Impact of A2 milk versus conventional milk on intestinal health: a proof-of-concept study in Irritable Bowel Syndrome patients

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PROTOCOL TITLE **'**Impact of A2 milk versus conventional milk on intestinal health: a proof-of-concept study in Irritable Bowel Syndrome patients'

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

AE	Adverse Event
AVG	Algemene Verordening Gegevensbescherming (in English; EU General Data Protection Regulation)
A2 milk	Cow's milk including only A2 β-casein
BCM	Bioactive β casamorphin
BMI	Body Mass Index
BSS	Bristol Stool scale
Conventional milk	Cow's milk including A1 and A2 β-casein
CRP	C-reactive protein
DPP-4	Dipeptidyl peptidase-4
eCRFs	Electronic case report forms
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GCP	Good Clinical Practice
GI	Gastrointestinal
HBD	Human beta-defensins
IBS	Irritable bowel syndrome
IBS-C	Irritable bowel syndrome with constipation
IBS-D	Irritable bowel syndrome with diarrhea
IBS-M	Irritable bowel syndrome with both constipation and diarrhea
IBS-SSS	Irritable Bowel Syndrome Severity Scoring System
IC	Informed Consent
METC	Medical research ethics committee (MREC); in Dutch: medisch-ethische toetsingscommissie (METC)
MUMC+	Maastricht University Medical Centre+
NA	Not applicable
PoC	Proof-of-concept
(S)AE	(Serious) Adverse Event
SD	Standard Deviation
sIgA	secretory Immunoglobulin A
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.

UAVG	Uitvoeringswet AVG (in English; the Dutch Act on Implementation of the General Data Protection Regulation)
VAS	Visual analogue scale
WMO	Medical Research Involving Human Subjects Act; in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen

SUMMARY

Rationale: Dairy products such as cow's milk are widely produced and an important component of the human diet. Beta-casein is a major protein component of cow's milk and includes A1 and/or A2 β-casein. Most cows produce a mixture of A1 and A2 β-casein (conventional milk), whereas some cows produce only A2 β-casein (A2 milk). A1 β-casein and A2 β-casein differ in amino acid composition at position 67 being histidine or proline, respectively. During gastrointestinal (GI) digestion, presence of histidine in A1 β-casein results in release of bioactive β casamorphin (BCM) opioid peptides, including BCM-7. These compounds show μ opioid agonistic activity and thereby may induce inflammation and GI symptoms. As A2 milk is not releasing these BCMs, it is found to be better tolerated than conventional milk, merely based on some acute (*i.e.* 1 day) and mid-term (*i.e.* 14 days) studies performed in China and New Zealand in healthy adults with a high rate of lactose malabsorbers, and in Australian children and healthy adults. The significant effects of A2 milk have been shown to be more pronounced in the acute studies compared with the mid-term studies. However, further studies are needed on the comparison between A2 milk and conventional milk on prevalent GI symptoms in various geographical populations, and especially in relevant subgroups, such as A1 β-casein that trigger GI symptoms and/or inflammation in irritable bowel syndrome (IBS) patients. In Western populations, IBS is a prevalent (*i.e.* 10%) functional bowel disorder characterized by abdominal pain and altered bowel habits. According to the Rome IV criteria, IBS subtypes include IBS with constipation (IBS-C), IBS with diarrhea (IBS-D) and mixed IBS with both constipation and diarrhea (IBS-M). Although the pathophysiology is incompletely understood, it is generally regarded as a multifactorial disorder involving host factors such as low-grade immune activation, altered intestinal barrier function and defense (*e.g.* by Human beta-defensins (HBD)). Environmental factors, including diet, are also suggested to play a role. With respect to cow's milk, it remains to be determined whether there are indeed specific food components such as A1 β-casein that trigger GI symptoms immediately after intake (*i.e.* acute effects) IBS patients. Lastly, it has been shown that GI symptoms, pathophysiological factors such as low-grade immune activation, and diet differ between IBS-C and IBS-D subtypes. Therefore, in this proof-of-concept study, we will explore the acute and mid-term effects of one day of A2 milk versus one day of conventional milk on 1) GI symptoms and 2) BCM-7 levels in separate groups of IBS-C and IBS-D patients. We hypothesize that in individuals with IBS-C and IBS-D, A2 milk will lead to lower GI symptom scores and lower BCM-7 levels compared with conventional milk.

Objective: The primary objective of this study is explore the acute effects of A2 milk versus conventional milk on overall GI discomfort in separate groups of individuals with IBS-C and

IBS-D. Furthermore, this study has four secondary objectives: First, explore the mid-term effects of one day of A2 milk versus conventional milk on overall GI discomfort in both groups. Second, to explore the acute and mid-term effects of one day A2 milk versus conventional milk on single GI symptoms and stool characteristics in both groups. Third, to explore the acute effects of A2 milk versus conventional milk on BCM-7 levels in both groups. And fourth, to explore the effect of different baseline characteristics (i.e. stool characteristics, GI immune and defense markers, microbiome and bodyweight) on before mentioned objectives.

Study design: The study conforms to a randomized double-blind cross-over design per IBS-subgroup (*i.e.* IBS-C and IBS-D).

Study population: Individuals (male and female) with IBS, 18-70 years of age.

Intervention: Each subject will undergo two different 1-day on-site intervention periods, during which 200 ml A2 milk or 200 ml conventional milk will be consumed twice that day, with a wash-out period in between.

Main study parameters/endpoints: The primary outcome is acute change in overall GI discomfort score after conventional vs A2 milk intervention

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: Some burden can be experienced by the volunteers during this study. After the screening visit, participants will have to visit the Maastricht University Medical Centre+ two times for one test days. In total, a participant will spend approximately eighteen hours at the university facility. Another small burden that participants can experience is the time that they will invest: *i.e.* by filling out questionnaires at home and by spending approximately eighteen hours (screening visit and two test days (±8,5 hours each test day)) at MUMC+. Another potential burden is to refrain from other dairy products as well as pre- and probiotics supplements. Moreover, we will ask participants to record three-day food records before each test days. Throughout the study, subjects will bring fecal samples twice (to both the test day), which will be collected and frozen at home. Collection of fecal samples does not yield any further risk. Lastly, twice during each intervention day, blood will be collected by venepuncture via an evacuated tube system. Venepunctures can occasionally cause a transitory local hematoma or bruise. Some participants report pain during venepuncture. A total of 28 mL blood will be sampled per subject during the entire study period of 19 days.

