MICROBIAL, METABOLOMIC, AND MOLECULAR DETERMINANTS OF NEONATAL MORTALITY IN THE RURAL AREAS OF LIMPOPO PROVINCE, SOUTH AFRICA: UMBILICAL CORD BLOOD METABOLOMIC PROFILE AND BACTERIAL ANALYSIS AND THEIR ASSOCIATION WITH PERINATAL COMPLICATIONS

A DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCES DEGREE IN MICROBIOLOGY (MNMMMS)

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DECLARATION

I, innocent Moagi hereby declare that the research titled " Microbial, metabolomic, and molecular determinants of neonatal mortality in the rural areas of Limpopo Province, South Africa: umbilical cord blood metabolomic profile and bacterial analysis and their association with perinatal complications " is the result of my original work and has not been previously submitted for any degree, diploma, or other qualifications. I affirm that all sources used in this research have been properly acknowledged and cited. Any contributions from other individuals or organizations have been duly recognized in the acknowledgments section.

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DEDICATION

This research is dedicated to the pursuit of knowledge and understanding, driven by the collective effort of all those who seek to unravel the mysteries of the universe and contribute to the betterment of humanity. Therefore, dedicated to Almighty Lord, my mother Dora Thifhelimbilu Nematswerani, my siblings, my sisters Thingahangwi and Conny, my brothers Khabaya, Clement and Mpho. In memory of the innocent souls whose lives were cut short before they could even begin, this research is dedicated to the neonates who left this world too soon. May their brief presence serve as a reminder of the fragility of life and inspire us to strive for a future where every child has the opportunity to thrive and fulfill their potential.

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LIST OF ABBREVIATIONS

16S rRNA	16 Small ribosomal Ribonucleic acid
AEBREC	Animal, Environment and Biosafety Research Ethics Committee
AK	Amikacin
AMP	Ampicillin
ANC	Antenatal care
ANOVA	Analysis of variance
APBOs	Adverse Pregnancy and birth outcomes
BHI	Brain Heart Infusion
CAZ	Ceftazidime
CIP	Ciprofloxacin
CN	Gentamicin
CoNS	Coagulase-negative staphylococci
DNA	Deoxyribonucleic acid
E	Erythromycin
EDTA	Ethylenediaminetetraacetic acid
FDR	False discovery rate
FGR	Fetal growth restriction

FOX	Cefoxitin
g	Grams
GDM	Gestational diabetes mellitus
GNP	Gram-negative bacteria
GPB	Gram-positive bacteria
HCA	Hierarchical Clustering Analysis
HIV	Human immunodeficiency virus
HMDB	Human metabolome database
I	Intermediate
Inter	Interpretation
IPM	Imipenem
IUGR	Intrauterine growth restriction
KEGG	Kyoto Encyclopedia of Genes and Genomes
LBW	Low birth weight
LC-MS	Liquid chromatography mass spectrophotometry
LC-q-TOF-MS	Liquid Chromatography-Quadrupole-Time-of-flight Mass Spectrometry
MDR	Multidrug-resistant
MeSH	Medical subject heading.

MHA	Muller Hinton agar
MHB	Muller Hinton broth
NOS	Newcastle-Ottawa Scale
OPLS-DA	Orthogonal Partial Least Squares Discriminant Analysis
PCA	Principal Component Analysis
PCR	Polymerase chain reaction.
PE	Pre-eclampsia
PRISMA	Preferred Reporting Items Systematic Review and Meta-Analysis
PROM	Premature rapture of membrane
PTB	Preterm birth
R	Resistant
RNA	Ribonucleic acid
S	Susceptible
SDGs	Sustainable Development Goals
sPTB	spontaneous Preterm birth
v/v	volume per volume
VIP	Variable importance in projection
WHO	World Health Organization

PUBLICATIONS EMANATING FROM THE RESEARCH

From the present study two manuscripts have so far been developed. One has been published while one is in the process.

The following manuscript has been developed from the study and will be submitted soon.

Innocent Moagi, Lawrence Mabasa, Sonto Maria Maputle, Thivhulawi Malwela, Amidou Samie. A Comprehensive Systematic Review: Unraveling Novel Biomarkers for Early Detection and Prediction of Adverse Pregnancy and Birth Outcomes (APBOs) through Integrative Metagenomic and Metabolomic Approaches. To be submitted to Frontiers in Endocrinology.

The following article has been published.

Moagi I, Mabasa L, Maputle SM, Ndwandwe D, Raliphaswa NS, Netshikweta LM, Malwela T, Samie A. The impact of DNA methylation as a factor of Adverse Pregnancy and Birth Outcomes (APBOs): a systematic review protocol. Syst Rev. 2024 Jan 2;13(1):4. doi: 10.1186/s13643-023-02416-w.PMID: 38167510.

ABSTRACT

BACKGROUND: Neonatal mortality continues to pose a substantial public health challenge globally, particularly in developing countries. Some primary contributors are microbial infections, adverse pregnancy and birth outcomes (APBOs), and labor or obstetric complications. Despite this recognition, the underlying molecular mechanisms of these infections and perinatal complications are poorly understood. Interestingly, microbial and metabolomic approaches have emerged as effective techniques for the early detection and identification of biomarkers for microbial infections and perinatal complications, shedding more light on understanding the underlying molecular mechanisms of these adverse outcomes. Hence, this study employed microbial analysis (through 16S rRNA PCR assay and antibiotic susceptibility test profiling) and metabolomic profiling of umbilical cord blood to identify potential biomarkers for perinatal complications.

METHODS: A cross-sectional study utilizing purposive sampling was conducted at selected district hospitals in the Vhembe district from May 2023 to September 2023. Following participants' consent, questionnaires were used for data collection and umbilical cord blood samples were collected from 129 participants. Blood culture was done, and antibiotic susceptibility testing was conducted on the isolates. Additionally, genomic DNA was isolated from the blood samples for the detection of pathogenic bacterial strains, using a conventional PCR assay. Untargeted metabolomic profiling approach using Liquid Chromatography-Quadrupole-Time-of-flight Mass Spectrometry (LC-q-tof-MS) was conducted. The data were entered into REDCap and exported to IBM Statistical Package for the Social Sciences (SPSS) software, version 26, for analysis. Descriptive analysis and multivariate logistic regression analysis were conducted, with statistical significance set at a p-value less than 0.05 at a 95% confidence level. For metabolomic analysis, multivariate analysis approaches were employed to identify signals that differed between perinatal complications and uncomplicated pregnancies. Metabolic pathway analysis of perturbed metabolites was conducted using MetaboAnalyst 6.0.

RESULTS: Among 129 participants, the overall occurrence of perinatal complications stood at 35/129 (27.13%). The majority of participants, accounting for 55/129 (42.64%), fell within the age range of 20 to 29 years, while a significant portion (83.7%), were unemployed. Factors such as maternal age, birth weight, maternal blood pressure, anesthesia use, and delivery mode showed associations with perinatal complication risk, with corresponding P-values of 0.032, 0.027, 0.041, <0.001, and <0.001, respectively. Additionally, bloodstream infection (bacteremia) prevalence, as detected by culture-dependent and 16S rRNA PCR assays, was recorded at 30.23% and 26.36%, respectively. The majority of culture-confirmed bacterial isolates (58.06%) were gram-positive, with *S. epidermidis* (36.73%) and *S. aureus* (20.43%) being the predominant strains. Notably, *E. coli* infections showed a significant association with perinatal complications (P = 0.001). Bloodstream infection (Bacteremia) correlated significantly with maternal educational level, maternal blood pressure at birth, gestational booking stage, and pre-pregnancy BMI. Most culture-confirmed isolates exhibited high levels of antibiotic resistance to ampicillin, ceftazidime, and cefoxitin, while gentamicin, imipenem, amikacin, and ciprofloxacin proved effective against both gram-positive and gram-negative isolates. Furthermore, 93.38% of the tested isolates displayed multidrug resistance (MDR). Through untargeted metabolomic profiling analysis and multivariate analysis using the orthogonal partial least squares discriminant analysis (OPLS-DA) model, 107 metabolites were identified, showcasing differences between perinatal complications and uncomplicated pregnancies. Among the 107 perturbed metabolites, univariate analysis highlighted 50 upregulated and 57 downregulated metabolites in perinatal complications at P<0.05. Furthermore, the affected metabolic pathways, including ethyl lipid metabolism, sphingolipid metabolism, and glycerophospholipid metabolism, were identified as statistically significant.

CONCLUSION: Our study revealed a high occurrence rate of perinatal complications (25.58%), with maternal high blood pressure, maternal age (10-19 years), low birth weight, delivery mode, and anesthesia for C-sections identified as significant contributors. Proactive healthcare interventions during antenatal care visits are crucial to minimize complications. Additionally, when looking at microbial analysis, there was no significant association between bacteremia and perinatal complications but highlighted a higher prevalence of bloodstream infections, linked to factors like maternal education level and BMI. Furthermore, upon conducting metabolic profiling, it was evident that specific umbilical cord blood processes were closely associated with perinatal complications, indicating their potential as biomarkers for assessment, prediction, and early intervention strategies.

Keywords: *Neonatal mortality, Metabolomics, Adverse pregnancy outcomes, perinatal complications, Biomarkers, Microbiome.*

CHAPTER ONE: GENERAL INTRODUCTION AND RATIONALE

1.1. BACKGROUND

Pregnancy is a highly complex biological process that involves the interaction between the mother, placenta, and the developing foetus, which is of paramount importance for the health and well-being of both mother and foetus (Berti et al., 2016). During pregnancy, umbilical cord blood plays a crucial role as it serves as a direct link between the placenta and the developing foetus (Basta and Lipsett., 2020). Consequently, umbilical cord blood is a valuable source of biological information due to the combination of genetic and environmental elements, which have the potential to impact the foetus's development and future health consequences (Rager et al., 2020). Hence, recent studies have recognized umbilical cord blood as a potential reservoir for microbial communities that may impact foetal development and subsequent health, challenging its former perception as a sterile conduit (Vander Haar et al., 2022; Heil and Bruno et al., 2020).

These highlight the concept of the Developmental Origins of Health and Disease (DOHaD) hypothesis which suggests that intrauterine exposure to adverse environmental conditions may increase susceptibility to the risk of disease later in life (Susirarjo, M., 2016). Remarkably, this concept has been supported by several studies which demonstrated that intrauterine exposure either to bacterial pathogens, impaired nutrients, or chemicals can disrupt placental function (Forgie et al., 2020; Stiemsma et al., 2018). Furthermore, this disruption may cause permanent functional and structural impairments during cell replication and differentiation or induce an inflammatory response, consequently contributing to perinatal complications including gestational diabetes mellitus (GDM), prematurity low birth weight (LBW), intrauterine growth restriction (IUGR), stillbirth, or neonatal sepsis (Khot et al., 2017; Mandy and Nyirenda et al., 2018; Tomlinson et al., 2019; Ye et al., 2023).

However, the underlying mechanistic pathways for the associations between adverse environmental exposure with adverse pregnancy outcomes or perinatal complications are poorly understood (Ivorra et al., 2012; Lorente-Pozo et al., 2021). Understanding the molecular pathways of APBOs is crucial, and interestingly, omics techniques including metabolomics and metagenomics (16S rRNA sequencing) have recently been proposed as prognostic or diagnostics tools for early detection and as tools that may provide insight into understanding the molecular pathways of infections and pregnancy complication (Dhas et al., 2015; Deodati et al., 2020; Van de Berg et al., 2017). Additionally, it also plays a role in identifying the potential biological markers for early detection of either bacterial infections or adverse pregnancy and birth outcomes (APBOs), highlighting the importance of integrative metabolomics and bacterial analysis in understanding the mechanistic pathways for APBOs (Ciborowski et al., 2014; Tendl et al., 2013).

Recently, studies have demonstrated that umbilical cord blood metabolites, either derived from gut microbiota or synthesized by the host, are significantly associated with the pathogenesis of several APBOs including LBW, PTB and IUGR (Rahman et al., 2023; Li et al., 2018; Wu et al., 2021; Dessia et al., 2014; Metrustry et al., 2018; Noto et al., 2016). Simultaneously, emerging evidence has illustrated that dysbiosis in the human microbiome, particularly gastrointestinal microbiota might be associated with the pathogenesis of several APBOs (Li et al., 2022). For instance, a recent study on the integration of untargeted metabolomic and metagenomic analysis in GDM has demonstrated that an increase in L-alpha-aminobutyric acid and 2-hydroxybutyric acid, decreased in allantoin, methionine sulfoxide, and dopamine, and reduced level of *Eubacterium siraeum, Bacteroides coprophilus, Faecalibacterium prausnitzii, Prevotella stercorea*, and *Prevotella copri*, were significantly associated with GDM (Ye et al., 2023).

Prenatal antibiotic exposure has been associated with dysbiosis in infants or foetus microbiome, ultimately leading to the increased risk of APBOs (Nguyen et al., 2022; Fish-Williamson et al., 2022). Although early antibiotic intervention is effective in preventing infections and enhancing survival rates, emerging evidence has demonstrated that most pathogenic microbes (*E. coli, Enterobacter spp*., and *K. pneumoniae*) associated with APBOs have emerged resistant to multiple drugs, making them difficult to treat and reduce the incidence of APBOs (Saturio et al., 2023; Samarra et al., 2023; Ngong, et al., 2021; Ali et al., 2022). Hence, the present study also investigated the impact of AMR on perinatal complications.

In general, metabolomics is primarily concerned with quantifying small molecules, known as metabolites present in biological systems, including body fluid, tissues, or cells (Quinones and Kaddurah-Daouk., 2009; Hu et al., 2020). These metabolites are the final products of cellular processes and provide valuable information about the biochemical pathways and dynamic changes occurring within an organism (Guo et al., 2019). Concurrently, bacterial analysis through metagenomics and antibiotic susceptibility patterns could provide insight into the microbial populations within umbilical cord blood, opening new opportunities for comprehending the interactions between maternal and fetal microbes that influence APBOs (Miko et al., 2022). Hence, in the present study metabolomics analysis, 16S rRNA PCR assay, and antibiotic susceptibility test were employed to investigate the metabolic and bacterial signatures in APBOs or perinatal complications.

1.2. STUDY RATIONALE

Despite the efforts of the past 20 years with Sustainable Development Goals in reducing childhood mortality by 12 deaths per 1000 live births between 2020-2030 (Hoang et al., 2021), neonatal mortality, remains a major public health challenge worldwide particularly in developing countries (Atnafu et al., 2020; Ntuli et al., 2020). Child Survival and Sustainable Development Goals (SDGs) speculated that more than 60 countries are at risk of missing the SGDs target on neonatal mortality, 80% of these deaths would occur in sub-Saharan Africa (UNICEF, 2015).

It is estimated that about 28 million newborns will die between 2020-2030 (UNICEF., 2015). Epidemiological studies have reported that approximately 3.3 million neonatal deaths occur around the world, wherein 41% (1.2 million) of the deaths take place in Sub-Saharan African countries (Kananura et al., 2016). Thus, about 13000 deaths occur per day. In South Africa alone, research has indicated that neonatal mortality constitutes 44% of all infant deaths, translating to 27 deaths per 1000 live births (Rhoda et al., 2018).

Among the causes of neonatal deaths, unfavourable pregnancy and birth outcomes including preterm birth, low birth weight, IUGR, GDM, stillbirth, and neonatal sepsis, are recognized as the major causes of neonatal mortality (Darmstadt et al., 2011; Kananura et al., 2016; Maitre et al., 2014). Hence, epidemiological studies have demonstrated that preterm birth, intrapartum-related events, and infections account for 47.9%, 24.3%, and 11.6% of neonatal deaths in South Africa respectively (Rhoda et al., 2018). According to the District Health Information System (DHIS) report, neonatal deaths caused by the above-mentioned pregnancy complications are more frequently reported in district hospitals than in central hospitals in Limpopo and Mpumalanga Provinces (CoMMiC., 2021). The report has guided the present study's choices of the sampling site, which focuses on selected district hospitals in Vhembe district, Limpopo Provinces, the district with a neonatal mortality rate of 12.8 deaths per 1000 live births.

Although studies have illustrated that early initiation of perinatal care facilitates the timely detection and treatment of APBOs caused by pathogenic microbes to improve the health of both maternal and foetus, antimicrobial drug resistance poses a global health threat due to the rapid emergence of multidrug-resistant bacteria (Tesfaye et al., 2017; Tessema et al., 2023; Chelkeba et al., 2022; Murray et al., 2022). Multi-omics techniques on the other hand could provide insight on a comprehensive understanding of the molecular mechanistic pathways of several APBOs (Diatchenko et al., 2022; Jaremek et al., 2021). However, the molecular mechanism of pathogenesis of APBOs including Preterm birth, neonatal sepsis, stillbirth, and IUGR remains unclear (Maitre et al., 2014; Tung et al., 2021; Vander Haar et al., 2022).

The present study aims to investigate the metabolomic and bacterial analyses of umbilical cord blood to comprehensively explore the association between the metabolic profile and microbial composition with perinatal complications in Vhembe district, Limpopo province. Under microbial analyses, the present study specifically focused on bacterial profile and antibiotic susceptibility patterns. Unveiling the novel potential biomarkers for early detection of these outcomes could ultimately contribute to neonatal death. Thus, understanding these associations is important for implementing effective preventive strategies and improving maternal and child health.

1.3. RESEARCH QUESTION

- What are the microbial and metabolomic determinants influencing neonatal mortality in the rural areas of Limpopo province, South Africa, with a specific focus on the umbilical cord blood metabolomic profile and bacterial analysis, and how do these factors relate to perinatal complications?

1.4. HYPOTHESIS

- The present study hypothesizes that the umbilical cord blood metabolomic profile and bacterial composition are associated with perinatal complications, ultimately contributing to the increased risk of neonatal mortality in the Vhembe district.

1.5. THE PRIMARY AND SECONDARY OBJECTIVES OF THE STUDY

1.5.1. Primary objectives

- The primary aim of this study is to investigate the umbilical cord blood metabolomics profile and bacterial contamination/infection and their associations with perinatal complications in the selected healthcare facilities in Vhembe district of Limpopo Province.

1.5.2. Specific objectives

- To investigate the presence and diversity of bacterial strains in umbilical cord blood samples, emphasizing the significance of their isolation through the use of various selective media.

- To evaluate the antibiotic resistance profiles of the bacterial strains isolated from umbilical cord blood, using the Kirby-Bauer disk diffusion method, in order to assess their potential impact on perinatal health.

- To detect the presence of bacterial organisms in the umbilical cord blood based on the amplification of the 16S rRNA gene and to identify key bacterial species present in umbilical cord blood.

- To employ untargeted metabolomics techniques to identify and quantify a broad spectrum of metabolites present in umbilical cord blood using LC-q-tof-MS.

- To explore variations in metabolic pathways and concentrations associated with several adverse pregnancy and birth outcomes.
- To establish associations between the identified metabolites and perinatal complications, including preterm birth, low birth weight, GMD, stillbirth, and labor or obstetric complications.
- To identify potential biomarkers within the umbilical cord blood metabolomic profile and bacterial composition that may serve as indicators or predictors of perinatal complications.

CHAPTER TWO: A SYSTEMATIC REVIEW

Exploring New Biomarkers for Early Identification and Prediction of Adverse Pregnancy and Birth Outcomes (APBOs) via Integrative Metagenomic and Metabolomic Methods.

2.1. INTRODUCTION

Despite the implementation of Sustainable Development Goals 3 (SDGs-3) (Good health and well-being), which targets the reduction of worldwide maternal and neonatal mortality rates while eradicating avoidable deaths among newborns and children under the age of 5 years (Scanlon et al., 2022), adverse pregnancy and birth outcomes (APBOs) remain significant global health concerns, posing risk to both maternal and infant well-being (Kuppusamy et al., 2023). APBOs consist of a broad range of health complications and unfavorable events that can occur during pregnancy and childbirth including low birth weight, preterm birth (PTB), premature membrane rapture (PROM), intrauterine growth restriction (IUGR), stillbirth, preeclampsia, gestational diabetes mellitus (GDM), and maternal complications such as hemorrhage or infections (Degno et al., 2021; Kaforau et al., 2022). Additionally, these outcomes have been identified as important indicators of maternal and infant health as well as the major causes of neonatal and maternal mortality around the world (Mihretie and Habitamu, 2022).

Epidemiological evidence has shown that the majority of APBOs occur and affect newborns in low- and middle-income countries (LMICs) (Younger et al., 2022). According to WHO, approximately 15 million infants around the world are delivered preterm, thus they are delivered before 37 gestational weeks and about one million cases of preterm birth contribute to the global neonatal deaths annually (WHO, 2023). Furthermore, an estimated 60% of preterm birth (PTB) cases occur in Africa and Asia (Degno et al., 2021). Low birth weight (LBW), defined as a birth weight less than 2500 grams and Stillbirth (delivery of fetus at or after 22 gestational weeks with no sign of life) on the other side account for 15-20% and 1.91% cases per 1000 live birth around the word respectively (Thapa et al., 2022; Mbongozi., 2023).

Emerging evidence has demonstrated that APBOs including PTB, LBW, and IUGR are associated with a higher risk of physiological complications and well-being later in life, emphasizing the concept of fetal programming (Mihretie and Habitamu, 2022; Nobile et al., 2022). Early detection and prediction of APBOs is essential for timely intervention and improved health outcomes (Tadese et al., 2022; Gülmezoglu et al., 2016). However, the precise pathophysiological mechanisms underlying the onset and progression of these adverse pregnancy and birth outcomes (APBOs) remain unclear (Kumar et al., 2021). Interestingly, integrated multi-omics approaches, encompassing metagenomics and metabolomics, have emerged as valuable tools offering an unbiased perspective to delve into the intricate molecular mechanisms linked with these adverse pregnancy and birth outcomes (APBOs) (Tao et al., 2023). These methodologies provide the potential for identifying microbiome and metabolome biomarkers, facilitating early prediction and diagnosis of various APBOs (Moschino et al., 2022; Clos-Garcia et al., 2019).

Metagenomics refers to a branch of genomics focused on examining and interpreting genetic material obtained directly from environmental and clinical samples through sequencing methods (Zhang et al., 2021). It serves as a potent tool for comprehending the diversity and functional roles of microbiomes (Thomas et al., 2012; Zhang et al., 2021). Although the human microbiome is recognized for its critical biological function in immune system development and host metabolism maintenance (Safari-Alighiarloo et al., 2023), accumulating evidence suggested that dysbiosis in microbiota is strongly associated with novel biomarkers for diseases and APBOs including gestational diabetes mellitus (GDM), preeclampsia, and PTB (Ottman et al., 2012; Zhuang et al., 2022; Neu and Pammi., 2018; Gudnadottir et al., 2022).

Evidence suggests that alterations in gut microbiota dynamics during early pregnancy significantly vary between women experiencing adverse pregnancy and birth outcomes (APBOs) compared to those with normal delivery (Zheng et al., 2020). For example, a case-control study linked a high abundance of Fusobacterium and Veillonella, along with a low abundance of Faecalibacterium and Akkermansia, with pre-eclampsia (Chen et al., 2020). Additionally, another study demonstrated distinct dysbiosis in the gut microbiome of women experiencing PTB compared to those delivering at term. This study reported an increase in opportunistic pathogens, particularly *Fusobacterium*, *Streptococcus*, *Veillonella*, and *Porphyromonas*, alongside a significant decrease in *Coprococcu*s and *Gemmiger* (Yin et al., 2021).

Growing evidence further demonstrated that alterations in the human microbiome, particularly gastrointestinal microbiota are pivotal in addressing the increasing energy and nutritional demands of the developing fetus (Liang et al., 2022; Dicks et al., 2018). Thus, the gut microbiota function as bioreactors possessing substantial metabolic capabilities, collaborating with the host to engage in various biological activities crucial for maintaining physiology and homeostasis (Agus et al., 2021; Krautkramer et al., 2021). The vital contribution of the gut microbiota to host physiology primarily occurs through the regulation of gastrointestinal metabolites, encompassing their production, fermentation, digestion, and secondary metabolism (Hosseinkhani et al., 2021). Some metabolites including carbohydrates (Short-chain fatty acids (SCFAs)) are directly produced through microbial fermentations and these microbiota-derived metabolites circulate within the serum, intestines, and several organs, regulating physiological processes (Tao et al., 2023; Safari-Alighiarloo et al., 2023).

Recently, studies have strongly associated changes in circulating microbiota-derived metabolites (metabolome) including SCFAs, bile acids, and indoles with most adverse pregnancy complications including GDM and IUGR (Ye et al., 2023; Tang et al., 2022). Although metagenomic sequence data has emerged as a potent tool, offering insights into the biological functions of microbes (Liu et al., 2021), it alone is insufficient to identify the gut microbiome and its mechanisms in influencing the host's metabolic profile (Safari-Alighiarloo et al., 2023; Wang et al., 2021). Hence, metabolomics has emerged as a potent approach enabling the characterization of metabolic profiles, identification, and quantification of metabolites produced by the human microbiome, as well as the elucidation of the biochemical pathways of these metabolites (Vernocchi et al., 2016; Wu et al., 2021). Therefore, integrated approaches using metabolomics and metagenomic sequencing have advanced our understanding of various aspects of APBOs-related diseases and the roles of several factors, including nutrition, in shaping the health of the offspring (Moschino et al., 2022), thereby contributing to a broader comprehension of the symbiotic interaction between the gut microbiota and host (Hashimoto et al., 2021). This integrated approach is essential for inferring how the microbiome influences host physiology by examining the role of bioactive metabolites (Safari-Alighiarloo et al., 2023).

Despite several systematic reviews that have respectively examined the associations between the microbiome and metabolome with various APBOs (Juliana et al., 2020; Carter et al., 2019; Rold., 2022), to the best of our knowledge, there are no published or ongoing systematic reviews that have investigated the integration of metagenomics and metabolomics analyses in APBOs. Therefore, this systematic review holds importance by aiming to unravel novel biomarkers through a comprehensive integration of microbial and metabolomic data, with the potential to advance early detection, improve predictive models, inform clinical practice, contribute to scientific knowledge, and have a positive impact on public health related to adverse pregnancy and birth outcomes.

2.2. METHODOLOGY

In the present study, a systematic review was undertaken, and as such ethical permission or approval was not deemed necessary since it did not involve direct interaction with participants but instead focused on extracting data from previously published studies. The systematic review adhered to the recommended "Preferred Reporting Items for Systematic Reviews and Meta-Analyses 2020 (PRISMA-2020) guideline (Page et al., 2021). The study protocol for this review is registered with the international Prospective Register of Systematic Review; PROSPERO (https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42024493411).

2.2.1. Eligibility criteria

2.2.1.1. Inclusion criteria

The eligibility criteria for this systematic review were defined and guided by the PI(E)COS framework, wherein P is for Population, I/E intervention/exposure, C for comparator/ control, O for outcomes, and S for the study designs. Thus, identify relevant studies and develop eligibility criteria for the systematic review. Eligible studies were those reporting on the integrative analysis of untargeted metabolomics and microbiome (using a culture-independent approach) in pregnant women and neonates, investigating their correlation with adverse pregnancy and birth outcomes (APBOs) such as preterm birth (PTB), premature rupture of membranes (PROM), low birth weight (LBW), gestational diabetes mellitus (GDM), intrauterine growth restriction (IUGR), neonatal mortality, and stillbirth/miscarriage. The systematic review included original research studies, including observational studies (such as cohort studies, case-control studies, and cross-sectional studies), and clinical trials. Conference abstracts or proceedings were only included if they contained substantial and relevant data. Only human studies written in English or with available English translations were included.

2.2.1.2. Exclusion criteria

Since the systematic review specifically intended to report on studies focusing on the integrated untargeted metabolomic and metagenomic analysis in pregnant women or neonates with APBOs, it is worth noting that studies that solely investigated gut microbiota without considering the metabolome, or vice versa, along with those focusing solely on microbial metabolites, were excluded. Furthermore, reviews, editorials, commentaries, and letters, non-peer-reviewed publications, studies published more than 10 years ago were also excluded.

2.3. INFORMATION SOURCE AND SEARCH STRATEGY

A comprehensive literature search was conducted on the following electronic databases: PubMed, MEDLINE EBSCOhost, and Web of Sciences, considering studies published from 2013-2023 using the advanced Boolean search strategies with at least the following MeSH terms and free text word: (((((("infant, newborn"[MeSH Terms] OR "fetus"[MeSH Terms] OR "pregnancy"[MeSH Terms] OR "fetal"[Text Word]) AND ("metabolom*"[MeSH Terms]) OR ("metabolite profil*"[Text Word]))) OR ((("metagenom*"[MeSH Terms]) OR ("microbiota"[MeSH Terms]) OR ("microbiota"[MeSH Terms]) OR ("microbiota"[MeSH Terms]) OR ("microbial"[Text Word])) AND ((("pregnancy complications"[MeSH Terms]) OR ("pregnancy outcome"[MeSH Terms]) OR ("birth outcomes"[Text Word]) OR ("adverse pregnancy outcomes"[Text Word])))). Additional studies were manually searched from the reference lists of included studies; however, no search for grey literature sources was conducted. The search did not include the geographic restrictions. The Mendeley desktop file was used to import data, auto-removal deduplicates, and manage retrieved records.

2.4. SELECTION PROCESS AND DATA RETRIEVAL

After the removal of deduplicates, two reviewers (IM and AS) independently screened the title and abstract read the full text of the potentially relevant studies and read the consensus on the included study. In case of disagreement and discrepancy between IM and AS about the included studies, they were resolved in consultation with a third reviewer (LM). Two reviewers (IM and AS) independently extracted data from all eligible papers. Each reviewer extracted the following data from the potentially relevant studies: author details (name and publication date), study design, socioeconomic characteristics of the population, type specimen, methods, microbiota, metabolites, APBOs, statistical tool, statistical correlation, and major findings.

2.5. ASSESSMENT OF BIAS IN INDIVIDUAL STUDIES (QUALITY EVALUATION)

Two reviewers (IM and AS) independently evaluated the quality and risk of bias of each included study using the Newcastle-Ottawa Scale (NOS), a tool designed for assessing the quality of non-randomized studies like case-control and cohort studies. This scale evaluates study quality across three parameters: selection, comparability, and outcomes (cohort studies) or exposure (case-control studies). These parameters are subdivided into eight specific items, each scored with one or two points, yielding a total score of nine (Wells et al., 2014; Peterson et al., 2011; Ma et al., 2020). Any discrepancies between the two reviewers (IM and AS) were resolved through consultation with a third reviewer (LM). Based on the total score, studies were categorized as having a low risk of bias (scores ranging from 7 to 9), moderate risk (scores ranging from 5 to 6), or high risk of bias (scores ranging from 1 to 4). The studies were scored based on how well they described and investigated the difference between cases and controls.

2.6. BIOINFORMATIC ANALYSIS

Metabolites showing perturbations in adverse pregnancy and birth outcome (APBO) cases were extracted from the eligible studies to uncover potential patterns and underlying molecular mechanisms. We employed the Human Metabolome Database (HMDB) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) to identify each metabolite's chemical class and subclass with corresponding metabolic pathways and processes using the online tool: MetaboAnalyst6 (https://www.metaboanalyst.ca) (Carter et al.,2019; Mayrink et al., 2022). We further searched KEGG and HMDB databases thus, to increase the possibility of identifying potential biomarkers or metabolites that might be missed or recognized through MetaboAnalyst 6.0.

2.7. RESULTS

2.7.1. Study Selection

Figure 2.1 shows the PRISMA flowchart of identification and inclusion of publications in this systematic review. After the removal of 1664 duplicates, database searches produced a total of 2379 records (Figure 2.1). Following the title and abstract screening, 2022 publications were eliminated, resulting in the remaining 357 articles. Furthermore, publications were screened for full text to assess whether they met the eligibility criteria, and a total of 340 studies were eliminated. Therefore, the review extracted data from 17 publications that met inclusion criteria, and the characteristics of each study and findings are summarized in Table 2.1. Thus, seventeen studies have reported potential microbial and metabolomics biomarkers that could be used to differentiate between cases of various Adverse Pregnancy and Birth Outcomes (APBOs) and healthy controls.

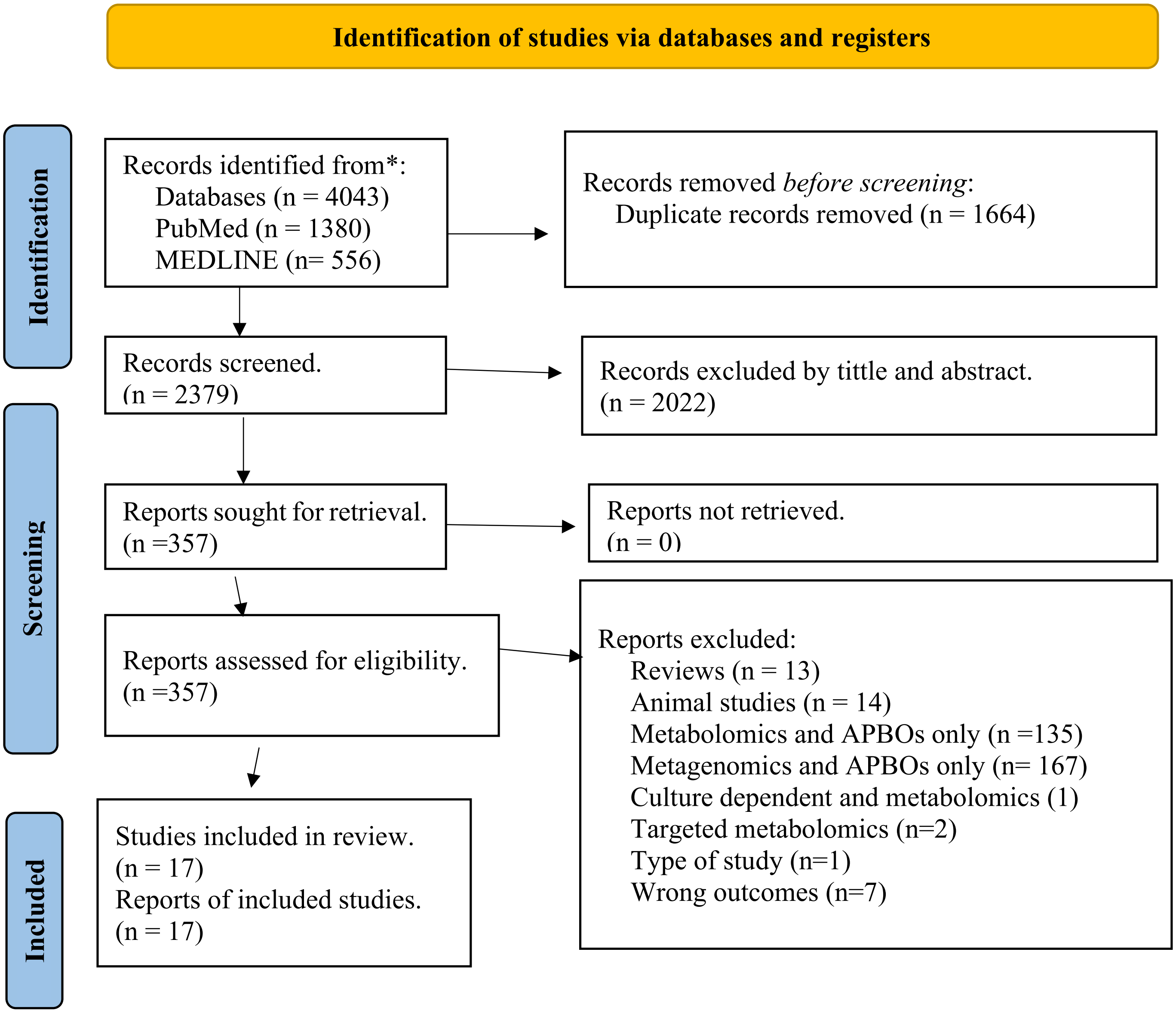


Figure 2.1: A detailed PRISMA flow diagram of identification and inclusion of publications.

2.7.2. Study Characteristics

Data extracted from the 17 studies included in the review demonstrated considerable variety in terms of the socioeconomic characteristics of participants, study design, specimen type, and statistical analysis tools (Table 2.1 below).

2.7.2.1. Study Design

In brief, the included studies were published between January 2013 to December 2023. Among the included publications (13 cohort studies and 4 case-control studies), the majority of the studies originated in China (7/17) (Ye et al., 2023; Liu et al., 2021; Yang et al., 2022; Tao et al., 2023; Liu et al., 2021; Wang et al., 2020; Chen et al., 2021), followed by the United State of America with 5 studies (Gershuni et al., 2021; Dougherty et al., 2020; Gerson et al., 2021; Kindschuh et al., 2023; Wandro et al., 2018). Other studies included the United Kingdom (2/17) (Flaviani et al., 2021; Pruski et al., 2021), Mexico (1/17) (Benítez-Guerrero et al., 2022), Israel (Pinto et al., 2023), and Ireland (Hill et al., 2013).

2.7.2.2. Characteristics of participants

The demographic and clinical characteristics of participants in Table 2.1 have shown that a total of 6,780 participants were recruited in the included studies. However, metabolomics and microbial analysis were conducted in 328 cases of gestational diabetes (Ye et al., 2023; Wang et al., 2020; Chen et al., 2021; Pinto et al., 2023; Benítez-Guerrero et al., 2022), 306 cases of preterm (Gerson et al., 2021; Flaviani et al., 2021; Dougherty et al., 2020; Gershuni et al., 2021; Hill et al., 2013; Kindschuh et al., 2023; Pruski et al., 2021; Wandro et al., 2018), PROM in 36 cases (Liu et al., 2021a), 41 miscarriage cases (Liu et al., 2021b), 75 FGR cases (Yang et al., 2022; Tao et al., 2023), 8 Pre-eclampsia cases (Benítez-Guerrero et al., 2022), and 8 neonatal sepsis (Wandro et al., 2018). Moreover, two studies included multiple cases (Wandro et al., 2018; Benítez-Guerrero et al., 2022). Most of the studies included healthy or normal pregnancy controls (complicated pregnancy, or outcomes that affect the metabolome or microbiome composition were excluded), except a study by Wandro et al., (2018) which considered healthy preterm birth as control and preterm birth who developed neonatal sepsis and necrotizing enterocolitis as cases.

Gestational diabetes cases were diagnosed using the standardized 75-g Oral Glucose Tolerance Test (OGTT) administered between 23 and 28 weeks of pregnancy. The diagnostic criteria for GDM in the included studies were as follows: fasting blood glucose levels ≥ 92 mg/dL (5.1 mmol/L), 1-hour plasma glucose levels ≥ 180 mg/dL (10.0 mmol/L), and 2-hour plasma glucose levels ≥ 153 mg/dL (8.5 mmol/L) (Ye et al., 2023; Wang et al., 2020; Chen et al., 2021; Pinto et al., 2023; Benítez-Guerrero et al., 2022). Since this review retrieved several cases of preterm cases, we classified preterm cases into two categories: Spontaneous Preterm Birth (sPTB) (Gerson et al., 2021; Flaviani et al., 2021; Gershuni et al., 2021; Kindschuh et al., 2023) and medically indicated preterm birth (PTB) (Dougherty et al., 2020; Hill et al., 2013; Kindschuh et al., 2023). Preterm birth (PTB) was defined as delivery occurring before 37 weeks of gestation, accompanied by cervical dilation or premature rupture of membranes. Additionally, PROM cases were diagnosed considering the guidelines of "ACOG Practice Bulletin No. 188: Prelabor Rupture of Membranes" (Liu et al., 2021). FGR on the other hand, is defined as birth weight less than 10th percentile for gestational age based on the following criteria: umbilical cord abnormalities or placental disorders by postnatal confirmation, consistent standards of birth weight discordance in dichorionic-diamniotic (DCDA) and monochorionic-diamniotic (MCDA) twins, and consensus-based diagnostic criteria (Yang et al., 2022; Tao et al., 2023). Pre-eclampsia (PE) cases were diagnosed after 20 weeks of gestation, with consideration given to a systolic blood pressure of 140 mmHg or higher and a diastolic blood pressure of 90 mmHg or higher, recorded on more than two occasions with at least 4 hours' difference on the same day. Additionally, the presence of proteinuria was also considered (Benítez-Guerrero et al., 2022).

2.7.2.3 Data sources/measurement

The most collected samples for both metabolomics and microbial analyses in the included studies were fecal specimens. Additionally, 5 studies measured metabolic and microbial signatures from vaginal samples including vaginal swabs (Liu et al., 2021b; Kindschuh et al., 2022), Cervicovaginal fluid samples, (Flaviani et al., 2021; Gerson et al., 2021), and Cervicovaginal swabs (Pruski et al., 2021). However, few studies collected multiple samples for microbial and metabolomics analyses. Thus, Stool for microbiota and blood for metabolomics profiling (Ye et al., 2023), Fecal samples (microbiota) and serum/plasma & fecal samples (metabolomics) (Tao et al., 2023; Gershuni et al., 2021), fecal samples (Microbial analysis) and Urine (Metabolomics) (Hill et al., 2017), and Stool samples (metagenomics), fecal and urine (metabolomics) (Wang et al., 2020).

2.7.2.4. Microbial and metabolomics approaches

The included studies were highly different regarding the techniques used for the analyses of both microbiome and metabolome composition in several APBOs (Table 2.1). Almost all studies employed 16S rRNA gene sequencing for microbiome analyses, except a few publications which performed metagenomic sequencing through the Illumina Seq platform, (Ye et al., 2023; Wang et al., 2020), Rolling circle replication and a DNA nanoball sequencing method (Liu et al., 2021b). Furthermore, most studies used LC-MS-based techniques for metabolomics analyses, except studies XploreMET platform, FT-ICR MS, and Metabolon, Inc. respectively (Ye et al., 2023; Benítez-Guerrero et al., 2022; Gershuni et al., 2021). Two studies used GC-MS approaches for profiling the metabolites in the biological samples (Wang et al., 2020; Yang et al., 2022). Additionally, only one study performed metabolomics using ^{1}H-NMR (Flaviani et al., 2021).

2.7.2.5. Statistical method/tools

The studies included in the analysis utilized a varied range of statistical tools to assess the associations between metabolome and microbiome with APBOs (Table 2.1). Several studies have employed Spearman’s rank test to assess the strength and direction of monotonic relationships between metabolomic and microbial profiles with the outcomes (Liu et al., 2021a; Tao et al., 2023; Liu et al., 2021b; Wang et al., 2020; Chen et al., 2021; Flaviani et al., 2021; Pinto et al., 2023). Two studies used the Pearson correlations test to assess a linear relationship between intervention and APBOs (Liu et al., 2021a; Wandro et al., 2018). Furthermore, four studies compared two independent groups using Mann-Whitney U tests (Dougherty et al., 2020; Gerson et al., 2021; Pinto et al., 2023), and Wilcoxon’s rank-sum test (Gershuni et al., 2021) separately. Few studies applied multiple statistical tools to integrate microbiome and metabolomic data to identify associations. Thus, Kenward–Roger approximation, Benjamini–Hochberg false discovery rate correction (Pruski et al., 2021), Spearman’s rank test or Pearson’s correlation test, and Benjamini–Hochberg (BH) FDR correction (Liu et al., 2021a), Wilcoxon rank-sum test and Benjamini–Hochberg method (Ye et al., 2023), Spearman’s rank correlation, canonical correlation analysis (CCA), and the Mantel test (Liu et al., 2021b), Mann–Whitney U tests, false discovery rate (FDR) correction, Spearman’s rank test, and Student’s t-tests (Pinto et al., 2023), Welch’s test, with Bonferroni correction (Benítez-Guerrero et al., 2022), and MIMOSA and mmvec (Yang et al., 2022). Analysis Variance (ANOVA) tests were used to analyze the differences among groups (Hill et al., 2017). PERMANOVA tests were used to analyze the differences among groups in multivariate data (Kindschuh et al., 2022).

