CLINICAL, SOCIODEMOGRAPHIC AND PLACENTAL HISTOLOGICALFEATURES IN PRETERM BIRTHS WITH PLACENTAL MALARIA AND HIV COINFECTION IN BUNGOMA COUNTY REFERRAL HOSPITAL, A RETROSPECTIVE COHORT STUDY

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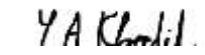
A dissertation presented in partial fulfilment of the requirement for the Degree of Master of Medicine in Department of Obstetrics and Gynecology, Faculty of Health Sciences, University of Nairobi.

Declaration

I hereby confirm that this dissertation is my original work and has not been presented elsewhere for examination

Signature:  **Date:28/11/2021**

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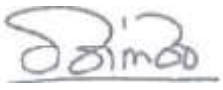
Signature:  **Date:3rd December,2021**

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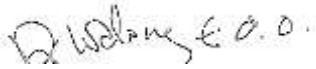
Signature:  **Date:03/12/2021**

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Dedication

Dedicated to my wife Dahabo Yusuf

My daughter

Mariam (Maya)

My parents

My mother Mariam Amin

My father Adam Khalil

My siblings

Adia, Idris, Khadija and Khalil

To my role model

The late Prof Mohammad Ahmad Mahjub Haj Nur

To my teachers, colleagues, and friends

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List of Abbreviation

ART	Antiretroviral Therapy
BCRH	Bungoma County Referral Hospital
CD	Cluster of Differentiation
ERC	Ethics and Review Board
HIV	Human Immune Deficiency Virus
ICAM	Intercellular Adhesion Molecules
KEMRI	Kenya Medical Research Institute
KNH	Kenyatta National Hospital
PTBi-EA	East Africa Preterm Birth Initiative
SDG	Sustainable Development Goals
UoN	University of Nairobi
WHO	World Health Organisation

Operational definitions

1. Malaria and HIV coinfection- the presence of both infections in a person.
2. Preterm birth: Delivery before 37 completed weeks. In this study preterm birth strictly means spontaneous preterm delivery.
3. HIV infection in this study means HIV patients on treatment
4. Malaria infection in this study means treated malaria
5. Morphometry is the quantitative analysis of form, a concept that encompasses size and shape, it entails the analysis of 2D images
6. Fiji is an image analysis software
7. Exposure group: Placentae of women with malaria and HIV coinfection with preterm births.
8. Non-exposure group: Placentae of women without malaria and HIV coinfection delivering preterm.
9. Maternal vascular malperfusion is a constellation of placental pathological findings resulting from pathological blood flow in the spiral arteries
10. Accelerated villous maturity is a histological finding of MVM consisting of syncytial knotting and fibrin deposition
11. Distal villous hypoplasia is a histological finding of MVM consisting of long slender terminal villi with increased intervillous space
12. Altshuler's criteria are the criteria that are used to diagnose villous hypervascularity, 10 microscopic fields each containing 10 terminal villi with 10 or more capillaries and seen under magnification 10X. 0 to 1 capillary is hypervascularity, 2 to 6 is normal vascularity, 7 to 9 is hypervascularity and 10 or more is chorangiosis
13. 2014 Amsterdam Consensus Criteria, are the criteria that used standardised terminologies for placental diagnosis, and it is generally categorised into three groups; vascular-stromal features, inflammatory immune features and others.

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Abstract

Background

Prematurity is the largest killer of children under 5 years of age. Malaria and HIV are both associated with prematurity due to partial maternal vascular malperfusion. This may be due to the altered angiogenesis associated with the two diseases. The structural changes associated with malaria and HIV coinfection in the placentae of premature births have largely not been described. This study aimed at determining the clinical, sociodemographic and histological differences in the parenchyma of placentae of preterm births with malaria and HIV coinfection compared to those without.

Methods

Twenty-five placentae of preterm birth with malaria and HIV coinfection were randomly selected and compared to twenty-five of those without malaria and HIV coinfection. Clinical data were abstracted. Light microscopy was used to determine histological features by a qualified pathologist while histomorphometric features of the terminal villous were analyzed using Fiji® image analysis software. Quantitative data were analysed using IBM Statistical Package for Social Sciences version 26 and results were presented in tables; a significance level of 0.05% was considered.

Results

Women with malaria and HIV coinfection were younger (26 years vs 29 years)and had a lower parity (2 vs 3) and level of education (most in the primary level) when compared to women without malaria and HIV coinfection. Placentae of malaria and HIV coinfection compared to those without were significantly associated with partial maternal vascular malperfusion with a RR of 2.10 CI (1.26-3.49). Placental weight, villous perimeter, and area were significantly lower in cases as compared to controls (454g vs. 488g (119.32μm vs. 130.47μm) and (937.93μm^2vs. 1132.88μm^2) respectively. The results were verified by a blinded independent pathologist.

Conclusion

Partial maternal vascular malperfusion was significantly higher in preterms with Malaria and HIV coinfection and should therefore be considered an important aspect of the pathophysiology of preterm births in these two conditions. Public health interventions targeting young pregnant women as well as increasing the level of education would reduce the burden of these two diseases and preterm births.

1 INTRODUCTION

Preterm birth is delivery before 37 completed weeks(1).It is of huge global concern because it is the largest killer of children under 5 yearsof age and sadly, about one million preterm babies die annually within the first month of life(2). In Kenya, prematurity causes 35% of neonatal deaths (3). Prematurity has also been associated with multi-systemic morbidities(4–13).According to WHO the incidence of preterm birth across its member countries varies between 5% to 18% and in Kenya,it is 12.3% (14,15). Malaria and HIV infection have both been associated with preterm births with higher incidence rates of 44.6% and 30.4% respectively (3). The possible mechanism for this association is due to the maternal vascular malperfusion(16) caused by both diseases as a result of the ischemia caused by the intervillous inflammation and decidual vasculopathy that results in occlusion of decidual maternal vessels(17,18).Sociodemographically in Kenya, malaria infection is more among women with low parity and low educational status while HIV is more in the young age group, married couples and those with low educational status (19–25). In the placenta parenchyma,maternal vascular malperfusion has two histological patterns. The first pattern is global/partial maternal vascular malperfusion which histological presents in two forms; distal villous hypoplasia or accelerated villous maturity and this results from partial occlusion of maternal decidual vessels. The second pattern is segmental/complete maternal vascular malperfusion which histologically presents as villous infarction and this is due to complete occlusion of maternal decidual vessels(26,27). The placental structure, therefore, can be considered as a record of in-utero events that is a valuable reference when explaining poor neonatal outcomes like prematurity (27,28).

The placenta is a materno-fetal organ that forms an interface for exchange between the mother and the fetus as well as carrying out important endocrine functions that initiate and maintain the physiological adaptations of pregnancy(29,30). These important functions are supported by a well-organised histological arrangement. If this delicate histoarchitecture is destroyed, then placental functions are impaired and adverse pregnancy outcomes result

from the unfavourable intrauterine environment (31). Microscopically, the placenta is composed of the basal plate on the maternal side and the chorionic plate on the fetal side. These two layers sandwich the villous trees which are bathed by the maternal blood contained in the intervillous spaces(29). The villi and the intervillous space are collectively known as placenta parenchyma which wasanalyzed in this study(32). The villous trees which form the functional units of the placenta must also arborize and mature normally for functional efficiency to be achieved. Both Malaria and HIV can destroy this delicate histoarchitecture by sequestration of respective causative agents in the placenta which in turn attract inflammatory cells that damage the placenta (33–35). Malaria and HIV infection have both been associated with placental inflammatory and vascular structural changes like villitis and accelerated maturation of the villi(36,37).

There is a strong push towards reducing the burden of preterm birth through research as evidenced by the birth of global and regional initiatives like the Preterm Birth Initiative of the University of California San Francisco and the Kenya Medical Research Institute (KEMRI) in East Africa. Since there are no studies that have investigated the histological placental changes caused by Malaria and HIV coinfection in the African population, we propose to investigate the parenchymal histological changes of these placentae in prematurity. The histological changes of interest were those related to partial maternal vascular malperfusion since that is our hypothesized cause of preterm birth in Malaria and HIV co-infection. We hope that the outcome of this study will provide specific explanations for preterm birth, identify structural changes associated with high risks of recurrence and provide information to guide the management of future pregnancies. This, in turn, will guide us in our obstetric care as to how early to start antenatal care, how frequently to visit the clinic and what medications and procedures might be used during pregnancy to reduce the burden of preterm births. We propose to conduct our analysis on bio-banked placentae specimens that were collected for the study titled, “Rapid and multiplex diagnostics for maternal infections”,this study aimed to develop rapid kits to diagnose asymptomatic neonatal

infections by conducting tests on the placentae to detect placental infections. A positive placental infection would justify the treatment of neonates for presumed asymptomatic infections and hence reduce neonatal mortality and morbidity. Another objective which concerns our study was to collect a large number of placentae specimens for the establishment of a Biobank that would provide specimens for future studies like ours. Placentae for the biobank were collected in Bungoma County which has a high Malaria and HIV infection burden as well as a high rate of hospital deliveries. In our study, we shall use the placentae specimens from the established biobank toanalyze the placentae of preterm birth with Malaria and HIV co-infection as compared to the placenta of preterm birth without Malaria and HIV coinfection.

