





BMJ Open Association between gut Microbiota, GROWth and Diet in peripubertal children from the TARGet Kids! cohort (The MiGrowD) study: protocol for studying gut microbiota at a community-based primary healthcare setting

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ABSTRACT

Introduction The gut microbiota interacts with diet to affect body health throughout the life cycle. Critical periods of growth, such as infancy and puberty, are characterised by microbiota remodelling and changes in dietary habits. While the relationship between gut microbiota and growth in early life has been studied, our understanding of this relationship during puberty remains limited. Here, we describe the Microbiota, GROWth and Diet in peripubertal children (The MiGrowD) study, which aims to assess the tripartite growth-gut microbiota-diet relationship at puberty.

Methods and analysis The MiGrowD study will be a cross-sectional, community-based study involving children 8–12 years participating in the TARGet Kids! cohort. TARGet Kids! is a primary healthcare practice-based research network in Canada. Children will be asked to provide a stool sample, complete two non-consecutive 24-hour dietary recalls and a pubertal self-assessment based on Tanner Stages. Anthropometry will also be conducted. The primary outcome is the association between gut microbiota composition and longitudinal growth from birth until entry into the study. Anthropometrics data from birth will be from the data collected prospectively through TARGet Kids!. Body mass index z-scores will be calculated according to WHO. The secondary outcome is the association between gut microbiota, diet and pubertal stage.

Ethics and dissemination Ethics approval has been obtained by the Hospital for Sick Children and St. Michael's Hospital—Unity Health, and the University of Toronto. Results will be disseminated in the public and academic sector, including participants, TARGet Kids! primary healthcare physicians teams, scientists via participation in the TARGet Kids! science and physician meetings, conferences and publications in peer-reviewed journals. The MiGrowD study results will help researchers understand the relationships underlying growth, gut microbiota and pubertal maturation in children.

STRENGTHS AND LIMITATIONS OF THIS STUDY

- ⇒ The Microbiota, GROWth and Diet in peripubertal children (The MiGrowD) study aims to investigate the relationship between microbiota and puberty, a sensitive but neglected period of growth.
- ⇒ The MiGrowD study is adapted for remote participant enrollment and data collection, thus providing a flexible research model, including applicability during a pandemic.
- ⇒ MiGrowD study procedures are participatory and generalisable to studies with different populations and communities, designs and children age groups.
- ⇒ MiGrowD is a cross-sectional study and can, thus, only reveal associations; a longitudinal study design with serial microbiota assessments during the pubertal period would confirm the dynamic changes in gut microbiota and diet.
- ⇒ MiGrowD is embedded within a prospective cohort study allowing for the future implementation of longitudinal studies and assessment of causal relationships.

BACKGROUND

Childhood is characterised by two periods of rapid growth and development: infancy and puberty.¹ Infancy lasts from birth until 2 years of age, and it is identified by rapid mental and physical development.^{1–3} Puberty starts around 8–9 years of age and corresponds to rapid development at the skeletal, neural levels and sexual maturation. During these periods, environmental impacts can be profound and have a permanent effect on development.^{4–6} Factors such as gestational age,^{7,8} ethnicity,^{9,10} sex,¹¹ medical history,^{10,12}

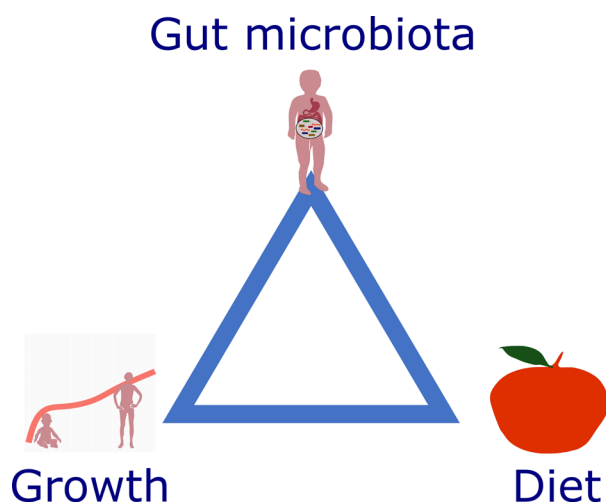


Figure 1 The conceptual framework of the MiGrowD study. The tripartite relationship between gut Microbiota, GROWth and Diet. The objective of the MiGrowD study is to investigate the association between gut microbiota, growth since birth, and diet in early adolescent children in an ongoing community-based primary healthcare setting.

