

## Project Title

“Trajectory of microbiota maturation in healthy Bern infants – a network approach”

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Research legislation:      Ordinance on human research with the exception of Clinical trials (HRO) [1].

Type of Research Project:      Research project involving human subjects

Risk Categorisation:      Risk Category A

### Project Leader

Prof. Dr. med. Benjamin Misselwitz  
Universitätsklinik für Viszerale Chirurgie und Medizin  
Inselspital Bern, Freiburgstrasse 18, 3010 Bern  
[benjamin.misselwitz@insel.ch](mailto:benjamin.misselwitz@insel.ch)

### Co-Investigator Kinderspital

PD Dr. med. Christiane Sokollik  
Universitätsklinik für Kinderheilkunde, Inselspital Bern  
Freiburgstrasse 15, 3010 Bern  
[christiane.sokollik@insel.ch](mailto:christiane.sokollik@insel.ch)

### Co-Investigator DBMR

Prof. Dr. Stephanie Ganal-Vonarburg  
Department for BioMedical Research  
Universitätsklinik für Viszerale Chirurgie und Medizin  
Murtenstrasse 35, 3008 Bern  
[Stephanie.ganal@dbmr.unibe.ch](mailto:Stephanie.ganal@dbmr.unibe.ch)

## PROTOCOL SIGNATURE FORM

Study Title

*Trajectory of microbiota maturation in healthy Bern infants – a network approach*

The project leader has approved the protocol version 7.0; 19.04.2022 and confirms hereby to conduct the project according to the protocol, the Swiss legal requirements [1, 2], current version of the World Medical Association Declaration of Helsinki [3] and the principles of Good Clinical Practice.

### Project leader and sponsor:

Prof. Dr. med. Benjamin Misselwitz  
Universitätsklinik für Viszerale Chirurgie und Medizin, Inselspital Bern  
Freiburgstrasse 18, 3010 Bern  
[benjamin.misselwitz@insel.ch](mailto:benjamin.misselwitz@insel.ch)

Date: Bern, 30. 5. 2022 Signature: 

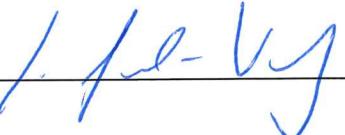
### Co-Investigator Kinderspital

PD Dr. med. Christiane Sokollik  
Universitätsklinik für Kinderheilkunde, Inselspital Bern  
Freiburgstrasse 15, 3010 Bern  
[christiane.sokollik@insel.ch](mailto:christiane.sokollik@insel.ch)

Date: Ber 02. 06. 2022 Signature: 

### Co-Investigator DBMR

Prof Dr. Stephanie Ganal-Vonarburg  
Department for BioMedical Research  
Universitätsklinik für Viszerale Chirurgie und Medizin  
Murtenstarsse 35, 3008 Bern  
[Stephanie.ganal@dbmr.unibe.ch](mailto:Stephanie.ganal@dbmr.unibe.ch)

Date: Ber, 8. 6. 2022 Signature: 

### Klinikdirektor, Leiter Labor:

Prof. Dr. med. Andrew Macpherson  
Klinikdirektor  
Klinik für Viszerale Chirurgie und Medizin  
Inselspital Bern  
Freiburgstrasse 18, 3010 Bern  
[andrew.macpherson@insel.ch](mailto:andrew.macpherson@insel.ch)

Date: 27. 6. 22 Signature: 

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## **GLOSSARY OF ABBREVIATIONS**

<i>BASEC</i>	<i>Business Administration System for Ethical Committees</i>
<i>CRF</i>	<i>Case report form</i>
<i>FOPH</i>	<i>Federal Office of Public Health</i>
<i>HRA</i>	<i>Human Research Act</i>
<i>HRO</i>	<i>Ordinance on Human</i>

## 1 BACKGROUND AND PROJECT RATIONALE

The human intestine represents a large interface between the host and the environment, mediating absorption of nutrients, minerals and water and defence against pathogens. Intestinal functionality is critically shaped by the intestinal microbiota, comprising bacteria, eukaryotes, viruses and archaea colonizing the gastrointestinal tract [1, 2]. The intestinal microbiota maintains a mutually beneficial relationship with the host which together form a superorganism of which the microbiota contributes more than 99% of the combined genomic content [3].

Until recently, the complexity and diversity of the microbiota overwhelmed classical culture techniques. These limitations have now been surmounted by the extreme analytic depth and high-throughput of 'omics techniques (genomics, proteomics, metabolomics). The resultant data attest to the instability of the microbiota in early life, its later variability between individuals according to lifestyle, nutrition, ethnicity and culture [4] and its association with incidence and severity of human disease [5, 6].

