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Study protocol for the **ABERRANT** study: Antibiotic-induced disruption of the maternal and infant microbiome and adverse health outcomes

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3 1 **Study protocol for the ABERRANT study: Antibiotic-induced disruption of the**
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5 2 **maternal and infant microbiome and adverse health outcomes**
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3 24 **ABSTRACT**
4

5 25 **Introduction:** There is compositional overlap between the maternal intestinal microbiome,
6
7 26 the breast milk microbiome and the infant oral and intestinal microbiome. Antibiotics cause
8
9 27 profound changes in the microbiome. However, the effect of intrapartum and early-life
10
11 28 antibiotics on the maternal intestinal and breast milk microbiome, and the infant oral and
12
13 29 intestinal microbiome, and whether effects are only short-term or persist long-term remain
14
15 30 uncertain.
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18
19 31 **Methods and analyses:** In this prospective cohort study, we will use metagenomic
20
21 32 sequencing to determine: (i) the effect of *intrapartum antibiotics* on the composition of the
22
23 33 breast milk, and the infant oral and intestinal microbiome, including the development and
24
25 34 persistence of antibiotic resistance; (ii) the effect of *antibiotic exposure in the first year of life*
26
27 35 on the composition of the infant oral and intestinal microbiome, including the development
28
29 36 and persistence of antibiotic resistance; (iii) the effect of disruption of the infant oral and
30
31 37 intestinal microbiome on health outcomes; and (iv) the compositional overlap between the
32
33 38 maternal intestinal microbiome, the breast milk microbiome and the infant oral and intestinal
34
35 39 microbiome.
36
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38
39 40 **Ethics and dissemination:** The ABERRANT study has been approved by the Commission
40
41 41 cantonale d'éthique de la recherche sur l'être humain (CER-VD) du Canton de Vaud (#2019-
42
43 42 01567). Outcomes will be disseminated through publication and will be presented at scientific
44
45 43 conferences.
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49 44 **Trial registration number:** The U.S. National Institutes of Health NCT04091282.
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45 **STRENGTHS AND LIMITATIONS OF THIS STUDY**

- 46 • This study will use metagenomic sequencing to comprehensively determine the effect
47 of intrapartum and early-life antibiotics on the composition of the maternal breast milk
48 and the infant oral and intestinal microbiome.
- 49 • High quality clinical data combined with cutting-edge microbiome analyses will
50 enable the identification of bacterial species, together with resistance genes and other
51 important components of the microbiome such as archaea, eukaryotes (fungi) and
52 viruses.
- 53 • The study includes investigation of the association between the early-life intestinal
54 microbiome and clinical health outcomes.
- 55 • The knowledge gained by this study will form the basis for the development of
56 evidence-based interventions to prevent adverse outcomes in situations where
57 antibiotics cannot be avoided, including modifying the intestinal microbiome with
58 directed pre- and probiotics, or bacteriophages.
- 59 • Meticulous precautions will be used to avoid contamination of potentially low
60 microbial biomass breast milk samples, such as working in a laminar flow cabinet and
61 including negative controls to identify microbial DNA signals from the environment
62 or extraction and sequencing kits.

63 INTRODUCTION

64 Antibiotics are amongst the most commonly used drugs, especially in infants and children.

65 Even before birth, more than 40% of infants are exposed to antibiotics.¹ Additionally, more

66 than two-thirds of children receive antibiotics before reaching the age of two years.^{2 3} The

67 human intestine is the habitat for a large community of microbes, the intestinal microbiome.

68 Colonisation of the intestinal tract increases rapidly after birth and the microbiome of the

69 maternal intestine and breast milk are an important source for the infant intestinal

70 microbiome.⁴ Conversely, as retrograde flow of breast milk into mammary ducts has been

71 documented,⁵ the infant oral microbiome might be responsible for colonising the mammary

72 ducts and therefore could contribute to the breast milk microbiome. Consequently, there is

73 compositional overlap between the maternal intestinal microbiome, the breast milk

74 microbiome and the infant oral and intestinal microbiome.^{6 7}

75 Growing evidence shows that the composition of the intestinal microbiome in infants plays an

76 important role in the development and regulation of the immune system, especially in the

77 early-life 'critical window' during which the microbiome and the immune response develop

78 concurrently.

79 Antibiotics cause profound changes in the microbiome.^{8 9} However, the magnitude of the

80 effect of intrapartum and early-life antibiotics on the breast milk, and the infant oral and

81 intestinal microbiome, and whether effects are only short-term or persist long-term remain

82 uncertain. Preliminary studies suggest that disruption of intestinal microbiome in the early-life

83 period is associated with the development of a number of immune- and non-immune-

84 mediated diseases, including allergies,¹⁰ eczema,¹⁰ asthma,¹⁰ chronic inflammatory bowel

85 disease,¹¹ obesity¹² and diabetes mellitus.¹³ Antibiotic exposure *in utero* and during infancy

86 has been associated with an increased risk for the same diseases¹⁴⁻¹⁶ and it is likely that the

87 association between antibiotic exposure and the subsequent development of these diseases is

88 mediated through changes in the infant microbiome. However, the features and composition

1
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3 89 characteristics of the intestinal microbiome associated with the development of these
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5 90 conditions are unclear.

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10 92 Importantly, antibiotic exposure can also lead to the development of antibiotic resistance,⁸
11
12 93 resulting in infections that are more difficult and costly to treat, often requiring longer
13
14 94 duration of hospital stay, and the use of antibiotics with more adverse effects. Currently, in
15
16 95 children, there is sparse data available on the effect of antibiotic exposure on the development
17
18 96 and persistence of antibiotic resistance in their intestinal microbiome.

19
20
21 97 In this prospective cohort study, we will use metagenomic sequencing to determine (i) the
22
23 98 effect of *intrapartum antibiotics* on the composition of the breast milk, and the infant oral and
24
25 99 intestinal microbiome (including the development and persistence of antibiotic resistance); (ii)
26
27 100 the effect of *antibiotic exposure in the first year of life* on the composition of the infant oral
28
29 101 and intestinal microbiome (including the development and persistence of antibiotic
30
31 102 resistance); (iii) the effect of disruption of the infant oral and intestinal microbiome on health
32
33 103 outcomes; and (iv) determine the compositional overlap between the maternal intestinal
34
35 104 microbiome, the breast milk microbiome and the infant oral and intestinal microbiome.

36
37 105 Determining the relationship between antibiotic exposure and changes in the breast milk, and
38
39 106 the infant oral and intestinal microbiome, and their potential association with adverse health
40
41 107 outcomes, will provide stronger evidence for strict antibiotic stewardship. Additionally, it will
42
43 108 form the basis for designing studies to investigate interventions to prevent adverse outcomes
44
45 109 in situations where antibiotics cannot be avoided, including modifying the intestinal
46
47 110 microbiome with directed pre- and probiotics, or bacteriophages.

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50 112 **OBJECTIVES**

51 113 **METHODS AND ANALYSIS**

52 114 **Study design**

1
2
3 115 A prospective single-centre cohort study of 400 mother-infant pairs.
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7
8 117 **Aims**
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10 118 **Aim 1:** To determine the extent to which, and for how long, intrapartum antibiotics affect the
11
12 119 composition of the breast milk microbiome and the infant oral and intestinal microbiome, as
13
14 120 well as the prevalence of antibiotic resistance genes.

15
16
17 121 **Aim 2:** To determine the extent to which, and for how long, antibiotics in the first year of life
18
19 122 affect the composition of the oral and intestinal microbiome in infants, as well as the
20
21 123 prevalence of antibiotic resistance genes.

22
23
24 124 **Aim 3:** To determine health outcomes (Table 1) in children up to the age of 2 years who have
25
26 125 or have not been exposed to intrapartum antibiotics or antibiotics in the first year of life and
27
28 126 determine whether there is an association with the composition of the oral and intestinal
29
30 127 microbiome.

31
32
33 128 **Aim 4:** To determine the degree to which the maternal intestinal and the breast milk
34
35 129 microbiome affect the composition of the infant oral intestinal microbiome and the prevalence
36
37 130 of antibiotic resistance genes.

38
39
40 131 **Aim 5:** To determine the degree to which the infant oral microbiome affects the composition
41
42 132 of the breast milk microbiome.
43

44 133

45
46
47 134 **Outcomes**
48

49 135 **Primary endpoints:**
50

- 51 136 - Composition of the maternal intestinal and breast milk microbiome, and the infant oral
52
53 137 and intestinal microbiome and the prevalence of antibiotic resistance genes within the
54
55 138 infant microbiome at birth and when infants are 7 days, 1, 2, 4, 6, 12 and 24 months of
56
57
58 139 age.
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- 1
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3 140 - Number of episodes of lower respiratory tract illnesses and acute otitis media in the
4
5 141 first two years of life.
6
7 142 - Prevalence of allergic sensitisation and eczema at 1 and 2 years of age.
8
9
10 143 - Weight at 1 and 2 years of age.
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13 144

14 145 **Setting and participants**

16 146 **Sampling frame and study sample:** Women who give birth at the Hôpital fribourgeois (HFR)
17
18 in Fribourg, Switzerland and their infants will be followed over a two-year period. If
19
20 147 recruitment is slow a second study site will be added.
21
22 148

23 149 **Recruitment:** Pregnant women attending the antenatal clinic will be given information about
24
25 the study by a research study nurse or doctor and asked to consider enrolling themselves and
26
27 150 their infant in the study.
28
29 151

30 152 **Blinding of outcome assessment:** Doctors and study nurses will be blinded to the group of
31
32 infants (control or antibiotic-exposed) when outcomes are measured.
33
34 153

35 154

37 155 **Eligibility criteria**

39 156 **Inclusion criteria:** Healthy babies born at 37 weeks or more gestation who are breastfed.

41 157 **Exclusion criteria:** Women with the following criteria: HIV, hepatitis B or C infection or
42
43 unscreened for these infections, antibiotics or probiotics in pregnancy or postpartum period
44
45 158 other than during delivery. Infants with the following criteria: low birth weight (<2500 g) or
46
47 159 severe congenital abnormality.
48
49 160

51 161

53 162 **Study outcome measures**

55 163 We will use internationally accepted validated measures for clinical outcomes. The study
56
57 164 protocol is depicted in **Table 2**.

58
59 165 **Diary:** Parents will be given a structured diary where they can record information about their
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1
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3 166 infant's diet (introduction of formula and new foods), illnesses, medical visits, hospital
4
5 167 admissions, use of antibiotics and other medications. This will help them when filling in the
6
7 168 questionnaires at the required time points.

9
10 169 **Questionnaire:** We will do computer-assisted interviews at birth, and when infants are 1, 6,
11
12 170 12 and 24 months of age using best practice international protocols. The following data will
13
14 171 be recorded: demographic variables including parental ancestry and education, family history
15
16 172 of atopy, eczema, asthma and other immune disorders, antenatal variables such as maternal
17
18 173 age, weight, smoking habits, underlying diseases, medication and supplementation use (e.g.
19
20 174 probiotics and vitamins). In addition, we will collect data on delivery history, perinatal course
21
22 175 (e.g. hospitalisation, infections, antibiotics or oxygen administration), breast-feeding
23
24 176 (including episodes of mastitis and maternal antibiotic and probiotic use), age of introduction
25
26 177 of formula and new foods, administration of probiotics and vitamins, use of antibiotics,
27
28 178 antacids and other medications, GP and other medical visits, illnesses including infections and
29
30 179 hospital admissions, number of siblings, child care attendance, parental smoking habits, pet
31
32 180 ownership, suspected food allergy and eczema (presence, medications). Data will be stored
33
34 181 using the Research Electronic Data Capture (REDCap Consortium) database.¹⁷

35
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37
38
39 182 **Clinical examination:** Participants will be reviewed at 12 and 24 months of age in a specially
40
41 183 designated clinic at the HFR by a study nurse or doctor using a structured interview and
42
43 184 clinical eczema assessment.

44
45
46 185 **Weight:** Weight will be assessed during the clinical examination at 12 and 24 months of age.
47
48 186 The WHO Child Growth Standards will be used as a reference for percentiles.¹⁸

49
50 187 **Lower respiratory tract illness (LRTI) and acute otitis media (AOM):** Symptoms of acute
51
52 188 lower respiratory illness (such cough and wheeze) and the number of episodes of LRTI and
53
54 189 AOM will be recorded by parents, and specific questions will be asked in the questionnaires.
55
56 190 We will use the definitions for LRTI developed by Oddy *et al* and Kusel *et al*.^{19 20}
57
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3 191 **Eczema:** Prevalence of eczema at 12 and 24 months of life will be assessed by the Williams'
4
5 192 UK diagnostic criteria of the International Study of Asthma and Allergy in Childhood
6
7 193 (ISAAC).²¹ This will be assessed by using parent-reported eczema from diary information and
8
9 194 from the clinical examinations at 12 and 24 months of age. We will assess the severity of
10
11 195 eczema using the SCORAD.²² We will also collect data on age of onset of eczema,
12
13 196 distribution of eczema, use of eczema medications, and medical consultations and hospital
14
15 197 admissions.

16
17 198 **Skin prick tests:** Sensitisation to the following panel of allergens will be assessed at 24
18
19 199 months of age: cow's milk, egg, peanut, sesame, house dust mite (*Dermatophagoides*
20
21 200 *pteronyssinus 1*), cat, dog and grass pollen. Skin prick allergy testing will be performed
22
23 201 according to standard guidelines.²³ A positive skin prick test will be defined as an average
24
25 202 wheal diameter at least 3 mm greater than that produced by a negative control solution at 15
26
27 203 minutes.²⁴

28
29 204 **Blood sampling:** We will collect maternal blood at time of delivery. We also will obtain cord
30
31 205 blood at birth. During the clinical examination at 12 and 24 months of age, blood will be
32
33 206 collected by personnel trained in infant venipuncture, whose parents consent to this
34
35 207 component of the study. The 5-10 ml volume required is safe and within limits for weight
36
37 208 recommended by the US-based Office of Human Research Protections guidelines for blood
38
39 209 collection from healthy infants.

40
41 210 **Breast milk samples:** Study nurses will collect one colostrum sample as soon as possible after
42
43 211 birth. Mothers will be asked to collect breast milk (with date and time recorded) from their
44
45 212 first feed of the day (a minimum of 2 hours required to the previous feed) when infants are 7
46
47 213 days, 1, 2, 4, and 6 months old. If breastfeeding is ceased earlier than 6 months, breast milk
48
49 214 will be collected before breastfeeding is discontinued. Mothers will be instructed to wash their
50
51 215 hands and breasts meticulously and to then extract breast milk manually without touching the
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216 areola. The first few drops will be discarded. Reminders will be sent by SMS. Sterile
217 containers will be provided.

