Version: 2.0 Date: 04 Aug 2021

Elucidating the Dynamics and Impact of the Gut Microbiome on Maternal Nutritional Status During Pregnancy

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Funded by: Canadian Institutes of Health Research (CIHR)

Aug 4, 2021

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1 PROTOCOL SUMMARY

1.1 SYNOPSIS

Title	Elucidating the Dynamics and Impact of the Gut Microbiome on Maternal Nutritional Status during Pregnancy.
Study Short Title	MMIP (Microbiome and Malnutrition in Pregnancy)
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Co-Investigators	Dr. John Parkinson, PhD (Senior Scientist at the Hospital for Sick Children) Dr. Sajid Bashir Soofi, MBBS, FCPS (Center of Excellence in Women and Child Health, Aga Khan University) Dr. Junaid Iqbal, PhD (Centre of Excellence in Women and Child Health, Aga Khan University) Mr. Yaqub Wasan, MPH MSc (Center of Excellence in Women and Child Health, Aga Khan University) Dr. Jessie Hulst, MD, PhD (Staff Pediatrician in the Division of Gastroenterology, Hepatology and Nutrition at the Hospital for Sick Children) Dr. Shazeen Suleman, MSc, MPH, MD (Assistant Professor in the Department of Pediatrics at the University of Toronto, and an Associate Scientist at the Li Ka Shing Knowledge Institute) Dr. Jo-Anna Baxter, PhD, MSc (Centre for Global Child Health, Hospital for Sick Children) Dr. Robert Bandsma, MD, PhD (Pediatric Gastroenterologist, Hepatologist and Nutritionist, at the Hospital for Sick Children) Ms. Carolyn Spiegel-Feld, MMASc (Translational Medicine at the Hospital for Sick Childen)
Collaborators	Dr. Arthur Mortha PhD (Assistant Professor in the Department of Immunology at the University of Toronto Dr. Ben Willing, PhD (Associate Professor and Tier 2 Canada Research Chair in Microbiology of Nutrigenomics at the University of Alberta) Dr. Marie Claire Arrieta Mendez, PhD (Assistant Professor at the University of Calgary) Dr. Elena Comelli, PhD (Associate Professor and the Lawson Family Chair in Microbiome Nutrition Research in the Department of Nutritional Sciences at the University of Toronto) Dr. Michael Grigg, PhD (Chief of Molecular Parasitology at the NIH)

	Dr. Andrew Roger, PhD (Professor and Canada Research Chair in Comparative Genomics at Dalhousie University)
Study Design	Prospective, longitudinal observational study design
Objectives	**Primary Objective:** To assess if alterations of the microbiota in the maternal gut (dysbiosis) is associated with maternal gestational weight gain. To determine the association between maternal microbiome dysbiosis during pregnancy and birth outcomes, infant growth, infant nutritional status and health in the first year of life. **Secondary Objectives:** To link the maternal microbiome to dietary intake, with a focus on calories and macronutrients. To integrate maternal anthropometric factors, morbidity and mortality with microbiome data to reveal key modulators (microbial taxa and metabolites) of dietary intake during pregnancy and the post-partum period. To determine the impact of the maternal microbiome during pregnancy, including the exposure to pathogens and parasites, on the development of the infant microbiome. To investigate the maternal microbiome's exposure to pathogens and parasites, and the association with intestinal inflammation. **Exploratory Objectives:** To explore the role of the maternal gut microbiome during pregnancy and to identify gut community dynamics in pregnant women and how this impacts differences between dietary intake and nutritional status. To investigate socio-economic factors; including gender, poverty, exclusion and empowerment, and their influence on the health of a mother's microbiome (assessed by alpha and/or beta diversity, and absence of pathogens). To explore the role of the human microbiota on nutritional status by performing fecal microbiota transplants in germ free mice and sterile piglets.
Recruitment and Follow-up	1) MV1: 10-14 weeks post conception 2) MV2: 30-34 weeks post conception 3) MV3/ IV1: 24-72 hours after birth 4) MV4/IV2: 3 months post-partum 5) MV5/ IV3: 6 months post-partum

	6) MV6/IV4:12 months post-partum *MV: Maternal Visit IV: Infant Visit*
Study Population:	Pregnant, married women aged 17 to 24 years from the Matiari district, Pakistan and their infants.
No. of Subjects	400 women and their infants. • 200 subjects with normal BMI (18.5-24.9) at conception • 200 subjects with low BMI (less than 18.5) at conception
Study Duration:	The study duration will be approximately 3 years.
Participant Duration:	Participants will be enrolled in the study from 10-14 weeks post-conception until 12 months post-partum. The post-conception and post-partum phase of the study will last between 17-19 months for participants. The infants will be followed for 12 months, from their birth until 12 months of age.
Collaborating Institutions:	Institute for Global Health & Development, AKU Centre for Global Child Health, Hospital for Sick Children

1.2 SCHEMA

Figure 1A: Maternal Study Flow Diagram

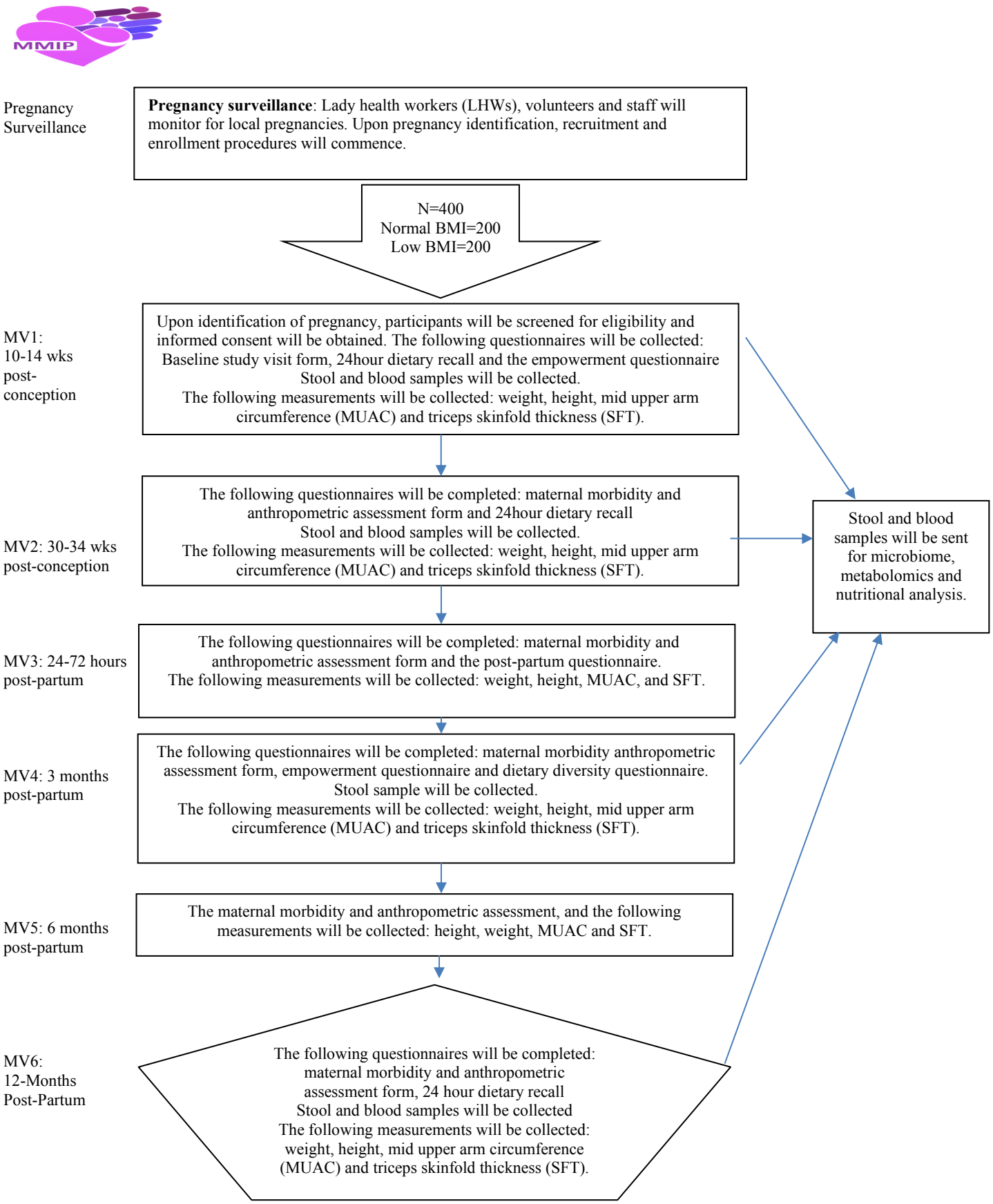
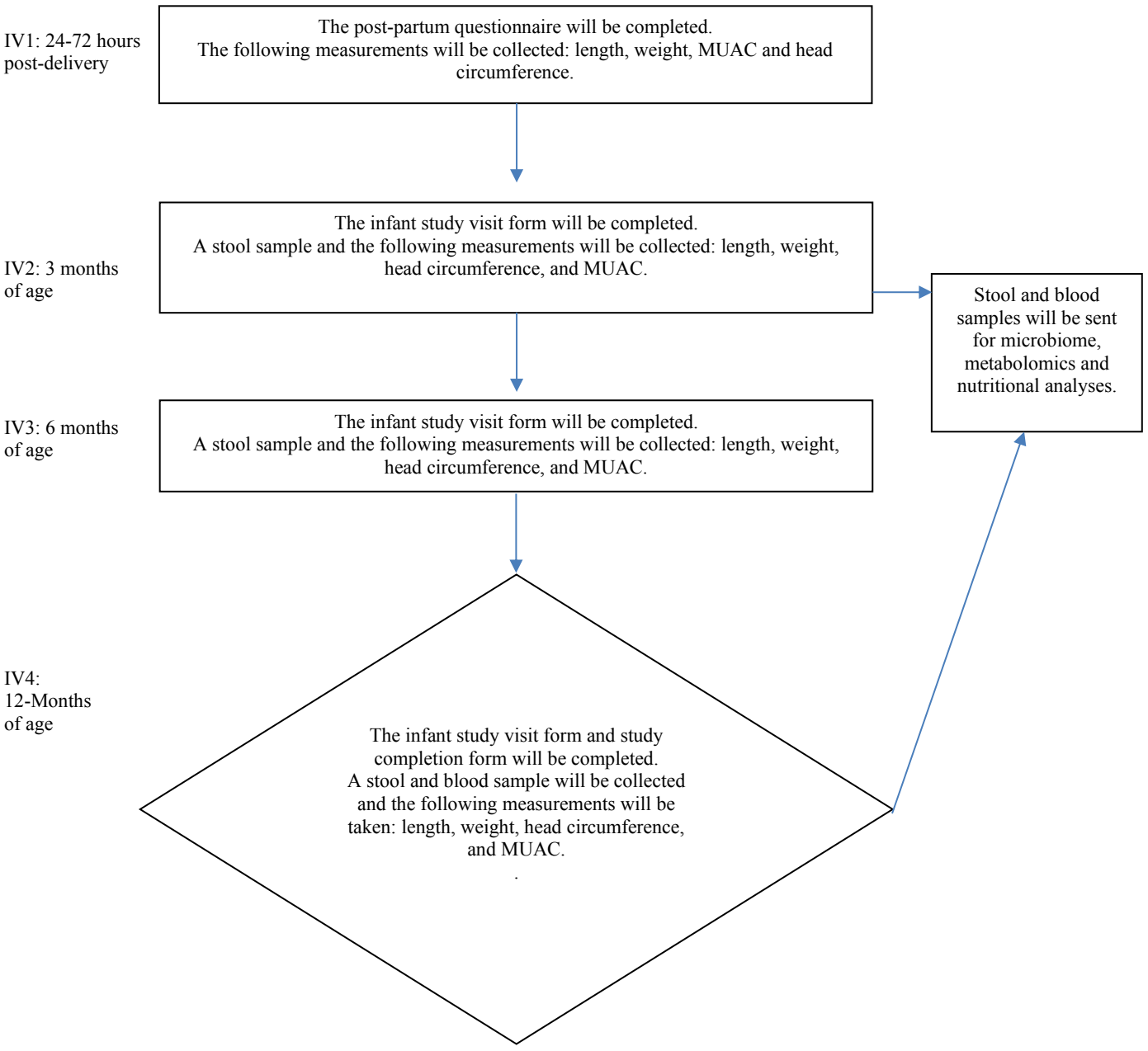

Pregnancy Surveillance
Pregnancy surveillance: Lady health workers (LHWs), volunteers and staff will monitor for local pregnancies. Upon pregnancy identification, recruitment and enrollment procedures will commence.
N=400
Normal BMI=200
Low BMI=200
MV1: 10-14 wks post-conception
Upon identification of pregnancy, participants will be screened for eligibility and informed consent will be obtained. The following questionnaires will be collected: Baseline study visit form, 24hour dietary recall and the empowerment questionnaire
Stool and blood samples will be collected.
The following measurements will be collected: weight, height, mid upper arm circumference (MUAC) and triceps skinfold thickness (SFT).
MV2: 30-34 wks post-conception
The following questionnaires will be completed: maternal morbidity and anthropometric assessment form and 24hour dietary recall
Stool and blood samples will be collected.
The following measurements will be collected: weight, height, mid upper arm circumference (MUAC) and triceps skinfold thickness (SFT).
Stool and blood samples will be sent for microbiome, metabolomics and nutritional analysis.
MV3: 24-72 hours post-partum
The following questionnaires will be completed: maternal morbidity and anthropometric assessment form and the post-partum questionnaire.
The following measurements will be collected: weight, height, MUAC, and SFT.
MV4: 3 months post-partum
The following questionnaires will be completed: maternal morbidity anthropometric assessment form, empowerment questionnaire and dietary diversity questionnaire.
Stool sample will be collected.
The following measurements will be collected: weight, height, mid upper arm circumference (MUAC) and triceps skinfold thickness (SFT).
MV5: 6 months post-partum
The maternal morbidity and anthropometric assessment, and the following measurements will be collected: height, weight, MUAC and SFT.
MV6: 12-Months Post-Partum
The following questionnaires will be completed: maternal morbidity and anthropometric assessment form, 24 hour dietary recall
Stool and blood samples will be collected
The following measurements will be collected: weight, height, mid upper arm circumference (MUAC) and triceps skinfold thickness (SFT).

