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

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1 PROTOCOL SUMMARY

1.1 SYNOPSIS

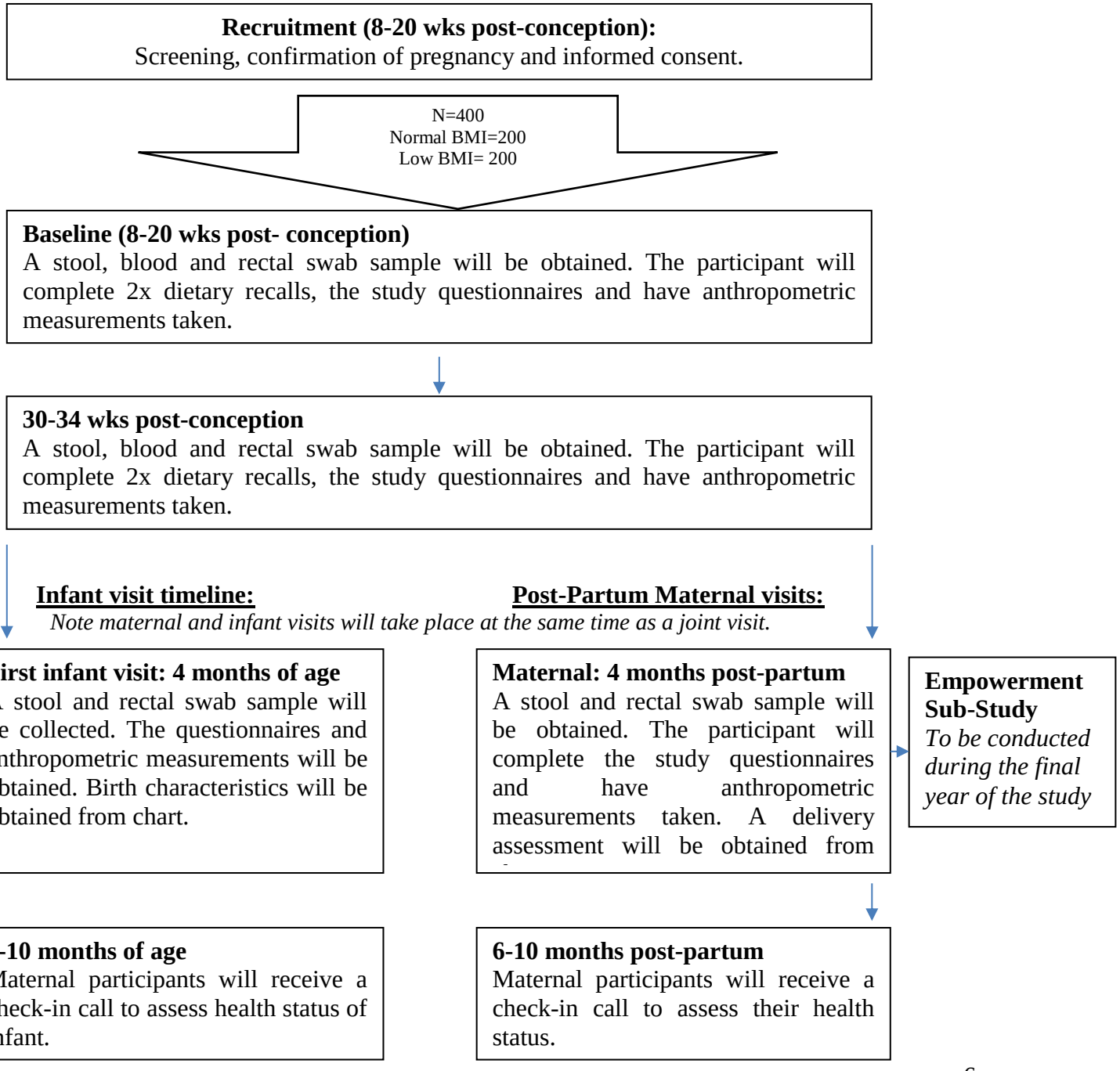
Title	Elucidating the Dynamics and Impact of the Gut Microbiome on Maternal Nutritional Status during Pregnancy.
Study Short Title	MMIP Study (Microbiome and Malnutrition in Pregnancy Study)
Principal Investigator	Dr. John Parkinson, PhD (Senior Scientist at the Hospital for Sick Children)
Co-Investigators	Dr. Jessie Hulst, MD, PhD (Associate Professor Department of Pediatrics and Nutritional Sciences at the University of Toronto; Staff Pediatrician in the Division of Gastroenterology, Hepatology and Nutrition at the Hospital for Sick Children) Dr. Shazeen Suleman, MD, Msc, MPH (Assistant Professor in the Department of Pediatrics at the University of Toronto, and an Associate Scientist at the Li Ka Shing Knowledge Institute) Dr. Liana Kaufman, MSc. MD (Department of Family and Community Medicine, St. Michael's Hospital) Dr. Rachel Spitzer, MD, MPH (Associate Staff, Department of Gynaecology at Sickkids and Assistant Professor, University of Toronto) Dr. Ashley Vandermorris, MD (Staff Physician, Department of Paediatrics at Sickkids Hospital) Dr. Nirmala Chandrasekaran, MD (Department of Obstetrics, St. Michael's Hospital) Dr. Zulfiqar Bhutta, PhD, MBBS, FRCPCH, FAAP (Co-Director of Research Centre for Global Child Health, Senior Scientist the Hospital for Sick Children) Dr. Robert Bandsma, MD, PhD (Pediatric Gastroenterologist, Hepatologist and Nutritionist, at the Hospital for Sick Children) Dr. Jo-Anna Baxter, PhD, MSc (Centre for Global Child Health, Hospital for Sick Children)
Collaborators	Linda Moscovitch, RM, Riverdale Community Midwives (Privileges at St. Michael's Hospital) Fariba Shodjaie, RM, Community Midwifes of Toronto (Privileges at St. Michael's Hospital) Tia Sarkar, RM, Riverdale Community Midwives (Privileges at St. Michael's Hospital) 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)
Research Coordinators	Gowshigga Thamotharampillai, (Molecular Medicine at Hospital for Sick Children and Department of Pediatrics, Unity Health Toronto, St. Michael's Hospital) Bronwyn Barker, (Department of Pediatrics, Unity Health Toronto, St. Michael's Hospital)
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, nutritional status and morbidity 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 and morbidity, 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.
Follow-up Periods	Baseline/Initial Recruitment: 8-20 weeks post conception 2nd time point: 30-34 weeks post conception 3rd time point: 4 month post-partum 4th and final time point: 12 months post-partum

Study Population:	Pregnant women 28 years and younger living in the Toronto and Greater Toronto Area and their infants.
No. of Subjects	**Total: 800 participants:** 400 women and their infants. • 200 maternal subjects with normal BMI (20-24.9) • 200 maternal subjects with low BMI (less than 20)
Study Duration:	The recruitment target should be met within four years. The study duration should be approximately 5 years.
Participant Duration:	• Participants will be in the study for approximately 17-19 months. • Participants will join the study 8-20 weeks post-conception and end their participation in the study at 12 months post-partum. • The infants will be followed for 12 months, from their birth until 12 months of age.
Collaborating Institutions:	The Hospital for Sick Children St. Michael's Hospital University of Toronto St. Michael's Midwifery Division Aga Khan University Dalhousie University University of Calgary University of Alberta The NIH

1.2 SCHEMA

Figure 1: Study Flow Diagram



Final visit: 12 months of age
A stool, blood and rectal swab sample will be collected. The questionnaires and anthropometric measurements will be obtained.

Final visit: 12 months post-partum
A stool, blood and rectal swab sample will be obtained. The participant will complete 2x dietary recalls, the study questionnaires and have anthropometric measurements taken.

Workshops Development and Implementation
To be conducted during the final year of the study

(SOA)

ctivities,

Procedures	Prior to Initial Base Visit at ~10-16 We Post-Conception	Enrollment/Basel Visit 1: 8-20 Wee Post-Conceptio	Study Visit 2: 30-34 Weeks Post-Conception	Study Visit 3: 4 Months Post-Pa	Infant Study Visit Months (*same visi mom*)	Maternal Check in 6-10 Months Pos Partum	Infant Check in Ca 10 Months of Ag	Maternal Study Vis 12-Months Post-Pa	Infant Study Visit 4: 12- Months (*same visi mom*)
Invitation Email and/ or Letter	X								
Screening for Eligibility and Pregnancy Confirmation		X							
Informed Consent		X							
Baseline demographics		X							
Dietary Recall x 2 (ASA24®)		X	X					X	
MDD-W *Note: this will be administered to the participant. For the other time points the research staff will calculate the MDD-W from the dietary recall data*				X					
Stool Sample		X	X	X	X			X	X
Blood Sample		X	X					X	X
Rectal Swab		X	X	X	X			X	X
Anthropometric Measurements		X	X	X	X			X	X
Empowerment Questionnaire		X		X					
Empowerment Sub-Study Interview *This will be for a subset of participants during the final year of the study.*				X					
Maternal Health Assessment		X	X	X		X		X	
Maternal Birth History and Post-Partum Questionnaire				X					
Birth Assessment					X				
Infant Feeding Assessment + Minimum Dietary Diversity Score					X				X

Procedures	Prior to Initial Baseline Visit at ~10-16 Weeks Post-Conception	Enrollment/Baseline Visit 1: 8-20 Weeks Post-Conception	Study Visit 2: 30-34 Weeks Post-Conception	Study Visit 3: 4 Months Post-Partum	Infant Study Visit 3: 4 Months (*same visit as mom*)	Maternal Check in Call: 6-10 Months Post-Partum	Infant Check in Call: 6-10 Months of Age	Maternal Study Visit 4: 12-Months Post-Partum	Infant Study Visit 4: 12-Months (*same visit as mom*)
Infant Health Assessment					X		X		X
NutricheQ questionnaire and food insecurity assessment									X
Study Exit								X	X

**Blue columns: women; grey columns: infant*

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	Recruitment visit	To obtain informed consent	Research Staff
ICF-2	Informed consent documentation	Recruitment visit	To document informed consent discussion	Research Staff
Invitation/ Instructions for Participants (I)				
I-1	Invitation email	Prior to the initial visit, an email or letter will go out to the potential participant from the clinical team.	The email will briefly introduce the study, and will contain the contact information for the study team.	N/A- for participant's information
I-2	Invitation letter	Prior to the initial visit, a letter or email will go out to the potential participant from the clinical team.	The email will briefly introduce the study, and will contain the contact information for the study team.	N/A- for participant's information

