PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	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
AUTHORS	Vanhatalo, Sanja; Munukka, Eveliina; Sippola, Suvi; Jalkanen, Sirpa; Grönroos, Juha; Marttila, Harri; Eerola, Erkki; Hurme, Saija; Hakanen, Antti; Salminen, Paulina

VERSION 1 - REVIEW

REVIEWER	David A Talan Olive View-UCLA Medical Center; The David Geffen School of Medicine at UCLA
REVIEW RETURNED	02-May-2019

GENERAL COMMENTS	This article describes an ambitious research plan by experienced clinical trialists for ancillary studies to ongoing clinical trials of the microbiota associated with the existence of uncomplicated vs. complicated appendix, and the effects of antibiotic treatment of appendicitis, and association of microbiota with antibiotic response initially and with recurrence over 1 year. Rectal and appendiceal swabs, appendicolith, and appendiceal biopsies will be analyzed by standard and molecular (NGS) testing. Serum will also be taken for studies of immune reaction. The authors are challenged to coherently bring together for the average reader a description of the themes of appendicitis pathophysiology and the hypotheses that might be generated from this exploratory study that collects myriad bacteriology and immunology data. As written, it seems that about 1/2 the paper describes the background and methods for clinical trials at the expense of more relevant detail about the study at hand. Under "Strengths," the authors refer to "the etiology of appendicitis," which is unclear, but some previous studies suggest a role of bacterial infection. Not everyone gets appendicitis, so host factors may come into play. But the etiology, i.e., what causes appendicitis, cannot be fully understood without a control population of people without appendicitis. For example, if one finds a certain bacterium (e.g., Fusobacterium) in flora/tissue and response varies with its antibiotic treatment and organism susceptibility, then one can hypothesize a role in appendicitis. But since everyone carries Fusobacterium, this does not help us

	understand why that person got a Fusobacterium infection. We also do not know why some people have uncomplicated vs. perforated appendicitis. The common belief was that the untreated disease progressed but there is also evidence of differential host immune factors that may drive this (Rivera-Chavez FA. Am Surg. 2004;240:269-77). The authors need to make this clear. Because of confusion as to the exact definition of "complicated" appendicitis (and its converse), this reviewer suggests the term "perforated" as more accurate and descriptive. Importantly, and which needs to be stated clearly, is that this is an exploratory study to, at best, generate hypotheses for future studies. No hypotheses are offered here, although some broadly exist (see above) and should be proffered to help the average reader. Also, no pilot study experience is presented so in many ways this is just a pilot study. Perhaps the authors should state that this is an exploratory study of possible associations of microbiota and host immune characteristics with uncomplicated vs. complicated appendicitis and antibiotic response among patients in clinical trials treated with and without antibiotics with the goal to generate hypotheses to better understand the role of disease progression and host susceptibility. The abstract should mention studies of immune response. Under "Strengths" is the assertion that NSG and cultures will provider reliable information about microbiota in the etiology of uncomplicated and complicated appendicitis. How would one know this with much confidence before doing the study? It is fairly likely that the results will not be reliable, e.g., contamination of specimens with non-etiologic bowel flora. Bacteria, if indeed they are involved in infection, may reside in the tissue alone, which may be impossible to isolate from colonizing flora especially with overly sensitive molecular assays. The first part of the Introduction focuses on the foundation for antibiotic treatment (and multiple references), when the primary focus should be our understanding of what causes appendicitis and appendicitis perforation and existing evidence to support these theories. This reviewer thinks that antibiotic treatment can be mentioned with later study objectives as part describing the clinical trials to which this is an ancillary study (just before the Methods). The theory themes (obstruction, infection, host factors) based on what is known need to be more clearly laid out. It is unclear what recitation of bowel flora or referring to the novelty of applying of NGS methods add to the introduction, but stating "studies of these suggest" would help frame the questions for which your exploratory study may provide future testable hypotheses. Again, the long description of the clinical trials seems excessive for this study's focus. The Methods have about 5 pages describing each of the contributing clinical trials. The focus should be on this study's methods, which are extensive enough, and the characteristics of the clinical trials should be summarized in one paragraph and either referenced or included with a supplemental appendix. Also, mention of things like patient withdrawal seem irrelevant. The specific assays should be described in sufficient detail so they can be replicated. For example, it is unclear what will be done with serum samples collected for immunological and inflammatory