Table 2.1: Characteristics of included studies.

Author Details	Study Design	Characteristics of Participants	Specimens	Analysis Techniques	Statistical tools	Study summary
Ye et al., 2023 (China)	Cohort	No:104 Cases:50 GDM Controls:54 NGT Eligibility: excluded diseases that might affect glucose metabolism or microbiome composition.	Blood (metabolites), Stool (microbiota).	Metagenomic sequencing (Illumina HiSeq 4000 platform) and Untargeted metabolomics profiling (XploreMET platform).	Wilcoxon rank-sum test and Benjamini–Hochberg method	The study found that microbial and metabolic signatures were closely associated with clinical parameters of glucose metabolism in GDM compared to NGT
Liu et al., 2021a (China)	Cohort	No: 60 Cases: 41 miscarriages Controls: 19 controls Eligibility: Pre-pregnancy women aged ≤ 35 years with no successful pregnancies, whose current male partner had normal semen testing	Fecal samples	16S rRNA gene sequencing, UHPLC coupled with Triple TOF 6600 System	Spearman's rank test or Pearson's correlation test and Benjamini–Hochberg (BH) FDR correction	The study highlighted the network among gut microbiota, fecal metabolites, and Th1/Th17-mediated immune response in miscarriage patients and explored the potential predictive values of two fecal metabolites for recurrent miscarriages.
Yang et al., 2022 (China)	Cohort	No: 150 Cases: 30 Selective FGR twins Eligibility: 2 live neonates from MCDA and DCDA twin pregnancies, cesarean delivery, and agreed to follow-up. Excluded antibiotic usage in 3rd trimester and complications	Fecal samples	High-throughput 16S rRNA gene sequencing (The V3-V4) PE250 sequencing on the Illumina HiSeq 2500 platform and Metagenomic sequencing (PE150 on the HiSeq 2500 platform Untargeted metabolomics profiling (gas chromatography-mass spectrometry (GC–MS))	MIMOSA and moves.	Dysbiotic microbiota profiles and pronounced metabolic alterations are associated with selective FGR affected by adverse intrauterine environments.

Tao et al., 2023 (China)	Cohort	No: 70 Cases: 35 FGR, Controls: 35 normal pregnancies Eligibility: estimated fetal weight or abdominal circumference less than the 10th percentile and placental disorders or umbilical cord abnormalities by postnatal confirmation	Fecal samples (Microbiota)and serum & fecal samples (metabolomics)	16S rRNA sequencing (Illumina Nova 6000 platform) Untargeted metabolomics (Liquid chromatography-tandem mass spectrometry (LC-MS/MS))	Spearman's correlation test	Integrated multi-omics analysis revealed the interactions among gut microbiota, metabolites, and clinical measurements.
Gershuni et al., 2021 (USA)	Prospective cohort	No: 301 Cases: 16 SPTB, Control: (18 obese and 32 nonobese) Eligibility: excluded multiple gestation, fetal chromosomal abnormality	Fecal samples (microbiota), plasma & fecal samples (metabolomics)	16S tagged gene sequencing (QIIME 2) Untargeted metabolomics by Metabolon (Metabolon, Inc.)	Wilcoxon's rank-sum test	Reduced α-diversity of the gut microbiota and higher excretion of omega-3 (n–3) fatty acids in stool may provide a novel biomarker signature predicting SPTB.
Liu et al., 2021b (China):	Cohort (pilot study)	No: 163 Cases: 36 PROM cases, Controls: 87 Healthy, 40 lost during follow-up. Eligibility: included singleton pregnancy, cephalic presentation, delivered virginally, and excluded pregnancy complications.	Vaginal swabs	Metagenomics sequencing (Rolling circle replication and a DNA nanoball sequencing method) Untargeted liquid chromatography-tandem mass spectrometry (LC-MS/MS).	Spearman's rank correlation, canonical correlation analysis (CCA), and the Mantel test	Downregulation of glycolysis substrates (GalNAc and sucrose) and increase of related pathogenic microorganisms (Streptococcus and Chlamydia) could serve as early warning biomarkers of PROM.
Wang et al., 2020 (China):	Case-control study	No: 107 Cases: 59 GDM, Eligibility: without a history of diabetes, without any other	Stool samples (metagenomics), fecal and	Metagenomic sequencing (Illumina MiSeq platform).	Spearman's correlation (SPSS V.19.0).	The study showed that the microbial and metabolic signatures of GDM patients were significantly different from those of HCs.

		complications, diarrhea, or any gastrointestinal symptoms.	urine (metabolomics).	untargeted metabolomics (Gas chromatography-mass spectrometry (GC/MS).		
Chen et al., 2021 (China)	Case-control study	No: 418 Cases: 105 GDM neonates, 59 GDM mothers, Controls: 199 neonates, 256 mothers Eligibility: excluded pre-existing diabetes, pre-existing metabolic diseases, antibiotics usage within 3 months, alcohol or substance abuse, and chronic diseases	Meconium samples (microbiota), serum from mother & meconium (metabolomics)	16S rRNA gene sequencing (Illumina MiSeq Sequencer) Metabolomic analysis (UPLC-QE).	Spearman's correlation	GDM could alter the serum metabolome and is associated with the neonatal meconium microbiota and metabolome, highlighting the importance of maternal factors on early-life metabolism.
Flaviani et al., 2021 (UK)	Cohort	No: 353 Cases: 60 sPTB or late miscarriage, Controls: 286 Eligibility: sPTB was identified if women had a spontaneous onset of labor or had experienced PROM and delivered before 37 weeks of gestation. Excluded: IUD pregnancies and iatrogenic deliveries.	Cervicovaginal fluid samples.	16S rRNA sequencing (Illumina MiSeq) Metabolome (1H-NMR).	Spearman's correlation	integration of metabolite and bacterial community composition has significant potential for enhancing our understanding of the contribution of the vaginal environment to sPTB.
Dougherty et al., 2020 (USA)	Cohort	No: 17 Cases: 10 Controls: 7 Eligibility: N/A	Stool/meconium samples.	16S rRNA gene sequencing (Illumina MiSeq) Global untargeted profiling (UHPLC).	Mann-Whitney U tests.	The study indicated that microbial metabolites from the more diverse gut microbiome associated with pre-term infants facilitate stem cell proliferation.

Gerson et al., 2021 (USA)	Nested case control from cohort	No: 2000 Cases: 20 PTB Eligibility: excluded major fetal anomaly, HIV seropositive status, history of organ transplant, chronic steroid use, enrollment into the study during a previous pregnancy, or multiple gestations.	Cervicovaginal fluid samples	16S rRNA gene sequencing Metabolomics analysis (UPLC-MS/MS and GC/MS).	Mann–Whitney U tests	The study raises the possibility that metabolites could distinguish women with greater risk of sPTB among those with similar cervicovaginal microbiota, and that metabolites within the amino acid and carbohydrate pathways may play a role in this distinction.
Pinto et al., 2023 (Israel)	Cohort	No: 394 Cases: 44 GDM, Controls: 350 Eligibility: pregnant women aged 18–40 years recruited between gestational ages (weeks+ days) 11+0–13+6.	Fecal Samples	16S rRNA gene sequencing (Illumina MiSeq platform) Untargeted metabolomics (LC-MS/MS).	Mann–Whitney U tests, false discovery rate (FDR) correction, Spearman's rank test, and Student's t-tests	The study showed that the gut microbiome appears to play a role in inflammation-induced GDM pathogenesis, with interleukin-6 as a potential contributor to pathogenesis.
Hill et al., 2017. (Ireland)	Cohort	No: 192 Cases: 35 PT-CS and 4 PT-SVD Controls: 70 FT-CS and 83 FT-SVD Eligibility: N/A	Fecal samples (Microbial analysis) and Urine (Metabolomics).	The V4–V5 amplicons for Illumina sequencing Untargeted LC-MS assays.	ANOVA statistical test.	The study findings confirm that mode of delivery and gestational age both have significant effects on early neonatal microbiota composition. There is also a significant difference between the metabolite profile of FT and PT infants.
Kindschuh et al., 2022. (USA)	Cohort	No: 2000 Cases: 80 sPTB Controls: 152 Terms birth Eligibility: Singleton pregnancy and presented before 20 weeks of gestation; Exclusion: (1) major fetal anomaly, (2) HIV positive status, (3) history of organ	Vaginal swabs.	16S rRNA gene amplicon sequencing (V3–V4 region) Metabolon, using an untargeted liquid chromatography–tandem mass spectrometry (LC-MS/MS) platform.	PERMANOVA tests.	The study results demonstrate the potential of vaginal metabolites as early biomarkers of sPTB and highlight exogenous exposures as potential risk factors for prematurity.

		transplant, and (4) chronic steroid use.				
Pruski et al., 2021 (UK)	Cohorts	No: 365 Cases: 73 controls: 292 Eligibility criteria were pregnant women with a singleton pregnancy, with and without risk factors for preterm birth. Exclusion criteria included women under 18 years of age, sexual intercourse within 72 h of sampling, vaginal bleeding in the preceding week, antibiotic use in the preceding 2 weeks, multiple pregnancies, and HIV or hepatitis C-positive status.	Cervicovaginal swabs	16S rRNA amplicon sequencing (Illumina MiSeq platform) Metabolic profiling (DESI-MS and LC-MS analysis).	Kenward–Roger approximation, Benjamini–Hochberg false discovery rate correction.	The study found that vaginal microbiota instability and innate immune activation, as predicted using DESI-MS, were associated with preterm birth, including in women receiving cervical cerclage for preterm birth prevention.
Wandro et al., 2018 (Orange County, California).	Cohort	No: 32 Cases: 8 Neonatal sepsis and 3 necrotizing enterocolitis Control: 21 PT infants (Healthy) Eligibility: N/A	Fecal sample	16S rRNA gene sequencing Metabolome (gas chromatography-mass spectrometry).	Pearson correlations	The study found that preterm infant microbiomes are shaped by shared exposures, especially to antibiotics, leading to the dominance of antibiotic-resistant facultative anaerobes, such as Enterococcus spp.
Benítez-Guerrero et al., 2022 (Mexico)	An observational, retrospective, case-control study	No: 54 Cases: 11 gestational diabetes (GD), 8 pre-eclampsia (PE), and 5 pre-pregnancy diagnoses of type 1 or 2 diabetes mellitus (PD). Controls: 30 healthy pregnant women Eligibility: N/A	Stool samples.	High-throughput DNA sequencing of V3-16S rRNA gene libraries Metabolomics (FT-ICR MS).	Welch's test, with Bonferroni correction.	The study found that GD, PD, and PE are associated with fecal bacterial microbiota profiles, with distinct predictive metagenomes

2.7.3. Quality Assessment

As illustrated in Table 2.2: all included studies, whether cohort or case-control studies, underwent assessment for risk of bias using the Newcastle-Ottawa Scale (NOS). The quality score reflects the robustness of the studies in investigating the effects of adverse pregnancy and birth outcomes (APBOs) on the microbiome and metabolome of exposed women and their neonates compared to non-exposed cases (healthy controls). When assessing the quality of selection, all case-control studies included selected cases from the health care facilities as healthy controls (Wang et al., 2020; Chen et al., 2021; Gerson et al., 2021; Benítez-Guerrero et al., 2022) and non-exposed cohort from all cohorts' studies were drawn from the same community as the exposed cohort. Regarding the risk of bias, 12 studies indicated low risk, 3 moderate, and 2 studies were high risk (Table 2.2). Both studies with a high risk of bias were cohort studies (Wandro et al., 2018; Dougherty et al., 2020). These studies exhibited high-risk bias primarily due to factors such as selection bias and measurement bias. Notably, they did not match or adjust controls for potential confounding variables such as age, body mass index (BMI), and gestational age at sampling. Consequently, the quality scores for these studies were 3 (Dougherty et al., 2020) and 4 (Wandro et al., 2018).

Table 2.2: Assessment of the included publications risks of bias.

Author Details	**Study Design**	**Selection**	**Comparability**	**Exposure**	**Outcome**	**NOS Score**	**Final remarks**
Ye et al., 2023	Cohort	***	**		*	6	Moderate
Liu et al., 2021	Cohort	****	**		***	9	low risk
Yang et al., 2022	Cohort	****	*		***	8	low risk
Tao et al., 2023	Cohort	***	*		**	6	Moderate
Gershuni et al., 2021	Prospective cohort	***	*		***	7	low risk
Liu et al., 2021	Cohort (pilot study)	****	**		**	8	low risk
Wang et al., 2020	Case-control study	***	*	**		6	Moderate
Chen et al., 2021	Case-control study	***	**	**		7	low risk
Flaviani et al., 2021	Cohort	***	*		***	7	low risk
Dougherty et al., 2020	Cohort	*	*		*	3	High risk
Gerson et al., 2021	Nested case-control	****	*	**		7	low risk
Pinto et al., 2023	Cohort	****	**		****	9	low risk
Hill et al., 2017	Cohort	****	*		****	8	low risk
Kindschuh et al., 2022	Cohort	****	*		****	8	low risk
Pruski et al., 2021	Cohorts	****	*		****	8	low risk
Wandro et al., 2018	Cohort	**			**	4	High risk
Benítez-Guerrero et al., 2022	Case-control	****	*	**		7	low risk

2.7.4. Human Microbiome about Adverse Pregnancy and Birth Outcomes

Seventeen (17) articles met the criteria for inclusion in this category, as detailed in Table 2.3. Despite variations in the timing of analysis during different stages of pregnancy across studies, a consistent pattern of changes in the microbiome was observed in adverse pregnancy and birth outcome (APBO) cases compared to control groups. Notably, considerable alterations in the phylum *Bacteroidetes/Bacteroidota, Firmicutes, Spirochaetes, Fibrobacteres, Actinobacteria, Proteobacteria*, and *Tenericutes* were recorded in the microbiome of mothers and neonates diagnosed with APBOs. Regarding the different phylum, Firmicutes were increased in APBOs including PT, PE, GDM, and miscarriage (Liu et al., 2021a; Hill et al., 2017; Benítez-Guerrero et al., 2022). Certainly, the phylum Firmicutes demonstrated notable changes in the microbiome of pregnant women and neonates with various APBOs, predominantly showing an increase.

Proteobacteria demonstrated distance in few cases of APBOs such as miscarriage, sPTB, GDM, PT, PE, and GDM (Gershuni et al., 2021; Chen et al., 2021; Hill et al., 2017; Benítez-Guerrero et al., 2022). Thus, Proteobacteria patterns were observed to be increased in PT (Hill et al., 2017), and PE (Benítez-Guerrero et al., 2022), but a decreased in their abundance was seen in sPTB (Gershuni et al., 2021), GDM (Chen et al., 2021; Benítez-Guerrero et al., 2022), and PE (Benítez-Guerrero et al., 2022). Furthermore, this inconsistency was also reported in the phyla Bacteroidetes/Bacteroidota, with an increase noted in miscarriage (Liu et al., 2021a) and PE (Benítez-Guerrero et al., 2022), and it was decreased in cases of GDM (Benítez-Guerrero et al., 2022). Regarding the Actinobacteriota, a study by Benítez-Guerrero et al., (2022) expressed an increase in PE groups, but its abundance was decreased in GDM groups. In the study conducted by Liu et al. (2021a), an investigation into the microbiome composition in cases of miscarriage compared to healthy control groups revealed a noteworthy increase in the abundance of Phylum

Spirochaetae, Fibrobacteres, and *Tenericutes*. This observation suggests a potential association between the elevated presence of *Spirochaetae, Fibrobacteres*, and *Tenericutes* and the occurrence of miscarriage.

Certainly, the variability in abundance changes at the genus level among pregnant women or neonates with various adverse pregnancy outcomes (APBOs) has been recorded in several studies. These fluctuations in microbial composition may indicate the dynamic nature of the microbiome during pregnancy and its potential association with different health outcomes. Prevotella expression demonstrated a significant alteration in various cases, two studies observed a lower abundance in miscarriage and GDM respectively (Liu et al., 2021a; Pinto et al., 2023). Conversely, Benítez-Guerrero et al. (2022) documented a higher prevalence of Prevotella in cases of gestational diabetes mellitus (GDM). Only two studies examined the Lactobacillus genus level, both indicating that an elevation in the abundance of this genus was linked to the risk of fetal growth restriction (FGR) and GDM, respectively (Tao et al., 2023; Benítez-Guerrero et al., 2022). In a study conducted by Liu et al., (2021a), they found that the genus Prevotella_1, Roseburia, Prevotellaceae_UCG_003, and Selenomonas content were lower in women with miscarriage.

In participants with FGR the following demonstrated a significant alteration in their abundance when compared with health controls: a decline in Enterococcus and Acinetobacter (Yang et al., 2022) and an increase in Catenibacterium (V38) (Tao et al., 2023). An increase in the abundance of conditional pathogens including *Streptococcus, Chlamydia, Staphylococcus, Mycobacterium*, and *Enterobacter* was demonstrated in PROM groups compared to normal pregnancy outcomes (Liu et al., 2021b). In cases of gestational diabetes mellitus (GDM), the abundance of various microbial genera exhibited diverse trends. According to the study conducted by Benítez-Guerrero et al. (2022), distinct alterations were observed in the abundance of several genera. Specifically, there was an increase in the abundance of Paraclostridium, Bosea, and Escherichia. Conversely, a decline in the abundance of Cutibacterium, Micrococcus, Variovorax, Achromobacter, and Alistipes was noted. These findings suggest a dynamic shift in the composition of the microbial community in individuals with GDM. Another study by Ye et al., (2023) also demonstrated that the following genera, Faecalibacterium, Prevotella, and Streptococcus, declined in GDM. Notably, a decrease in *Streptococcus* was also observed in PTB compared to healthy controls (Dougherty et al., 2020). Pre-eclampsia (PE) is also associated with a dynamic alteration in several microbial genera. Corynebacterium, Methylobacterium, Paracoccus, Acinetobacter, Muribaculaceae, Helicobacter, Enterococcus, Gemella, Finegoldia, and Staphylococcus were found to be dominant, while Reyranella exhibited a decline (Benítez-Guerrero et al., 2022).

At the species and family level, various studies have demonstrated a dynamic alteration in the abundance of species and families in several cases of Adverse Pregnancy and Birth Outcomes (APBOs) compared to normal pregnancy outcomes. Overall, the relative abundance of *Lactobacillus species* in the cases of PTB was observed to differ in several studies (Flaviani et al., 2021; Gerson et al., 2021; Kindschuh et al., 2022; Pruski et al., 2021). *L. acidophilus* (Flaviani et al., 2021), *L. crispatus* (Gerson et al., 2021), *L. crispatus, L. gasseri*, and *L. iners* (Kindschuh et al., 2022), and *L. crispatus*, *L. gasseri, L. iners, L. jensenii*, and another *Lactobacillus spp.* (Pruski et al., 2021) were elevated in cases of PTB. In contrast, Flaviani et al., (2021) and Gerson et al., (2021) observed a decline in the abundance of *L. crispatus* and some *Lactobacillus species,* respectively. On the contrary, in the case of GDM, a reduction in the prevalence of the species *Prevotella stercorea*, *Bacteroides coprophilus, Faecalibacterium prausnitzii, Prevotella copri,* and *Eubacterium siraeum* was noted (Ye et al., 2023). Microorganisms from various families exhibited a dynamic alteration in abundance as well. Bacteria from the Ruminococcaceae family were observed to decrease in both FGR groups (Tao et al., 2023) and GDM groups (Wang et al., 2020). On the contrary, the level of Enterobacteriaceae was observed to be differed in the cases of GDM, PTB, and late-onset sepsis (Wang et al., 2020; Dougherty et al., 2020; Wandro et al., 2018). Thus, its abundant increased in the case of PTB (Dougherty et al., 2020) and decreased in GDM Wang et al., 2020). In the cases of GDM, both Lachnospiraceae and Streptococcaceae were increased, as reported by Wang et al. (2020) and Chen et al. (2021) respectively. Furthermore, Veillonellaceae were found to be dominant in PTB compared to healthy controls, as reported by Dougherty et al. (2020).

2.7.5. Differential Metabolites as Potential Biomarkers in Adverse Pregnancy and Birth Outcomes

The study design of the included studies aimed at determining the differential metabolites in APBOs compared to healthy pregnancy outcomes. Of all 17 studies included employing untargeted metabolomic techniques for the identification of potential biomarkers for several APBOs, three studies were unable to detect differential metabolites in PTB (Pruski et al., 2021), PTB and late-onset sepsis (Wandro et al., 2018), as well as PE and GDM (Benítez-Guerrero et al., 2022) (Table 2.3). The remaining 14 studies demonstrated that no identifiable patterns in metabolite regulations were found across plasma, vaginal fluid, swab, meconium, fecal, and urine samples. Thus, nearly all studies presented potential biomarkers (metabolites) that were not replicated in other studies, with only Allantoin and Riboflavin appearing twice. Allantoin exhibited a dynamic concentration pattern in two distinct cases; it was observed to be upregulated in FGR (Tao et al., 2023) and downregulated in GDM (Ye et al., 2023). In both cases of GDM and Preterm, Riboflavin was upregulated, as reported by Chen et al. (2021) and Hill et al. (2017), respectively.

Comparing APBOs with healthy or normal pregnancy outcomes, the potential biomarkers exhibiting differential expression included a series of compounds such as Carboxylic Acid and Derivatives, Bile acid and Derivatives, Carbohydrate metabolism and derivatives, Amino acid and Derivatives, Indole derivative, Vitamins, Amine, tetrapyrroles, and Nucleosides (Table 2.4) (Ye et al., 2023; Liu et al., 2021a; Tao et al., 2023; Gershuni et al., 2021; Wang et al., 2020; Chen et al., 2021; Flaviani et al., 2021; Dougherty et al., 2020; Gerson et al., 2021; Hill et al., 2017; Kindschuh et al., 2022; Flaviani et al., 2021;

Pinto et al., 2023; Liu et al., 2021b). As illustrated in Table 2.4, carboxylic acids and derivatives were the most frequently reported differential metabolites in several APBOs, totaling 35 occurrences. They were followed by carbohydrate metabolism and derivatives with 16 instances, amino acids and derivatives with 15 instances, bile acid and derivatives with 14 instances, amine with 11 instances, indole and derivatives with 2 instances, and tetrapyrroles and nucleosides with 1 instance each. It is noteworthy that approximately five differential metabolites, namely, methyl dihydrophaseate, Pregnandione-GLC, N-Methylglumatic acid, Cystemine-Dehydrated, and Ca^2, were not assigned specific chemical classes.

Table 2.3: Regulated metabolites and microbiome and their associated APBOs compared to Healthy controls.

Level of Metabolites in cases	Level of Microbiome in cases	APBOs	Integrative analysis	Author Details
↑ 2-hydroxybutyric acid, L-alpha-aminobutyric acid ↓ methionine sulfoxide, allantoin, and dopamine.	↓ Genera: Faecalibacterium, Prevotella, and Streptococcus ↓ Species: *Bacteroides coprophilus, Eubacterium siraeum, Faecalibacterium prausnitzii, Prevotella copri, and Prevotella stercorea,*	GDM	*Faecalibacterium prausnitzii* = allantoin *Bacteroides finegoldii, Bacteriodides unniformis,* and *Protevella stecorea* = dopamine.	Ye et al., 2023
↑hyocholic acid, methyl dihydrophaseate, 3a, 7a, 12b-Trihydroxy-5b-cholanoic acid, 3a, 6a, 7b-Trihydroxy-5b-cholanoic acid, 3alpha-Hydroxy-5beta-chola-8,14-dien-24-oic acid, 3,8-Dihydroxy-6-methoxy-7(11)-eremophilen-12,8-olide, D-Urobilinogen, 1b,3a,7b-Trihydroxy-5b-cholanoic acid, Tetratydroacridin-9-amine (THA) or Tacrine, and chenodeoxycholic acid sulfate	↓At the genus level: Prevotella_1, Roseburia, Prevotellaceae_UCG_003 and Selenomonas. ↑ at phylum level: Bacteroidetes, Firmicutes, Spirochaetae, Fibrobacteres, and Tenericutes.	Miscarriage	*Prevotella_1 and Prevotellaceae*_UCG_003 = 7-Hydroxy-3-oxocholanoic acid Selenomonas = 7-Hydroxy-3-oxocholanoic acid	Liu et al., 2021a
↓methionine, dipicolinic acid and cysteine.	↓ Enterococcus and Acinetobacter	FGR	*Acinetobacter baumannni, E. faecium* and *E. faecalis* = methionine, D-aspartate, norvaline, beta-alanine and cysteine	Yang et al., 2022
↓ Maltose, malic acid, and 9-hexadecenoic. ↑Allantoin, pinitol, nicotinic acid, and lyxonic acid	↑ Lactobacillus (V32) and Catenibacterium (V38). ↓ Ruminococcaceae (V22), Bacteroides uniformis (V30), Mollicutes RF39 (V52), and Alistipes onderdonkii (V57)	FGR	*Ruminococcaceae (V22), Bacteroides uniformis (V30), Lachnospiracaceae (V51), and Lactobacillus* (V33) = Glyceollin IV *Pasteurellaceae (V29) and Catenibacterium (V38)* = Allantoin Catenibacterium (V38) and *Enterobacterriaceae shigella* (V5) = Glycolic-acid-2-phosphate *Erysipelotrichaceae* (V8) = Dodecanoic acid	Tao et al., 2023
↑ Eicosapentaenoic acid (EPA), and Docosahexaenoic acid (DHA)	↓Betaproteobacteria	sPTB	N/A	Gershuni et al., 2021

↓ N-acetyl-D-galactosamine (GalNAc) and sucrose	↑ conditional pathogens: *Prevotella, Streptococcus, Chlamydia, Staphylococcus, Mycobacterium and Enterobacter* ↓ *Lactobacillus* including *Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillu jensernii and Lactobacillus iners*	PROM	*Lactobacillus* = N-acetyl-D-galactosamine	Liu et al., 2021b
Fecal metabolites: ↑Valine and Allontoic acid ↓ Kymurenic acid and 5-Hydroxyindoleacetic acid. Urine metabolites: ↑D-Galactose, 3-Methoxyrosine and D-Glucose ↓ 2-O-Methyl-L-Ascorbic acid, Citraconic Acid, N-Methylglumatic acid, Cystemine-Dehydrated, Glycine, Galactitol, Trehalose, Isocitric acid, 2-Hydroxyhexanoic acid, Nicotinic acid, and Dehydroshikimic Acid.	↑ Lachnospiraceae ↓ Enterobacteriaceae and Ruminococcaceae.	GDM	*Lachnospiraceae* = Kymurenic acid *Enterobacteriaceae* = Valine, glutamine dehydrated, Trehalose, Citraconic Acid, Dehydroshikimic Acid, and Methylglumatic acid.	Wang et al., 2020
Neonatal meconium: ↓Glycerophosphocholine, glycocholic acid, and rhamnose. Maternal blood: ↑riboflavin and taurine.	↑ Firmicutes, Streptococcaceae ↓ Proteobacteria.	GDM	N/A	Chen et al., 2021
↑Lactate. ↓ acetate, Ca2+, betaine, glucose, and succinate.	↑ L. acidophilus. ↓ L. crispatus	sPTB	*Lactobacillus crispatus* = Lactate	Flaviani et al., 2021
↑pantothenic acid, pantetheine, histamine, and putrescine. ↓Glycodeoxycholic acid (GCDCA) and tauroursodeoxycholic acid.	↑Enterobacteriaceae, Veillonellaceae ↓ Streptococcus.	PTB	On class level: Clostridia and Gammaproteobacteria = succinate, putrescine, pyridoxine, nicotinate, isobutyric-acid, and uracil *Enterobacteriaceae, Enterococcsceaae*, and *Tissierellacea* = Succinate, pyridoxine, nicotinate, sarcosine, and uracil.	Dougherty et al., 2020

↑ Maltotriose, glucose 6-phosphate, 3-methylhistidine, 2-keto-3-deoxygluconate, Tartarate, Adenosine, triethanolamine, and kynurenate. ↓ Maleate, glutarate, nervonate, ceramide, Phosphoenolpyruvate, hippurate, and spermine.	↓ Lactobacillus species ↑ L. crispatus	sPTB	N/A	Gerson et al., 2021
↓ tyrosine, phenylalanine and alanine	↓ Prevotella	GDM	N/A	Pinto et al., 2023
↑ (Paracetamol sulfate, Paracetamol Glucuronide, Sulfosalicylic acid), (riboflavin, CECH or pyridoxic acid), indoxyl sulphate, indole acetic, acetyl tyrosine, acetylphenylalanine, (Glyco-tetrahydroxycholanic acid, Dehydro-glycocholic acid, Pregnandione-GLC, Glycocholic acid, Glycoursodeoxycholic sulfate), glutamic acid (dicarboxylic species). ↓ hydroxyphenylalanine sulphate, succinic acid and its derivates (Succinylacetone).	↑Proteobacteria and Firmicutes	PT	Microbiota at OUT level = Metabolome	Hill et al., 2017
↑Ethyl β-glucopyranoside, tartrate, and diethanolamine. ↓ choline	↑ Lactobacillus spp. (*Lactobacillus crispatus, Lactobacillus gasseri, and Lactobacillus iners*)	sPTB	*Enterococcus faecalis* = tyramine *G. vaginalis* and *corynebacterium aurimucosum* = Choline	Kindschuh et al., 2022
No specific Metabolites associated with APBOs.	↑ L. crispatus, L. gasseri, L. iners, L. jensenii, and another Lactobacillus spp. (Lactobacillus-dominated), Bifidobacterium breve and Gardnerella vaginalis (Lactobacillus-depleted).	PTB	Lactobacillus-dominated = Thiomalic acid and leucyl-serine Bifidobacterium breve and Gardnerella vaginalis = docosanoic acid and lignoceric acid	Pruski et al., 2021
No specific Metabolites associated with APBOs.	↓ Bifidobacterium ↑ Enterobacteriaceae, Enterococcus, and Staphylococcus	PTB and late-onset sepsis	Staphylococcus = sugar metabolites, organic acids, and central metabolites Enterobacteriaceae and Bacteroides= Fatty acids, lipids metabolism, and amino acids	Wandro et al., 2018

No specific Metabolites associated with APBOs.	The GDM group: ↑ Paraclostridium, Lactobacillus (Firmicutes), UCG-001 (family Prevotellaceae, Bacteroidota), Bosea, Escherichia (Proteobacteria) ↓ Cutibacterium, Micrococcus (Actinobacteriota), Variovorax, Achromobacter (Proteobacteria), and Alistipes (Bacteroidota). PE group: ↑ Corynebacterium (Actinobacteriota), Methylobacterium, Paracoccus, Acinetobacter (Proteobacteria), Muribaculaceae (Bacteroidota), Helicobacter (Campilobacterota), Enterococcus, Gemella, Finegoldia, and Staphylococcus (Firmicutes) ↓Reyranella (Proteobacteria)	PE and GDM	N/A	Benítez-Guerrero et al., 2022

↑ represent the increase in level of potential biomarkers (metabolites and microbiome) in cases compared to controls while ↓ represent the decrease. Adverse Pregnancy and birth Outcomes (APBOs) included involves: Gestational Diabetes Mellitus (GDM), spontaneous Preterm Birth (sPTB), Pre-eclampsia (PE), Preterm cases (PT), Premature membrane rupture (PROM), fetal growth restriction (FGR) and late-onset sepsis. = under integrative analysis indicating the significant association between metabolites and microbes. N/A demonstrating no associations.

2.7.5. Pathway Enrichment Analysis of Differential Potential Biomarkers

We systematically summarized the metabolic potential biomarkers from the 14/17 included studies, and 104 potential biomarkers for various APBOs were extracted. Among them 23 were not recognised by MetaboAnalyst6.0. Finally, metabolic pathways analysis was performed on 82 compounds recognised by MetaboAnalyst6.0 and their identification code are provided on Table 4. The enriched metabolic pathway and their impact scores analysed by MetaboAnalyst demonstrated that they were mainly 37 pathways involved; 13 pathways were significantly associated with extracted metabolites from the included studies at $P \leq 0.05$ (Table 2.5 and Figure 2.2).

Furthermore, among the 13 statistically significant pathways, 6 metabolomic pathways, including Starch and sucrose metabolism, Neomycin, kanamycin, and gentamicin biosynthesis, Pantothenate and CoA biosynthesis, Citrate cycle (TCA cycle), Pyruvate metabolism, and Phenylalanine, tyrosine, and tryptophan biosynthesis, demonstrated statistical significance when the P-value was adjusted for FDR < 0.05. Of the 6 metabolic pathways that remained statistically significant after adjusting the P-value, only 4 pathways exhibited an impact factor > 0.1. These pathways include Starch and sucrose metabolism, Citrate cycle (TCA cycle), Pyruvate metabolism, and Phenylalanine, tyrosine, and tryptophan biosynthesis (Table 5).

Among the six statistically significant (FDR < 0.05) metabolic pathways, we observed matches for five metabolites in Starch and sucrose metabolism, two in Neomycin, kanamycin, and gentamicin biosynthesis, three in Pantothenate and CoA biosynthesis, three in the Citrate cycle (TCA cycle), four in Pyruvate metabolism, and two in Phenylalanine, tyrosine, and tryptophan biosynthesis. Furthermore, the metabolites identified in different metabolic pathways exhibited varied regulatory levels across the included studies. Detailed information regarding the reported differential potential metabolic biomarkers and enriched pathways is illustrated in Table 2.4.

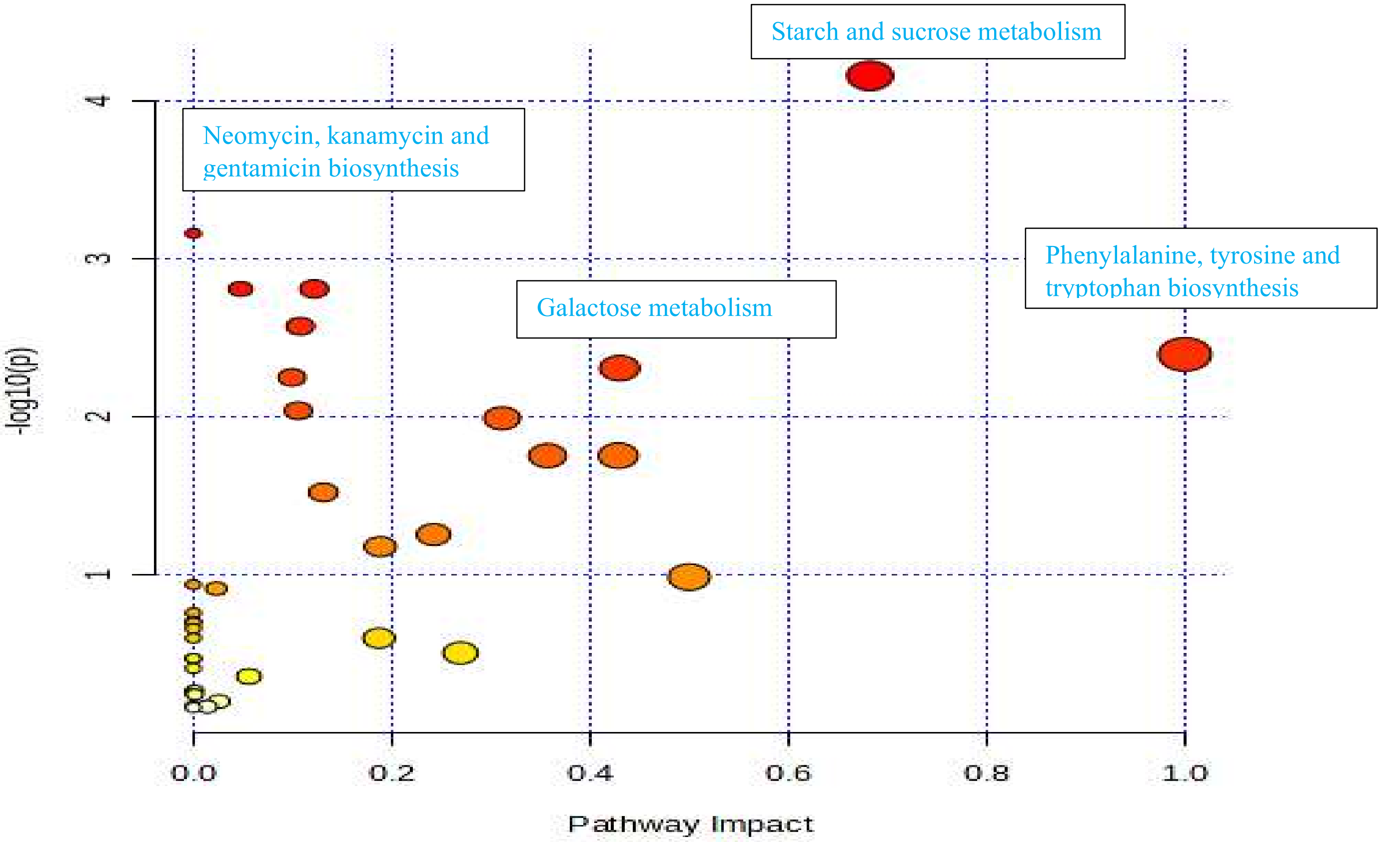


Figure 2.2: Figure illustrating the overall 42 Metabolic pathways of potential biomarkers of various adverse pregnancy and birth outcomes (APBOs) analysed using MetaboAnalyst6.0.

The "Pathway Impact Score" on the x-axis signifies the impact of the enriched pathways derived from topology analysis (colour of circle indicated a significant level of enrichment analysis and circle size illustrate the pathway impact value from topology analysis). On the y-axis, "-log P" denotes the negative natural logarithm of the original P-value resulting from the statistical analysis of the pathway.

Table 2.4: Differential Metabolites in APBOs and their respective chemical class, HMDB and KEGG IDs obtained from MetaboAnalyst 6.0.