I-3	Instructions for stool collection	1)Baseline visit post-conception 2)30-34 weeks post-conception 3) 4 months post-partum 4)12-months post-partum	The instructions will be given to each participant prior to stool collection, to advise them on how to properly collect stool and how to return to staff.	N/A- for participant's information
I-4	Instructions for rectal swab collection	1)Baseline visit post-conception 2)30-34 weeks post-conception 3) 4 months post-partum 4)12-months post-partum	The instructions will be given to each participant prior to rectal swab collection, to advise them on how to properly do so.	N/A- for participant's information
Maternal study visit forms (M)				
M-1	Baseline eligibility screening form	Recruitment visit: baseline 8-20 weeks post-conception	To confirm eligibility	Research Staff
M-2	Pregnancy confirmation and clinical assessment	Baseline post-conception visit	To confirm pregnancy	Research Staff
M-3	Baseline visit form	Baseline post-conception visit	To obtain baseline participant information. To record maternal anthropometric measurements. To assess maternal health through questions regarding medication use, health seeking behavior etc	Research Staff

M-4	Empowerment questionnaire	1)Baseline post-conception visit 2)4 months post-partum visit	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, including food and housing insecurity, and demographic data.	Research Staff
M-5	Post ASA24® survey	Baseline post-conception visit	This will be an optional survey for participants to complete after the second ASA24® recall for the baseline visit. The questionnaire will assess their experience with the ASA24® .	Research Staff
M-6	Dietary diversity questionnaire	4 months post-partum visit	Participants will complete a dietary diversity score at the 3rd visit .	Research Staff
M-7	Maternal study visit form	1)30-34 weeks post-conception 2) 4-months post-partum 3)12-months post-partum	To record maternal anthropometric measurements. To assess maternal health through questions regarding medication use, health seeking behavior etc.	Research Staff
M-8	Maternal and infant check-in call form	6-10 month post-delivery check-in call	To assess maternal and infant morbidity through questions regarding medication use, health seeking behavior etc.	Research Staff

M-9	Study Completion/ withdrawal form	The 12 month post-partum visit or earlier as required.	To document end of study participation.	Research Staff
M-10	The Edinburgh Postnatal Depression Scale (EPDS)	This questionnaire is often administered clinically; in the event that it cannot be obtained from chart review it will be administered at the 3 month post partum visit.	Screening questions that can indicate whether the maternal participant has symptoms that are common in women with depression and anxiety during pregnancy and in the year following the birth of a child	
Maternal Chart Review Forms (CM)				
CM-1	Chart review maternal baseline visit	Chart review for the baseline post-conception visit	To obtain baseline participant information. To assess maternal health through questions regarding medication use, health seeking behavior etc	Research Staff
CM-2	Chart review maternal second visit	Chart review for 30-34 week post-conception visit	To assess maternal health through questions regarding medication use, health seeking behavior etc	Research Staff
CM-3	Chart review birth and labor form	Chart review of birth history	To gather information about the birth and labor history	Research Staff
CM-4	Chart review maternal post partum visits	Chart review for the 4 and 12 month post-partum visit	To assess maternal health through questions regarding medication use, health seeking	Research Staff

			behavior etc	
Infant study visit forms (IN)				
IN-1	Infant study visit 1	4 months of age	To assess the amount and mode of feeding, (ie, mother's milk or formula; complementary feeding). To record infant anthropometric measurements. To assess infant health through questions regarding medication use, health seeking behavior etc.	Research Staff
IN-2	Infant study visit 2	12-months of age	To assess infant health through questions regarding medication use, health seeking behavior etc. To assess the infants diet, with a focus on markers of inadequate or excessive intake and dietary imbalances[1], minimum dietary diversity and to assess the annual food insecurity. To record infant anthropometric measurements.	Research Staff

Ages and Stages Questionnaires (ASQ)	Infant study visit 1 and 2 Infant follow up phone call ~6 months of age	3 months of age 12 months of age.	Developmental screening tool that assesses developmental progress for the infant participants.	Research Staff
Nipissing Questionnaire	Infant study visit 1 and 2 Infant follow up phone call ~6 months of age	This questionnaire is often administered clinically; in the event that it cannot be obtained from chart review it will be administered at 3, 6 and 12 month visit. If the questionnaire is administered clinically throughout the first year of the infant participants life it will be collected through chart review.	A checklist used to monitor infant development.	Research Staff
Infant chart review form (CIM)				
CIM-1	Chart review infant visits	1) 4 months of age 2) 12 months of age	To assess infant morbidity through questions regarding medication use, health seeking behavior etc	Research staff
Adverse Event (AE)				
AE	Adverse event form	As needed.	To document adverse events.	Research staff

2 INTRODUCTION

Nutritional status during pregnancy plays an important role in maternal health and birth outcomes[2, 3]. 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[4-10]. 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 Toronto cohort of the study, which will focus on refugee and young adult obstetric clinics in Toronto, a population of specific relevance to undernutrition[11]. This cohort is expected to yield insights into the influence of eukaryotic microbes that are often viewed as asymptomatic. To better define our target demographic, we focus on young mothers, 28 years of age and younger, in the Toronto and Greater Toronto Area. We will focus on this younger demographic due to our lack of knowledge on the microbiome of young women, and their vulnerability to undernutrition[11]. A second complementary cohort will be based in the Matiari district of Pakistan. 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[12]. 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[13] and can result in complications that impact maternal morbidity and mortality[14-18]. 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)[19]. Undernutrition prior to or during pregnancy may also have long term impact for the offspring (developmental origins of health and disease hypothesis)[20-22]. 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[23]. 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[24]. **At significant risk are young women (18-28 years of age), the target demographic of this study.**

The dietary requirements of young women are as high or higher than older age groups[25] and adequate nutrition at this key stage of growth is essential as up to 50% of adult weight is gained from the ages of 15-19 years. Undernutrition in this demographic has considerable consequences, with an increased risk of mortality from pregnancy and childbirth and a higher likelihood of LBW infants[13, 26-28]. While the health of young women has traditionally been neglected, they are recognized as a critical target for strategic interventions, as global health initiatives seek to break the intergenerational cycle of undernutrition and poor health[29-31], **A key challenge is understanding the factors that predispose young women to nutritional deficits (undernutrition) and identifying interventions that improve nutritional status during pregnancy.**

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[13, 28]. 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[32]. Pre-existing micronutrient deficiencies may be exacerbated during pregnancy as a result of the increased metabolic requirements[18]. 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[12].

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 [12]. Nutrition and growth in adolescence is particularly important to 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[13]. 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[12]. 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[33]. Specifically, adolescent pregnancy is associated with a 50% increased risk of stillbirths and neonatal deaths, and increased risk of preterm birth, LBW, and asphyxia [14, 15, 34]. The prevalence of anemia is suggested to be as high in adolescents as women 20–28 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[35], our understanding on the interactions relating host genetics with the gut microbiome in the context of undernutrition remains limited[36, 37]. Indeed, twin based studies have identified few genetic factors that predispose to undernutrition[5]. Furthermore, undernutrition is not simply a consequence of food insecurity resulting in macro- and micronutrient[12, 38-40]. Instead, the intestinal microbiome has emerged as a key factor defining nutritional status, with impaired maturation driving undernutrition[4-10]. An immature microbiome may impact the extraction of critical nutrients such as vitamins and short chain fatty acids (SCFAs)[41-45], and/or result in the production of metabolites that inhibit metabolism or increase host cell turnover[5]. During pregnancy, dramatic changes in gut microbiota occur, with a decrease in individual (alpha) diversity but an increase in population (beta) diversity[46]. 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[47], raising interest in dietary supplements to improve pregnancy outcomes[48]. **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**.

2.2.1 GENDER IMPACTS NUTRITIONAL STATUS AND THE MICROBIOME

It is increasingly apparent that biological sex impacts the gut microbiome, beginning in infancy[49]. 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[50-52]. 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[53]. **Addressing gender roles, specifically with respect to maternal empowerment, has the capacity to directly impact intestinal health in infants.**

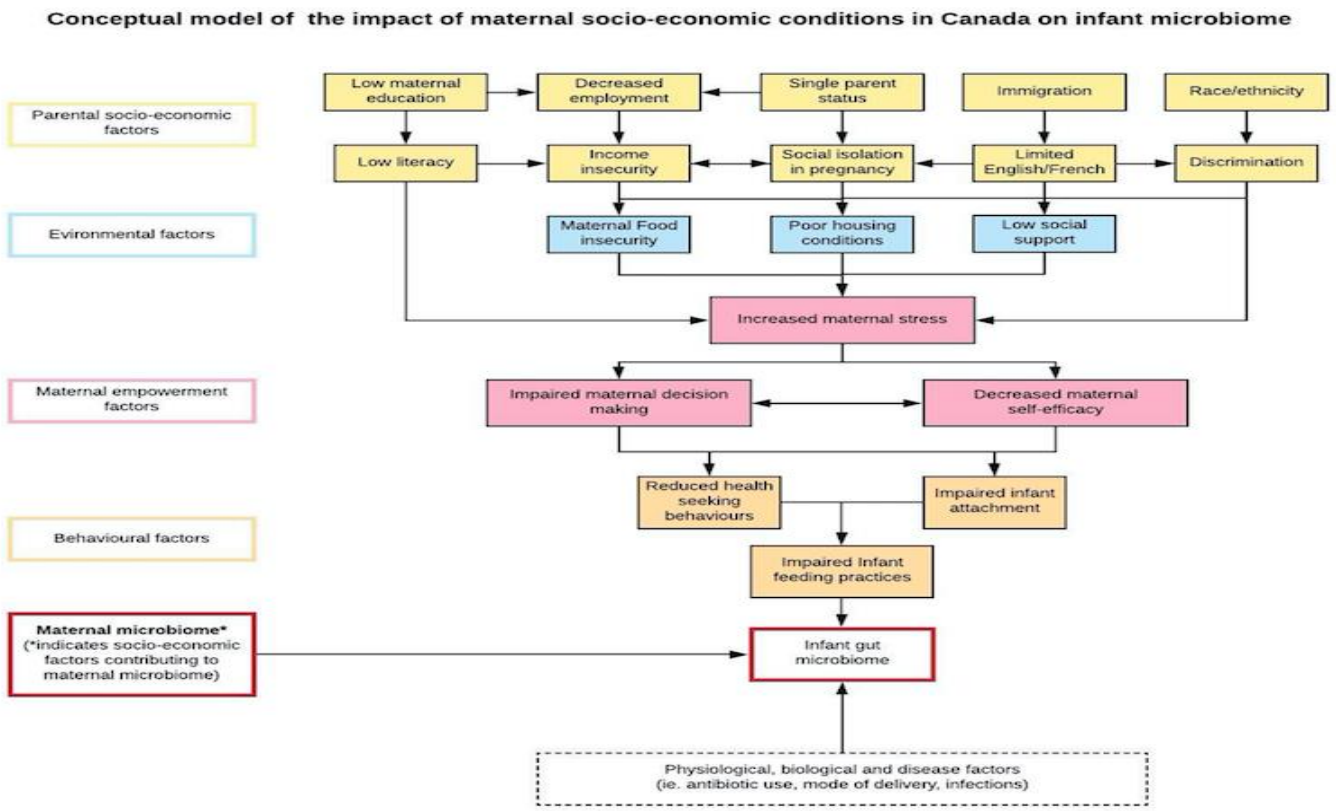


Figure 2: Conceptual Model of Maternal Socio-Economic Conditions Impact on the Infant Microbiome (Adapted from Herd *et al,* 2018)[54]

