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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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Keywords:	appendicitis, appendectomy, appendicitis etiology, appendicolith, microbiota, antimicrobial resistance

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

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3 34
45 35 **ABSTRACT**

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8 36 **Introduction:** Based on epidemiological and clinical data acute appendicitis can present either
9
10 37 as uncomplicated or complicated. Recent studies have shown the safety, efficacy and cost-
11
12 38 effectiveness of antibiotics for CT-confirmed uncomplicated acute appendicitis. The etiology of
13
14 39 these different appendicitis forms remains unknown. Complicated acute appendicitis most
15
16 40 often requires emergency appendectomy. Despite appendicitis being one of the most common
17
18 41 surgical emergencies, there are very few reports on appendicitis etiology and pathophysiology
19
20 42 focusing on the differences between uncomplicated and complicated appendicitis. MAPPAC
21
22 43 trial aims to evaluate microbiological etiology of these different forms also assessing both
23
24 44 antibiotics non-responders and recurrent appendicitis after successful antibiotic treatment.
25
26 45 MAPPAC also aims to determine antibiotic and placebo effects on gut microbiota composition
27
28 46 and antimicrobial resistance.

29 47 **Methods and analysis:** MAPPAC is a prospective clinical trial with both single- and multicentre
30
31 48 arm conducted in close synergy with concurrent trials APPAC II (p.o. vs. i.v+p.o. antibiotics,
32
33 49 NCT03236961) and APPAC III (double-blind trial placebo vs. antibiotics, NCT03234296)
34
35 50 randomized clinical trials further aiming to optimise the non-operative treatment of
36
37 51 uncomplicated acute appendicitis. Based on the enrolment for these trials, patients with CT-
38
39 52 confirmed uncomplicated acute appendicitis are recruited also to the MAPPAC study. In
40
41 53 addition to conservatively treated uncomplicated acute appendicitis patients, MAPPAC will
42
43 54 recruit patients with uncomplicated and complicated appendicitis undergoing appendectomy.
44
45 55 Rectal and appendiceal swabs, appendicolith, faecal, and serum samples, appendiceal biopsies,
46
47 56 and clinical data are collected during the hospital stay for microbiological and immunological
48
49 57 analysis in both study arms with the longitudinal study arm collecting samples also during
50
51 58 follow-up up to 12 months after appendicitis treatment.
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3 59 **Ethics and dissemination:** This study has been approved by the Ethics Committee of the
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5 60 Hospital District of Southwest Finland (Turku University Hospital) and the Finnish Medicines
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8 61 Agency (Fimea). Results of the trial will be published in peer-reviewed journals.
9

10 62 **Trial registration:** Eudra-CT: 2016-003655-29 (2017-01-12), clinicaltrials.gov NCT03257423.
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16 65 **KEYWORDS**

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18 66 Appendicitis, appendectomy, appendicitis etiology, appendicolith, microbiota, antimicrobial
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71 **Strengths and limitations of this study**

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

96 INTRODUCTION

97 Acute appendicitis is one of the most common causes of abdominal pain in emergency
98 departments. The lifetime risk of acute appendicitis in males is 8.6% and 6.7% in females [1]
99 with recent meta-analysis showing an increasing trend in appendicitis incidence in the
100 industrialised countries [2]. Appendectomy has unquestionably been the standard treatment
101 for acute appendicitis for over a century with more than 300 000 appendectomies performed
102 annually in the United States [3]. Although appendectomy is generally well tolerated, it is a
103 major surgical intervention potentially associated with postoperative morbidity [4-6]. Based
104 on epidemiological and clinical data, acute appendicitis can present either as uncomplicated
105 or complicated with the majority of cases being uncomplicated. Increasing amount of evidence
106 shows that the majority of patients with uncomplicated acute appendicitis may be treated
107 with antibiotics alone instead of surgery [7-13]. The original APPAC (APPendicitis ACuta) trial
108 reported that at long-term follow-up, the majority of patients with computed tomography
109 (CT) confirmed uncomplicated acute appendicitis were successfully treated with antibiotics,
110 and those patients that required later appendectomy did not have increased or major
111 complications [10, 14]. Antibiotics alone is an efficient and a safe first-line treatment for CT-
112 confirmed uncomplicated acute appendicitis [8, 15-17]. In addition to decreased morbidity,
113 antibiotic therapy was shown to offer significant cost savings compared to surgery [18].
114 The different epidemiological trends of uncomplicated and complicated acute appendicitis
115 indicate different pathophysiology [3]. Despite the high incidence of acute appendicitis, there
116 are very few reports on appendicitis etiology and pathophysiology especially focusing on the
117 possible differences between uncomplicated and complicated acute appendicitis. Complicated
118 acute appendicitis, defined as a finding of perforation, appendicolith, abscess or a suspicion of
119 tumor, requires emergency appendectomy. Appendicolith is a calcified faecal concretion in
120 the appendix and it is the most common form of complicated acute appendicitis. Even though

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121 the first thorough study on appendicoliths was already reported in 1966 [19], information
122 about appendiceal calculi is scarce. Obstruction of the appendiceal lumen caused by an
123 appendicolith, lymphoid hyperplasia, or swelling has been evaluated to be the primary cause
124 of appendicitis and bacterial overgrowth has been considered a consequence [20]. However,
125 bacterial infection has also been proposed as the primary cause of appendicitis [21, 22].
126 *Bacteroides* species are reported to be one of most common bacterial findings in appendicitis
127 [23, 24]. Further, certain members of the *Fusobacteria*, especially *F. nucleatum* and *F.*
128 *necrophorum*, are present in most appendicitis samples [21]. The most common aerobic
129 bacteria organism detected by culturing is *Escherichia coli*, but also *Klebsiella pneumoniae*,
130 *Streptococcus spp.*, *Enterococcus spp.* and *Pseudomonas aeruginosa* have been reported [25,
131 26]. To our knowledge, only one study with a very small number of patients has characterised
132 the adult appendiceal microbiota with next generation sequencing (NGS) methods. Appendix
133 seems to have diverse microbiota including both commensal species from gut microbiota
134 (GM) and opportunistic pathogens [27]. Since the interindividual variability in the microbial
135 composition of the appendix samples is high [27], a larger number of appendicitis patients is
136 needed to draw conclusions.

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

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3 146 The MAPPAC (Microbiology APPendicitis ACuta) trial patient enrolment is based on the
4
5 147 ongoing APPAC II and APPAC III randomised multicentre clinical trials of our study group.
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8 148 APPAC II trial aims to optimise antibiotic treatment for CT confirmed uncomplicated acute
9
10 149 appendicitis in order to both shorten the hospital stay and restrict the antibacterial spectrum.
11
12 150 The APPAC III trial aims to assess symptomatic treatment of uncomplicated acute appendicitis
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14
15 151 and the role of antibiotics in the resolution of uncomplicated appendicitis. To our knowledge,
16
17 152 there are so far no similar large microbiological studies focusing on acute appendicitis
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20 153 performed in conjunction with large clinical trials with prospective access to both
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22 154 uncomplicated and complicated appendicitis patients.
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25 155 MAPPAC trial aims to evaluate the possible role and differences in the microbiological etiology
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28 156 of complicated and uncomplicated appendicitis with a special reference to the presence of an
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30 157 appendicolith. In addition, MAPPAC aims to evaluate the immunological and microbiological
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33 158 factors involved in appendicitis recurrence after successful initial antibiotic therapy. In the
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35 159 longitudinal study arm we also aim to assess the effects of antibiotic and placebo treatment on
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37 160 the GM profile and the effects of hospital stay duration on the AMR reservoir of the GM.
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167 **METHODS AND ANALYSIS**

168 **Study Design**

169 MAPPAC is a prospective clinical trial with a single-centre and a multicentre arm. The single-
170 centre study arm at Turku University Hospital, aims to determine the possible differences in
171 the etiology of complicated and uncomplicated acute appendicitis with a special reference to
172 the role and etiology of appendicolith. In the multi-centre longitudinal arm, MAPPAC trial
173 concentrates on assessment of possible immunological and microbiological factors involved
174 in initial non-responders or appendicitis recurrence after successful initial antibiotic therapy
175 at long-term follow-up. In this longitudinal multicentre study arm we also aim to evaluate the
176 effects of antibiotic and placebo treatment on the GM and the effects of the duration of the
177 hospital stay on the possible AMR reservoir within the GM. MAPPAC protocol was drafted in
178 accordance with the SPIRIT statement [40]. The trial has been registered at both EudraCT
179 (2016-003655-29) and clinicaltrials.gov (NCT03257423).

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

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3 192 and III trials will be invited to participate in the MAPPAC trial. Patients recruited for the
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5 193 APPAC II or III trial are asked to sign a separate consent form for the MAPPAC trial allowing
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8 194 for the use of their data and collection of microbiological samples. The study flow is illustrated
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10 195 in Figure 2.

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14 15 197 **Patient selection**

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17 198 Eligible for inclusion are all adult patients 18 – 60 years old admitted to the emergency
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20 199 department with suspected acute appendicitis in whom a CT-confirmed uncomplicated or
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22 200 complicated appendicitis is diagnosed or patients presenting with a suspected recurrent
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24 201 appendicitis after initial successful antibiotic therapy for uncomplicated acute appendicitis.
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26 202 The optimized low-dose and standard CT protocols from our OPTICAP trial [43] are used at
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29 203 all trial hospitals. MAPPAC single-centre arm will enroll patients with both uncomplicated
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31 204 (both APPAC II and III trials and patients declining to participate in these trials undergoing
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33
34 205 appendectomy) and complicated acute appendicitis (patients excluded from APPAC II and
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36 206 APPAC III trials) at Turku University Hospital. The enrolment of uncomplicated acute
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38 207 appendicitis patients participating in the APPAC III trials will be performed at all APPAC III
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41 208 hospitals (all five Finnish university hospitals: Turku, Helsinki, Tampere, Oulu, and Kuopio).
42
43 209 In addition, all of the APPAC II and III trial patients having to undergo appendectomy either
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45 210 for a treatment failure during the primary hospitalisation or for suspected recurrence after a
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48 211 successful initial non-operative treatment will be enrolled in the MAPPAC trial at all ten study
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50 212 hospitals (all five university and five central hospitals in Finland: Jyväskylä, Pori, Mikkeli,
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52 213 Seinäjoki and Rovaniemi).

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55 214 All adult patients with a clinical suspicion of acute appendicitis will be studied carefully by
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57 215 attending surgeons at the emergency departments of the participating hospitals. Clinical
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59 216 history, physical investigation, and laboratory tests are evaluated and if these indicate acute

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217 appendicitis, the patient will undergo CT imaging with either optimised low-dose (BMI<30
218 kg/m²) or standard CT (BMI>30kg/m²).

220 Inclusion criteria

221 MAPPAC inclusion criteria follow mainly the criteria of APPAC II and APPAC III trials enrolling
222 patients with CT-confirmed uncomplicated acute appendicitis. In addition, MAPPAC includes
223 patients with complicated acute appendicitis and patients under or over the age limit of 18 to
224 60 years of the APPAC II and APPAC III trials in the single-centre study arm at Turku
225 University Hospital. Inclusion criteria: signed informed consent, CT scan confirmed diagnosis
226 of either uncomplicated acute appendicitis or complicated acute appendicitis (appendicolith,
227 perforation, abscess, suspicion of a tumor), suspected treatment failure of antibiotic therapy
228 for uncomplicated acute appendicitis during the primary hospitalisation of APPAC II or III
229 patients, and suspected recurrent appendicitis after a successful initial antibiotic therapy for
230 uncomplicated acute appendicitis. Patients with uncomplicated acute appendicitis
231 undergoing appendectomy based on the inclusion criteria and declining to participate in the
232 APPAC II or III trials, may also be enrolled in the MAPPAC trial.