diet¹³ and social-economic status¹⁴ act independently or in a synergistic manner¹⁰ to influence growth. However, these factors do not fully explain variability in growth. In fact, an emerging body of evidence suggests that the assembly of microorganisms living in the gut, defined as gut microbiota,^{15–18} plays an important role.^{19–20} A causal relationship between intestinal microbiota immaturity, undernutrition and growth impairment has been observed in younger children of age 6 months and 18 months²⁰, while there is an inverse association between gut microbiota diversity and stunting severity in stunted or severely stunted children.²¹ At the other end of the malnutrition spectrum, children and young adults with obesity (average age range 9.93–22.1 years) show different gut microbiota composition compared with children without obesity.^{22–24} Various mechanisms have been proposed to support this relationship,^{25–27} including microbial processing of dietary components, metabolite production and microbiota compositional and functional capability and adaptations. However, less is known about the association between microbiota and longitudinal growth patterns with nutritional status and diet in children.

The gut microbiota evolves as the host grows. The perinatal and postnatal processes of gut microbiota seeding and maturation during the first years of life have been well characterised, and this community is considered to be mature by the age of 3 years.^{17–28–30} While there is a consensus that the microbiota composition remains stable during the following childhood years, a limited number of studies suggest that it may undergo a final reshaping during adolescence. Indeed, differences have been found in the relative abundance of core taxa, such as *Bacteroides* and *Ruminococcaceae*, between 7 and 12 years of children and adults³¹ and bifidobacteria were shown to be

more highly represented in adolescents versus adults.^{32–33} Moreover, a recent study including 89 Chinese children from 5.5 to 14.5 years of age suggests that differences exist in the gut microbiota between sexes, which become more pronounced during puberty; discriminatory taxa were identified in pubertal men (*Dorea* and *Megamonas*) and women (*Bilophila*, *Parabacteroides* and *Phascolarctobacterium*).³⁴ However, it remains unknown if the gut microbiota varies among the five stages of puberty in men and women. The effect of growth since birth on the microbiota at the time of puberty onset is also unknown as well as its relationship with remodelling during puberty. Importantly, puberty is also the time when dietary patterns can undergo significant modifications as they transition into adult type.^{35–36} While these modifications vary across geographies, in North America, where this study will be conducted, they include decreased intake of dairy products, certain vegetables and fruits.³⁷ The 2015 Canadian Community Health Survey showed that both men and women 9–13 years of age eat fewer servings of total fruits and vegetables in comparison to younger children (2–8 years) and adults (19–30 years).³⁸ Moreover, evidence indicates that dietary trends in adolescents may be sex specific, with the daily intake of fruits and vegetables decreasing by 0.7 servings among girls and by 0.4 servings among boys during the transition from early to middle adolescence.³⁷ Since diet has been consistently shown to alter microbiota composition,^{39–42} it is important to extend current studies along time and include analyses of both diet and microbiota composition during that period of life. The low-quality diet often observed in adolescents, characterised by high intake of fat or sugar and low intake of fruits and vegetables,^{37–43–44} may affect gut microbiota composition and metabolism and makes dietary assessment essential during adolescent years. Puberty may offer a last window of opportunity to beneficially impact the gut microbiota composition and function, potentially through dietary alterations. Based on these premises, the objective of the MiGrowD study is to investigate the association between gut microbiota, growth since birth and diet, in children at different pubertal stages (figure 1).

METHODS

Study design

The MiGrowD study is an observational study using longitudinal growth analysis approaches. We previously studied growth since birth and until 12 years of age in 1134 children from the TARGeT Kids! cohort. Briefly, using latent class mixed modelling, we identified three patterns for Body Mass Index (BMI)-for-age z-score growth trajectories, which we labelled as (1) normal, (2) gradually increasing, developing overweight and (3) rapid increase, developing obesity.⁴⁵ We also identified three (lower normal, higher normal and increasing) and two (lower normal and higher normal increasing) patterns for height-for-age z-score and weight-for-age z-score trajectories, respectively. The same approach will

