ROTA-BIOME Study

The role of the intestinal microbiome in enteric and systemic vaccine immune responses

PROTOCOL TITLE '**The role of the intestinal microbiome in enteric and systemic vaccine immune responses**

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	**ABR form, General Assessment and Registration form, is the application form that is required for submission to the accredited Ethics Committee (In Dutch, ABR = Algemene Beoordeling en Registratie)**
AE	**Adverse Event**
AIGHD	**Amsterdam Institute for Global Health and Development**
AMC	**Academic Medical Center**
AR	**Adverse Reaction**
CEMM	**Center for Experimental and Molecular Medicine**
CCMO	**Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek**
CV	**Curriculum Vitae**
EU	**European Union**
GCP	**Good Clinical Practice**
IB	**Investigator's Brochure**
IC	**Informed Consent**
Ig	**Immunoglobulin**
IMP	**Investigational Medicinal Product**
IMPD	**Investigational Medicinal Product Dossier**
METC	**Medical research ethics committee (MREC); in Dutch: medisch ethische toetsing commissie (METC)**
PCR	**Polymerase Chain Reaction**
PBMC	**Peripheral Blood Mononuclear Cell**
RV	**Rotavirus**
RVV	**Rotavirus Vaccine**
(S)AE	**(Serious) Adverse Event**
SPC	**Summary of Product Characteristics (in Dutch: officiële productinfomatie IB1-tekst)**
Sponsor	**The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.**
SUSAR	**Suspected Unexpected Serious Adverse Reaction**
TLR	**Toll-like receptor**

Wbp	**Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgevens)**
WMO	**Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**

SUMMARY

Rationale: Rotavirus (RV) is the leading cause of diarrhea-related death in children under five years of age, particularly in Africa and Asia. Rotavirus vaccines (RVV) have the potential to dramatically reduce rotavirus morbidity and mortality, however rotavirus vaccine efficacy is lowest in the poorest countries with the highest child mortality rate.

We hypothesize that differences in gut microbiome colonization, composition and diversity might be contributing to the diminished RVV efficacy observed in developing countries. We propose evaluating the effects of intestinal microbiota differences obtained through antibiotics on RVV immune responses. To obtain a better mechanistic understanding of the relationship between the intestinal microbiota and vaccine immune responses, this study also proposes evaluating the effect of antibiotic microbiota manipulation on two well-studied and classic systemic vaccines: the tetanus vaccine and the pneumococcal vaccine (Pneumo23).

Primary Objective: to investigate the role of the gut microbiota in RVV immune response

Secondary Objectives: To investigate the role of the gut microbiota in tetanus and pneumococcal vaccine immune responses

Study design: Randomized, controlled intervention study in adult human volunteers

Study population: 3 groups of 21 healthy male volunteer subjects, 18-35 years of age

Intervention: Arm 1 (control): no antibiotic depletion then Rotarix™, Tetanus and Pneumococcus vaccination; **Arm 2**: broad-spectrum antibiotic depletion (ciprofloxacin, vancomyin, metronidazole) then Rotarix™, Tetanus, and Pneumococcus vaccination; **Arm 3:** Gram-positive depletion (oral vancomycin) then Rotarix™, Tetanus, and Pneumococcus vaccination

Main study parameters/endpoints: The main study endpoint is the 28-day post vaccination anti-RV IgA serum response. Secondary study parameters are the height, slope and time to positivity of the post-vaccination anti-RV IgA, anti-pneumococcal antibodies, anti-tetanus toxoid antibodies, and fecal rotavirus antigen shedding days 1-7 post vaccination and extent of peripheral blood mononuclear cell (PBMC) stimulation pre- and post- vaccination using ELISA spot with rotavirus, tetanus and pneumococcal vaccines.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: The burden of this study includes vaccination (one oral two intramuscular), oral antibiotics, and 6 visits (including screening) to the hospital, spread out over 5 ½ weeks. Intramuscular vaccinations can give local irritation, pain, muscle soreness and aching. Vaccination can give transient malaise and infrequently, fever. Oral antibiotic use could lead to gastrointestinal symptoms and (rarely) allergic reactions. The results of this study might lead to interventions that could improve the immunogenicity of rotavirus vaccine in developing countries, potentially preventing hundreds of thousands of deaths due to

rotavirus disease over the next 15 years. It could also elucidate important immune mechanisms involved in oral and systemic vaccination.

1. INTRODUCTION AND RATIONALE

Rotavirus gastroenteritis

Rotavirus is the leading cause of diarrhea-related death in children worldwide, causing an estimated 450,000 deaths in children under five each year, and hospitalizing millions more.[1] While all children are vulnerable to rotavirus infection, 95% of rotavirus deaths occur in low-income countries in Africa and Asia. Rotavirus vaccines have the potential to dramatically reduce the morbidity and mortality caused by rotavirus infection. However, rotavirus vaccines demonstrate lower efficacy in African and Asian countries with high under-five mortality rates.[2,3] Studies conducted in Africa with the Rotarix™ (South Africa and Malawi) and Rotateq™ (Ghana, Kenya, Mali) vaccines demonstrated a combined vaccine efficacy of 61% and 39% respectively.[4,5] This compares to an efficacy of 85-98% against severe rotavirus gastroenteritis in Rotarix™ and Rotateq™ trials conducted in Latin America, Europe, and the United States.[6,7,8] Understanding the pathophysiology of these marked differences in rotavirus vaccine efficacy are critical, as even small improvements in vaccine effectiveness could increase the number of children's lives saved by the vaccine by hundreds of thousands over the next 15 years.[9]

Several hypotheses have been posited to explain the decreased rotavirus vaccine efficacy seen in low-income countries including neutralizing maternal antibodies in breast milk, malnutrition and micronutrient deficiencies in infants, as well as gut enteropathy and early enteric co-infections.[10,11] Differences in the infant gut microbiota at the time of vaccination may be an important, under-explored explanation for the differences in vaccine efficacy between poor and wealthy nations.

The gut microbiome

The Nobel Laureate Joshua Lederberg first described the human microbiome, calling it the collective genome of our indigenous microbes.[12] The adult gut hosts ~10^{14} bacteria from 500-1000 different species, and can be understood as an exteriorised organ that exerts numerous functions including protection against epithelial cell injury, optimization of host immune responses and resistance against colonisation by pathogens. The development, maturation and function of the infant's gut microbiome are incompletely understood but are likely critical in training immune responses.

Microbiome and viral infection

A growing body of evidence points to the importance of the gut microbiota in mediating viral infections. When antibiotic-depleted mice are infected with rotavirus there is diminished intestinal rotavirus replication and infectivity, suggesting that rotavirus are dependent upon enteric bacteria for replication.[13] Other enteric viruses have also been shown to interact with and require enteric bacteria for replication or pathogenicity. Poliovirus has lower levels of infectivity and replication when the gut microbiota is depleted and the mouse mammary tumor virus uses the gut microbiota to induce an immune evasion pathway to evade mouse immune detection.[14],[15] Human norovirus infection of B cells *in vitro* also appears to require the presence of HBGA-expressing enteric bacteria.[16]

Not only may rotavirus, like other enteric viruses, depend on gut bacteria for efficient replication and infectivity, but the host immune system may also rely on the gut microbiota commensals for adequate rotavirus clearance. When given to rotavirus-infected mice, flagellin, (a motility and virulence factor for many enteric bacteria) was able to cure chronic rotavirus infection. Protection depended on flagellar binding to Toll-like receptor 5 (TLR5) and NOD-like receptor C4 (NLR C4), thereby initiating an innate immune response to rotavirus infection.[17] Thus, bacterial components, by inducing innate immune responses, can augment adaptive immunity to viral pathogens, like rotavirus.