2.2.1 THE TORONTO POPULATION

This study will target pregnant women aged 28 years and younger, living in Toronto. Some of our potential participants are refugees and newcomers to Canada. In previous studies conducted

at St. Michael's Hospital, one of our participating sites, it was found that refugee women experienced violence, homelessness, poor health and nutrition during pregnancy [55]. This study also found that refugee women were more likely compared to non-refugee controls to give birth to low birth weight infants. A scoping review examining maternal health in immigrant and refugee women in Canada, found that immigrant women gained less weight during pregnancy compared to Canadian born women. In addition, this immigrant population was less likely to gain the recommended weight advised by Health Canada during pregnancy [56]. Differences in maternal morbidity was also apparent, with immigrants from Sub-Saharan African at a higher risk for severe maternal morbidity, with common diagnoses including uterine rupture and eclampsia [56]. T**ogether, these findings further highlight the need to examine factors that potentially impact maternal weight gain during pregnancy, and the impact on birth outcomes, a key outcome of this study.**

2.2.2 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[57, 58]. 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[59-61]. EED is thought to be triggered by dysbiosis (where beneficial microbes are replaced by pathobionts with a loss of diversity[62, 63]), 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[64], 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[7]. **While maternal EED during pregnancy has been shown to adversely impact birth outcomes[65], our understanding of the impact of enteropathogens and dysbiosis on maternal gut community dynamics and nutritional status is limited.**

2.2.3 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[66-69]. However not all parasitic infections cause disease; a study of Toronto daycares revealed a ~5% incidence of largely asymptomatic *Giardia* infections[70]. Thus, disease is a consequence of the complex interplay between the eukaryotic and bacterial microbiome and the host immune system[71, 72]. For example, *Prevotella copri*, is increased in patients with diarrheagenic *E. Histolytica* infections[69], while indole-producing bacteria such as *E. coli* CFT073 protect

against *Cryptosporidium*[73]. Conversely, eukaryotic microbes may have neutral or even beneficial roles; *Blastocystis*, for example, has been associated with increased microbiome diversity[74, 75]. 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.**

2.2.4 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[76-78]. 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)[79]. **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.

2.2.5 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[80-89]. For example, Dr. Parkinson used metatranscriptomics to reveal how changes in fat absorption in mice impact metabolic pathway expression in the gut microbiome[90]. 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[91]. 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.**

2.3 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 and taking a rectal swab, detailed instructions and research staff will be available to provide support. If participants are uncomfortable taking a rectal swab, they will have the option to opt out of this portion of the study and to still participate. However, the stool samples are an essential component of the study, as such it is a required component of participation. Detailed instructions will be provided for both the stool and rectal swabs and research staff members will be available to answer any questions.

Participants may also experience psychological and emotional distress when completing questionnaires and/ or answering questions about their socio-economic status, their health, and 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 leave questions blank if they are uncomfortable, and research staff members will be on hand if a participant experience's physiological and/ or emotional distress during a study visit/ study procedures. If this occurs the study staff members will bring in members of the clinical team to assist as necessary.

Participants will also have the option to consent to future genetic testing of their blood samples. Genetic information can never be full 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. 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) are associated with maternal gestational weight gain.	The primary endpoint will be maternal gestational weight gain (GWG) during pregnancy, measured between the first (8-20 weeks post-conception) and second time point (30-34 weeks post conception). 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.	Weight gain is a key predictor of adverse birth outcomes[92, 93].
To determine the association between maternal microbiome dysbiosis during pregnancy and birth outcomes, infant growth, nutritional status and morbidity in the first year of life.	The specific birth outcomes evaluated will include preterm birth, birth weight, SGA, LGA, head circumference at birth, and 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, APGAR score, and antibiotic usage. The infant's health status and morbidity 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 4-months and 12-months. To monitor microbiome dynamics,	This will allow for an examination of the association between the maternal microbiome and birth outcomes.

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
	16S and 18S rDNA surveys will be applied to the maternal stool samples to monitor the bacterial and eukaryotic components of the microbiome.	
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. To better relate to individual nutrient intake, recalls will be implemented on two separate days (separated by 3-10 days) for each time point. Food insecurity will be assessed through the use of a food insecurity questionnaire administered at time of recruitment and 4 months post-partum. The quality of the diet will be assessed through dietary diversity scores.	Quantitative measures of nutritional status will provide parameters to better inform microbiome analyses.
To integrate maternal anthropometric factors and morbidity, 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 blood samples will be collected and analyzed for microbiome composition, micronutrients and 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

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
		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 surveys will be conducted on the stool samples.	These markers will reflect gut health and pathogen challenge.
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 4-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.

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
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 because of impaired nutrient absorption, leading to corresponding negative birth outcomes.

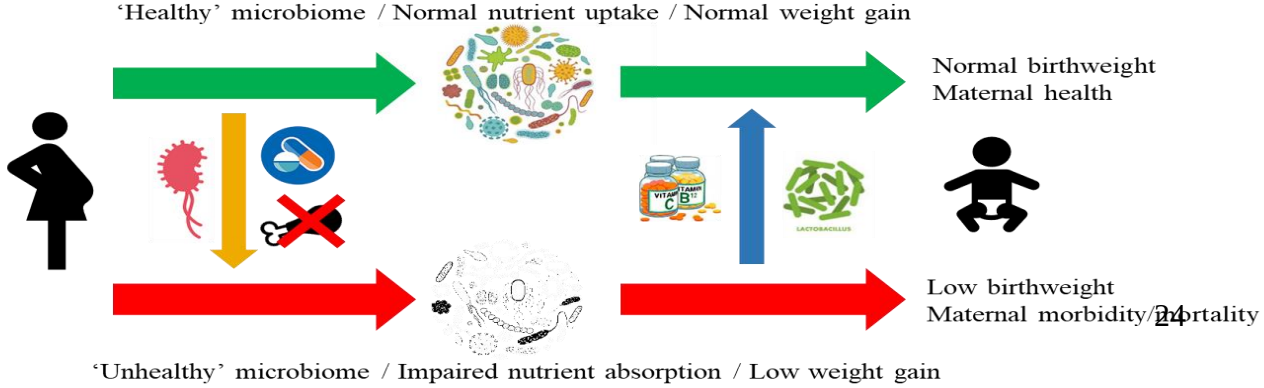


Figure 3: Project Hypothesis. During the course of pregnancy a shift to an 'unhealthy' microbiome, perhaps triggered by exposure to pathogens, antibiotics or poor diet, results in impaired nutrient absorption, low weight gain and poor birth outcomes.

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, 28 years of age and younger in the Toronto and Greater Toronto Area. The study will aim to recruit 400 women into two groups based on BMI at time of recruitment (Normal BMI will be defined as between 20 and 24.9 kg/m^2 and Low BMI will be defined as less than 20 kg/m^2). With a goal of having 200 participants within the normal BMI group and 200 participants within the low BMI group. Although this our recruitment aim, in the event that we are unable to recruit 200 women with a low BMI, we will recruit more women that fall within the normal BMI range. 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 PARTICIPATING SITES

For this study, we will recruit from the St. Michael's Hospital Family Medicine Obstetrics Department, the St. Michael's Hospital Obstetrics and Gynecology Department and St. Michael's Hospital Midwifery Clinics. The Department of Family and Community Medicine at St. Michael's Hospital, provides care to over 45,000 individuals annually, including prenatal care, with a focus on underserved inner-city populations. We expect to recruit around 75 women per year from this population. We also have a partnership with Dr. Rachel Spitzer, the Medical Director of the Young Prenatal Program (YPP) at SickKids and Dr. Ashley Vandermorris from the SickKids Young Families Clinic, we will be recruiting participants from the YPP. From the YPP we expect to recruit around 15-20 women per year. We therefore expect to meet our recruitment target within the first four years of the project.

See the figure below for the anticipated participant numbers at each site, and participant's potential paths through the health care system from pre-pregnancy to a year after child birth.