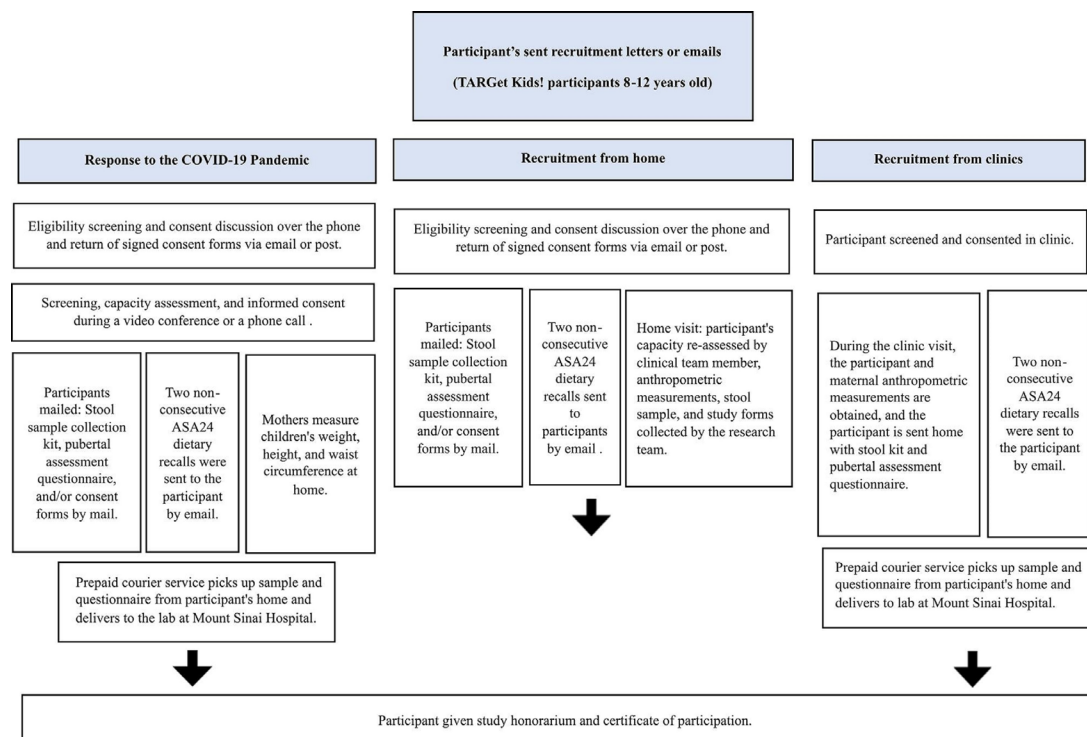


Figure 2 MiGrowD study procedures. Abbreviations: ASA24, Automated Self-Administered 24-hour; TARGet Kids!, The Applied Research Group for Kids!

be used in MiGrowD. Data on gut microbiota, diet and pubertal stages of growth will be obtained from a primary healthcare-based cohort study. Recruitment from the participating primary care practices started in January 2019, and recruitment from home started in April 2020 (figure 2).

Study population and setting

MiGrowD will be conducted through the TARGet Kids! practice-based research network in Canada. TARGet Kids! recruits children from paediatric and family medicine primary care practices in the Ontario and Quebec provinces. Children are enrolled between ages 0 and 5 years, and they are followed up prospectively for annual health supervision visits.⁴⁶ As of March 2021, the cohort recruited 11 488 participants. Children with developmental delay, chronic health conditions (except for asthma and high functioning autism) or conditions that affect physical development at enrollment are excluded.⁴⁶ Anthropometric measurements (weight, height, waist circumference) are obtained during the visits by trained research personnel following previously described standardised procedures.⁴⁷ In addition, questionnaires are administered to obtain data on participants' socioeconomic status, ethnicity and health status. The TARGet Kids! study was previously approved by the Research Ethics Board at SickKids and St. Michael's Hospital and is currently approved through Clinical Trials Ontario. More information on the cohort can be found at Carsley *et al*⁴⁶ and at <http://www.targetkids.ca/contact-us/>.

Sample size

The lack of previous data makes sample size calculation challenging. The study design, the microbiota metrics of interest (such as diversity measures) and their distribution in the population of interest play a role in specifying the minimum number of participants to enroll.⁴⁸ Since we lack this information, to estimate sample size, we used data from a published study that displays some similarities with ours, including classification of 6–12-year-old children into three different groups, that is, normal weight, overweight or with obesity, based on growth metrics.²⁴ This approach has been previously proposed for microbiome studies.^{48 49} We used microbial α -diversity, measured by the Shannon index,⁵⁰ distribution data from the Moran-Ramos *et al* study²⁴ as the outcome and multivariate analysis of variance via computer simulations⁴⁹ to evaluate the impact of growth on α -diversity while controlling for diet. The generated model estimated that 240 children will be required based on an alpha of 0.05 to achieve 80% power.