Microbiome and rotavirus vaccine.

Preliminary work suggests that the composition of the gut microbiome is different in infants who respond and who do not respond to rotavirus vaccine. In a recently completed study in Karachi, Pakistan, we compared the microbiome of infants with and without a rotavirus-specific IgA response to rotavirus vaccine (Rotarix™). (Clinical trials.gov identifier NCT02220439) Rotavirus vaccine response was significantly correlated with higher levels of Gram-negative bacteria and Proteobacteria, specifically bacteria related to *Escherichia coli*. Proteobacteria are potentially pathogenic bacteria that are often flagellated, therefore our research suggests that robust immune responses in infants to the rotavirus vaccine may depend upon flagellated bacteria in poor settings.

Rotavirus infection and vaccination in adults

In this study, we hypothesize that the composition of the gut microbiome, particularly the presence of flagellated, Proteobacteria, modulates innate and adaptive immune responses to rotavirus vaccination. In order to test this hypothesis, we are proposing an *in vitro* adult volunteer study, in which we modify the gut microbiome and measure broad innate and specific adaptive immune responses to rotavirus vaccination. We are choosing to perform this study in adults because it would be unethical and too burdensome to perform in infants

or young children. Adults have more developed immune systems, very mild or no symptoms following rotavirus infection and higher baseline anti rotavirus immunoglobulin titers.

Knowledge of adult rotavirus vaccination is hindered by limited clinical data, however rotavirus vaccines using attenuated bovine, human or recombinant strains appear to be safe in adult populations with only mild symptoms of nausea or diarrhea reported. (see 6.3 for details) Adults also appear to have post-vaccination rotavirus shedding and develop immune responses to the vaccine, particularly adults with low pre-vaccination anti-rotavirus IgA levels or serum neutralizing antibodies (albeit less reliable titer rises than infants).

Systemic vaccines and the gut microbiome

In addition to a possible role for the gut microbiome in responses to oral vaccines, the gut microbiome may also influence systemic immune (vaccine) responses. Evidence exists that the enteric microbiome can prime bone-marrow-derived neutrophils though chronic translocation of peptidoglycans from the gut, thereby improving immune responses to bacteria such as *S. pneumonia* and *S. aureus* in a murine model.[18] There is also evidence that systemic vaccine-specific responses are influenced by the intestinal microbiota. In a murine model studying the immunogenicity of influenza vaccine, TLR-5 mediated sensing of flagellin in the enteric microbiota impacted plasma cell differentiation and influenza-vaccine antibodies by stimulating lymph node macrophages to produce plasma cell growth factors. Therefore, the intestinal microbiota likely has an underappreciated role in both enteric and systemic vaccinations.

The tetanus and pneumococcal vaccines are logical choices by which to test the hypothesis that the gut microbiome influences systemic vaccine-specific immune responses in a human adult volunteer model. Both are routinely used as immune system challenges in the evaluation of potential immunodeficiencies in adults. The tetanus vaccine, a protein antigen vaccine, tests T-cell mediated immunity whereas the polysaccharide antigens in the pneumococcal vaccine induce B-cell driven antibody responses. The immunology laboratory in the AMC has experience in measuring and interpreting pre- and post-vaccination titers to both of the vaccines with great consistency. Both vaccines are associated with minimal side effects, and will provide valuable insight into the relationship between the enteric microbiota and classic vaccine immune responses alongside rotavirus vaccine responses.

2. OBJECTIVES

Primary Objective:

Is to investigate the role of the gut microbiota in mediating immune responses to oral rotavirus vaccine.

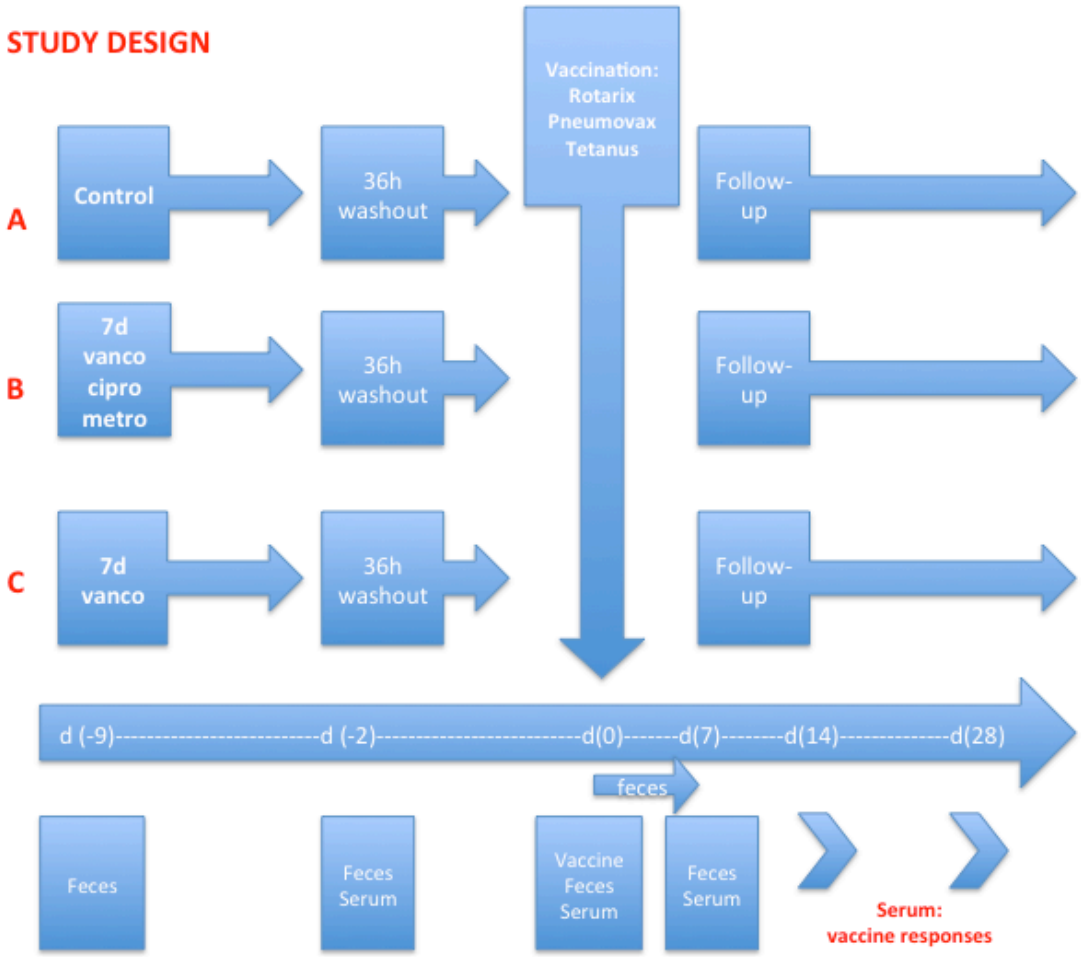
The primary endpoint is the difference in 28-day post vaccination anti-RV IgA serum response between adults treated with broad-spectrum, narrow-spectrum and no antibiotics.

