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Supplementation with *Bifidobacteria longum* subspecies *infantis* EVC001 for mitigation of type 1 diabetes autoimmunity - The GPPAD-SINT1A Study Protocol

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Word count: 4672

Keywords: type 1 diabetes, islet autoantibody, probiotic *Bifidobacteria longum* subspecies *infantis* EVC001, GPPAD, SINT1A, prevention trial

Timeline of the study:
Recruitment: 3.0 years
Start (FPFV): April 2021
Intervention: *B. infantis* EVC001
Intervention period: Until age 12 months

Follow-up after intervention: 2.5-5.5 years
Intended End (LPLV): October 2027
Protocol: V 1.0 November 09th, 2020

Abbreviations:

AE	Adverse Events
ADA	American Diabetes Association
B. infantis	*Bifidobacterium longum* subspecies *infantis* EVC001
CC	Coordinating Centre
CFU	Colony Forming Units
CI	Confidence Interval
DSMB	Data Safety Monitoring Board
eCRF	electronic Case Report Form
FPG	Fasting Plasma Glucose
GRS	Genetic risk score
GPPAD	Global Platform for the Prevention of Autoimmune Diabetes
HLA	Human Leukocyte Antigen
OGTT	Oral glucose tolerance test
POInT	Primary Oral Insulin Trial
SAE	Serious Adverse Events
SCFAs	short chain fatty acids
SINT1A	Supplementation with *B. infantis* for mitigation of type 1 diabetes autoimmunity
SNP	Single nucleotide polymorphism
T1D	Type 1 diabetes

ABSTRACT

Introduction: The GPPAD-SINT1A Study is designed as a randomised, placebo-controlled, double blind, multicentre, multinational, primary prevention study aiming to assess whether daily administration of *B. infantis* from age 7 days to 6 weeks until age 12 months to children with elevated genetic risk for type 1 diabetes reduces the cumulative incidence of beta-cell autoantibodies in childhood.

Methods and analysis: Infants aged 7 days to 6 weeks from Germany, Poland, Belgium, UK and Sweden are eligible for study participation if they have a >10.0% expected risk for developing multiple beta-cell autoantibodies by age 6 years as determined by genetic risk score or family history and HLA genotype. Infants are randomized 1:1 to daily administration of *B. infantis* or placebo until age 12 months, and followed for a maximum of 5.5 years thereafter. The primary outcome is the development of persistent confirmed multiple beta-cell autoantibodies. Secondary outcomes are 1. Any persistent confirmed beta-cell autoantibody, defined as at least one confirmed autoantibody in two consecutive samples, including IAA, GADA, IA-2A or ZnT8A, 2. Diabetes, 3. Transglutaminase autoantibodies associated with celiac disease, 4. Respiratory infection rate in first year of life during supplementation, 5. Safety. Exploratory outcomes include allergy, antibody response to vaccines, alterations of the gut microbiome or blood metabolome, stool pH and calprotectin.

Ethics and dissemination: The study is approved by the ethical committees of all participating clinical sites. The results will be disseminated through peer-reviewed journals and conference presentations and will be openly shared after completion of the study.

Registration: clingov id: NCT04769037

Strengths and limitations of this study

- This is the first adequately powered placebo controlled study to test the supplementation with *B. infantis* for mitigation of type 1 diabetes autoimmunity.
- Targets the immune system of the oral and gut mucosa which is considered important for preventing immune-mediated diseases such as type 1 diabetes.
- Includes other health outcomes such as celiac autoimmunity, respiratory infections, allergy, antibody response (IgG titres) to vaccines, alterations of the gut microbiome or blood metabolome, measurement of stool pH and calprotectin.
- The requirement to identify eligible at-risk infants by genetic screening does not allow the study to introduce *B. infantis* supplementation in the first days of life or during pregnancy, when it may be most beneficial for establishing and maintaining a healthy gut microbiome and immune status.
- There could be advantages in using multiple probiotic strains with complementary metabolic capacities.

- It may have been advantageous to add a prebiotic to the *B. infantis* formulation to compensate for the possibility that some mothers could stop breast feeding early into the trial, thereby reducing the availability of the oligosaccharides in breast milk that *B. infantis* metabolises for its health benefits.

INTRODUCTION

Type 1 diabetes (T1D) results from an immune-mediated destruction of the pancreatic islet beta-cells resulting in insulin deficiency. This process is clinically silent and can be identified by circulating autoantibodies to beta-cell antigens (glutamic acid decarboxylase (GADA), islet tyrosine phosphatase 2 (IA-2A), insulin (IAA) and zinc transporter 8 (ZnT8A) (1). Beta-cell autoantibodies occur early in life with a peak incidence period between age 9 months and 3 years (2, 3, 4), and the risk to develop multiple beta-cell autoantibodies exponentially declines with age (5, 6). On the basis of these findings, it is concluded that any interventional therapy given as a primary prevention strategy must be started early in life.

This randomised, placebo-controlled study will evaluate whether supplementation with a daily dose of a probiotic in the first year of life can reduce the risk of developing beta-cell autoimmunity in children identified by the Global Platform for the Prevention of Autoimmune Diabetes (GPPAD) as being genetically at risk for developing T1D (previously described in detail in 7 and 8). The rationale for this study combines the most recent findings concerning the specific role that the commensal bacteria, microbiota, and their genes, the microbiome, could play in the induction of peripheral tolerance to insulin (9, 10, 11, 12), and builds on knowledge of the reported health and clinical benefits of early probiotic supplementation in peanut allergy prevention (13) and in lowering mortality owing to sepsis in children (14). Although it is widely accepted that the induction of the state of tolerance to beneficial bacteria during early life is critical for a newborn's survival, the clinical benefit for the prevention of immune-related diseases is only now being explored. It is believed that tolerance is achieved when the innate and adaptive immune cells promote tolerogenic immune responses to dietary and commensal antigens as well as reactions to a variety of beneficial metabolites produced by commensal microbes, in particular the short chain fatty acids (SCFAs) (15, 16, 17, 18, 19). The SINT1A study follows the ongoing Primary Oral Insulin Trial (POInT) (8), which administers insulin orally to train and sensitize the immune system at an early stage via gut exposure so that autoimmunity against insulin does not occur.

Evidence for dysbiosis in children prior to the development of type 1 diabetes

Evidence that microbiome changes may alter the risk for T1D is presented by a number of prospective studies which have shown that changes in the microbiome precede the development of beta-cell autoimmunity and T1D. These include the BABYDIET study where alterations in microbial interaction networks were observed at age 0.5 and 2 years in children who developed beta-cell autoimmunity (9), and studies from Finland where higher abundances of *Bacteroides dorei* (20) and a decrease in microbial diversity were described in children with genetic predisposition to T1D (21). The TEDDY study

confirmed these alterations and found that the microbiome of children who did not develop beta-cell autoimmunity contained more genes that were related to fermentation and the biosynthesis of short-chain fatty acids (SCFA) supporting the protective effects of SCFA in early-onset human T1D (10). Furthermore, the TEDDY study has reported that probiotic supplementation in the first 27 days of life, and only in this early period (documented by questionnaires and diary records) was associated with a decreased risk of beta-cell autoimmunity providing the first evidence that correcting dysbiosis in infants may be beneficial for children at risk of T1D (11). A recent finding links microbial metabolism to insulin-specific immune responses: the microbial enzymes belonging to the transketolase superfamily contain the primary insulin autoantigenic epitope (INS B:9-25). The microbial transketolase upregulation reflects the adaptation of the microbiome to digest sugar polymers during weaning and matches the time of autoantibody appearance against insulin. It has been suggested that an immune response to insulin mimotopes due to commensal dysbiosis is a possible primary cause of T1D (12).
Altogether, these results suggest that correcting dysbiosis in early life could help to promote immune tolerance and thus inhibit the initiation of beta-cell autoimmunity.

Previous clinical studies using B. *infantis* in children

A substantial body of evidence has connected gut inflammation with improper immune programming and the subsequent development of autoimmune conditions including T1D, atopic dermatitis, food allergies and asthma (19, 22). Bifidobacteria and in particular *Bifidobacterium longum* subspecies *infantis* (*B. infantis*) have positive properties that potentially counteract the development of gut inflammation in the first few months of life (23, 24). Depleted numbers of *Bifidobacteria* are associated with immune-related diseases such as allergy (25, 26). *Bacteroides* species, which are naturally outcompeted by *Bifidobacteria*, are present at higher numbers within the microbiota of children with high susceptibility to autoimmunity (19). There is additional evidence that Bifidobacterium abundance in early infancy increases protective efficacy of vaccines by enhancing immunologic memory (27). Lactic acid bacteria and bifidobacteria are increasingly administered to pregnant women and infants with the intention of improving health. A number of clinical studies have been conducted to document the safety and health benefits of dietary supplementation with bacterial strains (probiotics). Previous clinical studies using *B. infantis* are summarized in **Table 1**.

Table 1: Clinical studies on *B. infantis*

Reference	Number of participants*	Main results
(28)	80	Safe consumption and good tolerance of *B. infantis*; stools significantly fewer and better formed
(29)	66	Significant changes to faecal microbiome composition; colonization with *B. infantis*; higher abundance of faecal short chain fatty acids; lower stool pH
(30)	40	Lower fecal calprotectin levels; lower enteric inflammation

* participants in total, meaning the group of children *B. infantis* fed and the group placebo fed (for all: treatment from day 7 on, dose 1.8-2.8 × 10^{10} CFU (colony forming units)), all participants were breast-fed infants

These studies show first evidence that supplementation with *B. infantis* is safe (28, 29, 30). The IMPRINT study demonstrates that supplementation with *B. infantis* (1.8-2.8 × 10^{10} CFU) for 14 days (from day 7 to day 21) is well tolerated (28). Stools are fewer and better formed in infants in the supplementation group compared to the non-supplemented group. A follow-up study indicates that supplementation results in significant changes of the faecal microbiome composition (29) including evidence of persistent colonization of the probiotic organism. Infants colonized by Bifidobacteriaceae have 4-fold-lower faecal endotoxin levels, consistent with observed lower levels of Gram-negative Proteobacteria and Bacteroidetes, lower stool pH, and higher faecal concentrations of short chain fatty acids (29). In addition, pro-inflammatory cytokines are significantly lower in *B. infantis*-fed infants (30). The mentioned results suggest that correcting dysbiosis in early life could help to promote immune tolerance and thus inhibit the initiation of beta-cell autoimmunity. In this context, a supplementation with *B. infantis* seems promising.

Primary Objective

To determine whether daily administration of *B. infantis* from age 7 days to 6 weeks until age 12 months to children with elevated genetic risk for T1D reduces the cumulative incidence of beta-cell autoantibodies in childhood.

METHODS

Outcome measures

Primary outcome

The primary outcome of SINT1A is the elapsed time from random treatment assignment to the development of persistent confirmed multiple beta-cell autoantibodies. For subjects who developed persistent confirmed multiple beta-cell autoantibodies, the elapsed time will be from the random treatment

assignment to the first confirmed autoantibody positive sample used in defining the persistent confirmed multiple beta-cell autoantibody positive status. It is expected that beta-cell autoantibodies will be detected prior to T1D diagnosis; however, the presence of diabetes in the absence of multiple beta-cell autoantibodies is also considered as a primary outcome endpoint, and in this case, the date of diagnosis is the time of the end point.

The study primary outcome is realized with either persistent confirmed multiple beta-cell autoantibodies or Oral Glucose Tolerance Test (OGTT) criteria for diabetes or clinical criteria for diabetes.