The development of the intestinal microbiota is incompletely understood. However, understanding the trajectory of microbiota maturation is likely key to the understanding of the pathogenesis of many pathologies in childhood. The intestine is almost sterile in utero. Postnatally mammals become colonized with successive waves of microbes seeding from the maternal microbiota. As a first step, within the first days after birth, in healthy full-term newborns the intestine is colonized by facultative anaerobic bacteria, corresponding to the initial oxygen availability of the newborn gut [7, 8]. Relevant species include *Enterococcus* spp., *Escherichia coli*, *Staphylococcus* spp. and *α-hemolytic streptococci*. Oxygen consumption by these bacteria enables further microbiota maturation with acquisition of *Bacteroides*, *Clostridium* spp and *Bifidobacterium* which later dominate the healthy infant microbiota [7-9]. The introduction of solid food leads to a progressively increased diversity and the presence of Eubacteria, Veillonella, Fusobacteria, Peptostreptococci and Ruminococci [8].

The early-life microbiota is unstable as incoming microbes initially populate open ecological niches, within a variable environment shaped by nutrition, xenobiotics [10, 11] and protective factors [12, 13]. Subsequently, the microbiota matures and reaches far greater stability provided the environmental conditions are constant [14].

Various factors are known to influence the infant microbiota [9, 15]: i) low gestational age leads to a dominant colonization with Gram-negative bacilli which in turn predispose infants to infections [16]. On the other hand, gestational age also determined the timing of intestinal colonization by bifidobacteria [17]. ii) feeding crucially affects microbiota composition with higher levels of Bifidobacteria in breast-fed infants and higher levels of bacteroides in formula-fed infants [18]. Vice versa, weaning was associated with acquisition of a more adult microbiota [19, 20]. iii) C-section leads to an infant intestinal microbiota more similar to the maternal cutaneous microbiota [21] and differences persist also at 12 months after delivery [20]. iv) the number of siblings also influenced the infant microbiota and the presence of older siblings was associated with higher bacteria diversity [22]. v) early hospitalization and antimicrobial therapy were shown to affect the infant microbiota [9, 15].

Lactation critically impacts on the health and development of infants. Human milk contains oligosaccharides [23] which will reach and shape the infant intestinal microbiota [24], resulting in an enrichment of *Bifidobacterium* species which can represent 70-90% of the faecal community [25]. Besides maternal skin bacteria, Bifidobacteria were also identified in breast milk, suggesting transfer. Neither the impact of maternal bacteria transferred by breastfeeding nor variations of milk constituents on infant intestinal microbiota and well-being have been sufficiently studied [24].

It is widely accepted that microbiota composition critically impacts on human health. One important benefit of the microbiota is based on the concept of 'colonisation resistance': the

healthy, beneficial intestinal microbiota competes with potentially pathological microbes and thus limits vulnerability to intestinal pathogens [26]. Reduced microbiota diversity on the other hand decreases resistance against pathogens such as *Enterococci* [27, 28]. Such microbiota fails to provide the beneficial properties of a functional microbiota and may therefore be considered 'dysbiotic'. Via transplantation of a healthy microbiota by stool transplantation, this dysbiotic state can be revoked and diseases like *Clostridium difficile* colitis can be cured [29]. Additional beneficial microbiota effects include vitamin synthesis, bile salt metabolism and xenobiotic detoxification [30].

Early microbiota perturbations in infants have been associated with subsequent pathology including autoimmune diseases such as type 1 diabetes mellitus [31], childhood atopy and asthma [32], childhood obesity [33] and intestinal conditions such as inflammatory bowel diseases [34]. The infant microbiota might also impact on the development of motor, social and cognitive functions [35] as well as abdominal pain.

On a global perspective, the most important set of microbiota associated problems for human health is in child development. Over 3 million children globally die annually before the age of 5 years [36]. Further, ~200 million children annually do not reach their developmental potential [37]. Malnutrition is especially important in children <2 years [38], and stunts the physical growth and cognitive development of survivors; for girls, it also limits the ability to deliver and raise healthy children later in life [39]. Childhood malnutrition is to a large degree resistant to simple caloric supplementation [24]. Poor health due to low-resources with inadequate nutrition and poor hygiene is accompanied by chronic intestinal maladaptive and inflammatory alterations, summarized as environmental enteropathy (EE). EE is also characterized by a delayed and aberrant maturation of the intestinal microbiota [40, 41], ultimately resulting in dysfunction and failure of the microbiota metabolic organ [42].