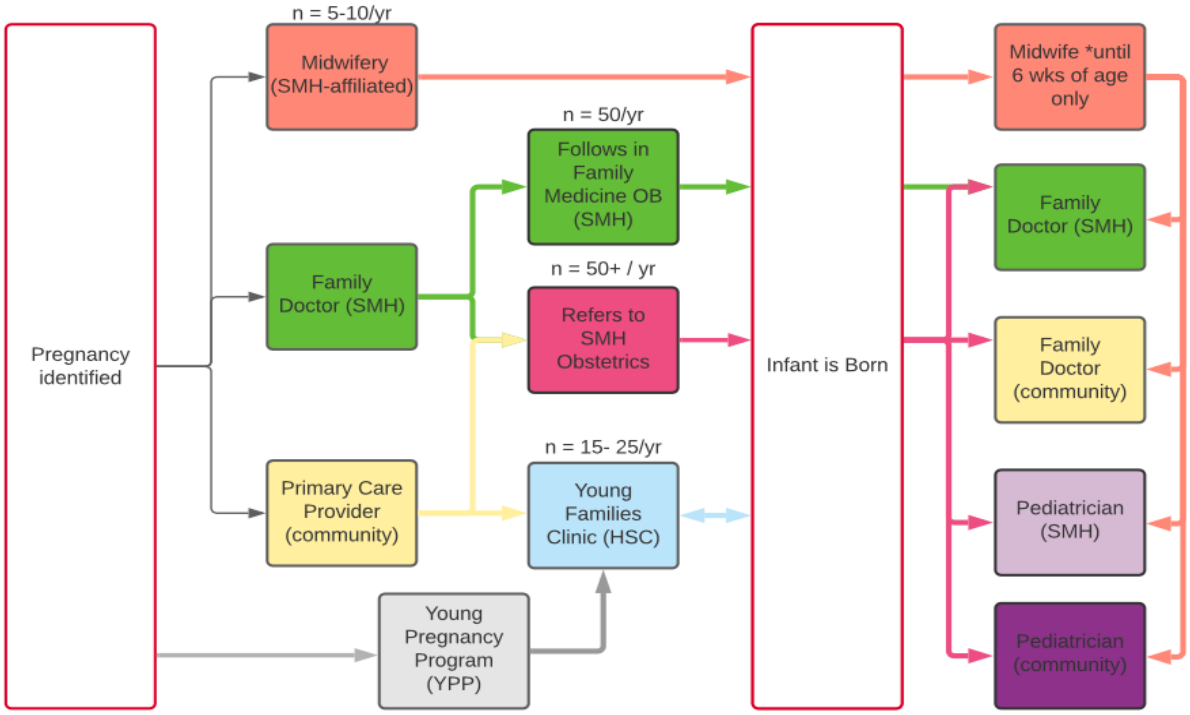


Figure 4: Pathways to Care for Pregnant Women and Their Infants in the Pregnancy and Microbiome Study

4.4 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 who completes all study visits will be approximately 17-19 months in duration, from 8-20 weeks into their pregnancy, until around one year post-partum.

It is estimated that it will take 4 years for the study to reach its recruitment target, and approximately 5.5 years from when the study opens to the final participant study visit.

5 STUDY POPULATION

The study population will consist of pregnant women, 28 years of age and younger, living in the Toronto and Greater Toronto Area. We will focus on younger women, due to our lack of knowledge about their microbiome and their vulnerability to undernutrition[11]. Many of the participants in our target population do not speak English; the main languages we anticipate are: Spanish, Arabic, Cantonese, Bengali, Tamil and French. During our study visits/ check-in call we will have a translator present, either, in person or over the phone. In addition, we will also have our study consent documents translated into the six languages indicated above. Once our initial consent form is approved by REB, we will have the consent forms translated by a certified translator. The translated versions will be back-translated into English and compared

with the original to ensure accuracy. These documents will then be submitted to the REB as an amendment and will include the translator's seal/attestation/certificate with the translations.

In addition, we anticipate that many individuals in our target population may have low literacy rates. Our study forms and documents will be written in easy to understand language, and we will have study staff members available to assist study participants with any questions they may have while filling out the forms.

5.1 INCLUSION CRITERIA

In order to be eligible to participate in this study, an individual must meet all of the following criteria:

1. Consent provided
2. Participant is between 8-20 weeks post-conception
3. Female aged 28 years of age and younger
4. Confirmation of pregnancy
5. 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:

1. Women who do not meet the enrollment age criteria
2. Women who are 20 + weeks post-conception
3. Women who have taken antibiotics within the past 3 months

Note: it is common practice to give the mother penicillin in perinatal period if they are GBS positive; because this is standardized across the board it would not act as an exclusion factor.

5.3 STRATEGIES FOR RECRUITMENT AND RETENTION

5.3.1 RECRUITMENT AND OBTAINING CONSENT

Recruitment may take place in person, or virtually via Microsoft Teams, Zoom Healthcare or if preferred by phone. Our study will employ recruitment posters in the waiting rooms of participating clinics at SickKids, St. Michael's Hospital and at participating midwifery clinics. In addition, prior to the clinic visit, participants will be sent an email or a letter introducing them to the study. In the event that a participant is not sent the recruitment letter/ email ahead of their clinic visit, they can still be approached by a member of the study team, as long as they agree to be approached.

In person Recruitment:

When the potential participant arrives for their clinic visit, someone within the participant's circle of care will ask the participant if the research team can approach them about the study. If the potential participant agrees, a member of the research team will approach the patient to explain the study and screen the participant for eligibility. If the participant is eligible and agrees to participate, the research team member will obtain informed consent. As part of the eligibility screening, the research staff member will obtain a confirmation of pregnancy and the participants BMI (based on their height and weight) from the clinical team and/ or the participant's chart.

Remote Recruitment:

When the participant arrives for their clinic visit (either remote or in-person), the clinical team will ask the participant if they can be approached by someone from the research team. If the participant agrees, their contact information will be sent to the research team. The research team will reach out to the participant to set up a time for the video call by Zoom Healthcare or Microsoft Teams, or if the participant prefers, the research member will conduct the visit over the phone. During the remote recruitment visit the research member will confirm the identity of the potential participant and first briefly explain the study, if the participant agrees the research member will then confirm participant eligibility and go through the consent document with the participant. No audio or video recording will be done. If the participant is eligible and agrees to participate, the participant will be able to either send in their signed consent document via email or regular mail.

The consent document will be written in simplified, easy-to-understand language to ensure participant understanding. Many of our potential participants have low literacy rates and/ or will not speak English. The main languages we anticipate our participants to speak are: Spanish, Arabic, Cantonese, Bengali, Tamil and French. If a participant does not speak English, a translator will be present either in person or via a phone call translation service. In addition, the consent documents will be translated into the six main languages encountered. Participants will sign the consent document in English as well as the translated version. The participant will be provided with a signed copy of both the English and translated version for their records.

In a systematic review conducted to investigate potential tools to enhance strategies for comprehension in the informed consent process, it was found that extended discussion with participants had the most significant impact on a participant's understanding[94]. To ensure informed consent and participant understanding, the research team member obtaining informed consent will engage in extended verbal discussion of the study procedures and the informed consent form. The participants understanding will be probed and documented, by having the participant answering the following questions in their own words:

- What is the purpose of the study?
- What samples will we be collecting during the study?
- How long is your participation in the study?

The participant's responses to these questions, as well as any questions the participant raises, will be documented. If an interpreter is present for the informed consent discussion, the research member will document their presence as well as their name on the informed consent documents.

The informed consent form will contain three additional sections for the participant to either provide consent for or to opt out of.

1. **Empowerment sub-study**, the participant will be asked to indicate whether or not they consent to be approached/ to participate in an empowerment sub-study. A subset of participants (around 20-30) who consent to this additional sub-study will be selected to participate using non-random purposive sampling (*more information on this sub-study can be found in section 8).* The empowerment sub-study will run during the final year of the study, only participants involved in the final year of the study will be approached for the sub-study. This section will be marked as 'non-applicable' for participants recruited in previous years.
2. **Workshops,** a final section of the consent form will ask for participants consent to be approached for future workshops that will be designed to provide knowledge translation on some of the key findings of the study (*more information on the workshops can be found in section 9).*
3. **Genetic testing:** Participants will also have the option to sign an additional section of the consent form for future genetic testing of their blood samples. With an additional option for their infant's blood samples. Participants will have the option to opt out of this future testing for themselves and their infant. If they sign this section of the consent form, the samples will be stored to further dissect patterns of microbiome genotype associated traits. (*More information about genetic testing can be found in section 12.4.1)*

The study staff will keep a record of all the study recruitment attempts, including participants who decline. The study recruitment file will be stored on the St. Michael's shared drive for St. Michael's participants, and on Onedrive for SickKids participants in a password protected excel file.

5.4 STUDY HONOURARIUM

As a token of appreciation for the time and effort spent on the study, participants will receive a grocery gift card after each study visit. The grocery gift cards will be given either as physical gift cards or as electronic gift cards (sent via email); this will be dependent on the participant's preference. Participants will receive $20 for the first and final study visits, as these visits will be longer in duration. For the 2 visits in between, participants will receive $10 grocery gift cards.

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 *Form M-9 Study Completion/ Withdrawal and Exit Form*. 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 a participant fails to return to the clinic for a required study visit:

- The site 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, 3 contact attempts, through telephone calls, emails and a voicemail. These contact attempts will 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

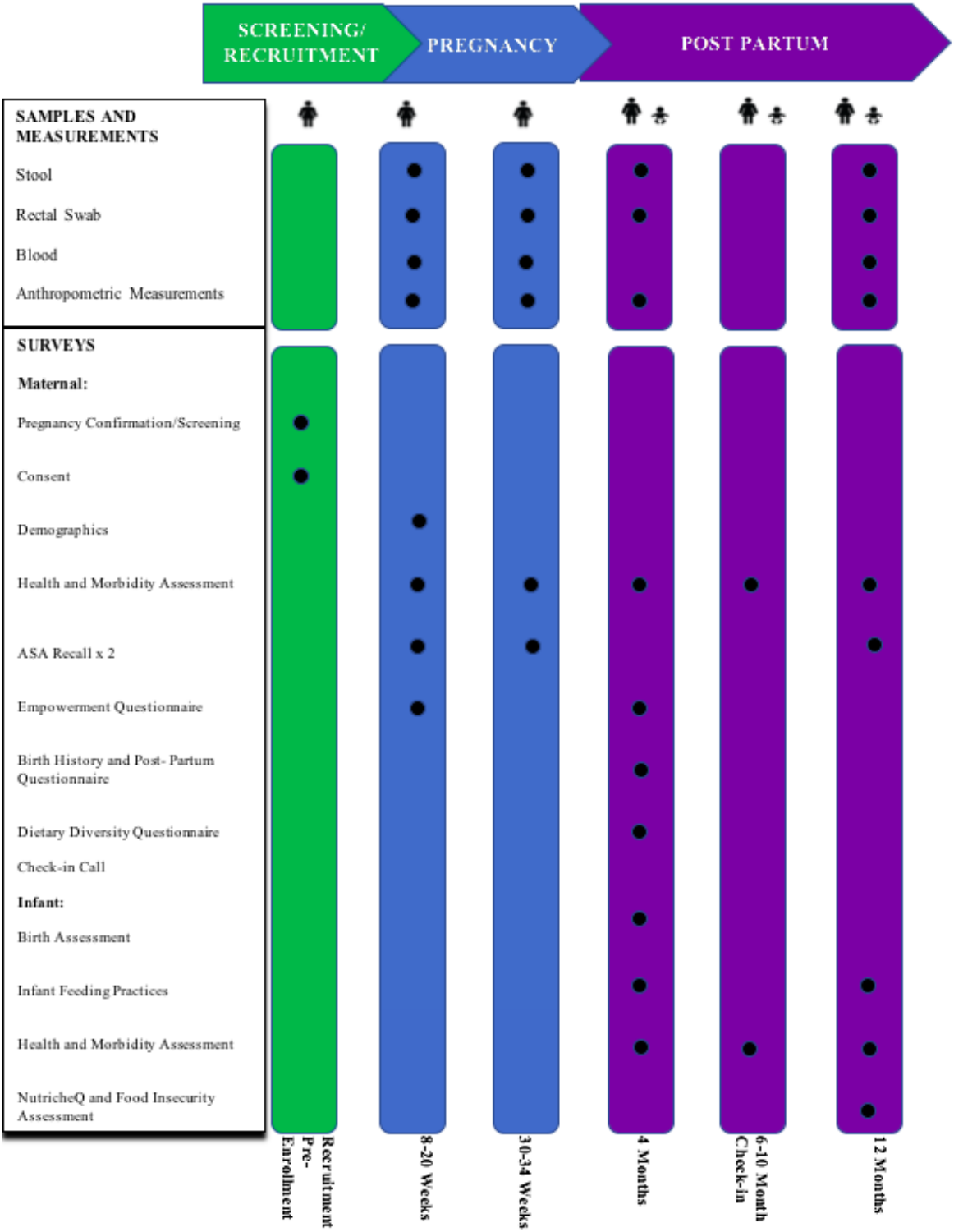


Figure 5: Overview of Study Visits Including Sample Collection, Measurements and Surveys