Secondary Objective(s):

Is to investigate the role of the gut microbiome in mediating immune responses to tetanus and pneumococcal vaccines

The secondary endpoints are the differences in height, slope and time to positivity of the post-vaccination anti-RV IgA, anti-pneumococcal antibodies, anti-tetanus toxoid antibodies, and fecal rotavirus antigen shedding days 1-7 post-vaccination between adults treated with broad-spectrum, narrow-spectrum and no antibiotics, and extent of peripheral blood mononuclear cell (PBMC) stimulation pre- and post- vaccination using ELISA spot with rotavirus, tetanus and pneumococcal vaccines.
An additional secondary endpoint is to correlate self-reported diet to microbiome composition pre and post-vaccination.

3. STUDY DESIGN



Study design summary:
The study is a randomized, controlled exploratory intervention study.

Adult, healthy male volunteers (age 18-35 years) will undergo

screening
randomization
antibiotic treatment (or control)
vaccination
follow-up

Overall, the study duration will span 5 ½ weeks (37 days), with 5 outpatient visits to the AMC after screening, (6 visits including screening).

Study procedures:

Screening

Prior to engaging in any study procedure, each subject must sign and date an informed consent form. At this time the subject will be given a screening number.

The screening period will be used to determine if the patient is eligible for study enrollment. The following procedures will be performed during the screening period, which is to occur in the 28 days prior to the subject receiving the first dose of antibiotics.

- Informed consent
- Medical history including prior/concomitant medications, allergies to medications, drug and alcohol history and demographic information
- Complete physical examination including vital signs (pulse rate, blood pressure, respiration rate and temperature), height and body weight
- Baseline blood samples for clinical laboratory assessment:
    - Immunology: anti-rotavirus Ig A, anti-tetanus toxoid Ig G, anti-pneumococcal polysaccharide Ig G; whole blood for baseline peripheral blood mononuclear cell stimulation
    - Hematology: Hemoglobin, Complete white blood cell count and differential, Platelets
    - Clinical chemistry: Sodium, Potassium, Blood urea nitrogen, Creatinine, Glucose, Alanine aminotransferase, Aspartate aminotransferase, Total bilirubin, Alkaline phosphatase, Albumin, CRP

- Assessment of Eligibility for study inclusion

Randomization

Subjects who have signed an informed consent and meet all inclusion- and exclusion criteria will be enrolled in the study and undergo randomization to one of three pre-treatment groups.

Pre-treatment

Group A: *Control group* – subjects will receive no antibiotics

Group B: *Broad-spectrum antibiotics* – subjects will receive 7 days of pre-treatment (days -9 to -3) with:

- Ciprofloxacin 500mg 2dd1
- Vancomycin 250mg 3dd2
- Metronidazole 500mg 3dd1

Group C: *Narrow-spectrum antibiotics* – subjects will receive 7 days of pre-treatment (days -9 to -3) with:

- Vancomycine 250mg 3dd2

Vaccination

After a 36-hour washout period for the antibiotics (days -2 to -1), subjects will be required to return to the AMC for vaccinations on day 0. All subjects will then be given an oral dose of the rotavirus vaccine, Rotarix™, and intramuscular injections of the Tetanus vaccine and Pneumococcal vaccine, Pneumo 23.

Post-vaccination

From day 1 to day 7 post-vaccination, patients will collect a daily fecal sample for rotavirus antigen shedding and store this at -20C at home. They will bring these samples to their 7-day clinic visit.

Follow-up

All patients will be asked to return to the AMC after vaccination for weekly blood draws on days 7, 14, and 28 days post-vaccination to measure vaccination uptake.

All patients will be asked to fill in a 4-day online diet log. This log can be obtained at https://mijn.voedingscentrum.nl/nl/registreren/. See appendix 1.

Study Measurements (all study subjects)

Day(s)		**Serum and whole blood**	**Feces**
-37 to -9	Screening	Chemistry (4mL), Hematology (4mL), Immunology (12mL)	
-9	Pre- ABx treatment		microbiome
0 = vaccination	Post ABx treatment	Chemistry (4mL), Hematology (4mL)	microbiome
+1,2,3,4,5,6	Post-vaccination		RV antigen
+7	Post-vaccination	Chemistry (4mL), Hematology (4mL), Immunology (12mL)	RV antigen, microbiome
+ days 14 and 28,	Follow-up	Immunology* (12mL)	

ABx, antibitoics; RV, rotavirus

Chemistry:	Sodium, Potassium, Blood urea nitrogen, Creatinine, Glucose, Alanine aminotransferase, Aspartate aminotransferase, Total bilirubin, Alkaline phosphatase, Albumin, CRP
Hematology:	Hemoglobin, complete white blood cell count and differential, platelets
Immunology:	anti-rotavirus Ig A, anti-tetanus toxoid Ig G, anti-pneumococcal polysaccharide Ig G; peripheral blood mononuclear cell stimulation with vaccines. * Day 28 Immunology will include anti-RV serum neutralizing antibodies
Feces:	Fecal microbiome analysis and rotavirus antigen shedding
Diet log	All patients will be asked to complete a 4-day diet log, via https://mijn.voedingscentrum.nl/nl/registreren/

4. STUDY POPULATION

4.1. Population (base) : Healthy male eligible subjects (volunteers), 18-35 years of age and recruited by advertisement, will be screened. The total number of enrolled study subjects will be 63.

4.2. Inclusion criteria: In order to be eligible to participate in this study, a subject must meet all of the following criteria:

- Healthy, as determined by a responsible physician, based on a medical evaluation including medical history, physical examination and laboratory tests carried out within 28 days prior to starting antibiotics (day -9). A subject with a clinical abnormality or laboratory parameter outside the reference range may be included if the investigator agrees that the finding is unlikely to introduce additional risk factors and will not interfere with the study procedures
- Male between 18 and 35 years of age, inclusive at the time of signing the informed consent
- Capable of giving written informed consent and able to comply with the requirements and restrictions listed in the informed consent form
- Normal defecation pattern (defined as ≤3x/ day and ≥3x/week)

4.3. Exclusion criteria: A potential subject who meets any of the following criteria will be excluded from participation in this study:

- Baseline anti-rotavirus immunglobluin A level greater than 20 IU/mL or equivalent geometric mean titer.
- Subject has had a major illness in the past 3 months or any significant chronic medical illness that the investigator would deem unfavorable for enrollment, including inflammatory diseases.
- Subject with any history of immunodeficiency
- Subjects with a history of any type of malignancy
- Subject with a history of thrombocytopenia or bleeding disorder
- Subject has a past or current gastrointestinal disease which may influence the gut microbiota
- Current or chronic history of liver disease, or known hepatic or biliary abnormalities (with the exception of Gilbert's syndrome or asymptomatic gallstones)
- History of alcoholism and/or drinking more than an average of 5 units of alcohol per day
- The subject has received an investigational product within three months of day 0 of the current study