	marker analysis and metabolomics approach. Also further description is need for, "biomarker analysis of numerous different cytokines, chemokine and growth factors as well as serum metabolome will be analyzed." Please explain how tissue/appendicolith specimens will be separated from contaminating colonic flora. To this reviewer, the supplemental methods should be the primary methods and the clinical trial methods should be supplemental. The journal may have a statistical reviewer, but this reviewer is unsure if all possible projected types of data summary and association testing methods need to described in this preliminary expedition. The Discussion is devoted to the clinical trials, not the ancillary study's aims, further background for the reader, and projected possible hypotheses that might be generated. This reviewer thinks the flow diagram provides unnecessary details of the clinical trials and distracts from the specific study being described. Perhaps these could be simplified and more schematically show where each specimen and testing of microbiota and immune response will be done over time. Additional references that may be of interest include Rivera-Chavez FA. Am Surg. 2004;240:269-77 (host response), Bennion RS. Ann Surg. 1990; 211:165-71 (bacteriology of microtomed tissue), Rogers MB. Clin Infect Dis 2016:63:71-78 (RNA sequencing/Fusobacterium), Salö M. Int J Colorectal Dis 2017;32:19-28 (RNA sequencing of microbiome), Guinane CM. MBio 2013;4(1) (RNA sequencing of microbiome), Schulin S. Medicine 2017;52 (DNA sequencing of microbiome), and Peeters T. Fut Microbiol 2019;14 (RNA sequencing of microbiome).

REVIEWER	Mauro Podda Department of Surgical Science, General and Emergency Surgery Unit, Cagliari University Hospital Italy
REVIEW RETURNED	16-May-2019

GENERAL COMMENTS	Vanhatalo et al presented an interesting study protocol for a prospective observational study on the appendicitis etiology and pathophysiology, focusing on the differences between uncomplicated and complicated appendicitis from a biological point of view. The protocol is well-structured, although very complex. However, the Authors focalized their attention on one of the most debated issues when talking about NOM for acute appendicitis: who to distinguish during the patient's assessment, those who might respond well to antibiotics or observation alone from those who will require surgery. I have just a few questions for the authors: 1. How do the Authors think the results of their research could be translated into the daily clinical practice? 2. Why the authors did decide to include appendicitis with appendicolith among the complicated forms? Recent meta-analyses and prospective trials reported that the failure rate of NOM in patients affected by uncomplicated appendicitis with appendicolith is high (40-60%), confirming that patients with

	evidence of appendicolith on imaging have an initial failure rate of NOM more than twice that of patients without an appendicolith [Mahida JB, et al. High failure rate of nonoperative management of acute appendicitis with an appendicolith in children. J Pediatr Surg. 2016;51:908–911; Tanaka Y, et al. Long-term outcomes of operative versus nonoperative treatment for uncomplicated appendicitis. J Pediatr Surg. 2015;50:1893–1897; Podda M, et al. Antibiotic Treatment and Appendectomy for Uncomplicated Acute Appendicitis in Adults and Children: A Systematic Review and Meta-analysis. Ann Surg 2019, doi: 10.1097/SLA.0000000000003225]. However, if we consider the most recognized definition of complicated appendicitis (Presence of gangrene/abscess/diffuse peritonitis), the presence of appendicolith alone cannot be considered as complicated form.

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1

Reviewer Name: David A Talan

Institution and Country: Olive View-UCLA Medical Center; The David Geffen School of Medicine at UCLA

1. This article describes an ambitious research plan by experienced clinical trialists for ancillary studies to ongoing clinical trials of the microbiota associated with the existence of uncomplicated vs. complicated appendix, and the effects of antibiotic treatment of appendicitis, and association of microbiota with antibiotic response initially and with recurrence over 1 year. Rectal and appendiceal swabs, appendicolith, and appendiceal biopsies will be analyzed by standard and molecular (NGS) testing. Serum will also be taken for studies of immune reaction. The authors are challenged to coherently bring together for the average reader a description of the themes of appendicitis pathophysiology and the hypotheses that might be generated from this exploratory study that collects myriad bacteriology and immunology data. As written, it seems that about 1/2 the paper describes the background and methods for clinical trials at the expense of more relevant detail about the study at hand.