Criteria for persistent confirmed beta-cell autoantibodies

Criteria are based on the measurement of beta-cell autoantibodies against insulin (IAA), GAD65 (GADA), IA-2 (IA-2A), and ZnT8 (ZnT8A) tested in the GPPAD central autoantibody laboratory and, if positive, confirmed in the GPPAD confirmatory laboratory.

Confirmed IAA is defined as sample positive for IAA in both the GPPAD central and confirmatory laboratories. Confirmed GADA is defined as sample positive for GADA in both the GPPAD central and confirmatory laboratories. Confirmed IA-2A is defined as sample positive for IA-2A in both the GPPAD central and confirmatory laboratories. Confirmed ZnT8A is defined as sample positive for ZnT8RA or ZnT8WA in both the GPPAD central and confirmatory laboratories.

The status persistent confirmed beta-cell autoantibody-positive is defined as confirmed IAA, confirmed GADA, confirmed IA-2A, or confirmed ZnT8A in two consecutive samples. Persistent confirmed multiple beta-cell autoantibodies (primary outcome) is defined as confirmed IAA, confirmed GADA, confirmed IA-2A, or confirmed ZnT8A in two consecutive samples, AND a confirmed second antibody from these four antibodies in one sample. Persistent confirmed beta-cell autoantibodies that are considered maternally derived are NOT included as positive for the primary outcome.

Criteria for T1D diagnosis

Diabetes may be diagnosed in a small number of children before a persistent confirmed multiple islet autoantibody positive status is achieved as the multiple autoantibody outcome requires two consecutive positive samples. In these cases, the primary outcome status is assigned to the child.

Criteria for T1D diagnosis are, as defined by the American Diabetes Association (ADA), based on glucose testing, or the presence of unequivocal hyperglycaemia with acute metabolic decompensation (diabetic ketoacidosis). One of the following criteria must be met on two occasions as soon as possible but no less than 1 day apart for diabetes to be defined:

1. Symptoms of diabetes and a casual plasma glucose ≥200 mg/dL (11.1mmol/L).

Casual is defined as any time of day without regard to time since last meal. The classic symptoms of diabetes include polyuria, polydipsia, and unexplained weight loss.

OR

2. Fasting plasma glucose (FPG) ≥126 mg/dL (7 mmol/L). Fasting is defined as no caloric intake for at least 8 hours.

OR

3. Two-hour plasma glucose (PG) ≥200 mg/dL (11.1 mmol/L) during an OGTT. The test should be performed using a glucose load containing the equivalent of 1.75g/kg body weight to a maximum of 75g anhydrous glucose dissolved in water. It is preferred that at least one of the two testing occasions involve an OGTT.

Cases diagnosed with T1D will be adjudicated by the Endpoint Committee. Study participation will be terminated if T1D is reached.

Secondary outcomes

Secondary outcomes of the study are: 1. The development of any persistent confirmed beta-cell autoantibody, defined as at least one confirmed autoantibody in two consecutive samples, including IAA, GADA, IA-2A or ZnT8A, 2. Diabetes, 3. The development of persistent confirmed transglutaminase antibodies associated with celiac disease, defined as confirmed autoantibody in two consecutive samples, 4. Respiratory infection rate in first year of life during supplementation and 5. Safety.

Exploratory outcomes

The following exploratory outcomes may be assessed or in part assessed on a portion of the participants. They may not necessarily be included in the primary outcome analysis and publication: 1. Allergy, 2. Antibody response (IgG titres) to vaccines, 3. Alterations of the stool microbiome or 4. Blood metabolome, 5. Stool pH and 6. Stool calprotectin concentration.

Study design and organisation

SINT1A will be conducted as an investigator-initiated, randomized, placebo-controlled, double-blind multi-center intervention study through GPPAD, a network of collaborating clinical study centres from European countries with sites in Belgium (Leuven), Germany (Dresden, Hannover, Munich), Poland (Warsaw), Sweden (Malmö), and UK (Newcastle, Cambridge). The Trial Coordinating Centre (GPPAD CC) is located at the Institute of Diabetes Research, Helmholtz Zentrum München. It manages coordination and communication between the SINT1A clinical study sites, and oversees the collection, analysis and storage of clinical data; also the supervision of regulatory activities, clinical research organization activities, the manufacturer of the active supplement, and the central laboratories is provided by the CC.

GPPAD was founded in 2015 with the aim to provide an international infrastructure to enable T1D primary prevention trials, identify infants with an elevated genetic risk of developing T1D and offer participation in randomized controlled trials aiming to reduce the incidence of T1D in children (8, 31).

Until March 2021, more than 251,000 infants have been screened and had their genetic risk of developing T1D evaluated using a combination of family history and 47 single nucleotide polymorphisms (SNPs) (7). From these, over 2,000 infants have been identified as having a 10% probability or greater of developing multiple beta-cell autoantibodies by 6 years of age, making them eligible for GPPAD primary prevention trials (7). The first GPPAD trial POInT (Primary Oral Insulin Trial) has now (March/2021) completed enrollment (1,050 participants) (8). SINT1A will commence in April 2021 with the first patient first visit.

Study population

Infants are tested for genetic risk of T1D based on advanced risk scores derived from 51 SNPs that define HLA-DR3, HLA-DR4, and HLA-DQ8 alleles as well as SNPs from HLA class I, and non-HLA T1D susceptibility genes, and from HLA class II protective alleles, as previously described (7, 32). Infants with a predicted risk of >10% to develop multiple beta-cell autoimmunity by age 6 years and who fulfil the inclusion criteria as stated below are eligible to participate in the GPPAD-SINT1A Study.
A total of 1,144 infants will be enrolled and randomized 1:1 (*B. infantis* or placebo) in the SINT1A study (see **Figure 1**). Children with T1D susceptible genotypes also have a marked risk of around 10% for autoimmunity found in celiac disease as shown in the TEDDY study (33).

Inclusion and exclusion criteria

Participants must meet all entry criteria for the protocol as outlined below.

- Infants between the ages of 7 days and 6 weeks (+14 days in case of illness or COVID-19 related issues or unexpected delay in result reporting) at the time of randomisation.
- A 10% or higher genetic risk to develop multiple beta-cell autoantibodies by age 6 years:
    a. For infants without a first-degree family history of T1D, high genetic risk is defined as a DR3/DR4-DQ8 or DR4-DQ8/DR4-DQ8 genotype and a genetic risk score that is in the upper 25th centile (>14.4) (34) or a DR3/DR4-DQ8 genotype with a GRS between the upper 50th (14.0) and 25th centile and a GG genotype at the rs3763305 SNP. These represent around 1% of all newborns.
    b. For infants with a first-degree family history of type 1 diabetes, high genetic risk is defined as having HLA DR4 and DQ8, and none of the following protective alleles: DRB1*1501, DQB1*0503, DRB1*1303. These represent around 30% of infants with a first-degree family history of T1D.
- Written informed consent signed by the custodial parent(s).

Participants may not enter the study if ANY of the following apply:

- Any medical condition, concomitant disease or treatment that may interfere with the assessments or may jeopardize the participant's safe participation in the study, as judged by the Investigators.
- Preterm delivery < 36 weeks of gestation.
- Proven immunodeficiency.
- Any condition that could be associated with poor compliance.
- Diagnosis of diabetes at the time of recruitment.

Informed Consent

The GPPAD-SINT1A Study will be described to the custodial parent(s) of potential participants by qualified GPPAD study personnel. The custodial parent(s) will have the opportunity to read the consent document and to discuss any questions concerning the consent or study participation. The families will be given enough time to consider whether or not to participate. The custodial parent(s) will then be asked to sign and date an informed consent form prior to or at the baseline visit. Date and signature of the study Investigator (or other authorized study personnel, if applicable) will also be obtained on the consent form. A copy of the informed consent form will be handed out to the families. The custodial parent(s) of the prospective participant will be told that being in the study is voluntary and that the participant may withdraw from the study at any time, for any reason.

Patient and Public Involvement

Patients were not involved in the study design but in the prioritization of the research question of T1D prevention. Patients support recruitment through dissemination, and participation in press conferences. Participating families will be informed about the outcome of the trial via webcast, letter, and personal communication upon the completion of the trial.

Randomisation

Subjects will be centrally randomised in a 1:1 ratio to one of the two intervention arms at the baseline visit. Siblings within one household will be randomised to the same intervention arm to avoid mix-up of supplementation. Randomisation will be stratified for whether the child is still breast-fed at the date of randomization and study centre.

Study timeline

The study is expected to take 6.5 years to complete. This includes an intervention phase of 12 months, and minimum 2.5 (last participant enrolled) to maximum 5.5 years of follow-up (first participant enrolled) after intervention (Figure 1). The enrolment period is projected to be 3.0 years.

Study assessment

The baseline visit includes the collection of information on medical history, C-section, breast-feeding, proton pump inhibitor therapy, infections, probiotic use and antibiotic treatment prior to enrolment and the collection of a stool sample. Families will be instructed in the administration and storage of the blinded food product (*B. infantis* or placebo). Mothers will be encouraged to make their best effort to maintain breastfeeding for at least the first 3-4 months, and they will be encouraged to continue breastfeeding for as long as possible during the first year of the infant's life. Two more intervention visits (visit 2 and 3) are planned after 6 months and 12 months (± 14 days). At these visits venous blood and a stool sample is collected, electronic questionnaires are discussed. The assessment of weight and height is performed at all visits. After the intervention period, study visit 4 will be conducted at age 2 years (± 30 days), subsequent follow-up study visits will be conducted every year (± 30 days) until the end of the study. A detailed table explaining study visits, and data and sample collection is shown in **Supplementary File 1**. All study relevant subject data and laboratory results are documented in corresponding electronic Case Report Forms (eCRFs).

E-diaries and Allergy questionnaires

Throughout the intervention period, parents will be asked to complete e-diaries fortnightly to collect information on breast-feeding, respiratory infections, antibiotic treatment and treatment with proton pump inhibitors. Additional questionnaires to obtain information about allergies will be collected every 12 months starting at age 12 months ± 14 days until the end of the study. The information given by the parents will be captured in a central database and reviewed and discussed during the study visits and phone calls between the visits.

For participants who develop positive beta-cell or transglutaminase autoantibodies

Participants who have confirmed positive beta-cell- or transglutaminase autoantibodies during the study are asked to donate a confirmation sample within 4-12 weeks. If the participant has persistent confirmed beta-cell autoantibodies, the custodial parent(s) are informed and asked to participate in an educational program informing about the diagnosis of beta-cell autoantibody positivity and possible symptoms of hyperglycemia and metabolic decompensation. The child remains in the study and continues to be treated

or followed as planned until the child has developed T1D or end of study. Home monitoring of blood glucose will be recommended 2-monthly if a child is considered at risk for a rapid progression to diabetes (eg IA-2A positive, very high titers of antibodies, or impaired blood glucose values). In case of persistent confirmed positivity for transglutaminase autoantibodies, an intestinal biopsy maybe recommended to clarify the diagnosis of celiac disease. These children will continue to receive *B. infantis*/placebo and will be followed in the study for continued monitoring of diabetes development and safety assessments.

Intervention

Participants are randomized in a 1:1 ratio to receive either *B. infantis* or placebo. Each dose of the active supplement is provided as one sachet with *B. infantis*, 8 x 10^9 colony forming units (CFU) in lactose. The placebo consists of lactose, identical in appearance and taste to the active supplement.
B. infantis/placebo will be administered orally, once a day, using single-dose sachets. The content (powder) of the sachets is poured into a small bowl and mixed with 3-5ml of breast milk, infant formula, or water. The solution will be administered using a feeding syringe, preferably in the morning. Parent(s) will be instructed in the administration and storage of the sachets (should be kept frozen until use) at or prior to their baseline visit. The genome of *B. infantis* is available in the NCBI accession number NZ_LR655210 under the strain name USA001_1 (35).