MyGrowD eligibility criteria and screening procedures

The MiGrowD study will invite children aged 8–12 years (96–155 months), aiming to capture the initial stages of puberty. In fact, in Canada, 68% of women between 11.53 and 13.91 years of age have reached Tanner stage 5,⁵¹ while 5% of the women and 3% of the men reported having entered puberty by age 10.⁵² Potential participants will be screened with a specifically designed screening tool (online supplemental material section 1) for factors affecting gut microbiota composition and excluded based on (1) diagnosis of chronic gastrointestinal disorders

such as coeliac disease, chronic diarrhoea and (2) dietary restrictions, allergies or major changes in diet 3 months before recruitment. Children who used antibiotics, probiotics or laxatives 3 months before stool sample collection⁵³ or were diagnosed with common acute disorders will be temporarily excluded and reinvited 3 months later.

Recruitment

MiGrowD will use two recruitment approaches that are aligned with the established research procedures of the TARGet Kids! cohort study⁴⁶ (figure 2). In the first approach, called *recruitment from clinics*, participants with a prescheduled clinic visit will be introduced to the MiGrowD study by a recruitment letter or email invitation from the child's paediatric clinic sent 2 weeks before their visit. On arrival at the clinic, participants will be approached by the on-site research assistant to undergo screening and, if eligible, a capacity assessment and consent discussion. Participant anthropometric measurements are then taken (height, weight and waist circumference), and the participant is sent home with a stool sample collection kit and a pubertal assessment questionnaire to be self-completed or completed with parental assistance. Posters and information sheets will be available in waiting rooms.

The second recruitment approach, called *recruitment from home*, will target potential participants with no upcoming clinic visits. Families will be approached by email or by phone. If they are interested, screening and consent discussions will take place over the phone. Participants will then return their signed consent documents by email or post. On receipt of the signed consent documents, participants will be sent the study supplies by mail.

Stool sample collection

Stool samples will be collected with the Stool Nucleic Acid Collection and Preservation Tubes collection kit (Cat. 63700, NORGEN BIOTEK CORP, Thorold, Ontario).⁵⁴ We have developed written stool collection instructions (online supplemental material section 2) and videos designed to target this study population. Instructions will be provided in both written and video formats with animated graphics. Stool samples will be transported to the laboratory facilities by prepaid courier service or the research assistant (figure 2), where they will be deidentified, labelled, barcoded and stored until further analysis.

Gut microbiota analysis

DNA will be extracted from the collected stool with the ZymoBIOMICS DNA Miniprep Kit (Zymo Research Corp, Irvine, California) and used for deep shotgun metagenomics. Data will be used for both taxonomic and functional analysis (HUMAN2).⁵⁵ Negative and positive controls, including commercial and in-house synthetic communities, will be included. Sequencing will be run at the Centre for the Analysis of Genome Evolution and Function at UToronto.

Dietary assessment

Children's diet will be assessed using the Automated Self-Administered 24-hour (ASA24) Dietary Assessment Tool for Canada, developed by the National Cancer Institute of the National Institutes of Health (NIH).⁵⁶ ASA24-Canada-2018 is an adaptation of ASA24 to reflect Canadian food availability and serving sizes, and it is automatically linked to the Canadian Nutrient File 2015.⁵⁷ Food group equivalents will be used as diet indicators in this study. Children will be asked to provide two non-consecutive recalls with parent assistance. A recent TARGet Kids! study including parents of children aged 4–15 years from the cohort showed the feasibility of using ASA24 for parent proxy-reporting of children's intake and yielded intake estimates comparable to those from national surveillance.⁵⁸ Prior research suggested that children between the ages of 9 and 11 years may not be able to successfully complete the recall alone.⁵⁹ Though, a study in children aged 10–13 showed that they can report some foods and beverages using ASA24-Canada-2018 without assistance in 41 min.⁶⁰ Accuracy has been found to vary with age, with children aged 8–9 years being less accurate reporters of past diet than older children (10–11 years).⁶¹ Studies in preschoolers show that parents can accurately report children's diet, especially when they are present during the meal.^{62 63} However, children's dietary intake changes during adolescence³⁵ and the accuracy of parental reporting might be different for older children.