Environmental enteropathy is associated with abnormally dense microbial consortia, low-grade inflammation, and poor intestinal function [43], which further aggravates infant malnutrition [42, 44]. Therefore, malnutrition and its consequences of stunted growth and impaired cognitive development cannot be attributed solely to food insecurity: early life microbial colonization is critical [45, 46] and severe acute malnutrition can be limited and recovery accelerated by antibiotics to supplement therapeutic food [47]. Detailed associations with mutualists and pathobionts leading to EE are unclear and it is unknown whether these organisms are transmitted from the environment or from the mother at birth or during lactation.

In any case, considering microbiota complexity and functional redundancy, species composition alone (on its own) is an insufficient descriptor for metabolic activities [48]. Therefore, recent work utilized bacterial full genome metagenomic sequencing to additionally identify bacterial genes present and expressed in relevant contexts. In combination with mass spectrometry, the analysis can then be constrained to the actual small molecule metabolites present to even further specify relevant mechanisms [49]. Finally, advanced network analysis can subsequently provide a comprehensive view of active metabolic pathways [13, 50-52]. Such an approach has not yet been applied to the development of the microbiota.

In summary, there is excellent evidence that microbiota composition is fundamental to human health and that the microbiota undergoes critical changes within the first two years of life. We are planning to perform a comprehensive analysis using advanced microbiota features and small molecule composition of stool samples as well as features of maternal microbiota and maternal milk together with clinical characteristics and parameters for infant nutrition and hygiene to understand the development of the normal infant microbiota within the first two years of life. Our results will allow a better understanding of microbiota pathologies in many conditions including environmental enteropathy.

## 2 PROJECT OBJECTIVES AND DESIGN

### 2.1 Hypotheses

Primary hypothesis: Maturation of the intestinal microbiota within the first two years of life is characterized by step-wise acquisition of bacteria with an increasing complexity and its trajectory can be followed by a comprehensive network analysis of bacteria metabolic activity and metabolites.

Secondary hypothesis:

- 1) Infant nutrition and socioeconomic status predict microbiota development in healthy children
- 2) Maternal microbiota is transferred via maternal milk and/ or skin contact to the newborn baby
- 3) Infant microbiota maturation characteristics predict:
  - a. Growth and weight gain of healthy children
  - b. Neurodevelopment of healthy children
  - c. Adverse outcomes including obesity, abdominal pain, infectious diseases and allergies
- 4) In direct comparison with children from the University of Zimbabwe birth cohort, trajectories of the microbiota maturation of Swiss infants will resemble healthy Zimbabwean children but will strongly differ from African children with malnutrition and stunted growth.
- 5) Intestinal microbiota composition of the mother determines composition of the breast milk regarding its immune-modulatory properties. This affects immune maturation in the newborn.
- 6) The maternal placenta is expected to be sterile and therefore has no impact on intestinal microbiota composition of the newborn.

### 2.2 Objectives and Endpoints

The objectives are explorative in nature and the study should be considered a pilot study. The endpoints are not assessed by fixed parameters but are defined by the achievement of said objectives. Therefore, objectives and endpoints were combined into one single section.

#### Primary objective and primary endpoint

We are aiming for a deep understanding of the maturation of a healthy infant intestinal microbiota considering composition, diversity and metabolic activities. We will characterize the composition, metabolic potential and activity at various time points by advanced techniques (16S sequencing, metagenomics shotgun sequencing and mRNA sequencing) and the metabolites present by mass spectrometry. Using this information, we will estimate networks of metabolic activity of the microbiota. Network analysis can be informed by information regarding small molecules present. The trajectories shared by the microbiota of most healthy infants will be considered normal (for details see 3.3 Study procedures).

#### Secondary objectives and secondary endpoints

- 1) To understand the impact of variations of the normal environment in Switzerland on microbiota development. To this end we will use parameters for nutrition and socioeconomic status, microbiota characteristics, and metabolomics and correlate those with parameters for infant development and immunity.
- 2) To understand the transfer of the maternal microbiota to the infant. We will identify bacterial species (or operational taxonomic units, OTU) in the maternal microbiota in maternal stool, maternal skin, vaginal environment, maternal placenta, as well as

maternal milk at various points in time and correlate these parameters to identified species/ OTU in the intestinal and skin microbiota of the infant at various points in time.