Answer: We fully agree with the reviewer that the emphasis of the manuscript may be too much on the clinical background and methods. However, for the general reader, this clinical background needs to be acknowledged for the importance of potential gut microbiota effects on the appendicitis severity and recurrence. We have now revised this emphasis throughout the manuscript to better reflect the current study details and we have omitted and revised some of the clinical background data into a more concise form, as suggested by the reviewer.

2. Under "Strengths," the authors refer to "the etiology of appendicitis," which is unclear, but some previous studies suggest a role of bacterial infection. Not everyone gets appendicitis, so host factors may come into play. But the etiology, i.e., what causes appendicitis, cannot be fully understood without a control population of people without appendicitis. For example, if one finds a certain bacterium (e.g., Fusobacterium) in flora/tissue and response varies with its antibiotic treatment and organism susceptibility, then one can hypothesize a role in appendicitis. But since everyone carries Fusobacterium, this does not help us understand why that person got a Fusobacterium infection. We

also do not know why some people have uncomplicated vs. perforated appendicitis. The common belief was that the untreated disease progressed but there is also evidence of differential host immune factors that may drive this (Rivera-Chavez FA. Am Surg. 2004;240:269-77). The authors need to make this clear. Because of confusion as to the exact definition of "complicated" appendicitis (and its converse), this reviewer suggests the term "perforated" as more accurate and descriptive.

Answer: We thank the reviewer for pointing out this very important factor that we have acknowledged, but which has not been stated in the manuscript. We have now revised both the first “strength of the study” and the Introduction according to the reviewer suggestion on this topic.

Study strengths: “To our knowledge, MAPPAC (Microbiology APPendicitis ACuta) is the first prospective trial comparing the role of microbiology and immunology including immune response in the etiology of uncomplicated and complicated acute appendicitis in a large patient cohort consisting of CT-diagnosed patients also specifically evaluating appendicoliths and recurrent appendicitis after initial successful conservative treatment.”

Introduction: “Since most of the species identified from the appendix with culturing and NGS methods can also be part of normal gut microbiota it is challenging to determine their role in the infection [14]. In addition, the immune response and predisposition for infection by specific bacteria varies between individuals. Consequently, innate immunity may be a contributing factor in the development complicated of appendicitis [15].” We also added the suggested reference by Rivera-Chavez et al. (ref# 15) and Bennion et al (ref# 14).

We understand the terminology suggestion made by the reviewer, but the term and concept of complicated acute appendicitis includes other forms of complicated acute appendicitis in addition to perforated acute appendicitis as stated in the inclusion criteria. Furthermore, we have used this same terminology and similar definitions throughout our study group trials and to avoid unnecessary confusion, we shall continue using the terms uncomplicated and complicated.

3. Importantly, and which needs to be stated clearly, is that this is an exploratory study to, at best, generate hypotheses for future studies. No hypotheses are offered here, although some broadly exist (see above) and should be proffered to help the average reader. Also, no pilot study experience is presented so in many ways this is just a pilot study. Perhaps the authors should state that this is an exploratory study of possible associations of microbiota and host immune characteristics with uncomplicated vs. complicated appendicitis and antibiotic response among patients in clinical trials treated with and without antibiotics with the goal to generate hypotheses to better understand the role of disease progression and host susceptibility.

Answer: We agree on the pilot study nature as there are no previous studies assessing the microbiological and immunological differences between uncomplicated and complicated acute appendicitis patients. We have revised the Discussion section accordingly as follows:

Discussion: “In many aspects, MAPPAC is an exploratory study of possible associations of microbiota and host immune characteristics with uncomplicated vs. complicated appendicitis and antibiotic response among patients in clinical trials treated with and without antibiotics. MAPPAC trial aims to generate hypotheses to better understand the role of disease progression and host susceptibility for future studies; i.e. determination of one primary outcome…”

“One of the main hypotheses of the MAPPAC study is that the microbial composition of both appendix and gut differs between CT differentiated complicated and uncomplicated appendicitis. Therefore, a strong element of the study is that all patients included in the study are imaged with CT protocol.”