2 LITERATURE REVIEW

2.1 Literature review

2.1.1 General introduction: Preterm, Malaria, HIV and Placenta.

Preterm birth is delivery before 37 completed weeks(1). It is a leading cause of mortality and morbidity in neonatal, childhood and even mid-adulthood periods. Prematurity is the largest killer of children under 5 years. Globally about one million preterm babies die every year within the first 4 weeks of life(38). In Kenya, prematurity accounts for 35% of neonatal deaths (3). Prematurity is associated with both acute, short-term and chronic complications(39). These sequelae include; chronic pulmonary diseases like Asthma, Pulmonary Arterial Hypertension, Hypertension, Chronic Kidney disease and other cardiovascular disorders(5–8), a long-term reduced Bone Mineral Density(9), increase cancer risk in both childhood and adulthood(10), impaired neurodevelopment(11), Autism Spectrum Disorder(13), and all these have high-cost implications(40). In Sub-Saharan Africa, because of the burden associated with prematurity, there is a global push for the reduction of the incidence of preterm birth, especially in Sub-Saharan Africa (39). According to the World Health Organisation (WHO), the incidence of preterm in its member states ranges from 5% to 18%, in Kenya, the incidence is 12.3% (14,15). One of the most important causes of preterm birth is Malaria and HIV infection which is also of global concern and especially in the Sub Saharan Africa (39,41) Globally malaria accounted for 216 million cases and 445,000 deaths in 2016 and 90% of the burden was in Sub Saharan Africa whereas HIV accounted for 36.7 million cases and 1 million deaths in the same year, 70% of this burden was in Sub Saharan Africa. The prevalence of Malaria inHIV-infected pregnant women is upto 37% in Sub-Saharan Africa (39). In Kenya malaria accounted for 6.7 million new cases and 4000 deaths in 2015, especially in the Western part of the country. Kenya is the fourth largest in terms of the HIV epidemic globally with an estimated 1.6 million cases and 44,000 HIV-related deaths in 2016. The infection rates in the Western part of the country are up to 25.7%(41). These two conditions are associated with even higher incidences of

preterm birth 44.6% and 30.4% for malaria and HIV respectively (3).The incidence of preterm birthsin pregnancies affected with malaria and HIV co-infectionmay be higher because the two infections are synergic and bidirectional(39). A possible mechanism by which malaria and HIV cause preterm birth is by damaging the delicate placenta structural integrity. This may result from the partial maternal vascular malperfusion(16) that is a consequence of hypoxia and ischemia caused by inflammation and vasculopathy caused by the causative organisms of malaria and HIV (17,18). This is manifested by specific microscopic placental changes (36,37,42–44). This results in impairment of the placental function which is vital for a successful pregnancy. The placenta is a temporary feto-maternal organ that supports pregnancy by allowing feto-maternal exchange and carrying out important endocrine functionsthat initiate and maintain the physiological adaptations during pregnancy(30,45). These physiological functions are enabled by a well-organised placental histoarchitecture. Preterm birth, therefore, occurs as a response to the resultant adverse in utero conditions(31).Sociodemographically in Kenya, malaria infection is more among women with low parity (primigravidae and secundigravidae) and low educational status (below secondary education level)while HIV is more in the young age group (15 years to 24 years), married couples and those with low educational status, below secondary education level (19–25).

2.1.2 Histological features of placental parenchyma in Malaria and HIV

Microscopically, the placenta is formed by the basal plate, the chorionic plate and the inter-villous space with the placental villous trees in between. In this study, we shall only analyze the histological changes of the placental parenchyma (villi and the intervillous space) with emphasis on histological changes of partial maternal vascular malperfusion (27,32). The placenta villous tree has the following four structures from outside to inside; STB later or syncytium (multinucleated), a non-continuous CTB layer (Langerhans cells); villous stroma containing mesenchymal cells, collagen fibres, Hofbauer cells (stromal macrophage) and ground substance (hyaluronic acid) and finally the villous capillaries and sinusoids lined by

endothelial cells. There are five types of placental villi, mesenchymal villi, immature intermediate villi, stem villi, mature intermediate villi, and terminal villi. These can be differentiated by their physical characteristics and staining (45,46).

The 2014 Amsterdam Placental Workshop Group Criteria classified Histological changes of maternal vascular malperfusion into two patterns. Firstly, global/partialmaternal malperfusion has two forms the severe form of distal villous hypoplasia and accelerated villous maturity. Histologically distal villous hypoplasia is seen as long, slender distal villi that are sparsely occupying the relatively increased intervillous space. Accelerated villous maturity is characterized histologically by increased syncytial knots for gestational age, reduced size of villi and fibrin deposition (47). The second pattern of maternal vascular malperfusion is complete or segmental which is associated with villi infarction. All these findings have been seen separately in malaria and HIV infection (36,37,42–44). Our study focuses on partial maternal vascular malperfusion because it is associated with up to 25% risk of recurrence of preterm births (27). It appears that these histological changes are initiated by sequestration in the placentae of HIV by the Hofbauer cell (48) and Plasmodium infection of RBCs through attachment to Lewis antigen, Intercellular adhesion molecules(ICAM) and Cluster of Differentiation(CD) (CD36 and CD54(ICAM 1) and chondroitin sulfate A(33,49). This is followed by the attraction of inflammatory cells with resultant damage to the syncytiotrophoblast layer and subsequently the aforementioned structural changes. Malaria and HIV co-infection is synergic and bidirectional, and this worsens the effect on the placenta. This is because malaria infection activates T lymphocytes which in turn provide a favourable environment for HIV replication and progression while on the other hand, HIV destroys cellular immunity which is vital for malaria clearance(39,50,51). Though histological changes associated with Malaria and HIV separately have been well studied and documented, histological changes associatedwith co-infection have not been studied.Since partial maternal vascular malperfusion is associated with a high risk of recurrence of preterm births in subsequent pregnancies, their identification would guide future obstetrics

management with the aim of reducing the burden of preterm births(27).These histological changes in this study will be analyzed by light microscopy.

2.1.3 Histomorphometric analysis of the Placenta in Malaria and HIV

Proper arborization and maturation of the villous tree are of vital importance in the normal functioning of the placenta. This ensures that there is an adequate surface area for fetal-maternal exchange and provides a sufficient placenta reserve. This maturation process entails thinning of the placenta barrier that brings the maternal and fetal circulations closer for efficient feto-maternal exchange.

The development and structure of the placental villous tree are in part influenced by fetal genetics (52) and partly by the placenta microenvironment. The latter is affected by placental ageing, oxygen tension, immunological status, maternal nutritional status, and maternal chronic disease and infections e.g. Malariaand HIV.

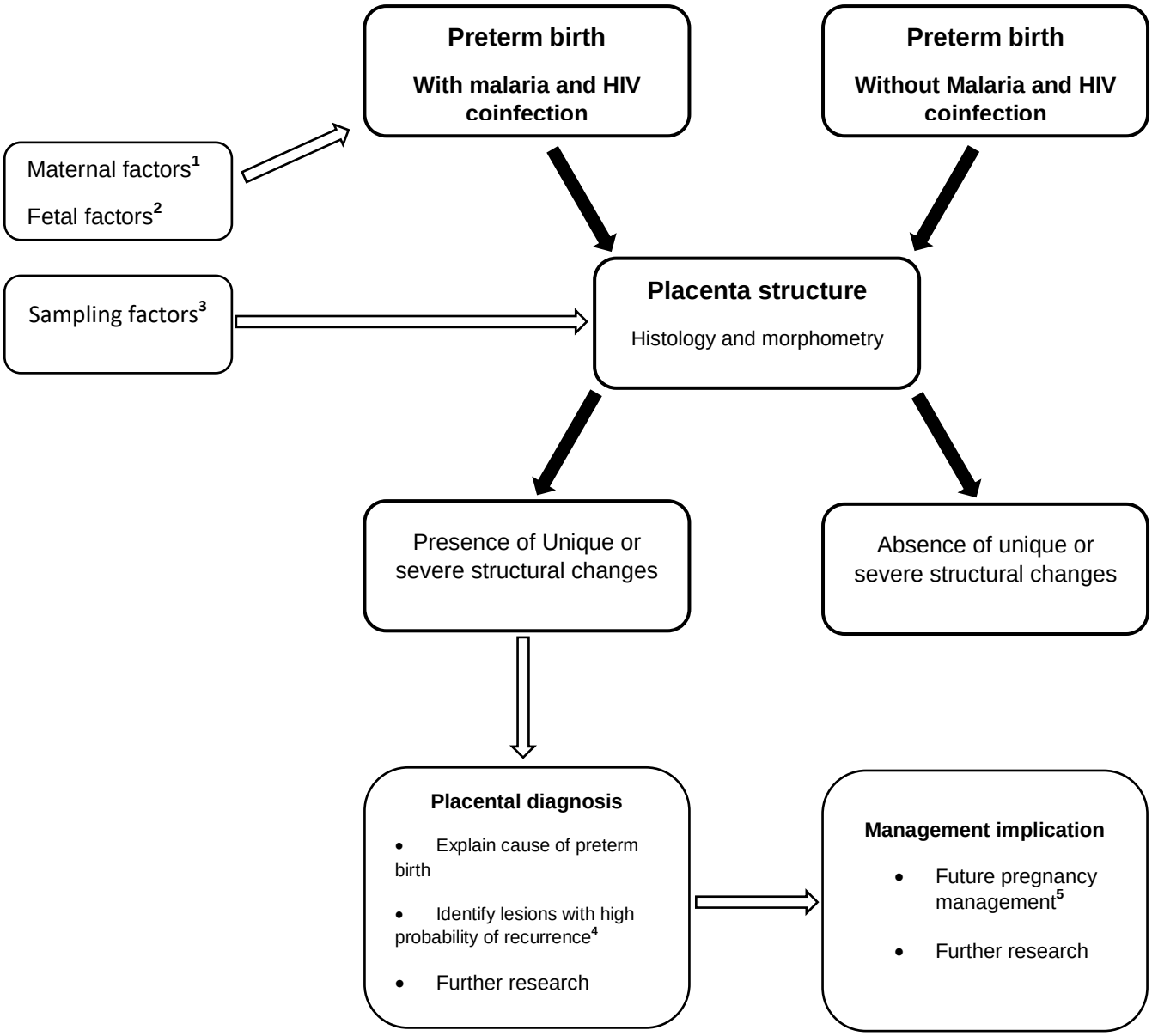
Morphological quantitative analysis was done through morphometric analysis which is the scientific analysis of two-dimensional (2D) images (53). Morphometry features include diameter, perimeter, cross-sectional area of the terminal villi and counting of structures e.g., villous capillaries.Some of the morphometric changes that are seen with placental infections like malaria and HIV are; a decrease in villous size, perimeter and vascularity which would effectively reduce the surface area of feto-maternal exchange per villous (42). These changes are likely due to partial maternal vascular malperfusion. It is known that Malaria and HIV infection are both associated with altered angiogenesis (16,54). Morphological changes in malaria and HIV co-infection have not been studied in relation to gestation age at birth. The morphometric analysis was conducted using Fiji/ImageJ.