233 Exclusion criteria

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

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3 242 around the appendix). CT criteria for complicated acute appendicitis include appendicolith (>
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5 243 3mm stone within the appendix), abscess (periappendiceal walled of collection with
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8 244 enhancing walls), perforation (appendiceal wall enhancement defect and
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10 245 periappendiceal excess of fluid and/or infectious phlegmon and/or extraluminal air), or
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12 246 tumor suspicion (tumor-like prominence of the appendix).
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17 248 **Study setting and feasibility**

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19 249 The study was initiated at Turku University Hospital in April 2017, with the study
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22 250 commencing at all study centres by fall 2017. The majority of MAPPAC patient enrolment is
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24 251 evaluated to be completed in conjunction with the APPAC II and III trials latest by fall 2020.
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26 252 Patient recruitment in the MAPPAC trial will continue for a minimum of five years after APPAC
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29 253 II and III trial enrolment completion based on the planned microbiological assessment of late
30
31 254 appendicitis recurrence.
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36 256 **Interventional groups**

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38 257 Four interventional groups within MAPPAC are defined as follows:

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41 258 1. Patients with uncomplicated acute appendicitis participating in the APPAC II trial at
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43 259 Turku University Hospital (MAPPAC single-centre treatment arm) receiving either i.v.
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45 260 antibiotic therapy followed by p.o. antibiotics (i.v. + p.o.) or patients receiving p.o.
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48 261 antibiotic monotherapy (p.o.). *I.v. + p.o.* group receives intravenous ertapenem 1 g once
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50 262 daily for two days followed by p.o. levofloxacin 500 mg once daily and metronidazole
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52 263 500 mg three times per day for 5 days. *P.o.* group receives *p.o.* moxifloxacin 400 mg
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55 264 once daily for seven days.
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60 266 2. Patients with uncomplicated acute appendicitis participating in the double-blinded
APPAC III trial at all five university hospitals (MAPPAC multicentre treatment arm)

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3 267 receiving either antibiotic therapy or placebo treatment. *Antibiotic treatment* is
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5 268 intravenous ertapenem 1 g once per day for three days followed by p.o. levofloxacin
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8 269 500 mg once per day and metronidazole 500 mg three times per day for four days.
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10 270 *Placebo treatment* entails intravenous placebo once per day for three days followed by
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12 271 per oral placebo three times per day for four days.

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15 272 3. Patients with uncomplicated acute appendicitis or suspected recurrent appendicitis
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17 273 undergoing appendectomy

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20 274 The patients in this group will undergo laparoscopic appendectomy either after
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22 275 declining to participate in the APPAC II or APPAC III trials (MAPPAC single-centre
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24 276 treatment arm at Turku University Hospital) or after suspected treatment failure of
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26
27 277 non-operative treatment or after presenting with suspected appendicitis recurrence
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29 278 after initial successful non-operative treatment (MAPPAC multicentre treatment arm,
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31 279 all APPAC II and APPAC III hospitals)

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33
34 280 4. Patients with complicated acute appendicitis undergoing appendectomy

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36 281 The patients in this group will undergo laparoscopic appendectomy and are eligible for
37
38 282 enrolment only in the MAPPAC single-centre study arm at Turku University Hospital.
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42 43 284 **Sample collection**

44
45 285 **Rectal swabs** are collected from all patients in the emergency department prior to antibiotic
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48 286 treatment or surgery (timepoint 0). Further, rectal swabs are collected on the 1st day for
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50 287 APPAC II patients and for APPAC III patients on the 1st day (Turku University Hospital) and
51
52 288 on the 3rd day (all five university hospitals). At each time point, two rectal swabs are collected:
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55 289 Amies-Coal transport swab for culturing methods (Sarstedt, Nümbrecht, Germany) and
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57 290 Puritan DNA/RNA swab (Puritan Medical products, Guilford, Maine) with shield fluid (Zymo
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3 291 Research, Irvine, Canada) for microbial DNA extraction. The shield fluid in this collection tube
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5 292 allows the transportation at room temperature.

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8 293 **Samples from the appendix** are collected from patients undergoing appendectomy for
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10 294 complicated or uncomplicated acute appendicitis and from patients with suspected disease
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12 295 progression during the primary hospitalisation or appendicitis recurrence after successful
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15 296 initial non-operative therapy. Samples include routine histology as well as specific trial swabs
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17 297 and biopsies. The appendix is opened vertically with a sterile scalpel and two swabs are taken
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19
20 298 similar to the rectal swab (one for culture and one for DNA extraction). Three biopsies are
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22 299 taken at a distance of approximately 2 cm from the base of the appendix. All biopsies are
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24 300 stored at -75 °C, one immediately and the rest two are first embedded in RNAlater solution
25
26 301 (Thermo Fisher Scientific, Waltham, MA, USA). Possible appendicoliths are collected into a
27
28
29 302 sterile container, frozen and stored at -75 °C. If appendectomy is performed during office
30
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32 303 hours, appendiceal samples are collected by study personnel and an additional swab for
33
34 304 anaerobic culture is then collected and cultured in connection with collection and
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36 305 immediately transferred into an anaerobic jar. During on call hours, the samples are collected
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39 306 by surgeons and the -75 °C appendiceal biopsy may not be collected. These appendiceal
40
41 307 samples are collected only in the Turku University Hospital and at the other study hospitals
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43 308 only a swab sample (transport tube with DNA shield fluid) from the appendix of non-
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45
46 309 responders and patients with appendicitis recurrence is collected.

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48 310 **Additional serum samples** are collected from all the patients recruited in MAPPAC trial at
49
50 311 Turku University Hospital and for APPAC III trial at all five study hospitals for future
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53 312 immunological and inflammatory marker analysis and metabolomics approach. The serum
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55 313 samples are divided into six aliquots prior freezing at -75 °C.

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57 314 **Questionnaire**

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3 315 MAPPAC patients are asked to fill a primary questionnaire during the initial hospitalisation
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5 316 covering topics possibly affecting their GM composition: chronic diseases, special diets,
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8 317 smoking and alcohol consumption, travel history, antibiotic intake, other medications (12
9
10 318 months prior the sampling), consumption of probiotics and other dietary supplements, recent
11
12 319 diarrhoea and/or vomiting, Bristol stool form scale estimate [44] measuring stool consistency
13
14
15 320 (1 for very hard stool to 7 for diarrhoea) and the average number of defecations per day.
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17 321 **Follow-up during the hospitalisation**

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19 322 During the hospitalisation the following parameters will be recorded every 24 hours: pain
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22 323 assessed by Visual Analogue Scale (VAS), leukocyte count, CRP, temperature and clinical
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24 324 findings at patient reassessment. If the patient is suspected of not responding to the
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27 325 randomized therapy based on clinical deterioration signs combined with laboratory findings
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29 326 (signs of peritonitis, persisting fever, increasing pain, white blood cell count or CRP), the
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31 327 patient will be operated based on the surgeon's decision and the reasons for proceeding to
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33 328 appendectomy will be recorded. For appendectomy, laparoscopic approach is recommended.
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36 329 The operative findings and the histopathology of the appendix will be recorded. After the
37
38 330 initial hospitalisation, recurrent acute appendicitis will be diagnosed on a clinical basis and
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41 331 patients with a suspected recurrence of appendicitis will undergo a laparoscopic
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43 332 appendectomy.
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45 333 **Follow-up**

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48 334 The specific MAPPAC trial follow-up is conducted for patients also participating in the APPAC
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50 335 II and III trials with collection of three faecal samples at home (at one week, six months and
51
52 336 one year). Follow-up samples at home are not collected in MAPPAC interventional groups
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54
55 337 undergoing appendectomy. The follow-up faecal sample is collected into 10 ml DNA/RNA
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57 338 Shield Faecal Collection Tube (Zymo Research) enabling transportation and storage at room
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59 339 temperature prior to DNA extraction. Patients also deliver a faecal sample into a gel tube for
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3 340 culture purposes. A follow-up questionnaire is collected at 6 and 12 months consisting of the
4
5 341 same questions as the preliminary questionnaire covering the time between hospital
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8 342 discharge and the follow-up time point. Other follow-up for patients in APPAC II trial will
9
10 343 include a phone interview at one week after discharge, APPAC III trial patients have their first
11
12 344 follow-up call 2-4 days after discharge from the hospital and then at 10 days, both trials at two
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14
15 345 months and at one, three, five and ten years. The follow-up for APPAC III patients will include
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17 346 laboratory tests (leukocyte count, CRP, additional follow-up serum samples).

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21 22 348 **Outcome parameters**

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24 349 Based on the MAPPAC trial design, no specific primary endpoint can be determined. The
25
26 350 following parameters will be recorded for all patients: age, gender, BMI, clinical findings on
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29 351 admission (tympanic temperature, nausea, pain or tenderness in the right lower abdominal
30
31 352 quadrant, pain migration, VAS pain scores, white blood cell count, CRP, findings on CT
32
33 353 imaging), and data on primary and follow-up questionnaires. Blood cultures will be obtained
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35
36 354 from patients with complicated acute appendicitis and for APPAC II and III trial patients at
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38 355 Turku University Hospital.

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41 356 The following outcome parameters will be assessed based on the sample types collected:

42
43 357 *Parameters from the appendix i.e. patients undergoing appendectomy*

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45 358 Operative details and histopathology of the appendix, host transcriptomics, proteomics, and
46
47
48 359 immunohistochemical analysis from appendiceal biopsy, possible appendicolith composition,
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50 360 morphology, and classification.

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52 361 *Microbiological parameters from the rectal and appendiceal swabs, faecal and appendiceal*
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54
55 362 *samples*

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57 363 Microbial profile, metagenome and metatranscriptome, name of different identified bacterial
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59 364 species, number of species identified both by NGS and culture methods, antimicrobial
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3 365 susceptibility test results, the presence of AMR related genes by molecular analysis methods,
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5
6 366 bacterial genome data of AMR or virulence genes, all microbial data combined with clinical and
7
8 367 additional data.

10 368 *Serum samples*

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13 369 In order to compare possible differences between patients with successful antibiotic therapy
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15 370 to patients with either failed antibiotic therapy or complicated acute appendicitis, the serum
16
17 371 samples will be analysed to identify possible inflammatory or immunological markers.
18
19
20 372 Biomarker analysis of numerous different cytokines, chemokines and growth factors as well
21
22 373 as serum metabolome will be analysed. Additional analysis include the level and activity of
23
24 374 CD73 and soluble vascular adhesion protein-1 (VAP-1).

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27 375 Laboratory methods used in the trial to analyse the collected samples are described in detail
28
29 376 in the online supplementary material.

31 377 **Statistical analysis**

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34 378 Based on the explorative research nature of the MAPPAC study, there is not enough
35
36 379 information available about the study aims to enable sample size calculations. Categorical
37
38 380 variables of the study will be characterised using frequencies and percentages. For continuous
39
40
41 381 variables means and standard deviations or medians with range and 25th and 75th
42
43 382 percentiles will be used. In case of categorical outcomes, groups will be compared using
44
45 383 Pearson's Chi-squared -test and if further analyses will be needed, logistic regression models
46
47
48 384 will be used. Group differences in continuous variables will be evaluated using independent
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50 385 samples t-test, analysis of variance (ANOVA) or non-parametric methods, if needed.
51
52 386 Associations between continuous variables will be evaluated using correlation coefficients
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54
55 387 and linear regression analysis and if adjustments are needed, linear models will be used.
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57 388 Continuous outcomes measured in several time-points will be analysed using linear mixed
58
59 389 models. For categorical outcomes with repeated measurements generalised linear mixed
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3 390 models will be used. The assumptions of the methods will be checked for justification of the
4
5 391 analyses and transformations will be used for the variables, if needed. The study site
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7
8 392 differences will be evaluated in statistical models and if major differences are detected, more
9
10 393 complicated statistical models will be used in the analyses. Two-sided p-values will be used
11
12 394 and p-values less than 0.05 will be considered statistically significant. The measurements with
13
14
15 395 missing data will automatically be excluded from the analyses of the variables in concern.
16
17 396 Statistical analyses will be performed using SAS System for Windows, Version 9.4 and JMP Pro
18
19 397 13 or later versions (SAS Institute, Cary, NC, USA).
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24 399 **Patients and public involvement**

26 400 The MAPPAC research questions and outcome measures were based on the results of original
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28
29 401 APPAC trial [10] and the study protocol was developed together with the study group
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31 402 surgeons, clinical microbiologists and immunologists. Patients were not directly involved in
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34 403 the design of this study and the burden of study participation was not assessed by patients
35
36 404 themselves. Upon recruitment, patients are well informed of all aspects of the trial including
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38 405 antibiotic or symptomatic therapy of CT-confirmed uncomplicated acute appendicitis,
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41 406 difference between complicated and uncomplicated acute appendicitis, treatment success,
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43 407 possible late recurrence, and safety in order to help patients make an informed decision about
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45 408 trial participation. Patients also receive additional instructions in a phone call made prior to
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48 409 follow-up sample collection at 6 and 12 months. After completion of data collection and
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50 410 analysis, the patients will be informed of the study results and they will be provided with an
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52 411 opportunity to ask further questions.
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415 **ETHICS AND DISSEMINATION**

416 **Ethics**

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

420 **Data collection and confidentiality**

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

428 **Withdrawal**

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

431 **Dissemination plan**

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

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3 440 **DISCUSSION**

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5 441 As non-operative treatment for uncomplicated acute appendicitis has been shown to be
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8 442 efficient and safe also at long-term [10, 13-17] and cost-effective [18], understanding both the
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10 443 etiology of the different appendicitis forms and potentially predicting the recurrences has
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12 444 become of utmost clinical importance in order to thoroughly evaluate all the optimization of
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15 445 the different treatment options. The MAPPAC trial aims to assess this largely unknown
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17 446 microbiological etiology of the different disease forms of acute appendicitis. In addition, the
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19
20 447 effects of antibiotic and placebo treatments on the GM composition and the effects of the
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22 448 duration of the hospital stay on the AMR reservoir of the GM will be assessed. With the
23
24 449 longitudinal study design, both the immediate and long-term effects of the antibiotic treatment
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26
27 450 on GM can be analysed. As the hospital stay differs in APPAC II and APPAC III patients, the
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29 451 effects of the duration of the hospital stay on the emergence of resistant bacteria and AMR
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31 452 reservoir can be evaluated. Moreover, the comparison of the effects of antibiotic treatment on
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34 453 GM between two different administration routes (p.o. and i.v. + p.o.) is possible. This study
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36 454 design carried out simultaneously with APPAC II and APPAC III clinical trials allows us to collect
37
38 455 a unique set of microbiological samples in a large prospective clinical setting, which may
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40
41 456 provide clinically relevant new knowledge of appendicitis microbiology and immunology
42
43 457 potentially having an impact on the treatment of acute appendicitis patients.