Figure 1B: Infant Study Flow Diagram



1.3 SCHEDULE OF ACTIVITIES (SOA)

Table 1: Overview of study visits, visit activities, and data collection

Procedures	Pregnancy Surveillance	MV1: 10-14 Weeks Post-Conception	MV2: 30-34 Weeks Post-Conception	MV3: Maternal 24-72 Hours Post- Partum	IV1: Infant 24-72 Hours Post-delivery (same visit as mom)	MV4: Maternal 3-months post-partum	IV2: Child 3-Months (same visit as mom)	MV5: Maternal 6 Months Post-Partum	IV3: Infant 6 Months (same visit as mom)	MV6: Maternal 12-Months Post-Partum	IV4: Child 12-Months (same visit as mom)
Pregnancy Surveillance	X										
Screening and Confirmation of Pregnancy		X									
Informed Consent		X									
Dietary Recall		X	X							X	
Dietary Diversity Questionnaire						X					
Infant feeding practices							X		X		X
Stool Sample		X	X			X	X			X	X
Blood Sample		X	X							X	X
Anthropometric Measurements		X	X	X	X	X	X	X	X	X	X
Empowerment		X				X					
Birth and Labour History				X	X						
Health and Medication Use Assessment			X	X	X	X	X	X	X	X	X
Study Exit										X	X

Purple: Pregnancy Surveillance
Blue: Post Conception
Orange: Maternal Post-Partum
Green: Infant Visits

Table 2 Overview of the Study Forms

Study Form Code	Study Form Name	Study Visit Form Used	Purpose	Person Responsible
Consent Forms (ICF)				
ICF	Consent Form	MV1	To obtain informed consent	Research Staff
Maternal study visit forms (M)				
M-1	Screening form-post conception baseline visit	MV1	To confirm eligibility	Research Staff
M-2	Baseline visit form	MV1	To obtain baseline participant information. To record maternal anthropometric measurements. To assess maternal morbidity and mortality through questions regarding medication use, health seeking behavior etc	Research Staff
M-3	Ultrasound assessment	N/A: participants will be referred for ultrasound at baseline. This form will only be filled out for participants that have ultrasound completed	To record details from the ultrasound assessment.	Research Staff
M-4	Empowerment questionnaire	1)MV1 2)MV4	To assess gender related variables focused on five main metrics: perceived maternal self-efficacy, perceived social support, decision making, perceived stress in addition to socio-economic factors,	Research Staff

			including food and housing insecurity, and demographic data.	
M-5	24 Hour dietary recall.	1) MV1 2) MV2 3) MV6	To link the microbiome to nutritional status and nutritional intake, with a focus on calories and macronutrients.	Research Staff
M-6	Dietary diversity questionnaire	MV4	Participants will complete a dietary diversity score at the 3rd visit . *Note the research staff will calculate the dietary diversity score from the dietary recalls filled out at MV1, MV2 and MV6*	Research Staff
M-7	Maternal morbidity and anthropometric assessment form	1)MV2 2)MV4 3)MV5 4)MV6	To record maternal anthropometric measurements. To assess maternal morbidity and mortality through questions regarding medication use, health seeking behavior etc.	Research Staff
M-8	Post-Partum Questionnaire	MV3	To gather additional information, including birth characteristics (vaginal/ cesarean section), gestational age, birthweight and other measurements at birth (head circumference and length), placental insufficiency and antibiotic use. To collect maternal anthropometric measurements.	Research Staff
M-9	Food Recipe Record Form	1) MV1 2) MV2 3) MV6	This form is used to record new recipes	Research Staff

			observed during the 24 hour dietary recall.	
Infant study visit forms (IV)				
IV-1	Infant Study Visit Form	1)IV2 2)IV3 3)IV4	To assess the amount and mode of feeding, (ie, mother's milk or formula; complementary feeding). To record infant anthropometric measurements. To assess infant morbidity and mortality through questions regarding medication use, health seeking behavior etc. IV4 visit only: To assess potential markers of inadequate or excessive intake and dietary imbalances through the use of the NutricheQ questionnaire.	Research Staff
Adverse Event (AE)				
SAE & AE	Adverse Event Form	As needed.	To document serious/adverse events.	Research staff

2 INTRODUCTION

Nutritional status during pregnancy plays an important role in maternal health and birth outcomes[1, 2]. While few factors impacting nutritional status during pregnancy have been identified, studies of undernutrition in children have revealed a key role for the gut microbiome[3-9]. Remarkably, studies examining the dynamics of the maternal gut microbiome

before and during pregnancy and its impact on birth outcomes are limited. Further, relatively little is known concerning the influence of enteric eukaryotic microbes, such as parasites, on the bacterial microbiome and host nutrition. Here our goal is to define the relationships between host nutritional status and microbiome dynamics during pregnancy and how they contribute to birth outcomes and growth in infancy.

2.1 STUDY RATIONALE

This project represents the first systematic investigation of the impact of the microbiome on nutritional status during pregnancy in young women and directly aligns with global health initiatives focused on this vulnerable cohort. Here our goal is to define the relationships between host nutritional status and microbiome dynamics during pregnancy and how they contribute to birth outcomes. The gut microbiome has a profound influence on host nutritional status. Dysbiosis (loss of diversity/beneficial microbes and gain of pathobionts) has emerged as a major factor in the development of undernutrition. Despite the importance of nutrition during pregnancy, few studies have examined the role of the microbiome on maternal health and birth outcomes. Further, little is known concerning the influence of enteric eukaryotic microbes, such as parasites, on the bacterial microbiome and host nutrition.

At the core of this study are two complementary cohorts of young women that provide an exceptional opportunity to obtain longitudinal samples to monitor the dynamic relationships between microbiome community structure and function with gut health and host nutritional status. This protocol will discuss the Pakistan cohort of the study, which builds off the partnership with Aga Khan University and Dr. Zulfiqar Bhutta at SickKids. This cohort is expected to be exposed to a multitude of nutritional risks as well as pathogens and parasites associated with intestinal disease. To better define our target demographic, we focus on young mothers, 17-24 years of age, in the Matiari district of Pakistan, who are especially vulnerable to the impact of undernutrition[10]. A second complementary cohort will be based in Toronto, focused on refugee and young pregnant women in Toronto. This project will yield unprecedented insights into the relationships between prokaryotic and eukaryotic microbes in the gut and their associations with maternal health and birth outcomes.

2.2 BACKGROUND

2.2.1 UNDERNUTRITION BEFORE AND DURING PREGNANCY DRAMATICALLY IMPACTS MATERNAL HEALTH, BIRTH AND LIFE OUTCOMES

Maternal and child undernutrition, is a tremendous burden on global health. Globally, undernutrition contributes to 45% of all deaths in children under 5 years[11]. Deficiencies in macro- (e.g. proteins, lipids and carbohydrates) and/or micronutrients (e.g. minerals and vitamins) can result in stunting (low height-for-age) and/or wasting (low weight-for-height) or

thinness in adolescents and adults (low Body Mass Index – BMI). Pregnant women are particularly vulnerable due to the high nutrient demands during development of the fetus. Consequently undernutrition during pregnancy is associated with increased risk of poor birth outcomes, intra-uterine growth restriction of the fetus[12] and can result in complications that impact maternal morbidity and mortality[13-17]. In terms of perinatal outcomes, women with low BMI are at increased risk for stillbirths and neonatal deaths, preterm birth, low birth weight (LBW; <2500g) and delivering babies that are small for gestational age (SGA)[18]. Undernutrition prior to or during pregnancy may also have long term impact for the offspring (developmental origins of health and disease hypothesis)[19-21]. For example, studies of the 1944 Dutch winter famine found women exposed to famine during late gestation did not gain weight in the third trimester and had babies with reduced birth weights, who were shorter and had smaller heads and placentas than unexposed babies[22]. Mothers exposed to famine only in early -mid gestation however, gained more weight than non-exposed mothers did. The offspring of mothers with mid gestation famine exposure were lighter, shorter and had smaller heads, whereas the babies that were exposed during early gestations were heavier and longer at birth. but had higher rates of obesity[23]. **At significant risk are young women (17-24 years of age), the target demographic of this study.**

These findings broadly support the hypothesis that chronic diseases originate through adaptations made by the fetus in response to undernutrition. The long-term effects of intrauterine undernutrition, however, depend upon its timing during gestation and on the tissues and systems undergoing critical periods of development at that time.

A woman's nutritional status is important both prior to and during pregnancy, as it plays an important role in reproductive health and pregnancy outcomes[12, 24]. Prior to pregnancy, having sufficient nutrient intake and nutrient stores affect a woman's ability to maintain obligatory physiological functioning, and support fetal development in the case of pregnancy. During the periconceptional period and throughout pregnancy, sufficient nutrient intake is necessary since different nutrients influence pregnancy outcomes by altering both maternal and fetal metabolism via roles in modulating oxidative stress, enzyme function, signal transduction, and transcription pathways[25]. Among women in LMICs, several micronutrient deficiencies often co-exist because a woman's dietary intake is insufficient to meet her physiological requirements[12]. Pre-existing micronutrient deficiencies may be exacerbated during pregnancy as a result of the increased metabolic requirements[17]. Ultimately, maternal undernutrition during pregnancy often leads to fetal growth restriction, which increases the risk of neonatal deaths and childhood stunting by 2 years of age[11].