2.1.4 What is known about Malaria and HIV coinfection, placenta structure and prematurity

There is a huge global awareness and push for the reduction of preterm birth. This awareness is emphasized every year during World Prematurity Day marked annually on the 17thof November. Agenda 3 of the Sustainable Development Goals (SDG 3) aims to reduce preterm birth by a third, prevent the death of under-fives and end the HIV and Malaria epidemic by 2030, these goals have also been adopted by Africa Agenda(55,56). In the past two decades, the rate of increase in preterm research has remained higher than the rate of increase in overall research. Most of the research done on preterm is about epidemiology and clinical pathology (50). Despite the increase in preterm global awareness and research, the incidence has remained the same over the years. This has seen the formation of the Preterm Birth Initiative in California and East Africa (PTBi-EA) which aim to build capacity in preterm birth research(2). Very few studies have been done about preterm birth and placenta structure, especially in the context of Malaria and HIV coinfection which is a huge burden in Sub-Saharan Africa and a major contributor to preterm birth in this region. It is well known that malaria and HIV both cause partial maternal vascular malperfusion which results from hypoxia and ischemia caused by the associated inflammation and vasculopathy (17,18,57). The histological changes associated with maternal vascular malperfusion have not been studied in the context of malaria and HIV coinfection. Similarly, the specific placental structural changes have not been related to gestation age. It is also known that some of these structural changes are also associated with a high risk of recurrence in the subsequent pregnancy and their identification would help in future obstetrics management to reduce the burden of prematurity. These placental structural changes have not been investigated in Malaria and HIV co-infection. Some of the management implications for future pregnancies (subject to further studies) that may arise from identifying features of partial maternal vascular malperfusion are; preconception care, early start of antenatal care, frequent visits, evaluation of cardiovascular system and

diabetes, use of aspirin, antenatal steroids and early delivery. If the incidence of preterm birth is to be reduced then an all-round and integrated knowledge of Malaria and HIV co-infection from epidemiology, clinical pathology, immunology and most importantly placenta changes(50). The answer to our study question, “What are the clinical, sociodemographic and placental histological differences in placentae of preterm births among women with Malaria and HIVand those without?” will hopefully add more information on Malaria and HIV co-infection in pregnancy and help reduce the burden of prematurity. The placenta samples for our study were obtained from bio-banked placenta specimens that were collected from Bungoma County (a place with a high burden of Malaria and HIV as well as high rates of hospital deliveries) between January 2018 and December 2019. These placentae were collected for a study titled; “Rapid and Multiplex Diagnosis of Maternal infections”. A study aimed at developing test kits for diagnosing asymptomatic neonatal infection by carrying out tests on placentae after birth to institute antibiotic therapy early and to reduce neonatal mortality and morbidity.Another objective of the study was to collect a large number of placenta specimens for a Biobank that would provide specimens for future studies like ours

2.2 Conceptual Framework



1. **Maternal factors** include sociodemographics and maternal diseases e.g. Age, parity, level of education, hypertension, diabetes, anaemia, chorioamionitis, malnutrition etc.
2. **Fetal factors** include Fetal malformation, multiple gestations etc.
3. **Sampling factors** include Time of placenta collection, time of cord clamping, sample preparation
4. Partial maternal vascular malperfusion
5. Preconception care, early antenatal care, frequent antenatal care, frequent fetal surveillance, aspirin, antenatal steroids. early delivery etc.

NB: Solid arrows and boxes represent the main study pathway

2.3 Problem Statement

The global preterm birth incidence ranges from 5% to 18%, and in Kenya, the incidence is 12.3%(14). Prematurity is the largest killer of children under 5 years and in Kenya accounts for 35% of all neonatal deaths (3).Prematurity is also associated with morbidities seen even in mid-adulthood ages and comes with a great cost of care. The incidence of preterm birth is even higher for Malaria and HIV-affected pregnancies, 44.6% and 30.4% respectively(3), this figure might even be higher for malaria and HIV co-infection. These two conditions pose a huge burden in Sub-Saharan Africa where the prevalence of Malaria in HIV-infected pregnant women is upto 37%(39). In Kenya, the western region where Bungoma County lies has also a huge burden of Malaria and HIV(41). Malaria and HIV infection are known to cause preterm births by their effect on the placenta due to maternal malperfusion caused by hypoxia and ischemia caused by inflammation and vasculopathy(17,18). There are no studies that related these placental changes to the gestation age especially studies that identify placental histological changes associated with a high risk of recurrence in future pregnancies.

2.4 Justification

Because of the huge mortality and morbidity associated with prematurity, there has been a strong global push for awareness and efforts aimed at reducing the burden of prematurity. This is evidenced by SDG 3 and African Agenda 2063 which aim to reduce the incidence of prematurity. Research is one of the ways that have been fronted to reduce this burden. There have been numerous studies done on Malaria and HIV epidemiology, clinical pathology, immunology and its association with preterm births. However, there are relatively fewer studies done on the structural effects on the placenta as a cause of preterm birth and especially so in the context of Malaria and HIV co-infection. A study on the placental histological changes would help identify the immediate cause of preterm birth and identify histological features associated with a high risk of recurrence which could guide on management of future pregnancies to reduce the incidence of prematurity as well as assist

in the future development of markers which will indicate the predisposed fetuses to premature delivery.

2.5 Significance

Knowledge of placental histological changes associated with a high risk of recurrence will guide the management of future pregnancies of women with Malaria and HIV coinfection to reduce the burden of prematurity. For instance, to prevent the recurrence of preterm birth or even to reduce the death and associated morbidities, a pregnant woman with Malaria and HIV infection may be advised to start antenatal care early and to make frequent visits. The care provider may also increase the frequency of fetal surveillance, evaluate for other conditions that may contribute to poor outcomese.g., cardiovascular system, start the pregnant mother's medication e.g. aspirin, antenatal steroids for those at risk of preterm birth and plan for early delivery for those at risk of stillbirth.

2.6 Research question

What are the clinical, sociodemographic and placental histological differences in the placentae of preterm births among women with Malaria and HIV co-infection and those without?

2.7 Hypothesis

2.7.1 Null hypothesis

There are no clinical, sociodemographic and placental histological differences in placentae of preterm births among women with Malaria and HIV co-infection and those without.

2.8 Broad objective

To compare the clinical, sociodemographic and placental histological features in placentae of preterm births among women with Malaria and HIV co-infection and those without.

2.9 Specific objectives

2.9.1 To describe the differences in the clinical and sociodemographic characteristicsof preterm births among women with Malaria and HIV coinfection and those without.

2.9.2 To describe the differences in theparenchyma (villi and intervillous space) of placentae of preterm births among women with Malaria and HIV co-infection and those without.

2.9.3 To analyze the differences in the histomorphometricfeatures of parenchyma (villi and intervillous space) of placentae of preterm births among women with Malaria and HIV co-infection and those without.

3 METHODOLOGY

3.1 Study design

This was aretrospective cohort study in which the clinical and sociodemographic characteristics and histological differences of placentae of women with preterm birth and Malaria & HIV co-infection(exposed group) were compared with those and those withoutthe co-infection(the unexposed group). The investigators were blinded to Malaria and HIV co-infection status.The blinding was enabled by a dedicated Research Assistant who performedthe random selection, decoded and recoded the specimen and later decoded them after analysis. This Research Assistant was not involved in the analysis stage. The diagnosis of HIV and malaria was based on the historical record of the patient and the time of preterm delivery, most participants in the exposure group were diagnosed with HIVat booking around 12 weeks and malaria diagnosis was made mostly around the secondvisit at 18 weeks. Appropriate treatment was instituted.

3.2 Study site and setting

In this study, bio-banked placentae specimenswere analysed at the Basic Clinical and Translational Research Laboratory (BCTRL) based at the Chiromo Campus in the Department of Human Anatomy at UoN. The specimens were collected from the Bungoma County Referral Hospital (BCRH)between January 2018 and December 2019 to fulfil one of the objectives of the larger study titled Rapid and multiplex diagnosis of maternal infections, to establish a placental biobank. The Ethical Review Committee number is MKU/ERC/0543 (see attached Appendix 6). One of my supervisors is a coinvestigator in this larger study

The Basic Clinical and Translational Research Laboratory is located in the Department of Human Anatomy at the Chiromo Campus of the University of Nairobi. It has a biorepository of placentae specimen. It has a digital light microscope, the Richter Optica, which is interphased with the Moticam BTU camera system. It is manned by a full-time Research and

Pathology technician who assists researchers in retrieving specimens, specimen preparation and basic analysis.

BCRH is the main referral hospital in Bungoma County with a maternity capacity of 40 beds. It has an active labour ward carrying out approximately 600 deliveries monthly. Thecatchment areas of BCRH are drawn from Bungoma, Kakamega, Busia and Trans-Nzoia Counties all parts of the former western province of Kenya.Located 324km North-West of Nairobi, Bungoma County has a big burden of Malaria and HIV infection. The HIV infection rate in the Western part of the country is up to 25.7%(41). Though there is no data about Malaria and HIV coinfection in Bungoma, it is reasonable to believe that since the burden of both diseases is high the coinfection rates are also high and if this happens in pregnancy then this will contribute to preterm births.

3.3 Study population

Bio-banked placentae specimens of women who delivered at BCRH wereanalyzed. The study specimens were grouped into exposure and non-exposure groups. The exposure group were placentae of women with Malaria and HIV co-infection delivering preterm babies at gestations between 28 weeks plus 0 days to 36 weeks plus 6 days while the non-exposure groupwere placentae of womenalso delivering preterm birth without Malaria and HIV coinfection.Participants were recruited after delivering preterm and the diagnosis of malaria and HIV was obtained from the patient antenatal notes. Most participants were diagnosed with HIV by the time they started antenatal care at around 12 weeks and malaria diagnosis was made by blood smear test at an average gestation of 18 weeks.