1. INTRODUCTION AND RATIONALE

Dairy products such as cow's milk are widely produced and consumed in the Netherlands (1), are a common source of macro- and micronutrients and an important component of the human diet. Beta-casein is a major protein component of cow's milk and includes A1 and/or A2 β-casein (2). The relative concentrations of A1 and A2 is genetically determined and varies between cow species. Most cows produce a mixture of A1 and A2 β-casein (**conventional milk**), whereas some cows produce only A2 β-casein (**A2 milk**) (2). A1 β-casein and A2 β-casein differ with regard to the amino acid at position 67, being histidine or proline, respectively (3). During gastrointestinal (GI) digestion, presence of histidine in A1 β-casein results in release of bioactive β casamorphin (BCM) opioid peptides, including BCM-7 (4). These compounds show μ opioid agonistic activity and thereby may induce GI symptoms and/or inflammation (4). As A2 milk is lacking these potential harmful compounds, it is suggested to be better tolerated than conventional milk. However, the evidence is humans is still limited, as recently reviewed by Küllenberg *et al.* (2019) (2). These investigators showed that there is moderate evidence (with low-to moderate quality studies) for digestive effects, mainly with regard to lower GI symptoms such as flatulence, bloating, abdominal pain, stool frequency and stools consistency after A2 milk versus conventional milk consumption. These acute (*i.e.* 1 day) and mid-term (*i.e.* 14 days) studies have been performed in China and New Zealand in healthy adults with a high rate of lactose malabsorbers (5-7), in Australian children (8) and in Australian healthy adults (9). The significant effects of A2 milk have been shown to be more pronounced in the acute studies (5, 7) compared with the mid-term studies (6, 8, 9). In the systematic review, they also concluded that these studies need to be reproduced in other research or clinical settings (2). Therefore, further studies are needed on the comparison between A2 milk and conventional milk on prevalent GI symptoms in various populations. We noticed that studies on GI symptoms and inflammatory markers have been registered in a clinical study database (ClinicalTrials.gov) and are currently ongoing in the United Kingdom and United States of America (*i.e.* in healthy adults with self-reported milk intolerance). However, a particularly vulnerable population of individuals with GI symptoms, subjects with irritable bowel syndrome (IBS), has not been taken into account. IBS is a common functional bowel disorder characterized by abdominal pain and altered bowel habits, with reported prevalences up to 10% in the Western population (10, 11). According to the Rome IV criteria, IBS subtypes include IBS with constipation (IBS-C), IBS with diarrhea (IBS-D) and mixed IBS with both constipation and diarrhea (IBS-M) (11). Although the pathophysiology is incompletely understood, it is generally regarded as a multifactorial disorder involving various host factors such as low-grade immune activation, altered intestinal barrier function and defense markers (*e.g.* by Human beta-defensins (HBD)(12,

13). Environmental factors, including diet, are also suggested to play a role. Individuals with IBS often indicate that diet is a key factor in symptom generation, and these patients tend to exclude food products from their diet to aim for symptom relief (14). Dairy products are frequently reported to trigger GI symptoms in IBS patients, even when lactose intolerance is excluded or treated with lactase supplementation (15). Consequently, several elimination diets have been studied with the goal to reduce GI symptoms, but mechanistic and clinical evidence is scarce on single components potentially triggering these symptoms (16). With respect to cow's milk, it remains to be determined whether there are indeed specific food components such as A1 β-casein that trigger GI symptoms immediately after intake (*i.e.* acute effects) in IBS patients. Such research question can be answered in the setting of a study comparing intake of A2 milk versus conventional milk. It has been shown that GI symptoms, pathophysiological factors such as low-grade immune activation, and diet differ between IBS-C and IBS-D subtypes. Therefore, in this proof-of-concept study, we will explore the acute and mid-term effects of one day of A2 milk versus one day of conventional milk on 1) GI symptoms and 2) BCM-7 levels in separate groups of IBS-C and IBS-D patients. We hypothesize that in individuals with IBS-C and IBS-D, A2 milk will lead to lower GI symptom scores and lower BCM-7 levels compared with conventional milk.

2. OBJECTIVES

Primary Objective:
To explore the acute effects (*i.e.* day 1) of A2 milk versus conventional milk on overall GI discomfort in separate groups of individuals with IBS-C and IBS-D.
We hypothesize that the acute intake of A2 milk causes less GI discomfort when compared with conventional milk in individuals with IBS-C as well as in individuals with IBS-D.

Secondary Objective(s):
1. To explore the mid-term effects (*i.e.* up to 3 days) after 1 day of A2 milk versus conventional milk on overall GI discomfort in separate groups of individuals with IBS-C and IBS-D.
We hypothesize that the intake of A2 milk causes less GI discomfort in the following days when compared with conventional milk in both groups.

2. To explore the acute (*i.e.* day 1) and mid-term (*i.e.* up to 3 days) effects after 1 day of A2 milk versus conventional milk on single GI symptoms and stool characteristics in separate groups of individuals with IBS-C and IBS-D.
We hypothesize that the intake of A2 milk causes less GI symptoms and improve stool characteristics when compared with conventional milk in both groups.

3. To explore the acute effects (*i.e.* day 1) of A2 milk versus conventional milk on BCM-7 in separate groups of individuals with IBS-C and IBS-D.
We hypothesize that the intake of A2 milk causes a lower release of BCM-7 when compared with conventional milk in both groups.

4. To explore the effect of baseline characteristics (*i.e.* stool characteristics, GI immune and defense markers, microbiome and bodyweight) on above mentioned objectives.
We hypothesize that baseline characteristics can be evaluated to predict the effect of A2 milk versus conventional milk on different outcomes.

3. STUDY DESIGN

In this study, the effects of A2 milk will be explored based on a randomized double-blind cross-over design per IBS-subgroup (*i.e.* IBS-C and IBS-D). After the run-in period, each subject will undergo two different intervention periods of one day (Figure 1), during which 200 ml A2 milk or 200 ml conventional milk will consumed twice daily that day, with a wash-out period of 7 days in between. The wash-out period can eventually be prolonged up to 14 days to plan the second intervention day to the convenience of the subject. The choice for a cross-over design is based on the high heterogeneity in GI symptoms between subjects. The order of intervention will be decided by a randomized procedure.