Among young women, and especially among adolescents, ensuring sufficient nutrient intake is critical to facilitate the rapid physiological growth and maturation that occurs during the transition to adulthood [11]. Nutrition and growth in adolescence is particularly important to a one's health and adult stature. Many of the risk factors that impact maternal and newborn health, such as nutritional deficiencies, exist from adolescence. Becoming pregnant during this sensitive life stage has been found to slow and stunt one's growth[12]. In some countries, as many as half of adolescents are already stunted which further increases the risk of poor perinatal outcomes in their off spring[11]. Pregnancies that occur among adolescents are additionally associated with a higher risk of complication, maternal and child mortality, and poor birth outcomes than

pregnancies in older women[26]. Specifically, adolescent pregnancy is associated with a 50% increased risk of stillbirths and neonatal deaths, and increased risk of preterm birth, LBW, and asphyxia [13, 14, 27]. The prevalence of anemia is suggested to be as high in adolescents as women 20–24 years of age. **Collectively, this makes adolescent nutrition of substantial public health importance.**

2.2.2 THE GUT MICROBIOME IMPACTS MATERNAL AND INFANT NUTRITION

While both genetic and environmental factors are known to impact BMI[28], our understanding on the interactions relating host genetics with the gut microbiome in the context of undernutrition remains limited[29, 30]. Indeed, twin based studies have identified few genetic factors that predispose to undernutrition[4]. Furthermore, undernutrition is not simply a consequence of food insecurity resulting in macro- and micronutrient[11, 31-33]. Instead, the intestinal microbiome has emerged as a key factor defining nutritional status, with impaired maturation driving undernutrition[3-9]. An immature microbiome may impact the extraction of critical nutrients such as vitamins and short chain fatty acids (SCFAs)[34-38], and/or result in the production of metabolites that inhibit metabolism or increase host cell turnover[4]. During pregnancy, dramatic changes in gut microbiota occur, with a decrease in individual (alpha) diversity but an increase in population (beta) diversity[39]. Relative to the 1st trimester, microbiota in the 3rd trimester exhibit higher abundances of *Proteobacteria*, typically associated with obesity in humans, and when transplanted to mice, result in increased adiposity and insulin insensitivity. Such adaptations may increase energy extraction from the diet to support pregnancy[40], raising interest in dietary supplements to improve pregnancy outcomes[41]. **While these limited studies suggest an important role for the gut microbiome during pregnancy, there is a lack of data on gut community dynamics in pregnant women and how this impacts host nutrient acquisition.**

2.2.3 PATHOGEN EXPOSURE CONTRIBUTES TO ENVIRONMENTAL ENTERIC DYSFUNCTION

Recently, data from the Malnutrition and Enteric Diseases multisite international study revealed that cumulative pathogen exposures confer a high risk for poor growth, with the prokaryotic enteroaggregative *Escherichia coli* and the protozoan *Giardia lamblia* most commonly detected[42, 43]. Exposure to pathogens such as these can contribute to EED, a condition characterized by chronic inflammation, poor nutrient absorption and reduced gut epithelial barrier function[44-46]. EED is thought to be triggered by dysbiosis (where beneficial microbes are replaced by pathobionts with a loss of diversity[47, 48]), initiated by nutrient deficiencies, antibiotic treatment and/or pathogen exposure. A ‘healthy’ microbiome normally limits pathogen invasion through: antimicrobial compounds; competition for nutrients; and direct stimulation of the immune system[49], but dysbiosis may exacerbate pathogen colonization, impair development of the mucosal immune system and disrupt, by as yet unknown mechanisms, metabolic processes that supply nutrients and energy for normal growth[6]. While maternal EED during pregnancy has been shown to adversely impact birth outcomes[50], **our understanding**

of the impact of enteropathogens and dysbiosis on maternal gut community dynamics and nutritional status is limited.

2.2.4 EUKARYOTIC MICROBES ARE A KEY COMPONENT OF THE MICROBIOME

Typically, studies of the microbiome focus on bacterial components and tend to neglect the diverse array of eukaryotic microbiota. These include many considered to be parasites such as *Giardia*, *Cryptosporidium* and *Entamoeba*, each a significant global healthcare burden[51-54]. However not all parasitic infections cause disease; a study of Toronto daycares revealed a ~5% incidence of largely asymptomatic *Giardia* infections[55]. Thus, disease is a consequence of the complex interplay between the eukaryotic and bacterial microbiome and the host immune system[56, 57]. For example, *Prevotella copri*, is increased in patients with diarrheagenic *E. Histolytica* infections[54], while indole-producing bacteria such as *E. coli* CFT073 protect against *Cryptosporidium*[58]. Conversely, eukaryotic microbes may have neutral or even beneficial roles; *Blastocystis*, for example, has been associated with increased microbiome diversity[59, 60]. **While current knowledge of the interactions between bacteria and eukaryotic microbiota and their host is limited, recent advances, discussed below, provide the molecular tools required to dissect these interactions.**

2.2.5 MARKER GENE SURVEYS COUPLED WITH ADVANCES IN NEXT GENERATION SEQUENCING PROVIDE UNPRECEDENTED INSIGHTS INTO MICROBIAL COMMUNITIES

To date, microbiome studies have largely relied on marker gene surveys (e.g.16S rRNA sequences) to profile community structure and dynamics[61-63]. In a typical application, PCR reactions are used to amplify marker genes from a DNA sample. Sequencing is then performed to yield tens of thousands of sequences which are subsequently compared against a database of known marker genes to yield readouts of the abundance of each taxon in the sample. Key to this technology is the identification of a variable genetic region that allows unambiguous identification of taxonomy, flanked by highly conserved regions that allow universal primers to amplify the marker genes. For bacterial surveys, hypervariable regions of the 16S rRNA gene are typically targeted. Recently, Dr. John Parkinson and Dr. Robert Bandsma, targeted 18S and 28S rRNA genes to perform equivalent surveys for eukaryotic microbes (Table 3)[64]**. In combination, 16S, 18S and 28S rRNA markers reveal the bacterial and eukaryotic components of the microbiome.**

Organism	Incidence in 44 children N (%)
Blastocystis	15 (34)
Cryptosporidium	12 (27)
Eimeriorina (unknown genus)	24 (55)
Giardia	14 (34)[a]
Trichomonas	37 (84)
Tritrichomonas	38 (86)
Pentatrichomonas	2 (5)
Trichomonad (unknown genus)	2 (5)
Tetramitia (unknown genus)	2 (5)

a. Clinical diagnostic test of 41 patients

Table 3. Protozoa in children with severe acute malnutrition from Malawi using 18S biomarker technology and clinical diagnostics.

2.2.6 METAGENOMICS, METRANSCRIPTOMICS AND METABOLOMICS DELIVER READOUTS OF MICROBIOME FUNCTION

Beyond sequence surveys, whole microbiome DNA and RNA sequencing (metagenomics and metatranscriptomics), together with metabolomics, offer more mechanistic insights, detailing the biochemical functionalities encoded and expressed by the microbiome[65-74]. For example, Dr. Parkinson used metatranscriptomics to reveal how changes in fat absorption in mice impact metabolic pathway expression in the gut microbiome[75]. Further, metabolomics has been transformed by improved technologies for the sensitive detection of metabolites. For example, our group recently revealed significant changes in metabolic pathways involved in lipid and protein metabolism in stunted and severely malnourished children relative to controls[76]. However, the underlying mechanisms explaining how the microbiome affects intestinal and whole-body metabolic function is just starting to be elucidated. **These tools support the functional interrogation of microbiomes to identify microbial pathways that may modulate health and disease.**

2.2.7 GENDER IMPACTS NUTRITIONAL STATUS AND THE MICROBIOME

It is increasingly apparent that biological sex impacts the gut microbiome, beginning in infancy[77]. While all pregnant individuals have the same biological sex, the experience of being a mother is related to gender roles and identity. Intriguingly, recent research is beginning to reveal how gender-related variables such as socio-economic status, food security, early marriage and maternal empowerment contribute to nutritional status and the development of a healthy intestinal microbiome[78-80]. For example, in low-income settings, maternal empowerment may play a role in improving breastfeeding, diversity and quality of diet in children, setting up the opportunity for a healthy microbiome[81]. Addressing gender roles, specifically with respect to maternal empowerment, has the capacity to directly impact intestinal health in infants.

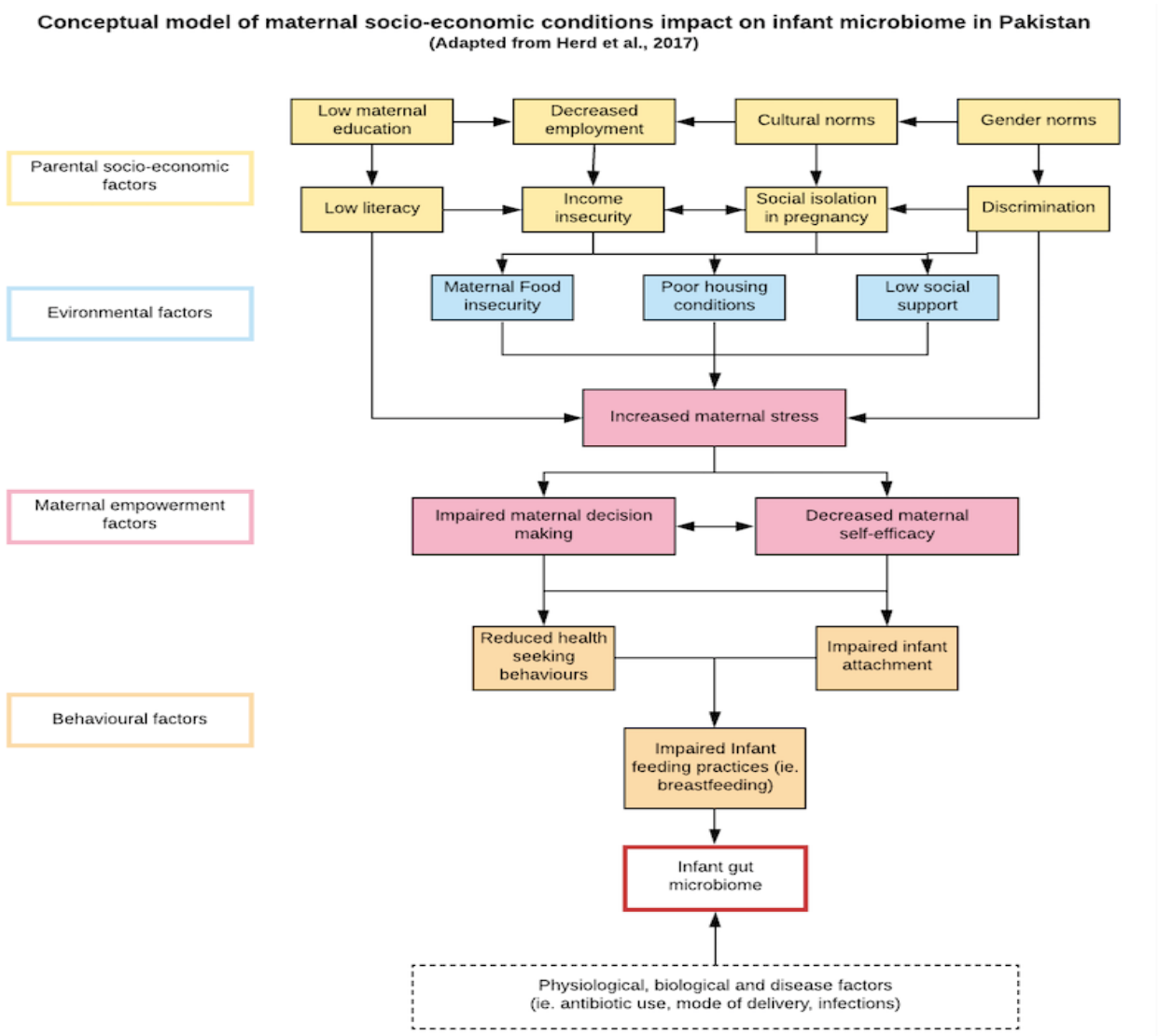


Figure 2: Conceptual Model of Maternal Socio-Economic Conditions Impact on the Infant Microbiome in Pakistan (Adapted from Herd *et al,* 2018[82]).

2.3 RISK AND BENEFITS

This is an observational study. Potential risks to participants include the general risks of giving blood, including: light-headedness, bruising, minor bleeding where the needle is inserted into the arm, pain, and a very low chance of infection. Participants may feel discomfort providing a stool sample, detailed instructions and research staff will be available to provide support. The stool samples are an essential component of the study, and as such are a required component of participation.

Participants may also experience psychological and emotional distress when completing questionnaires and/ or answering questions about their socio-economic status, their health, their infants' health. In addition, some participants may go through postpartum issues that may impact their mental state during the study period. Participants will have the option to leave questions

blank/unanswered if they are uncomfortable, research staff members will be on hand if a participant experience's physiological and/ or emotional distress during a study visit or during study procedures. If this occurs, the study staff members will refer and transport the participant to the government health care facility.

Participants will also have the option to consent to future genetic testing of their serum samples. Genetic information can never be fully de-identified. Although procedures will be put in place to de-identify genetic information, there is a risk that the information gained from genetic research can be linked to participants. While this is very unlikely at this time, rapid scientific advances mean that re-identification may be more likely in the future. There is also a risk of unintentional release of information that could lead to loss of privacy and to possible future discrimination against participants and/ or their biological relatives. The potential future use of genetic information is unknown and therefore not all potential future risks are known. The potential psychological and social risk of participating and receiving genomic information is not fully known at this time. It may be upsetting to learn about genetic causes and medically actionable findings.

There are no direct benefits to participants in the study. However, all participants will be assessed for anemia, and ongoing interaction with the Lady Health Worker Program (LHWs) will be promoted. This provides a small benefit to the study population. All study participants will benefit from receiving appropriate standard of care, which may be better than the average care received in the health system. Participants identified with severe anemia will be referred to nearest public health facility for further evaluation and treatment.

The societal benefits of the proposed study are that it is expected to yield insights into the relationships between prokaryotic and eukaryotic microbes in the gut and their association with maternal health and birth outcomes. The study results are expected to have an impact beyond the study setting. The actual risk of harm caused by a non- interventional trial is expected to be low, and the potential benefits in terms of knowledge generation outweigh the theoretical risks.