218 **Oral swabs:** Oral swabs will be taken from infants as soon as possible after birth by a study
219 nurse. Additionally, parents will then be asked to collect buccal swabs (with date and time
220 recorded) before the first feed of the day when infants are 7 days, 1, 2, 4, and 6 months old.
221 Sterile containers will be provided. Reminders will be sent by SMS.

222 **Stool samples:** Mothers will be asked to collect stool from their first bowel movement of the
223 day at 38 weeks of pregnancy and on the day after delivery (with date and time recorded). A
224 meconium sample will be collected from infants as soon as possible after birth by a study
225 nurse. Parents will then be asked to collect stool samples from their infants when they are 7
226 days, 1, 2, 4, 6, 12 and 24 months old. Reminders will be sent by SMS. To minimise
227 variation, parents are asked to collect stool from the first bowel movement of the day (with
228 date and time recorded). Sterile containers will be provided.

229 **Storage of samples:** Parents will be instructed to freeze breast milk, oral swabs and stool
230 samples in sealed bags in their domestic freezer at -20°C until collection by the research team.
231 Samples will be kept frozen during transportation to the laboratory where they will be
232 aliquoted and stored at -80°C.

233 **DNA extraction and sequencing:** DNA from breast milk, oral swabs and stool samples
234 (approximately 200 mg) will be extracted using the FastDNA Spin Kit for soil (MP
235 Biomedicals, Santa Ana, California, USA). DNA concentrations will be quantified using a
236 Qubit 4.0 fluorometer (ThermoFisher Scientific, Waltham, Massachusetts, USA) and high
237 sensitivity DNA reagents. Bacterial DNA will be quantified by broad-range bacterial qPCR.
238 The library preparation will be done using Nextera DNA Flex Library Preparations Kits.
239 Extracted DNA will be indexed with IDT Illumina Nextera DNA Unique Dual Indexes to
240 allow analysis of pooled samples. 150-bp pair-end sequencing will be done using the Illumina
241 NextSeq. The required sequencing depth to provide adequate coverage of microbial

1
2
3 242 communities for taxonomic profiling will be determined by rarefaction curves. We will aim
4
5 243 for a minimum yield of 2×10^6 read-pairs per sample. Appropriate negative controls (including
6
7 244 controls from sterile containers, extraction kits etc.) and positive controls of mock
8
9 245 communities will be included. These controls will be sequenced together with the samples to
10
11 246 identify potential environmental and laboratory contaminants.
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14 247 Researchers carrying out the microbial analyses will be blinded to the group identity of
15
16 248 infants (control or antibiotic exposed group).
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19 249 ***Storage of blood samples:*** Peripheral blood mononuclear cells will be separated from whole
20
21 250 blood and stored in liquid nitrogen. Plasma will be stored frozen at -80°C . These will be
22
23 251 retained for future analysis in projects to evaluate the effect of microbiome on the immune
24
25 252 system, including immunophenotyping and analysis of markers of immune function.
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30 254 **Bioinformatics and statics plan**

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33 255 ***Bioinformatics:*** Sequences will be trimmed according to quality scores and sequencing
34
35 256 adaptors will be removed using Trimmomatic.²⁵ Host sequences will be removed by mapping
36
37 257 against the Human genome with Bowtie2.²⁶ High-quality sequences will be used to create
38
39 258 taxonomic and functional profiles using Metaphlan2²⁷ and HUMAnN2²⁸, respectively.
40
41 259 Antibiotic resistance genes will be identified using ResFinder.²⁹ The outputs will be tables
42
43 260 with taxonomically classified sequence counts and gene abundances.
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45
46

47 261 ***Statistical and association analysis:*** Metrics describing and summarising the different
48
49 262 dimensions of microbiome composition will be considered for statistical analyses. Relative
50
51 263 abundances of bacterial and non-bacterial (archaea, eukaryotes and viruses) taxa, as well as
52
53 264 metabolic functional and antibiotic resistance genes profiles, will be directly integrated for
54
55 265 some analyses. Microbial abundances will also be summarised in alpha-diversity indexes to
56
57 266 describe the number of different taxa (Chao richness) and their distribution (Simpson
58
59 267 diversity) within each sample. Inter-samples distances will be described in standard beta-
60

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3 268 diversity indexes matrices (Jaccard, Bray-Curtis, UniFrac and weighted UniFrac). All these
4
5 269 different metrics will be studied in appropriate statistical analyses to investigate the
6
7
8 270 relationships between sample groups of interest and correlation between clinical metadata and
9
10 271 microbiome composition.

11
12 272 Statistical analyses will compare these metrics in different sample-groups of interest.

13
14 273 Antibiotics exposed samples will be compared to non-exposed samples for significant
15
16
17 274 changes in relative abundance of bacterial taxa, antibiotic resistance genes content and alpha-
18
19 275 diversity by Pearson χ^2 test and logistic regression.

20
21 276 Infant age, demographics, delivery mode and feeding method will be modeled in
22
23
24 277 permutational multivariate analysis of variance (PERMANOVA, adonis2 in VEGAN
25
26 278 package, R Foundation) using beta-diversity distance matrices to identify significant
27
28 279 contributors to the infant stool microbiome composition. To compare paired maternal stool,
29
30 280 breast milk, and infant stool samples, we will do clustering analysis and perform a Wilcoxon
31
32
33 281 rank sum test on beta-diversity distances between true mother-infant pairs and randomly
34
35 282 paired mothers and infants matched by infant age. The same will be done for comparison of
36
37
38 283 the breast milk and the infant oral microbiome. These different analyses should allow to
39
40 284 describe the relationships between samples of different origins and identify determinants of
41
42 285 microbiome composition.

43
44 286

45
46
47 287 To further characterise the mother-to-infant microbiome transmission, the StrainPhlAn
48
49 288 software will be used.³⁰ This software enables same-species sequence comparisons at the
50
51 289 single nucleotide polymorphism (SNPs) level to define sample-specific strains. Combined
52
53
54 290 results for shared species allow precise description of the phylogenetic distance between
55
56 291 samples. Added to the beta-diversity analyses, this will allow to better disentangle the
57
58 292 hypothesised seeding from maternal stool and breast milk to the infant oral and stool
59
60 293 microbiome.

1
2
3 294 For integrated analysis of the microbiome data and clinical outcomes, allergic sensitisation,
4
5 295 eczema, and overweight cases will be defined using the prospectively collected outcome
6
7 296 measures. The relationship between bacterial taxa and these clinical outcomes will be
8
9
10 297 determined by hierarchical clustering of communities using heatmaps and principal
11
12 298 component analysis (PCA). Significance of grouping of clinical categories using
13
14 299 permutational multivariate analysis of variance (PERMANOVA). Microbes that have
15
16 300 significantly different abundance between the clinical outcome groups will be identified using
17
18 301 the multiple testing (“mt”) function in phyloseq.³¹ The potential influence of antenatal and
19
20 302 postnatal factors on the microbiome or clinical outcomes will be accounted for in all analyses
21
22 303 by PERMANOVA and unsupervised hierarchical clustering.
23
24
25
26 304

28 305 **ETHICS AND DISSEMINATION**

30 306 **Ethics approval**

31
32
33 307 The ABERRANT study has been approved by the Commission cantonale d’éthique de la
34
35 308 recherche sur l’être humain (CER-VD) du Canton de Vaud (#2019-01567).
36
37
38 309

39 310 **Recruitment and consent**

40
41
42 311 Written informed consent will be obtained from all participants included in the trial.
43
44 312 Participants will be informed that they are not obliged to take part in the study and are free to
45
46 313 withdraw at any time without any impact on their future care.
47
48
49 314

50 315 **Data collection and storage**

51
52 316 Data will be sourced from medical maternal and infant records, as well as by questionnaire
53
54 317 from parents. Data will be de-identified and entered in to a secure, web-based electronic
55
56 318 database.
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3 320 **Safety**
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5 321 No serious adverse reactions are anticipated but these will be checked for by the Data Safety
6
7 322 and Monitoring Committee.
8
9

10 323

11
12 324 **Dissemination of results**
13

14 325 Outcomes will be disseminated through publication according to the SPIRIT statement and
15
16 326 will be presented at scientific conferences.
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18

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20
21 328 **Study duration**
22

23 329 We aim to recruit participants over a two-year period.
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25

26 330

27
28 331 **DISCUSSION**
29

30 332 The intestinal microbiome is crucial in the development of the immune system and regulation
31
32 333 of immune responses, especially during infancy, when the intestinal microbiome and the
33
34 334 immune response develop concurrently.³² The development of intestinal microbiome is easily
35
36 335 disrupted by external factors and perturbation during this vulnerable period may have a large
37
38 336 influence on immune development. A number of factors influence the development of the
39
40 337 infant intestinal microbiome, including gestational age, delivery mode, feeding, and maternal
41
42 338 and infant antibiotic exposure (**Figure 1**).³³ While the effect of delivery mode and feeding
43
44 339 methods on the establishment of microbial communities has been well studied, much less is
45
46 340 known about the effects of intrapartum and early-life antibiotic exposure on the establishment
47
48 341 of microbial communities in the intestinal microbiome.⁹
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51 342 A number of commonly used antibiotics have profound effects on specific bacteria within the
52
53 343 intestinal microbiome, as detailed in a recent systematic review.⁸ This ‘collateral damage’
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55 344 includes changes in abundance of microbial taxa, a decrease in ‘colonisation resistance’
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57 345 (protection against colonisation with potentially pathogenic organisms) and the development
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3 346 of antibiotic resistance. To date, most studies on the effect of antibiotic exposure on the
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5 347 intestinal microbiome have been done in adults.⁸ The main findings of these studies are that
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7 348 antibiotics often lead to a decreased bacterial diversity, a decrease in the abundance of
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10 349 anaerobic bacteria, an increase in abundance of *Enterobacteriaceae* other than *E. coli* and an
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12 350 increase in the abundance of yeast.⁸ These studies show that changes in the intestinal
13
14 351 microbiome after just one course of antibiotics can persist up to four years.⁸ However, the
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16
17 352 clinical consequences of changes in the composition of the intestinal microbiome with
18
19 353 antibiotic treatment are unknown. An increase in *Enterobacteriaceae*, which are often resistant
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21 354 to beta-lactam and other antibiotics, might render the host more susceptible to infections with
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23 355 antibiotic-resistant bacteria. This phenomenon has been observed in infants in neonatal
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25
26 356 intensive care units, who become more often colonised with *Klebsiella* spp., *Enterobacter*
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28 357 spp. and *Citrobacter* spp., when treated with antibiotics.³⁴
29
30 358 Intrapartum antibiotic prophylaxis (IAP) has become common practice in obstetric medicine
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33 359 and is used in up to 40% of deliveries, which makes it the most common source of antibiotic
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35 360 exposure in neonates.¹ IAP is routinely used in both elective and emergency Caesarean
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37 361 section (CS). It is also routinely used in women who are colonised with group B streptococcus
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40 362 (GBS). Despite the benefits, the risks associated with exposing a large number of infants to
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42 363 antibiotics, especially the long-term effects on health through changes in the microbiome,
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44 364 remain unclear. Infants who were exposed to IAP have been reported to have a lower alpha-
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46 365 diversity, a lower relative abundance of Actinobacteria, especially *Bifidobacteriaceae*, and a
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48 366 larger relative abundance of Proteobacteria in their intestinal microbiome compared to non-
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51 367 exposed infants.⁹
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53 368 Breastfeeding has been shown to be beneficial in preventing many communicable and non-
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56 369 communicable diseases.³⁵ Despite intensive research into the positive health effects of
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58 370 breastfeeding, the underlying mechanisms are still not understood. However, a large part of
59
60 371 the beneficial effects of breast milk is likely mediated through the microbiome and its

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3 372 associated immunomodulatory, anti-inflammatory and antimicrobial components. The breast
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5 373 milk microbiome also plays a large part in shaping the infant's intestinal microbiome.⁴

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10 375 There is relatively little data about the effects of IAP on the composition of the breast milk
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12 376 microbiome.^{36 37} Mothers who receive IAP have been reported to have a lower abundance or
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14 377 even an absence of the beneficial bacteria *Bifidobacterium* spp. in their breast milk.^{36 37} There
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16 378 is also some evidence suggesting that mothers who receive IAP have a higher bacterial
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18 379 richness and diversity in their breast milk microbiome compared with mothers who do not
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20 380 receive antibiotics.³⁶ However, these findings have to be interpreted with caution: it could be
21
22 381 that antibiotics lead to lower bacterial numbers and therefore signals from contamination, e.g.
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24 382 bacteria found in DNA extraction or sequencing kits might be amplified more leading to a the
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26 383 detection of a higher diversity. Use of broad-range qPCR to quantify bacterial load in milk
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28 384 samples will allow to assess this potential bias.

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31 385 Interestingly, recent preliminary studies have also shown that delivery mode affects the
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33 386 composition of the breast milk microbiome.^{36 38 39} However, during suckling, a high degree of
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35 387 retrograde flow of milk into the mammary ducts can occur,⁵ transferring bacteria from the
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37 388 infant to the mother, as postulated for GBS.⁴⁰ It is therefore possible that the differences in the
38
39 389 breast milk microbiome observed with different delivery modes are mediated through
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41 390 differences in the oral microbiome of infants.