- Use of prescription or non-prescription drugs and herbal and dietary supplements within 6 months unless in the opinion of the investigator the medication will not interfere with the study procedures or compromise subject safety
- Recent (< 12 months) use of antibiotics (any kind, except for dermal antibiotics)
- Known allergy to antibiotics (any kind)
- Allergy to any vaccine components (any kind, including allergy to egg white, thiomersal and phenol) or past adverse reaction of any kind to a tetanus or pneumococcal vaccination
- Subject with a history of thrombocytopenia or bleeding disorder
- Subject has difficulty in donating blood or accessibility of a vein in left or right arm
- Subject has donated more than 350 mL of blood in last 3 months
- Difficulty swallowing pills
- Body mass index >28 kg/m2
- Any other issue that, in the opinion of the investigator, could be harmful to the subject or compromise interpretation of the data

4.4. Sample size calculation

The primary objective of this study is to determine if rotavirus vaccine immune response, is affected by microbiota modulation. A formal sample size determination is not required for such an exploratory study. Few studies have been performed in adult populations with reporting of anti-rotavirus immunoglobulin response and distribution. Lawrence *et al* described the response to rotavirus vaccination in an elderly population in 36 individuals.[19] The authors used the RV5 (Merck) vaccine in elderly adults and reported post-vaccination anti-rotavirus IgA antibodies using a different ELISA than this study will use. Nevertheless they describe a pre-vaccination geometric mean titer of 679.6 dilution units with a 95% Confidence Interval of 464-994. Post-one rotavirus dose geometric mean titer was 1026 dilution units (95% CI 621-1696). This implies a standard deviation of 970 dilution units in the post dose group. We assume that the standard deviation will be smaller and the immune response to vaccination will be higher in a young adult population pre-screened for low anti-rotavirus immunoglobulin levels. When a two-tailed test is employed with alpha = 0.05 and a power of 0.80 is used, to find a difference in anti-rotavirus immunoglobulin levels in plasma of 35%, assuming a standard deviation of 400 dilution units, 21 individuals per group will need to be included. All inferential statistics will be two-tailed in nature with statistical significance being claimed when the obtained alpha is equal to or less than 0.05. As this is an exploratory study no adjustments for multiplicity will be employed.

5. TREATMENT OF SUBJECTS

5.1. Investigational product/treatment

Ciprofloxacin:	Tablet 500mg
Vancomycin:	Capsule 250mg
Metronidazole:	Tablet 500mg
Rotarix:	oral suspension, 1.5mL with human rotavirus RIX4414 strain (live, attenuated) ≥ 1.000.000 $CCID_{50}$ (Cell Culture Infective Dose 50%)
Tetanus vaccine:	injection fluid 80IE/mL, 0,5mL, i.m.
Pneumo23:	injection fluid 50 microg/ml, 0,5mL. i.m.

5.2. Use of co-intervention (if applicable)

As far as possible, the study subject should refrain from using antibiotics other than the study antibiotics during the study period.

5.3. Escape medication (if applicable)

The patient may use paracetamol (maximum 3 dd 1000mg) for any gastrointestinal pain due to antibiotic or rotavirus vaccine use or pain at the injection site secondary to intramuscular injection.

6. INVESTIGATIONAL PRODUCTS –

Not applicable

7. PRODUCTS USED IN STUDY

7.1. Name and description of products used in study

Ciprofloxacin:	Tablet 500mg
Vancomycin:	Capsule 250mg
Metronidazole:	Tablet 500mg
Rotarix:	oral suspension, 1.5mL with human rotavirus RIX4414 strain (live, attenuated) ≥ 1.000.000 $CCID_{50}$ (Cell Culture Infective Dose 50%)
Tetanus vaccine:	injection fluid 80IE/mL, 0,5mL, i.m.
Pneumo23:	injection fluid 50 microg/ml, 0,5mL. i.m.

7.2. Summary of findings from non-clinical studies

All antibiotics and vaccines have been in routine use for several years, therefore we refer to section 5.5.
For full information, see the relevant Summary of Product Characteristics (SPC)s (submitted as D2) of the antibiotics and vaccines

7.3. Summary of findings from clinical studies

For full information, see also the relevant Summary of Product Characteristics (SPC)s of the antibiotics and vaccines

Vancomycin is a bactericide antibiotic which targets the bacterial cell wall. It targets Gram positive bacteria (*Stahylococci* and *Streptococci*) and *Clostridium difficile*. It is used mainly in hospital settings to treat serious infections. Reported side effects include overgrowth of bacteria and fungi (1-10%); nausea, fever, rash, pruritus, loss of hearing (0,1-1%); anafylaxia, hypersensitivity reactions, tinnitus, blood pressure lowering, exfoliative dermatitis, urticaria, interstitial nephritis, blushing (0,01-0,1%); stevens-johnson syndrome, toxic epidermal necrolysis (<0,01%). Resorption is very low. Elimination happens mostly with faeces. Half-life: 5–11 hours.

Ciprofloxacin is a bactericide fluoriquinolon, which influences bacterial DNA synthesis by inhibiting DNA gyrase. It targets both Gram negative and Gram positive bacteria.
It is contraindicated in children younger than 16 and people with hypersensitivity for quinolones, combined liver- and kidney failure or tendon problems caused by quinolones.

Reported side effects include nausea and diarrhoea (1-10%); abdominal pain, vomiting, dyspepsia, anorexia, flatulence, candidiasis, allergic reactions (rash, pruritus, urticaria), elevated plasma levels of liver enzymes, urea, creatinin and bilirubin, eosinophilia, leucopenia, joint pain, headache, vertigo, agitation, confusion, sleeping disorders, changed taste, renal insufficiency, asthenia, fever (0,1-1%); palpitations, fainting, vasodilatation, hypotension, hepatitis, cholestatic icterus, pseudomembranous colitis, differential blood count changes, allergic and anaphylactic reactions, edema, hyperglycemia, muscle ache, swollen face, hallucinations, sweating, paresthesias, fear, abnormal dreams, depression, tremor, convulsions, dyspnea, fotosensibilisation, tinnitus, reversible hearing problems, vision problems, touch-, smell- and taste disorders, hematuria, cristaluria, interstitial nephritis (0,01-0,1%); vasculitis, petecchias, erythema multiforme, erythema nodosum, liver cell necrosis, pancreatitis, tendinitis, tendon ruptures, exacerbation of myasthenia gravis, intracranial hypertension, Stevens-johnson syndrome, lyell syndrome, ataxia, hypertonia, psychotic reactions, agranulocytosis, pancytopenia, bone marrow depression (< 0,01%). Resorption is high and fast. Elimination occurs 2/3 with urine and 1/3 with feces, mostly unchanged. Half-life: 4–7 hours.

