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***Periodontal disease and alterations in the oral and vaginal microbiome communities among gravidae chewing xylitol-gum in Malawi: Microbiome Alterations with Xylitol (MAX) in pregnancy pilot STUDY***

A research proposal submitted in fulfillment of a mentored research fellowship

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# BACKGROUND, PROBLEM STATEMENT AND JUSTIFICATION.

Oral diseases present a growing global health burden. By 2022, the WHO estimated that 3.5 billion people had oral disease three-quarter of whom reside in low-and middle-income countries.1 It further estimated that at least 19% of the global adult population had a severe form of periodontal disease i.e. clinical loss of tooth attachment. Periodontal disease or periodontitis is a chronic inflammation of teeth supportive structures that may result into loss of teeth as well as systemic consequences. Although there have been variations on its definition in research, of recent, it’s become widely acceptable by researchers and dental societies that it is a clinical diagnosis characterized by bleeding gums on probing, presence of pockets or clinical loss of attachment. 1, 2 A recent meta-analysis that included studies that defined periodontal disease using standardized diagnostic criteria estimated a general prevalence of 40% in pregnant women. 3 Prevalence was highest (67%) by bleeding on probing followed by 42% and 24% by pocket depth ≥4mm and clinical attachment loss of ≥4mm respectively. Pregnant women are at higher risk of periodontal disease, and this is thought to be due to the excess oestrogen and progesterone through immune modulation. 4

Periodontal disease (PD) in pregnancy adversely affects pregnancy outcomes. While more studies have shown a significant association with adverse pregnancy outcomes such as preeclampsia, eclampsia, preterm birth and low-birthweight, others have failed to establish a significant association.5-7 This is attributed to inconsistencies in measurements, and weak-evidence. Similar controversies also appear in studies that have evaluated periodontal disease therapy and prevention of adverse pregnancy outcomes. 6, 8-12

Periodontal disease is thought to affect pregnancy outcomes through two mechanisms: directly through systemic dissemination of periodontal disease bacteria such *Fusobacterium nucleatum* and *Porphyromonas gingivalis* to the utero-placental unit where they cause local inflammation, and indirectly activation of acute phase response and systemic inflammation by pro-inflammatory mediators locally produced in the affected periodontium in response to pathogenic bacteria or dysbiosis. 13, 14

Despite a general global reduction in deaths in children under-five years of age and neonates, neonatal mortality (deaths within 28 days of life) contributes the most. Neonatal mortality contributed to 47% of all 5 million under-five deaths in 2019, being a 40% increase from the year 1990.15 The majority (43%) of these deaths occurred in Sub-Saharan Africa. Yet, Malawi’s estimated neonatal mortality rate of 29 per 1000 livebirths in 2017-2020 is relatively higher than that of Sub-Saharan Africa (27 per 1000 livebirths) and that of the world (17.5 per 1000 livebirths) in 2019.15, 16 Preterm birth, birth asphyxia and other intrapartum-related complications, infections and congenital defects are the most common causes of neonatal mortality.17

Preterm birth (PTB), defined as birth before 37 completed weeks of pregnancy is a leading cause of perinatal mortality worldwide, occurring most commonly in low- and middle-income countries (LMICs).17,18, 19 The World Health Organization (WHO) estimates that 13.4 million preterm births occurred in the year 202020, 90% of which is thought to occur in low-and-middle income countries.15, 21 Preterm birth and related complications were the most prevalent cause of all under-five deaths ranging from 6.3-25.7% amongst ten countries with the highest number of under-five deaths. 22 Multiple mechanisms and risk factors such as uterine overdistention, vascular disorders, stress, decline in progesterone, cervical diseases and maternal infections, including periodontal disease to mention a few, have been proposed to lead to PTB.23,24-26

Malawi is the country with the highest PTB rate in the world, occurring in up to 29.7% of all births.18, 19, 27, 28 Gravidae in Malawi also are known to have high rates (76.7%) of periodontal disease (PD), a chronic infection of the gingival tissue, highlighting the importance of developing innovative strategies to treat maternal dental infections and inn return, prevent associated PTB.29, 30

Studies evaluating the traditional treatment (*e.g.* dental scaling and root planing) of PD during pregnancy have demonstrated inconsistencies in preventing PTB and other adverse pregnancy outcomes.6, 9 Furthermore, our literature search failed to yield studies that have evaluated the impact of xylitol on periodontal disease severity in pregnancy while studies evaluating how xylitol affects periodontal disease have been conducted in the general, non-gravid populations at large. 31-34 Xylitol is a natural sugar alcohol with prebiotic properties known to prevent dental caries by reducing *Streptococcus mutans* growth.35, 36 Additional studies have shown that xylitol prevents dysbiosis of the oral microbiome and subsequently improves oral health. Aagaard and colleagues studied an innovative treatment, xylitol-containing chewing gum in pregnancy in Malawi (PPaX trial)37.

In the PPaX trial, Aagaard et al. found gravidae who chewed xylitol gum had significantly lower occurrence of PTB and low birthweight (<2500 gram) offspring with a concurrent significantly reduced maternal PD burden compared to gravidae who did not chew xylitol gum. Yet, the mechanism of action for how xylitol led to these beneficial pregnancy effects is unknown and can only be hypothesized as no biologic specimens were obtained during the PPaX trial.

Given the ground-breaking findings of the PPaX trial, we seek to explore the impact of xylitol-containing chewing gum during pregnancy on periodontal disease severity and the associated oral and vaginal microbiome alterations. Additionally, we will also evaluate its impact on gut microbiome of their newborns. Alterations in the maternal microbiome would provide further biologic evidence explaining the results from the PPaX trial and underscore xylitol as an economical and impactful intervention to prevent PTB.

# HYPOTHESES

We hypothesize that in gravidae who chew xylitol-containing chewing gum three times daily throughout the course of pregnancy starting prior to 20 weeks’ gestation through delivery, as compared to gravidae who chew a placebo, non-xylitol-containing chewing gum of same frequency and duration will have:

1. Improved periodontal disease severity in the third trimester of pregnancy and at 6 weeks postpartum.
2. Altered oral microbiome communities, specifically a reduction in abundance of pathogenic periodontopathic and cariogenic bacteria (i.e., *Porphyromonas gingivalis*, *Streptococcus mutans*, among others).
3. Changes within the diversity of the gut microbiome communities of the newborns at 6 weeks postpartum.
4. Reduced localized inflammatory mediators at the gingival level, as assessed via gingival crevicular fluid.
5. Higher abundance of vaginal bacterial commensal species (i.e., Lactobacillus) and reduced abundance of vaginal bacteria pathogenic species (i.e. Gardnerella).