- 3) To understand the impact of the microbiota on child development and health. We will correlate infant microbiota characteristics from the primary endpoint with
  - a. Parameters for physical development (size, weight, percentiles, head circumference, mid upper arm circumference, waist to hip ratio)
  - b. Parameters for neurodevelopment and child behaviour
  - c. Onset/ occurrence of pathology (obesity, abdominal pain, new onset of allergies, asthma, eczema, number of infectious complications, physician consultations outside regular preventive medical check-ups).
- 4) To understand the impact of low resources with poor nutrition and poor hygiene in developing countries on the maturation of the intestinal microbiota. Children from the University of Zimbabwe birth cohort were followed in a similar manner as planned for the children from the Bern infant microbiota study with the same acquisition of biosamples. We will use microbiota characteristics from endpoint 1 to compare microbiota maturation in healthy Swiss infants to microbiota maturation in healthy Zimbabwean children as well as children with environmental enteropathy and stunted growth.
- 5) To understand the extent to which the maternal microbiota and maternal diet affects the immunomodulatory properties of breast milk and how those properties in turn influence immune maturation in the newborn, we will analyse the composition of stool and breast milk samples in regard to metabolites, cellular components, cytokines and miRNA. We will further use the material to test the impact of maternal milk *in vitro* (cell culture) and *in vivo* (mice). Immunomodulatory mechanisms will be identified and correlating changes in development and maturation of the immune system of the newborn will be analysed.

### 2.3 Project design

We will perform a non-interventional, single center exploratory study. With the help from local gynecologists and pediatricians we will enroll mothers from week 20 of pregnancy until birth. Participants will be followed as mother-baby pairs until 10 years after birth. Using questionnaires, we will assess mother and infant nutrition, clinical parameters, sociodemographic parameters and parameters for child development. For clinical outcomes we will mainly rely on the results of regular preventive medical checkups performed by pediatricians ("Vorsorgeuntersuchungen") but also acquire anthropometric data (mid-upper arm circumference, MUAC, and waist to hip ratio) as well as information regarding mother and infant nutrition using a standard weighted food record protocol conducted over 1-3 days as well as validated food frequency questionnaires, infectious complications, allergies, abdominal pain and psychological outcomes by questionnaires. Biosampling will be performed at enrolment (maternal stool and skin swabs, optional maternal vaginal swabs) and during follow-up (infant stool, infant skin swabs, maternal stool, maternal skin swabs, maternal milk).

## 3 PROJECT POPULATION AND STUDY PROCEDURES

### 3.1 Project population, inclusion and exclusion criteria

We are aiming to recruit 250 pregnant participants which will be followed as mother baby pairs.

General inclusion criteria:

- Signed informed consent.
- Ability to understand and follow study procedures and understand informed consent
- From week 20 of pregnancy until birth

- General good health, i.e. absence of major severe medical/ surgical/ psychiatric condition requiring ongoing management. Minor well controlled conditions (e.g. medically controlled arterial hypertension, occupational asthma, gestational diabetes mellitus) may be present.
- Absence of known severe embryonal pathology, expected normal pregnancy (e.g. minor conditions including twin/ triplet pregnancy, final pelvic position may be present)
- Age 18-45 years.

General exclusion criteria:

- Participation in another clinical study interfering with study procedures.

### 3.2 Recruitment, screening and informed consent procedure

Patients will be recruited by the project team and co-workers in collaboration with pediatricians and gynecologists at the Inselspital and in the Bern area. We will preferentially include mothers known to local pediatricians during a next pregnancy.

The patient will be provided with information regarding the study. If the patient shows interest, the investigator will explain the study protocol, answer questions, check inclusion and exclusion criteria and inquire further about the patient's current symptoms, medication and medical history. In case all criteria are met, patients will be asked to sign the informed consent.

At the last time point, when the age of the child is 10 years, the study will be explained to the child in appropriate terms. If the child does not want to provide information/ biosamples at this point in time, this will be respected. Information and biosamples up to this time point will be evaluated and statistically analyzed.

Patients will be compensated for any additional expenses related to the study (e.g. travel). For this study, no compensatory fee will be payed.

### 3.3 Study procedures

#### 3.3.1. Visits and procedures

The project duration for each participant will be from inclusion during pregnancy to 10 years after birth.

