PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Evaluating the Alimentary and Respiratory Tracts in Health and disease (EARTH) research program: a protocol for prospective, longitudinal, controlled, observational studies in children with chronic disease at an Australian tertiary paediatric hospital.
AUTHORS	Coffey, Michael; McKay, Isabelle; Doumit, Michael; Chuang, Sandra; Adams, Susan; Stelzer-Braid, Sacha; Waters, Shafagh A; Kasparian, N; Thomas, Torsten; Jaffe, Adam; Katz, Tamarah; Ooi, Chee Y.

VERSION 1 – REVIEW

REVIEWER	Steve Turner University of Aberdeen, UK
REVIEW RETURNED	11-Oct-2019

GENERAL COMMENTS	This is a detailed protocol which is easily read. I have a few thoughts/suggestions that the authors might consider 1. It is not clear whether all samples and all questionnaires will be collected from all participants. For example will sputum be obtained from children with Hirschsprung's disease (I presume so)? Will the GI symptom module be completed by participants with CF (I am not so sure)? I think that table 2 identifies which questionnaires are for which patient but the table is spread over three pages and is very hard to interpret. This could all be made more clear to the reader. 2. Antibiotic treatment can affect the respiratory and GI microbiome. Please can the protocol clarify whether samples will be collected after a "wash out" period post antibiotics? This might be tricky for CF participants but clarification will be helpful (sorry if the clarification is there and I have missed it). 3. Please can the protocol state the start and end dates for enrolment? 4. I had not heard of the gut-brain axis before reading the protocol. It would be helpful to readers such a myself to have a brief explanation of what the gut and brain outcomes will be (I presume microbiome and behaviour respectively). 5. Sputum is hard to collect and also unpleasant. We found no benefit of sputum over a throat/mouth swab for microbiome studies (An, S., Warris, A., & Turner, S. (2018). Microbiome characteristics of induced sputum compared to bronchial fluid and upper airway samples. Pediatric Pulmonology, 53(7), 921-928. The authors might consider whether it is worth omitting the sputum induction. 6. There is no mention of deprivation or poverty as a confounder. Page 17 line 33 mentions "sociodemographic factors such as ethnicity". Please can the authors specify an index of deprivation/poverty? 7. I was surprised to see obstructive sleep apnoea and Hirschsprung's disease included given that their underlying

	mechanisms seem to be independent of microbiome. Any dysbiosis in these conditions (to me) seems likely to be secondary to interventions or the underlying condition themselves. I absolutely appreciate that this is a platform for testing hypotheses but I would have anticipated conditions to be included where “infection” seems to be important to causation and exacerbation such as asthma and inflammatory bowel disease, and possibly immune deficiencies. 8. Is this just a cross sectional study or is there a plan to follow up the participants? e.g collect more samples, repeat the questionnaires? Having permission/consent to link the data collected to previous or subsequent clinical events would add a potentially useful longitudinal component to the data collected. 9. I appreciate that this is a platform for future research but some consideration to the analysis at this stage would be useful. The researchers will have tens or hundreds or thousands of data points for each individual and false positive findings will be abundant. Consideration of how to reduce multiple testing and an a priori significant p value (or “q value” see p 27 line 5) would be useful. Directed acyclic graphs may be useful for the researchers in their analysis plan. 10. Will the dietary data from the food frequency questionnaire data be adjusted for energy intake (this is standard practice but needs to be explicitly stated)

REVIEWER	Karina Colonetti Hospital de Clínicas de Porto Alegre, Brazil
REVIEW RETURNED	28-Nov-2019