7.1 STUDY VISITS

Study participants will be involved in four main study visits. We aim to conduct these four visits at: 8-20 weeks post conception, 30-34 weeks post-conception, 4 months post-partum and 12 months post-partum. However, in the event that participants miss their study visits, we will still conduct visits and collect participant samples/data outside of these windows. We will also have a check-in call that will fall between 6-10 months post-partum.

7.1.1.1 BASELINE ASSESSMENT: 8- 20 WEEKS POSTCONCEPTION

A member of the clinical team will ask the participant if they can be approached for a research study. If participants are interested, the research staff member will approach the participant to explain the study, screen for eligibility/ confirm pregnancy and obtain informed consent (see section 5.3 for more information). Following consent, research staff will administer the baseline enrolment form, the pregnancy confirmation form, the first ASA24® dietary recall and the empowerment questionnaire. The research staff and/ or the healthcare staff will obtain anthropometric measurements, a blood sample and give participants the stool collection kit. The participant will collect a rectal swab during this visit after detailed instructions and will return it to the research staff.

Study staff will follow-up with participants over the phone 3-10 days after this baseline visit to administer the second ASA24® dietary recall. After completing this recall, the research staff member will ask the participant if they are willing to answer some questions about their experience with the ASA24® . The questions will revolve around the participant's experience with the ASA24® ; were they able to find all of the food items they looked for, how long it took, was the recall easy/ difficult to fill out etc. The survey questions will be pilot tested with a convenience sample, and based on participant responses to the questionnaire, we may revise the questions, and submit the revised questions as an amendment. Participants will have the option to decline answering these questions.

The mother's chart will be reviewed for pregnancy details and a health and medication assessment using the chart review form *CM-1 Chart Review Maternal Baseline Visit.* The purpose of the chart review is to minimize participant burden during the visit in order to keep the visit as short and convenient for the participant as possible.

7.1.1.2 STUDY VISIT 2: 30-34 WEEKS POSTCONCEPTION

During this visit, research staff will administer the first ASA24® dietary recall and the study visit form. The research staff and/or the health care staff will obtain anthropometric measurements, a blood sample and give participants the stool collection kit. The participant will collect a rectal swab during this visit and will return it to the research staff. Study staff will follow-up over the phone 3-10 days after this visit to administer the second ASA24® dietary recall for this time point.

The mother's chart will be reviewed for pregnancy details and a health and medication assessment using the chart review form *CM-2 Chart Review Maternal 2nd visit.*

7.1.1.3 STUDY VISIT 3: 4 MONTHS POST PARTUM

This will be the third maternal time point and the first infant time point. The research staff will aim to conduct the visit at around 4 months post-partum, however this time point will be flexible to allow participants to come in at a time convenient to them that aligns with a clinic visit i.e. "well-baby visit". This visit will be conducted within 6 wks-4 months post-partum. The research staff will administer the maternal study visit form and the infant study visit form, the empowerment questionnaire, and the dietary diversity questionnaire. The research staff and/ or the health care staff will obtain anthropometric measurements from the infant and mother. If the infant arrives in a freshly soiled diaper and/ or they soil their diaper during the visit, then stool will be taken from the freshly soiled diaper. However, if the infant does not have a freshly soiled diaper, a rectal swab will be taken and the participant will be sent home with a stool collection kit. The mother will also take a rectal swab and will be sent home with a stool collection kit.

Note at this visit, participants will not provide a blood sample or complete the 2 ASA24® recalls.

Delivery details, a birth assessment and infant and maternal health details will be obtained from the mother and infant's charts using the chart review forms, *CM- Birth and Labor History, CIM-1 Chart Review Infant Visits and CM-4 Chart Review Maternal Post-Partum Visits.* In the event that a mother gave birth outside of SickKids and/ or St. Michael's Hospital she will be asked to bring in her discharge summary to collect birth and labor details. The Edinburgh Postnatal Depression Scale (EPDS)[95], Ages and Stages Questionnaire [96], and Nipissing Development Questionnaire [97] will be obtained from the participant's chart, if the questionnaires are unavailable from the chart, they will be administered to the participant.

7.1.1.4 CHECK IN CALL: 6-10 MONTHS POST-PARTUM

There is a large gap between the 3rd study visit and the final study visit, the purpose of this call is to have a check-in midway through, to check in with the participant and to keep them engaged with the study. The call will take place between 6-10 months post-partum; with the timing of the call dependent on when the third visit took place. For instance, if the third visit took place at 6 weeks post-partum, we will aim to call earlier around 4 months, alternatively if the 3rd visit took place later around 4 months, then we would call later into the post-partum period around 10 months. The research staff member will call the participant to check in and administer the check-in call form with questions addressed to both the mother and infant.

7.1.1.5 STUDY VISIT 4: 12 MONTHS POST PARTUM

In order to coincide with the participant's clinic visits, this visit can be scheduled within 11-13 months post-partum. This will be the final time point for both the mother and infant. The research staff will administer the study visit forms, and the first ASA24® recall for this time point. The research staff and/ or the health care staff will obtain anthropometric measurements and blood samples from the infant and mother. If the infant arrives in a freshly soiled diaper and/ or they soil their diaper during the visit, then stool will be taken from the freshly soiled diaper. However, if the infant does not have a freshly soiled diaper, a rectal swab will be taken and the participant will be sent home with a stool collection kit. The mother will also take a rectal swab and will be sent home with a stool kit. The research staff will follow-up 3-10 days after this visit to administer the second ASA24® dietary recall over the phone. The research staff will fill out the study completion form at this time point as well.

The mother's chart will be reviewed for a post-partum health and medication assessment using the chart review form *CM-4 Chart Review Post-Partum Visit* and the infant's chart will be reviewed for health and medication use using the chart review form *CIM-1 Chart Review Infant Visits.* The Ages and Stages Questionnaire [96], and Nipissing Development Questionnaire [97] will be obtained from the participant's chart, if the questionnaires are unavailable from the chart, they will be administered to the participant.

7.2 STUDY ASSESSMENTS AND SAMPLE COLLECTION

7.2.1.1 BLOOD SAMPLE COLLECTION

For Maternal Participants:
A venous blood sample of 5 mL will be collected; 0.6 ml in SST Serum, 4.4 ml in EDTA tubes at enrolment, 30-34 weeks post conception and 12-months post-partum. When possible the bloodwork will take place at the same time as the clinical bloodwork to avoid the participant requiring an additional poke.

For Infant Participants:
A venous blood sample of 3 mL; 0.6 ml in SST Serum and 2.4 ml in EDTA tubes at 12 months. When possible the bloodwork will take place at the same time as the clinical bloodwork to avoid the participant requiring an additional poke.

All blood samples will be labelled with the participant's name, DOB, the study time point, and the date and time of collection. This, and other identifiable data of participants from both sites will be shared with SickKids hospital along with the samples for processing. Once the blood samples are collected 1.1 ml (0.5 ml in EDTA tube and 0.6 ml in SST serum) will be analyzed for CBC with a focus on HB + MCV, ferritin, and CRP. Bloodwork will be analyzed at SickKids laboratory or St. Michael's Laboratory depending on the timing and logistics of the visit. The remaining sample will then be transported to the Parkinson lab where they will be flash frozen and stored at -80^{o}C. Samples will be brought to the lab by the research team member, if the team member is not present at the visit, the blood samples will be transported via Canada Post pre-

labelled envelopes or through the KJV courier service. Upon arrival in the Parkinson lab, the sample will be de-identified to include only the participant ID, study visit time point (Baseline will be marked as visit 1, 30-34 weeks post-conception will be marked as visit 2, and 12 months post-partum will be marked as visit 4*) and the date and time of collection. Blood samples from individuals who do not complete the study will be processed and their data analyzed, as possible, for selected outcomes.

**Note there is no blood sample collection at the third visit, 4 months post-partum.*
See section 11 for more information on blood sample analysis.

7.2.1.2 STOOL SAMPLE COLLECTION

Participants will receive a stool sample collection kit with illustrated pictorial and written instructions that explain the procedure during each study visit. The samples will be collected using the OMNiGene fecal collection tube. The materials will be provided to the participant by the research team during their scheduled research visits, these supplies can also be sent to participants by mail. Upon collection of the stool samples, participants will place the sealed tube containing the stool sample in a Ziploc bag, inside the biohazard bag, and mail it to the Parkinson lab using the pre-labelled FEDex envelope or send it in via KJV, a prepaid courier service, whichever is their preference. For follow-up visits participants will be provided with stool kits ahead of time and will be instructed to bring their stool sample in with them to the study visit. If preferred these samples can also be sent in via prepaid courier or pre-labelled FEDex envelope. Participants will receive detailed instructions for the collection and shipment of their stool samples.

A subset of participants will split their stool sample into two containers; the first is the container indicated above (the OMNiGene kit which allows for DNA/ RNA analysis) and a second: the OMNImetGUT for Metabolomics kit. As our study is focused on recruiting 200 participants with a low BMI and 200 participants with a normal BMI. Our approach will be to have the first 50 participants with a BMI below 19.5 and the first 50 participants with a BMI above 19.5 collect stool using both kits and then for all participants after that to collect their stool samples using only the DNA/ RNA OMNiGene kit. This would allow us to conduct the more advanced analysis on a subset of participants, while remaining within our budget.