Metronidazole is a nitro-imidazole derivative targeting anaerobe bacteria, *Trichomonas vaginalis, Giardia lamblia* and *Entamoeba histolytica*. Reported side effects include a metallic taste, nausea, vomiting, diarrhoea, abdominal pain, stomatitis, urticaria, angio-edema, rash, pruritus, fever; confusion, hallucinations, vertigo, headache, ataxia, coordination problems, somnolence, temporary vision defects, myalgia, artralgia (frequencies unknown); anafylaxia, disulfiram-effect, pancreatitis (reversible) (<0.001%). Side effects with a frequency of <0.0001%: see SPC metronidazole. Resorption is high (>90%). Elimination of metronidazole occurs mostly with faeces, that of metronidazolebenzoate mostly with urine. It is contra-indicated in patients with blood dyscrasias, neurological diseases and hypersensitivity to nitro-imidazolederivatives. Half-life: ca. 8 hours.

Rotarix is a live, attenuated human rotavirus vaccine. The vaccine is produced using Vero-cells. Normal vaccination includes two doses spaced (at least) 4 weeks apart, given above the age of 6 weeks. Reported side-effects in infants often (1-10%) include diarrhea and irritability. Sometimes (0.1-1%) abdominal plain, flatulence and dermatitis. Sporadic reports of apnea (particularly in premature children), bloody feces, and intussusception. Peak titer responses are expected 28 days post vaccination.

Few clinical studies have been published describing rotavirus infection and vaccination in adults. Two viral challenge studies conducted in the 1980s and 90s showed that approximately 68% of healthy adults developed rotavirus infection when challenged with the live unattenuated virus – either through rotavirus shedding or seroconversion. Approximately 40% of these adults actually developed illness (mild diarrhea and or nausea). The probability of rotavirus infection or illness after challenge was inversely correlated with the height of pre-challenge antibodies, specifically serum neutralizing antibodies and serum anti-rotavirus IgG and IgA levels. [20], [21], [22] When these same subjects were re-challenged one year after their infection, they were extremely well protected against infection or illness, with only 3 of 20 (15%) of subjects demonstrating rotavirus antigen shedding or seroconversion. Again, pre-challenge antibody titer predicted the probability of infection.

Three studies have published data on the safety and efficacy of rotavirus *vaccination* in adult subjects. A recent randomized industry-sponsored double-blind study used the Rotateq (RV5) vaccine in healthy elderly ($\geq$ 65 years old) patients. 44 patients received RV5 and 22 received placebo. Study subjects were more likely to report gastrointestinal disorders than subjects in the placebo group (34% vs. 18%), particularly diarrhea (25% vs. 9%) and nausea (11 vs. 4.5%). No vaccine-related serious adverse events were reported in the study.[23] Serum anti-rotavirus IgA and serum neutralizing antibodies were augmented in the population after a single dose of the vaccine, with approximately 27% of subjects in the RV5 group and 0% of the placebo demonstrating a $\geq$ 3-fold geometric mean fold titer rise in anti-rotavirus IgA.

A small study looked at the safety of the attenuated bovine rotavirus pentavalent Serum Institute vaccine in adults, currently being evaluated for use in India. 18 adults received the vaccine and reported no serious adverse events. No detailed data was presented, but adults and toddlers did not show an immune response.[24]

Finally, in the 1990s, when Rotarix was being developed, there was also a small sub-study evaluating the safety and efficacy of the vaccine in adults. 20 adult subjects received one dose of the vaccine (10^5 plaque forming units) and 14 subjects received placebo. No significant differences were found in the number of adverse events reported. One study subject receiving the vaccine reported mild diarrhea for one day. No subject shed detectable rotavirus and two (10%) of the vaccine recipients developed a significant rise in serum rotavirus IgA antibody. These recipients also had the lowest pre-existing

rotavirus IgA antibodies. Stool rotavirus IgA was detected in four (20%) vaccine recipients.[25]
This study will be in adults and using the Rotarix™ vaccine – an attenuated strain, which is registered for neonates. Based on the above trials, and the response rate among adults described by Lawrence et al, we will use one oral dose of the vaccine. We hope to get an increased response rate in our volunteers by pre-selecting those volunteers with a baseline low anti-RV IgA level, as those volunteers have the highest likelihood of seroconverting and boosting IgA levels.

Tetanus vaccine contains tetanus toxoid, the conservation fluid is thiomersal and it contains aluminum phosphate. The vaccine is indicated for protection against the toxin formed by *Clostridium tetani.* It is recommended in all infant vaccination schemes as well as for all children between the ages of 9-12 years. It is routinely given after injury. The Landelijks Coordinatie Reizigersadvisering advises all travelers to developing countries to receive the vaccination. The side effects include: sometimes (0.1-1%) redness or swelling at the injection site. Malaise. Seldom (0,01-0,1%): fever. Very seldom (<0,01%) neuopathy. Allergic reaction to thiomersal is possible. One vaccination usually provides protection against *Clostridium tetani* for 10 years. The duration of immunity is unknown. Indications in adults include: age > 60years, immunocompetent adults

Pneumo 23 is a pneumococcal vaccine contains 23 serotypes of *Streptococcus pneumonia* that represent ≥90% of the invasive pneumococcal serotypes in developed and developing countries. Indications for pneumococcal vaccination in adults include: age > 60years, immunocompetent adults with chronic illness such as cardiovascular disease, lung disease, diabetes, liver cirrhosis or alcoholis; immunodeficient patients with asplenia, sickle cell disease, Hodgkins lymphoma, multiple myeloma, chronic renal insufficiency, nephrotic syndrome or organ transplantation; HIV positivity; cerebrospinal fistels; living or working in an environment with an increased risk of pneumococcal infections. The most important reported side effects include: very often (>10%): local reactions such as erythema, edema, induration and pain. Often (1-10%): fever (rarely ≥ 39,5 C, and they disappear within 24 hours). Sometimes (0,1-1%) immune complex mediated type III hypersensitivity reaction (particularly with high pneumococcal baseline antibodies). Further reported side effects include: cellulitis at the site of injection, peripheral edema in extremities, nausea, vomiting, asthenia, malaise, exhaustion, arthralgia, arthritis, febrile epilepsy, paresthesias, Guillain-Barre syndrome, headaches, allergy-like syndromes (skin rash, erythema, urticarial), anaphylaxis including shock, angioedema, serum illness,

lymphadenopathy (often in combination with local reactions, lymphadenitis, leukocytosis, vasculitis. The vaccine is given once, preferably intramuscularly.

7.4. Summary of known and potential risks and benefits

See section 6.3 and chapter 13. The antibiotics are routinely used and have mild side effects. There is unlikely to be a treatment benefit in using them. Rotavirus vaccine is very unlikely to give any serious side effects and can protect patients against subsequent rotavirus infection and disease, particularly if they frequently come into contact with small children. The tetanus vaccine is well tolerated and will provide 10 years of protection against tetanus. The pneumococcal vaccine is also well tolerated and will provide protection (unknown duration) against invasive pneumococcal disease.