GENERAL COMMENTS	After carefully reviewing the article, these are the key points that I recommend to be better developed in the text provided by the authors: 1- Metabolome technique was not mentioned in the abstract, as well as inflammatory markers measurement. The authors should inform that the sequencing partial (V3-V4 region) in the table 1 content. 2- The authors state that bacterial communities will be evaluated through 16S rRNA or MSS. I understand that authors are given the option of partial 16S rRNA sequencing or shotgun sequencing, but for sake of the reader, they should explain why they are stating both options. They intend to use which one, and why? They should keep in mind the fact that these methods are not entirely interchangeable. This should be stated clearly. 3- The authors are going to consider ACAES to the adults, and it is adequate. However, for children, the authors should consider a 3-day food record or 3 independent 24-hour food recall for children that already are consuming solid food. A single 24-hour food recall might be a biased tool to estimate nutrient intake. 4- The authors must record and evaluate the interference of antibiotics and medicine intake in the period of study. At last 3 months of antibiotic usage should asked and the usage of proton pumps, anti-inflammatory and other drugs also should be accessed. Vaccination should be considered as interleukins will be measured. 5- Healthy controls should be better described. Acute/infectious diseases can be easily found in infants, and the inclusion/exclusion criteria must be clear about this issue. Colds and flu, as well as vaccination should be considered in this matter.

	6- The authors must define a maximum difference based on age. This will be of extreme importance in younger patients. In that case, 6 months can represent a huge difference for gut microbiome. The authors must mention and refer references about this issue. I strongly recommend: Muriel Derrien, Anne-Sophie Alvarez, Willem M. de Vos. The Gut Microbiota in the First Decade of Life. Trends in Microbiology, Volume 27, Issue 12, 2019, Pages 997-1010, ISSN 0966-842X, https://doi.org/10.1016/j.tim.2019.08.001. Christian Milani, Sabrina Duranti, Francesca Bottacini et al. The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. Microbiology and Molecular Biology Reviews Nov 2017, 81 (4) e00036-17; DOI: 10.1128/MMBR.00036-17 7- The group range from 0 to 5 yr is inappropriate. Intragroup comparison of children until 2-3 yr is very problematic. This issue should be considered. How the authors intend to describe/compare these participants should be explained, if they intend to do that. 8- The authors should consider to ask for participants to keep the sample frozen (immediately after collection until transport it to the facility), to avoid bacterial reproduction and metabolite degradation.

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1

This is a detailed protocol which is easily read. I have a few thoughts/suggestions that the authors might consider

1. It is not clear whether all samples and all questionnaires will be collected from all participants. For example will sputum be obtained from children with Hirschsprung's disease (I presume so)? Will the GI symptom module be completed by participants with CF (I am not so sure)? I think that table 2 identifies which questionnaires are for which patient but the table is spread over three pages and is very hard to interpret. This could all be made more clear to the reader.

All samples, questionnaires and data will be collected from all participants involved in the study. We had added a description to the Section: Outcome Measures (page 15), and legends for Table 1 and Table 2 to clarify this point: "All samples, questionnaires and data will be collected from all participants at each time-point." We agree Table 2 being spread over three pages is hard to interpret, but we feel it summarises a vast array of information in a reasonable format.

2. Antibiotic treatment can affect the respiratory and GI microbiome. Please can the protocol clarify whether samples will be collected after a "wash out" period post antibiotics? This might be tricky for CF participants but clarification will be helpful (sorry if the clarification is there and I have missed it).

We thank the Reviewer for highlighting this important issue. We have clarified that where practical, samples will be collected at least four weeks after completion of oral/intravenous antibiotic therapy (excluding prophylactic antibiotics) (page 20).

3. Please can the protocol state the start and end dates for enrolment?

The study start date was April 18, 2018. The estimated completion date for the current list of included conditions is March 13, 2023. We have included these dates on page 2.

4. I had not heard of the gut-brain axis before reading the protocol. It would be helpful to readers such a myself to have a brief explanation of what the gut and brain outcomes will be (I presume microbiome and behaviour respectively).

A detailed definition of the gut-brain and gut-lung axes has been included in Introduction (page 8). Additionally, we have included descriptions of the Outcome Measures used to explore these axes (pages 17-18).

