Research report

Menopause-related changes in the gut microbiome and their association with cardiometabolic diseases in women from four sub-Saharan African countries

by

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MSc (Med) in Genomic Medicine

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A **research report (in the format of a "submissible" paper)** submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of **Master of Science in Medicine (Genomic Medicine)**.

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RE: A RESEARCH REPORT SUBMITTED TO THE FACULTY OF HEALTH SCIENCES BY PHEHELLO CHAUKE IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICINE IN GENOMIC MEDICINE

Dear Examiner,

Thank you for agreeing to examine the following research report.

This research report, entitled "Menopause-related changes in the gut microbiome and their association with cardiometabolic diseases in women from four sub-Saharan African countries", is being submitted by the candidate, Phehello Chauke, in partial fulfilment of the degree, Master of Science in Medicine in Genomic Medicine. The research report is being submitted in the form of a "submissible" paper and contributes towards 30% of the degree.

The candidate, with approval from her supervisors, has selected the peer-reviewed journal, *Frontiers in Microbiomes*, for submission to the Faculty of Health Sciences. *Frontiers in Microbiomes* was selected as it is aimed at advancing the understanding of how microbiomes impact the host and the environment. The research report was written in accordance with the *Frontiers* Author Guidelines, which has been attached for ease of reference (see Appendix 4). The *article* type selected was the format of original research. The *Frontiers* Author Guidelines state that figures must be individually submitted in the order in which they are referred to in the manuscript, and that tables should be inserted at the end of the manuscript. However, for ease of reference and for marking purposes these guidelines have been deviated from. The tables and figures will appear in text in the order in which they are referred to, as opposed to being submitted separately. In Appendix 1, the approved research protocol has been attached to serve as an extended literature review and to provide further context for the submitted research. The research protocol is not being submitted for examination as it was previously assessed and approved by the Faculty of Health Sciences.

Yours sincerely,

Phehello Chauke	Dr. Dylan Maghini	Dr. Luicer Olubayo	Prof. Scot Hazelhurst
Candidate	Co-supervisor	Co-supervisor	Supervisor
			Scott Hazelhurst (Feb 26, 2025 18:36 GMT+2)

Declaration

I, Phehello Chauke declare that this Research Report (in the format of a "submissible paper") is my own unaided work. It is being submitted for the degree of Master of Science in Medicine (Genomic Medicine) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University

Signature of candidate

06/06/25

Contribution of candidate to the paper

Declaration: Student's contribution to article (s) and agreement of co-author

I, **Phehello Selaelo Chauke**, student number **2302562**, declare that this Research Report is my own work and that I contributed adequately towards research findings presented in the paper stated below, intended for publication.

Signature of student:

Date: 26/02/2025

Name of Primary Supervisor: Professor Scott Hazelhurst

Signature of Primary Supervisor:
Scott Hazelhurst (Feb 26, 2025 18:37 GMT+2)

Date: 26/02/2025

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of this article by the student as part of her Research Report.

Article title: Menopause-related changes in the gut microbiome and their association with cardiometabolic diseases in women from four sub-Saharan African countries

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To my beloved parents in heaven and my dear aunt Kgabo, I dedicate this paper to you, with all my heart. I want you to be proud of me, always.

Presentations arising from this research

1. Young and Early career Geneticists Group (YEGG) symposium 2024, 26 October, University of the Witwatersrand, Johannesburg (Poster presentation)

2. Southern African Society for Human Genetics (SASHG) Biennial Congress 2024, 27-29 October, Sun city, Northwest (Poster presentation)

3. International Society for Computational Biology (ISCB)-Africa conference 2025, 14-17 April, Lagoon Beach hotel, Cape Town (Oral Presentation).

Abstract

Menopause is associated with an increased risk of cardiometabolic diseases (CMDs). Existing research suggests that the gut microbiome (GM) may mediate this relationship through its response to changing hormone levels. However, this association remains understudied in African populations, where CMD burden is increasing. This study aimed to investigate compositional differences in the GM between pre- and postmenopausal women in sub-Saharan Africa and their association with CMDs. The study analysed the gut microbial composition of 1232 women from six sites across four sub-Saharan African countries. Microbial diversity was assessed using Inverse Simpson index and Bray-Curtis dissimilarity and differentially abundant taxa between menopausal groups were identified using Linear discriminant analysis Effect Size. Our analysis revealed that CMD status emerged as a stronger determinant of gut microbial diversity than menopausal status. Women with CMDs showed significantly lower microbial diversity regardless of menopausal status. Geographic location also significantly influenced GM composition, with substantial variations across study sites. Taxonomically, premenopausal women were enriched with beneficial short-chain fatty acid-producing bacteria, including *Faecalibacterium prausnitzii*, *Bacteroides fragilis*, and *Prevotella*, while postmenopausal women exhibited both beneficial (*Ruminococcus champanellensis*) and potentially harmful species (*Collinsella bouchesdurhonensis*). Rather than menopause directly altering the GM and subsequently increasing the risk of CMDs, our findings suggest that the higher prevalence of CMDs in postmenopausal women may be driving of the observed changes in the microbiome. These findings highlight the importance of population-specific approaches to women's health research and understanding the complex interplay between hormonal status, GM, and metabolic health in African women.

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List of abbreviations

AWI-Gen	Africa Wits-INDEPTH Partnership for Genomic Research
BMI	Body mass index
CMD	Cardiometabolic disease
DBP	Diastolic blood pressure
E_2	Estradiol
GM	Gut microbiome
FSH	Follicle stimulating hormone
H3Africa	Human Hereditary and Health in Africa
LDA	linear discriminant analysis
LEfSe	linear discriminant analysis effect size
PCoA	Principal Coordinate analysis
PERMANOVA	Permutational multivariate analysis of variance
SAMRC	South African Medical Research Council
SBP	Systolic blood pressure
SHBG	Sex hormone-binding globulin
SCFA	Short-chain fatty acids
SSA	Sub-Saharan Africa

A research report in the format of a ‘submissible’ paper.

This work has been written up as a paper for submission to **Frontiers in Microbiomes**. The author guidelines adhered to can be found in **Appendix 4**. Tables and figures have been included in the text for ease of reference.

Menopause-related changes in the gut microbiome and their association with cardiometabolic diseases in women from four sub-Saharan African countries.

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Abstract for journal submission

Menopause is associated with an increased risk of cardiometabolic diseases (CMDs). Existing research suggests that the gut microbiome (GM) may mediate this relationship through its response to changing hormone levels. However, this association remains understudied in African populations, where CMD burden is increasing. This study aimed to investigate compositional differences in the GM between pre- and postmenopausal women in sub-Saharan Africa and their association with CMDs. The study analysed the gut microbial composition of 1232 women from six sites across four sub-Saharan African countries. Microbial diversity was assessed using Inverse Simpson index and Bray-Curtis dissimilarity and differentially abundant taxa between menopausal groups were identified using Linear discriminant analysis Effect Size. Our analysis revealed that CMD status emerged as a stronger determinant of gut microbial diversity than menopausal status. Women with CMDs showed significantly lower microbial diversity regardless of menopausal status. Geographic location also significantly influenced GM composition, with substantial variations across study sites. Taxonomically, premenopausal women were enriched with beneficial short-chain fatty acid-producing bacteria, including *Faecalibacterium prausnitzii*, *Bacteroides fragilis*, and *Prevotella*, while postmenopausal women exhibited both beneficial (*Ruminococcus champanellensis*) and potentially harmful species (*Collinsella bouchesdurhonensis*). Rather than menopause directly altering the GM and subsequently increasing the risk of CMDs, our findings suggest that the higher prevalence of CMDs in postmenopausal women may be driving of the observed changes in the microbiome. These findings highlight the importance of population-specific approaches to women's health research and understanding the complex interplay between hormonal status, GM, and metabolic health in African women.

Keywords: African, Cardiometabolic disease (CMD), Gut microbiome (GM), Menopause, Microbial diversity.

1 Introduction

Menopause is a natural biological transition characterised by the cessation of menstruation due to decreased ovarian function after 12 consecutive months without menstruation (OlaOlorun & Shen, 2020). This transition is part of normal biological aging and is accompanied by a decline in ovarian sex hormones such as estrogen (particularly estradiol (E_2)), progesterone, and sex hormone-binding globulin (SHGB) (Liu et al., 2022a). It is also marked by elevated levels of the follicle stimulating hormone (FSH) (Harlow et al., 2012). These hormonal shifts contribute to various physiological changes, including an increased risk of cardiometabolic diseases (CMDs) such as hypertension, type 2 diabetes, hypercholesterolaemia and obesity (Chikwati et al., 2023; Peters et al., 2022).

However, the underlying mechanism between the menopausal transition and CMDs remains poorly understood. A significant challenge in this field is isolating the specific effects of menopause from the effects of aging itself (El Khoudary et al., 2020). It remains unclear whether the increased risk of CMDs in postmenopausal women is due to reproductive or chronological aging. However, some studies suggest that menopause has an influence on several CMD biomarkers beyond the effects of chronological aging (Wang et al., 2018). Additionally, the prevalence of metabolic syndrome and its biomarkers have been shown to increase with menopause independent of the effects of chronological aging (El Khoudary et al., 2020). Given the increasing life expectancy, women now spend approximately three decades of their lives in the postmenopausal phase (World Health Organization, 2022), underscoring the importance of understanding the long-term health implications of menopause.

Emerging evidence suggests that the gut microbiome (GM)— a collection of genes from trillions of microbes that inhabit the human body, including bacteria, archaea, viruses, and eukaryotes— may be a mediator between menopause and increased cardiometabolic risk factors (Liu et al., 2022b; Peters et al., 2022; The Human Microbiome Project Consortium, 2012). The GM plays an important role in many physiological processes, including digestion, metabolism, and immune function and it is continuously shaped by interactions between the host genetics, diet, lifestyle, age, medication, and environmental factors (The Human Microbiome Project Consortium, 2012). Notably, sex-specific differences are observed in GM studies, with distinct differences between males and females that appear to be hormone-dependent (Yurkovetskiy et al., 2013). This hormonal influence is demonstrated in mouse studies, where sex-specific microbial differences disappear after ovariectomy or castration (Yurkovetskiy et al., 2013). Similar patterns exist in humans, where sex-based differences in gut microbiota emerge post-puberty (Korpela et al., 2021), and postmenopausal women show microbial

profiles more similar to men than premenopausal women (Santos-Marcos et al., 2018). These findings suggest that the decline in estrogen during menopause may contribute to alteration in GM composition.