7.5. Description and justification of route of administration and dosage

All antibiotics will be administered orally, as the gut flora is the main target. The following dosages (as used in daily practice) will be prescribed:

Ciprofloxacin 500mg 2dd1
Vancomycin 250mg 3dd2
Metronidazole 500mg 3dd1

One dose of each of the vaccines will be given as described below, justified by clinical trial dosages, as recommended for licensure, and as described in the Farmacotherapeutisch Kompas:

Rotarix™:	oral suspension, 1.5mL with human rotavirus RIX4414 strain (live, attenuated) ≥ 1.000.000 $CCID_{50}$ (Cell Culture Infective Dose 50%)
Tetanus vaccine:	injectie vloeistof 80IE/mL, 0,5mL, i.m.
Pneumo 23:	injectie vloeistof 50 microg/ml, 0,5mL. i.m.

7.6. Dosages, dosage modifications and method of administration

See section 6.3 and 6.5

7.7. Preparation and labelling of products used in study

Products with marketing authorization will be purchased by the Kenniscentrum Geneesmiddelenonderzoek of the department of pharmacy of the AMC. Antibiotics will be additionally labeled by the pharmacy according the GMP annex 13 as these products will be taken by the healthy volunteers at home. As the vaccines are administered by the investigator and/or study nurse in the AMC, no additional information will be on the vaccines.

7.8. Drug accountability

The Kenniscentrum Geneesmiddelenonderzoek will perform drug accountability of antibiotics and vaccines. The investigators will perform drug accountability of returned products.

8. METHODS

8.1. Study parameters/endpoints

8.1.1. Main study parameter/endpoint

Height of the serum anti-rotavirus IgA response 28 days post-vaccination

8.1.2. Secondary study parameters/endpoints

- Time to positivity for serum anti-rotavirus Immunoglobulin IgA and IgG response, and when plotted, area under the curve and slope of anti-rotavirus immunoglobulin response curve (d0 through d28)
- Differences in anti-RV serum neutralizing antibodies 28 days post-vaccination
- Height, time to positivity, area under the curve and slope of serum tetanus toxoid IgG response (d0 through d28)
- Height, time to positivity, area under the curve and slope of serum pneumococcal poly-saccharide-specific IgG for all vaccine strains (d0 through d28)
- Differences in the diversity and composition of the fecal microbiome before and after antibiotics
- Differences in anti-RV serum neutralizing antibodies 28 days post-vaccination
- Differences in fecal rotavirus antigen shedding days 1-7 post-vaccination

- Extent of peripheral blood mononuclear cell (PBMC) stimulation pre- and post-vaccination using ELISA spot with rotavirus, tetanus and pneumococcal vaccine stimulation
- Correlation between self-reported diet and microbiome composition

8.2. Randomisation, blinding and treatment allocation

This will be a randomized, un-blinded, controlled study. Subjects will be randomized 1:1:1 into one of the three arms. Randomization will be performed after inclusion using sealedenvelope.com.

8.3. Study procedures

Screening

Potential subjects who respond to study advertisements will undergo a brief telephone or e-mail screening to determine potential eligibility for the study. No data collected from this initial screening will be considered study data. Potential subjects who express interest in participating in the study will be provided the information sheet and consent form and an official screening appointment at the AMC will be scheduled. Potential subjects will be given an opportunity to have any questions about the study or their participation in it answered by the investigators. Prior to engaging in any study procedure, each potential subject must sign and date an informed consent form. When the consent form is signed, each subject will be assigned a unique screening number. The screening visit is to occur within 28 days prior to the subject receiving antibiotics or 36 days prior to vaccination.

Following informed consent, all the procedures, including blood collection, listed in section 3 will be performed. If patients are deemed eligible they will undergo the study procedures described below.

Day -9

- Eligible subjects will be invited to return to the research unit in the morning to start the study.
- Assessment of prior and concomitant medications
- Assessment for any changes in health from the screening visit
- Randomization into pretreatment groups: broad-spectrum antibiotic depletion, Gram-positive antibiotic depletion, or no antibiotic depletion.
- Both antibiotic depletion groups' subjects will receive antibiotics and details about antibiotic use

- Feces will be sampled from all subjects and stored (or already collected frozen feces sample will be stored)

Day -8 to -2

- Subjects allocated to pre0treatment will self-administer antibiotics as prescribed (see chapter 6).

Day -2 to -1

- 36-hour washout period of antibiotics

Day 0

- Participants will return to the research unit early in the morning
- Blood will be drawn to check for complications of antibiotic treatment prior to vaccination (see also 7.3)
- Feces sample collection and storage (or already collected frozen feces sample will be stored)) (see also 7.3)
- Assessment of any changes in health since day -8 visit

Day 1-7

- Daily fecal sample collection at home and storage

Days 7, 14 and 28

- Subjects will be asked to return to the research unit for blood draw
- Assessment of any changes in health since last visit

All study participants will be asked to complete a 4-day diet log describing their diet via https://mijn.voedingscentrum.nl/nl/registreren/. This may be completed at any time during or after the study and is not mandatory.

8.4. Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons.

- Adverse event
- Subject request
- Failure to return for follow-up
- Administrative reasons

- General non-compliance with the protocol

8.5. Replacement of individual subjects after withdrawal

Additional subjects will be recruited to achieve a total of sixty-three (3x21) subjects.

8.6. Follow-up of subjects withdrawn from treatment

Not applicable

8.7. Premature termination of the study

New scientific insights and/ or volunteer-related factors can lead to premature termination of the study, after in-house consultation of the senior staff of CEMM. In case the study is ended prematurely, the sponsor will notify the accredited METC and the competent authority within 15 days of termination, including the reasons for the premature termination.

9. SAFETY REPORTING

9.1. Section 10 WMO event

In accordance to section 10, subsection 1, of the WMO, the investigator will inform the subjects and the reviewing accredited METC if anything occurs, on the basis of which it appears that the disadvantages of participation may be significantly greater than was foreseen in the research proposal. The study will be suspended pending further review by the accredited METC, except insofar as suspension would jeopardise the subjects' health. The investigator will take care that all subjects are kept informed.

9.2. AEs, SAEs and SUSARs

9.2.1. Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to the experimental intervention. All adverse events reported spontaneously by the subject or observed by the investigator or his staff will be recorded. Side effects to antibiotics and vaccinations will be that are already listed in the SPC will be collected in CRFs but will not be reported as adverse events to the METC during the trial.

9.2.2. Serious adverse events (SAEs)

A serious adverse event is any untoward medical occurrence or effect that at any dose:

- results in death
- is life threatening (at the time of the event)
- requires hospitalisation or prolongation of existing inpatients' hospitalisation
- results in persistent or significant disability or incapacity
- is a congenital anomaly or birth defect
- Any other important medical event that may not result in death, be life threatening, or require hospitalization, may be considered a serious adverse experience when, based upon appropriate medical judgement, the event may jeopardize the subject or may require an intervention to prevent one of the outcomes listed above.

The sponsor will report the SAEs through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within 15 days after the sponsor has first knowledge of the serious adverse reactions.

SAEs that result in death or are life-threatening will have expedited reporting. The expedited reporting will occur not later than 7 days after the responsible investigator has first knowledge of the adverse reaction. This is for a preliminary report with another 8 days for completion of the report.

9.2.3. Suspected unexpected serious adverse reactions (SUSARs)

Adverse reactions are all untoward and unintended responses to an investigational product related to any dose administered.