5. Sputum is hard to collect and also unpleasant. We found no benefit of sputum over a throat/mouth swab for microbiome studies (An, S., Warris, A., & Turner, S. (2018). Microbiome characteristics of induced sputum compared to bronchial fluid and upper airway samples. Pediatric Pulmonology, 53(7), 921-928. The authors might consider whether it is worth omitting the sputum induction.

We thank the Reviewer for highlighting this important point. We included sputum as many older children with cystic fibrosis at our centre already have induced sputum collected for clinical purposes. In these few instances, we elected not to perform a second oropharyngeal swab given the characteristics are comparable (as you correctly highlighted). The aim of this was to reduce study/investigation burden in these children.

6. There is no mention of deprivation or poverty as a confounder. Page 17 line 33 mentions "sociodemographic factors such as ethnicity". Please can the authors specify an index of deprivation/poverty?

Locally we use the Socio-Economic Indexes for Areas (SEIFA) 2016 from the Australian Bureau of Statistics as a measure of relative socio-economic advantage and disadvantage. We have included this factor in the secondary outcomes/variables (page 18) and Table 1.

7. I was surprised to see obstructive sleep apnoea and Hirschsprung's disease included given that their underlying mechanisms seem to be independent of microbiome. Any dysbiosis in these conditions (to me) seems likely to be secondary to interventions or the underlying condition themselves. I absolutely appreciate that this is a platform for testing hypotheses but I would have anticipated conditions to be included where "infection" seems to be important to causation and exacerbation such as asthma and inflammatory bowel disease, and possibly immune deficiencies.

As discussed in the introduction (pages 7-8), in children with obstructive sleep apnoea (OSA) there have been reports of cardiovascular (metabolic syndrome is also linked with intestinal dysbiosis), neurocognitive and behavioural consequences.[1] Additionally in adults with OSA there have been reports of gut epithelial damage,[2] and nasal dysbiosis and inflammation.[3] Additionally murine studies suggest intermittent hypoxia, hypercapnia and sleep fragmentation promote intestinal dysbiosis, increased visceral fat mass, systemic inflammation and atherosclerosis.[4-7] Regarding Hirschsprung's disease, children with and without Hirschsprung-associated enterocolitis often have an altered intestinal microbiome[8] and altered composition of short chain fatty acids.[9] We acknowledge that the disease mechanisms themselves are independent of the intestinal and/or respiratory microbiome but these conditions and their management appear to affect these environments. We agree that other chronic gastrointestinal and/or respiratory conditions including inflammatory bowel disease and asthma would be important to investigate, as stated in the introduction (page 6) these conditions were chosen as examples due to the clinical and/or research expertise of the authors. Translation to additional conditions is exactly within the scope of this research program.

8. Is this just a cross sectional study or is there a plan to follow up the participants? e.g collect more samples, repeat the questionnaires? Having permission/consent to link the data collected to previous or subsequent clinical events would add a potentially useful longitudinal component to the data collected.

This study is longitudinal in design, as described in the protocol (page 14) and stated in the title.

9. I appreciate that this is a platform for future research but some consideration to the analysis at this stage would be useful. The researchers will have tens or hundreds or thousands of data points for each individual and false positive findings will be abundant. Consideration of how to reduce multiple testing and an a priori significant p value (or "q value" see p 27 line 5) would be useful. Directed acyclic graphs may be useful for the researchers in their analysis plan.

We acknowledge that false positive results are definitely an issue with this type of exploratory platform. As stated in the statistical methods section (pages 27 -28), a two-tailed $p<0.05$ is considered statistically significant. In all instances where multiple testing is relevant, a Benjamini & Hochberg (i.e. a false discovery rate) correction will be applied. A q-value is used to describe the adjusted p-value.