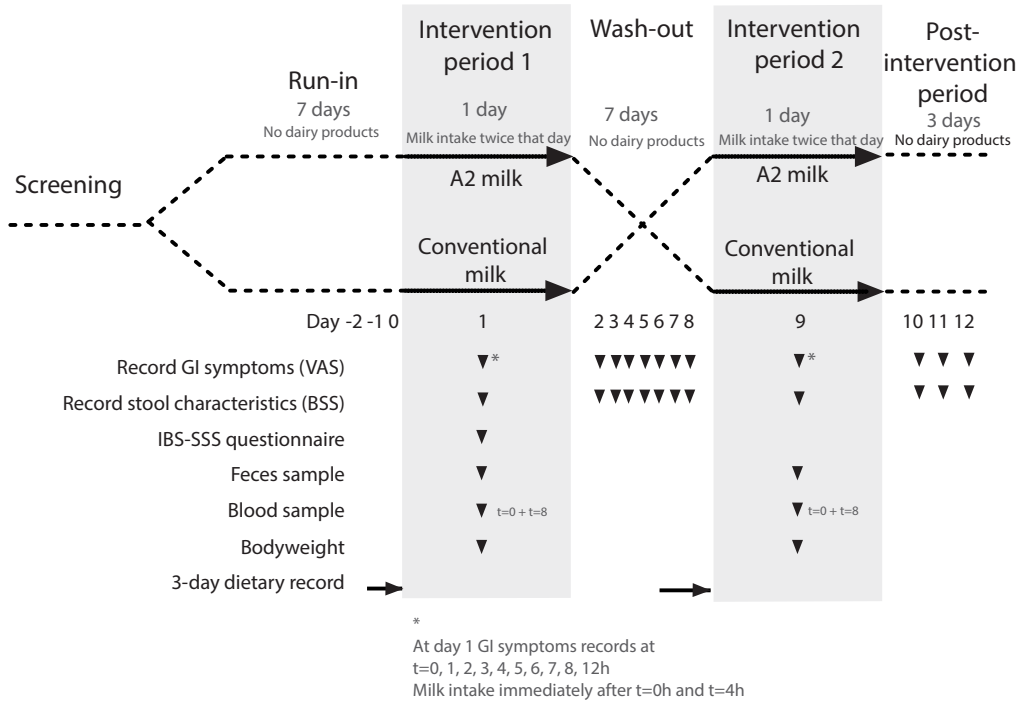

Figure 1. Proposed flow chart of the study design, and timing of data- and sample collection

Subject recruitment will start after the Medical research ethics committee (METC) azM/UM, Maastricht has approved the study according to the Guidelines of the revised version of the Guidelines of the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013). The study will be executed according to International Conference on Harmonisation and Good Clinical Practice (GCP) guidelines.

Volunteers will be recruited using posters in the hospital and university, advertisements in local newspapers, and via the Maastricht IBS cohort (*i.e.* in case an individual gave written permission to approach for future studies). After contact with the researcher by e-mail or telephone, potentially interested subjects will receive detailed oral and written information.

Furthermore, participants will be recruited via the outpatient clinic of the division Gastroenterology-Hepatology of the Maastricht University Medical Centre+ (MUMC+). The treating physician will briefly inform potential eligible patients about the study. If the patient is interested, detailed oral and written information will be provided by the researcher. If the volunteer is interested (as indicated by e-mail or phone), an appointment will be made for a first screening visit. Additional information about goals and study procedures is provided during this visit. If the volunteer is willing to participate, the informed consent (IC) form will be signed (and a copy will be given to the volunteer). Subsequently, screening criteria will be assessed by means of questionnaires, measuring length and body weight. The screening criteria will be completed in a secured, electronic environment (*e.g.* Castor), expect for the personal information (this latter will be completed on paper). The visit will require 30-60 minutes and will take place at MUMC+.
If a subject seems to be eligible for participation, a lactose breath test to check for lactose intolerance (*i.e.* if not done for clinical purposes) can be performed during a second screening visit. The day before this visit, subjects will have to refrain from dairy products, and arrive at the University Hospital in fasting state. This visit will require approximately 3 hours. In an exceptional situation where participant explicitly asks for it, the lactose breath test can also be performed at home. In this case, participants will receive the materials and instructions on how to perform the test during the screening visit. In addition to this, instructions on paper will be given to the participant (see document 'F4_Informatie_lactose-ademtest_V1.0_2022'). The investigator is available for questions via a telephonic consultation when the test is performed at home.
In case there will be any abnormality found during the screening regarding the participant's medical-physiological state, the participant will be excluded, and the medical researcher executing the screening will advise the participant to consult a general practitioner.

The total duration of the study will be 19 days: 1 week run-in period (*i.e.* dairy-free diet), 1 day intervention, 1 week wash-out period (*i.e.* dairy-free diet), 1 day intervention and 3 day post-intervention period (*i.e.* dairy-free diet).

Preceding the first intervention day, the subject starts a run-in period of seven days. During this run-in period, subjects will be instructed to maintain a dairy-free diet. Food records will be completed during the last three days of the run-in period.

Each subject will undergo two intervention days. During each on-site intervention day, after an overnight fast, bio samples will be collected (*i.e.* feces (collected at home) and blood at both intervention days). In addition, bodyweight and length will be measured. Baseline GI

symptoms and stool characteristics will be recorded at the study site via digital tools. The Irritable Bowel Syndrome Severity Scoring System (IBS-SSS) questionnaires will be completed only at the first intervention day at the study site via digital tools. After this, subjects will consume the study product, 200 ml A2 milk or 200 ml conventional milk, twice that day, with a breakfast and lunch brought by the subject to the intervention day (to prevent them from getting any complaints from a standard meal provided by us, as products in this meal might be avoided in daily life), according to the randomization scheme. The participant and the investigator will both be blinded for the intervention. At different time points, GI symptoms will be recorded. At the end of the on-site intervention day, another blood collection will take place at t=8, before the subject can go home. Exact timing of data- and sample collection is shown in figure 1.

After the first intervention day, a wash-out period of 7 days will be applied, before crossing over to the next intervention day. A wash-out period of 7 days is chosen, which ensures a sufficient wash-out period to avoid any carry-over effects and to monitor the mid-term effects (i.e. up to 3 days) of the intake of the study product. The wash-out period will generally last 7 days, but to give some flexibility to the participants' agenda, the duration of the wash-out period can eventually be prolonged up to 14 days. During the wash-out period, the subjects will be asked to continue their dairy-free diet. GI symptoms and stool characteristics will be recorded once daily during the wash-out period. Food records will be completed during the last three consecutive days of the wash-out period (i.e. before the start of the second intervention day).

After the second intervention day, also a post-intervention period of 3 days will be applied, before ending of the study. A post-intervention period of 3 days is chosen, which ensures a sufficient period to monitor eventual mid-term effects of the intake of the study product. During the post-intervention period, the subjects will be asked to continue their dairy-free diet. GI symptoms and stool characteristics will be recorded once daily during the post-intervention period to monitor the mid-term effects of the intake of the study product.

4. STUDY POPULATION

4.1 Population (base)

Individuals (male and female) with IBS will be recruited from the general population, the outpatient clinic, as well as from the Maastricht IBS cohort (NL24160.068.08/METC 08-2-066).