“The lack of healthy control group is a limitation in the study regarding both the etiology and in determining the effects of antibiotics on GM,”

4. The abstract should mention studies of immune response.

Answer: This has been added to the abstract according to the suggestion as follows: “MAPPAC trial aims to evaluate microbiological and immunological aspects including immune response in the etiology of these different forms also assessing both antibiotics non-responders and appendicitis recurrence.”

5. Under "Strengths" is the assertion that NSG and cultures will provide reliable information about microbiota in the etiology of uncomplicated and complicated appendicitis. How would one know this with much confidence before doing the study? It is fairly likely that the results will not be reliable, e.g., contamination of specimens with non-etiologic bowel flora. Bacteria, if indeed they are involved in infection, may reside in the tissue alone, which may be impossible to isolate from colonizing flora especially with overly sensitive molecular assays.

Answer: We understand the reviewer’s point of view and we have revised these sentences by both adding the information about bowel microbiota challenges and omitting the word “reliable” and using ”extensive” instead as the point was that by having both NGS and traditional culture method data, we will have more extensive knowledge that by just using either one of the methods. Further, inclusion of the microbiological analysis of both biopsy and lumen enables the differentiation of possibly invaded bacterial species. In addition, our aim is not only to isolate certain appendiceal pathogenic bacterial species, but to assess the impact of whole normal gut microbiota community on the different disease forms. Thus, gut microbiota profiling is included in the analysis and the purpose is to characterize the differences in the whole appendiceal and gut microbiota entities in these two forms of appendicitis. Further, the use of metagenomics approach will provide the opportunity to detect microbiota components that are not detected with culturing methods, i.e. for example fungi, yeasts, and viruses. We have now used the word microbiota profile throughout the text and added this information also to the discussion.

Strengths and limitations: ” -The application of next generation sequencing combined with traditional culturing methods will provide extensive reliable information about the microbiological factors in the etiology of complicated and uncomplicated acute appendicitis also presenting a challenge in differentiating between etiologic and non-etiologic bowel microbiota in the specimens. “

Discussion: “Further, the whole microbial entity, i.e. profile both in the appendix and in gut is studied, and their role in the disease severity is assessed.”

6. The first part of the Introduction focuses on the foundation for antibiotic treatment (and multiple references), when the primary focus should be our understanding of what causes appendicitis and appendicitis perforation and existing evidence to support these theories. This reviewer thinks that antibiotic treatment can be mentioned with later study objectives as part describing the clinical trials to which this is an ancillary study (just before the Methods). The theory themes (obstruction, infection, host factors) based on what is known need to be more clearly laid out. It is unclear what recitation of bowel flora or referring to the novelty of applying of NGS methods add to the introduction, but stating "studies of these suggest" would help frame the questions for which your exploratory study may provide future testable hypotheses. Again, the long description of the clinical trials seems excessive for this study's focus.

Answer: This is a very relevant point and as described already in the first comment, we have now omitted a large part of the clinical background from the Introduction section and revised the order of the Introduction according to the reviewer suggestion. However, as the MAPPAC trial aims to also compare the effect of antibiotics and placebo on gut microbiota composition and antimicrobial resistance in addition to assessing the antibiotic non-responders and recurrences, we feel that the reader also needs the basic background information to better understand the trial rationale.

6. The Methods have about 5 pages describing each of the contributing clinical trials. The focus should be on this study's methods, which are extensive enough, and the characteristics of the clinical trials should be summarized in one paragraph and either referenced or included with a supplemental appendix. Also, mention of things like patient withdrawal seem irrelevant. The specific assays should be described in sufficient detail so they can be replicated. For example, it is unclear what will be done with serum samples collected for immunological and inflammatory marker analysis and metabolomics approach. Also further description is need for, "biomarker analysis of numerous different cytokines, chemokine and growth factors as well as serum metabolome will be analyzed." Please explain how tissue/appendicolith specimens will be separated from contaminating colonic flora. To this reviewer, the supplemental methods should be the primary methods and the clinical trial methods should be supplemental. The journal may have a statistical reviewer, but this reviewer is unsure if all possible projected types of data summary and association testing methods need to described in this preliminary expedition.

