STUDY protocol: MAPPAC (Microbiology Appendicitis Acuta) trial

Acute appendicitis and microbiota – etiology and effects of the antimicrobial treatment

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1. Background

Appendectomy has unquestionably been the standard treatment for acute appendicitis for over a century. More than 300.000 appendectomies are performed annually in the United States[1]. Although appendectomy is generally well tolerated, it is a major surgical intervention and can be associated with postoperative morbidity [2, 3].

Since the time Fitz described the relationship between the appendix and pelvic abscess and McBurney demonstrated reduced morbidity from pelvic infections attributable to appendectomy, it has been thought that acute appendicitis invariably progresses to perforation. This line of thinking underlies the belief that emergency appendectomy is required when a diagnosis of appendicitis is made [4, 5]. Fitz and McBurney's publications predated the availability of antibiotics by 40 years. In the absence of antibiotics, appendectomy saved lives by reducing the risk of uncontrolled pelvic infection when appendicitis was present.

Even though appendectomy has been the mainstay treatment for appendicitis, relatively soon after antibiotics were available, Coldrey reported treating 471 acute appendicitis patients with antibiotic therapy in 1959. Mortality was low (0.2 %) and recurrent appendicitis occurred only in 14.4 % of patients [6]. More recently, the notion of treating appendicitis with antibiotics was tested in 3 randomized clinical trials[7-9].Their results were summarized in a Cochrane analysis [10] and several meta-analyses. [11-15] Each of these previous trials had limitations and appendectomy has remained the standard approach for treating appendicitis.

1.1. The APPAC trial

In order to compare antibiotic therapy with appendectomy in the treatment of CT-scan confirmed uncomplicated acute appendicitis, we conducted the APPAC trial enrolling patients from November 2009 to June 2012. The APPAC trial [16] is a multicenter, randomized, open-label, non-inferiority trial conducted in Finland enrolling 530 patients 18 to 60 years of age with a CT scan confirmed uncomplicated acute appendicitis. Patients were randomly assigned to early appendectomy or antibiotic treatment with a follow-up of one year. Antibiotic therapy was intravenous ertapenem for three days followed by seven days of oral levofloxacin and metronidazole treatment; patients randomized to the operative treatment group underwent standard, open appendectomy. The primary endpoint for surgical intervention was the successful completion of an appendectomy. The primary endpoint for antibiotic treated patients was discharge from the hospital without the need for surgery and no recurrent appendicitis during a follow-up of one-year.

There were 273 patients in the operative group and 257 in the antibiotic group. The majority (73%), of patients with uncomplicated acute appendicitis were successfully treated with antibiotics. None of the patients, who initially were treated with antibiotics that later had appendectomy, had major complications. These results suggest that CT-proven uncomplicated acute appendicitis is not a surgical emergency and antibiotic therapy is a safe first-line treatment of uncomplicated acute appendicitis. With the development of more precise diagnostic capabilities like CT and effective broad-spectrum antibiotics, appendectomy may be unnecessary for uncomplicated appendicitis, which occurs in the majority of acute appendicitis cases. Patients should be able to make an informed decision between antibiotic treatment and appendectomy and focus should also be on taking into account the patient-centric outcomes. Future studies should focus both on early identification of complicated acute appendicitis patients needing surgery and to prospectively evaluate the optimal use of antibiotic treatment in patients with uncomplicated acute appendicitis.

1.2. The diagnosis and treatment of acute appendicitis

Acute appendicitis is the most common cause of abdominal pain in emergency departments and appendectomy is the most common emergency abdominal surgery. The lifetime risk of acute appendicitis in males is 8.6% and 6.7% in females. [17] In Finland according to Stakes data there were 6 377 appendectomies (3242 in males, 3135 in females, median age 35 years) performed in 2010. The total number of days in hospital care was 16 111 days and the mean length of hospital stay was three days.