4.2 Inclusion criteria

In order to be eligible to participate in this study, a subject must meet the following criteria:

- Age 18-70 years
- Based on the Rome IV criteria, IBS with constipation (IBS-C) or with diarrhea (IBS-D) being predominant
- Self-reported indication that dietary components (*e.g.* milk) trigger GI symptoms
- Body Mass Index (BMI) < 30 kg/m2
- Weight-stable for at least 90 days prior to participation (no change in bodyweight, *i.e.* < 3kg).
- Willing to be informed in case of unexpected findings.

4.3 Exclusion criteria

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

- History of any disease or surgery interfering with the study aims, limiting participating or completing the study protocol
- Self-admitted human immunodeficiency virus-positive state.
- Disease with a life expectancy shorter than 5 years.
- Abdominal surgery interfering with gastrointestinal function (to be decided by the medical doctors of the study team)
- Based on anamnesis, patients with lactose intolerance will be excluded. If not tested before, a lactose breath test can be performed to check for lactose intolerance.
- Self reported cow's milk allergy
- Use of antibiotics within 30 days prior to the study
- Use of other medication interfering with study outcomes, as will be reviewed by the medical doctors of the study team, who will decide on in- or exclusion based on the drug(s) used
- Administration of probiotic or prebiotic supplements, investigational drugs or participation in any scientific intervention study, which may interfere with this study (to be decided by the medical doctors of the study team), in the 14 days prior to the study

- Known pregnancy or lactation
- Plan to lose weight or follow a specific diet within the study period
- Alcohol intake: female >7 units/week for females, male >14 units/week
- Blood donation within 1 month prior to the study
- Insufficient fluency of the Dutch language

4.4 Sample size calculation

Because this proof-of-concept study is of explorative nature, and as no studies have been performed on the acute effects of A2 milks on overall GI discomfort in Western vulnerable populations such as IBS patients, we based our sample size calculation on a cross-over study investigating the acute effects of another dietary component (*i.e.* gluten) in non-celiac gluten sensitive subjects (17). A change in overall GI symptoms of 15 mm (ranging from 0 to 100 mm) and standard deviation (SD) of 12.8 mm (data obtained by requesting raw data) are used for the sample size calculation, using a randomized cross-over design. Because the current study will be performed in two IBS-subgroups, α will be 0.025 (0.05/2). Power will be 80%. As a result, a sample size of 9 participants is required per IBS subtype. To account for possible dropouts of 10%, we would like to include 10 individuals with IBS-C and 10 individuals with IBS-D.

5. TREATMENT OF SUBJECTS

5.1 Investigational product/treatment

The investigational products in this study are A2 milk and conventional milk (placebo). Both A2 milk and conventional milk are processed under controlled conditions according to conventional method guidelines, and will be prepared from 100% food-grade ingredients suitable for human consumption at VecoZuivel (Zeewolde, the Netherlands). Both products will appear and taste similar.

5.2 Use of co-intervention

Subjects will be asked to refrain from intake of dairy products (apart from the specified A2 milk and conventional milk) as well as pre- and probiotics supplements from the start of the run-in period until the end of the last intervention period. Nutritional supplements like vitamin or mineral supplements are allowed in a stable dose from the start of the run-in period until the end of the last intervention period. Instructions on the consumption of alcohol will be given, so that total intake will not be exceed 14 alcoholic units per week. To be able to evaluate habitual food and beverage intake, we will ask subjects to record their food and beverage intake three days prior to the start of each intervention period. Further, subjects will be asked to maintain their habitual diet.

5.3 Escape medication

Medication such as anti-diarrhoea, laxatives or pain medication can be used after contacting the researcher (which will consult the medical doctors of the study team), and should be noted on paper, and will be taken into account in the analyses. In case pain medication is deemed necessary by the participant, paracetamol is recommended.

6. INVESTIGATIONAL PRODUCT

6.1 Name and description of investigational product(s)

The A2 milk which will be investigated is A2 milk (Organic A2 Dairy, Heino, the Netherlands). The conventional milk which will be used is VecoZuivel organic pasteurized semi-skimmed milk (Zeewolde, the Netherlands). Both A2 milk and VecoZuivel organic pasteurized semi-skimmed milk are natural food products. Both types of milk must be stored in the refrigerator, with a maximum temperature of 7 °C. The appearance (white liquid) and taste (light sweet) of A2 milk is comparable to conventional milk. The specification of both products is similar. Microbiological specifications of both products show a concentration of Enterobacteriaceae <1 cfu/g (colony-forming unit / gram) and is therefore safe for human consumption. The quality of each delivery of the milk products is suitable according to the requirements and is similar to the milk products available in the supermarket.

6.2 Summary of findings from non-clinical studies

Not applicable (NA)

6.3 Summary of findings from clinical studies

Some previous scientific studies investigated the effects of A2 milk versus conventional milk on GI symptoms (*i.e*. flatulence, bloating, abdominal pain, stool frequency and stools consistency) in healthy adults (9), healthy adults with a high rate of lactose malabsorbers (5-7), and in Australian children (8). In those studies, A2 milk and conventional milk intake up to 750 ml/day have been shown to be safe.

6.4 Summary of known and potential risks and benefits

In subjects in which lactose intolerance and cow's milk allergy has been excluded, there are no risks known to be associated with or expected from the intake of A2 milk or conventional milk. Milk is a widely consumed food item, and milk is included in dietary guidelines of a healthy diet.

6.5 Description and justification of route of administration and dosage

A2 milk (400 ml/day) and conventional milk (400 ml/day) will be taken as oral drinks.

6.6 Dosages, dosage modifications and method of administration

After the run-in period of one week, in which subjects are instructed to refrain from dairy products, subjects will start the intake of A2 milk or conventional milk. A2 milk (200 ml/dosage) and conventional milk (200 ml/dosage) will be taken two times during the intervention day. We expect intake of A2 milk (400 ml/day) and conventional milk (400

ml/day) to be safe. During the intervention day, subjects will ingest the study products at the study site. During the intervention day, we will instruct the subjects to ingest the study products at t=0 hours and t=4 hours, in order to make sure that the time of ingestion of the study product corresponds with the frequent questionnaires. The study products will be ingested with a breakfast and lunch brought by the subject (to prevent them from getting any complaints from a standard meal provided by us, as products in this meal might be avoided in daily life), respectively. Subjects are asked to bring equal meals to both test days. What is taken during breakfast and lunch is noted by the researcher. The milk will be packed in 0.5L milk cartons. Study product intake continues until all measurements are finished.

6.7 Preparation and labelling of Investigational Medicinal Product

A2 milk and conventional milk will be provided in milk cartons. Blinding is ensured by the fact that the content and packaging of both products look identical. All products must be stored in the refrigerator, with a maximum temperature of 7 °C.