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43 391 This study will determine the effect of intrapartum and early-life antibiotics on the
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45 392 composition of the breast milk, and the infant oral and intestinal microbiome and antibiotic
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47 393 resistance. The knowledge gained by this study will make an important contribution to the
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49 394 growing field of research investigating the importance of the immunological role of the breast
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51 395 milk microbiome and the infant intestinal microbiome on infant health. It will form the basis
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53 396 for investigating the interplay between the microbiome and the regulation of the human
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3 397 immune system and possible adverse health outcomes, such as the development of immune
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5 398 and non-immune mediated diseases, including allergic diseases.
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7 399 The results of this study will also build a stronger evidence base for strict antibiotic
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9 400 stewardship and form the basis for development of evidence-based interventions to prevent
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11 401 adverse outcomes in situations where antibiotics cannot be avoided, including modifying the
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14 402 intestinal microbiome with directed pre- and probiotics or bacteriophages.
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3 403 **Contributors** VS, LF, NC and PZ were responsible for study conception and design. JW, AL
4
5 404 and PZ were responsible for funding acquisition and implementation. MV drafted the
6
7 405 manuscript and coordinated the manuscript preparation and revision. PZ has developed the
8
9 406 statistical analysis plan. MV and PZ have developed the online questionnaires and database
10
11 407 set-up in REDCap, based on the database setup by the Melbourne Infant Study: BCG for
12
13 408 Allergy and Infection Reduction (MIS BAIR).⁴¹ All authors provided critical evaluation and
14
15 409 revision of manuscript and have given final approval of the manuscript accepting
16
17 410 responsibility for all aspects.
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26 546 *press* 2019

Table 1 Clinical outcomes for aim 3

Outcome	Main measure	Timing
Lower respiratory tract illness	Number of episodes & hospitalisations ^{19 20}	first 2 years of life
Acute otitis media	Number of episodes ^{19 20}	first 2 years of life
Allergic (atopic) sensitisation	Prevalence (positive skin prick test) ²³	at 2 years of age
Eczema	Prevalence (Williams criteria) ²¹	at 1 and 2 years of age
Weight	Centile (WHO Child Growth Standards) ¹⁸	at 1 and 2 years of age

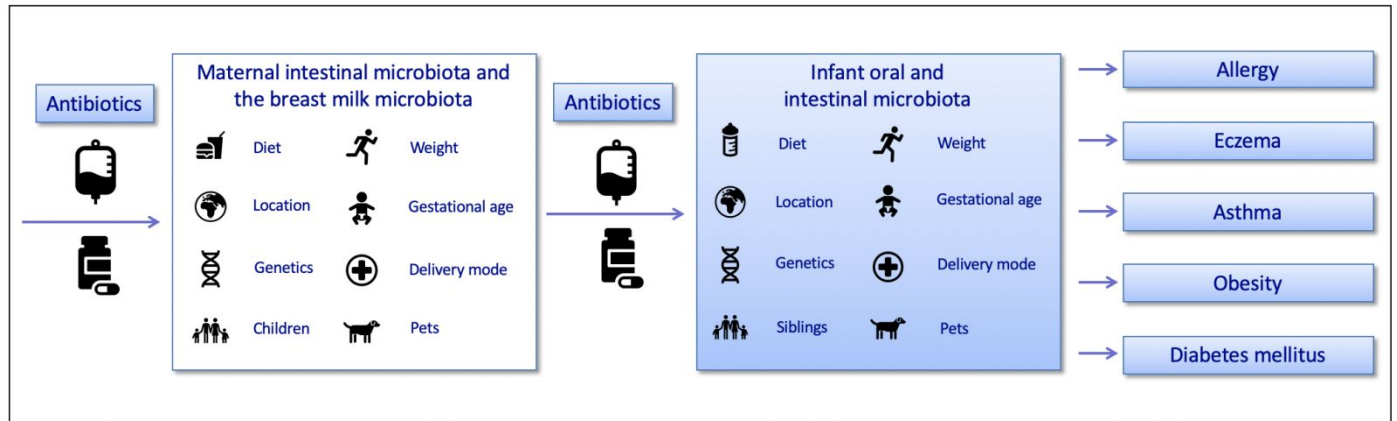
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Table 2 Study protocol

Time	Ant	Birth	7d	1m	2m	4m	6m	12m	24m
Diary			✓	✓	✓	✓	✓	✓	✓
Questionnaire			✓		✓	✓	✓	✓	✓
Maternal blood sample		✓							
Maternal stool sample	✓	✓							
Breast milk sample		✓ ^{col}	✓	✓	✓	✓	✓ [*]		
Infant oral swab		✓	✓	✓	✓	✓	✓		
Infant stool sample		✓ ^{mec}	✓	✓	✓	✓	✓	✓	✓
Clinical examination								✓	✓
Skin prick test									✓
Blood sampling (optional)		✓ ^{cb}						✓	✓

Ant = antenatal; col = colostrum; mec = meconium; ³cb = cord blood;
^{*}or before breastfeeding is discontinued if earlier than 6 m

Figure 1 Summary of factors that might influence the composition of the maternal intestinal and breast milk microbiome, and the infant oral and intestinal microbiome together with possible associated adverse health outcomes.



BMJ Open

Study protocol for the **ABERRANT** study: Antibiotic-induced disruption of the maternal and infant microbiome and adverse health outcomes - A prospective cohort study among children born at term

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3 1 **Study protocol for the ABERRANT study: Antibiotic-induced disruption of the**
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5 2 **maternal and infant microbiome and adverse health outcomes - A prospective cohort**
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7 3 **study among children born at term**
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3 25 **ABSTRACT**
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5 26 **Introduction:** There is compositional overlap between the maternal intestinal microbiome,
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7 27 the breast milk microbiome and the infant oral and intestinal microbiome. Antibiotics cause
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9 28 profound changes in the microbiome. However, the effect of intrapartum and early-life
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11 29 antibiotics on the maternal intestinal and breast milk microbiome, and the infant oral and
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13 30 intestinal microbiome, and whether effects are only short-term or persist long-term remain
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15 31 uncertain.
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21 33 **Methods and analyses:** In this prospective cohort study, we will use metagenomic
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23 34 sequencing to determine: (i) the effect of *intrapartum antibiotics* on the composition of the
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25 35 breast milk, and the infant oral and intestinal microbiome, including the development and
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27 36 persistence of antibiotic resistance; (ii) the effect of *antibiotic exposure in the first year of life*
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29 37 on the composition of the infant oral and intestinal microbiome, including the development
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31 38 and persistence of antibiotic resistance; (iii) the effect of disruption of the infant oral and
32
33 39 intestinal microbiome on health outcomes; and (iv) the compositional overlap between the
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35 40 maternal intestinal microbiome, the breast milk microbiome and the infant oral and intestinal
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37 41 microbiome.
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44 43 **Ethics and dissemination:** The ABERRANT study has been approved by the Commission
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46 44 cantonale d'éthique de la recherche sur l'être humain (CER-VD) du Canton de Vaud (#2019-
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48 45 01567). Outcomes will be disseminated through publication and will be presented at scientific
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50 46 conferences.
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56 48 **Trial registration number:** The U.S. National Institutes of Health NCT04091282.
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49 STRENGTHS AND LIMITATIONS OF THIS STUDY

- 50 • The use of metagenomic sequencing to comprehensively determine the effect of
51 intrapartum and early-life antibiotics on the composition of the maternal breast milk
52 and the infant oral and intestinal microbiome.
- 53 • The possibility to identify bacterial species, together with resistance genes and other
54 important components of the microbiome such as archaea, eukaryotes (fungi) and
55 viruses.
- 56 • The investigation of the association between the early-life intestinal microbiome and
57 clinical health outcomes.
- 58 • The potential for contamination of low microbial biomass such as breast milk or
59 meconium samples from the environment or extraction and sequencing kits.

60 INTRODUCTION

61 Antibiotics are amongst the most commonly used drugs, especially in infants and children.

62 Even before birth, more than 40% of infants are exposed to antibiotics.¹ Additionally, more

63 than two-thirds of children receive antibiotics before reaching the age of two years.^{2,3} The

64 human intestine is the habitat for a large community of microbes, the intestinal microbiome.

65 Colonisation of the intestinal tract increases rapidly after birth and the microbiome of the

66 maternal intestine and breast milk are likely important source for the infant intestinal

67 microbiome.⁴ Conversely, as retrograde flow of breast milk into mammary ducts has been

68 documented,⁵ the infant oral microbiome might be responsible for colonising the mammary

69 ducts and therefore could contribute to the breast milk microbiome. Consequently, there is

70 compositional overlap between the maternal intestinal microbiome, the breast milk

71 microbiome and the infant oral and intestinal microbiome.^{6,7}

72
73 Growing evidence shows that the composition of the intestinal microbiome in infants plays an

74 important role in the development and regulation of the immune system, especially in the

75 early-life 'critical window' during which the microbiome and the immune response develop

76 concurrently.

77
78 Antibiotics cause profound changes in the microbiome.^{8,9} However, the magnitude of the

79 effect of intrapartum and early-life antibiotics on the breast milk, and the infant oral and

80 intestinal microbiome, and whether effects are only short-term or persist long-term remain

81 uncertain. Preliminary studies suggest that disruption of intestinal microbiome in the early-life

82 period is associated with the development of a number of immune- and non-immune-

83 mediated diseases, including allergies,¹⁰ eczema,¹⁰ asthma,¹⁰ chronic inflammatory bowel

84 disease,¹¹ obesity¹² and diabetes mellitus.¹³ Antibiotic exposure *in utero* and during infancy

85 has been associated with an increased risk for the same diseases¹⁴⁻¹⁶ and it is likely that the

1
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3 86 association between antibiotic exposure and the subsequent development of these diseases is
4
5 87 mediated through changes in the infant microbiome. However, the features and composition
6
7 88 characteristics of the intestinal microbiome associated with the development of these
8
9 89 conditions are unclear.

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14 91 Importantly, antibiotic exposure can also lead to the development of antibiotic resistance,⁸
15
16 92 resulting in infections that are more difficult and costly to treat, often requiring longer
17
18 93 duration of hospital stay, and the use of antibiotics with more adverse effects. Currently, in
19
20 94 children, there is sparse data available on the effect of antibiotic exposure on the development
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22 95 and persistence of antibiotic resistance in their intestinal microbiome.

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28 97 In this prospective cohort study, we will use metagenomic sequencing to determine (i) the
29
30 98 effect of *intrapartum antibiotics* on the composition of the breast milk, and the infant oral and
31
32 99 intestinal microbiome (including the development and persistence of antibiotic resistance); (ii)
33
34 100 the effect of *antibiotic exposure in the first year of life* on the composition of the infant oral
35
36 101 and intestinal microbiome (including the development and persistence of antibiotic
37
38 102 resistance); (iii) the effect of disruption of the infant oral and intestinal microbiome on health
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40 103 outcomes; and (iv) determine the compositional overlap between the maternal intestinal
41
42 104 microbiome, the breast milk microbiome and the infant oral and intestinal microbiome.

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48 106 Determining the relationship between antibiotic exposure and changes in the breast milk, and
49
50 107 the infant oral and intestinal microbiome, and their potential association with adverse health
51
52 108 outcomes will provide stronger evidence for strict antibiotic stewardship. Additionally, it will
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54 109 form the basis for designing studies to investigate interventions to prevent adverse outcomes
55
56 110 in situations where antibiotics cannot be avoided, including modifying the intestinal
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58 111 microbiome with directed pre- and probiotics, or bacteriophages.

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5 113 **OBJECTIVES**6
7 114 **METHODS AND ANALYSIS**8
9 115 **Study design**10 116 A prospective single-centre cohort study of 400 mother-infant pairs.
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14 11715
16 118 **Aims**17
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19 119 ***Aim 1:*** To determine the extent to which, and for how long, intrapartum antibiotics affect the
20
21 120 composition of the breast milk microbiome and the infant oral and intestinal microbiome, as
22
23 121 well as the prevalence of antibiotic resistance genes.
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26 12227
28 123 ***Aim 2:*** To determine the extent to which, and for how long, antibiotics in the first year of life
29
30 124 affect the composition of the oral and intestinal microbiome in infants, as well as the
31
32 125 prevalence of antibiotic resistance genes.
33
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35 12636
37 127 ***Aim 3:*** To determine health outcomes (**Table 1**) in children up to the age of 2 years who have
38
39 128 or have not been exposed to intrapartum antibiotics or antibiotics in the first year of life and
40
41 129 determine whether there is an association with the composition of the oral and intestinal
42
43 130 microbiome.
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46 13147
48 132 ***Aim 4:*** To determine the degree to which the maternal intestinal and the breast milk
49
50 133 microbiome affect the composition of the infant oral intestinal microbiome and the prevalence
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52 134 of antibiotic resistance genes.
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55 13556
57 136 ***Aim 5:*** To determine the degree to which the infant oral microbiome affects the composition
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59 137 of the breast milk microbiome.
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Outcomes

Primary endpoints:

- Composition of the maternal intestinal and breast milk microbiome, and the infant oral and intestinal microbiome and the prevalence of antibiotic resistance genes within the infant microbiome at birth and when infants are 7 days, 1, 2, 4, 6, 12 and 24 months of age.
- Number of episodes of lower respiratory tract illnesses and acute otitis media in the first two years of life.
- Prevalence of allergic sensitisation and eczema at 1 and 2 years of age.
- Weight at 1 and 2 years of age.

Setting and participants

Sampling frame and study sample: Women who give birth at the Hôpital fribourgeois (HFR) in Fribourg, Switzerland and their infants will be followed over a two-year period. If recruitment is slow a second study site will be added.

Recruitment: Pregnant women attending the antenatal clinic will be given information about the study by a research study nurse or doctor and asked to consider enrolling themselves and their infant in the study. *Either both parents or only mothers are present during the antenatal consent interview. We explicitly encourage caregivers to discuss participation with their partners, other family members, doctors and midwives (this is clearly stated in the consent form). We will re-evaluate the willingness to participate when mothers are admitted to the hospital for delivery. Paediatricians will be informed about the children's participation in the study.*

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3 164 **Blinding of outcome assessment:** Doctors and study nurses will be blinded to the group of
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5 165 infants (control or antibiotic-exposed) when outcomes are measured.
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10 167 **Patient and Public Involvement**

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12 168 Patients and public were not involved in the design of this study. The results of this study will
13
14 169 be disseminated to parents of the study participants via a participant newsletter distributed by
15
16 170 email.
17
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20
21 172 **Eligibility criteria**

22
23 173 **Inclusion criteria:** Healthy infants born at 37 weeks or more gestation who are breastfed.

24 174 Mothers will be asked at an antenatal consent interview if they intend to breastfeed. This will
25
26 175 be reassessed at delivery. Mothers will only be included if they breastfeed their infants.