A key mechanism by which sex hormones influence the GM is through estrogen. Estrogen plays a significant role in regulating the gut microbiota, and research has shown a bidirectional relationship between the GM and menopause. While the liver is primarily responsible for metabolising endogenous estrogen by conjugating it with glucuronide or sulfate groups, the GM contains an "estrobolome"—a collection of microbial genes capable of deconjugating estrogen and other sex hormones (Ervin et al., 2019; Kwa et al., 2016). The estrobolome reactivates estrogen, allowing it to be reabsorbed into the enterohepatic and systemic circulations. Estrogen not only plays a role in the reproductive system, but it is also responsible for maintaining homeostasis in the gut by influencing the intestinal barrier function and microbial composition (Kwa et al., 2016). Therefore, deficiency in estrogen due to menopause disrupts the balance of the GM and affects the microbial diversity and composition, leading to dysbiosis —imbalances in the GM (Peters et al., 2022). The impact of menopause on the GM is further illustrated by changes in specific microbial taxa linked to metabolic health. Studies have shown that postmenopausal women exhibit a decreased abundance of beneficial microbes such as *Akkermansia muciniphila* and *Faecalibacterium* which play crucial roles in enhancing insulin sensitivity and supporting glucose metabolism (Zhao et al., 2019). These shifts in microbial composition may represent a mechanistic link between menopause and cardiometabolic risk, as the reduction of these beneficial microbes coincides with increased prevalence of CMDs in postmenopausal women. This further highlights the complex relationship between hormonal changes, the GM, and metabolic health.

Despite the growing body of research on the GM and its role in human health, African populations remain largely underrepresented in these studies. Large cohort studies such as the Human Microbiome Projects (HMP), Metagenomics of the Human Intestinal Tract (MetaHIT), and the Dutch Microbiome Project have played a pivotal role in our understanding of the GM in disease and lifestyle factors, shedding light on associations between socioeconomic and environmental influences, however, these initiatives have primarily focused on European populations (Allali et al., 2021; Pheeha et al., 2023). Initiatives such as the Human Hereditary and Health in Africa (H3Africa) Consortium have played an important role in promoting GM research among African populations, notably through collaborations like the Africa Wits-INDEPTH Partnership for Genomics studies (AWI-Gen study) (Ali et al., 2018). Despite these efforts, however, there is still a lack of studies examining the specific impact of menopause on the GM in African women. This gap highlights the need for further research to understand how menopausal changes may interact with unique genetic, environmental, and lifestyle

factors in African populations, influencing the composition and function of the GM. Such research could not only provide valuable findings into the relationship between menopause and CMDs but also help inform culturally relevant health strategies and interventions tailored to African women. This study aims to address this gap by investigating compositional differences of the GM between pre- and postmenopausal women in SSA and their association with CMDs. Specifically, the objectives of the study were to: (1) characterise alpha and beta microbial diversity patterns between menopausal groups, (2) identify differentially abundant bacterial taxa between menopausal groups, and (3) determine how these microbial patterns may be linked to CMDs. This multi-site study represents the first comprehensive analysis of menopause-related GM changes in African populations, expanding our understanding of the relationship between the gut microbiota, menopause and CMDs.

2 Methods and Materials

2.1 Study participants

AWI-Gen is a longitudinal study that recruited participants in wave 1 (2013–2015) and followed them up in wave 2 (2018–2023). Participants were recruited from five Health and Demographic Surveillance System (HDSS) sites across Burkina Faso (Nanoro), Ghana (Navrongo), Kenya (Nairobi), and South Africa (Agincourt-Bushbuckridge, DIMAMO). The sixth site, Soweto (South Africa), is managed by the SAMRC/Wits Developmental Pathways to Health Research Unit. The study sites span diverse settings, including agricultural communities (Nanoro, Burkina Faso, and Navrongo, Ghana), rural areas undergoing urbanisation (Agincourt and DIMAMO, South Africa), urban informal settlements (Nairobi, Kenya), and a post-industrialised urban township (Soweto, South Africa). Detailed site descriptions can be found in the supplementary materials of Maghini et al. (2025). This microbiome study, nested within wave 2 of AWI-Gen, included 1801 semi-randomly selected women aged 42–86 years. Trained field workers from AWI-Gen guided participants through a comprehensive questionnaire, capturing key aspects of their demographic background, reproductive history, overall health, and disease status. In addition, participants completed a microbiome-specific questionnaire designed to capture environmental and lifestyle factors that may influence the GM composition. Participants provided stool samples for the microbiome study, while urine and blood biomarkers were collected as part of the AWI-Gen wave 2. These data were integrated to provide a comprehensive perspective on the broader factors shaping microbiome diversity.

Ethics approval

The ethics clearance for this sub-study was granted by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (Ethics certificate No. M240765) (Appendix 2).

2.2 Study design and classification

This was a cross-sectional data analysis study that utilised existing quality-controlled data from the AWI-Gen 2 microbiome study (Maghini et al., 2025). The original data generation by the microbiome study included DNA extraction from faecal samples and shotgun metagenomic sequencing using Illumina technology. The metagenomic reads were processed using an in-house Nextflow pipeline that performed deduplication, quality trimming, and alignment to the human genome build (GRCh38) using BWA v0.7.17. Maghini et al. (2025) performed taxonomic profiling using mOTUs v3.0.3 (Milanese et al., 2019).

2.3 Menopause status

Menopausal status was determined through self-reported menstrual patterns. The participants were asked about their menstruation patterns and were then grouped into the different menopausal groups: premenopause— regular menstruation, perimenopause— irregular menstruation in the last 12 months and postmenopause— no menstruation for 12 or more consecutive months. Perimenopausal women were excluded as this transitional phase is associated with extreme fluctuating hormone levels, and these could potentially hinder the identification of distinct differences in the GM across well-defined menopausal stages (Harlow et al., 2012). Women who had undergone hysterectomy were excluded to prevent potential misclassification of menopausal status, as menstrual cessation after hysterectomy can occur regardless of ovarian function. Additionally, women using contraceptives were excluded from the analysis to isolate the effects of natural menopause and avoid confounding by altered sex hormones.

2.4 Cardiometabolic disease (CMD) classification

The AWI-Gen study defined cardiometabolic conditions using clinical measurements and/or medical history (Ali et al., 2018). The following criteria were used: obesity (Body mass index (BMI) ≥ 30 kg/m^2) (World Health Organization, 2000), hypertension (Systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg) (James et al., 2014), diabetes (fasting glucose ≥ 7.0 mmol/l) (American Diabetes Association, 2013), and hypercholesterolaemia (total cholesterol ≥ 5.0 mmol/l) (Grundy, 2002). For the latter three conditions, either medication use, or prior diagnosis by a healthcare professional also met the criteria. In this current study, to create an overall CMD profile, a simple scoring system was developed. A score of 1 was assigned for each condition present and the total score ranged from 0-4. Individuals with none of these conditions (score = 0) were categorised as without CMDs (CMD -), while those with any of these conditions (score ≥ 1) were categorised as with CMDs (CMD +).

2.5 Diversity measures

2.5.1 Alpha diversity

To measure alpha diversity, a measure of microbial diversity within an individual, the species counts were rarefied to 7500 using the rrarefy function from the vegan R package version 2.6-6.1 (Oksanen et al., 2024). The alpha diversity was measured using the Inverse Simpson index which accounts for richness— number of species present and evenness — the relative abundance of the species (Simpson, 1949). The statistical analysis of alpha diversity differences was assessed using a linear mixed-effects model from the lmerTest R package version 3.1-3 (Kuznetsova et al., 2017) with site as a random effect for combined site analysis, while standard linear regression models were applied for individual site analyses. Women were stratified by CMD status, and differences between menopausal groups within each CMD category also assessed using a linear mixed-effects model accounting for variability across sites. The interaction between menopausal status and CMDs was examined to evaluate its effect on alpha diversity. Age and CMD status were then tested separately as potential confounding variables and added into the model.

2.5.2 Beta diversity

Beta diversity, or the similarity between microbiomes, for each site was measured using Bray-Curtis distance and analysed through non-metric multidimensional scaling (NMDS) using the vegan R package (Oksanen et al., 2024). The adonis function from the vegan package in R, which implements permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2017), was used to assess the independent effects of menopausal status and CMD on beta diversity. The analysis included three models: a univariate model testing the effects of menopausal status on microbial composition; an additive model testing whether menopausal status and CMD independently explained variance in microbiome composition; and an interaction model assessing whether the effect of menopause on microbiome composition is modified by CMD status.

2.6 Differentially abundant taxa between menopausal groups

Differentially abundant species between menopausal groups at each site were identified using linear discriminant analysis (LDA) effect size (LEfSe) approach (Segata et al., 2011). Using a combination of Kruskal-Wallis and Wilcoxon Rank tests, LEfSe identified significantly different taxa between groups, with an LDA score threshold of 2.

2.7 Statistical analysis

All statistical analyses were carried out in R v4.3.2 and all the statistical tests used are provided in the respective method section. Multiple testing was corrected using the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995) via the p.adjust function in base R. The following packages were used for data visualisation: ggplot2 (v3.5.1) (Wickham, 2016), cowplot (v1.1.3) (Wilke, 2020), and tidyverse (v2.0.0)(Wickham et al., 2019).

3 Results

3.1 Study participants

The initial study cohort consisted of 1801 women. Those classified as perimenopausal (n = 411), had undergone hysterectomy (n = 66), or used birth control (n = 69) were excluded. Following integration of metadata and microbiome data the final dataset consisted of 1232 women (n= 352 premenopausal, n = 880 postmenopausal). Twenty-five women remained unclassified for CMDs and were excluded from further analyses. Table 1 presents demographic, clinical, and socioeconomic characteristics of premenopausal and postmenopausal women. Significant differences were observed across multiple parameters. Postmenopausal women were older (median age 57 years, IQR 10.00) and had increased cardiometabolic risk factors, including higher BMI, total cholesterol, glucose levels, and blood pressure when compared to premenopausal women. A higher proportion of postmenopausal women had CMDs (74%) compared to premenopausal women (39%). Physical activity levels were lower among postmenopausal women and most participants belonged to the lower socioeconomic quintiles (quintiles 1-3).