If the infant arrives to the study visit with a soiled diaper, or they soil their diaper during the study visit, a study team member will collect stool from the diaper into a labelled stool collection tube. The stool collection tube will be transported by the study team member to the Parkinson Lab, in the event that a study team member is not present at the study visit, then a health care staff member or the mother will collect stool from the diaper into the labelled stool collection tube, and the sample will be shipped to the Parkinson Lab through a pre-labelled FEDEx envelope or through the KJV courier service.

The stool sample will be labelled with the participant's name, study ID, DOB, the visit number and the date and time of collection. Upon arrival at the lab the sample will be de-identified, to

only include the study ID, the visit time point and the date and time of collection. The sample will then be stored in the Parkinson lab -80 Celsius freezer, until sample analysis. Stool samples from individuals who do not complete the study will be processed and their data analyzed, as possible, for selected outcomes.

See section 11 for more information on stool sample analysis.

7.2.1.3 RECTAL SWABS

We anticipate that some participants may not return their stool samples, so in addition to stool samples, maternal participants will also provide a rectal swab at baseline, 30-34 weeks post-conception, at 4 month's post-partum and at 12 months post-partum. If infants do not have a soiled diaper available, they will also have a rectal swab taken at 4 months and 12 months. However, if participants are uncomfortable with the collection of the rectal swabs, they can opt out of having them taken.

Maternal participants will receive detailed verbal and written instructions on how to safely and properly collect the rectal swab. Maternal participants will receive all of the necessary equipment, including; biohazard bags, plastic Ziploc bags, paper towel and the COPNAN Floqswab rectal swab kit transported in eNAT medium.. Maternal participants will collect the rectal swab on their own, once they collect the rectal swab, they will place the swab in a pre-labelled tube. The tube will be labelled with the participant's full name, date of birth, study ID, visit time point, and the date and time of collection. The tube will be placed in the Ziploc bag, which will be placed in the biohazard bag. If a study team member is present at the study visit, they will transport the rectal swab to the Parkinson lab. If a study team member is not present at the study visit, the study team member will call the KJV courier service to pick up the rectal swab, where it will be transported to the Parkinson lab.

Infant participants will have a rectal swab taken if they do not have a soiled diaper available, the swab will be taken by a health care provider or by a trained research member. The rectal swab will be taken according to the study rectal swab SOP. Once the swab is taken it will be placed in a pre-labelled tube. The tube will be labelled with the participant's full name, date of birth, study ID, and the date and time of collection. The tube will be placed in the Ziploc bag, which will be placed in the biohazard bag. If a study team member is present at the study visit, they will transport the rectal swab to the Parkinson lab. If a study team member is not present at the study visit, the study team member will call the KJV courier service to pick up the rectal swab, where it will be transported to the Parkinson lab.

We will be following the SOP developed by the CHAIN network, *Chain Rectal Collection SOP v1.04*[98].

See section 11 for more information on stool sample analysis.

7.2.1.4 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 collected by the study staff member and/ or the health care provider, using standardized procedures.

Infant anthropometry will include measurement of weight, length, MUAC, SFT, and head circumference; these measurements will be conducted using standardized procedures: a digital weight scale, infantometer, and measuring tape and skinfold caliper. All infant measurements will be collected by study personnel and/ or health care providers, using standardized procedures.

The staff member taking the anthropometric measurements will take one measurement for weight, length, height, MUAC and head circumference. The staff member will take 3 measurements for SFT, and will take the average of the three measurements.

Note if visits are conducted remotely, available measurements will be obtained from the chart, the rest will be marked on the study form as unavailable.

7.2.1.5 STUDY FORMS

The information collected will depend on the visit, for the maternal participant, the forms collected will include; a pregnancy confirmation and clinical assessment, a baseline and enrolment questionnaire, a dietary diversity questionnaire, an empowerment questionnaire (*see section 8 for more information)*, a health/ medication assessment questionnaire and a birth and labor history form. A summary of all the study questionnaires and visit-specific forms that will be used throughout the study is provided in *Table 2*.

The baseline study visit form 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, fluency of English and/ or French, race/ ethnicity, country of origin, immigration status, income, # of people this income supports, education and housing. The maternal study visit form will collect anthropometric measurements (*described in the previous section)* as well as a health assessment to document morbidity and medication usage. This form will also document the first three digits of the participants postal code. The postal code will be used to conduct GIS mapping, in order to gain a better understanding of the environmental impacts of the microbiome and pregnancy and birth outcomes

The birth and labor history form will gather information through chart review of both maternal and infant charts including: delivery characteristics (vaginal/cesarean section), gestational age, birthweight and other measurements at birth (head circumference and length), placental insufficiency, APGAR score and antibiotic use.

At 30-34 weeks post-conception, 4 months post-partum and 12-months post-partum, a health assessment of the mother and child will be administered. The assessment will include questions

related to the use of medications, including antibiotics, and the presence of illnesses and other health problems leading to doctor's visits/hospitalizations.

For the infant an anthropometric, feeding and health assessment will be completed at both visits and during the phone follow-up. The form will collect the infant anthropometric measurements (*described in the previous section)*, a breastfeeding assessment, and an infant feeding assessment as well as a health assessment to assess infant health status, morbidity and medication usage. At 12 months the Nutricheq form will also be administered, this form assesses potential dietary risk factors in children aged 1-3 years of age[1]. The feeding assessment is discussed further in the section below.

The participants charts will be reviewed for information on participant's health, medication use and birth and labor details. The chart review forms detail the exact information that will be obtained from the chart. For SickKids participant's EPIC will be reviewed and for SMH participant's both the family medicine EMR and the hospital EMR will be reviewed to ensure the most robust data. Participant's that deliver outside of SickKids and St. Michael's hospital, will be asked to bring in their discharge summary. The Edinburgh Postnatal Depression Scale (EPDS)[95], Ages and Stages Questionnaire [96], and Nipissing Development Questionnaire [97] will be obtained from the participant's chart, if the questionnaires are unavailable from the chart, they will be administered to the participant. Note if the ASQ questionnaire and Nipissing Development Questionnaire are administered clinically throughout the first year of the infant participant's life it will be collected through chart review, even if administered outside the research visit windows.

7.2.1.6 DIETARY RECALLS AND FEEDING ASSESSMENT

To link the microbiome to nutritional status and nutritional intake, with a focus on calories and macronutrients, we will use the Automated Self-Administered 24-Hour Dietary Assessment Tool (ASA24®-Canada2018)[99]. The ASA24 has been used in multiple cohort studies in Canada and includes details of all food items available in Canada; moreover, it is also found to be useful among low-income females[100, 101] and pregnant women[102]. Participants will complete the recall, with assistance from a research staff member, at both time points during pregnancy and at 1 year post-partum. To better capture reliable individual nutrient intake, recalls will be completed on two separate days (separated by 3-10 days). The first recall for each time point will be completed in person, or virtually over Microsoft Teams or Zoom Healthcare and assisted by the research team. The second recall will take place either virtually through Zoom HealthCare or Microsoft teams. If the participant prefers they can also complete their visits via phone call. If required, an interpreter will also be present on the video call / phone call. Each participant's usual intake will be obtained through averaging the two 24-hr dietary intakes per time point. The ASA24 is linked to the Canadian Nutrient File and provides a detailed analysis of diet[99].

The participants will receive the links to complete the ASA recalls via email. The recall works on all web browsers, and can be used on smartphones, tablets, laptops and desktops. ASA24 is linked to the Canadian Nutrient File and provides a detailed analysis of diet (macronutrients and micronutrients)[99]. Dietary data is saved on the National Institute of Health (NIH) website, the NIH has no access to them.

At the 3rd visit, which will take place around 4 months post-partum, 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[103]. 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 ASA recalls completed at baseline, 30-34 weeks post-conception and at 12 months.

For the infant, feeding assessments will take place at the 4 month and 12 month visits. At 4 month's the form will include an assessment of feeding initiation, and the amount and mode of breastfeeding/formula feeding and dietary supplements. At 12 month's this form will also include an assessment of young child feeding, with questions to address the introduction of complementary foods (solid, semi-solid and soft foods), meal frequency and the minimum dietary diversity score (MDD, see appendix X) will be assessed to estimate quality of food intake. Overall, 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)"*[104]

At the 12 month visit, the research staff will also 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[1]. Also, household annual food insecurity will be assessed based on a validated 2 question score[105].

7.3 CONTACT WITH STUDY PARTICIPANTS

Study participants will be recruited at around 8-20 week's post-conception, with our aim to recruit participants around 12-16 weeks post-conception. Participants will be followed from recruitment, until 12 months post-partum. Study staff will aim to schedule research visits at the same time as clinic visits and infant check-ups to avoid having the participant come in for additional visits. Study participants can contact the Pregnancy and Microbiome study coordinator and research team for any question or concerns they might have. The study team will communicate with participants via email and/ or phone for study visit/ procedure reminders, to follow-up about outstanding study tasks, to send study information (like the links for the ASA dietary recalls) and to answer any participant questions. The expected number and duration of visits is detailed in table 7 below.

All participant contact, including follow-up calls/ emails will be documented in a study contact log. The study contact log will include the participant ID, the participant contact information and a log of all the participant contact. The contact log will be stored on the St. Michael's shared drive for St. Michael's participants and on Onedrive for SickKids participants, in password protected excel files.

Table 7: Overview of Study Visit Duration

Visit	Visit Duration
Enrolment and baseline	~1.5-2 hours
2nd baseline dietary recall	30-40 minutes
30-34 weeks post conception	1-1.5 hours

2nd 30-34 week dietary recall	30-40 minutes
Empowerment sub-study around 4-12months post-partum	30-45 minutes
Maternal visit 4 months post-partum	1-1.5 hours (This visit will be completed at the same time as the infant visit)
Infant visit within 4 months of birth	1-1.5 hours (This visit will be completed at the same time as the maternal visit)
6-10 month check- in call	10-20 minutes.
Maternal visit 12-months post-partum	1-1.5 hours (This visit will be completed at the same time as the infant visit)
Infant visit 12 months post-partum	1-1.5 hours (This visit will be completed at the same time as the maternal visit)
2nd 12 month post-partum dietary recall	30-40 minutes

7.4 SAFETY AND ADVERSE EVENTS

Although study-related adverse events are not anticipated, the participants may encounter pregnancy-related or other unrelated adverse events, including, but not limited to, miscarriages, abortions, stillbirths and maternal and newborn deaths. These events will be recorded (*AE – Adverse event form*). Women who experience clinical events during study participation will be referred to the appropriate health facility. Serious adverse events will be reported to the Hospital for Sick Children and St. Michael's REB 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 St. Michael's Hospital or The Hospital for Sick Children 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 St. Michael's Hospital or The Hospital for Sick Children to arrange referral to the appropriate specialist as needed.