**Table 1: Timeline of study procedures**

Time ± tolerance interval	Enrollment	Birth	Days after birth			Weeks after birth							Years after birth	
			0	0-3 ±3	10 ±5	6 ±2	10 ±4	14 ±4	24 ±6	36 ±8	48 ±8	96 ±12	5 ±1	10 ±1
<i>Information about the study</i>	+													
<i>Check inclusion/ exclusion criteria</i>	+													
<i>Signing of written informed consent</i>	+													
<i>Medical history</i>	+													
<i>Baseline questionnaire</i>	+					+		+	+					
<i>In-depth dietary questionnaires of mothers</i>														

<i>(optional) – 1-3 days weighed food record</i>											
<i>food frequency questionnaire of the mother</i>	+				+	+	+				
<i>In-depth dietary questionnaires of infant (optional) – 1-3 days weighed food record</i>									+	+	+
<i>food frequency questionnaire of the infant</i>									+	+	+
<i>Anthropometric measures</i>				+	+	+	+	+	+	+	+
<i>Follow-up questionnaire</i>			+	+	+	+	+	+	+	+	+
<i>Maternal stool samples</i>	+		+	+	+	+	+	+	+	+	+
<i>Maternal skin swab</i>	+				+		+	+	+	+	+
<i>Maternal vaginal samples</i>	+										
<i>Maternal placenta sample</i>		+									
<i>Maternal milk samples, if nursing</i>			+	+	+	+	+	+	+		
<i>Infant stool samples</i>			+	+	+	+	+	+	+	+	+
<i>Infant skin swabs</i>			+	+	+	+	+	+	+	+	+
<i>Child Behaviour Check list (CBCL)</i>									+		+

### **3.3.2. Material sampling**

Sampling will be performed/ assisted by the study team.

- For maternal stool samples, a sampling kit will be provided to the participant.
- If specific consent is given, vaginal swabs will be retrieved.
- At birth, small samples will be taken from the placenta.
- Infant stool samples will be acquired from infant diapers. In case sampling is not possible during the visit, a sampling kit with instructions for sampling will be provided.
- Maternal and infant skin swabs will be acquired in the inguinal region
- For acquisition of milk samples, the breast will first be cleaned followed by acquisition of 1-2ml of milk by squeezing of the breast. The first drops of acquired milk will be discarded.

### **3.3.3. Methods for sample analysis:**

For the **primary objective and outcome/ endpoint** of our study, bacterial content of infant stool will be analyzed by:

- Mass spectrometry to assess **intestinal content (metabolome)**. Techniques have been established in the laboratory of Prof. U. Sauer who already collaborated with our group in previous studies.
- 16S rRNA sequencing for **bacterial species composition** as well as microbial diversity.
- Bacterial full genome metagenomics shotgun sequencing to identify bacterial genes present (**metabolic potential** of the microbiota).
- Bacterial mRNA sequencing to assess transcription and a functional role of the microbiota (**metabolic activity** of microbiota).
- Analysis of the **intestinal virome and eukaryotic intestinal populations** by appropriate sequencing or culturing techniques.

- Analysis of IgA antibodies in human milk and stool and the interaction of antibodies with intestinal bacteria.
- For the secondary endpoints identical analyses will be performed in skin swabs, maternal milk, maternal vaginal swabs, maternal placenta probes and maternal stool. Parameters for infant growth, neurodevelopment, immune maturation and potential occurrence of pathology will be assessed at every visit. Maternal and infant nutrition, hygiene, socioeconomic status and clinical history will be assessed by questionnaires at every visit. All methods described above have already been used for microbiota analyses in human and murine samples for previous unrelated questions [13, 50-52]. Milk samples will further be analyzed for their cellular contents by flow cytometry and single cell RNA-sequencing, as well as for cytokines and exosome-based miRNAs. All biosamples will be analyzed by mass spectrometry to assess impact of the environment on infant metabolism and physiology.

Further follow-up experiments with the acquired samples are possible. Specifically, individual bacteria strains can be isolated and cultured in vitro and also tested alone or in combination in experimental animals.

Bacterial sequencing by the methods described above will also inevitably identify maternal or infant DNA sequences, since metagenomic shotgun sequencing cannot differentiate between bacterial and human DNA. These human DNA sequences will not be analyzed within the scope of this project. However, these sequences might be the subject of future studies. Study participants will therefore be asked for permission to analyze human DNA from mother and/ or child at the page for “further analyses” within the consent form. An option to “opt out” for human DNA analysis will be provided and refusal will not lead to exclusion from the study.

Any findings of clear relevance to the health of the participant (i.e. mother or child) will be reported to the participant in collaboration with their treating pediatrician. Participants will need to inform the study team if they do not wish to be informed.

