DETERMINATION OF ANTIGEN LEVELS: T-PLASTIN, TRANSGELIN AND CA-125 AS POTENTIAL BIOMARKERS IN BABOONS WITH INDUCED ENDOMETRIOSIS

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Determination of antigen levels: t-plastin, transgelin and ca-125 as potential biomarkers in baboons with induced endometriosis

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DECLARATION

This thesis is my original work and has not been presented elsewhere for a degree award in any university.

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To my husband and friend Hamilton G. Nyamu and my lovely children, Isaac, Sarah Joyce and Victor with all my love. For intentionally providing moral and emotional support to me. I'm forever grateful.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

3Rs	Reduce, Replace, Refine
AAGL	American Association of Gynecological Laparoscopists
AFS	American fertility society
AMH	Anti-Mullerian hormone
ASRM	American society of reproductive medicine
CA-125	Cancer (Carcinoma) Antigen 125
CA-19-9	Carbohydrate antigen-19-9
CRP	C - reactive protein
EFI	Endometriosis Fertility Index
ELISA	Enzyme –Linked Immunosorbent Assays
EIA	Enzyme immunoassays
ESHRE	European Society of Human Reproduction and Embryology
IL-	Interleukin
IFN	Interferon
Ig	Immunoglobulin

IPR	Institute of Primate Research
JKUAT	Jomo Kenyatta University of Agriculture and Technology
LV	Lymphatic Vessels
LN	Lymphatic nodes
MCP-1	Monocyte Chemotactic Protein-1
MIF	Migratory Inhibitory Factor
MMP	Matrix metalloproteinase
MRI	Magnetic Resonance Imaging
NHP	Non-Human Primates
NK	Natural killer cells
PBS	Phosphate Buffered Saline
PF	Peritoneal fluid
PB	Peripheral blood
PID	Pelvic Inflammatory Disease
RANTES	Regulated on activation normal T-cell expressed and secreted
ROC	Receiver operating Characteristics
SOP	Standard operation procedures

TGF	Transforming growth factor
TNF	Tumor Necrosis Factor
Trans	Transgelin
TVU	Transvaginal Ultra Sound
VEGF	Vascular endothelial growth factor

ABSTRACT

Endometriosis is defined as the presence of endometrial-like tissue outside the uterine cavity and is associated with chronic intra-pelvic inflammation. Its symptoms, which are often severe, have a negative impact on a woman's quality of life. They include chronic pelvic pain and infertility. It is estimated that about 10% of women in their reproductive ages are affected by the disease globally. Currently, diagnostic methods are laparoscopy with histological confirmation and non-invasive methods such as ultrasound and Magnetic Resonance Imaging (MRI) for advanced disease. Serum biomarkers such as CA-125 is known to be elevated in endometriosis, however, it does not have sufficient diagnostic power as a single biomarker of endometriosis. Due to this fact, there is a need to determine other antigens that would act as potential biomarkers for endometriosis. It has been observed that Transgelin and T-Plastin are upregulated in endometriosis lesions and endometrium during the secretory phase respectively in women. There is insufficient data available showing the concentration of T-plastin in serum or plasma in the development of endometriosis. The main objective of this study was to determine whether T-plastin, Transgelin and CA-125 are potential biomarkers in early diagnosis of endometriosis using baboon model for endometriosis. The baboon model represents clinically relevant research models for endometriosis. This study compared the levels of T-plastin, Transgelin and CA-125 in the peripheral blood and peritoneal fluid of baboons before and after induction of endometriosis. In this experimental design study, ten female baboons (*Papio anubis*, 9-15 kg each) of proven fertility, that had at least one menstruation during captivity, were induced by intra-pelvic injection of menstrual endometrium on day 1 or 2 of menstruation. This was followed by staging laparoscopy. Serum and peritoneal fluid samples were collected prior to induction to serve as controls. T-Plastin, Transgelin and CA-125 were measured using commercially available ELISA Kits. Data was analyzed using non-parametric test and level of significance determined at $p<0.005$. T-Plastin and Transgelin had insignificant p values in both peripheral blood and peritoneal fluid. However CA-125 showed a p- value of 0.0003 in peripheral blood between pre induction and 1st post induction sampling while the p-

value in peritoneal fluid was equal to 0.0279. Diagnostic performance of individual biomarkers was determined by Receiver Operating Characteristic (ROC) with the Area under the curve (AUC) equal to 0.5 showing T-Plastin and Transgelin in both serum and peritoneal fluid were unreliable for use as biomarkers in endometriosis unless combined with other antigen. Levels of CA-125 in peritoneal fluid had an AUC of 0.7900 indicating it can be used as potential biomarkers for endometriosis in combination with other antigens. An evaluation of the three antigens using different body fluids and different methods would enhance possibility of them being used as potential biomarkers.