3.4 Inclusion criteria

Bio-banked placentae specimen of women;

1. Aged between 18 and 40 years
2. Singleton pregnancy
3. Gestational age of between 28 weeks and 36 weeks plus 6 days with Malaria and HIV co-infection for the exposure group.
4. Women were sure of the date of the first day of the last normal menstrual period and confirmation of the dates was done by an obstetrics ultrasound scan before 16 weeks.
5. Bio-banked specimen for which consent was obtained for their use

3.5 Exclusion criteria

Bio-banked placentae specimens of women with the following medical and obstetric complications:

1. Hepatitis B and syphilis,
2. Chronic hypertension,
3. Preeclampsia,
4. Diabetes Mellitus,
5. Cardiovascular diseases
6. Malnutrition.
7. Preterm Premature Rupture of Membrane
8. Congenital anomalies of the baby
9. During sampling, the paraffin blocks missing labels or clinical data, as well as those missing tissues or those with exposed tissues, were excluded
10. During sectioning, the blocks that were not sectionable due to excessive brittleness were excluded
11. During staining, the blocks that were over-stained, under-stained or washed out were excluded

3.6 Sample size and sampling procedures

3.6.1 Sample size

To calculate the sample size, we usedthe findings of a study by Obimbo et al(36) that found the prevalence ofvillous hypervascularityat 32% amongplacentae of HIV-positive women versus 1% in HIV-negative placentae. We used the percentages as the proportion for the exposed and non-exposed respectively to calculate the sample size.

$$N_{Kelsey} = \frac{(z_{\alpha/2} + z_{\beta})^2 p(1-p)(r+1)}{r(p_0 - p_1)^2}$$

Variable Notations:

α The probability of type I error (significance level) is the probability of rejecting the true null hypothesis =0.05

β The probability of type II error (1 – the power of the test) is the probability of failing to reject the false null hypothesis =0.20. The power of this study is 80% (0.8)

P The mean of $\mathbf{P_0}$ and $\mathbf{P_1}$

$\mathbf{P_0}$ The proportion of disease in population 1(Exposure group) = 0.32

$\mathbf{P_1}$ The proportion of disease in population 2 (Non-exposure group) =0.01

r The ratio of population 2 to population 1 (population 2 to 1 population 1)= 1

$\mathbf{N_{Kelsey}}$ Required sample size for the population one group using Kelsey formula

This formula will give a sample size of 23 placentae for the exposure group and 23 placentae for the non-exposure group. A 10% margin was added in case of placental damage. The final sample size is 25 placentae for each group.

3.6.2 Sampling procedures

A random online number generator was used to randomly select 25 paraffin blocks for each of the exposure and non-exposure group from the eligible paraffin blocks in the Biobank. A Research Assistant who was not involved in the analysis decoded and recoded the specimens. The placenta blocks were prepared as follows, after delivery of placenta cord was cut at 5 cms from the disk and occluded to preserve vascular architecture. The placenta was fixed in 10% buffered formalin for 24 hours, the membrane removed and 6 placenta blocks were taken 4 from the peripheries and 2 from centrally on either side of the cord attached to the disk. These blocks were then dehydrated in incremental concentrations of alcohol 70% to 100%) then infiltrated in molten paraffin for 12 hours and finally embedded in molten paraffin to make paraffin placental blocks. These were then transported from Bungoma to the Biobank in the Basic and Clinical Translation laboratory on the Chiromo campus.

Our study used a central block from each of the randomly selected placentae as this is where maternal vascular malperfusion is best analyzed (26). For analysis, a Leitz Wetzler microtome was used to cut 7µm sections. 6 sections were cut from each placenta block, floated in warm water and mounted on slides and stored at 40˚C in a dry heat oven overnight. Then they were deparaffinizedand rehydrated at decreasing concentration of ethanol, with xylene used to clear the paraffin wax. These were then stained with hematoxylin and eosin and mason's trichome as per protocol. Then they were dehydrated again at an increasing concentration of ethanol and mounted using DPX (Distyrene, Plasticizer and Xylene). Two sections from each block were subjected to analysis as follows;

1. For light microscopy Richter Optica XU-1T plan Achromatic digital microscope was used for analysis at a total magnification of 100X and 400X. Features like distal villous hypoplasia, fibrin deposition and villous vascularity were best analyzed at a total magnification of 100X. For villous vascularity, the criteria of Altshuler were used(58). The rest of the features like

syncytial knotting and villous infarctions were analyzed at a total magnification of 400X. (A total of 100 slides for light microscopy; 50 H&E and 50 Masson's Trichome).

2. For morphometric analysis, microphotographs were captured at resolution 1280x800 pixels at a total magnification of 400X by the MoticamBTU8 camera system which was calibrated to a known scale and saved as BMP files. Fiji/Image J was used to estimate the villous diameter (µm), perimeter(µm) and cross-sectional area (μm^2) of the terminal villous. The image was first opened in Fiji and converted to an 8-bit image and a scale set then analysis was done to give measurements. A total of 150 microphotographs were taken randomly from the 50 H&E stained slides including both exposure and non-exposure group.

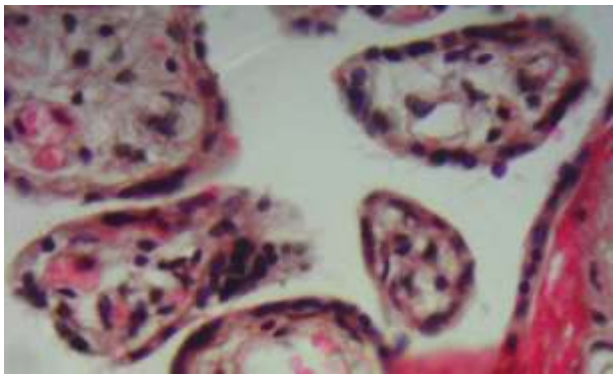


Figure 8: Red Blue Green (RGB) BMP file as uploaded on FIJI/Image J software

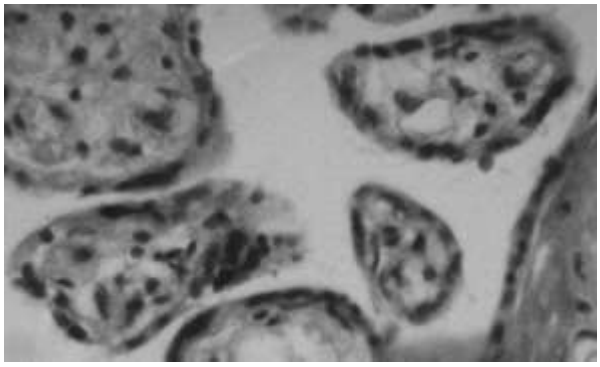


Figure 9: The image in figure 1 converted to an 8-bit greyscale image, a form that is required for FIJI/Image J morphometric analysis

Figure 10: Image in figure 2 segmented using the auto threshold method to enable measurements by FIJI/Image J

Clinical data of every specimen was retrieved from the archives and analyzed appropriately.

3.7 Quality assurance

3.7.1 Internal quality assurance

Only the placenta tissue specimens that were undamaged and collected, processed and stored according to internationally accepted standards were used for analysis. During the analysis stage, the specimenswere prepared and analyzed according to the predetermined standard operating procedure. To acquire basic working knowledge and skills for histological evaluation, the Principal Investigator undertooka 2-week on-bench mentorship practical sessions at the histology lab in the Department of Human Anatomy at Chromo Campus, these sessions were supervised by the Chairman of the Human Anatomy Department who is one of the Principal Investigator's mentors. He has vast experience in placental biology after his PhD and Postdoc work. The sessions equipped the Principal Investigator with basic skills

in; tissue preparation for histopathology evaluation, sectioning specimens, staining specimens, mounting on slides and basic analysis of tissues under the microscope to identify the basic histological organization of the placenta as well as knowledge of morphometric quantification methods. Together with his mentors, a Pathologist and an Anatomist who were blinded to malaria and HIV coinfection statusthe histological diagnosis was made. After the analysis, the data was recorded on a specifically designed data collection form which was secured by the Principal Investigator.

3.7.2 External quality assurance

10% of the results after analysis (randomly selected) were verified by an Independent Pathologist.

3.8 Data variables

SPECIFIC OBJECTIVES	EXPOSURE VARIABLE	OUTCOME VARIABLE	SOURCE OF DATA
To describe clinical data of women with Malaria and HIV delivering preterm.	1. Age 2. Gestational age 3. Parity 4. Marital status 5. level of education	1. Preterm Birth with Malaria and HIV coinfection 2. Preterm birth without malaria and HIV coinfection	The pre-filled clinical data collection form
To describe histological changes in the placental parenchyma	1. Preterm Birth with Malaria and HIV coinfection 2. Preterm birth without malaria and HIV coinfection	1. General structural organization of the placental parenchyma 2. Syncytial knotting 3. distal villous hypoplasia 4. villous infarction 5. Villous capillaries 6. villitis 7. fibrin deposition	Light microscopic examination of the serial 7µmsections
To describe the morphometric changes of the terminal villi	1. Preterm Birth with Malaria and HIV coinfection 2. Preterm birth without malaria and HIV coinfection	1. The diameter of the terminal villous(µm) 2. The perimeter of terminal villous(µm) 3. The cross-sectional area of terminal villous(μm^2) 4. Number of fetal capillaries in the villous stromal core	microphotographs analyzed by computed assisted morphologic quantification (Fiji/Image J)

3.9 Data collection and management

The data on histologyand morphometricfeatures were recorded by the investigator on specifically designed data entry forms (see appendix 4). Additionally, microphotographs of the histological feature were taken and saved as BMP files. Numerical and categorical data were collected in excel sheets. All data were stored on an external hard disk in password-

protected folders that were in the custody of the Principal investigator. Backup storage of data was also arranged in another external hard disk.

3.10 Data analysis

Data was saved in an excel sheet, cleaned and uploaded in SPSS version 26 and analysed as follows;

For objective 1, to compare the clinical characteristics

For numerical data, the mean and the standard deviation were calculated and compared using the student t-test while for categorical data the numbers and the percentages were calculated and compared using the X^2 test/Fisher's Exact test.

For Objective 2, to compare the histology of placental parenchyma

All data was categorical and the numbers and the percentages were compared using the X^2 test/Fisher's Exact test as appropriate. The strength of association of the diagnosis of partial maternal vascular malperfusion was analysed using a 2x2 table with Relative Risk and 95% CI calculated.

For objective 3, comparing morphometric analysis

All data was numerical, and the mean and the standard deviation were calculated and compared using the student t-test/Mann-Whitney U test as appropriate. The strength of association of the diagnosis of villous hypervascularity was analysed using a 2x2 table with Relative Risk and 95% CI calculated.

The significance level was set at 0.05 for all tests

3.11 Protection of human subjects (Ethical considerations)

3.11.1 *Human subjects' involvement and characteristics of the study population*

While the bio-banked placentae specimen are not Human subjects, theyrepresented the study population in this research. The human subjects whose placentae were collected

consented and their information was treated confidentially.The placenta specimen for this study were collected for another placenta study and transported to the Basic Clinical and Translational Laboratory at Chiromo Campus. This study population represented the Catchment areas of BCRH which are drawn from Bungoma, Kakamega, Busia and Trans-Nzoia Counties. All the data of t

3.11.2 Ethical approval

The study that established the biobank was titled, “Rapid and Multiplex Diagnosis of maternal infections” and its ethical approval was obtained from the Mount Kenya University Ethical Review Committee (see appendix 6). Consent to use the bio-banked specimen was obtained (see appendix 7) Approval for this study wasobtained from the KNH/UoN Ethical and Scientific Review Committee P/406/08/2020 (see appendix 8).