Safety

As the study intervention is not considered a medicinal product, safety reporting obligations as for IMP clinical trials do not apply. However, AEs and serious adverse events (SAEs) up to 30 days after the last administration of the food product are assessed and captured in the eCRF. Adverse events will be graded as mild, moderate, severe, life-threatening or death according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (NCI-CTCAE) Version 5.0.
Physical examinations including measurement of height and weight are performed at all visits.

ANALYSIS

All efficacy analyses will be conducted under the Intention-To-Treat principle whereby all effectiveness outcome data in all randomised subjects who have received at least one dose of *B. infantis* supplement or placebo will be included in all analyses as appropriate. Subjects who drop-out of the study will not be replaced. All data acquired prior to termination will be included in the primary analysis unless a participant withdraws consent.

Primary outcome and analysis

The cumulative incidence of multiple beta-cell autoantibodies over time since randomization within each treatment group will be estimated from a Kaplan-Meier estimate of the "beta-cell autoantibody-free" survival function. The difference between groups in the cumulative incidence functions, and the associated hazard functions, will be tested at the 0.05 level, two-sided, using Cox regression including site as covariate. With respect to the primary outcome, the hazard ratio of *B. infantis* to placebo will be given together with its 95% confidence interval. The final alpha is 0.05 (two-sided). In case the assumptions of the sample size estimation hold, it will be possible to reject the null hypothesis of equal hazard rates with the power of 80%, if 914 children will be uniformly randomised over 3 years and afterwards, all 914 children will be followed for another 3.5 years (6.5 years total duration after first enrollment). We have assumed a drop-out rate of 20%, and therefore we need to randomise 1,144 children to support an 80% power by a complete follow-up of 914 children ranging from 3.5 to 6.5 years.

Sub-group analyses of Primary Outcome

It is reasonable to consider that *B. infantis* colonization, breast-feeding status, and secretor (FUT2 gene) status may impact the outcome (36, 37). *B. infantis* colonization will be measured in stool samples collected at visit age 3 months. A multiple Cox regression analysis will be performed on the primary outcome including treatment group and colonization as a continuous variable. This will also be performed with a treatment group-colonization interaction term, and with colonization as a categorical variable. Three categories representing no colonization, low and high colonization based on the distribution of values in the children will be used. Breast-feeding will be encouraged. Nevertheless, a portion of the children will not be receiving breast milk when they start. Breast-feeding will, therefore, be included as a categorical variable together with treatment in a Cox regression. An interaction analysis between colonization and breast-feeding status will be performed, and a subgroup analysis for the primary outcome will be performed in children categorized by their FUT2 gene genotype as secretors and non-secretors. Additional variables that will be analysed by multivariable Cox regression include 1. Children categorized as having a mother with diabetes versus the remainder; 2. Children who have HLA DR3/4-DQ8 versus the remainder; 3. Children who have the T1D susceptible INS AA genotype versus the remainder; 4. Sex; 5. Caesarean section versus vaginal birth; 6. BMI at age 1 year as tertiles; 7. Genetic risk score tertiles.

Secondary and exploratory outcomes and analyses

For the secondary outcomes, the treatment arms will be compared on the corresponding incidence rates of each secondary outcome using the log rank statistic. Other secondary outcomes will be analysed by

comparison between the *B. infantis* supplementation and placebo supplementation groups using appropriate statistics in dependence on the outcome of interest. Subgroup analyses analogous to those described for the beta-cell autoantibodies endpoint will be conducted on the secondary outcome endpoints. Exploratory analyses will examine the associations between *B. infantis* supplementation and allergy, mouth and stool organisms (microbiome), and blood markers such as the metabolome, lipidome, or inflammatory proteins and ancillary study measurements that specific sites may undertake.

Study power and accrual target

For the sample size estimation, the following scenario was chosen:

- Overall alpha level = 0.05 (two-sided).
- Overall beta level = 0.2, i.e. power = 0.8.
- In the placebo group, at 3.5 years (approximate age of participants, 4 years), an event probability of 7.5% was assumed. Based on the exponential distribution, this leads to a hazard of 0.02227.
- For the active treatment, it is assumed that the hazard rate will be halved.
- Accrual time is 3 years.
- Follow-up time is 3.5 years.
- A dropout rate of 20% was taken into account.

The study has been designed to provide 80% power to detect a 50% risk reduction in the hazard rate of the event of confirmed persistent beta-cell autoantibodies using a two-sided test at the significance level 0.05 after 6.5 years of study duration. Decisive test will be the Wald test for the hazard ratio between the two groups within a Cox PH model. It is expected that the hazard is halved by active treatment. According to the assumptions described in above scenario, n=914 patients should be randomised between the two groups. With an assumed drop-out rate of 20%, n=1,144 children will need to be randomised.

Benefits and Risks

Benefits

The potential benefit for a participating child is the prevention (or delay in onset) of beta-cell autoantibodies and diabetes, celiac disease, childhood infections, and allergy. For all participating children, including children who receive placebo, testing blood samples will allow early recognition of pre-symptomatic T1D and celiac disease before the child shows the typical symptoms, and an appropriate therapy could be started immediately, potentially reducing complications later in life. Furthermore, information about other ongoing prevention trials or available treatments and intervention studies can be given to families.

Risks

So far, there have been no reports of risks and side effects associated with the use of *B. infantis*. Several studies show that various strains of *B. infantis* have been administered to numerous newborns and especially to premature babies without complications. In breastfed babies, *B. infantis* is one of the most common bacteria found in the intestine. Typical risks or complaints associated will taking a blood sample cannot be excluded. The volume of blood drawn for the trial endpoints is <1% of the total blood volume, within the suggested limits from the European guidelines for a paediatric population (38).

A data safety monitoring board (DSMB) is established. A DSMB is an independent group of experts responsible to safeguard the well-being and safety of the study participants. The DSMB will meet six-monthly during the intervention phase and 12-monthly during the follow-up phase. Before each DSMB meeting, the DSMB will receive a report with all relevant information on recruitment rate, data completeness and safety data, including beta-cell- and transglutaminase autoantibody and diabetes outcomes.

ETHICS AND DISSEMINATION

Ethics

The study was approved by the local ethical committees and regulatory authorities of the Technische Universität München, Medical Faculty (715/20 S), the Technische Universität Dresden SR+BO-44012021), the Medizinische Hochschule Hannover (9589_BO_S_2021), the Medical University of Warsaw (KB/5/2021) and the Institute of Mother and Child in Warsaw, the UK Health Research Authority, EC Research UZ Leuven (322) and the Swedish ethical review authority (dnr 2021-01210).

Dissemination

GPPAD is committed to sharing of data and biosamples in compliance with all applicable European and GPPAD Consortium Member State, Data Protection and Privacy Protection laws, rules and regulations. Pseudonymized data collected through clinical studies will be made available for scientific and/or medical research no later than twelve months after the completion and publication of the clinical study. GPPAD provides access to biobank material gathered from study participants to external investigators, respecting study participants' privacy rights.

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Authors' contributions:
AGZ, MLP and JAT conceived the study, and led the protocol team. All authors (AGZ, SA, AK, PA, RB, EB, KC, HEL, MG, JH, OK, ML, MO, MLP, MP, MDS, AS, JAT) contributed to protocol development. EB, JH, and MP developed the statistical design for the study and wrote the statistical section of the protocol. EB and PA developed the outcome definition of the trial and wrote the autoantibody and outcome section of the protocol. AGZ, SA, AK, and MG drafted the manuscript. All authors reviewed the protocol as well as this manuscript.

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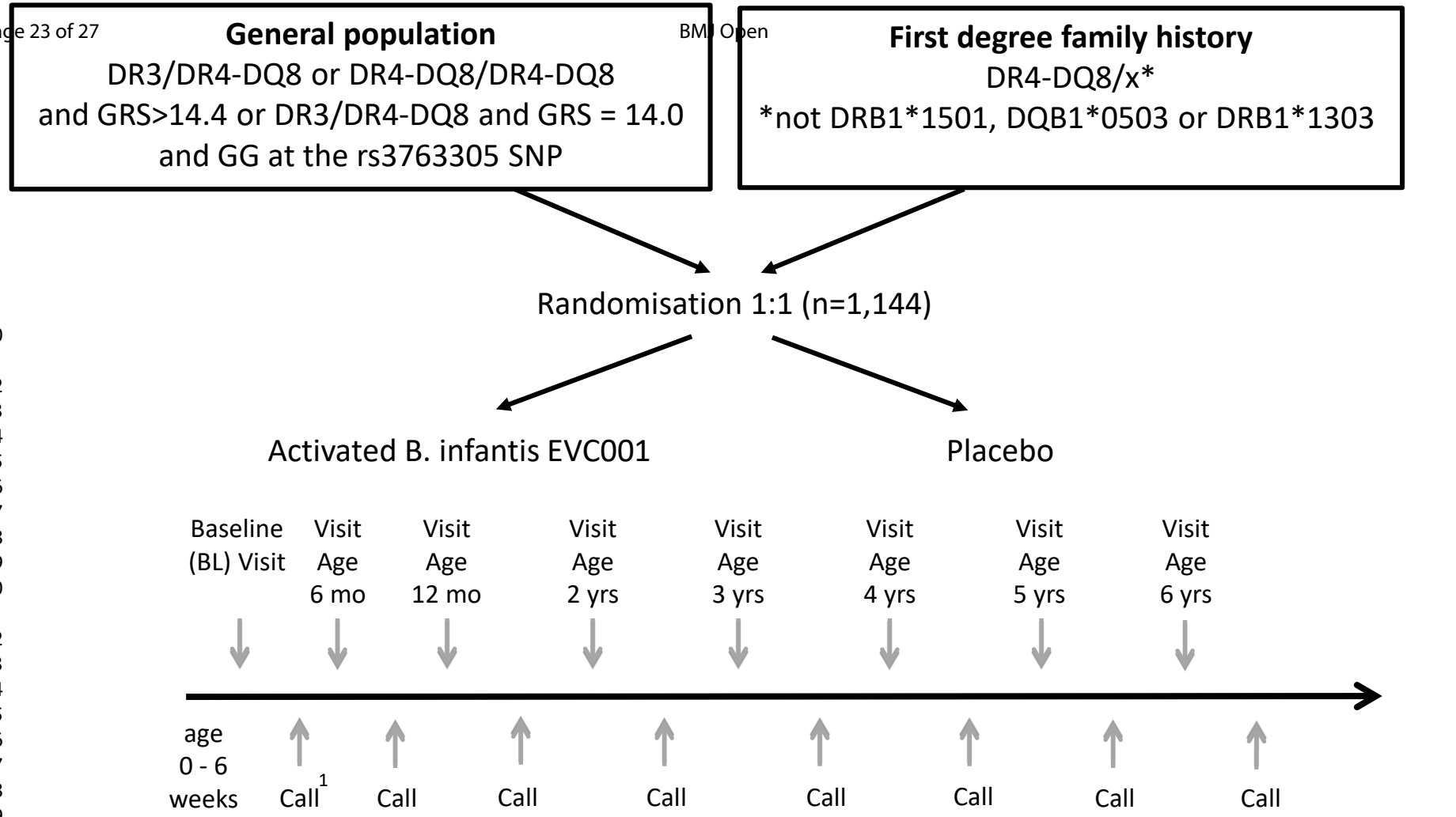
Competing interests
JAT is a member of the Scientific Advisory Board of Precion Ltd and of the Human Genetics Advisory Board of GSK.