Table 1: Demographic, clinical, and socioeconomic characteristics of premenopausal and postmenopausal women

Parameters	Premenopausal	Postmenopausal
N	352	880
Age (years)	49.00 (4.0)	57 (10.0)
Sites (%)		
Soweto	30 (21%)	114 (79%)
Agincourt	44 (12%)	335 (88%)
DIMAMO	17 (24%)	53 (76%)
Nairobi	36 (25%)	106 (75%)
Navrongo	32 (17%)	160 (83%)
Nanoro	193 (63%)	112 (37%)
BMI (kg/m^2)	22.1 (8.9)	26.7 (11.0)
Total cholesterol (mmol/L)	4.1 ± 1.1	4.8 ± 1.1
Glucose (mmol/L)	5.4 (0.7)	5.5 (1.0)
Systolic BP (mm Hg)	114 (24)	129 (31)
Diastolic BP (mm Hg)	74 (14)	80 (16)
MVPA (minutes/week)	2680 (2539)	812 (1860)
Cholesterol medication (%)	6 (29%)	15 (71%)
High cholesterol = Yes (%)	10 (28%)	26 (72%)
Diabetes status = Yes (%)	28 (17%)	135 (83%)
Diabetes medication (%)	10 (14%)	59 (86%)
Hypertension status = Yes (%)	81 (14%)	507 (86%)
Hypertension medication (%)	26 (8%)	292 (92%)
Total Employed (%)	274 (45%)	333 (55%)
CMD status (%)		
With CMDs	144 (39%)	661 (74%)
Without CMDs	201 (59%)	201 (24%)
Unclassified*	7(2 %)	18 (2%)
Socioeconomic status (%)		
1 (poorest)	43 (12%)	186 (21%)
2 (poorer)	246 (70%)	540 (62%)
3 (poor)	48 (14%)	102 (12%)
4 (less poor)	5 (1%)	21 (2%)
5 (wealthiest)	9 (3%)	29 (3%)

Data is presented as Median (Interquartile range) or Mean± SD for continuous variables and as count (percentage) for categorical variables. For sites, values show count (percentage) distribution of menopausal status within each distinct geographical location. BMI; Body mass index, BP; Blood pressure, CMD; cardiometabolic disease, MVPA; Moderate-vigorous intensity activity. Women that reported as perimenopausal, had a hysterectomy or used birth control were excluded from this analysis. *These women had missing variables that prevented CMD classification.

3.2 Diversity measures

3.2.1 Alpha diversity

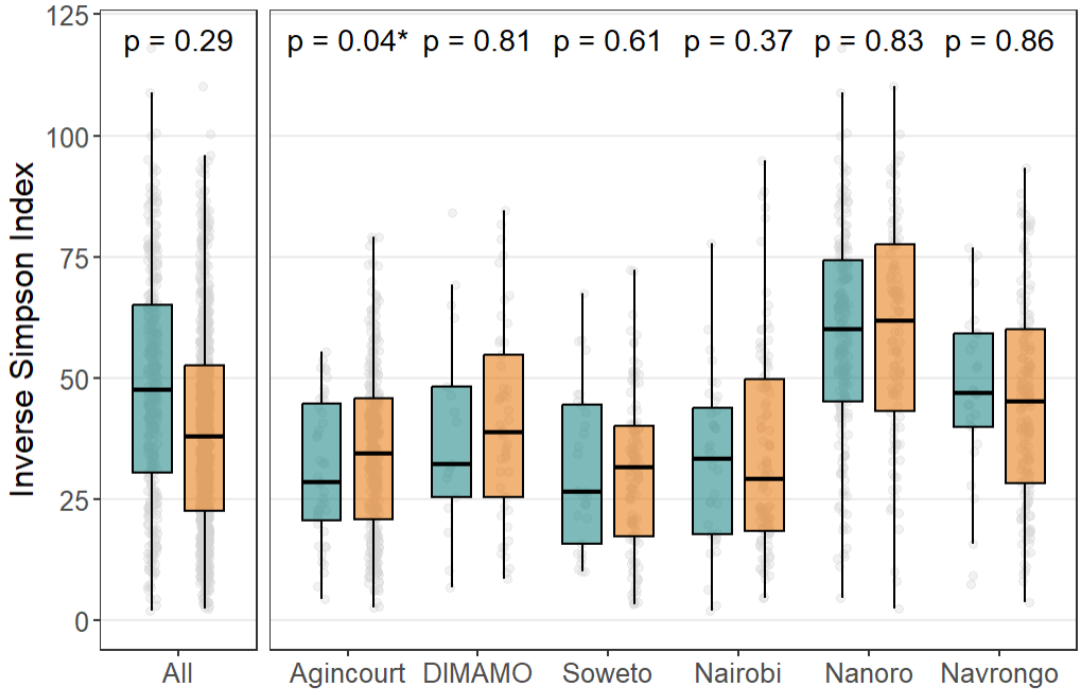
The Inverse Simpson Index revealed site- and menopause-dependent differences in microbial diversity (Figure 1A). Women from Nanoro and Navrongo exhibited the highest diversity, while those from Soweto had the lowest. When all sites were combined, premenopausal women had slightly higher microbial diversity than postmenopausal women, though the differences were not significant ($p = 0.29$). In contrast, site-specific analyses showed the opposite trend, with postmenopausal women generally exhibiting higher diversity, though this difference was statistically significant in Agincourt ($p = 0.04$) only. Nairobi was the only site where premenopausal women had higher diversity, aligning with the overall combined-site pattern, although the difference were not significant.

Analysis of continuous cardiometabolic traits including BMI, glucose levels, SBP, DBP and total cholesterol identified statistically significant correlations with alpha diversity in both pre- and postmenopausal women, with the exception of glucose levels, which showed no significant correlation in postmenopausal women (Supplementary Figure 1). The strength and direction of these relationships varied by geographic location (Supplementary Figure 2). These findings reveal complex interactions between microbial diversity and cardiometabolic traits that differ by menopausal status and geographic region.

Stratification by CMD status revealed that women with CMDs had lower microbial diversity than those without CMDs (Figure 1B). Within each CMD group, no significant differences were observed between pre- and postmenopausal women, suggesting that CMD status may be a stronger determinant of microbial diversity than menopausal status.

When CMD was analysed as a confounder, the analyses revealed a statistically significant negative relationship between individuals with CMD ($p = 0.01$) and microbial diversity. Further, the interaction between CMD and menopause was not significant ($p = 0.21$), and age was not significantly associated with microbial diversity ($p = 0.35$). These findings suggest that CMD status impacts microbial diversity, but menopause and age do not significantly influence it, nor is there a combined effect of CMD and menopausal status.

(A)



(B)

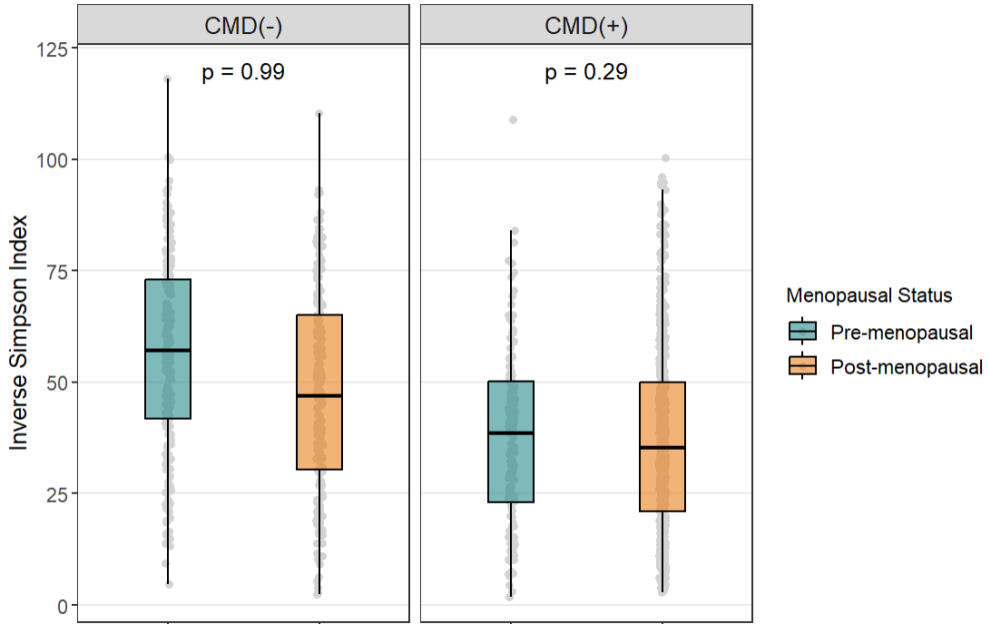


Figure 1: Microbial diversity by menopausal status, study site, and cardiometabolic disease (CMD) status. Figure 1A: Microbial diversity —measured by Inverse Simpson index which accounts for richness and evenness —is compared between menopausal groups across all sites combined and at individual sites. A linear mixed-effects model, accounting for site as a random effect, was used for the combined analysis, while standard linear regression was applied for site-specific analyses. * Indicates statistically significant differences. Figure 1B: Microbial diversity is compared by menopausal status within each CMD group. The differences between pre- and postmenopausal women were also assessed using a linear mixed-effects model accounting for site variability.

3.2.2 Beta diversity

This analysis investigated the effects of menopause status, CMD status, and their interaction on beta diversity across six sites. The findings revealed that the effect of menopause is not consistent across sites (Figure 2)**.** Menopause significantly affected beta diversity in Nanoro (p= 0.01) and showed borderline significance in Agincourt (p = 0.05), however, at both sites, the effect of menopause explains a very small percentage of the overall variance (~0.4 – 0.6%) in GM composition. In Nanoro CMD status also significantly influenced beta diversity (p =0.01**)**, and an interaction between menopause and CMDs is present (p = 0.03). In contrast, no significant effect of menopause, CMD or their interaction were observed in DIMAMO, Nairobi, Soweto, or Navrongo. These findings highlight that menopause-related microbial shifts are site-dependent and may be influenced by local environmental, dietary, or metabolic factors.