Although acute appendicitis is the most common reason for surgical emergency department visit, its diagnosis still remains challenging. The clinical diagnosis has previously been based on patient history, physical examination and laboratory findings as well as the clinical surgical diagnosis. Several scoring systems have been created to aid in the diagnosis of acute appendicitis[18-20], but the accuracy of clinical diagnosis without preoperative imaging is about 76 – 80 % for combined patient groups of males and females[21, 22]. As acute appendicitis has historically been thought to always progress to perforation requiring emergency appendectomy, high negative appendectomy rates even up to 40 % in some

patient populations have been previously accepted as good surgical practice. For the last two decades, the use of dedicated imaging in acute abdomen in general and also in acute appendicitis has led to improved diagnostic accuracy.

1.2.1. Uncomplicated and complicated acute appendicitis

Based on large epidemiological studies, we now know that complicated (perforated) and uncomplicated (non-perforated) appendicitis have followed different epidemiological trends. These unassociated epidemiologic trends suggest different pathophysiology for the two form of appendicitis. However, the etiology of the different forms of appendicitis remains unknown. The differential diagnosis is essential as patients with an uncomplicated acute appendicitis may not require surgical intervention and might experience even spontaneous resolution without perforation.[23] The majority (approximately 80 %) of acute appendicitis cases are of uncomplicated nature. Complicated acute appendicitis defined as a finding of a perforation, appendicolith, abscess or a suspicion of a tumor, requires emergency appendectomy with the exception of cases with abscess as they are often managed conservatively.

Appendicolith is a calcified fecal concretion in the appendix resulting in internal luminal obstruction and it is the most common form of complicated acute appendicitis. In the first randomized study by Vons et al.[9] comparing operative treatment and antibiotic therapy using CT as a diagnostic inclusion criterion, the presence of an appendicolith in preoperative CT scan was the only factor that significantly increased the risk of complicated appendicitis and it was also the only factor associated with the failure of antibiotic therapy for acute appendicitis. Indeed, if Vons et al[9] had excluded the patients with an appendicolith from their analysis, no significant difference in the incidence of post-intervention peritonitis between the treatment groups would have been noticed in their study.

1.2.2. Computed tomography (CT) in diagnosing acute appendicitis

CT imaging is the primary imaging modality and the golden standard in the diagnosis of acute appendicitis as it establishes the diagnosis with almost perfect diagnostic accuracy. The advantages of CT imaging are high accuracy, availability, ease of performance and interpretation, and that it is rarely affected by bowel gas, severe abdominal pain or extreme body habitus. The increased use of preoperative CT has been shown to markedly reduce the number of unnecessary appendectomies. The main disadvantage of CT is exposure to radiation. The favorable diagnostic performance of CT imaging has encouraged optimization of the protocol to minimize exposure to radiation through the development of low-dose CT protocols. Low-dose protocols balance with as low as reasonably achievable-principle while maintaining diagnostic accuracy[24]. We have initiated a prospective observational study (OPTICAP trial, NCT02533869) in order to optimize a low-dose CT scan for both diagnosing acute appendicitis and to differentiate uncomplicated acute appendicitis from a complicated acute appendicitis. The OPTICAP clinical phase results will be analyzed by October 2016 and the most optimal imaging protocol will be selected for use in the APPAC II and III trials enabling also the initiation of the MAPPAC trial.

1.2.3. Treatment of acute appendicitis

For over a century appendectomy has been the standard treatment for all patients with acute appendicitis. However, the results of our APPAC trial[16] have now shown that the majority (73%) of patients with uncomplicated acute appendicitis were successfully treated with antibiotics alone. We also showed that none of the patients treated initially with antibiotics and later undergoing appendectomy had major complications or increased morbidity defining antibiotic therapy as a safe first-line treatment. Patients with a complicated acute appendicitis require emergency appendectomy

and early identification of these patients is of vital importance. Laparoscopic appendectomy has become the golden standard for appendectomy providing lower morbidity and faster recovery compared with open appendectomy. For patients with uncomplicated acute appendicitis, the time has come to evaluate abandoning routine appendectomy and evaluating the optimal use of antibiotic therapy.