Primary outcome: Persistent confirmed multiple beta-cell autoantibodies
Secondary outcomes: Any persistent confirmed beta-cell autoantibody, diabetes, transglutaminase antibodies, respiratory infection rate, safety
Exploratory outcomes: Allergy, antibody response to vaccines, alterations of the gut microbiome or blood metabolome, stool pH and calprotectin

[1] interim telephone calls with families to asses AEs and support trial adherence

Supplementation with *Bifidobacteria longum* subspecies *infantis* EVC001 for mitigation of type 1 diabetes autoimmunity - The GPPAD-SINT1A Study Protocol

Anette-Gabriele Ziegler, Stefanie Arnolds, Annika Kölln, Peter Achenbach, Reinhard Berner, Ezio Bonifacio, Kristina Casteels, Helena Elding Larsson, Melanie Gündert, Joerg Hasford, Olga Kordonouri, Markus Lundgren, Mariusz Oltarzewski, Marcin L. Pekalski, Markus Pfirrmann, Matthew D. Snape, Agnieszka Szypowska, John A. Todd and the GPPAD Study group

Supplementary File 1: GPPAD-SINT1A Study: Visit–schedule (Study Flow Chart)

	Intervention				
Visits	**Baseline Visit age 7 days - 6 weeks**	**Call age 3months**	**Visit age 6 months**	**Call age 9 months**	**Visit age 12 months**
Visit window	+14d	- 14d	± 14d	± 14d	+ 14d
Study visit	1		2		3
Study call		1		2	
Informed consent, Review Incl./Excl. Criteria	X				
Randomization	X				
Medical History	X				
Intervention					
Dispense supplement and compliance data sheet *(do not administer supplement at study site, only at home)*	X		X		
Local investigations & measurements					
Physical examination (height, weight)	X		X		X
Assessment of AEs and SAEs[A]		X	X	X	X
Assessment of rotavirus and MMR vaccination schedule using official records			X		X
Blood glucose[B]			X		X
HbA1c					X
Sample collection					
<200 µl capillary or venous blood for glucose			X		X
< 1ml EDTA blood for HbA1c					X
2ml blood for serum samples for central antibody measurement [C, D]			X		X
2ml EDTA blood for plasma samples for mechanistic studies (inflammation, metabolomics)			X		X
2 ml EDTA blood for DNA sample [C]					X

Visits	Intervention				
	Baseline Visit age 7 days - 6 weeks	**Call age 3months**	**Visit age 6 months**	**Call age 9 months**	**Visit age 12 months**
Stool sample for microbiome 16S	X	X	X		X
Stool sample for colonization		x			
Stool sample for stool pH & calprotectin *(in selected participants)*			X		
Central measurements					
IAA; GADA; IA-2A; ZnT8RA; ZnT8WA			X		X
TGA			X		X
Stool PCR for B. infantis colonization		X			
Antibody responses to rotavirus vaccine			X		
Microbiome 16s [E]	X	X			X
Mechanistic markers (inflammation, metabolomics) [E]			X		X
Electronic questionnaires completed by families					
Questionnaire about breast-feeding and antibiotics	every 2 weeks until age 12 months				
Questionnaire about infections and vaccinations	every 2 weeks until age 12 months				
Questionnaire about allergies					X
Ancillary assessments					
Whole blood FACS[F] *(Dresden and Munich only)*			X		X

[A] AEs/SAEs will be noted and reported as under intervention phase for 30 days after end of treatment day

[B] by handmeter or haemocue

[C] if there is left over material and a signed biobank consent, the left over serum and DNA will be stored in the IBBL or local biobank

[D] venous or capillary blood for the AAB confirmation sample can be obtained by a local physician

[E] measurements may partly be done as exploratory project after unblinding and analysis of main outcomes

[F] to assess maturation of immune cell composition and response

Visits	Follow-up (minimum 2.5 years; maximum up to 5.5 years after end of intervention)			
	Call age 18 months	**Visit age 2 years**	**Call every 12 month** (in the middle of yearly visits)	**Visit every 12 months[G]**
Visit window	± 30d	± 30d	± 30d	± 30d
Study visit		4		5+
Study call	3		4+	
Local investigations and measurements				
Physical examination (height, weight)		X		X
Assessment of AEs and SAEs[A]	X			
Assessment of MMR vaccination schedule using official records		X		
Blood glucose[B]		X		X
Sample collection				
<200 µl capillary or venous blood for glucose		X		X
2ml blood for serum samples for central antibody measurement [C, D]		X		X
2ml EDTA blood for plasma samples for mechanistic studies (inflammation)		X		
Central measurements				
IAA; GADA; IA-2A; ZnT8RA; ZnT8WA		X		X
TGA		X		X
Antibody Responses to MMR vaccine		X		
Mechanistic markers (inflammation) [E]		X		
Electronic questionnaires completed by families				
Questionnaire about allergies	every 12 months until end of study			
Ancillary assessments				
Whole blood FACS *(Dresden and Munich only)*		X		

[A] AEs/SAEs will be noted and reported as under intervention phase for 30 days after end of treatment day

[B] by handmeter or haemocue

[C] if there is left over material and a signed biobank consent, the left over serum and DNA will be stored in the IBBL or local biobank

[D] venous or capillary blood for the AAB confirmation sample can be obtained by a local physician

[E] measurements may partly be done as exploratory project after unblinding and analysis of main outcomes

[F] to assess maturation of immune cell composition and response

[G] Final visit must be performed within the last 6 months before last enrolled child completed 2.5 years of follow-up

PRISMA-P 2015 Checklist

This checklist has been adapted for use with protocol submissions to *Systematic Reviews* from Table 3 in Moher D et al: Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. *Systematic Reviews* 2015 **4**:1

Section/topic	#	Checklist item	Information reported		Page number(s)
			Yes	No	
ADMINISTRATIVE INFORMATION					
Title					
Identification	1a	Identify the report as a protocol of a systematic review	x	☐	Title
Update	1b	If the protocol is for an update of a previous systematic review, identify as such	☐	☐	Not applicable
Registration	2	If registered, provide the name of the registry (e.g., PROSPERO) and registration number in the Abstract	x	☐	End of Abstract, page 3
Authors					
Contact	3a	Provide name, institutional affiliation, and e-mail address of all protocol authors; provide physical mailing address of corresponding author	x	☐	Title page
Contributions	3b	Describe contributions of protocol authors and identify the guarantor of the review	x	☐	Authors' contributions, page 17,18
Amendments	4	If the protocol represents an amendment of a previously completed or published protocol, identify as such and list changes; otherwise, state plan for documenting important protocol amendments	☐	☐	Not applicable
Support					
Sources	5a	Indicate sources of financial or other support for the review	x	☐	Funding, page 18
Sponsor	5b	Provide name for the review funder and/or sponsor	x	☐	Methods, study organisation, page 6
Role of sponsor/funder	5c	Describe roles of funder(s), sponsor(s), and/or institution(s), if any, in developing the protocol	x	☐	Methods, study organisation, page 6, Authors' contributions, page 17,18

Section/topic	#	Checklist item	Information reported		Page number(s)
			Yes	No	
INTRODUCTION					
Rationale	6	Describe the rationale for the review in the context of what is already known	x	☐	Introduction, Rationale for use of oral insulin…, page 5
Objectives	7	Provide an explicit statement of the question(s) the review will address with reference to participants, interventions, comparators, and outcomes (PICO)	x	☐	Introduction, page 4-5
METHODS					
Eligibility criteria	8	Specify the study characteristics (e.g., PICO, study design, setting, time frame) and report characteristics (e.g., years considered, language, publication status) to be used as criteria for eligibility for the review	x	☐	Methods, page 6-11
Information sources	9	Describe all intended information sources (e.g., electronic databases, contact with study authors, trial registers, or other grey literature sources) with planned dates of coverage	☐	☐	Not applicable
Search strategy	10	Present draft of search strategy to be used for at least one electronic database, including planned limits, such that it could be repeated	☐	☐	Not applicable
STUDY RECORDS					
Data management	11a	Describe the mechanism(s) that will be used to manage records and data throughout the review	x	☐	eCRFs, page 9
Selection process	11b	State the process that will be used for selecting studies (e.g., two independent reviewers) through each phase of the review (i.e., screening, eligibility, and inclusion in meta-analysis)	☐	☐	Not applicable
Data collection process	11c	Describe planned method of extracting data from reports (e.g., piloting forms, done independently, in duplicate), any processes for obtaining and confirming data from investigators	☐	☐	Not applicable
Data items	12	List and define all variables for which data will be sought (e.g., PICO items, funding sources), any pre-planned data assumptions and simplifications	x	☐	Analysis, Primary outcome and analysis, Secondary outcomes and

Section/topic	#	Checklist item	Information reported		Page number(s)
			Yes	No	
					analyses, page 11,12
Outcomes and prioritization	13	List and define all outcomes for which data will be sought, including prioritization of main and additional outcomes, with rationale	x	☐	Analysis, Primary outcome and analysis, Secondary outcomes and analyses, page 11,12
Risk of bias in individual studies	14	Describe anticipated methods for assessing risk of bias of individual studies, including whether this will be done at the outcome or study level, or both; state how this information will be used in data synthesis	☐	☐	Not applicable
DATA					
Synthesis	15a	Describe criteria under which study data will be quantitatively synthesized	☐	☐	Not applicable
	15b	If data are appropriate for quantitative synthesis, describe planned summary measures, methods of handling data, and methods of combining data from studies, including any planned exploration of consistency (e.g., I^2, Kendall's tau)	☐	☐	Not applicable
	15c	Describe any proposed additional analyses (e.g., sensitivity or subgroup analyses, meta-regression)	x	☐	Analysis, Primary outcome and analysis, Secondary outcomes and analyses, page 11,12
	15d	If quantitative synthesis is not appropriate, describe the type of summary planned	☐	☐	Not applicable
Meta-bias(es)	16	Specify any planned assessment of meta-bias(es) (e.g., publication bias across studies, selective reporting within studies)	☐	☐	Not applicable
Confidence in cumulative evidence	17	Describe how the strength of the body of evidence will be assessed (e.g., GRADE)	x	☐	Study power and accrual target, page 12

BMJ Open

Supplementation with Bifidobacteria longum subspecies infantis EVC001 for mitigation of type 1 diabetes autoimmunity - The GPPAD-SINT1A randomised controlled trial protocol

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Keywords:	General diabetes < DIABETES & ENDOCRINOLOGY, IMMUNOLOGY, Diabetes & endocrinology < INTERNAL MEDICINE, PAEDIATRICS, DIABETES & ENDOCRINOLOGY, EPIDEMIOLOGY

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Supplementation with *Bifidobacteria longum* subspecies *infantis* EVC001 for mitigation of type 1 diabetes autoimmunity - The GPPAD-SINT1A randomised controlled trial protocol

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Word count: 4672

Keywords: type 1 diabetes, islet autoantibody, probiotic *Bifidobacteria longum* subspecies *infantis* EVC001, GPPAD, SINT1A, prevention trial

Timeline of the study:
Recruitment: 3.0 years
Start (FPFV): April 2021
Intervention: *B. infantis* EVC001
Intervention period: Until age 12 months

Follow-up after intervention: 2.5-5.5 years
Intended End (LPLV): October 2027
Protocol: V 1.0 November 09th, 2020

Abbreviations:

AE	Adverse Events
ADA	American Diabetes Association
B. infantis	*Bifidobacterium longum* subspecies *infantis* EVC001
CC	Coordinating Centre
CFU	Colony Forming Units
CI	Confidence Interval
DSMB	Data Safety Monitoring Board
eCRF	electronic Case Report Form
FPG	Fasting Plasma Glucose
GRS	Genetic risk score
GPPAD	Global Platform for the Prevention of Autoimmune Diabetes
HLA	Human Leukocyte Antigen
OGTT	Oral glucose tolerance test
POInT	Primary Oral Insulin Trial
SAE	Serious Adverse Events
SCFAs	short chain fatty acids
SINT1A	Supplementation with *B. infantis* for mitigation of type 1 diabetes autoimmunity
SNP	Single nucleotide polymorphism
T1D	Type 1 diabetes

ABSTRACT

Introduction: The GPPAD-SINT1A Study is designed as a randomised, placebo-controlled, double blind, multicentre, multinational, primary prevention study aiming to assess whether daily administration of *B. infantis* from age 7 days to 6 weeks until age 12 months to children with elevated genetic risk for type 1 diabetes reduces the cumulative incidence of beta-cell autoantibodies in childhood.