8 EMPOWERMENT SUBSTUDY

8.1 EMPOWERMENT QUESTIONNAIRE

Gender-related variables that may influence how mothers understand, promote and maintain intestinal microbiome health will be explored. While there may be sex-specific interactions between the microbiome and hormones, here we focus explicitly on gender. Validated modules will be administered by a trained member of the research team to explore maternal education status[106], household food insecurity[107], generalized self-efficacy[108] and decision making[106], with modifications made and beta-tested for internal validity. Other gender-related variables including self-identified race and ethnicity, religion, primary language, immigration status and income will be captured. Validated modules that have been used in the MaPPS cohort will allow for comparison between the cohort based in Pakistan and the Toronto cohorts, to account for cultural factors that further intersect with gender norms in their specific context.

Participants will fill out an empowerment questionnaire at baseline and 4-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 from the

Pakistan Demographic and Health Survey (PDHS)[106]; food insecurity will be assessed using the Household Food Insecurity Access Scale (HFIAS)[107]. Self-efficacy will be measured using the Generalized Self-Efficacy scale, developed by Schwarzer and Jerusalem[108]. Perceived social support will be measured using the Multi-dimensional Scale of Perceived Social Support (MSPSS), developed by Zimet and colleagues[109]. Perceived parental stress will be measured using the Perceived Stress Scale (PSS-10)[110].

8.2 QUALITATIVE IN-DEPTH INTERVIEWS

A sub-set of Toronto cohort study participants will be invited to participate in qualitative in-depth interviews around 4 -12 months post-partum. In order to get a broader perspective of empowerment, this sub-study will also be open to participant's who are not involved in the main study. These participants will be selected using non-random purposive sampling in line with recommended sampling strategies. Using grounded theory methodology[111], we will conduct in-depth interviews with mothers in the Toronto cohort to further explore knowledge and attitudes toward intestinal gut health. Interviews will be recorded and transcribed and coded for themes using an inductive coding strategy. Thematic analysis will yield opportunities for knowledge translation to empower young mothers to improve gut health.

8.2.1 RECRUITMENT

We will approach participants who provided written consent to be approached to participate in the empowerment interview when they enrolled to the main (MMiP) study. Potential participants will receive the study information by phone. If they express their interest to participate in the empowerment interview, an interview will be scheduled at a date and time that is convenient for them. The visit will take around 30-45 minutes, these interviews will be conducted in person or over the phone or videoconference using a Zoom Healthcare. The researcher conducting the interview will explain the study, its purpose, and go through the informed consent process, followed by obtaining signed consent. Participants who do not speak English will be given the option to participate by using an interpreter. At the time of obtaining consent, we will provide the participant with the option for interpretation; in some cases, the participant may choose to decline the interpretation. Participants who have limited English literacy, both in speaking and in reading, will have the option to have the consent read to them by the research assistant. In person interviews will be held at either of the participating institutions. The consent discussion will happen remotely over videoconference using a Zoom Healthcare or phone for the participants who prefer to attend the interview over videoconference using a Zoom Healthcare or by phone accordingly. Following the consent discussion, an REB approved consent form will be emailed to participants with password protection and participants will email or mail back a signed and dated consent. Participants will be provided with a signed copy of consent. As a token of appreciation for the time and effort spent on the study, the participant will be given a $50 gift card at the end of the study.

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8.2.2 SAMPLE SIZE

The exact number of interviews depends on theoretical saturation of information (typically after 10-15 interviews), which will occur when subsequent interviews do not provide any new insights, nor reveal any new properties of the core themes. We will aim for data richness and reaching meaningful data. As such, we expect 20-30 participants

8.2.3 DATA COLLECTION

Interviews will be conducted using a semi-structured interview guide by a trained research assistant in person or, by telephone or videoconference using a Zoom Healthcare account. In person interviews will be held at the participating clinics at either SickKids, St. Michael's Hospital. Interviews will be conducted in English or with an interpreter for all other languages. This approach will ensure we are inclusive, and language will not be a barrier to participate in this study across different ethno-racial groups. At the time of obtaining consent, we will provide the participant with the option for interpretation; in some cases, the participant may choose to decline the interpretation. During the interview, we will use a secure form of Zoom (i.e., Zoom for healthcare) to transcribe our conversation. The interviews will be audio recorded by the interviewer, and the audiotapes will be used by the research team to ensure all information transcribed by Zoom is accurate and complete. Neither de-identified interview data nor audio recordings will be sent to any external organizations.Audio recordings will be back translated to English by a member of the multi-lingual study team when possible and uploaded into Dedoose software[132].

Participants can withdraw from the study at any time without having to provide a reason. If a participant chooses to withdraw from the study, they are encouraged to contact the research coordinator of the study using the information provided in the consent form. Also, participant may withdraw their permission to use the interview data and audio recordings that were collected about them for this study at any time by letting the research team know. However, this would also mean that they withdraw from the study. Information that was recorded before the participant withdrew will be used by the researchers for the purposes of the study, but no information will be collected after participant withdraw their permission.

8.2.4 DATA ANALYSIS

All transcriptions will be uploaded into a data management software (Dedoose). As per the Consolidated Criteria for Reporting Qualitative Research (COREQ) Checklist, transcripts will be returned to participants for comment and correction. This will be done by a mailed copy, with a

follow-up phone call to obtain feedback. For participants with limited English literacy or fluency, the research assistant will offer to read the transcript to the participant, with the support of an interpreter if required. At the time of obtaining consent, we will provide the participant with the option for interpretation; in some cases, the participant may choose to decline the interpretation. This will be done after the recording is transcribed and before data analysis begins[133].

Transcripts will be analyzed using reflexive thematic analysis (RTA), which includes open-coding, category development, and quote abstraction[134]. In keeping with RTA, members of the research team will open-code the same select number of transcripts, then discuss their codes and reasoning in research team meetings. Differences and similarities in coding will be discussed, and a draft codebook will be created for language and similarity purposes. Transcripts will then be assigned to multiple people in the research team, and discrepancies will be resolved by collective agreement.[135]Once all transcripts are coded, the coding team will meet to compare the codes across participant types; matrices of the core codes will be created with relevant themes in each. To ensure trustworthiness of data, we will use a peer debriefing strategy to verify coding approach and interpretation of findings, in accordance with the RATS (relevancy, appropriateness, transparency and soundness) guidelines for qualitative research[133].

Dedoose qualitative software will be used for data management. We will only upload de-identified data to this software. For data reporting, the most illustrative quotes will be selected for each theme. We will follow the Consolidated Criteria for Reporting Qualitative Research (COREQ) checklist, to ensure all important aspects of the research team, study methods, context of the study, findings, analysis, and interpretations are reported[133]. For demographic information, we will use descriptive statistics.

8.2.5 ETHICS

Data handling and participant confidentiality will be maintained in accordance with the procedures outlined in the main study.

We expect that the impact of gender-related variables may vary by cohort as cultural expectations of gender roles widely differ. We anticipate our qualitative investigation may identify that young mothers lack knowledge concerning gut health and may benefit from targeted education opportunities early in pregnancy.

9 WORKSHOPS

We will work with our partner clinics in Toronto as well as our participants, to identify gaps in knowledge and to integrate findings from our gender focused investigations into accessible material that can be delivered as workshops to promote the importance of a healthy gut microbiome during pregnancy and beyond. We will develop two kinds of workshops:

1. The first workshop will be directed at clinicians and healthcare providers. It will deliver tangible material on how to improve patients gut health in general. The content of the workshop will be designed based on participant and clinician feedback. These workshops will be delivered during the final year of the study, once the clinicians and healthcare providers have completed their participation in the study.
2. The other workshop will be directed at participants who have completed their participation in the study. These workshops will be designed/ delivered during the final year of the study, and will be developed based on participant identified gaps in knowledge. Potential topics may include how to improve gut health, how to develop a healthy gut microbiome in toddlers, etc.

These workshops will be centered on patient experiences and knowledge translation, once we design the workshop content, we will submit an amendment application to our REB. We will only deliver the workshops once we have approval from the REB.

10 STOOL AND BLOOD SAMPLE ANALYSIS

10.1 PROFILING MICROBIAL COMMUNITY STRUCTURE AND FUNCTION

To assess nutritional status, metabolomics targeting minerals and general metabolites will be applied to the blood samples. To monitor microbiome dynamics, 16S and 18S rDNA surveys will be applied to the maternal and infant stool samples. To gain more mechanistic insights into the relationship between microbiome function and maternal health and birth outcomes, we will deploy metatranscriptomics, metabolomics, proteomics and markers of inflammation.

10.1.1 PROFILING MICROBIAL COMMUNITYSTRUCTURE

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 [112-114] and the V4V5 region of the 18S rRNA gene to capture eukaryotic taxa. DNA library preps will include error-correcting barcodes[115] 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.

Prior to implementing these lab analyses, a pilot experiment will be performed using 10 previously sequenced DNA samples derived from human stool at the Integrated Microbiome Resource (IMR) facility, which applies a modified approach to preparation of DNA sequencing libraries compared to that used in our previous studies, to ensure a comparable quality of output.

The pilot experiment will involve PCR amplification of the 18S rRNA variable regions 4 and 5 using primers V4-1 and V4-4[130,79], which will be fused to Illumina adapter sequences unlike in previously published analyses. Sequencing will be performed using Illumina MiSeq v3 chemistry, 300 cycles, as previously, and the quality and number of sequenced reads as well as

success of detecting protozoa will be compared to outputs from the published data. As the DNA extraction procedure in the current study differs from that of our previous studies, we will include 5 archived DNA samples from each of two different cohort studies (NCT00705445 and NCT02308917), where DNA samples were extracted using two different kits to minimize extraction bias. We will also submit the same 10 DNA samples for 16S rRNA variable region 4 sequencing to IMR, which uses slightly modified versions of EMP primers we previously used (515FB instead of 515F, and 806RB instead of 806R) to ensure reproducibility of results.