Metabolites name	Chemical class	HMDB ID	KEGG ID	Increased/Decreased	References
2-hydroxybutyric acid	Carboxylic Acid and Derivatives	HMDB0000008	C05984	Increased	Ye et al., 2023
L-alpha-aminobutyric acid	Carboxylic Acid and Derivatives	HMDB0000452	C02356	Increased	Ye et al., 2023
hyocholic acid	Bile acid and Derivative	HMDB0000760	NA	Increased	Liu et al., 2021a
methyl dihydrophaseate		HMDB0039376	NA	Increased	Liu et al., 2021a
3a,7a, 12b-Trihydroxy-5b-cholanoic acid	Bile acid and Derivative	NA	NA	Increased	Liu et al., 2021a
3a,6a,7b-Trihydroxy-5b-cholanoic acid	Bile acid and Derivative	NA	NA	Increased	Liu et al., 2021a
3alpha-Hydroxy-5beta-chola-8	Bile acid and Derivative	NA	NA	Increased	Liu et al., 2021a
3,8-Dihydroxy-6-methoxy-7(11)-eremophilen-12,8-olide	Bile acid and Derivative	HMDB0041551	NA	Increased	Liu et al., 2021a
Urobilinogen	tetrapyrroles	HMDB0004158	C05791	Increased	Liu et al., 2021a
1b,3a,7b-Trihydroxy-5b-cholanoic acid	Bile acid and Derivative	HMDB0013158	NA	Increased	Liu et al., 2021a
Tetrahydroaminacrine	Amine	HMDB0014526	C01453	Increased	Liu et al., 2021a
chenodeoxycholic acid sulfate	Bile acid and Derivative	HMDB0002522	NA	Increased	Liu et al., 2021a
Allantoin	Carboxylic Acid and Derivatives	HMDB0000462	C01551	Increased	Tao et al., 2023
Pinitol	Carbohydrate metabolism and derivate	HMDB0034219	C03844	Increased	Tao et al., 2023
nicotinic acid	Carboxylic Acid and Derivatives	HMDB0001488	C00253	Increased	Tao et al., 2023
lyxonic acid	Carbohydrate metabolism and derivatives	HMDB0060255	C05412	Increased	Tao et al., 2023
Eicosapentaenoic acid	Carboxylic Acid and Derivatives	HMDB0001999	C06428	Increased	Gershuni et al., 2021
Docosahexaenoic acid	Carboxylic Acid and Derivatives	HMDB0002183	C06429	Increased	Gershuni et al., 2021
Valine	Amino acid and Derivatives	HMDB0000883	C00183	Increased	Wang et al., 2020

Allontoic acid	Carbohydrate metabolism and derivatives	NA	NA	Increased	Wang et al., 2020
D-Galactose	Carbohydrate metabolism and derivatives	HMDB0000143	C00984	Increased	Wang et al., 2020
3-Methoxyrosine	Amino acid and Derivatives	NA	NA	Increased	Wang et al., 2020
D-Glucose	Carbohydrate metabolism and derivatives	HMDB0000122	C00221	Increased	Wang et al., 2020
Riboflavin	Vitamin	HMDB0000244	C00255	Increased	Chen et al., 2021
Taurine	Amino acid and Derivatives	HMDB0000251	C00245	Increased	Chen et al., 2021
Lactate	Carboxylic Acid and Derivatives	HMDB0000190	C00186	Increased	Flaviani et al., 2021
pantothenic acid	Vitamin	HMDB0000210	C00864	Increased	Dougherty et al., 2020
pantetheine	Carboxylic Acid and Derivatives	HMDB0003426	C00831	Increased	Dougherty et al., 2020
histamine	Amine	HMDB0000870	C00388	Increased	Dougherty et al., 2020
putrescine	Amine	HMDB0001414	C00134	Increased	Dougherty et al., 2020
Maltotriose	Carbohydrate metabolism and derivatives	HMDB0001262	C01835	Increased	Gerson et al., 2021
glucose 6-phosphate	Carbohydrate metabolism and derivatives	HMDB0001401	C00092	Increased	Gerson et al., 2021
3-methylhistidine	Amino acid and Derivativess	HMDB0000479	C01152	Increased	Gerson et al., 2021
2-keto-3-deoxygluconate	Carboxylic Acid and Derivatives	NA	NA	Increased	Gerson et al., 2021
Tartarate	Carboxylic Acid and Derivatives	HMDB0000956	C00898	Increased	Gerson et al., 2021
Adenosine	Nucleosides	HMDB0000050	C00212	Increased	Gerson et al., 2021
Triethanolamine	Amine	HMDB0032538	C06771	Increased	Gerson et al., 2021
Kynurenate	Carboxylic Acid and Derivatives	HMDB0000715	C01717	Increased	Gerson et al., 2021
Paracetamol sulfate	Amine	HMDB0059911	NA	Increased	Hill et al., 2017

Paracetamol Glucuronide	Amine	HMDB0010316	NA	Increased	Hill et al., 2017
Sulfosalicylic acid	Carboxylic Acid and Derivatives	HMDB0011725	C16199	Increased	Hill et al., 2017
riboflavin	Vitamin	HMDB0000244	C00255	Increased	Hill et al., 2017
pyridoxic acid	Carboxylic Acid and Derivatives	HMDB0000017	C00847	Increased	Hill et al., 2017
indoxyl sulphate	Indole derivate	HMDB0000682	NA	Increased	Hill et al., 2017
indole acetic	Indole derivate	NA	NA	Increased	Hill et al., 2017
acetyl tyrosine	Amino acid and Derivatives	NA	NA	Increased	Hill et al., 2017
acetyl phenylalanine	Amino acid and Derivatives	NA	NA	Increased	Hill et al., 2017
Glyco-tetrahydroxycholanic acid	Bile acid and Derivative	NA	NA	Increased	Hill et al., 2017
Dehydro-glycocholic acid	Bile acid and Derivative	NA	NA	Increased	Hill et al., 2017
Pregnandione-GLC		NA	NA	Increased	Hill et al., 2017
Glycocholic acid	Bile acid and Derivative	HMDB0000138	C01921	Increased	Hill et al., 2017
Glycoursodeoxycholic sulfate	Bile acid and Derivative	NA	NA	Increased	Hill et al., 2017
glutamic acid	Amino acid and Derivatives	HMDB0000148	C00025	Increased	Hill et al., 2017
Ethyl Î²-glucopyranoside	Carbohydrate metabolism and derivatives	NA	NA	Increased	Kindschuh et al., 2022
tartrate	Carboxylic Acid and Derivatives	HMDB0000956	C00898	Increased	Kindschuh et al., 2022
diethanolamine	Amine	HMDB0004437	C06772	Increased	Kindschuh et al., 2022
methionine sulfoxide	Amino acid and Derivatives	HMDB0002005	C02989	Decreased	Ye et al., 2023
allantoin	Carboxylic Acid and Derivatives	HMDB0000462	C01551	Decreased	Ye et al., 2023
dopamine	Amine	HMDB0000073	C03758	Decreased	Ye et al., 2023
methionine	Amino acid and Derivatives	HMDB0000696	C00073	Decreased	Yang et al., 2022
dipicolinic acid	Carboxylic Acid and Derivatives	HMDB0033161	NA	Decreased	Yang et al., 2022
cysteine	Amino acid and Derivatives	HMDB0000574	C00097	Decreased	Yang et al., 2022
N-acetyl-D-galactosamine	Carbohydrate metabolism and derivatives	HMDB0000212	C01074	Decreased	Liu et al., 2021b

Sucrose	Carbohydrate metabolism and derivatives	HMDB0000258	C00089	Decreased	Liu et al., 2021b
Kymurenic acid	Carboxylic Acid and Derivatives	NA	NA	Decreased	Wang et al., 2020
5-Hydroxyindoleacetic acid	Carboxylic Acid and Derivatives	HMDB0000763	C05635	Decreased	Wang et al., 2020
2-O-Methyl-L-Ascorbic acid	Carbohydrates metabolism and derivatives	NA	NA	Decreased	Wang et al., 2020
Citraconic Acid	Carboxylic Acid and Derivatives	HMDB0000634	C02226	Decreased	Wang et al., 2020
N-Methylglumatic acid		NA	NA	Decreased	Wang et al., 2020
Cystemine-Dehydrated		NA	NA	Decreased	Wang et al., 2020
Glycine	Amino acid and Derivatives	HMDB0000123	C00037	Decreased	Wang et al., 2020
Galactitol	Carbohydrate metabolism and derivatives	HMDB0000107	C01697	Decreased	Wang et al., 2020
Trehalose	Carbohydrate metabolism and derivatives	HMDB0000975	C01083	Decreased	Wang et al., 2020
Isocitric acid	Carboxylic Acid and Derivatives	HMDB0000193	C00311	Decreased	Wang et al., 2020
2-Hydroxyhexanoic acid	Carboxylic Acid and Derivatives	HMDB0001624	NA	Decreased	Wang et al., 2020
Nicotinic acid	Vitamin	HMDB0001488	C00253	Decreased	Wang et al., 2020
Dehydroshikimic Acid	Carboxylic Acid and Derivatives	NA	NA	Decreased	Wang et al., 2020
Glycerophosphocholine (GPC)	Carboxylic Acid and Derivatives	HMDB0000086	C00670	Decreased	Chen et al., 2021
glycocholic acid	Bile acid and Derivatives	HMDB0000138	C01921	Decreased	Chen et al., 2021
rhamnose	Carbohydrate metabolism and Derivatives	HMDB0000849	C00507	Decreased	Chen et al., 2021
acetate	Carboxylic Acid and Derivatives	HMDB0000042	C00033	Decreased	Flaviani et al., 2021
Ca2		NA	NA	Decreased	Flaviani et al., 2021
Betaine	Amine	HMDB0000043	C00719	Decreased	Flaviani et al., 2021

Glucose	Carbohydrate metabolism and derivates	HMDB0000122	C00221	Decreased	Flaviani et al., 2021
Succinate	Carboxylic Acid and Derivatives	HMDB0000254	C00042	Decreased	Flaviani et al., 2021
Glycodeoxycholic acid	Bile acid and Derivative	HMDB0000631	C05464	Decreased	Dougherty et al., 2020
tauroursodeoxycholic acid	Bile acid and Derivative	HMDB0000874	NA	Decreased	Dougherty et al., 2020
Maleate	Carboxylic Acid and Derivatives	HMDB0000176	C01384	Decreased	Gerson et al., 2021
Glutarate	Carboxylic Acid and Derivatives	HMDB0000661	C00489	Decreased	Gerson et al., 2021
Nervonate	Carboxylic Acid and Derivatives	HMDB0002368	C08323	Decreased	Gerson et al., 2021
Ceramide	Carboxylic Acid and Derivatives	HMDB0004947	C00195	Decreased	Gerson et al., 2021
Phosphoenolpyruvate	Carboxylic Acid and Derivatives	HMDB0000263	C00074	Decreased	Gerson et al., 2021
Hippurate	Carboxylic Acid and Derivatives	HMDB0000714	C01586	Decreased	Gerson et al., 2021
spermine	Amine	HMDB0001256	C00750	Decreased	Gerson et al., 2021
Tyrosine	Amino acid and Derivatives	HMDB0000158	C00082	Decreased	Pinto et al., 2023
phenylalanine	Amino acid and Derivatives	HMDB0000159	C00079	Decreased	Pinto et al., 2023
alanine	Amino acid and Derivatives	HMDB0000161	C00041	Decreased	Pinto et al., 2023
hydroxyphenylalanine sulphate	Amino acid and Derivatives	NA	NA	Decreased	Hill et al., 2017
succinic acid	Carboxylic Acid and Derivatives	HMDB0000254	C00042	Decreased	Hill et al., 2017
Succinylacetone	Carboxylic Acid and Derivatives	HMDB0000635	NA	Decreased	Hill et al., 2017
choline	Amine	HMDB0000097	C00114	Decreased	Kindschuh et al., 2022
Maltose	Carbohydrate metabolism and derivatives	HMDB0000163	C00208	Decreased	Tao et al., 2023
Malic acid	Carboxylic Acid and Derivatives	HMDB0000156	C00149	Decreased	Tao et al., 2023
9-hexadecenoic	Carboxylic Acid and Derivatives	NA	NA	Decreased	Tao et al., 2023

N/A: Indicating non-recognised HMDB and KEGG ID from MetaboAnalyst 6.0.

Table 2.5: Pathway analysis of potential biomarkers of several APBOs.

Pathway Name	Matched metabolites (refer)	P	-log(p)	Holm p	FDR	Impact
Starch and sucrose metabolism.	D-Glucose (Wang et al., 2020) Maltose (Tao et al., 2023) Sucrose (Liu et al., 2021b) Trehalose (Wang et al., 2020) Glucose 6-phosphate (Gerson et al., 2021)	7.0182E-5	4.1538	0.0056145	0.0056145	0.6825
Neomycin, kanamycin and gentamicin biosynthesis	D-Glucose (Wang et al., 2020) Glucose 6-phosphate (Gerson et al., 2021)	6.9462E-4	3.1583	0.054875	0.027785	0.0
Pantothenate and CoA biosynthesis	Pantetheine (Dougherty et al., 2020) Valine (Wang et al., 2020) Cysteine (Yang et al., 2022)	0.0015543	2.8085	0.12123	0.031086	0.04762
Citrate cycle (TCA cycle)	(S)-Malate (Tao et al., 2023) Succinate (Flaviani et al., 2021) Phosphoenolpyruvate (Gerson et al., 2021)	0.0015543	2.8085	0.12123	0.031086	0.12183
Pyruvate metabolism	Phosphoenolpyruvate (Gerson et al., 2021) (S)-Malate (Tao et al., 2023) Lactate (Flaviani et al., 2021) Acetate (Flaviani et al., 2021)	0.0026799	2.5719	0.20367	0.042878	0.10781
Phenylalanine, tyrosine and tryptophan biosynthesis	Phenylalanine (Pinto et al., 2023) Tyrosine (Pinto et al., 2023)	0.0040277	2.3949	0.30208	0.053703	1.0
Galactose metabolism	Sucrose (Liu et al., 2021b) Galactitol (Wang et al., 2020) D-Glucose (Wang et al., 2020) D-Galactose (Wang et al., 2020)	0.0049148	2.3085	0.36369	0.056169	0.43001
Glutathione metabolism	Glycine (Wang et al., 2020) Cysteine (Yang et al., 2022) Glutamic acid (Hill et al., 2017) Putrescine (Dougherty et al., 2020) Spermine (Gerson et al., 2021)	0.0056238	2.25	0.41054	0.056238	0.09935
Glyoxylate and dicarboxylate metabolism	(S)-Malate (Tao et al., 2023) Cysteine (Yang et al., 2022) Acetate (Flaviani et al., 2021)	0.0091411	2.039	0.65816	0.081254	0.10582

Pathway Name	Matched metabolites (refer)	P	-log(p)	Holm p	FDR	Impact
	Glutamic acid (Hill et al., 2017)					
Glycine, serine and threonine metabolism	Choline (Kindschuh et al., 2022) Betaine (Flaviani et al., 2021) Glycine (Wang et al., 2020) Cysteine (Yang et al., 2022)	0.010203	1.9913	0.72439	0.081621	0.3113
Phenylalanine metabolism	Phenylalanine (Pinto et al., 2023) Tyrosine (Pinto et al., 2023)	0.017561	1.7555	1.0	0.11707	0.35714
Taurine and hypotaurine metabolism	Taurine (Chen et al., 2021) Cysteine (Yang et al., 2022)	0.017561	1.7555	1.0	0.11707	0.42857
Glycolysis / Gluconeogenesis	Lactate (Flaviani et al., 2021) Phosphoenolpyruvate (Gerson et al., 2021) Acetate (Flaviani et al., 2021)	0.029932	1.5239	1.0	0.1842	0.13108
Cysteine and methionine metabolism	Methionine (Yang et al., 2022) Cysteine (Yang et al., 2022) L-alpha-aminobutyric acid (Ye et al., 2023)	0.055237	1.2578	1.0	0.31564	0.24217
Histidine metabolism	histamine (Dougherty et al., 2020) 3-methylhistidine (Gerson et al., 2021)	0.065788	1.1819	1.0	0.35087	0.18852
Riboflavin metabolism	Riboflavin (Chen et al., 2021; Hill et al., 2017)	0.10257	0.98898	1.0	0.51285	0.5
Propanoate metabolism	Succinate (Flaviani et al., 2021) 2-hydroxybutyric acid (Ye et al., 2023)	0.11464	0.94066	1.0	0.53937	0.0
Primary bile acid biosynthesis	Taurine (Chen et al., 2021) Glycocholic acid (Hill et al., 2017) Glycine (Wang et al., 2020)	0.12136	0.91593	1.0	0.53937	0.02321
Thiamine metabolism	Cysteine (Yang et al., 2022)	0.17268	0.76275	1.0	0.72709	0.0
Valine, leucine and isoleucine biosynthesis	Valine (Wang et al., 2020)	0.19484	0.71032	1.0	0.75886	0.0
Porphyrin metabolism	Glycine (Wang et al., 2020) D-Urobilinogen (Liu et al., 2021a)	0.1992	0.70071	1.0	0.75886	0.0
Vitamin B6 metabolism	Pyridoxic acid (Hill et al., 2017)	0.21642	0.6647	1.0	0.78699	0.0
Biosynthesis of unsaturated fatty acids	Docosahexaenoic acid (Gershuni et al., 2021) Eicosapentaenoic acid (Gershuni et al., 2021)	0.249	0.6038	1.0	0.83001	0.0

Pathway Name	Matched metabolites (refer)	P	-log(p)	Holm p	FDR	Impact
Arginine and proline metabolism	🟩 Putrescine (Dougherty et al., 2020) 🟨 Spermine (Gerson et al., 2021)	0.249	0.6038	1.0	0.83001	0.18721
Tyrosine metabolism	🟨 Tyrosine (Pinto et al., 2023) 🟨 Dopamine (Ye et al., 2023)	0.30932	0.5096	1.0	0.98981	0.26944
Butanoate metabolism	🟨 Succinate (Flaviani et al., 2021)	0.33453	0.47556	1.0	0.99121	0.0
Nicotinate and nicotinamide metabolism	🟨 Nicotinic acid (Wang et al., 2020)	0.33453	0.47556	1.0	0.99121	0.0
Ubiquinone and other terpenoid-quinone biosynthesis	🟨 Tyrosine (Pinto et al., 2023)	0.38688	0.41242	1.0	1.0	0.0
beta-Alanine metabolism	🟨 Spermine (Gerson et al., 2021)	0.4352	0.36131	1.0	1.0	0.05597
Alanine, aspartate and glutamate metabolism	🟨 Alanine (Pinto et al., 2023)	0.53395	0.2725	1.0	1.0	0.0
Lipoic acid metabolism	🟨 Glycine (Wang et al., 2020)	0.53395	0.2725	1.0	1.0	0.0017
Inositol phosphate metabolism	🟩 Glucose 6-phosphate (Gerson et al., 2021)	0.55892	0.25265	1.0	1.0	0.0
Purine metabolism	🟩 Adenosine (Gerson et al., 2021) 🟨 Allantoin (Ye et al., 2023) 🟩 Allantoin (Tao et al., 2023)	0.56568	0.24743	1.0	1.0	0.00158
Glycerophospholipid metabolism	🟨 Choline (Kindschuh et al., 2022)	0.62625	0.20325	1.0	1.0	0.02582
Valine, leucine and isoleucine degradation	🟨 Alanine (Pinto et al., 2023)	0.66544	0.17689	1.0	1.0	0.0
Tryptophan metabolism	🟨 5-Hydroxyindoleacetic acid (Wang et al., 2020)	0.6746	0.17096	1.0	1.0	0.0139
Amino sugar and nucleotide sugar metabolism	🟨 N-Acetyl-D-galactosamine (Liu et al., 2021b)	0.68351	0.16526	1.0	1.0	0.0

🟩 🟨

indicating increased metabolites in APBOs cases as compared to controls and demonstrating decreased metabolites. *P* value was considered statistically significant when p ≤0.005. FDR was statistically significant at q<0.05.

2.7.6. Integrated Metabolome and Microbiome Analysis

The purpose of Integrated Metabolome and Microbiome analysis in adverse pregnancy and birth outcomes is to comprehensively investigate and understand the interconnected roles of metabolites and microbial communities in contributing to or indicating adverse conditions during pregnancy and childbirth (Zhao at el., 2020; Nguyen et al., 2021). This systematic review included 17 studies which attempted to explore both metabolomic and microbiome signature to underline the potential impact of metabolic pathways and dysbiosis microbiome on pregnancy and birth complications as compared to healthy controls. However, approximately 5 publications were unable to establish this integration (Gershuni et al., 2021; Chen et al., 2021; Gerson et al., 2021; Pinto et al., 2023; Benítez-Guerrero et al., 2022).

Among the included studies that demonstrated integration between the microbiome and metabolome, only one failed to specify which metabolites and microorganisms were associated with either cases or controls. In this particular study, it was only indicated that microbiota data at the OTU level were significantly associated with the metabolomic dataset (Hill et al., 2017). The integrative analysis of eligible studies revealed several microbial taxa associated with specific metabolites. For instance, Ye et al. (2023) discovered that *Faecalibacterium prausnitzii* is linked to allantoin, while they also indicated a strong correlation between the following species: *Bacteroides finegoldii, Bacteriodides unniformis*, and *Protevella stecorea* with dopamine. Consequently, a reduction in the abundance of microbial taxa was associated with a decrease in the concentration of allantoin and dopamine in cases of gestational diabetes mellitus (GDM) compared to controls.

Comparing miscarriage cases with normal pregnancy controls, Liu et al. (2021a) reported a significant association between an increase in the abundance of 7-Hydroxy-3-oxocholanoic acid and a decrease in the genera *Selenomonas, Prevotella_1,* and *Prevotellaceae_UCG_003*. Yang et al. (2022) demonstrated a strong association between various amino acids, such as methionine, D-aspartate, norvaline, beta-alanine, and cysteine, with Enterococcus and Acinetobacter (*Acinetobacter baumannni, E. faecium*, and E. faecalis). Within this integrated framework, amino acids such as methionine and cysteine, along with *Enterococcus* and *Acinetobacter*, were found to be downregulated in cases of fetal growth restriction (FGR).

In a cohort study comparing fetal growth restriction (FGR) cases with healthy pregnancy controls conducted by Tao et al. (2023), multiple microbial taxa demonstrated a robust association with various metabolites. Specifically, *Ruminococcaceae (V22), Bacteroides uniformis (V30), Lachnospiracaceae (V51)*, and *Lactobacillus (V33)* showed positive associations with Glyceollin IV. Additionally, *Pasteurellaceae (V29) and Catenibacterium* (V38) were associated with Allantoin, *while Catenibacterium (V38)* and *Enterobacterriaceae shigella (V5)* exhibited a strong correlation with Glycolic-acid-2-phosphate. Lastly, *Erysipelotrichaceae (V8)* was associated with Dodecanoic acid. Interestingly, an increase in *Catenibacterium (V38)* was linked to the upregulation of Allantoin in FGR cases.

Several studies have demonstrated a direct association between *Lactobacillus species* with several metabolites in various adverse pregnancy outcomes and healthy controls. In cases of Premature Rupture of Membranes (PROM), Liu et al. (2021b) found that a decrease in the abundance of *Lactobacillus species*, including *Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus jensernii*, and *Lactobacillus iners*, was positively associated with a decrease in N-Acetyl-D-galactosamine. Furthermore, a reduction in the relative abundance of *Lactobacillus species*, particularly *Lactobacillus crispatus*, exhibited a direct correlation with an increased concentration of Lactate in cases of spontaneous preterm birth (sPTB) (Flaviani et al., 2021). Through integrative analysis, Pruski et al. (2021) indicated a positive correlation between Lactobacillus-dominated species (*L. crispatus, L. gasseri, L. iners, L. jensenii, and other Lactobacillus spp*.) and Thiomalic acid and leucyl-serine. In contrast, Lactobacillus-depleted species (*Bifidobacterium breve and Gardnerella vaginalis*) showed a correlation with docosanoic acid and lignoceric acid. However, the study was unable to determine the differential metabolites associated with spontaneous preterm birth (sPTB) and controls.

The integration between the metabolome and microbiome in cases of gestational diabetes mellitus (GDM), compared to normal pregnancy outcomes, revealed a direct correlation. Wang et al. (2020) demonstrated that an increase in the abundance of the bacterial family *Lachnospiraceae* was associated with a decrease in Kymurenic acid in fecal samples. In the same study, *Enterobacteriaceae* showed positive associations with various amino acids and carbohydrate metabolism in both fecal and urine samples. Consequently, a decrease in *Enterobacteriaceae* was directly correlated with an increase in Valine in fecal samples and a decrease in glutamine dehydrated, Trehalose, Citraconic Acid, Dehydroshikimic Acid, and Methylglumatic acid in urine samples.

In all studies that attempted to explore the integration of metabolomics and microbial profiles in preterm birth cases, including spontaneous preterm birth, late-onset sepsis, and normal controls, several microbial classes and families were strongly associated with various metabolites from different chemical classes (Dougherty et al., 2020; Kindschuh et al., 2022; Wandro et al., 2018). However, none of these studies were able to demonstrate whether the positively correlated metabolites had any impact on the relative abundance of several microbiomes in cases compared to normal controls or versa-versa. Thus, either the metabolites or the microbiome showed elevation or downregulation in cases or controls (Table 3).

2.8. DISCUSSION

Adverse pregnancy and birth outcomes (APBOs) persist as the leading and yet inadequately understood causes of neonatal deaths worldwide (Han et al., 2020). Consequently, there has been a growing interest in the quest for non-invasive prognostic and diagnostic biomarkers aimed at reducing the incidence of neonatal mortality. This interest has been encapsulated and summarized in several systematic reviews (Gomes et al., 2019; Lim et al., 2021; Ronde et al., 2021; Moschino et al., 2022). However, the multifactorial pathogenesis of several APBOs such as PROM, IUGR, GDM, PE, LBW, PTB, and miscarriage, poses a significant challenge in unraveling a unique potential biomarker for APBOs (Valenzuela et al., 2012; Bisson et al., 2023; Mautner et al., 2013). To date, detection and identification of APBOs remain quite challenging, primarily due to the asymptomatic nature of many complications or the presence of symptoms that overlap with those of normal pregnancies. Thus, they are often recognized during ANC service attendance using fetal monitoring or ultrasound examinations as primary tools (Moschino et al., 2022). Even with these measures in place, accurate identification of the severity of APBOs remains a challenge (Ng et al., 2015; Wang et al., 2019). Hence, there is a need for rapid advancement in diagnostic technologies to enable the comprehensive detection and identification of APBOs (Roberts et al., 2022).

Interestingly, omics technologies have emerged as advanced techniques and high-throughput techniques, which may unveil a better understanding of the underlying molecular mechanism for APBOs (Dai and Shen., 2022; Gracie et al., 2011). One notable example lies in the synergistic analysis of metagenomics and metabolomics data. This integrated approach has shed light on the complex interplay between host and microbial communities, providing novel perspectives on the impact of factors such as nutrition, antibiotic interventions, and immunomodulation on maternal health. By elucidating these intricate interactions, researchers aim to pave the way for preventive strategies aimed at mitigating the risk of pregnancy complications in the future (Moschino et al., 2022). Hence, our review summarized recent studies published between 2013-2023 cohort and case-control studies on the integration of metabolomics and metagenomics in various pregnancy complications around the world, identifying various biomarkers for predicting GDM, miscarriage, FGR, sPTB, PTB, PROM, PE, and neonatal sepsis.

Among the omics techniques, metabolomics displays several advantages as compared to other techniques by presenting the end products of cellular processes, reflecting the dynamic interplay between genes, proteins, and environmental factors (Akyol et al., 2023; Resurreccion and Fong., 2022). Additionally, metabolomics profiles capture the real-time metabolic status of a biological system or encompass a range of chemical entities, including small molecules, lipids, and intermediates of various metabolic pathways, as compared to genomic, transcriptomic, or proteomic profiles, which provide static snapshots (e.g., DNA, RNA, and proteins) (Onuh and Qiu., 2021). At last, metabolites reflect downstream effects of genetic and environmental influence, offering a direct link to observable traits such as disease manifestation, drug response, or physiological changes. This connection facilitates the identification of biomarkers for disease diagnosis, prognosis, and therapeutic monitoring (Resurreccion and Fong, 2022).

The detection of these metabolites in any type of sample has been through 1H-nuclear magnetic resonance spectroscopy or mass spectrometry, and recently they have been separated using a variety of techniques, including LC-MS and GC-MS (Moschino et al., 2022). Comparing with recently used methods for separating metabolites, NMR spectroscopy had a relatively lower sensitivity, typically requiring larger sample volumes, and was inherently limited to the detection of nuclei with nonzero nuclear spin (Ghini et al., 2023). Conversely, metagenomics enables the investigation of genetic material obtained directly from environmental samples, offering insights into the genetic diversity and functional capabilities of entire microbial communities (Zhong et al., 2021). Numerous studies have employed advanced techniques to characterize the genetic diversity and functional potential of microbial communities directly from environmental samples, thereby identifying biomarkers associated with adverse pregnancy outcomes (Lozupone et al., 2012; Koester et al., 2021).

Furthermore, the commonly utilized high-throughput sequencing technologies include next-generation sequencing (NGS), 16S rRNA gene sequencing, shotgun metagenomic sequencing, meta transcriptomics, meta proteomics, single-cell genomics, and functional metagenomics (Chen et al., 2021; Zhang et al., 2021; Liao et al., 2023). These methods can be used alone or in combination to explore different aspects of microbial communities, including taxonomic composition, functional potential, gene expression, protein activities, and metabolic interactions (Abram., 2015; Aguiar-Pulido et al., 2016). Through this integrative analysis, numerous studies have successfully identified distinctive biomarkers within umbilical cord serum, stools, vaginal fluid, and urine (easily collected and feasible to explore samples), offering valuable insights for diagnosing various pregnancy complications (Koh et al., 2022; Ponzi et al., 2021).

Remarkably, our systematic review comprehensively summarized studies that applied metagenomics and metabolomics to identify unique biomarkers for various APBOs, wherein we observed a wide variability in the eligible population in terms of number of participants, type of APBOs examined, and the type of samples analyzed. Notably, these APBOs were measured in accordance with the recommended standards by WHO. In consistent to previous systematic reviews, we observed 16S rRNA gene sequencing and LC-MS-based techniques were the frequently utilized metagenomics and metabolomics approaches for profiling changes in APBOs and normal pregnancies outcomes (Safari-Alighiarloo et al., 2023; Peelen et al., 2023; Carter et al., 2019; Huynh et al., 2014).

The reason that may be attributed to the frequent use of these approaches may be that 16S rRNA gene sequencing is generally more cost-effective compared to whole-genome shotgun sequencing, particularly for large-scale studies involving numerous samples. In addition, the reason why LC-MS-based techniques may be due to their higher sensitivity, and their ability to detect and quantify more than 5000 metabolites, even at low concentrations as compared to NMR spectroscopy (Emwas et a., 2013). Furthermore, our review revealed stool or fecal samples as the most commonly utilized non-invasive biological samples for both metabolomics and metagenomics analysis. The reason for this is indicated in a systematic review by Moschino et al., (2022) which demonstrated that fecal samples may better reflect the host-microbiota interaction. However, it is important to note that some APBOs including extremely preterm infants (particularly in the initial days of life) and miscarriages, may not consistently produce stool, (Erben et al., 2021). Thus, while stool samples remain valuable for many studies, researchers must consider the specific characteristics and needs of their study population when selecting sample types and collection protocols.

It is important to note that the majority of the included studies demonstrated low risk of publication bias as assessed using Newcastle-Ottawa Scale (NOS). The minimal risk of publication bias indicates that the studies that were part of the evaluation were probably carried out and reported in an impartial and rigorous way, which improves the validity and reliability of the conclusions that the systematic review synthesis.

Although the specific molecular mechanisms driving alterations in the microbiome during pregnancy remain poorly understood, emerging evidence suggest that these alterations may arise as normal consequences of physiological reactions during pregnancy or represent an independent manifestation with its own role, an area ongoing research (Farhat et al., 2022). In our review, we also summurized focused on the dysiobis in mocrobiome in APBOs cases as compared to normal pregnancy groups.

Hence, most of the included studies in our systematic review demonstrated that dysbiosis microbiomes which significantly associated with the increased risk of APBOs compared to normal pregnancies controls. Thus, several studies suggested a predominance of phyla *Firmicutes, Actinobacteriota, Bacteroidetes* or *Bacteroidota, Proteobacteria, Spirochaetae, Fibrobacteres*, and *Tenericutes* was associated with elevated risk of PT, PE, GDM, and miscarriage (Liu et al., 2021a; Hill et al., 2017; Benítez-Guerrero et al., 2022). In contrast, reduced abundance of *Proteobacteria, Bacteroidetes* or *Bacteroidota*, and *Actinobacteriota* was observed in cases of sPTB, GDM, PE (Gershuni et al., 2021; Chen et al., 2021; Benítez-Guerrero et al., 2022), and (Benítez-Guerrero et al., 2022). These findings suggest that some of changes in human microbiome may occur during pregnancy and after birth (Nuriel-Ohayon et al., 2016), further implying that specific phylum is associated not with just one, but several negative pregnancy outcomes.

Consistent with the findings from systematic reviews by Dualib et al., (2021), Colonetti et al., (2023) and Zimmermann et al., (2019), our review observed dynamic alteration in the abundance of species and families in several cases of APBOs compared to normal pregnancy outcomes. In case of increased abundance, most *Lactobacillus species* were associated with elevated risk of PTB (Kindschuh et al., 2022; Pruski et al., 2021; Gerson et al., 2021). In contrast, few studies indicated decline in the relative abundance of Lactobacillus species (Flaviani et al., 2021; Gerson et al., 2021). Furthermore, reduced relative abundance of *Eubacterium siraeum, Bacteroides coprophilus, Prevotella copri*, *Faecalibacterium prausnitzii,* and *Prevotella stercorea* was noted in cases of GDM (Ye et al., 2023). Interestingly, different microbial families including *Ruminococcaceae, Enterobacteriaceae, Lachnospiraceae, Veillonellacea*, and *Streptococcaceae* also exhibited a dynamic alteration (Tao et al., 2023; Wang et al., 2020; Dougherty et al., 2020; Wandro et al., 2018; Dougherty et al., 2020; Chen et al. 2021).

In general, factors such as changes in dietary habits, metabolisms, antibiotic use during pregnancy may contributed to alterations in the abundance of microbiome observed in our review (Kunasegaran et al., 2023). Hence, Firmicutes and Bacteroidetes are known respectively for their role in the production of butyrate and acetate or/and propionate involved in short-chain-fatty acids (SCFAs) formulation (Farhat et al., 2022). This highlights the need for integrative metabolome and microbiome analysis to understand the intricate host-microbe crosstalk. Therefore, our review further summarizes metabolomic profiling in APBOs from similar studies as well as explores the integration the metabolome and microbiome in various cases of APBOs (discussed below).

Despite the disagreement with previous systematic reviews on metabolomics profiling in APBOs, our systematic review has identified some reassuring patterns emerging from several metabolites. Thus, among 104 perturbated metabolites retrieved, potential signatures as determined through pathway enrichment could include decreased level of Starch and sucrose metabolism, Citrate cycle (TCA cycle), Pyruvate metabolism and Phenylalanine, tyrosine and tryptophan biosynthesis. Additionally, we obseverd enhanced metabolites related to Neomycin, kanamycin and gentamicin biosynthesis and Pantothenate and CoA biosynthesis (FDR<0.05). These findings corroborate with other reviews suggesting that the metabolome hold promise as a tool for the predicting the incidence and prognosis of APBOs (Carter et al., 2019). However, the heterogeneity among the included studies, sample size was highlighted as issue associated with the discovery of uniform patterns of potential biomarkers.

The purpose of this review was to summarize studies on determine the associations between dynamic alteration microbiome and metabolomes in APBOs. Recently Short-chain fatty acids including acetate, propionates and butyrate has gained interest compounds produced by gut microbiota, particularly *Firmicutes* and *Bacteroidete*s that play an important role in health and disease prevention (Parada Venegas et al., 2019). In this review we retrieved only was regulated in APBOs, however we did not observe the associations between these compounds with any bacteria species. Interestingly, we did find associations between regulation of certain metabolites with the abundance of *Bacteroidetes, Protevella, Enterococcus* and *Acinetobacter, Ruminococcaceae, Lachnospiracaceae*, and *Lactobacillus.* This underscores the significant to research of this specific association as way to determined novel predictive indictors of APBOs.

2.9. STRENGTH AND LIMITATION OF THIS SYSTEMATIC REVIEW

The strength of this review is that all included were assessed for publications bias using Newcastle-Ottawa Scale (NOS) and most studies indicated low risk of bias. All adverse pregnancy and birth outcomes (cases) in the included studies were measured as per recommended standard of WHO, enhancing the credibility of the findings. However, several limitations are noted from this review. Firstly, human microbiomes and metabolome are prone to several factors in genetical, social and environmental factors that pregnancy women exposed to; therefore, it is difficult to identify a uniform pattern of microbiome and metabolome. Secondly, the absence of accurate and detailed metadata analysis, stemming from the lack of precise quantitative parameters for confounding factors, may impact the interpretation and validity of the findings. These parameters are frequently missing from both the methods and results sections of the studies. Thirdly, some of the included studies may have suffered from limited sample sizes, which may restrict the generalizability of the metabolomics and microbiome profiles to the broader study population. Small sample sizes can undermine the statistical power and reliability of the findings. Finally, the utilization of different methodologies for profiling metabolomes and microbiomes across the included studies introduces variability and potential biases. Each profiling method carries its own limitations, further complicating the synthesis and interpretation of results.

2.10. CONCLUSION

Based on the findings of the systematic review, it appears that there is evidence supporting the idea that the complex interaction between hosts and microbes (host-microbe crosstalk) may contribute to understanding the molecular mechanisms underlying the development of various adverse pregnancy and birth outcomes. The integration of metagenomics and metabolomics approaches in the analysis seems to hold promise for identifying and discovering novel biomarkers associated with these outcomes.

CHAPTER THREE: RESEARCH METHODOLOGY

3.1. STUDY DESIGN AND SETTING

A cross-sectional study design with purposive sampling was conducted from pregnant mothers recruited in selected health facilities in the Vhembe districts of the Limpopo province, South Africa. Limpopo is the northernmost province of South Africa, having international borders with Zimbabwe, Mozambique, and Botswana, making it the ideal entrance to Africa. The region comprises 123,910 square kilometres, making up 10.2% of the entire land area of the country (Bradshaw et al., 2005). The province is comprised of five district municipalities: Vhembe, Mopani, Sekhukhune, Waterberg, and Capricorn which are further divided into 25 local municipalities. Most of the population uses local public health services that are provided free of charge. The estimated population of this province is 6, 572,721 with the annual population change of 1.9% (Census, 2022). As of 2020, the population of the Vhembe district is 1,402,779, with 54% being females and 46% males. The district is comprised of seven public hospitals, and the monthly recorded number of deliveries in the Vhembe district is 1,921 (Makhado et al., 2022; Vhembe District Municipality, 2020).

3.2. ELIGIBITY CRITERIA

The study included all pregnant women who were at the latent stage of labor at the selective district health care facilities of Vhembe district including Elim, Donald Fraser, Malamulele, and Siloam Hospitals (this hospitals were selected based on their geographical representation and mortality rates as per DHIS). Only participants who were at the right state of mind to understand the research purpose and benefits of the study were considered. The study included both women who delivered either through normal vaginal delivery (NVD) and Caesarean section. However, it is worth noting that the enrolment of the participants did not influence standard of care concerning site management of labor and delivery. Female participants under the age of 18 were included in the study only if their parents or guardians provided consent, and the adolescent herself provided assent. All pregnant women who chose not to participate and females under the age of 18 without accompanying parents or legal guardians were excluded from the study.

3.3. ETHICAL CLEARANCE AND ETHICAL CONSIDERATIONS

The study adhered to chapter 3, section 3.3 of the National Department of Health Research Guidelines of 2015. This commitment ensures the fair and respectful treatment of South Africa's people by researchers. The continuous updating and strengthening of South Africa's research ethics systems and infrastructure aim to uphold ethical standards in all research conducted within the country. This contributes to the overarching project of ensuring that research is conducted in alignment with the highest ethical norms and standards. The ethical clearance to conduct this study was obtained from the Animal, Environment and Biosafety Research Ethics Committee (AEBREC) at the University of Venda, with the ethical clearance number: FSEA/22/BMY/02/1807 (Annexure 1). The permission to access the health care facilities for sample collection (umbilical cord blood) and to interact with the participants was granted by the Department of Health of Limpopo Province (LP_202211_012), the Department of Health in Vhembe district (S5/4/2/3), as well as the relevant hospital managements, each granted their respectively approvals (see annexures below).

3.4. INFORMED CONSENT

Prior to sample collection, participants were provided with an informed consent form which they are expected to sign. Permission to interview the participants was obtained from all key participants. Sufficient information regarding the study was provided in the informed consent letter. Furthermore, researcher also explained the aim and objectives of the study, as well as the participant’s rights (explained that they are not compelled to participate, and they would not be judged if they chose not to participate) before they gave their consent. Given that the Vhembe district in Limpopo is multi-racial region, language barriers were addressed by translating consent forms into English, Sepedi, Tshivenda, and Xitsonga.

Additionally, to improve data accuracy, interviews were conducted with participants, regardless of literacy level, in their respective preferred languages (Annexure 2).

3.5. PARTICIPANTS PRIVACY, ANONYMITY, AND CONFIDENTIALITY

Throughout the duration of this study, strict adherence to the provisions outlined in the Protection of Personal Information Act 4 of 2013 was paramount. This legislative framework was rigorously observed to safeguard participants against any form of unauthorized collection, retention, dissemination, or utilization of personal information. Fundamental to this endeavour was the protection of participants' rights to privacy and anonymity, ensuring that their personal information remained confidential throughout the study process. To uphold these principles, both the questionnaire and consent forms were treated with utmost confidentiality. Each participant was assigned with a unique code that was to be used on the questionnaires and the samples.

Interview questionnaires were completed, and data was collected and stored using an online platform called REDCap hosted at the SAMRC (Annexure 3). The platform employs an encrypted database stored locally on each mobile device, where all user data is securely housed. Each mobile user's data is isolated within their own designated area, ensuring that only the respective individual can access it. Data transmission occurs through a secure SSL connection to the original REDCap server where the project originated. Users have the flexibility to select and send modified data, if any, to the server. Notably, the server remains unaware of the status of mobile values and records until data transmission takes place. The platform enables seamless offline data collection, ensuring privacy for each mobile user. While data can be transmitted to the REDCap server, it remains exclusive to the individual user and cannot be shared among multiple REDCap Mobile App users. Data collected on REDCap online platform can be stored for 15 years, for consultation and data analysis.

3.6. PARTICIPANT ENROLMENT TO THE STUDY

The enrolment of participants and sample collection took place from May 2023 to September 2023. Initially the study recruited 159 pregnant women in four selective health care facilities, about 15 refused to be part of study, 7 adolescents expressed their willingness to participate in the study, unfortunately, they were subsequently excluded from participation due to the absence of consent from their parents or guardians, and 2 females were identified as experiencing mental health issues. Lastly, 6 participants gave consent, however, due to complications experienced during labor they ended up being transferred to provincial hospital before sample collection. With this framework in place, a total of 129 participants provided consent to participate in the study. They underwent interviews aimed at gathering information on various socio-demographic characteristics, as well as current and past morbidity, including details on their intake of prescribed and over-the-counter medications and dietary supplements both before and during pregnancy. Additionally, medical records from the delivery period were reviewed to corroborate certain details obtained from the self-administered questionnaire. These records also provided additional maternal peri-partum information, such as blood pressure and body temperature, as well as details on the offspring (Figure 3.1). The participants were classified into the following categories: (1) Normal or Healthy pregnancy outcomes, Caesarean section delivery, and adverse pregnancy and birth outcome (APBO). Furthermore, C-section groups was further subdivided into two categories based on the reasons that led to C-section: Labor and obstetric complications and previous C-section(s) (Figure 3.1).

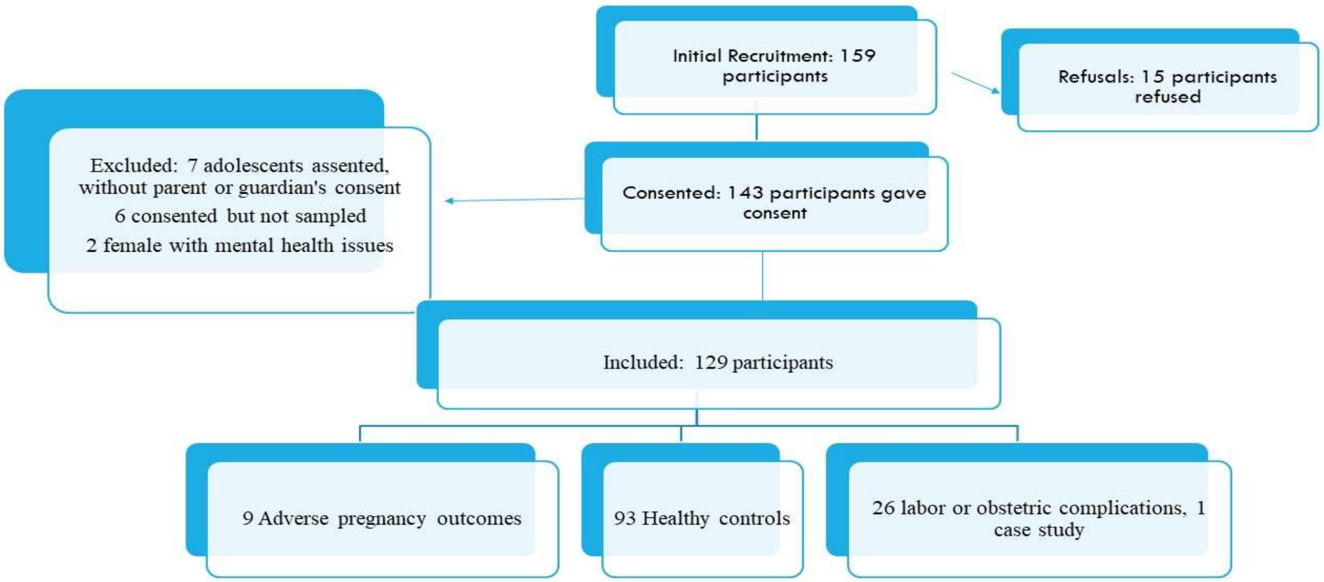


Figure 3.1: Schematic representation of a flow diagram of the participant's recruitment to the study.

3.6.1. Definition of terms

Adverse pregnancy and birth outcomes (APBOs): described as unfavourable and possibly harmful events or circumstances that arise during pregnancy or childbirth and have a detrimental impact on the health and well-being of both the mother and the newborn (Tadese et al., 2022). These outcomes may include complications such as preterm birth (PTB), low birth weight (LBW), maternal infections, gestational diabetes (GDM), and pre-eclampsia (PE). The present study therefore sampled from following APBOs cases: 1 GDM, 1 PTB, 2 LB-PTB, 1 LBW, 1 miscarriage, 2 stillbirth/Macerated, and 1 Large for gestational age (LGA). Preterm birth was defined as delivery of a baby before 37 weeks of pregnancy.

Labor and obstetric complications: refer to difficulties and challenges that arise during childbirth. "Labor" is the physiological process of contractions within the uterus leading to the fetus and placenta being expelled, whereas "obstetric complications" refers to a variety of problems and challenges that can occur throughout pregnancy, labor, and delivery (Hoque, M., 2011). Prolonged labor, fetal distress, breech presentation, problems with the umbilical cord, and placenta abnormalities are merely some of the complications that may occur. In this study we collected umbilical cord blood from participants who had the following obstetric and labor complications: Fetal distress, Poor movement, meconium stain/ aspiration, Cephalon pelvic disproportion (CPD), Oblique lie, Posterior presentation, Failed induction of labor (IOL), Delay first stage of labor, and slow progression, Twin pregnancy, and big for abdomen.

3.6.2. Maternal pre-pregnancy BMI and Blood pressure

Data were retrieved from the medical birth records of the participants. Pre-pregnancy Body Mass Index (BMI) was calculated using height and weight verified at the initial visit to the antenatal care clinic, employing the formula: BMI = weight (kg) / [height (m)] ^2 (Misra and Dhurandhar, 2019). Maternal antenatal blood pressures at birth were also collected from the medical birth register and categorized according to WHO guidelines as follows: normal blood pressure (120/< 80), elevated blood pressure (120-129/< 80), hypertension stage 1 (130-139/80-89), hypertension stage 2 (140 or >/ >90), and hypertensive crisis (>180/ >120).