# OBJECTIVES

## Main objective

* To determine the impact on periodontal disease severity, describe associated alterations in oral and vaginal microbiome communities, evaluate changes in localized inflammation at the gingival level, and evaluate changes in the neonatal stool and oral microbiomes comparing gravidae who chew xylitol-containing chewing gum as compared to a placebo (non-xylitol-containing chewing gum) initiated in the first 20 weeks of pregnancy.

## Specific objectives

1. To determine the impact of chewing xylitol-containing gum on periodontal disease severity in pregnancy using a periodontal disease score, and
2. Evaluate alterations in oral microbiome communities in pregnant women who chewed xylitol-gum compared to controls via 16S gene sequence analysis, and
3. Evaluate alterations in vaginal microbiome communities in pregnant women who chewed xylitol-gum compared to control via 16S gene sequence analysis, and
4. Explore alterations in the relative abundance and diversity within the oral and gut microbiome communities in newborns whose mothers chewed xylitol- containing gum via 16S gene sequence analysis as compared to those born to mothers who chewed the control (placebo) gum, and
5. To examine the impact of chewing xylitol-containing gum on localized inflammatory mediators at the gingival level, as assessed via gingival crevicular fluid in gravidae who chewed xylitol-containing gum compared to control.

# LITERATURE REVIEW

Oral diseases are prevalent in adult populations. In spite variations in populations, how studies define and measure periodontal disease, periodontal disease is highly prevalent in the pregnant population and ranges from 0-61%.6 Of late, it has become widely acceptable that periodontal disease can be diagnosed clinically with presence of either bleeding gums on probing, pocket depth ≥4mm or/and clinical attachment loss of ≥3mm.2 In addition, while bleeding on probing may be acceptable clinically as a sign for gum inflammation, this may be inaccurate and saliva biomarkers and advanced imaging may increase early detection where available. A recent meta-analysis estimated a general prevalence of 40% of periodontal disease in pregnancy by reviewing studies that used standardized diagnostic criteria above. 3 Several studies have evaluated effect of periodontal therapy on periodontal disease status and adverse pregnancy outcomes and have yielded conflicting results. 6, 8-12 While there appears to be an improvement in oral health with PD treatment, this does not appear to significantly prevent adverse pregnancy outcomes. This can be attributed to the type and timing of therapy initiation, usually in the second trimester when inflammatory mediators may already have reached the utero-placental unit. Non-surgical methods such as root planning and scaling are the common therapies that have been evaluated in pregnancy population, but our literature search did not find studies that evaluated xylitol as therapy for PD in pregnancy. Xylitol is a natural sugar alcohol with prebiotic properties known to prevent dental caries by reducing *Streptococcus mutans* growth and preventing dysbiosis thus improving periodontal disease and oral health in the general population.34-36 Due to lack of literature on xylitol as PD therapy in pregnancy, we aim to evaluate its impact on PD severity and associated alterations in oral microbiomes.

Preterm birth is a global burden with significant contribution to under-five mortality worldwide, two-thirds of which occur spontaneously.19, 20 Several pathologic mechanisms such as uterine overdistention, cervical disease, vascular disorders, decidual senescence, decline in progesterone action, stress, infection and idiopathic causes amongst others have been shown to lead to PTBs.23 Infections may lead to an inflammatory cascade triggered by recognition of microbials and their products by pattern recognition receptors such as toll-like receptors leading to increased production of chemokines, cytokines, prostaglandins and proteases and thus a common pathway to labour and delivery. Vaginal and oral microbiomes have been found in amniotic fluid and placentae of gravidae who developed spontaneous preterm births therefore suggesting a causal association.24, 30, 38-41 The link between vaginal microbiome is further elucidated by a more recent meta-analysis in which pregnant women presenting with low-lactobacilli (increased diversity of anaerobic or a mixture of aerobe and facultative anaerobe bacteria) vaginal microbiome had 1.69 higher risk of preterm delivery compared to those with *Lactobacillus crispatus* dominance.40 In addition, Aagard and colleagues24 have previously documented that the placental microbiome profile tends to be most similar to the oral microbiome. Several other studies have found similar findings and an association between periodontal disease and risk of preterm birth. 25, 26, 30, 39, 42 Other adverse effects of periodontal disease in pregnancy include low birthweight, intra-uterine growth restriction, small for gestation age, preeclampsia, and perinatal mortality. 9, 42

Numerous studies have been conducted to identify interventions for prevention of preterm labour and births, and recently, large, randomised studies brought a confusion regarding progestogens in preventing PTBs in singleton pregnancy with a previous history where they found no significant association.43-45 In the setting where periodontal disease is a risk factor, conventional treatment of periodontal disease has produced controversial findings.8, 10-12 While some of these studies showed significant improvement in periodontal disease and reduced occurrence of PTB and/or low birthweight, other studies’ results did not support this association.8, 10-12 This is thus an area needing more quality research to identify safe, accessible, and affordable treatment options.

Xylitol-containing chewing gum has recently been shown to be a novel, affordable and accessible treatment option for periodontal disease in pregnancy that significantly improves periodontal disease and reduces risk of PTB and/or low-birthweight when initiated below 20 weeks of pregnancy.37 Yet, the mechanisms through which xylitol affect these associations have not been documented. A recent meta-analysis has shown that habitual use of xylitol-chewing gum may have plaque-accumulation reducing effects and improve overall oral health. 33 However, further rigorous studies are warranted to confirm this.

# METHODOLOGY

## Study design

This is a triple-blinded (data collectors, data analysts and participants) pilot study where pregnant women <20 weeks of pregnancy will be individually randomized to receive daily xylitol (intervention arm) or sorbitol (control arm) chewing gum for the remainder of their pregnancy. Dental evaluation (including oral microbiome and inflammatory mediator sampling) and vaginal sampling will be conducted at study enrolment (<20 weeks’ gestation), 28-30 weeks’ gestation, at delivery/within 1 week of delivery and 4-6 weeks after delivery. In addition, newborns’ meconium/stool and oral swab samples will be taken at birth/within 48 hours and at 4-6 weeks after birth.

Individual randomization will be done by participants using a group of opaque envelopes, inside of which there will be study arm allocations to either A or B. A or B will be the designation to receive either xylitol or sorbitol chewing gum, and only the PI will be aware of which group is intervention and which is placebo. A list will be kept secure in an online, encrypted database that is not accessible to the data collectors, data analysts or participants to ensure robust methods are being utilized to prevent accidental unblinding.

## Place of Study

The study will take place at Area 25 health centre (HC) which serves a population of 250,000 people. Area 25 HC was a cluster within the original, parent PPaX trial and, thus, includes gravidae with the same demographics. Area 25 HC provides obstetric surgeries and consultant services with at least 3500 deliveries annually.

## Study population and eligibility

Gravidae attending antenatal care services at Area 25 HC will form the study population.