3 STUDY OBJECTIVES

Table 4: Study Objectives

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
Primary		
To assess if alterations of the microbiota in the maternal gut (dysbiosis) is associated with maternal gestational weight gain.	The primary endpoint will be maternal gestational weight gain (GWG) during pregnancy, measured between the first (10-14 weeks post-conception) and second time point (30-34 weeks post conception). To monitor microbiome dynamics, 16S and 18S rDNA surveys will be	Weight gain is a key predictor of adverse birth outcomes[83, 84].

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
	applied to the maternal stool samples to monitor the bacterial and eukaryotic components of the microbiome.	
To determine the association between maternal microbiome dysbiosis during pregnancy and birth outcomes, infant growth, nutritional status and health status in the first year of life.	The specific birth outcomes evaluated will include preterm birth, birth weight, SGA, LGA, head circumference at birth, mortality, morbidity. The specific infant outcomes evaluated will include the following growth and nutritional status parameters; WHO z-scores for weight, length, head circumference and MUAC during the first year. Birth characteristics will be collected, including information on vaginal or cesarean birth (elective, emergency), placental insufficiency and antibiotic usage. Infant morbidity and mortality will be assessed at follow-up visits, with questions regarding care-seeking, hospitalizations and treatments for any morbidity; including antibiotic usage. For the infants an assessment of amount and mode of feeding will be obtained at 3-months, 6-months and 12-months. To monitor microbiome dynamics, 16S and 18S rDNA surveys will be applied to the maternal stool samples to monitor the bacterial and eukaryotic components of the microbiome.	This will allow for an examination of the association between the maternal microbiome and birth outcomes.
Secondary		
To link the maternal microbiome to dietary intake, with a focus on calories and macronutrients.	The maternal participants will complete a 24 hour dietary recall at both time points during pregnancy and at 1-year post-partum.	Quantitative measures of nutritional status will provide parameters to better inform microbiome analyses.

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
	Food insecurity will be assessed through the use of a food insecurity questionnaire administered at time of recruitment and 3 months post-partum. To assess the quality of the diet through dietary diversity scores.	
To integrate maternal anthropometric factors, morbidity and mortality with microbiome data to reveal key modulators (microbial taxa and metabolites) of dietary intake during pregnancy and the post-partum period.	Maternal morbidity will be assessed at follow-up visits, with questions asked about illness or complications during pregnancy, childbirth and the post-partum period, care-seeking, hospitalizations and treatments for any morbidity; including antibiotic usage. Maternal anthropometric measurements will include maternal height, weight, MUAC, and SFT.	Integration with clinical information will allow for the analysis of microbiome data in the context of patient nutritional status and health outcomes.
To determine the impact of the maternal microbiome during pregnancy, including the exposure to pathogens and parasites, on the development of the infant microbiome.	Maternal and child stool and serum samples will be collected and analyzed for microbiome composition, micronutrients in the serum and stool macronutrients.	These analyses will inform on the role of the maternal microbiome during pregnancy to impact the developing child's microbiome. Our hypothesis is that exposure to pathogens and/or parasites during pregnancy will negatively impact the development of the child's microbiome with downstream consequences for nutrient uptake.
To investigate the maternal microbiome's exposure to pathogens and parasites, and the association with intestinal inflammation.	The maternal stool samples will be examined for markers of intestinal mass, inflammation, and gut permeability. To monitor the bacterial and eukaryotic components of the microbiome, 16S and 18S rDNA	These markers will reflect gut health and pathogen challenge.

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
	surveys will be conducted on the stool samples.	
Exploratory		
To explore the role of the maternal gut microbiome during pregnancy and to identify gut community dynamics in pregnant women and how this impacts differences between dietary intake and nutritional status.	To gain more mechanistic insights into the relationship between microbiome function and maternal health and birth outcomes, metatranscriptomics, metabolomics and markers of inflammation will be selectively deployed on the stool samples.	The output of these analyses are concentrations of metabolites that are expected to correlate with pathway expression data; linked to readouts of microbial gene expression detailing biochemical activity and the taxa responsible.
To investigate socio-economic factors; including gender, poverty, exclusion and empowerment, and their influence on the health of a mother's microbiome (assessed by alpha and/or beta diversity, and absence of pathogens).	Participants will answer questionnaires at two time points: baseline and 3-months post-partum. The questionnaire will assess gender related variables focused on five main metrics: perceived maternal self-efficacy, perceived social support, decision making, perceived stress in addition to socio-economic factors, including food and housing insecurity, and demographic data.	The use of previously validated questionnaires within this population, will allow for comparison between the Pakistan and Toronto cohorts, to account for gender related variables that impact maternal and infant gut health.
To explore the role of the human microbiota on nutritional status by performing fecal microbiota transplants in germ free mice and sterile piglets.	The pregnancy rates, birth outcomes, microbiome dynamics, health outcomes, host nutritional status, inflammation and barrier integrity will be collected and assessed.	Exploiting animal models will allow us to define causal interactions between diet, microbiome, pathogen exposure and nutritional status during pregnancy. Through recapitulating patient phenotypes with fecal microbiome transplants, our animal studies will set the stage for developing new therapeutic strategies that promote gut health.

4 STUDY DESIGN

4.1 STUDY HYPOTHESIS

The central hypothesis of the study is that alterations of the microbiota in the maternal gut (dysbiosis) exacerbated by nutritional status or pathogen exposure during pregnancy, impacts weight gain during pregnancy because of impaired nutrient absorption, leading to corresponding negative birth outcomes.

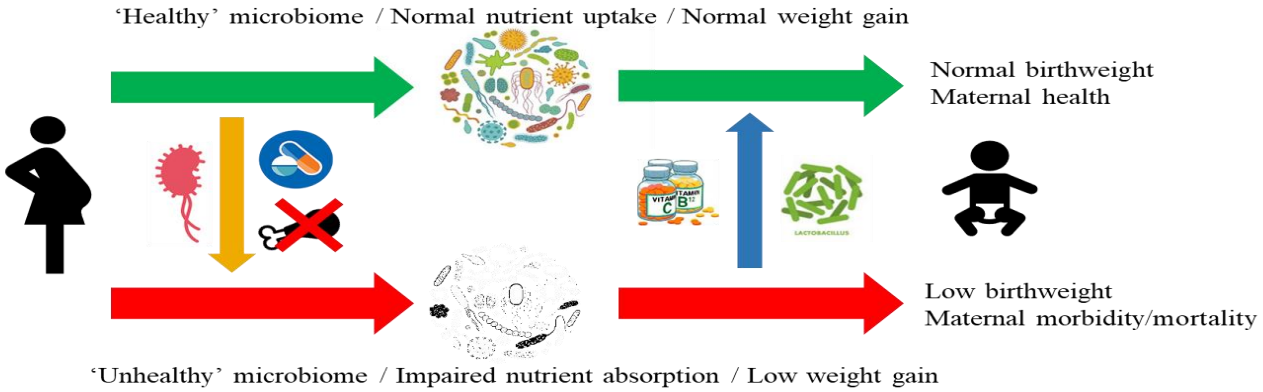


Figure 3: Project Hypothesis. During the course of pregnancy a shift to an 'unhealthy' microbiome, perhaps triggered by exposure to pathogens, antibiotics or poor diet, results in impaired nutrient absorption, low weight gain and poor birth outcomes.

4.2 STUDY DESIGN

The study will be a prospective, longitudinal observational study to investigate the impact and relationship between prokaryotic and eukaryotic microbes in the gut and their association with maternal health and birth outcomes among young women, 17-24 years of age in Matiari District, Pakistan. The study will aim to recruit 400 women into two groups based on BMI at time of recruitment (Normal: BMI between 18.5 and 24.9 kg/m^2 and Low: BMI less than 18.5 kg/m^2, as defined by WHO guidelines[85]). Our goal is to recruit 200 participants with normal BMI and 200 participants with low BMI into the study. However, in the event that we have difficulty reaching this recruitment target, we will recruit more women into the normal weight BMI. The study will follow women and their infants over the course of their pregnancy and for a year post-partum, collecting stool and blood samples, nutritional information, heath assessments, anthropometric measurements and empowerment metrics at different time points.

4.3 END OF STUDY DEFINITION

A participant is considered to have completed the study if she has completed all phases of the study including the last visit or the last scheduled procedure shown in *Section 1.3, Schedule of Activities (SoA)*. The duration of participation for each individual participant will vary depending on how long they are in the pregnancy monitoring cohort, upon conception the participants will

be followed for approximately 17-19 months in duration, from 10-14 weeks into their pregnancy, until one year post-partum.

It is estimated that it will take approximately 6 -8 months for the study to reach its recruitment target, and approximately 3 years from when the study opens to the final participant study visit.

5 STUDY POPULATION

This study will examine young, married women, including newlyweds, 17-24 years of age, living in Matiari District, Pakistan. We will focus on this younger demographic due to our lack of knowledge on the microbiome of young women, and their vulnerability to undernutrition[10]. This will be the first systematic investigation of the impact of the microbiome on nutritional status during pregnancy in young women and directly aligns with global health initiatives focused on this vulnerable cohort.

Upon identification of pregnancy, participants will be screened for eligibility and their pregnancy will be confirmed. If the woman is between 10-14 (+ 2 weeks) post-conception she will be invited to join the study. If she agrees to participate written informed consent will be obtained. Only one woman per household will be eligible to participate in the study.

5.1 INCLUSION CRITERIA

In order to be eligible to participate in the pregnancy surveillance cohort phase, an individual must meet all of the following criteria:

1. Consent provided
2. Married female aged 17-24 years
3. In good general health, with no comorbidities
4. Absence of COVID19 symptoms
5. Women who are 14 weeks post-conception or less (+ 2 weeks)
6. Intend to comply with study procedures and follow up

5.2 EXCLUSION CRITERIA

An individual who meets any of the following criteria will be excluded from participation in this study at the pregnancy surveillance cohort phase of the study:

1. Women who do not meet the enrollment age criteria
2. Women participating in interventional clinical trials
3. Women who intend to leave the study area
4. Women who cannot comply with study procedure's and follow-up
5. Illness and other co-morbidities
6. Signs of potential COVID19 infection
7. BMI higher than 24.9 kg/m^2
8. Women who already have a member of their household participating
9. Women who have taken antibiotics within the past 3 months
10. Women who are past 16 weeks post- conception

5.3 STUDY SETTING

This study will be conducted in the rural settings of Matiari District in Sindh province, Pakistan. Matiari is situated in North Eastern part of Sindh, about 200 km away from Karachi. Including 1,418 villages and a population of about 776,542, around 78,000 residents are young women 15-24 years (based on regular surveillance data). Approximately 20% of these women are married. The study setting has a well-established community and health system liaison, basic demographic surveillance, and field centers for research. This site has also been selected because it has been the location of several large cluster randomized trials, meaning that households are well mapped with household level GIS. This district is representative of typical conditions in Pakistan, and there is a close working relationship with community, civic society leaders, and public health department facilities. Select epidemiological features of the Matiari site are highlighted below in (Table 4**)** and findings to date from the household survey have been provided in (Table 5). These tables were taken from the MaPPS trial protocol. The current MaPPS baseline household listing data shows that the proportion of young women 15-19 and 20-24 years each comprise 5% of the population. The MaPPS trial was a prospective, cluster randomized evaluation of the effectiveness of supplementation with multiple micronutrients and life skills development education provided from preconception on health and birth outcomes among young, reproductive-age Pakistani women (15-24 years).

Table 4. Epidemiological information from Matiari district (estimated from regular surveillance data)

Characteristic	Matiari-specific observations
Population size	776,542
Villages	1,418
Households	112,542
Number of women 15-45 years of age	178,993
Number of women 15-24 years of age	77,740
Number of females 15-19 years of age	39,417
Mean (±SD) age at 1st pregnancy (years)	20.3 ± 3.2
Mean (±SD) age at marriage (years)	17.7 ± 2.8
Number births in last year to women 15-45 years of age	27,178
Number of births to women 15-24 years of age	5979
Number of births to women 15-19 years of age	715
Birth rate (per 1000 population)	35

Table 5. Key statistics to date from the MaPPS household survey (from data collected up to end of March 2017, including population size of 158,019)

Characteristic	MaPPS catchment area-specific observations		
	15-24 years	15-19 years	19-24 years
Number of women	17,164	9,286	9,054
Number of pregnant women	983	226	813
Pregnancy rate (%)	6	2	9
Ratio of women to pregnancies	17.5	41.1	11.1

5.4 STRATEGIES FOR RECRUITMENT AND RETENTION

This study builds off the MaPPS trial partnership between Aga Khan University in Pakistan and Dr. Bhutta at SickKids. Led by Principal Investigator Dr. Bhutta, this trial is an exciting partnership between SickKids' Centre for Global Child Health and the Institute for Global Health and Development, AKU. Here we will leverage the clinical network established by the MaPPS trial, to extend the trial to recruit hundreds of young mothers to investigate host-microbiome interactions in pregnancy.

