Comparison of clinical and sociodemographic characteristics and placental microbiome in women with undernutrition versus normal nutritional status at Bungoma County referral Hospital

(A Comparative Cross-Sectional Study)

This dissertation is submitted in partial fulfillment for the Award of Degree in Master of Medicine in Obstetrics and Gynecology at the College of Health Sciences, University of Nairobi.

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DEDICATION

Special dedication to my sons Johnson and Jeremy,

My parents, John kihagi and Margaret Wangari,

My lovely siblings Ann, Grace,Regina, Janet, Beatrice, Samie, Anthony, and Charles,

My spiritual director Fr George,

and my teachers, colleagues, and friends who have supported me immensely during the dissertation project

ACKNOWLEDGEMENT

With utmost gratitude and humility of heart, I thank Almighty God for guiding me through this research. I also would love to thank my supervisors' Dr. Diana Ondieki and Prof Moses Obimbo, who have guided me in the application of Basic Sciences in Research in Obstetrics and Gynecology. I also thank Dr. Jesse Gitaka of Mount Kenya University, who has greatly helped me to understand the interpretation of the genetic sequencing data. I am also thankful to Mr. Patrick Mwaura (KAVI technician) and Mr. Mohammed Abdi (statistician), who have been of invaluable assistance in my study. Lastly, I thank the entire faculty and colleagues for their various contributions.

DEFINITION OF OPERATIONAL TERMS

Metagenomics Study of the entire genetic material of organisms isolated from environmental samples by use of culture dependent or independent principles

Microbiome The entire genetic material within the entire collection of microorganisms in a specific niche (in this study the placenta)

Undernutrition Lack of proper nutrition caused by inadequate dietary intake or a diet lacking essential nutrients evidenced by a BMI of less than 18.5 kg/m^2 and a MUAC less than 210 mm with a recent weight loss.

LIST OF ABBREVIATIONS

BMI	Body Mass Index
BCT	Basic Clinical and Translational Laboratory
BCRH	Bungoma County Referral Hospital
CI	Confidence Interval
DNA	Deoxyribonucleic Acid
IL	Interleukin
KAVI	Kenya AIDS Vaccine Initiative
KDHS	Kenya Demographic Health Survey
MUAC	Mid Upper Arm Circumference
OTU	Operational Taxonomic Unit
QIIME	Quantitative Insights into Microbial Ecology
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
SOP	Standard Operating Procedures
TNF	Tumor Necrosis Factor
WHO	World Health Organization

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ABSTRACT

Background: Undernutrition remains a major burden in low-income countries and is considered a significant risk factor for morbidity and mortality among pregnant women and younger children. It impacts the immune system, especially through thymus gland atrophy with subsequent reduction in immature T-cells. During pregnancy, the placenta plays a critical role of transfer of nutrients from maternal blood to the fetus, hormone synthesis, and immune protection, among others. The effect of undernutrition on placental biology is little studied. Several bacteria in the phyla Tenericutes and Proteobacteria have been identified in the placenta,endometrium and the membranes and their impact is not clearly understood. The study aimed at comparing the placental microbiome in pregnant women of normal nutritional status versus pregnancy affected with undernutrition.

Objective: To compare the clinical and sociodemographic characteristics and the microbiome from placentas of women with undernutrition versus those with normal nutritional status in pregnancy.

Methodology: In this comparative cross-sectional study we studied 23 bio banked placentas from pregnant women with undernutrition and 25 from pregnant women with normal nutritional status. DNA extraction from 25 mg of thawed and minced placenta per sample was done at the Kenya AIDS Vaccine Initiative (KAVI) laboratory strictly following Burton's protocol. The DNA samples were coded and transported to Macrogen Laboratory in Amsterdam, Netherlands for the next generation sequencing of the 16s RNA gene .The data on the clinical, sociodemographic and reproductive characteristics was derived from the clinical data forms. This data was entered into password protected excel sheets and analyzed using SPSS version 26. The Chi square test of association and/or Fisher's test for the categorical variables while independent sample T-test/Mann Whitney test was used for continuous variables. A P-value of <0.05 was considered statistically significant.

Results

The level of education, placental and neonatal weights were significantly lower in pregnant women with undernutrition, while parity, age, and marital status were not significantly different between the 2 groups. The odds of being anemic, given that one is undernourished in pregnancy was high with an ODDs ratio of 17(3.84-76.43)and a p-value of <0.001. No microbiome was demonstrated in placentas from both groups.

Conclusion

Next Generation sequencing of the 16s RNA gene from placentas with undernutrition and those with normal nutrition fails to demonstrate presence of a microbiome. Undernutrition however is associated with low levels of education and anemia and thus there is need for nutritional counselling in pregnancy to help avert anemia which is a major indirect cause of maternal mortality.

Key Words: Microbiome, Placenta, sequencing, Undernutrition

CHAPTER ONE: INTRODUCTION

1.1 Background and Epidemiology of Under-Nutrition

Undernutrition still remains a major burden in low income countries and is considered a significant risk factor for mortality and morbidity mainly affecting hundreds of millions of pregnant women and young children (1).A pregnant woman is considered undernourished if they have a pre pregnancy BMI of less than 18.5 kg/m² and MUAC of less than 210 mm with a positive history of recent weight loss (2).Globally, there is paucity of data on the trends and incidences of undernutrition in pregnancy. In a study in Ethiopia by Kumera et al in 2018, it was established that the prevalence of undernutrition in pregnant women was 16.2%. The Kenya Demographic Health Survey 2014 indicated that 10% of women in the reproductive age were undernourished with a BMI of less than 18.5. Bungoma County had a similar prevalence of 10% (3).

Sociodemographic factors have been shown to affect maternal nutrition in pregnancy. Of the sociodemographic factors studied, lack of formal education, low level of education, extreme maternal age, not being married or the state of being married but not living with the spouse and high parity have been shown to cause an increased risk of undernutrition pregnancy (4)(5)(6)(7)

As per the World Health Statistics Report 2002, undernutrition during pregnancy was found to cause adverse pregnancy outcomes including low birth weight babies, increased susceptibility to infections, developing anemia, pregnancy losses and intrauterine growth restriction(8). Undernutrition is thought to alter the immune system as demonstrated by atrophy of the thymus gland(9). The thymus plays the role of differentiation T-cells of bone marrow origin into CD4 or CD8 cells. The differentiated T-cells play a critical role in modulating the immunology of pregnancy and this may explain the increased vulnerability to infection in the undernourished pregnant mothers. (9)

1.2 Causes of Under-Nutrition among Pregnant Women

1.2.1 Clinical Causes

The body is considered to have malnutrition when it is receiving inadequate nutrients. To maintaine adequate nutrition, the body requires water, proteins, carbohydrates, minerals,fats,

vitamins, and fiber (10). The body must be able to digest, absorb, and utilize these nutrients effectively for them to be beneficial. Infections and health conditions such as cancer, diarrheal diseases, and HIV can limit the body's ability to take in adequate nutrients and calories , making a person vulnerable to malnutrition (11) .

Another condition that can lead to deteroriation of the nutritional status is poor dental hygiene, leading to tooth decay. This limits the amount and type of food eaten (11).

1.2.2 Socio-demographic Factors

Sociodemographic factors that affect nutrition include level of education, employment status, parity, place of residence and marital status (12)(7). Pregnant women between the ages of 15-19 have been shown to be at a higher risk of undernutrition due to inadequate food intake except for meat(5).In terms of level of education, primary education or no education amongst the pregnant women has been shown to increase the risk of undernutrition 2 fold when compared with the women with secondary and tertiary education (13).The impact of education level on undernutrition has been shown to be closely associated with the place of residence. Women with basic education but living in the urban areas had a lower risk of undernutrition (12).

Women who have never been married or were initially married but currently divorced or separated have been shown to be more prone to undernutrition as compared to those women living with their spouses, probably due to better food security in the married group (7).Parity has also been shown to affect nutritional status in pregnancy. Multiparas have a 2.5 times higher risk of undernutrition as compared with their nulliparous counterparts. Other sociodemographic factors associated with undernutrition include women’s decision making autonomy, food insecurity and skipping meals(6)

1.2.3 Environmental Causes

There are higher levels of malnutrition in the rural areas as compared with the urban regions (10). Rainfall patterns, access to agricultural tools, agricultural knowledge, and human capital affect food security(11). Poor sanitation can lead to diarrhoeal diseases that lead to malabsorption which impairss nutrient absorption.

1.3 Assessment of Nutritional Status in Pregnancy (Anthropometric Measures)

There are four methods used to assess a person's nutritional status: anthropometry, biochemical assessment, clinical assessment, and dietary intake assessment. Anthropometric measurements include height, weight, mid upper arm circumference(MUAC) and body mass index(BMI)(2). Surveillance of these anthropometrics of a population identifies the major nutritional issues of the population and aids in offering interventions such as supplementation and therapeutic feeding of those affected(2).In pregnancy,MUAC measurement is more objective with consistent results since it controls for the variations that arise from the weight gain in pregnancy(2).