Methods and analysis: Infants aged 7 days to 6 weeks from Germany, Poland, Belgium, UK and Sweden are eligible for study participation if they have a >10.0% expected risk for developing multiple beta-cell autoantibodies by age 6 years as determined by genetic risk score or family history and HLA genotype. Infants are randomized 1:1 to daily administration of *B. infantis* EVC001 or placebo until age 12 months, and followed for a maximum of 5.5 years thereafter. The primary outcome is the development of persistent confirmed multiple beta-cell autoantibodies. Secondary outcomes are 1. Any persistent confirmed beta-cell autoantibody, defined as at least one confirmed autoantibody in two consecutive samples, including IAA, GADA, IA-2A or ZnT8A, 2. Diabetes, 3. Transglutaminase autoantibodies associated with celiac disease, 4. Respiratory infection rate in first year of life during supplementation, 5. Safety. Exploratory outcomes include allergy, antibody response to vaccines, alterations of the gut microbiome or blood metabolome, stool pH and calprotectin.

Ethics and dissemination: The study was approved by the local ethical committees of the Technical University Munich, Medical Faculty, the Technische Universität Dresden, the Medizinische Hochschule Hannover, the Medical University of Warsaw, EC Research UZ Leuven, and the Swedish ethical review authority. The results will be disseminated through peer-reviewed journals and conference presentations and will be openly shared after completion of the study.

Registration: clingov id: NCT04769037

Strengths and limitations of this study

- This is the first adequately powered placebo controlled study to test the supplementation with *B. infantis* for mitigation of type 1 diabetes autoimmunity.
- Includes other health outcomes such as celiac autoimmunity, respiratory infections, allergy, antibody response (IgG titres) to vaccines, alterations of the gut microbiome or blood metabolome, measurement of stool pH and calprotectin.
- The requirement to identify eligible at-risk infants by genetic screening does not allow to introduce *B. infantis* supplementation in the first days of life or during pregnancy, when it may be most beneficial.

- There could be advantages in using multiple probiotic strains with complementary metabolic capacities.
- It may have been advantageous to add a prebiotic to the *B. infantis* formulation to compensate for the possibility that some mothers stopped breast feeding early in the trial.

INTRODUCTION

Type 1 diabetes (T1D) results from an immune-mediated destruction of the pancreatic islet beta-cells resulting in insulin deficiency. This process is clinically silent and can be identified by circulating autoantibodies to beta-cell antigens (glutamic acid decarboxylase (GADA), islet tyrosine phosphatase 2 (IA-2A), insulin (IAA) and zinc transporter 8 (ZnT8A) (1). Beta-cell autoantibodies occur early in life with a peak incidence period between age 9 months and 3 years (2, 3, 4), and the risk to develop multiple beta-cell autoantibodies exponentially declines with age (5, 6). On the basis of these findings, it is concluded that any interventional therapy given as a primary prevention strategy must be started early in life.

This randomised, placebo-controlled study will evaluate whether supplementation with a daily dose of a probiotic in the first year of life can reduce the risk of developing beta-cell autoimmunity in children identified by the Global Platform for the Prevention of Autoimmune Diabetes (GPPAD) as being genetically at risk for developing T1D (previously described in detail in 7 and 8). The rationale for this study combines the most recent findings concerning the specific role that the commensal bacteria, microbiota, and their genes, the microbiome, could play in the induction of peripheral tolerance to insulin (9, 10, 11, 12), and builds on knowledge of the reported health and clinical benefits of early probiotic supplementation in peanut allergy prevention (13) and in lowering mortality owing to sepsis in children (14). Although it is widely accepted that the induction of the state of tolerance to beneficial bacteria during early life is critical for a newborn's survival, the clinical benefit for the prevention of immune-related diseases is only now being explored. It is believed that tolerance is achieved when the innate and adaptive immune cells promote tolerogenic immune responses to dietary and commensal antigens as well as reactions to a variety of beneficial metabolites produced by commensal microbes, in particular the short chain fatty acids (SCFAs) (15, 16, 17, 18, 19). The SINT1A study follows the ongoing Primary Oral Insulin Trial (POInT) (8), which administers insulin orally to train and sensitize the immune system at an early stage via gut exposure so that autoimmunity against insulin does not occur.

Evidence for dysbiosis in children prior to the development of type 1 diabetes

Evidence that microbiome changes may alter the risk for T1D is presented by a number of prospective studies which have shown that changes in the microbiome precede the development of beta-cell autoimmunity and T1D. These include the BABYDIET study where alterations in microbial interaction networks were observed at age 0.5 and 2 years in children who developed beta-cell autoimmunity (9), and studies from Finland where higher abundances of *Bacteroides dorei* (20) and a decrease in microbial diversity were described in children with genetic predisposition to T1D (21). The TEDDY study

confirmed these alterations and found that the microbiome of children who did not develop beta-cell autoimmunity contained more genes that were related to fermentation and the biosynthesis of short-chain fatty acids (SCFA) supporting the protective effects of SCFA in early-onset human T1D (10). Furthermore, the TEDDY study has reported that probiotic supplementation in the first 27 days of life, and only in this early period (documented by questionnaires and diary records) was associated with a decreased risk of beta-cell autoimmunity providing the first evidence that correcting dysbiosis in infants may be beneficial for children at risk of T1D (11). A recent finding links microbial metabolism to insulin-specific immune responses: the microbial enzymes belonging to the transketolase superfamily contain the primary insulin autoantigenic epitope (INS B:9-25). The microbial transketolase upregulation reflects the adaptation of the microbiome to digest sugar polymers during weaning and matches the time of autoantibody appearance against insulin. It has been suggested that an immune response to insulin mimotopes due to commensal dysbiosis is a possible primary cause of T1D (12).
Altogether, these results suggest that correcting dysbiosis in early life could help to promote immune tolerance and thus inhibit the initiation of beta-cell autoimmunity.

Previous clinical studies using B. *infantis* in children

A substantial body of evidence has connected gut inflammation with improper immune programming and the subsequent development of autoimmune conditions including T1D, atopic dermatitis, food allergies and asthma (19, 22). Bifidobacteria and in particular *Bifidobacterium longum* subspecies *infantis* (*B. infantis*) have positive properties that potentially counteract the development of gut inflammation in the first few months of life (23, 24). Depleted numbers of *Bifidobacteria* are associated with immune-related diseases such as allergy (25, 26). *Bacteroides* species, which are naturally outcompeted by *Bifidobacteria*, are present at higher numbers within the microbiota of children with high susceptibility to autoimmunity (19). There is additional evidence that Bifidobacterium abundance in early infancy increases protective efficacy of vaccines by enhancing immunologic memory (27). Lactic acid bacteria and bifidobacteria are increasingly administered to pregnant women and infants with the intention of improving health. A number of clinical studies have been conducted to document the safety and health benefits of dietary supplementation with bacterial strains (probiotics). Previous clinical studies using *B. infantis* are summarized in **Table 1**.

Table 1: Clinical studies on *B. infantis*

Reference	Number of participants*	Main results
(28)	80	Safe consumption and good tolerance of *B. infantis*; stools significantly fewer and better formed
(29)	66	Significant changes to faecal microbiome composition; colonization with *B. infantis*; higher abundance of faecal short chain fatty acids; lower stool pH
(30)	40	Lower fecal calprotectin levels; lower enteric inflammation

* participants in total, meaning the group of children *B. infantis* fed and the group placebo fed (for all: treatment from day 7 on, dose 1.8-2.8 × 10^{10} CFU (colony forming units)), all participants were breast-fed infants

These studies show first evidence that supplementation with *B. infantis* is safe (28, 29, 30). The IMPRINT study demonstrates that supplementation with *B. infantis* (1.8-2.8 × 10^{10} CFU) for 14 days (from day 7 to day 21) is well tolerated (28). Stools are fewer and better formed in infants in the supplementation group compared to the non-supplemented group. A follow-up study indicates that supplementation results in significant changes of the faecal microbiome composition (29) including evidence of persistent colonization of the probiotic organism. Infants colonized by Bifidobacteriaceae have 4-fold-lower faecal endotoxin levels, consistent with observed lower levels of Gram-negative Proteobacteria and Bacteroidetes, lower stool pH, and higher faecal concentrations of short chain fatty acids (29). In addition, pro-inflammatory cytokines are significantly lower in *B. infantis*-fed infants (30). The mentioned results suggest that correcting dysbiosis in early life could help to promote immune tolerance and thus inhibit the initiation of beta-cell autoimmunity. In this context, a supplementation with *B. infantis* seems promising.

Primary Objective

To determine whether daily administration of *B. infantis* EVC001 from age 7 days to 6 weeks until age 12 months to children with elevated genetic risk for T1D reduces the cumulative incidence of beta-cell autoantibodies in childhood.

METHODS

Outcome measures

Primary outcome

The primary outcome of SINT1A is the elapsed time from random treatment assignment to the development of persistent confirmed multiple beta-cell autoantibodies. For subjects who developed

persistent confirmed multiple beta-cell autoantibodies, the elapsed time will be from the random treatment assignment to the first confirmed autoantibody positive sample used in defining the persistent confirmed multiple beta-cell autoantibody positive status. It is expected that beta-cell autoantibodies will be detected prior to T1D diagnosis; however, the presence of diabetes in the absence of multiple beta-cell autoantibodies is also considered as a primary outcome endpoint, and in this case, the date of diagnosis is the time of the end point.

The study primary outcome is realized with either persistent confirmed multiple beta-cell autoantibodies or Oral Glucose Tolerance Test (OGTT) criteria for diabetes or clinical criteria for diabetes.

Criteria for persistent confirmed beta-cell autoantibodies

Criteria are based on the measurement of beta-cell autoantibodies against insulin (IAA), GAD65 (GADA), IA-2 (IA-2A), and ZnT8 (ZnT8A) tested in the GPPAD central autoantibody laboratory and, if positive, confirmed in the GPPAD confirmatory laboratory.

Confirmed IAA is defined as sample positive for IAA in both the GPPAD central and confirmatory laboratories. Confirmed GADA is defined as sample positive for GADA in both the GPPAD central and confirmatory laboratories. Confirmed IA-2A is defined as sample positive for IA-2A in both the GPPAD central and confirmatory laboratories. Confirmed ZnT8A is defined as sample positive for ZnT8RA or ZnT8WA in both the GPPAD central and confirmatory laboratories.

The status persistent confirmed beta-cell autoantibody-positive is defined as confirmed IAA, confirmed GADA, confirmed IA-2A, or confirmed ZnT8A in two consecutive samples. Persistent confirmed multiple beta-cell autoantibodies (primary outcome) is defined as confirmed IAA, confirmed GADA, confirmed IA-2A, or confirmed ZnT8A in two consecutive samples, AND at least one other confirmed antibody from these four antibodies in one sample. Persistent confirmed beta-cell autoantibodies that are considered maternally derived are NOT included as positive for the primary outcome.