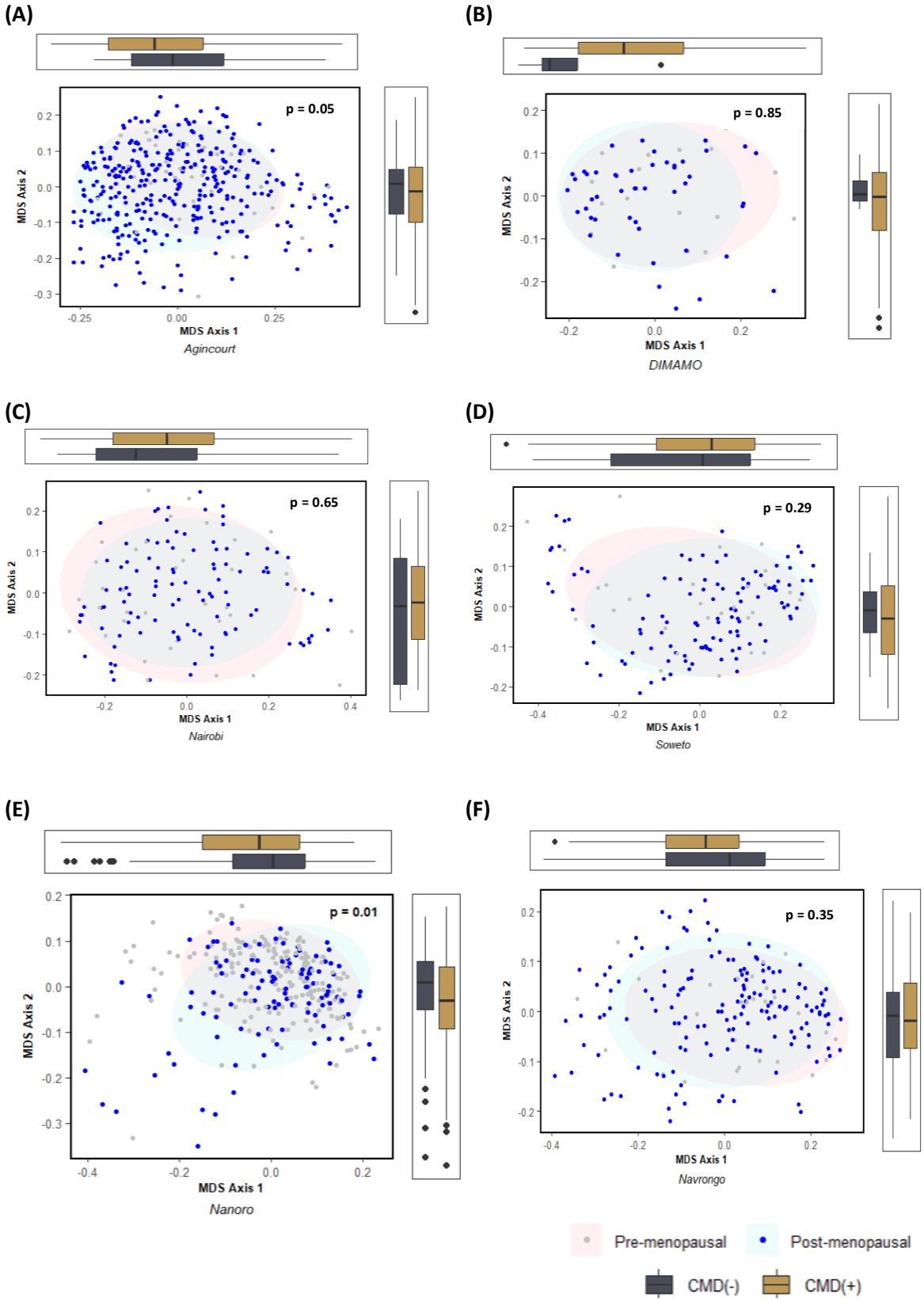


Figure 2. Beta diversity comparisons across six study sites by menopausal and cardiometabolic disease (CMD) status. Non-metric multidimensional scaling (NMDS) plots display Bray-Curtis distance matrix for women at six study sites: (A) Agincourt, (B) DIMAMO, (C) Nairobi, (D) Soweto, (E) Nanoro, and (F) Navrongo. Points are coloured by menopausal status. Ellipses represent the 75% confidence

interval for each menopausal group. Box plots summarise the distribution of MDS axis 1 (top) and axis 2 (right), stratified by CMD status. The p-values (PERMANOVA) shown indicate the effects of menopause on beta diversity at each site.

3.3 Differential abundance between menopausal groups

LEfSe identified site-specific differences in bacterial taxa between pre- and postmenopausal women (Figure 3). However, LEfSe analysis for DIMAMO failed, likely due to the small sample size limiting statistical power for detection of differentially abundant taxa. Differentially abundant taxa varied across sites, highlighting the influence of geographic factors on GM composition. Premenopausal women were enriched with taxa such as *Faecalibacterium prausnitzii, Bacteroides fragilis, Bifidobacterium dentium*, and *Prevotella* whereas, postmenopausal women showed increased abundance of taxa such as, *Fusicatenibacter saccharivorans, Ruminococcus_F. champanellensis*, *Collinsella bouchesdurhonensis* and *Clostridium saudiense*. These results reveal geographic variation in menopause-associated bacterial signatures, with each site showing unique patterns of taxonomic abundance.

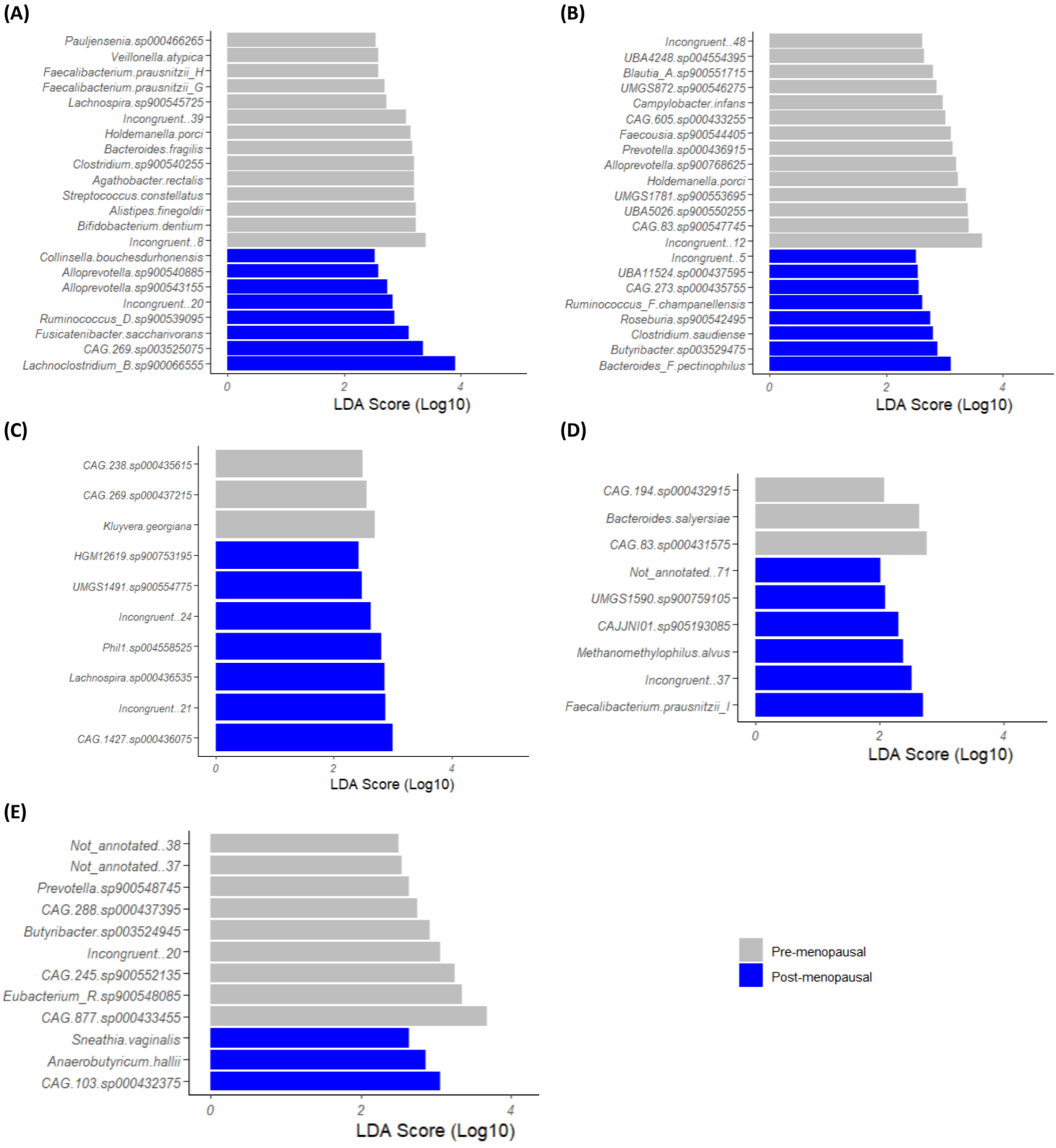


Figure 3: Linear discriminant analysis identifying differentially abundant bacterial taxa in pre- and postmenopausal women across five study sites. (A) Agincourt, (B) Nanoro, (C) Soweto, (D) Nairobi, (E) Navrongo. The LDA scores (x-axis) indicate the effect size of the differences in relative abundance. Only taxa with an LDA score >2 and a p-value <0.05 are shown.

4 Discussion

This study represents the first investigation of menopause-related changes in the GM and their association with CMDs in African women. By analysing data from women across six sites in four SSA countries, our study provides novel insights into the determinants of GM composition in African women. Our key findings reveal that cardiometabolic health status emerge as a stronger determinant of microbial diversity than menopausal status. Additionally, geographic location significantly influenced microbial diversity and composition patterns with substantial variation across study sites. Despite the predominant influence of CMD status, taxonomic analysis revealed differentially abundant bacterial taxa between pre- and postmenopausal women, suggesting that hormonal factors may still play a role in shaping specific aspects of the GM.