27
28 176 However, if breastfeeding is stopped before the infant reaches six months of age, this will not
29
30 177 be a reason for exclusion. All the breast milk samples up to that point, as well as stool
31
32 178 samples and oral swabs collected afterwards will be analysed.
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37 180 **Exclusion criteria:** Women with the following criteria: HIV, hepatitis B or C infection or
38
39 181 unscreened for these infections, antibiotics or probiotics in pregnancy or postpartum period
40
41 182 other than during delivery. Infants with the following criteria: low birth weight (<2500 g) or
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43 183 severe congenital abnormality.
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49 185 **Study outcome measures**

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51 186 We will use internationally accepted validated measures for clinical outcomes. The study
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53 187 protocol is depicted in **Table 2**.
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59 189 **Diary:** Parents will be given a structured diary where they can record information about their
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3 190 infant's diet (introduction of formula and new foods), illnesses, medical visits, hospital
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5 191 admissions, use of antibiotics and other medications. This will help them when filling in the
6
7
8 192 questionnaires at the required time points.
9

10 193
11
12 194 **Questionnaire:** We will do computer-assisted interviews at birth, and when infants are 1, 6,
13
14 195 12 and 24 months of age using best practice international protocols. The following data will
15
16 196 be recorded: demographic variables including parental ancestry and education, family history
17
18 197 of atopy, eczema, asthma and other immune disorders, antenatal variables such as maternal
19
20 198 age, weight, smoking habits, underlying diseases, medication and supplementation use (e.g.
21
22 199 probiotics and vitamins). In addition, we will collect data on delivery history, perinatal course
23
24 200 (e.g. hospitalisation, infections, antibiotics or oxygen administration), breast-feeding
25
26 201 (including episodes of mastitis and maternal antibiotic and probiotic use), age of introduction
27
28 202 of formula and new foods, administration of probiotics and vitamins, use of antibiotics,
29
30 203 antacids and other medications, GP and other medical visits, illnesses including infections and
31
32 204 hospital admissions, number of siblings, child care attendance, parental smoking habits, pet
33
34 205 ownership, suspected food allergy and eczema (presence, medications). Data will be stored
35
36 206 using the Research Electronic Data Capture (REDCap Consortium) database.¹⁷
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44 208 **Clinical examination:** Participants will be reviewed at 12 and 24 months of age in a specially
45
46 209 designated clinic at the HFR by a study nurse or doctor using a structured interview and
47
48 210 clinical eczema assessment.
49

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51 211
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53 212 **Weight:** Weight will be assessed during the clinical examination at 12 and 24 months of age.
54
55 213 The WHO Child Growth Standards will be used as a reference for percentiles.¹⁸
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3 215 **Lower respiratory tract illness (LRTI) and acute otitis media (AOM):** Symptoms of acute
4
5 216 lower respiratory illness (such cough and wheeze) and the number of episodes of LRTI and
6
7 217 AOM will be recorded by parents, and specific questions will be asked in the questionnaires.
8
9
10 218 We will use the definitions for LRTI developed by Oddy *et al* and Kusel *et al*.^{19 20}

11
12 219
13
14 220 **Eczema:** Prevalence of eczema at 12 and 24 months of life will be assessed by the Williams'
15
16 221 UK diagnostic criteria of the International Study of Asthma and Allergy in Childhood
17
18 222 (ISAAC).²¹ This will be assessed by using parent-reported eczema from diary information and
19
20 223 from the clinical examinations at 12 and 24 months of age. We will assess the severity of
21
22 224 eczema using the SCORAD.²² We will also collect data on age of onset of eczema,
23
24 225 distribution of eczema, use of eczema medications, and medical consultations and hospital
25
26 226 admissions.

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31 227
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33 228 **Skin prick tests:** Sensitisation to the following panel of allergens will be assessed at 24
34
35 229 months of age: cow's milk, egg, peanut, sesame, house dust mite (*Dermatophagoides*
36
37 230 *pteronyssinus 1*), cat, dog and grass pollen. Skin prick allergy testing will be performed
38
39 231 according to standard guidelines.²³ A positive skin prick test will be defined as an average
40
41 232 wheal diameter at least 3 mm greater than that produced by a negative control solution at 15
42
43 233 minutes.²⁴

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46 234
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48 235 **Blood sampling:** We will collect maternal blood at time of delivery. We also will obtain cord
49
50 236 blood at birth. During the clinical examination at 12 and 24 months of age, blood will be
51
52 237 collected by personnel trained in infant venipuncture, whose parents consent to this
53
54 238 component of the study. The 5-10 ml volume required is safe and within limits for weight
55
56 239 recommended by the US-based Office of Human Research Protections guidelines for blood
57
58 240 collection from healthy infants.

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5 242 **Breast milk samples:** Study nurses will collect one colostrum sample as soon as possible after
6
7 243 birth. Mothers will be asked to collect breast milk (with date and time recorded) from their
8
9 244 first feed of the day (a minimum of 2 hours required to the previous feed) when infants are 7
10
11 245 days, 1, 2, 4, and 6 months old. If breastfeeding is ceased earlier than 6 months, breast milk
12
13 246 will be collected before breastfeeding is discontinued. Mothers will be instructed to wash their
14
15 247 hands and breasts meticulously and to then extract breast milk manually without touching the
16
17 248 areola. The first few drops will be discarded. Reminders will be sent by SMS. Sterile
18
19 249 containers will be provided.

20
21 250 **Oral swabs:** Oral swabs will be taken from infants as soon as possible after birth by a study
22
23 251 nurse. Additionally, parents will then be asked to collect buccal swabs (with date and time
24
25 252 recorded) before the first feed of the day when infants are 7 days, 1, 2, 4, and 6 months old.
26
27 253 Sterile containers will be provided. Reminders will be sent by SMS.

28
29 254
30
31 255 **Stool samples:** Mothers will be asked to collect stool on or after the day of the delivery (with
32
33 256 date and time recorded). A meconium sample will be collected from infants as soon as
34
35 257 possible after birth by a study nurse. Parents will then be asked to collect stool samples from
36
37 258 their infants when they are 7 days, 1, 2, 4, 6, 12 and 24 months old. Reminders will be sent by
38
39 259 SMS. To minimise variation, parents are asked to collect stool from the first bowel movement
40
41 260 of the day (with date and time recorded). Sterile containers will be provided.

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43 261
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45 262 **Storage of samples:** Parents will be instructed to freeze breast milk, oral swabs and stool
46
47 263 samples in sealed bags in their domestic freezer at -20°C until collection by the research team.
48
49 264 Samples will be kept frozen during transportation to the laboratory where they will be
50
51 265 aliquoted and stored at -80°C.

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3 267 ***DNA extraction and sequencing:*** DNA from breast milk, oral swabs and stool samples
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5 268 (approximately 200 mg) will be extracted using the FastDNA Spin Kit for soil (MP
6
7 269 Biomedicals, Santa Ana, California, USA). DNA concentrations will be quantified using a
8
9 270 Qubit 4.0 fluorometer (ThermoFisher Scientific, Waltham, Massachusetts, USA) and high
10
11 271 sensitivity DNA reagents. Bacterial DNA will be quantified by broad-range bacterial
12
13 272 quantitative polymerase chain reaction (qPCR). The library preparation will be done using
14
15 273 Nextera DNA Flex Library Preparations Kits. Extracted DNA will be indexed with IDT
16
17 274 Illumina Nextera DNA Unique Dual Indexes to allow analysis of pooled samples. 150-bp
18
19 275 pair-end sequencing will be done using the Illumina NextSeq. The required sequencing depth
20
21 276 to provide adequate coverage of microbial communities for taxonomic profiling will be
22
23 277 determined by rarefaction curves. We will aim for a minimum yield of 2×10^6 read-pairs per
24
25 278 sample. Appropriate negative controls (including controls from sterile containers, extraction
26
27 279 kits etc.) and positive controls of mock communities will be included. These controls will be
28
29 280 sequenced together with the samples to identify potential environmental and laboratory
30
31 281 contaminants.

32
33 282 Researchers carrying out the microbial analyses will be blinded to the group identity of
34
35 283 infants (control or antibiotic exposed group).
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44 285 ***Storage of blood samples:*** Peripheral blood mononuclear cells will be separated from whole
45
46 286 blood and stored in liquid nitrogen. Plasma will be stored frozen at -80°C . These will be
47
48 287 retained for future analysis in projects to evaluate the effect of microbiome on the immune
49
50 288 system, including immunophenotyping and analysis of markers of immune function.
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290 **Statistical power calculation**

56
57
58 291 The analysis for the a priori computation of the required sample size was conducted using the
59
60 292 G*Power 3.1 software. For the power analysis, the repeated measurements MANOVAs were

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2
3 293 considered. The calculation was based on F-tests for the interaction effect of the between-
4
5 294 subject factor antibiotics vs no antibiotics and the within-subject factor time (8 time points).
6
7 295 We chose a small to medium effect size of 0.22 for the calculation. With a significance level
8
9
10 296 of 5% per test and a power of 80% the power analysis yields a sample size of 304. While we
11
12 297 will attempt to retain and follow up all participants, we are powering our study on ‘a worst-
13
14 298 case scenario’ assumption that complete 24-month data will be available for 76% of
15
16
17 299 participants (this corresponds to a drop-out rate of 12% per year). We will therefore recruit
18
19 300 approximately 400 infants in total (plus their mothers, therefore 800 participants).
20
21 301 Approximately 40-50% of infants are exposed to prophylactic intrapartum antibiotics at our
22
23 302 institution for colonisation with Group B *streptococcus* or premature rupture of membranes.
24
25 303 Additional 50-60% of children are expected to be exposed to antibiotics during the first two
26
27 304 years of life.
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306 **Bioinformatics**

307 Sequences will be trimmed according to quality scores and sequencing adaptors will be
308 removed using Trimmomatic.²⁵ Host sequences will be removed by mapping against the
309 Human genome with Bowtie2.²⁶ High-quality sequences will be used to create taxonomic and
310 functional profiles using Metaphlan2²⁷ and HUMAnN2²⁸, respectively. Antibiotic resistance
311 genes will be identified using ResFinder.²⁹ We will share our metagenomic data through the
312 European Nucleotide Archive (ENA).
313

314 **Statistical analysis**

315 ***Considered metrics:***

316 Alpha-diversity: Alpha-diversity indexes are descriptive of the intra-sample richness (number
317 of taxonomic functional features), evenness (features distribution) or diversity (richness
318 weighted by evenness).³⁰ We will use Chao richness (number of different taxa) and Simpson

1
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3 319 diversity (distribution of taxa) to summarise the alpha-diversity for each sample.
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7 321 Beta-diversity: For beta-diversity, samples are compared for their composition in features of
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9
10 322 interest (taxonomic or functional units).³¹ All-versus-all distances between samples are
11
12 323 computed in pairwise comparisons and summarised in distance-matrices. We will use indices,
13
14 324 with distance equal to 1-index value, to capture different dimensions of microbial structures,³¹
15
16 325 restricting ourselves to non-Euclidean indices and excluding the “joint absences” as sharing of
17
18 326 unseen features would have doubtful significance in this context. Feature presence/absence will
19
20 327 be described by Jaccard indices and quantitative overlap by Bray-Curtis indices. In taxonomic
21
22 328 comparisons, Unifrac (presence/absence) and Weighted Unifrac (quantitative) will be used to
23
24 329 account for features phylogenetic distances.³² Statistics on distances matrices require adapted
25
26 330 methods to assess for significant differences in average location (centroid) of the samples of
27
28 331 groups of interest. For this, we will use the PERMANOVA approach implemented in the
29
30 332 Adonis2 function of the vegan R package. PERMANOVA can be sensitive to variance
31
32 333 heterogeneity in unbalances groups. Thus, variance homogeneity will be tested by ANOVA to
33
34 334 centroids.
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42 336 Differential abundance testing: Metagenomics data are compositional due to technical
43
44 337 limitations.^{33 34} Relative abundances of bacterial and non-bacterial (archaeal, eukaryotic and
45
46 338 viral) taxa and antibiotic resistance genes, will be directly integrated for analyses. For
47
48 339 bacteria, we will transform observed proportions into absolute quantities by multiplying
49
50 340 proportions (%) by measured microbial loads, quantified by broad-range qPCR. Thus, we will
51
52 341 explore correlation abundance testing of transformed counts for taxa and bacterial resistance
53
54 342 genes. Statistical challenges of metagenomics data are the high number of features (and
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56 343 related multiple-testing false-discovery) and features sparsity (a given features – species or
57
58 344 genes – will be observed only in a few samples, leading to a high proportion of zeros in count
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3 345 tables). These limitations in metagenomics statistics were only recently recognised and the
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5 346 developments of methods accounting for these is a field of active research and publication.
6
7 347 Thus, we will follow on future developments, recommendation and consensuses in the field
8
9 348 regarding these challenges. Currently identified solutions accounting for these limitations are
10
11 349 MetagenomSeq, Aldex2 and Maaslin2, which all integrate normalisation and correlation
12
13 350 testing to account for the pre-mentioned limitations while trying to identify differentially
14
15 351 abundant features (genes or speices) between tested groups.
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19 352
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21 353 *Statistical plan for aim 1 and 2:* Antibiotics exposed samples will be compared to non-
22
23 354 exposed samples (grouped per received drug) for differences in alpha-diversity metrics and
24
25 355 abundances of bacterial and non-bacterial taxa and antibiotic resistance genes with significant
26
27 356 changes defined as a $> 0.5\%$ change in abundance between groups. Alpha-diversity metrics
28
29 357 are continuous numeric values. Normality of the data will be checked by the Kolmogorov-
30
31 358 Smirnov test and QQ-plots. For normal variables, a one-way ANOVA will be used. If
32
33 359 normality cannot be reached by transformation, differences between groups will be analysed
34
35 360 using a nonparametric Kruskal-Wallis test, or in case of pairwise comparisons, a Mann-
36
37 361 Whitney U-test. For comparison of abundance Pearson χ^2 tests will be used. Infant age,
38
39 362 demographics, delivery mode, feeding method and antibiotic type and dose will be modeled
40
41 363 in permutational multivariate analysis of variance (PERMANOVA, adonis2 in VEGAN
42
43 364 package, R Foundation) using beta-diversity distance matrices to identify significant
44
45 365 contributors to the microbiome composition.
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53 367 *Statistical plan for aim 3:* For integrated analysis of the microbiome data and clinical
54
55 368 outcomes, allergic sensitisation, eczema, and overweight cases will be defined using the
56
57 369 prospectively collected outcome measures. The relationship between alpha-diversity and
58
59 370 clinical outcomes will be investigated using logistic regression. The relationship between taxa
60

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3 371 and these clinical outcomes will be determined by hierarchical clustering of communities
4
5 372 using heatmaps and principal component analysis (PCA). Significance of grouping of clinical
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7 373 categories using permutational multivariate analysis of variance (PERMANOVA). Microbes
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10 374 that have significantly different abundance between the clinical outcome groups will be
11
12 375 identified using Aldex2.³⁵ The potential influence of antenatal and postnatal factors on the
13
14 376 microbiome or clinical outcomes will be accounted for in all analyses by PERMANOVA and
15
16
17 377 unsupervised hierarchical clustering.