3.6.3. Covariates

Covariates derived from the medical health record included maternal age, parity status, neonatal birth weight, newborn's sex, anesthesia, maternal blood pressure, delivery mode, as well as the reason for C-section. Maternal educational status, household income, employment status, and newborn's position in the family were self-reported by the participants during the interview. Furthermore, maternal substance use (smoking and alcohol consumption) before and during pregnancy was also self-reported.

3.7. SAMPLE COLLECTION PROCEDURE

Within the first 5 minutes of delivery and before the delivery of the placenta, a professional midwife (Nurse assistant) carefully aspirated umbilical cord blood either from the umbilical vein or through free draining using a 10mm needle. The umbilical cord blood (approximately 6ml) was collected into BD vacutainer SSTII advanced tubes and appropriately labelled according using unique code assigned to that particular participant at the selected health facilities. Immediately after sampling, the samples were carefully kept in the cooler box with ice and transported to the University of Venda where they were processed. Thus, some portion of the sample was used for blood culture and the remaining sample was centrifuged at 1400g for 10 minutes to separate plasma and buffy coat. Both serum and buffy coat were stored in -70°C prior to metabolomic and microbial assays.

3.8. UMBLICAL CORD BLOOD BACTERIAL ANALYSIS

3.8.1. Blood culture, bacterial identification and antibiotic susceptibility testing

Upon arrival in the Lab, 200µl of fresh blood were inoculated in Brain Heart Infusion (BHI) broth prepared following manufactures instructions and incubated in aerobic conditions at 37°C for 24 hours. After 24 hours, the inoculum was then sub-cultured in Nutrient Agar (to observe colony morphology and isolate pure cultures) and different selective media including Eosin Methylene Blue (EMB) agar (for isolating Gram-positive bacteria, lactose fermenters, and non-fermenters), MacConkey agar (to isolate Gram-positive bacteria, lactose fermenters, and non-fermenters), and Mannitol salt agar (MSA) (o isolate and differentiate *Staphylococcus* species based on their ability to ferment mannitol) and incubated at 37°C for 24 hours. All these media were prepared considering the manufacturer's protocol. After 24 hours, the culture results were observed for the identification of possible microbial growth. The culture was reported sterile if there was no growth observed with the period of 5 days. For mixed colonies, single bacterial colony was picked using the sterile loop and sub-culture was performed on the media where the growth was observed. Bacterial identification was achieved through morphological characteristics observation.

Antibiotic susceptibility testing was performed using the Kirby-Bauer disc diffusion technique on Muller Hinton agar (MHA) (Condalab, South Africa), following the recommendations of the Clinical and Laboratory Standard Institute (CLSI) guidelines (CLSI, 2023). In brief, 3-5 colonies of the test microorganism were inoculated into a tube containing 3 ml of Muller Hinton broth (MHB) (Condalab, South Africa) and gently shaken until the suspension became turbid. The tube was then incubated for 2–8 hours until visible growth was observed. The suspension was adjusted to meet the 0.5 McFarland standards. Sterile MHB was used to standardize bacterial count estimations against the 0.5 McFarland standards in the Kirby-Bauer disc diffusion method. The bacterial inoculum adjusted to McFarland standards was uniformly spread on MHA plates using a sterile cotton swab. The following antimicrobial agents were employed: Gentamicin (10 µg), Ampicillin (10 µg), Erythromycin (15 µg), Cefoxitin (30 µg), Ceftazidime (30 µg), Ciprofloxacin (5 µg), Imipenem (10 µg), and Amikacin (30 µg) (Oxaid Ltd). According to CLSI guidelines, the diameter of growth inhibition (zone of inhibition) around the antibiotic disk was measured and interpreted as susceptible (Zone of inhibition ≥ 20 mm), intermediate (Zone of inhibition = 15-19 mm), or resistant (Zone of inhibition ≤ 14 mm). Multiple drug resistance (MDR) was defined as a bacterial strain that was resistant to at least two or more antibiotics (Eshetu et al., 2020).

3.8.2. Genomic DNA isolation

Prior 16S rRNA PCR amplification, genomic DNA was extracted from the whole blood using NucleoSpin kit considering the manufacturer's protocol. Briefly, 25µl of Proteinase K and 200µl equilibrated to room temperature were added into 1.5ml microcentrifuge tubes. Lysis buffer B3 was added to the sample and vortexed for 10-20 seconds and then incubated at 70°C for 10 minutes. 210µl of 99.99% ethanol was to each sample and vortexed again, to adjust DNA binding conditions. To bind DNA, each sample was loaded on NucleSpin Blood column and centrifuged for 1 minutes at 11 000×g. For first wash, 500ul of Buffer BW was added into NucleSpin Blood column and centrifuged for 1 minute at 11 000×g. The second wash was perfomed by adding 600ul of wash Buffer B5 into NucleSpin Blood column and centrifuged for 1 minute at 11 000×g. After discarding of the flow-through, NucleSpin Blood column was placed back into the 2ml collection tubes and centrifuged for 1 minute at 11 000×g thus, to dry silica membrane. Finally, DNA was eluted from the NucleSpin Blood column and adding 100µl preheated (70°C) Buffer BE and centrifuged for 1 minute at 11 000×g. The DNA was quantified using Nanodrop and the concentration of the DNA ranged from 17.5 to 135 ng/ul.

3.8.3. 16S rRNA PCR Amplification

For the detection of microbiome present in the blood, the extracted genomic DNA was subjected to conventional-PCR based on the amplification 16S rRNA gene using universal primers 27F and 1492R (table 3.1). The amplification mixture consisted of (25µl) of 12.5µl one Taq 2X master mix, 0.5µl of 5pmole of primer 27F, 0.5µl of 10pmole primer 1492R, 6,25µl nuclease free water, 5µl genomic DNA, and 0.25µl BSA buffer. The amplification was carried out on Bio-Rad T100 thermal cycler with the following cycling conditions: initial denaturation at 94°C for 30 seconds, denaturation at 94°C for 15 seconds, annealing at 57°C for 30 seconds, extension at 68°C for 1 minutes, and final extension at 68°C for 5 minutes. The amplified fragments were visualized on 1% agarose gel stained with Ethidium bromide under ultraviolet light using Omega Flour Gel Documentation system, Vacutec.

Table 3.1: Table showing universal 16S rRNA primers used for the detection of blood microbial DNA.

Gene	Primers sequence	Target	Reference
16S rRNA Forward	27F: 5′-AGAGTTTGATCCTGGCTCAG-3′	1500-bp	Salamandane et al., 2022
16S rRNA Reverse	1492R: 5-CGGTTACCTTGTTACGACTT-3		

3.9. UMBILICAL CORD SERUM METABOLOMIC PROFILING

3.9.1. Metabolite Extraction

All serum samples were maintained at 4°C throughout the analytical process. Protein precipitation from the serum was achieved using a cold organic solvent, acetonitrile (AcN). Specifically, an aliquot of 100 µl of serum/plasma was combined with 300 µl of cold acetonitrile (3:1, v:v), followed by thorough vortexing for 60 seconds to precipitate proteins. The precipitated proteins were subsequently removed by centrifugation at 13,000×g for 5 minutes, and the resulting supernatant was collected. After filtration using a 0.22 µm nylon filter (Stargate Scientific, South Africa), the supernatant was transferred into sterile HPLC vials and stored at 4°C until metabolomic analysis on LC-q-TOF-MS. LC-MS grade acetonitrile and water were purchased at Sigma-Aldrich (Johannesburg, South Africa).

3.9.2. Liquid Chromatography Mass Spectrometry Analysis

The prepared samples were analyzed on a Liquid Chromatography-Quadrupole-Time-of-flight Mass Spectrometry (LC-q-TOF-MS) instrument. A Shim-pack Scepter C18 column (100 × 2.1 mm, 1.9 µm) (Shimadzu Corporation in Kyoto, Japan), was utilized for the chromatographic separation of 5 µl of the sample. The column was consistently maintained at 55 °C, and the separation process involved a binary mobile phase gradient elution with a flow rate of 0.4 mLmin^{-1}. Mobile phase A consisted of 0.1% (v/v) formic acid in ultrahigh purity water, while mobile phase B was composed of methanol. A 53-minute gradient was utilized under the following separation conditions: maintaining 10% of mobile phase B for the initial 3 minutes, gradually increasing from 10% to 60% B between 3 and 40 minutes, holding at 60% B from 40 to 43 minutes, transitioning to 90% B from 43 to 45 minutes, and maintaining 90% B for the subsequent 3 minutes. The gradient was then reverted to the initial conditions between 48 and 50 minutes, followed by a 3-minute column equilibration time. Mass spectral analysis was carried out using a q-TOF high-resolution mass spectrometer equipped with an electrospray interface (ESI), operating in positive ionization mode.

The MS instrument parameters were set as follows: an interface voltage of 4.0 kV, an interface temperature of 300 °C, nebulization and dry gas flow at 3 L/min, a heat block temperature of 400 °C, a desolvation line (DL) temperature of 280 °C, a detector voltage of 1.8 kV, and a flight tube temperature of 42 °C. Sodium iodide (NaI) served as the calibration solution to ensure high mass accuracy, containing NaI clusters with high masses to facilitate calibration of higher m/z. Both MS1 and MS2 (data-dependent acquisition, DDA) were simultaneously collected for all ions within an m/z range of 100 to 1000 Da, surpassing an intensity threshold of 5000 counts. Fragmentation experiments were conducted using argon as a collision gas at a collision energy of 30 eV with a spread of 5 eV. Additionally, sample acquisition was randomized, and a quality control (QC) sample was analyzed after every 10 injections to monitor and correct any changes in the instrument response.

3.9.3. Data Processing and Multivariate Data Analysis

The data obtained from the Shimadzu LCMS 9030 were first converted into an open-source mzML file. Subsequently, various automated procedures such as peak identification, alignment, peak exploration, filtering out low-quality features, data editing, filling in missing values, integration, and data normalization were performed using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/), which is implemented with the freely available R statistical language (Xia et al., 20211). Spectra processing using MetaboAnalyst 6.0 was restricted to the following parameters peak peaking algorithms centWave-auto method, peak alignment minFraction 0.8, positive ionization with elimination of missing data (contaminant removal (Ramabulana et al., 2021). A list of ion intensities for every detected peak was generated, utilizing retention time (RT) and m/z data pairs as distinct identifiers for each ion.

The processed matrices were exported in Excel format, formatted to be compatible with MetaboAnalyst. Subsequently, they were uploaded to MetaboAnalyst 6.0 for analysis and remodeling, aiming to delve deeper into the data structures and uncover hidden patterns (Mashabela et al., 2022). Within MetaAnalyst 6.0, we applied various statistical techniques, including missing value estimation, log transformation, and Pareto scaling. Pareto scaling was employed to mitigate the disproportionate impact of high values originating from abundant metabolites while partially preserving the data structure. Furthermore, Two-sample T-test and Wilcoxon rank sun test, a nonparametric univariate method, was executed to quantify and identify metabolites that exhibited significant changes between adverse pregnancy and birth outcomes (APBOs) or labor and obstetric complications, and healthy controls. The results were subsequently corrected using a false discovery rate (FDR) at $P<0.05$ to ensure the reproducible detection of metabolite peaks (Luan et al., 2013). The results from nonparametric univariate (up or down regulated metabolites) were represented on the Volcano plot.

Multivariate statistical analyses (MVA), such as PCA and OPLS-DA, were conducted to distinguish APBOs and labor complication samples from control subjects on MetaboAnalyst (Feng et al., 2016). Principal component analysis (PCA), a technique in unsupervised Multivariate Data Analysis (MVDA), was utilized to condense the data's dimensionality and effectively capture the essence of the information contained in the datasets. These yields "compact indices" that are easily visualized and analyzed. In contrast, Orthogonal-partial least square discriminant analysis (OPLS-DA), a supervised MVDA method, was utilized to extract maximum information from the datasets, specifically focusing on significant variables. PLS-DA facilitates the elimination of systematic variation from the experimental data (X variables) that is not correlated with discriminant classes (Y). The Hotelling's region, depicted by the ellipse, defines the 95% confidence interval (Mhlongo et al., 2020). The effectiveness of OPLS-DA models was assessed by examining R^2 for goodness of fitness and Q^2 for predictive capability (Das et al., 2022). Additionally, data deconvolution was explored through statistical analysis and the generation of quantitative heatmaps to visually represent the relatively perturbed and imbalanced metabolic states observed between APBOs, Labor complications samples, and control subjects (Mashabela et al., 2022; Feng et al., 2016).

3.9.4. Metabolite Annotation

For the identification of metabolites (potential biomarkers) detected by LC-q-TOF-MS spectrometry, comparison was made with compounds stored in databases such as KEGG (https://genome.jp/kegg), PubChem, CheMBI (https://www.ebi.ac.uk/chebi/), and the Human Metabolome Database (https://hmdb.ca/) through MetaboAnalyst 6.0. Annotation of metabolites of particular interest was confirmed by comparing their retention times, accurate mass spectra, and high-resolution MS/MS information to those of authentic chemical standards. This process adheres to the Metabolomic Standards Initiative (MSI) metabolite identification (MI) level 2 criteria (Chitiva et al., 2023; Van Der Hooft et al., 2016).

3.9.5. Pathway Analysis of Serum Metabolites

The potential biomarkers (Metabolites) for APBOs identified from KEGG and HMDB databases were subjected to computational platform MetaboAnalyst 6.0 for metabolic pathway enrichment and topology analysis, thus, to better understand the functional impact of several APBOs or labor and obstetric complications on human metabolic processes. Topology and enrichment analyses were both performed in this analysis. While the topological analysis evaluated the effect factors, the enrichment analysis investigated the overrepresentation of metabolites within pathways. To select metabolic pathways that were significantly associated with several pregnancy and labor complications, screening parameters of $p < 0.05$ or an impact factor greater than 0.2 were utilized.

3.10. STATISTICAL ANALYSES OF ALL DATA

Prior to statistical analysis, the participants were classed into groups of perinatal complications (APBOs + labor/ obstetric complications) and normal pregnancy outcomes. Data management and coding was conducted on REDcap and exported to IBM SPSS (Statistical Package for the Social Sciences) software, version 26 for analysis. The characteristics of the population and potential confounding factors were described using descriptive summary statistics. Pearson Chi-Square tests were performed to evaluate the association between the umbilical cord blood microbial infections with clinical characteristic of participants. Maternal pre-pregnancy BMI, age and newborn's sex were considered when adjusting the measured levels blood microbial infections and antimicrobial resistance patterns. *P*-values of 0.05 were considered statistically significant at as 95% confidence interval. The research findings were presented by using graphs, tables, as well as figures based on the type of data.

CHATPER FOUR: RESEARCH FINDINGS

4.1. DEMOGRAPHIC, CLINICAL, AND LIFESTYLE CHARACTERISTICS OF PARTICIPANTS

In this study, we recruited a total of 129 participants, comprising 9/129 (6.98%) APBOs cases, 26/129 (20.15%) incidences of labor and obstetric complications, and 93/129 (72.09%) participants with normal pregnancy outcomes. Among the APBOs, the cases encompassed 1 instance of Gestational Diabetes Mellitus (GDM), 1 Preterm Birth (PTB), 2 incidences of Late Preterm Birth (LB-PTB), 1 Low Birth Weight (LBW), 1 miscarriage, 2 cases of stillbirth/macerated, and 1 case of Large for Gestational Age (LGA). Labor and obstetric complications included various types, such as 7 cases of prolonged labor (Failed induction of labor (IOL), 1 case of Delay in the first stage of labor, 2 cases of foetal distress, 1 case of Poor movement, 4 cases of meconium stain/aspiration. For foetal positioning issues there were 1 case of Cephalopelvic disproportion (CPD), 1 case of Oblique lie, and 1 case of Posterior Presentation. There were 4 cases of twin pregnancies and 4 cases of being big for abdomen. Neonatal medical information collected was 74/129 (57.36%) males and 55/129 (42.64%) females.

During the course of the study, we encountered 1/129 (0.78%) participant with complications related to social factors, which we treated as a case study. The participant was involved in either a fight, an accident, or an incident of gender-based violence. Unfortunately, the participants could not provide any information on the cause of these complications. The participant presented with multiple complications that necessitated a delivery via caesarean section, including bowel injury, bladder injury, haemorrhage, infection, and the need for a total abdominal hysterectomy. The normal pregnancy outcomes were defined by pregnancies without complications, leading to normal vaginal deliveries or C-sections due to previous Caesarean section. These normal births typically resulted in newborns weighing between 2,500g to 4,000g, with estimated fetal weights (EFW) falling within the 10th to 90th percentile range.

The descriptive demographic and perinatal characteristics of the included participants are presented in terms of frequency, as outlined in Table 4.1. Among the participants, 55/129 (42.6%) were aged between 20 to 29 years, and 7.8% (n = 10) were aged 40 and above. Many of the mothers were single 73/129 (56.6%), while few participants reported being married as second wives 5/129 (3.9%). Additionally, 108/129 (83.7%) were unemployed, 89/129 (69%) depended on social grants as their sole source of monthly income, and 90/129 (69.8%) had attended high school, with a minority of 2/129 (1.6%) reporting as illiterate. Furthermore, the descriptive analyses of the clinical features revealed a wide variety in the gravidity of the participants. Among them, 44/129 (34.1%) were nulliparous, 35/129 (27.1%) were primiparous, 21/129 (18.6%) were multiparous (third gravid), 17/129 (13.2%) were multiparous (fourth gravid), and 9/129 (7%) were grand multiparous.

Regarding the gestational booking age, most pregnant women 62/129 (48.1%) booked during the first trimester, while 27/129 (20.9%) booked during the third trimester. Additionally, 21/129 (16.3%) of the participants were HIV positive (Table 4.1). On the other hand, regarding their pre-pregnancy BMI, 2/129 (1.6%) were underweight, 27/129 (20.9%) had normal weight, 34/129 (26.4%) were overweight, and 27/129 (20.9%) were classified as obese. Additionally, 105/129 (81.4%) participants exhibited normal blood pressure, while 4/129 (3.1%) presented with hypertensive crisis. Most participants 70/129 (54.3%) delivered through natural unassisted childbirth, followed by C-section childbirth, accounting for 51/129 (39.5%). Approximately, 8/129 participants (6.2%) delivered through natural assisted methods. Finally, more than half of participants 67/129 (51.9%) in our study had late antennal care (ANC) attendance, thus, 40/129 (31%) and 27/129 (20.9%) participants booked for ANC during the second and third trimesters, respectively.

As illustrated in Table 4.2, the univariate association analyses were conducted to assess the correlations between perinatal complications and risk factors. Demographic and clinical factors, including maternal age, birth weight, maternal blood pressure, anesthesia use, and delivery mode, were found to be significantly associated with perinatal complications, with the following *P*-values: 0.032, 0.027, 0.041, <0.001, and <0.001, respectively. As expected, experiencing complications during labor and delivery was significantly correlated with perinatal complications, with a *P*-value of 0.011. Factors including marital status, parity status, employment status, income status, educational status, HIV status, BMI class, baby sex, smoking tobacco during pregnancy, as well as alcohol consumption were not significantly associated with perinatal complications.

Table 4.1: Socio-demographic and clinical characteristics of the study participants in selective health care facilities in the Vhembe district, May 2023-September 2023.

Variables	Number	Percentage
Age groups		
10 to 19 years	17	13,2
20 to 29 years	55	42,6
30 to 39 years	47	36,4
40 and above	10	7,8
Marital status		
Married first wife	21	16,3
Married second wife	5	3,9
Single	73	56,6
Living with a partner	30	23,3
Number of Childrens		
Nullipara	44	34,1
Primipara	35	27,1
Multipara (3)	24	18,6
Multipara (4)	17	13,2
Grand Multipara	9	7
Employment status		
Unemployment	108	83,7
Self-employment	11	8,5
Private employment	6	4,7
Government employment	4	3,1
Income status		
SASSA grant	89	69
less than R3000	16	12,4
R3000-5000	10	7,8
R5100-10 000	1	0,8
R10 100- 15 000	1	0,8
Missing	12	9,3
Educational status		
Illiterate	2	1,6
Primary school	11	8,5
High school	90	69,8
Tertiary school	24	18,6
Postgraduate	1	0,8
Missing	1	0,8
HIV status		
Positive	21	16,3
Negative	108	83,7
Body Mass Index class		
Underweight	2	1,6
Normal weight	27	20,9
Overweight	34	26,4
Obese	27	20,9
Missing	39	30,2
Blood pressure		

Normal blood pressure 120/< 80	105	81,4
Elevated blood pressure 120-129/< 80	13	10,1
Hypertension stage 1 130-139/80-89	4	3,1
Hypertension stage 2 140 or >/ >90	2	1,6
Hypertensive crisis >180/ >120	4	3,1
Missing	1	0,8
Delivery mode		
Natural unassisted childbirth	70	54,3
Natural assisted childbirth	8	6,2
C-section childbirth	51	39,5
Gestational booking stage		
First trimester	62	48,1
Second trimester	40	31
Third trimester	27	20,9

Table 4.2: The factors associated with perinatal complications in the neonates and mothers of the Vhembe district.

Socio-demographic and clinical Characteristics	**Perinatal complications**		**P-value**
	n (%) negative	n (%) positive	
Maternal Age			
10 to 19 years	9 (52,9%)	8 (47.1%)	0.032
20 to 29 years	47 (85,5%)	8 (14,5%)	
30 to 39 years	32 (68.1%)	15 (31,9%)	
40 and above	8 (80,0%)	2 (20,0%)	
Marital status			
Married first wife	16 (76.2%)	5 (23.8%)	0.539
Married second wife	4 (80.0%)	1 (20.0%)	
Single	51 (69.9%)	22 (30.1%)	
Living with a partner	25 (83,3%)	5 (16.7%)	
Parity status			
Nullipara	31 (70,5%)	13 (29.5%)	0.707
Primipara	29 (82,9%)	6 (17.1%)	
Multipara (3)	17 (70.8%)	7 (29.2%)	
Multipara (4)	13 (76.5%)	4 (23.5%)	
Grand Multipara	6 (66.7%)	3 (33.3%)	
Employment status			
Unemployment	78 (72.2%)	30 (27.8%)	0.063
Self-employment	11 (100.0%)	0 (0.0%)	
Private employment	3 (50.0%)	3 (50.0%)	
Government employment	4 (100.0%)	0 (0.0%)	
Income status			
SASSA grant	65 (73.0%)	24 (27.0%)	0.866
less than R3000	13 (81.0%)	3 (18.8%)	
R3000-5000	7 (70.0%)	3 (30.0%)	
R5100-10 000	1 (100%)	0 (0.0%)	
R10 100- 15 000	1 (100%)	0 (0.0%)	
Educational status			
Illiterate	2 (100%)	0 (0.0%)	0.225
Primary school	6 (54.5%)	5 (45.5%)	
High school	65 (72.2%)	25 (27.8%)	
Tertiary school	21 (87.5%)	3 (12.5%)	
Postgraduate	1 (100%)	0 (0.0%)	
HIV status			
Positive	13 (61.9%)	8 (38.1%)	0.151
Negative	83 (76,9%)	25 (23.1%)	

BMI class			
Underweight	2 (100.0%)	0 (0.0%)	0.840
Normal weight	22 (81.5%)	5 (18.5%)	
Overweight	26 (76.5%)	8 (23.5%)	
Obese	22 (81.5%)	5 (18.5%)	
Baby sex			
Male	53 (71.6%)	21 (28.4%)	0.398
Female	43 (78.2%)	12 (21.8%)	
Birth weight			
<2.5kg	7 (46.7%)	8 (53.3%)	0.027
2.5-3.5 kg	78 (77.2%)	23 (22.8%)	
>3.5 kg	11 (84.6%)	2 (15.4%)	
Blood pressure			
Normal blood pressure 120/< 80	84 (80.0%)	21 (20.0%)	0.041
Elevated blood pressure 120-129/< 80	8 (61.5%)	5 (38.5%)	
Hypertension stage 1 130-139/80-89	2 (50.0%)	2 (50.0%)	
Hypertension stage 2 140 or >/ >90	1 (50.0%)	1 (50.0%)	
Hypertensive crisis >180/ >120	1 (25.0%)	3 (75.0%)	
Anaesthesia use			
Yes	22 (48.2%)	23 (51,1%)	<0.001
No	39 (88.6%)	5 (11.4%)	
Delivery Mode			
Natural unassisted childbirth	64 (91.4%)	6 (8.6%)	<0.001
Natural assisted childbirth	8 (100.0%)	0 (0.0%)	
C-section childbirth	24 (47.1%)	27 (52.9%)	
Gestational booking stage			
First trimester	49 (51.04%)	13(39.39%)	0.125
Second trimester	31 (32.29%)	9 (27.27%)	
Third trimester	16 (16.67%)	11 (33.33%)	
Smoking tobacco during pregnancy			
No	95 (75.4%)	31 (24.6%)	0.411
Yes	1 (50.0%)	1 (50.0%)	
Did you Consumption alcohol?			
Not at all	70 (74.5%)	24 (25.5%)	0.895
Before pregnancy	24 (77.4%)	7 (22.6%)	
During pregnancy	0 (0.0%)	0 (0.0%)	
Before and during pregnancy	2 (66.7%)	1 (33.3%)	

4.2. THE ANALYSIS OF BACTEREMIA IN WHOLE-BLOOD SAMPLES

4.2.1. Prevalence of bacterial culture-confirmed Isolates in Perinatal complications

Figure 4.1 presents various bacterial isolates on MSA and EMB agars. The umbilical cord blood culture results revealed that 39/129 (30.23%) participants were positive for bacterial growth, with several samples showing mixed infection with two or more bacterial organisms. The proposition of 54 (58.06%) were categorized as Gram-positive bacteria (GPB) and 39 (41.93%) as Gram-negative bacteria (GNB) (Table 4.3). The total number of bacterial isolates identified in the present study was ninety-three (93).

Among GPB, *S. epidermidis* was the most dominant isolates 34 (36.56%) followed by *S. aureus* 19 (20.43%). In contrast, GNB were predominantly represented by *Klebsiella pneumoniae* with 19 (20.43%), followed by *Escherichia coli* with 15(16.13%) cases (figure 4.2). In comparison to normal pregnancy outcomes, perinatal complications were significantly associated with *E. coli* infection, accounting for 9(60.0%), showing a significant *P*-value (*P*=0.001) (Table 4.3). Most isolates were identified in normal pregnancy outcomes, comprising a high proportion of 55(59.14%) cases (Table 4.3).

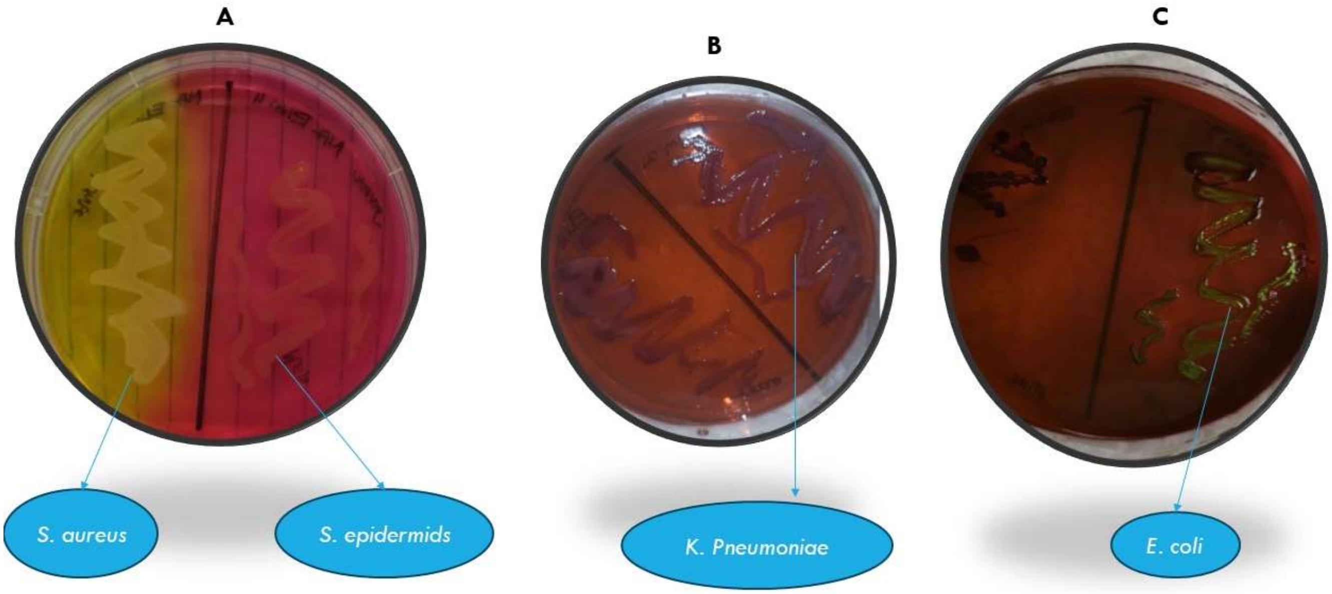


Figure 4.1: Umbilical cord blood culture plates: Plate A showing the *Staphylococcus* spp. grown on MSA, the yellow species surrounded by yellow zone being *S. aureus* and the pink colonies with pink zone demonstrating *S. epidermidis*. Plate B showing *K. pneumoniae* as pink, mucoid colonies on EMB agar. Plate C showing *E. coli*, metallic green colonies on EMB agar.

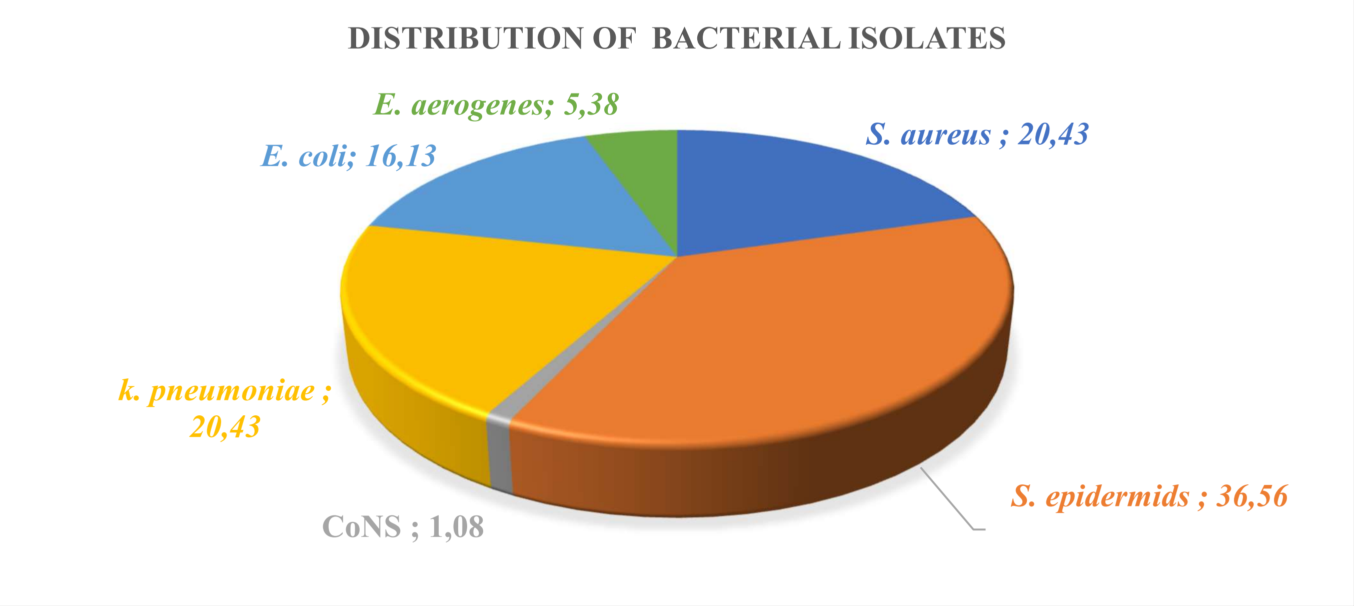


***Figure 4.2*:** Frequency (%) of bacterial isolates from umbilical cord blood collected in four district Hospitals in Vhembe district.

Table 4.3: Distribution of bacterial culture-confirmed isolated from the participants: classified by the types of pregnancy outcomes: Perinatal complications, as well as Normal control.

	Prevalence of culture confirmed isolates in Pregnancy outcomes			
Isolates (93)	**Perinatal complications N=33, n (%)**	**Normal controls N=95, n (%)**	**Total number of Mos = 93 (%)**	***P*-value**
Gram positive bacteria				
S. aureus	4(21.1%)	15(78.9%)	19(20.43%)	0.610
S. epidermidis	9(26.5%)	25(73.5%)	34(36.56%)	0.915
CoNS	1(100%)	0(0.0%)	1(1.08%)	
Sub-total	14(15.05%)	40(43.01%)	54(58.06%)	
Gram-negative bacteria				
k. pneumoniae	8(42.1%)	11(57.9%)	19(20.43%)	1.76
E. coli	9(60.0%)	6(40.0%)	15(16.13%	0.001*
E. aerogenes	1(20.0%)	4(80.0%)	5(5.38%)	0.77
Sub-total	18(19.35%)	21(22.58%)	39(41.93%)	

CoNS=Coagulase-negative staphylococci, Mos=Microorganism

4.2.2. Detection of Bacteraemia Based on 16S rRNA Gene Amplification by PCR.

A Conventional PCR was conducted based on the amplification of 16S rRNA gene to detect the presence of umbilical cord blood bacterial infections in pregnant mother and neonates in the Vhembe district. The overall bacteremia rate detected by PCR in the pregnant women and neonates in the Vhembe district was 34/129 (26.36%). The positive samples identified by conventional PCR were confirmed using gel electrophoresis to separate DNA fragments based on their molecular size (1500bp) as illustrated in figure 4.3.

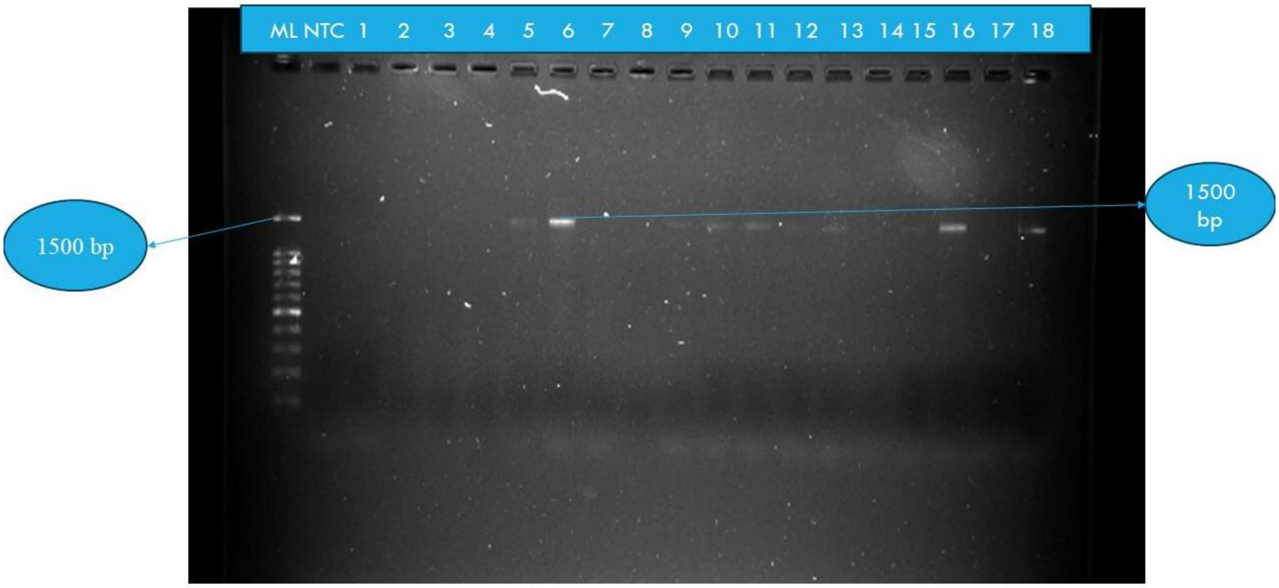


Figure 4.3: Gel electrophoresis picture illustrating bands of the PCR amplicons of the 16S rRNA gene. Lane 1 100bp molecular ladder (ML) from 100bp to 1500bp. NTC (Lane 2) =Non template control (negative control). Lane 5,6,9,10,11,12,15,16, and18 are showing amplified PCR at 1500 bp. Negative samples were detected in lane 1,2,3,4,7,8,13,14, and 17.

4.2.3. Comparison of blood culture and 16S rRNA gene PCR amplification results.

The comparison between bacterial culture and PCR assays findings revealed relative discordance between the two detection methods (Table 4.4). Of all the 34 participants who were positive for bacterial infection detected based on 16S rRNA PCR assays, 22 (64.71%) also tested positive on blood culture. Conversely, 12 (35,29%) had negative blood culture results, suggesting the potential for PCR to detect infections missed by blood culture, leading to false-negative blood culture results. Among the 90 participants who tested negative on PCR assays, a portion, 17(17.89%) had positive blood culture results (false-positive), and 78 (82.11%) had negative blood culture results. Comparing with blood culture, PCR exhibited a 22/39 (56.41%) sensitivity, 78/95 (82.11%) specificity, 22/34 (64.71%) positive predictive value, and 78/95 (86.67%) negative predictive value. Remarkably, the results of statistical analysis revealed significant associations between PCR assays and blood culture findings, with a *P*-value (*P*<0.001) (Table 4.4).

Table 4.4: Comparison between PCR and blood culture results of Bacteremia in pregnancy (Pearson Chi-Square Test).

	Blood culture results		**Total**	**P-value**
	Positive	Negative		
PCR Positive	22(64.71%)	12(35.29%)	34	<0.001*
PCR Negative	17(17.89%)	78(82.11%)	90	
Total	39	95	129	

PCR= polymerase chain reaction.

4.2.4. Factors Associated with the Occurrence of Blood Bacterial Infection in Pregnancy.

A univariate model employing the Pearson Chi-Square test, was utilized to access potential associations between PCR positivity and blood culture positivity with various predetermined factors among mother and neonates in the Vhembe district (Table 4.5). Four variables: maternal educational level (*P*=0.02), Maternal blood pressure at birth (*P*=0.006), gestational booking stage (*P*=4.43E^{-04}), and pre-pregnancy BMI (*P*=0.01) showed a statistically significant correlation with bacteraemia detected based on the PCR amplification of 16S rRNA gene. Specifically, maternal blood pressure at birth exhibited a significant association with blood culture results, with *P*-value of 0.04. However, no statistically significant associations were found between bacteraemia with perinatal complications, HIV status, maternal age, marital status, baby sex, employment status, monthly income, as well as delivery mode.

Table 4.5: Association between the results of PCR assays and blood culture regarding perinatal complications and sociodemographic and medical factors among mother and neonates.

PCR results			Blood culture results			Characteristics
P-value	**Negative, N (%)**	**Positive, N (%)**	**P-value**	**Negative, N (%)**	**Positive, N (%)**	
						Complications
0.13	74(77.89%)	21(22.11%)	0.37	69(76.67%)	27(69.23%)	Normal pregnancy outcomes
	22(64.71%)	12(35.29%)		21(23.33%)	12(30.77%)	Perinatal complication
						Maternal Age
0.65	12(12.63%)	5(14.71%)	0.92	12(13.33%)	5(12.82%)	10 to 19 years
	43(45.26%)	12(35.29%)		39(43.33%)	16(41.03%)	20 to 29 years
	34(35.79%)	13(38.24%)		33(36.33%)	14(35.9%)	30 to 39 years
	6(6.32%)	4(11.76%)		6(6.67%)	4(10.26%)	40 and above
						HIV status
0.43	81(85.26%)	14(14.74%)	0.39	77(85.55%)	31(79.49%)	Negative
	27(79.41%)	7(20.59%)		13(14,44%)	8(20.51%)	Positive
						Marital status
0.44	13(13.68%)	8(23.53%)	0.098	12(13.33%)	9(23.08%)	Married first wife
	3(3.16%)	2(5.88%)		2(2.22%)	3(7.69%)	Married second wife
	55(57.89%)	18(52.94%)		51(56.67%)	22(56.41%)	Single
	24(25.26%)	6(17.65%)		25(27.78%)	5(12.82%)	Living with a partner
						Employment status
0.91	79(83.16%)	29(85.29%)	0.47	73(81.11%)	35(89.75%)	Unemployment
	9(9.47%)	2(5.88%)		8(8.89%)	3(7.69%)	Self-employment
	4(4.21%)	2(5.88%)		5(5.56%)	1(2.56%)	Private employment
	3(3.16%)	1(2.94%)		4(4.44%)	0(0.0%)	Government employment
						Income Status
0.91	65(68.42%)	24(72.73%)	0.87	61(67.78%)	28(73.68%)	SASSA grant
	11(11.59%)	5(15.15%)		12(13.33%)	4(10.53%)	less than R3000
	8(8.42%)	2(6.06%)		8(8.89%)	2(5.26%)	R3000-5000
	1(1.05%)	0 (0.0%)		1(1.11%)	0(0.0%)	R5100-10 000
	1(1.05%)	0 (0.0%)		1(1.11%)	0(0.0%)	R10 100- 15 000
						Educational status
0.02*	0 (0.0%)	2(5.88%)	0.063	0(0.0%)	2(5.13%)	Illiterate
	5(5.26%)	6(17.65%)		6(6.67%)	5(12.82%)	Primary school
	67(70.53%)	23(67.65%)		61(67.78%)	29(74.36%)	High school
	21(22.11%)	3(8.82%)		21(23.33%)	3(7.69%)	Tertiary school
	1(1.05%)	0 (0.0%)		1(1.11%)	0(0.0%)	Postgraduate
						Maternal Blood pressure
0.006*	84(88.42%)	21(61.11%)	0.04*	79(87.78%)	26(66.67%)	Normal blood pressure 120/< 80
	8(8.42%)	5(14.71%)		6(6.67%)	7(17.95%)	Elevated blood pressure 120-129/< 80
	1(1.05%)	3(8.82%)		3(3.33%)	1(2.56%)	Hypertension stage 1 130-139/80-89
	1(1.05%)	1(2.94%)		1(1.11%)	1(2.56%)	Hypertension stage 2 140 or >/ >90
	1(1.05%)	3(8.82%)		1(1.11%)	3(7.69%)	Hypertensive crisis >180/ >120
						BMI classes
0.01*	1(1.05%)	1(2.94%)	0.23	1(1.11%)	1(2.56%)	Underweight
	21(22.11%)	6(17.65%)		22(24.44%)	5(12.82%)	Normal weight
	28(29.47%)	6(17.65%)		20(22.22%)	14(35.9%)	Overweight
	13(13.68%)	14(41.18%)		17(18.89%)	10(25.64%)	Obese
						Gestational booking stage

4,43E-04	49(51.58%)	13(38.24%)	0.92	44(48.89%)	18(46.15%)	First trimester
	34(35.79%)	6(17.65%)		28(31.11%)	12(30.77%)	Second trimester
	12(12.63%)	15(44.12%)		18(20.0%)	9(23.08%	Third trimester
						Delivery mode
0.48	49(51.58%)	21(61.76%)	0.77	47(52.22%)	23(58.97%)	Natural unassisted childbirth
	7(7.37%)	1(2.94%)		6(6.67%)	2(5.13%)	Natural assisted childbirth
	39(41.05%)	12(35.29%)		37(41.11%)	14(35.9%)	C-section childbirth
						Baby sex
0.31	57(60.0%)	17(50.0%)	0.09	56(62.22%)	18(46.15%)	Male
	38(40.0%)	17(50.0%)		34(37.78%)	21(53.85%)	Female

PCR= polymerase chain reaction, BMI=body mass index, HIV=human Immunodeficiency virus.