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CHAPTER ONE

INTRODUCTION

1.1 Background Information

1.1.1 Endometriosis

Endometriosis is a common non-threatening chronic and multifactorial complex gynecological disease which is estrogen dependent (NICE guidlines, 2017; Giudice , 2010). It is characterized by chronic pelvic pain, dysmenorrhea (painful and heavy menstruation) and an inability to conceive. Other symptoms of endometriosis include deep pain during or after sexual intercourse what is referred to as dyspareunia and painful bowel or bladder movement (Koninckx, *et al*., 2020). It is defined by presence of endometrial-like tissue in form of stroma and glands outside the uterine cavity (Gruber & Mechsner, 2021). Endometriosis may be asymptomatic in as many as 20-25% patients (Yela, *et al*., 2020).

Endometriosis clinically presents itself in three varying forms namely; peritoneal endometriosis, ovarian endometriotic cyst and deep infiltrating endometriosis (Capezzuol, et al., 2020). About 10-15% of women in the reproductive age globally are reported to suffer from endometriosis (Mehedintu, *et al.*, 2014). This is approximately 176 million women globally (Adamson, *et a*l., 2010). The incidence of endometriosis rises to 40-60% of women with infertility and chronic pain (Vermeuleri, *et al*., 2014). According to National Institute for Health and Care guidelines (NICE, 2017) and American Society of Reproductive Medicine report (ASRM, 1996) on classification and staging of endometriosis, there are four classes/stages of endometriosis, minimum, mild, moderate and severe (1-4 stages). The symptoms such as pelvic pain and infertility do not necessarily correlate with the stage of the disease.

Despite the much work done on endometriosis, there is still no clear understanding of the etiology). Several theories have been hypothesized; the most popular and well

accepted being Sampson's 1927 retrograde menstruation. In this theory, viable cells or tissues of endometrium flow back in menstrual flow via fallopian tubes into the peritoneum where they implant (Wang, *et al*., 2020). A good environment in the peritoneum allows the disease to develop and thrive. Inflammatory cytokines such as IL-1β, IL-2, IL-6, IL-8, and TNF-α (expressed by macrophages) are found increased in endometriosis and therefore associated to development of the disease. A damaged immunosurveillance which fails in defending the body from the endometrial cells or tissues that have flowed back with menstrual flow in women allows development of endometriosis (Roumandeh, *et al*., 2019).

Studies have shown that transgelin is highly increased in lesions in endometriosis (Kyama, *et al*., 2006). Another study (Hidalgo *et al*., 2011) also found the Transgelin gene in the endometriotic lesions in women with endometriosis. Transgelin has been associated with development and maintenance of endometriosis.

T-Plastin is upregulated in the secretory phase of women in endometriosis (Kyama, *et al*., 2011).

Cancer Antigen 125 (CA-125) is also elevated in endometriosis and used as a serum marker in endometriosis (Irungu, *et al*., 2019). It however lacks sufficient diagnostic power as a single biomarker. The gold standard for diagnosis is laparoscopy which has to be followed by histological confirmation (Taylor , 2019). This usually leads to a delay of 8-11 years (Fassbender, *et al*. 2015). It is also an expensive procedure and more than 100 million women in their reproductive age worldwide may not afford. Although laparoscopy has been considered the gold standard diagnostic tool, misdiagnosis of the disorder due to delays involved has been reported (Wellbery, 1999). Other factors leading to delay in diagnosis of endometriosis include overlapping of symptoms with other conditions, individual concerns where a woman is embarrassed to seek medical advice as well as lack of proper knowledge and understanding and awareness of this disease which has a diversity of symptoms (Weintraub, 2016).

Non -invasive methods for diagnosis of endometriosis are Magnetic Resonance imaging (MRI) and Trans Vaginal Ultra sound (TVU) which are expensive and not affordable to many (Alcazar, *et al*., 2017). They are unreliable for diagnosis of minimum and mild endometriosis. Several biomarkers have been used in diagnosis of endometriosis although they are not yet validated most important one being CA-125 (Nisenblat, *et al.*, 2016; Fassbender, *et al*., 2015; May, *et al*., 2010). There is a need for non-invasive diagnostic test that is simple, low-cost, fast, accurate and reliable and which will solve the delay and help in treatment or management at early stages of the disease (Tamaresis, *et al*., 2014).