The study will aim to recruit 400 married women into two groups based on BMI at time of recruitment (Normal: BMI between 18.5 and 24.9 kg/m^2 and Low: BMI less than 18.5 kg/m^2, as defined by WHO guidelines[*[85]*). Our goal is to recruit 200 women with normal BMI and 200 women with low BMI. However, in the event that we are unable to reach this recruitment target, we will recruit more women in the normal BMI category. With an annual birth rate of 2% and Matiari Surveillance data revealing proportions of normal and low BMI individuals to be 49.7% and 29.8% respectively, we anticipate meeting our target sample size in the first year of the study.

5.4.1 METHOD FOR MONITORING NEW PREGNANCIES

From the MaPPS trial we found that women typically report their pregnancy to study personnel near the end of the first trimester or start of their second trimester. Field team shall implement multiple strategies to identify new pregnancies:

1) The MaPPS study was implemented in the LHW-covered area within the district, and LHWs are required to visit their catchment population once a month. LHWs are required to meet with their respective lady health supervisors (LHSs) in the first week of each month to report pregnancies and share data on several other indicators. Research field staff will join this meeting and get information about new pregnancies.
2) Research field staff will get contact numbers of women/families and make regular calls inquiring as to whether they think that they might be pregnant.
3) Research field staff will also identify volunteers from within local villages and primary health care facilities to notify them about new pregnancies. A communication card of Rs.100 will be provided to the volunteer against a valid pregnancy notification.
4) Research field staff will also employ random check of households and villages and meet with LHWs and volunteers in the field to get information on pregnancies.

Once potential pregnancies are identified by LHWs and volunteers, eligible individuals will be asked by them to provide verbal consent to have a research staff member contact them about the study. If the individual provides this verbal consent to contact, they will be contacted by study personnel at their home to confirm their eligibility. This will include the administration of a pregnancy test to confirm the pregnancy, questions to determine the gestational age (if the getstational age is 16+ weeks the individual will be excluded) and the collection of anthropometric measurements to confirm their BMI is equal to or below 24.9 kg/m^2. The aim will be to recruit participants during the 10-14 week period post-conception, however, women

can be enrolled in the study up to 16 weeks post-conception. Only one participant per household can be enrolled in the study.

5.4.2 OBTAINING INFORMED CONSENT

The investigators at AKU have well established protocols for informed consent and will share information about the project with local government leaders, health workers, and members from all participating villages, as appropriate.

The study staff will obtain a verbal consent to take the potential participant's height and weight, this will be taken to determine eligibility for the study (BMI below 24.9 kg/m^2) and to conduct a pregnancy test to confirm pregnancy. If the participant meets the eligibility criteria, the research staff member will verbally explain the study to the participant; participation components; potential benefits and harms; and obtain informed consent, Women will be asked to consent to (1) provide stool samples at four time points during the study, and upon delivery for her infant to provide stool samples at 2 times points (2) provide blood samples at 3 time points during the study and upon delivery for her infant to provide a blood sample at 1 time point (3) have anthropometric measurements conducted, and upon delivery upon her infant as well (4) participate in periodic study follow-up including health assessments and study questionnaires, and, upon delivery, assessment of both herself and infant. Participants will be provided with information about the study both verbally and through a written consent form.

Following an objective assessment of understanding of the project, the women will be invited to join the study and to provide written informed consent. In all instances, participants will be informed of the right to withdraw from the study without prejudice.

Participants will have the option sign an additional section of the consent form for future genetic testing of their blood samples. Participants will have the option to opt out of this future testing. If they sign this section of the consent form, the samples will be stored for future genetic testing to further dissect patterns of microbiome genotype associated traits.

5.4.1 STUDY HONOURARIUM

To thank participants for their time and effort in the study, participant's will be given a thank-you gift three times during the study. 1) Non-food items i.e. suit for mother at enrollment 2) baby hygiene kit at birth 3) toys for baby at the end of participation. Within the available budget these items can be changed if families would prefer other items of equal value.

6 DISCONTINUATION AND WITHDRAWAL

Participants are free to withdraw from participation in the study at any time upon request.
An Investigator may discontinue or withdraw a participant from the study for the following reasons:

- Withdrawal of informed consent (participant can withdraw for any reason)

- If any clinical Adverse Event (AE), laboratory abnormality, or other medical condition or situation occurs such that continued participation in the study would not be in the best interest of the participant.
- Significant study non-compliance
- If the participant meets an exclusion criterion (either newly developed or not previously recognized) that precludes further study participation

The reason for participant discontinuation or withdrawal from the study will be recorded in the study file. The data from participants who are withdrawn or discontinued from the study will be used in the analysis unless the participant requests otherwise.

6.1 LOST TO FOLLOW-UP

A participant will be considered lost to follow-up if they are unable to be contacted by the study site staff. If a participant misses one of the scheduled visits, they will still be included/ contacted for future study visits.

The following actions must be taken if an attempt to contact participants at their homes for a required study visit fails:

- The study staff will attempt to contact the participant and reschedule the missed visit and counsel the participant on the importance of maintaining the assigned visit schedule and ascertain if the participant wishes to and/or should continue in the study.
- Before a participant is deemed lost to follow-up, the Principal Investigator or designee will make every effort to regain contact with the participant, where possible, including 3 telephone calls and, if necessary, an in-person visit. These contact attempts should be documented in the participant's study file.
- Should the participant continue to be unreachable, they will be considered to have withdrawn from the study with a primary reason of lost to follow-up.

6.2 REMOVAL OF PARTICIPANTS

Efforts will be made to obtain complete follow-up data from enrolled participants. If a participant experiences a serious adverse event, follow-up and scheduled data collection will continue to the extent that is possible. However, study activities will be stopped for a participant when any one of the following events occurs:

- Consent for follow-up is withdrawn.
- Participant moves residences and is lost to follow-up. Loss to follow-up will be considered to have occurred if study personnel receive information that the participant has moved away and will not return to follow-up at any time during the remainder of the study.

7 STUDY PROCEDURES

7.1 STUDY VISITS

7.1.1.1 MV1: 10-14 WEEKS POST-CONCEPTION BASELINE ASSESSMENT:

Research staff will administer the screening form. If the participant is eligible, the research staff member will obtain written, informed consent. In the case that a participant cannot write her name, ink will be used to get an imprint of her left thumb. Once consent is obtained, the following forms will be administered: the baseline visit form, 24-hour dietary recall and the empowerment questionnaire. The research staff will obtain anthropometric measurements, a blood sample and give participants the stool collection kit. Study participants will arrange with study field staff a day to collect stool sample, and the field team will facilitate the transport of the sample to the field office-based laboratory.

7.1.1.2 MV2: 30-34 WEEKS POST-CONCEPTION

During this visit, research staff will administer the 24-hour dietary recall and the maternal morbidity and anthropometric assessment form. The research staff will obtain anthropometric measurements, a blood sample and give participants the stool collection kit. Study participants will arrange with study field staff a day to collect stool sample, and the field team will facilitate the transport of the sample to the field office-based laboratory.

7.1.1.3 MV3/IV1: POST-PARTUM 24-72 HOURS POST DELIVERY

Study research staff will aim to complete this visit with 24-48 hours after birth but can complete the visit up to 72 hours post-delivery. During this visit the research staff will administer the maternal morbidity and anthropometric assessment form and the post-partum questionnaire, obtain birth characteristics and will obtain infant and maternal anthropometric measurements.

7.1.1.4 MV4/IV2: 3 MONTHS POST PARTUM

The research staff will administer the maternal morbidity and anthropometric assessment form, the empowerment questionnaire, the dietary diversity questionnaire, and the infant study visit form. The research staff will obtain anthropometric measurements from both the mother and infant; and will provide the stool sample kit to both. Study participants will arrange with study field staff a day to collect stool sample, and the field team will facilitate the transport of the sample to the field office-based laboratory.

7.1.1.5 MV5/IV3: 6 MONTHS POST PARTUM

Study staff will collect infant and maternal anthropometric measurements and will administer the maternal morbidity and anthropometric assessment form and the infant study visit form.

7.1.1.6 MV6/IV4: 12 MONTHS POST PARTUM

This will be the final time point for both the mother and infant. The research staff will administer the maternal morbidity and anthropometric assessment form and the infant study visit form and the 24-hour recall. The research staff will obtain the following from both the mother and infant; anthropometric measurements, blood samples and will provide the stool sample kit. Study participants will arrange with study field staff a day to collect the stool sample, and the field team will facilitate the transport of the sample to the field office-based laboratory.

7.2 STUDY ASSESSMENTS AND SAMPLE COLLECTION

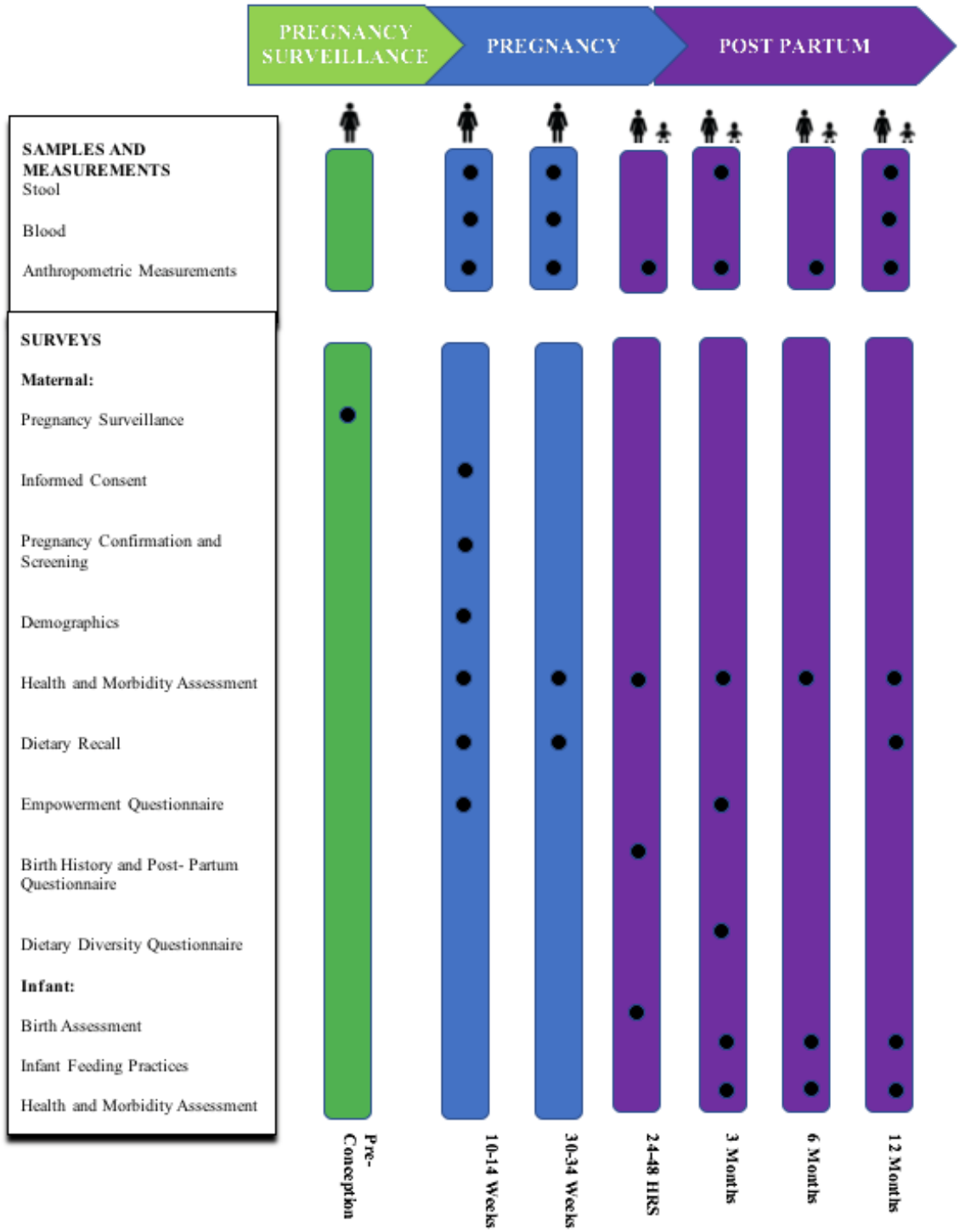


Figure 4: Overview of Sampling, Analyses and Surveys

7.2.1.1 BLOOD SAMPLE COLLECTION

A venous blood specimen of 5 mL will be collected from the mother at enrolment, 30-34 weeks post conception and 12-months post-partum. The blood will be divided into two tubes, 0.6 ml in SST Serum, for ferritin and CRP, the remaining sample will be collected in EDTA tube for HB and MCV, and for storage for future analysis. The infants will have a venous blood specimen of 3 mL collected into two tubes, 0.6 ml in SST Serum, for ferritin and CRP, the remaining sample will be collected in EDTA tube for HB and MCV, and for storage for future analysis at 12 months.