4.2.5. Antimicrobial susceptibility profile of culture-confirmed bacterial isolates.

Antibiotic Susceptibility testing plays a significant role in highlighting the resistance patterns of pregnancy related GPB and GNB isolates as well as guiding the health care practitioner in selecting antibiotics for treating Gram-positive and Gram-negative bacterial infections. Hence, the present study profiled the antimicrobial susceptibility patterns in 63 culture-confirmed bacterial isolates from the participants enrolled in the Vhembe district. All isolates were tested on 8 antimicrobial agents. In this study, Gram-positive and Gram-negative bacteria exhibited a notable degree of resistance to certain antibiotics.

Thus, Gram-positive bacteria (35/36 (97.22%)) demonstrated a high proportion of resistance to AMP, FOX, and CAZ. Furthermore, GPB showed a higher sensitivity to CN (97.22%), IMI (88.89%), AK (88.89%), and CIP (75.0%) (Table 4.4). However, they exhibited relatively low resistance to CN (2.78%), AK (5.56%), CIP (8.33%), and IMI (11.11%). Among the GPB tested, *S. epidermidis (23)* and *CoNS (1)* represented 100% resistance rate to AMP, FOX, and CAZ, except, *S. aures* which account to 11(91.67%) resistance rate in AMP, FOX, and CAZ.

GNB on the other hand exhibited a very strong sensitivity to AK (88.87%), CN (88.87%), CIP (77.78%), and IMI (66.67%) but higher degree of resistance in E (100%), AMP (88.87%), FOX (77.78%), and CAZ (74.07%). Almost all tested GPB and GNB isolates showed high sensitivity to Gentamicin, Ciprofloxacin, Imipenem, and Amikacin. However, only *E. coli* and *S. aureus* indicated comparatively lower resistance rate for Gentamicin at 2/9(22.22%) and 1/12(8.33%) respectively. K. *pneumoniae, E. coli*, and *E. aerogenes* demonstrated resistance to Erythromycin at 100% (Table 4.6). Furthermore, only *S. epidermidis, K. pneumoniae,* and *E. aerogenes* were very susceptible to Amikacin at 100% rate. Figure 4.4 depicting an example of organisms subjected to antibiotic susceptibility test.

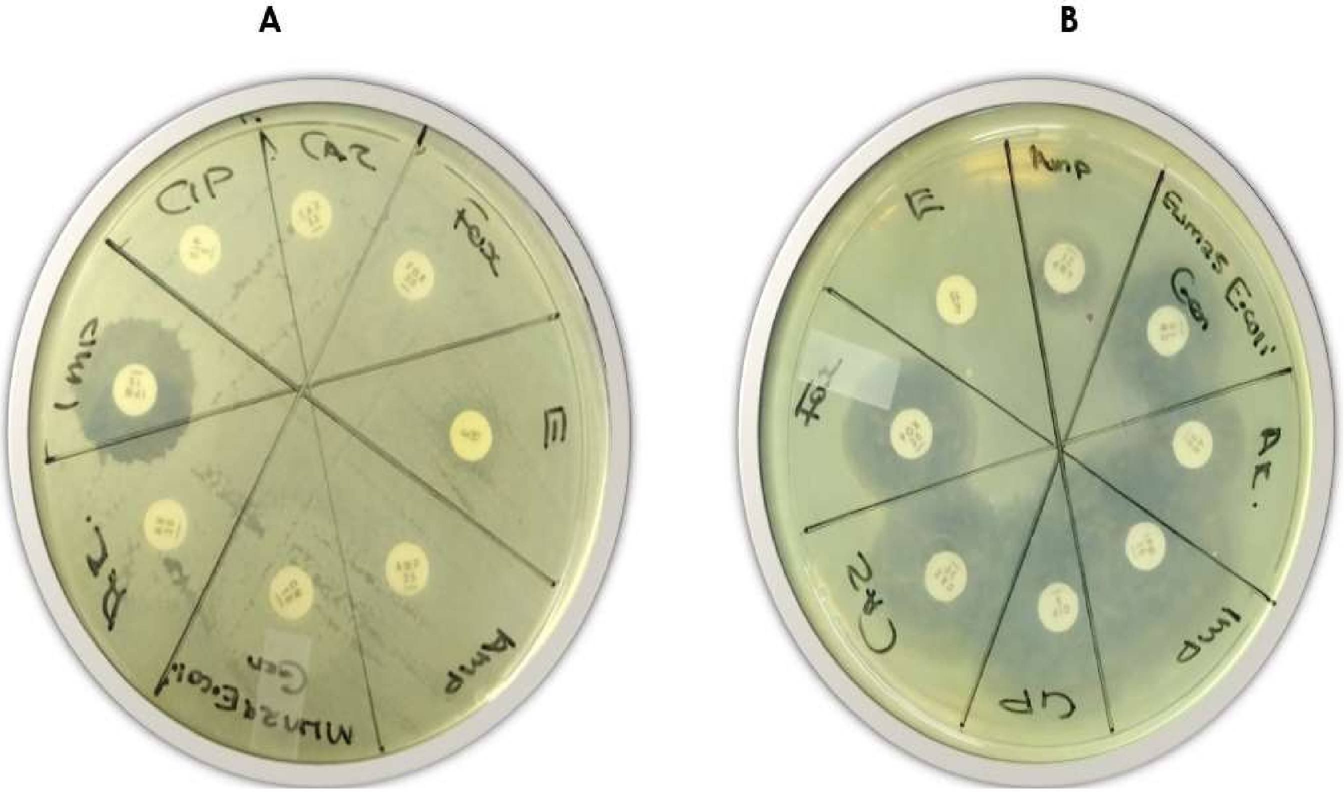


Figure 4.4: MHA Disk diffusion plate. Plate A Showing Multiple drug resistant E. coli with no zone of growth inhibition observed around the following disks: AMP, CAZ, FOX, E, AMP, CN, as well as AK. Plate B showing a plate for *E. coli* strain where the is no zone of growth inhibition observed around the E disk.

Table 4.6. Antibiotic susceptibility profile of culture-confirmed of both gram-positive and negative bacterial isolates from participants at selective Hospital in Vhembe district (n = 63).

Isolates (no.)	**Antimicrobial Agents Tested**								
	Inter	CN	AMP	E	FOX	CAZ	CIP	IMI	AK
S. aureus (12)	S	11	1	3	1	0	7	11	9
	I	0	0	3	0	1	4	0	2
	R	1	11	6	11	11	1	1	1
S. epidermidis (23)	S	23	0	12	0	0	19	20	22
	I	0	0	2	0	0	2	0	0
	R	0	23	9	23	23	2	3	1
CoNS (1)	S	1	0	1	0	0	1	1	1
	I	0	0	0	0	0	0	0	0
	R	0	1	0	1	1	0	0	0
Sub-total (36)	**S (%)**	**35 (97.22)**	**1 (2.78)**	**16 (44.44)**	**1 (2.78)**	**0 (0.0)**	**27 (75.0)**	**32 (88.89)**	**32 (88.89)**
	I (%)	**0 (0.0)**	**0 (0.0)**	**5 (13.89)**	**0 (0.0)**	**1 (2.78)**	**6 (16.67)**	**0 (0.0)**	**2 (5.56)**
	R (%)	**1 (2.78)**	**35 (97.22)**	**15 (41.67)**	**35 (97.22)**	**35 (97.22)**	**3 (8.33)**	**4 (11.11)**	**3 (5.56)**
K. pneumoniae (16)	S	16	0	0	1	1	14	10	16
	I	0	0	0	0	1	0	4	0
	R	0	16	16	15	14	2	2	0
E. coli (9)	S	6	0	0	4	3	7	6	6
	I	1	3	0	0	2	1	2	1
	R	2	6	9	5	4	1	1	2
E. aerogenes (2)	S	2	0	0	1	0	0	2	2
	I	0	0	0	0	0	0	0	0
	R	0	2	2	1	2	2	0	0
Sub-total (27)	**S (%)**	**24 (88.87)**	**0 (0.0)**	**0 (0.0)**	**6 (22.22)**	**4 (14.81)**	**21 (77.78)**	**18 (66.67)**	**24 (88.87)**
	I (%)	**1 (3.70)**	**3 (11.11)**	**0 (0.0)**	**0 (0.0)**	**3 (11.11)**	**1 (3.70)**	**6 (22.22)**	**1 (3.70)**
	R (%)	**2 (7.41)**	**24 (88.87)**	**27 (100.0)**	**21 (77.78)**	**20 (74.07)**	**5 (18.52)**	**3 (11.11)**	**2 (7.41)**

Inter=Interpretation as per CLSI, S= Susceptible (Zone of inhibition ≥ 20 mm), I=Intermediate (Zone of inhibition = 15-19 mm), R=Resistance (Zone of inhibition ≤ 14 mm), CN=Gentamicin, AMP=Ampicillin, E=Erythromycin, FOX=Cefoxitin, CAZ=Ceftazidime, CIP=Ciprofloxacin, IPM=Imipenem, AK=Amikacin.

4.2.6. Multiple Drug Resistance profiling of bacterial isolates.

Among the 63 tested isolates for susceptibility, a notable 93.38% exhibited resistance to three or more classes of antibiotics. The prevalence of MDR among gram-positive and gram-negative bacterial isolates were 97.22% and 96.30%, respectively. Specifically, 36.51% of the bacteria demonstrated resistance to three different antimicrobials, while a substantial 69.84% displayed resistance to more than three classes of antibiotics. In the case of *S. aureus* and *E. coli,* 91.67% and 55.55% of isolates, respectively, were identified as multidrug-resistant (MDR) respectively, indicating resistance to three and more antibiotics agents. The study also revealed a high prevalence of multidrug resistance among Coagulase-Negative Staphylococci (CoNS), *S. epidermidis, K. pneumoniae*, and *E. aerogenes* at 100% as detailed in Table 4.7.

Table 4.7: Multiple Drug Resistance profile of Culture-confirmed Bacterial Isolates at four selective district Hospital in Vhembe District, Limpopo.

Bacterial Isolates	**Drug Resistance Pattern**									**Total MDR (%)**
	R0(%)	R1(%)	R2(%)	R3(%)	R4(%)	R5(%)	R6(%)	R7(%)	R8(%)	
S. aureus (n=12)	0(0.0%)	1(8.33%)	0(0.0%)	5(41.33%)	4(33.33%)	2(16.67%)	0(0.0%)	0(0.0%)	0(0.0%)	12(100%)
S. epidermids (n=23)	0(0.0%)	0(0.0%)	0(0.0%)	13(56.52%)	6(26.09%)	3(13.04%)	1(4.35%)	0(0.0%)	0(0.0%)	23(100%)
CoNS (n=1)	0(0.0%)	0(0.0%)	0(0.0%)	1(100%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	1(100%)
K. pneumoniae (n=16)	0(0.0%)	0(0.0%)	1(6.25%)	1(6.25%)	12(75%)	2(12.5%)	0(0.0%)	0(0.0%)	0(0.0%)	16(100%)
E. coli (n=9)	0(0.0%)	1(11.11%)	3(33.33%)	2(22.22%)	1(11.11%)	1(11.11%)	0(0.0%)	0(0.0%)	1(11.11%)	9(100%)
E. aerogenes (n=2)	0(0.0%)	0(0.0%)	0(0.0%)	1(50.0%)	1(50.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	2(100%)
Total (n=63)	0(0.0%)	2(3.17%)	4(6.35%)	23(36.51%)	24(38.1%)	8(12.7%)	1(1.59%)	0(0.0%)	1(1.59%)	63(100%)

Key: R0 =Represent susceptibility to all antimicrobial agents; R1-8 Resistance to 1,2,3,4,5,6,7,8, antibiotics, MDR=Multi drug resistance.

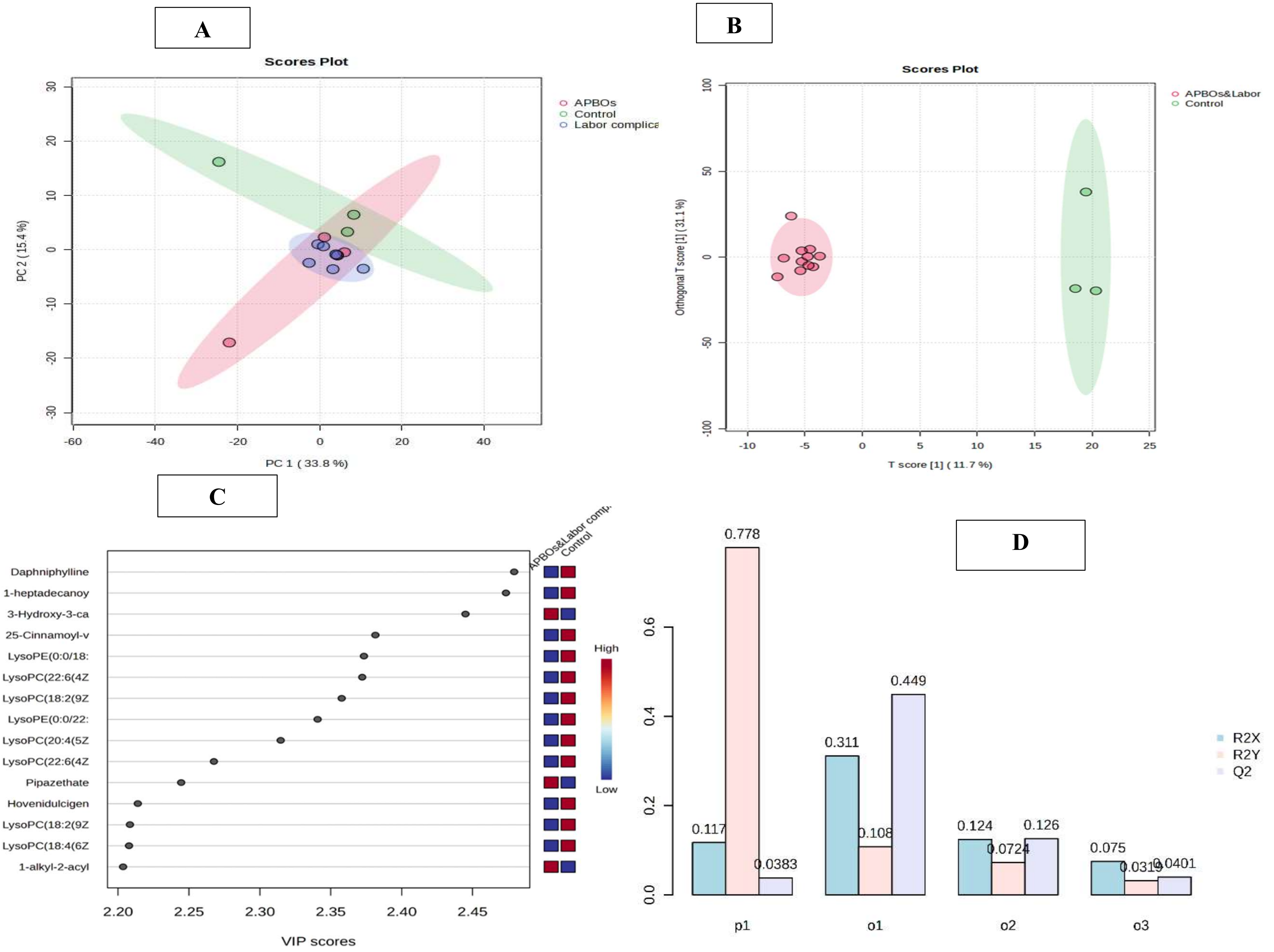
4.3. METABOLOMIC SIGNATURE IN CORD BLOOD IN PREGNANCY

4.3.1 Multivariate data analysis of metabolite profiles from perinatal complications and controls

Taking into account the research budget and the representative nature of the samples, only 14 samples were used for metabolomic analysis. From these 14 samples, 4 APBOs cases, 7 labor and obstetric complications and 3 normal pregnancy outcomes were selected. Multivariate analysis of metabolomic data simplifies intricate and interpret complex datasets that involve multiple variables (metabolites) simultaneously. This type of analysis is employed to identify patterns, trends, and relationships within the data that may not be apparent when examining individual variables in isolation (Mashabela et al., 2022). By applying multivariate analysis techniques, researchers can gain a comprehensive understanding of the metabolic changes associated with various conditions, such as diseases or exposure to different environmental factors (Pretorius, et al., 2021). Multivariate Principal component analysis (PCA) was performed for positive ion, to explore the clustering patterns within the metabolome profile in perinatal complications and normal controls. The first (PC1) and second (PC2) component explained 33.8% and 15.4% of the variance between perinatal complications and normal pregnancy outcomes, respectively and no outliers in the data (Figure 4.4A).

For the identification of metabolic alterations in umbilical cord serum from poor and normal pregnancy outcomes classes, multivariate analyses was conducted. All APBOs and labor or obstetric complications were grouped together in case groups (perinatal complications). In the supervised Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) depicted in Figure 4.4B, a clear separation is evident with minimal risk of overfitting. The OPLS-DA score plot indicated that the threshold for significant metabolite differences between the two groups was set at 11.7%. Furthermore, the threshold for intra-group differences in metabolites within the two groups was determined to be 31.1%.

Figure 4.4C illustrates the variable importance in projection (VIP) for top 15 metabolites with value greater than 1.5 (metabolites responsible for class separation). The heatmap on right side of the plot (Figure 4.4C) indicates if the metabolite is highly and lower contracted in cases compared to controls. Permutation plots (Figure 4.4E) were used for the validations of the results and the model obtained by OPLS-DA showed a high performance was achieved when four latent variables were used, resulting in accuracy ($R^2X=0.311$, $R^2Y=0.108$) and a strong predictive ability ($Q^2=0.440$) (Figure 4.4D). The prediction rate was more than 0.50, ($Q^2=0.653$), which further proved the higher prediction accuracy of the OPLS-DA model. Additionally, the *P* values of the model were statistically significant ($P<0.0205$).


A
Scores Plot
APBOs
Control
Labor complica
PC 1 (33.8 %)
PC 2 (15.4 %)
-60
-40
-20
0
20
40
-30
-20
-10
0
10
20
30
B
Scores Plot
APBOs&Labor
Control
T score [1] (11.7 %)
Orthogonal T score [1] (31.1 %)
-10
-5
0
5
10
15
20
25
-100
-50
0
50
100
C
APBOs&Labor comp.
Control
Daphniphylline
1-heptadecanoy
3-Hydroxy-3-ca
25-Cinnamoyl-v
LysoPE(0:0/18:
LysoPC(22:6(4Z
LysoPC(18:2(9Z
LysoPE(0:0/22:
LysoPC(20:4(5Z
LysoPC(22:6(4Z
Pipazethate
Hovenidulcigen
LysoPC(18:2(9Z
LysoPC(18:4(6Z
1-alkyl-2-acyl
High
Low
2.20
2.25
2.30
2.35
2.40
2.45
VIP scores
D
0.778
0.449
0.311
0.117
0.108
0.0383
0.124
0.126
0.0724
0.075
0.0319
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0.4
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p1
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R2X
R2Y
Q2

E

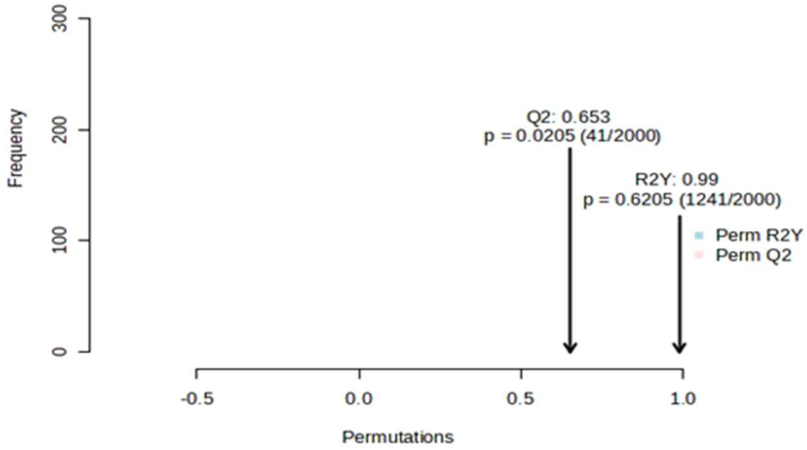


Figure 4.4: Multivariate statistical analysis of metabolites in perinatal complications and healthy controls. (A) A Two-dimension score plot generated through Principal Component Analysis (2D-PCA) illustrates the distribution of APBOs (red dots), labor and obstetric complications (blue dots) and healthy controls (green dots). (B) Two-dimension Orthogonal Partial Least Squares Discriminant Analysis (2D-OPLS-DA) score plot reveals a distinct separation between the labor and obstetric complications + APBOs and healthy controls, indicating notable differences in their metabolic. (C) Variable importance in projection (VIP) plot (top 15 most metabolites that contribute to group separations identified through OPLS-DA) indicating the most discriminating umbilical cord serum metabolites between APBOs + labor and obstetric complications and health controls. On the right, red and blue boxes show whether the average metabolite abundance is increased (red) or decreased (blue) in cases compared to healthy controls. (D) Performance of metabolomics model obtained by OPLS-DA. (E) The permutation test involved 2000 permutations, resulting in a test statistic based on the model's prediction accuracy. The observed statistic yielded a P value of 0.0205. The arrows in the OPLS-DA model indicate the positioning of the model's accuracy. R2X and R2Y denote the explanatory rates of the constructed model for the X and Y matrices, respectively, while Q2 represents the predictive capability of the model. The model's effectiveness is higher when the values of R2 and Q2 are closer to 1.

4.3.2. Identification of Discriminative Metabolites in Perinatal Complications and Normal control.

Based to the untargeted metabolomics profiling data, 2485 peaks were initially identified by LCMS-QTOF in positive mode. A total of 963 metabolic features were found after statistical analysis that included a univariate significant analysis at a *P* value < 0.05, and a fold change cut-off of ≥2.0 or ≤0.8 between perinatal complications and healthy controls. A nonparametric univariate analysis (Two-sample t-tests & Wilcoxon rank-sum tests) revealed 107 differential metabolites in umbilical cord blood associated with perinatal complications (Table 4.8), among which 57 metabolites were up regulated and 50 down regulated in perinatal complications and healthy controls, identified by the volcanic map, which is made up of different fold changes (log FC) and -log10 (p) (P<0.05) (Figure 4.6).

The hierarchical cluster analysis method, employed as an unsupervised multivariate analysis approach (illustrated in Figure 4.5), was utilized to assess how well 107 metabolic peaks differentiate between participants with perinatal complications and control groups. We conducted exploratory statistical differences in metabolites. A heatmap generated through Unsupervised Hierarchical Clustering Analysis (HCA) illustrates the expression patterns of the top 50 differentially expressed metabolites in individuals with perinatal complications compared to healthy controls. The heatmap visually represents the significant up-regulation or down-regulation of these metabolites, as depicted in Figure 4.5.

Notably, seven metabolites emerge as the most discriminative metabolites, retaining significance even after false discovery rate (FDR) adjustment (q<0.05) (Table 4.9). Among significant metabolic features, compound **G** was the only metabolite that was increased in perinatal complications compared to healthy controls (Figure 4.7A). The six metabolites (**A-F)** that were down regulated in cases after false discovery rate (FDR) adjustment (q<0.05) are shown in figure 4.7B1-6.

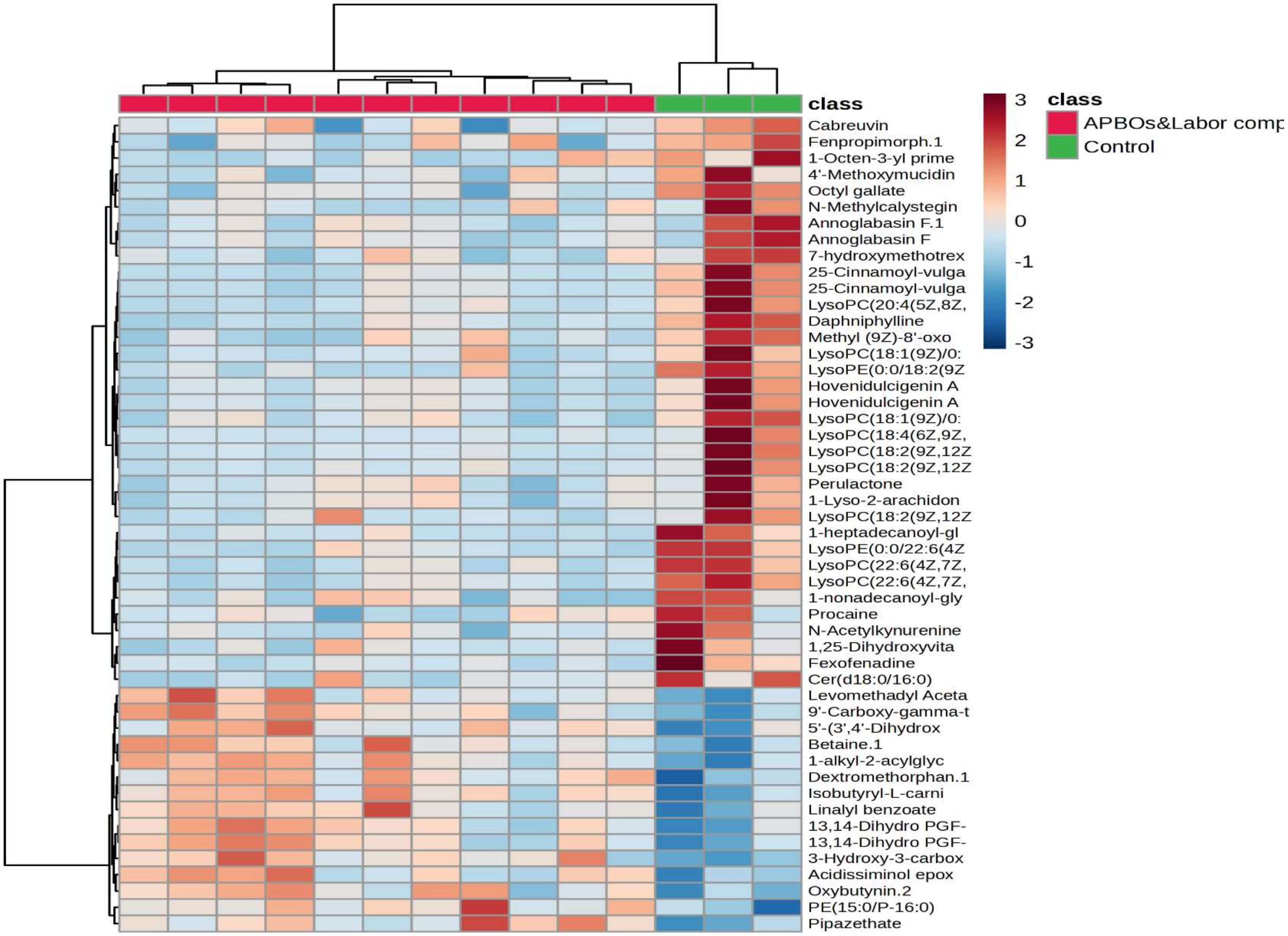


Figure 4.5: Unsupervised Hierarchical clustering analysis (HCA)-heatmap plot generated using MetaboAnalyst highlighting the top 50 differentially expressed metabolites in perinatal complications (red), and healthy controls (green) in umbilical cord serum, analyzed by T-test/ANOVA. Blue color indicates a decrease in expression, while red indicates an increase in expression. The intensity of the color reflects the magnitude of the corresponding abundance difference. Metabolites and samples are organized in a tabular format, where rows correspond to metabolites, and columns correspond to samples. The clustering of both metabolites and samples is achieved using Euclidean distance and Ward linkage.

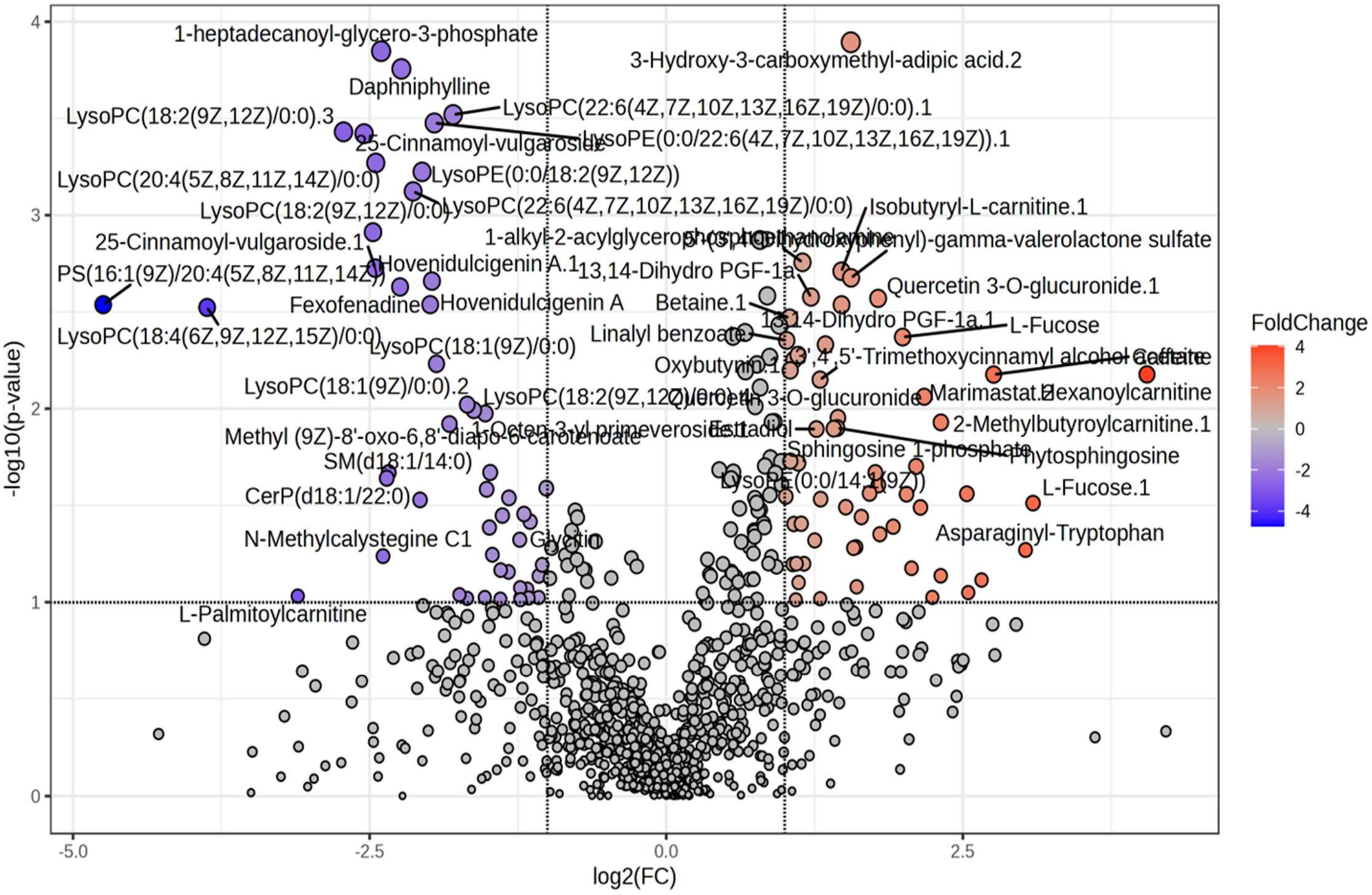


Figure 4.6: Volcano plot illustrating the significant potential biomarkers (metabolites) that discriminates between perinatal complications and healthy groups. In the plot, non-significant variables, as well as significant up-regulated and down-regulated variables, are depicted in gray, red, and blue, respectively, with the criteria of $P < 0.05$ and fold change threshold of 2.0.

Table 4.8: The significant up and down regulated metabolite (Potential biomarkers) in perinatal complications compared to healthy controls.

Name of compounds	Level of metabolites	Precursor m/z	RT	log2(FC)	raw.pval	-log10(p)
3-Hydroxy-3-carboxymethyl-adipic acid	Increased	221.066589876148	1,813932545	1.556	1.278E-4	3.8935
1-heptadecanoyl-glycero-3-phosphate	Decreased	425.270397382798	4,73867494	-2.4034	1.4208E-4	3.8475
Compound D	Decreased	634.312676004453	7,062566951	-2.2324	1.7535E-4	3.7561
LysoPC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)	Decreased	568.341035234795	7,047147965	-1.796	3.0239E-4	3.5194
LysoPE(0:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	Decreased	526.294690439693	3,92813118	-1.9546	3.3384E-4	3.4765
LysoPC(18:2(9Z,12Z)/0:0).3	Decreased	520.342629129969	7,083535062	-2.7202	3.7083E-4	3.4308
25-Cinnamoyl-vulgaroside	Decreased	566.32451240191	7,069710714	-2.5469	3.7919E-4	3.4211
LysoPC(20:4(5Z,8Z,11Z,14Z)/0:0)	Decreased	544.342596953387	7,048502872	-2.4483	5.3693E-4	3.2701
LysoPE(0:0/18:2(9Z,12Z))	Decreased	478.295377708962	6,893620395	-2.058	5.9722E-4	3.2239
LysoPC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)	Decreased	568.341035234795	7,047147965	-2.1335	7.5321E-4	3.1231
LysoPC(18:2(9Z,12Z)/0:0)	Decreased	542.324972249456	7,085954248	-2.4717	0.0012244	2.9121
1-alkyl-2-acylglycerophosphoethanolamine	Increased	243.048760367804	1,809014323	1.1484	0.0017526	2.7563
25-Cinnamoyl-vulgaroside	Decreased	567.328433648204	7,056012651	-2.4525	0.0018784	2.7262
Isobutyryl-L-carnitine	Decreased	232.155420607922	1,091751246	1.477	0.0019488	2.7102
5'-(3',4'-Dihydroxyphenyl)-gamma-valerolactone sulfate	Increased	289.042021336224	1,81959277	1.5581	0.0021086	2.676
Hovenidulcigenin A	Decreased	544,3407264	7,485741901	-1.9748	0.0021913	2.6593
Fexofenadine	Decreased	524,2778238	6,896696455	-2.2438	0.00235	2.6289
13,14-Dihydro PGF-1a	Increased	358,2720473	3,479245908	1.22	0.0026492	2.5769
Quercetin 3-O-glucuronide	Increased	240,0404735	1,814839787	1.786	0.0026914	2.57
Hovenidulcigenin A	Decreased	544,3407264	7,485741901	-1.9893	0.0028993	2.5377
PS(16:1(9Z)/20:4(5Z,8Z,11Z,14Z))	Decreased	782,5066384	8,577709626	-4.7458	0.002901	2.5375
13,14-Dihydro PGF-1a	Increased	358,2720473	3,479245908	1.4778	0.0029045	2.5369
LysoPC(18:4(6Z,9Z,12Z,15Z)/0:0)	Decreased	516,3091476	6,839847375	-3.8698	0.0029983	2.5231
Betaine	Increased	157,0500426	1,82108984	1.0399	0.0033983	2.4687
L-Fucose	Increased	187,0584554	0,644616822	1.9928	0.0042657	2.37
Linalyl benzoate	Increased	259,1665443	3,370900925	1.0157	0.004442	2.3524
3,4-Dihydroxymandelic acid	Increased	185,0450869	1,817633441	1.3421	0.0046684	2.3308
Acidissiminol epoxide	Increased	410,2278195	3,632883342	1.1115	0.0052855	2.2769
3-Hydroxy-3-carboxymethyl-adipic acid	Increased	175,0607288	1,814022404	1.0758	0.0055932	2.2523
LysoPC(18:1(9Z)/0:0)	Decreased	522,288745	6,016302271	-1.9366	0.0058617	2.232
Oxybutynin	Increased	358,2418873	4,084549123	1.045	0.0063419	2.1978
Hexanoylcarnitine	Increased	260,1862204	3,646296808	4.0522	0.006651	2.1771
Caffeine	Increased	195,0883811	3,637771943	2.7599	0.0066642	2.1763
3',4',5'-Trimethoxycinnamyl alcohol acetate	Increased	267,121594	4,093215997	1.2966	0.0070796	2.15
Marimastat	Increased	331,2108899	4,299948726	2.1746	0.0086775	2.0616

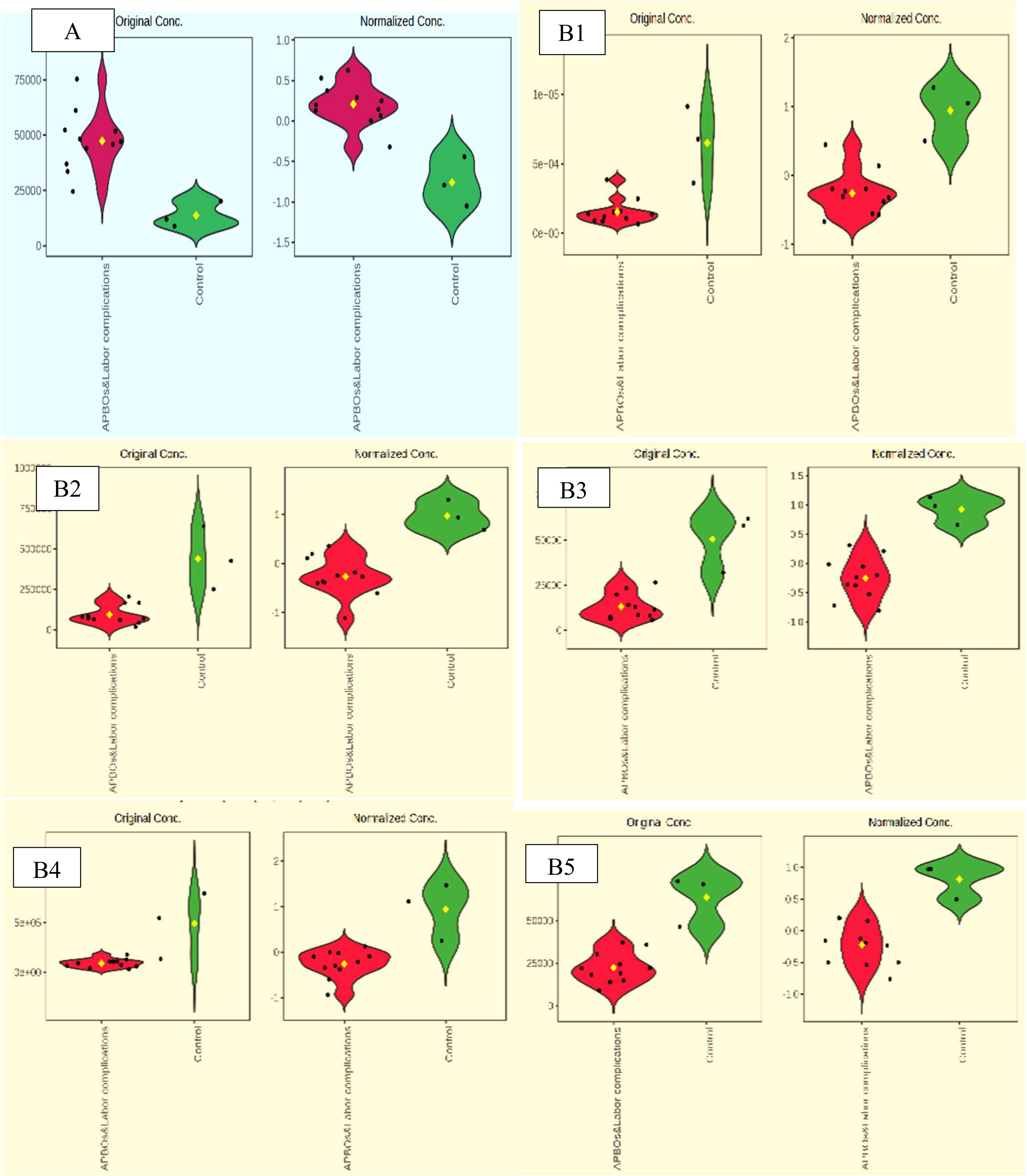
LysoPC(18:1(9Z)/0:0)	Decreased	522,35857	7,493280607	-1.6774	0.0095266	2.0211
LysoPC(18:2(9Z,12Z)/0:0)	Decreased	542,3250024	6,902246271	-1.6218	0.010178	1.9923
1-Octen-3-yl primeveroside	Decreased	423,2298766	3,859761183	-1.525	0.010583	1.9754
Quercetin 3-O-glucuronide	Increased	479,0738797	1,810131187	1.4469	0.011145	1.9529
2-Methylbutyroylcarnitine	Increased	246,1711429	2,198276041	2.3152	0.01177	1.9292
Methyl (9Z)-8'-oxo-6,8'-diapo-6-carotenoate	Decreased	353,2106874	4,504086347	-1.827	0.012005	1.9207
Phytosphingosine	Increased	354,2422726	5,702033957	1.4404	0.012587	1.9001
Sphingosine 1-phosphate	Increased	380,2536836	3,476536946	1.4125	0.012683	1.8968
Estradiol	Increased	273,1824296	3,698911812	1.266	0.012741	1.8948
PE(18:3(6Z,9Z,12Z)/P-18:1(11Z))	Increased	724,5367086	3,842692253	1.0462	0.018666	1.729
LysoPE(0:0/14:1(9Z))	Increased	424,2432614	3,745146636	1.1135	0.019095	1.7191
Neoacrimarine H	Increased	568,2036765	3,773379955	2.106	0.019874	1.7017
LysoPC(18:2(9Z,12Z)/0:0)	Decreased	520,3427898	6,89822606	-1.4836	0.021359	1.6704
(-)-alpha-Narcotine	Increased	413,1440192	3,393282571	1.7629	0.021399	1.6696
SM(d18:1/14:0)	Decreased	675,5476044	7,666383022	-2.3395	0.021413	1.6693
CerP(d18:1/22:0)	Decreased	701,5635787	8,607337746	-2.3559	0.022883	1.6405
Isobutyryl-L-carnitine	Increased	232,1554206	1,091751246	1.788	0.024797	1.6056
1-nonadecanoyl-glycero-3-phosphate	Decreased	232,1554869	3,388716234	-1.0108	0.025751	1.5892
S-Adenosylhomocysteine	Decreased	385,132144	4,426441264	-1.5138	0.026096	1.5834
Phenylalanylthreonine	Increased	267,1324071	3,709953571	1.714	0.027388	1.5624
Raltitrexed	Increased	459,1330638	3,643873461	2.5337	0.027522	1.5603
Marimastat	Increased	332,2168656	6,489286424	2.0253	0.027655	1.5582
Kiwiionoside	Increased	407,2273772	3,588837471	1.0078	0.028408	1.5466
N-Acetylkynurenine	Decreased	251,1037533	3,615151485	-1.3261	0.02891	1.5389
Nifedipine	Increased	347,1257148	3,680463417	1.3014	0.029334	1.5326
Cer(d18:0/16:0)	Decreased	540,5383527	7,975776504	-2.075	0.029608	1.5286
L-Fucose	Increased	203,0327702	0,642141156	3.0918	0.030769	1.5119
Dihydrofukinolide	Increased	393,2269067	4,572918548	1.5136	0.032292	1.4909
Avocadienofuran	Increased	247,2027839	0,631590221	2.1429	0.032393	1.4896
1-Lyso-2-arachidonoyl-phosphatidate	Decreased	459,2509403	7,335100571	-1.1988	0.035019	1.4557
4'-Methoxymucidin	Decreased	289,1425849	4,99864754	-1.3812	0.035681	1.4476
10,11-Dihydro-12R-hydroxy-leukotriene E4	Increased	458,2598703	4,47388583	1.6447	0.036303	1.4401
Glycitin	Decreased	573,1634329	7,868167608	-1.1468	0.03835	1.4162
Avocadyne	Increased	285,2428501	3,501653108	1.1384	0.039321	1.4054
Isoleucyl-Valine	Increased	231,171352	2,061072173	1.076	0.039435	1.4041
Gentian Violet	Increased	374,2558454	3,751913065	1.9129	0.040723	1.3902
Octyl gallate	Decreased	283,1553841	5,316013293	-1.4905	0.041134	1.3858
2-(2-Phenylacetoxy)propionylglycine	Increased	288,0858228	3,593795484	1.8007	0.044547	1.3512
Perulactone	Decreased	518,3248691	7,336462688	-1.2348	0.047578	1.3226
Goyaglycoside c	Increased	701,3967938	4,051539975	1.2515	0.047939	1.3193
N-Benzoylaspartic acid	Increased	221,0431358	0,64250588	1.5991	0.05194	1.2845

Bopindolol	Increased	381,2195378	3,668042783	1.5788	0.05252	1.2797
Asparaginyl-Tryptophan	Increased	319,138105	3,486646721	3.0286	0.053722	1.2698
SM(d18:1/16:0)	Increased	703,5793471	10,63285646	-1.4657	0.056868	1.2451
N-Methylcalystegine C1	Decreased	206,1012984	1,53373558	-2.3869	0.05782	1.2379
TG(10:0/13:0/8:0)	Increased	569,4783005	3,499429847	1.0974	0.063244	1.199
1,7-Dimethylguanosine	Increased	312,1319057	1,49724089	1.1613	0.063284	1.1987
L-Isoleucine	Increased	154,0843533	0,8951027	1.0668	0.063771	1.1954
Cinitapride	Decreased	403,2413025	6,567497861	-1.0455	0.064132	1.1929
Droperidol	Increased	380,1836488	3,686357304	2.0676	0.066756	1.1755
Sphingosine 1-phosphate	Decreased	380,2536836	3,476536946	-1.3958	0.068265	1.1658
Bisnorcholic acid	Decreased	381,2612975	6,572005399	-1.3293	0.069739	1.1565
2,8-Dihydroxyquinoline-beta-D-glucuronide	Increased	338,0890261	3,491465548	2.3139	0.073052	1.1364
N-lactoyl-Tryptophan	Increased	277,1197435	3,756332399	-1.0743	0.073217	1.1354
Octaethylene glycol	Increased	371,2294522	3,761832464	2.6577	0.076824	1.1145
Glycerophosphocholine	Increased	280,0934632	0,575403072	1.1157	0.079158	1.1015
LysoPC(18:1(9Z)/0:0)	Increased	522,35857	7,493280607	1.6051	0.08328	1.0795
LysoPC(16:0/0:0)	Decreased	534,2984664	7,337556348	-1.231	0.084265	1.0744
Indolelactic acid	Decreased	206,0820247	3,720794261	-1.1718	0.085623	1.0674
Hexaethylene glycol	Decreased	283,1763342	3,521405267	2.5457	0.089211	1.0496
1,25-Dihydroxyvitamin D3-26,23-lactone	Decreased	427,2861672	4,83953313	-1.7432	0.091519	1.0385
L-Palmitoylcarnitine	Decreased	400,3443904	6,225972788	-3.106	0.093036	1.0313
Syndesine	Increased	305,1586757	3,521899666	2.243	0.094419	1.0249
Methylscopolamine	Decreased	319,1799351	4,028794104	-1.075	0.094522	1.0245
Annoglabasin F	Decreased	378,2425486	6,102933918	-1.5295	0.094603	1.0241
Fenpropimorph	Decreased	304,2626401	6,807268957	-1.2293	0.094916	1.0227
PE(14:0/20:2(11Z,14Z))	Decreased	716,5271732	7,845755495	-1.1608	0.095153	1.0216
5-Bromotryptophan	Decreased	283,0092158	3,695454206	-1.6787	0.095535	1.0198
6-Keto-decanoylcarnitine	Increased	330,2292062	3,982591913	1.2981	0.095986	1.0178
Androsterone	Decreased	291,2320717	4,770732106	-1.3982	0.096436	1.0158
SM(d18:1/18:1(11Z))	Increased	729,5950664	7,858224677	-1.229	0.097037	1.0131
1-(9Z-tetradecenoyl)-glycero-3-phosphate	Increased	381,201437	3,797491647	1.0924	0.097244	1.0121

Increased and decreased indicates that metabolites were up regulated and down regulated in perinatal complications respectively. LysoPC; Lyso-phosphatidylcholines, LysoPE; Lyso-phosphatidylethanolamine, PC; phosphatidylcholine, PE; phosphatidylethanolamine, SM; Sphingomyelin, Cer; Ceramides.