1.1.2 Baboon Model as a tool for research on Endometriosis

Many non- human primates have been used in medical research because of their relatedness to human beings. Much has been achieved about diseases, disorders, prevention and treatments (Abee, *et al*.,2012). Rodents also have been used in their immunocompromised state to study endometriosis (Asally, *et al*., 2015). Non-human primates (NHP) such as the Olive baboon, cynomolgus monkeys, the pigtail macaque and De Brazza monkey have been used as models for endometriosis (Nishimoto-Kakiuchi, *et al*., 2016). The baboon model is quite a better representative of clinical models for endometriosis because of the close relatedness to baboons and humans in their phylogeny (D'Hooghe, *et al*., 2009 ; Braundmeier & Fazleabas, 2009). It has also been known that endometriosis occurs only in menstruating females and this is true of baboons with spontaneous menstruation (Kyama, *et al*., 2007). In the same review Kyama has observed that baboons can be easily followed up in their 33 days menstrual cycle because of the characteristic penial skin inflation and deflation which corresponds to their cycle phases. More so baboons usually breed through out their life even in captivity and due to their big size, hence big cervix,its easy and comfortable to carryout transvaginal ultra sound as well as laparoscopy. There are several ethical limitations and impracticalities of performing studies in women and therefore baboons becoming the option. For instance it is almost impractical to control for the pelvic condition in

endometriosis and in normal pelvis or in instances where there are other pelvic pathology. Symptoms such as pain and sub fertility or the combined pain and sub fertility cannot completely be followed in humans unlike in non-humans (Nishimoto-Kakiuchi, *et a*l., 2018). Baboons are able to develop spontaneous endometriosis which is macroscopically and microscopically similar to what occurs in humans (Hastings & Fazleabas, 2006). Intra-pelvic seeding of menstrual endometrium leads to development of endometriosis within 25 days. In Kenya baboons are not endangered but are actually a menace in agricultural zones where they are said to be in conflict with humans.

1.1.3 T-plastin, Transgelin and CA-125

Antigens T-plastin, Transgelin and CA-125 are said to be elevated during endometriosis. T-Plastin was reported by Kyama and Giudice as regulated upwards in secretory phase endometrium of women in minimum and mild endometriosis (Giudice,*et al*., 2012, Kyama, *et al*., 2011). Transgelin was reported upregulated in endometriosis in lesions when compared with normal peritoneum (Kyama, *et al*., 2006). Antigen T-plastin, Transgelin and CA-125 in serum and peritoneal fluid as a panel, would serve as the long awaited diagnostic indicator, upon verification. The goal of this study was to compare T-plastin, Transgelin and CA-125 levels in baboons before and after induction of endometriosis in the peripheral blood and peritoneal fluid.

1.2 Statement of the Problem

Endometriosis is a common benign gynecological disease affecting approximately ten percent of women in their reproductive age. It is characterized by endometrial-like tissue outside the uterus, with common symptoms as chronic pelvic pain, bleeding and infertility. It has a negative impact on the general well-being of a person. The lack of an early and reliable noninvasive diagnostic test for endometriosis results to delay in diagnosis and treatment. The available and gold standard diagnostic test for endometriosis is the semi invasive visual laparoscopy inspection which has to be confirmed histologically. The Magnetic Resonance Imaging (MRI) and transvaginal

ultrasound (TVU) are often used for diagnosis but are not specific, failing to differentiate from other gynecological carcinomas. The peripheral CA-125 is the only available non-invasive diagnostic tests but lacks the diagnostic power as a single biomarker. T-Plastin and Transgelin are said to be upregulated in endometriosis in women but have not been explored .There is an urgent need to identify a noninvasive diagnostic test. This will aid diagnosis and management of endometriosis.