Our study revealed differences in health profiles between menopausal groups, with postmenopausal women exhibiting a higher prevalence of CMDs and associated risk factors such as elevated BMI, blood pressure, cholesterol, and glucose levels. These findings are consistent with previous studies that have reported increased cardiometabolic risk factors in postmenopausal women (Arthur et al., 2013; Chikwati et al., 2023; El Khoudary et al., 2020). Stratification by CMD status revealed that women with CMDs had lower microbial diversity than those without CMDs, regardless of menopausal status. Within each CMD group, no significant differences were observed between menopausal groups. Our findings revealed that CMD status is an important factor influencing microbial diversity, with the presence of CMDs associated with lower diversity, while age showed no significant association. While previous research has established an association between menopause-related GM dysbiosis and increased cardiometabolic risk (Peters et al., 2022; Zhao et al., 2019), our findings indicate that CMD status exerts a stronger influence on microbiome diversity than menopause, with CMD presence associated with lower diversity independent of menopausal status. Although menopause may not be the primary driver of microbiome changes in our study, it likely contributes indirectly by increasing CMD risk, which in turn impacts microbial diversity, highlighting a complex and interrelated pathway. The absence of a significant age effect in our study further supports the notion that hormonal and metabolic shifts, rather than chronological aging alone, drive microbiome changes. Future studies exploring mediation models are needed to disentangle these relationships.

The stronger influence of CMD status over menopausal status in shaping the GM contrasts with previous studies in Western and Asian populations, which have reported more consistent associations between menopause and reduced microbial diversity (Peters et al., 2022; Zhao et al., 2019). Given that hormone levels were not measured in this study, the relationship between hormones and the GM warrants further investigation. The literature indicates that estrogen plays a key role in maintaining gut

homeostasis by regulating intestinal barrier function and microbial composition (Kwa et al., 2016). Additionally, the gut microbiota can metabolise estrogens via the estrobolome, potentially affecting both hormone levels and host physiology (Ervin et al., 2019). Future studies incorporating hormone measurements alongside microbiome data would help clarify these relationships in African women.

Beyond the effects of CMD status, geographic location emerged as another important determinant of microbial diversity. Regional differences in GM diversity across our study sites indicate that local factors including dietary habits, lifestyle practices, and environmental conditions may substantially influence microbial community structures. Sites like Nanoro and Navrongo demonstrated greater microbial diversity, potentially reflecting traditional diets high in plant fibers and fermented foods, while Soweto's lower diversity might be attributed to urban living patterns and adoption of Western dietary practices. These findings highlight the need for region-specific analyses to elucidate how local exposures interact with menopause and CMD status to influence the GM. While some studies have revealed that premenopausal women have higher alpha diversity than postmenopausal women (Peters et al., 2022; Zhao et al., 2019), others reported that there were no differences in microbial diversity between menopausal groups (Santos-Marcos et al., 2018). In our study, premenopausal women showed higher microbial diversity than postmenopausal women overall, though these differences were not statistically significant. While premenopausal women exhibited higher alpha diversity overall, site-specific analysis revealed the opposite pattern, with postmenopausal women showing higher diversity in most sites except Nairobi. This discrepancy likely stems from differences in richness and evenness, where premenopausal women had higher richness but lower evenness, leading to an overall pattern of higher diversity despite local site differences. The observed variability emphasizes the importance of considering both richness and evenness when interpreting microbial diversity metrics. The contrasting findings across studies on menopause-related shifts in alpha diversity further suggest that these microbial changes may not be universal but rather shaped by population-specific factors.

Prior research in Western and Asian populations has reported significant differences in beta diversity between menopausal groups (Peters et al., 2022; Santos-Marcos et al., 2018; Zhao et al., 2019). However, our findings suggest that the impact of menopause on GM composition is not uniform across populations. The observed site-dependent effects indicate that menopause-related microbial shifts may be modulated by region-specific factors such as diet, environmental exposures, or genetic background. Notably, while menopause was significantly associated with beta diversity in Nanoro and marginally in Agincourt, the proportion of variance explained was minimal, suggesting that additional determinants contribute to microbial differences. Importantly, Nanoro had the highest over-

representation of premenopausal women, whereas the other sites had a greater proportion of postmenopausal participants. These imbalances in menopausal group representation may have influenced the strength and direction of associations detected at each site. Further, the interaction between CMD status and menopause observed in Nanoro highlights the complexity of these relationships, suggesting that the metabolic consequences of menopause may be more pronounced in certain settings. The absence of significant associations in other sites warrants further investigations into the relative contributions of menopause, CMDs, and local environmental influences on the GM.

Despite the stronger influence of CMD status, our taxonomic analysis revealed differentially abundant taxa between pre- and postmenopausal women, although these patterns varied by geographical location. Premenopausal women were enriched with SCFA-producing bacteria such as *Faecalibacterium prausnitzii*, *Bacteroides fragilis*, and *Prevotella.* SCFA have been shown to play a role in the regulation of host metabolism, modulating immune responses and delaying the progression of CMDs such as obesity, hypertension, and type 2 diabetes (Tagliamonte et al., 2021; Yang et al., 2018; Zhao et al., 2018). The reduced abundance of *Faecalibacterium prausiitzii*, a butyrate producer has been observed in conditions such as obesity and hypertension (Bier et al., 2018; Yang et al., 2023), suggesting its potential involvement in CMD progression. *Bacteroides fragilis* has been shown to protect the intestinal barrier and support gut homeostasis in murine models (Sofi et al., 2021). While the role of *Prevotella* in human health remains complex due to its functional diversity, its higher abundance in non-industrialised populations suggests a possible protective effect against metabolic diseases (Hitch et al., 2022; Prasoodanan et al., 2021). These findings highlight the potential protective role of specific microbial taxa in premenopausal women and their relevance to metabolic health.

In contrast to previous studies reporting decreased SCFA producers in postmenopausal women (Peters et al., 2022; Zhao et al., 2019), our analysis revealed distinct taxonomic patterns. Postmenopausal women showed enrichment of both potentially beneficial and harmful species. *Ruminococcus champanellensis* specialises in cellulose degradation—a major component of plant-based foods—leading to the production of SCFAs such as acetate and succinate, which play key roles in gut health (Ben David et al., 2015; Chassard, et al., 2012). Higher levels of the *Ruminococcus* genus have been linked to lower odds of type 2 diabetes, suggesting that its presence may confer metabolic benefits (Wang et al., 2024). Similarly, *Fusicatenibacter saccharivorans produces beneficial SCFAs such as acetate and lactate which* enhance gut health (Takada et al., 2013). However, other enriched species may have detrimental effects. *Collinsella bouchesdurhonensis*, though understudied, belongs to a genus associated with increased intestinal permeability, potentially contributing to metabolic dysfunction and CMD development (Chen et al., 2016). Additionally, an increased abundance of the

Collinsella genus has also been linked to type 2 diabetes (Lambeth et al., 2015). *Clostridium saudiense* was first isolated from a faecal sample of an individual with obesity (Angelakis et al., 2014) however, its specific metabolic implications require further investigation. This mixed profile of beneficial and potentially harmful bacteria indicates that the GM changes during menopause may be more complex than previously understood and potentially influenced more by CMD status than by menopausal status directly.

These taxonomic shifts may reflect both hormonal changes and the higher prevalence of CMDs in postmenopausal women. The differential abundance of certain bacterial taxa between menopausal groups suggests that hormonal factors may still play a role in shaping specific aspects of the GM, even if overall diversity is more strongly influenced by CMD status. The loss of estrogen during menopause may create a metabolic environment that alters the competitive dynamics between different bacterial species, while the presence of CMDs may further modify these interactions. The site-specific variations in taxonomic patterns suggest that local dietary habits, environmental exposures, lifestyle and cultural practices may significantly modify how both CMD status and menopausal status affect the GM.

Strengths and Limitations

Our study has several strengths. First, it represents the largest investigation of menopause-related GM changes in African women to date, with a substantial sample size of 1232 women across multiple sites in SSA, allowing for a more comprehensive understanding of geographic and population-specific microbiome determinants. Second, our comprehensive health data, including CMD risk factors, offers deeper understanding into the relationship between cardiometabolic health, menopausal status, and GM composition. Third, our methodology advances the field by studying menopause-microbiome relationships in the context of CMD risk across diverse African populations, revealing regional variations and complex interactions that could not be captured in previous studies of more homogeneous populations.

Despite these strengths, our study has several limitations that should be considered in future research. The over-representation of postmenopausal women at most study sites, except Nanoro where premenopausal women predominated, is a notable limitation. This imbalance may have influence microbial comparisons between the groups and highlights the need for adequate and balanced sampling. The failure of LEfSe analysis in DIMAMO likely driven by the small and uneven sample size, further reinforces the importance of achieving balanced sample sizes for reliable subgroup analyses.

While age and CMD prevalence increase concurrently in postmenopausal women, our findings suggest that CMD status, rather than chronological age alone, plays a dominant role in shaping the

microbiome. The lack of a significant effect of age in our statistical models further supports this interpretation. However, future research with stratified age groups or age-matched cohorts is warranted to refine these associations.

The cross-sectional design inherently limits our ability to establish causality between microbiome changes, menopausal transition, and CMD development. Without longitudinal data, we cannot determine whether observed microbiome alterations precede or follow menopausal transitions and CMD onset. Additionally, while the use of self-reported menstrual patterns has been deemed appropriate for identifying the different menopausal stages, incorporating sex hormone measurements would have expanded our understanding of the bidirectional relationship between hormonal fluctuations and GM composition in menopause. The use of a composite CMD score, while useful for providing an overall measure of cardiometabolic health, may have masked important associations between individual conditions and specific microbial patterns. Moreover, our reliance on LEfSe analysis, while useful for identifying differentially abundant taxa, has limitations as it does not account for crucial confounding factors such as age, lifestyle factors, socio-economic status, and medication use, all of which can independently influence the GM composition and potentially bias the observed associations.

Future recommendations

These limitations point to several promising directions for future research. Future investigations should expand beyond taxonomic identification to explore the functional implications of differentially abundant taxa between menopausal groups. Detailed functional profiling and pathway analysis could investigate whether these taxa share common functional features or metabolic pathways, potentially revealing conserved biological mechanisms underlying menopause-related microbiome changes despite taxonomic variations across different sites.

Future studies should also consider age-matched male cohorts, or alternatively, age-stratified female cohorts, to better disentangle hormonal vs. aging effects. However, given sex differences in GM composition prior to menopause, male controls may not fully capture menopause-specific changes. Additionally, the inclusion of sex hormone measurements such as estradiol, FSH, and SHBG may clarify whether the overall non-significant relationship observed between menopausal status and microbial diversity could be attributed to hormonal differences.