Answer: We understand the reviewer’s criticism on the emphasis of the trial methods and we have now revised the Methods accordingly. The information of the essentially contributing clinical trials is now concise and summarized in one paragraph as suggested by the reviewer. In addition, we have specified the serum sample analysis in the supplementary file by adding methodology and a list if cytokine panel analytes. We feel that the essential part of the protocol is to description of the sample collection and due to the word limit, we have left the analysis methods in to the supplementary files. Patient withdrawal is part of the standard ethical conduct and we have thus left the information in the manuscript, but this can naturally be omitted based on editorial discretion.

7. The Discussion is devoted to the clinical trials, not the ancillary study's aims, further background for the reader, and projected possible hypotheses that might be generated.

Answer: We have revised the discussion section accordingly by omitting some of the information regarding the clinical trials APPAC II and III. In addition, we have provided the main hypotheses together with strengths and limitations regarding the methods and study design, please see below.

Discussion: “In many aspects, MAPPAC is an exploratory study of possible associations of microbiota and host immune characteristics with uncomplicated vs. complicated appendicitis and antibiotic response among patients in clinical trials treated with and without antibiotics. MAPPAC trial aims to generate hypotheses to better understand the role of disease progression and host susceptibility for future studies; i.e. determination of one primary outcome…”

“One of the main hypothesis of the MAPPAC study is that the microbial composition of appendix differs between CT differentiated complicated and uncomplicated appendicitis. Therefore, strong element of the study is that all patients included in the study are imaged with CT protocol.”

8. This reviewer thinks the flow diagram provides unnecessary details of the clinical trials and distracts from the specific study being described. Perhaps these could be simplified and more

schematically show where each specimen and testing of microbiota and immune response will be done over time.

Answer: We have now markedly simplified the flowcharts according to the reviewer suggestion. As this manuscript describes the study protocol, it is important for the reader to understand the patient recruitment for the MAPPAC trial as the three trials are performed in conjunction with each other providing synergy for the topic at hand. With the simplified flowcharts, we hope that the reader now has easy access to the essential information without unnecessary clinical trial details. We feel that it would be beneficial to present the flowcharts in the actual manuscript, but these can also be presented as supplementary online material, if the editors prefer that option.

Reviewer: 2

Reviewer Name: Mauro Podda

Institution and Country: Department of Surgical Science, General and Emergency Surgery Unit, Cagliari University Hospital, Italy

Vanhatalo et al presented an interesting study protocol for a prospective observational study on the appendicitis etiology and pathophysiology, focusing on the differences between uncomplicated and complicated appendicitis from a biological point of view. The protocol is well-structured, although very complex. However, the Authors focalized their attention on one of the most debated issues when talking about NOM for acute appendicitis: who to distinguish during the patient's assessment, those who might respond well to antibiotics or observation alone from those who will require surgery.

I have just a few questions for the authors:

1. How do the Authors think the results of their research could be translated into the daily clinical practice?

Answer: We thank the reviewer for the question as some of the MAPPAC study aims are not directly applicable to clinical practice, but the main hypotheses and results may markedly contribute to further understanding of acute appendicitis etiology and associated immunological factors and thus have a clear clinical relevance, please see below.

The relevance of our previous APPAC trial has been substantial in initiating worldwide discussion and evaluation of the optimal treatment for uncomplicated acute appendicitis as the time has come to abandon routine appendectomy for all in the treatment of CT-confirmed uncomplicated acute appendicitis. The changes in the treatment paradigm for CT-proven uncomplicated acute appendicitis will require these APPAC II and III, and MAPPAC trials and also many other further prospective studies.

The differences in the microbiological etiology or the immunology of the different forms of appendicitis would be of significant value in evaluating the different treatment options for acute appendicitis patients and could be used to further guide the antibiotic treatment. The comparison of the antibiotic therapy effect on the gut microbiota and AMR in APPAC III patients enrolled in this MAPPAC trial would be of profound interest as the APPAC III setting is ideal in this respect (antibiotic therapy vs. placebo). Further the results could help the clinicians to distinguish patients that are possible non-responders or who will later develop recurrent appendicitis.