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45 458 In the 5-year follow up of the original APPAC trial, 39 % (n=100) of the antibiotic group patients
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48 459 underwent appendectomy for either during the primary hospitalization or for suspected late
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50 460 appendicitis recurrence. Out of these hundred patients, 85 were operated on for suspected
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52 461 recurrence and 78 patients had a histopathologically confirmed acute appendicitis [14].
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55 462 Understanding the pathophysiology and contributing factors in recurrent appendicitis are of
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57 463 vital clinical importance in evaluating the optimal treatment paradigms for uncomplicated
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59 464 acute appendicitis. Therefore, MAPPAC trial is assessing both initial non-responders to
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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.

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3 489 The study limitations include the difficult challenge of conducting prospective clinical trials in
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5 490 the emergency setting. It is expected that all eligible patients may not be evaluated for
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8 491 enrolment or some patients may not have all study samples available as the recruitment is
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10 492 performed by a large number of surgeons on call. The lack of healthy control group is a
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12 493 limitation in the study when determining the effects of antibiotics on GM, as the results cannot
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15 494 be fully distinguished from the effects of acute appendicitis.

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For peer review only

514 **Authors' contributions**

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

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

537 **Competing interests**

538 Authors declare they have no competing interests.

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4
5 540 Due to the multicentre nature of the trial, not all supporting researchers are mentioned by
6
7
8 541 name in the protocol article. In addition, we acknowledge all supporting surgeons,
9
10 542 radiologists, emergency medicine physicians, nurses and technical staff in the laboratory.

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663 Abbreviations

664 **AMR:** Antimicrobial resistance

665 **BMI:** Body mass index

666 **CRP:** C-reactive protein

667 **CT:** Computed tomography

668 **GM:** Gut microbiota

669 **i.v.:** Intravenous

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

671 **MS:** Mass spectrometry

672 **NGS:** Next generation sequencing

673 **p.o.:** Per os

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

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

676 **VAS:** Visual analogue scale

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FIGURE TITLES

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

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684 Figure 2. Flow chart of the study protocol

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For peer review only

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Patients with a clinical suspicion of acute appendicitis

Optimized low dose CT imaging (OPTICAP trial)

Uncomplicated acute appendicitis

Complicated acute appendicitis

refusing to participate in the APPAC II or III trials

MAPPAC enrolment at Turku University Hospital 24/7

APPAC II patient enrolment 24/7

MAPPAC at Turku University Hospital

Randomisation

- i.v. + p.o.
- p.o.

APPAC III patient enrolment on week days from 8 am to 2 pm

MAPPAC at all APPACIII study hospitals

Randomisation

- antibiotics
- placebo

Laparoscopic appendectomy

- complicated appendicitis
- uncomplicated appendicitis
- recurrent appendicitis

treatment failure or recurrent appendicitis

Microbiological and immunological samples and questionnaire

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Clinical suspicion of acute appendicitis

BMI < 30 -> low-dose CT scan
BMI > 30 -> standard dose CT scan

Exclusion:
• inclusion criteria not met
• declined to participate

Uncomplicated acute appendicitis

Complicated acute appendicitis

Declined to participate in APPAC II and III

Consent for MAPPAC

Consent for APPAC II and MAPPAC

Consent for APPAC III and MAPPAC

Rectal swab and pre-questionnaire at timepoint 0

Randomisation

Randomisation

i.v. + p.o. group
i.v. ertapenem 1 g/day for two days continued by p.o. levofloxacin 500 mg/day + metronidazole 1.5 g/day for five days

p.o. group
p.o. moxifloxacin 400 mg per day for seven day

Antibiotic group
i.v. ertapenem 1 g/day for three days continued by p.o. levofloxacin 500 mg/day + metronidazole 1.5 g/day for four days

Placebo group
i.v. placebo for three days continued by p.o. placebo for four days

Rectal swab at timepoint 1 (1 day)

Clinical status, temperature, VAS daily

Deteriorating in clinical status

Appendectomy

Rectal swab at timepoint 2 (2-3 days) only from APPAC III patients

Discharge from the hospital

Recurrence of appendicitis

Microbiological samples from appendix PAD, blood culture

Discharge from the hospital

Follow up:
• Phone (2-4 days, 2 weeks, 2 months and 1,3,5 and 10 years)
• Laboratory tests
Fecal sample and questionnaire at timepoints 7 days, 6 and 12 months

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 a new 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,

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2
3 California, USA). DNA is divided into two aliquots and stored at -75°C for later analysis. Microbial
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5 profile of samples will be analysed with appropriate methods including NGS approach.
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10 **Analyses of transcriptome and proteome from appendiceal biopsy**

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12 Total RNA and proteins from the appendiceal biopsy are extracted with appropriate methods.
13
14 Transcriptome is analysed with RNA sequencing using Illumina Hiseq system. In addition, the
15
16 expression of specific genes is quantified with quantitative real-time PCR. Proteome is
17
18 characterized using mass spectrometry-based methods with qualitative and quantitative
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20 approach.
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27 **Culture methods**

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29 Appendiceal swabs are cultured with both aerobic and anaerobic culture methods. For aerobic
30
31 culture the following growth media are used: CHROMagar Orientation (Becton Dickinson,
32
33 Franklin Lakes, New Jersey, USA), Trypticase soy agar with 5% sheep blood (Becton Dickinson),
34
35 Yersinia selective agar and Streptococcus selective agar (in house production). If the
36
37 appendectomy is performed during the office hours, an additional anaerobic culture is made in
38
39 connection with the sample collection. Samples for anaerobic culturing are collected with a sterile
40
41 cotton swab from the appendix and cultured for fastidious anaerobe agar with blood (FAA) and
42
43 kanamycin-vancomycin laked blood agar (KVLB) (both in house production), which are
44
45 immediately transferred into anaerobic jar with anaerobic gas generating sachet (Anaerogen 3,5
46
47 l, Thermo Fisher Scientific).
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3 Cultures are incubated at +35 °C for 18-24 hours. Morphologically distinct colonies are
4
5 subcultured until a pure culture is obtained. Isolated species are frozen in a mixture of skim milk
6
7 and glycerol at -75 °C.
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10 11 12 **MALDI-TOF mass spectrometry**

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14 The identification of isolated bacteria is done with Bruker matrix-assisted laser
15
16 desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). Isolates are inoculated
17
18 and cultured for 18-48 hours at +35 °C before the analysis. For the analysis, colony is applied
19
20 either directly or through ethanol/formic acid extraction on a steel plate and HCCA matrix (α -
21
22 cyano-4-hydroxycinnamic acid in 50% acetonitrile-2.5% trifluoroacetic acid (Bruker Daltonics,
23
24 Bremen, Germany)) is added according to the manufacturer's instructions. The identification is
25
26 done using Microflex LT instrument and MALDI Biotyper software (Bruker Daltonics). If isolates
27
28 cannot be identified due to the absence of reference peaks in the database, the isolate is identified
29
30 with sequencing the 16S rRNA gene.
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38 **Antimicrobial susceptibility testing**

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40 Phenotypic antimicrobial susceptibility testing for the isolated aerobic and anaerobic bacteria is
41
42 performed by disk diffusion and MIC methods, according to the European Committee on
43
44 Antimicrobial Susceptibility Testing (EUCAST) guidelines. For certain bacteria, the Clinical and
45
46 Laboratory Standards Institute (CLSI) guidelines can be used.
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50 Further molecular analysis for antimicrobial resistance or virulence genes using e.g., PCR, Sanger
51
52 sequencing and whole genome sequencing (WGS) are also performed for selected isolates.
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55 **Appendicolith analysis**

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3 Appendicoliths are assessed both on the surface and cross-sectional morphology. Based on the
4 morphological characteristics and a degree of hardness, all appendicoliths will be classified. The
5 morphological characteristics and a degree of hardness, all appendicoliths will be classified. The
6 composition of selected appendicoliths are analysed with physical and chemical methods.
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10 11 12 **Immunological analysis** 13

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

1 2 3 4 5 6 7 8 9 10 11	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
12 13 14 15 16 17		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
18 19 20 21 22 23 24 25	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
26 27 28 29 30 31	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
32 33 34		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	17
35 36 37 38 39 40		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
41 42	Methods: Monitoring			
43 44 45 46 47 48 49 50 51 52	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
53 54 55 56 57 58 59 60		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](https://creativecommons.org/licenses/by-nc-nd/3.0/)" 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**

Journal:	<i>BMJ Open</i>
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Date Submitted by the Author:	20-Jun-2019
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Primary Subject Heading:	Gastroenterology and hepatology
Secondary Subject Heading:	Infectious diseases
Keywords:	appendicitis, appendectomy, appendicitis etiology, appendicolith, microbiota, antimicrobial resistance

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Manuscripts

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5 2 **A Prospective Multicenter Cohort on Acute Appendicitis and Microbiota – Etiology and**
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8 3 **Effects of Antimicrobial Treatment:**
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10 4 **Study protocol for the MAPPAC (Microbiology Appendicitis Acuta) Trial**
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17 7 Vanhatalo S., MSc ^{1,2}, Munukka E., PhD ^{3,4}, Sippola S., MD ^{2,5}, Jalkanen S., MD, PhD ⁶, Grönroos J.,
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58 34 Word count: 4145
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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.

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3 59 **Ethics and dissemination:** This study has been approved by the Ethics Committee of the
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5 60 Hospital District of Southwest Finland (Turku University Hospital) and the Finnish Medicines
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8 61 Agency (Fimea). Results of the trial will be published in peer-reviewed journals.
9

10 62 **Trial registration:** Eudra-CT: 2016-003655-29 (2017-01-12), clinicaltrials.gov NCT03257423.
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16 65 **KEYWORDS**

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18 66 Appendicitis, appendectomy, appendicitis etiology, appendicolith, microbiota, antimicrobial
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71 **Strengths and limitations of this study**

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

96 INTRODUCTION

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

103 The different epidemiological trends of uncomplicated and complicated acute appendicitis
104 indicate different pathophysiology [3]. Despite the high incidence of acute appendicitis, there
105 are very few reports on appendicitis etiology and pathophysiology especially focusing on the
106 possible differences between uncomplicated and complicated acute appendicitis. Complicated
107 acute appendicitis, defined as a finding of perforation, appendicolith, abscess or a suspicion of
108 tumor, requires emergency appendectomy. Appendicolith is a calcified faecal concretion in
109 the appendix and it is the most common form of complicated acute appendicitis. Even though
110 the first thorough study on appendicoliths was already reported in 1966 [4], information
111 about appendiceal calculi is scarce. Obstruction of the appendiceal lumen caused by an
112 appendicolith, lymphoid hyperplasia, or swelling has been evaluated to be the primary cause
113 of appendicitis and bacterial overgrowth has been considered a consequence [5]. However,
114 bacterial infection has also been proposed as the primary cause of appendicitis [6, 7].
115 *Bacteroides* species are reported to be one of most common bacterial findings in appendicitis
116 [8, 9]. Further, certain members of the *Fusobacteria*, especially *F. nucleatum* and *F.*
117 *necrophorum*, are present in most appendicitis samples [6]. The most common aerobic
118 bacteria organism detected by culturing is *Escherichia coli*, but also *Klebsiella pneumoniae*,
119 *Streptococcus spp.*, *Enterococcus spp.* and *Pseudomonas aeruginosa* have been reported [10,
120 11]. To our knowledge, only one study with a very small number of patients has characterised