18 378

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21 379 *Statistical plan for aim 4:* To compare paired maternal stool, breast milk, and infant stool
22
23 380 samples, we will do clustering analysis and perform a Wilcoxon rank sum test on beta-
24
25 381 diversity distances between true mother-infant pairs and randomly paired mothers and infants
26
27 382 matched by infant age. These different analyses should allow to describe the relationships
28
29 383 between samples of different origins and identify determinants of microbiome composition.

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33 385 To further characterise the mother-to-infant microbiome transmission, the StrainPhlAn
34
35 386 software will be used.³⁶ This software enables same-species sequence comparisons at the
36
37 387 single nucleotide polymorphism (SNPs) level to define sample-specific strains. Combined
38
39 388 results for shared species allow precise description of the phylogenetic distance between
40
41 389 samples. Added to the beta-diversity analyses, this will allow to better disentangle the
42
43 390 hypothesised seeding from maternal stool and breast milk to the infant oral and stool
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45 391 microbiome.

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49 393 *Statistical plan for aim 5:* The same as for aim 4 will be done for comparison of the breast
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51 394 milk and the infant oral microbiome.

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60 396 **Missing data**

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3 397 If the fraction of missing data is less than 5%, the primary analysis will be a complete case
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5 398 analysis. If not, the rate and patterns of missing data will be examined and, if
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7 399 appropriate, multiple imputation models will be applied for the outcome variables.
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11 401 **ETHICS AND DISSEMINATION**

12 402 **Ethics approval**

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17 403 The ABERRANT study has been approved by the Commission cantonale d'éthique de la
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19 404 recherche sur l'être humain (CER-VD) du Canton de Vaud (#2019-01567), Switzerland.
20

21 405

22 406 **Recruitment and consent**

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26 407 Written informed consent will be obtained from all participants included in the trial.

27
28 408 Participants will be informed that they are not obliged to take part in the study and are free to
29
30 409 withdraw at any time without any impact on their future care.
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33 411 **Data collection and storage**

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37 412 Data will be sourced from medical maternal and infant records, as well as by questionnaire
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39 413 from parents. Data will be de-identified and entered in to a secure, web-based electronic
40
41 414 database.
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44 416 **Safety**

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49 417 No serious adverse reactions are anticipated but these will be checked for by the Data Safety
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51 418 and Monitoring Committee.
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54 420 **Dissemination of results**

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58 421 Outcomes will be disseminated through publication according to the SPIRIT statement and
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60 422 will be presented at scientific conferences.

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5 424 **Study duration**6
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8 425 We aim to recruit participants over a two-year period.9
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12 427 **DISCUSSION**13
14 428 The intestinal microbiome is crucial in the development of the immune system and regulation

15 429 of immune responses, especially during infancy, when the intestinal microbiome and the

16
17 430 immune response develop concurrently.³⁷ The development of intestinal microbiome is easily18
19 431 disrupted by external factors and perturbation during this vulnerable period may have a large20
21 432 influence on immune development. A number of factors influence the development of the22
23 433 infant intestinal microbiome, including gestational age, delivery mode, feeding, and maternal24
25 434 and infant antibiotic exposure (**Figure 1**).³⁸ While the effect of delivery mode and feeding26
27 435 methods on the establishment of microbial communities has been well studied, much less is28
29 436 known about the effects of intrapartum and early-life antibiotic exposure on the establishment30
31 437 of microbial communities in the intestinal microbiome.⁹32
33 43834
35 439 A number of commonly used antibiotics have profound effects on specific bacteria within the36
37 440 intestinal microbiome, as detailed in a recent systematic review.⁸ This ‘collateral damage’38
39 441 includes changes in abundance of microbial taxa, a decrease in ‘colonisation resistance’40
41 442 (protection against colonisation with potentially pathogenic organisms) and the development42
43 443 of antibiotic resistance. To date, most studies on the effect of antibiotic exposure on the44
45 444 intestinal microbiome have been done in adults.⁸ The main findings of these studies are that46
47 445 antibiotics often lead to a decreased bacterial diversity, a decrease in the abundance of48
49 446 anaerobic bacteria, an increase in abundance of *Enterobacteriaceae* other than *E. coli* and an50
51 447 increase in the abundance of yeast.⁸ These studies show that changes in the intestinal52
53 448 microbiome after just one course of antibiotics can persist up to four years.⁸ However, the

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2
3 449 clinical consequences of changes in the composition of the intestinal microbiome with
4
5 450 antibiotic treatment are unknown. An increase in Enterobacteriaceae, which are often resistant
6
7 451 to beta-lactam and other antibiotics, might render the host more susceptible to infections with
8
9 452 antibiotic-resistant bacteria. This phenomenon has been observed in infants in neonatal
10
11 453 intensive care units, who become more often colonised with *Klebsiella* spp., *Enterobacter*
12
13 454 spp. and *Citrobacter* spp., when treated with antibiotics.³⁹
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18
19 456 Intrapartum antibiotic prophylaxis (IAP) has become common practice in obstetric medicine
20
21 457 and is used in up to 40% of deliveries, which makes it the most common source of antibiotic
22
23 458 exposure in neonates.¹ IAP is routinely used in both elective and emergency Caesarean
24
25 459 section (CS). It is also routinely used in women who are colonised with group B streptococcus
26
27 460 (GBS). Despite the benefits, the risks associated with exposing a large number of infants to
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29 461 antibiotics, especially the long-term effects on health through changes in the microbiome,
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31 462 remain unclear. Infants who were exposed to IAP have been reported to have a lower alpha-
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33 463 diversity, a lower relative abundance of Actinobacteria, especially *Bifidobacteriaceae*, and a
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35 464 larger relative abundance of Proteobacteria in their intestinal microbiome compared to non-
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37 465 exposed infants.⁹ Furthermore, they have been reported to have a higher number of beta-
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39 466 lactamase encoding genes.⁴⁰
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44 467 Breastfeeding has been shown to be beneficial in preventing many communicable and non-
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46 468 communicable diseases.⁴¹ Despite intensive research into the positive health effects of
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48 469 breastfeeding, the underlying mechanisms are still not understood. However, a large part of
49
50 470 the beneficial effects of breast milk is likely mediated through the microbiome and its
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52 471 associated immunomodulatory, anti-inflammatory and antimicrobial components. The breast
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54 472 milk microbiome also likely plays a part in shaping the infant's intestinal microbiome,⁴ to
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56 473 which extent we will be investigated in this study.
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3 475 There is relatively little data about the effects of IAP on the composition of the breast milk
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5 476 microbiome.⁴²⁻⁴⁴ Mothers who receive IAP have been reported to have a lower abundance or
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7 477 even an absence of the beneficial bacteria *Bifidobacterium* spp. in their breast milk.^{42 43}
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10 478 Furthermore, in a small study IAP has been shown to increase antibiotic resistance genes in
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12 479 the breast milk microbiota.⁴⁵ There is also some evidence suggesting that mothers who
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14 480 receive IAP have a higher bacterial richness and diversity in their breast milk microbiome
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16 481 compared with mothers who do not receive antibiotics.⁴² However, these findings have to be
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18 482 interpreted with caution: it could be that antibiotics lead to lower bacterial numbers and
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20 483 therefore signals from contamination, e.g. bacteria found in DNA extraction or sequencing
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22 484 kits might be amplified more leading to a the detection of a higher diversity. Use of broad-
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24 485 range qPCR to quantify bacterial load in milk samples will allow to assess this potential bias.
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26 486 Interestingly, recent preliminary studies have also shown that delivery mode affects the
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28 487 composition of the breast milk microbiome.^{42 44 46 47} However, during suckling, a high degree
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30 488 of retrograde flow of milk into the mammary ducts can occur,⁵ transferring bacteria from the
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32 489 infant to the mother, as postulated for GBS.⁴⁸ It is therefore possible that the differences in the
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34 490 breast milk microbiome observed with different delivery modes are mediated through
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36 491 differences in the oral microbiome of infants.
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40 492 This study will determine the effect of intrapartum and early-life antibiotics on the
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42 493 composition of the breast milk, and the infant oral and intestinal microbiome and antibiotic
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44 494 resistance. The knowledge gained by this study will make an important contribution to the
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46 495 growing field of research investigating the importance of the immunological role of the breast
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48 496 milk microbiome and the infant intestinal microbiome on infant health. It will form the basis
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50 497 for investigating the interplay between the microbiome and the regulation of the human
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52 498 immune system and possible adverse health outcomes, such as the development of immune
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54 499 and non-immune mediated diseases, including allergic diseases.
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3 501 The results of this study will also build a stronger evidence base for strict antibiotic
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5 502 stewardship and form the basis for development of evidence-based interventions to prevent
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7 503 adverse outcomes in situations where antibiotics cannot be avoided, including modifying the
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9 504 intestinal microbiome with directed pre- and probiotics or bacteriophages.
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14 506 **Contributors** PZ is the lead investigator. VS, LF, NC and PZ were responsible for study
15
16 507 conception and design. JW, AL and PZ were responsible for funding acquisition and
17
18 508 implementation. MV drafted the manuscript and coordinated the manuscript preparation and
19
20 509 revision. PZ and VS has developed the statistical analysis plan. MV and PZ have developed
21
22 510 the online questionnaires and database set-up in REDCap. WJ will be responsible for sample
23
24 511 analysis. MV, VS, WJ, DB, VDM, ALB, JW, LF and NC provided critical evaluation and
25
26 512 revision of manuscript and have given final approval of the manuscript accepting
27
28 513 responsibility for all aspects.
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33 514
34
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36
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38
39 517 F. Taroni). None of the funders had a role in designing the study or in the study conduct and
40
41 518 they will not be involved in the publication of the results from the study.
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46 520 **Competing interests** None.
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51 522 **Provenance and peer review** Not commissioned; externally peer reviewed.
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3 **523 References**
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Table 1 Clinical outcomes for aim 3

Outcome	Main measure	Timing
Lower respiratory tract illness	Number of episodes & hospitalisations ^{19 20}	first 2 years of life
Acute otitis media	Number of episodes ^{19 20}	first 2 years of life
Allergic (atopic) sensitisation	Prevalence (positive skin prick test) ²³	at 2 years of age
Eczema	Prevalence (Williams criteria) ²¹	at 1 and 2 years of age
Weight	Centile (WHO Child Growth Standards) ¹⁸	at 1 and 2 years of age

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Table 2 Study protocol

Time	Birth	7d	1m	2m	4m	6m	12m	24m
Diary		✓	✓	✓	✓	✓	✓	✓
Questionnaire		✓		✓	✓	✓	✓	✓
Maternal blood sample	✓							
Maternal stool sample	✓							
Breast milk sample	✓ ^{col}	✓	✓	✓	✓	✓ [*]		
Infant oral swab	✓	✓	✓	✓	✓	✓		
Infant stool sample	✓ ^{mec}	✓	✓	✓	✓	✓	✓	✓
Clinical examination							✓	✓
Skin prick test								✓
Blood sampling (optional)	✓ ^{cb}						✓	✓

col = colostrum; mec = meconium; ³cb = cord blood;

*or before breastfeeding is discontinued if earlier than 6 m

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3 **Figure 1** Summary of factors that might influence the composition of the maternal intestinal and
4 breast milk microbiome, and the infant oral and intestinal microbiome together with possible
5 associated adverse health outcomes
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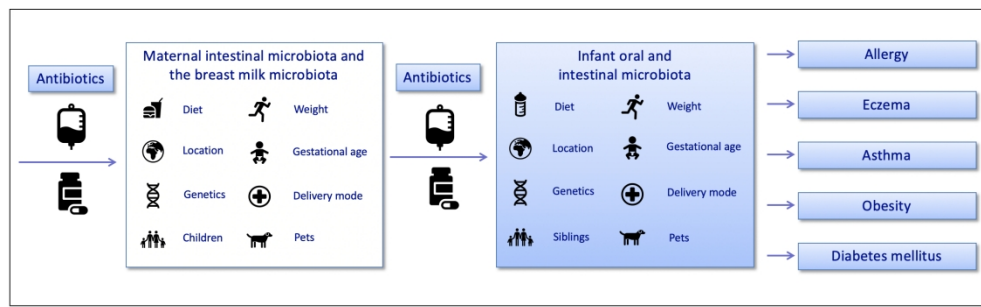


Figure 1

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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	Page Line
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1 1-2
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	2 46
	2b	All items from the World Health Organization Trial Registration Data Set	Available at Clinical trials.gov
Protocol version	3	Date and version identifier	Approved by the CER-VD
Funding	4	Sources and types of financial, material, and other support	18 450
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1 5-20 18 441-4448
	5b	Name and contact information for the trial sponsor	1 22-24
	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	18 452-453
	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)	18 441-448
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	4-5

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2	6b	Explanation for choice of comparators	4-5
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4	Objectives	7	Specific objectives or hypotheses
5			6-7
6	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)
7			7
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12	Methods: Participants, interventions, and outcomes		
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14	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
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18	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)
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23	Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered
24			no intervention
25			
26		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)
27			no intervention
28			
29			
30		11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)
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35		11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial
36			NA
37			
38	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
39			8-9
40			Table 1
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46	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)
47			Table 2
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51	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
52			11-12
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55	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size
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58	Methods: Assignment of interventions (for controlled trials)		
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2	Allocation:			
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4	Sequence	16a	Method of generating the allocation sequence (eg, computer-generated	NA
5	generation		random numbers), and list of any factors for stratification. To reduce	
6			predictability of a random sequence, details of any planned restriction (eg,	
7			blocking) should be provided in a separate document that is unavailable to	
8			those who enrol participants or assign interventions	
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11	Allocation	16b	Mechanism of implementing the allocation sequence (eg, central telephone;	NA
12	concealment		sequentially numbered, opaque, sealed envelopes), describing any steps to	
13	mechanism		conceal the sequence until interventions are assigned	
14				
15	Implementatio	16c	Who will generate the allocation sequence, who will enrol participants, and	NA
16	n		who will assign participants to interventions	
17				
18	Blinding	17a	Who will be blinded after assignment to interventions (eg, trial participants,	7
19	(masking)		care providers, outcome assessors, data analysts), and how	
20				
21		17b	If blinded, circumstances under which unblinding is permissible, and	7
22			procedure for revealing a participant's allocated intervention during the trial	
23				
24				
25	Methods: Data collection, management, and analysis			
26				
27	Data collection	18a	Plans for assessment and collection of outcome, baseline, and other trial	8-9
28	methods		data, including any related processes to promote data quality (eg, duplicate	
29			measurements, training of assessors) and a description of study	
30			instruments (eg, questionnaires, laboratory tests) along with their reliability	
31			and validity, if known. Reference to where data collection forms can be	
32			found, if not in the protocol	
33				
34				
35		18b	Plans to promote participant retention and complete follow-up, including list	8-9
36			of any outcome data to be collected for participants who discontinue or	
37			deviate from intervention protocols	
38				
39	Data	19	Plans for data entry, coding, security, and storage, including any related	14
40	management		processes to promote data quality (eg, double data entry; range checks for	
41			data values). Reference to where details of data management procedures	
42			can be found, if not in the protocol	
43				
44				
45	Statistical	20a	Statistical methods for analysing primary and secondary outcomes.	11-13
46	methods		Reference to where other details of the statistical analysis plan can be	
47			found, if not in the protocol	
48				
49		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	NA
50				
51		20c	Definition of analysis population relating to protocol non-adherence (eg, as	14
52			randomised analysis), and any statistical methods to handle missing data	
53			(eg, multiple imputation)	
54				
55				
56	Methods: Monitoring			
57				
58				
59				
60				