The study inclusion criteria are:

* + Age ≥ 18 years old, able to provide informed consent.
  + A singleton at <20 weeks’ gestation (based on ultrasound or best obstetric measurement)
  + Planning to deliver at Area 25 health center.
  + Willing to chew two pieces of gum thrice daily for 10 minutes after the morning, day and evening meals throughout pregnancy.
  + Willing to undergo at least two dental exams including oral microbiota sampling at study enrolment <20 weeks of pregnancy, 28-30 weeks, at delivery/within 48 hours and 4-6 weeks after giving birth.
  + Willing to have at least two vaginal sampling at study enrolment <20 weeks of pregnancy, 28-30 weeks, at delivery/within 48 hours and 4-6 weeks after giving birth.
  + Able to speak Chichewa or English.
  + Cognitively aware enough to be able to participate in the study.
  + Willing to consent to all required aspects of protocol including allowing collection of placenta specimens, infant oral swab and meconium/stool sampling at birth/within 48 hours and 4-6 weeks after.

Exclusion criteria

* + Not meeting the inclusion criteria as listed above.
  + Those who upon screening and enrolment but dislike the taste of the gum and state they will not chew the gum throughout pregnancy.
  + Gravidae with known or suspected non-viable pregnancy (including life threatening congenital anomalies such as cardiac, neurological or others).
  + Pregnant individual has a life-threatening diagnosis such as cancer requiring treatment during pregnancy.
  + Pregnant women with a known or suspected morbidly adherent placenta (such as placenta accrete, increta and percreta).

## Timing and order of visits

Participants will be enrolled and followed-up as per below study schematic. Dental assessments will be done in accordance to the WHO Oral health Assessment Form Adults (Appendix 4).



Visit 1: Recruitment, consent, and enrolment:

* Recruitment will take place in the antenatal clinic at area 25 where all attending gravidae at gestation age < 20 weeks will be approached by study team member.
* Consent will then be obtained which will include consenting for oral, vaginal and placental samples of mother and meconium and stool sampling of the neonate.
* Those approving the taste of the gum and consent to the study, will then be individually randomized into either arm and enrolled into the study. A sticker will be placed on their health passports for ease of identification in subsequent visits/hospital interactions e.g., in case of preterm labor.
* Vaginal sampling (including microbiome assessments from the introitus and posterior fornix and vaginal pH assessments) will be performed by a study staff while dental assessment including subgingival plaque, oral swab and gingival crevicular fluid sampling will be done by study dental officer.
* An enrolment questionnaire which includes demographic and medical data, and dental assessment will be filled and later entered in REDCap electronic database.
* A 1 month’s supply of gum in an unlabeled container (for blinding) will be supplied in every visit, including routine non-study visits.

Visit 2: Follow-up visit at 28-30 weeks of pregnancy:

* Vaginal sampling (including microbiome assessments from the introitus and posterior fornix and vaginal pH assessments) will be performed by a study staff while dental assessment including subgingival plaque, oral swab and gingival crevicular fluid sampling will be done by study dental officer.
* A follow-up questionnaire with above details including adherence check will be filled.
* A 1-month re-fill of gum supply will be offered.

Visit 3: Within 48 hours of delivery:

* Vaginal microbiome sampling (both at the introitus and posterior fornix) and only the maternal oral swab, neonatal oral swab and meconium sample will be collected. The placenta specimens will be obtained by a study staff member.
* A follow-up questionnaire with above details including details on labor and delivery will be filled.
* A 6-week appointment date will then be scheduled, and a reminder phone call/text will be done a week prior.

Visit 4: 4-6 weeks post delivery

* Vaginal sampling (including microbiome assessments from the introitus and posterior fornix and vaginal pH assessments) will be performed by a study staff while dental assessment including subgingival plaque, oral swab and gingival crevicular fluid sampling will be done by study dental officer.
* A 4-6 week follow-up questionnaire with the above details, mother’s health and that of the infant will be filled.

## Sample size and sampling technique

The primary outcome will be maternal periodontal disease (any periodontitis or gingivitis) diagnosed at 28-30 weeks’ gestation. The decision to not assess outcomes at 6 weeks’ postpartum is due to the known impact of pregnancy-induced gingivitis that rapidly changes after delivery. Therefore, study subjects that deliver preterm will likely have arbitrarily decreased rates of periodontal disease compared to those who deliver at term simply due to less duration of pregnancy. By assessing at 28-30 weeks’ gestation, we will evaluate a change from study entry to the third trimester with daily xylitol use, assessing >8 weeks of xylitol use during pregnancy. We have chosen a sample size and allocation ratio of 1:1 (xylitol-containing chewing gum:non-xylitol gum base) so that we have 80% power to detect a 50% relative difference in prevalence of periodontal disease between groups at 28-30 weeks’ gestation using the ACOG-published findings that the composite oral disease rates approach 77% in Malawi pregnant and recently postpartum women  (as well as  and based upon published data demonstrating 54% reduction in dental caries with xylitol dosing >4 grams/day.33, 46, 47 Notably, the PPaX trial demonstrated a 30% reduction in periodontal disease with 2 grams/day of xylitol, and meta-analysis demonstrated trials using a xylitol dose <3 grams/day demonstrated substantially reduced efficacy (~17% reduction) compared to using dosages >5g/day.37 Therefore, it is consistent and biologically plausible to have a >50% reduction in periodontal disease prevalence (*i.e.,* periodontal disease 75% control compared to 36% in xylitol group at 28 weeks’ gestation) with our planned 6g/day xylitol dosing.

## Data collection techniques and tools

Eligible subjects will be informed of the scope and purpose of the study, and those that are interested in participating will be consented, enrolled, and given a study number at enrollment.

Specific subject information will be collected via review of the medical record(s) routinely collected at the antenatal clinic, admission and delivery at Area 25 Hospital, and referral hospitals (i.e. Kamuzu Central Hospital or Bwaila) for those who will be referred there for delivery and filled into health questionnaires associated with this study (appendix 3).

Samples will be collected as outlined below (in specific sections) and will be stored and extracted at the Baylor Center of Excellence, Lilongwe, Malawi.

All sequencing and data analysis will occur at the University of Washington and/or Baylor College of Medicine.