3.11.3 Sources of material

Research material was bio-banked placenta specimens that were collected for thestudy titled Rapid and Multiplex Diagnosis of Maternal infections. The specimens were collected at BCRH. **Linkages to subjects**

Confidentiality was maintained during the selection, preparation and analysis of the biospecimens at the Basic Clinical and Translational Research Laboratory. All specimens and data were de-identified and coded.

3.11.4 Potential risks

There was a risk of alteration of the placentae data due to collection, processing and storage factors. A standard protocol was used during the collection, processing and storage of the samples to avoid these variations. Because these specimens were de-identified, therefore confidentiality was maintained and hence there was no risk to the mothers whose placentae were used for this study.

3.11.5 Recruitment of study participants and consent process

Random selection from the bio-banked placenta specimen was done according to the inclusion and exclusion criteria of this study. Consent from the study participants was

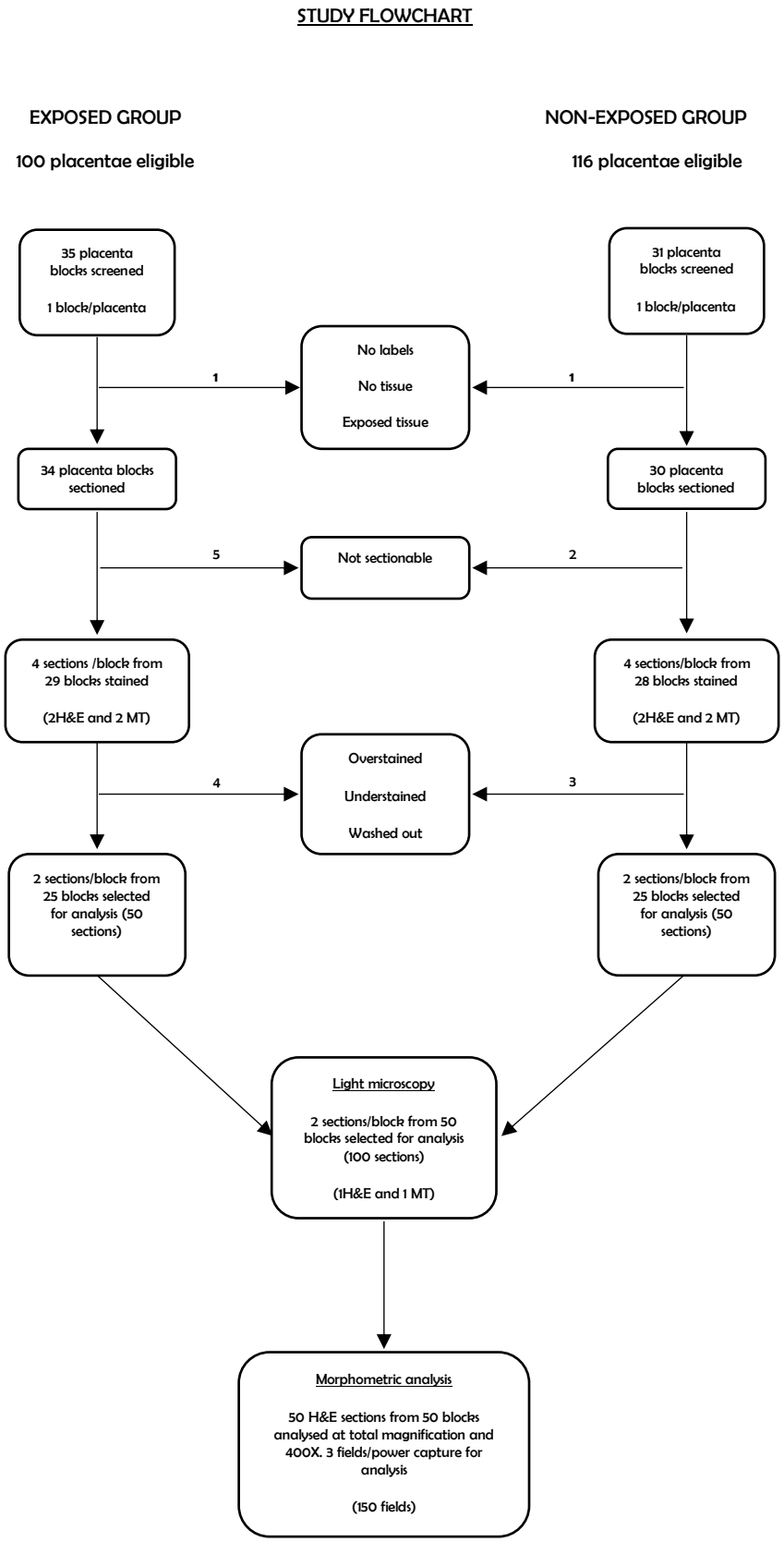
already obtained by the investigators of the study that established the biobank (see appendix 2).

3.11.6 Potential benefits and importance of the knowledge gained

This study was aimed at identifying the clinical, sociodemographic and histologicaldifferences in the placentae of preterm births among women with Malaria and HIV coinfection and those without. This will add to the knowledge of what placenta structural features are associated with preterm and inturn help in coming up with interventions to reduce the burden of Preterm birthor at least the study will form a foundation for future studies. These results will be disseminated by sharing with Bungoma County Referral Hospital, Kenyatta National Hospital, and the University of Nairobi and published in Medical Journals.

4 RESULTS AND DISCUSSION

4.1 Results



4.1.1 clinicaland sociodemographic characteristics of women with preterm birth and malaria and HIV coinfection compared to those without

Table 1: Clinical and sociodemographic characteristics of women delivering preterms.

(HIV-Human immunodeficiency virus; m-mean; SD-standard deviation)

Clinical and social demographic features	Malaria and HIV coinfection n=25	Malaria and HIV negative n=25	p-value
Age(years)[m(SD)]	26(5.147)	29(4.482)	0.029$^{\alpha}$
Age categories(years)			
15-24 [n(%)]	10(40%)	4(16%)	0.059$^{\beta}$
25-40 [n(%)]	15(60%)	21(84%)	
Parity [m(SD)]	2(1.173)	3(0.997)	0.012$^{\alpha}$
Parity categories			
Primipara [n(%)]	8(32%)	0(0%)	0.004$^{\gamma}$
Multipara [n(%)]	16(64%)	23(92%)	
Grand Multipara [n(%)]	1(4%)	2(8%)	
Gestational age at delivery(weeks)[m(SD)]	34(1.159)	35(1.118)	0.113$^{\alpha}$
Level of education			
Primary school	12(48%)	2(8%)	0.007$^{\gamma}$
High School	9(36%)	15(60%)	
College	4(16%)	8(32%)	

α- Student t-test
β- Chi-square test
γ-Fisher Exact test

Table 1, summarizes the clinical characteristics of patients. The mean gestational age for preterms in the exposure group was 34(1.159) weeks and 35(1.118) weeks in the non-exposure group. Parity was treated as both a numerical variable and categorical variable broken down into primipara, multipara and grand multipara. The majority of the patients were multipara in both groups.

Women with malaria and HIV coinfection were younger (26 years vs 29 years p-value 0.029) and had a lower parity (2 vs 3 p-value of 0.012) and level of education (most in the primary level p-value 0.07) when compared to women without malaria and HIV coinfection. Marital status and gestational age were not statistically significant.

4.1.2 Comparison of theplacental parenchyma histological features between preterm placentae with malaria and HIV coinfection and those without

Table 2: Comparison of the histological features of placental parenchyma among preterm placentae.

(HIV-Human immunodeficiency virus)

Placental histological findings	Malaria and HIV coinfection n=25	Malaria and HIV negative n=25	
	n(%)	**n(%)**	**p-value**
Accelerated Villous Maturity			
Absent	13(52%)	20(80%)	0.037$^{\beta}$
Present	12(48%)	5(20%)	
Distal Villous Hypoplasia			
Absent	17(68%)	18(72%)	0.758$^{\beta}$
Present	8(32%)	7(28%)	
Villous Necrosis			
Absent	25(100%)	21(84%)	0. 111$^{\alpha}$
Present	0(0%)	4(16%)	
Syncytial knots (for gestational age)			
Absent	13(52%)	20(80%)	0.037$^{\beta}$
Present	12(48%)	5(20%)	
Thickening of Villous Membrane			
Absent	25(100%)	23(92%)	0.490$^{\alpha}$
Present	0(0%)	2(8%)	
Villous stromal fibrosis			
Absent	25(100%)	21(84%)	0. 111$^{\alpha}$
Present	0(0%)	4(16%)	
Fibrin deposition			
Absent	20(80%)	4(16%)	<0.001$^{\beta}$
Present	5(20%)	21(84%)	
Villous Vascularity			
Decreased	1(4%)	11(44%)	0.004$^{\alpha}$
Normal	14(56%)	12(48%)	
Increased	10(40%)	2(8%)	
Overall impression			
No partial maternal vascular malperfusion	4(16%)	15(60%)	0.004$^{\alpha}$
Partial maternal vascular malperfusion	21(84%)	10(40%)	

α-Fisher's Exact test
β-Chi-Square test

Table 2, summarizes the histological changes in the placenta. There was no presence of delayed villous maturity, villitis, intervillositis, or massive histiocytic intervillositis, in both groups and only one presence of villous oedema in the non-exposure group, there was no significant difference between the groups to test. Fibrin deposition was 20(80%) absent among the exposure group and 21(84%) present among the non-exposure group. Villous necrosis presence and absence correlate to that of villous stromal fibrosis in both groups.

There was a significant difference between the groups with regards to accelerated villous maturity, increased or decreased syncytial knots for gestational age, fibrin deposition, villous vascularity and the final diagnosis of partial maternal vascular malperfusion.

4.1.3 Comparison of morphometrical features of the terminal villi among preterm placenta with malaria and HIV coinfection and those without

Table 3: Morphometrical parameters of the terminal villi among preterm placentae.