1.2.4. Microbiological etiology of appendicitis

Gut microbiota is an extremely complex ecosystem with both high bacterial density and diversity. Recent scientific evidence emphasizes that the symbiosis between the host and the balanced gut microbiota supports good health, and contributes to various biochemical and metabolic functions occurring in host's body.[25] The possible role of the somehow distorted gut microbiota composition in addition to its metabolites in the etiopathogenesis of many diseases such as allergy, inflammatory bowel disease, type 1 diabetes and obesity related disorders, has been recently proposed[25, 26]. Further, detected alterations and perturbations both in the gut microbiota composition and functionality have been linked to the development of various malignancies such as colorectal cancer, gastric cancer and hepatocellular carcinoma[25, 26]. To date, the role of the microbes and especially the members of the commensal microbiota with their structural compartments and metabolites in the pathogenesis and etiology of appendicitis have not been clarified in detail, despite the recent knowledge that uncomplicated acute appendicitis could be treated by antibiotic treatments alone[16]. Further, there is only limited amount of evidence on the appendix microbial composition in humans.

Microbial overgrowth has been speculated to serve as a secondary consequence in appendicitis[27]. However, recent accumulating evidence suggests that primary bacterial infection may actually be an initiating event in the pathogenesis of disease[28]. Interestingly, it has been postulated that the appendix could serve as a microbial reservoir for repopulating the gastrointestinal tract in times of necessity thus gut microbiota may act as a source for these pathogenic intruders[29]. Further it has been reported that certain members of the gram negative *Fusobacteria* especially *F. nucleatum* and *necrophorum* are present in most appendicitis samples[28, 30].

1.2.5. Antibiotics and microbiota – evaluation of the effects of the antimicrobial treatment

Antimicrobial resistance (AMR) is an increasing global threat. According WHO, in 2050s more people will be killed by AMR bacteria than by all cancers.The use of antimicrobials in human and especially in animal health care and production industry is the major cause of increasing AMR worldwide; the prudent use of antimicrobials is essential to prevent increasing AMR. Antimicrobials are known to decrease the gut microbiota diversity, richness and species variation and cause the perturbation of its overall balance[30] and even a short-term antimicrobial treatment has a long-term impact on its composition[31] underlining the importance of evaluating both short- and long-term effects of the antimicrobial treatment in old and new indications.

2. Aims of the study

The aims of this study are: 1. To evaluate the possible role and differences in the microbiological etiology of complicated and uncomplicated appendicitis. The bacterial composition of the complicated appendix will be compared to the gut microbiota composition determined from the fecal sample collected from the same individual. Additionally, these results will be compared to the gut microbiota composition of patients with uncomplicated acute appendicitis.

2. To determine the effects of both antibiotic and placebo treatment on the composition of gut microbiota, and to evaluate how it recovers after the appendicitis-related antimicrobial treatment (AMT). The bacterial composition and

AMR reservoir of the gut microbiota will be evaluated both pre and post treatment in patients receiving antibiotic or placebo treatment for uncomplicated acute appendicitis. Additionally, the recovery of gut microbiota composition and disappearance of AMR will be evaluated. We will compare two variations (i.v.and p.o.) of antibiotic treatment with the placebo treatment. 3. To evaluate the effects of the duration of the hospital stay on the AMR reservoir of the gut microbiota. According to the study protocols of the APPAC II and III trials, patients will spend either 1 or 3 days in the hospital in order to receive treatment before continuing the selected treatment at home. We will evaluate the effects of length of stay on the AMR reservoir of gut microbiota as well as evaluating if and when possible colonization occurs.

3. Study design, patients and methods

3.1. Study design

The MAPPAC trial has been designed as a prospective multicenter trial regarding the effects of both antibiotic and placebo treatment on the gut microbiota composition, and to evaluate how it recovers after the appendicitis-related AMT in a longitudinal study design. In a single-center study design (Turku University Hospital) the MAPPAC trial aims to determine the composition of gut microbiota in the different forms of appendicitis.