Malnutrition is evident on physical examination with signs like pedal edema due to pregnancy fluid retention and visible wasting. There are some biochemical tests that can be done on blood or urine tests to determine lipid, vitamin, mineral, and protein concentrations (8).Physical assessment of food intake in a certain duration of time can accurately identify the quality and quantity of an individuals' diet; though very invasive, expensive, and time-consuming.this would also need expansive biochemical and laboratorial infrastructure put in place(8).

In women of reproductive age (15−44 years),use of BMI is recommended to assess the prevalence of maternal underweight (2).

In this study, the cut-off for categorizing low nutritional status in mothers as a risk factor for perinatal deaths was chosen to be a BMI < 18.5 kg/m2, and a MUAC of <210mm based on the cut-off used in the WHO meta-analysis that derived the odds ratio (2). These measurements in our study were taken during the third trimester antenatal clinic at 36 weeks.

CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction

In the past, the intrauterine environment was thought to be sterile, but current research is focusing on the human tissue microbiome that has been demonstrated in the endometrium, basal plate, placenta and the membranes whose impact on pregnancy has not been widely studied (14)(15). Several types of bacteria in the Phyla of Firmicutes, Tenericutes, Proteobacteria, Bacteroides and Fusobacteria have been isolated from healthy placentas without histological evidence of infection or inflammation and are thought to be normal flora. This microbiome is comparable to oral cavity microbiome and is believed to spread hematogenously(16). This is further supported by the increase in rate of preterm births that has been shown in some studies to occur in pregnant women with periodontal disease(17)(16). Presence of a microbiome however remains controversial to date as some studies have failed to demonstrate presence of microbiome using culture independent methods; for instance, Sterpu et al 2021 (24) conducted a cross-sectional study on placentas harvested after term vaginal delivery and also those harvested after elective ceserean delivery and failed to demonstrate microbiome from the two groups while using next generation sequencing of the 16s RNA gene.

The placenta plays a major role in exchange of nutrients in the fetal-maternal interface as well as endocrine function. A healthy placental micro-environment is necessary for the health of the pregnancy. Dysbiosis in the placental microorganisms has been associated with adverse pregnancy outcomes such as but not limited to preterm births and low birth weight. Several factors have been shown to cause a change in the placental microbiome though their mechanisms are unclear. These include maternal obesity, excess gestational weight gain in pregnancy, gestational diabetes mellitus and the use of antibiotics and probiotics(18).

Undernutrition has been demonstrated to cause atrophy of the thymus gland. This gland has a role in immunity in terms of differentiation of T-cells into CD4 and CD8. These cells play a role in the immunology of the placenta. It can thus be postulated that atrophy of the thymus gland may predispose to infection at the placenta. There are studies that have linked undernutrition to certain sociodemographic characteristics including low level of education, maternal age less than 24 years,

not being married, multi parity and low level of income(7)(6)(12)(13).The effects of undernutrition on the placental microbiome has however not been studied.

2.2 Assessment of Placental Microbiome

Culture-independent sequencing technologies have been used to provide insight into the diversity of microbial communities that inhabit the human body as well as other ecosystems such as soil and oceans (19). Studies derived from the Human Microbiome Project indicate that different human body sites are populated by site-specific microbiota ("the assemblage of microorganisms present in a defined niche or environment" (19).

Two main approaches have been employed in the determination of tissue microbiomes: Cultivation techniques and DNA sequencing technologies. The results from these techniques are largely consistent, qualitatively(i.e. although molecular surveys of these sites typically capture far more microbial diversity than culture-based surveys, many of the prominent microbes in the molecular surveys have also been recovered through culture from these same sites (20). Samples derived from sites with a low microbial biomass such as the placenta can however give results that are difficult to distinguish from DNA present in reagents used for extraction, amplification, and sequence library preparation for molecular microbiology studies (20).

Bacterial culture of placental tissues entails inoculation of placental tissue on growth media (e.g., trypticase soy agar with 5% sheep blood, chocolate agar, MacConkey's agar) under aerobic and anaerobic conditions and used in an assay for genital mycoplasmas. This is followed by DNA extraction to identify bacteria with molecular microbiologic techniques such as the 16S rRNA gene, used widely as a phylogenetic marker to identify bacterial types present in clinical samples and metagenomic surveys that entail sequencing all of the genes in a clinical sample and assigning the protein-coding genes of bacterial origin to particular bacterial taxa.

Some of the limitations of using this approach, however, include chances of contamination during processing; this can however be limited by regular use of DNA clearing solutions on the working surface (19).

2.3 Placental microbiome composition

The sterility of the intrauterine environment and specifically the placenta still remains controversial.

In 2014, Aagard et al undertook a cross-sectional study whose objective was to characterize the microbiome isolated from the endometrium, placenta and the membranes(16). Sterile placental specimens were used.16s ribosomal DNA and whole-genome short-gun metagenomic studies were carried out to identify isolated micro-organisms.

From placentas of women who had delivered at term and had no history of ante partum infection, pathogenic bacteria belonging to the Tenericutes phylum (including ureaplasma and mycoplasma) and those from proteobacteria phylum were isolated. These bacteria were identical to bacteria isolated from the oral cavity and are thought to reach the placenta through hematogenous spread. A rich and diverse gram positive and gram negative bacteria populations, with a predominance of the lactobacillus species have since been isolated from healthy placentas using cultivation-independent techniques such as PCR and 16s RNA gene sequencing that demonstrate that placenta harbors some commensal microbiome(21)(22)(18).

Several studies recently carried out recently have failed to demonstrate presence of a placental microbiome. Theis et al 2019 undertook a cross-sectional study on 29 placentas obtained during elective caesarean delivery utilizing quantitative PCR, 16s RNA gene sequencing and metagenomics. 28 out of the 29 samples failed to demonstrate a placental microbiome (23). Similar results have been quoted by Sterpu et al 2021 and Lieby et al 2018, while utilizing similar analytical methods (19) (24)

Alteration of placental microbiome has however been shown to cause adverse pregnancy outcomes and this has been postulated to be as a result of production of pro-inflammatory mediators (22). For instance, Enterobacter, Enterococcus, Tannerella, Streptococcus and Acinetobacter species were isolated from placentas of patients who had preterm deliveries and those who had a remote

ante partum infection. This indicates that dysbiosis of placental microbiome can alter the placental micro-environment and have adverse effects on the placenta (25)(16)(26)(27).

Some of the factors that have been shown to alter placental microbiome include maternal pre pregnancy obese state, more than normal weight gain during pregnancy, gestational diabetes and use of antibiotics and probiotics(18).Their mechanisms have not been clearly understood but has been thought to be through modulation of the immune system(18). There is paucity of data available for review on the effects and/or associations of undernutrition and placental microbiome alteration. However, undernutrition in other studies has been shown to alter gut microbiome (28)(29)and also to cause atrophy of the thymus gland that has a big role in immunology of the placenta(9)(30).

The objectives of this study therefore were to determine whether a microbiota exists in term placentas, using multiple complementary modes of microbiologic inquiry: quantitative real time polymerase chain reaction (qPCR), 16S rRNA gene sequencing, and metagenomics and to compare these with the clinical and reproductive characteristics in pregnant women with undernutrition versus those with normal nutritional status.

2.4 Conceptual Framework

Narrative for the Conceptual Framework

In this study, the independent variables compared included the nutritional status indices (MUAC and BMI) of women who were undernourished and those with normal nutrition during the antenatal clinic visit at 36 weeks. The sociodemographic characteristics of these women including age, parity, marital status and level of education were also analyzed. The dependent variable analyzed was the placental microbiome isolated from both groups of women. Gestational diabetes, H.I.V and obesity, as shown in a study by Pelzer et al 2017, can directly cause alteration in placental microbiome and thus placentas from women with these conditions were excluded in our study. Gestational diabetes if complicated with gastroparesis and H.I.V infection especially if patient is not yet started on ART can cause undernutrition.