1				
2	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	15
3				
4				
5				
6				
7				
8				
9		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
10				
11				
12				
13	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	NA
14				
15				
16				
17				
18	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	NA
19				
20				
21	Ethics and dissemination			
22				
23	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	14
24				
25				
26	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)	NA
27				
28				
29				
30				
31	Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	14
32				
33				
34		26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	14
35				
36				
37	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	Approved by the CER-VD
38				
39				
40				
41				
42	Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	19
43				
44				
45	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	Approved by the CER-VD
46				
47				
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49	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	Approved by the CER-VD
50				
51				
52				
53				
54	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	7
55				
56				
57				
58				
59		31b	Authorship eligibility guidelines and any intended use of professional writers	NA
60				

- 1
2 31c Plans, if any, for granting public access to the full protocol, participant-level 14
3 dataset, and statistical code
4

5 **Appendices**
6

- 7 Informed 32 Model consent form and other related documentation given to participants Attachment
8 consent and authorised surrogates
9 materials
10
11 Biological 33 Plans for collection, laboratory evaluation, and storage of biological 9-11
12 specimens for genetic or molecular analysis in the current trial and for future
13 use in ancillary studies, if applicable
14
15

16 *It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013
17 Explanation & Elaboration for important clarification on the items. Amendments to the
18 protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT
19 Group under the Creative Commons "[Attribution-NonCommercial-NoDerivs 3.0 Unported](#)"
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BMJ Open

Study protocol for the **ABERRANT** study: Antibiotic-induced disruption of the maternal and infant microbiome and adverse health outcomes - A prospective cohort study among children born at term

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3 1 **Study protocol for the ABERRANT study: Antibiotic-induced disruption of the**
4
5 2 **maternal and infant microbiome and adverse health outcomes - A prospective cohort**
6
7 3 **study among children born at term**
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3 25 **ABSTRACT**
4

5 26 **Introduction:** There is compositional overlap between the maternal intestinal microbiome,
6
7 27 the breast milk microbiome and the infant oral and intestinal microbiome. Antibiotics cause
8
9 28 profound changes in the microbiome. However, the effect of intrapartum and early-life
10
11 29 antibiotics on the maternal intestinal and breast milk microbiome, and the infant oral and
12
13 30 intestinal microbiome, and whether effects are only short-term or persist long-term remain
14
15 31 uncertain.
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21 32
22 33 **Methods and analyses:** In this prospective cohort study, we will use metagenomic
23
24 34 sequencing to determine: (i) the effect of *intrapartum antibiotics* on the composition of the
25
26 35 breast milk, and the infant oral and intestinal microbiome, including the development and
27
28 36 persistence of antibiotic resistance; (ii) the effect of *antibiotic exposure in the first year of life*
29
30 37 on the composition of the infant oral and intestinal microbiome, including the development
31
32 38 and persistence of antibiotic resistance; (iii) the effect of disruption of the infant oral and
33
34 39 intestinal microbiome on health outcomes; and (iv) the compositional overlap between the
35
36 40 maternal intestinal microbiome, the breast milk microbiome and the infant oral and intestinal
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38 41 microbiome.
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44 43 **Ethics and dissemination:** The ABERRANT study has been approved by the commission
45
46 44 cantonale d'éthique de la recherche sur l'être humain (CER-VD) du Canton de Vaud (#2019-
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48 45 01567). Outcomes will be disseminated through publication and will be presented at scientific
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50 46 conferences.
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56 48 **Trial registration number:** The U.S. National Institutes of Health NCT04091282.
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STRENGTHS AND LIMITATIONS OF THIS STUDY

- The use of metagenomic sequencing to determine the effect of intrapartum and early-life antibiotics on the composition of the maternal breast milk and the infant oral and intestinal microbiome.
- The possibility to identify bacterial species, together with resistance genes and other important components of the microbiome such as archaea, eukaryotes (fungi) and viruses.
- The investigation of the association between the early-life intestinal microbiome and clinical health outcomes.
- The potential for contamination of low microbial biomass such as breast milk or meconium samples from the environment or extraction and sequencing kits.

60 INTRODUCTION

61 Antibiotics are amongst the most commonly used drugs, especially in infants and children.

62 Even before birth, more than 40% of infants are exposed to antibiotics.^{1 2} Additionally, more

63 than two-thirds of children receive antibiotics before reaching the age of two years.^{3 4} The

64 human intestine is the habitat for a large community of microbes, the intestinal microbiome.

65 Colonisation of the intestinal tract increases rapidly after birth and the microbiome of the

66 maternal intestine and breast milk are likely important source for the infant intestinal

67 microbiome.⁵ Conversely, as retrograde flow of breast milk into mammary ducts has been

68 documented,⁶ the infant oral microbiome might be responsible for colonising the mammary

69 ducts and therefore could contribute to the breast milk microbiome. Consequently, there is

70 compositional overlap between the maternal intestinal microbiome, the breast milk

71 microbiome and the infant oral and intestinal microbiome.^{7 8}

72

73 Growing evidence shows that the composition of the intestinal microbiome in infants plays an

74 important role in the development and regulation of the immune system, especially in the

75 early-life 'critical window' during which the microbiome and the immune response develop

76 concurrently.⁹⁻¹¹

77

78 Antibiotics cause profound changes in the microbiome.^{12 13} However, the magnitude of the

79 effect of intrapartum and early-life antibiotics on the breast milk, and the infant oral and

80 intestinal microbiome, and whether effects are only short-term or persist long-term remain

81 uncertain. Preliminary studies suggest that disruption of intestinal microbiome in the early-life

82 period is associated with the development of a number of immune- and non-immune-

83 mediated diseases, including allergies,¹⁴ eczema,¹⁴ asthma,¹⁴ chronic inflammatory bowel

84 disease,¹⁵ obesity¹⁶ and diabetes mellitus.¹⁷ Antibiotic exposure *in utero* and during infancy

85 has been associated with an increased risk for the same diseases¹⁸⁻²⁰ and it is likely that the

1
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3 86 association between antibiotic exposure and the subsequent development of these diseases is
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5 87 mediated through changes in the infant microbiome. However, the features and composition
6
7 88 characteristics of the intestinal microbiome associated with the development of these
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10 89 conditions are unclear.

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14 91 Importantly, antibiotic exposure can also lead to the development of antibiotic resistance,¹²
15
16 92 resulting in infections that are more difficult and costly to treat, often requiring longer
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18 93 duration of hospital stay, and the use of antibiotics with more adverse effects. Currently, in
19
20 94 children, there is sparse data available on the effect of antibiotic exposure on the development
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22 95 and persistence of antibiotic resistance in their intestinal microbiome.
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28 97 In this prospective cohort study, we will use metagenomic sequencing to determine (i) the
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30 98 effect of *intrapartum antibiotics* on the composition of the breast milk, and the infant oral and
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32 99 intestinal microbiome (including the development and persistence of antibiotic resistance); (ii)
33
34 100 the effect of *antibiotic exposure in the first year of life* on the composition of the infant oral
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36 101 and intestinal microbiome (including the development and persistence of antibiotic
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38 102 resistance); (iii) the effect of disruption of the infant oral and intestinal microbiome on health
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40 103 outcomes; and (iv) determine the compositional overlap between the maternal intestinal
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42 104 microbiome, the breast milk microbiome and the infant oral and intestinal microbiome.
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48 106 Determining the relationship between antibiotic exposure and changes in the breast milk, and
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50 107 the infant oral and intestinal microbiome, and their potential association with adverse health
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52 108 outcomes will provide stronger evidence for strict antibiotic stewardship. Additionally, it will
53
54 109 form the basis for designing studies to investigate interventions to prevent adverse outcomes
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56 110 in situations where antibiotics cannot be avoided, including modifying the intestinal
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58 111 microbiome with directed pre- and probiotics, or bacteriophages.
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5 113 **OBJECTIVES**6
7 114 **METHODS AND ANALYSIS**8
9 115 **Study design**10 116 A prospective single-centre cohort study of 400 mother-infant pairs.
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16 118 **Aims**17
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19 119 ***Aim 1:*** To determine the extent to which, and for how long, intrapartum antibiotics affect the
20
21 120 composition of the breast milk microbiome and the infant oral and intestinal microbiome, as
22
23 121 well as the prevalence of antibiotic resistance genes.
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26 12227
28 123 ***Aim 2:*** To determine the extent to which, and for how long, antibiotics in the first year of life
29
30 124 affect the composition of the oral and intestinal microbiome in infants, as well as the
31
32 125 prevalence of antibiotic resistance genes.
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35 12636
37 127 ***Aim 3:*** To determine health outcomes (**Table 1**) in children up to the age of 2 years who have
38
39 128 or have not been exposed to intrapartum antibiotics or antibiotics in the first year of life and
40
41 129 determine whether there is an association with the composition of the oral and intestinal
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43 130 microbiome.
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46 13147
48 132 ***Aim 4:*** To determine the degree to which the maternal intestinal and the breast milk
49
50 133 microbiome affect the composition of the infant oral intestinal microbiome and the prevalence
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52 134 of antibiotic resistance genes.
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55 13556
57 136 ***Aim 5:*** To determine the degree to which the infant oral microbiome affects the composition
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59 137 of the breast milk microbiome.
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Outcomes

Primary endpoints:

- Composition of the maternal intestinal and breast milk microbiome, and the infant oral and intestinal microbiome and the prevalence of antibiotic resistance genes within the infant microbiome at birth and when infants are 7 days, 1, 2, 4, 6, 12 and 24 months of age.
- Number of episodes of lower respiratory tract illnesses and acute otitis media in the first two years of life.
- Prevalence of allergic sensitisation and eczema at 1 and 2 years of age.
- Weight at 1 and 2 years of age.

Setting and participants

Sampling frame and study sample: Women who give birth at the Hôpital fribourgeois (HFR) in Fribourg, Switzerland and their infants will be followed over a two-year period. If recruitment is slow a second study site will be added.

Recruitment: Pregnant women attending the antenatal clinic will be given information about the study by a research study nurse or doctor and asked to consider enrolling themselves and their infant in the study. Either both parents or only mothers are present during the antenatal consent interview. We explicitly encourage caregivers to discuss participation with their partners, other family members, doctors and midwives (this is clearly stated in the consent form). We will re-evaluate the willingness to participate when mothers are admitted to the hospital for delivery. Paediatricians will be informed about the children's participation in the study.

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3 164 **Blinding of outcome assessment:** Doctors and study nurses will be blinded to the group of
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5 165 infants (control or antibiotic-exposed) when outcomes are measured.
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10 167 **Patient and Public Involvement**

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12 168 Patients and public were not involved in the design of this study. The results of this study will
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14 169 be disseminated to parents of the study participants via a participant newsletter distributed by
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17 170 email.
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21 172 **Eligibility criteria**

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23 173 **Inclusion criteria:** Healthy infants born at 37 weeks or more gestation who are breastfed.

24 174 Mothers will be asked at an antenatal consent interview if they intend to breastfeed. This will

25
26 175 be reassessed at delivery. Mothers will only be included if they breastfeed their infants.

27
28 176 However, if breastfeeding is stopped before the infant reaches six months of age, this will not

29
30 177 be a reason for exclusion. All the breast milk samples up to that point, as well as stool

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32 178 samples and oral swabs collected afterwards will be analysed.
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37 180 **Exclusion criteria:** Women with the following criteria: HIV, hepatitis B or C infection or

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39 181 unscreened for these infections, antibiotics or probiotics in pregnancy or postpartum period

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41 182 other than during delivery. Infants with the following criteria: low birth weight (<2500 g) or

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43 183 severe congenital abnormality.
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49 185 **Study outcome measures**

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51 186 We will use internationally accepted validated measures for clinical outcomes. The study

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53 187 protocol is depicted in **Table 2**.
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60 189 **Diary:** Parents will be given a structured diary where they can record information about their