3.2. APPAC II and APPAC III trials

APPAC II and III trials constitute a major part of MAPPAC trial enrollment. The aim of APPAC II trial is to optimize the antibiotic therapy for uncomplicated acute appendicitis by evaluating the success of treatment in both study groups and by comparing intravenous antibiotic therapy followed by per oral antibiotics with per oral antibiotic monotherapy. The aim of APPAC III is to compare antibiotic therapy with placebo in the treatment of uncomplicated acute appendicitis to evaluate the role of antibiotic therapy in the resolution of acute uncomplicated appendicitis. APPAC II and APPAC III trials are separate studies regarding the applied study permissions (Fimea, Tukija, the Ethical committee of Turku University Hospital). In practice these two studies will be performed in close conjunction with each other as the enrolled patient population is identical in both studies and the study chosen for enrollment will be based mainly on the time of day (based on study design APPAC III enrollment is only possible between 8 a.m. and 2 p.m.) and secondly on patient preference (if the patient is unwilling to participate in APPAC III, they will be informed and invited to participate in APPAC II trial). After 2 p.m. until 8 a.m. all of the eligible patients will be invited to participate in APPAC II trial.

3.3. MAPPAC participants

Patients presenting with suspected acute appendicitis will be enrolled in APPAC trial from nine participating Finnish hospitals; four university hospitals (Turku, Oulu, Tampere, Kuopio) and four central hospitals (Jyväskylä, Mikkeli, Hämeenlinna, Vaasa). APPAC III trial will enroll patients in all university hospitals (Turku, Oulu, Tampere, Helsinki, Kuopio). Out of these hospitals, MAPPAC trial will enroll patients in all APPAC III hospitals (Turku, Oulu, Tampere, Helsinki, Kuopio). Regarding APPAC II trial patients and the patients with complicated appendicitis, the MAPPAC enrollment will be performed in Turku.

Based on APPAC II and III trials all adult patients (aged 18 – 60 years) admitted to the emergency department with a clinical suspicion of uncomplicated acute appendicitis will undergo a low-dose CT scan optimized for the diagnosis of acute appendicitis (OPTICAP trial, please see chapter 1.2.2.). Clinical history, physical investigation, VAS pain scores (visual analogue scale) and laboratory tests will be recorded for all of the APPAC and MAPPAC trial evaluated patients

in a prospective online database (BCB Medical APPAC-database developed by our study group). MAPPAC trial patient data will be recorded in a separate excel file based on data collection sheets from all of the participating hospitals. A signed informed consent will be obtained from all of the patients.

In addition to APPAC II and III trial patients with uncomplicated acute appendicitis, the MAPPAC trial will enroll patients with complicated acute appendicitis at Turku University Hospital.

MAPPAC inclusion criteria follow the criteria of APPAC II and III trials: 1) Signed informed consent, 2) Age 18 – 60 years, 3) CT scan confirmed diagnosis of uncomplicated acute appendicitis or complicated acute appendicitis (appendicolith, perforation, abscess, suspicion of a tumor). MAPPAC exclusion criteria follow APPAC II and III criteria: 1) Age <18 or > 60 years, 2) Pregnancy or lactating, 3) Allergy to contrast media or iodine, 4) Allergy or contraindication to antibiotic therapy 5) Renal insufficiency, 6) Metformine medication, 7) Severe systemic illness (for example malignancy, medical condition requiring immunosuppressant medications), 8) Inability to co-operate and give informed consent.

According to modern normal clinical practice all patients admitted to the emergency room with suspected acute appendicitis will undergo CT imaging, as CT has become the golden standard imaging in diagnosing acute appendicitis. Based on our OPTICAP-trial, the CT scan protocol used for acute appendicitis will be optimized for radiation exposure. If complicated acute appendicitis is diagnosed on CT, patients will undergo a laparoscopic appendectomy within eight hours (the patients will be classified as “requiring surgery within 0- 8 hours” in an acute care surgery criteria used in the operating theatre). Patients with a CT diagnosis of uncomplicated acute appendicitis will be evaluated for enrollment in APPAC II and III studies depending on the time of the day (please see 3.2.)