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3 121 the adult appendiceal microbiota profile with next generation sequencing (NGS) methods.
4
5 122 Appendix seems to have diverse microbiota including both commensal species from gut
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8 123 microbiota (GM) and opportunistic pathogens [12, 13]. Since the interindividual variability
9
10 124 in the microbial composition of the appendix samples is high [12], a larger number of
11
12 125 appendicitis patients is needed to draw the microbiological conclusions. Since most of the
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15 126 species identified from the appendix with both culturing and NGS methods can also be part of
16
17 127 normal gut microbiota it is challenging to determine their role in the infection [14]. In
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19
20 128 addition, the immune response and predisposition for infection by specific bacteria varies
21
22 129 between individuals. Consequently, innate immunity is considered to be a contributing factor
23
24 130 in the development of complicated appendicitis [15]. In conclusion, several factors have been
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26
27 131 proposed to take part in the development of appendicitis, most importantly obstruction of the
28
29 132 lumen and bacterial infection with this research largely focusing on complicated acute
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31 133 appendicitis [5-7, 16, 17].
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33
34 134 Antimicrobials decrease GM diversity, richness and species variation, and cause a perturbation
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36 135 in its overall balance. Particularly broad-spectrum antibiotics have been shown to have a long-
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39 136 term effect on GM profile. [18, 19] A prolonged disturbance in GM and the following imbalance
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41 137 with the host and its immune system have been associated with a variety of diseases, such as
42
43 138 inflammatory bowel disease [20] and type 2 diabetes [21]. Antibiotic use can further lead to
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46 139 increased prevalence of antibiotic resistant bacteria and antibiotic resistance genes [22-24].
47
48 140 Although the effects of antibiotic treatment on the development of antimicrobial resistance
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50 141 (AMR) development is less clear in countries with lower prevalence of resistant bacteria [25,
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53 142 26], the evaluation of antibiotic treatment effects on GM is essential in the treatment of acute
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55 143 appendicitis.
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144 Appendectomy has unquestionably been the standard treatment for acute appendicitis for over
145 a century with more than 300 000 appendectomies performed annually in the United States
146 [3]. The original APPAC (APPendicitis ACuta) trial reported that at long-term follow-up, the
147 majority of patients with computed tomography (CT) confirmed uncomplicated acute
148 appendicitis were successfully treated with antibiotics, and those patients that required later
149 appendectomy did not have increased or major complications [27, 28]. Antibiotic therapy for
150 CT-confirmed uncomplicated acute appendicitis has been shown to be safe, efficient and cost-
151 effective both in adult and paediatric patients [29-33]. APPAC II trial aims to optimise antibiotic
152 treatment for CT confirmed uncomplicated acute appendicitis in order to both shorten the
153 hospital stay and restrict the antibacterial spectrum. The APPAC III trial aims to assess
154 symptomatic treatment of uncomplicated acute appendicitis and the role of antibiotics in the
155 resolution of uncomplicated appendicitis. The MAPPAC (Microbiology APPendicitis ACuta) trial
156 patient enrolment is based on the ongoing APPAC II and APPAC III randomised multicentre
157 clinical trials of our study group. To our knowledge, there are so far no similar large
158 microbiological studies focusing on acute appendicitis performed in conjunction with large
159 clinical trials with prospective access to both uncomplicated and complicated appendicitis
160 patients. MAPPAC trial aims to evaluate the possible role and differences in the microbiological
161 etiology of complicated and uncomplicated appendicitis with a special reference to the
162 presence of an appendicolith. In addition, MAPPAC aims to evaluate the immunological and
163 microbiological factors involved in appendicitis recurrence after successful initial antibiotic
164 therapy. In the longitudinal study arm we also aim to assess the effects of antibiotic and placebo
165 treatment on the GM profile and the effects of hospital stay duration on the AMR reservoir of
166 the GM.

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168 **METHODS AND ANALYSIS**

169 **Study Design**

170 MAPPAC is a prospective clinical trial with a single-centre and a multicentre arm. The single-
171 centre study arm at Turku University Hospital, aims to determine the possible differences in
172 the etiology of complicated and uncomplicated acute appendicitis with a special reference to
173 the role and etiology of appendicolith. In the multi-centre longitudinal arm, MAPPAC trial
174 concentrates on assessment of possible immunological and microbiological factors involved
175 in initial non-responders or appendicitis recurrence after successful initial antibiotic therapy
176 at long-term follow-up. In this longitudinal multicentre study arm we also aim to evaluate the
177 effects of antibiotic and placebo treatment on the GM and the effects of the duration of the
178 hospital stay on the possible AMR reservoir within the GM. MAPPAC protocol was drafted in
179 accordance with the SPIRIT statement [34]. The trial has been registered at both EudraCT
180 (2016-003655-29) and clinicaltrials.gov (NCT03257423).

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

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193 **Patient selection**

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

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211 **Study setting and feasibility**

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

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218 **Interventional groups**

219 Four interventional groups within MAPPAC are defined as follows:

- 220 1. Patients with uncomplicated acute appendicitis participating in the APPAC II trial at
221 Turku University Hospital (MAPPAC single-centre treatment arm) receiving either i.v.
222 antibiotic therapy followed by p.o. antibiotics (i.v. + p.o.) or patients receiving p.o.
223 antibiotic monotherapy (p.o.). *I.v. + p.o.* group receives intravenous ertapenem 1 g once
224 daily for two days followed by p.o. levofloxacin 500 mg once daily and metronidazole
225 500 mg three times per day for 5 days. *P.o.* group receives *p.o.* moxifloxacin 400 mg
226 once daily for seven days.
- 227 2. Patients with uncomplicated acute appendicitis participating in the double-blinded
228 APPAC III trial at all five university hospitals (MAPPAC multicentre treatment arm)
229 receiving either antibiotic therapy or placebo treatment. *Antibiotic treatment* is
230 intravenous ertapenem 1 g once per day for three days followed by p.o. levofloxacin
231 500 mg once per day and metronidazole 500 mg three times per day for four days.
232 *Placebo treatment* entails intravenous placebo once per day for three days followed by
233 per oral placebo three times per day for four days.
- 234 3. Patients with uncomplicated acute appendicitis or suspected recurrent appendicitis
235 undergoing appendectomy
236 The patients in this group will undergo laparoscopic appendectomy either after
237 declining to participate in the APPAC II or APPAC III trials (MAPPAC single-centre
238 treatment arm at Turku University Hospital) or after suspected treatment failure of
239 non-operative treatment or after presenting with suspected appendicitis recurrence
240 after initial successful non-operative treatment (MAPPAC multicentre treatment arm,
241 all APPAC II and APPAC III hospitals)
- 242 4. Patients with complicated acute appendicitis undergoing appendectomy

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3 243 The patients in this group will undergo laparoscopic appendectomy and are eligible for
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5 244 enrolment only in the MAPPAC single-centre study arm at Turku University Hospital.
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10 246 **Sample collection**

11
12 247 **Rectal swabs** are collected from all patients in the emergency department prior to antibiotic
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14
15 248 treatment or surgery (timepoint 0). Further, rectal swabs are collected on the 1st day for
16
17 249 APPAC II patients and for APPAC III patients on the 1st day (Turku University Hospital) and
18
19 250 on the 3rd day (all five university hospitals). At each time point, two rectal swabs are collected:
20
21
22 251 Amies-Coal transport swab for culturing methods (Sarstedt, Nümbrecht, Germany) and
23
24 252 Puritan DNA/RNA swab (Puritan Medical products, Guilford, Maine) with shield fluid (Zymo
25
26 253 Research, Irvine, Canada) for microbial DNA extraction. The shield fluid in this collection tube
27
28
29 254 allows the transportation at room temperature.

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31 255 **Samples from the appendix** are collected from patients undergoing appendectomy for
32
33 256 complicated or uncomplicated acute appendicitis and from patients with suspected disease
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36 257 progression during the primary hospitalisation or appendicitis recurrence after successful
37
38 258 initial non-operative therapy. Samples include routine histology as well as specific trial swabs
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41 259 and biopsies. The appendix is opened vertically with a sterile scalpel and two swabs are taken
42
43 260 similar to the rectal swab (one for culture and one for DNA extraction). Three biopsies are
44
45 261 taken at a distance of approximately 2 cm from the base of the appendix. All biopsies are
46
47
48 262 stored at -75 °C, one immediately and the rest two are first embedded in RNAlater solution
49
50 263 (Thermo Fisher Scientific, Waltham, MA, USA). Possible appendicoliths are collected into a
51
52 264 sterile container, frozen and stored at -75 °C. If appendectomy is performed during office
53
54
55 265 hours, appendiceal samples are collected by study personnel and an additional swab for
56
57 266 anaerobic culture is then collected and cultured in connection with collection and
58
59
60 267 immediately transferred into an anaerobic jar. During on call hours, the samples are collected

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3 268 by surgeons and the -75 °C appendiceal biopsy may not be collected. These appendiceal
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6 269 samples are collected only in the Turku University Hospital and at the other study hospitals
7
8 270 only a swab sample (transport tube with DNA shield fluid) from the appendix of non-
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10 271 responders and patients with appendicitis recurrence is collected.

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13 272 **Additional serum samples** are collected from all the patients recruited in MAPPAC trial at
14
15 273 Turku University Hospital and for APPAC III trial at all five study hospitals for the
16
17 274 identification of possible disease-form specific inflammatory biomarkers in serum. The serum
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19
20 275 samples are divided into six aliquots prior freezing at -75 °C.

21 22 276 **Questionnaire**

23
24 277 MAPPAC patients are asked to fill a primary questionnaire during the initial hospitalisation
25
26
27 278 covering topics possibly affecting their GM profile: chronic diseases, special diets, smoking
28
29 279 and alcohol consumption, travel history, antibiotic intake, other medications (12 months
30
31 280 prior the sampling), consumption of probiotics and other dietary supplements, recent
32
33 281 diarrhoea and/or vomiting, Bristol stool form scale estimate [37] measuring stool consistency
34
35
36 282 (1 for very hard stool to 7 for diarrhoea) and the average number of defecations per day.

37 38 283 **Follow-up during the hospitalisation**

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41 284 During the hospitalisation the following parameters will be recorded every 24 hours: pain
42
43 285 assessed by Visual Analogue Scale (VAS), leukocyte count, C-reactive protein (CRP),
44
45 286 temperature and clinical findings at patient reassessment. If the patient is suspected of not
46
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48 287 responding to the randomized therapy based on clinical deterioration signs combined with
49
50 288 laboratory findings (signs of peritonitis, persisting fever, increasing pain, white blood cell
51
52 289 count or CRP), the patient will be operated based on the surgeon's decision and the reasons
53
54
55 290 for proceeding to appendectomy will be recorded. For appendectomy, laparoscopic approach
56
57 291 is recommended. The operative findings and the histopathology of the appendix will be
58
59
60 292 recorded. After the initial hospitalisation, recurrent acute appendicitis will be diagnosed on a

1
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3 293 clinical basis and patients with a suspected recurrence of appendicitis will undergo a
4
5 294 laparoscopic appendectomy.

8 295 **Follow-up**

10 296 The specific MAPPAC trial follow-up is conducted for patients also participating in the APPAC
11
12 297 II and III trials with collection of three faecal samples at home (at one week, six months and
13
14
15 298 one year). Follow-up samples at home are not collected in MAPPAC interventional groups
16
17 299 undergoing appendectomy. The follow-up faecal sample is collected into 10 ml DNA/RNA
18
19 300 Shield Faecal Collection Tube (Zymo Research) enabling transportation and storage at room
20
21
22 301 temperature prior to DNA extraction. Patients also deliver a faecal sample into a gel tube for
23
24 302 culture purposes. A follow-up questionnaire is collected at 6 and 12 months consisting of the
25
26 303 same questions as the preliminary questionnaire covering the time between hospital
27
28
29 304 discharge and the follow-up time point. Other follow-up for patients in APPAC II trial will
30
31 305 include a phone interview at one week after discharge, APPAC III trial patients have their first
32
33 306 follow-up call 2-4 days after discharge from the hospital and then at 10 days, both trials at two
34
35
36 307 months and at one, three, five and ten years. The follow-up for APPAC III patients will include
37
38 308 laboratory tests (leukocyte count, CRP, additional follow-up serum samples).

43 310 **Outcome parameters**

45 311 Based on the MAPPAC trial design, no specific primary endpoint can be determined. The
46
47 312 following parameters will be recorded for all patients: age, gender, Body mass index, clinical
48
49
50 313 findings on admission (tympanic temperature, nausea, pain or tenderness in the right lower
51
52 314 abdominal quadrant, pain migration, VAS pain scores, white blood cell count, CRP, findings on
53
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55 315 CT imaging), and data on primary and follow-up questionnaires. Blood cultures will be
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57 316 obtained from patients with complicated acute appendicitis and for APPAC II and III trial
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59 317 patients at Turku University Hospital.