### 3.4 Withdrawal and discontinuation

If an individual withdraws informed consent during the investigation he/she will be withdrawn from the project. Additional reasons for withdrawal include clear cases of non-compliance with study procedures where the integrity of the samples/questionnaires is clearly in question.

Samples that belong to patients that discontinue/ drop-out during the study (e.g. attend to only to initial visit but fail to attend some of the following visits) or withdraw informed consent will still be considered for study purposes and analyzed accordingly. This approach allows the utilization of an ‘intention to treat’ analysis. By using all collected samples, we aim to reduce confounding factors which would otherwise critically compromise the validity of the generated data considering the small number of participants.

Further, considering the length of the project it is also very likely that at the time point of withdrawal generated data from the first study period have already been analyzed and/ or published which cannot be undone.

Subjects who discontinue from the study or withdraw consent should always be asked about the reason(s) for their discontinuation and about the presence of any adverse events. Adverse events should be followed up.

At 10 years of age the child will be informed about study participation in appropriate terms. If the child expresses unwillingness to continue the last time point of the study this will be respected. If the child expresses the wish for withdrawal of the informed consent provided by the mother, this will also be respected.

## 4. STATISTICS AND METHODOLOGY

### 4.1. Statistical analysis plan

Only very limited knowledge exists regarding networks of bacterium-metabolite interactions in the small intestine. Therefore, a formal power analysis cannot be performed and the current study should be considered a pilot study. The number of study participants has been chosen for practical reasons for this pilot study, also considering capacities of our lab for the expected analyses.

Data from the current study will enable to build hypothesis and perform power analyses for future trials. Observed effects and interactions will be statistically substantiated by parametric and non-parametric tests and/ or multivariate analyses. A p-value  $<0.05$  will be considered significant. Infant intestinal microbiota will be compared to maternal microbiota in stool samples, milk samples, skin swabs, vaginal swabs and maternal placenta probes. Therefore, a microbiota clustering analysis based on a Bray-Curtis dissimilarity principal coordinate analysis (PCoA) will be performed.

A pipeline for detailed assessment of sequence data (QIIME v1.9.1 and several R packages including phyloseq, ggplot2, shape, ape, vegan and Maaslin) and further measures including  $\alpha$ -,  $\beta$ -diversity and Bray-Curtis dissimilarity principal coordinate analysis (PCoA) has been established at DBMR, University of Bern and will be available for this project.

### 4.2. Handling of missing data

The nature of this exploratory study allows for some tolerance regarding missing data. Patients with a limited number of missing samples will not be replaced as long as the missing data does not compromise assessment of microbiota characteristics. Due to the explorative nature of our study, a specific threshold for participant exclusion/ replacement cannot be provided. Patients with an insufficient number of biosamples can be replaced by newly recruited individuals. Dropouts within the first 2 years can also be replaced by newly recruited individuals, if feasible until the final number of individuals indicated at 3.1 will be reached.

## 5 REGULATORY ASPECTS AND SAFETY

### 5.1 Local regulations / Declaration of Helsinki

This research project will be conducted in accordance with the protocol, the Declaration of Helsinki [3], the principles of Good Clinical Practice, the Human Research Act (HRA) and the Human Research Ordinance (HRO) [1] as well as other locally relevant regulations. The Project Leader acknowledges his responsibilities as both the Project Leader and the Sponsor.

### 5.2 Notification of safety and protective measures (HRO Art. 20)

The project leader/ sponsor is promptly notified (within 24 hours) if immediate safety and protective measures have to be taken during the conduct of the research project. The Ethics Committee will be notified via BASEC of these measures and of the circumstances necessitating them within 7 days.

### 5.3 Serious events (HRO Art. 21)

If a serious event occurs, the research project will be interrupted and the Ethics Committee notified on the circumstances via BASEC within 7 days according to HRO Art. 21<sup>1</sup>.

#### **5.4 Radiation**

Not applicable (patients will not be exposed to radiation for this study).

#### **5.5 Amendments**

Substantial changes to the project set-up, the protocol and relevant project documents will be submitted to the Ethics Committee for approval according to HRO Art. 18 before implementation. Exceptions are measures that have to be taken immediately in order to protect the participants.

#### **5.6 End of project**

All encrypted biological materials will be stored in a biobank at Inselspital Bern for 10 years under the responsibility of the sponsor. Clinical data (questionnaires and digitalized material) will be stored by Prof. Dr. med. Benjamin Misselwitz for 10 years after end of study or publication of data analysis (whatever comes last).