1. **Screening and enrolment** of eligible pregnant women attending antenatal clinic in area 25 HC will be approached by research staff. Using a translator, if necessary, study personnel will inform eligible subjects of the purpose of this study. The sample collection and questionnaire involved will be described and the subjects will be asked if they would like to participate. The subjects will be made aware that their care will not be altered because of their participation. No access to care will be withheld or changed if subjects decline participation. Full disclosure of the nature of participating in the study will be made. In addition, the consent form (Appendices 1 and 2) will include a statement regarding long term storage of the samples. For interested participants, consents will be obtained, dental evaluation and the initial samples collected. Participants will be given a month’s supply of gum and refills will be done at their regular antenatal visits. They will also be given appointments for follow-up visit at 28-30 weeks.
2. **The intervention and placebo:** Participants will be randomized into either arm ([1] 6.36grams of daily xylitol; Epic dental gum, 1.06 grams Xylitol per piece of gum; chew two pieces for 10 minutes after meals, thrice a day or [2] Gum base; Epic sorbitol-containing gum (placebo); chew two pieces for 10 minutes after meals, thrice a day) by randomly picking from a group of opaque, sealed envelopes containing group allocation. Participants, data collectors (i.e. dental officers, study staff members, clinical officers), and data analysts will be blinded (triple-blinded) to group allocation throughout the study.
3. **Maternal** **Oral cavity sampling**: This will be done at enrolment (or the next day), at 28-30 weeks, at birth/within 1 week of delivery and after 4-6 weeks of delivery by a dental officer. However, subgingival plaque and gingival crevicular fluid sampling will not be done at birth/within 1 week of delivery. Dental evaluation will be done in accordance to Malawi guidelines, similar to the WHO guidelines for dental assessment in adults (Appendix 4).48 Three samples will be collected in each visit, an oral swab, a sub gingival plaque and gingival crevicular fluid except a birth. Gingival crevicular fluid sampling will done first using paper points (after first removing supragingival plaque), followed by oral swab and subgingival plaque sampling. The dental evaluation will occur after the collection of samples to prevent any potential contamination that may occur due to disruption of the plaque or blood contamination of the GCF paper during the oral and periodontal assessment.
   1. Collection: Gingival Crevicular Fluid (GCF)
4. Instruct the participants not to eat, drink (even water), chew gum or brush teeth for at least one hour prior to appointment.
5. Label collection tubes.
6. GCF samples will be collected at each study visit from both control and test sides.
7. GCF samples will be collected from mesiobuccal and mesiopalatal surfaces of 3 teeth per side.
8. At each study visit, a total of six samples per study side will be collected and pooled into a single sterile tube. Specifically, we will use the mandibular quadrants for this examination (2 quadrants per participant per examination).
9. The sites to be sampled will be isolated with cotton rolls to avoid the contamination of the samples with saliva.
10. GCF samples collected with sterile paper points which will be inserted slowly using a sterile cotton plier into the gingival sulcus until mild resistance is felt.
11. The paper point will be left for 30 seconds, then it will be carefully removed without touching the adjacent tissues.
12. Then paper points will be placed into a sterile collection tube.
13. Paper points visibly contaminated with saliva and blood will be excluded from the study.
14. Samples will be transported to the lab on ice.
15. Samples will be eluted in 200 μl sample diluent (Bio-Rad Laboratories, Hercules, CA, USA) with 0.5% bovine serum albumin (Blocker™ BSA (10X) in PBS; Waltham, MA, Thermo Scientific, USA) and then stored at -80˚C until processing.
16. GCF samples will be assayed using commercially available Bead-based Multiplex Immunoassay, Bio-Plex Pro™ Human Chemokine Panel (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's protocol.
    1. Collection: Subgingival Plaque
17. Wear a surgical mask and eye protection (goggles or a face shield) to avoid contact with the splatter of dental plaque, calculus, or saliva.
18. Ask participant to rinse their mouth with water to remove any food particles.
19. Sampling will be performed in three maxillary (upper quadrant) molars that have the largest pocket depths. Subsequent sampling at future visits will be performed on these same teeth to evaluate for progression or improvement in disease status or oral health.
20. Isolate the sites from the buccal mucosa by placing sterile dental cotton rolls in the buccal vestibule related to the tooth. Note that the buccal vestibule is the junction between the teeth and the interior of the cheeks or lips.
21. Before collecting the samples, scrape the supragingival plaque or tooth surface biofilm above the gingiva to avoid cross- contamination with the subgingival plaque sample.
22. For each site to be sampled, using a sterile swab, swab the buccal (facial/ outer/external) surface of the tooth as deep as possible into the gingival sulcus, the thin space between the tooth and the surrounding gingiva. Start at the nearest part of the sulcus to the jaw midline (mesiobuccal) and slowly pass to the farthest part of the sulcus away from the midline (distobuccal) .
23. Place the swabs from all the sampled teeth into same PowerBead tube containing 750 μL of MoBio buffer in an appropriately labeled specimen collection tube. The swab should be pressed against the tube wall multiple times for 20 seconds to ensure transfer of bacteria from swab to solution.
24. Store all the tubes in ice chest and deliver to the clinical laboratory within approximately two hours.
    1. Collection: Oral Swab
25. Swab over the entire gingival line (the most superior aspect of the gingivum in the lower jaw and most inferior in the upper jaw) twice and the entire buccal mucosa (left and right) four times, being careful to cover all surfaces of the swab.
26. Immediately after swabbing, the swab is swirled in 750 μL of specimen collection buffer in an appropriately labeled PowerBead tube. The swab should be pressed against the tube wall multiple times for 20 seconds to ensure transfer of bacteria from swab to solution. The timing will be done using digital watches.
27. Store all the tubes in ice chest and deliver to the clinical laboratory within approximately two hours.
28. **Infant oral swab:** This will be done at delivery/within 1 week of giving birth and at 4-6 weeks after. The oral swab will be done by swabbing the entire gingival line (both top and bottom) twice and swabbing the entire buccal mucosa (left and right) 4 times. Samples will immediately be placed in MoBio DNA extraction tubes and homogenized for 30 seconds to transfer bacteria for extraction.
29. **Vaginal sampling:** Two vaginal swabs; one from the vaginal introitus and posterior fornix via a Puritan® sterile foam tipped applicators, will be collected by clinical providers at enrolment at < 20 weeks of pregnancy, at 28-30 weeks, at birth/within 1 of delivery and 4-6 weeks after delivery. The nucleic acid will be stored in PowerBead tube containing MoBio buffer media and transferred to the laboratory for extraction.

Collection: Aseptic technique will be used for collection of all specimens:

* + - * 1. Collect clinical information.
        2. Spread labia to visualize vaginal introitus immediately posterior to hymenal ring/tissue.
        3. Determine vaginal pH at the introitus using Litmus paper.
        4. Proceed with obtaining vaginal introitus specimen: place one Sterile Puritan® Sterile Foam Tipped Applicator (Puritan Medical Products Company LLC, USA) at the vaginal introitus posterior to the hymenal ring/tissue and rotate swab along the lumen with a circular motion five times. Immediately after swabbing, the swab is swirled in 750 μL of buffer in the labeled specimen collection tube (PowerBead tube). The swab should be pressed against the tube wall multiple times for 20 seconds to ensure transfer of bacteria from swab to solution.
        5. Insert speculum. It is preferable that the speculum be used without lubricant or water. If, in the opinion of the clinician, it is not feasible to insert the dry speculum without causing extreme discomfort to the participant, the exterior of the tip of the speculum may be moistened with tap water, taking care to use as little water as possible to avoid diluting the vaginal fluids being collected and to avoid any impact on pH measurement.
        6. Visualize cervix and posterior fornix.
        7. Proceed with obtaining posterior fornix specimen: place one Puritan® Sterile Foam Tipped Applicator (Puritan Medical Products Company LLC, USA) in the posterior fornix and rotate the swab along the lumen with a circular motion five times. Immediately after swabbing, the swab is swirled in 750 μL of collection buffer in an appropriately labeled PowerBead tube. The swab should be pressed against the tube wall multiple times for 20 seconds to ensure transfer of bacteria from swab to solution.
        8. Store all the tubes in ice chest and deliver to the clinical laboratory within approximately two hours.