Criteria for T1D diagnosis

Diabetes may be diagnosed in a small number of children before a persistent confirmed multiple islet autoantibody positive status is achieved as the multiple autoantibody outcome requires two consecutive positive samples. In these cases, the primary outcome status is assigned to the child.

Criteria for T1D diagnosis are, as defined by the American Diabetes Association (ADA), based on glucose testing, or the presence of unequivocal hyperglycaemia with acute metabolic decompensation (diabetic ketoacidosis). One of the following criteria must be met on two occasions as soon as possible but no less than 1 day apart for diabetes to be defined:

1. Symptoms of diabetes and a casual plasma glucose ≥200 mg/dL (11.1mmol/L).

Casual is defined as any time of day without regard to time since last meal. The classic symptoms of diabetes include polyuria, polydipsia, and unexplained weight loss.

OR

2. Fasting plasma glucose (FPG) ≥126 mg/dL (7 mmol/L). Fasting is defined as no caloric intake for at least 8 hours.

OR

3. Two-hour plasma glucose (PG) ≥200 mg/dL (11.1 mmol/L) during an OGTT. The test should be performed using a glucose load containing the equivalent of 1.75g/kg body weight to a maximum of 75g anhydrous glucose dissolved in water. It is preferred that at least one of the two testing occasions involve an OGTT.

Cases diagnosed with T1D will be adjudicated by the Endpoint Committee. Study participation will be terminated if T1D is reached.

Secondary outcomes

Secondary outcomes of the study are: 1. The development of any persistent confirmed beta-cell autoantibody, defined as at least one confirmed autoantibody in two consecutive samples, including IAA, GADA, IA-2A or ZnT8A, 2. Diabetes, 3. The development of persistent confirmed transglutaminase antibodies associated with celiac disease, defined as confirmed autoantibody in two consecutive samples, 4. Respiratory infection rate in first year of life during supplementation and 5. Safety.

Exploratory outcomes

The following exploratory outcomes may be assessed or in part assessed on a portion of the participants. They may not necessarily be included in the primary outcome analysis and publication: 1. Allergy, 2. Antibody response (IgG titres) to vaccines, 3. Alterations of the stool microbiome or 4. Blood metabolome, 5. Stool pH and 6. Stool calprotectin concentration.

Study design and organisation

SINT1A will be conducted as an investigator-initiated, randomized, placebo-controlled, double-blind multi-center intervention study through GPPAD, a network of collaborating clinical study centres from European countries with sites in Belgium (Leuven), Germany (Dresden, Hannover, Munich), Poland (Warsaw), Sweden (Malmö), and UK (Newcastle, Cambridge). The Trial Coordinating Centre (GPPAD CC) is located at the Institute of Diabetes Research, Helmholtz Zentrum München. It manages coordination and communication between the SINT1A clinical study sites, and oversees the collection, analysis and storage of clinical data; also the supervision of regulatory activities, clinical research organization activities, the manufacturer of the active supplement, and the central laboratories is provided by the CC.

GPPAD was founded in 2015 with the aim to provide an international infrastructure to enable T1D primary prevention trials, identify infants with an elevated genetic risk of developing T1D and offer

participation in randomized controlled trials aiming to reduce the incidence of T1D in children (8, 31). Until March 2021, more than 251,000 infants have been screened and had their genetic risk of developing T1D evaluated using a combination of family history and 47 single nucleotide polymorphisms (SNPs) (7). From these, over 2,000 infants have been identified as having a 10% probability or greater of developing multiple beta-cell autoantibodies by 6 years of age, making them eligible for GPPAD primary prevention trials (7). The first GPPAD trial POInT (Primary Oral Insulin Trial) has now (March/2021) completed enrollment (1,050 participants) (8). SINT1A will commence in April 2021 with the first patient first visit.

Study population

Infants are tested for genetic risk of T1D based on advanced risk scores derived from 51 SNPs that define HLA-DR3, HLA-DR4, and HLA-DQ8 alleles as well as SNPs from HLA class I, and non-HLA T1D susceptibility genes, and from HLA class II protective alleles, as previously described (7, 32). Infants with a predicted risk of >10% to develop multiple beta-cell autoimmunity by age 6 years and who fulfil the inclusion criteria as stated below are eligible to participate in the GPPAD-SINT1A Study.
A total of 1,144 infants will be enrolled and randomized 1:1 (*B. infantis* or placebo) in the SINT1A study (see **Figure 1**). Children with T1D susceptible genotypes also have a marked risk of around 10% for autoimmunity found in celiac disease as shown in the TEDDY study (33).

Inclusion and exclusion criteria

Participants must meet all entry criteria for the protocol as outlined below.

- Infants between the ages of 7 days and 6 weeks (+14 days in case of illness or COVID-19 related issues or unexpected delay in result reporting) at the time of randomisation.
- A 10% or higher genetic risk to develop multiple beta-cell autoantibodies by age 6 years:
    a. For infants without a first-degree family history of T1D, high genetic risk is defined as a DR3/DR4-DQ8 or DR4-DQ8/DR4-DQ8 genotype and a genetic risk score that is in the upper 25th centile (>14.4) (34) or a DR3/DR4-DQ8 genotype with a GRS between the upper 50th (14.0) and 25th centile and a GG genotype at the rs3763305 SNP. These represent around 1% of all newborns.
    b. For infants with a first-degree family history of type 1 diabetes, high genetic risk is defined as having HLA DR4 and DQ8, and none of the following protective alleles: DRB1*1501, DQB1*0503, DRB1*1303. These represent around 30% of infants with a first-degree family history of T1D.
- Written informed consent signed by the custodial parent(s).

Participants may not enter the study if ANY of the following apply:

- Any medical condition, concomitant disease or treatment that may interfere with the assessments or may jeopardize the participant's safe participation in the study, as judged by the Investigators.
- Preterm delivery < 36 weeks of gestation.
- Proven immunodeficiency.
- Any condition that could be associated with poor compliance.
- Diagnosis of diabetes at the time of recruitment.

Informed Consent

The GPPAD-SINT1A Study will be described to the custodial parent(s) of potential participants by qualified GPPAD study personnel. The custodial parent(s) will have the opportunity to read the consent document and to discuss any questions concerning the consent or study participation. The families will be given enough time to consider whether or not to participate. The custodial parent(s) will then be asked to sign and date an informed consent form prior to or at the baseline visit. Date and signature of the study Investigator (or other authorized study personnel, if applicable) will also be obtained on the consent form. A copy of the informed consent form will be handed out to the families. The custodial parent(s) of the prospective participant will be told that being in the study is voluntary and that the participant may withdraw from the study at any time, for any reason.

Patient and Public Involvement

Patients were not involved in the study design but in the prioritization of the research question of T1D prevention. Patients support recruitment through dissemination, and participation in press conferences. Participating families will be informed about the outcome of the trial via webcast, letter, and personal communication upon the completion of the trial.

Randomisation

Subjects will be centrally randomised in a 1:1 ratio to one of the two intervention arms via IVRS/IWRS at the baseline visit. The participant and the treating physician and the central research team will be blinded. The study product packages will not indicate whether the content is B. infantis or placebo, but kit numbers. The IVRS/IWRS will assign the appropriate kit numbers for each participant following a randomisation list. Emergency unblinding will be available through the IVRS/helpdesk. Siblings within one household will be randomised to the same intervention arm to avoid mix-up of supplementation. Randomisation will be stratified for whether the child is still breast-fed at the date of randomization and study centre.

Intervention

Participants are randomized in a 1:1 ratio to receive either *B. infantis* or placebo. Each dose of the active supplement is provided as one sachet with *B. infantis* EVC001 at a minimum concentration of 8 x 10^9 colony forming units (CFU) in lactose. The dose was selected according to the previous IMPRINT study (28). The actual concentration as per batch certificate of analysis ranged from 13.8 x 10^9 to 15.8 x 10^9 CFU per sachet; the shelf-life is 15 months. The placebo consists of lactose, identical in appearance and taste to the active supplement. *B. infantis*/placebo will be administered orally, once a day, using single-dose sachets. It is recommended to administer the product in the morning (7-10am), preferably together with some breast-milk. The content (powder) of the sachets is poured into a small bowl and mixed with 3-5ml of breast milk, infant formula, or water. The solution will be administered using a feeding syringe, preferably in the morning. Parent(s) will be instructed in the administration and storage of the sachets (should be kept frozen until use) at or prior to their baseline visit. The genome of *B. infantis* is available in the NCBI accession number NZ_LR655210 under the strain name USA001_1 (35). Active and placebo products are provided by Evolve Biosystems, USA. Blinding, packing, and distribution to clinical study sites is performed by the pharmacy, University of Heidelberg, Germany.

Study timeline

The study is expected to take 6.5 years to complete. This includes an intervention phase of 12 months, and minimum 2.5 (last participant enrolled) to maximum 5.5 years of follow-up (first participant enrolled) after intervention (Figure 1). The enrolment period is projected to be 3.0 years.

Study assessment

The baseline visit includes the collection of information on medical history, C-section, breast-feeding, proton pump inhibitor therapy, infections, probiotic use and antibiotic treatment prior to enrolment and the collection of a stool sample. Families will be instructed in the administration and storage of the blinded food product (*B. infantis* or placebo). Mothers will be encouraged to make their best effort to maintain breastfeeding for at least the first 3-4 months, and they will be encouraged to continue breastfeeding for as long as possible during the first year of the infant's life. Two more intervention visits (visit 2 and 3) are planned after 6 months and 12 months (± 14 days). At these visits venous blood and a stool sample is collected, electronic questionnaires are discussed. The assessment of weight and height is performed at all visits. After the intervention period, study visit 4 will be conducted at age 2 years (± 30 days), subsequent follow-up study visits will be conducted every year (± 30 days) until the end of the study. A detailed table explaining study visits, and data and sample collection is shown in

Supplementary File 1. All study relevant subject data and laboratory results are documented in corresponding electronic Case Report Forms (eCRFs).

E-diaries and Allergy questionnaires

Throughout the intervention period, parents will be asked to complete e-diaries fortnightly to collect information on breast-feeding, respiratory infections, antibiotic treatment and treatment with proton pump inhibitors. Additional questionnaires to obtain information about allergies will be collected every 12 months starting at age 12 months ± 14 days until the end of the study. The information given by the parents will be captured in a central database and reviewed and discussed during the study visits and phone calls between the visits.

For participants who develop positive beta-cell or transglutaminase autoantibodies

Participants who have confirmed positive beta-cell- or transglutaminase autoantibodies during the study are asked to donate a confirmation sample within 4-12 weeks. If the participant has persistent confirmed beta-cell autoantibodies, the custodial parent(s) are informed and asked to participate in an educational program informing about the diagnosis of beta-cell autoantibody positivity and possible symptoms of hyperglycemia and metabolic decompensation. The child remains in the study and continues to be treated or followed as planned until the child has developed T1D or end of study. Home monitoring of blood glucose will be recommended 2-monthly if a child is considered at risk for a rapid progression to diabetes (e.g. IA-2A positive, very high titers of antibodies, or impaired blood glucose values). In case of persistent confirmed positivity for transglutaminase autoantibodies, an intestinal biopsy maybe recommended to clarify the diagnosis of celiac disease. These children will continue to receive *B. infantis*/placebo and will be followed in the study for continued monitoring of diabetes development and safety assessments.

Safety

As the study intervention is not considered a medicinal product, safety reporting obligations as for IMP clinical trials do not apply. However, AEs and serious adverse events (SAEs) up to 30 days after the last administration of the food product are assessed and captured in the eCRF. Adverse events will be graded as mild, moderate, severe, life-threatening or death according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (NCI-CTCAE) Version 5.0.

Physical examinations including measurement of height and weight are performed at all visits.