The package will contain the following information:
- Study name (A2 Milk PoC study)
- Subject code (‘PP-01’, ‘PP-02’ etc), thereby assigning the participant blindly to one intervention order. The code is linked with the name, address, date of birth and telephone number of the subject.
- Date and time of intake
- Expiration date

6.8 Drug accountability

The products will be shipped by delivery courier to the MUMC+. Products will be labelled according to the randomization scheme and stored at the Clinical or Metabolic Research Unit Maastricht. At the intervention day, subjects will receive the 200ml milk twice from the investigator on site. The total volume of milk intake for one subject is 400ml per intervention day. Therefore, only one milk carton of 0.5L has to be delivered. However, to be sure, one spare milk carton will be deliveredin case of spillage and will be stored at the Clinical or Metabolic Research Unit as a backup.

7. NON-INVESTIGATIONAL PRODUCT

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

NA

7.2 Summary of findings from non-clinical studies

NA

7.3 Summary of findings from clinical studies

NA

7.4 Summary of known and potential risks and benefits

NA

7.5 Description and justification of route of administration and dosage

NA

7.6 Dosages, dosage modifications and method of administration

NA

7.7 Preparation and labelling of Non Investigational Medicinal Product

NA

7.8 Drug accountability

NA

8. METHODS

8.1 Study parameters/endpoints

8.1.1 Main study parameter/endpoint

Overall GI discomfort score

The primary outcome is change in overall GI discomfort score after a specific milk intervention in which participants will consume A2 milk or conventional milk. Overall GI discomfort score will be measured as a separate single item using a visual analogue scale (VAS), anchored at the low end (score of 0) with the absence of overall symptoms and at the other end (score of 100) with severe symptoms, the worst it has ever been. A relevant effect is defined as at least a 15mm difference between interventions. During the intervention days, this item will be frequently (*i.e.* at t=0, 1, 2, 3, 4, 5, 6, 7, 8 and 12 hours) completed at day 1 to determine the acute effects. Subsequently, this item will be completed once daily during the wash-out and post-intervention period to determine mid-term effects of one-day intervention.

8.1.2 Secondary study parameters/endpoints

Single GI symptom scores

- Abdominal pain
- Abdominal discomfort
- Belching
- Bloating
- Flatulence
- Diarrhoea
- Constipation
- Urge to empty bowel
- Fullness
- Nausea

All GI symptoms will be measured using a visual analogue scale (VAS), anchored at the low end (score of 0) with the absence of overall symptoms and at the other end (score of 100) with severe symptoms, the worst it has ever been. During the intervention days, single GI symptoms will be frequently (*i.e.* at t=0, 1, 2, 3, 4, 5, 6, 7, 8 and 12 hours) completed at day 1 to determine the acute effects. Subsequently, GI symptoms will be completed once daily during the wash-out and post-intervention period to determine mid-term effects of one-day intervention.

Stool characteristics
Stool characteristics including stool consistency and stool frequency, will be scored by the participants using the Bristol Stool Scale (BSS). During the intervention days, wash-out period and post-intervention period, stool characteristics will be completed once daily for each stool to determine acute and mid-term effects. At the lab, stool consistency will also be measured by determining the fecal dry weight content. For that, samples will be collected at home before both intervention days (during the three-day dietary intake diary) to evaluate baseline characteristics.

Symptom severity
Abdominal pain intensity, abdominal pain frequency, abdominal distension, dissatisfaction with bowel habits, and influence of IBS on life in general ("life interference") during the preceding week, will be scored by the participants using the IBS-SSS questionnaire. The IBS-SSS questionnaire will be completed at day 1 of the first intervention period to evaluate the baseline symptom severity and obtain an extensive characterization at baseline.

GI immune and defense markers, and BCM-7
Several parameters of GI immune and defense including calprotectin, sIgA and HBD in feces, and C-reactive protein (CRP), cytokines and BCM-7 in blood. Samples will be collected at both intervention days to evaluate baseline characteristics (feces will be collected at home before the intervention day (during the three-day dietary intake diary), blood will be collected at t=0 hours at the intervention day). In addition, blood samples to evaluate BCM-7 will also be collected a second time during both intervention days (at t=8 hours) to evaluate acute effects on this parameter.

8.1.3 Other study parameters (if applicable)

Food and beverage intake
Three-day dietary intake will be recorded by completing diary's three days prior to the start of each intervention period, to check for the adherence to the dairy-free diet and to compare data on habitual dietary intake before each intervention day.

Bodyweight
Bodyweight will be determined at the beginning of both intervention days to evaluate baseline characteristics.

8.2 Randomisation, blinding and treatment allocation

Within each group of IBS subtypes (i.e. IBS-C and IBS-D), subjects will be randomized in a double-blind cross-over design to one of the following experimental conditions in each intervention period:

1. A2 milk
2. Conventional milk

The randomization lists will be generated by two co-workers of the same department as the principal investigator and who is not further involved in the study, using a publically available procedure through the internet (https://www.sealedenvelope.com/). The investigator has no access to the randomization lists that conceals the treatment code. The head of the division of Gastroenterology-Hepatology, Prof. dr. D. Keszthelyi, will receive sealed envelopes, to reveal the treatment in case of medical emergency. The condition numbers as allocated by the co-worker, who will carry out the randomization, are registered by the investigator in the screening and enrolment log. Furthermore, the same co-worker will keep the randomizations lists. The 'randomization key' will be provided to the investigator after finishing all experimental and data analyses.

This is a double-blind study. Both study products are supplied in identical packaging. Only by means of the batch number it can be determined whether it concerns conventional milk or A2 milk. The employees of the manufacturer will communicate this information to the two co-workers involved in the randomisation (who are not further involved in the study). The principal investigator (Prof. dr. D. Jonkers), coordinating investigator (Drs. M. Bosman) and subinvestigator (Prof. Keszthelyi) will not be aware of this. The two co-workers involved in the randomization will store this information (including the certificates of study products) and will be provided this to the investigator after finishing all experimental and data analyses.

8.3 Study procedures

GI symptoms and stool characteristics

Participants will be asked to rate their daily GI symptoms on 100-mm VAS scales as well as rating their stool characteristics (*i.e.* stool frequency and stool consistency) during the intervention days, the wash-out and post-intervention period. The diary includes five sections: 1. Stool frequency and consistency - Bristol Stool Scale, 2. Single GI Symptoms, 3. Overall GI discomfort, 4. Medication, 5. Comments. Furthermore, at the each intervention day, sections 2. Single GI symptoms and 3. Overall GI discomfort will be completed more frequently (*i.e.* at t=0, 1, 2, 3, 4, 5, 6, 7, 8 and 12 hours). All diaries

will be completed in a secured, electronic environment (*e.g.* Castor) that can be accessed via the internet by electronic devices (telephone, tablet, or computer).