1. **Infant meconium/stool sampling:** Samples of meconium at delivery or within 48 hours after birth and stool at 4-6 weeks after delivery. Mothers will be instructed to notify or bring diapers/local cloth used once meconium/stool has passed. Using sterile swab, the assistant will scoop the meconium/stool and place it in a MoBio DNA extraction tube.
2. **Placenta specimens:** Around the time of birth and under sterile conditions, we will collect three cuboidal samples comprising of a 1 cm x 1 cm x 1 cm sections of the cord, the decidua, and the chorion (3 samples per placenta). These will then be put into 2 mL screwtop tubes and then stored at -80C as a biorepository until future analyses are funded.
3. **DNA extraction:** All microbiome samples will be stored and extracted at the Baylor Center of Excellence in Area 33. DNA will be extracted using the MoBio PowerSoil DNA isolation kit by study personnel using well-established protocols (MOP available upon request). As the resources for DNA sequencing and analysis are not available at the Baylor Center of Excellence, extracted DNA samples will be shipped to Baylor College of Medicine in Houston, Texas, USA for metagenomic sequencing and analysis. Samples will be shipped as extracted DNA labelled in a deidentified fashion to protect subject confidentiality.

*Sequencing overview:* The predominant metagenomics sequencing strategy will focus on 16S rDNA 454 sequencing. This takes advantage of existing and fully functional technology and pipelines with proven reproducibility and quality reads in a self-sampled pediatric population. In addition to bacteria, archaea are surveyed by the addition of different primer sets to amplify archaeal 16S rDNA sequences, and fungi are identified by 18S rRNA gene/ITS-based sequencing. Sequencing of the bacterial 16S rRNA gene targets will be performed as we have previously extensively published on the Roche 454 GS Titanium sequencing platform. A detailed presentation of metagenomics DNA sequencing, informatics/biostatistics, and DNA sequencing approaches is outside the scope of this protocol but suffice it to say we are employing our present and proven methodology for sequencing, informatics pipelining, and analysis. All data are maintained on secure servers, in a coded (deidentified) fashion.

*Bioinformatics overview:* Read data will be filtered and analysed so that accurate taxonomic assignments may be made. These are then mapped onto know taxonomic or phylogenetic trees or identified as novel based on consensus elements. Further details are provided in the data analysis aspects of this protocol.

Key outcomes of interest**:** The primary outcome of interest will be the changes in periodontal disease at 28-30 weeks’ gestation. Secondary outcomes will include the following: (1) periodontal disease at 6 weeks postpartum, (2) alteration in the maternal oral microbiome communities or their inferred metabolic function at (a) 28-30 weeks and (b) delivery, (3) microbial community shifts in mother’s oral microbiome, (4) inflammatory mediator changes in the maternal gingival crevicular fluid, (5) alterations within the maternal vaginal microbiome communities, (6) change within the infants’ oral microbiome and (7) gut microbiome communities.

Analysis plan**:** To measure periodontal disease severity, we will create scaled periodontal disease score comprising of sum of scores for gingival bleeding (+1 if bleeding was present, per tooth), gingival pockets (+1 for pockets of 4-5 mm and +2 for 6 mm or deeper, per tooth), and loss of attachment (+1 for 4-5 mm loss, +2 for 6-8 mm, +3 for 9-11 mm, and +4 for 12 mm or more, per tooth with values recorded for index teeth) divided by the number of teeth present will be created for every dental visit.37 A score of >0 will indicate presence of periodontal disease.

We will utilize generalized linear mixed methods (GLMM) with binomial distribution to examine the association between study primary outcome of periodontal disease and assignment to the study intervention group (xylitol-containing chewing gum or placebo gum). A separate model will be fit for each study secondary outcome and corresponding relative risk with 95% confidence intervals (CI) estimated. Full information maximum likelihood estimation (FIML) allows for analyses to be performed with the full sample, under conditions of Missing Completely at Random (MCAR) and Missing at Random (MAR). In these analysis the fixed effects consist of treatment (xylitol vs placebo), time (before, after treatment) will be modeled as random effects. Robust SEs calculated through sandwich estimators were presented to account for the different measurement variance across time points (before and after treatment). In the presence of a significant F test for time and treatment interaction, Scheffe’s-like adjustment will be used for multiple comparison. Generalized linear mixed method analyses will be conducted in SAS, R, or STATA using the GLIMMIX procedure.

**For microbiome and metagenomic analyses, we will use a two-layered analysis strategy.** Bayesian and multipartite analysis will alternately be employed for determination of inferred causation.

We will collect microbial specimens. We will extract nucleic acids from at the time of specimen collection and subsequently subject such immediate extractions to flash freezing for later batched-run metagenomic sequencing and analysis.

*Creating input data*: QIIME is utilized to produce OTU tables from the quality-filtered sequences. The generated OTU tables combined with the clinical metadata comprised the data matrix used as input for alpha diversity (biodiversity within a group of samples), beta diversity (biodiversity between groups of samples), and machine learning pipelines. Quality filtered sequences are analyzed using three standard microbiome analysis techniques: OTU generation, phylogenetic tree construction, and taxonomic binning of classified sequences. Unique reads are classified with the MSU RDP classifier v2.2, and normalized data are produced from the relative abundance of taxa present in each sample based on a naïve Bayesian classifier. Output sequences are classified as domain, phylum, family and genus. Beta diversity analysis will be employed on incorporating non-phylogenetic and phylogenetic distance metrics calculated based on normalized OTU table. Each distance metrix will be analyzed by Principal Coordinates Analysis (PCoA).

*Supervised learning approaches for 16S analysis:* We have demonstrated that machine learning algorithms are useful in determining the strength of meta data clusters as well as listing the most important variables involved in discriminating two groups of samples (feature selection). The algorithm randomForest enables classification of groups of samples by constructing a classification tree, randomly sampling the predictors, choosing the best splitting variables, and predicting new data by combining the predictions from all trees to estimate the error rate and list the highest performing variables, then applying R package Boruta to explicitly perform feature selection. Linear discriminate analysis effect size (LEfSe) is a novel method developed to support high dimensional class comparisons in metagenomics analysis. LEfSe combines the standard tests for statistical significance (Kruskal-Wallis test and pairwise Wilcoxon test) with linear discriminate analysis for feature selection.