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

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

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

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

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

330 *Serum samples*

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

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

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3 343 **Statistical analysis**

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5 344 Based on the explorative research nature of the MAPPAC study, there is not enough
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8 345 information available about the study aims to enable sample size calculations. Categorical
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10 346 variables of the study will be characterised using frequencies and percentages. For continuous
11
12 347 variables means and standard deviations or medians with range and 25th and 75th
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14
15 348 percentiles will be used. In case of categorical outcomes, groups will be compared using
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17 349 Pearson's Chi-squared -test and if further analyses will be needed, logistic regression models
18
19
20 350 will be used. Group differences in continuous variables will be evaluated using independent
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22 351 samples t-test, analysis of variance (ANOVA) or non-parametric methods, if needed.
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24 352 Associations between continuous variables will be evaluated using correlation coefficients
25
26 353 and linear regression analysis and if adjustments are needed, linear models will be used.
27
28
29 354 Continuous outcomes measured in several time-points will be analysed using linear mixed
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31 355 models. For categorical outcomes with repeated measurements generalised linear mixed
32
33 356 models will be used. The assumptions of the methods will be checked for justification of the
34
35
36 357 analyses and transformations will be used for the variables, if needed. The study site
37
38 358 differences will be evaluated in statistical models and if major differences are detected, more
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41 359 complicated statistical models will be used in the analyses. Two-sided p-values will be used
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43 360 and p-values less than 0.05 will be considered statistically significant. The measurements with
44
45 361 missing data will automatically be excluded from the analyses of the variables in concern.
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48 362 Statistical analyses will be performed using SAS System for Windows, Version 9.4 and JMP Pro
49
50 363 13 or later versions (SAS Institute, Cary, NC, USA).

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55 365 **Patients and public involvement**

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57 366 The MAPPAC research questions and outcome measures were based on the results of original
58
59 367 APPAC trial [27] and the study protocol was developed together with the study group
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3 368 surgeons, clinical microbiologists and immunologists. Patients were not directly involved in
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5 369 the design of this study and the burden of study participation was not assessed by patients
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8 370 themselves. Upon recruitment, patients are well informed of all aspects of the trial including
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10 371 antibiotic or symptomatic therapy of CT-confirmed uncomplicated acute appendicitis,
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12 372 difference between complicated and uncomplicated acute appendicitis, treatment success,
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15 373 possible late recurrence, and safety in order to help patients make an informed decision about
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17 374 trial participation. Patients also receive additional instructions in a phone call made prior to
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20 375 follow-up sample collection at 6 and 12 months. After completion of data collection and
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22 376 analysis, the patients will be informed of the study results and they will be provided with an
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24 377 opportunity to ask further questions.
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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.

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393 **Withdrawal**

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

396 **Dissemination plan**

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

For peer review only

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60**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

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

444 **Strengths and limitations**

445 MAPPAC has a strong synergy between APPAC II and III studies enabling the enrolment of both
446 uncomplicated and complicated acute appendicitis patients in a large prospective series with
447 associated clinical data to be assessed in conjunction with the microbiological and
448 immunological findings. In many aspects, MAPPAC is an exploratory study of possible
449 associations of whole microbial community and host immune characteristics with
450 uncomplicated vs. complicated appendicitis and antibiotic response among patients in clinical
451 trials treated with and without antibiotics. MAPPAC trial aims to generate hypotheses to better
452 understand the role of disease progression and host susceptibility for future studies; i.e.
453 determination of one primary outcome is insufficient for the study, as several factors are
454 indispensable for the understanding of etiology and the effects of antibiotics on GM all provided
455 with this unique set of microbiological samples. To our knowledge, only one previous study
456 [12] has characterized the adult appendix microbiota during appendicitis in adult patients
457 using NGS technique and to date no reports on the structure and physicochemical contents of
458 appendicoliths exists. Using these assessments is a strength in our study. Further, to our
459 knowledge this is the first trial aiming to prospectively assess the possible microbiological
460 and/or immunological etiology of appendicitis recurrence after a successful initial conservative
461 treatment with antibiotics or symptomatic therapy and primary non-responders to
462 conservative treatment of uncomplicated acute appendicitis. One of the main hypothesis of the
463 MAPPAC study is that the microbial composition of appendix differs between CT differentiated
464 complicated and uncomplicated appendicitis. Therefore, strong element of the study is that all
465 patients included in the study are imaged with CT protocol. CT scan is the gold standard for

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3 466 acute appendicitis imaging and this near perfect accuracy of CT adds to the reliability of the
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5 467 clinical data and appendicitis severity also in the patients with uncomplicated acute
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8 468 appendicitis without a histopathological confirmation of the appendix.

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10 469 The study limitations include the difficult challenge of conducting prospective clinical trials in
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12 470 the emergency setting. It is expected that all eligible patients may not be evaluated for
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15 471 enrolment or some patients may not have all study samples available as the recruitment is
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17 472 performed by a large number of surgeons on call. The lack of healthy control group is a
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20 473 limitation in the study regarding both the etiology and in determining the effects of antibiotics
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22 474 on GM, as the results cannot be fully distinguished from the effects of acute appendicitis.

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491 **Authors' contributions**

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

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

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513 researcher oriented trial.

514 **Competing interests**

515 Authors declare they have no competing interests.

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5 517 Due to the multicentre nature of the trial, not all supporting researchers are mentioned by
6
7
8 518 name in the protocol article. In addition, we acknowledge all supporting surgeons,
9
10 519 radiologists, emergency medicine physicians, nurses and technical staff in the laboratory.

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22 633

23 634 **Abbreviations**

24

25 635 **AMR:** Antimicrobial resistance

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27 636 **CRP:** C-reactive protein

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30 637 **CT:** Computed tomography

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32 638 **GM:** Gut microbiota

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34 639 **i.v.:** Intravenous

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37 640 **MALDI-TOF:** Matrix Assisted Laser Desorption/Ionisation time-of-flight mass spectrometry

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39 641 **MS:** Mass spectrometry

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41 642 **NGS:** Next generation sequencing

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44 643 **p.o.:** Per os

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46 644 **SPIRIT:** Standard Protocol Items: recommendations for Interventional Trials

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48 645 **VAP-1:** Vascular adhesion protein-1

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51 646 **VAS:** Visual analogue scale

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57 649 **FIGURE LEGENDS**

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59 651 Figure 1. The synergy between MAPPAC, APPAC II and APPAC III studies.

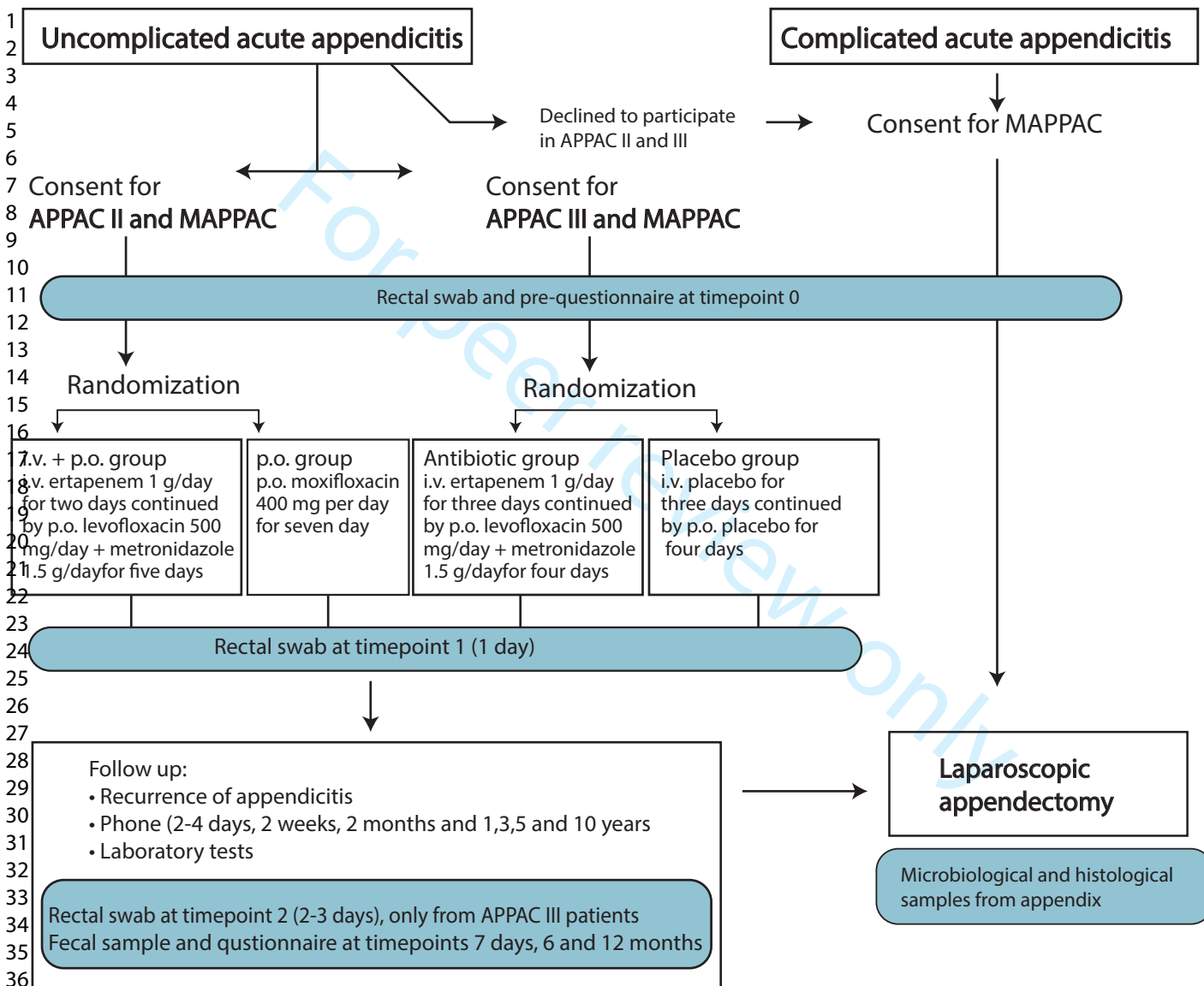
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652 Figure 2. Flow chart of the study protocol

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For peer review only



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Clinical suspicion of acute appendicitis

CT imaging

Uncomplicated acute appendicitis

Complicated acute appendicitis

refusing to participate in the APPAC II or III trials

APPAC II patient enrollment

APPAC III patient enrollment

Laparoscopic appendectomy

MAPPAC at Turku University Hospital

During hospital pharmacy office hours
MAPPAC at all APPACIII study hospitals

- complicated appendicitis
 - uncomplicated appendicitis
 - recurrent appendicitis
- MAPPAC enrollment at Turku University Hospital

Randomization

Randomization

i.v. + p.o.

p.o.

antibiotics

placebo

treatment failure or recurrent appendicitis

Microbiological and immunological samples and questionnaire

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 a new 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

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2
3 profile of samples will be analysed with NGS approach using appropriate methods and Illumina
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5 Miseq system.
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10 **Analyses of metagenome, transcriptome and proteome from appendiceal biopsy**

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12 Total RNA and proteins from the appendiceal biopsy are extracted with appropriate methods.
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14 Transcriptome and metagenome are analysed with Illumina Hiseq system. In addition, the
15
16 expression of specific genes is quantified with quantitative real-time PCR. Proteome is
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18 characterized using mass spectrometry-based methods with qualitative and quantitative
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20 approach.
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26 **Culture methods**

27
28 Appendiceal swabs are cultured with both aerobic and anaerobic culture methods. For aerobic
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30 culture the following growth media are used: CHROMagar Orientation (Becton Dickinson,
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32 Franklin Lakes, New Jersey, USA), Trypticase soy agar with 5% sheep blood (Becton Dickinson),
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34 Yersinia selective agar and Streptococcus selective agar (in house production). If the
35
36 appendectomy is performed during the office hours, an additional anaerobic culture is made in
37
38 connection with the sample collection. Samples for anaerobic culturing are collected with a sterile
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40 cotton swab from the appendix and cultured for fastidious anaerobe agar with blood (FAA) and
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42 kanamycin-vancomycin laked blood agar (KVLB) (both in house production), which are
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44 immediately transferred into anaerobic jar with anaerobic gas generating sachet (Anaerogen 3,5
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46 l, Thermo Fisher Scientific).
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3 Cultures are incubated at +35 °C for 18-24 hours. Morphologically distinct colonies are
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5 subcultured until a pure culture is obtained. Isolated species are frozen in a mixture of skim milk
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7 and glycerol at -75 °C.
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12 **MALDI-TOF mass spectrometry**

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

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40 Phenotypic antimicrobial susceptibility testing for the isolated aerobic and anaerobic bacteria is
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42 performed by disk diffusion and MIC methods, according to the European Committee on
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44 Antimicrobial Susceptibility Testing (EUCAST) guidelines. For certain bacteria, the Clinical and
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46 Laboratory Standards Institute (CLSI) guidelines can be used.
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50 Further molecular analysis for antimicrobial resistance or virulence genes using e.g., PCR, Sanger
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52 sequencing and whole genome sequencing (WGS) are also performed for selected isolates.
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55 **Appendicolith analysis**