2. Why the authors did decide to include appendicitis with appendicolith among the complicated forms? Recent meta-analyses and prospective trials reported that the failure rate of NOM in patients

affected by uncomplicated appendicitis with appendicolith is high (40-60%), confirming that patients with evidence of appendicolith on imaging have an initial failure rate of NOM more than twice that of patients without an appendicolith [Mahida JB, et al. High failure rate of nonoperative management of acute appendicitis with an appendicolith in children. J Pediatr Surg. 2016;51:908–911; Tanaka Y, et al. Long-term outcomes of operative versus nonoperative treatment for uncomplicated appendicitis. J Pediatr Surg. 2015;50:1893–1897; Podda M, et al. Antibiotic Treatment and Appendectomy for Uncomplicated Acute Appendicitis in Adults and Children: A Systematic Review and Meta-analysis. Ann Surg 2019, doi: 10.1097/SLA.0000000000003225]. However, if we consider the most recognized definition of complicated appendicitis (Presence of gangrene/abscess/diffuse peritonitis), the presence of appendicolith alone cannot be considered as complicated form.

Answer: We agree with the reviewer that the definitions of uncomplicated and complicated acute appendicitis are not yet clear, and this also involves a timeline, i.e. the mere presence of an appendicolith may not yet have progressed into a more traditional definition of complicated acute appendicitis. In our original APPAC trial, the presence of an appendicolith was an exclusion criterion and at that time most of the data supporting this approach was from pediatric studies. However, the presence of an appendicolith has since then been shown to be associated with a more complicated course of the disease as stated above by the reviewer. A very good example of this is the French RCT by Vons et al. as if they had excluded the patients with an appendicolith, there would have been no difference between the study groups (antibiotics vs. appendectomy)1. We have also added the most recent meta-analysis in the references.

1.Vons C, Barry C, Maitre Set al. Amoxicillin plus clavulanic acid versus appendicectomy for treatment of acute uncomplicated appendicitis: an open-label, non-inferiority, randomised controlled trial. Lancet. 2011;377(9777):1573-1579.

VERSION 2 – REVIEW

REVIEWER	David Talan Olive View-UCLA Medical Center, USA
REVIEW RETURNED	25-Jun-2019

GENERAL COMMENTS	The paper is much improved compared to the original submission. The sentence stating complicated appendicitis "requires" emergency appendectomy in the abstract and text body should be deleted since it is both untrue as a general statement and unnecessary to mention in an exploratory study of how uncomplicated and complicated appendicitis (prefer ruptured and ruptures) differs in terms of microbiology and immune response.

REVIEWER	Mauro Podda Department of Surgical Science, University of Cagliari (Italy).
REVIEW RETURNED	03-Jul-2019

GENERAL COMMENTS	I am very happy with the clarifications provided by the Authors in this R1 version of the manuscript. I would suggest the acceptance of the manuscript in the current version, and I wish the full success of the study.

VERSION 2 – AUTHOR RESPONSE

Reviewer: 1

Reviewer Name: David A Talan

Institution and Country: Olive View-UCLA Medical Center; The David Geffen School of Medicine at UCLA

1. The paper is much improved compared to the original submission. The sentence stating complicated appendicitis "requires" emergency appendectomy in the abstract and text body should be deleted since it is both untrue as a general statement and unnecessary to mention in an exploratory study of how uncomplicated and complicated appendicitis (prefer ruptured and ruptures) differs in terms of microbiology and immune response.

Answer: We agree with the reviewer that regarding the topic of this exploratory trial, this information is not necessary, and we have now removed the suggested sentence from the abstract and introduction according to the reviewers comment.

“Complicated acute appendicitis in this trial is defined as a finding of perforation, appendicolith, abscess or a suspicion of tumor.”

Reviewer: 2

Reviewer Name: Mauro Podda

Institution and Country: Department of Surgical Science, General and Emergency Surgery Unit, Cagliari University Hospital, Italy

1. I am very happy with the clarifications provided by the Authors in this R1 version of the manuscript. I would suggest the acceptance of the manuscript in the current version, and I wish the full success of the study