All blood specimens will be coded to link with an individual subject's records using a specimen ID system. Blood specimens from individuals who did not complete the study will be processed and their data analyzed, as possible, for selected outcomes. Blood samples will be analyzed for Ferritin, CRP and HB + MCV. Blood samples will be flash frozen and stored at -80ºC. Samples will be transported to the Nutrition Research Lab at the AKU main campus on dry ice, where they will be further stored at -80ºC until analysis.

See section 8 for more information on blood sample analysis and refer to the blood sample collection SOP.

7.2.1.2 STOOL SAMPLE COLLECTION

Stool samples will be requested prior to visits by field staff, with families provided with stool container, cold box and ice packs. At data collection visits, study participants will be provided with a stool container, cold box, and ice pack to collect the stool sample. They will arrange with study field staff a day to collect stool sample, and the field team will facilitate the transport of the sample to the field office-based laboratory.

For maternal stool samples, the participants will be asked to hand over freshly passed stool in sterile container to field staff. The samples will be transported to the field office lab on ice packs and transferred to a -80ºC freezer. Samples will be transported to the Nutrition Research Lab at the AKU main campus on dry ice, where they will be further stored at -80ºC.

For infant stool samples, freshly soiled diapers will be collected from infants and transferred into a Ziploc bag and quickly transported to field lab with ice packs (4ºC), there with the help of sterile applicator two cryovials will be three quarter filled and immediately transferred to -80ºC freezer.

From the Matiari field lab stored samples will be shipped in batches on dry ice to NRL, AKU. They will be stored in a -80 freezer until they are subjected to nucleic acid extraction. The stool samples will be coded to link with the individual's subject records using a specimen ID system.

See section 8 for more information on stool sample analysis and refer to the stool sample SOP.

7.2.1.3 ANTHROPOMETRIC DATA COLLECTION

Maternal height and weight will be measured using a digital floor scale and stadiometer; MUAC will be determined using a measuring tape. A triceps skinfold thickness (SFT) measurement will be taken using a skinfold caliper. All measurements will be conducted in duplicate by two study personnel using standardized procedures, as specified in the anthropometric data collection SOP. The average (mean) of acceptable paired measures will be used in analysis.

Infant anthropometry will include measurement of weight, length, MUAC, and head circumference; these measurements will be conducted using standardized procedures: a digital weight scale, infantometer, and measuring tape. All infant measurements will be collected in duplicate, by two study personnel, using standardized procedures, as specified in the anthropometric data collection SOP. The average (mean) of acceptable paired measures will be used in analysis.

Refer to the infant and maternal anthropometric measurement SOP.

7.2.1.4 QUESTIONNAIRES

For all structured interviews with the study participants, study personnel will use tablet-based questionnaires to guide data collection. The information collected will depend on the visit. A summary of all the study questionnaires and visit-specific forms that will be used throughout the study is provided in *Table 2.*

The forms will include information on participant's baseline demographics, anthropometrics and health assessments that will collect participant information regarding the participant's age, gender, sex, number of pregnancies, number of children, marital status, the language spoken in the home, languages spoken in the home, religion, country of origin, income, # of people this income supports, education and housing. The morbidity assessment section of the forms will document mortality, morbidity and medication usage.

Participants will fill out an empowerment questionnaire at baseline and 3-months post-partum. The five main metrics will be: perceived maternal self-efficacy, perceived social support, decision making, perceived stress in addition to socio-economic factors, including food and housing insecurity, and demographic data. These metrics align with major social constructs contributing to infant feeding practices and suspected microbiome health (Figure 4). These constructs will be measured using validated questionnaires that have been previously translated and utilized with this study population. Questions pertaining to perceived decision-making, and demographic data including maternal education, income, housing and gender norms are adapted from the Pakistan Demographic and Health Survey (PDHS)[86]; food insecurity will be assessed using the Household Food Insecurity Access Scale (HFIAS)[87]. Self-efficacy will be measured using the Generalized Self-Efficacy scale, developed by Schwarzer and Jerusalem[88]. Perceived social support will be measured using the Multi-dimensional Scale of Perceived Social Support (MSPSS), developed by Zimet and colleagues[89]. Perceived parental stress will be measured using the Perceived Stress Scale (PSS-10)[90].

The birth and labour history questionnaire will be administered at the post-conception visit, 24-72 hours post-partum and will gather additional information including: birth characteristics (vaginal/cesarean section), gestational age, birthweight and other measurements at birth (head circumference and length), placental insufficiency, antibiotic use, among other birth characteristics.

For the infant, the study forms will assess breastfeeding and infant feeding as well as a health assessment's to assess infant mortality, morbidity and medication usage.

7.2.1.5 DIETARY RECALLS AND INFANT FEEDING PRACTICES

To link the microbiome to nutritional status and nutritional intake, with a focus on calories and macronutrients, a semi-quantitative 24-hour paper-based dietary recall will take place at both time-points during pregnancy and at 1-year post-partum. This will be done based on an interactive 24-hour recall method[91], that will be administered by the research staff, and aided with a food kit.

At the 3 months post-partum visit, maternal participants will complete a dietary diversity questionnaire using the Minimum Dietary Diversity Score for Women (MDD-W). The MDD-W is a population level indicator for dietary diversity for women aged 15-49, based on 10 food groups[92]. The MDD-W will reflect what they have eaten over the previous 24 hours, and they will be asked at the end of the questionnaire whether this reflects their diet over the previous 3 months. The research team will calculate the MDD-W from the dietary recalls completed at baseline, 30-34 weeks post-conception and at 12 months.

For the infant, an assessment of initiation, amount and mode of breastfeeding, as well as an assessment of young child feeding, including questions to address the introduction of solid, semi-solid and soft foods, the minimum dietary diversity, meal frequency and acceptable diet will be administered at 3 and 6 months and 1 year post-partum. The questionnaire will be designed based on the guidance developed by the WHO in 2010, in the "*Indicators for assessing infant and young child feeding practices (Part 2 Measurement)"*[93]

At the 12 month visit, the research staff will administer the NutricheQ questionnaire, a tool designed for toddlers aged 1 to 3 years of age, with a focus on markers for inadequate or excessive intake and dietary imbalances[94]. The mother and infant will both answer two food insecurity questions, that will assess their annual food insecurity[87].

7.3 CONTACT WITH STUDY PARTICIPANTS

The majority of the study visits will occur at the participants' homes. The expected duration of visits is detailed in the table below.

Table 7: Overview of Study Visits and Expected Visit Duration

Visit	Description of Visit	Visit Duration
MV1: Baseline post-conception visit 10-14 weeks post-conception	Confirmation of eligibility and written informed consent to participate in the study will be conducted. The participant will	~1.5-2 hours

	complete the study forms specified in table 2, the 24-hour paper-based dietary recall, anthropometry, fecal sample and blood draw.	
MV2: 30-34 weeks post conception	The participant will complete the study forms specified in table 2, maternal anthropometric measurements will be collected. The maternal participant will complete a 24-hour dietary recall and a stool and blood sample will be collected.	1-1.5 hours
MV3/IV1: 24-72 Hours post-delivery	The participant will complete the study forms specified in table 2, infant and maternal anthropometric measurements will be collected.	20-30 minutes
MV4/IV2: 3-Months post-partum	The participant will complete the study forms specified in table 2, infant and maternal anthropometric measurements will be collected. A stool sample will be collected from both the mother and infant.	1-1.5 hours
MV5/IV3: 6- Months post-partum	The participant will complete the study forms specified in table 2, infant and maternal anthropometric measurements will be collected.	30-40 minutes
MV6/IV4: 12-Months post-partum	The participant will complete the study forms specified in table 2, infant and maternal anthropometric measurements will be collected. The maternal participant will complete a 24-hour dietary recall. A stool and blood sample will be collected from both the mother and infant.	1-1.5 hours

7.4 SAFETY AND ADVERSE EVENTS

Safety will be monitored by study personnel tri-monthly throughout the study progression. This includes, but is not limited to, death, hospitalizations, miscarriages, abortions, stillbirths, and maternal and newborn deaths.

Although study-related serious adverse events and adverse events are not anticipated, the participants may encounter pregnancy-related or other unrelated adverse events. These events will be recorded in the form: *AE-10 Adverse Event Form*. Women who experience clinical events during study participation will be referred to the appropriate public health facility. Adverse events will be reported to the AKU and Hospital for Sick Children REBs according to their respective institutional requirements.

Serious adverse events include the following events, irrespective of their association with study procedures:

- Death
- Life-threatening complication, such that death was averted by medical or surgical interventions (the term "life-threatening" in this context refers to an event in which the patient was at risk of death at the time of the event; it does not refer to an event, which hypothetically might have cause death, had it been more severe)
- Inpatient hospitalization or prolongation of an existing hospitalization
- Persistent or significant disability/incapacity following the resolution of the acute event.
- Reporting of certain events (e.g., suspected child abuse) is mandatory because of the study population.

7.4.1 REPORTING EVENTS TO PARTICIPANTS

Participants will be informed in a timely manner of any new information, including safety information, that is relevant to that participant's willingness to continue participation. The communication of this information will be documented through a revised REB approved Informed Consent Form, where possible, based on the timeliness of the information.

In the event that a study procedure detects a new clinically important secondary finding/incidental finding, the qualified physician will notify the Most Responsible Physician (MRP) at AKU or request the participant's family doctor's name and contact information in order to arrange medical follow-up to interpret the significance of the findings.

In the event of discovering a medically actionable incidental finding, or if any new clinically important information about the participant's health is obtained as a result of participation in the study and where a second sample is already available, the test result will be validated clinically in an accredited laboratory. For this study, the presence of a pathogen might be identified, in the event of this finding, the follow up would include clinical microbiology. The qualified physician will work with the participant, their family physician and MRP at AKU to arrange referral to the appropriate specialist as needed.

8 STOOL AND BLOOD SAMPLE ANALYSIS

Time-points	Blood		Stool		Hemoglobin	
	Mother	Child	Mother	Child	Mother	Child
MV1: 10-14 Weeks Post-Conception	yes	-	yes	-	yes	-
MV2: 30-34 Weeks Post- Conception	yes	-	yes	-	yes	-
MV4/ IV2: 3-months post-partum	-	-	yes	yes	-	-
MV6/ IV4: 12-Months Post-Partum	yes	yes	yes	yes	yes	-

Overview of Sample Collection Time Points

Sample type	Times
Mother Spot Hemoglobin	X 3
Mother Blood Samples	X 3
Mother Stool Samples	X 4
Child Blood Samples	X 1
Child Stool Samples	X 2

Overview of Collection of Sample Types

Assay	Sample size	Subject
Whole blood		
Hemoglobin + MCV	400 X 4	3 X Mother + 1 X child
Hemoglobin	400 X 3	Mother
Serum		
CRP	400 X 4	3 X Mother + 1 X child
Ferritin	400 X 4	3 X Mother + 1 X child
Stool		
Extraction of DNA	400 X 6	4 X Mother + 2 X child
Extraction of RNA	400 X 2	2 X Mother
Calprotectin	200 x 2	2 X 200 Mothers
Lipocalin	200 x 2	2 X 200 Mothers
Claudin 15	200 x 2	2 X 200 Mothers

Overview of Sample Analysis to be Conducted at AKU

8.1 PROFILING MICROBIAL COMMUNITY STRUCTURE AND FUNCTION

8.1.1 STOOL SAMPLE PROCESSING

Stool samples will be extracted for DNA and RNA; maternal stool samples from all 4 time points

(M1, M2, M4 and M6) will be extracted for DNA and infant stool samples from both time points (IV2 and IV4) will be extracted for DNA. RNA will be extracted from the maternal stool samples collected during the two pregnancy visits (MV1 and MV2). Stool will be stored at AKU in a -80 freezer until analysis. Stool samples will be analyzed for inflammatory markers at AKU. The stool samples and extracted DNA and RNA will then be batch shipped to the Parkinson lab on dry ice for downstream analysis. The stool samples will be biobanked at SickKids.

8.1.2 PROFILING MICROBIAL COMMUNITY STRUCTURE

Microbial communities will be analyzed through 16S and 18S rDNA surveys using established methods that target the V4 region of the 16S rRNA gene to capture bacterial taxa [95-97] and the V4V5 region of the 18S rRNA gene to capture eukaryotic taxa. DNA library preps will include error-correcting barcodes[98] for multiplexing of samples. Sequencing will be performed to generate ~50,000 2x300bp paired end reads generated per sample. To define taxonomic diversity, species profiles from 16S and 18S rDNA data will be clustered to identify differences in community structure across samples. We will utilize the QIIME2 platform[99], MOTHUR[100], multivariate approaches such as Permutation Multivariate Analysis of Variance (i.e. PERMANOVA-S a method that can associate microbiome changes with outcome measures while accounting for confounders[101]) among other state of the art tools. Differences between groups in microbiome community structure will be tested by analysis of similarities (ANOSIM) and co-occurrence analysis[102]. To better define bacterial pathogen burden, we will apply TaqMan array card technology for the simultaneous detection of 19 common enteropathogens[103].

8.1.3 PROFILING MICROBIAL COMMUNITY FUNCTION

After total RNA extraction and rRNA depletion (RiboZero Gold Kit, Illumina, San Diego, Ca, or equivalent), libraries will be constructed and Illumina-based sequencing will be performed to generate ~30 million 2x150bp paired end reads per sample (our rarefaction analyses have previously shown such sequencing depth is sufficient to identify the vast majority of species and enzymes present in the samples[75]). Reads will be processed for quality and contaminants using established pipelines (e.g. [66, 68, 104]). Reads will be assembled using appropriate state of the art tools such as SPAdes[105] and subsequently annotated with taxonomic and functional assignments Expression will be normalized to Reads per Kilobase of transcript per million mapped reads. Annotations are mapped onto biochemical pathways and complexes such as those defined by the Kyoto Encyclopedia of Genes and Genomes [104]. The output of these analyses are readouts of microbial gene expression detailing biochemical activities as well as the taxa responsible.

8.1.4 PROFILING OF PATIENT AND MICROBIOME METABOLITES AND INFLAMMATORY MARKERS

To gain more mechanistic insights into the relationship between microbiome function and maternal health and birth outcomes, we will deploy metatranscriptomics, metabolomics and markers of inflammation.

We will obtain host readouts that reflect gut health and pathogen challenge, by examining markers in the stool for Calprotectin, Lipocalin and Claudin 15. These markers will be examined

in the maternal stool samples collected during the 2 pregnancy visits (MV1 and MV2) for 200 of the participants. We will target this analysis at the 200 participants representing the two extremes of weight gain; the 100 participants with the highest and 100 participants with the lowest WHZ scores.

Stool samples will be sent from SickKids to The Metabolomics Innovation Centre (TMIC) for analysis of general metabolites (e.g. SCFAs, amino acids, intermediates in glycolysis and nucleotide metabolism, among other metabolites), using the TMIC Prime Metabolomics platform[105]. This analysis will be targeted at the maternal stool samples collected during pregnancy (MV1 and MV2); with a focus on the 200 participants at the two extremes of weight gain: the 100 participants with the highest and 100 participants with the lowest WHZ scores.

Blood samples will be analyzed at AKU for Hemoglobin + MCV, Ferritin, and CRP. Blood samples will be shipped on dry ice to SickKids and then to the TMIC Centre, where they will be analyzed for Metallomics using the TMIC Metallomics platform[106]. The output of these analyses will be concentrations of metabolites that are expected to correlate with pathway expression data. Blood samples will be biobanked at SickKids.

Blood proteomics will allow for a deeper investigation into the relationship between the maternal microbiome and maternal weight gain and the effects on offspring. Proteomics will be conducted at the Sickkids SPARC (Sickkids Proteomics, Analytics, Robotics and Chemical Biology Centre)[107]

8.2 POTENTIAL PROBLEMS, MITIGATIONS AND EXPECTED RESULTS

We and others have already established the feasibility of obtaining DNA, proteomics and metabolomics datasets from LMIC settings (e.g.[4-6, 76, 108]). With the outstanding facilities available at AKU, we are therefore confident we will generate these datasets. In the unlikely event we find RNA yields are poor, we will revert to performing whole shotgun DNA sequencing (metagenomics), which also has the capacity to provide functional insights.

Our expectation is that: 1) study participants exhibiting positive maternal health and birth outcomes will be associated with more diverse communities than those exhibiting poor maternal health and/or birth outcomes; 2) pregnancies associated with poor nutritional status at the start of pregnancy, will exhibit a greater incidence of dysbiosis (e.g. loss of microbial diversity and decrease in otherwise dominant stool taxa e.g. Firmicutes and/or Bacteroidetes) throughout pregnancy; 3) pregnancies associated with pathogen exposure will result in dysbiosis; and 4) poor nutritional health will correlate with altered fecal metabolite profiles and changes in the expression of metabolic *pathway* enzymes encoded by the microbiome. Since analysis of microbiome data is a rapidly evolving field, to ensure data analysis is in line with best practices, we will adopt current state of the art bioinformatics tools for sequence processing, assembly, annotation and analysis. In case of delays or other issues that may be experienced at the Metabolomics Innovation Centre, we may rely on the SPARC Biocentre, hosted by the Hospital for Sick Children, to perform equivalent analyses.

As previous[39], we may expect specific taxa (prokaryotic or eukaryotic) will directly correlate

with nutritional status during pregnancy and birth outcomes. The core deliverables will be an unprecedented view of dynamic changes in microbial communities that contribute to nutritional status during pregnancy, together with the prediction of biochemical pathways and taxa responsible.

9 STATISTICAL CONSIDERATIONS

9.1 POWER CALCULATION

The adequacy of the sample size was verified using the 'pwr' package (version 1.2-2) in R (version 3.6.1). Calculations were based on the correlation between α-diversity (Shannon index) and weight gain during pregnancy. Assuming 400 participants are recruited into the study, and a type I error rate of 0.05, there will be 80% power to detect a correlation coefficient (r) >0.14. This is conventionally considered a small effect size[109]. Thus, we expect to be powered to reveal significant association between weight gain during pregnancy and diversity. Further, a study of 123 lean and 24 obese individuals, revealed 96% power to detect differences in Bacteroidetes abundance and 80% power for Firmicutes[110], while a cohort of 44 Malawian malnourished children was sufficient to detect significant differences in bacterial α-diversity in the presence and absence of *Blastocystis*.

9.2 SUBGROUP ANALYSIS

To reduce study complexity and to avoid confounding effects due to micronutrient deficiencies, we will focus specifically on interactions between macronutrient deficiencies with host microbiome and health. Given the high prevalence of micronutrient deficiencies experienced within the study population, the effect of micronutrient deficiencies of public health importance (i.e. anemia, iron deficiency, iron deficiency anemia, vitamin A deficiency and vitamin D deficiency) will be explored within a subgroup analysis. We will achieve this by monitoring for anemia as well as serum mineral content when participants are recruited into the study.

9.3 STATISTICAL ANALYSIS

We will work with the research core in Calgary to apply multivariate analyses and other integrative methods to analyze microbiome data in the context of patient nutritional status and health outcomes. Our initial strategy is to apply the Similarity Network Fusion framework[111] to combine the various datatypes (i.e. metabolomics, microbiome, and clinical). In this approach networks are first constructed from each datatype, with nodes representing patients and links representing similarities between participants (e.g. based on correlations between microbiome or metabolite profiles). Networks are then fused using a method based on message-passing theory to identify links shared between patients supported by multiple datatypes, eliminating poorly supported links and strengthening links supported by multiple datatypes. This allows the integration of all available datasets to uncover their global substructures that can be associated to relevant outcomes. In an alternative approach, we will also employ Random Forests to identify combinations of predictors (microbial and/or metabolic factors) that best correlate with our clinical outcomes. Beyond integrative methods we will also apply simple statistical tests (e.g.

Wilcoxon, t-test, Spearman's rank correlation, PERMANOVA) to examine pairwise interactions (e.g. presence of pathogens vs. bacterial diversity and/or abundance of specific taxa; metabolic pathway expression vs. inflammation; and stool metabolites vs. serum metabolites). Further, we will also attempt to include additional quantitative measures of nutritional status as well as gender-related variables from the empowerment questionnaires, as covariates where appropriate. These analyses will reveal relationships between taxonomic composition, microbiome diversity (both prokaryotic and eukaryotic), metabolomics and the primary and secondary outcome measures gathered.

9.4 PLAN FOR STATISTICAL ANALYSIS

The following details the plans for evaluating the aims of this study. Table 8 lists the variables to be considered in the analyses.

Table 8. List of variables collected in the study to be utilized in analysis

Clinical Variables

1. Maternal BMI, MUAC and SFT (continuous variables)
2. Maternal gestational weight gain (continuous)
3. Maternal markers of inflammation (continuous)
4. Infant sex
5. Infant morbidity and mortality
6. Infant weight, height, head circumference (continuous)
7. Infant gestational age
8. Infant birth weight, Kg (continuous)
9. Breastfeeding duration (continuous)
10. Maternal age years
11. Maternal use of antibiotics
12. Infant use of antibiotics
13. Maternal calorie and macronutrient intake
14. Maternal incidence of pathobionts
15. Infant incidence of pathobionts

Microbiome Variables

1. Maternal gut bacteria profile
2. Maternal eukaryotic microbe profile
3. Infant gut bacteria profile
4. Infant eukaryotic microbe profile
5. Maternal metabolomic profile of stool
6. Maternal metabolic pathway expression profile
7. Maternal bacterial gene expression profile

Gender-related Variables

1. Food insecurity
2. Self-efficacy

3. Perceived decision making
4. Perceived social support
5. Perceived parental stress

9.4.1 ADDRESSING THE PRIMARY OBJECTIVES

This study has two primary objectives: 1) To assess if alterations of the microbiota in the maternal gut (dysbiosis) are associated with maternal gestational weight gain; and 2) To determine the association between maternal microbiome dysbiosis during pregnancy and birth outcomes, infant growth, nutritional status and health status in the first year of life.

The first objective will be addressed through the following approaches. First, maternal gut bacteria and eukaryotic profiles will be used to calculate Bray-Curtis dissimilarity metrics between individual samples which will be leveraged in principal co-ordinate analyses to determine the extent samples collected at the first or third trimester, exhibiting similar gestational weight gains, co-cluster. Permutational multivariate analysis of variance (PERMANOVA) tests will assess the degree of overlap between samples exhibiting low gestational weight gain versus samples exhibiting high gestational weight gain. A lack of clustering in the first trimester, followed by clustering of samples on the basis of similar gestational weight gain would indicate that alterations in the maternal microbiota during the course of pregnancy, impacts gestational weight gain. Next, we will attempt to correlate changes in the alpha diversity (as measured by the Shannon and Simpson indices) of the gut microbiome samples between the first and third trimester, with gestational weight gain. A decrease in alpha diversity (indicative of dysbiosis) associated with low gestational weight gain, would again indicate that dysbiosis is associated with maternal weight gain. To examine the influence of individual taxa on gestational weight gain, we will perform bivariate analyses that examine the correlation (Pearson, Spearman) of each taxon with gestational weight gain. Correlations will examine taxon abundance in first and third trimester, in addition to the change in abundance between the two trimesters. The Benjamini-Hochberg procedure will be applied to correct p-values while controlling for false-discovery rates.

To complement these analyses, we will also undertake an integrative modeling strategy based on Similarity Network Fusion framework[112] to analyze the contribution of each variable (clinical, microbiome and gender-related) on gestational weight gain. In this approach we construct a series of networks (one for each variable) in which nodes represent patients and links represent similarities between patients (e.g. based on correlations between e.g. microbiome or metabolite profiles). Networks are then fused using a method based on message-passing theory to identify links shared between patients supported by multiple datatypes, eliminating poorly supported links and strengthening links supported by multiple datatypes. This allows the integration of all available datasets to uncover their global substructures that can be associated with gestational weight gain. In an alternative approach, we will also employ Random Forests to identify

combinations of variables (clinical, microbiome and gender-based; Table 8) that correlate with gestational weight gain.

9.4.2 ADDRESSING THE SECONDARY OBJECTIVES

This study has the following secondary objectives: 1) To link the maternal microbiome to dietary intake, with a focus on calories and macronutrients; 2) To integrate maternal anthropometric factors, morbidity and mortality with microbiome data to reveal key modulators (microbial taxa and metabolites) of dietary intake during pregnancy and the post-partum period; 3) To determine the impact of the maternal microbiome during pregnancy, including the exposure to pathogens and parasites, on the development of the infant microbiome; and 4) To investigate the maternal microbiome's exposure to pathogens and parasites, and the association with intestinal inflammation.

To assess these secondary objectives, we will employ general linear models. In a first approach we will perform PERMANOVA and probe the association of microbial community structure with the clinical variables. Pair-wise differences in microbiome structure between patient samples at similar time-points are defined by abundance-weighted pair-wise differences using the Bray-Curtis dissimilarity metric. DESeq2, a method for differential analysis of count data, will subsequently be applied to investigate both associations of specific taxa with the clinical variables, together with the strength of those associations. DESeq2 will utilize default settings, and q-values calculated with the Benjamini-Hochberg procedure to correct p-values while controlling for false-discovery rates. These analyses will reveal which clinical variables (including exposure to pathogens and parasites) correlate with the maternal microbiome from a taxonomic perspective.

To probe the association of clinical variables with the function of the microbiome, PERMANOVA and DESeq2 analyses will be performed replacing microbial community structure metrics with microbial community function metrics, specifically: 1) metatranscriptomic profiles; and 2) stool metabolomic profiles. Profile differences will be defined through Spearman rank differences for metatranscriptomic data and Euclidean distance metrics for metabolomic profiles. These analyses will reveal microbial genes, metabolites and pathways that correlate with clinical variables.

9.4.3 ADDRESSING THE EXPLORATORY OBJECTIVES

This study has the following exploratory objectives: 1) To explore the role of the maternal gut microbiome during pregnancy and to identify gut community dynamics in pregnant women and how this impacts differences between dietary intake and nutritional status; 2) To investigate socio-economic factors; including gender, poverty, exclusion and empowerment, and their influence on the health of a mother's microbiome (assessed by alpha and/or beta diversity, and absence of pathogens); 3) To explore the role of the human microbiota on nutritional status by performing fecal microbiota transplants in germ free mice and sterile piglets.

For the first exploratory objective, we will apply the same statistical framework as we utilized for the secondary objectives here, however, we are concerned with changes in the microbiome within a patient from the first to third trimester. Consequently, microbiome structural and functional profiles will be generated from the difference in: 1) taxonomic abundances; 2) gene expression; and 3) metabolite concentrations, between the first and third trimesters. Profile differences will then be utilized in the PERMANOVA and DESeq2 approaches as described above to identify associations between clinical variables and changes in microbiome structure and function.

9.5 FUTURE USE OF STORED SPECIMENS AND DATA

Data collected for this study will be analyzed and stored at AKU and SickKids. After the study is completed, the de-identified, archived data will be transmitted to and stored at SickKids. Permission to transmit data to SickKids will be included in the informed consent.

With the participant's approval and as approved by the local Research Ethics Boards (REBs), de-identified biological samples will be biobanked at SickKids. Samples will be analyzed at AKU, SickKids and sent to research collaborators outside Pakistan. Samples will not be sold. These samples will be used to research the causal relationships between microbiome dynamics, pathogen exposure, nutritional status during pregnancy, undernutrition, its complications and other conditions for which study participants are at increased risk, and to improve treatment. SickKids will also be provided with a code-link that will allow for the linking of the biological specimens with the phenotypic data from each participant, maintaining the blinding of the identity of the participant.

Established animal models will be used to study the casual relationships between microbiome dynamics, pathogen exposure and nutritional status during pregnancy and to examine whether manipulation of the microbiome can improve nutritional status. The gnotobiotic facilities are located at the University of Toronto and the University of Alberta. Human fecal microbiome transplants (FMTs) have been effectively applied to animals to study the role of human microbiota on nutritional status. Here we will perform FMT into germ-free mice and sterile piglets to first, recapitulate the disease-associated phenotypes in animals and second, to set the stage for therapeutic intervention strategies. We will investigate 10 patient microbiomes: correlated with positive and negative nutritional status, as indicated by primary and secondary outcomes. This is consistent with previous animal studies that identified key taxonomic associations with undernutrition[4]. Extracted microbes will also be sent to our collaborator Dr. Michael Grigg, the Chief of Molecular Parasitology at the NIH, Bethesda, and an expert at host-parasite interactions. Dr. Grigg will oversee mouse experiments with the extracted microbes to investigate the parasite-microbiome interactions. Extracted microbes will also be sent to Dalhousie to investigate parasite-microbiome interactions.

In addition to publishing findings in open access journals, we will ensure all sequences and metabolomics datasets are deposited in appropriate public repositories. SOPs, pathogen samples and statistical methods developed through this project will be shared with the IMPACTT research core.

9.5.1 FUTURE GENETIC TESTING

Participants will have the option to sign an additional consent form for future genetic testing of their blood samples and their infant's blood samples. Participants will have the option to opt out of this future testing, or to have genetic testing on their own samples but not on their infant's. If they sign the consent form, the samples will be stored for future genetic testing to further dissect patterns of microbiome genotype associated traits.

The focus of the current application is on the investigation of interactions between the bacterial and eukaryotic components of the microbiome and their downstream impact on host nutritional status. However, with the availability of host DNA, opportunities will exist to examine the interactions between host genetic factors and gut microbial communities. For example, defects in these interactions can result in imbalances in the microbial community, such as the loss of beneficial commensal microbes and also lead to impaired pathogen clearance[113]. In our own studies applying metatranscriptomics to fecal samples from mice, we showed that knockout of the gene encoding Perilipin-2, a protein involved in lipid absorption, in mice results in significant shifts in the expression of microbial genes involved in energy production [75]. Another study focused on gene-microbiome interactions in healthy individuals used genotyping to identify 58 single nucleotide polymorphisms associated with the relative abundance of 33 taxa in their gut microbiomes[114]. Although not funded as part of this study, we envision that the availability of DNA samples from the mothers and children enrolled in this study, provides an unparalleled opportunity to apply whole genome sequencing or targeted resequencing methodology, to examine how the interactions between host genetic factors and the gut microbiome contribute to host nutritional status and birth outcomes. Such studies would form the basis of future grant applications.

During the conduct of the study, an individual participant can choose to withdraw consent to have biological specimens stored for future research. However, withdrawal of consent with regard to biosample storage may not be possible after the study is completed.

10 SUPPORTING DOCUMENTATION AND OPERATIONAL CONSIDERATIONS

10.1 REGULATORY, ETHICAL, AND STUDY OVERSIGHT CONSIDERATIONS

Research ethics committee/institutional review board approval will be obtained from the Aga Khan University, The National Bioethics Committee in Pakistan and the Hospital for Sick Children. All standard procedures related to consent will be followed.

Informed consent will be obtained from all study participants. The trial will be verbally explained to participants by study personnel, with formal consent being taken following a standardized interview with the team supervisor/medical officer to ensure prospective participants' understanding of the project and its implications. Written informed consent will then be obtained and participants will be informed that they have the right to withdraw from the

study at any time without any penalty in terms of care. Withdrawal from the study or refusal to participate will not in any way affect their ability to receive any services offered to the community by the study. All signed informed consent forms will be retained in the study files and a copy supplied to the Ethics Review Committee of AKU.

10.2 STUDY DISCONTINUATION AND CLOSURE

This study may be temporarily suspended or prematurely terminated if there is sufficient reasonable cause. Written notification, documenting the reason for study suspension or termination, will be provided by the suspending or terminating party to study participants, the Investigator, the CIHR, and regulatory authorities. If the study is prematurely terminated or suspended, the PI will promptly inform study participants and the REB and will provide the reason(s) for the termination or suspension. Study participants will be contacted, as applicable, and be informed of changes to the study visit schedule.

Circumstances that may warrant termination or suspension include, but are not limited to:

- Determination of unexpected, significant, or unacceptable risk to participants
- Insufficient compliance to protocol requirements
- Data that is not sufficiently complete and/or evaluable

Study may resume once concerns about safety, protocol compliance, and data quality are addressed, and satisfy the PI and REB

10.3 CONFIDENTIALITY AND PRIVACY

Confidentiality of all the data collected from the population will be guaranteed through existing systems of data storage and de-identified of forms. Participant privacy and confidentiality in electronic and printed data, publications, and reports during and following completion of the study will be fully ensured. The field site offices and central storage facilities at AKU are fully secure with electronic access and monitoring around the clock.

Participant confidentiality and privacy is strictly held in trust by the participating Investigators and their staff. This confidentiality is extended to cover testing of biological samples and genetic tests in addition to the clinical information relating to participants. Therefore, the study protocol, documentation, data, and all other information generated will be held in strict confidence. Any research information obtained about the patient in this study will be kept confidential. A patient will not be identified by name, only by unique study ID number. The patient's name or any identifying information will not appear in any reports published as a result of this study.

All research activities will be conducted in as private a setting as possible.

The study auditor, authorized representatives of CIHR, and representatives of the Research Ethics Board (REB), may inspect all documents and records required to be maintained by the Investigator, including but not limited to, medical records for the participants in this study. The clinical study site will permit access to such records.

Study participant research data, for the purposes of statistical analysis and scientific reporting, will be transmitted to and stored at SickKids. This will not include the participant's contact or identifying information. Rather, individual participants and their research data will be identified by a unique study identification number. The study data entry and study management systems used by clinical sites and by SickKids research staff will be secured and password protected. At the end of the study, all study databases will be de-identified and archived at SickKids.

10.4 QUALITY ASSURANCE AND QUALITY CONTROL

To ensure proper implementation the field, supervisors will make spot checks and will arrange refresher group sessions for the study personnel, in which the problems encountered will be discussed and resolved. In addition, the data collection activity will be further monitored by field supervisors who will perform a check on a subset (5%) of households. These quality checks would occur at least once per month for LHWs and research staff. Similarly, the quality of all laboratory procedures will be assessed by periodically rechecking results and ensuring the standardization of procedures. The Micronutrient Research Laboratories at AKU has a quality assurance system in place in collaboration with the biochemistry laboratories of icddr,b and with the Q-Rad system in UK.

10.4.1 DATA HANDLING AND RECORD KEEPING

Each participating site will maintain appropriate medical and research records for this trial, in compliance with ICH GCP and regulatory and institutional requirements for the protection of confidentiality of participants.

10.4.1.1 DATA COLLECTION AND MANAGEMENT RESPONSIBILITIES

Data collection is the responsibility of the clinical trial staff at the site under the supervision of the site Investigator. The Investigator is responsible for ensuring the accuracy, completeness, legibility, and timeliness of the data reported. All source documents should be completed in a neat, legible manner to ensure accurate interpretation of data.

Data collection tablets are collected from the study personnel on a daily basis so that the questionnaire data can be uploaded to the AKU data management unit. The tablets include built in logic and range checks to ensure data quality. Paper-based questionnaire forms will be visually checked by field site supervisors for completeness before being sent to the AKU data management unit on a weekly basis for entry into the database. Double data entry is used to reduce data entry errors and manual checking of frequencies during data cleaning (e.g., consistency, range, and internal validity checks) as applicable. All collected data is kept under lock and key and de-identified through the use of nine-digit participant identification codes. Data entered into the database is password protected.

10.4.1.2 STUDY RECORDS RETENTION

To enable evaluations and/or audits, the Principal Investigator agrees to keep records, including the identity of all participating patients (sufficient information to link records, CRFs and hospital records), all original signed informed consent forms, copies of all CRFs, source documents, and detailed records in a secure location for a minimum of 7 years in accordance with SickKids policy.

If the Principal Investigator relocates, retires, or for any reason withdraws from the study, then the study records must be transferred to an acceptable designee, such as another Investigator or another institution.

10.5 PROTOCOL DEVIATIONS

A protocol deviation is any noncompliance with the study protocol. The Principal Investigator will assure that no deviation from, or changes to the protocol will take place without documented approval from the Research Ethics Board (REB), except where necessary to eliminate an immediate hazard(s) to the trial participants. The noncompliance may be either on the part of the participant, the Investigator, or the study site staff. As a result of deviations, corrective actions are to be developed by the site and implemented promptly. All protocol deviations will be documented; the Principal Investigator will assess each protocol deviation to determine the impact to the patient's rights, safety or welfare, study efficacy and data integrity. Protocol deviations must be sent to the reviewing REB in accordance with their policies. The Principal Investigator is responsible for knowing and adhering to the reviewing REB requirements.

10.6 CONFLICT OF INTEREST

The research team declares no conflict of interest.

10.7 ABBREVIATIONS

AE	Adverse Event
AKU	Aga Khan University
ANCOVA	Analysis of Covariance
CIOMS	Council for International Organizations of Medical Sciences
CLIA	Clinical Laboratory Improvement Amendments
CONSORT	Consolidated Standards of Reporting Trials
CRF	Case Report Form
eCRF	Electronic Case Report Forms
GCP	Good Clinical Practice
ICH	International Council on Harmonisation
ICMJE	International Committee of Medical Journal Editors
LHW	Lady Health Worker
MOP	Manual of Procedures
MRP	Most Responsible Physician
MUAC	Mid Upper Arm Circumference
PI	Principal Investigator
QA	Quality Assurance
QC	Quality Control
REB	Research Ethics Board
SAE	Serious Adverse Event
SFT	Triceps Skinfold Thickness
SoA	Schedule of Activities
SOP	Standard Operating Procedure

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