Incorporating other potential confounding variables such as physical activity, diet, socioeconomic status, and regional factors through appropriate statistical adjustments would better capture the extent of the effects of CMDs and menopause on the GM. Analysing individual CMD traits—such as

fasting glucose, SBP, DBP, cholesterol levels, and waist circumference—separately rather than using composite scores could reveal more specific microbiome associations with particular cardiometabolic parameters. This trait-specific approach may uncover more precise biological mechanisms connecting the GM to distinct aspects of cardiometabolic health.

Research should also prioritise balanced sample sizes by implementing recruitment methods that allow equal representation across pre- and postmenopausal groups. Additionally, interventional studies targeting the GM in postmenopausal women with and without CMDs could help determine whether modifying the microbiome can improve cardiometabolic health outcomes in this population. Longitudinal studies following women through the development of CMDs and the perimenopausal transition would also help establish temporal relationships, clarifying whether CMD-related GM changes precede or follow hormonal changes.

5 Conclusion

This study provides the first comprehensive investigation of menopause-related GM changes in African women across multiple sites in SSA. Our findings contrast with prior research in non-African populations by demonstrating that rather than menopause directly altering the GM and subsequently increasing CMD risk, the higher prevalence of CMDs in postmenopausal women may be driving the observed microbiome changes. Geographic location also emerged as a significant determinant, underscoring the role of region-specific factors such as dietary patterns, lifestyle behaviours, environmental exposures, and socioeconomic conditions in shaping microbial communities. While we observed distinct taxonomic differences between pre- and postmenopausal women, these patterns varied by location and included both beneficial and potentially harmful bacteria in postmenopausal women. To further elucidate these complex relationships, future research should prioritise longitudinal studies following women through menopausal transition and CMD development, employ more robust methodological approaches including age-matched controls and hormone measurements, and expand beyond taxonomic analysis to functional profiling. Additionally, interventional studies targeting the GM in postmenopausal women could determine whether microbiome modification might improve CMD outcomes. This study underscores the complex interplay between hormonal status, geographic factors, and metabolic health, emphasizing the need for population-specific approaches to women's health research and clinical interventions targeting the gut-hormone-metabolism axis in African women.

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7 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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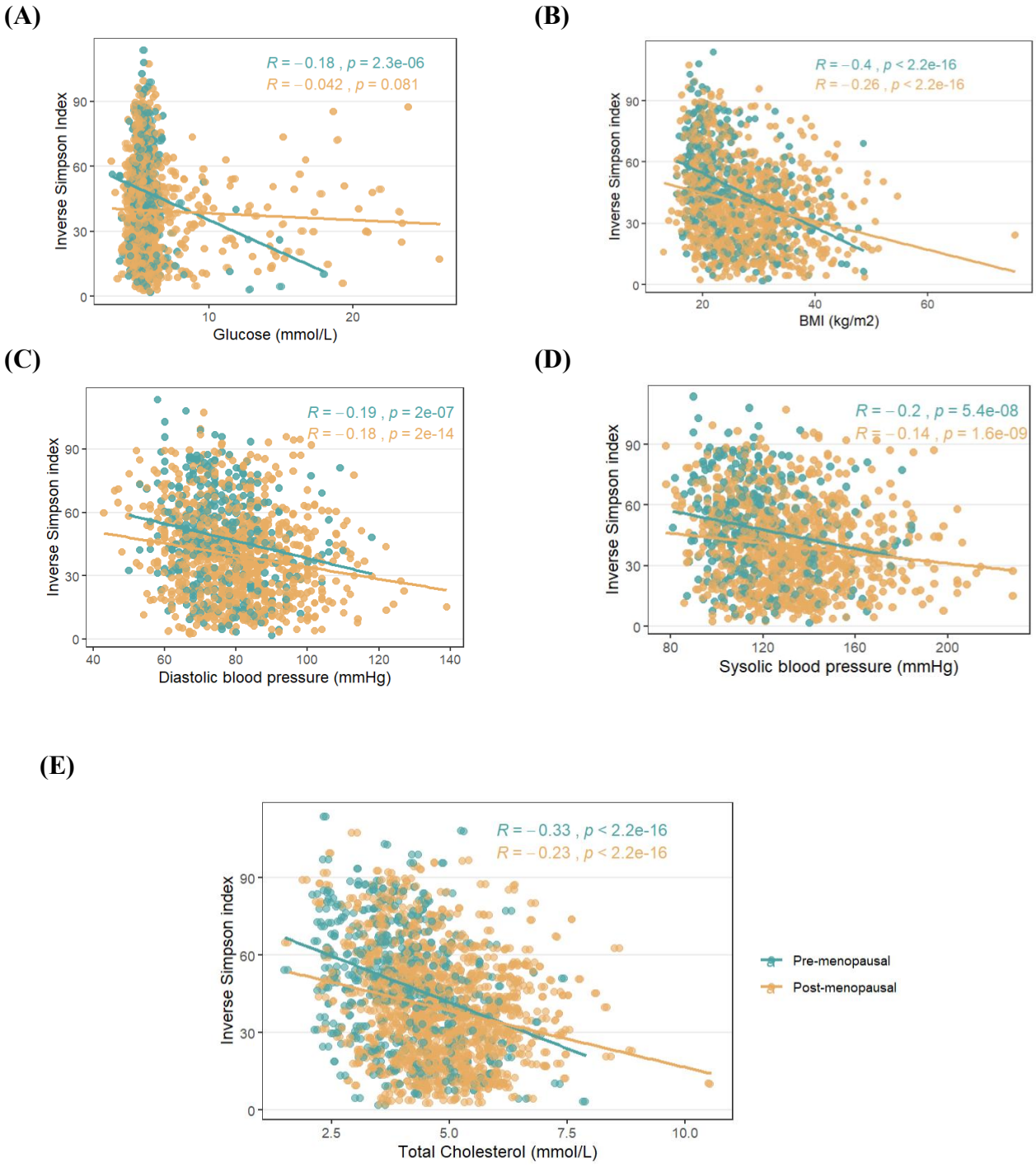
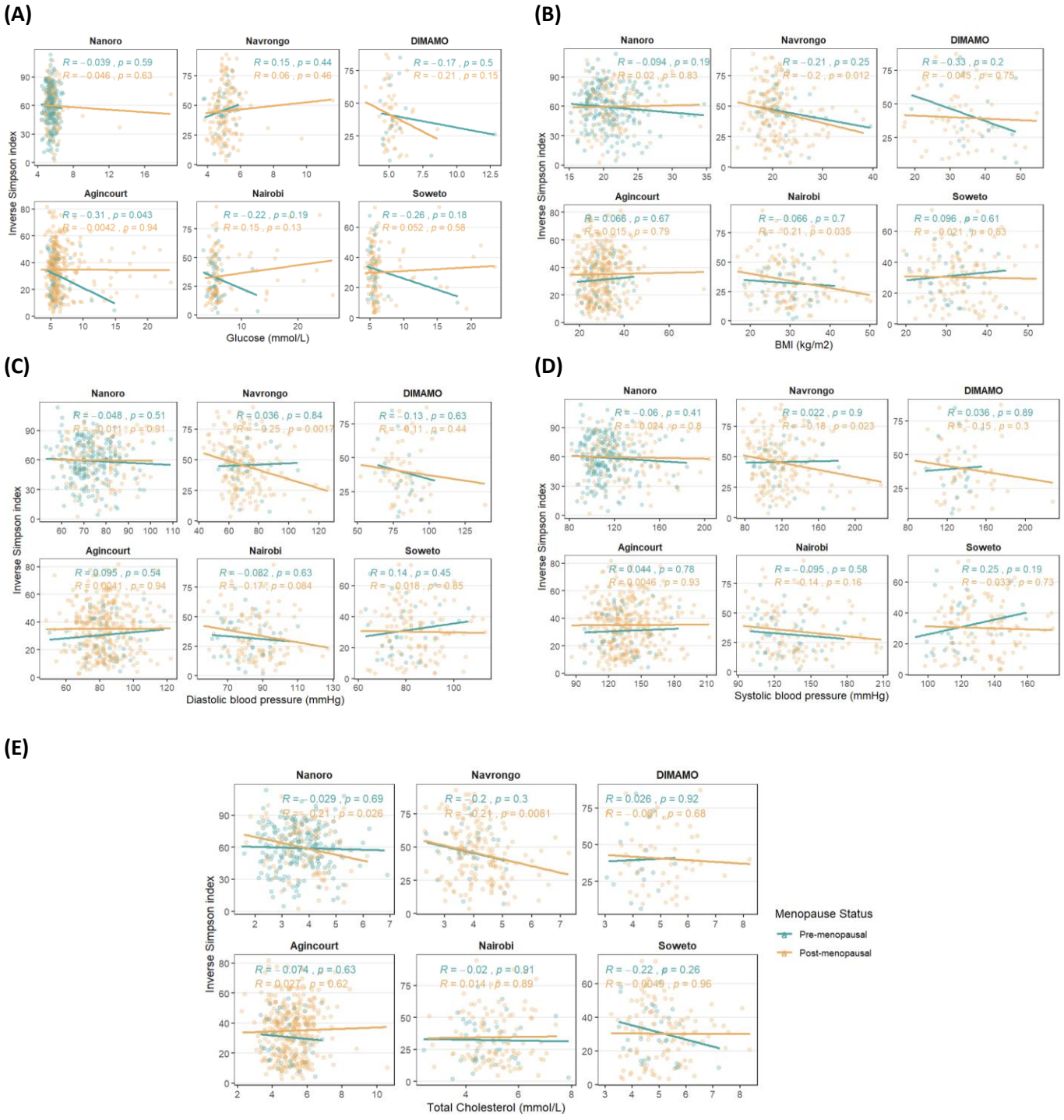
9 Supplementary materials



Supplementary Figure 1: Alpha diversity by menopausal status and cardiometabolic risk traits This figure illustrates the relationship between alpha diversity and cardiometabolic traits, including (A) glucose, (B) body mass index (BMI), (C) diastolic blood pressure (DBP), (D) systolic blood pressure (SBP), and (E) total cholesterol.



Supplementary Figure 2: Alpha diversity variations in cardiometabolic risk traits across menopausal status and study Sites. This figure explores alpha diversity relationships with cardiometabolic traits [(A) glucose, (B) body mass index (BMI), (C) diastolic blood pressure (DBP), (D) systolic blood pressure (SBP), and (E) total cholesterol] comparing pre- and postmenopausal women across six study sites.