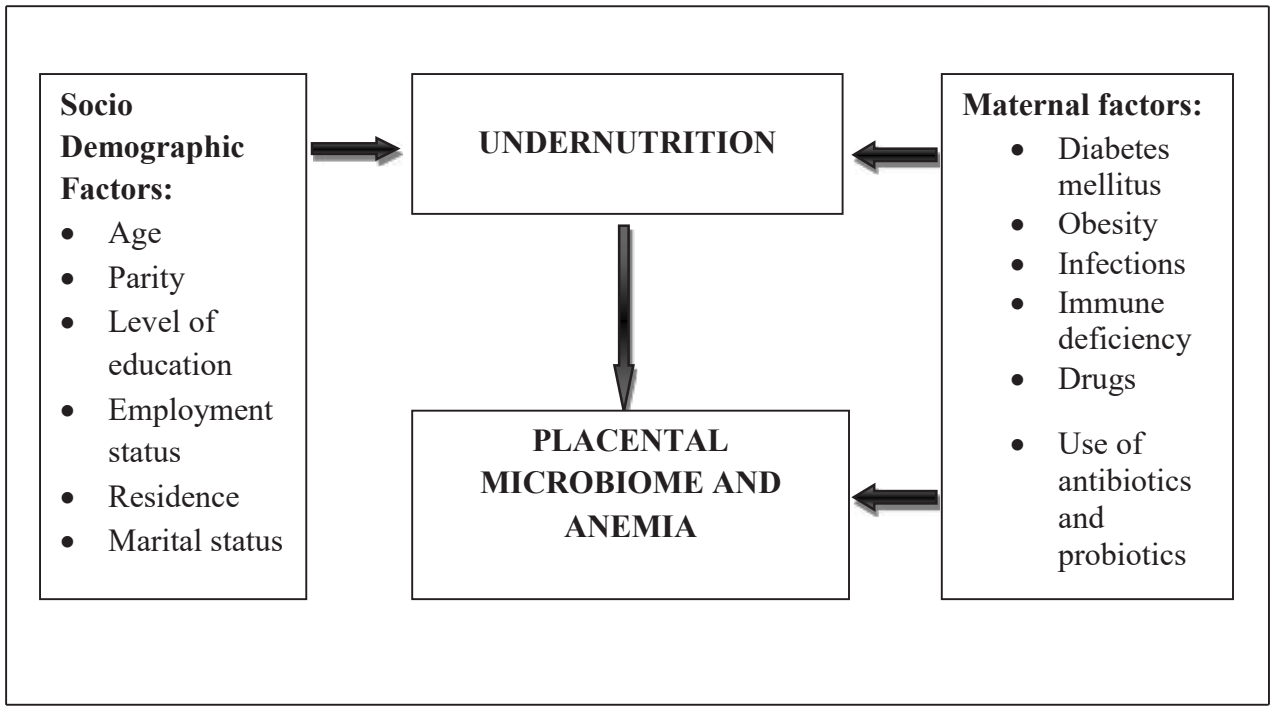


Figure 2.1: Figurative Presentation of the Conceptual Framework

2.5 Problem Statement

The prevalence of undernutrition in KENYA as per the KDHS 2014 was 10%, and 10% in Bungoma County among women in the reproductive age group. This problem more so has been shown to affect women with low level of education, low levels of income and early marriages (4)(7) and thus there is need to evaluate the impact of undernutrition on these women .

The placenta has a key function in the intrauterine fetal development including exchange of nutrients and immunity against acquisition of infections.Since the placenta has been shown by some studies to harbour some microbiome, it is important to investigate their impact on the pregnancy. Despite undernutrition being shown to cause atrophy of the thymus gland that usually plays a vital role in immunity and altering gut microbiome, its effects on placental microbiome has not been studied.

2.6 Justification

The paradigm of sterility of the intra uterine environment has been studied and the results are still inconclusive. Some studies done fail to demonstrate a placental microbiome (24) (19) (23) Others indicate that the placenta, endometrium and the membranes harbor a unique nonpathogenic microbiome whose importance has not been very well studied(16)(18). Some of the bacteria isolated from healthy placentas belong to the Phyla Tenericutes, Firmicutes, Proteobacteria, Fusobacteria and Bacteroides.

Undernutrition has been shown to increase the risk of acquisition of infection through alteration of the immune system. It has been associated with atrophy of the thymus and severe reduction in bone marrow. Wasting away of the thymus gland causes severe decrease in T-cells that modulate the immunology of placenta. Reduction in bone marrow mass causes a significant reduction in TNFα and IL-6(9)(31). These cytokines also are critical in the immunology of placenta during pregnancy. It is not clear however if undernutrition alters the placental microbiome composition as there is no data on this for review. In some studies, prevalence of undernutrition in pregnancy has been found to be as high as 16%(4).In our local set up, the prevalence of undernutrition as per the KDHS 2014 was at 10%.

There is paucity of data to associate undernutrition and pattern of placental microbiome globally.Moreover, although some studies like Aagard et al 2014 have demonstrated presence of microbiome in placenta, membranes and endometrium, the impact of this microbiome on pregnancy has remained an under-studied area.Through this research we therefore aimed to assess the influence of undernutrition on placental microbiome in Bungoma County and also assess the impact certain sociodemographic and clinical characteristics may have on the placental microbiome. The results of this study will provide novel information from our local set up that will guide future prospective studies on this problem.

2.7 Research Question

What are the differences in the clinical and sociodemographic characteristics and the placental microbiome among women with undernutrition versus those with normal nutritional status as seen at the Bungoma County Referral Hospital?

2.8 Null Hypothesis

There is no difference in the clinical and sociodemographic characteistics and the placental microbiome population in women with undernutrition versus those with normal nutritional status in Bungoma County Referral Hospital

2.9 Objectives

2.9.1 Broad Objective

To compare the clinical and sociodemographic characteristics and the placental microbiome amongst women with undernutrition and those with normal nutritional status as seen at the Bungoma County Referral Hospital between January 2018 to December 2019.

2.9.2 Specific Objectives

Among pregnant women with undernutrition versus those with normal nutritional status delivered at the Bungoma County Referral Hospital between January 2018 to December 2019

1. To compare the clinical and sociodemographic characteristics
2. To compare the microbiome composition of their placentas

CHAPTER THREE: METHODOLOGY

3.1 Study design

This was a comparative cross-sectional study design, to describe and compare the clinical and sociodemographic characteristics and the placental microbiome from bio-banked placental specimen from pregnant women both with undernutrition versus those with normal nutritional status who delivered at the Bungoma County Referral Hospital between January 2018 and December 2019.

In this study,those with concomitant medical conditions for instance diabetes, pre-eclampsia, malaria and H.I.V were excluded.

3.2 Study area and site description

The placentas for the study participants were obtained from the bio-banked specimens from a study on "Rapid and Multiplex Diagnosis of Maternal Bacterial Infections". This was a study whose aim was early diagnosis of subclinical bacterial infection that usually cause neonatal sepsis using placental specimen to allow prompt treatment of these infections to reduce neonatal morbidity and mortality. These women were delivered at the Bungoma County Referral Hospital.

Bungoma County Referral Hospital is a 216-bed capacity hospital with surgical, pediatric, medical, dental, ophthalmic, nutritional and obstetric departments. On average, a total of 1200 women are delivered at the hospital annually. This is the teaching hospital for the Kenya Medical Training College (KMTC) and Kibabii University,Bungoma county is generally cosmopolitan inhabited mainly by the Bukusu, Batura, Saboat, Iteso and Tachoni communities. DNA extraction from the bio banked placental specimen was done at the KAVI laboratory in Kenyatta National Hospital and the samples sent for 16s RNA gene sequencing at the Macrogen laboratory in Amsterdam, Netherlands.

3.3 Study population

The study population was the placental specimens collected from Bungoma County Referral Hospital in Bungoma Kenya from women with undernutrition in pregnancy versus those with normal nutritional status in pregnancy. The nutritional status of the pregnant women was assessed

by use of MUAC, measured at the midpoint between the tip of the shoulder and the tip of the elbow) and BMI during the third trimester visit as documented during the study. In this study, women with MUAC of less than 210 mm and a BMI of less than 18.5 were considered undernourished. These measurements were taken at 36 weeks at the antenatal clinic for those who met the inclusion criteria and consented to take part in the study.

3.3.1 Inclusion Criteria

The entire bio-banked placental specimens from women aged > 18 years who had consented for the study with history of undernutrition and those with normal nutritional status was eligible for this study.

3.3.2 Exclusion Criteria

Placentas from women with preexisting medical conditions including;

1. Diabetes mellitus,
2. Human Immune Deficiency Virus
3. Pre-eclampsia,
4. Malaria,
5. Premature pre-labor rupture of membranes

3.4 Sample Size Determination

The desired sample size calculation was adopted from Kelsey et al, 1996 (32).There were no studies for review on impact of undernutrition on placental microbiome from which we could make assumptions.

$$n=\frac{1.96^2\, p\,(1-p)(r+1)}{r\,(p1-p2)}$$

P1=percentage of exposed with outcome (assumed 50%)

P2=percentage of unexposed with outcome (assumed 50%)

R=ratio of population 1 to 1

P=Power assumed 80%

10% attrition

This gave a sample size of 29 per arm with a total sample size of 58. We analysed 23 samples from the undernutrition group and 25 samples from the normal nutrition group as explained in the study flow. This current sample yielded a power of 31% using the assumptions of the expected sample.

3.5 Sampling procedure

Purposive sampling of all the placental blocks from women with undernutrition in pregnancy and those from women with normal nutritional status until the desired sample size was achieved.