*Inferred metagenomics:* A hybrid approach that marries 16S rRNA gene sequence data with functional gene databases, allowing for the inference of metagenome function from 16S gene sequence data, is known as “inferred metagenomics” (http://picrust.sourceforge.net/). Using our rich collection of 16S rRNA gene libraries on all samples and shotgun metagenomes on more limited samples we will evaluate the potential of 16S-based functional inference in the context of obstetric fistula, before and after surgical repair. Using our annotated shotgun metagenomes as a “gold standard”, we will evaluate the ratio of inferred vs. predicted (annotated in the metagenome) KEGG orthologs, modules, and pathways. Should the 16S-based inferred functions be a reasonable match, we will compare their performance relative to their matched metagenomes in our functional metagenome correlation- and network-based analyses (see Data Integration below).

*Whole genome shotgun (WGS) sequencing:* Typically, the Illumina HiSeq library and sequence production and QC pipeline in the Baylor College of Medicine Human Genome Sequencing Center can generate data for 1-100 metagenomic samples over a four-week span depending on the sample type and multiplexing/sequencing depth desired; ergo, sequencing capacity will not limit this project. Pipeline controls will be in place to monitor the integrity of samples and data. DNA from a Mock Community and PhiX will also be sequenced as a periodic pipeline control. These measures minimize bias in DNA sequencing and post-sequencing analyses.

Metabolic reconstruction identifies microbial gene pathways. In our WGS work flow pipeline, HUMAnN (the HMP Unified Metabolic Analysis Network) determines the presence/absence and abundance of microbial pathways from metagenomic data. The reconstruction of network is accomplished by mapping the protein coding genes onto reference pathway collections, such as eggNOG and KEGG orthologous groups, based on their homology to reference genes with previously characterized functions. The raw BLAST hits will be further refined by applying BLAST against genes to get weighted sum of hits. MinPath adopts integer programming algorithm to reconstruct “minimal pathway”, which is defined as given a list of functions annotated for a set of genes, find the minimal set of pathways that include all given functions. This approach avoids the problem of identification of spurious pathways and overestimation by microbial abundance (“data normalization and smoothing”); filtering at the pathway level will remove organism pathways not in the original sequences. Missing rare genes in the abundant pathways will be imputed in manual curation to fill in the gaps as we have previously described. Finally, pathway coverage (relative confidence of each pathway being present in the sample) and pathway abundance (relative "copy number" of each pathway in the sample) will be generated and organized into matrix like format for post processing. Although the current version of HUMAnN is based on KEGG Orthology, any catalog or orthologs can be employed to represent presence/absence and abundance of pathways (such as eggNOG or metaCYC).

*Data integration:* The clinical phenotype data has the potential to inform how obstetric fistula affects the vaginal microbiome and may be used in the future to understand the role of the vaginal microbiome in overall health and healing.

However, these high-level traits represent the consequences of a complex array of molecular, genetic, microbial, and environmental factors acting over time. The integration of multi’omics data therefore is needed to elucidate the molecular basis of the physiology of the condition of interest (obstetric fistula), as well as the microbiome “trait” (species, genus, or composite profile) which is or is not associated affected by obstetric fistula. While the pair-wise correlation analysis between each level of omics data tell us about bidirectional correlations, they cannot get at the more sophisticated points of complex networking and certainly not at multi-directional predictions over time and with environmental or treatment perturbations. To take the most out of multilevel omics data, we will employ several advanced data integration approaches in addition to correlation analysis.

Weighted co-expression networks can be applied to large-scale ‘omics data. By organizing patterns of correlations into groups in which the component parts are more interconnected with each other than they are with components outside of the group, high-dimensional data will be organized into coherent groups that are very useful for further interpreting the data. For example, different groups of genes/metabolites (commonly referred to as network modules) that emerge from this analysis can inform specific pathways and omics features that associate with at-risk clinical traits (obstetric fistula before and after repair) and with intermediate phenotypes (such as a differential expressed microbial gene or metabolite). Modules having biomarkers with similar patterns may function similarly enabling inferred function based on the same module “neighborhood markers”. For each module, network of various types of multi’omics data will be constructed to visualize the relationships among them. These more advanced views simultaneously consider all possible relationships among diverse types of data to provide the most informative molecular features with respect to clinical traits, and as a result the biomarkers of interest may be more informative than those obtained from the simple pairwise results.

*Bi- and tri-partite analysis:* In addition to robust statistical analysis, bipartite network based visual analyses enable us to acquire an intuition of the molecular and phenotypic relationships in the data, while reducing the overhead and bias of assumptions inherent in most quantitative methods. This intuition has been shown to rapidly lead to insights about the underlying biological mechanisms involved in the disease. The visual patterns will then be used to guide the selection of quantitative methods whose assumptions match the patterns observed in the data.

To move beyond association into finding high-confidence causality targets, Bayesian network algorithms which integrates bacterial taxa/ metagenomes/RNA/metabolites/clinical traits is one effective way to treat metagenomic variations as the systematic source of variation from which causal relationships can be inferred. Using these approaches, we can understand not only that sets of traits are highly interconnected with one another, but we can resolve how they are causally related. In order to avoid inherent bias of WGS interpretation by annotation platform, as an alternative to MetaPhlan and HUMAnN we will also use the automated analysis platform metagenomics-RAST server (MG-RAST) to process the WGS data. Granger Causality modeling over a small number of time points (3 in this cohort) with very high dimensional data scored (like multi’omics combined with clinical data), can be employed as an alternative to Bayesian networks to resolve direction of information flow.

# Dissemination of results

We plan to publish the results in a peer-reviewed medical journal where other physicians providing care for women with preterm birth and/or periodontal disease may benefit from reading the information. Results will also be presented in national and international scientific conferences. All participant information will be de-identified when published.

# Ethical consideration

A full disclosure of the nature of participating in the study will be made and there will be no change in accessibility and routine standard of care. If a participant chooses not to continue involvement after enrolment, she will have the option to express this and she will continue to receive care at the center as needed. Should a participant withdraw from the study before the 28-30 weeks follow-up, a new participant will be recruited. In addition, the participants will be compensated $10 at enrolment, at 28-30 weeks, at delivery, and at the completion of the study (4-6 weeks postpartum), per NHSRC guidelines.