ANALYSIS

All efficacy analyses will be conducted under the Intention-To-Treat principle whereby all effectiveness outcome data in all randomised subjects who have received at least one dose of *B. infantis* supplement or placebo will be included in all analyses as appropriate. Subjects who drop-out of the study will not be replaced. All data acquired prior to termination will be included in the primary analysis unless a participant withdraws consent.

Primary outcome and analysis

The cumulative incidence of multiple beta-cell autoantibodies over time since randomization within each treatment group will be estimated from a Kaplan-Meier estimate of the "beta-cell autoantibody-free" survival function. The difference between groups in the cumulative incidence functions, and the associated hazard functions, will be tested at the 0.05 level, two-sided, using Cox regression including site as covariate. With respect to the primary outcome, the hazard ratio of *B. infantis* to placebo will be given together with its 95% confidence interval. The final alpha is 0.05 (two-sided). In case the assumptions of the sample size estimation hold, it will be possible to reject the null hypothesis of equal hazard rates with the power of 80%, if 914 children will be uniformly randomised over 3 years and afterwards, all 914 children will be followed for another 3.5 years (6.5 years total duration after first enrollment). We have assumed a drop-out rate of 20%, and therefore we need to randomise 1,144 children to support an 80% power by a complete follow-up of 914 children ranging from 3.5 to 6.5 years.

Sub-group analyses of Primary Outcome

It is reasonable to consider that *B. infantis* colonization, breast-feeding status, and secretor (FUT2 gene) status may impact the outcome (36, 37). *B. infantis* colonization will be measured in stool samples collected at visit age 3 months. A multiple Cox regression analysis will be performed on the primary outcome including treatment group and colonization as a continuous variable. This will also be performed with a treatment group-colonization interaction term, and with colonization as a categorical variable. Three categories representing no colonization, low and high colonization based on the distribution of values in the children will be used. Breast-feeding will be encouraged. Nevertheless, a portion of the children will not be receiving breast milk when they start. Breast-feeding will, therefore, be included as a categorical variable together with treatment in a Cox regression. An interaction analysis between colonization and breast-feeding status will be performed, and a subgroup analysis for the primary outcome will be performed in children categorized by their FUT2 gene genotype as secretors and non-secretors. Additional variables that will be analysed by multivariable Cox regression include 1. Children categorized as having a mother with diabetes versus the remainder; 2. Children who have HLA DR3/4-

DQ8 versus the remainder; 3. Children who have the T1D susceptible INS AA genotype versus the remainder; 4. Sex; 5. Caesarean section versus vaginal birth; 6. BMI at age 1 year as tertiles; 7. Genetic risk score tertiles.

Secondary and exploratory outcomes and analyses

For the secondary outcomes, the treatment arms will be compared on the corresponding incidence rates of each secondary outcome using the log rank statistic. Other secondary outcomes will be analysed by comparison between the *B. infantis* supplementation and placebo supplementation groups using appropriate statistics in dependence on the outcome of interest. Subgroup analyses analogous to those described for the beta-cell autoantibodies endpoint will be conducted on the secondary outcome endpoints. Exploratory analyses will examine the associations between *B. infantis* supplementation and allergy, mouth and stool organisms (microbiome), and blood markers such as the metabolome, lipidome, or inflammatory proteins and ancillary study measurements that specific sites may undertake.

Study power and accrual target

For the sample size estimation, the following scenario was chosen:

- Overall alpha level = 0.05 (two-sided).
- Overall beta level = 0.2, i.e. power = 0.8.
- In the placebo group, at 3.5 years (approximate age of participants, 4 years), an event probability of 7.5% was assumed. Based on the exponential distribution, this leads to a hazard of 0.02227.
- For the active treatment, it is assumed that the hazard rate will be halved.
- Accrual time is 3 years.
- Follow-up time is 3.5 years.
- A dropout rate of 20% was taken into account.

The study has been designed to provide 80% power to detect a 50% risk reduction in the hazard rate of the event of confirmed persistent beta-cell autoantibodies using a two-sided test at the significance level 0.05 after 6.5 years of study duration. Decisive test will be the Wald test for the hazard ratio between the two groups within a Cox PH model. It is expected that the hazard is halved by active treatment. According to the assumptions described in above scenario, n=914 patients should be randomised between the two groups. With an assumed drop-out rate of 20%, n=1,144 children will need to be randomised.

Benefits and Risks

Benefits

The potential benefit for a participating child is the prevention (or delay in onset) of beta-cell autoantibodies and diabetes, celiac disease, childhood infections, and allergy. For all participating children, including children who receive placebo, testing blood samples will allow early recognition of pre-symptomatic T1D and celiac disease before the child shows the typical symptoms, and an appropriate therapy could be started immediately, potentially reducing complications later in life. Furthermore, information about other ongoing prevention trials or available treatments and intervention studies can be given to families.

Risks

So far, there have been no reports of risks and side effects associated with the use of *B. infantis*. Several studies show that various strains of *B. infantis* have been administered to numerous newborns and especially to premature babies without complications. In breastfed babies, *B. infantis* is one of the most common bacteria found in the intestine. Typical risks or complaints associated will taking a blood sample cannot be excluded. The volume of blood drawn for the trial endpoints is <1% of the total blood volume, within the suggested limits from the European guidelines for a paediatric population (38).

A data safety monitoring board (DSMB) is established. A DSMB is an independent group of experts responsible to safeguard the well-being and safety of the study participants. The DSMB will meet six-monthly during the intervention phase and 12-monthly during the follow-up phase. Before each DSMB meeting, the DSMB will receive a report with all relevant information on recruitment rate, data completeness and safety data, including beta-cell- and transglutaminase autoantibody and diabetes outcomes.

Retention strategies

A special family friendly retention concept has been developed to make families feel as part of the research team. Special care and support is offered for families who participate in the study and small gifts for the children are given out during the visits. Families are reminded of the advantages of study participation such as free monitoring of the children's health status and potential benefit from the intervention. Strategies for retention also include newsletters and reports on islet- and celiac autoantibody testing, and activities on community building (Homepage, Facebook groups).

Further information can be found on the GPPAD homepage:

https://www.gppad.org/de-en/prevention-sint1a/

ETHICS AND DISSEMINATION

Ethics

The study was approved by the local ethical committees and regulatory authorities of the Technische Universität München, Medical Faculty (715/20 S), the Technische Universität Dresden (SR+BO-44012021), the Medizinische Hochschule Hannover (9589_BO_S_2021), the Medical University of Warsaw (KB/5/2021), EC Research UZ Leuven (322) and the Swedish ethical review authority (dnr 2021-01210).

Dissemination

GPPAD is committed to sharing of data in compliance with all applicable European and GPPAD Consortium Member State, Data Protection and Privacy Protection laws, rules and regulations. Pseudonymised data of the SINT1A Study (GPPAD-04) will be available to the scientific community after the publication of the trial analysis, which is anticipated in 2028 (please visit https://clinicaltrials.gov/, Identifier Number: NCT04769037). The SINT1A data will be available upon request.

UPDATE ON THE CURRENT STATUS

The first participant was enrolled in April 2021. By August, six study sites in Germany, Poland, Belgium and Sweden had been initiated stepwise and 78 participants have been enrolled.

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Authors' contributions:
AGZ, MLP and JAT conceived the study, and led the protocol team. All authors (AGZ, SA, AK, PA, RB, EB, KC, HEL, MG, JH, OK, ML, MO, MLP, MP, MDS, AS, JAT) contributed to protocol development. EB, JH, and MP developed the statistical design for the study and wrote the statistical section of the protocol. EB and PA developed the outcome definition of the trial and wrote the autoantibody and outcome section of the protocol. AGZ, SA, AK, and MG drafted the manuscript. All authors reviewed the protocol as well as this manuscript.

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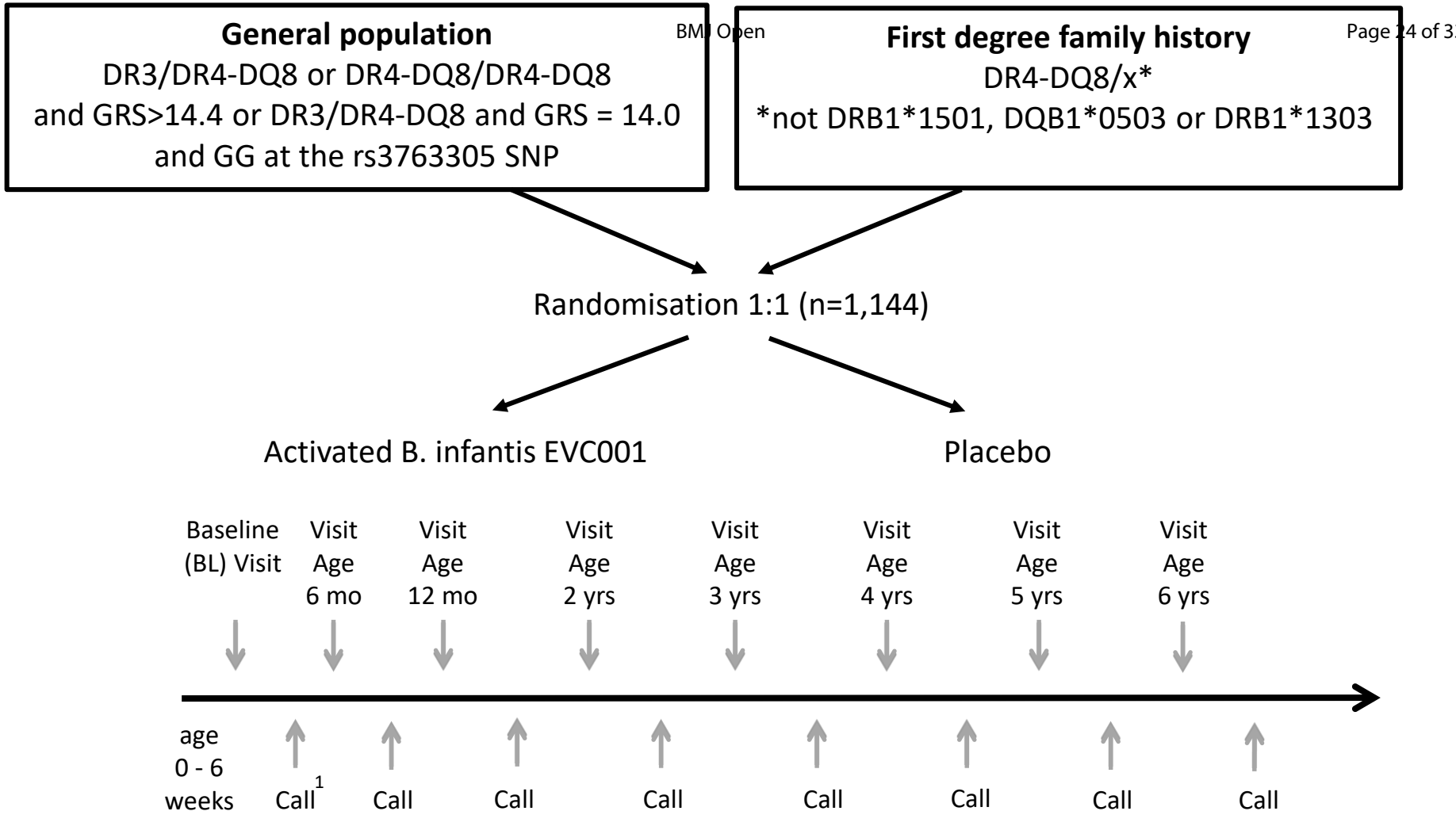
Competing interests
JAT is a member of the Scientific Advisory Board of Precion Ltd and of the Human Genetics Advisory Board of GSK.