(HIV-Human immunodeficiency virus; m-mean; SD-standard deviation)

histomorphometric features	**Malaria and HIV coinfection n=25**	**Malaria and HIV negative n=25**	
	***m*(*SD*)**	***m*(*SD*)**	**p-value**
Placental weight(g)	454(32)	488(36.7)	0.001^{α}
Diameter (µm)	41.24(4.406)	43.37(4.611)	0.102^{α}
Perimeter (µm)	119.32(9.2)	130.47(12.47)	0.001^{α}
Cross sectional area of villous (µm2)	937.93(148.6)	1132.88(235.85)	0.001^{α}
No of capillaries / villous	5.28(3.93)	3.24(2.48)	0.099^{β}

α-Student t-test

β-Mann Whitney U test

Table 3, summarizes the morphometric features of the placenta. **Placental weight(g), perimeter (µm) and Cross-sectional area of villous (µm2) were significantly different between the 2 groups.** The number of capillaries/ villous was similar in both groups.

Table 4: Relative Risk of partial maternal vascular malperfusion and villous hypervascularity among preterm placentae.

(HIV-Human immunodeficiency virus; RR-relative risk; CI-confidence interval)

Select histological findings	Malaria and HIV coinfection Yes	Malaria and HIV coinfection No		
	n(%)	**n(%)**	**p-value**	**RR(CI)**
Partial maternal vascular malperfusion				
Present	21(68%)	10(32%)	0.001	2.1(1.261-3.496)
Absent	4(21%)	15(79%)		
Villous hypervascularity				
Present	8(80%)	2(20%)	0.034	4.0 (0.941-17.00)
Absent	17(42%)	23(58%)		
p-values were obtained using Fisher's Exact test				

Table 4, Summarises the strength of association of two select histological features and malaria and HIV status. Partial maternal vascular malperfusion is present more in the exposure group than in the non-exposure group. Malaria and HIV coinfection doubled the risk of developing maternal vascular malperfusion. 7 or more capillaries/villous indicated hypervascularity. This was similar in both groups (see table 4)**.**

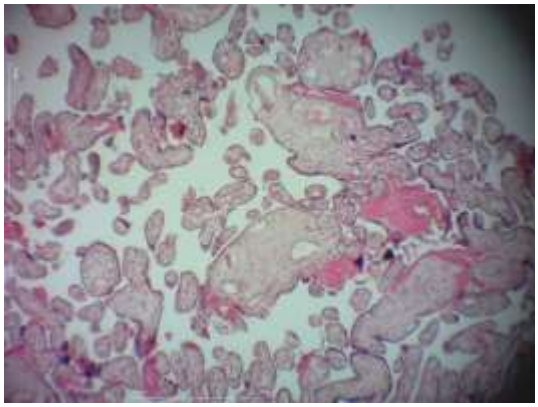


Figure 11(A): This is a photomicrograph taken at total magnification of 100X and stained by Haematoxylin and Eosin. the scale bar represents 100µm. it shows distal villous hypoplasia which is a scarcity of the terminal villi relative to the intervillous space.

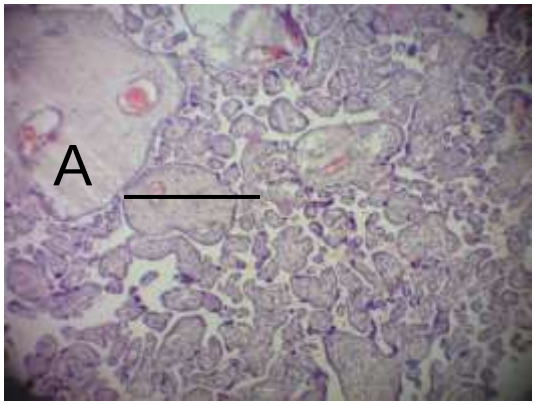


Figure 4(B): this photomicrograph was taken at a total magnification of 100X and stained with Hematoxylin and Eosin. The scale bar represents 100um. it shows the normal density of villi.

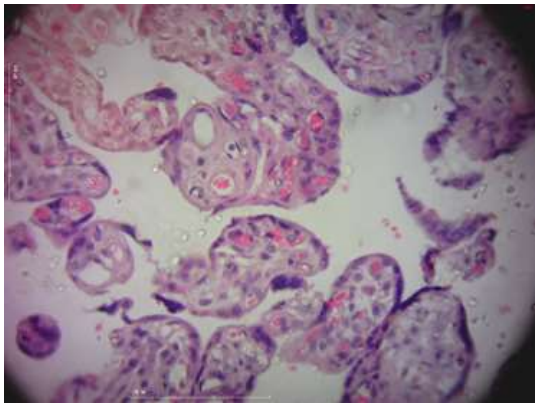


Figure 12(C): Microphotograph taken at total magnification of 400x and Haematoxylin and Eosin stained, the scale bar represents 100µm. this shows accelerated villous maturity demonstrated by increased syncytial knotting for gestation with vesiculosyncitial membrane. There is also normal villous vascularity.

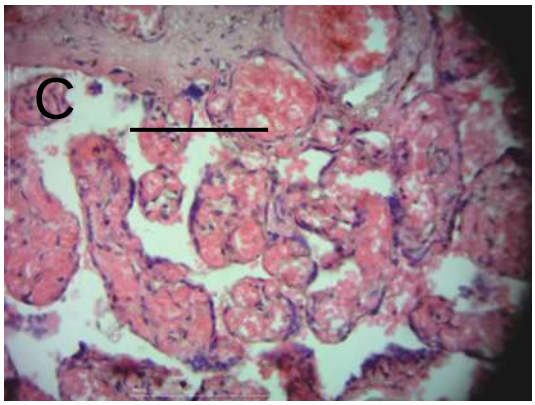


Figure 5(D): Microphotograph taken at total magnification 400X and stained with Hematoxylin and Eosin, the scale bar represents 100µm. It shows increased villous hypervascularity

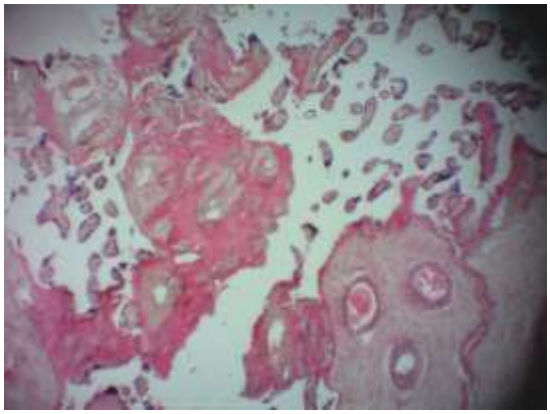


Figure 13: Microphotograph taken at total magnification 100X and stained with Haematoxylin and Eosin, the scale bar represents 100µm. It shows increased fibrin deposition

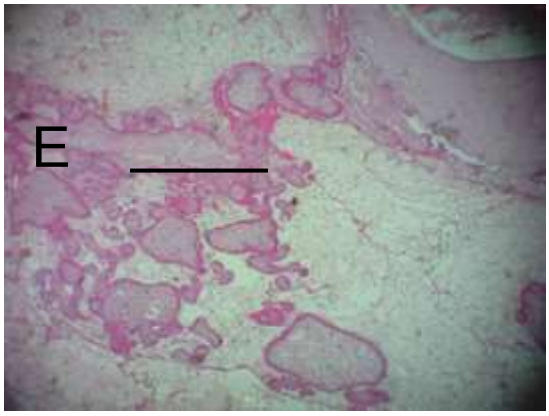


Figure 14:Microphotograph taken at total magnification 400X and stained with Hematoxylin and Eosin, the scale bar represents 100µm. It shows villous necrosis

4.1.4 Discussion

This study was able to compare the clinical, sociodemographic and placental parenchymal histology of preterm births with malaria and HIV infection and those without. In addition, it associated the coinfection with partial maternal vascular malperfusion and villous vascularity. Clinical and sociodemographic factors associated with malaria and HIV coinfection were younger age (26 years vs 29 years), low parity (2 vs 3) and low levels of education (below secondary level). Studies have shown that in Kenya, the rate of new HIV infections is high in the young age group(20,22).In ourstudy, women with malaria and HIV coinfection and preterm births in the young age group were 40% compared to the 16% of the women with preterm births but without the coinfection. In the young age group low perception of the risk of HIV, unprotected sex under the influence of alcohol and other drugs as well as forced sex and sexual violence are the most likely contributing factors(22). Of the women with preterm births and malaria coinfections, 88% were married. A Kenyan study has demonstrated that lack of mutual knowledge of partner HIV status, the misconception that if one partner is infected the other one is also infected, extramarital affairs and the high prevalence of ulcerative sexually transmitted disease are the likely contributing factors(20). This calls for more aggressive public health interventions targeting the young age group and the married couple to reduce the disease burden and subsequently reduce preterm births due to malaria and HIV coinfection. The young age group is particularly important because they are theschool-going youths, newly employed and economically productive. Women in the exposure group had relatively lower parity, this is in agreement with studies that show that malaria is more prevalent in primigravida and secundigravida and for HIV infection the parity may be lower because it is more prevalent in the young age group. Though higher parity is

associated with acquired immunity against malaria, it should be remembered that pregnant women are more susceptible to malaria infection than non-pregnant hence contraception use should be encouraged to reduce this vulnerability due to pregnancy(23,59). A higher level of education above the primary level appears to be protective against malaria and HIV coinfection and this is because an education level higher than primary education places a person in a better position of understanding the transmission and preventionof HIV and malaria, better access to health services, reduced social and economic vulnerability and higher levels of participation in programmes dealing with public health education, this has been shown by Kenyan studies referenced here(24,25). The Government should consider availing free secondary school education as part of its activities to reduce preterm births due to malaria and HIV infection and encourage education of the female youth who appear to be twice as likely as their male counterparts to acquire HIV infection(22).