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3 Appendicoliths are assessed both on the surface and cross-sectional morphology. Based on the
4 morphological characteristics and a degree of hardness, all appendicoliths will be classified. The
5
6 morphological characteristics and a degree of hardness, all appendicoliths will be classified. The
7
8 composition of selected appendicoliths are analysed with physical and chemical methods.
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10 11 12 **Immunological analysis**

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15 The appendiceal biopsies are analysed immunohistochemically by determining the presence of
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17 different cell types and blood vessel surface markers. Neutrophils, leukocytes and monocytes will
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19 be analysed and special interest will be focused on lymphocyte subtypes (i.e., CD4/CD8 and more
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21 detailed subgroup analyses such as regulatory T cells) and monocyte markers (i.e., macrophage
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23 M1/M2 / receptor MHCII). Moreover, certain inflammation induced markers on endothelium such
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25 as VAP-1, E-selectin and P-selectin will be evaluated. In order to compare possible differences
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27 between patients with successful antibiotic therapy to patients with failed antibiotic therapy or
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29 complicated acute appendicitis the serum samples will be screened to identify possible inflammatory
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31 or immunological markers for identifying the different forms of the disease. Cytokines, chemokines
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33 and growth factors will be tested using Bio-Plex Pro Human Cytokine 48-Plex Screening Panel (BIO-
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35 RAD) containing the following analytes: Basic FGF, CTACK, eotaxin, G-CSF, GM-CSF, GRO-a, HGF,
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37 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-
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39 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,
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41 MIG, MIP-1a, MIP-1b, b-NGF, PDGF-BB, RANTES, SCF, SCGF-b, SDF-1a, TNF-a, TNF-b, TRAIL, VCAM-1
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43 and VEGF-A. Moreover, we will use in house analyses to measure soluble VAP-1 (inflammatory) and
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45 CD73 (anti-inflammatory). In addition, metabolomics approach using nuclear magnetic resonance
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47 (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS) metabolomics
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49 platforms will be used for biomarker analysis. The results obtained from immunological analyses will
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51 be correlated to the clinical parameters and to the microbiological findings.
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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			

1 2 3 4 5 6 7 8 9 10 11	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
12 13 14 15 16 17		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
18 19 20 21 22 23 24 25	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
26 27 28 29 30 31	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
32 33 34		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	17
35 36 37 38 39 40		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
41 42	Methods: Monitoring			
43 44 45 46 47 48 49 50 51 52	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
53 54 55 56 57 58 59 60		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](https://creativecommons.org/licenses/by-nc-nd/3.0/)" 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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Secondary Subject Heading:	Infectious diseases
Keywords:	appendicitis, appendectomy, appendicitis etiology, appendicolith, microbiota, antimicrobial resistance

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5 2 **A Prospective Multicenter Cohort Trial on Acute Appendicitis and Microbiota – Etiology**
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8 3 **and Effects of Antimicrobial Treatment: Study protocol for the MAPPAC (Microbiology**
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10 4 **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. 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

1
2
3 60 ATMK:142/1800/2016) and the Finnish Medicines Agency (Fimea). Results of the trial will be
4
5
6 61 published in peer-reviewed journals.

7
8 62 **Trial registration:** Eudra-CT: 2016-003655-29 (2017-01-12), clinicaltrials.gov NCT03257423.
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12 64

13 65 **KEYWORDS**

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16 66 Appendicitis, appendectomy, appendicitis etiology, appendicolith, microbiota, antimicrobial
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18 67 resistance
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For peer review only

71 **Strengths and limitations of this study**

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

96 INTRODUCTION

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

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

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3 121 with next generation sequencing (NGS) methods. Appendix seems to have diverse microbiota
4
5 122 including both commensal species from gut microbiota (GM) and opportunistic pathogens
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7
8 123 [12, 13]. Since the interindividual variability in the microbial composition of the appendix
9
10 124 samples is high [12], a larger number of appendicitis patients is needed to draw the
11
12 125 microbiological conclusions. Since most of the species identified from the appendix with both
13
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15 126 culturing and NGS methods can also be part of normal gut microbiota it is challenging to
16
17 127 determine their role in the infection [14]. In addition, the immune response and
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19
20 128 predisposition for infection by specific bacteria varies between individuals. Consequently,
21
22 129 innate immunity is considered to be a contributing factor in the development of complicated
23
24 130 appendicitis [15]. In conclusion, several factors have been proposed to take part in the
25
26 131 development of appendicitis, most importantly obstruction of the lumen and bacterial
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29 132 infection with this research largely focusing on complicated acute appendicitis [5-7, 16, 17].
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32 133 Antimicrobials decrease GM diversity, richness and species variation, and cause a perturbation
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34 134 in its overall balance. Particularly broad-spectrum antibiotics have been shown to have a long-
35
36 135 term effect on GM profile. [18, 19] A prolonged disturbance in GM and the following imbalance
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39 136 with the host and its immune system have been associated with a variety of diseases, such as
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41 137 inflammatory bowel disease [20] and type 2 diabetes [21]. Antibiotic use can further lead to
42
43 138 increased prevalence of antibiotic resistant bacteria and antibiotic resistance genes [22-24].
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46 139 Although the effects of antibiotic treatment on the development of antimicrobial resistance
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48 140 (AMR) development is less clear in countries with lower prevalence of resistant bacteria [25,
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50 141 26], the evaluation of antibiotic treatment effects on GM is essential in the treatment of acute
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53 142 appendicitis.
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56 143 Appendectomy has unquestionably been the standard treatment for acute appendicitis for over
57
58 144 a century with more than 300 000 appendectomies performed annually in the United States
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3 145 [3]. The original APPAC (APPendicitis ACuta) trial reported that at long-term follow-up, the
4
5 146 majority of patients with computed tomography (CT) confirmed uncomplicated acute
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8 147 appendicitis were successfully treated with antibiotics, and those patients that required later
9
10 148 appendectomy did not have increased or major complications [27, 28]. Antibiotic therapy for
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12 149 CT-confirmed uncomplicated acute appendicitis has been shown to be safe, efficient and cost-
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15 150 effective both in adult and paediatric patients [29-33]. APPAC II trial aims to optimise antibiotic
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17 151 treatment for CT confirmed uncomplicated acute appendicitis in order to both shorten the
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20 152 hospital stay and restrict the antibacterial spectrum. The APPAC III trial aims to assess
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22 153 symptomatic treatment of uncomplicated acute appendicitis and the role of antibiotics in the
23
24 154 resolution of uncomplicated appendicitis. The MAPPAC (Microbiology APPendicitis ACuta) trial
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26 155 patient enrolment is based on the ongoing APPAC II and APPAC III randomised multicentre
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29 156 clinical trials of our study group. To our knowledge, there are so far no similar large
30
31 157 microbiological studies focusing on acute appendicitis performed in conjunction with large
32
33
34 158 clinical trials with prospective access to both uncomplicated and complicated appendicitis
35
36 159 patients. MAPPAC trial aims to evaluate the possible role and differences in the microbiological
37
38 160 etiology of complicated and uncomplicated appendicitis with a special reference to the
39
40
41 161 presence of an appendicolith. In addition, MAPPAC aims to evaluate the immunological and
42
43 162 microbiological factors involved in appendicitis recurrence after successful initial antibiotic
44
45 163 therapy. In the longitudinal study arm we also aim to assess the effects of antibiotic and placebo
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47
48 164 treatment on the GM profile and the effects of hospital stay duration on the AMR reservoir of
49
50 165 the GM.

57 167 **METHODS AND ANALYSIS**

59 168 **Study Design**

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3 169 MAPPAC is a prospective clinical trial with a single-centre and a multicentre arm. The single-
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6 170 centre study arm at Turku University Hospital, aims to determine the possible differences in
7
8 171 the etiology of complicated and uncomplicated acute appendicitis with a special reference to
9
10 172 the role and etiology of appendicolith. In the multi-centre longitudinal arm, MAPPAC trial
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13 173 concentrates on assessment of possible immunological and microbiological factors involved
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15 174 in initial non-responders or appendicitis recurrence after successful initial antibiotic therapy
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17 175 at long-term follow-up. In this longitudinal multicentre study arm we also aim to evaluate the
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20 176 effects of antibiotic and placebo treatment on the GM and the effects of the duration of the
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22 177 hospital stay on the possible AMR reservoir within the GM. MAPPAC protocol was drafted in
23
24 178 accordance with the SPIRIT statement [34]. The trial has been registered at both EudraCT
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26
27 179 (2016-003655-29) and clinicaltrials.gov (NCT03257423).
28
29 180 The three ongoing study entities (APPAC II, APPAC III, and MAPPAC) are strongly
30
31 181 interconnected having a common study aim and a patient enrolment population (Figure 1).
32
33
34 182 APPAC II trial is a randomised open-label, non-inferiority multicentre trial to compare
35
36 183 intravenous antibiotic therapy followed by per oral (p.o.) antibiotics with p.o. monotherapy
37
38 184 in the treatment of uncomplicated acute appendicitis (NCT03236961)[35]. APPAC III trial is a
39
40
41 185 randomised double-blind, placebo-controlled, superiority multicentre study to compare
42
43 186 antibiotic therapy with placebo in the treatment of uncomplicated acute appendicitis
44
45 187 (NCT03234296) [36]. All incoming patients are informed of all ongoing trials. All patients
46
47
48 188 invited to participate in APPAC II and III trials will be invited to participate in the MAPPAC
49
50 189 trial. Patients recruited for the APPAC II or III trial are asked to sign a separate consent form
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52 190 for the MAPPAC trial allowing for the use of their data and collection of microbiological
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55 191 samples. The study flow is illustrated in Figure 2.
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192 **Patient selection**

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

210 **Study setting and feasibility**

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

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217 **Interventional groups**

218 Four interventional groups within MAPPAC are defined as follows:

- 219 1. Patients with uncomplicated acute appendicitis participating in the APPAC II trial at
220 Turku University Hospital (MAPPAC single-centre treatment arm) receiving either i.v.
221 antibiotic therapy followed by p.o. antibiotics (i.v. + p.o.) or patients receiving p.o.
222 antibiotic monotherapy (p.o.). *I.v. + p.o.* group receives intravenous ertapenem 1 g once
223 daily for two days followed by p.o. levofloxacin 500 mg once daily and metronidazole
224 500 mg three times per day for 5 days. *P.o.* group receives *p.o.* moxifloxacin 400 mg
225 once daily for seven days.
- 226 2. Patients with uncomplicated acute appendicitis participating in the double-blinded
227 APPAC III trial at all five university hospitals (MAPPAC multicentre treatment arm)
228 receiving either antibiotic therapy or placebo treatment. *Antibiotic treatment* is
229 intravenous ertapenem 1 g once per day for three days followed by p.o. levofloxacin
230 500 mg once per day and metronidazole 500 mg three times per day for four days.
231 *Placebo treatment* entails intravenous placebo once per day for three days followed by
232 per oral placebo three times per day for four days.
- 233 3. Patients with uncomplicated acute appendicitis or suspected recurrent appendicitis
234 undergoing appendectomy
235 The patients in this group will undergo laparoscopic appendectomy either after
236 declining to participate in the APPAC II or APPAC III trials (MAPPAC single-centre
237 treatment arm at Turku University Hospital) or after suspected treatment failure of
238 non-operative treatment or after presenting with suspected appendicitis recurrence
239 after initial successful non-operative treatment (MAPPAC multicentre treatment arm,
240 all APPAC II and APPAC III hospitals)
- 241 4. Patients with complicated acute appendicitis undergoing appendectomy

1
2
3 242 The patients in this group will undergo laparoscopic appendectomy and are eligible for
4
5 243 enrolment only in the MAPPAC single-centre study arm at Turku University Hospital.
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8 244
9
10 245 **Sample collection**

11
12 246 **Rectal swabs** are collected from all patients in the emergency department prior to antibiotic
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14
15 247 treatment or surgery (timepoint 0). Further, rectal swabs are collected on the 1st day for
16
17 248 APPAC II patients and for APPAC III patients on the 1st day (Turku University Hospital) and
18
19
20 249 on the 3rd day (all five university hospitals). At each time point, two rectal swabs are collected:
21
22 250 Amies-Coal transport swab for culturing methods (Sarstedt, Nümbrecht, Germany) and
23
24 251 Puritan DNA/RNA swab (Puritan Medical products, Guilford, Maine) with shield fluid (Zymo
25
26 252 Research, Irvine, Canada) for microbial DNA extraction. The shield fluid in this collection tube
27
28
29 253 allows the transportation at room temperature.