#### **5.7 Insurance**

In the event of project-related damage or injuries, the liability of Inselspital Bern provides compensation, except for claims that arise from misconduct or gross negligence.

### **6 FURTHER ASPECTS**

#### **6.1 Overall ethical considerations**

The intestinal microbiota critically impacts on utilization of nutrients, host metabolism, immunity and defense. The importance of the host-microbiota interaction is increasingly recognized. The maturation of the intestinal microbiota is insufficiently understood and has not been sufficiently investigated with state-of-the-art analysis techniques and network analyses. However, microbiota maturation likely critically impacts on infant development, health and subsequent diseases (see introduction). Insights gained during this study might provide a solid framework for further investigations and development of future treatment and prevention strategies for many diseases related to the intestinal microbiota.

This observational study involves only minor burden to the participants, but does not require any modification of clinical care and does not pose any risk to participants (see 6.2., risk-benefit assessment). The study requires handling of their own stool by participants, which might be considered inconvenient or unpleasant by some participants. The study requires several study visits, which poses an inconvenience for the participants. During these visits, participants (mothers) will fill in questionnaires and non-invasive sampling of biomaterials will be performed. All procedures are not associated with risks to patients (see 6.2 risk-benefit assessment). The extensive sampling during this study is crucial for answering the primary and all secondary endpoints.

Individuals will be informed about any incidental finding with clear implications regarding the health of an individual. However, given the pilot character of the current study and the current

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<sup>1</sup> A serious event is defined as any adverse event where it cannot be excluded, that the event is attributable to the sampling of biological material or the collection of health-related personal data, and which:

- a. requires inpatient treatment not envisaged in the protocol or extends a current hospital stay;
- b. results in permanent or significant incapacity or disability; or
- c. is life-threatening or results in death.

understanding on the topic of study the microbiota information obtained during this study is unlikely to have an impact on individual patient health.

Overall, the very low risk of the study, only minor inconveniences associated with the study and the chance of helping to improve medical care for infants ensure an overall fair balance for each study participant.

## **6.2 Risk-Benefit Assessment**

None of the study procedures is associated with risks for relevant complications.

**Stool sampling:** Handling of one's own stool (for mothers) is not associated with any risk. Similarly, infant stool is not considered infectious and handling infant's stool (i.e. changing diapers) is part of daily routine baby care anyways.

**Skin swabs:** are considered to be entirely safe

**Sampling of maternal milk:** is also considered entirely safe

**Vaginal swabs:** are also considered to be entirely safe

**Placenta probes:** the collection of tissue samples from an expelled placenta is also considered risk-free

For these reasons (lack of risks, potential benefits for future patients) the potential benefit outweighs the potential risks in this study.

## **6.3 Rationale for the inclusion of vulnerable participants**

This study will be observing healthy pregnant women and healthy infants; information regarding healthy infant and maternal microbiota cannot be obtained by other means. When informed consent is obtained, we will carefully explain the right to refuse and to withdraw informed consent.

# **7 QUALITY CONTROL AND DATA PROTECTION**

## **7.1 Quality measures**

All individuals involved in the project will be trained regarding study procedures. The sponsor (Prof. Dr. B. Misselwitz) will continuously monitor the appropriate handling of data and samples.

In case the sponsor leaves the Inselspital Bern before completion of the project, the sponsorship will be transferred preferably to a member of the study team. If this should not be possible, the sponsorship will be transferred to a long-term employee of the Universitätsklinik für Viscerale Chirurgie und Medizin or the Universitätsklinik für Kinderheilkunde of the Inselspital Bern. However, as all participants of the study team are long-term employees of the Inselspital Bern, we are not expecting any change of sponsorship.

For quality assurance the Ethics Committee may visit the research sites. Direct access to the source data and all project related files and documents must be granted on such occasions.

## **7.2 Data recording and source data**

Study data is recorded in the worldwide used research electronic data capture application REDCapTM. This study database is run by the Clinical Trial Unit (CTU) of the University of Bern. Data is physically stored on servers of the University of Bern that are located in Bern, Switzerland.

Source data include the medical history of the patient, questionnaires filled out by the patient and our structured study documentation. Source data will be collected during screening and follow-up, coded and entered in the electronic database (electronic CRF, eCRF). During and at the end

of the project period data will be transferred into the electronic database. After the end of data collection the database will be frozen.