3.6 Data variables

Table 3.1: Data Variables

Variable	Type of variable	Coding	Source of data
Sociodemographic Factors			
Age	Exposure	<35 = 0 >35 = 1	Patient Biodata Form/File Records
Parity	Exposure	Primi para = 0 Multi para = 1	Patient Biodata Form/File Records
Marital status	Exposure	Single = 0 Married = 1	Patient Biodata Form/File Records
Level of education	Exposure	Basic = 0 Post basic = 1	Patient Biodata Form/File Records
Nutritional status			
BMI	Exposure	<18.5 = 0 >18.5 = 1	Data collection sheet
MUAC	Exposure	<210 mm = 0 >210 mm = 1	Data collection sheet
Placental Microbiome	Outcome	Presence Absence.	Bio-banked placental blocks
Anemia	Outcome	Normal =Hb ≥11g/dl Anemic =Hb <11g/dl	Patient biodata Form/File records

3.7 Study Flow

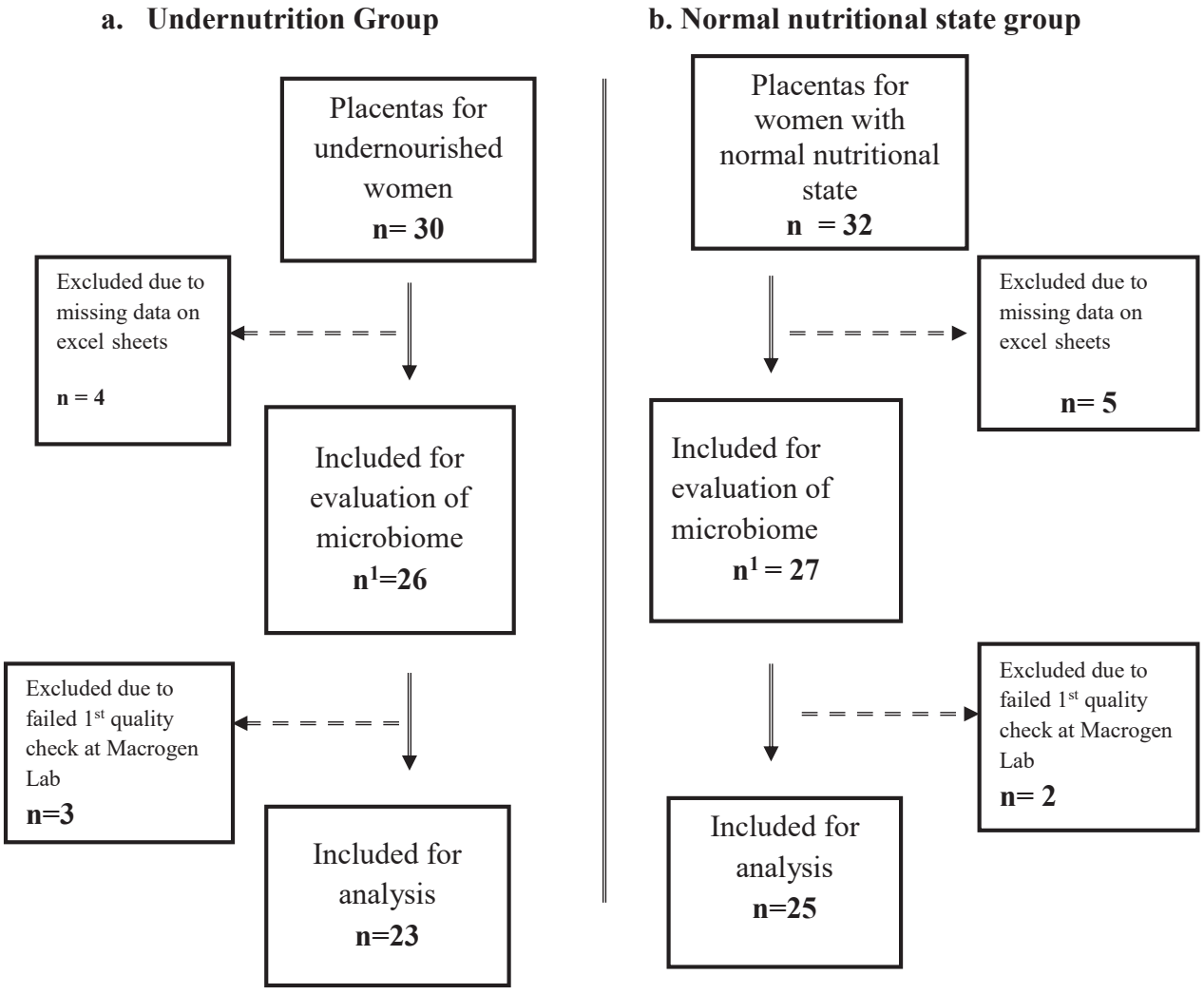


Figure 3. 1: Study Flow

Undernutrition in pregnancy was used for analysis of placental microbiome and compared to those with normal nutritional state. In the process of harvesting the placenta, Burton's protocol was observed to ensure that the possible confounders were eliminated like women with malaria infection, H,I.V and diabetes. Placental specimen blocks were harvested by qualified personnel to ensure guaranteed quality of the specimen that were included for analysis.

3.8 Study Procedure and Data Collection Procedures

3.8.1 Collection of the placenta samples

The placentas were collected from women who met the inclusion criteria in a sterile way by the team sanctioned by the principal investigators (Moses Obimbo, Jesse Gitaka). Clothed in sterile gowns and powder free gloves, they used sterile disposable scalpels and forceps to collect core biopsy placental samples from the amnion, chorion and basal plates. The sampling and collection procedure were as per the protocol described by Burton et al, 2014 (33). This entailed taking photographs of the chorionic and basal aspects of the placenta, taking a 2 cm membrane roll from the sire of rupture to the placental margin, trimming the cord and the membranes to 1 cm and trying the cord followed by weighing the placenta, sampling 4 sites of the placenta including one full thickness block,fetal membranes, umbilical cord and a large grape-size piece of villous tissue,putting material for RNA in a RNAlater buffer for stabilization before freezing and finally completing the placental biobank with relevant clinical information including maternal and fetal information. Samples collected were placed in a petri dish then transferred into a 5 mls conical tube and stored in dry ice at -80ºC within one hour of Collection. They were then flown from Bungoma to Nairobi for storage at the KAVI laboratory within the -80ºC freezer.

3.8.2 DNA extraction

The extraction was done from 25 mg of thawed and minced placental tissue sampled from the villous tree and the amnion, chorion and basal plates in a sterile environment using the DNeasy blood and Tissue Kit (Qiagen) as per the manufacturer's protocol both from the study and control groups. This was done by a trained laboratory technician and the principal investigator with guidance from one of the supervisors who is trained in DNA extraction processes. During this process, the surfaces were constantly wiped with the DNA-AWAY solution to avoid DNA contamination. The extracted DNA was put in micro-ampules labelled in unique codes per specimen and quantified using the Invitrogen machine into ng/ml.

3.8.3 PCR and illumina sequencing protocols

The DNA samples were transported to Macrogen Laboratory in Amsterdam, Netherlands at -20 ºC for the 16s RNA gene sequencing. Upon reception at the laboratory, the samples were subjected to a quality check. Two samples from the undernutrition group and three from the normal nutritional status group failed to pass the quality check. The sequencing of the V3-V4 and V4-V6

regions of the other samples was done using standard PCR and illumina MiSeq (San Diego,CA) protocols. This entailed a 3 minutes' incubation at 95ºC then thermocycling at 43ºC for 30seconds, at 72ºC for 30 seconds and finally at 72ºC for 5 minutes. Dilution was then done using nuclease-free water (promega) at a ratio of 1:15. Amplification and sequencing of the V4 region of the 16sRNA gene was done in line with the dual indexing sequencing strategy by Kozich et al,2013 (34).

3.8.4 Processing of data on 16s rRNA gene

The V3-V4 and V4-V6 libraries were then constructed.QIIME, an open source bioinformatics pipeline for conducting microbiome analysis was used to assemble paired-read contiguous sequesnces, to trim, filter and align sequences, to identify and align chimeras, to assign sequences to bacterial taxonomies and to cluster sequences into operational taxonomic units (OTUs) based on the percentage of nucleotide similarity(97% and 99%)

3.8.5 Metagenomic sequencing of the extracted DNA

This process entails three steps namely amplification, sequencing and analysis. Following creation of V3-V4 and V4-V6 libraries,adapters with the sequence complementary to the solid support, bar code sequence and binding site to the sequencing primer were introduced. As the fragmented DNA was washed over the flow cell, the appropriate adapter attached to the complementary solid support. The process that followed was bridge amplification with generation of clusters. Lastly, analysis was done by finding fragments with overlapping areas and lining them up in order to do variant identification. There was no placental microbiome picked following this sequencing process

3.9 Quality assurance procedures

The principle investigator and laboratory technicians had received some basic on bench mentorship on DNA extraction procedure from the Supervisor who is well versed in this, following the laid down standard operating procedures. Only placenta blocks that were undamaged and were collected following the strict Standard Operating Procedure were used in the analysis.