10. Will the dietary data from the food frequency questionnaire data be adjusted for energy intake (this is standard practice but needs to be explicitly stated)
Yes, the Australian Child and Adolescent Eating Survey (ACAES) provides an adjusted output by reporting the percentage of energy from each macro- and micro-nutrient. We have updated the description of the ACAES output to clarify this point (page 25).

Reviewer: 2
After carefully reviewing the article, these are the key points that I recommend to be better developed in the text provided by the authors:
1- Metabolome technique was not mentioned in the abstract, as well as inflammatory markers measurement. The authors should inform that the sequencing partial (V3-V4 region) in the table 1 content.
Due to the word count limit of the abstract, we were unable to specify all of the analytical techniques included in the protocol and generalised this to "Data on the intestinal and respiratory microbiomes". We have included the description of the V4 region in Table 1 and the outcome measures (page 15) as primers 515F and 806R primers amplify variable region 4.

2- The authors state that bacterial communities will be evaluated through 16S rRNA or MSS. I understand that authors are given the option of partial 16S rRNA sequencing or shotgun sequencing, but for sake of the reader, they should explain why they are stating both options. They intend to use which one, and why? They should keep in mind the fact that these methods are not entirely interchangeable. This should be stated clearly.

We thank the Reviewer for highlighting this important point. We have clarified in the section on sample and data processing techniques (page 24) that MSS will be performed as an alternative to 16S rRNA sequencing in instances where species resolution of bacterial communities is thought to be of benefit.

3- The authors are going to consider ACAES to the adults, and it is adequate. However, for children, the authors should consider a 3-day food record or 3 independent 24-hour food recall for children that already are consuming solid food. A single 24-hour food recall might be a biased tool to estimate

nutrient intake.
The ACAES is administered to children aged 2 to 18 years, has been validated against a 3 day food record and is validated for use in Australian Children.[10-13] For children under 2 years of age, a single 24-hour recall will be administered. We made a pragmatic decision for a single 24-hour food recall (compared to a 3-day food record or 3 independent 24-hour food recall) to reduce the burden for parents, especially given we are already collecting a lot of data and samples from our participants. We acknowledge that a single 24-hour food recall might introduce bias in this small subset of patients and this will be acknowledged in the limitations of any publications.

4- The authors must record and evaluate the interference of antibiotics and medicine intake in the period of study. At last 3 months of antibiotic usage should asked and the usage of proton pumps, anti-inflammatory and other drugs also should be accessed. Vaccination should be considered as interleukins will be measured.
As part of our Qualtrics survey we also collect a thorough medication history over the preceding 2 months, and we have amended Table 1 and the secondary outcomes/variables (page 18) to clarify this important point. The authors decided not to collect vaccination records as part of this study and given approximately 90-95% of Sydney children are vaccinated, we feel this factor will unlikely contribute significantly to our results.

5- Healthy controls should be better described. Acute/infectious diseases can be easily found in infants, and the inclusion/exclusion criteria must be clear about this issue. Colds and flu, as well as vaccination should be considered in this matter.
Healthy controls are defined as children who are free of any chronic disease. We have updated the study design section (page 14) to clarify this detail. Acute/infectious diseases are not an exclusion criterion for participation in this study, however data and samples will be collected at least four weeks after completion of oral/intravenous antibiotic therapy (excluding prophylactic antibiotics) or an acute infectious illness (page 20). Given the wide age range of eligible participants (0 to 18 years), vaccinations are not controlled for in the protocol design.

6- The authors must define a maximum difference based on age. This will be of extreme importance in younger patients. In that case, 6 months can represent a huge difference for gut microbiome. The authors must mention and refer references about this issue. I strongly recommend:

Muriel Derrien, Anne-Sophie Alvarez, Willem M. de Vos. The Gut Microbiota in the First Decade of Life. Trends in Microbiology, Volume 27, Issue 12, 2019, Pages 997-1010, ISSN 0966-842X, https://doi.org/10.1016/j.tim.2019.08.001.