Maternal vascular malperfusion is associated with the occurrence and recurrence of preterm births (27). It is a condition that not only results from low maternal blood flow in the placenta but also turbulent and high-pressure flow rather than eddy current and low-pressure flow in the intervillous space. The primary pathology involves the maternal decidual arterioles, which histologically manifests as atherosclerosis and persistent muscularization(26). In our case this may result from the altered angiogenesis associated with both malaria and HIV infection, the two conditions tilt the scale in favour of the antiangiogenic state e.g. reduced angiopoietin 1, reduced placental growth factor and increased soluble endoglin(16,57). The low maternal blood flow results in chronic hypoxia and oxidative stress while the turbulent flow results in mechanical stress to the placental villi. All the aforementioned results in the secondary pathology which was investigated in this study. The secondary pathology manifests grossly as reduced placental volume and weight and histological as a change in villous structure(26). This change in villous structure is categorised into two according to the 2014 Amsterdam Consensus Criteria; partial maternal vascular malperfusion which occurs when the decidual arterioles are partially occluded and complete maternal vascular

malperfusion which occurs when the decidual arterioles are completely occluded. This study concentrated on partial maternal vascular malperfusionwhich has two patterns; distal villous hypoplasia and accelerated villous maturity, these are also referred to as early and late partial maternal vascular malperfusion respectively. Histologically distal villous hypoplasia is seen as a scarcity of terminal villous relative to the intervillous space, the villi are long and slender, the accelerated villous maturity is characterised by smaller terminal villi, with increase syncytial knots and vesciculosyncitial membrane for gestation(27). Morphometrically these two patterns have reduced diameter, perimeter and area of terminal villous(36,42). Villous hypervascularity is a compensatory reaction to chronic hypoxia, this occurs to increase oxygen uptake from intervillous blood that has low oxygen content. Our study also investigated villous hypervascularity which is defined as 7 or more villous capillaries per villous and is diagnosed using Altshuler's criteria (58,60).

In this study, partial maternal vascular malperfusion was more in preterms with malaria and HIV coinfection compared to those without 84% compared to 40%. These findings were significantly higher in the coinfection than in either of the infection alone, which points towards synergism between these two diseases (36,37,39,41,42).This was seen grossly as reduced placental weight (454 g vs 488g), histologically as distal villous hyperplasia and accelerated villous maturity and morphometrically as reduced villous perimeter and cross-sectional area. The late partial maternal vascular malperfusion featured more in preterms with malaria and HIV coinfections. The relative risk of partial maternal vascular malperfusion2.1, CI (1.261-3.496) and villous hypervascularity4.0 CI (0.941-17.00)was higher in preterms with malaria and HIV coinfection compared to preterms without malaria and HIV coinfection. These findings suggest that malaria and HIV co-infection leads to chronic hypoxia which may explain preterm births.These findings however have to be interpreted with caution as the confidence interval was wide owing to the small sample size and in the case of hypervascularity, the wide range of variation in the numbers of capillaries, the lower limit of the confidence interval was also included the null value. Studies have

shown that maternal vascular malperfusion not only caused preterm births but is also associated with the recurrence of preterm births. Some studies have also suggested that the risk of recurrence can be reduced by employing the following strategies; early antenatal care. Frequent visits and fetal surveillance, aspirin therapy, early delivery after antenatal corticosteroids and optimisation of maternal cardiovascular function by controlling blood pressure and diabetes mellitus(26,27). In this study, neither the typical histological findings that are seen in malaria namely hemozoin deposition and intervillositis nor the villitis in HIV infection was demonstrated, this suggests that treatment had some beneficial effects not only clinically but also histologically(61,62). Given that all participants of this study were treated appropriately for both conditions, one may conclude that using the appropriate treatment for malaria and HIV is an important part of antenatal care for these patients.

5 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

1. Our study found out that women with malaria and HIV coinfection were younger and had lower parity and level of education when compared to women without malaria and HIV coinfection. This suggests that Public Health interventions that are directed toward preventing malaria and HIV infection in young aged women and those interventions that increase the level of education could lower the burden of malaria and HIV coinfection and preterm births resulting from the two conditions.

2. Our study also found that malaria and HIV coinfection doubled the risk of developing maternal vascular malperfusion and this finding suggests that chronic hypoxia, which is a known cause of maternal vascular malperfusion, contributes to preterm births in malaria and HIV coinfection.

3. This study ascertains that placental histology is important not only important in the diagnosis of preterm births but also in the determination of the risk of recurrence which is important in guiding themanagement of future pregnancies to avoid adverse outcomes.

5.2 Recommendations

1. Placental histology should be considered for every pregnancy with an adverse outcome as it may give important information that would diagnose the cause of the

adverse outcome as well as give information on the management of future pregnancies to avoid a repeat of the same adverse outcome.

2. We also recommend building up on the findings of this study by conducting studies that look at the ultrastructure and the biomarkers of inflammation and angiogenesis to develop a predictive model for preterm births.

5.3 Study strengths

1. It is the first study to investigate placentae of preterm births with malaria and HIV coinfection
2. It analyses placental histological features associated with the risk of recurrence in subsequent pregnancies and hence the study could lead to other studies to investigate management interventions

5.4 Study limitations and delimitations

1. Since this was a retrospective study, important data e.g. duration of disease, type of treatment, and history of preterm births was not available. We suggest conducting larger prospective studies that would investigate the effect of these.

2. This being a retrospective study that cannot show causality, we suggest conducting a larger prospective study.

3. The participants of this study were recruited from the western part of Kenya who may be different genetically from peoplein other parts of the country, we suggest conducting studies that include people from other regions of the country.

5.5 Source of funding

There were no External Funders for this study and all the funds were raised by the Principal investigator

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7 Appendices

Appendix 1: SOP placenta sample collection and preparation

Placenta sample collection and preparation for placental bank

Section 1: Describes the general collection of placental biopsies

1. Informed authorized consent
2. Harvest placental biopsies immediately after birth as described below
3. Harvest a full thickness placental biopsy 2cm by 2cm from six sites of the placenta
    a. 2 central biopsies from the sides of the cord insertion
    b. 4 peripheral biopsies taken at 12, 3, 6 and 9 O'clock positions of the disk
4. A membrane roll from the smooth chorion
5. 2 cord biopsies, one 1cm from the cord insertion and the other from the cut end of the cord
6. Wash the biopsies thoroughly in PBS until there is no more obvious blood
7. Cut the biopsy samples into three smaller samples A, B and C and prepare them as described in section 2 below.

Section 2: Describes processes of fixing the biopsies

Preparation of A: for fixed frozen section

1. After washing in PBS, fix the cut biopsy in 3% PFA for 12 hours
2. Transfer the biopsy and orientate into labeled biopsy cryomold containing OCT. The tissue should be covered with OCT
3. Freeze in dry ice/ -80° freezer
4. Cover in a labeled foil and store at -80° (freezer/dry ice)

Preparation of B: for electron microscopy

1. After washing in PBS, trim the tissue and separate it into two segments (the basal and chorion portions) each measuring 0.3cm by 0.3cm
2. Fix the biopsies in 3% glutaraldehyde at 2-8o for 12 hours
3. Ship for post-fixation in Osmium tetraoxide

Preparation of C: for paraffin blocks

1. After PBS wash, fix the biopsy samples in 10% formal saline solution
2. Transfer to the lab within 24 hours put them in 70% ethanol
3. Store at 4° for a maximum of 7 days
4. Start preparing blocks through standard procedure

In our case we need to ship in batches every three days for further processing

Appendix 2: Client information and consent form

This is the consent form that was used to recruit participants at BCRH for the Study that established the Biobank.

CLIENT INFORMATION AND CONSENT FORM
Study title

Rapid and Multiplex Diagnosis of Maternal Infections

Study no.....

Date __/__/__

Investigator : Dr Jesse Gitaka

Telephone contact: 0722425613

RESEARCHERS' STATEMENT

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

INTRODUCTION

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

PURPOSE AND BENEFITS

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

Procedure

Once you have agreed to participate in the study, you will sign this consent form to allow us to include information obtained from you in our data. Your personal details will not be included in this questionnaire so

as to protect your privacy. We will take a small portions of your delivered placenta for the purpose of this study. We will also look at your antenatal record to obtain more information which will remain confidential. You will continue to receive appropriate management while at the hospital. We also guarantee your safety during your participation in this study. If you agree to let the researchers collect specimens, the following will happen:

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

Confidentiality

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

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

Subject's statement

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

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

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

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

_____ _____

(Participant) *(Witness)*

Appendix 3: Certificate of Informed Consent

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

Participant Name: _____ (PRINT)

Signature of Participant _____

Or

Thumb print of participant

Date _____

Statement by the principal investigator/research assistant taking consent

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

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

Name of the person taking the consent _____ (PRINT)

Signature _____ Date _____

For any questions or concerns about the study contact

Dr Jesse Gitaka on 0722425613.

P.o.BOX 342-01000, Kenya.

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

Ethical review committee

P. O. Box 342-01000 Thika;

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

Appendix 3: Clinical Data Form

CLINICAL DATA FORM

Rapid and Multiplex Diagnosis of Maternal Bacterial Infections

Participant Study Number: | | | | | | |

Study group: | | |

Protocol Number:

CASE REPORT FORM

BASELINE DATA

MATERNAL PROFILE			
Participant Number			
ANC NUMBER	_____		
Study Site (Health Centre Name)	_____		
Inclusion/exclusion criteria *Patient must meet all criteria to eligible for the study	Met all ☐1.	Not met* ☐2	
Date of Informed Consent	D D M M M Y Y Y Y		
Date of Birth	D D M M M Y Y Y Y	Or estimated age ____	
Gravida			
Parity			
Estimated Gestational Age _____weeks			
Date of Enrolment	D D M M M Y Y Y Y		
Marital status	☐1. S	☐2. M	
Education	☐1.Primary	☐2.Secondary Sch	☐9. University

CASE REPORT FORM

Participant Number:					

Address			
Telephone			
Occupation			
Next of kin			RELATIONSHIP: _____
Next of Kin's contact/phone			

CASE REPORT FORM

Participant Number:					

MEDICAL HISTORY		
Malnutrition_____	Diabetes_____	Preeclampsia_____
HIV_____	Malaria_____	
Family History: Twins Y or N		

Participant Number: | | | | | |

PHYSICAL EXAMINATION (First Visit)
General_____

CVS_____ Resp._____

Breasts_____ Abdomen_____

Vaginal Examination_____ Discharge/GUD_____

Weight in kgs_____ Gestation in weeks_____

Antenatal Profile

Hb_____
Blood Group_____
Rhesus_____
Serology(VDRL/RPR)_____
TB Screening
HIV:
Reactive ☐
Non reactive ☐
Not tested ☐

Urinalysis_____

Bs for Mps_____

Participant Number:					

Neonatal outcome:

Live
YES

NO

If NO; tick appropriately
Fresh stillbirth _____

Macerated stillbirth _____

APGAR score

Neonatal weight

_____ **grams**

Appendix 4: Equipment

The following equipment will be used in this study

1. Leitz Wetzler Sledge Microtome

This will be used to slice the bio-banked placentae blocks into 5µm slices for staining.