Unique identifiers were assigned to all the study participants. Information filled on the data collection sheet was checked for any errors and corrected. Next generation sequencing was done

at Macrogen laboratory that has accreditation and certification from various international bodies and is also ISO certified

3.10 Ethical Considerations

Ethical approval to carry out the research was granted by the KNH/OUN Ethics and Research Committee (REF: P423/08/2020).

The initial Ethical approval for harvesting of the placentas for storage in the Biorepository was obtained from the Mount Kenya University Ethics Review Committee (REF.NO.MKU/ERC/0543) for use in the "RAPID AND MULTIPLEX DIAGNOSIS OF MATERNAL BACTERIAL INFECTIONS" project.

The participant's personal details were de-identified by use of an assigned unique identifier, only applicable to the study. This coded information was uploaded to the excel sheet and was password protected.

The results of this study will be communicated to the clinical team at the Bungoma County Referral Hospital at no cost.

3.11 Data Management and Analysis

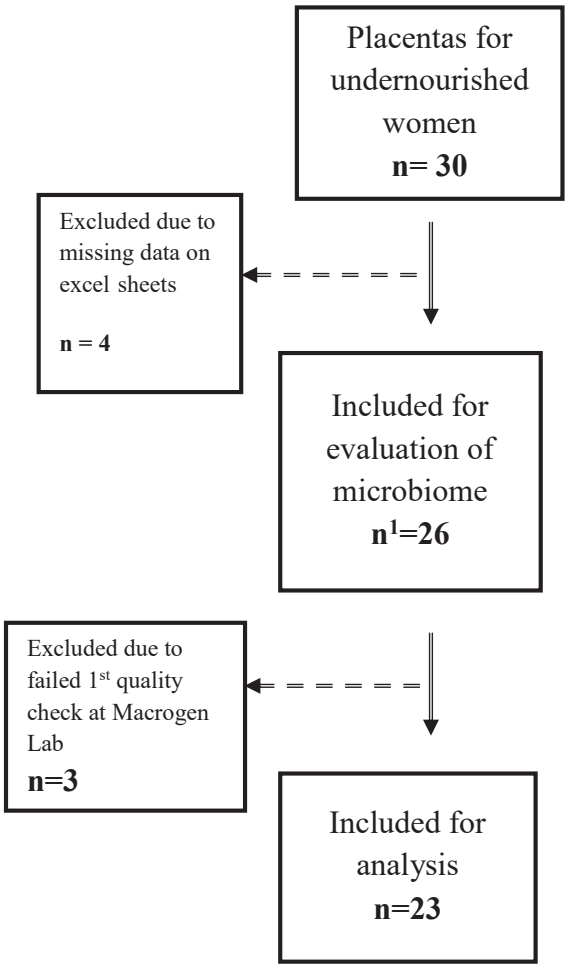
The data was analyzed using SPSS version 26. The patient's sociodemographic and clinical characteristics were analyzed descriptively with generation of summary tables. Categorical variables such marital status, level of education, parity, hemoglobin level and neonatal weight were presented as frequencies with percentages. Continuous variables such as age, gestation age(weeks), hemoglobin level,placental weight and neonatal weight were summarized with their means and standard deviation. The two groups, undernutrition and normal nutrition, were then compared using the Chi square test of association and/or Fisher's test for the categorical variables. The continuous variables were compared using the independent sample T-test/Mann Whitney test. A P-value of <0.05 showed statistically significant difference between the 2 groups. In our study, there was no placental microbiome isolated.

CHAPTER FOUR :RESULTS

4.1.1 Sociodemographic and Clinical Characteristics of the Pregnant Women

A total of 48 placentas were studied; 23 from the undernutrition group and 25 from the normal nutrition group. The mean age of the patients was 25(3.04) and 26(4.01) from the undernutrition and normal nutrition groups, respectively.

a. Undernutrition Group



b. Normal nutritional state group

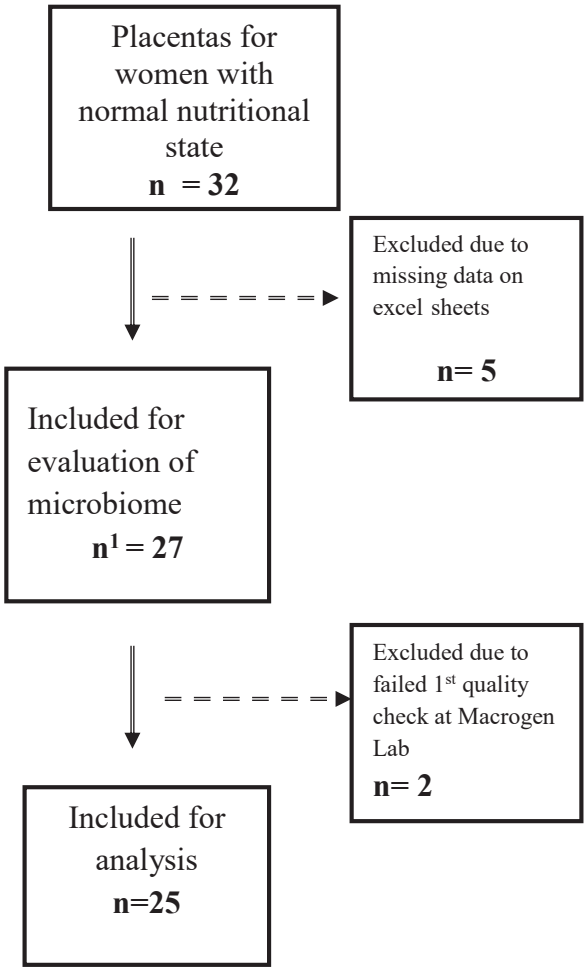


Table 4. 1: Sociodemographic and clinical characteristics of the pregnant women

		Undernutrition N= 23		Normal N= 25		
		n/Mean	%/SD	n/Mean	%/SD	P-value
Age		25	3.04	26	4.01	0.417
Marital status	Single	7	30%	5	20%	0.404
	Married	16	70%	20	80%	
Level of Education	Primary School	15	65%	6	24%	**0.004**
	High School	8	35%	14	56%	
	College	0	0%	5	20%	
Parity	Primipara	0	0%	5	20%	0.051
	Multipara	23	100%	20	80%	
Gestational Age (weeks)		38	2.17	39	1.21	**0.005**
Haemoglobin level(g/dl)		10.28	0.97	11.96	1.52	**<0.001**

Table 4.1 Summarises the sociodemographic and clinical characteristics of pregnant women from both the undernutrition and normal nutrition groups. There was no difference in the age, marital status and parity between the two groups. However, their level of education showed statistical difference between the 2 groups with those who happened to have normal nutrition having achieved higher education. The gestational age for the group with undernutrition was significantly lower at 38(2.17) compared to those with normal nutritional status 39(1.21) though this was not statistically significant.

Lastly, hemoglobin level was also significantly associated with the nutritional status with those in the undernutrition group having a mean hemoglobin of 10.28(0.97) and 11.92(1.52) for those with normal nutritional status.

Bivariate analysis was done in this study since the sample size was too small for multivariate analysis to be done.

Table 4: 2 Comparison of hemoglobin levels between the undernutrition and normal nutrition groups

	Undernutrition N= 23		Normal N= 25			
	N	**(%)**	**n**	**(%)**	**P-value**	**OR(CI)**
Hemoglobin(g/dl)					**<0.001**	
Anaemic (<11g/dl)	20	87%	7	28%		17.14(3.84 - 76.43)
Normal (≥11 g/dl)	3	13%	18	72%		

After further analysis of the hemoglobin level based on hemoglobin of < 11 g/dl representing anemic condition and that of ≥11 g/dl representing normal condition, it emerged that the ODDs ratio of being anemic given that one was suffering from undernutrition was 17.14(3.84 - 76.43) with a p-value of <0.001 as shown in the table

Table 4.3: Comparison of placental and neonatal weights between the undernutrition and normal nutrition groups

		Undernutrition N= 23		Normal N= 25		
		n/Mean	%/SD	n/Mean	%/SD	P-value
Placental weight		491.96	27.33	531.2	35.51	**<0.001**
Neonatal weight		2900.43	302.38	3122	309.64	**0.016**
	Underweight (<2500 grams)	3	13.00%	1	4.00%	0.338
	Normal (≥2500 grams)	20	87.00%	24	96.00%	

The placental and neonatal weights were both found to be significantly different between the two groups as shown by table 4.3. Categorization of neonatal weights into ≥2500 grams and <2500 grams however did not show any significant statistical difference between the two groups. The average neonatal weight being less among the undernutrition was not enough to classify them as underweight.