30
31 254 **Samples from the appendix** are collected from patients undergoing appendectomy for
32
33 255 complicated or uncomplicated acute appendicitis and from patients with suspected disease
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35
36 256 progression during the primary hospitalisation or appendicitis recurrence after successful
37
38 257 initial non-operative therapy. Samples include routine histology as well as specific trial swabs
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40
41 258 and biopsies. The appendix is opened vertically with a sterile scalpel and two swabs are taken
42
43 259 similar to the rectal swab (one for culture and one for DNA extraction). Three biopsies are
44
45 260 taken at a distance of approximately 2 cm from the base of the appendix. All biopsies are
46
47
48 261 stored at -75 °C, one immediately and the rest two are first embedded in RNAlater solution
49
50 262 (Thermo Fisher Scientific, Waltham, MA, USA). Possible appendicoliths are collected into a
51
52 263 sterile container, frozen and stored at -75 °C. If appendectomy is performed during office
53
54
55 264 hours, appendiceal samples are collected by study personnel and an additional swab for
56
57 265 anaerobic culture is then collected and cultured in connection with collection and
58
59
60 266 immediately transferred into an anaerobic jar. During on call hours, the samples are collected

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3 267 by surgeons and the -75 °C appendiceal biopsy may not be collected. These appendiceal
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6 268 samples are collected only in the Turku University Hospital and at the other study hospitals
7
8 269 only a swab sample (transport tube with DNA shield fluid) from the appendix of non-
9
10 270 responders and patients with appendicitis recurrence is collected.

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13 271 **Additional serum samples** are collected from all the patients recruited in MAPPAC trial at
14
15 272 Turku University Hospital and for APPAC III trial at all five study hospitals for the
16
17 273 identification of possible disease-form specific inflammatory biomarkers in serum. The serum
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19
20 274 samples are divided into six aliquots prior freezing at -75 °C.

21 22 275 **Questionnaire**

23
24 276 MAPPAC patients are asked to fill a primary questionnaire during the initial hospitalisation
25
26
27 277 covering topics possibly affecting their GM profile: chronic diseases, special diets, smoking
28
29 278 and alcohol consumption, travel history, antibiotic intake, other medications (12 months
30
31
32 279 prior the sampling), consumption of probiotics and other dietary supplements, recent
33
34 280 diarrhoea and/or vomiting, Bristol stool form scale estimate [37] measuring stool consistency
35
36 281 (1 for very hard stool to 7 for diarrhoea) and the average number of defecations per day.

37 38 282 **Follow-up during the hospitalisation**

39
40
41 283 During the hospitalisation the following parameters will be recorded every 24 hours: pain
42
43 284 assessed by Visual Analogue Scale (VAS), leukocyte count, C-reactive protein (CRP),
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45
46 285 temperature and clinical findings at patient reassessment. If the patient is suspected of not
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48 286 responding to the randomized therapy based on clinical deterioration signs combined with
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50 287 laboratory findings (signs of peritonitis, persisting fever, increasing pain, white blood cell
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53 288 count or CRP), the patient will be operated based on the surgeon's decision and the reasons
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55 289 for proceeding to appendectomy will be recorded. For appendectomy, laparoscopic approach
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57 290 is recommended. The operative findings and the histopathology of the appendix will be
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59
60 291 recorded. After the initial hospitalisation, recurrent acute appendicitis will be diagnosed on a

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3 292 clinical basis and patients with a suspected recurrence of appendicitis will undergo a
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5 293 laparoscopic appendectomy.

8 294 **Follow-up**

10 295 The specific MAPPAC trial follow-up is conducted for patients also participating in the APPAC
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12 296 II and III trials with collection of three faecal samples at home (at one week, six months and
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14
15 297 one year). Follow-up samples at home are not collected in MAPPAC interventional groups
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17 298 undergoing appendectomy. The follow-up faecal sample is collected into 10 ml DNA/RNA
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19 299 Shield Faecal Collection Tube (Zymo Research) enabling transportation and storage at room
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22 300 temperature prior to DNA extraction. Patients also deliver a faecal sample into a gel tube for
23
24 301 culture purposes. A follow-up questionnaire is collected at 6 and 12 months consisting of the
25
26 302 same questions as the preliminary questionnaire covering the time between hospital
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29 303 discharge and the follow-up time point. Other follow-up for patients in APPAC II trial will
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31 304 include a phone interview at one week after discharge, APPAC III trial patients have their first
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33 305 follow-up call 2-4 days after discharge from the hospital and then at 10 days, both trials at two
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36 306 months and at one, three, five and ten years. The follow-up for APPAC III patients will include
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38 307 laboratory tests (leukocyte count, CRP, additional follow-up serum samples).

43 309 **Outcome parameters**

45 310 Based on the MAPPAC trial design, no specific primary endpoint can be determined. The
46
47 311 following parameters will be recorded for all patients: age, gender, Body mass index, clinical
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49
50 312 findings on admission (tympanic temperature, nausea, pain or tenderness in the right lower
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52 313 abdominal quadrant, pain migration, VAS pain scores, white blood cell count, CRP, findings on
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55 314 CT imaging), and data on primary and follow-up questionnaires. Blood cultures will be
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57 315 obtained from patients with complicated acute appendicitis and for APPAC II and III trial
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59 316 patients at Turku University Hospital.

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

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

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

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

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

329 *Serum samples*

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

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

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3 342 **Statistical analysis**

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5 343 Based on the explorative research nature of the MAPPAC study, there is not enough
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8 344 information available about the study aims to enable sample size calculations. Categorical
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10 345 variables of the study will be characterised using frequencies and percentages. For continuous
11
12 346 variables means and standard deviations or medians with range and 25th and 75th
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14
15 347 percentiles will be used. In case of categorical outcomes, groups will be compared using
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17 348 Pearson's Chi-squared -test and if further analyses will be needed, logistic regression models
18
19 349 will be used. Group differences in continuous variables will be evaluated using independent
20
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22 350 samples t-test, analysis of variance (ANOVA) or non-parametric methods, if needed.
23
24 351 Associations between continuous variables will be evaluated using correlation coefficients
25
26 352 and linear regression analysis and if adjustments are needed, linear models will be used.
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29 353 Continuous outcomes measured in several time-points will be analysed using linear mixed
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31 354 models. For categorical outcomes with repeated measurements generalised linear mixed
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33 355 models will be used. The assumptions of the methods will be checked for justification of the
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36 356 analyses and transformations will be used for the variables, if needed. The study site
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38 357 differences will be evaluated in statistical models and if major differences are detected, more
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41 358 complicated statistical models will be used in the analyses. Two-sided p-values will be used
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43 359 and p-values less than 0.05 will be considered statistically significant. The measurements with
44
45 360 missing data will automatically be excluded from the analyses of the variables in concern.
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48 361 Statistical analyses will be performed using SAS System for Windows, Version 9.4 and JMP Pro
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50 362 13 or later versions (SAS Institute, Cary, NC, USA).

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55 364 **Patients and public involvement**

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57 365 The MAPPAC research questions and outcome measures were based on the results of original
58
59 366 APPAC trial [27] and the study protocol was developed together with the study group

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3 367 surgeons, clinical microbiologists and immunologists. Patients were not directly involved in
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5 368 the design of this study and the burden of study participation was not assessed by patients
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8 369 themselves. Upon recruitment, patients are well informed of all aspects of the trial including
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10 370 antibiotic or symptomatic therapy of CT-confirmed uncomplicated acute appendicitis,
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12 371 difference between complicated and uncomplicated acute appendicitis, treatment success,
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15 372 possible late recurrence, and safety in order to help patients make an informed decision about
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17 373 trial participation. Patients also receive additional instructions in a phone call made prior to
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20 374 follow-up sample collection at 6 and 12 months. After completion of data collection and
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22 375 analysis, the patients will be informed of the study results and they will be provided with an
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24 376 opportunity to ask further questions.
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28 29 378 **ETHICS AND DISSEMINATION**

30 31 379 **Ethics**

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34 380 This protocol has been accepted by the Ethical Committee of the Hospital district of Southwest
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36 381 Finland (Turku University Hospital) and Finnish Medicines Agency (Fimea). The trial will be
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38 382 conducted in compliance with the Declaration of Helsinki.
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40 41 383 **Data collection and confidentiality**

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43 384 All data and samples are handled confidentially and the information in the datasets is non-
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45 385 identifiable. Data are gathered during the emergency room visit, hospitalisation for acute
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48 386 appendicitis, clinical observations, and follow-up phone calls. The main investigators will be
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50 387 in charge of the common database with full access to the data which is, otherwise strictly
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52 388 limited. As the MAPPAC and APPAC II and III trials are based on the same patient population,
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55 389 the interventions partly overlap and the enrolled patients are informed about this overlapping
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57 390 of the trials and the acquired data.
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392 **Withdrawal**

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

395 **Dissemination plan**

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

For peer review only

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60**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

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3 441 these results may provide novel tools to predict the potential recurrence risk and thus help to
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5 442 assess the optimal treatment choice for patients with uncomplicated acute appendicitis.
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9 443 **Strengths and limitations**

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11 444 MAPPAC has a strong synergy between APPAC II and III studies enabling the enrolment of both
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13 445 uncomplicated and complicated acute appendicitis patients in a large prospective series with
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16 446 associated clinical data to be assessed in conjunction with the microbiological and
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18 447 immunological findings. In many aspects, MAPPAC is an exploratory study of possible
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20 448 associations of whole microbial community and host immune characteristics with
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23 449 uncomplicated vs. complicated appendicitis and antibiotic response among patients in clinical
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25 450 trials treated with and without antibiotics. MAPPAC trial aims to generate hypotheses to better
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27 451 understand the role of disease progression and host susceptibility for future studies; i.e.
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30 452 determination of one primary outcome is insufficient for the study, as several factors are
31
32 453 indispensable for the understanding of etiology and the effects of antibiotics on GM all provided
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34 454 with this unique set of microbiological samples. To our knowledge, only one previous study
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37 455 [12] has characterized the adult appendix microbiota during appendicitis in adult patients
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39 456 using NGS technique and to date no reports on the structure and physicochemical contents of
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42 457 appendicoliths exists. Using these assessments is a strength in our study. Further, to our
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44 458 knowledge this is the first trial aiming to prospectively assess the possible microbiological
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46 459 and/or immunological etiology of appendicitis recurrence after a successful initial conservative
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49 460 treatment with antibiotics or symptomatic therapy and primary non-responders to
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51 461 conservative treatment of uncomplicated acute appendicitis. One of the main hypothesis of the
52
53 462 MAPPAC study is that the microbial composition of appendix differs between CT differentiated
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56 463 complicated and uncomplicated appendicitis. Therefore, strong element of the study is that all
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58 464 patients included in the study are imaged with CT protocol. CT scan is the gold standard for
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3 465 acute appendicitis imaging and this near perfect accuracy of CT adds to the reliability of the
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6 466 clinical data and appendicitis severity also in the patients with uncomplicated acute
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8 467 appendicitis without a histopathological confirmation of the appendix.

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10 468 The study limitations include the difficult challenge of conducting prospective clinical trials in
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13 469 the emergency setting. It is expected that all eligible patients may not be evaluated for
14
15 470 enrolment or some patients may not have all study samples available as the recruitment is
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17 471 performed by a large number of surgeons on call. The lack of healthy control group is a
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20 472 limitation in the study regarding both the etiology and in determining the effects of antibiotics
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22 473 on GM, as the results cannot be fully distinguished from the effects of acute appendicitis.

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490 Authors' contributions

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

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502 Competing interests

503 Authors declare they have no competing interests.

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17 631 **Abbreviations**

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19 632 **AMR:** Antimicrobial resistance

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21 633 **CRP:** C-reactive protein

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23 634 **CT:** Computed tomography

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25 635 **GM:** Gut microbiota

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27 636 **i.v.:** Intravenous

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29 637 **MALDI-TOF:** Matrix Assisted Laser Desorption/Ionisation time-of-flight mass spectrometry

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31 638 **MS:** Mass spectrometry

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33 639 **NGS:** Next generation sequencing

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35 640 **p.o.:** Per os

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37 641 **SPIRIT:** Standard Protocol Items: recommendations for Interventional Trials

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39 642 **VAP-1:** Vascular adhesion protein-1

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41 643 **VAS:** Visual analogue scale

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46 646 **FIGURE LEGENDS**

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48 647 Figure 1. The synergy between MAPPAC, APPAC II and APPAC III studies.