A waiver of consent is being requested for the secondary use of the 5 samples from the study (cRCT NCT00705445, AKU (752-Peds/ERC-07)), that were previously approved by the research ethics board (REB) at SickKids (REB No. 1000054244) for a use in a retrospective analysis (NCT00705445). Currently, these 5 samples are stored in Dr. Bandsma's lab. Informed consent was obtained from the participants in the study (NCT02308917, REB 1000039604) to use their samples for future research. Participants in this study are allowed to withdraw their specimens. At the governance level, a sample can be withdrawn as long as it remains within the research network and has not been utilized. If participants choose to withdraw their specimen, they should contact the research coordinator of the study, who will ensure the specimen will no longer be used for research activities. However, there are some limitations. If the sample has been published as a part of grouping with other samples, or if it has been sent for third party analysis based on an agreement, and the participant has consented to future research activities, the sample cannot be withdrawn.

In this pilot experiment, only deidentified samples will be used for sequencing and analyzing the data, meaning all the personal information linking the samples to specific participants will be removed. DNA extracted from stool samples sent to IMR will be assigned a study ID number, ensuring that no patient identifying information will be shared with the IMR facility. The information that links the study ID to the participant's personal information will be stored in a separate, encrypted file in a password protected computer which can only be accessed by the principal investigator of the original study. This process ensures the privacy and confidentiality for all participants. The secure transfer process will involve packaging the deidentified samples in compliance with IATA P.I. 650 shipping standards, ensuring safe and secure transport. Additionally, a material transfer agreement (MTA) with IMR will be fully executed prior to transfer of de-identified samples, further ensuring that all legal and ethical guidelines for handling the de-identified samples are met.

At the end of the pilot testing, any unused samples will be destroyed at the IMR facility. The microbiome sequencing processing takes approximately 6-8 weeks, after which the unused samples will be destroyed. After a successful pilot, the stool samples from the MMIP study will be sent to IMR for 16s and 18s sequencing.

We will utilize the QIIME2 platform[116], MOTHUR[117], 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[118]) 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[119].

To better define bacterial pathogen burden, we will apply TaqMan array card technology for the simultaneous detection of 19 common enteropathogens[120].

10.1.2 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[90]). 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 [121]. The output of these analyses are readouts of microbial gene expression detailing biochemical activities as well as the taxa responsible.

10.1.3 PROFILING OF PATIENT AND MICROBIOME METABOLITES AND PROTEOMICS

To generate read outs of stool and blood metabolites, samples will be sent on dry ice to the Metabolomics Innovation Centre. Stool and blood samples will be analyzed for general metabolites (e.g. SCFAs, amino acids, intermediates in glycolysis and nucleotide metabolism, among other metabolites, using the TMIC Prime Metabolomics platform[122]). Blood samples will additionally be analyzed for vitamins and minerals through the TMIC center. The output of these analyses will be concentrations of metabolites that are expected to correlate with pathway expression data.

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)[123]

10.1.4 HOST MARKERS OF INFLAMMATION

In addition to microbiome function, we will also obtain host readouts that reflect gut health and pathogen challenge. Here we will examine markers in the stool for intestinal mass, inflammation, and gut permeability and circulating lipopolysaccharide, among other markers.

10.2 POTENTIAL PROBLEMS, MITIGATIONS AND EXPECTED RESULTS

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[46], 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.

11 STATISTICAL CONSIDERATIONS

11.1 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[124]. 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[125], 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*.

11.2 SUBGROUP ANALYSIS

To reduce study complexity and to avoid confounding effects due to existing micronutrient deficiencies, we will focus specifically on interactions between macronutrient deficiencies with the 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 (ie. 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. Patients exhibiting deficiencies will still be included in the study, but will be analyzed separately.

In addition, we may conduct further sub- group analysis for participants with documented substance use, this sub-group analysis will be dependent on the prevalence of substance use among our study population.

11.3 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[126] 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. blood 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.

11.4 PLAN FOR STATISTICAL ANALYSIS

The following section 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
6. Infant weight, height, head circumference z-scores (continuous)

7. Infant gestational age
8. Infant birth weight z-scores (continuous)
9. Breastfeeding duration (continuous)
10. Maternal age years (continuous)
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
16. Infant and maternal diet diversity

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

11.4.1 ADDRESSING THE PRIMARY OBJECTIVES

This study has two primary objectives: 1) To assess if alterations of the microbiota in the maternal gut (dysbiosis) is 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 morbidity 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. Equivalent approaches will be used to investigate other clinical outcomes in the first year of life.

To complement these analyses we will also undertake an integrative modeling strategy based on Similarity Network Fusion framework[127] 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.

11.4.2 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 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.

11.4.3 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.

12 SUPPORTING DOCUMENTATION AND OPERATIONAL CONSIDERATIONS

12.1 REGULATORY, ETHICAL, AND STUDY OVERSIGHT CONSIDERATIONS

Research ethics committee/institutional review board approval will be obtained through Clinical Trials Ontario. 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, who will then obtain informed consent. In the event that the participant does not speak English, a translator will be present either in person or through a phone call. 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.

12.2 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

12.3 CONFIDENTIALITY AND PRIVACY

12.3.1 PARTICIPANT ID SYSTEM

All participants will be assigned a unique study ID, the IDs will be assigned as follows:
Maternal ID:
Site ID- Participant number*
Infant ID:
Same as mom: Site ID- Participant number- 01

Participants from SickKids will receive an ID: 01-XX
Participants from St. Michael's clinics will receive an ID: 02-XX
Participants from St. Michael's Affiliated Midwifery clinics will receive an ID: 03-XX
**The participant number will be assigned consecutively.*

For example, the third participant enrolled at a St. Michael's clinic, will be assigned the participant ID: 02-03. If this mother had twins the infant IDs would be: 02-03-01 and 02-03-02.

Participant samples (stool, blood and rectal swabs), will be de-identified, and will only contain the participant ID, the visit number (**1**- baseline, **2**- 30-34 weeks post conception, **3**- 16wk-4 months post partum and **4**- 12 months post-partum), and the date and time of collection. The

master linking log, which will link the participant name to the participant ID, will be stored as a password protected excel file. The master linking log for SickKids participants will be stored on the SickKids Onedrive, and the master linking log for St. Michael's participants will be stored as a password protected excel file on the St. Michael's shared drive.

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.

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 identifying information will be kept behind 2 security measures or as per equivalent institutional policy, under the supervision of the study/site PI and will not be transferred outside of the hospital.

The PI, 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. However, in order to facilitate sample processing at SickKids from St. Michael's Hospital, identifiable labels on blood samples will be transferred to SickKids to assist with processing.

12.4 FUTURE USE OF STORED SPECIMENS AND DATA

Data collected for this study will be analyzed and stored at 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 Research Ethics Boards (REBs), de-identified biological samples will be stored at SickKids. Extracted DNA and RNA will be batch

shipped to SickKids from participating sites. 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. De-identified samples and de-identified study data will be sent to research collaborators outside SickKids and Canada for analysis. The samples will not be sold. Blood and stool samples will be biobanked at SickKids.

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[5]. 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.

12.4.1.1 FUTURE GENETIC TESTING

Participants will have the option to sign an additional section of the 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[128]. 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 [90]. 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[129]. 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.

12.4.1.2 DATA COLLECTION AND MANAGEMENT RESPONSIBILITIES

Data collection is the responsibility of the study research 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.

In the data analysis, growth measurements, medication usage, health assessments and birth characteristics will be obtained from the patient's clinical chart. Participants previous and recent data will be used to determine eligibility to participate in the study and/ or will be used as predictors, mediators, and/ or covariates in the analysis of their gut microbiota.

For SickKids study participants, participant logs will be stored on SickKids Onedrive, using password protected excel files. For St. Michael's participants, participant logs will be stored on the St. Michael's shared drive using password protected excel files.

Study data will be entered into REDCap (Research Electronic Data Capture), a secure, web-based application designed exclusively to support data capture for research studies. REDCap is developed and maintained by a team at Vanderbilt University and licensed free of charge by the Research Institute at The Hospital for Sick Children. The application and data are housed on servers provided by The Hospital for Sick Children. These servers are located within SickKids secure data center. Local support for REDcap is provided by SickKids Research IT. All of the information entered into REDcap will be de-identified.

The sample analysis information, including the sequencing data and metabolomics data will be de-identified and hosted on a network storage array in the Parkinson Lab. This will be protected by the Sickkids firewall. The patient sequence data will be removed, and the microbiome data will be uploaded on the National Centre for Biotechnology Information (NCBI). The NCBI facilitates the automated storage and analysis of databases for the research and medical community.

12.4.1.3 DATA SECURITY AND CONFIDENTIALITY OF PARTICIPANTS USING THE ASA24 SYSTEM

Researchers using the ASA24® recall system do not provide the NCI, Westat, or the ASA24® system with any identifying data for participants of their studies. Rather, researchers specify a unique numeric identifier for each Respondent and download system-generated usernames and encrypted passwords that they provide to respondents so that they may access the application.

The ASA24® system also does not collect any identifying data directly from respondents. However, IP address information is accessed for the purpose of routing information between the server and the respondent's computer—often the IP address is that of the user's Internet Service Provider (ISP). IP addresses are not stored or tracked by the ASA24® system. However, logs of connections are kept in the hosting environment for audit trail purposes. This information is not mined in any way but would be available if there were a legal obligation to release it.

Response data is secured at the hosting site using industry standard security controls, including firewalls and encryption. All data entered into the ASA24® system at the Respondent's computer is encrypted by the internet browser (e.g., Internet Explorer, Firefox) before they are transmitted to our servers using Secure Socket Layer (SSL) Technology. SSL allows for the authentication of the sending and receiving computers. Only a particular study's investigator(s) and the ASA24® operations team can access response data. Access is gained through usernames and strong passwords.

The company that hosts the online survey is called Westat. The servers are located in Rockville, MD. Encrypted backups are stored offline in MD and no data is transferred to any other server, domestic or foreign. Email address is obtained for the Researcher conducting data collection using ASA24® and it is stored in the database. IP addresses of Researchers and Respondents are captured and logged by the web server. ASA24® does not capture any PII related to Respondents. ASA24® is subject to FISMA moderate level controls that protect the integrity, confidentiality, and availability of the system. Data is not shared by the ASA24®NCI and Westat teams with other researchers. The system is a government system and subject to any lawful government purpose.

12.4.1.4 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.

12.5 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.

12.6 CONFLICT OF INTEREST

The research team declares no conflict of interest.

12.7 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
ICMJE	International Committee of Medical Journal Editors
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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