IBS-SSS questionnaire

At baseline the IBS-SSS questionnaire will be completed. This is a is a widely used questionnaire to assess the symptom severity (18). It measures abdominal pain intensity, abdominal pain frequency, abdominal distension, dissatisfaction with bowel habits, and influence of IBS on life in general ("life interference") during the preceding week on a 0-100 scale, with the total IBS-SSS score ranging between 0 and 500, with higher scores indicating more severe symptoms. The IBS-SSS questionnaire will be completed in a secured, electronic environment (*e.g.* Castor) that can be accessed via the internet by electronic devices (telephone, tablet, or computer).

Feces collection

Fresh fecal samples will be collected in dedicated feces collection buckets at home. Frozen at -20 °C in special safety bags, and will be handed in frozen by the subject to the investigator at the intervention days. One fecal sample will be obtained in the three days before both intervention days (during the three-day dietary intake diary). Feces will be stored at -80 °C until analysis for analysis of GI immune and defense markers. Quantification of fecal calprotectin, sIgA and HBD will be done by using enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions. Fecal dry weight will be determined as parameter of stool consistency. Samples will be dried by means of a vacuum drying procedure using an Eppendorf concentrator plus. Remaining samples will be kept at -80 °C for eventual future analyses of the microbiome as possible predictor of response.

Blood collection

Blood samples will be collected from an antecubital vein in the fore-arm. During blood sampling collection at the beginning of both intervention days 5 mL blood will be collected in a Serum-Separating tube (BD, Vacutainer®) for immunological parameters like CRP. 5 mL blood will be collected in a sodium heparin tube (BD, Vacutainer®) for cytokine analyses, specifically. Another 2 mL blood will be collected in a K2-ethylenediaminetetraacetic acid (EDTA) tube (BD™ P800, with additive DPP-IV Inhibitors) for analysis of BCM-7. Altogether, a total of 12 mL blood will be collected during the baseline blood sampling procedure. In addition, 2 mL blood will be collected in a K2-ethylenediaminetetraacetic acid (EDTA) tube (BD™ P800, with additive DPP-IV Inhibitors) for analysis of BCM-7 a second time during the first intervention day (at t=8

hours) and twice during the second intervention day (at t=0 hours and t=8 hours). Subsequently, all serum and plasma will be divided in separate aliquots, snap frozen in nitrogen, and stored at -80 °C until analysis. Thus, a total amount of 28 mL blood will be sampled per subject during the entire study.

Body weight

At the beginning of both intervention days body weight will be determined using a body scale.

Three-day food records

Before each intervention day, three-day food records will be completed by the subject.

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)

NA

8.5 Replacement of individual subjects after withdrawal

When participants withdraw from the study after signing IC but prior to the test day (before randomisation), or prove to be unsuitable on the basis of screening and therefore cannot participate in the study, an alternative candidate will be selected, in order to meet the calculated sample size/power. We anticipated dropouts (*i.e.* after randomisation) within our sample size calculation, in case participants withdraw from the study after inclusion (*i.e.* after start of the first intervention period). These participants will be included in the analysis, so no alternative candidate will be selected in case of dropout.

8.6 Follow-up of subjects withdrawn from treatment

Volunteers who dropped out of the study will be followed up by the investigator and by the person who is medically responsible for the study, depending on the reason of the drop-out. If drop out is induced by intolerable increase of GI symptoms and subjects will stop milk intake, we will ask them to record when intake was stopped and to continue completing symptom diaries, as part of the follow-up (*i.e.* to see if symptoms decrease).

8.7 Premature termination of the study

There are no expected reasons for premature termination of the study. However, if certain urgent medical problems occur during study (for example SAEs that result in death or are life threatening) the investigator (if necessary together with the medical committee) can decide to halt the study to check whether it is safe to proceed or if termination is preferred.

9. SAFETY REPORTING

9.1 Temporary halt for reasons of subject safety

In accordance to section 10, subsection 4, of the Medical Research Involving Human Subjects Act (WMO), the sponsor will suspend the study if there is sufficient ground that continuation of the study will jeopardise subject health or safety. The sponsor will notify the accredited METC without undue delay of a temporary halt including the reason for such an action. The study will be suspended pending a further positive decision by the accredited METC. The investigator will take care that all subjects are kept informed.

9.2 AEs and SAEs

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 study products (*i.e.* A2 milk or conventional milk). All adverse events, with the exception of IBS-related symptoms (i.e. abdominal pain and -discomfort, bloating, excessive belching, flatulence, diarrhea, constipation, bowel urgency, feeling of fullness and nausea) which the subject has experienced before, reported spontaneously by the subject or observed by the investigator or his staff will be recorded.

9.2.2 Serious adverse events (SAEs)

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

- 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; or
- any other important medical event that did not result in any of the outcomes listed above due to medical or surgical intervention but could have been based upon appropriate judgement by the investigator.

An elective hospital admission will not be considered as a serious adverse event.

The investigator will report all SAEs to the sponsor without undue delay after obtaining knowledge of the events, except for the following SAEs: SAEs not related to the intake of the study product or the study procedures. The principle investigator will decide whether an SAE is related to the study or not.

The sponsor will report the SAEs through the web portal ToetsingOnline to the accredited METC that approved the protocol, within 7 days of first knowledge for SAEs that result in death or are life threatening followed by a period of maximum of 8 days to complete the initial preliminary report. All other SAEs will be reported within a period of maximum 15 days after the sponsor has first knowledge of the serious adverse events.

SAEs that are not related to the study product or study procedures do not require (expedited) reporting to the accredited METC and will be listed and presented to the accredited METC in an annual line-listing. The line-listing will be presented to the METC together with the annual progress report, but as a separate document. In addition, one week after the last colonoscopy we will check the electronic patient file of the MUMC+ on AEs and/or SAEs.

9.3 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.

STATISTICAL ANALYSIS

Statistical analyses will be carried out using IBM SPSS for Macintosh, Version 25.0, Armonk, NY: IBM Corp. Baseline characteristics (including the outcomes of the questionnaires at baseline) will be analysed and summarized using descriptive statistics. The difference between A2 milk and conventional milk will be compared within-subjects per IBS-subtype (*i.e.* IBS-C and IBS-D). To check for carry-over effect, we will 1) compare the baseline values of the first intervention period with those of the second intervention period using the paired-samples t-test, and 2) compare difference in average of the mean outcomes over both periods between arms (*i.e.* intervention orders). Analyses will be done on an intention-to-treat principle.