Anthropometrics

Anthropometric measurements (weight, height and waist circumference) will be obtained from participants during a visit to each clinical site as per TARGet Kids! cohort participant enrollment and follow-up protocol⁴⁶ or during the study home visit following the same standardised procedures as at the clinical sites.⁶⁴ At home, measurements will be taken by a trained dietitian using a digital flat scale for mobile use (Seca model 876) and a stable stadiometer for mobile height measurements (Seca model 217). The anthropometry equipment that will be used in the MiGrowD study will have the same technical specifications as in the rest of the TARGet Kids! cohort. Waist circumference will be measured at the waist midway between the hip bones and the ribs using a measuring tape. Participants will be asked to remove their shoes and any heavy clothing before each measurement. Weight will be measured to the nearest 0.01 kg while height and waist circumference to the nearest 0.1 cm. BMI, weight and height-for-age z-scores will be calculated using the WHO growth standards and *igrowup* R package.⁶⁵

Pubertal assessment

Pubertal stages will be determined with a self-assessment tool based on Tanner Stages 1–5.^{66 67} The tool includes line drawings of breast, genital and male and female pubic hair for each of the five Tanner stages, with an accompanying description.⁶⁸ Separate versions have been created

for male and female participants that will be completed by children only or children with parental assistance.

Home visits

For participants recruited from home, two trained team members will go to the participant's home to conduct all study procedures and receive the stool sample (figure 2). A home visit procedure and safety manual were developed by the research team.

Consent and capacity assessment

Informed consent will be obtained from TARGet Kids! cohort participants who consented to be approached for future research. Eligible participants who attend scheduled clinic visits will be approached by the research assistant at each site. The research assistant will provide children and their parent/guardian with both a written and verbal description of the study, and they will obtain written informed consent and assent from the parent and children, respectively. Research assistants from all TARGet Kids! sites will be thoroughly trained on the MiGrowD study procedures, and they will also be introduced to the basic concepts around gut microbiota and health to be able to answer participant questions. Participants who have opted for a home visit will be asked to provide consent for the home visit and the MiGrowD study ahead of the visit.

Reimbursement

The family will be provided with a CAD \$10 gift card for their participation, and the children will receive a certificate of participation that will be sent on receipt of the stool sample, the pubertal questionnaire and the two 24-hour dietary recalls.

Response to the COVID-19 pandemic

In response to the COVID-19 pandemic, the home recruitment approach was developed, with a transition to entirely remote study procedures. The home visit will take place virtually using Zoom HealthCare for the consent/assent discussion and capacity assessment. Documentation of consent will be obtained either electronically or by regular mail. Participants will be mailed the study supplies. To minimise in-person visits, the collection of anthropometric measurements will be moved to a self-collection model (figure 2). Study procedures will be adapted using the home recruitment approach with the following modifications:

Recruitment

A stepwise recruitment model will be followed to approach participants remotely. First, the contact information of all potentially eligible participants will be located by the research manager of the cohort. TARGet Kids! research assistants will approach each eligible family over the phone and verbally provide information about the nature and the objectives of the study. If interested, a video conference will be scheduled between the child

and the parent/guardian and the two MiGrowD study coordinators.

Remote study visits

Screening, capacity assessment and informed consent will be conducted during the video conference or the phone call. Verbal consent will be obtained before each virtual study visit. Capacity assessment will be conducted remotely, respecting children's rights and liberty.^{69 70} Participants will sign the consent form electronically, and they will receive the stool sample collection kit, the study instructions and the pubertal assessment questionnaire via regular mail. Video conferences will be conducted as per SickKids institutional guidelines. Specifically, secure internet access will be used, and the audio and video will not be recorded. If families do not have internet access or if it is their preference, a phone call will be used for the capacity assessment and informed consent process. The consent form has been adapted to include the remote study procedures.

Anthropometrics

Mothers will measure children's weight, height and waist circumference at home. Measurements will be taken a total of four times for each measure on the same day and at the same time. Parents will be provided with an illustrated guide with detailed instructions on self measurements, which has been developed based on the University of Guelph's Guelph Family Health Study⁷¹ instructions for anthropometry at home and the practical guide for the identification, evaluation and treatment of overweight and obesity in adults from the NIH.⁷² Measurements will be collected within 3 months before or after stool sample collection.