Collecting the MAPPAC samples either in uncomplicated acute appendicitis (rectal swabs, possible histology and swab of the removed appendix, if appendectomy is required) or complicated acute appendicitis (rectal swabs, histology including separate histology specimen (small part of the removed appendix) stored separately for later analysis, swab of the removed appendix) will not affect the clinical treatment of the patients. Additionally, an extra serum sample will be collected for later immunological analyses in both patient groups.

3.4. Registration

Patients with uncomplicated acute appendicitis also taking part in the APPAC II trial will be randomized with a 1:1 equal allocation ratio to i.v. + p.o. or p.o. antibiotics group. Patients with uncomplicated acute appendicitis also taking part in the APPAC III trial will be randomized with a 1:1 equal allocation ratio to receive either antibiotic therapy or placebo. Patients with complicated acute appendicitis who are excluded from the APPAC II and III trials will be treated with surgery according to current treatment protocols. As the MAPPAC and APPAC trials will be ongoing simultaneously and 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. All MAPPAC patients will be enrolled simultaneously with and within the APPAC trials. Patients recruited for the APPAC II or III trial will asked to sign a separate consent form allowing for the use of their data and collection of microbiological samples for the MAPPAC study and the MAPPAC trial information is stated in the patient information for both APPAC trials.

All of the patients participating in the APPAC III trial will be invited to take part in the MAPPAC trial. All of the APPAC II trial patients at Turku University Hospital will be invited to take part in the MAPPAC trial and all of the patients presenting with complicated acute appendicitis at Turku University Hospital will be invited to take part in the MAPPAC trial. The aim is to enroll a large number of patients with confirmed acute appendicitis (both the

uncomplicated and complicated forms) to participate in this MAPPAC trial to gain a comprehensive view of acute appendicitis.

3.5. Interventions

In order to evaluate the first aim of the MAPPAC trial (the role of the gut microbiota, and possible differences in its composition in different forms of acute appendicitis), both patients with uncomplicated and complicated forms of acute appendicitis will be included. The patients with uncomplicated acute appendicitis will be enrolled from those participating in the APPAC II and III trials. The patients with complicated appendicitis will be recruited from those not participating in either of the APPAC trials, but have CT confirmed complicated acute appendicitis. Rectal swabs will be taken from all the patients prior the AMT, and additional samples from the appendix will be collected for analysis from both patients undergoing surgery for complicated acute appendicitis and also in cases of surgically treated uncomplicated appendicitis patients. In addition, possible appendicoliths at surgery for both complicated and uncomplicated acute appendicitis will be collected as samples in dry test tubes for further microbiological and other analysis. In order to evaluate the second aim of the MAPPAC trial (the effects of AMT as compared to placebo on the gut microbiota composition and its recovery), patients participating in the APPAC II and III trial will be enrolled. Rectal swabs will be taken from all the patients both pre and post treatment. Based on the study designs, we will have three groups: two of various antibiotic alternatives and one placebo. In order to evaluate the third aim of the MAPPAC trial (the effects of length of hospital stay on the composition of gut microbiota), patients from both of the APPAC trials will be invited to participate. Patients in the APPAC II trial spend one day hospitalized as described in the APPAC II protocol, and patients in the APPAC III trial spend three days hospitalized as described in the APPAC III protocol. Previously described rectal swabs taken to evaluate the other aims of this study will be analyzed in order to determine the effect of the duration of the hospital stay on the changes of gut microbiota as well as to evaluate possible colonization.

3.6. Outcome parameters

Microbial outcome is defined by evaluating the changes in the gut microbiota species variation after the AMT. Determining the possible correlation of gut microbiota and complicated appendicitis will be performed by comparing the species variation. In order to further understand both the etiology and specific features of uncomplicated acute appendicitis, we are collecting fecal samples for future analysis. The fecal samples will be stored according to regulations and will be used only for APPAC II/III trials. In cases of patients undergoing appendectomy for treatment failure, we will inform the patients, that further specialized histopathological analysis may be performed in addition to standard histopathological examination.

3.7. Data collection and follow-up

After signed consent to the MAPPAC trial, patients will be evaluated for acute appendicitis and enrolment is registered on paper sheets. The information from the paper sheets will later be added to an excel file containing the information for the MAPPAC trial.