# Personnel roles and institution

1. Gregory Valentine, University of Washington – Co-investigator/Co-Primary mentor
2. Jeffrey Wilkinson, MD OBS/GYN Baylor College of Medicine, Global Women’s health – Co-Primary mentor
3. Benjamin C Shayo, MD OBS/GYN BCM Kamuzu Central Hospital – Principal Investigator/mentee (research fellow)
4. Jessie Mlotha-Namarika, Head Department of Dentistry, Kamuzu Central Hospital – Co Investigator/mentor
5. Chikondi Chiweza MBBS OBS/GYN, Area 25 health Center – Co Investigator
6. Maxim Seferovic, PhD, baylor College of Medicine – Co-investigator/mentor
7. Peter Milgrom, DDS - University of Washington – Co-investigator/ mentor
8. Kris Kerns, PhD – University of Washington – Co-investigator

# Work plan

January – April 2024: Participant recruitment, and specimen collection.

March – November 2024: Follow-up visits and specimen collections.

January – February 2025: Gene sequencing and Data analysis

March - May 2025: Complete results write up and submit for publication.

# Budget

This project is part of mentored research fellowship that is conducted by the mentee as part of his fellowship. All specimen collection kits, extraction and analysis resources will be provided freely by Baylor College of Medicine and the University of Washington. In addition, all the mentors and co-investigators will not be receiving additional funds other than their institutional salaries. Transportation of extracted DNA specimens to the University of Washington, USA will follow established institutional mechanisms with the Baylor College of Medicine – Malawi (BCM). In total, 2,330$ will be required for this study.

**Study personnel**

Apart from the PIs and co-investigators, 2 research assistants who are clinicians in area 25 HC, are already employed and receive a salary through BCM. Hence, we will not be paying salaries or wages through the project. However, we will pay dental officers to perform dental examinations at a rate of 5$ per participant for a total of 250$

**Supplies**

The chewing gums are donated free of charge to Dr Valentine.

In addition, Dr Shayo is afforded secretarial services and stationeries by his employers (Baylor – Malawi) that can be used for the project as needed. He is also in possession of a laptop and can procure through his work, the necessary software required for analyses and manuscript write-up. However, there will be stationeries and pens required for field work for the assistants i.e., 80$

**Laboratory costs**

The 16S gene sequence analyses will be done in the University of Washington, DC. The samples will be stored locally and for free at a BCM local laboratory biobank in Baylor Centre of Excellence in area 33 before shipment to Washington following finalization of sample collection. DNA extraction will be done by Dr Shayo in collaboration with laboratory staff at the Centre of Excellence who are already receiving a salary from their employer.

**Participant compensation**

We will reimburse participants with 40$ each as compensation costs for involvement in the study i.e., 40$x50 = 2,000$References

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# Appendices

## Appendix 1: Consent Form (English)

**Background and Purpose**

Researchers from Baylor College of Medicine, the University of Washington and Area 25 Health Centre, Malawi are studying the connection between pregnant women using a specific type of chewing gum (one with a harmless sugar called xylitol) and the health of the teeth and mouth, as well as the bacteria that live in her mouth and vagina. We are also interested in seeing how chewing this gum during pregnancy impacts the development of the bacteria commonly residing in the mouth and stool of the baby.

Our bodies normally carry around trillions of bacteria – these bacteria live in many places on and inside our bodies, such as the skin, mouth, gut, and vagina. It is completely normal and healthy to have bacteria living inside you. While we still don’t know how they do it, many of these bacteria help keep us healthy, while others contribute to diseases such as inflammation of the tooth gums (also called periodontal disease) and are associated with preterm birth, low birthweight children, and other diseases of pregnancy that affect both the health of the mother and the baby.

The purpose of this study is to understand if chewing xylitol-gum before 20 weeks of pregnancy and continued until delivery affects the bacteria that are found in your mouth and vaginal cavity, the health of the tissues in the mouth, and the bacteria in the mouth and gut of newborns. Not all women who participate in this study will receive xylitol chewing gum, others will receive a non-xylitol chewing gum. The chance of you receiving xylitol or non-xylitol chewing gum is a flip of the coin (just as likely as not likely) but it will not be revealed to you whether you are receiving the gum with xylitol or the gum without xylitol. All women who are confirmed to be pregnant and before their 20th week may participate in this study.

**Procedures**

Participation in this study is completely voluntary (up to you). Choosing to participate or not participate will not affect your health care in any way. If you agree to participate in this study, you will need to do the following things:

1. You will randomly pick from a group of opaque envelopes, and in it will be the gum group (xylitol vs non-xylitol gum) allocation. This group allocation will be known ONLY to some of the researchers.
2. Provide information about your health history including some details regarding previous and current pregnancy and delivery. You will also be asked to provide your contact information as well as name and contacts of a person or relative that we may reach out to in case we cannot reach you for appointment reminders and other study related matters that may arise.
3. Participate in dental exams at Area 25 health center and allow oral samples to be taken by dental officers at four time points during and up to 4-6 weeks after your pregnancy. The first will be when you start the study and prior to initiating the chewing gum. The second will be during your 7th month of pregnancy (28-30 weeks of pregnancy), the third at delivery or within 48 hours of delivery, whereas the last dental exam and sampling will be done at 4-6 weeks after delivery.
4. Allow researchers to collect samples from your vagina at four time points through pregnancy to 4-6 weeks after delivery, just as the dental exams and sampling; and preferably at the same date.
5. Allow researchers to collect samples from your placenta at the time of delivery and store it for future analysis (Biorepository).
6. Allow researchers to collect oral, meconium (first stool) and stool samples from your baby. This will take place at birth or within 48 hours of delivery, and at 4-6 weeks after delivery, just as your last two visits as well. You will be given a stool sample collection container in your third visit (at birth or within 48 hours of delivery) so you can collect morning stool sample on the day of your fourth visit (4-6 weeks after delivery).
7. Chew two pieces of the provided gum throughout pregnancy for 10 minutes three times a day. To help keep the timing with your meals, we will ask you to chew two pieces of gum once in the morning after the morning meal, two pieces after lunch and lastly, two pieces of gum after the evening meal. Let gum be the last thing you eat at night. Gum will be provided to you free of charge on your every visit (monthly) scheduled with the research staff, which is in line with routine antenatal clinic visits. Please also continue to take care of your teeth and gums how you normally do.

*How will my (and my newborn’s) samples be stored?*

Samples and the DNA extracted from the samples will be stored for future analysis in a secured laboratory associated with the investigators in the study, and this may require transfer of the specimens to a laboratory in a different country such as the United States (such as the University of Washington and/or Baylor College of Medicine). To protect your confidentiality, samples will only be labeled with a unique study identification number (study ID). We will keep your study ID linked with your name in our local records (locked and secure) for up to 20 years after the end of the study.