Unexpected adverse reactions are SUSARs if the following three conditions are met:

1. the event must be serious (see chapter 9.2.2);
2. there must be a certain degree of probability that the event is a harmful and an undesirable reaction to the medicinal product under investigation, regardless of the administered dose;
3. the adverse reaction must be unexpected, that is to say, the nature and severity of the adverse reaction are not in agreement with the product information as recorded in:
   - Summary of Product Characteristics (SPC) for an authorised medicinal product;
   - Investigator's Brochure for an unauthorised medicinal product.

The sponsor will report expedited the following SUSARs through the web portal *ToetsingOnline* to the METC:

- SUSARs that have arisen in the clinical trial that was assessed by the METC;
- SUSARs that have arisen in other clinical trials of the same sponsor and with the same medicinal product, and that could have consequences for the safety of the subjects involved in the clinical trial that was assessed by the METC.

The remaining SUSARs are recorded in an overview list (line-listing) that will be submitted once every half year to the METC. This line-listing provides an overview of all SUSARs from the study medicine, accompanied by a brief report highlighting the main points of concern.
The expedited reporting of SUSARs through the web portal ToetsingOnline is sufficient as notification to the competent authority.

The sponsor will report expedited all SUSARs to the competent authorities in other Member States, according to the requirements of the Member States.

The expedited reporting will occur not later than 15 days after the sponsor has first knowledge of the adverse reactions. For fatal or life threatening cases the term will be maximal 7 days for a preliminary report with another 8 days for completion of the report.

9.3. Annual safety report

In addition to the expedited reporting of SUSARs, the sponsor will submit, once a year throughout the clinical trial, a safety report to the accredited METC.
This safety report consists of:

- a list of all suspected (unexpected or expected) serious adverse reactions, along with an aggregated summary table of all reported serious adverse reactions, ordered by organ system, per study;
- a report concerning the safety of the subjects, consisting of a complete safety analysis and an evaluation of the balance between the efficacy and the harmfulness of the medicine under investigation.

9.4. Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist. SAEs need to be reported till end of study within the Netherlands, as defined in the protocol

9.5. Data Safety Monitoring Board (DSMB) / Safety Committee

Due to the exploratory, open-label nature of this study no DSMB will be instituted. A safety committee will be established, consisting of three experienced staff members (Kees Hovingh, Max Nieuwdorp and Liffert Vogt), with whom all data and events can be discussed if unexpected events occur.

10. STATISTICAL ANALYSIS

10.1. Primary study parameter

- Height of the serum anti-rotavirus Immunoglobulin IgA response 28 days post-vaccination

10.2. Secondary study parameters

- Time to positivity for serum anti-rotavirus Immunoglobulin IgA and IgG response, and when plotted, area under the curve and slope of anti-rotavirus immunoglobulin response curve (d0 through d28)
- Anti-RV serum neutralizing antibodies at 28 days post-vaccination
- Height, time to positivity, area under the curve and slope of serum anti-RV IgG response (d0 through d28)
- Height, time to positivity, area under the curve and slope of serum tetanus toxoid IgG response (d0 d28)
- Height, time to positivity, area under the curve and slope of serum pneumococcal poly-saccharide-specific IgG for all vaccine strains (d0 through d28),
- Differences in the diversity and composition of the fecal microbiome before and after antibiotics
- Extent of peripheral blood mononuclear cell (PBMC) stimulation pre- and post-vaccination using ELISA spot with rotavirus, tetanus and pneumococcal vaccines.

The primary study outcome: height of the serum anti-rotavirus Immunoglobulin IgA response 28 days post-vaccination, will be presented quantitatively, as will all secondary outcomes excluding the fecal microbiome which will be presented quantitatively and qualitatively.

For serological data, chi-square and Fisher's exact tests will be used to compare baseline proportions above a cut-off between groups. One-way analysis of variance (ANOVA) with Bonferroni correction will be used to compare log-transformed antibody concentrations and rotavirus, pneumococcal and tetanus titres at baseline between groups. Chi-square will be used to compare proportions above a cut-off between groups at each time-point after immunization. McNemar's test will be used to detect changes in proportion from baseline within groups. Repeated-measures ANOVA and analysis of covariance will be used to compare log-transformed antibody concentrations between and within group. A value of *P* less than 0.05 will be considered statistically significant.

Descriptive statistics

Baseline data will be expressed as mean plus standard deviation for variables with a parametric distribution and median plus range for variables with a skewed distribution. Data will be checked for normal distribution and equal variances using the residuals. Depending on the results of these tests, data will be analyzed either parametrically or nonparametrically.

Univariate analysis

Between groups comparisons will be calculated using an independent t-test or ANOVA-test for continuous variables with a parametric or Mann-Whitney-U test (or Kruskal Wallis-test) for variables with a non-parametric distribution. A 2 sided p-value < 0.05 will be considered significant. These analyses will be performed using SPSS software (version 20.0, SPSS Inc., Chicago, IL, USA).

Microbiome analysis

The Shannon diversity index will be used to measure the diversity of the microbiota, including 80 richness and evenness, per sample, using the hybridization signal of all probes included in the HITChip [10]. Paired 2-tailed Student's t-tests were used to evaluate statistical significance. Comprehensive multivariate statistical analyses will be performed using Canoco 5.0 software for Windows [11]. Principal component (PCA) and redundancy analyses (RDA) will be used to determine if there are differences in the overall microbial composition between the study groups. The 130 genus-like bacterial groups targeted by the HITChip will be used as biological variables and environmental confounder variables will be the immunogenicity data. Monte Carlo permutation testing (MCPT) will assess the significance of the effect of these variables in the data set.

The relative abundance of specific bacterial groups in the fecal microbiota will be determined at the genus-like level and at the phylum level (class for the Firmicutes). Wilcoxon signed-rank test will be used to determine significant differences in composition and p-values will be corrected for false discovery rate (FDR) by the Benjamini-Hochberg method.

11. ETHICAL CONSIDERATIONS

11.1. Regulation statement

The study will be conducted according to the principles of the Declaration of Helsinki in the current version of Fortalezea Brazil, October 2013 and in accordance with the Medical Research Involving Human Subjects Act (WMO) and local guidelines

11.2. Recruitment and consent

Possible volunteers will be recruited by posting advertorials in journals supplied by our university (UvA) and by other universities/high schools in Amsterdam (VUmc, HvA) and pin-up boards. If necessary, volunteer-recruiting websites and advertorials in local newspapers may be used. A short screening will be performed by a medical doctor or study nurse to see if possible candidates meet the inclusion criteria. If so, they are invited for a visit during which the study design will be explained. Informed consent will be asked by the investigator or study nurse and signed by both the investigator/study nurse and the volunteer. Subjects will be given a week to consider their decision if desired, after which the screening will be performed. See also the patient information letter and informed consent form (E1/E2)

11.3. Benefits and risks assessment, group relatedness

The burden of this study involves

- a screening visit
- the ingestion of antibiotics for 2 groups. The antibiotics used in this study are well known and commonly used. Their side effects mainly include gastrointestinal symptoms and hypersensitivity reactions, the risk of the latter being minimalized by exclusion of subjects with known allergies to antibiotics.
- two intramuscular vaccinations and one oral vaccination
- possible side effects from vaccination including malaise, low-grade fever, injection site irritation, mild nausea and diarrhea (see also chapter 1)
- sampling of blood and collecting faeces.
- no smoking and no drinking during the antibiotic use and no travelling to tropical countries during the study
- screening including physical examination and laboratory parameters, which might reveal a previously unknown illness
- non-mandatory diet log for 4 days

Volunteers will not directly benefit from participating in this study except that they may have protection from rotavirus infection, tetanus, and pneumococcal disease. This research may lead to improvements in vaccine immunogenicity, particularly important in developing countries with a high burden of rotavirus and pneuoumococcal morbidity and mortality and where improvements in immunogenicity could save hundreds of thousands of lives over 10-20 years.