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3 190 infant's diet (introduction of formula and new foods), illnesses, medical visits, hospital
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5 191 admissions, use of antibiotics and other medications. This will help them when filling in the
6
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8 192 questionnaires at the required time points.
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12 194 **Questionnaire:** We will do computer-assisted interviews at birth, and when infants are 7 days,
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14 195 2, 4, 6, 12 and 24 months of age using best practice international protocols. The following
15
16 196 data will be recorded: demographic variables including parental ancestry and education,
17
18 197 family history of atopy, eczema, asthma and other immune disorders, antenatal variables such
19
20 198 as maternal age, weight, smoking habits, underlying diseases, medication and
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22 199 supplementation use (e.g. probiotics and vitamins). In addition, we will collect data on
23
24 200 delivery history, perinatal course (e.g. hospitalisation, infections, antibiotics or oxygen
25
26 201 administration), breast-feeding (including episodes of mastitis and maternal antibiotic and
27
28 202 probiotic use), age of introduction of formula and new foods, administration of probiotics and
29
30 203 vitamins, use of antibiotics, antacids and other medications, GP and other medical visits,
31
32 204 illnesses including infections and hospital admissions, number of siblings, child care
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34 205 attendance, parental smoking habits, pet ownership, suspected food allergy and eczema
35
36 206 (presence, medications). Data will be stored using the Research Electronic Data Capture
37
38 207 (REDCap Consortium) database.²¹
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47 209 **Clinical examination:** Participants will be reviewed at 12 and 24 months of age in a specially
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49 210 designated clinic at the HFR by a study nurse or doctor using a structured interview and
50
51 211 clinical eczema assessment.
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55 213 **Weight:** Weight will be assessed during the clinical examination at 12 and 24 months of age.
56
57 214 The WHO Child Growth Standards will be used as a reference for percentiles.²²
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3 216 **Lower respiratory tract illness (LRTI) and acute otitis media (AOM):** Symptoms of acute
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5 217 lower respiratory illness (such cough and wheeze) and the number of episodes of LRTI and
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7 218 AOM will be recorded by parents, and specific questions will be asked in the questionnaires.
8
9 219 We will use the definitions for LRTI developed by Oddy *et al* and Kusel *et al*.^{23 24}
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14 221 **Eczema:** Prevalence of eczema at 12 and 24 months of life will be assessed by the Williams'
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16 222 UK diagnostic criteria of the International Study of Asthma and Allergy in Childhood
17
18 223 (ISAAC).²⁵ This will be assessed by using parent-reported eczema from diary information and
19
20 224 from the clinical examinations at 12 and 24 months of age. We will assess the severity of
21
22 225 eczema using the SCORAD.²⁶ We will also collect data on age of onset of eczema,
23
24 226 distribution of eczema, use of eczema medications, and medical consultations and hospital
25
26 227 admissions.
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33 229 **Skin prick tests:** Sensitisation to the following panel of allergens will be assessed at 24
34
35 230 months of age in children whose parents consent to this component of the study: cow's milk,
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37 231 egg, peanut, sesame, house dust mite (*Dermatophagoides pteronyssinus 1*), cat, dog and grass
38
39 232 pollen. Skin prick allergy testing will be performed according to standard guidelines.²⁷ A
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41 233 positive skin prick test will be defined as an average wheal diameter at least 3 mm greater
42
43 234 than that produced by a negative control solution at 15 minutes.²⁷
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49 236 **Blood sampling:** We will collect maternal blood at time of delivery. We also will obtain cord
50
51 237 blood at birth. During the clinical examination at 12 and 24 months of age, blood will be
52
53 238 collected by personnel trained in infant venipuncture, whose parents consent to this
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55 239 component of the study. The 5-10 ml volume required is safe and within limits for weight
56
57 240 recommended by the US-based Office of Human Research Protections guidelines for blood
58
59 241 collection from healthy infants.
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5 243 **Breast milk samples:** Study nurses will collect one colostrum sample as soon as possible after
6
7 244 birth. Mothers will be asked to collect breast milk (with date and time recorded) from their
8
9 245 first feed of the day (a minimum of 2 hours required to the previous feed) when infants are 7
10
11 246 days, 1, 2, 4, and 6 months old. If breastfeeding is ceased earlier than 6 months, breast milk
12
13 247 will be collected before breastfeeding is discontinued. Mothers will be instructed to wash their
14
15 248 hands and breasts meticulously and to then extract breast milk manually without touching the
16
17 249 areola. The first few drops will be discarded. Reminders will be sent by SMS. Sterile
18
19 250 containers will be provided.
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25
26 252 **Oral swabs:** Oral swabs will be taken from infants as soon as possible after birth by a study
27
28 253 nurse. Additionally, parents will then be asked to collect buccal swabs (with date and time
29
30 254 recorded) before the first feed of the day when infants are 7 days, 1, 2, 4, and 6 months old.
31
32 255 Sterile containers will be provided. Reminders will be sent by SMS.
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38 257 **Stool samples:** Mothers will be asked to collect stool on or after the day of the delivery (with
39
40 258 date and time recorded). A meconium sample will be collected from infants as soon as
41
42 259 possible after birth by a study nurse. Parents will then be asked to collect stool samples from
43
44 260 their infants when they are 7 days, 1, 2, 4, 6, 12 and 24 months old. Reminders will be sent by
45
46 261 SMS. To minimise variation, parents are asked to collect stool from the first bowel movement
47
48 262 of the day (with date and time recorded). Sterile containers will be provided.
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53 264 **Storage of samples:** Parents will be instructed to freeze breast milk, oral swabs and stool
54
55 265 samples in sealed bags in their domestic freezer at -20°C until collection by the research team.
56
57 266 Samples will be kept frozen during transportation to the laboratory where they will be
58
59 267 aliquoted and stored at -80°C.
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5 269 ***DNA extraction and sequencing:*** DNA from breast milk, oral swabs and stool samples
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7
8 270 (approximately 200 mg) will be extracted using the FastDNA Spin Kit for soil (MP
9
10 271 Biomedicals, Santa Ana, California, USA). DNA concentrations will be quantified using a
11
12 272 Qubit 4.0 fluorometer (ThermoFisher Scientific, Waltham, Massachusetts, USA) and high
13
14 273 sensitivity DNA reagents. Bacterial DNA will be quantified by broad-range bacterial
15
16
17 274 quantitative polymerase chain reaction (qPCR). The library preparation will be done using
18
19 275 Nextera DNA Flex Library Preparations Kits. Extracted DNA will be indexed with IDT
20
21 276 Illumina Nextera DNA Unique Dual Indexes to allow analysis of pooled samples. 150-bp
22
23
24 277 pair-end sequencing will be done using the Illumina NextSeq. The required sequencing depth
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26 278 to provide adequate coverage of microbial communities for taxonomic profiling will be
27
28 279 determined by rarefaction curves. We will aim for a minimum yield of $2\text{-}5 \times 10^6$ read-pairs per
29
30 280 sample. Appropriate negative controls (including controls from sterile containers, extraction
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32
33 281 kits etc.) and positive controls of mock communities will be included. These controls will be
34
35 282 sequenced together with the samples to identify potential environmental and laboratory
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37 283 contaminants.
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40 284 Researchers carrying out the microbial analyses will be blinded to the group identity of
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42 285 infants (control or antibiotic exposed group).
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46 287 ***Storage of blood samples:*** Peripheral blood mononuclear cells will be separated from whole
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48 288 blood and stored in liquid nitrogen. Plasma will be stored frozen at -80°C . These will be
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50
51 289 retained for future analysis in projects to evaluate the effect of microbiome on the immune
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53 290 system, including immunophenotyping and analysis of markers of immune function.
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58 292 **Statistical power calculation**

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60 293 The analysis for the a priori computation of the required sample size was conducted using the

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3 294 G*Power 3.1 software. For the power analysis, the repeated measurements MANOVAs were
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5 295 considered. The calculation was based on F-tests for the interaction effect of the between-
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7 296 subject factor antibiotics vs no antibiotics and the within-subject factor time (8 time points).
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9
10 297 We chose a small to medium effect size of 0.22 for the calculation. With a significance level
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12 298 of 5% per test and a power of 80% the power analysis yields a sample size of 304. While we
13
14 299 will attempt to retain and follow up all participants, we are powering our study on ‘a worst-
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16 300 case scenario’ assumption that complete 24-month data will be available for 76% of
17
18 301 participants (this corresponds to a drop-out rate of 12% per year). We will therefore recruit
19
20 302 approximately 400 infants in total (plus their mothers, therefore 800 participants).
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23 303 Approximately 40-50% of infants are exposed to prophylactic intrapartum antibiotics at our
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25 304 institution for colonisation with Group B *streptococcus* or premature rupture of membranes.
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27 305 Additional 50 to 60% of children are expected to be exposed to antibiotics during the first two
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29 306 years of life.
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35 308 **Bioinformatics**

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37 309 Sequences will be trimmed according to quality scores and sequencing adaptors will be
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39 310 removed using Trimmomatic.²⁸ Host sequences will be removed by mapping against the
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41 311 Human genome with Bowtie2.²⁹ High-quality sequences will be used to create taxonomic and
42
43 312 functional profiles using Metaphlan2³⁰ and HUMAnN2³¹, respectively. Antibiotic resistance
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45 313 genes will be identified using ResFinder.³² We will share our metagenomic data through the
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47 314 European Nucleotide Archive (ENA).
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53 316 **Statistical analysis**

54 317 ***Considered metrics:***

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56 318 Alpha-diversity: Alpha-diversity indexes are descriptive of the intra-sample richness (number
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58 319 of taxonomic functional features), evenness (features distribution) or diversity (richness
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3 320 weighted by evenness).³³ We will use Chao richness (number of different taxa) and Simpson
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5 321 diversity (distribution of taxa) to summarise the alpha-diversity for each sample.
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10 323 Beta-diversity: For beta-diversity, samples are compared for their composition in features of
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12 324 interest (taxonomic or functional units).³⁴ All-versus-all distances between samples are
13
14 325 computed in pairwise comparisons and summarised in distance-matrices. We will use indices,
15
16 326 with distance equal to 1-index value, to capture different dimensions of microbial structures,³⁴
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18 327 restricting ourselves to non-Euclidean indices and excluding the “joint absences” as sharing of
19
20 328 unseen features would have doubtful significance in this context. Feature presence/absence will
21
22 329 be described by Jaccard indices and quantitative overlap by Bray-Curtis indices. In taxonomic
23
24 330 comparisons, Unifrac (presence/absence) and Weighted Unifrac (quantitative) will be used to
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26 331 account for features phylogenetic distances.³⁵ Statistics on distances matrices require adapted
27
28 332 methods to assess for significant differences in average location (centroid) of the samples of
29
30 333 groups of interest. For this, we will use the PERMANOVA approach implemented in the
31
32 334 Adonis2 function of the vegan R package. PERMANOVA can be sensitive to variance
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34 335 heterogeneity in unbalances groups. Thus, variance homogeneity will be tested by ANOVA to
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36 336 centroids.
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44 338 Differential abundance testing: Metagenomics data are compositional due to technical
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46 339 limitations.^{36 37} Relative abundances of bacterial and non-bacterial (archaeal, eukaryotic and
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48 340 viral) taxa and antibiotic resistance genes, will be directly integrated for analyses. For
49
50 341 bacteria, we will transform observed proportions into absolute quantities by multiplying
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52 342 proportions (%) by measured microbial loads, quantified by broad-range qPCR. Thus, we will
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54 343 explore correlation abundance testing of transformed counts for taxa and bacterial resistance
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56 344 genes. Statistical challenges of metagenomics data are the high number of features (and
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58 345 related multiple-testing false-discovery) and features sparsity (a given features – species or
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3 346 genes – will be observed only in a few samples, leading to a high proportion of zeros in count
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5 347 tables). These limitations in metagenomics statistics were only recently recognised and the
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7 348 developments of methods accounting for these is a field of active research and publication.
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9
10 349 Thus, we will follow on future developments, recommendation and consensuses in the field
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12 350 regarding these challenges. Currently identified solutions accounting for these limitations are
13
14 351 MetagenomSeq, Aldex2 and Maaslin2, which all integrate normalisation and correlation
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16 352 testing to account for the pre-mentioned limitations while trying to identify differentially
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18 353 abundant features (genes or species) between tested groups.
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24 355 *Statistical plan for aim 1 and 2:* Antibiotics exposed samples will be compared to non-
25
26 356 exposed samples (grouped per received drug) for differences in alpha-diversity metrics and
27
28 357 abundances of bacterial and non-bacterial taxa and antibiotic resistance genes with significant
29
30 358 changes defined as a > 0.5% change in abundance between groups. Alpha-diversity metrics
31
32 359 are continuous numeric values. Normality of the data will be checked by the Kolmogorov-
33
34 360 Smirnov test and QQ-plots. For normal variables, a one-way ANOVA will be used. If
35
36 361 normality cannot be reached by transformation, differences between groups will be analysed
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38 362 using a nonparametric Kruskal-Wallis test, or in case of pairwise comparisons, a Mann-
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40 363 Whitney U-test. For comparison of abundance Pearson χ^2 tests will be used. Infant age,
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42 364 demographics, delivery mode, feeding method and antibiotic type and dose will be modeled
43
44 365 in permutational multivariate analysis of variance (PERMANOVA, adonis2 in VEGAN
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46 366 package, R Foundation) using beta-diversity distance matrices to identify significant
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48 367 contributors to the microbiome composition.
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56 369 *Statistical plan for aim 3:* For integrated analysis of the microbiome data and clinical
57
58 370 outcomes, allergic sensitisation, eczema, and overweight cases will be defined using the
59
60 371 prospectively collected outcome measures. The relationship between alpha-diversity and

1
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3 372 clinical outcomes will be investigated using logistic regression. The relationship between taxa
4
5 373 and these clinical outcomes will be determined by hierarchical clustering of communities
6
7 374 using heatmaps and principal component analysis (PCA). Significance of grouping of clinical
8
9 375 categories using permutational multivariate analysis of variance (PERMANOVA). Microbes
10
11 376 that have significantly different abundance between the clinical outcome groups will be
12
13 377 identified using Aldex2.³⁸ The potential influence of antenatal and postnatal factors on the
14
15 378 microbiome or clinical outcomes will be accounted for in all analyses by PERMANOVA and
16
17 379 unsupervised hierarchical clustering.
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24 381 *Statistical plan for aim 4:* To compare paired maternal stool, breast milk, and infant stool
25
26 382 samples, we will do clustering analysis and perform a Wilcoxon rank sum test on beta-
27
28 383 diversity distances between true mother-infant pairs and randomly paired mothers and infants
29
30 384 matched by infant age. These different analyses should allow to describe the relationships
31
32 385 between samples of different origins and identify determinants of microbiome composition.
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36
37 387 To further characterise the mother-to-infant microbiome transmission, the StrainPhlAn
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39 388 software will be used.³⁹ This software enables same-species sequence comparisons at the
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41 389 single nucleotide polymorphism (SNPs) level to define sample-specific strains. Combined
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43 390 results for shared species allow precise description of the phylogenetic distance between
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45 391 samples. Added to the beta-diversity analyses, this will allow to better disentangle the
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47 392 hypothesised seeding from maternal stool and breast milk to the infant oral and stool
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49 393 microbiome.
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56 395 *Statistical plan for aim 5:* The same as for aim 4 will be done for comparison of the breast
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58 396 milk and the infant oral microbiome.
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3 398 **Missing data**
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5 399 If the fraction of missing data is less than 5%, the primary analysis will be a complete case

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7 400 analysis. If not, the rate and patterns of missing data will be examined and, if

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9 401 appropriate, multiple imputation models will be applied for the outcome variables.
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14 403 **ETHICS AND DISSEMINATION**
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17 404 **Ethics approval**
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19 405 The ABERRANT study has been approved by the commission cantonale d'éthique de la

20
21 406 recherche sur l'être humain (CER-VD) du Canton de Vaud (#2019-01567), Switzerland.
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26 408 **Recruitment and consent**
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28 409 Written informed consent will be obtained from all participants included in the trial.