Appendices

Appendix 1: Approved Research Protocol

Appendix 2: Ethics Clearance certificate (M240765)

Appendix 3: Plagiarism declaration and Turnitin originality report

Appendix 4: Frontier Author Guidelines

Appendix 1: Approved research protocol

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Dear Miss Phehello Chauke

Master of Science in Medicine: Approval of Title

We have pleasure in advising that your proposal entitled *Menopause-related changes in the gut microbiome and their association with cardiometabolic diseases in women from four sub-Saharan African countries* has been approved. Please note that any amendments to this title have to be endorsed by the Faculty's higher degrees committee and formally approved.

Yours sincerely

Ms Sandra Munesar
Faculty Registrar
Faculty of Health Sciences

Protocol Proposal

Menopause-related changes in the gut microbiome and their association with cardiometabolic diseases in women from four sub-Saharan African countries

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MSc (Med) in Genomic Medicine

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1. Background literature analysis and critique

1.1 Introduction

The average woman spends approximately three decades of their lives post-menopause (World Health Organization, 2022). Menopause is characterised by the cessation of menstruation resulting from a decreased ovarian function after 12 consecutive months without menstruation (OlaOlorun & Shen, 2020). This transition is part of normal biological ageing and is accompanied by a decline in ovarian sex hormones such as estrogen, progesterone, and sex hormone binding globulin (Liu et al., 2022a). The alteration in the levels of sex hormones is met with a myriad of symptoms that vary in severity across women; these symptoms include but are not limited to changes in metabolism, mood and sleep, vaginal atrophy, and hot flashes (World Health Organization, 2022).

Over the last 20 years, sub-Saharan Africa (SSA) has experienced a significant rise of non-communicable diseases (NCDs), primarily due to the growing prevalence of cardiometabolic risk factors like sedentary lifestyles, hypertension, obesity, diabetes, hypercholesterolaemia, among others (Forouzanfar et al., 2016). Moreover, research has shown an association between menopausal transition and an increase in cardiometabolic risk factors (Peters et al., 2022; Chikwati et al., 2023). The link between menopausal transition and cardiometabolic diseases is poorly understood however, research suggests that the gut microbiome (GM) is a mediator between menopause and increased risk of cardiometabolic diseases (Liu et al., 2022b; Peters et al., 2022).

The microbiome is a collection of genes from trillions of microbes that inhabit the human body, including bacteria, archaea, viruses, and eukaryotes (Human Microbiome Project Consortium, 2012). While the microbiome colonises various organs in the body, the gut is the most extensively colonised organ with 10-100 trillion microbes. The GM plays an important role in many physiological processes including digestion, metabolism, and immune function and it is continuously shaped by interactions between the host genetics, diet, lifestyle, age, medication, and environmental factors (Human Microbiome Project Consortium, 2012).

Emerging research suggests a bidirectional relationship between the GM and sex hormones. Moreover, there is growing recognition of the GM's involvement in modulating lipid metabolism, systemic inflammation and insulin resistance which are key variables in cardiometabolic health (Ramezani Tehrani et al., 2013). Given that postmenopausal women face increased risks of developing cardiovascular disease and metabolic disorders, understanding the association between the GM and menopause may hold significant implications for mitigating these health outcomes (El Khoudary et al., 2019).

1.2 Gut microbiome research in African populations

Large cohort studies such as the Human Microbiome Projects (HMP), Metagenomics of the Human Intestinal Tract (MetaHIT), and the Dutch Microbiome Project have played a pivotal role in our understanding of the GM with disease and lifestyle factors, shedding light on associations between socioeconomic and environmental influences. However, these initiatives have primarily focused on European countries, resulting in underrepresentation of African populations, as reviewed in Allali *et al*. (2021) and Pheeha *et al*. (2023). Initiatives such as the Human Hereditary and Health in Africa (H3Africa) Consortium have played a pivotal role in promoting GM research among African populations, notably through collaborations like the Africa Wits-INDEPTH Partnership for Genomics studies (AWI-Gen study) (Ali *et al*., 2018). Through these collaborative efforts and population-based cohorts, researchers aim to understand the interplay between the GM and the factors that influence it in African populations.

1.3 Gut microbiome and Menopause

1.3.1 The relationship between the gut microbiome and menopause

Studies suggest a bidirectional relationship between GM and menopause. While the liver is primarily responsible for metabolising endogenous estrogen through conjugation with glucuronide or sulfate groups, the GM harbours an estrobolome —a collection of microbial genes that can deconjugate

estrogen and other sex hormones (Kwa et al., 2016; Ervin et al., 2019). The estrobolome allows for the reactivation of sex hormones, promoting their reabsorption into the enterohepatic circulation and systemic circulation (Ervin et al., 2019). Estrogen does not only play a role in the reproductive system, but it is also responsible for maintaining homeostasis in the gut by influencing the intestinal barrier function and microbial composition (Kwa et al., 2016). Therefore, deficiency in estrogen due to menopause disrupts the balance of the GM and affects the microbial diversity and composition causing alterations of the GM (Ramezani Tehrani et al., 2013; Peters et al., 2022).

1.3.2 Gut microbiota changes in menopause: Evidence from studies

Research indicates that menopause is linked to a reduction in GM diversity, leading to a shift towards greater similarity with the male GM. Both animal and human studies support this alteration in the GM during menopause. Studies on mice have shown sex differences in the gut microbiota composition which were reversed when female mice were ovariectomised and when male mice were castrated, suggesting that sex hormones can alter the GM (Yurkovetskiy et al., 2013). Similarly, human studies observed sex differences in the gut microbiota emerging post- puberty. One study compared the gut microbiota of pre- and postmenopausal women, and the findings revealed that menopause results in a decreased GM diversity (Zhao et al., 2019). Another study observed that the GM make-up of postmenopausal women more closely resembled that of men compared to premenopausal women although no differences in diversity were noted (Santos-Marcos et al., 2018). These discrepancies in findings may stem from the limited sample sizes and variations in population groups, given that the GM is influenced by environmental and lifestyle factors.

1.3.3 Microbial taxa previously identified in pre- and postmenopausal women

The microbial taxa previously identified in pre- and postmenopausal women reveal intriguing insights into the association of the GM and menopause. Yang et al. 2022 reviewed three studies that explored the GM-menopause association and there were no differences in the abundance of Bacteroidetes and Firmicutes between pre- and postmenopausal women. However, in another study, postmenopausal women exhibited an increased abundance of Firmicutes and a decreased abundance of Bacteroidetes

resulting in a higher Firmicutes to Bacteroidetes ratio (Santos-Marcos et al., 2018). Conversely, another study reported that postmenopausal women have a decreased Firmicutes to Bacteroidetes ratio due to a decline in Firmicutes abundance and an increase in Bacteroidetes abundance (Zhao et al., 2019)

At the genus level, postmenopausal women displayed higher levels of *Bilophila* and *Odoribacter* (Yang et al., 2022). Contrastingly, Santos-Marcos et al. 2018 reported a reduced abundance of *Bilophila*. Another study reported a decreased abundance of *Akkermansia muciniphila, Faecalibacterium* and *Roseburia* in postmenopausal women, which play a role in generating short-chain fatty acids (SCFA) that enhance insulin sensitivity and improve glucose metabolism (Zhao et al., 2019). Thus, the reduced abundance of these bacteria in postmenopausal women may elevate the risks of cardiometabolic disorders (Zhao et al., 2019; Peters et al., 2022). Again, the inconsistencies in the findings may result from the limited sample sizes and differences in the populations.

1.4 The association between the GM, menopause, and cardiometabolic diseases

The phase preceding menopause, known as menopausal transition or perimenopause, is marked by menstrual irregularities and a decline in sex hormone levels (Harlow et al., 2012). Studies suggest that this transition is associated with increases in cardiometabolic risk factors. One study carried out in a cohort of 143 premenopausal and 107 postmenopausal Ghanaian women reported a higher occurrence of metabolic syndrome in postmenopausal women compared to the premenopausal women. Significant variations between the two groups were observed in blood pressure, fasting blood glucose, low density lipoprotein, and high-density lipoprotein cholesterol (Athur et al., 2013). Age is an important factor to consider when investigating the relationship between menopause, GM, and cardiometabolic disorders, as these variables are influenced by age (Forouzanfar et al., 2016; Odamaki et al., 2016). It remains unclear whether the increase in cardiometabolic risk factors among postmenopausal women is attributed to reproductive or chronological aging. However, some studies suggest that menopause has an influence on several cardiometabolic disease biomarkers beyond the effects of chronological aging (Wang et al., 2018). Additionally, the prevalence of metabolic syndrome and its biomarkers have been shown to increase with

menopause independent of the effects of chronological aging (El Khoudary et al., 2020). Peters et al. (2022) investigated the association between menopause, the GM, and its potential implications on cardiometabolic disease in Hispanic women, and their findings revealed that alterations in the GM as a sequelae of menopause results in adverse cardiometabolic profiles. They found that in postmenopausal women, Clostridium lactatifermentans, which is associated with lower risks of metabolic syndrome, was depleted. Additionally, postmenopausal women had increased levels of Sutterella wadsworthensis which is associated with elevated blood pressure—another variable of cardiometabolic disorder. However, this was a cross-sectional study and even though associations could be identified, causality cannot be inferred as there may be other factors influencing these relationships (Peters et al., 2022)

1.5 Rationale

Menopause is accompanied by alterations in the level of sex hormones such as estrogen and progesterone which can impact disease susceptibility. While research has established an association between the menopausal transition and the GM composition, understanding how these microbial communities influence menopause-associated health outcomes such as cardiometabolic disorders is limited. Moreover, much of the existing research has been conducted within European populations, limiting the generalizability of findings to diverse ethnicities and geographical regions. Thus, there is a need to extend this research to African cohorts, where unique genetic, environmental, and lifestyle factors may modulate the GM and menopausal health. By defining the microbiome signature of menopause, this study seeks to address this gap in knowledge, offering insights that could inform more nuanced and culturally relevant approaches to women's health management within the genomic medicine landscape across Africa.

2. Aims and objectives

2.1 Aim

To investigate how the gut microbiome composition differs in pre- and post-menopause and its

association with cardiometabolic diseases in African women.

2.2 Objectives

1. To describe the α and β microbial diversity in premenopausal and postmenopausal women with and without cardiometabolic disease.

2. To identify differentially abundant taxa in premenopausal and postmenopausal women with and without cardiometabolic disease.