11.4. Compensation for injury

The sponsor/investigator has a liability insurance which is in accordance with article 7, subsection 9 of the WMO. After METC consent this will be arranged by AMC Medical Research, according to usual AMC practice.

The sponsor (also) has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO and the Measure regarding Compulsory Insurance for Clinical Research in Humans of 23th June 2003). This insurance provides cover for damage to research subjects through injury or death caused by the study.

1. € 450.000,-- (i.e. four hundred and fifty thousand Euro) for death or injury for each subject who participates in the Research;
2. € 3.500.000,-- (i.e. three million five hundred thousand Euro) for death or injury for all subjects who participate in the Research;
3. € 5.000.000,-- (i.e. five million Euro) for the total damage incurred by the organisation for all damage disclosed by scientific research for the Sponsor as 'verrichter' in the meaning of said Act in each year of insurance coverage.

The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

11.5. Incentives

Subjects will receive a maximum payment of € 300,- for their participation in the trial (including travel expenses). Payment will be made in full if a subject is withdrawn from the study for any medical reason. Payment will be made on a pro-rata basis if a subject chooses to withdraw, or is withdrawn for any non-medical reasons, including non-compliance with any of the study procedures and conditions outlined in the study protocol or in the informed consent form.

12. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

12.1. Handling and storage of data and documents

The study will be conducted in accordance with the principles founded in the Declaration of Helsinki, Good Clinical Practices, ICH Guidelines and the appropriate regulatory requirement(s). For each patient a case report form (CRF) is completed. The CRF consists of a sequential set of instructions with provision for data recording. A subject number identifies all included volunteers. The investigator ensures that volunteers' anonymity is maintained. The investigator will safeguard the subject identification code list. At the end of the study all essential documents pertaining to the conduct of the study, (e.g., screening forms, CRFs, originals of test result reports, records of informed consent, etc) are archived by the investigator for a period of 15 years. Human material (blood and faeces) will be kept for maximal 5 years.

12.2. Monitoring and Quality Assurance

Not applicable

12.3. Amendments

A 'substantial amendment' is defined as an amendment to the terms of the METC application, or to the protocol or any other supporting documentation, that is likely to affect to a significant degree:

- the safety or physical or mental integrity of the subjects of the trial;
- the scientific value of the trial;
- the conduct or management of the trial; or
- the quality or safety of any intervention used in the trial.

All substantial amendments will be notified to the METC. Non-substantial amendments will not be notified to the accredited METC, but will be recorded and filed by the sponsor.

12.4. Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

12.5. End of study report

The sponsor will notify the accredited METC and the competent authority of the end of the study within a period of 90 days. The end of the study is defined as the last patient's last visit. In case the study is ended prematurely, the sponsor will notify the accredited METC and the competent authority within 15 days, including the reasons for the premature termination. Within one year after the end of the study, the investigator/ sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC and the Competent Authority.

12.6. Public disclosure and publication policy

After completion of the study, results will be made publically available.

13. STRUCTURED RISK ANALYSIS

13.1. Potential issues of concern

a. Level of knowledge about mechanism of action

The level of knowledge concerning the antibiotics used in the study is very high, as the antibiotics have been used in daily practice for many years. The level of knowledge concerning pneumococcal and tetanus vaccination is also very high as these vaccines have also been used for many years in adults in daily practice. The lever of knowledge about rotavirus vaccine use in adults is relatively low as the vaccine has had limited testing in adult populations. See Chapter 5

b. Previous exposure of human beings with the test product(s) and/or products with a similar biological mechanism

See chapter 1, chapter 5

c. Can the primary or secondary mechanism be induced in animals and/or in *ex-vivo* human cell material?

Human volunteers are essential to understand the influence of the microbiome in influencing systemic vaccine responses. Proof of concept studies in animals are already available, and evidence in low-income settings correlates the microbiome with vaccine responses. However, human volunteer studies are need for proof-of principle as well as a mechanistic understanding of the relationship between vaccination and immune responses.

d. Selectivity of the mechanism to target tissue in animals and/or human beings

Not applicable

e. Analysis of potential effect

Gastrointestinal side effects of antibiotics are likely; serious adverse effects are unlikely (see g. and chapter 5).

Tetanus and pneumococcal vaccine can give injection sight soreness and slight malaise.

Rotavirus vaccine may give diarrhea and/or slight nausea.

We expect that combining antibiotics and vaccination will influence immune functional tests but will not exacerbate vaccine-induced symptomatology. Cito laboratory

measurements on day 0 will ensure that no serious side effects from the antibiotic will have occurred, prior to the administration of the vaccines.

f. Pharmacokinetic considerations

Antibiotic dosages are prescribed as in daily practice, in accordance with pharmacokinetic properties of the used antibiotics. A washout period of 36 hours before vaccination is adequate for most of the antibiotics to be cleared.
Rotavirus oral dose and tetanus and pneumococcal vaccine i.m. doses are standard doses in accordance with daily practice. Duration of immunity is unknown in adult populations for rotavirus, likely 10 years for tetanus, and unknown but likely several years for pneumococcus.

g. Study population

The study population includes only healthy men and excludes all individuals with an allergy to any kind of antibiotic or vaccine. All risks in this area are therefore minimal.

h. Interaction with other products

Not applicable, subjects are not allowed to use any other medication.

i. Predictability of effect

The level of knowledge about the used antibiotics is very high, as they have been used in daily practice for many years. The level of knowledge about pneumococcal and tetanus vaccines in high. The level of knowledge about rotavirus vaccine in adults is lower and therefore less predictable.

j. Can effects be managed?

Subjects will self-administer antibiotics. Doctors or nurses will administer the vaccines. All side effects will be reported to the study investigators at weekly and then bi-monthly intervals.

13.2. Synthesis

All possible measures to minimize risk to the study volunteers have been taken. The study population is relatively small and will only include healthy men. The study volunteers will have no previous allergies to either antibiotics or vaccine components. The volunteers will not have gastrointestinal diseases or states of immunosuppression, further decreasing any risks from vaccination.

The remaining risk for volunteers is small, therefore we consider it acceptable for the amount of knowledge this study will gain.

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Appendix One:

Example of diet log that patients will be requested to complete via registration and eetmeter at https://mijn.voedingscentrum.nl/nl/dashboard/eetmeter/: