RESEARCH PROTOCOL RISE-UP study

The effect of Riboflavin

SupplemEntation on *Faecalibacteri*U*m*

*P*rausnitzii in Crohn's disease

RISE-UP Study

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PROTOCOL TITLE: The effect of riboflavin supplementation on *Faecalibacterium prausnitzii* in Crohn's disease

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The effect of riboflavin supplementation on *Faecalibacterium prausnitzii* in Crohn's disease

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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**
AR	**Adverse Reaction**
CA	**Competent Authority**
CCMO	**Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek**
DSMB	**Data Safety Monitoring Board**
EudraCT	**European drug regulatory affairs Clinical Trials**
GCP	**Good Clinical Practice**
IB	**Investigator's Brochure**
IC	**Informed Consent**
IMP	**Investigational Medicinal Product**
IMPD	**Investigational Medicinal Product Dossier**
METc	**Medical research ethics committee (MREC); in Dutch: Medisch Ethische Toetsing Commissie (METc)**
(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**
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**
IBD	**Inflammatory Bowel Disease**
F. prausnitzii	***Faecalibacterium prausnitzii***
M. Crohn	**Morbus Crohn**
E. coli	**Escherichia coli**
FISH	**fluorescent in situ hybridization**
UMCG	**University Medical Center Groningen**
SCFAs	**Short Chain Fatty Acids**

SUMMARY

Rationale
Recent studies show that in patients with Inflammatory Bowel Disease (IBD) a dysbiosis exists in the composition of the intestinal microbiota. In particular, the potentially pathogenic bacterium Escherichia coli (E. coli) is often more abundant in the bowel of IBD patients, and the anaerobic commensal *Faecalibacterium prausnitzii* (*F. prausnitzii*) is often reduced. This last mentioned bacteria is known to be abundant in the intestine of healthy individuals. It is known to produce butyrate, which stimulates the intestinal epithelium, and to secrete anti-inflammatory substances.
Riboflavin – also known as vitamin B2 – is required for a wide variety of cellular processes and has an important role in maintaining health in humans. In a pilot intervention with healthy volunteers it is shown that a riboflavin supplement increases the number of *F. prausnitzii* and results in a higher production of butyrate. In Crohn's disease patients, it is known that the amount of *F. prausnitzii* in the intestine is generally low. Furthermore, it is known that there is an association between the number of *F. prausnitzii* bacteria and the length of disease in remission.

This study will evaluate if supplementation of the diet with riboflavin in Crohn's disease patients will result in a similar increase in the amount of *F. prausnitzii* as in healthy volunteers. In this patient group, an increase in the number of *F. prausnitzii* bacteria in the bowel may result in a more favourable disease course. This will be assessed with faeces calprotectin and two questionnaires. Additionally we will assess if there is any modulation by riboflavin on the other intestinal bacteria, short chain fatty acids (SCFAs) (such as butyrate), and the pH of the faeces. Finally, the effect of the riboflavin on the permeability of the gut will be evaluated with a Chroom-EDTA test, and a number of different biomarkers of permeability.

Hypothesis
The hypothesis is that in Crohn's disease patients, supplementation of the diet with riboflavin results in an increase in the amount of *F. prausnitzii*, changes in microbial composition, increased fatty acid production, an increase in pH and a reduction of intestinal permeability. These changes might result in a more favourable disease course with less exacerbations.

Study design
Prospective clinical study.

Study population and sample size
In total 84 Crohn's disease patients will be included in this study, divided into two groups. Group 1 (n=42) will consist of patients with disease in remission (quiescent disease); group 2 (n=42) will consist of patients with active disease. In this study an adaptive design will be

used. First 12 patients in the disease in remission group will be analysed. The methods of analysis and safety aspects will be taken into account.

Intervention
Supplementation of the normal diet with 1 capsule of 100 mg riboflavin (vitamin B2) during three weeks.

Main study parameters
Primary Objective:
1). To investigate the effect of a riboflavin supplement on the number of *F. prausnitzii* bacteria in the faeces of active and quiescent Crohn's disease patients.

Secondary Objectives:
1). To evaluate the effect of a riboflavin supplement on the bacterial composition in the faeces of active and quiescent Crohn's disease patients.
2). To evaluate the effect of a riboflavin supplement on the production of short chain fatty acids (SCFAs) in the faeces of active and quiescent Crohn's disease patients.
3). To assess the effect of the riboflavin intervention on the disease severity (Harvey Bradshaw Index, IBD-Q and faeces Calprotectin) of active and quiescent Crohn's disease patients.
4). To evaluate the effect of the riboflavin intervention on permeability of the gut in active and quiescent Crohn's disease patients (Chroom-EDTA test, and different biomarkers of permeability).
5). To determine the effect of a riboflavin supplement on the faecal pH in active and quiescent Crohn's disease patients.
6). To evaluate whether riboflavin intervention leads to an increase in electrical current in the faeces, as a result of an increase in extracellular electron shuttling bacteria.

Nature and extent of the burden and the risks associated with participation, benefit and group relatedness
Participating in this study has a potential health benefit. It is known that in healthy subjects, the supplement riboflavin increases the amount of beneficial bacteria. In Crohn's disease there is often a dysbiosis of the bacterial composition, and the beneficial bacteria are depleted. When a similar increase in the beneficial bacteria occurs in Crohn's disease patients as seen in healthy volunteers, this may result in a more favourable disease outcome (staying in remission longer). Riboflavin is freely available, and commonly sold in health shops. There is no need for a prescription to buy this supplement, and its use is considered to be safe. The riboflavin supplement may give a (completely innocent) yellow discoloration of the urine several hours after ingestion. There is no need to discontinue the riboflavin supplementation. The adverse event of discoloration of urine is only temporary.

1. INTRODUCTION AND RATIONALE

Rationale

Bacterial dysbiosis in IBD

The most common types of Inflammatory Bowel disease (IBD) are M. Crohn and Ulcerative Colitis (UC). Although there are many differences between these diseases, both are characterized by an altered composition of the gut microbiome. (1)

Microbiota generally play an important physiological role in the digestion of nutrients. However, in patients with a particular genetic predisposition it has been suggested that an aberrant immune response against the intestinal microbiota takes place. (2) (3) We have previously shown that specific bacteria are essential in developing an experimental colitis (model of an experimental colitis). (4) In Crohn's disease it appears that pro-inflammatory and anti-inflammatory microbiota are out of balance. Recently we showed that this imbalance is also responsible for developing a humoral immune response against the own gut bacteria as is demonstrated by IgG coating of bacteria. (5) From a clinical practice it has long been known that an elemental diet can achieve remission of disease activity. (6) The exact mechanism that underlies this finding is yet to be discovered. We believe that this elemental diet changes the bacterial composition in the intestine and restores the dysbiosis.

The dysbiosis as seen in IBD is characterized by an increase in the number of potentially pathogenic bacterium Escherichia coli (E. coli) and a reduction of the anaerobic gram-positive commensals in the intestine (such as bacteria belonging to the Clostridium groups IV en XIVa). (7) (5) (8) (9) (10) These Clostridium groups are bacteria that convert complex carbohydrates and sugars into fatty acids such as butyrate. One of the bacteria of the Clostridium group IV (and the phylum of Firmicutes) is *Faecalibacterium prausnitzii* (*F. prausnitzii*), a bacterium that generally forms around 10% of our gut microbiota and occurs in almost every human being. (11)

F. prausnitzii can grow on sugars, pectin and degradation products of human mucus. From glucose and acetate it produces in particular butyrate, which stimulates the intestinal epithelium by influencing regeneration and gene expression, stimulating mucus production and by reducing the permeability of the gut. (12) Butyrate is also the main source of energy for the colonocytes in the intestine. Butyrate belongs to the group of short chain fatty acids (SCFAs). SCFAs are produced as a product of microbial fermentation of indigestible carbohydrates. (13) (14) (15) Moreover, *F. prausnitzii* produces soluble substances which have an anti-inflammatory effect on the immune system of the intestine. (7) Furthermore, in mice it is shown that oral administration of live *F. prausnitzii* or its supernatant markedly reduced the severity of TNBS colitis (murine model for colitis) and tended to correct the

dysbiosis associated with TNBS colitis models. (7) Although it is difficult to cultivate *F. prausnitzii*, due to its extreme sensitivity to oxygen, it was recently demonstrated that a culture medium of riboflavin and cysteine makes growth of this bacteria at low oxygen levels possible. (16)

Riboflavin – also known as vitamin B2 – is required for a wide variety of cellular processes, and has an important role in maintaining health in humans. Riboflavin enables the bacterium *F. prausnitzii* to use a form of anaerobic respiration, by use of external electron acceptors such as cysteine and oxygen. This results in the growth of *F. prausnitzii* in vitro at low oxygen levels. This may also be the case in the intestinal wall mucosa. (16) In a pilot intervention with healthy volunteers it is shown that a riboflavin supplement increases the number of *F. prausnitzii* in the faeces and results in a higher production of butyrate (results yet to be published, see Figure 1). It is shown that in Crohn's disease there is a depletion of vitamin B2 level as compared with healthy controls, and that the level of this vitamin showed a negative correlation with the Crohn's disease activity index. (17) Also, in Crohn's disease patients, it is known that the amount of *F. prausnitzii* in the intestine is generally low. There is an association between the number of *F. prausnitzii* in the bowel and the length of disease in remission. (see Figure 2).

Figure 1: A study in healthy volunteers shows an increase in the number of F. prausnitzii in the faeces during riboflavin supplement intake in the majority of the subjects.

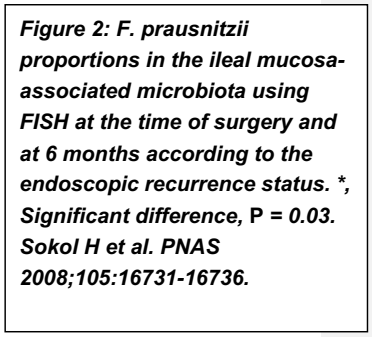

Figure 2: F. prausnitzii proportions in the ileal mucosa-associated microbiota using FISH at the time of surgery and at 6 months according to the endoscopic recurrence status. *, Significant difference, P = 0.03. Sokol H et al. PNAS 2008;105:16731-16736.

The present study will evaluate if supplementation of the diet with riboflavin in Crohn's disease patients will result in a similar increase in the amount of *F. prausnitzii* as seen in the healthy volunteers. Potentially an even larger effect can occur, because of the low starting amount. In this patient group, an increase in the number of *F. prausnitzii* in the bowel may result in a more favourable disease course. (7) A possible protective effect of *F. prausnitzii* in the development of IBD has been suggested in a number of studies. (8) (7) (11) (18) Our hypothesis is that supplementation of the diet with riboflavin will result in an increase in the amount of *F. prausnitzii*. We hypothesize that this will be more pronounced in the Crohn's disease patients with disease in remission, than in the group with active disease. This because, cytokines and mediators of inflammation might counteract the beneficial effect of the riboflavin on *F. prausnitzii*. Further, we hypothesize that as a result of the increase in *F. prausnitzii*, butyrate production will increase. (8) (18) (19) (20) Butyrate is thought to play a beneficial role on reducing the inflammation of the intestinal mucosa; the exact mechanism(s) are yet to be defined. (18) We expect the pH of the faeces to increase after the riboflavin intervention, since, the faeces pH is shown to be lower in inflammatory conditions, and riboflavin might result in a reduction of the inflammation. (21) Also, the permeability of the gut will be evaluated with a Chroom-EDTA test, and a number of different biomarkers of permeability. A decrease in permeability is hypothesized as a result of reduced inflammation. (7) (8) Finally Khan et al demonstrated that *F. prausnitzii* uses riboflavin for electron transfer. In this study it was shown that riboflavin is required for the extracellular shuttling of electrons by *F. prausnitzii* that metabolize glucose anaerobically. We will evaluate whether riboflavin intervention leads to an increase in electrical current, as a result of an increase in extracellular electron shuttling bacteria. (16)

Previous studies have analysed the effect of different nutritional supplements in Crohn's disease patients. In a group of patients with inactive or mild disease it was shown that a vitamin E and C supplement resulted in a significant reduction in oxidative stress. (22) In an article of Ramirez-

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Farias et al 2008 it is shown that the dietary supplement inulin is able to increase the number of *F. prausnitzii* bacteria in the bowel from 10.3% during the control period to 14.5% during inulin intake (increase of 41%) (23). Until now, no study has been performed that evaluated the effect of a vitamin B2 supplement on the intestinal microbiota.

In conclusion, the present study will evaluate the effect of the vitamin supplement riboflavin on the intestinal bacterial composition in Crohn's disease patients. Dysbiosis of the intestinal microbiota is generally seen in Crohn's disease. Restoring this dysbiosis may lead to a more favourable disease course with less exacerbations.

2. OBJECTIVES

Research Goals

Primary Objective:
1). To investigate the effect of a riboflavin supplement on the number of *F. prausnitzii* bacteria in the faeces of active and quiescent Crohn's disease patients.

Secondary Objectives:
1). To evaluate the effect of a riboflavin supplement on the bacterial composition in the faeces of active and quiescent Crohn's disease patients.
2). To evaluate the effect of a riboflavin supplement on the production of short chain fatty acids (SCFAs) in the faeces of active and quiescent Crohn's disease patients.
3). To assess the effect of the riboflavin intervention on the disease severity (Harvey Bradshaw Index, IBD-Q and faeces Calprotectin) of active and quiescent Crohn's disease patients.
4). To evaluate the effect of the riboflavin intervention on permeability of the gut in active and quiescent Crohn's disease patients (Chroom-EDTA test, and different biomarkers of permeability).
5). To determine the effect of a riboflavin supplement on the faecal pH in active and quiescent Crohn's disease patients.
6). To evaluate whether riboflavin intervention leads to an increase in electrical current in the faeces, as a result of an increase in extracellular electron shuttling bacteria.

3. STUDY DESIGN

Baseline measurements
At baseline a number of parameters will be assessed: Vital signs (blood pressure, pulse, respirations and temperature); weight (in kilograms, without shoes) and height (in cm, without shoes). Also a blood sample will be taken prior to the riboflavin intervention. After the three

weeks of intervention this blood sample will be repeated. Laboratory analysis will take place of a standard set of parameters routinely determined in IBD patients. The blood sample consists of: ALAT, ASAT, Alkaline phosphatase, amylase, BSE, CRP, ferritin, GGT, Hb, Ht, creatinine, leukocyte count, MCV, trombocyte count, TYBC and iron (9.0 ml of blood (EDTA 4.0 ml and Lithium Heparine 5.0 ml)). Additionally, both before and after the intervention two extra blood tubes (1 plasma and 1 serum tube) will be drawn for storage (2 x 10 ml = 20 ml blood). This blood will be used to determine a number of different biomarkers for permeability: I-FABP (plasma) and SM-22 (serum). The above means that 29 ml of blood will be drawn per time point. Also an urine sample will be collected. Claudin-3, a marker of permeability, will be measured in the urine.

	Baseline	Wk 1 of riboflavin intervention	Wk 2 of riboflavin intervention	Wk 3 of riboflavin intervention	2 wk after the intervention
Informed consent	X				
Clinical visit	X			X[4]	
HB-index [1]	X			X[4]	
IBD-Q questionnaire	X			X[4]	
Bloodsample	X			X[4]	
Urinesample	X[2]		X[3]	X[4]	X
Chroom-EDTA test	X			X[4]	
Faeces sample	X[2]		X[3]	X[4]	X
Separate faeces sample for calprotectin	X			X[4]	

Table 1: **Schedule of activities for participating patients:** ***1). HB - Index: Harvey Bradshaw Index; 2). Faeces and urine sample are taken at two time points prior to the riboflavin intervention. There will be at least six days between both time points. 3). Faeces and urine collected on day 10 after starting the intervention. 4). These measurements will take place on the last day of the riboflavin intervention.***

At two time points a Chroom-EDTA permeability test will take place to assess the permeability of the gut. The Chroom-EDTA test is used in this hospital before in the (by the ethical committee approved) COLIPI clinical trial (''*Identification of predictive parameters for colitis in melanoma patients treated with ipilimumab*'' (METc 2012/085)). Participants will be asked to come to the hospital fasted (without breakfast). Prior to drinking the Chroom-EDTA a pregnancy test will be performed in the urine of all female participants (dipstick test). When the pregnancy test is negative, participants will be asked to drink the Chroom-EDTA during the clinical visit. After drinking the Chroom-EDTA participants will remain fasted for an additional two hours. Hereafter participants will be asked to collect urine during 24 hours (see 'proefpersoneninformatie' for detailed instructions). Additionally, a Harvey Bradshaw Index (HBI) will be taken before and after the riboflavin intervention to quantify the severity of the symptoms of the disease, and to evaluate if any clinical relevant improvement occurs (see attachment 1). The HBI is based on five items: general wellbeing (0 = very well, 1 = slightly below average, 2 = poor, 3 = very poor, 4 = terrible); abdominal pain (0 = none, 1 = mild, 2 = moderate, 3 = severe); number of liquid stools per day; abdominal mass (0 = none, 1 = dubious, 2 = definite, 3 = tender); and complications (arthralgia, uveitis, erythema nodosum, aphthous ulcers, pyoderma gangrenosum, anal fissure, new fistula and abcess (complications, score 1 per item)). A total score of less than 5 is generally considered to represent clinical remission. A score of 5 – 7 is considered as mild disease, 8 – 16 as moderate disease and > 16 as severe disease. (24)

Riboflavin intervention

The study is designed in such a way that the participants are their own controls (control period). After signing the informed consent patients are asked to collect a stool sample to measure the amount of *Faecalibacterium prausnitzii* (*F. prausnitzii*). After 6 days a second sample is requested, where after the intervention with riboflavin can commence. These two samples prior to the riboflavin intervention are meant to give a reflection of the amount of *F. prausnitzii* without stimulation and the mean individual degree of fluctuation (control period). During the riboflavin intervention period, the participant is asked to take one capsule of riboflavin (100 mg) per day. After ten days, and on the final day of taking the supplement another stool sample will be requested (see check lists). This to measure the effect of riboflavin on the amount of *F. prausnitzii*. On the second clinical visit patients will be asked to bring the riboflavin container. The capsules will be counted as a control to assess if the patient has followed the dosing instructions. The riboflavin supplement is then stopped, and another stool sample will be requested two weeks after stopping. Furthermore, the changes in bacterial composition, and bacterial products (i.e. butyrate) before and after the riboflavin intervention will be assessed. The stool samples will be tested on *F. prausnitzii* and other (butyrate producing) bacteria, using fluorescent in situ hybridization (FISH) with specific probes. On two days (day 6 and day 28, see checklist) patients will be asked to divide de faeces over two containers instead of one. This extra container on these days contains a glycerol (20%), cysteine and pbs buffer (10 ml). This is used to preserve the bacteria for

analysis of the electrical current and culturing experiments. The concentration of certain fatty acids in the stool will be measured. In this butyrate is of particular interest. In addition, a portion of the faeces sample will be stored, for microbiome analysis. Microbiota will be analysed with pyrosequencing. All findings in the area of intestinal wellbeing, frequency of defecation, and the consistency of the faeces will be recorded in a questionnaire on two time points (IBD-Q, see F1, 'vragenlijst IBD-Q'). Also faeces will be collected for analysis of the faecal calprotectin. Faecal calprotectin is a neutrophilic protein in faeces, and considered to be a useful non-invasive tool, for differentiating IBD from non-organic disease. Also it is shown that repeated measurements of faecal calprotectin can predict disease course. (25) This measurement will be performed twice: at baseline and on the last day of the riboflavin intervention. For the complete schedule of activities and the different time points see Table 1 and the checklist of the participants. On two timepoints (day 17 and day 42) all samples will be collected (either with car) or participants will bring the samples to the hospital themselves.

4. STUDY POPULATION

4.1 Population (base)

The study population consists of 84 participants in total. All participants have been diagnosed with Crohn´s disease prior to inclusion. Two groups will be formed based on severity of disease. Disease activity will be primarily determined by faecal calprotectin. Participants in the first group (group 1) have a low calprotectin and are considered to be in remission. Participants in the second group (group 2) have a high calprotectin, and are considered to have (moderate) active disease. 42 participants will be included per group. The groups are described in detail below (Chapter 4.2). Of all 84 participants, the effect of the riboflavin intervention will be evaluated by analysing different samples at different time points (blood, urine, faeces, and a clinical severity disease score (HB-index) and the validated IBD-Q questionnaire).

4.2 Inclusion criteria

Group 1 (n=42)
- Patients diagnosed with Crohn´s disease in remission (calprotectin < 200 µg/g)
- Age 18-65 years

Group 2 (n=42)
- Patients diagnosed with Crohn´s disease with active disease (calprotectin > 200 µg/g)
- Age 18-65 years

Concomitant medication for Crohn's disease is allowed in all groups.

4.3 Exclusion criteria

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

- Swallowing disorders
- Pregnancy and lactation
- Use of antibiotic drugs, probiotics (i.e.Yakult, Vifit, Activia etc) or specific prebiotic supplements in the 3 weeks prior to the riboflavin intervention (for a list of probiotic, prebiotic and other supplements see attachment 2)
- Use of Methotrexate drugs
- Colonoscopy and colon cleansing in last 3 months (26)
- Use of a vitamin B2 supplement, or multivitamin complexes containing vitamin B (i.e. vitamin B-complex) in the 3 weeks prior to the riboflavin intervention
- Severe Crohn's disease (HBI > 12)

4.4 Sample size calculation

The primary objective in this study is to investigate whether the number of *F. prausnitzii* increases as result of a riboflavin intervention in patients with Crohn's disease. A power calculation has been performed (dr. I.M. Nolte). First the mean increase in *F. prausnitzii* as seen in the healthy volunteer riboflavin pilot study was calculated. In this study bacteria were measured per grams of faeces. The amount of bacteria were measured with FISH. The mean increase was 6.14 10^8 bacteria per gram of faeces per volunteer (SD: 1.56 10^9). In an article of Swidsinski et al 2008 the number of *F. prausnitzii* bacteria in the bowel was measured in 82 Crohn's disease patients using FISH. (27) The mean of *F. prausnitzii* was 5.6 10^9 bacteria / ml of faeces (SD: 5.9 10^9). In this same article, it is shown that the mean of *F. prausnitzii* in healthy volunteers (n=32) is much higher: 14.9 10^9 bacteria / ml faeces (SD: 4.5 10^9). In the article of Swidsinski, the number of *F. prausnitzii* is roughly 2.5 times higher in the healthy group as opposed to the group with Crohn's disease. We expect that the same ratio of *F. prausnitzii* between healthy volunteers and Crohn's disease patients will be the case in our population. A similar increase as seen in the healthy volunteer group is hypothesized to take place after the riboflavin intervention. This results in an expected mean increase of *F. prausnitzii* of 2.45 10^8 bacteria / grams of faeces in our Crohn's disease group (SD: 6.22 10^8). With a one sided test, with a power of 0.80 and an alpha of 0.05, this will result in a sample size of 42 patients for the group in remission. Since we want to study the effect in two experimental groups (one group in remission, and one group with active disease), 84 patients will be recruited.
In this study an adaptive design will be used. When the first 12 patients in the disease in remission group are included and have completed the study procedures, the collected faeces samples will be analyzed. The number of F. prausnitzii with FISH will be determined and compared on different timepoints (primary endpoint). Also the ratio of enterobacteriaceae / F. prausnitzii will be determined. When the increase of the F. prausnitzii bacteria is less than

the increase seen in the healthy volunteer study, we will reassess the performed power calculation. Also safety aspects will be taken into account continuously. The HB-index and the reported adverse events will be noted. If there are indications to believe that the safety of the participants are at risk, the study will be suspended, and we will reassess continuing the study.

5. TREATMENT OF SUBJECTS

5.1 Investigational product/treatment

See chapter 7.

5.2 Use of co-intervention (if applicable)

When patients use antibiotic drugs, probiotics (i.e. Yakult, Vifit, Activia etc), specific prebiotic supplements or specific vitamin supplements during the 3 weeks prior to the start of the riboflavin intervention they cannot participate in the study (see exclusion criteria). In addition participants are asked to refrain from specific supplements during the study period (see 'proefpersoneninformatie', attachment 8). In addition, participants are asked to refrain from alcohol, two days before test days. Participants are asked to refrain from smoking during the study period. Also participants are asked to refrain from intensive physical activity on the day before a test days.

5.3 Escape medication (if applicable)

Not applicable.

6. INVESTIGATIONAL PRODUCT

6.1 Name and description of investigational product(s)

Not applicable.

6.2 Summary of findings from non-clinical studies

Not applicable.

6.3 Summary of findings from clinical studies

Not applicable.

6.4 Summary of known and potential risks and benefits

Not applicable.

6.5 Description and justification of route of administration and dosage

Not applicable.

6.6 Dosages, dosage modifications and method of administration

Not applicable.

6.7 Preparation and labelling of Investigational Medicinal Product

Not applicable.

6.8 Drug accountability

Not applicable.

7. NON-INVESTIGATIONAL PRODUCT

7.1 Name and description of non-investigational product(s)

Riboflavin

The non-investigational product used in this study is vitamin B2 (riboflavin) produced by DSM Nutritional Products. The riboflavin product (hard gel capsules) will be consumed orally. The daily dose will be ingested by taking one capsule per day at breakfast. Riboflavin is readily absorbed in the gastro-intestinal tract. Absorption is increased by intake of food.

Chroom-EDTA

Chroom-EDTA oral solution (see IMPD, versie 2, 23 Dec 2015) is a permeability marker which is often used in studies with human subjects. Chroom-EDTA is an extremely stable and inert complex with very limited toxicity.

7.2 Summary of findings from non-clinical studies

Not applicable.

7.3 Summary of findings from clinical studies

Not applicable.

Riboflavin

The effect of riboflavin supplementation on *Faecalibacterium prausnitzii* in Crohn's disease

In a pilot-intervention study in healthy volunteers, it is shown that a riboflavin supplement increases the number of *F. prausnitzii*, and there appeared to be a positive effect on the production of butyrate (results not published yet).

Chroom-EDTA

The Chroom-EDTA test is used in this hospital before in the (by the ethical committee approved) COLIPI clinical trial (''*Identification of predictive parameters for colitis in melanoma patients treated with ipilimumab*'' (METc 2012/085)).

7.4 Summary of known and potential risks and benefits

Riboflavin

No adverse effects associated with riboflavin consumption from food or supplements have been reported. In a study by Schoenen and coworkers (1994) no short-term side effects were reported in 49 patients treated with 400 mg/day of riboflavin taken with meals for at least 3 months. The lack of harm resulting from high oral doses of riboflavin may be due to its limited solubility and limited capacity for absorption in the human gastrointestinal tract (Levy and Jusko, 1966; Stripp, 1965; Zempleni et al., 1996) and its rapid excretion in the urine (McCormick, 1994). Zempleni et al. (1996) showed that the maximal amount of riboflavin that was absorbed from a single oral dose was 27 mg. Similarly in a study by Stripp (1965) there was limited absorption of 50 to 500 mg of riboflavin with no adverse effects.

Based on the studies above as well as the widespread market presence of 200 mg riboflavin supplements without reported adverse effects, the (US) Council for Responsible Nutrition (CRN) published an opinion in 2013, setting a tolerable upper intake level (UL) of 200 mg/d riboflavin

Furthermore, the above (section 7.3) described finding of an increase in the number of *F. prausnitzii* and butyrate after riboflavin intake in healthy volunteers is promising. A similar (or even larger increase) in the number of *F. prausnitzii* (and butyrate) in the bowel of Crohn's disease patients by means of a simple supplement may result in the improvement of health of Crohn's disease patients. No adverse effects are known of this supplement, however vitamin B2 can cause a temporary and harmless bright yellow discoloration of the urine.

Chroom-EDTA

Chroom-EDTA oral solution is a safe diagnostic to measure the integrity of the intestines. To achieve better results in the research concerning the integrity of the intestines it is important that there are proper diagnostics. To achieve this the development of new markers is necessary. Without these markers it is more complicated to measure the effect of a therapy on the recovery of the intestines. As opposed to the labelled 51-Chroom-EDTA, the Chroom-EDTA solution used in this study has no gamma-radiation. The risk for the humans is very low. No side effects of Chroom-EDTA have been reported.

7.5 Description and justification of route of administration and dosage

Riboflavin

Riboflavin will be taken orally (conform the intended use).The product used in this study contains 100 mg of riboflavin per capsule. Participants will take 1 capsule of riboflavin per day at breakfast. The total daily dose is 100 mg. This is a commonly used dose for riboflavin supplementation (sold over the counter, without prescription). Participants will be advised to store the riboflavin product at room temperature in a dry place and to keep the product out of reach of children and pets.

Chroom-EDTA

Patients will consume the Chroom-EDTA after a night of fasting (last meal 10 p.m. one day prior to the clinical visit). After emptying the bladder, subjects will consume a Chroom-EDTA drink, followed by a glass (240 ml) of water. The first dose (at baseline) will be given under supervision of the study nurse/investigator. After an additional two hours of fasting, participants are allowed to eat and drink again without restrictions. From consuming of the Chroom-EDTA-drink onwards for 24 hours, participants will collect urine. Urine samples will be collected in plastic bottles containing 10 ml 6 mol/l hydrochloric acid to prevent bacterial deterioration.

7.6 Dosages, dosage modifications and method of administration

Riboflavin

No method of administration modifications will take place in the present study.

Chroom-EDTA

Chroom-EDTA solution is prepared (20 µmol/ml), using the method of Binnerts et al, in a food-grade environment and complete formation and stability is checked. Drinks will be filled with exact amounts of Chroom-EDTA, resulting in drinks containing 400 µmol Chroom-EDTA. The drinks will be produced in the pharmacy of A15 in accordance with GMP license (see attachment).

7.7 Preparation and labelling of Non Investigational Medicinal Product

Prior to the start of the study, DSM Nutritional Products will provide riboflavin and be responsible for packaging and labelling of the study supplement. The supplement will be packed into bottles of 23 capsules (21 days supplementation of 1 capsule per day + 2 spare capsules). The packages will be labelled in accordance with applicable laws and regulations. Labels on the study products will contain information required for usage and identification

purposes. The investigator will store the study product in a secure, limited access storage area at a cool dry place (between 15°C and 25°C), and protected from extremes of light and humidity.

7.8 Drug accountability

Riboflavin
Participants will receive a bottle of 23 capsules at the start of the study. When possible products of the same batch will be used in all subjects. Subjects should return all unused capsules to the investigator. The investigator will then throw away the capsules.

Chroom-EDTA
The Chroom-EDTA oral solution will be labelled by the pharmacy A15. Chroom-EDTA will be stored at the pharmacy of the UMCG (contact person dr. B.G.J. Dekkers, dr. S. Hofman, B.H.W. Molmans and M.C. Mollema). On the day of administration the investigator will collect the Chroom-EDTA at the pharmacy.

8. METHODS

8.1 Study parameters/endpoints

8.1.1 Main study parameter/endpoint

The main study parameter is the number of *F. prausnitzii* bacteria per gram of faeces. This will be measured with FISH and specific probes for *F. prausnitzii* and all bacteria will be counted. (28) This will be determined in the faeces samples at different time points. The number of bacteria from before and after the riboflavin intervention will be compared.

8.1.2 Secondary study parameters/endpoints

- Before and after the riboflavin intervention, faeces samples will be analyzed for:
    - The number of butyrate producing bacteria other than *F. prausnitzii* per gram faeces, in particular Roseburia species, as measured by FISH. The numbers before and after the intervention will be compared.
    - The number of Enterobacteriaceae, including E. coli per gram of faeces. The numbers before and after the intervention will be compared.
    - The ratio of Enterobacteriaceae / Faecalibacterium is a measure of the improvement in the dysbiosis in intestinal microbiota.
    - The concentration of fatty acids in the stool (i.e. butyrate).
    - The level of faecal calprotectin will be measured at different time points, as marker of activity of Crohn's disease.
    - Faeces pH.

- Microbiota analysis by pyrosequencing.
- The Harvey Bradshaw Index, a measure of disease severity, will be taken before and after the intervention (see paragraph 3 and attachment 1).
- The validated IBD-Q questionnaire will be taken before and after the intervention (see document F1: IBD-Q).
- Blood and a urine sample (see paragraph 3, study design) will be taken before and after the intervention.
- A Chroom-EDTA test will be performed at two time points.
- Biomarkers of gut permeability will be measured before and after the intervention.
- Electrical current measurements of faeces and cell culturing experiments.
- Adverse events (AE's), serious adverse events (SAE's) and suspected unexpected serious adverse reactions (SUSAR's).

8.1.3 Other study parameters

Not applicable.

8.2 Randomisation, blinding and treatment allocation

In this current study participants will not be randomized. Also no blinding will take place. The study is designed in such a way that participants are their own controls.

8.3 Study procedures

Standard faeces samples **(five time points: day 0, day 6, day 17, day 28 and day 42)** will be divided into three fractions:

- The first fraction (0.5 gram) will be fixed with 4% paraformaldehyde in Phosphate Buffered Saline (PBS) and used for FISH analysis with specific probes. (29)
- The second fraction (100 µgram) will be used for the analysis of volatile fatty acids.
- The third fraction (0.5 gram) will be stored for microbiome analysis by Next Generation Sequencing (microbiome analysis, by 16S RNA sequencing and MiSeq Illumina).
- The remainder is retained for dry weight determination, pH analysis and lactate analysis.

Faeces samples on glycerol, cysteine and PBS mix **(two time points: day 6 en day 28):**

-These samples will be used for the electrical current measurements of faeces and cell culturing experiments.

FISH analysis

Fluorescent probes for *F. prausnitzii* (F. prau), Clostridium group XIVa (Erec), *Roseburia* (rint), *Enterobacteriaceae* (EC1535) and total bacteria (EUB338) will be used to measure the effect of the riboflavin intervention. The hybridized bacteria will be counted with an epifluorescence microscope DMRA2 of Leica, automatic system, with a user modified version of Leica Q-win software (Leica, Wetzlar, Germany). (30)

Short Chain Fatty Acid analysis

HPLC ion chromatography system (Metrohm AG, Herisau, Switzerland) will be used for fatty acid analysis. The concentrations of lactate, formate, butyrate, propionate and acetate will be measured with a conductivity detector. The 6.1005.200 Metrosep Organic Acids column (Metrohm AG) with 5 mm particle size and dimensions of 7.8 mm by 250 mm will be used for differentiating fatty acids. The mobile phase is 5mM HNO3 and acetonitrile v/v ratio of 98:2, respectively, with a flow rate of 0.6 ml min^{-1}.

Chroom-EDTA test

Chroom-EDTA oral solution is a permeability marker which is often used in studies with human subjects. Chroom-EDTA is an extremely stable and inert complex with very limited toxicity. (31) Under normal physiological circumstances (i.e. healthy intestines) an amount of less than 2.5% of Chroom-EDTA is absorbed. When the intestine is more permeable, more Chroom-EDTA will enter the body passively by the intestinal paracellular route via tight junctions, resulting in an increased urinary Chroom-EDTA concentration. (32) In UC patients, Chroom-EDTA excretion reflects colonic permeability. (33)

No side effects of Chroom-EDTA have been reported. (33) For the Chroom-EDTA permeability test, subjects will consume a Chroom-EDTA drink at baseline and on the last day of the riboflavin intervention. After emptying the bladder, subjects will consume a Chroom-EDTA drink. The first dose (at baseline) will be given under supervision of the study nurse/investigator. From consuming of the Chroom-EDTA-drink onwards for 24 hours, participants will collect urine. Urine samples will be collected in plastic bottles containing 10 ml 6 mol/l hydrochloric acid to prevent bacterial deterioration.

Chroom-EDTA solution is prepared, using the method of Binnerts et al, in a food-grade environment and complete formation and stability is checked. Drinks will be filled with exact amounts of Chroom-EDTA, resulting in drinks containing 400 µmol Chroom-EDTA. The drinks will be produced in the pharmacy of A15 in accordance with GMP license (see attachment).

Faeces Calprotectin

Calprotectin (also known as MRP-8 / MRP-14 or S100A8/A9 complex) is widely used as a marker for inflammatory bowel disease. Calprotectin is released when neutrophils enter the gut wall and are activated during the inflammatory process. (34) Calprotectin is a very stable compound at room temperature and can be measured within hours with a simple ELISA test. Literature has shown that faecal calprotectin levels correlate well with endoscopic, as well as histological, disease activity of patients with IBD. (35)

Relevant biomarkers

Serum and plasma will be stored at two time points. This will allow us to look for a number biomarkers of permeability. Multiple biomarkers will be determined (see below).

I-FABP
Intestinal-Fatty acid binding proteins (I-FABP) is member of a class of three FABPs. I-FABP levels rise rapidly after enterocyte cell death. It is considered to be a promising marker of enterocyte injury. (36) (37) (38) I-FABP is determined in plasma.

SM-22
New promising marker of permeability (personal communication with (J.P.M. Derikx, MUMC)), determined in serum samples.

Urine Claudin-3
Urine Claudin-3 is a promising marker for intestinal tight junction loss. Recently it has been shown that in rats Claudin-3 urine levels increased rapidly in a rat hemorrhagic shock model (in this model intestinal barrier loss is observed rapidly after induction of hemorrhagic shock). This study also showed an association between claudin-3 stained biopsies and claudin-3 urine levels in IBD patients. (36) (39)

Measurement of electrical current and cell culturing experiments
The experiments of the current measurements will take place conform the methods of Khan et al. A two chambered microbial fuel cell will be used. The current before and after the riboflavin intervention will be measured. (16) In addition a small fraction of this sample will be used for cell culturing experiments.

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.

8.4.1 Specific criteria for withdrawal (if applicable)

Not applicable.

8.5 Replacement of individual subjects after withdrawal

In case of withdrawal or insufficient participation of a subject, he / she will be replaced by a newly recruited subject who matches the former subject. This is done in order to include the desired number of participants in each experimental group.

8.6 Follow-up of subjects withdrawn from treatment

Not applicable.

8.7 Premature termination of the study

The study will be suspended if there are indications to believe that the safety of the participants is at risk. However it is not expected that this supplement and the procedures in this study may lead to adverse events. In the case of premature termination of the current study the institution, regulatory authorities, CCMO and the METc of the UMCG will be informed.

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 investigational product. All adverse events reported spontaneously by the subject or observed by the investigator or his staff will be recorded.

The collection of faeces and urine samples entails no risks for the subjects. The Harvey Bradshaw Index can be determined by taking a short questionnaire (5 items). The IBD-Q is a validated questionnaire used to measure the influence of the inflammatory bowel disease on the quality of life and the impact of the disease.

No side effects of Chroom-EDTA have been reported.

Riboflavin is freely available, and commonly sold in health shops. There is no need for a prescription to buy this supplement, and its use is considered to be safe. The adverse event of discoloration of urine is only temporary.

A minor risk of this study is the development of a small hematoma ('blauwe plek') as a result of the blood sampling from the inside of the elbow. All together, we believe that the risks in this study are minor, and acceptable for the subjects participating in this study.

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 events.

SAEs that result in death or are life threatening should be reported expedited. The expedited reporting will occur not later than 7 days after the responsible investigator has first knowledge of the adverse event. 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, competent authority, and competent authorities of the concerned Member States.
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

Not applicable.

10. STATISTICAL ANALYSIS

The study population consists of two groups (an active disease group and a disease in remission group). The primary endpoint is number of F. prausnitzii bacteria per grams of faeces. The two groups will first be statistically analysed separately. A non-parametric statistical test will be used in which the number of F. prausnitzii bacteria prior to the intake of riboflavin (control period) will be compared with the number of bacteria after intake of riboflavin (i.e. day 28). This will be analyzed with the paired Wilcoxon test. For analysis between groups (active disease and disease in remission) the Mann-Whitney U test will be used. The MiSeq Illumina data will be analyzed with a multi-parametric test.

10.1 Primary study parameter(s)

- The number of *F. prausnitzii* per gram of faeces will be measured with FISH and specific probes for *F. prausnitzii*, both before and after the riboflavin intervention.

10.2 Secondary study parameter(s)

Both before and after supplementation the following parameters will be included:
- The number of other bacteria in the faeces. Of special interest are the number of butyrate producing bacteria other than *F. prausnitzii*, in particular Roseburia species. Additionally the number of Enterobacteriaceae in the stool, and the ratio of Enterobacteriaceae / Faecalibacterium will be determined.
- Microbiota in the faeces by pyrosequencing.
- The concentration of short chain fatty acids (SCFAs) in the faeces samples.
- Routine IBD blood test.
- Faeces pH.
- Faecal calprotecin.
- The clinical Harvey Bradshaw Index (index of disease severity).

- IBD-Q questionnaire.
- Chroom-EDTA test.
- Electrical current measured in faeces sample.
- Biomarkers of gut permeability (as described above).

10.3 Other study parameters

Not applicable.

10.4 Interim analysis

Not applicable.

11. ETHICAL CONSIDERATIONS

11.1 Regulation statement

This study will be conducted according to the principles of the Declaration of Helsinki (October 2013) and in accordance with the principles of with the Medical Research Involving Human Subjects Act (WMO). The study will start after approval of the medical ethical committee (METc) is obtained.

11.2 Recruitment and consent

The responsible physician will first approach eligible patients with general information about the study. When patients are interested, and agree to receive more information from the physician, the physician will supply a partipant information letter 'proefpersonen informatie' for the potential participant to read at home (see document E1 and E2). If the potential participant is interested to participate in the study, the participant will send back a signed 'toestemmingsformulier'.After this, the investigator can contact the participant to make an appointment at the outpatient clinic. At this time more information will be given about the research, its goals, the duration and procedures and the possible adverse events. Also, additional questions of the potential participant can be answered at this time. The investigator will stress that participating is voluntarily, and that they can quit at any time and for any reason, without any consequence for their health and further treatment. Also they will be informed about the strict confidentially concerning the acquired medical data and further patient details. Hereafter the investigator will also sign the informed consent form. If needed the potential participant can request additional information at any time by a second opinion (i.e. the independent expert). Potential participants will have one week to decide whether or not to enrol in this study. If the potential participant does not respond within one week, a reminder letter will be sent. After this, the participant have one more week to respond. If there is no reaction after this second letter, no more attempts will be made to try and contact

this patient for this study Documented informed consent must be obtained from all patients prior to inclusion in the study. Informed consent will be asked in writing, and in the following manner: the patient will sign and personal date the informed consent form first and send this form back to the investigator. When the investigator has signed and dated the consent form afterwards, the patient will receive a copy. Finally, the general physician of each patient will be informed about the enrolment of the patient to the study by a standard letter. The informed consent procedure takes place conform Good Clinical practice (GCP). After signing the informed consent sheet, the study procedures will commence (see paragraph 3, study design).

11.3 Objection by minors or incapacitated subjects (if applicable)

Not applicable.

11.4 Benefits and risks assessment, group relatedness

Participating in this study has a potential health benefit. It is know that in healthy subjects, the supplement riboflavin increases the amount of beneficial bacteria. In Crohn's disease there is often a dysbiosis of the bacterial composition, and the beneficial bacteria are depleted. When a similar increase in the beneficial bacteria occurs in Crohn's disease patients as seen in healthy volunteers, this may result in a more favourable disease outcome (staying in remission longer). The vitamin preparation containing vitamin B2, may give a (completely innocent) yellow discoloration of the urine several hours after ingestion. The collection of faeces samples entails no risks for the subjects.

11.5 Compensation for injury

The sponsor/investigator has a liability insurance, which is in accordance with article 7, subsection 6 of the WMO.

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 23rd 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.

11.6 Incentives

Travel costs will be compensated: the subjects will receive a compensation of 0.19 euro/km when travelling with car. In addition, participants will receive a ticket for free parking at the UMCG on study days. The total amount of money will be paid after completing the study, and after the last samples have been collected. The riboflavin supplement will be handed over to the participant free of charge. Participating in the study will not result in costs for the participant.

12. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

12.1 Handling and storage of data and documents

The handling of personal data complies with the Dutch 'Personal Data Protection Act' (Dutch: 'wet bescherming persoonsgegevens (wbp)'). All related documents and records will be kept and archived for at least 15 years. Data of participants will be handled confidentially and a coded identification number will be used to link the data to the specific patient. This specific data that can be linked to a patient will be stored separately. The coded identification number used in this study respectively consists of: the acronym: RISE-UP combined with the date and number of inclusion. For example the first participant included of the first of June 2014 would have the code identification number: RISE-UP/01-06-2014/01. The principal investigator will safeguard the key to the code.

The case record forms (CRF's) will be completed and signed for correctness by the investigators. By signing this CRF, the medical investigator is responsible for the legibility, completeness and correctness of the CRF.

Changes, errors and/or additions to the original CRFs shall be corrected by drawing a single line through the incorrect entry and writing as close to the original as possible. The correction must be initialled and dated by the authorized person making the changes. If necessary the reason for the changes must be given.

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.

Non-substantial amendments will not be notified to the accredited METc and the competent authority, but will be recorded and filed by the sponsor.

All substantial amendments will be notified to the METc and to the competent authority.

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 collection of the last samples of the last patient. 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

The sponsor of this study are the UMCG. The goal of this study is to contribute to research, and to publish the results. The results of this research will be disclosed unreservedly. Both positive and negative trial results must be disclosed. In general, the results of this research will be submitted for publication to peer-reviewed scientific journals.

13. STRUCTURED RISK ANALYSIS

13.1 Potential issues of concern

See section 13.2.

13.2 Synthesis

All subjects included in this study have been diagnosed with Crohn's disease. No children will be included in this study. Pregnancy is one of the exclusion criteria in this study.

In a pilot-intervention study in healthy volunteers, it is shown that a riboflavin supplement increases the number of *F. prausnitzii*, and there appeared to be a positive effect on the production of butyrate (results not published yet). A similar (or even larger increase) in the number of *F. prausnitzii* (and Butyrate) in the bowel of Crohn's disease patients by means of a simple supplement may have therapeutic consequences. Riboflavin is freely available, and commonly sold in health shops. There is no need for a prescription to buy this supplement, and its use is considered to be safe. The adverse event of discoloration of urine is only temporary. The Chroom-EDTA is safe and, no adverse effects are known. The collection of faeces samples entails no risks for the subjects. A minor risk of this study is the development of a small hematoma ('blauwe plek') as a result of the blood sampling from the inside of the elbow. All together, we believe that the risks in this study are minor, and acceptable for the subjects participating in this study.

Attachment 1. Harvey Bradshaw Index

(See document F1: (HB index) for the Dutch version of this questionnaire).

Date: _____

1. General well-being (yesterday)

- *Very well = 0* ❑
- *Slightly below par = 1* ❑
- *Poor = 2* ❑
- *Very poor = 3* ❑
- *Terrible = 4* ❑

2. Abdominal pain (yesterday)

- *None = 0* ❑
- *Mild = 1* ❑
- *Moderate = 2* ❑
- *Severe = 3* ❑

3. Number of liquid or soft stools per day (yesterday)

_____

4. Abdominal mass

- *None = 0* ❑
- *Dubious = 1* ❑
- *Definite = 2* ❑
- *Definite and tender = 3* ❑

5. Complications

(Check any that apply; score one per item, except for first box)

- *None* ❑
- *Arthralgia* ❑
- *Uveitis* ❑
- *Erythema nodosum* ❑
- *Aphtous ulcers* ❑
- *Pyoderma gangrenosum* ❑
- *Anal fissure* ❑
- *New fistula* ❑
- *Abcess* ❑

Total Harvey Bradshaw index score: _____

Remission < 5
Mild disease = 5 – 7
Moderate disease = 8 – 16
Severe disease > 16

Attachment 2. List of commercially available probiotic, prebiotic and other supplements that are not allowed in the current study

Probiotica:

1x TSglich LGG
40+ Acidophilus Vegicaps
Acidophilus (Hema)
AB Kultur Drink
ABC Dophilus
Acidobif
Acidophilasé
Acidophilus (Etos)
Acidophilus (Hema)
Acidophilus Plus
Acidophilus vegicaps
Acidophilus Yoghurt met smaakjes
Acidophilus, bifidus en bulgaricus
Acidophilus-4
Aciforce (Vogel)
Activia
Actimel (Danone)
Aktifit
A-Yoghurt
Beneflora
Bifilus
Bio
Biotic
Biomild
Bioghurt DiSt
Colon Balans
Colon Rein Naturel (Kruidvat)
Colonphyta
Cultura
Cyclops yoghurt
Darm conditioner
Darm Flora Optimaal (Kruitvat)
Darmclean
Darmflora Plus

Darmo Caps
Darmplus
Darmflora balans (DA)
Darm totaal (Etos)
Ecologic
Equiderm
Eugalan forte
Fidus
Fresh'n Fruity
Frucht Bighurt
Fysiq
Gefilus
GB-Bifidus halfvolle yoghurt
Lactobacillus acidophilus
Lactobacillus complex (Hema)
LGG+
Maxi Baby Dophilus
Milupino Kinder Trink-Frühstück
Mix Lactobacillus
Multi-Billion Dophilus
Multidophilus
Natufood Vitaal
Naturally acidophilus
Nature's biotics
Nite Cap
Non Dairy Acidophilus plus
Omnibionta 3
Onaka
Orthiflor Junior
Orthiflor Plus
Orthiflor
Power Dophilus
Power Dophilus II
Pro'Ac
ProBiotic
Prolife
ProViva
Rela Vruchtensap
R-LSttyoghurt
Ser con GG
Simfidus
Symbalance

Symbio (Flor)
Tofu Jog
Trenev Trio
Trifit
Vitamel
Vifit (Mona)
Vitelle Lactobacillus (Trekpleister)
Yakult
Yolac 5
Yosa
VSL#3

***** Tevens alle zuivelproducten waarbij vermeld staat dat er *Lactobacillen* en/of *Bifidobacteriën* aan zijn toegevoegd.

Prebiotica

Fructo-oligo-sacchariden (FOS)
Oligofructose (raftilose)
Inuline (raftiline) (bijv Colonclean ®)
Galactose-oligo-sacchariden (GOS)
Lactulose
Lactitol
Psyllium (Vlozaad)
Verschillende soorten vezeltabletten, bijvoorbeeld: Dagravit Vitentials ®,
Bio-Vezel tabletten ®, Vezel complex Forte ®, Famafiber ®, etc.

Overig

-**Vitaminesupplementen** (bijvoorbeeld multivitaminen tabletten, vitamine C, vitamine E, β-caroteen, zink.
-Supplementen die claimen het immuunsysteem of de gezondheid van de darmen te verbeteren.

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