Figure 1: SINT1A Study flow and time schedule for a participant with intervention until 12 months and maximum follow-up of 5.5 years.

Supplementary File 1: GPPAD-SINT1A Study: Visit–schedule (Study Flow Chart)



Primary outcome: Persistent confirmed multiple beta-cell autoantibodies

Secondary outcomes: Any persistent confirmed beta-cell autoantibody, diabetes, transglutaminase antibodies, respiratory infection rate, safety

Exploratory outcomes: Allergy, antibody response to vaccines, alterations of the gut microbiome or blood metabolome, stool pH and calprotectin

[1] interim telephone calls with families to asses AEs and support trial adherence

Supplementation with *Bifidobacteria longum* subspecies *infantis* EVC001 for mitigation of type 1 diabetes autoimmunity - The GPPAD-SINT1A randomized controlled trial protocol

Anette-Gabriele Ziegler, Stefanie Arnolds, Annika Kölln, Peter Achenbach, Reinhard Berner, Ezio Bonifacio, Kristina Casteels, Helena Elding Larsson, Melanie Gündert, Joerg Hasford, Olga Kordonouri, Markus Lundgren, Mariusz Oltarzewski, Marcin L. Pekalski, Markus Pfirrmann, Matthew D. Snape, Agnieszka Szypowska, John A. Todd and the GPPAD Study group

Supplementary File 1: GPPAD-SINT1A Study: Visit–schedule (Study Flow Chart)

	Intervention				
Visits	**Baseline Visit age 7 days - 6 weeks**	**Call age 3months**	**Visit age 6 months**	**Call age 9 months**	**Visit age 12 months**
Visit window	+14d	- 14d	± 14d	± 14d	+ 14d
Study visit	1		2		3
Study call		1		2	
Informed consent, Review Incl./Excl. Criteria	X				
Randomization	X				
Medical History	X				
Intervention					
Dispense supplement and compliance data sheet *(do not administer supplement at study site, only at home)*	X		X		
Local investigations & measurements					
Physical examination (height, weight)	X		X		X
Assessment of AEs and SAEs[A]		X	X	X	X
Assessment of rotavirus and MMR vaccination schedule using official records			X		X
Blood glucose[B]			X		X
HbA1c					X
Sample collection					
<200 µl capillary or venous blood for glucose			X		X
< 1ml EDTA blood for HbA1c					X
2ml blood for serum samples for central antibody measurement [C, D]			X		X
2ml EDTA blood for plasma samples for mechanistic studies (inflammation, metabolomics)			X		X
2 ml EDTA blood for DNA sample [C]					X

Visits	Intervention				
	Baseline Visit age 7 days - 6 weeks	**Call age 3months**	**Visit age 6 months**	**Call age 9 months**	**Visit age 12 months**
Stool sample for microbiome 16S	X	X	X		X
Stool sample for colonization		x			
Stool sample for stool pH & calprotectin *(in selected participants)*			X		
Central measurements					
IAA; GADA; IA-2A; ZnT8RA; ZnT8WA			X		X
TGA			X		X
Stool PCR for B. infantis colonization		X			
Antibody responses to rotavirus vaccine			X		
Microbiome 16s [E]	X	X			X
Mechanistic markers (inflammation, metabolomics) [E]			X		X
Electronic questionnaires completed by families					
Questionnaire about breast-feeding and antibiotics	every 2 weeks until age 12 months				
Questionnaire about infections and vaccinations	every 2 weeks until age 12 months				
Questionnaire about allergies					X
Ancillary assessments					
Whole blood FACS[F] *(Dresden and Munich only)*			X		X

[A] AEs/SAEs will be noted and reported as under intervention phase for 30 days after end of treatment day

[B] by handmeter or haemocue

[C] if there is left over material and a signed biobank consent, the left over serum and DNA will be stored in the IBBL or local biobank

[D] venous or capillary blood for the AAB confirmation sample can be obtained by a local physician

[E] measurements may partly be done as exploratory project after unblinding and analysis of main outcomes

[F] to assess maturation of immune cell composition and response

Visits	Follow-up (minimum 2.5 years; maximum up to 5.5 years after end of intervention)			
	Call age 18 months	**Visit age 2 years**	**Call every 12 month** (in the middle of yearly visits)	**Visit every 12 months[G]**
Visit window	± 30d	± 30d	± 30d	± 30d
Study visit		4		5+
Study call	3		4+	
Local investigations and measurements				
Physical examination (height, weight)		X		X
Assessment of AEs and SAEs[A]	X			
Assessment of MMR vaccination schedule using official records		X		
Blood glucose[B]		X		X
Sample collection				
<200 µl capillary or venous blood for glucose		X		X
2ml blood for serum samples for central antibody measurement [C, D]		X		X
2ml EDTA blood for plasma samples for mechanistic studies (inflammation)		X		
Central measurements				
IAA; GADA; IA-2A; ZnT8RA; ZnT8WA		X		X
TGA		X		X
Antibody Responses to MMR vaccine		X		
Mechanistic markers (inflammation) [E]		X		
Electronic questionnaires completed by families				
Questionnaire about allergies	every 12 months until end of study			
Ancillary assessments				
Whole blood FACS *(Dresden and Munich only)*		X		

[A] AEs/SAEs will be noted and reported as under intervention phase for 30 days after end of treatment day

[B] by handmeter or haemocue

[C] if there is left over material and a signed biobank consent, the left over serum and DNA will be stored in the IBBL or local biobank

[D] venous or capillary blood for the AAB confirmation sample can be obtained by a local physician

[E] measurements may partly be done as exploratory project after unblinding and analysis of main outcomes

[F] to assess maturation of immune cell composition and response

[G] Final visit must be performed within the last 6 months before last enrolled child completed 2.5 years of follow-up

SPIRIT

STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	3
	2b	All items from the World Health Organization Trial Registration Data Set	3
Protocol version	3	Date and version identifier	2
Funding	4	Sources and types of financial, material, and other support	21
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	19
	5b	Name and contact information for the trial sponsor	NA
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	9, 20
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	9, 19, 20, 21

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	__5, 6, 7_____
	6b	Explanation for choice of comparators	__6, 7_____
Objectives	7	Specific objectives or hypotheses	___7, 8_____
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	___9_____
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	__9, 10_____
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	__10, 11_____
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	____12_____
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	____13_____
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	____12, 13_____
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	____NA_____
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	____7, 8, 9_____
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Fig 1, Suppl file 1

Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	15
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	10
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	11
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	11
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	11
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	11
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	11
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	12, 13, 14, 15
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	16

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	___13, 14_____
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	____14, 15____
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	__14, 15_____
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	____NA_____
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	__20, 21_____
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	___NA_____
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	___13_____
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	__NA_____
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	___17_____
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	___20_____

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	____11_____
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	____11_____
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	____11, 12_____
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	____21_____
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	___17_____
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	____NA_____
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	___17_____
	31b	Authorship eligibility guidelines and any intended use of professional writers	____NA_____
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	____17_____
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	__attached___
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	___17_____

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated.

PRISMA-P 2015 Checklist

This checklist has been adapted for use with protocol submissions to *Systematic Reviews* from Table 3 in Moher D et al: Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. *Systematic Reviews* 2015 **4**:1

Section/topic	#	Checklist item	Information reported		Page number(s)
			Yes	No	
ADMINISTRATIVE INFORMATION					
Title					
Identification	1a	Identify the report as a protocol of a systematic review	x	☐	Title
Update	1b	If the protocol is for an update of a previous systematic review, identify as such	☐	☐	Not applicable
Registration	2	If registered, provide the name of the registry (e.g., PROSPERO) and registration number in the Abstract	x	☐	End of Abstract, page 3
Authors					
Contact	3a	Provide name, institutional affiliation, and e-mail address of all protocol authors; provide physical mailing address of corresponding author	x	☐	Title page
Contributions	3b	Describe contributions of protocol authors and identify the guarantor of the review	x	☐	Authors' contributions, page 17,18
Amendments	4	If the protocol represents an amendment of a previously completed or published protocol, identify as such and list changes; otherwise, state plan for documenting important protocol amendments	☐	☐	Not applicable
Support					
Sources	5a	Indicate sources of financial or other support for the review	x	☐	Funding, page 18
Sponsor	5b	Provide name for the review funder and/or sponsor	x	☐	Methods, study organisation, page 6
Role of sponsor/funder	5c	Describe roles of funder(s), sponsor(s), and/or institution(s), if any, in developing the protocol	x	☐	Methods, study organisation, page 6, Authors' contributions, page 17,18

Section/topic	#	Checklist item	Information reported		Page number(s)
			Yes	No	
INTRODUCTION					
Rationale	6	Describe the rationale for the review in the context of what is already known	x	☐	Introduction, Rationale for use of oral insulin…, page 5
Objectives	7	Provide an explicit statement of the question(s) the review will address with reference to participants, interventions, comparators, and outcomes (PICO)	x	☐	Introduction, page 4-5
METHODS					
Eligibility criteria	8	Specify the study characteristics (e.g., PICO, study design, setting, time frame) and report characteristics (e.g., years considered, language, publication status) to be used as criteria for eligibility for the review	x	☐	Methods, page 6-11
Information sources	9	Describe all intended information sources (e.g., electronic databases, contact with study authors, trial registers, or other grey literature sources) with planned dates of coverage	☐	☐	Not applicable
Search strategy	10	Present draft of search strategy to be used for at least one electronic database, including planned limits, such that it could be repeated	☐	☐	Not applicable
STUDY RECORDS					
Data management	11a	Describe the mechanism(s) that will be used to manage records and data throughout the review	x	☐	eCRFs, page 9
Selection process	11b	State the process that will be used for selecting studies (e.g., two independent reviewers) through each phase of the review (i.e., screening, eligibility, and inclusion in meta-analysis)	☐	☐	Not applicable
Data collection process	11c	Describe planned method of extracting data from reports (e.g., piloting forms, done independently, in duplicate), any processes for obtaining and confirming data from investigators	☐	☐	Not applicable
Data items	12	List and define all variables for which data will be sought (e.g., PICO items, funding sources), any pre-planned data assumptions and simplifications	x	☐	Analysis, Primary outcome and analysis, Secondary outcomes and

Section/topic	#	Checklist item	Information reported		Page number(s)
			Yes	No	
					analyses, page 11,12
Outcomes and prioritization	13	List and define all outcomes for which data will be sought, including prioritization of main and additional outcomes, with rationale	x	☐	Analysis, Primary outcome and analysis, Secondary outcomes and analyses, page 11,12
Risk of bias in individual studies	14	Describe anticipated methods for assessing risk of bias of individual studies, including whether this will be done at the outcome or study level, or both; state how this information will be used in data synthesis	☐	☐	Not applicable
DATA					
Synthesis	15a	Describe criteria under which study data will be quantitatively synthesized	☐	☐	Not applicable
	15b	If data are appropriate for quantitative synthesis, describe planned summary measures, methods of handling data, and methods of combining data from studies, including any planned exploration of consistency (e.g., I^2, Kendall's tau)	☐	☐	Not applicable
	15c	Describe any proposed additional analyses (e.g., sensitivity or subgroup analyses, meta-regression)	x	☐	Analysis, Primary outcome and analysis, Secondary outcomes and analyses, page 11,12
	15d	If quantitative synthesis is not appropriate, describe the type of summary planned	☐	☐	Not applicable
Meta-bias(es)	16	Specify any planned assessment of meta-bias(es) (e.g., publication bias across studies, selective reporting within studies)	☐	☐	Not applicable
Confidence in cumulative evidence	17	Describe how the strength of the body of evidence will be assessed (e.g., GRADE)	x	☐	Study power and accrual target, page 12