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3.7.1. Bacteriological specimens

3.7.1.1. Collection and storage

Briefly, MAPPAC fecal samples in APPAC III trial patients will be collected twice during the stay at the research hospital (time points 0 and 1 or 3 d) and three times at home (follow-up at one week, six months and one year). The MAPPAC fecal samples time points in APPAC II patients are 0 and 1 d. Briefly, in hospital (0 and 1d/3 d) fecal samples will be collected from the rectum. Two swabs, one for bacterial culture (1. gel tube) and one for the gut microbiota determination (2. FLOQSwab) will be taken at each time point and placed immediately into sterile collection tubes. Tube 1 is stored at room temperature and sent immediately by mail to the Department of Microbiology and Immunology. Tube 2 is stored at -20 °C in hospitals and sent frozen on dry ice to the Department of Medical Microbiology and immunology, University of Turku.

3.7.1.2. Bacterial sample collection from complicated appendicitis patients

Fecal samples will be collected from the rectum as described prior to appendectomy from the patients presenting with complicated appendicitis at Turku University Hospital. During the appendectomy the contents of the appendix will be collected into a sterile collection tube by swab after the surgical removal and then the contents will be immediately stored in a freezer. In addition, a piece of the appendix will be cut and stored in a sterile collection tube and stored in a freezer. All samples are transported frozen (on dry ice) to the Department of Microbiology and Immunology.

3.7.1.3. Analysis of the bacterial specimens

Fecal samples in tube 1 are sent to the Department of Medical Microbiology and Immunology, where bacteria from the samples will be cultured to the Chrom agar and lactose agar plates. Plates are incubated over night at 37 °C. Colonies of *E. coli* or *K. pneumoniae* morphology from Chrom agar plates will be picked and cultured as pure cultures after which antimicrobial susceptibility testing will be performed. In addition, DNA will be isolated by Chelex DNA-extraction method. The whole bacterial cell mass will be collected and frozen as such (in milk-glycerol tubes). The total bacterial DNA i.e. microbiome will be extracted from the frozen fecal samples collected in the tube 2 and appendix contents (4.6.1.2)) as previously described[32]. The DNA concentrations of the extracts are measured with Qubit 2.0 fluorometer (Life Technologies), and the DNA is stored at -75°C. DNA is further used in order to characterize the microbiota composition (16S rRNA analysis) and whole microbiome gene content by utilising so-called next generation sequencing (NGS) methodologies. Briefly, various variable regions of the bacterial 16S rRNA gene are amplified with barcoded primers that allow the identification of the products of each original sample (modified from [33]). The PCR products are purified with Agencourt AMPure XP Magnetic beads (Beckman Coulter, Inc.), utilizing the DynaMag™-96 magnetic plate (Life Technologies, CA, USA). The product length and DNA integrity are checked with TapeStation (Agilent Technologies Inc., CA, USA), and the final DNA concentrations will be measured with Qubit 2.0 fluorometer (Life Technologies). The sequencing will be performed with Illumina Miseq system in Turku Centre of Biotechnology (BTK) and Department of Genetics, University of Turku. The quality of the sequence data will be checked with fastQC program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and the data will be analyzed with QIIME 1.9 pipeline [34]. Bioinformatic analyses will be performed in BTK. Further, microbial metagenome will be analysed by Illumina HiSeq protocol in Centre for Public Health Research, FISABIO (Fomento de la Investigación Sanitaria y Biomédica de la Comunitat Valenciana), Valencia, Spain.

4. Statistical methods

Categorical variables of the study will be characterized by trial group using frequencies and percentages and for continuous variables means and standard deviations or medians with range and 25th and 75th percentiles will be used. Microbial outcomes will be analyzed using repeated measurements ANOVA or other repeated measurements methods if ANOVA is not suitable. The assumptions of the methods will be checked for justification of the analyses and transformations will be used for the variables if needed. The study site differences will be evaluated in statistical models and if major differences are detected more complicated statistical models will be used in the analyses. Two-sided p-values will be used and p-values less than 0.05 will be considered statistically significant. The measurements with missing data will automatically be excluded from the analyses of the variables in concern. Statistical analyses will be performed using SAS System for Windows, Version 9.4 or later (SAS Institute Inc., Cary, NC).