Table 4.9. Significant metabolites after *P*-value adjusted (False Discovery Rates)

Compounds Name	**log2(FC)**	**p. adjusted**	**-log10(p)**
A	-2.7202	0.052166	1.2826
B	-2.5469	0.052166	1.2826
C	-2.4034	0.052166	1.2826
D	-2.2324	0.052166	1.2826
E	-1.9546	0.052166	1.2826
F	-1.796	0.052166	1.2826
G	1.556	0.052166	1.2826
H	-2.4483	0.063903	1.1945
I	-2.058	0.063903	1.1945
J	-2.1335	0.072534	1.1395


A
Original Conc.
75000
50000
25000
0
APBOs&Labor complications
Control
Normalized Conc.
1.0
0.5
0.0
-0.5
-1.0
-1.5
APBOs&Labor complications
Control
B1
Original Conc.
1e-05
5e-04
0e-00
APBOs&Labor complications
Control
Normalized Conc.
2
1
0
-1
APBOs&Labor complications
Control
B2
Original Conc.
1000000
750000
500000
250000
0
APBOs&Labor complications
Control
Normalized Conc.
1
0
-1
APBOs&Labor complications
Control
B3
Original Conc.
50000
25000
0
APBOs&Labor complications
Control
Normalized Conc.
1.5
1.0
0.5
0.0
-0.5
-1.0
APBOs&Labor complications
Control
B4
Original Conc.
5e+05
0e+00
APBOs&Labor complications
Control
Normalized Conc.
2
1
0
-1
APBOs&Labor complications
Control
B5
Original Conc.
50000
25000
0
APBOs&Labor complications
Control
Normalized Conc.
1.0
0.5
0.0
-0.5
-1.0
APBOs&Labor complications
Control

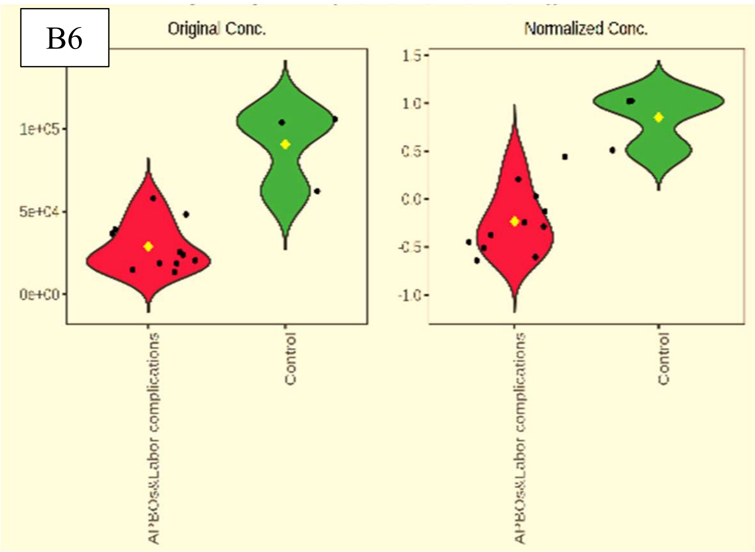


Figure 4.7: Violin plot of the significantly differentially expressed metabolites (potential biological markers) between perinatal complications and healthy groups, after *P* value adjusted. (A) indicates significantly up-regulated metabolite while (B1-6) indicating significantly down-regulated metabolites in perinatal complications compared to healthy controls.

4.3.3. Metabolomic Pathways Analysis of Differential Metabolite

MetaboAnalyst, Version 6.0 was utilized to conduct a more detailed analysis of the most relevant potential biomarkers (122 metabolites) pathways and enrichment analysis. Table 4.6 presents the ten pathways exhibiting the highest correlation, while the bubble graph in Figure 4.9 B displays the topological impact factors and enrichment analysis p-values associated with these pathways. By employing a screening criterion of either $p < 0.05$ or impact > 0.2, we identified three metabolic pathways demonstrating a significant influence on perinatal complications. The major pathways (statistically significant) identified were Ether lipid metabolism, Sphingolipid metabolism, and Glycerophospholipid metabolism. In addition, topology analysis demonstrated that ether lipid metabolism and glycerophospholipid metabolism had the highest enrichment coefficients. Among the three statistically significant pathway, two metabolites were identified in Ether lipid metabolism, two in Sphingolipid metabolism, and two in Glycerophospholipid metabolism (Figure 4.8C1-3 and Table 4.6).

Among the identified potential biomarkers for negative pregnancy and obstetric complications; both 1-Alkyl-2-acylglycerophosphoethanolamine, Sphingosine 1-phosphate, Phytosphingosine, Phosphatidylethanolamine, sn-Glycero-3-phosphocholine were decreased in cases compared to healthy controls (Figure 4.10). Furthermore, sn-Glycero-3-phosphocholine was involved in two statistically significant pathways. Utilizing metabolic pathway analysis based on the KEGG database, we employed topology analysis, in addition to classic enrichment analysis, to assess the impact of each pathway. Our findings revealed that the differential metabolites identified in the cases and control groups were enriched in several functional pathways. Among the top 25 pathways analyzed, Betaine, Estrone, and Methylhistamine metabolism were significantly enriched in perinatal complications and normal pregnancy outcomes with $P<0.05$ (Figure 4.9A).

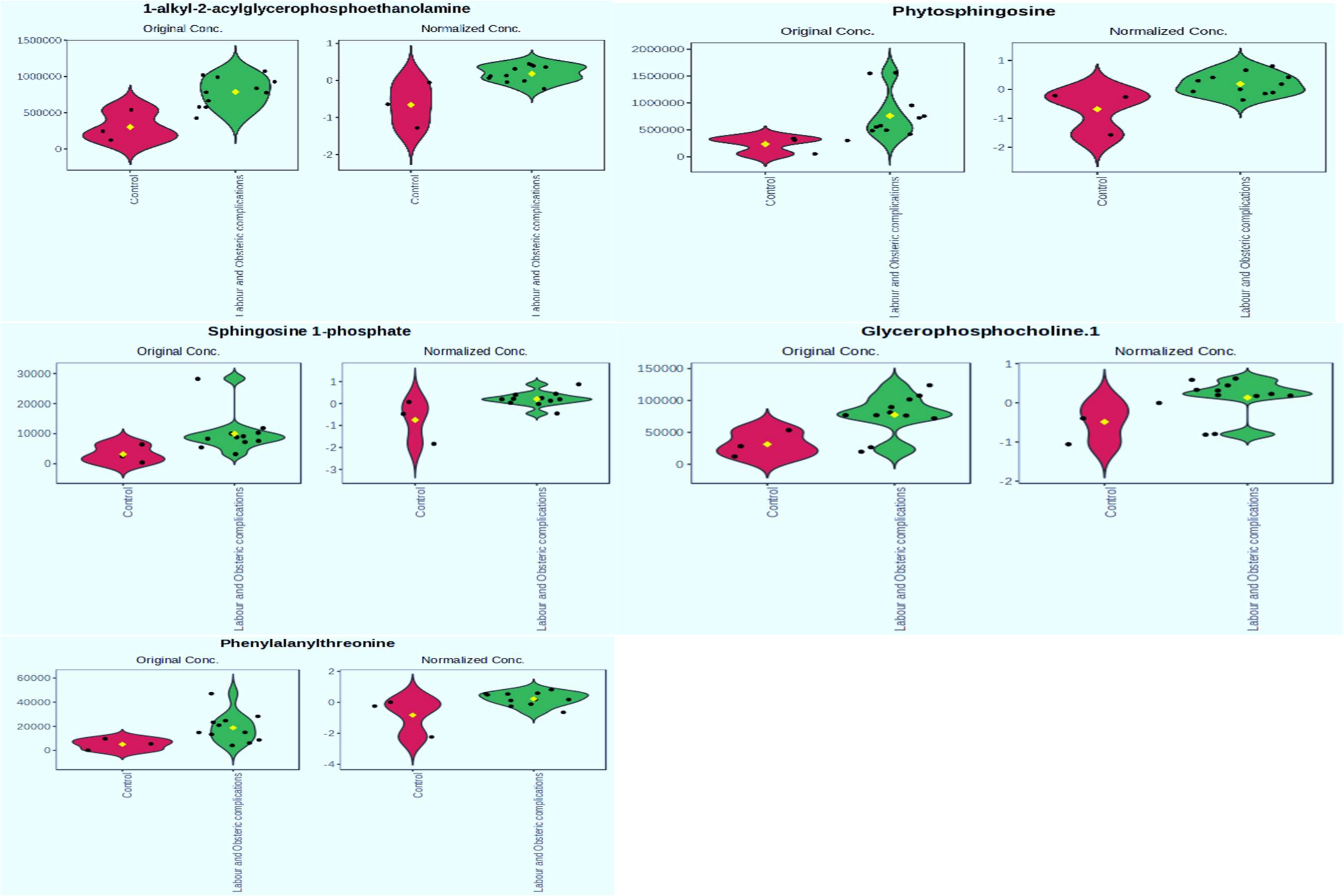


Figure 4.8. Violin plot of key metabolites involved in statistically significant metabolic pathways: abundance of differential metabolites in three metabolic pathways related to perinatal complications.

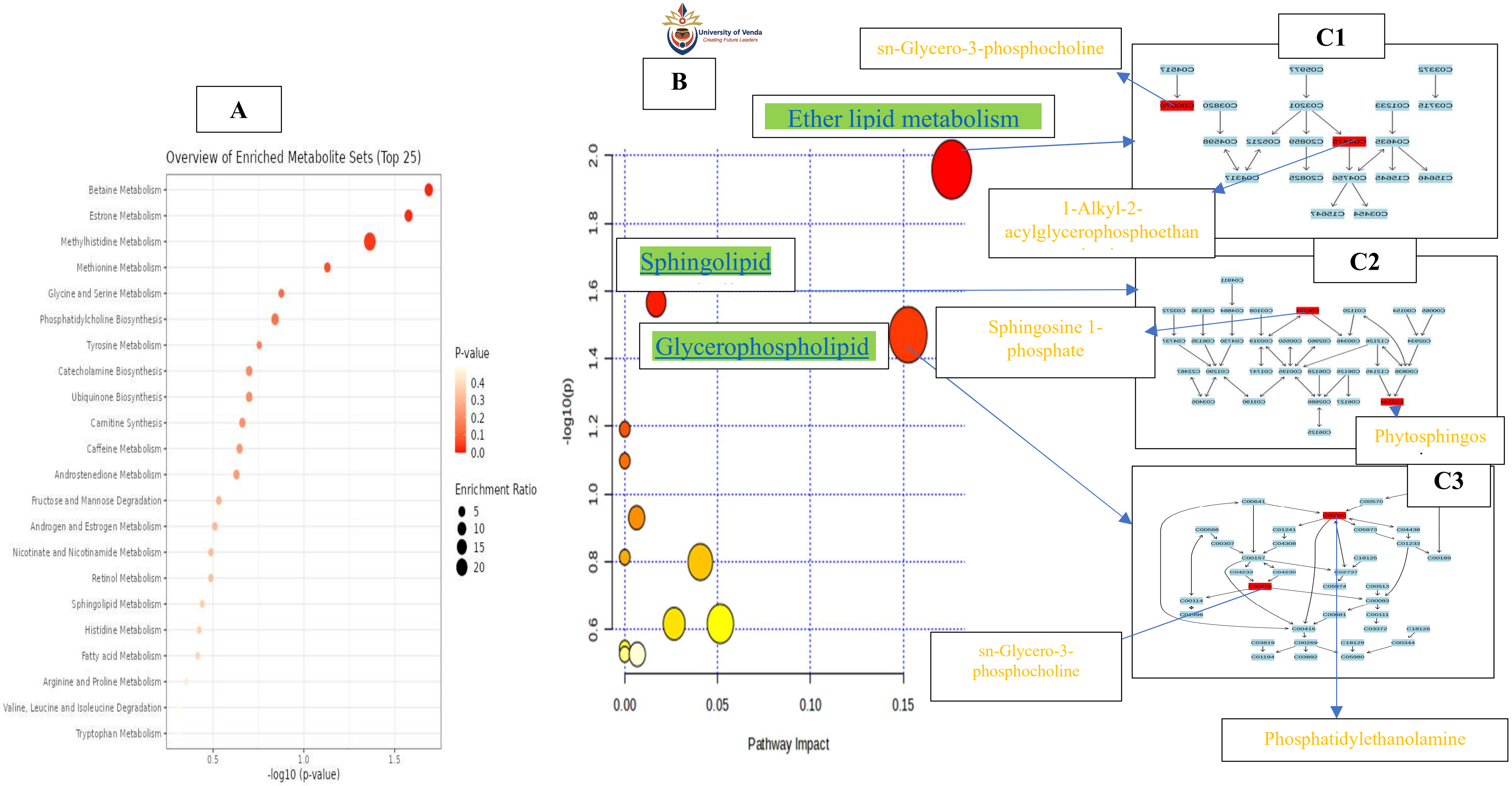


Figure 4.9: Functional and Pathway analysis of Umbilical cord serum Up and Down regulated potential biomarkers. MetaboAnalyst 6.0 was employed for both pathway enrichments in KEGG database (A) and the metabolome view map of relevant metabolic pathway analysis (B) of these distinct metabolites. The pathway impact values on the x-axis and the -log10 (p value) on the y-axis are utilized to illustrate the influencing factor in topological analysis and the p value from the pathway enrichment analysis, respectively. Node size signifies the influence factor in topological analysis, while node color indicates the p value in the enrichment analysis. Significance in vital metabolic pathways is attributed to those with a p value < 0.05 and an impact value exceeding 0. (C1, C2 and C3) Indication of the topological characteristics of the Ether lipid metabolism, Sphingolipid metabolism, Glycerophospholipid metabolism pathways (the 3 statistically significant pathways) with integrated matched metabolites respectively.

Table 4.10: Metabolic Pathways of significant up and down regulated metabolites perinatal complications.

Pathway Name	**Identified metabolites**	**p**	**-log(p)**	**Holm p**	**FDR**	**Impact**
Ether lipid metabolism	sn-Glycero-3-phosphocholine 1-Alkyl-2-acylglycerophosphoethanolamine	0.010993	1.9589	0.87941	0.87941	0.1761
Sphingolipid metabolism	Sphingosine 1-phosphate Phytosphingosine	0.027136	1.5665	1.0	0.90217	0.01688
Glycerophospholipid metabolism	Phosphatidylethanolamine sn-Glycero-3-phosphocholine	0.033832	1.4707	1.0	0.90217	0.15263
Valine, leucine and isoleucine biosynthesis	L-Isoleucine	0.064294	1.1918	1.0	1.0	0.0
Caffeine metabolism	Caffeine	0.07976	1.0982	1.0	1.0	0.0
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	Phosphatidylethanolamine;	0.1174	0.93034	1.0	1.0	0.00639
Fructose and mannose metabolism	6-Deoxy-L-galactose	0.15361	0.81358	1.0	1.0	0.0
Steroid hormone biosynthesis	Androsterone Estradiol-17beta	0.15864	0.79958	1.0	1.0	0.04071
Cysteine and methionine metabolism	S-Adenosyl-L-homocysteine	0.24145	0.61718	1.0	1.0	0.02659
Glycine, serine and threonine metabolism	Betaine;	0.24145	0.61718	1.0	1.0	0.05149
Valine, leucine and isoleucine degradation	L-Isoleucine	0.28518	0.54488	1.0	1.0	0.0
Amino sugar and nucleotide sugar metabolism	6-Deoxy-L-galactose	0.29724	0.5269	1.0	1.0	0.0
Tyrosine metabolism	3,4-Dihydroxymandelate	0.29724	0.5269	1.0	1.0	0.00674

Note: -log (p) represents the original *P*-value derived from the enrichment analysis, while Impact indicates the pathway impact value determined through pathway topology analysis.

CHAPTER FIVE: DISCUSSION AND CONCLUSION

5.1. DISCUSSION

Despite the national intervention and strategies aimed at early detection and identification of pregnancy and obstetric complications, aiming to reduce neonatal mortalities, neonatal mortality rates remain high in low- and middle-income countries (Gülmezoglu et al., 2016; Rambliere et al., 2023). Serum metabolomic profiling, PCR detection of bacteraemia, and antibiotic susceptibility profiling play a significant role in the diagnosis, treatment, and management of various pregnancy and birth complications, ultimately contributing to improved health outcomes (El-Amir et al., 2019; Straňák et al., 2022; Ussher et al., 2016).

Specifically, metabolomic profile analysis allows for the identification of unique metabolite signatures associated with several perinatal complications and can potentially lead to the discovery of biomarkers for these complications (Souza et al., 2019). Consequently, molecular detection of blood bacterial infection through 16S rRNA PCR assays facilitates the rapid and accurate identification of specific bacterial species causing infections in perinatal complications, thereby informing antibiotic selection and dosing strategies (Eichberger et al., 2022). Antibiotic susceptibility profiling, on the other hand, plays a crucial role in guiding antibiotic therapy by ensuring the selection of the most effective antibiotic for treating infections associated with perinatal complications while minimizing the risk of antibiotic resistance (Martinez de Tejada., 2014; Ibrahim et al., 2024).

To date, there has been limited scientific investigation into the associations between perinatal complications and both bacteraemia and umbilical cord blood metabolic profiles. The present study investigated the metabolomic profile, detection of bacteraemia, and antibiotic susceptibility profile in perinatal complications, aiming at reducing the risk of pregnancy complications and ultimately lowering neonatal mortality in both mothers and neonates in the Vhembe district.

5.1.1. The Prevalence of Perinatal Complications among the Participants

In all the studied populations, the overall occurrence rate of perinatal complications was determined to be 35/129 (27.13%). Among these, adverse pregnancy and birth outcomes (APBOs) were reported in 9 cases (6.98%) and labor or obstetric complications in 26 cases (20.15%). These figures are higher than the 16% rate observed in KwaZulu-Natal Province, South Africa (Hoque., 2011) and the 19.4% reported by Getaneh et al., (2021) in Northwest Ethiopia. This could imply that perinatal complications are still a public health issue, highlighting the need for interventions such as advising pregnant women to seek early initiation of antenatal care to minimize neonatal mortality.

In contrast, the prevalence rate was lower than figures found in study conducted by Hortence et al., (2022) (49.1%) and Melese et al., (2019) (46.5%). Notably, Hortence et al., (2022) found that complications were dominated by prematurity at a rate of 35%, a discrepancy from the present study's findings. Discrepancies in findings may be due to differences in the study population, diagnostic criteria used to define and diagnose perinatal complications, and disparities in healthcare infrastructure such as the availability of antenatal care, emergency obstetric services, and skilled birth attendants (Tamale et al., 2022; Getaneh et al., 2021). Additionally, confounding factors associated with perinatal complications may contribute to these disparities.

5.1.2. Sociodemographic and Clinical factors associated with Perinatal Complications.

The present study further explores the sociodemographic and clinical factors associated with perinatal complications among both mothers and neonates. Sociodemographic and clinical factors, including maternal blood pressure, maternal age, birth weight, anesthesia use, and delivery mode, were identified as significantly associated with perinatal complications in the present study.

Evidence has suggested that there is a paucity of data on the associations between perinatal complications and clinical factors such as maternal blood pressure (Ahenkorah et al., 2022). Hence, this cross-sectional study found that perinatal complications were significantly associated with maternal blood pressure. Compared with normal pregnancy complications, participants with abnormal blood pressure demonstrated a high risk of developing perinatal complications. Notably, perinatal complications also presented normal blood pressure. Although this study could not determine the significant associations between the gestational booking stage (antenatal care booking) and perinatal complications, our findings illustrated that most participants (20/33) who developed complications had a late ANC attendance, during the second and third trimesters. These could not be ruled out as one of the reasons for the significant associations between perinatal complications and maternal blood pressure. This highlights the significance of monitoring and maintaining the maternal blood pressure to a low level of less than 110 mmHg/<65 mmHg during ANC to minimize the risk of developing perinatal complications.

The present study's findings align with those of several other studies, including Parikh et al., (2017), Jung et al., (2022), Zhu et al., (20109), and Gu et al., (2022) which have identified a significant association between abnormal blood pressure during pregnancy and labor and the risk of perinatal complications. In contrast to the findings from the study in Swedish, Wikström et al., (2016), China, Li et al., (2016), and in New York, Thermidor et al., (2023) were unable to find these correlations. The disagreements might be due to the heterogeneity among study populations, timing of blood pressure measurement, and potential covariates including maternal age, BMI, and smoking status.

This study utilized data collected from the participants in the selected district hospitals of the Vhembe district, Limpopo Province, to examine the association between the maternal age group and the risk of perinatal complications. Consistent with previous studies conducted in the United States and China by Cavazos-Rehg et al. (2015) and Li et al. (2013), respectively, the findings revealed that both adolescent

(10–19 years) and older (30–39 years) maternal age at the time of delivery were positively associated with the risk of developing perinatal complications compared to adult maternal age (20–29 years). Scientific evidence has indicated that, as women get older the endothelial response to vasodilators diminishes, maximizing the risk of developing perinatal complications (Franklin et al., 2001; Mehariet al., 2020). This scientific evidence might be attributed to the present study's findings on older maternal ages and its association with perinatal complication. Ultimately, the reasons for this finding in the adolescent maternal age group might be multifactorial including physiological immaturity of teenagers, inadequate maternal nutrition, lack of prenatal care, increased risk behaviors, and psychosocial factors.

Contrary to the present study’s findings, in India, by Nagarwal et al., (2015), (Berenson et al., 1997) which failed to find significant association between maternal age and perinatal complications. The disagreement between these findings may be attributed to variations in sociodemographic and sample heterogeneity. Overall, the findings of this study highlight the importance of understanding the unique challenges and risk factors associated with different maternal age groups to develop targeted interventions and strategies to minimize the risk of perinatal complications.

In addition to the factors associated with perinatal complications, this study demonstrated that perinatal complications were associated with foetal weight. Thus, the present study revealed that mothers who gave birth to LBW (birth weight below 2500g) neonates had a 53.3% risk of developing perinatal complications compared to normal birth weight. In line with the findings from the study by Chen et al., (2016) in China, which found that the rate of caesarean section and incidences of neonatal stillbirth, asphyxia, and neonatal death were significantly higher in LBW infants compared to normal birth weight, another study in Ethiopia also supported the present study’s findings by showing a 63% higher risk of developing adverse pregnancy outcomes among mothers who delivered LBW neonates (Tadese et al., 2022).

These consistent findings across various studies and regions underscore the need for foetal weight as a determinant of perinatal complications. LBW serves as an indicator of increased risk for complications that negatively affect foetal growth and lead to perinatal complications. The reason for these results might be due to poor maternal nutrition during pregnancy, gestational age at delivery, and genetic and epigenetic factors.

The present study examined whether Caesarean section, vaginal birth, and anaesthesia for C-section are associated with perinatal complications, in addition to factors associated with perinatal complications. As expected, Caesarean section delivery mode and anaesthesia for C-section appear to be strongly associated with perinatal complications in the present study compared to vaginal birth. These findings are in accordance with the findings from other studies in Australia (Shamsa et al., 2013) and Thailand (Kongwattanakul et al., 2020), which demonstrated that Caesarean section delivery was significantly associated with increased risk factors of maternal death as well as postpartum haemorrhage compared to vaginal delivery, respectively.

As a means to reduced mortalities associated with perianal complications, a C-section is recommended as a crucial intervention that can hinder severe perinatal complications that could lead to maternal and neonatal mortality at birth (Kiruja et al., 2023). This recommendation may explain the reason for the strong associations between C-sections and perinatal complications in the present study. During the preparation of the Caesarean section delivery procedure, pregnant women are given either general or spinal anaesthesia agents to further minimize the risks associated with the delivery method (Balinskaite et al., 2017). Consistent with the findings of Bao et al., (2022), our study also demonstrated the strong correlations between anaesthesia for C-section and perinatal complications, likely due to the routine administration of anaesthesia agents to pregnant women before C-section procedures. This study's findings highlight the importance of these interventions in minimizing adverse outcomes during childbirth.

5.1.3. Bacterial infections in Umbilical Cord Blood after Delivery and associated the risk factors.

Scientific evidence has indicated that umbilical cord infection can be preventable and reduced through interventions including avoiding harmful practices, practicing clean delivery, and maintaining clean cord care (Coffey et al., 2017; Turyasiima et al., 2020). In this study, we employed conventional blood culture and a 16S rRNA PCR assay to detect and identify bacterial pathogens in umbilical cord blood after delivery of complicated and non-complicated pregnancies at district hospitals in the Vhembe district, Limpopo Province.

In our study, the positivity rate of bacteraemia determined by conventional blood culture method was 30,23% (39/129), conflicting with the higher positivity rate from previous studies in Ethiopia (Shitaye et al., 2010), Tanzania (Blumenröder et al., 2023), and another study in Tanzania (Kayange et al., 2010) which reported rates of 60%, 57%, and 47%, respectively. The present study findings are comparable to the 31.75% rate reported in Zimbabwe by Nathoo et al., (1993) and another study conducted by Shah et al., (2012) which demonstrated a 31.57% positivity rate.

However, other studies have demonstrated lower positivity rates, such as the study conducted by Hayder Hamad et al., (2023) which reported a rate of 7.06%, and the study by Fanaroff et al., (2007) which reported a rate of 8.7%. These disparities in positivity of blood culture results may be attributed to variations in blood culture techniques such as differences in incubation times, culture media utilized, and sampling techniques. Additionally, antimicrobial agents administered prior to sample collection may also suppress bacterial growth, resulting in a false-negative result. Potential misdiagnosis or false positives due to possible contamination during sample handling may also contribute to these disagreements.

The findings of the present study revealed that the predominant bacterial isolates were gram-positive bacteria, accounting for 58.06%, followed by gram-negative bacteria with a frequency of 26.36%. Similarly, several studies have demonstrated that the most frequent aetiological agents were gram-positive bacteria (Draz et al., 2013; Jordan and Durso., 2005; Kohli-Kochhar et al., 2011; Lealet al., 2012). Contrary to our findings, studies conducted in Tanzania (Kayange et al., 2010), Iraq (Hayder Hamad et al., 2023), and India (Bhat et al., 2011) illustrated that gram-negative bacteria were the predominant bacterial isolates, with positivity rates of 61.1%, 83.34% and 90.8%, respectively. The reasons mentioned above under positivity rate may also contributed to the variation in the occurrences of gram-positive and gran-negative bacteria.

Regarding the most common bacterial pathogen, the present study identified *S. epidermidis* as the most common isolate (36.56%) responsible for bacteremia which was in contrast with the findings of previous studies that reported different pathogens. For instance, Draz et al., (2023), Sriram et al., (2011), and Karthikeyan et al., (2001) found that the most common pathogen responsible for septicemia was *S. aureus*. Furthermore, the disagreement was also observed in a study conducted by Hayder Hamad et al. (2023) and Haque et al., (2014) which found that *K. pneumoniae* and CoNS were responsible for bacteremia, respectively.

The predominance of *S. epidermidis* among the participants in our study may be attributed to its presence in the normal flora of human skin and mucous membranes, making it prone to contaminating blood streams during sampling procedures (Lee and Anjum., 2023; Kleinschmidt et al., 2015). Additionally, surgery and invasive procedures might have contributed to *S. epidermidis* being a major cause of bacteremia, as these procedures disrupt the skin barrier and introduce bacteria into the bloodstream (Ehlers et al., 2028).

In connection with perinatal complications, our culture results revealed that infection with *E. coli* was significantly associated with the risk of developing perinatal complications. This is consistent with the results of studies by McClure and Goldenberg., (2009) and Obiero et al., (2022) which demonstrated that infection with *E. coli* was associated with stillbirth and neonatal death, respectively. However, contrary to our findings, Goldenberg et al., (2008) found that infection with *Ureaplasma urealyticum* and *Mycoplasma hominis* as detected through umbilical cord blood culture, was significantly associated with the risk of having preterm and adverse neonatal outcomes. Additionally, Sweeney et al., (2016) exhibited that *Ureaplasma spp.* was associated with late preterm. The disparities in the findings highlight the complexity of bacterial infections in the context of perinatal complications, suggesting that various bacterial pathogens may have different effects on pregnancy and neonatal outcomes. Hence, further research utilizing advanced biological techniques may be needed to fully understand the mechanisms and implications of these associations.

Despite conventional blood culture methods being considered the gold standard for diagnosing bacteremia, growing evidence suggests that this remains challenging (Huber et al., 2020). Notably, the process may take more than 48 hours to obtain the results, and previous exposure to antibiotic agents often interferes with blood culture growth (Dutta et al., 2009). Interestingly, a systematic review by Pammi et al., (2011) found that molecular assays offer a promising alternative, particularly in neonatal populations, providing rapid detection times compared to conventional blood culture methods. In this study, broad range 16S rRNA PCR analysis of umbilical cord blood sample from neonates and mothers revealed that 34/129 (26.36%) samples were positive for the presence of bacterial DNA. This was lower than 30,23% (39/129) detection rate by using blood culture methods. Contrary to the findings of several studies that reported higher detection rates using PCR assays compared to traditional blood culture methods (Draz et al., 2023; Draz et al., 2013; Yadav et al., 2005), the results of our study did not support this trend. However, our findings are consistent with those of the study by Van den Brand et al., (2018) which reported 58% of episodes of neonatal sepsis by multiplex PCR and 66% by blood culture. Despite this agreement, possible contamination cannot be ruled out as a potential reason for any discordant results observed between the two methods.

Although there was a significant difference in positivity rates between blood culture and 16S rRNA PCR assays (*P*<0.001) in the present study, the sensitivity, specificity, positive predictive value, and negative predictive value of the 16S rRNA PCR assay were 56.41%, 82.11%, 64,71%, and 86.67%, respectively. These findings are comparable with those of a study by Draz et al., (2013), which demonstrated a sensitivity of 71.42%, a specificity of 31.81%, a positive predictive value of 57.14%, and a negative predictive value of 46.66%. However, much better findings were reported by Van de Brand., (2018), with a sensitivity, specificity, positive predictive value, and negative predictive value of 77%, 81%, 87%, and 68%, respectively. Contrary to our finding, the above-mentioned PCR results yielded no significant association in detection rate compared to blood culture (*P* = 0.17) (Van de Brand., 2018). These disparities between PCR and blood culture results may be attributed to the use of different primers with varying sensitivities for detecting bacteremia. Additionally, the lack of standardized clinical tests specifically designed for detecting pathogenic nucleic acids in umbilical cord blood could also contribute to these discrepancies. Therefore, further research and development are necessary to address these challenges and enhance diagnostic capabilities for pathogen detection at the nucleic acid level.

Although the present study failed to demonstrate the associations between bacteremia and perinatal complications (*P* = 0.13), there is growing evidence that there are strong associations between blood bacterial infections and adverse pregnancy and neonatal outcomes. For instance, Li et al., (2021) demonstrated the significant associations between an infection with *Helicobacter pylori* with the risk of developing GDM, while a study by Ye et al., (2022) have associated an infection with *Neisseria spp.* with preterm low birth weight. These disagreements in the findings from the present study and other studies may be attributed to the difference in the studies sample size, heterogeneity of pathogens, and inaccuracies in the measurement of perinatal complications.

Furthermore, this study examined the potential risk factors associated with bacteremia, providing valuable insights for the early detection and management of risk factors associated with bacteremia. Hence, sociodemographic, and clinical factors including maternal age, HIV status, marital status, maternal employment status, maternal monthly income status, educational status, maternal blood pressure, pre-pregnancy BMI, gestational booking stage, delivery mode, and neonate sex were maternal and neonatal factors for bacteremia included in the study. Interestingly, maternal education level, maternal blood pressure, pre-pregnancy BMI, and gestational booking stages were all significantly associated with blood bacterial infections in the present study.

Regarding the maternal education level, the present study exhibited that 23 (67.65%) of mothers who attended high school education were significantly associated with an increased risk of bacteremia either at delivery or during pregnancy. These findings contrast with previous studies by Koch et al., (2014) and Burton et al., (2010), which found that individuals with lower education levels were significantly associated with a higher risk of bacteremia and bacteremic pneumonia, respectively. Likewise, this is consistent with the findings from a study by Oestergaard et al., (2017) and Flory et al., (2009) which illustrated that having a high school education was associated with a high risk of having *S. aureus* bacteremia and pneumococcal pneumonia, respectively. The disagreement between the study findings might be explained by the differences in the sample sizes of the studies. Hence, the present study included a limited number of individuals without a high school education, which might have influenced the observed associations.

In addition to identifying risk factors associated with bacteremia among mothers and their neonates, our study revealed a positive correlation between bacteremia and pre-pregnancy BMI at a P-value (P = 0.01). Notably, bacterial blood infection was prevalent among the obese group (41.18%). Consistent with our findings, Bang et al., (2014) revealed the associations between bacteremia and BMI with a P-value (P = 0.04), with obesity identified as a risk factor. Another study in Finland by Huttunen et al., (2007) also reported a strong association between obesity and cases of deaths linked to bacteremia. Despite the sample size and study populations in all these studies findings collectively suggest obesity is an emerging independent risk factor for bacteremia and mortality after infections, emphasizing the need for future studies (Milner. and Beck., 2012).

Recently, the WHO has recommended that pregnant women undergo a minimum of 8 antenatal care (ANC) visits to minimize the risk of infections and APBOs going undetected and untreated (Fagbamigbe et al., 2021). Thus, pregnant women should initiate ANC early during the first trimester. Hence, the present study explores the associations between the gestational first booking stage and the risk of bacteraemia. As expected, our findings reveal a significant association between gestational booking stage and with the risk of umbilical cord bacteraemia ($P = 4{,}43\times^{-04}$). Specifically, booking during the third trimester was associated with a notably high risk of bacteraemia (44.12%). These results highlight the importance of timely initiation of ANC in reducing the risk of neonatal bacteraemia and its associated complications. In support of our findings, a study by Teshome et al., (2022) reported that the number of ANCs was significantly associated with the risk of developing early onset neonatal sepsis; thus, mothers who initiated ANC early and received adequate follow-up during their pregnancy were less likely to have neonates with early onset neonatal sepsis. One potential explanation for the association observed in our study is that who initiated their ANC early might be aware of the risk factors or infections might be detected and treated early.

5.1.4. Antibiotic Susceptibility Profile of Bacterial isolates in Mothers and Neonates

Bacteraemia during pregnancy remains a significant complication worldwide, with implications for both developed and developing countries (Suegers et al., 2014; Reimer et al., 2020). The incidence of bacteraemia during pregnancy varies, with studies indicating a threat to foetal life, including a reported 10% incidence of foetal death (Surger et al., 2013). In response to this threat, WHO recommends two empiric antibiotic regimens for the treatment of suspected bacteraemia (Suegers et al., 2014). However, the effectiveness of these interventions is increasingly challenged by the emergence of antibiotic-resistant strains among bacteraemia organisms (Ibrahim et al., 2024). Consequently, the present study further explores the antibiotic susceptibility pattern of commonly used antibiotics against the causative organism of bacteraemia isolated from umbilical cord blood.

Our study analysed the antimicrobial susceptibility profile of gram-positive and gram-negative isolates in accordance with WHO-recommended first- and second-line antibiotics. Conversely, our overall findings of the antimicrobial susceptibility profile showed that most bacterial isolates were resistant to ampicillin, cefoxitin, and ceftazidime, while displaying a high degree of sensitivity to gentamicin, imipenem, amikacin, and ciprofloxacin. In alignment with previous studies in Ethiopia, Egypt, and India (Weldu et al., 2020; Almohammady et al., 2020, and Mohakud et al., 2022). These can indicate that gentamicin, imipenem, amikacin, and ciprofloxacin could be a potential choice for empiric treatment of bacteraemia in the region.

The present study revealed that among the gram-positive isolates, a significant proportion (97.22%) of these isolates exhibited resistance to ampicillin, ceftazidime, and cefoxitin. However, there was notably higher sensitivity towards gentamicin, imipenem, amikacin, and ciprofloxacin, with sensitivity rates of 97.22%, 88.89%, and 75%, respectively. These findings align with a study conducted in Nepal by Yadav et al., (2018), where gentamicin and ampicillin emerged as the most effective and least effective antibiotics against gram-positive isolates, respectively. Additionally, a study by Gyawali and Sanjana., (2013) also identified gentamicin, amikacin, and ciprofloxacin as highly effective antibiotics for gram-positive isolates, aligning with the present study's findings. Similarly, Mythri et al., (2016) reported findings consistent with ours, indicating resistance among gram-positive isolates to cefoxitin.

Although, gram-positive isolates show an approximately average (44.44%) sensitivity or medium to lower resistance (41, 67%) towards erythromycin, this underscores the potential challenges in using it for the treatment of bacteremia in neonates or their mothers, as it might lead to prolonged antibiotic treatment. Our findings are somewhat comparable to those of Birru et al., (2021), who reported lower resistance (30.7%) of gram-positive isolates to erythromycin. These findings highlight the importance of careful antibiotic selection and monitoring in clinical practice.

In contrast to our observations of the antimicrobial susceptibility profile of gram-positive bacterial isolates, it is noteworthy that gram-negative bacteria exhibited resistance to an additional antibiotic, erythromycin, at a rate of 100%, in addition to ampicillin, ceftazidime, and cefoxitin. Emphasizing the known scientific evidence ruling out the use of erythromycin for the treatment of bacteremia or neonatal sepsis caused by the gram-negative organism (Dong et al., 2017). However, the present study finding was in congruence with Muley et al., (2015), who demonstrated resistance of gram-negative bacteremia to ampicillin and ceftazidime in India. Interestingly, our findings are partly consistent, and partly in contrast with a similar study by Viswanathan et al., (2012), where the gram-negative isolates were reported to be highly resistant to gentamicin and ampicillin. The discrepancy in these results may be attributed to the diversity of antibiotic resistance genes in different regions, making it difficult to determine the antibiotic susceptibility profile worldwide.

The overall findings of our study highlight the need to reconsider the ampicillin + gentamicin combination as the first line of drug therapy in terms of ampicillin. This high resistance may potentially be attributed to the inappropriate and indiscriminate use of these antibiotics or could be explained by the emergence of antimicrobial resistance genes in the bacterial isolates. These results highlight the importance of future research focusing on the molecular detection and identification of resistance genes in the study population.

Recently, studies have demonstrated an increase in the emergence of multiple drug resistance in bacterial isolates responsible for bacteremia in neonates. This trend poses significant challenges to the management and treatment of these infections (Pokhrel et al., 2018). Therefore, the present study explores the profile of multiple drug resistance in bacterial isolates from umbilical cord blood. The present study reported the highest prevalence of bacteremia due to MDR strains at a rate of 93.38%. MDR among gram-positive and gram-negative bacterial isolates was 97.22% and 96.30%, respectively, in our study, which is comparable to the prevalence observed in Nepal and Ethiopia (Pokhrel et al., 2018; Birru et al., 2021).