4.1.2 Comparison of Placental microbiome

The Next generation sequencing of the V3-V4 and V4-V6 regions of the RNA gene isolated from placental specimens of women with history of undernutrition and those with normal nutritional status in pregnancy did not pick any microbiome. The samples indicated multiple peaks as demonstrated by the random figures showed below

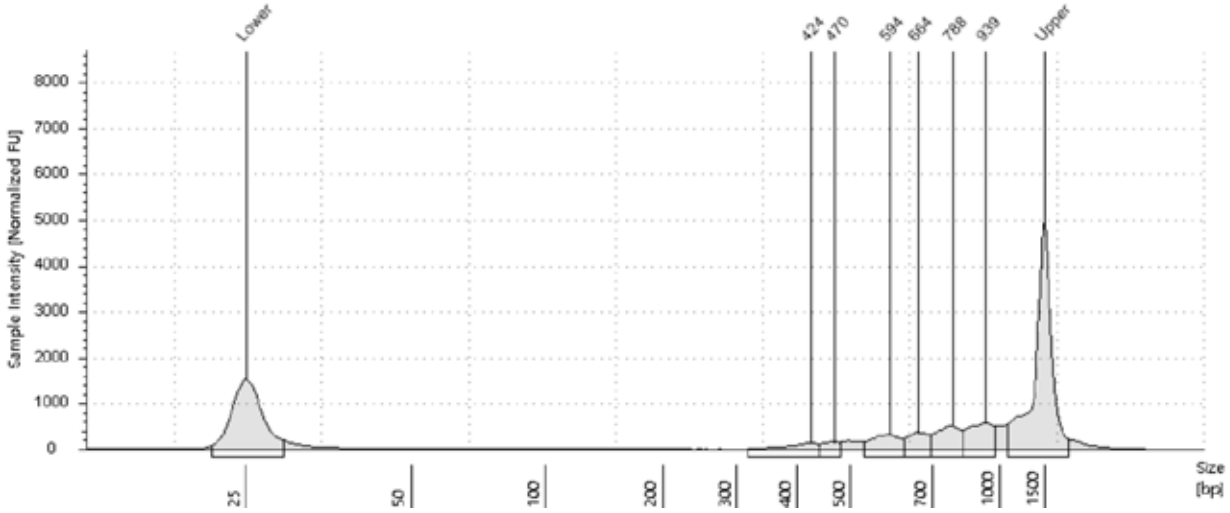


Figure 4. 1: Sample sequencing results of a placenta from normal nutrition

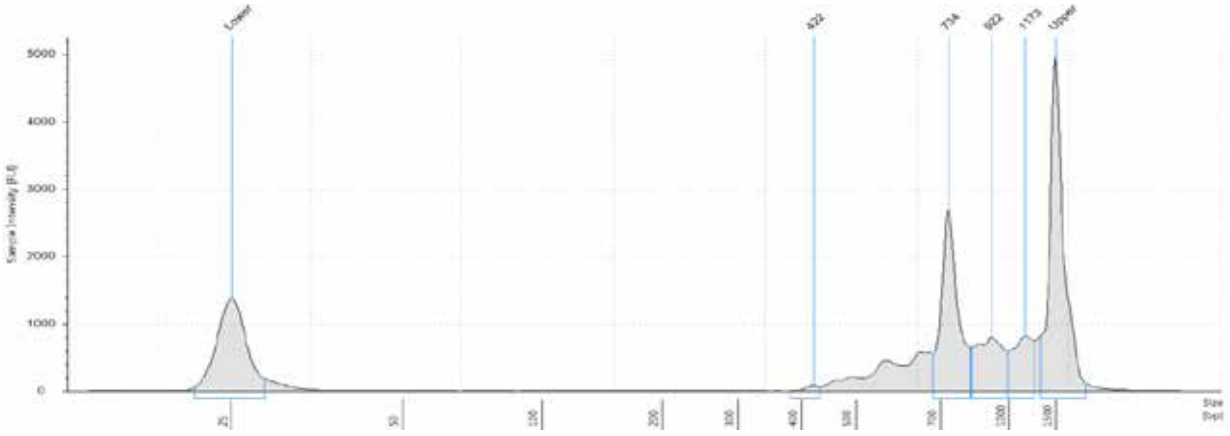


Figure 4. 2: Sample sequencing results of a placenta from under nutrition

The peaks represent parsimoniously amplified short oligonucleotides that were not suffiecietly long enough to be assigned to a particular taxa. The apparent higher peak observed for women with undernutrition at 700 bp may represent more abundant diversity of template nucleic material compared to normal nutrition, possibly due to 'leaked' bacterial genomes or alternatively degraded DNA material by placental endonucleases.

CHAPTER FIVE:DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

In this study, the clinical characteristics associated with undernutrition in our study were anemia (Hb of 10.28 vs 11.92), lower placental weights (491.96 grams vs 531.2 grams) and neonatal weights(2900 grams vs 3200 grams). Behanu et al had similar clinical outcome whereby undernourished women were 3.83 times more likely to be anemic than the well nourished counterparts . Kumera et al 2018 found a 25 % prevalence of anemia in undernourished women when compared to those who were well nourished (13). Beckami et al 2010 also lower mean placental and neonatal weights amongst women who were undernourished (30). This is likely to be due to reduced dietary intake leading to micronutrient deficiency and with time leading to reduced maternal-fetal transfer of nutrients with resultant intrauterine growth restriction and diminished placental health. This calls for proactive nutritional assessment with necessary nutritional interventions to ensure normal nutritional status in pregnancy to avert these negative effects of undernutrition.

Having lower levels of education in our study had an association with undernutrition. Those who had attained post basic education in the normal nutrition group were 20 % while there were none in the undernutrition group. Even if not statistically significant in our study, this was similar to the findings of the study by Kumera et al 2018 that found that having no education or just attaining basic primary education put someone at 2 times risk of undernutrition (13). This may be attributable to increased knowledge on good nutritional practices that one gets through formal education or in part due to assumed better financial status with rising education levels that comes with better nutritional bargaining power. This calls for the government to promote education to the girl child and to also facilitate incorporation of nutrition in the schools curricula so as to improve nutritional status of the women in the reproductive ages.

The mean age in our study was comparable in the two groups (25 years and 26 years in the undernutrition and normal nutrition groups respectively). This is in contrast to other studies that have demonstrated a difference in age in the two groups. Serbesa et al 2019 found that women aged less than 30 years were at a higher risk of undernutrition than those aged more than 30 years (12). In their study, this was attributable to lower socio-economic status of these women aged less

than 30 years. Our study may have failed to show a difference in age owing to the small sample size. The recommendation would be use of larger prospective studies.

Parity in this study did not have any association with undernutrition. An Ethiopian study by Serbesa had found multiparas to have a 2.5 times higher risk of undernutrition than the primiparas (12). This may be due to progressive nutrient deficiency in each consecutive pregnancy. However, if women receive proper nutritional education and counselling during pregnancy regardless of their parity, this difference can be averted.

In our study, there was no microbiome picked from placental specimen of the women with undernutrition and those with normal nutritional status using the 16s DNA gene sequencing. This is comparable to studies by Sterpu et al 2020 (24), Lieby et al 2018 (19) and Theis et al 2020 (23) that utilized next generation sequencing of the 16 s DNA and also failed to pick any microbiome. Aagard et al 2014 had however used the same method and demonstrated Firmicutes,Fusobacteria and Proteobacteria in otherwise normal placentas (16). The absence of placental microbiome in our study despite the undernutrition status suggests the ability of the placenta to maintain sterity with protection of the developing fetus from infection. This is despite the known fact that undernutrition predisposes one to infection due to the demonstrated atrophy of the thymus gland. There was however a difference in the peaks read around two areas.This may be due to leaked genomes into the placenta maybe from gut or vagina bacteria that maybe were vastly degraded not to allow for a clear amplification or maybe due to altered activity of placental endonucleases in women with undernutrition that alternatively degraded the nucleic materials and hence the additional peak at 700 bp.

5.2 Conclusion

From our study, the level of education significantly affects the nutritional status in pregnancy with undernutrion more in women with low levels of education with 65% of pregnant women in the undernutrition group and 24% in the normal nutritional status group having achieved only primary school education.Undernutrition is also associated with anemia with the odds of being anemic given that one is undernourished being 17.14(3.84-76.43) with a p-value of <001 and there is thus need for nutritional counseling in pregnancy to help avert anemia, which is a major indirect cause of maternal mortality.

Next Generation Sequencing of the 16 s RNA gene from placentas with undernutrition and those with normal nutritional status did not pick any microbiome. This might suggest the ability to maintain placental sterility despite undernutrition.

5.3 Recommendations

Based on the findings of this work, we recommend the following:

- Larger prospective cohort studies that can establish causality between sociodemographic characteristics and clinical aspects with undernutrition since some of the sociodemographic factors like age and parity,that were significantly associated with nutritional status in pregnancy from studies with larger sample size were not statistically significant in our study. This may be due to our small sample size
- Prospective cohort studies to allow measurement of the BMI through the pre-pregnancy state to the pregnancy and for control of some confounders including use of antibiotics in pregnancy and duration of undernutrition as these have an impact on interpretation of nutritional status assessment in pregnancy.