Primary study parameter(s)

Overall GI discomfort score

To determine the acute effects of the intervention on the overall GI discomfort score, linear mixed model analysis will be conducted using an random intercept and/or slope structure. Fixed factors comprise time (0, 1, …, 8, 12 hours), intervention (A2 milk vs. conventional milk) and period (period 1 vs. period 2). Interactions between these factors will be tested as well.

Secondary study parameter(s)

Overall GI discomfort score

To determine the mid-term effects of the intervention on the overall GI discomfort score, linear mixed model analysis will be conducted using an random intercept and/or slope structure. Fixed factors comprise time (1, 2, 3 days), intervention (A2 milk vs. conventional milk) and period (period 1 vs. period 2). Interactions between these factors will be tested as well.

Single GI symptom scores

To determine the acute and mid-term effects of the intervention on single GI symptom scores, linear mixed model analysis will be conducted using an random intercept and/or slope structure. Fixed factors comprise time (0, 1, …, 8, 12 hours for acute effects; and 1, 2, 3 days for mid-term effects), intervention (A2 milk vs. conventional milk) and period (period 1 vs. period 2). Interactions between these factors will be tested as well.

Baseline characteristics: stool characteristics, symptom severity, GI immune and defence markers and bodyweight

Baseline characteristics will be described as means and standard deviations (in case of normal distribution) or medians and interquartile ranges (in absence of a normal distribution) for continuous variables and frequencies for categorical variables to evaluate the baseline characteristics and obtain an extensive characterization at baseline. In addition, a comparison of most of the baseline characteristics (stool characteristics, GI immune and defence markers and bodyweight) between responders versus non-responders (i.e. effects of the intervention on the overall GI discomfort score versus no effects of the overall GI discomfort score) will be made to evaluate if a prediction can be made of the effect of baseline characteristics on the outcome of each intervention day.

BCM-7

To determine the acute effects of the intervention on BCM-7, linear mixed model analysis will be conducted using an random intercept and/or slope structure. Fixed factors comprise time (t=0 and t=8), intervention (A2 milk vs. conventional milk) and period (period 1 vs. period 2). Interactions between these factors will be tested as well.

Other study parameters

Food and beverage intake

Three-day dietary intake will be recorded to check for the adherence to dairy-free diet and have data on habitual diet. Data will be described as means and standard deviations (in case of normal distribution) or medians and interquartile ranges (in absence of normal distribution).

ETHICAL CONSIDERATIONS

9.4 Regulation statement

This study has to be approved by the METC. The study will be conducted according to the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013), the WMO and the Food and Drug Administration guidelines for the conduct of clinical trials. The general principles of IC, ethics review and data management will be in line with GCP.

9.5 Recruitment and consent

After approval of the study by the METC of AZM/UM subjects will be recruited from the general population by means of advertisements in local newspaper, on social media, posters on notice boards at Maastricht University. If a subject is interested in participation in this study, he or she can contact the researcher by e-mail or telephone, as given by the advertisement. The researcher will provide oral as well as written information. Subsequently, a time period of at least one week is provided to decide whether the volunteer would like to participate.

Furthermore, participants will be recruited via the Maastricht IBS cohort and the outpatient clinic of the division Gastroenterology-Hepatology of the MUMC+. In the latter case, the treating physicians will give short information to potential eligible IBS patients and will ask them if contact information can be forwarded to the researcher to contact them once if they are interested in receiving further information regarding the study. Further information will be provided verbally by the researcher and the written information brochure will be sent by regular mail or email. Subsequently, a time period of one week (minimum) is provided to decide whether the participant would like to participate. The participant is asked to contact the investigator by telephone or e-mail if he or she would like to participate. If the participant does not contact the researcher however, the researcher will contact the participant once (at the earliest 7 days after sending the written information brochure, and only if the participant has given permission to do so during the first conversation). Thereafter, an appointment with the researcher will be made to sign an IC. The treating physician will not be involved in this IC procedure.

All subjects will have to sign an IC prior to the check of screening criteria (see appendix ´Toestemmingsverklaring´). The IC form has to be filled out completely. Both the participant and researcher have to sign the form at the same time. Date of assignment is necessary. The participant will receive a copy of the signed IC form.

Privacy of the subjects will be protected, which means that the study results will be related to an identification code. Codes and related participant information will not be

approachable to individuals by others than the investigator and the project leader. Subjects can, at any time, make an appeal to the independent doctor that has been appointed to this study. Subjects will be informed that their decision to participate is totally voluntary and they can withdraw at any time without giving a reason. Subjects will have the opportunity to be informed about the group results at the end of the study.

9.6 Objection by minors or incapacitated subjects (if applicable)

NA

9.7 Benefits and risks assessment, group relatedness

Participants will not have any direct benefits as a result of participating in this study. The results of this study will be used to better understand whether A2 milk will induce less GI symptoms compared with conventional milk in subjects with IBS.

Milk itself is a harmless food product, which is widely consumed on a daily basis by the general population. However, volunteers may or may not experience mild to moderate GI symptoms after the consumption of the study milk, since the included participants suffer from IBS. Another small burden that participants can experience is the time that they will invest: the screening visit (approximately 1 hour), the two on-site intervention days (each 8,5 hours) and at home by filling out questionnaires. Another potential burden is the dietary and healthy regimen which is required. Participants will be asked to refrain from other dairy products as well as pre- and probiotics supplements. Moreover, we will ask participants to record three-day food records before of each intervention day.

Although the risk of consuming milk in this population is considered to be very low, we still would like to limit any potential risk by excluding participants with lactose intolerance. Furthermore, the inclusion of such patients may lead to a bias in the study and the expected effects. Therefore, volunteers will only be eligible when not self-reported lactose intolerant or if in doubt, a lactose breath test can be performed to rule out lactose intolerance.

Fecal sample collection

Collection of fecal samples does not yield any risk.

Blood sampling

During each intervention day blood will be collected twice by venepuncture via an evacuated tube system. Venepunctures can occasionally cause a transitory local hematoma or bruise. Some participants report pain during venepuncture.

9.8 Compensation for injury

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

The sponsor (also) has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO). This insurance provides cover for damage to research subjects through injury or death caused by the study.

• € 650.000,-- (*i.e.* six hundred and fifty thousand Euro) for death or injury for each subject who participates in the Research;
• € 5.000.000,-- (*i.e.* five million Euro) for death or injury for all subjects who participate in the Research;
• € 7.500.000,-- (*i.e.* seven million five hundred thousand 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.

9.9 Incentives

In compensation for participating, subjects will receive 150 EUR in case of fully completing the study. Proportional amounts will be given in case of incomplete participation. Subjects don't receive payment after completing the screening only. The screening can be seen as a general health check, which would provide beneficial information for the subject. Furthermore, we will reimburse travel expenses for coming to the MUMC+ for screening and test days.

10. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

10.1 Handling and storage of data and documents

Data will be handled confidentially (coded) and the privacy of the participants will be guaranteed, according to the Algemene Verordening Gegevensbescherming (AVG; in English; EU General Data Protection Regulation) and the Uitvoeringswet AVG (UAVG; in English; the Dutch Act on Implementation of the General Data Protection Regulation). All electronic case report forms (eCRFs) (*e.g*. in Castor) will be coded in such a way that no personal information about the participant can be derived.

All samples and data will be coded in such a way that no personal information about the participant can be derived. Each subject will be allocated to an individual code, which will be visible on biological samples. The code contains a participant number in chronological order and a test day number in chronological number. Furthermore, the label of each sample will contain the date of collection. The key to the identification code will also be safeguarded by the coordinating investigator (Drs. M. Bosman) and is also accessible to the principle investigator (Prof. dr. D. Jonkers). All primary documents and data shall be kept for possible inspection for 15 years after the end of the experimental phase of the study and is accessible to the principal investigator (Prof. dr. D. Jonkers), coordinating investigator (Drs. M. Bosman), subinvestigator (Prof. dr. D. Keszthelyi), the Dutch Health Care Inspectorate (Inspectie Gezondheidszorg en Jeugd) and monitors during the study. Samples taken from the subjects during the study will be kept for 15 years after the end of the experimental phase for possible additional analysis. The principal investigator (Prof. dr. D. Jonkers), coordinating investigator (Drs. M. Bosman) and subinvestigator (Prof. dr. D. Keszthelyi) have access to the stored samples. In the IC, subjects indicate whether they give consent for storing and keeping these samples, which may be used for additional analyses in the line of the current investigation. If subjects deny this consent, all samples will be destructed after carrying out the analyses as described in this protocol. The electronic CRF, electronic diary and electronic questionnaires (with use of Castor and LDot) will be developed taking into account current regulations.

10.2 Monitoring and Quality Assurance

A qualified monitor of the Clinical Trial Center Maastricht will monitor the conduct of the study. A specific monitoring plan will be created after classification of the risk of this study in line with local guidelines.

10.3 Amendments

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given. All amendments will be notified to the METC that gave a favourable opinion.

10.4 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, SAEs, other problems, and amendments.

10.5 Temporary halt and (prematurely) end of study report

The investigator/sponsor will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last patient's last visit. The sponsor will notify the METC immediately of a temporary halt of the study, including the reason of such an action. In case the study is ended prematurely, the sponsor will notify the accredited METC 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.

10.6 Public disclosure and publication policy

All trial results, both positive and negative, will be disclosed in agreement with the Centrale Commissie Mensgebonden Onderzoek statement on publication policy. Based on the results of the study, at least one publication will be submitted for publication to a peer-reviewed scientific journal. The authorship of the article shall be determined in appropriate consultation based on a considerable contribution to the set-up and execution of the study and an active participation in publication. Further, this study will be registered in a public trial registry before the first volunteer will be recruited.

11. STRUCTURED RISK ANALYSIS

11.1 Potential issues of concern

a. Level of knowledge about mechanism of action

The study products include A2 milk and conventional milk. Both products originate from cow's milk. Cow's milk is composed of various macronutrients (*e.g.* fats, lactose, whey protein and casein), micronutrients (*e.g.* calcium, potassium) and water. Macronutrients are digested and subsequently absorbed in the small intestine to provide energy (*i.e.* to maintain body functions). Micronutrients and water are absorbed in the small intestine and colon. The difference between A2 milk and conventional milk is in the β-casein fractions. Beta-casein includes A1 and/or A2 β-casein (2). The relative concentrations of A1 and A2 is genetically determined and varies between cow species. Most cows produce a mixture of A1 and A2 β-casein, whereas some cows produce only A2 β-casein (2). A1 β-casein and A2 β-casein differ with regard to the amino acid at position 67 being histidine or proline, respectively (3). During GI digestion, presence of histidine in A1 β-casein results in release of BCM opioid peptides, including BCM-7 (4). These compounds show μ opioid agonistic activity and thereby may induce inflammation and GI symptoms (4). As A2 milk is lacking these potential harmful compounds, it is suggested to be better tolerated than conventional milk. However, the evidence is humans is still limited merely based on studies performed in China and New Zealand in healthy adults with a high rate of lactose malabsorbers, and in Australian children healthy adults. However, further studies are needed on the comparison between A2 milk and conventional milk on prevalent GI symptoms in various geographical populations, and especially in relevant subgroups, such as A1 β-casein that trigger GI symptoms and/or inflammation in irritable bowel syndrome (IBS) patients.

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

The use of conventional milk by consumers is widespread. A2 milk is on the market and used by consumers. No side effects have been reported. The use is considered to be safe in the amounts used in the current study.

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

No, in this study, effects of A2 milk on GI symptoms, and immune and defense markers in IBS-C and IBS-D patients *in vivo* are primarily of interest. This is not feasible to test in for instance animals, since mice are not representative for this specific target population.

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

e. Analysis of potential effect
The dosage of milk used in this study are commonly used and can be considered to be safe for human use.

f. Pharmacokinetic considerations
NA

g. Study population
Individuals (male and female, 18-70 years of age) with IBS (diarrhea- or constipation-prodominant according to the Rome IV criteria) will be recruited, who indicate dietary factors to trigger their symptoms. People with lactose intolerance and cow's milk allergy will be excluded from the study.

h. Interaction with other products
The test products are food products, which are consumed as part of a regular human diet as well. No interaction with other products, posing a health risk to the individual of any kind, are expected.

i. Predictability of effect
Most GI symptoms and markers, which are chosen in this study have been used previously in scientific studies to investigate the effects of A2 milk. Based on those studies, we hypothesize that A2 milk will lead to lower GI symptom scores. However it is difficult to predict the effects of A2 milk in individuals with IBS as specific target group.

j. Can effects be managed?
The risk associated with participating in the intervention is low since the expected effects (*i.e.* GI symptomatology) are only mild-moderate and transient. Participants will be asked to report their symptoms and side effects daily (in the electronic diary). If participants are unable to continue the consumption of the study products due to intolerable symptoms, they can stop consuming the milk, but will be asked to contact the study team. In case of emergency access to adequate medical support is available as the study will be

conducted in the MUMC+ under supervision of Prof. dr. D. Jonkers, researcher at NUTRIM School for Nutrition, Toxicology and Metabolism and head of department of Gastroenterology-Hepatology.

11.2 Synthesis

There are no significant risks expected regarding the study products. The milk products are commercially available and impose no risk to IBS patients apart from temporary symptom induction. Overall, any side effects are expected to be mild and transient.

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