1.3 Justification.

The gold standard available test for diagnosis of endometriosis is laparoscopy which is invasive and ideally has to be confirmed histologically. This leads to the delay of diagnosis. The lack of a non-invasive method for diagnosis of endometriosis has weighed down efforts to study the aetiopathogenesis of the disease among women of reproductive age and complicates treatment. Biomarkers that have been studied have not been validated and the most studied CA-125 lacks the sufficient diagnostic power for diagnosis of endometriosis. So far no study has evaluated transgelin and T-plastin (which are known to be increased in endometriosis in women) as potential biomarkers in diagnosis of endometriosis in baboon model. In baboons, intra-pelvic injection of menstrual endometrium in the pelvic cavity leads to development of endometriosis whose macroscopic lesions resemble those of human endometriosis. This study sought to evaluate levels of antigens T plastin, transgelin and CA-125 in plasma and peritoneal fluid as potential biomarkers for early diagnosis of endometriosis and contribute towards improvement of immunodiagnosis and treatment of endometriosis.

1.4 Hypothesis

T-Plastin, Transgelin and CA-125 are not potential biomarkers in baboons with induced endometriosis.

1.5 Objectives

1.5.1 General objective

To evaluate the levels of T-plastin, Transgelin, and CA-125 as potential biomarkers in baboons with induced endometriosis.

1.5.2 Specific objectives

i. To determine T-plastin, Transgelin and CA-125 levels in plasma samples of baboons with induced endometriosis.
ii. To determine T-plastin, Transgelin, and CA-125 levels in peritoneal fluid of baboons with induced endometriosis.
iii. To evaluate T-plastin, Transgelin and CA-125 as biomarkers in the diagnosis of endometriosis in induced baboon.

CHAPTER TWO

LITERATURE REVIEW

2.1 Endometriosis

Endometriosis is a gynecological and inflammatory disorder common to women of reproductive age. It is defined as the presence of abnormal growth, endometrial –like tissue in the form of stroma and glands outside the uterine cavity. It creates a huge burden on the quality of life of women (Eisenberg, *et al*., 2017). Medically endometriosis is defined by chronic pelvic pain and heavy painful menstruation and infertility (Fassbender, *et al.*, 2015; Sasson, 2008). Other signs and symptoms which ordinarily impact much on general well-being of patients can be non-specific and include: severe dysmenorrheal, deep dyspareunia, ovulation pain, cyclical or premenstrual symptoms (e.g. bowel or bladder associated) with or without abnormal bleeding and chronic fatigue. Many women (20-25%) with endometriosis may be asymptomatic (Mehedintu, *et al.*, 2014).

Usually the endometriotic lesions are found in the lining of peritoneum, on ovaries and other pelvic organs and on uterosacral ligaments all outside the uterine cavity as shown in Figure 2.1 In rare occasions they occur in brain ,lungs and other body regions (Bhat, et al., 2020). A few cases of male endometriosis have been described (Sourial, *et al*., 2014).

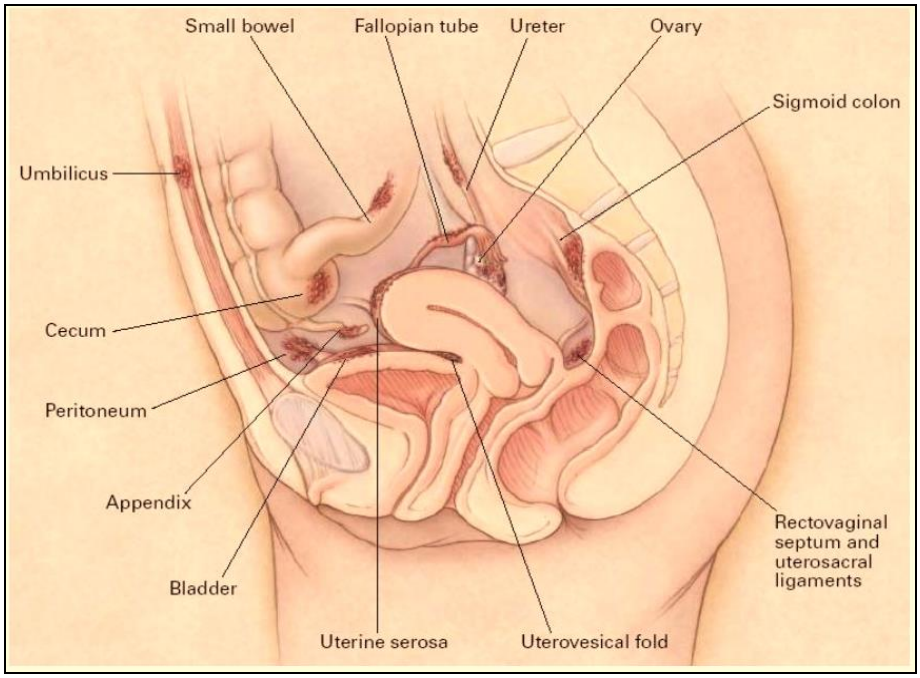


Figure 2.1: The common sites for endometriosis (Valencia, 2010)

