obtained from immunological analyses will be correlated to the clinical parameters and to the microbiological findings.

SPIRIT
STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

| Section/item | Item No | Description | Page Number on which item is reported |
|---|---|---|---|
| **Administrative information** | | | |
| Title | 1 | Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym | 1 |
| Trial registration | 2a | Trial identifier and registry name. If not yet registered, name of intended registry | 3 |
| | 2b | All items from the World Health Organization Trial Registration Data Set | n/a |
| Protocol version | 3 | Date and version identifier | n/a |
| Funding | 4 | Sources and types of financial, material, and other support | 22 |
| Roles and responsibilities | 5a | Names, affiliations, and roles of protocol contributors | 1,22 |
| | 5b | Name and contact information for the trial sponsor | 1 |
| | 5c | Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities | 22 |
| | 5d | Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee) | 7-8 |
| **Introduction** | | | |

| | | | |
|---|---|---|---|
| Background and rationale | 6a | Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention | 5-7 |
| | 6b | Explanation for choice of comparators | 6-7 |
| Objectives | 7 | Specific objectives or hypotheses | 7 |
| Trial design | 8 | Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory) | 7 |
| **Methods: Participants, interventions, and outcomes** | | | |
| Study setting | 9 | Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained | 8 |
| Eligibility criteria | 10 | Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists) | 9-11 |
| Interventions | 11a | Interventions for each group with sufficient detail to allow replication, including how and when they will be administered | 11-12 |
| | 11b | Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease) | 14 |
| | 11c | Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests) | 14-15, 17 |
| | 11d | Relevant concomitant care and interventions that are permitted or prohibited during the trial | n/a |
| Outcomes | 12 | Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended | 15-16 |

| | | | |
|---|---|---|---|
| Participant timeline | 13 | Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure) | 12-15, figure 2 |
| Sample size | 14 | Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations | 16, n/a |
| Recruitment | 15 | Strategies for achieving adequate participant enrolment to reach target sample size | 11, 17 |
| **Methods: Assignment of interventions (for controlled trials)** | | | |
| Allocation: | | | n/a |
| Sequence generation | 16a | Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions | n/a |
| Allocation concealment mechanism | 16b | Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned | n/a |
| Implementation | 16c | Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions | n/a |
| Blinding (masking) | 17a | Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how | n/a |
| | 17b | If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial | n/a |
| **Methods: Data collection, management, and analysis** | | | |

| | | | |
|---|---|---|---|
| Data collection methods | 18a | Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol | 12-17, supplementary material |
| | 18b | Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols | 17 |
| Data management | 19 | Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol | 18 |
| Statistical methods | 20a | Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol | 16 |
| | 20b | Methods for any additional analyses (eg, subgroup and adjusted analyses) | 17 |
| | 20c | Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation) | n/a |
| **Methods: Monitoring** | | | |
| Data monitoring | 21a | Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed | 22 |
| | 21b | Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial | n/a |

| | | | |
|---|---|---|---|
| Harms | 22 | Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct | 17, 18 |
| Auditing | 23 | Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor | n/a |
| **Ethics and dissemination** | | | |
| Research ethics approval | 24 | Plans for seeking research ethics committee/institutional review board (REC/IRB) approval | 18 |
| Protocol amendments | 25 | Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators) | n/a |
| Consent or assent | 26a | Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32) | 8-9 |
| | 26b | Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable | 9, 18 |
| Confidentiality | 27 | How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial | 18 |
| Declaration of interests | 28 | Financial and other competing interests for principal investigators for the overall trial and each study site | 22 |
| Access to data | 29 | Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators | 18 |
| Ancillary and post-trial care | 30 | Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation | n/a |
| Dissemination policy | 31a | Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions | 18 |

| | | | |
|---|---|---|---|
| | 31b | Authorship eligibility guidelines and any intended use of professional writers | n/a |
| | 31c | Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code | n/a |
| **Appendices** | | | |
| Informed consent materials | 32 | Model consent form and other related documentation given to participants and authorised surrogates | in finnish only |
| Biological specimens | 33 | Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable | 12-15, supplementary material |

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.