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50 648 Figure 2. Flow chart of the study protocol

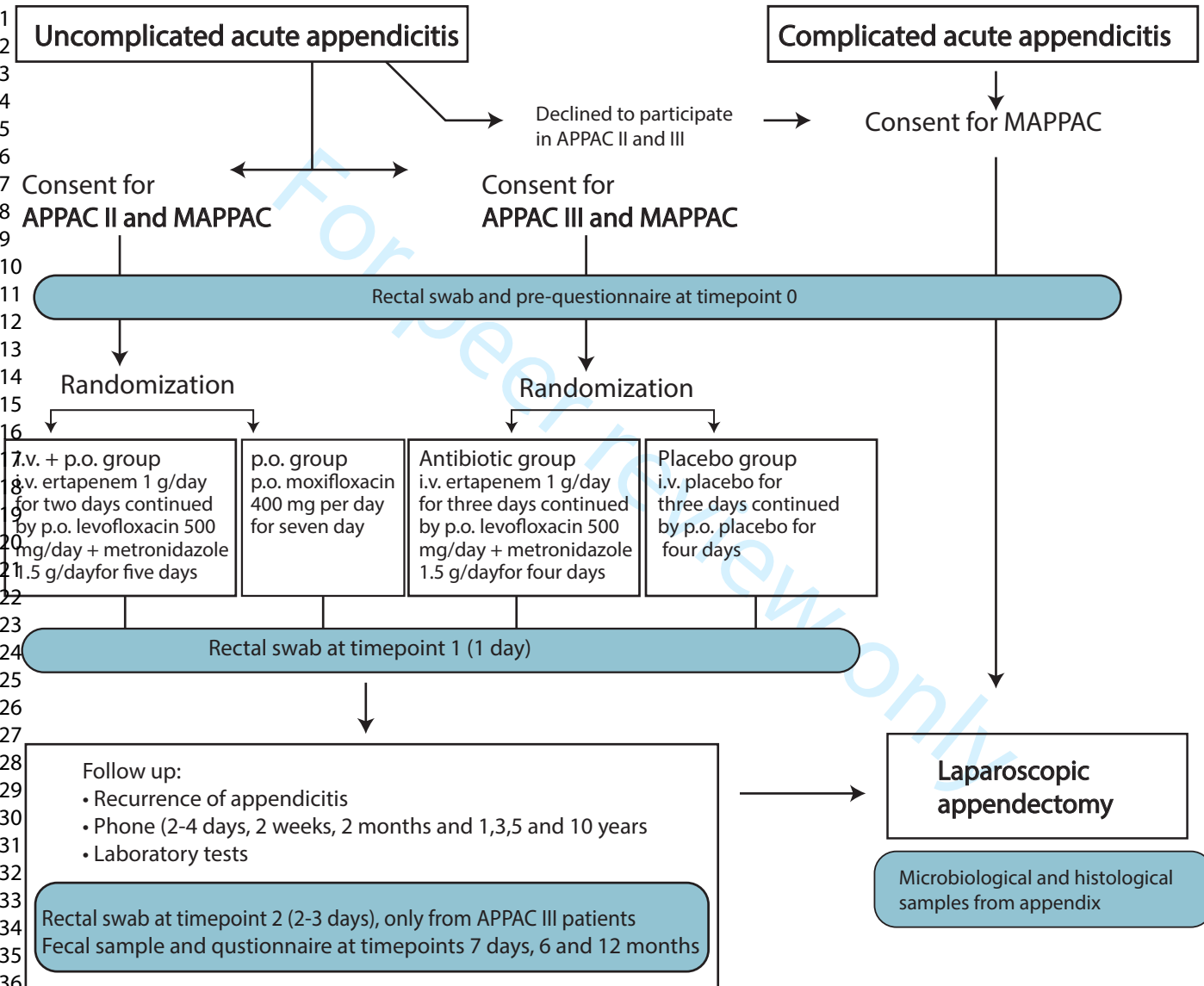
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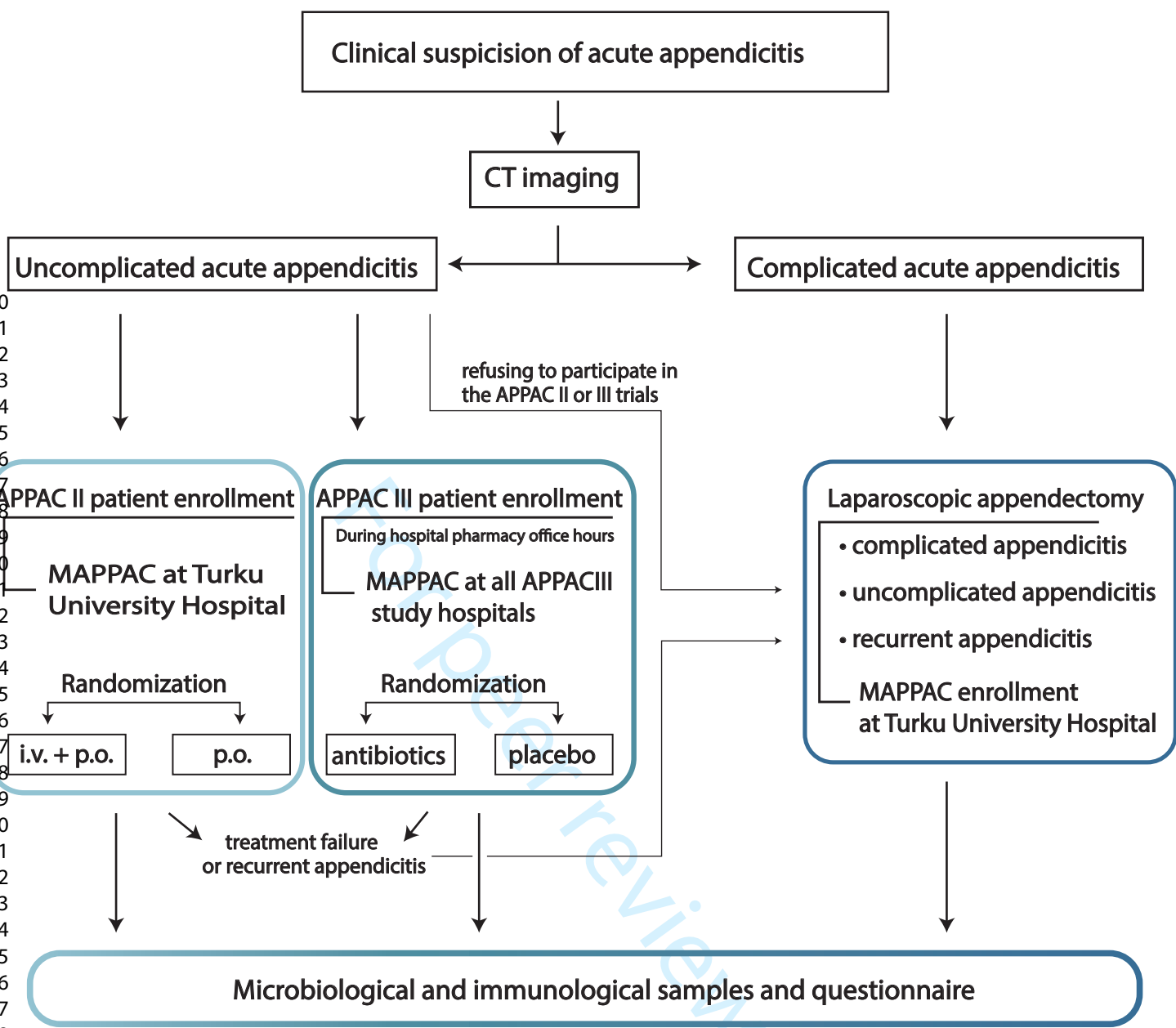
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SUPPLEMENTARY FILE

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 a new 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

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2
3 profile of samples will be analysed with NGS approach using appropriate methods and Illumina
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5 Miseq system.
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10 **Analyses of metagenome, transcriptome and proteome from appendiceal biopsy**

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12 Total RNA and proteins from the appendiceal biopsy are extracted with appropriate methods.
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14 Transcriptome and metagenome are analysed with Illumina Hiseq system. In addition, the
15
16 expression of specific genes is quantified with quantitative real-time PCR. Proteome is
17
18 characterized using mass spectrometry-based methods with qualitative and quantitative
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20 approach.
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26 **Culture methods**

27
28 Appendiceal swabs are cultured with both aerobic and anaerobic culture methods. For aerobic
29
30 culture the following growth media are used: CHROMagar Orientation (Becton Dickinson,
31
32 Franklin Lakes, New Jersey, USA), Trypticase soy agar with 5% sheep blood (Becton Dickinson),
33
34 Yersinia selective agar and Streptococcus selective agar (in house production). If the
35
36 appendectomy is performed during the office hours, an additional anaerobic culture is made in
37
38 connection with the sample collection. Samples for anaerobic culturing are collected with a sterile
39
40 cotton swab from the appendix and cultured for fastidious anaerobe agar with blood (FAA) and
41
42 kanamycin-vancomycin laked blood agar (KVLB) (both in house production), which are
43
44 immediately transferred into anaerobic jar with anaerobic gas generating sachet (Anaerogen 3,5
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46 l, Thermo Fisher Scientific).
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3 Cultures are incubated at +35 °C for 18-24 hours. Morphologically distinct colonies are
4
5 subcultured until a pure culture is obtained. Isolated species are frozen in a mixture of skim milk
6
7 and glycerol at -75 °C.
8
9

12 **MALDI-TOF mass spectrometry**

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15 The identification of isolated bacteria is done with Bruker matrix-assisted laser
16
17 desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). Isolates are inoculated
18
19 and cultured for 18-48 hours at +35 °C before the analysis. For the analysis, colony is applied
20
21 either directly or through ethanol/formic acid extraction on a steel plate and HCCA matrix (α -
22
23 cyano-4-hydroxycinnamic acid in 50% acetonitrile-2.5% trifluoroacetic acid (Bruker Daltonics,
24
25 Bremen, Germany)) is added according to the manufacturer's instructions. The identification is
26
27 done using Microflex LT instrument and MALDI Biotyper software (Bruker Daltonics). If isolates
28
29 cannot be identified due to the absence of reference peaks in the database, the isolate is identified
30
31 with sequencing the 16S rRNA gene.
32
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38 **Antimicrobial susceptibility testing**

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40 Phenotypic antimicrobial susceptibility testing for the isolated aerobic and anaerobic bacteria is
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42 performed by disk diffusion and MIC methods, according to the European Committee on
43
44 Antimicrobial Susceptibility Testing (EUCAST) guidelines. For certain bacteria, the Clinical and
45
46 Laboratory Standards Institute (CLSI) guidelines can be used.
47
48

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50 Further molecular analysis for antimicrobial resistance or virulence genes using e.g., PCR, Sanger
51
52 sequencing and whole genome sequencing (WGS) are also performed for selected isolates.
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55 **Appendicolith analysis**

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3 Appendicoliths are assessed both on the surface and cross-sectional morphology. Based on the
4 morphological characteristics and a degree of hardness, all appendicoliths will be classified. The
5
6 morphological characteristics and a degree of hardness, all appendicoliths will be classified. The
7
8 composition of selected appendicoliths are analysed with physical and chemical methods.
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10 11 12 **Immunological analysis**

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15 The appendiceal biopsies are analysed immunohistochemically by determining the presence of
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17 different cell types and blood vessel surface markers. Neutrophils, leukocytes and monocytes will
18
19 be analysed and special interest will be focused on lymphocyte subtypes (i.e., CD4/CD8 and more
20
21 detailed subgroup analyses such as regulatory T cells) and monocyte markers (i.e., macrophage
22
23 M1/M2 / receptor MHCII). Moreover, certain inflammation induced markers on endothelium such
24
25 as VAP-1, E-selectin and P-selectin will be evaluated. In order to compare possible differences
26
27 between patients with successful antibiotic therapy to patients with failed antibiotic therapy or
28
29 complicated acute appendicitis the serum samples will be screened to identify possible inflammatory
30
31 or immunological markers for identifying the different forms of the disease. Cytokines, chemokines
32
33 and growth factors will be tested using Bio-Plex Pro Human Cytokine 48-Plex Screening Panel (BIO-
34
35 RAD) containing the following analytes: Basic FGF, CTACK, eotaxin, G-CSF, GM-CSF, GRO-a, HGF,
36
37 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-
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39 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,
40
41 MIG, MIP-1a, MIP-1b, b-NGF, PDGF-BB, RANTES, SCF, SCGF-b, SDF-1a, TNF-a, TNF-b, TRAIL, VCAM-1
42
43 and VEGF-A. Moreover, we will use in house analyses to measure soluble VAP-1 (inflammatory) and
44
45 CD73 (anti-inflammatory). In addition, metabolomics approach using nuclear magnetic resonance
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47 (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS) metabolomics
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49 platforms will be used for biomarker analysis. The results obtained from immunological analyses will
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51 be correlated to the clinical parameters and to the microbiological findings.
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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			

1 2 3 4 5 6 7 8 9 10 11	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
12 13 14 15 16 17		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
18 19 20 21 22 23 24 25	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
26 27 28 29 30 31	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
32 33 34		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	17
35 36 37 38 39 40		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
41 42	Methods: Monitoring			
43 44 45 46 47 48 49 50 51 52	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
53 54 55 56 57 58 59 60		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](https://creativecommons.org/licenses/by-nc-nd/3.0/)" license.