*What will my (and my newborn’s) samples and information be used for?*

We will use your samples to understand how chewing gum shapes the bacteria that are found inside you and your baby. To determine which bacteria are in your samples, we will extract the bacterial DNA and sequence (determine the genetic code) of the DNA. The results of research, including gene sequences of bacterial DNA, may be stored in a way that the public can access them. When we extract and sequence the DNA from your samples, some of your human DNA may be included also. While we will make every effort to remove your human DNA from the bacterial genetic data to make it very hard for anyone who looks at the data to access your human DNA, there is a chance some that your human DNA will be made available on restricted or publicly available websites. Some limited health information may also be stored with the results, like your age, and gender. This may help researchers all over the world understand what factors influence the bacteria living inside the human body. No identifying personal information (like your name or address) will be stored with the results. It is also possible that your sample will not be used in any other research.

*How long will my (and my newborn’s) samples be kept? Will I have access to the results from these samples?*

Your samples will be kept until they are used up. Samples will not be sold but may be shared with other researchers around the world. If they are, no identifying information that could link the samples to you will be shared. We will not be able to provide the results for individual subjects participating in this study.

If you agree to have the DNA sequences from your samples publicly released, neither your name nor any other information about you will be released. Nobody will be able to know just from looking at the public database that the DNA sequence belongs to your sample. However, because some of your human DNA may remain in your sample and because the genetic code in your DNA is unique to you, there is a small chance that someone could trace the information back to you or your close biological relatives and determine information about you and your health. There are only a few ways this could be done. First, if somebody thought your information might be in the database and they were able to get another sample from you, they could do many tests on those samples and compare the genetic information from those tests with the information in the database. Second, if a relative thought your information might be in the database, they could compare their own DNA, which is similar to yours, with the information in the database and may be able to trace the information back to you or your family. Finally, if a hacker violated the security of the computer that stores the codes linking your information to your name or a researcher accidentally discloses the codes, somebody might be able to access your information. The risk of any of these things happening is very small but could possibly grow in the future. However, releasing your sample information into the database also can helps the scientific community to speed the scientific research process.

Participant Costs and Payments

You will not be asked to pay any costs related to this research. If you choose to participate in this study, you will receive 10,000MWK at each visit required for completion of your participation in the study. This can partly be used as your transport reimbursement to/from Area 25 health center for your study visits.

PLEASE INDICATE (TICK) WHETHER YOU AGREE TO UNRESTRICTED USE OF YOUR DEIDENTIFED SAMPLES AND HEALTH INFORMATION FOR FUTURE RESEARCH:  
\_\_\_\_\_\_\_\_\_ I consent to unrestricted use of my oral, vaginal and placental samples (and my newborn’s oral and meconium/stool samples) as well as health information.  
\_\_\_\_\_\_\_\_\_ I do not consent to unrestricted use of my oral, and vaginal samples (and my newborn’s oral and meconium/stool samples) as well as health information.

\_\_\_\_\_\_\_\_\_ I consent to giving my contact and secondary contact information.

Your signature (or thumb print) on this consent form means that you have received the information about this study and that you voluntarily agree to participate in this research study.

Signature\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_Date\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Research Staff Name: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_Signature:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

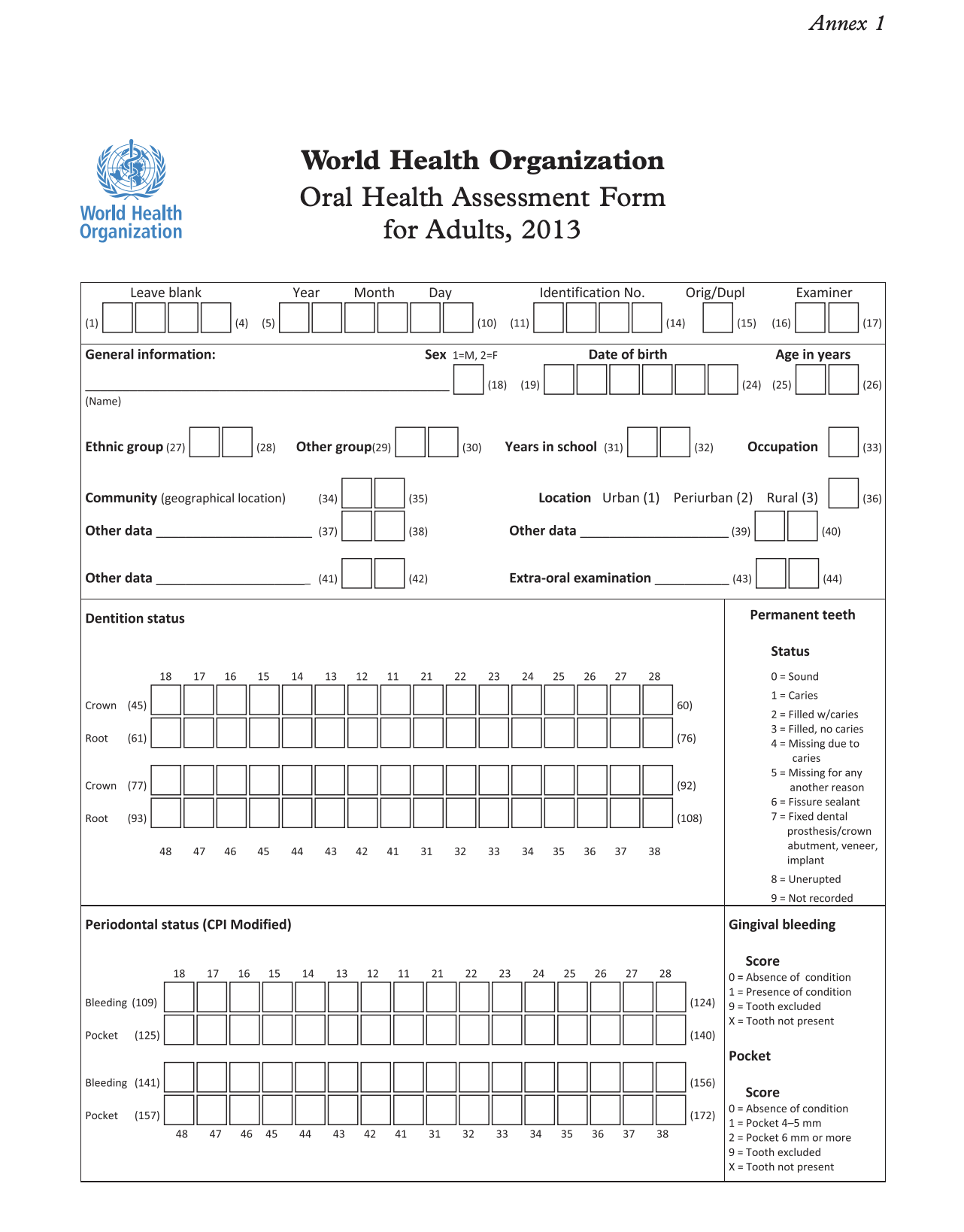
## Appendix 2: Consent Form (Chichewa)

See attached separately.

## Appendix 3: Study Questionnaire

See attached separately.

## Appendix 4: WHO Dental Assessment in Adults

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