Endometriosis mainly occurs as peritoneal disease (Figure 2.2) when the peritoneum cavity has been affected. Lesions or what is referred to as the endometrial implants develop on the lining of the peritoneum of the pelvic region (Riazi, *et al*., 2015). The other type is called endometriotic ovarian cysts (Figure 2.3) which occurs when the pelvic organs such as the ovaries, fallopian tubes, rectovaginal septum, and uterovesical folds are affected. Deep infiltrating rectovaginal endometriosis (DIE) occurs when lesions of about 5 mm deep into the peritoneum occur, (figure 2.4) (D'Alterio, *et al*., 2021). Endometriosis is a hormone (estrogen) dependent inflammatory disorder which is associated with increased secretions of pro-inflammatory cytokines and neoangiogeneis resulting to pelvic pain and infertility (Bhatia, *et al.*, 2020).

The black arrows showing highly vascularized lesions while the blue arrows point at relatively healed lesions white in color see below in figure 2.2). (Wang, Nicholes & Shih, 2020)

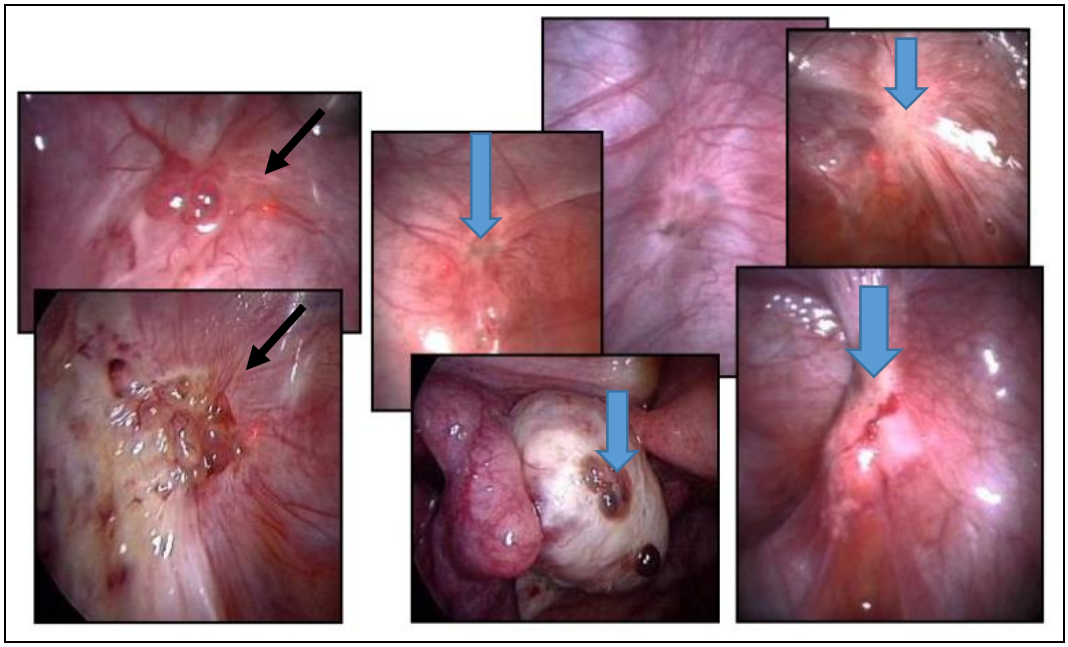


Figure 2.2: Peritoneal endometriosis, clinical presentation.