5.4 Strength

1. Use of next generation sequencing of the 16sDNA. The 16s DNA subunit has several hypervariable regions that are very species- specific. This makes this method very accurate (92-95% accuracy) in identification of micro-organisms. Due to its thoroughput

amplification, this next generation sequencing allows for detection and identification of otherwise “difficult to culture” micro-organisms

5.5 Limitations

1. Some important data for instance duration of use of antibiotics was not available since it was not captured during data collection. There are important confounders that may have an impact on the results obtained. We suggest use of larger prospective studies in the future that would ensure these important confounders are captured.
2. Prepregnancy nutritional assessment that usually forms a baseline for nutritional assessment during pregnancy was not available. Prospective studies that allow follow up of women from the prepregnancy state through the pregnancy would be recommended to eliminate this major limitation.
3. The current sample size yielded a power of 31% using the assumptions of the expected sample size. The results therefore may not be generalizable. We recommend future studies with larger sample size from which inference can be made.

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ANNEXES

Annex 1: Classes of Samples that were collected for the placental biorepository

1. Samples from women with malnutrition/undernutrition in pregnancy
    a. Malnutrition/undernutrition will be defined with the following clinical observations:
    b. BMI < 18.5 and/or MUAC measurement of <210mm with recent weight loss
2. Samples from HIV positive and negative women with term and preterm deliveries
    a. Term delivery is defined as birth after 37 completed weeks of gestation
    b. Preterm birth in our case will be defined birth between 28 and 37 weeks of gestation
    c. Number of samples 25 in each group
3. Samples from women with history of malaria in pregnancy
    a. Both from term and preterm deliveries
    b. In the data sheet history of treatment and fetal outcomes will be recorded
    c. Both clinical and laboratory diagnosis of malaria will be needed for samples to be collected
4. Samples from women with preeclampsia
    a. 25 samples from mothers with all spectrum of preeclampsia from mild to severe
5. Samples from mothers with Gestational diabetes and diabetes in pregnancy
    a. 25 samples from mothers with either diabetes in pregnancy and or gestational diabetes

Annex 2: Methodology of DNA extraction

Collection of the placenta samples

The placentas were collected from women who met the inclusion criteria in a sterile way by the principal study personnel (Moses Obimbo, Jesse Gitaka, Daniel Wanjala). Clothed in sterile gowns and powder free gloves, they used sterile disposable scalpels and forceps to collect core biopsy placental samples from the amnion, chorion and basal plates. The sampling and collection procedure were as per the protocol described by Burton et al, 2014.Samples collected were placed in a petri dish then transferred into a 5 mls conical tube and stored in dry ice at -80ºC within one hour of Collection. They were then flown from Bungoma to Nairobi for storage at the KAVI laboratory within the -80ºC freezer.

DNA extraction

The extraction will be done from the villous tree and the amnion, chorion and basal plates in a sterile environment using the DNeasy blood and Tissue Kit (Qiagen) as per the manufacturer's protocol both from the study and control groups.

PCR and illumina sequencing protocols

16s RNA gene sequencing of the extracted DNA from the placental specimen will be done using standard PCR and illumina MiSeq (San Diego,CA) protocols. After a 5 minutes' incubation at 95ºC, thermocycling will be done at 94ºC for 30seconds, then at 50ºC for 30 seconds and finally at 72ºC for 120 seconds. Dilution will then be done using nuclease-free water (promega) at a ratio of 1:15. Amplification and sequencing of the V4 region of the 16sRNA gene will be done at the KAVI laboratory in line with the dual indexing sequencing strategy by Kozich et al,2013. Sequencing will be done on the illumine MiSeq platform with the MiSeq Reagent kit V2 (500-cycle format; MS102-2003; illumina) as per the manufacturer's instructions. Each of the PCR reaction will contain 1.0 µM of each primer, 2.5µL of template DNA 0.15µL of AccuPrime HiFi polymerase and DNase-free water to make up a total of 20µL.This PCR reaction will be done at 95ºC for 2 minutes then 30 cycles at 95ºC for 20 seconds, 55ºC for 30 seconds and 72ºC for 5 minutes. This will be followed by an additional elongation at 72ºC for 10 minutes. Sequencing libraries will be prepared according to Illumina's protocol. FASTQ files generated will be paired end reads.

APPENDICES

Appendix 1: Consent form for the original study

CLIENT INFORMATION AND CONSENT FORM

Study title

Rapid and Multiplex Diagnosis of Maternal Infections

Study no.....

Date __/__/__

Investigator: Dr Jesse Gitaka

Telephone contact: 0722425613

RESEARCHERS' STATEMENT

We are asking you to participate in a research study. The purpose of this consent form is to give you the information you will need to help you decide whether you should be in this study or not. Please read the form carefully. You may ask questions about the purpose of the research, what we would ask you to do, the possible risks and benefits, your rights as a volunteer, and anything else about the research or this form that is not clear to you. When we have answered all your questions, you can decide if you want to be in the study or not. This process is called 'informed consent.' We will give you a copy of this form for your records.

INTRODUCTION

Rapid and multiplex detection during pregnancy of bacteria that cause still births, preterm deliveries and neonatal infections can enable prompt treatment improving outcomes. There is increasing evidence that bacterial infections that are mostly subclinical contribute significantly to the inflammatory processes that underlie still births and preterm labour and jeopardise the new-born. This study aims at reducing neonatal mortality rate.

PURPOSE AND BENEFITS

We would like to come up with a novel diagnostic tool that will detect bacterial infections simultaneously in mothers. There will be additional benefits to you as a participant in this study. There will be treating of those infected and information obtained would contribute to overall improvement of neonate's health and well –being nationally.

Procedure

Once you have agreed to participate in the study, you will sign this consent form to allow us to include information obtained from you in our data. Your personal details will not be included in this questionnaire so as to protect your privacy. We will take a small portion of your delivered placenta for the purpose of this study. We will also look at your antenatal record to obtain more information which will remain confidential. You will continue to receive appropriate management

while at the hospital. We also guarantee your safety during your participation in this study. If you agree to let the researchers collect specimens, the following will happen:

- There will be no mutilation of the placenta
- Measurements will be taken with the organ intact and only small blocks will be extracted for histology
- The tissue blocks will be stored in a placental biorepository for further and future research.

Confidentiality

All the information obtained from you will be treated with utmost confidentiality. Your name will not appear on the questionnaire. A study number will be used instead.

You may choose to withdraw from the study or refuse to answer questions at any point of this study. Your decision will not affect your care at while at the hospital.

Subject's statement

I, the undersigned have been explained to and have understood the above and willingly accept to participate in the research study. I understand that participation in the study does not entail financial benefit. I have been assured that any information obtained will be treated with utmost confidentiality and my treatment will not be compromised if i decline to participate in or withdraw from the study.

I have had a chance to ask questions and if other questions arise, I can ask the researcher.

No coercion has been used to influence my decision to participate in the study whose nature, benefits and risks have been explained to me by Dr/Mr./Mrs./Ms.....

Signature/ Left thumbprint **Signature of the witness**

_____ _____

(Participant) *(Witness)*

Certificate of Informed Consent

The above information has been read and explained to me. I also had the opportunity to ask questions regarding the study and I have been answered satisfactorily. I consent voluntarily to participate in this study.

Participant Name: _____ (PRINT)

Signature of Participant _____

Or

Thumb print of participant

Date _____

Statement by the principal investigator/research assistant taking consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands the study protocol.

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that this consent has been given voluntarily without any coercion.

Name of the person taking the consent _____ (PRINT)

Signature _____ Date _____

For any questions or concerns about the study contact

Dr Jesse Gitaka on 0722425613.

P.O.BOX 342-01000, Kenya.

For any questions pertaining to rights of as a research participant, contact the secretary

Ethical review committee

P. O. Box 342-01000 Thika;

Tel: 0725809429.email: research@mku.ac.ke

Appendix 2: ERC approval for the original study

Mount Kenya University

SEPTEMBER 25, 2017

Ref. No. MKU/ERC/0543

CERTIFICATE OF ETHICAL CLEARANCE

This is to certify that the proposal titled **"RAPID AND MULTIPLEX DIAGNOSIS OF MATERNAL BACTERIAL INFECTIONS"**, whose Principal Investigator is Dr Jesse Gitaka has been reviewed by Mount Kenya University Ethics Review Committee (ERC), and found to adequately address all ethical concerns.