Data management

All data and questionnaires will be captured and curated^{73 74} online using the Research Electronic Data Capture (REDCap) application⁷⁵ hosted on the St. Michael's Hospital server. A new MiGrowD study REDCap project will be hosted in the Applied Health Research Centre of the Li Ka Shing Knowledge Institute of St. Michael's Hospital—Unity Health, the data coordination centre of the TARGet Kids! cohort.⁴⁶ The database stores information from all the participating TARGet Kids! sites, allowing research assistants to enter data directly into the database. Instruments (ie, data entry forms in REDCap) will be separately created to capture participant demographics, screening information, pubertal assessment, stool sample tracking and protocol deviations. Quality control procedures will be implemented to ensure the accuracy of the study records. These procedures include warning and reminder messages, range control, the use of stop actions or branching logic to specify instrument fields based on the answers to specified questions. Data collected using paper-based questionnaires will be reviewed by the MiGrowD study coordinator and manually entered in REDCap. ASA24-Canada-2018 responses

will be stored on the NIH research portal (<https://asa24.nci.nih.gov/researchersite/>).

Statistical analysis

As mentioned above, we have previously identified growth patterns in this population. The corresponding growth data and trajectory features (eg, slope, area under the curve) will be integrated with diet and microbiota data using general linear models, allowing for multivariable associations, including Microbiome Multivariable Associations with Linear Models.⁷⁶ Additional exploratory analyses will be performed to investigate the relationship between specific bacteria taxa, growth, pubertal development, and diet. The statistical analysis plan will be adjusted to incorporate the latest advances in gut microbiota data analysis.

Ethics and dissemination

The MiGrowD study has been approved by the Hospital for Sick Children (REB number 1000059644), St. Michael's Hospital—Unity Health (REB number 18–252) and The University of Toronto (REB number 00036627). Our dissemination plan includes sharing the results with (a) participants and their caregivers, (b) TARGeT Kids! primary healthcare providers, (c) the TARGeT Kids! research team, (d) our funding agencies (Joannah and Brian Lawson Center for Child Nutrition, Faculty of Medicine, University of Toronto) and (e) the academic community. The MiGrowD study results will be shared with the TARGeT Kids! primary healthcare providers and research team. Finally, study results will be disseminated through peer-reviewed publications and conference presentations.

Patient and public involvement

Patients are not involved in the design, or conduct of our study, but they were included in our result dissemination plans. Specifically, stool sample analysis results will be summarised and given to participants by the TARGeT Kids! primary healthcare providers scientists via participation in the TARGeT Kids! science and physician meetings. Furthermore, participant safety is a priority; we provide detailed instructions for safe, and convenient stool sample collection (online supplemental material section 2) and we adapted our study approaches as a response to the COVID-19 pandemic (figure 2).

DISCUSSION

The MiGrowD study will examine the relationships between diet, microbiota and growth during puberty in a large prospective cohort. The study will follow two procedures; an in-person visit (conducted either in a clinic or through a home visit) or, alternatively, a remote study visit as a response to the COVID-19 pandemic. This is a cross-sectional study and we are inviting children who will mainly be in the initial stages of puberty. Though, taking advantage of an ongoing longitudinal cohort,⁴⁶ MiGrowD will be, to our knowledge,

the first investigation linking growth patterns since birth with gut microbiota at puberty onset. This is important because the maturation of the gut microbiota and its host proceed in parallel, but investigation on how they influence each other has focused largely on the first years of life. In the specific population under study, we have identified three trajectories for body mass index-for-age z-score,⁴⁵ which we expect to be reflected in the MiGrowD population and which will allow us to better understand the relationship between the gut microbiota with overweight and obesity during adolescence. This is particularly important since childhood obesity rates tripled over the last 30 years and 2 million children aged 5–19 years are currently living with overweight or obesity. In Canada, obesity rates increase during the pre and early teen years, from 9 (8.5%) to 14 (13%) years of age.⁷⁷