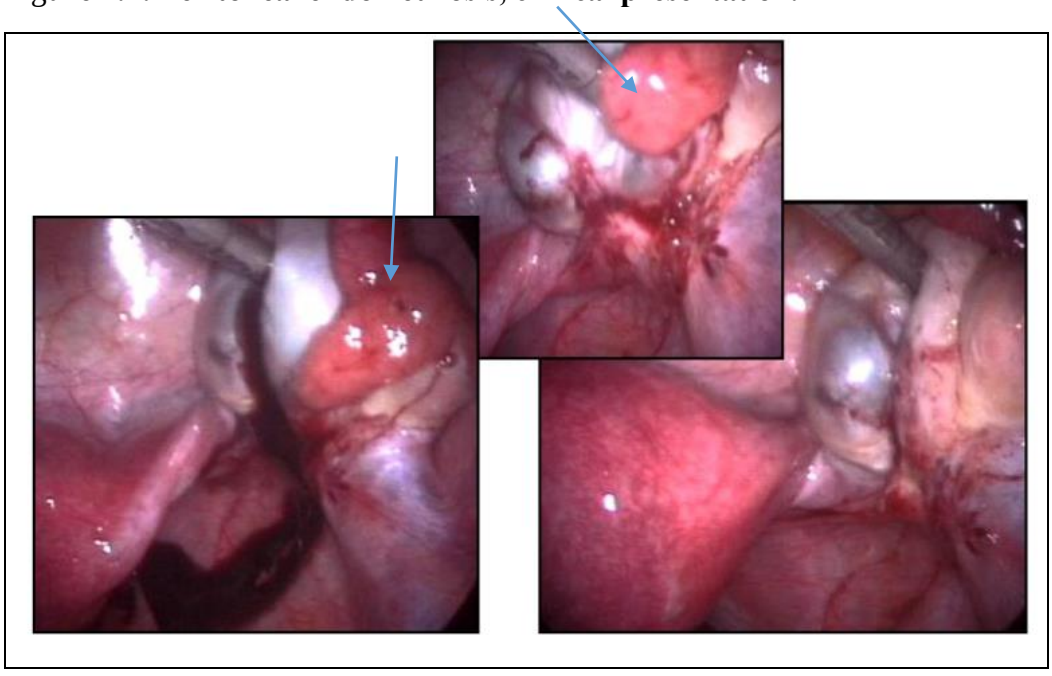


Figure 2.3: Ovarian cyst endometriosis. (Wang, Nicholes & Shih, 2020).

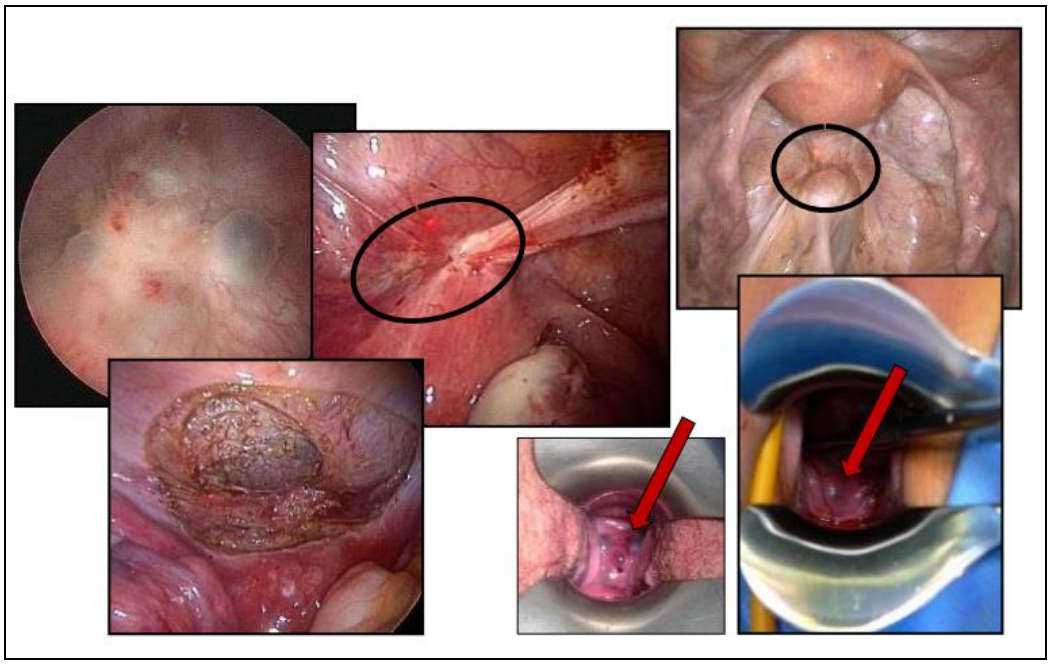


Figure 2.4: Deep infiltrating endometriosis (Wang, Nicholes & Shih, 2020)

Women with endometriosis show elevated numbers of activated macrophages, cytokines and growth factors including IL-1, IL-6, IL-8 and IL- 12, TNFα, VEGF, and RANTES in their serum and peritoneal fluid (Roumandeh, *et al*., 2019; Fassbender, *et al.*, 2011).