### **7.3 Confidentiality and coding**

**Project data** will be handled with uttermost discretion and is only accessible to authorized personnel who require the data to fulfil their duties within the scope of the research project. On the CRFs and other project specific documents, participants are only identified by a unique participant number.

Access to source documents will be permitted for purposes of monitoring and audits. All involved parties must keep the participant data strictly confidential. Data protection will be based on local and national guidelines and applicable local and national law. The anonymity of the participants will be guaranteed when presenting the data at scientific meetings or publishing them in scientific journals. Individual subject medical information obtained as a result of this study is considered confidential and disclosure to third parties is prohibited.

All data entered into the eCRF are transferred to the database using Secure Sockets Layer (SSL) encryption. Each data point has attributes attached to it identifying the user who entered it with the exact time and date. Retrospective alterations of data in the database are recorded in an audit table. Time, table, data field and altered value, and the person are recorded (audit trail).

The server hosting the EDC system and the database is kept in a locked server-room. Only the system administrators have direct access to the server and back-up tapes. A role concept with personal passwords (site investigator, statistician, monitor, administrator etc.) regulates permission for each user to use the system and database as he/she requires.

A multi-level back-up system is implemented. Back-ups of the whole system including the database are run internally several times per day and externally backed up once a day.

At interim or final analyses, data files will be extracted from the database into statistical packages to be analyzed. The status of the database at this time is recorded in special archive tables. The study database with all archive tables will be securely stored by CTU Bern for at least 10 years. The sponsor also keeps the Trial Master File and interim and final reports both in electronic and in hard copy form for at least 10 years.

The investigator will create and Trial Master File and is responsible to keep this Investigator Site File updated and available for review by authorized persons. All paper documents will be stored at the investigator's sites. All documents pertaining to the conduct of the study must be kept by the investigator for a period of 10 years.

The investigator is responsible for treating patients' study information as confidential. The investigator ensures that identifying patient data (name, date of birth, initials) will not be made publicly available. All patient records are identified by the patient identification number (that must not contain patient's data of birth or initials) to maintain patient's confidentiality. Identification code lists that link the subjects' names to the subjects' identification number must be stored in the Investigator Site File.

**Biological material** in this project is not identified by participant name but by a unique study ID. Biological material is appropriately stored in a restricted area only accessible to authorized personnel.

The samples will be stored at the Mucosal Immunology Laboratory, Gastroenterology Group from Benjamin Misselwitz, Stephanie Ganal-Vonarburg and Andrew Macpherson, in Bern and the PI, Benjamin Misselwitz and collaborators from the lab who are actively involved in the study will have access to them.

All freezers have temperature control implemented (temperature is continuously monitored) and are alarm protected. Most analyses will be performed in the facilities of Bern University. Mass spectrometry will be performed in the laboratory of Prof. U. Sauer, Institute for Molecular Systems Biology, ETH Zürich. Otto-Stern-Weg 3, 8093 Zürich. Appropriate storage of samples and participant confidentiality is also guaranteed by our collaborators.

#### **7.4 Retention and destruction of study data and biological material**

The data and samples collected during this study are stored for 10 years after the end of the study. It is possible that the data and samples will be reused at a later date for other examinations (projects), or later be sent to a different database/biobank in Switzerland or abroad for further studies not yet defined (reuse). This other database/biobank must meet the same standards as the database/biobank for this study.

After termination of the study, samples will be autoclaved and put to the waste which will subsequently be burned. These procedures will be documented in our study documentation.

#### **7.5 Infrastructure**

The necessary infrastructure for this project including clinical care of participants, equipment for sequencing, bacterial culturing and further sample analysis is available at the Department of Visceral Surgery and Medicine and the Department of Biomedical Research, Inselspital Bern.

### **8 FUNDING / PUBLICATION / DECLARATION OF INTEREST**

This study will be funded by Prof. Dr. med. Benjamin Misselwitz at Inselspital Bern and Prof. Dr. S. Ganal-Vonarburg, DBMR. Prof. Misselwitz and Prof. Ganal-Vonarburg will apply for additional funding for this project and follow-up projects (for instance at the Schweizerischer Nationalfonds (SNF)). Additional costs will be covered by the Department of biomedical research (DBMR) of the University of Bern.

There are no conflicts of interest from the sponsor and the other investigators of this study.

We are aiming to publish data from this project in a peer-reviewed journal. Authorships of all publications will reflect the relative contribution of a given investigator to this project and agreed on by mutual consent. Following current standard for scientific publishing, we will aim to make raw-data of this project available as electronic supplemental material to a publication.

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