MiGrowD will also help to establish relationships between these specific patterns of growth with gut microbiota phenotypes at different pubertal stages. Pubertal children with different trajectories of growth since birth may carry a microbiota at different stages of maturation. The MiGrowD study may provide a 'pubertal baseline' to be followed up prospectively within the TARGeT Kids! cohort throughout adolescence and beyond. However, potential limitations exist in that, while sex-specific questionnaires will be used, self-reporting of pubertal development may be less reliable than clinical assessment⁷⁸ and may, thus, introduce bias. A recent microbiota study (N=89) using Tanner staging assessment grouped children into prepuberty and puberty.³⁴ In comparison, our study will include a higher number of children. Additional bias may also be introduced in the anthropometric measures since these may be taken either at the clinic or home by a trained research assistant or by parents. Though, while the same standardised protocol will be applied as explained in the Methods section, the TARGeT Kids! cohort has found agreement between measurements taken by trained research staff and parents at home (unpublished). It should also be acknowledged that our sample size calculation only provides an estimate of the number of children that may be needed. As is common to several microbiome studies, previous data are not available in this cohort, and thus we based our calculation on a published study, as explained above. However, we will have the possibility to run an internal pilot study using the first 100 stool samples collected. A stop-and-go approach⁷⁹ has been proposed for pilot studies to inform trials and can potentially be implemented in microbiome studies as well.

In conclusion, this work will provide the opportunity to investigate the relationship between growth patterns across the full spectrum of early and mid-childhood and gut microbiota at puberty. Our approach may be generalisable to other settings, including low-income and middle-income settings, since the stool sample collection system used here simplifies the collection, transportation and storage of samples. MiGrowD may establish a foundation to design strategies targeting the microbiome to support health throughout childhood.

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1 **Supplementary material section 1: MiGrowD study screening questionnaire****MiGrowD Study Screening Questionnaire**

Subject study ID: _____

Date: ____/____/____
Month Day Year

Pre-consent was obtained?

☐ Yes ☐ No

In the following questionnaire we would like to ask you a number questions about your child's health during the last 6 months. You will need approximately 4 minutes to complete it.

To be enrolled in this study, the participant needs to meet all the following criteria. If you reply "Yes" to any of the exclusion criteria you might be temporarily excluded and invited again to participate, 1-3 months later.

Inclusion Criterion

1. Is the child enrolled in TARGet Kids?
2. Is your child between the ages 8-12 years?

☐ Yes ☐ No☐ Yes ☐ No**Exclusion Criteria**

1. Has your child used antibiotics (i.e. Amoxil, Vibramycin, Augmentin) during the last 3 months?
2. Has your child recently used any stool softeners or laxatives (treatments for childhood constipation i.e. PEG 3350, Lactulose, Milk of magnesia, Senna) during the last 3 months?
3. Has your child used probiotics or prebiotics during the last 3 months*?
Other supplements.....

☐ Yes ☐ No☐ Yes ☐ No☐ Yes ☐ No

Has your child had any episodes of abdominal pain or gastrointestinal disorders in the last 3 months?

☐ Yes ☐ No

4. Does your child follow a special diet or has any dietary restrictions?

☐ Yes ☐ No

If yes indicate what kind of diet (i.e. weight loss program, gluten free diet etc) or restriction (i.e. lactose, gluten, etc.).....and for how long.....

Is the child eligible to participate in the study? (filled by the study personnel)☐ Yes ☐ No

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7 **Supplementary material section 2: Stool collection instructions**

8

Collection Instructions


 *Before you start:*


- ✓ Write the date and time of collection on the tube and on the label requisition form
- ✓ If possible, urinate (pee) in the toilet first
- ✓ Wash your hands


 *Keep in mind that you should **NOT**:*


- ✓ Mix toilet paper, water, urine or soap with the sample
- ✓ Do **NOT** drink the liquid from the tube. If any of the liquid touches your skin or eyes, rinse thoroughly with water


Are you ready to start?


 **Step 1:** Put on the gloves. Ms. Poop doesn't enjoy being touched!


 **Step 2:** Put the cellophane over the toilet seat.


 **Step 3:** Ms. Poop is eventually here! Now, let's make sure she has a safe trip! Once you are ready, you can poop directly onto the cellophane!


 *Don't forget that you have to poop on the cellophane!*

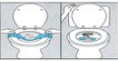
 **Step 4:** Carefully unscrew the cap of the tube without spilling the liquid.


 **Step 5:** Use this spoon to scoop 3 small samples from different areas of Ms. Poop.

 **Step 6:** Transfer Ms. Poop into the tube until the liquid reaches the line where it says "Fill To This Line" (**Do not over fill**).

 **Step 7:** Tighten the cap and shake gently until **Ms. Poop** is thoroughly mixed with the liquid.

 **Step 8:** Clean up any mess on the outside of the tube with the Alcohol Swab

 **Step 9:** Throw away the cellophane.

 *Wash your hands!!!*

9