5. Ethical considerations and study relevance

Based on epidemiological studies we know that complicated (perforated) and uncomplicated (non-perforated) appendicitis have followed different epidemiological trends. These unassociated epidemiologic trends suggest different pathophysiology for the two form of appendicitis. However, the etiology of the different forms of appendicitis remains unknown and the possible difference in the microbiological etiology of these different disease forms of this very common surgical emergency would be of great value in evaluating the different treatment options for acute appendicitis patients. In addition, the comparison of the antibiotic therapy effect on the gut microbiota in APPAC III patients enrolled in this MAPPAC trial would be of profound interest as the patient groups in APPAC III trial setting are ideal in this respect with one group receiving antibiotic therapy and the other placebo.

6. Study costs

The MAPPAC trial sample collection, sample storing and all analyses including both material expenses and personnel costs (technical laboratory personnel and researchers) are evaluated to add up to approximately 495 000 € and the number of possible collected samples is based on available funding by research grants. The detailed description on costs is described in the separate cost analysis including research months for three main researchers, hiring technical personnel to handle the samples and collection, DNA-storage and analysis and genomics analysis. Based on the maximum limit of EVO application funding, the required 16S Illumina analyses have been left out of the total costs.

7. Study schedule

APPAC II/III trial protocols have been accepted in the local ethics committee and APPAC II (EudraCT 2015-003633-10) also already has the Fimea approval. APPAC III (EudraCT 2015-003634-26) will be sent for Fimea evaluation after the hospital pharmacy contracts and preparation of the i.v. and p.o. antibiotics and placebo have been thoroughly defined by the Turku University Hospital pharmacy. APPAC II trial enrollment is evaluated to start after the OPTICAP clinical trial phase results are available; most likely APPAC II trial will start enrolling in November 2016. APPAC III trial will be initiated in January 2017 after finalizing the Fimea approval and hospital pharmacy contracts in each of the participating hospitals. MAPPAC (EudraCT 2016-003655-29) application to TUKIJA has been referred by TUKIJA to the local ethical committee and the ethical committee application will be submitted by the next possible deadline on the 29th of September 2016. MAPPAC trial will be initiated accordingly along with the APPAC II and III trials. MAPPAC

trial is evaluated to last for approximately two years (until the end of year 2018) and the primary endpoint will available for analysis after completing the MAPPAC enrollment.

8. Study hospitals and investigators

MAPPAC trial will be a national multicenter study and Turku University Hospital will be the main research center and the primary investigator will be Paulina Salminen. Study will be conducted at all five university hospitals (Turku, Tampere, Oulu, Helsinki and Kuopio) and Jyväskylä central hospital. The investigators at each research hospital: 1) Turku (Paulina Salminen, MD, PhD, Juha Grönroos, MD, PhD, Johanna Virtanen MD, PhD, Suvi Sippola, MD, PhD student, Harri Marttila, MD, PhD,), 2) Tampere (Pia Nordström, MD, PhD, Johanna Laukkarinen MD, PhD, Irina Rinta-Kiikka, MD, PhD), 3) Oulu (Tero Rautio MD, PhD, Jari Mällinen MD), 4) Helsinki (Ari Leppäniemi, MD, PhD, Ville Sallinen, MD, PhD) 5) Kuopio (Hannu Paajanen MD, PhD, Tuomo Rantanen MD, . The study statistician is Saija Hurme, MSc (University of Turku). The investigators at the Department of Microbiology and Immunology at University of Turku will be Antti Hakanen MD, PhD, Eveliina Munukka, PhD, Marianne Gunell, PhD, Erkki Eerola MD, PhD, Jaana Vuopio MD, PhD, Anniina Rintala, MSc, PhD student and Sanja Vanhatalo, BSc.

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