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30 410 Participants will be informed that they are not obliged to take part in the study and are free to

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32 411 withdraw at any time without any impact on their future care.
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37 413 **Data collection and storage**
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39 414 Data will be sourced from medical maternal and infant records, as well as by questionnaire

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41 415 from parents. Data will be de-identified and entered in to a secure, web-based electronic

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43 416 database.
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48 418 **Safety**
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50 419 No serious adverse reactions are anticipated but these will be checked for by the Data Safety

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52 420 and Monitoring Committee.
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57 422 **Dissemination of results**
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3 423 Outcomes will be disseminated through publication according to the SPIRIT statement and
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5 424 will be presented at scientific conferences.
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10 426 **Study duration**

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12 427 We aim to recruit participants over a two-year period.
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17 429 **DISCUSSION**

18
19 430 The intestinal microbiome is crucial in the development of the immune system and regulation
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21 431 of immune responses, especially during infancy, when the intestinal microbiome and the
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23 432 immune response develop concurrently.⁴⁰ The development of intestinal microbiome is easily
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25 433 disrupted by external factors and perturbation during this vulnerable period may have a large
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27 434 influence on immune development. A number of factors influence the development of the
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29 435 infant intestinal microbiome, including gestational age, delivery mode, feeding, and maternal
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31 436 and infant antibiotic exposure (**Figure 1**).⁴¹ While the effect of delivery mode and feeding
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33 437 methods on the establishment of microbial communities has been well studied, much less is
34
35 438 known about the effects of intrapartum and early-life antibiotic exposure on the establishment
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37 439 of microbial communities in the intestinal microbiome.¹³
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44 441 A number of commonly used antibiotics have profound effects on specific bacteria within the
45
46 442 intestinal microbiome, as detailed in a recent systematic review.¹² This ‘collateral damage’
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48 443 includes changes in diversity and abundance of microbial taxa, a decrease in ‘colonisation
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50 444 resistance’ (protection against colonisation with potentially pathogenic organisms) and the
51
52 445 development of antibiotic resistance. To date, most studies on the effect of antibiotic exposure
53
54 446 on the intestinal microbiome have been done in adults.¹² The main findings of these studies
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56 447 are that antibiotics often lead to a decreased bacterial diversity, a decrease in the abundance of
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58 448 anaerobic bacteria, an increase in abundance of *Enterobacteriaceae* other than *E. coli* and an
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3 449 increase in the abundance of yeast.¹² These studies show that changes in the intestinal
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5 450 microbiome after just one course of antibiotics can persist up to four years.¹² However, the
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7 451 clinical consequences of changes in the composition of the intestinal microbiome with
8
9 452 antibiotic treatment are unknown. An increase in Enterobacteriaceae, which are often resistant
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11 453 to beta-lactam and other antibiotics, might render the host more susceptible to infections with
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13 454 antibiotic-resistant bacteria. This phenomenon has been observed in infants in neonatal
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15 455 intensive care units, who become more often colonised with *Klebsiella* spp., *Enterobacter*
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17 456 spp. and *Citrobacter* spp., when treated with antibiotics.⁴²
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23
24 458 Intrapartum antibiotic prophylaxis (IAP) has become common practice in obstetric medicine
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26 459 and is used in up to 40% of deliveries, which makes it the most common source of antibiotic
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28 460 exposure in neonates.¹ IAP is routinely used in both elective and emergency Caesarean
29
30 461 section (CS). It is also routinely used in women who are colonised with group B streptococcus
31
32 462 (GBS). Despite the benefits, the risks associated with exposing a large number of infants to
33
34 463 antibiotics, especially the long-term effects on health through changes in the microbiome,
35
36 464 remain unclear. Infants who were exposed to IAP have been reported to have a lower alpha-
37
38 465 diversity, a lower relative abundance of Actinobacteria, especially *Bifidobacteriaceae*, and a
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40 466 larger relative abundance of Proteobacteria in their intestinal microbiome compared to non-
41
42 467 exposed infants.¹³ Furthermore, they have been reported to have a higher number of beta-
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44 468 lactamase encoding genes.⁴³
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49 469 Breastfeeding has been shown to be beneficial in preventing many communicable and non-
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51 470 communicable diseases.⁴⁴ Despite intensive research into the positive health effects of
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53 471 breastfeeding, the underlying mechanisms are still not understood. However, a large part of
54
55 472 the beneficial effects of breast milk is likely mediated through the microbiome and its
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57 473 associated immunomodulatory, anti-inflammatory and antimicrobial components. The breast
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3 474 milk microbiome also likely plays a part in shaping the infant's intestinal microbiome,⁵ to
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5 475 which extent we will be investigated in this study.
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10 477 There is relatively little data about the effects of IAP on the composition of the breast milk
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12 478 microbiome.⁴⁵⁻⁴⁷ Mothers who receive IAP have been reported to have a lower abundance or
13
14 479 even an absence of the beneficial bacteria *Bifidobacterium* spp. in their breast milk.^{45 46}
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17 480 Furthermore, in a small study IAP has been shown to increase antibiotic resistance genes in
18
19 481 the breast milk microbiome.⁴⁸ There is also some evidence suggesting that mothers who
20
21 482 receive IAP have a higher bacterial richness and diversity in their breast milk microbiome
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23 483 compared with mothers who do not receive antibiotics.⁴⁵ However, these findings have to be
24
25 484 interpreted with caution: it could be that antibiotics lead to lower bacterial numbers and
26
27 485 therefore signals from contamination, e.g. bacteria found in DNA extraction or sequencing
28
29 486 kits might be amplified more leading to a the detection of a higher diversity. Use of broad-
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31 487 range qPCR to quantify bacterial load in milk samples will allow to assess this potential bias.
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33 488 Interestingly, recent preliminary studies have also shown that delivery mode affects the
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35 489 composition of the breast milk microbiome.^{45 47 49 50} However, during suckling, a high degree
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37 490 of retrograde flow of milk into the mammary ducts can occur,⁶ transferring bacteria from the
38
39 491 infant to the mother, as postulated for GBS.⁵¹ It is therefore possible that the differences in the
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41 492 breast milk microbiome observed with different delivery modes are mediated through
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43 493 differences in the oral microbiome of infants.
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49 494 This study will determine the effect of intrapartum and early-life antibiotics on the
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51 495 composition of the breast milk, and the infant oral and intestinal microbiome and antibiotic
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53 496 resistance. The knowledge gained by this study will make an important contribution to the
54
55 497 growing field of research investigating the importance of the immunological role of the breast
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57 498 milk microbiome and the infant intestinal microbiome on infant health. It will form the basis
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59 499 for investigating the interplay between the microbiome and the regulation of the human
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3 500 immune system and possible adverse health outcomes, such as the development of immune
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5 501 and non-immune mediated diseases, including allergic diseases.
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10 503 The results of this study will also build a stronger evidence base for strict antibiotic
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12 504 stewardship and form the basis for development of evidence-based interventions to prevent
13
14 505 adverse outcomes in situations where antibiotics cannot be avoided, including modifying the
15
16 506 intestinal microbiome with directed pre- and probiotics or bacteriophages.
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21 508 **Contributors** PZ is the lead investigator. VS, LF, NC and PZ were responsible for study
22
23 509 conception and design. PZ, JW and ALB were responsible for funding acquisition and
24
25 510 implementation. MV drafted the manuscript and coordinated the manuscript preparation and
26
27 511 revision. PZ and VS has developed the statistical analysis plan. MV and PZ developed the
28
29 512 online questionnaires and database set-up in REDCap. WJ will be responsible for sample
30
31 513 analysis. MV, VS, WJ, DB, VDM, ALB, JW, LF and NC provided critical evaluation and
32
33 514 revision of manuscript and have given final approval of the manuscript accepting
34
35 515 responsibility for all aspects.
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45
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47
48 520 they will not be involved in the publication of the results from the study.
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53 522 **Competing interests** None.
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58 524 **Provenance and peer review** Not commissioned; externally peer reviewed.
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Table 1 Clinical outcomes for aim 3

Outcome	Main measure	Timing
Lower respiratory tract illness	Number of episodes & hospitalisations ^{23 24}	first 2 years of life
Acute otitis media	Number of episodes ^{23 24}	first 2 years of life
Allergic (atopic) sensitisation	Prevalence (positive skin prick test) ²⁷	at 2 years of age
Eczema	Prevalence (Williams criteria) ²⁵	at 1 and 2 years of age
Weight	Centile (WHO Child Growth Standards) ²²	at 1 and 2 years of age

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Table 2 Study protocol

Time	Birth	7d	1m	2m	4m	6m	12m	24m
Diary		✓	✓	✓	✓	✓	✓	✓
Questionnaire		✓		✓	✓	✓	✓	✓
Maternal blood sample	✓							
Maternal stool sample	✓							
Breast milk sample	✓ ^{col}	✓	✓	✓	✓	✓*		
Infant oral swab	✓	✓	✓	✓	✓	✓		
Infant stool sample	✓ ^{mec}	✓	✓	✓	✓	✓	✓	✓
Clinical examination							✓	✓
Skin prick test (optional)								✓
Blood sampling (optional)	✓ ^{cb}						✓	✓

col = colostrum; mec = meconium; ³cb = cord blood;

*or before breastfeeding is discontinued if earlier than 6 m

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Figure 1 Summary of factors that might influence the composition of the maternal intestinal and breast milk microbiome, and the infant oral and intestinal microbiome together with possible associated adverse health outcomes

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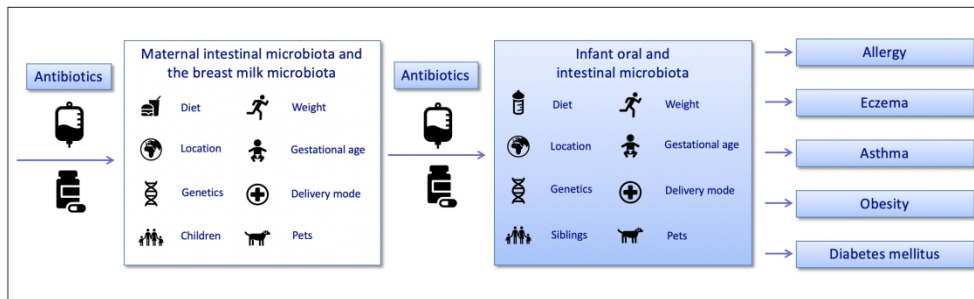


Figure 1



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

Section/item	Item No	Description	Page Line
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1 1-2
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	2 46
	2b	All items from the World Health Organization Trial Registration Data Set	Available at Clinical trials.gov
Protocol version	3	Date and version identifier	Approved by the CER-VD
Funding	4	Sources and types of financial, material, and other support	18 450
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1 5-20 18 441-4448
	5b	Name and contact information for the trial sponsor	1 22-24
	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	18 452-453
	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)	18 441-448
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	4-5

1			
2	6b	Explanation for choice of comparators	4-5
3			
4	Objectives	7	Specific objectives or hypotheses
5			6-7
6	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)
7			7
8			
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11			
12	Methods: Participants, interventions, and outcomes		
13			
14	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
15			7
16			
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18	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)
19			7-8
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23	Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered
24			no intervention
25			
26		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)
27			no intervention
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29			
30		11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)
31			8
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35		11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial
36			NA
37			
38	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
39			8-9
40			Table 1
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46	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)
47			Table 2
48			
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51	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
52			11-12
53			
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55	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size
56			7
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58	Methods: Assignment of interventions (for controlled trials)		
59			
60			

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2	Allocation:			
3				
4	Sequence	16a	Method of generating the allocation sequence (eg, computer-generated	NA
5	generation		random numbers), and list of any factors for stratification. To reduce	
6			predictability of a random sequence, details of any planned restriction (eg,	
7			blocking) should be provided in a separate document that is unavailable to	
8			those who enrol participants or assign interventions	
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11	Allocation	16b	Mechanism of implementing the allocation sequence (eg, central telephone;	NA
12	concealment		sequentially numbered, opaque, sealed envelopes), describing any steps to	
13	mechanism		conceal the sequence until interventions are assigned	
14				
15	Implementatio	16c	Who will generate the allocation sequence, who will enrol participants, and	NA
16	n		who will assign participants to interventions	
17				
18	Blinding	17a	Who will be blinded after assignment to interventions (eg, trial participants,	7
19	(masking)		care providers, outcome assessors, data analysts), and how	
20				
21		17b	If blinded, circumstances under which unblinding is permissible, and	7
22			procedure for revealing a participant's allocated intervention during the trial	
23				
24				
25	Methods: Data collection, management, and analysis			
26				
27	Data collection	18a	Plans for assessment and collection of outcome, baseline, and other trial	8-9
28	methods		data, including any related processes to promote data quality (eg, duplicate	
29			measurements, training of assessors) and a description of study	
30			instruments (eg, questionnaires, laboratory tests) along with their reliability	
31			and validity, if known. Reference to where data collection forms can be	
32			found, if not in the protocol	
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35		18b	Plans to promote participant retention and complete follow-up, including list	8-9
36			of any outcome data to be collected for participants who discontinue or	
37			deviate from intervention protocols	
38				
39	Data	19	Plans for data entry, coding, security, and storage, including any related	14
40	management		processes to promote data quality (eg, double data entry; range checks for	
41			data values). Reference to where details of data management procedures	
42			can be found, if not in the protocol	
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45	Statistical	20a	Statistical methods for analysing primary and secondary outcomes.	11-13
46	methods		Reference to where other details of the statistical analysis plan can be	
47			found, if not in the protocol	
48				
49		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	NA
50				
51		20c	Definition of analysis population relating to protocol non-adherence (eg, as	14
52			randomised analysis), and any statistical methods to handle missing data	
53			(eg, multiple imputation)	
54				
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56	Methods: Monitoring			
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2	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	15
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9		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
10				
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13	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	NA
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18	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	NA
19				
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21	Ethics and dissemination			
22				
23	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	14
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25				
26	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)	NA
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31	Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	14
32				
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34		26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	14
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37	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	Approved by the CER-VD
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42	Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	19
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45	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	Approved by the CER-VD
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49	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	Approved by the CER-VD
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54	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	7
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59		31b	Authorship eligibility guidelines and any intended use of professional writers	NA
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2 31c Plans, if any, for granting public access to the full protocol, participant-level 14
3 dataset, and statistical code
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5 **Appendices**
6

- 7 Informed 32 Model consent form and other related documentation given to participants Attachment
8 consent and authorised surrogates
9 materials
10
11 Biological 33 Plans for collection, laboratory evaluation, and storage of biological 9-11
12 specimens for genetic or molecular analysis in the current trial and for future
13 use in ancillary studies, if applicable
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15

16 *It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013
17 Explanation & Elaboration for important clarification on the items. Amendments to the
18 protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT
19 Group under the Creative Commons "[Attribution-NonCommercial-NoDerivs 3.0 Unported](#)"
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