Figure 8: Leitz Wetzler Sledge microtome

2. Hot Air oven

Will be used to dry the stained specimen slices at 40ºC

Figure 9: Hot air oven

3. Richter Optica Achromatic Plan XU-T1 digital microscope interphased with Moticam BTU 10 Camera systems connected to a computer with the Motic Images 3.0 software open

Figure 10: Richter Optica Achromatic Plan XU-T1 digital microscope interphased with Moticam BTU 10 Camera systems connected to a computer with the Motic Images 3.0 software open

Appendix 5: Data Collection Sheet

Appendix 1 – Data Collection Sheet

Study Number: _____

Sex: F

Maternal age ☐ years

Gestation in weeks ☐

HIV status Positive ☐ Negative ☐

CD4 count ☐

HIV viral load ☐

ART Treatment Yes ☐ No ☐

Route of delivery: Vaginal ☐ Caesarean section ☐

Areas of infarction Yes ☐ No ☐

Areas of thrombosis: Yes ☐ No ☐

Site of cord insertion: Central ☐ Eccentric ☐ Marginal ☐ Velamentous ☐

Cord diameter: _____ mm

Cord length: _____ cm

Shape of the placenta: Discoid ☐ Annular ☐ Circular ☐ horseshoe ☐

Color of the membranes and chorionic plate

Maroon ☐ Green-brown ☐ Yellow-gray ☐

Areas of calcification Yes ☐ No ☐

Cord colour; White ☐ dark brown ☐ black green ☐

Number of vessels in the cord; one ☐ two ☐ three ☐ more than three ☐

Umbilical cord hemorrhages. Yes ☐ No ☐

Weight of the placenta (gms) ☐

Diameter of the placenta (cms) in three dimensions; Greatest ☐ Major ☐ Minor ☐

Thickness of the placenta (cms); Greatest ☐ Minor ☐

Histomorphology Placenta

Central sections 1 and 2

1_____

2 _____

Peripheral sections 1, 2, 3, 4, 5, 6

1_____

2_____

3_____

4_____

5_____

6_____

Histomorphology, umbilical cord taken in two sections

1 _____

2 _____

Results of Giemsa staining

_____

Appendix 6: Ethical Review Committee Approval

Mount Kenya University

SEPTEMBER 25, 2017

Ref. No. MKU/ERC/0543

CERTIFICATE OF ETHICAL CLEARANCE

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

Mr Francis W. Makokha
Secretary, Mount Kenya University ERC

Sign: _____ **Date:** 26.09.2017

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

Sign: _____ **Date:** 26.09.2017

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

Appendix 7: Approval to use Biobanked specimen

Mount Kenya University

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

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

We make reference to the above matter.

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

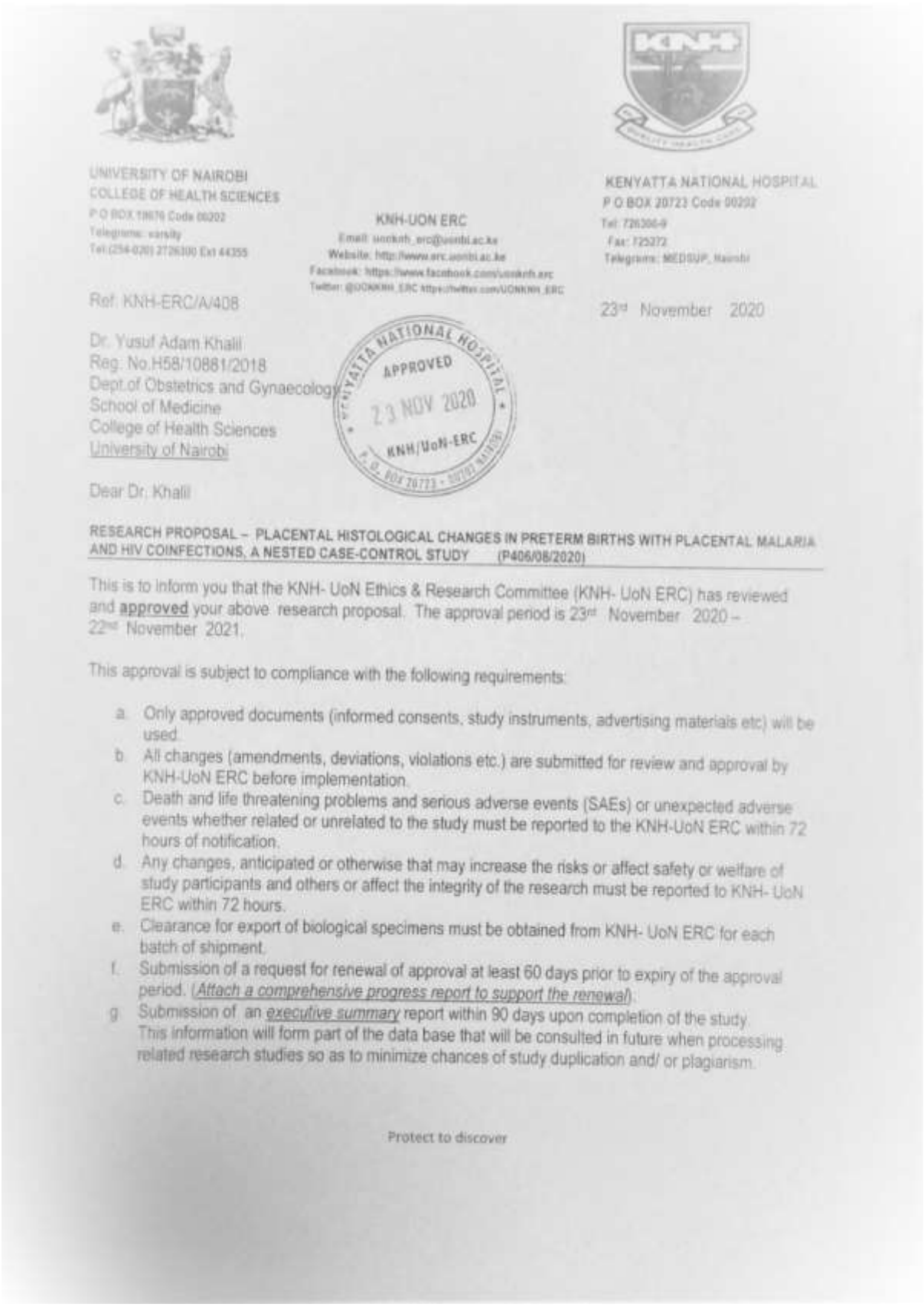
INVESTIGATOR'S NAME	COURSE
Dr. Consolata Wangeci Kihagi;	Comparison of placental microbiome in women with undernutrition and those with normal nutritional state at Bungoma County Referral Hospital.
Dr. Yusuf Adam Khalil;	Placental histological changes in preterm births with placental malaria and HIV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

Kindly accord them the necessary assistance
Thank you in Advance.

Yours Faithfully;

.....
Dr. Jesse Gitaka, MD, MTM, PhD

Appendix 8: KNH-UON Ethical approval for the study



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KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref: KNH-ERC/A/408

23rd November 2020

Dr. Yusuf Adam Khalil
Reg. No.H58/10881/2018
Dept.of Obstetrics and Gynaecology
School of Medicine
College of Health Sciences
University of Nairobi

KENYATTA NATIONAL HOSPITAL
APPROVED
23 NOV 2020
KNH/UoN-ERC
P. O. BOX 20723 - 00202 NAIROBI

Dear Dr. Khalil

RESEARCH PROPOSAL – PLACENTAL HISTOLOGICAL CHANGES IN PRETERM BIRTHS WITH PLACENTAL MALARIA AND HIV COINFECTIONS, A NESTED CASE-CONTROL STUDY (P406/08/2020)

This is to inform you that the KNH- UoN Ethics & Research Committee (KNH- UoN ERC) has reviewed and **approved** your above research proposal. The approval period is 23rd November 2020 – 22nd November 2021.

This approval is subject to compliance with the following requirements:

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

Protect to discover

Appendix 9: Gannt Chart

	SEP 2019	OCT 2019	to	MAY 2020	JUN 2020	JUL 2020	AUG 2020	to	NOV 2020	DEC 2020	MAY 2021	SEP 2021
CONCEPT NOTE DEVELOPMENT												
PROPOSAL DEVELOPMENT												
PROPOSAL MARKING												
PROPOSAL PRESENTATION												
ETHICS APPROVAL												
DATA COLLECTION												
RESULT PRESENTATION												
MANUSCRIPT WRITING												
PUBLISHING												

Appendix 10: Study Budget

BUDGET		
STAFF COST		
Research assistant	KES 20,000.00	
Statistician	KES 30,000.00	
Sub-total	KES 50,000.00	KES 50,000.00
OFFICE COST		
Transport	KES 20,000.00	
Airtime	KES 5,000.00	
Internet	KES 5,000.00	
Sub-total	KES 30,000.00	KES 30,000.00
OPERATIONAL COST		
Stain	KES 10,000.00	
ERC fees	KES 2,000.00	
Printing	KES 5,000.00	
Binding	KES 5,000.00	
Sub-total	KES 22,000.00	KES 22,000.00
GRAND TOTAL		**KES 102,000.00**

Appendix 11: STROBE Checklist

STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	i
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	vii
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	1-12
Objectives	3	State specific objectives, including any prespecified hypotheses	12-13
Methods			
Study design	4	Present key elements of study design early in the paper	14
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	14-15
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	15-23
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	N/A
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	xiii
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	22
Bias	9	Describe any efforts to address potential sources of bias	18-21
Study size	10	Explain how the study size was arrived at	17
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	23
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	23
		(*b*) Describe any methods used to examine subgroups and interactions	23
		(*c*) Explain how missing data were addressed	N/A
		(*d*) If applicable, explain how loss to follow-up was addressed	N/A
		(*g*) Describe any sensitivity analyses	N/A
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	26
		(b) Give reasons for non-participation at each stage	26
		(c) Consider use of a flow diagram	26
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	27
		(b) Indicate number of participants with missing data for each variable of interest	N/A
		(c) Summarise follow-up time (eg, average and total amount)	N/A
Outcome data	15*	Report numbers of outcome events or summary measures over time	

1

Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	27-32
		(*b*) Report category boundaries when continuous variables were categorized	27-32
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	N/A
Discussion			
Key results	18	Summarise key results with reference to study objectives	27-32
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	40
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	35-38
Generalisability	21	Discuss the generalisability (external validity) of the study results	40
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	40

*Give information separately for exposed and unexposed groups.

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