Christian Milani, Sabrina Duranti, Francesca Bottacini et al. The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. Microbiology and Molecular Biology Reviews Nov 2017, 81 (4) e00036-17; DOI: 10.1128/MMBR.00036-17

We thank the Reviewer for highlighting this very important point. As stated in the participants section (page 15), we will match participants with a condition to healthy controls for gender and age (as closely as possible). We acknowledge that there are rapid changes in the intestinal microbiota during the first 3 years of life, after which it becomes relatively stable.[14] Although we aim to match participants as closely as possible, our criteria for acceptable matching is as follows:

- For children less than one year, age to be matched within 3 months,
- For children aged one to three years, age to be matched within 6 months,
- For children aged four years and older, age to be matched within 2 years.

We have updated the section on confounding factors to address this issue and have included the suggested references (page 26).

7- The group range from 0 to 5 yr is inappropriate. Intragroup comparison of children until 2-3 yr is very problematic. This issue should be considered. How the authors intend to describe/compare these participants should be explained, if they intend to do that.
This comment is addressed in the previous comment (Reviewer #2, Comment 6).

8- The authors should consider to ask for participants to keep the sample frozen (immediately after collection until transport it to the facility), to avoid bacterial reproduction and metabolite degradation.

We thank the Reviewer for their suggestion however, we prefer samples not to be frozen in domestic freezers prior to transport and rather be transported in a cooler bag (with a -18°C ice pack) to the hospital laboratory within 24 hours of collection. This was decided as it avoided a freeze-thaw cycle which was inevitable when aliquoting samples (particularly stool) prior to storage at -80 °C.

VERSION 2 – REVIEW

REVIEWER	Karina Colonetti Hospital de Clínicas de Porto Alegre, Brazil
REVIEW RETURNED	26-Dec-2019

GENERAL COMMENTS	I would like to comment one point: I understand that there is a vaccination schedule and the authors consider that it will not significantly interfere with the results of the study (and probably they are right). However, vaccines may influence short-term cytokine outcome (1). This is a point that may interfere with results of collections made during times of influenza vaccination, for example (2). Having at least one idea if the participant received any vaccine can add confidence to the results, as reproducibility is one of the challenges in microbiome studies (3). Also, because it is a convenience sampling, there is a tendency for participants to already be at the center for some reason, such as receiving vaccines. Asking about the timing of the last vaccine does not substantially change the protocol, or represents an additional big burden to participants. Cytokines change rapidly, and if any correlation is made with the microbiome, this question may justify excluding subjects from the analysis, or to compute vaccination as confounding variable, yielding more reliable results.

VERSION 2 – AUTHOR RESPONSE

Reviewer: 2

I would like to comment one point: I understand that there is a vaccination schedule and the authors consider that it will not significantly interfere with the results of the study (and probably they are right). However, vaccines may influence short-term cytokine outcome (1). This is a point that may interfere with results of collections made during times of influenza vaccination, for example (2). Having at least one idea if the participant received any vaccine can add confidence to the results, as reproducibility is

one of the challenges in microbiome studies (3). Also, because it is a convenience sampling, there is a tendency for participants to already be at the center for some reason, such as receiving vaccines. Asking about the timing of the last vaccine does not substantially change the protocol, or represents an additional big burden to participants. Cytokines change rapidly, and if any correlation is made with the microbiome, this question may justify excluding subjects from the analysis, or to compute vaccination as confounding variable, yielding more reliable results.

We appreciate the excellent argument made by Reviewer 2 and have amended the protocol to incorporate the collection of vaccination status and timing for all participants. This change is reflected in Table 1 and the Secondary outcomes/variables (page 18). This record will allow for the evaluation of vaccines as a confounding variable for cytokine and microbiome analyses. We have updated the discussion on confounders to reflect this (page 26).

Additionally, Associate Professor Nadine Kasparian has recently changed affiliations and this has been amended.