5.1.5. Metabolome profile in perinatal complications and normal pregnancy outcomes

Despite intervention programs such as antenatal care (ANC) aimed at early detection and management of perinatal complications to minimize the risk of maternal and neonatal mortality, the burden of perinatal complications remains significantly high in developing countries (Konje et al., 2018; Vallely et al., 2023). Given that pregnancy is a period of rapid metabolic change, the etiology of many adverse pregnancy and neonatal outcomes remains poorly understood (Mitro et al., 2021). Consequently, metabolomics has emerged as a promising diagnostic or prognostic tool for evaluating and identifying metabolic biomarkers for the identification of pregnancy-related complications (Phillips et al., 2018). Therefore, the present study utilized LC-Q-TOF-MS untargeted metabolomics profiling to identify metabolites (potential biomarkers) in umbilical cord blood that might be useful in identifying perinatal complications.

In this cross-sectional study, we identified 107 significant differential metabolites associated with the risk of perinatal complications using multivariate statistical analysis. Among these, seven metabolic fingerprint demonstrated significant differences between the perinatal complications and non-complicated pregnancies groups after adjustment for false discovery rate (FDR<0.05) using *P*-value adjusted analysis. Our findings suggest that in early pregnancy, the serum level of compound G is significantly rise and that of Compound **A**, compound **B**, compound **C**, Compound **D**, Compound **E**, and Compound **F** are significantly decreased. These findings suggest that these metabolites may serve as potential biomarkers for perinatal complications.

Our study revealed that a significant proportion of the differentially expressed metabolic fingerprints were lipid compounds, specifically glycerophospholipid (compound C and Lysophospholipid), which were reduced in cases of perinatal complications. These findings are partly consistent with a study by Ciborowski et al. (2014), which observed that lipid compounds such as Lysophospholipid could distinguish mothers at risk of delivering large neonates (macrosomia). In further agreement with our results, studies by Ross et al. (2019) and Parrott et al. (2002) conducted in the United States of America demonstrated that an increase in the level of Lysophospholipid is associated with a reduced risk of perinatal complications compared to healthy controls. Lysophospholipid metabolites have long been recognized for their role in cell-to-cell signal transmission and regulation of various aspects of reproduction and parturition, including trophoblast function and placentation (Nagamatsu et al., 2014). These findings underscore the significance of elevated lysophospholipids levels during early pregnancy as a potential indicator of heighted risk for perinatal complications. In general, lysophospholipids are lipid derivatives resulting from the removal of one or both acyl groups through hydrolysis (Ross et al. 2019).

Consistent with our findings, a review by Stubbs and Stubbs, (1978), reported that reducing sn-glycero-3-phosphate compounds is associated with an increased risk of having gestational diabetes mellitus (GDM).

Additionally, Rosenblatt and Bryant-Greenwood., (2010) suggested that sn-glycero-3-phosphate could serve as predictive tool for identifying the risk of preterm birth. Interestingly, on the contrary, Zhou et al., (2017) in China and Ferreira et al., (2021) in Portugal demonstrated that downregulated levels of sn-glycero-3-phosphate were also associated with the risk of human breast cancer and multiple sclerosis, respectively. This suggests a diverse role for sn-glycero-3-phosphate plays in pathogenesis and its potential to serves as a metabolic biomarker (diagnostic tool) for various health conditions. Generally, sn-glycero-3-phosphate is a key intermediate in glycolysis and glycerophospholipid metabolism, playing essential roles in direct regulation of cellular structure, function, and signalling (Faris., 2013; Ahmmed et al., 2020).

Although metabolomics procedures have been applied to study a range of perinatal complications, our literature search reveals a limitation of metabolomics studies addressing the regulation of compound B, compound G, and compound D in this context. These metabolites hold a promise as novel biomarkers for the diagnosing of various perinatal complications. Interestingly, previous studies indicate that elevated levels of compound B are associated with an increased risk of obstructive sleep apnea (Pinilla et al., 2022). Conversely, animal studies have shown a decrease in the regulation of compound G in male suckling piglets exposed to maternal cysteine intake (Ni et al., 2021).

Compound D belongs to the class of organic compounds known as compound D-type alkaloids, which are commonly found in herbs and spices from the genus Daphniphyllum (Liang et al., 2021; Nwankwo et al., 2023). In contrast, compound B is a specific chemical compound belonging to the class of cinnamoyl esters, derived from natural glycoside compounds commonly found in plants (Deshaies et al., 2022). Recent research has highlighted the potential applications of compound B in traditional medicine and pharmaceuticals due to its diverse biological effects (Sánchez et al., 2022), implying that its presence in umbilical cord blood may attributed to the use of pharmaceutical products that containing this compound during pregnancy. On the other hand, compound G is an organic acid derived from adipic acid which is commonly found in certain foods and beverages (Liu et al., 2020; Yang et al., 2008). Finding the presence of compound D, compound B, and compound G in umbilical cord blood in our study suggests the significant of maternal consumption of herbs, beverages, and spice foods during pregnancy. However, the limited scientific evidence for these metabolites underscores the importance of further metabolomics research to explore their roles and associations with perinatal complications.

In this study, perturbed metabolites were utilized to conduct metabolic pathway analysis. A total of 13 metabolic pathways were obtained through KEGG enrichment analysis for both perinatal complications and uncomplicated pregnancies. Among these pathways, we focused on three statistically significant metabolic pathways: ether lipid metabolism, sphingolipid metabolism, and glycerophospholipid metabolism, from the broader category of lipid metabolism. These findings suggest that variations in lipid metabolism have the potential to serve as a preconception molecular signature distinguishing between perinatal complications and normal pregnancy outcomes. The disturbed lipid metabolism in perinatal complications was also investigated by Chen et al., (2021) who indicated that altered lipid metabolism, specifically declining trends in glycerophospholine, is associated with risk of developing GDM. These results are consistent with our findings. Similarly, Lizewska et al., (2018) reported decreased level of lipid metabolism as a perturbed pathway in sPTB, further supporting our findings. Additionally, Feng et al. (2017) demonstrated that alterations in lipid metabolism in pregnant women were associated with an increased risk of miscarriage, providing further validation of our results.

Evidence suggests that lipid metabolism and their levels in the microenvironment can significantly impact embryogenesis and pregnancy outcomes (Marei et al., 2010; Burton et al., 2016). During pregnancy, the placenta plays a crucial role in converting circulating maternal lipids into free fatty acids. These fatty acids are then taken up and processed by trophoblast cells to meet metabolic demands, produce hormones for pregnancy maintenance, and facilitate transfer to the developing fetus (Wild and Feingold, 2023). The biological activities described above provide insight into the alterations observed in our study and are supported by related research.

5.2. CONCLUSIONS

In conclusion, the present study showed that the occurrence of perinatal complications was high, at a rate of 27.13% in the region. Factors including maternal blood pressure, maternal age, birth weight, delivery mode, and the administration of anaesthesia for C-sections were identified as significant contributors to the incidences of perinatal complications among the study participants. These findings underscore the importance of proactive healthcare interventions during antenatal care visits to minimize the prevalence of perinatal complications in the study area.

Additionally, through comprehensive microbial analysis and metabolomics profiling, we can uncover additional insights. Contrary to expectations, our microbial analysis did not find a significant association between bacteraemia and perinatal complications. However, it did reveal a higher prevalence rate of bacteraemia (bloodstream infection). Notably, maternal education level, maternal blood pressure, pre-pregnancy BMI, and gestational booking stages emerged as significant factors associated with blood bacterial infections in our study. Importantly, our study contributes to the understanding of bacterial isolates with a high rate of resistance to commonly used antimicrobial agents. Therefore, these findings emphasize the importance of continued surveillance and management strategies to address antimicrobial resistance. Subsequently, our metabolomics profiling results unveiled a significant correlation between the alterations in umbilical cord blood metabolites during delivery and the occurrence of perinatal complications. Specifically, metabolites including compound G, Compound A, compound B, compound C, Compound D, compound E and Compound F demonstrated close association with perinatal complications. These findings suggest that lipid metabolism, represented by these metabolites, may potentially serve as useful biomarkers to develop strategies for assessment, prediction, and diagnosis, as well as for the identification of promising targets for early intervention in cases of perinatal complications.

5.3. STRENGTH AND LIMITATIONS

The strength of this study lies in its adherence to the most recent approaches recommended by the World Health Organization (WHO) for measuring perinatal complications. Additionally, the collection of umbilical cord blood by skilled midwives using sterile equipment, followed by immediate processing upon arrival at the laboratory, enhances the reliability of the data. However, several limitations were encountered during the study.

Firstly, delays in obtaining ethical clearance from the university and permission to access healthcare facilities from the Department of Health in Limpopo Province hindered planned metagenomic sequencing, impacting our ability to fully understand the microbiome profile's influence on perinatal complications and the participants' metabolic profiles.

Secondly, cultural and religious beliefs in the Vhembe district posed challenges in recruiting participants, with many expressing fears regarding the misuse of their blood samples for sacrificial purposes.

Thirdly, metabolomic profiling was conducted using a smaller number of normal pregnancy controls compared to cases, potentially introducing bias.

Fourthly, in our investigation of bacteremia detection, we employed both the 16S rRNA PCR assay and a culture-dependent method, each presenting distinct limitations. The PCR assay, targeting conserved regions of the 16S rRNA gene, may struggle to differentiate closely related bacterial species or strains, potentially leading to inaccuracies (Peker et al., 2019). Moreover, PCR assays have the propensity to detect DNA from non-viable bacteria, potentially inflating the perceived bacterial load in the sample (Winand et al., 2019). Conversely, culture-dependent techniques may exhibit lower sensitivity compared to PCR assays, particularly for detecting low-level bacteremia. Furthermore, these methods may overlook fastidious or non-culturable organisms present in the sample (Skvarc et al., 2013). Finally, the incidence of perinatal complications in the Vhembe district may be higher than reported, as data collection was limited to selective district hospitals. Additionally, the lack of an overall occurrence rate for the population hinders the generalizability of the results to other settings.

5.4. RECOMMENDATIONS

Based on our findings, it is recommended that a large cohort study be undertaken to establish a broad and diverse dataset. Such an approach would facilitate a comprehensive understanding of the intricate interplay between maternal factors, microbial communities, and metabolic profiles in the context of perinatal complications. By encompassing a larger sample size, this study would boost statistical power, enhance the generalizability of findings across diverse populations, and enable longitudinal analysis. This longitudinal approach is vital for tracking temporal changes in both the microbiome and metabolome, shedding light on their dynamic roles in perinatal health outcomes.

Further research is needed employing high-performance metagenomic sequencing to profile the microbiome in pregnancy, thus allowing for the identification of specific microbial taxa, functional pathways, and the detection of antibiotic resistance genes associated with both normal pregnancies and perinatal complications. Furthermore, we recommend an integrated cross-omics framework to better understand the link between the microbiome and metabolome in the context of perinatal complications. Thus, it enables the identification of specific microbial taxa and metabolic pathways associated with perinatal complications, elucidating the underlying biological mechanisms.

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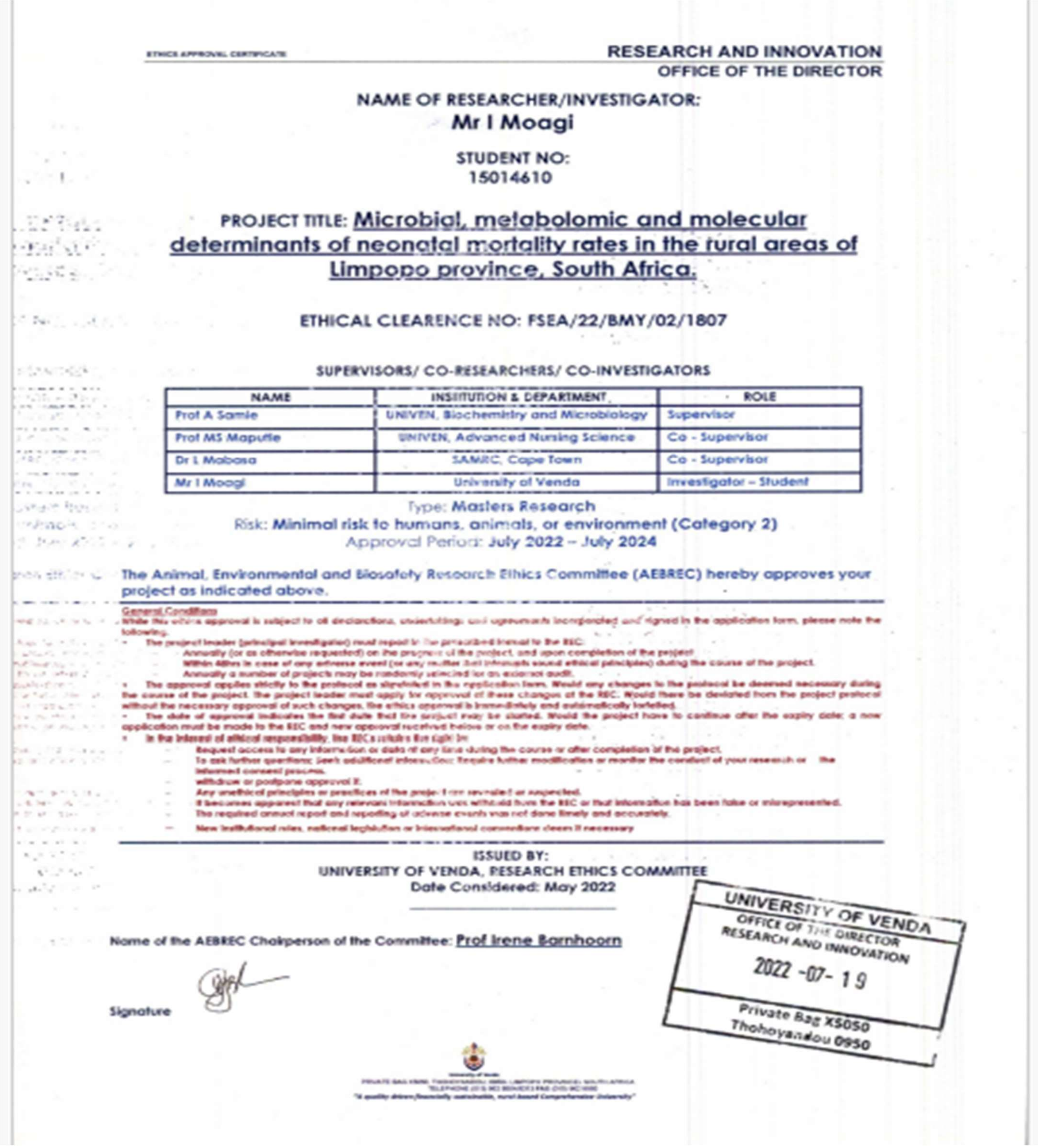
ANNEXURES

Annexure 1: Ethical Clearance and Approval Letters



ETHICS APPROVAL CERTIFICATE

RESEARCH AND INNOVATION
OFFICE OF THE DIRECTOR

NAME OF RESEARCHER/INVESTIGATOR:
Mr I Moagi

STUDENT NO:
15014610

PROJECT TITLE: Microbial, metabolomic and molecular determinants of neonatal mortality rates in the rural areas of Limpopo province, South Africa.

ETHICAL CLEARENCE NO: FSEA/22/BMY/02/1807

SUPERVISORS/ CO-RESEARCHERS/ CO-INVESTIGATORS

NAME	INSITITUTION & DEPARTMENT	ROLE
Prof A Samie	UNIVEN, Biochemistry and Microbiology	Supervisor
Prof MS Maputle	UNIVEN, Advanced Nursing Science	Co - Supervisor
Dr L Mabasa	SAMRC, Cape Town	Co - Supervisor
Mr I Moagi	University of Venda	Investigator – Student

Type: **Masters Research**
Risk: **Minimal risk to humans, animals, or environment (Category 2)**
Approval Period: **July 2022 – July 2024**

The Animal, Environmental and Biosafety Research Ethics Committee (AEBREC) hereby approves your project as indicated above.

General Conditions

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following.

- The project leader (principal investigator) must report in the prescribed format to the REC:
  - Annually (or as otherwise requested) on the progress of the project, and upon completion of the project
  - Within 48hrs in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.
  - Annually a number of projects may be randomly selected for an external audit.
- The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the REC. Would there be deviated from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.
- The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date; a new application must be made to the REC and new approval received before or on the expiry date.
- **In the interest of ethical responsibility, the REC retains the right to:**
  - Request access to any information or data at any time during the course or after completion of the project.
  - To ask further questions; Seek additional information; Require further modification or monitor the conduct of your research or the informed consent process.
  - withdraw or postpone approval if:
  - Any unethical principles or practices of the project are revealed or suspected.
  - It becomes apparent that any relevant information was withheld from the REC or that information has been false or misrepresented.
  - The required annual report and reporting of adverse events was not done timely and accurately.
  - New institutional rules, national legislation or international conventions deem it necessary

ISSUED BY:
UNIVERSITY OF VENDA, RESEARCH ETHICS COMMITTEE
Date Considered: May 2022

UNIVERSITY OF VENDA
OFFICE OF THE DIRECTOR
RESEARCH AND INNOVATION
2022 -07- 19
Private Bag X5050
Thohoyandou 0950

Name of the AEBREC Chairperson of the Committee: **Prof Irene Barnhoorn**

Signature

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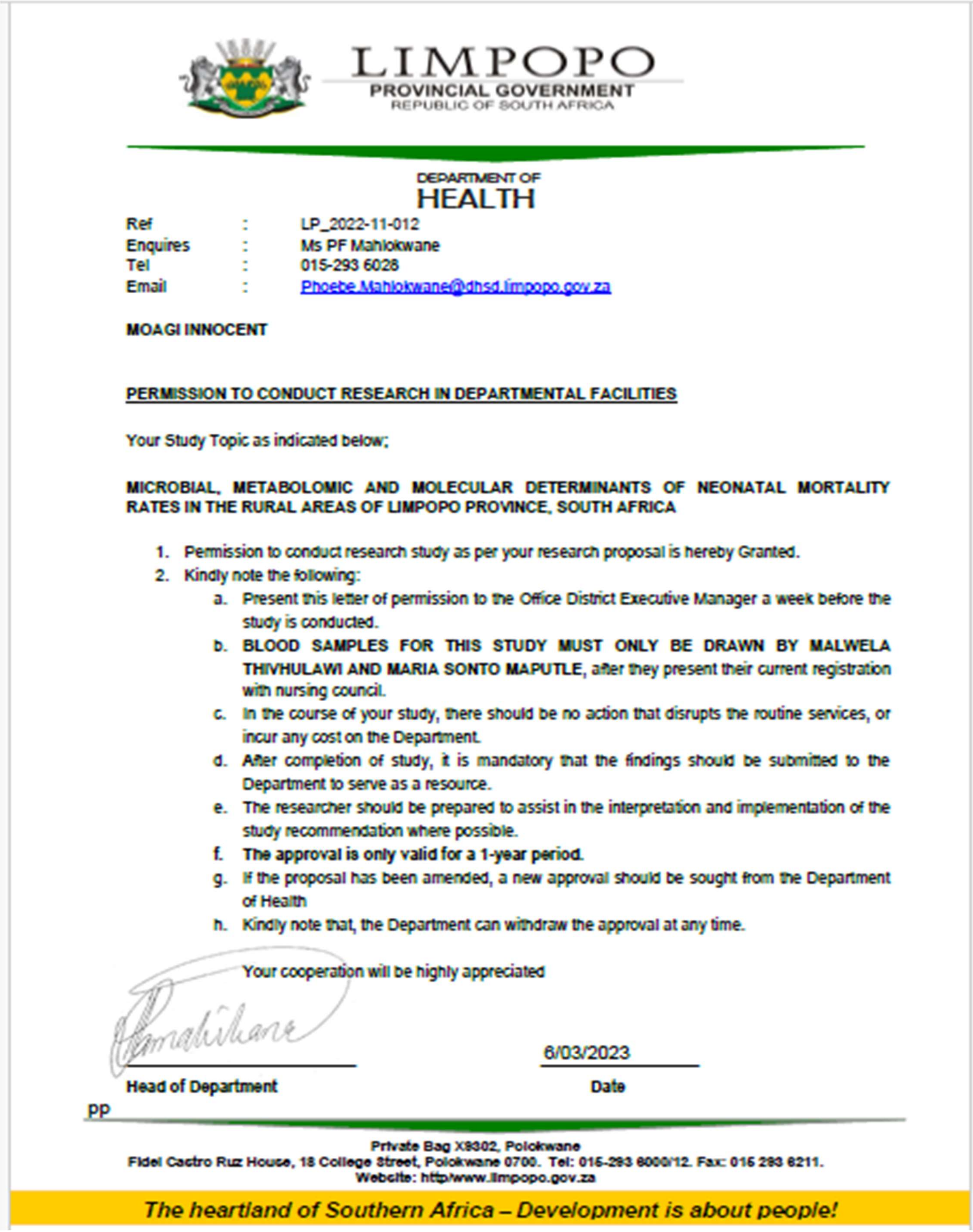


LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF
HEALTH

Ref : LP_2022-11-012
Enquires : Ms PF Mahlokwane
Tel : 015-293 6028
Email : Phoebe.Mahlokwane@dhsd.limpopo.gov.za

MOAGI INNOCENT

PERMISSION TO CONDUCT RESEARCH IN DEPARTMENTAL FACILITIES

Your Study Topic as indicated below;

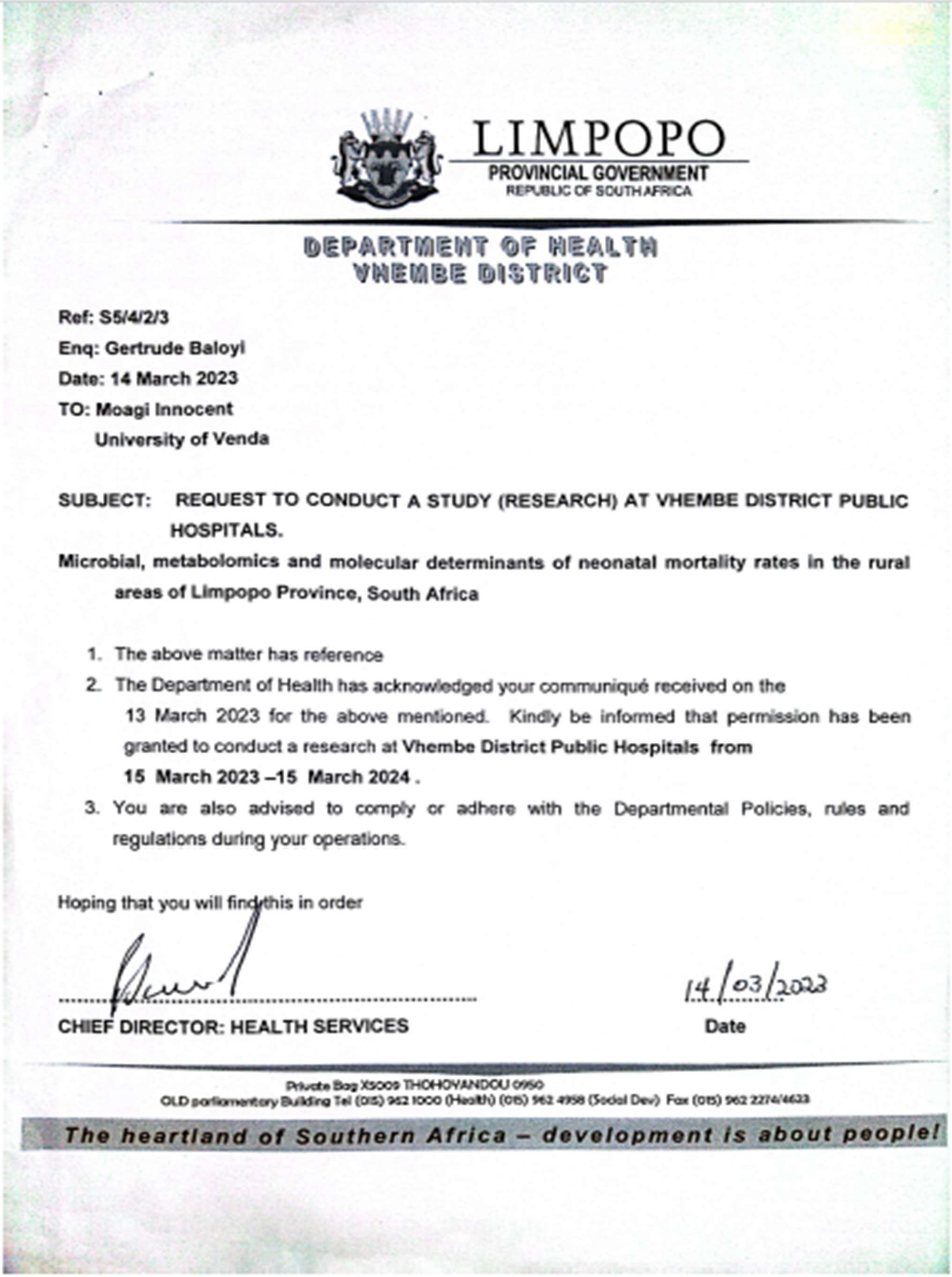
MICROBIAL, METABOLOMIC AND MOLECULAR DETERMINANTS OF NEONATAL MORTALITY RATES IN THE RURAL AREAS OF LIMPOPO PROVINCE, SOUTH AFRICA

1. Permission to conduct research study as per your research proposal is hereby Granted.
2. Kindly note the following:
   a. Present this letter of permission to the Office District Executive Manager a week before the study is conducted.
   b. **BLOOD SAMPLES FOR THIS STUDY MUST ONLY BE DRAWN BY MALWELA THIVHULAWI AND MARIA SONTO MAPUTLE**, after they present their current registration with nursing council.
   c. In the course of your study, there should be no action that disrupts the routine services, or incur any cost on the Department.
   d. After completion of study, it is mandatory that the findings should be submitted to the Department to serve as a resource.
   e. The researcher should be prepared to assist in the interpretation and implementation of the study recommendation where possible.
   f. **The approval is only valid for a 1-year period.**
   g. If the proposal has been amended, a new approval should be sought from the Department of Health
   h. Kindly note that, the Department can withdraw the approval at any time.

Your cooperation will be highly appreciated

Head of Department 6/03/2023
Date

pp



LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH
VHEMBE DISTRICT

Ref: S5/4/2/3

Enq: Gertrude Baloyi

Date: 14 March 2023

TO: Moagi Innocent
University of Venda

SUBJECT: REQUEST TO CONDUCT A STUDY (RESEARCH) AT VHEMBE DISTRICT PUBLIC HOSPITALS.

Microbial, metabolomics and molecular determinants of neonatal mortality rates in the rural areas of Limpopo Province, South Africa

1. The above matter has reference
2. The Department of Health has acknowledged your communiqué received on the 13 March 2023 for the above mentioned. Kindly be informed that permission has been granted to conduct a research at **Vhembe District Public Hospitals from 15 March 2023 –15 March 2024**.
3. You are also advised to comply or adhere with the Departmental Policies, rules and regulations during your operations.

Hoping that you will find this in order

....
CHIEF DIRECTOR: HEALTH SERVICES

14/03/2023
Date

Private Bag X5009 THOHOYANDOU 0950
OLD parliamentary Building Tel (015) 962 1000 (Health) (015) 962 4958 (Social Dev) Fax (015) 962 2274/4633

The heartland of Southern Africa – development is about people!

Annexure 2: Consent forms and information sheet

RESEARCH ETHICS COMMITTEE

UNIVEN Informed Consent

Appendix B

LETTER OF INFORMATION

Title of the Research Study : Microbial, metabolomic and molecular determinants of high neonatal mortality rates in the rural areas of Limpopo province, South Africa

Principal Investigator/s/ researcher : (Moagi Innocent, MSc)

Co-Investigator/s/supervisor/s : (Prof A Samie (PhD), Prof MS Maputle (PhD) and Dr L Mabasa (PhD)

Brief Introduction and Purpose of the Study: I am doing a study about the determinants of high neonatal mortality rate in the rural areas in Mopani, Vhembe, and Capricorn districts of Limpopo province, South Africa. This study is a research project to obtain a master's degree in microbiology. Therefore, I want to collect information about your knowledge and attitude on the factors associated with neonatal mortality.

Outline of the Procedures : You may participate in this project if you are a mother who is about to give birth or if you just gave birth. Clinical Information of neonates whose parents are willing to participate will be considered.

As part of this study, I am going to hand you an informed consent that you will sign and questionnaire that you will have to fill, which will help me get the information mentioned above. Showing that you are among other volunteers who agreed to be part of the study. After you finish the questionnaire, I ask that you allow us to collect urine and blood samples. The information you provided and the results of from both blood and urine samples will help us to determine several factors leading to high neonatal mortality.

Risks or Discomforts to the Participant : (There will be no risks involved by taking part in the study)

Benefits : By participating in this study, you will not receive any benefit. However, the information obtained from you will help to increase our understanding on the several factors contributing to high neonatal mortality rate. I hope that the results of the study will address the Sustainable Development Goal 3 (Good health and well-being), which aims to reduce childhood mortality rate by 12 deaths per 1000 live birth by 2030.

Reason/s why the Participant May Be Withdrawn from the Study: Participant are completely free to take part in this study or to refuse to do so. Although participants agreed to be a part of the study, they will be free to leave the discussion any time they wish. The decision to not to participate or to withdraw will not affect any future aspects of thiers.

Remuneration : (The participant will not receive any remuneration for

participating on the study*)*

Costs of the Study : (No costs are expected to be covered by the

participant on the study*)*

Confidentiality : (Confidentiality will be achieved by using codes on

the samples that will be collected from study participants and the questionnaires will be also be allocated the same code as the samples)

Research-related Injury : (It is highly unlikely that there will be any injury as the investigators will not be handling the participants themselves but the professional neonatal nurses and midwives.)

Persons to Contact in the Event of Any Problems or Queries:

(Prof Samie Amidou. samieamidou@yahoo.com) Please contact the researcher (tel no. 071 271 8585), my supervisor (tel no. 015 962 8186) or the University Research Ethics Committee Secretariat on 015 962 9058. Complaints can be reported to the Director: Research and Innovation, Prof GE Ekosse on 015 962 8313 or Georges Ivo.Ekosse@univen.ac.za

General:

Potential participants must be assured that participation is voluntary and the approximate number of participants to be included should be disclosed. A copy of the information letter should be issued to participants. The information letter and consent form must be translated and provided in the primary spoken language of the research population

CONSENT

Statement of Agreement to Participate in the Research Study:

- I hereby confirm that I have been informed by the researcher, (*Moagi Innocent*), about the nature, conduct, benefits and risks of this study - Research Ethics Clearance Number: _,
- I have also received, read and understood the above written information (*Participant Letter of Information*) regarding the study.
- I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report.
- In view of the requirements of research, I agree that the data collected during this study can be processed in a computerized system by the researcher.
- I may, at any stage, without prejudice, withdraw my consent and participation in the study.
- I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.
- I understand that significant new findings developed during the course of this research which may relate to my participation will be made available to me.

Full Name of Participant	Date	Time	Signature
I, …………………….	……………	…………	…………………..

(*Name of researcher*) herewith confirm that the above participant has been fully

Informed about the nature, conduct and risks of the above study.

Full Name of Researcher

……………………. Date………………. Signature…………………

Full Name of Witness (If applicable)

…………………….. Date **……………….** Signature**…………………..**

Full Name of Legal Guardian (If applicable)

………………… Date**………………** Signature**…………………….**

Please note the following:

Research details must be provided in a clear, simple and culturally appropriate manner and prospective participants should be helped to arrive at an informed decision by use of appropriate language (grade 10 level- use Flesch Reading Ease Scores on Microsoft Word), selecting of a non-threatening environment for interaction and the availability of peer counseling (Department of Health, 2004)

If the potential participant is unable to read/illiterate, then a right thumb print is required and an impartial witness, who is literate and knows the participant e.g. parent, sibling, friend, pastor, etc. should verify in writing, duly signed that informed verbal consent was obtained (Department of Health, 2004).

If anyone makes a mistake completing this document e.g. a wrong date or spelling mistake, a new document has to be completed. The incomplete original document has to be kept in the participant's file and not thrown away, and copies thereof must be issued to the participant.

References:

Department of Health: 2004. *Ethics in Health Research: Principles, Structures and Processes*

http://www.doh.gov.za/docs/factsheets/guidelines/ethnics/

Department of Health. 2006. *South African Good Clinical Practice Guidelines*. 2nd Ed. Available at:

http://www.nhrec.org.za/?page_id=14

ANNEXURE D

INFOMARTION SHEET

Dear Respondent

I am a master's student (Microbiology) at the University of Venda in the Faculty of Sciences, Engineering and Agriculture.

My research topic is: **Microbial, metabolomic and molecular determinants of neonatal mortality rates in the rural areas of Limpopo province, South Africa.** The purpose of the study is to investigate microbial, metabolomic and molecular determinants of high neonatal mortality rates in the rural areas of Limpopo. The study will determine factors contributing to neonatal mortality will help produce the most reliable diagnostic test that can detect disease at early stages. The outcome of this study will address the Sustainable Development Goal 3 (Good health and well-being), which aims to reduce childhood mortality rate by 12 deaths per 1000 live birth by 2030. Therefore, you can take a part in the study if you're a pregnancy woman who is attending either their 3rd antenatal care (ANC) visits at 32nd week or the last ANC Visit between 36–38 weeks of gestation and you are willing to give birth at one of the selected health care facilities.

Before taking part to this study, you should know that there will be no financial or any form of reward for participating in the study and there is no financial gain for the researcher. There will be minimal, or no risks expected as only umbilical cord blood will be collected during birth or no administration of treatment to participants. The interview will be conducted using an online platform REDCap and the answers will not be traced back to you, the information is anonymous. The information will be confidential, only the researcher and supervisors will have access to it. They will be no tape recorder.

You are not forced to take part in this study, you have to agree or refuse to take part. However, I would appreciate it if you agree to be part on the study. There will be no adverse effect even if you refuse take part in the study. If you agree to take part in the study and change your in the middle of the interview, you will be allowed to withdraw without any penalties. Ask questions if anything is not clear or if you would like more information. Take time to decide whether or not to take part

Your cooperation will be highly appreciated

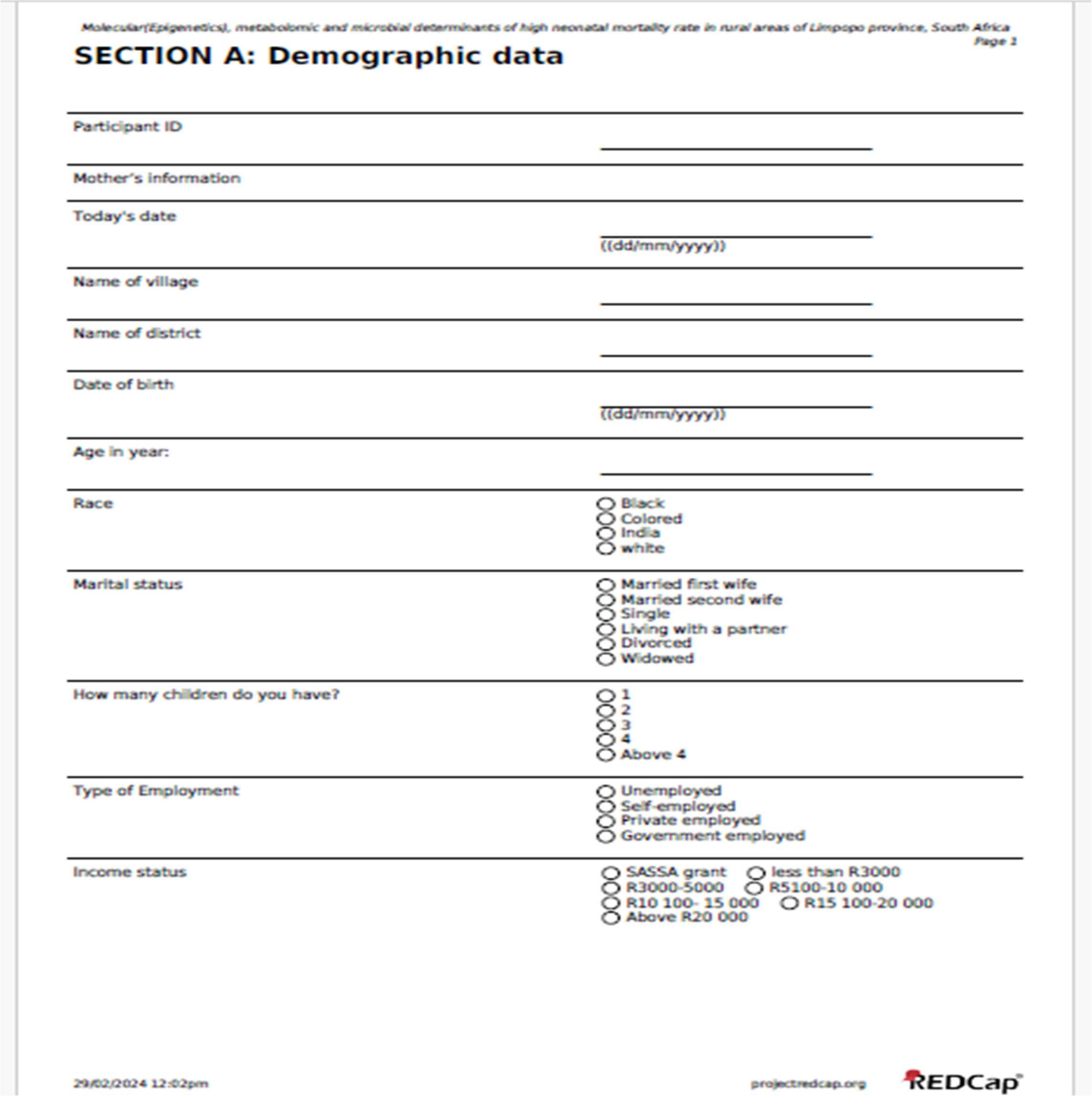
Thank you

Signature: ______________________________

Mr Moagi I

Participant signature: ___________________________________Date:

Annexure 3: Data collections tool (Questionnaire)



Molecular(Epigenetics), metabolomic and microbial determinants of high neonatal mortality rate in rural areas of Limpopo province, South Africa
Page 1

SECTION A: Demographic data

Participant ID	____________________
Mother's information	
Today's date	____________________ ((dd/mm/yyyy))
Name of village	____________________
Name of district	____________________
Date of birth	____________________ ((dd/mm/yyyy))
Age in year:	____________________
Race	○ Black ○ Colored ○ India ○ white
Marital status	○ Married first wife ○ Married second wife ○ Single ○ Living with a partner ○ Divorced ○ Widowed
How many children do you have?	○ 1 ○ 2 ○ 3 ○ 4 ○ Above 4
Type of Employment	○ Unemployed ○ Self-employed ○ Private employed ○ Government employed
Income status	○ SASSA grant ○ less than R3000 ○ R3000-5000 ○ R5100-10 000 ○ R10 100- 15 000 ○ R15 100-20 000 ○ Above R20 000

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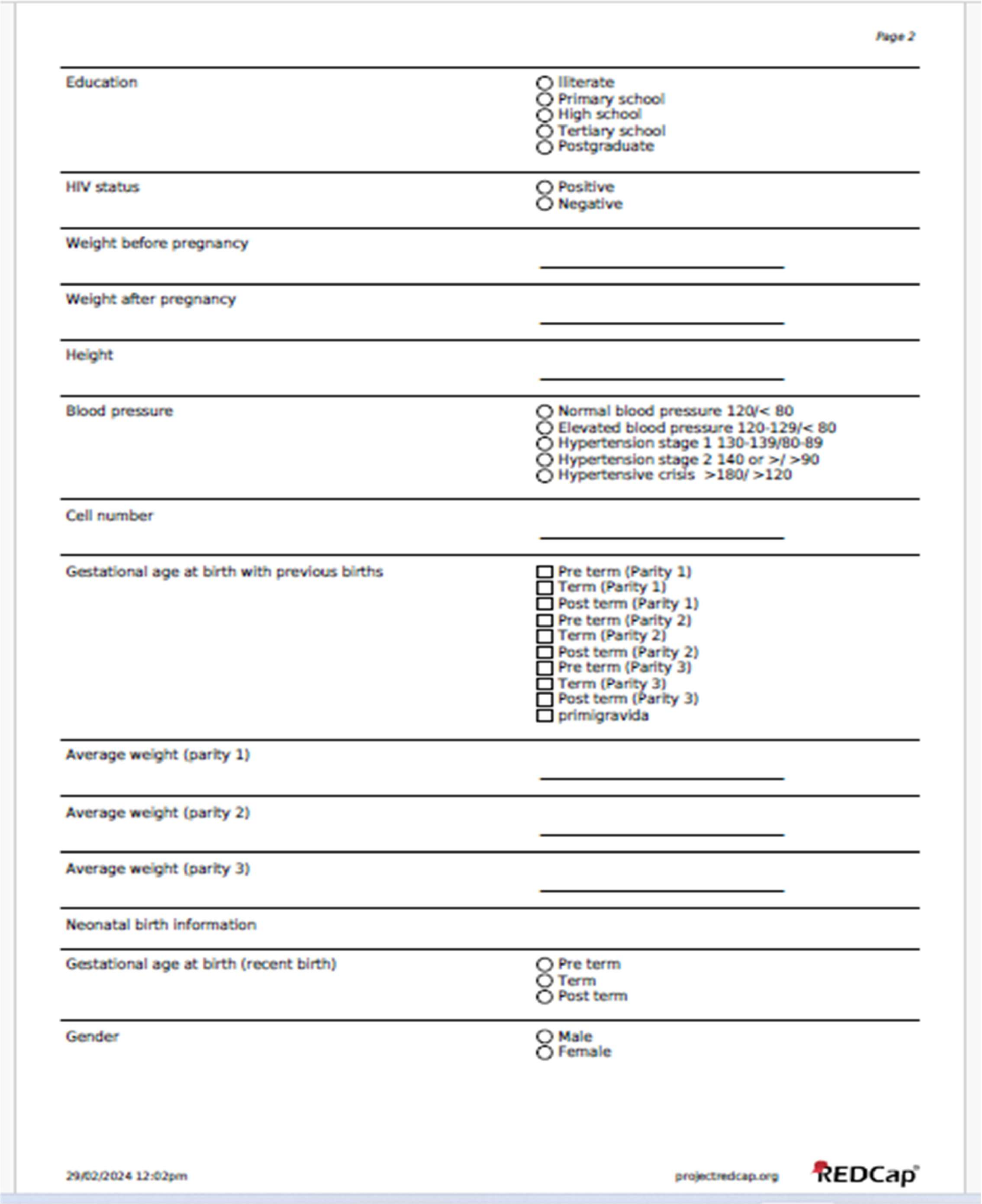

Page 2

Education	○ Illiterate ○ Primary school ○ High school ○ Tertiary school ○ Postgraduate
HIV status	○ Positive ○ Negative
Weight before pregnancy	__________________________
Weight after pregnancy	__________________________
Height	__________________________
Blood pressure	○ Normal blood pressure 120/< 80 ○ Elevated blood pressure 120-129/< 80 ○ Hypertension stage 1 130-139/80-89 ○ Hypertension stage 2 140 or >/ >90 ○ Hypertensive crisis >180/ >120
Cell number	__________________________
Gestational age at birth with previous births	☐ Pre term (Parity 1) ☐ Term (Parity 1) ☐ Post term (Parity 1) ☐ Pre term (Parity 2) ☐ Term (Parity 2) ☐ Post term (Parity 2) ☐ Pre term (Parity 3) ☐ Term (Parity 3) ☐ Post term (Parity 3) ☐ primigravida
Average weight (parity 1)	__________________________
Average weight (parity 2)	__________________________
Average weight (parity 3)	__________________________
Neonatal birth information	
Gestational age at birth (recent birth)	○ Pre term ○ Term ○ Post term
Gender	○ Male ○ Female