Mr Francis W. Makokha
Secretary, Mount Kenya University ERC

Sign: _____ **Date:** 26.09.2017

Prof. Francis W. Muregi
Chairman, Mount Kenya University ERC

Sign: _____ **Date:** 26.09.2017

The Chairman
Mount Kenya University
Ethics Review Committee
P. O. Box 342 - 0100, Thika

Appendix 3: Authorization letter from the principal investigator of the Rapid Multiplex Diagnosis of Maternal Bacterial project

Mount Kenya University

TO:
KNH-UoN ERC
Email: uonknh_erc@uonbi.ac

RE: CONSENT TO THE USE OF BIOBANKED PLACENTA SPECIMENSACQUIRED FOR *"RAPID AND MULTIPLEX DIAGNOSIS OF MATERNAL BACTERIAL INFECTION"*PROJECT(REFERENCE NUMBER: MKU/ERC/0543)

We make reference to the above matter.

I, **Dr. Jesse Gitaka** , the Principal Investigator of the above named study do give my consent to the use of the Biobanked Placenta Specimen to the following investigators in the University of Nairobi Obstetrics and Gynecology Department:-

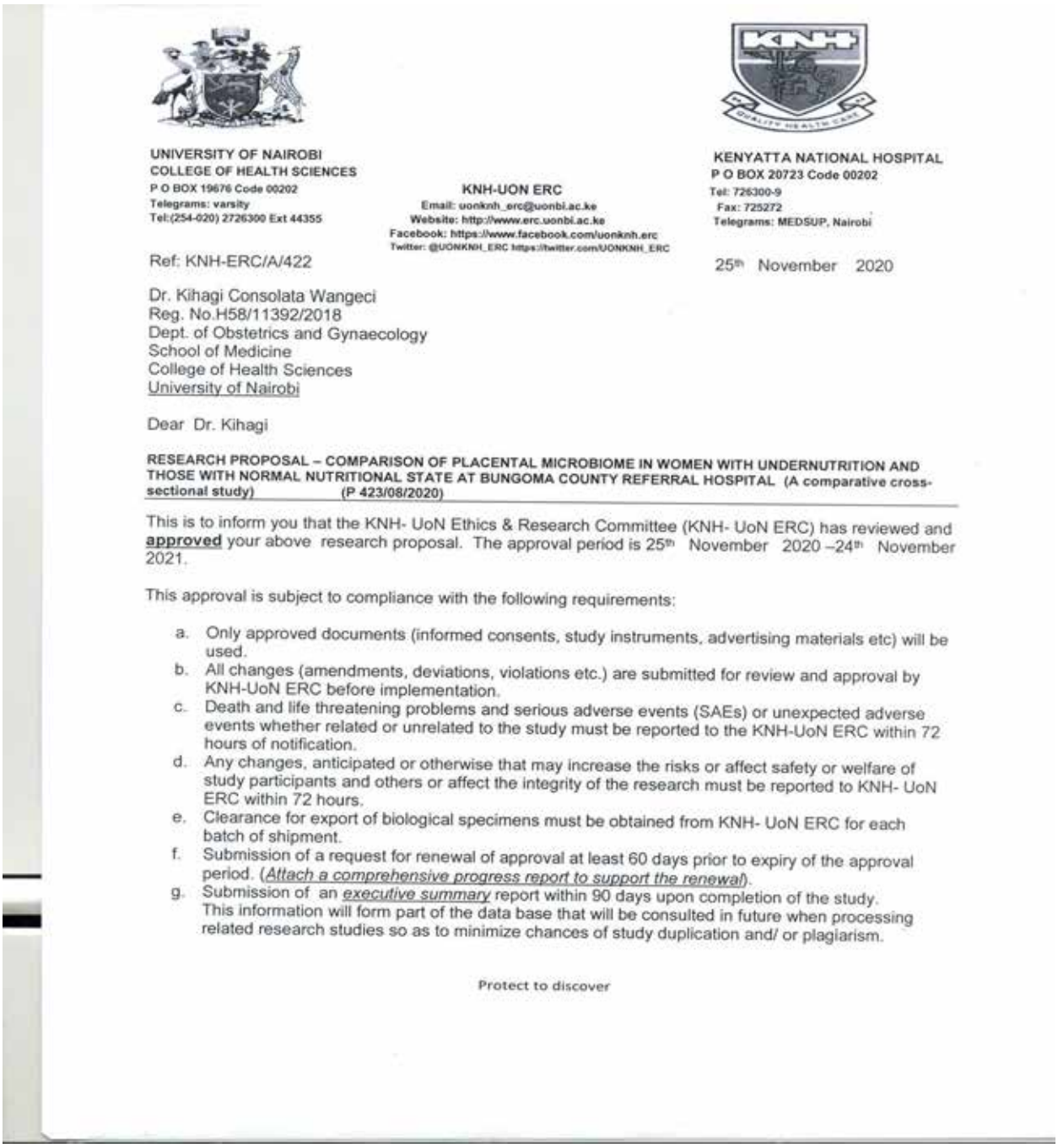
INVESTIGATOR'S NAME	**COURSE**
Dr. Consolata Wangeci Kihagi;	Comparison of clinical, sociodemographic characteristics and placental microbiome in women with undernutrition and those with normal nutritional status at Bungoma County Referral Hospital.
Dr. Yusuf Adam Khalil;	Placental histological changes in preterm births with placental malaria and HIV coinfection.
Dr. Everett Lamulungi;	Structural differences in placentas of women with malaria-preeclampsia comorbidity in healthy pregnancies
Dr. John Kamau Mwangi;	The vaginal microbiome of women with preterm births versus women with term births who attended ANC at Thika Level 5 County Referral Hospital between January 2019 and March 2019
Dr. Stephen Lutukayi Marumbu	Comparison of placental morphology and perinatal outcomes in women with and without GDM among low income rural population in Kenya.
Dr. Maero Deogracious Moses	Comparison of placental structure in pregnant women with undernutrition and those with normal nutrition delivering at Bungoma County Referral Hospital.

Kindly accord them the necessary assistance
Thank you in Advance.
Yours Faithfully

.....

Dr. Jesse Gitaka, MD, MTM, PhD

Appendix 4: KNH-ERC ethics approval



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Ref: KNH-ERC/A/422

25th November 2020

Dr. Kihagi Consolata Wangeci
Reg. No.H58/11392/2018
Dept. of Obstetrics and Gynaecology
School of Medicine
College of Health Sciences
University of Nairobi

Dear Dr. Kihagi

RESEARCH PROPOSAL – COMPARISON OF PLACENTAL MICROBIOME IN WOMEN WITH UNDERNUTRITION AND THOSE WITH NORMAL NUTRITIONAL STATE AT BUNGOMA COUNTY REFERRAL HOSPITAL (A comparative cross-sectional study) (P 423/08/2020)

This is to inform you that the KNH- UoN Ethics & Research Committee (KNH- UoN ERC) has reviewed and **approved** your above research proposal. The approval period is 25th November 2020 –24th November 2021.

This approval is subject to compliance with the following requirements:

a. Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b. All changes (amendments, deviations, violations etc.) are submitted for review and approval by KNH-UoN ERC before implementation.
c. Death and life threatening problems and serious adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH-UoN ERC within 72 hours of notification.
d. Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH- UoN ERC within 72 hours.
e. Clearance for export of biological specimens must be obtained from KNH- UoN ERC for each batch of shipment.
f. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
g. Submission of an *executive summary* report within 90 days upon completion of the study. This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/ or plagiarism.

Protect to discover

Appendix 5: Data abstraction tool

Study Number: _____

Social demographic data

Sex: F

Maternal age [ ] years

Parity primipara [ ] multipara [ ]

Marital status married [ ] unmarried [ ]

Education level basic [ ] post basic [ ]

Nutritional assessment

Weight in Kg [ ] Height in Cm [ ] BMI [ ]

Hemoglobin level in g/dl [ ]

Gestational age in weeks [ ]

Neonatal weight in grams [ ]

Placental weight in grams [ ]

Appendix 6: STROBE checklist

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group

Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time
		Case-control study—Report numbers in each exposure category, or summary measures of exposure
		Cross-sectional study—Report numbers of outcome events or summary measures
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included

		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

Appendix 7: Itemized Budget

Budget Item	**Cost of Item**	**No of Items**	**Total Cost in Ksh**
Printing of draft proposals	1,000	4	4,000
KNH/UON ERC	2,000	1	2,000
DNA extraction kits	1,500	60	90,000
PCR kits	1,200	60	72,000
Cost of performing the tests	100,000	1	100,000
Transport costs to Korea	80,000	1	80,000
Research Assistant	5,000 per day	10 days	50,000
Statistician	30,000	1	30,000
Draft thesis printing	1,500	2	3,000
Miscellaneous	**10% of the budget**		20,000
		Total	**Ksh451,000**

Appendix 8: Timeline

Activity	May 2020	Aug 2020	Oct & Nov 2020	Dec 2020	Jan 2021	Feb 2021	Mar 2021	April 2021	May 2021	June 2021	July 2021
Proposal development	■										
Proposal presentation		■									
Ethics committee review			■								
Data collection and analysis including metagenomics in Amsterdam, Netherlands				■	■	■	■	■	■	■	
Results presentation											■