3. To contextualise the microbiome signatures of menopause and cardiometabolic disease through functional profiling

3 Methods

3.1 Study participants

The study cohort consists of 1,803 women aged 42-86 years from Burkina Faso, Ghana, Kenya, and South Africa; Agincourt-Bushbuckridge, Dikgale (also called DIMAMO), and Soweto. These women were semi-randomly selected from the broader participant pool of the AWI-Gen 2 study. The AWI-Gen study spanned 10 years and it encompasses two phases: phase 1 (2013- 2016) and phase 2 (2018-2023). The primary objective of this study is to delve into the interaction between genetic and environmental factors shaping disease prevalence, with a specific emphasis on cardiometabolic disorders among African populations. Phase 2 entailed revisiting phase 1 participants to evaluate longitudinal changes in their health and exposures, while also capturing new data on their exposure to pollutants, respiratory health, sleep, and life stressors. Trained field workers or staff from AWI-Gen guided participants through a questionnaire covering details like their demographic profile, family structure, education and employment, general health, pregnancy, menopause, hysterectomy, and the use of contraceptives amongst others.

3.1.1 Inclusion and exclusion criteria

For this substudy, only individuals who were assigned female at birth and were participants of AWI-Gen 1 and 2 were included. The AWI-Gen 1 and 2 studies excluded pregnant females, first degree relatives of existing participants, and individuals that had been residents for less than 10 years in each region.

3.2 Study design

This is a cross-sectional data analysis study that will utilise already existing data generated from shotgun metagenomic sequencing. The data have already undergone quality control processes and taxonomic analysis and are ready for downstream analysis. DNA extraction was performed on faecal samples followed by shotgun metagenomic sequencing using the Illumina platform. Subsequently, an in-house Nextflow pipeline was utilised to analyse the metagenomic reads, which involved deduplication, trimming, and alignment to the Human genome build 38 (Hg38) using BWA v0.7.17, followed by taxonomic profiling of the metagenomic reads using mOTUs v3.0.3 to allow for downstream analysis.

3.2.1 Menopausal staging

For menopause staging, the participants were asked about their menstruation patterns and were then grouped into the different menopausal groups: premenopause— regular menstruation, perimenopause— irregular menstruation in the last 12 months and postmenopause— no menstruation for 12 or more consecutive months. Perimenopausal women will be excluded as this transitional phase is associated with extreme fluctuating hormone levels (Harlow et al., 2012). These fluctuations could potentially hinder the identification of distinct differences in the GM across well-defined menopausal stages. Excluding the perimenopausal group ensures that there's a clear differentiation between women who have undergone the menopausal transition (Postmenopausal) and those who have not (Premenopausal). Additionally, women with surgery-induced menopause (i.e., hysterectomy) and those on hormonal contraceptives will be excluded to isolate the effects of natural menopause from surgical menopause

and to avoid confounding from altered sex hormones. The use of self-reported menstruation patterns has been deemed appropriate for identifying the different menopausal stages (Harlow et al., 2012). However, we are awaiting additional hormone data from AWI-Gen and there may be re-evaluations if the hormone data comes on time. The hormone data will be used as a continuous variable instead of relying on self-reported categories to allow for more direct menopausal status measurement.

3.2.2 Classification of cardiometabolic disorders

The AWI-Gen study calculated body mass index (BMI) using the participant's weight (kg) and height ($m2$) defining obesity as BMI $\geq$ 30 kg/$m2$ (Ali et al., 2018). Hypertension was defined as systolic blood pressure (SBP) > 140 mmHg and/or diastolic blood pressure (DBP) > 90 mmHg, taking hypertension medication, or a previous diagnosis from a healthcare professional. For this study, these variables will be analysed as continuous rather than categorical variables to maintain statistical power.

4. Data analysis

To achieve the set objectives, the demographic and clinical characteristics including age, and site will be stratified based on self-reported menopausal status. The GM data will be integrated with the metadata including cardiometabolic disease status to allow for proper alignment. Using the integrated microbiome-metadata, the study population will be stratified into premenopause and postmenopause groups and associations between microbiome features and continuous BMI and blood pressure variables will be performed within and between groups.

The alpha diversity, or a measure of microbial diversity within an individual, will be measured as prokaryotic richness or the Shannon/Simpson index using the vegan package in R. Then beta diversity, or the similarity between microbiomes, will be measured using Bray-Curtis distance using the vegdist function from the vegan package on R. For differential abundance analysis, a linear mixed-effect model implemented through the lmerTest R package will be used. There are several factors that may affect the composition of the GM. For example, the composition of the GM changes with age leading to changes

in the in the microbial profiles that may be incorrectly attributed to menopausal status. Additionally, the risk of developing cardiometabolic diseases increases with age. Given that the participants in this study are older women (42-86 years), age could confound the analysis, making it difficult to distinguish whether the observed health outcomes are due to menopause or chronological aging. Failure to account for age in the linear mixed effect model could lead to misleading conclusions about the effects of menopause on the GM and cardiometabolic disease. Therefore, we will adjust for confounding variables, such as age, site, and HIV status. These covariates will be included based on preliminary analysis using Pearson's correlation coefficient to identify phenotype data that are strongly correlated with menopausal status, BMI, systolic blood pressure, or diastolic blood pressure.

R v4.3 (RStudio, PBC, Boston, https://www.rstudio.com/) will be used for all the statistical analysis. The Benjamini-Hochberg procedure implemented using the p. adjust function in base R will be employed to correct for multiple testing. The level of significance for the statistical analyses will be set at $p < 0.05$ and results will be visualized with the ggplot2 package in R.

Functional profiling of the GM will be conducted to understand its metabolic potential and associations with menopause and cardiometabolic risk. This will involve the annotation of metagenomic reads with functional information such as gene families, metabolic pathways, and enzyme functions. The HMP Unified Metabolic Analysis Network (HUMAnN) 4 bioinformatics tool that leverages databases such as the MetaCyc and Uniprot Reference Clusters (UniRef) will be employed. HUMAnN 4 will be accessed via the ZA-Wits-Core Cluster (PI: Prof Scott Hazelhurst) and it will quantify the abundance of gene families, enzymes, and metabolic pathways present in the metagenomic data then output files will be processed to generate plots summarising the functional profiles of the GM. The functional analysis results will be interpreted to identify key metabolic pathways and functions associated with menopause and cardiometabolic disorders.

5. Ethics

This study involves analysis of data that was collected and generated as part of the AWI-Gen 2 (M2210108). No contact will be made with the participants from whom the data was collected from. Permission to access the data was granted on the 8th of March 2024 (PI: Michele Ramsay) and a substudy ethics application was submitted to the Human Research Ethics Committee (Medical) of the University of Witwatersrand on the 15th of March 2024 and is pending approval.

6. Timing

This study will span a duration of 10 months as shown in Table 1.

Table 1: Gantt chart indicating the time allocations for this proposed study.

	2024										2025
	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Feb
Literature Review											
Ethics Application											
Protocol Write-up											
Protocol Assessment											
Data Analysis											
Research Write-up											
Research report submission and presentation											
External moderation											

7. Funding

AWI-Gen is funded by the National Human Genome Research Institute (NHGRI) & National Institute of Environmental Health Sciences (NIEHS) of the National Institutes of Health under award number U54HG006938**.** This substudy is a data analysis study and all primary data that will be analysed has

already been generated therefore, funding is not required. Additional hormone data are being generated by AWI-Gen; however, funding is already in place.

8. Limitations

- This is a cross-sectional study and while it may identify associations between microbiome composition, menopause status, and cardiometabolic disorders, establishing causality would require additional evidence. Thus, it is important to interpret the findings cautiously to avoid overinterpreting correlation as causation.

- Despite the efforts to control for confounding variables, unmeasured variables may still influence the observed associations between the GM, menopause, and cardiometabolic disorders thus impacting the validity of our results.

- Within African populations, there are differences in the genetic backgrounds, cultural practices, and environmental exposures. This study includes participants from six sites in four African countries. Once we stratify by menopause, cardiometabolic disorders, and by site we may run into statistical power issues.

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Appendix 2: Ethics Clearance Certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research and Innovation)

TO:	Ms P Chauke School of Pathology Division of Human Genetics Faculty of Health Sciences E-mail: 2302562@students.wits.ac.za
CC:	Supervisor: Professor S Hazelhurst; Drs L Olubayo and D Maghini Scott.Hazelhurst@wits.ac.za and <HREC-Medical Research Office@wits.ac.za>
FROM:	Mr Iain Burns Human Research Ethics Committee (Medical) Tel: 011 717 1252 E-mail: Iain.Burns@wits.ac.za
DATE:	27 August 2024
REF:	R14/49
PROTOCOL NO:	**M240765** (This is your ethics application reference number. Please quote it in all enquiries, oral or written, relating to this study.)
PROJECT TITLE:	*The gut microbiome association with menopausal status and cardiometabolic disorders in African populations*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to Government funding of the University.

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UNIVERSITY OF THE
WITWATERSRAND
JOHANNESBURG

R49 Ms P Chauke

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M240765

NAME: (Principal Investigator) Ms P Chauke **DEGREE:** MSc

DEPARTMENT: School of Pathology
Division of Human Genetics
Faculty of Health Sciences

TITLE: *The gut microbiome association with menopausal status and cardiometabolic disorders in African populations*

DATE CONSIDERED: Ad hoc

DECISION: Approved unconditionally

CONDITIONS: Sub-study under M22/10/108

NOTE: If contact information regarding student study participants is required, please contact the Registrar's office <Nicoleen.Potgieter@wits.ac.za>

SUPERVISORS: Professor S Hazelhurst; Drs L Olubayo and D Maghini

APPROVED BY:

Professor P Ruff, Chairperson, HREC (Medical)

DATE OF APPROVAL: 27 August 2024 **EXPIRY DATE:** 26 August 2029

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report** in the format available at https://wits.ac.za/research/researcher-support/research-ethics/ethics-committees/. This report is due annually, for the duration of the Clearance Certificate, beginning on the first anniversary of Date of Approval above. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator

05/11/24
Date

Appendix 3: Plagiarism Declaration and Turnitin Originality Report

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I **Phehello Selaelo Chauke** (Student number: **2302562**) am a student registered for the degree of MSc (Med) Genomic Medicine in the academic year **2025**.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/orwithout acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided workexcept where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection)software indicating the level of plagiarism in my research document.

Signature: _____ Date: 26/02/25

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