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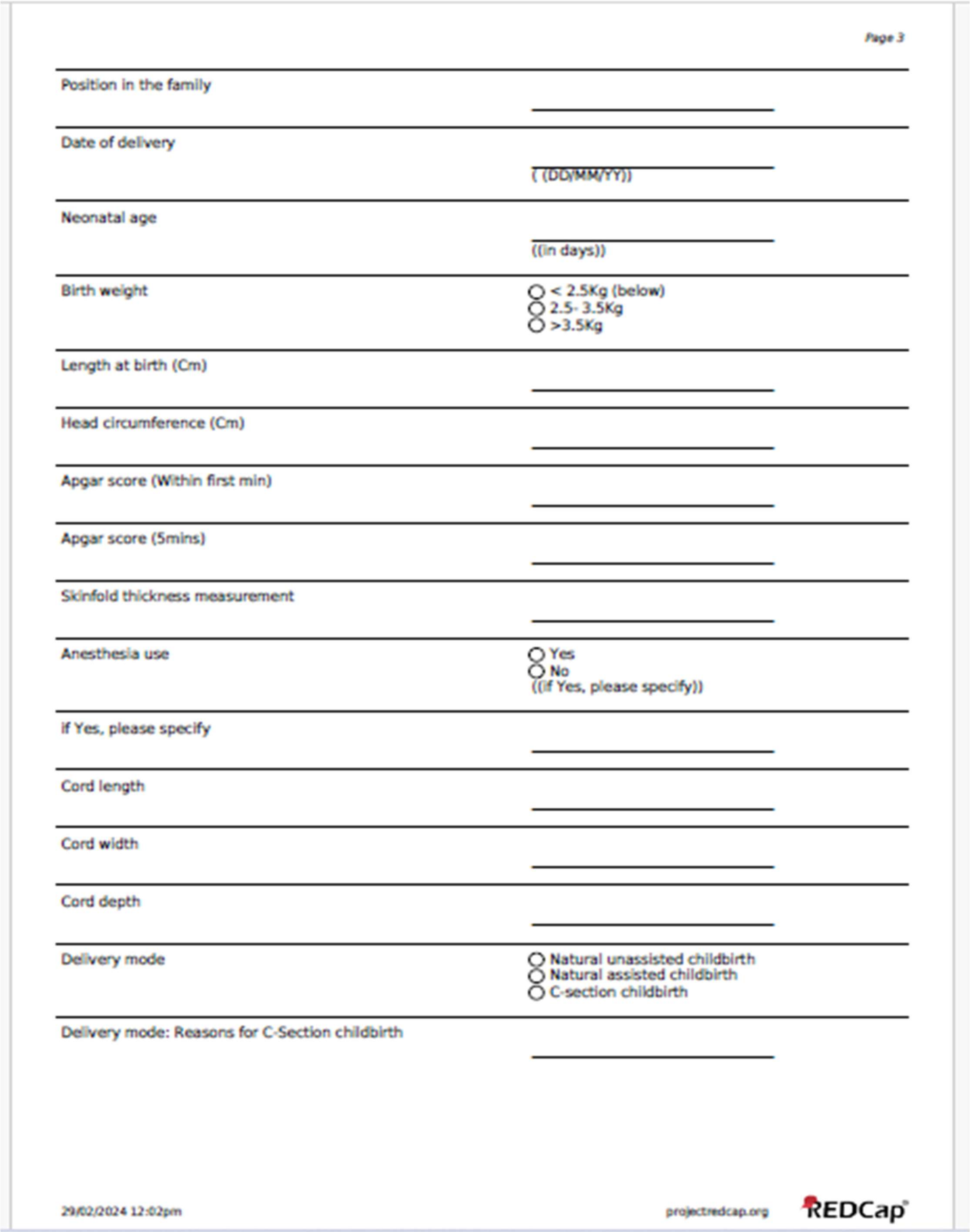

Page 3

Position in the family	________________
Date of delivery	________________ ((DD/MM/YY))
Neonatal age	________________ ((in days))
Birth weight	○ < 2.5Kg (below) ○ 2.5- 3.5Kg ○ >3.5Kg
Length at birth (Cm)	________________
Head circumference (Cm)	________________
Apgar score (Within first min)	________________
Apgar score (5mins)	________________
Skinfold thickness measurement	________________
Anesthesia use	○ Yes ○ No ((If Yes, please specify))
if Yes, please specify	________________
Cord length	________________
Cord width	________________
Cord depth	________________
Delivery mode	○ Natural unassisted childbirth ○ Natural assisted childbirth ○ C-section childbirth
Delivery mode: Reasons for C-Section childbirth	________________

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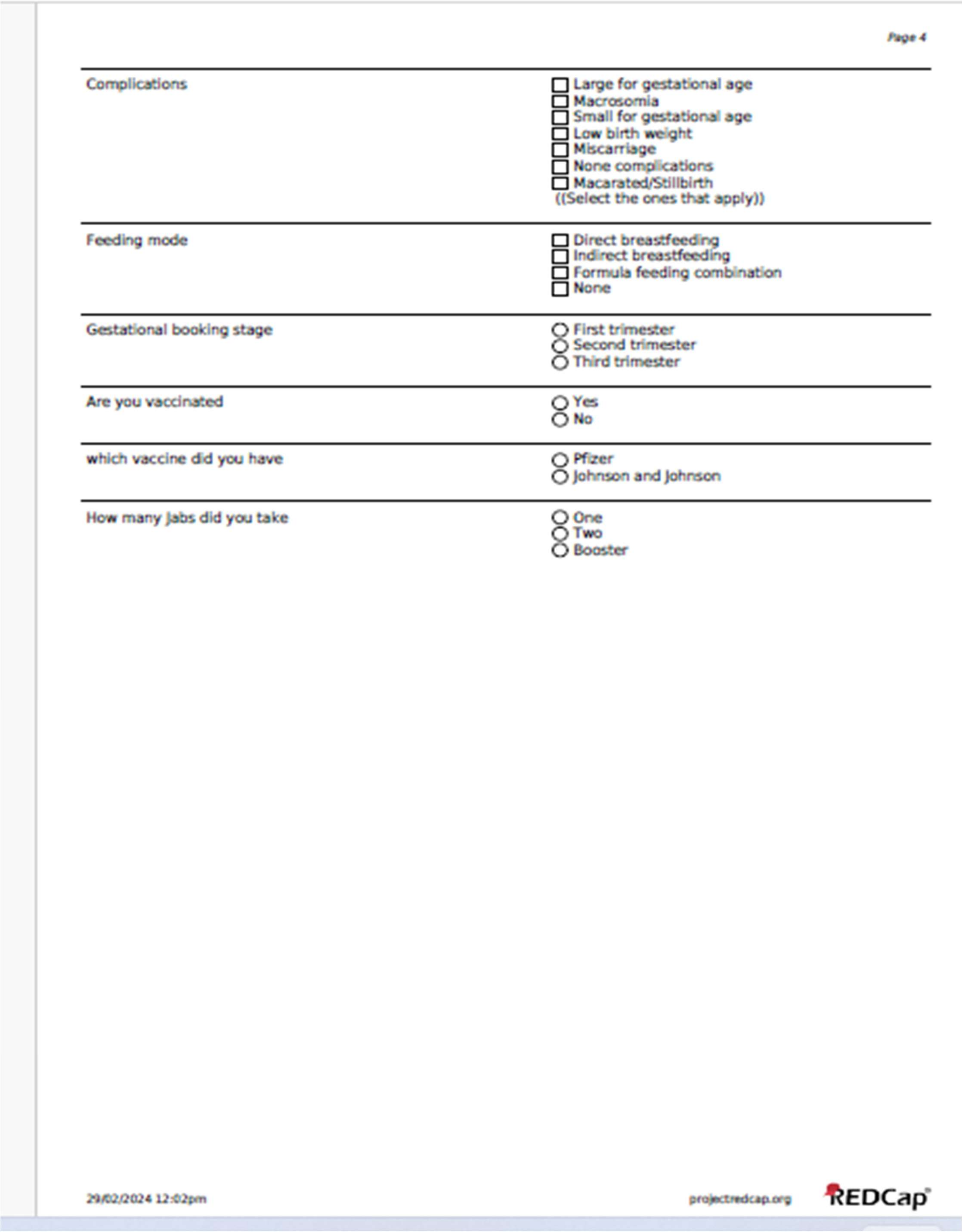

Page 4

Complications	☐ Large for gestational age ☐ Macrosomia ☐ Small for gestational age ☐ Low birth weight ☐ Miscarriage ☐ None complications ☐ Macarated/Stillbirth ((Select the ones that apply))
Feeding mode	☐ Direct breastfeeding ☐ Indirect breastfeeding ☐ Formula feeding combination ☐ None
Gestational booking stage	○ First trimester ○ Second trimester ○ Third trimester
Are you vaccinated	○ Yes ○ No
which vaccine did you have	○ Pfizer ○ Johnson and Johnson
How many Jabs did you take	○ One ○ Two ○ Booster

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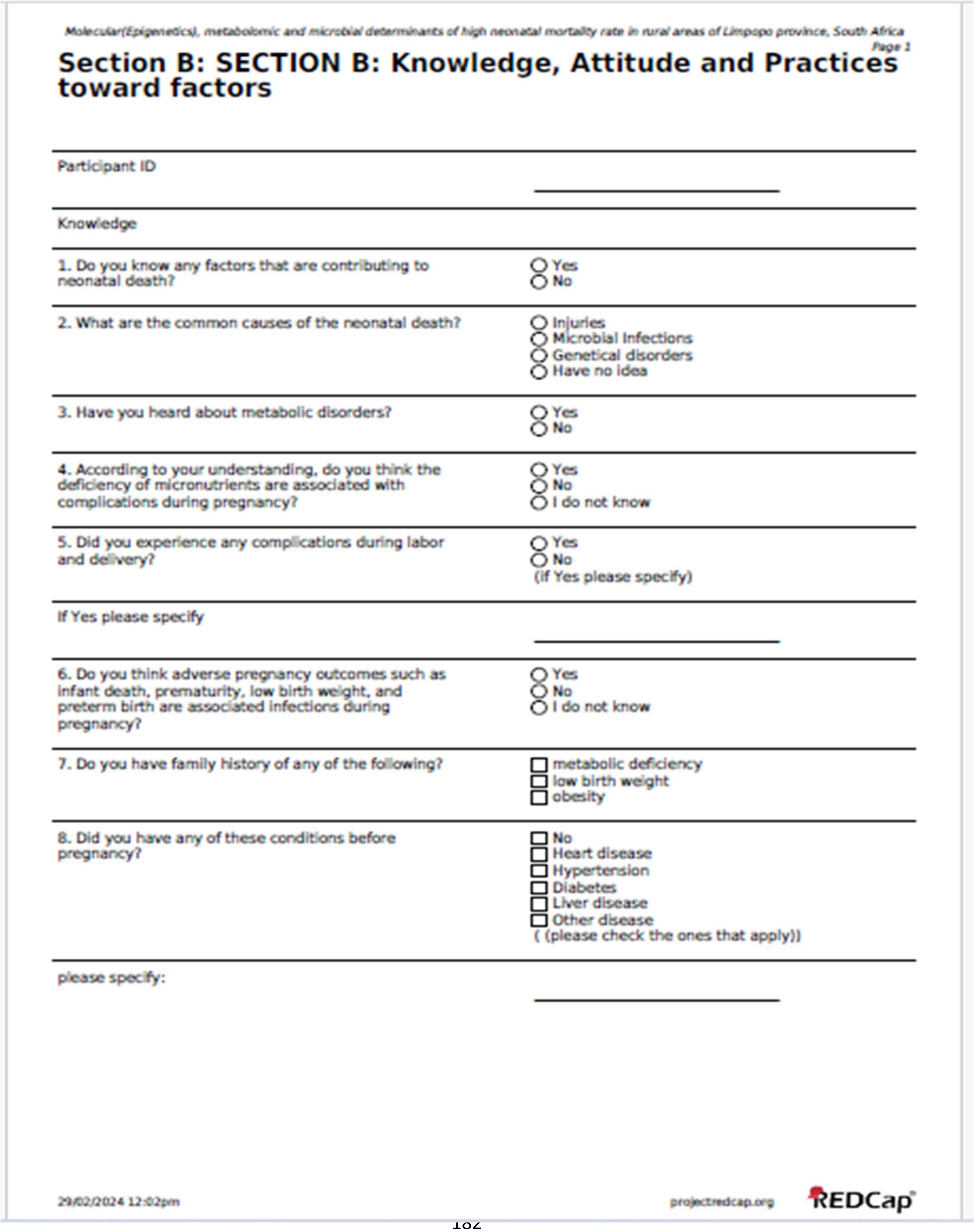

Molecular(Epigenetics), metabolomic and microbial determinants of high neonatal mortality rate in rural areas of Limpopo province, South Africa
Page 1

Section B: SECTION B: Knowledge, Attitude and Practices toward factors

Participant ID	__________________________________
Knowledge	
1. Do you know any factors that are contributing to neonatal death?	○ Yes ○ No
2. What are the common causes of the neonatal death?	○ Injuries ○ Microbial Infections ○ Genetical disorders ○ Have no idea
3. Have you heard about metabolic disorders?	○ Yes ○ No
4. According to your understanding, do you think the deficiency of micronutrients are associated with complications during pregnancy?	○ Yes ○ No ○ I do not know
5. Did you experience any complications during labor and delivery?	○ Yes ○ No (If Yes please specify)
If Yes please specify	__________________________________
6. Do you think adverse pregnancy outcomes such as infant death, prematurity, low birth weight, and preterm birth are associated infections during pregnancy?	○ Yes ○ No ○ I do not know
7. Do you have family history of any of the following?	☐ metabolic deficiency ☐ low birth weight ☐ obesity
8. Did you have any of these conditions before pregnancy?	☐ No ☐ Heart disease ☐ Hypertension ☐ Diabetes ☐ Liver disease ☐ Other disease ((please check the ones that apply))
please specify:	__________________________________

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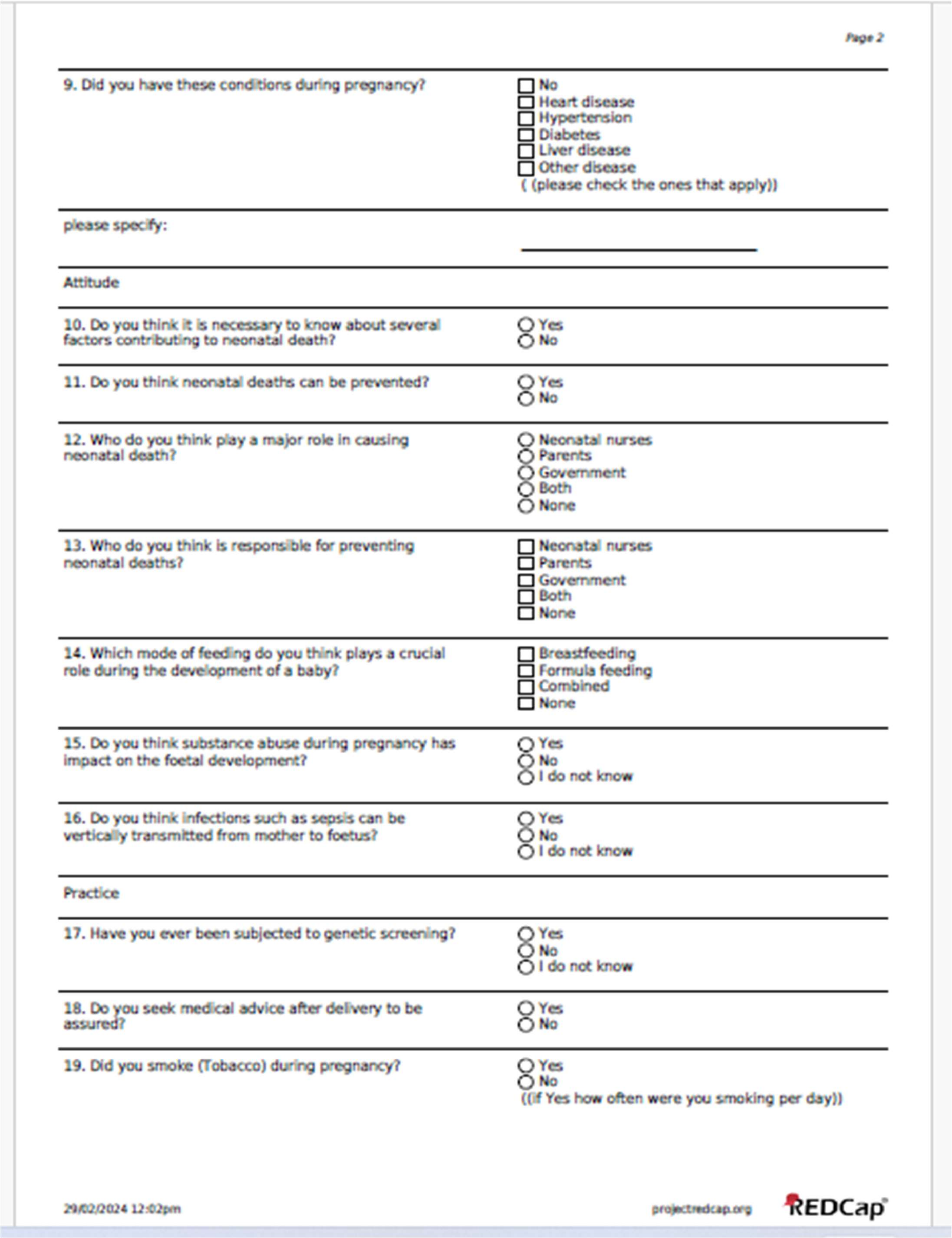
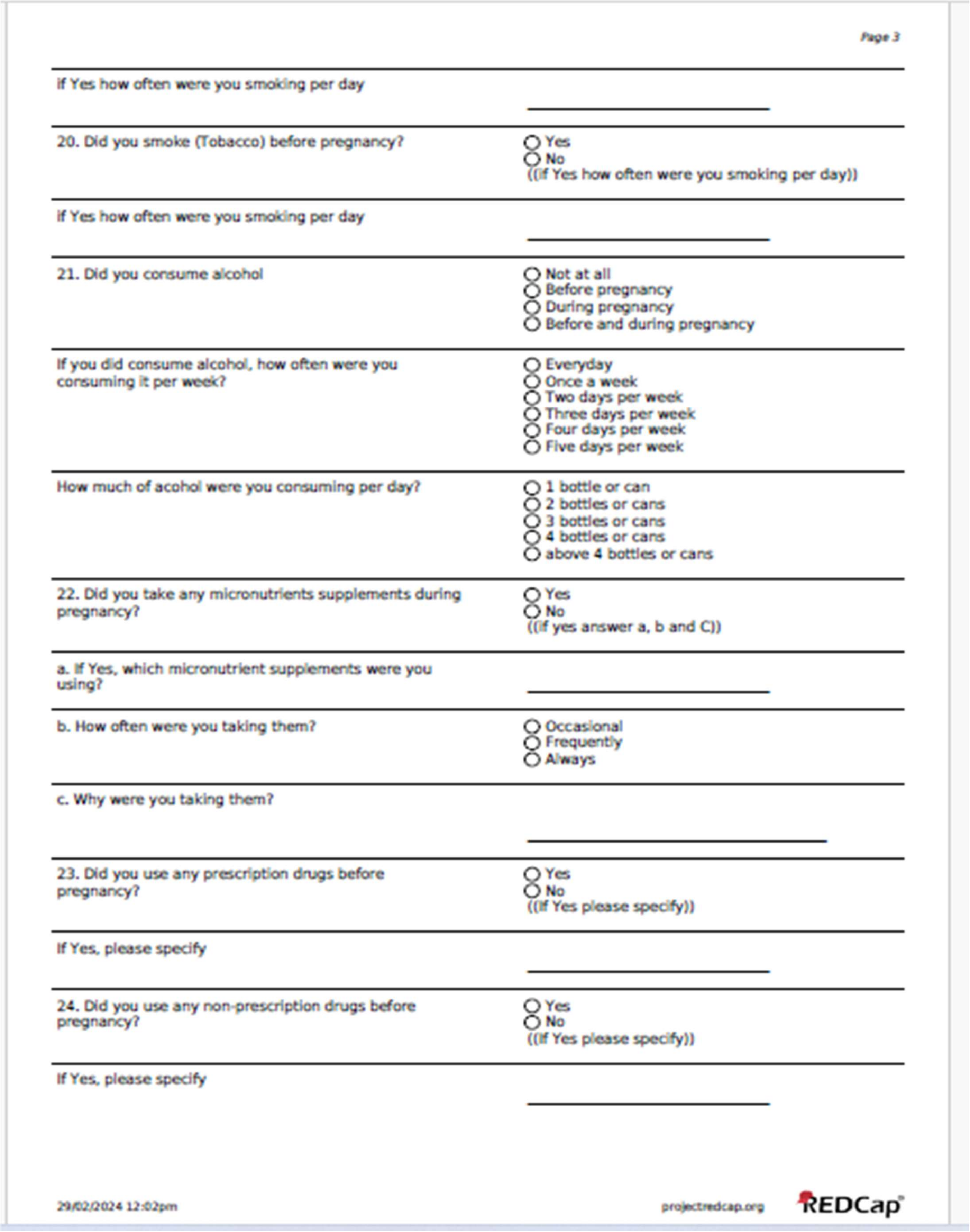


9. Did you have these conditions during pregnancy?	☐ No ☐ Heart disease ☐ Hypertension ☐ Diabetes ☐ Liver disease ☐ Other disease ((please check the ones that apply))
please specify:	__________________________
Attitude	
10. Do you think it is necessary to know about several factors contributing to neonatal death?	○ Yes ○ No
11. Do you think neonatal deaths can be prevented?	○ Yes ○ No
12. Who do you think play a major role in causing neonatal death?	○ Neonatal nurses ○ Parents ○ Government ○ Both ○ None
13. Who do you think is responsible for preventing neonatal deaths?	☐ Neonatal nurses ☐ Parents ☐ Government ☐ Both ☐ None
14. Which mode of feeding do you think plays a crucial role during the development of a baby?	☐ Breastfeeding ☐ Formula feeding ☐ Combined ☐ None
15. Do you think substance abuse during pregnancy has impact on the foetal development?	○ Yes ○ No ○ I do not know
16. Do you think infections such as sepsis can be vertically transmitted from mother to foetus?	○ Yes ○ No ○ I do not know
Practice	
17. Have you ever been subjected to genetic screening?	○ Yes ○ No ○ I do not know
18. Do you seek medical advice after delivery to be assured?	○ Yes ○ No
19. Did you smoke (Tobacco) during pregnancy?	○ Yes ○ No ((if Yes how often were you smoking per day))



Page 3

If Yes how often were you smoking per day	______________________
20. Did you smoke (Tobacco) before pregnancy?	○ Yes ○ No ((if Yes how often were you smoking per day))
If Yes how often were you smoking per day	______________________
21. Did you consume alcohol	○ Not at all ○ Before pregnancy ○ During pregnancy ○ Before and during pregnancy
If you did consume alcohol, how often were you consuming it per week?	○ Everyday ○ Once a week ○ Two days per week ○ Three days per week ○ Four days per week ○ Five days per week
How much of acohol were you consuming per day?	○ 1 bottle or can ○ 2 bottles or cans ○ 3 bottles or cans ○ 4 bottles or cans ○ above 4 bottles or cans
22. Did you take any micronutrients supplements during pregnancy?	○ Yes ○ No ((if yes answer a, b and C))
a. If Yes, which micronutrient supplements were you using?	______________________
b. How often were you taking them?	○ Occasional ○ Frequently ○ Always
c. Why were you taking them?	____________________________
23. Did you use any prescription drugs before pregnancy?	○ Yes ○ No ((If Yes please specify))
If Yes, please specify	______________________
24. Did you use any non-prescription drugs before pregnancy?	○ Yes ○ No ((If Yes please specify))
If Yes, please specify	______________________

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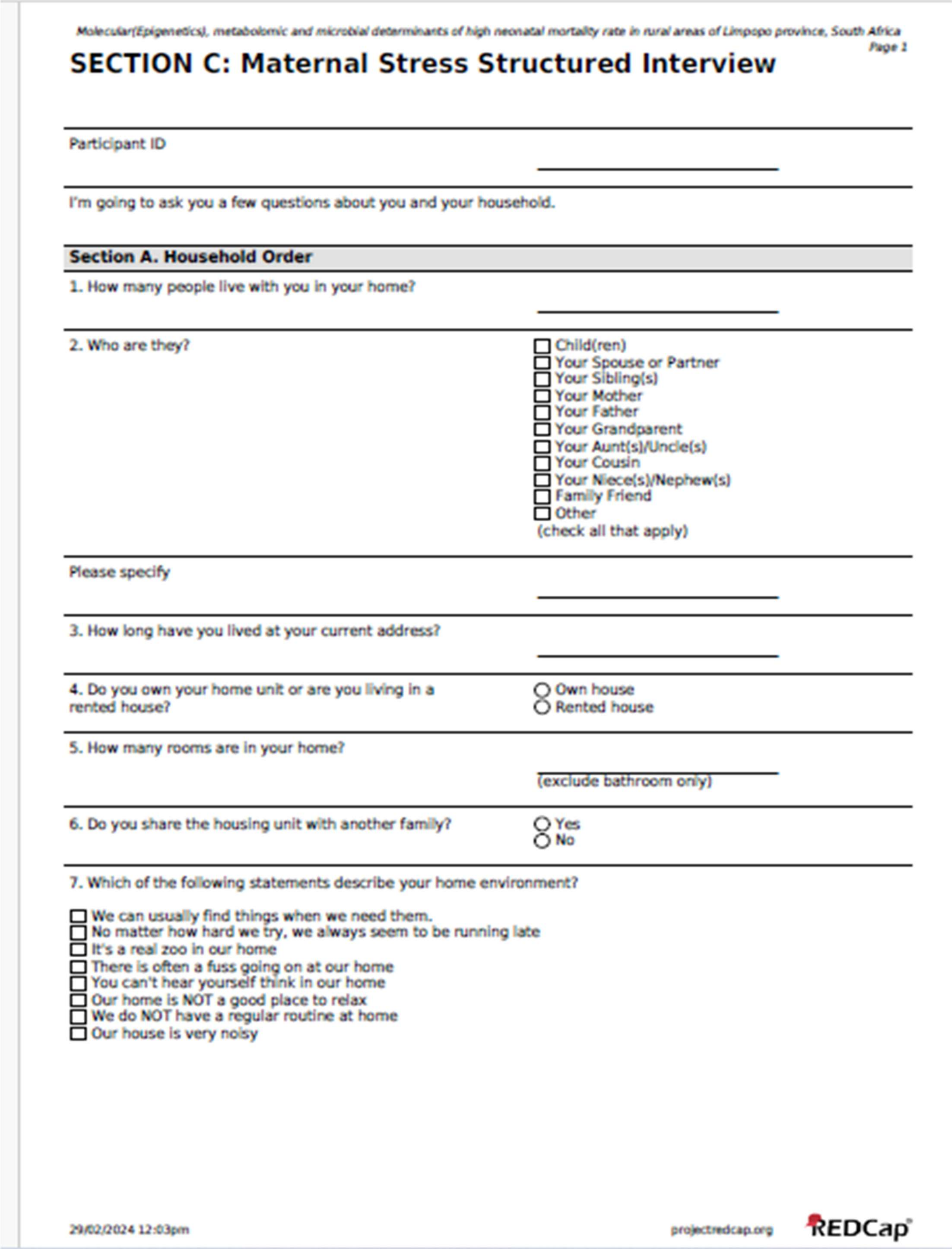

Molecular(Epigenetics), metabolomic and microbial determinants of high neonatal mortality rate in rural areas of Limpopo province, South Africa

Page 1

SECTION C: Maternal Stress Structured Interview

Participant ID	__________________________________
I'm going to ask you a few questions about you and your household.	
Section A. Household Order	
1. How many people live with you in your home?	__________________________________
2. Who are they?	☐ Child(ren) ☐ Your Spouse or Partner ☐ Your Sibling(s) ☐ Your Mother ☐ Your Father ☐ Your Grandparent ☐ Your Aunt(s)/Uncle(s) ☐ Your Cousin ☐ Your Niece(s)/Nephew(s) ☐ Family Friend ☐ Other (check all that apply)
Please specify	__________________________________
3. How long have you lived at your current address?	__________________________________
4. Do you own your home unit or are you living in a rented house?	○ Own house ○ Rented house
5. How many rooms are in your home?	__________________________________ (exclude bathroom only)
6. Do you share the housing unit with another family?	○ Yes ○ No

7. Which of the following statements describe your home environment?

☐ We can usually find things when we need them.
☐ No matter how hard we try, we always seem to be running late
☐ It's a real zoo in our home
☐ There is often a fuss going on at our home
☐ You can't hear yourself think in our home
☐ Our home is NOT a good place to relax
☐ We do NOT have a regular routine at home
☐ Our house is very noisy

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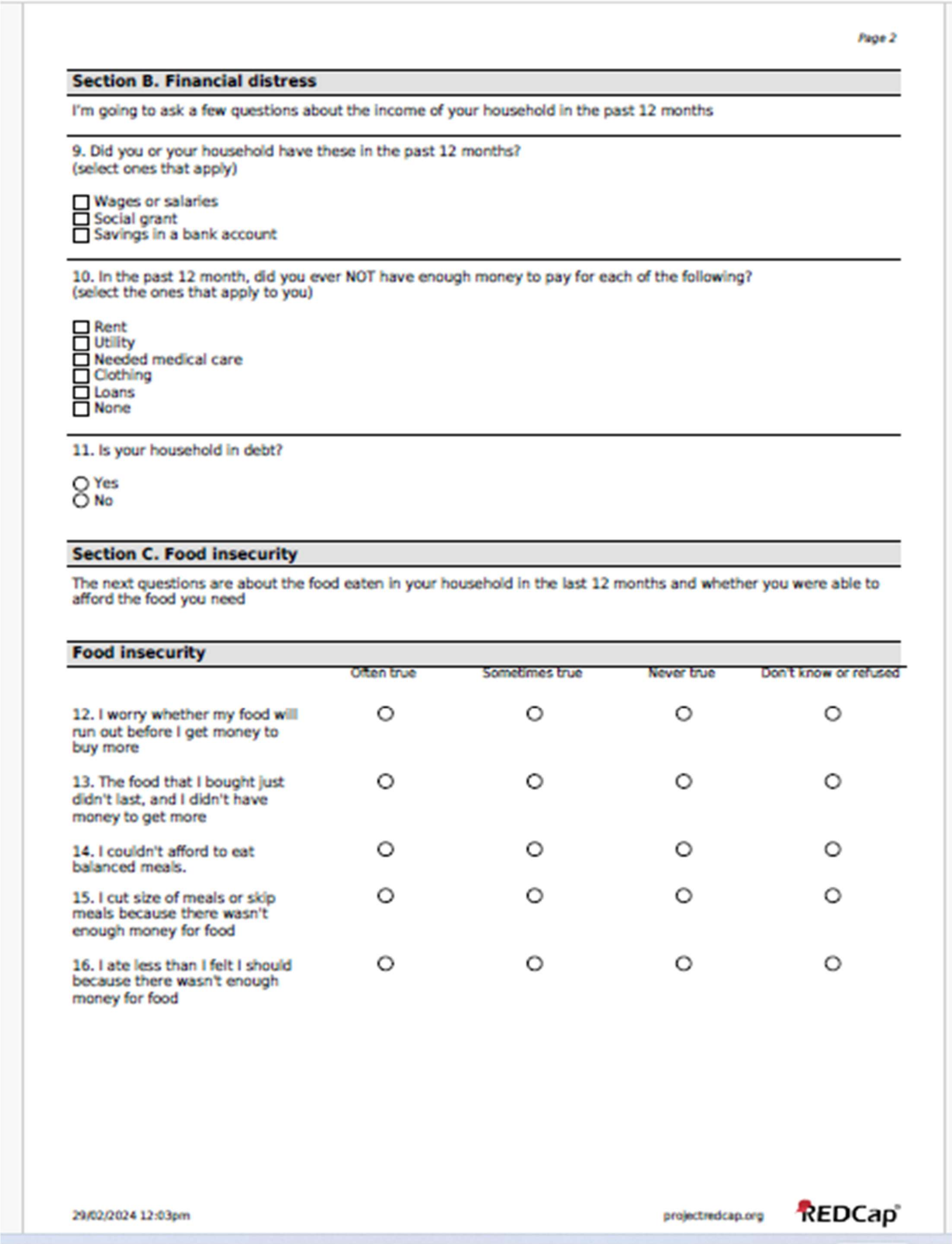

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Section B. Financial distress

I'm going to ask a few questions about the income of your household in the past 12 months

9. Did you or your household have these in the past 12 months?
(select ones that apply)

☐ Wages or salaries
☐ Social grant
☐ Savings in a bank account

10. In the past 12 month, did you ever NOT have enough money to pay for each of the following?
(select the ones that apply to you)

☐ Rent
☐ Utility
☐ Needed medical care
☐ Clothing
☐ Loans
☐ None

11. Is your household in debt?

○ Yes
○ No

Section C. Food insecurity

The next questions are about the food eaten in your household in the last 12 months and whether you were able to afford the food you need

Food insecurity

	Often true	Sometimes true	Never true	Don't know or refused
12. I worry whether my food will run out before I get money to buy more	○	○	○	○
13. The food that I bought just didn't last, and I didn't have money to get more	○	○	○	○
14. I couldn't afford to eat balanced meals.	○	○	○	○
15. I cut size of meals or skip meals because there wasn't enough money for food	○	○	○	○
16. I ate less than I felt I should because there wasn't enough money for food	○	○	○	○

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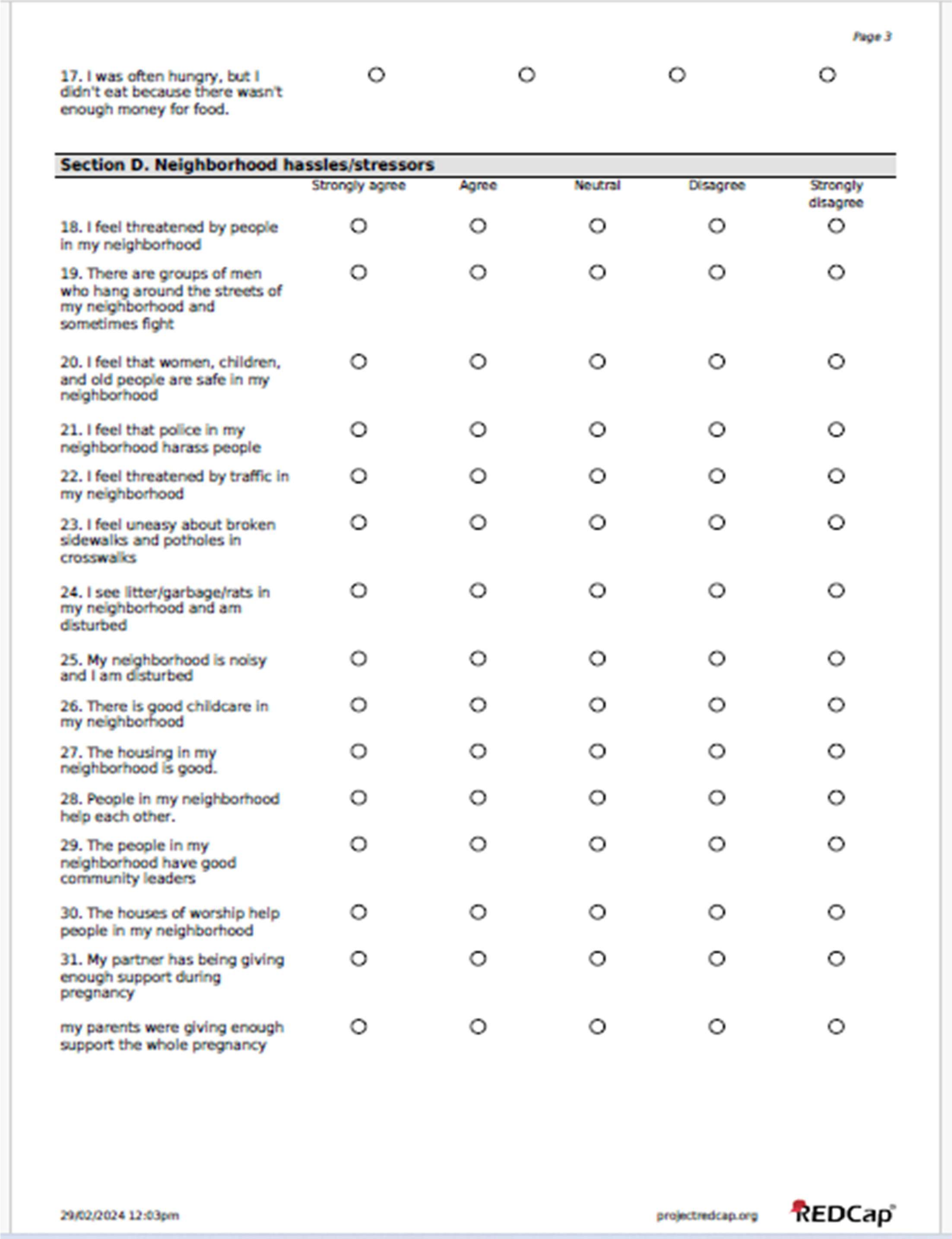
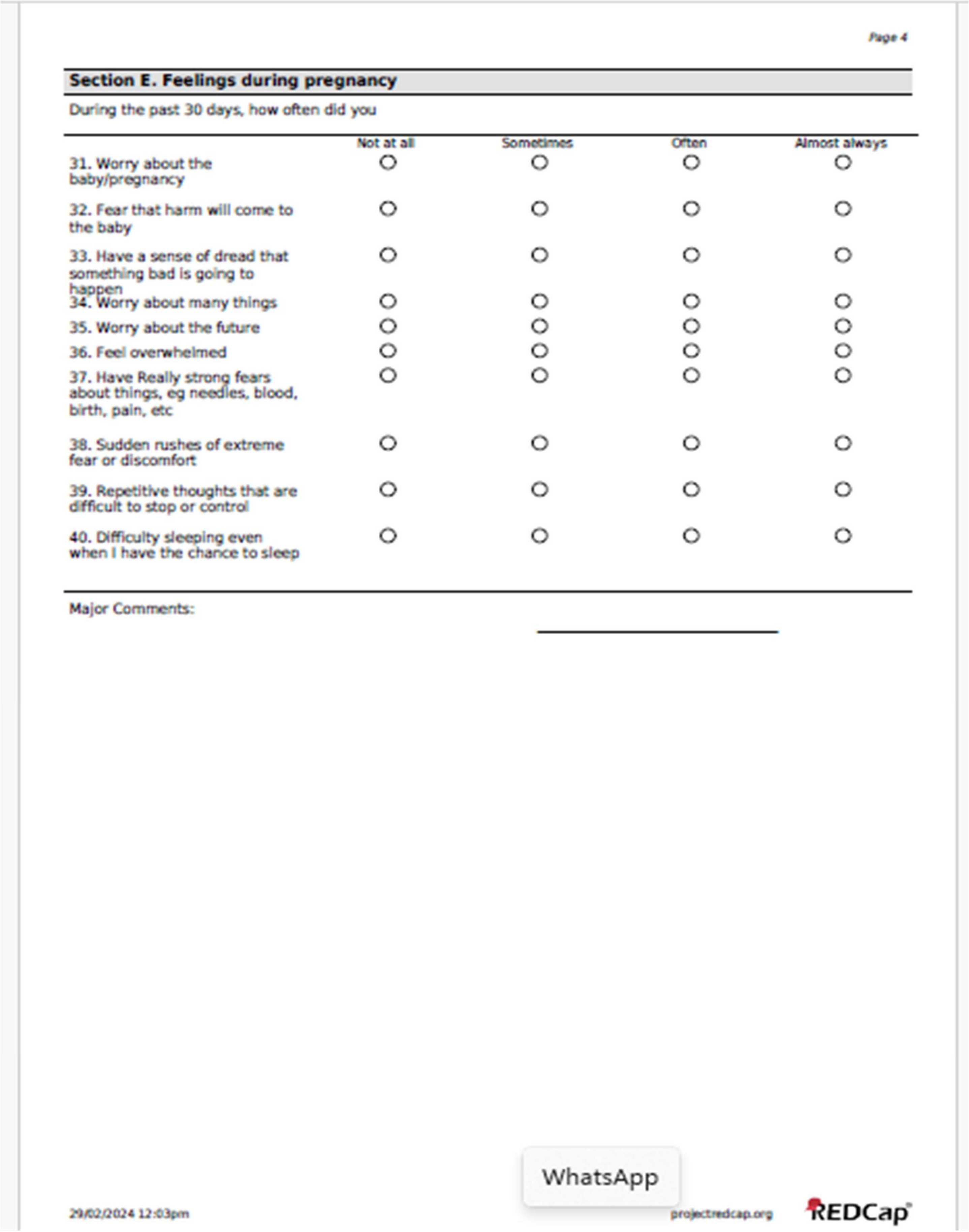


17. I was often hungry, but I didn't eat because there wasn't enough money for food.	○	○	○	○

Section D. Neighborhood hassles/stressors

	Strongly agree	Agree	Neutral	Disagree	Strongly disagree
18. I feel threatened by people in my neighborhood	○	○	○	○	○
19. There are groups of men who hang around the streets of my neighborhood and sometimes fight	○	○	○	○	○
20. I feel that women, children, and old people are safe in my neighborhood	○	○	○	○	○
21. I feel that police in my neighborhood harass people	○	○	○	○	○
22. I feel threatened by traffic in my neighborhood	○	○	○	○	○
23. I feel uneasy about broken sidewalks and potholes in crosswalks	○	○	○	○	○
24. I see litter/garbage/rats in my neighborhood and am disturbed	○	○	○	○	○
25. My neighborhood is noisy and I am disturbed	○	○	○	○	○
26. There is good childcare in my neighborhood	○	○	○	○	○
27. The housing in my neighborhood is good.	○	○	○	○	○
28. People in my neighborhood help each other.	○	○	○	○	○
29. The people in my neighborhood have good community leaders	○	○	○	○	○
30. The houses of worship help people in my neighborhood	○	○	○	○	○
31. My partner has being giving enough support during pregnancy	○	○	○	○	○
my parents were giving enough support the whole pregnancy	○	○	○	○	○



Section E. Feelings during pregnancy

During the past 30 days, how often did you

	Not at all	Sometimes	Often	Almost always
31. Worry about the baby/pregnancy	○	○	○	○
32. Fear that harm will come to the baby	○	○	○	○
33. Have a sense of dread that something bad is going to happen	○	○	○	○
34. Worry about many things	○	○	○	○
35. Worry about the future	○	○	○	○
36. Feel overwhelmed	○	○	○	○
37. Have Really strong fears about things, eg needles, blood, birth, pain, etc	○	○	○	○
38. Sudden rushes of extreme fear or discomfort	○	○	○	○
39. Repetitive thoughts that are difficult to stop or control	○	○	○	○
40. Difficulty sleeping even when I have the chance to sleep	○	○	○	○

Major Comments: ______________________