2.2 Prevalence of endometriosis

Endometriosis is reported in nearly ten percent of women, translating to approximately 176 million women worldwide who are in their reproductive age (Koninckx *et al*., 2020; Rogers, *et al.*,2009). This percentage rises to between 40 and 60 in women with infertility and chronic pelvic pain (Vermeuleri, *et al.*, 2014). Recurrence is often observed after surgery and after the cessation of medical suppressive therapy, especially in women with moderate to severe endometriosis. However endometriosis has been reported in 20-25 % of asymptomatic women (Mehedintu, *et al*., 2014). They may have known that they have endometriosis when by coincidence went for laparoscopy for tubal ligation rather than for endometriosis. African women have been thought to have lower risks than others. Little is known about endometriosis and diagnosis in African woman

and its likely underreported (Kyama, *et al*., 2007) due to two main reasons; the African indigenous woman would get married or pregnant very early in life and therefore endometriosis may have gone unnoticed. Secondly laparoscopy is a relatively expensive procedure which very few people in Africa would afford or access. Many of the signs and symptoms of endometriosis are mistaken for simply painful menstruation and therefore ignored (Hudson, *et al.,* 2020). The lifestyle of African woman has increased risk of pelvic inflammatory disease and blockage of fallopian tubes both conditions that resemble endometriosis in characteristics and therefore masking diagnosis of endometriosis that may be there (Kyama, et al., 2004). The actual prevalence has not been clearly put. However a recent study shows endometriosis would be higher than thought of, requiring and deserving more attention than being accorded to avoid progression of the disease to severe pain and infertility (Kagia, *et al*., 2018). The prevalence of endometriosis in kenya has not been documented but an article in one magazine entitled, "The Big Read: Endometriosis,The Monister silently tormenting women" indicates endometriosis as largely unknown and misdiagnosed as heavy bleeding with severe abdominal cramps (Star, 28 March 2019-06:00). A woman's risk for endometriosis increases with increased exposure to endometrial material, this is in shorter menstrual cycles, longer bleeding periods and in early menarche (Saffarieh & Sharami, 2020). The cost of this disease was estimated at US$22 billion in 2012 (Simoens, *et al.,* 2012) and an indirect cost of a mean of 5.3 days in a month was estimated for 78 % of the women with endometriosis in United Kingdom (Hummelshoj *et al*. 2006). In Kenya there is no data that exists on cost of endometriosis treatment but a magazine quoted approximate cost at Ksh.355,000/= per woman per year. (Wanjala & Nyawira, 2019)

2.3 Classification and staging of endometriosis

According to National Institute for Health and Care Excellence (NICE) guidelines (NICE, 2017) and revised classification system of American Society of Reproductive Medicine report (rASRM, 1997), endometriosis has been classified into four stages

namely minimal, mild, moderate and severe (stages I,II,III,IV). The classification is mainly based on the amount and size of lesions, location and depth of same as well as the severity of the lesions. However, the severity of the disease, pelvic pains and or other symptoms such as infertility is not correlated with the stage (Mehedintu, *et al*., 2014). There are other systems for classification of endometriosis including ENZIAN classification, which was developed to classify deep infiltrating endometriosis, endometriosis fertility index (EFI) and American Association of Gynecological Laparoscopists (AAGL). Classification that would be developed without necessarily going through surgery will be very much helpful in development of diagnostic tools and plans and therapeutic approach (Lee, *et al*., 2021; Capezzuol, *et al*., 2020).

Endometrial lesions are either on the peritoneal surface or deep infiltrating lesions (about 5mm deep in the peritoneum) or on ovaries to occur as ovarian endometriotic cysts.

Lesions appear as red, black/blue or white depending on the stage. The Red lesions indicating high vascularization and active are considered in the first stage while the white lesions are considered healed and latent (Chamie, *et al*., 2011; Kyama, *et al.*, 2007; D' Hooghe, *et al.*, 1992).

2.4 Pathogenesis of endometriosis

Endometriosis is a disease that is not fully understood. Several theories have been put up to explain its aetiology. The most widely held theory is the Sampson's retrograde menstruation (transplantation or implantation) theory of 1927. Menstrual flow is said to flow backwards via Fallopian tubes into the peritoneal cavity or ectopic sites (Yovich, *et al*., 2020). It has been theorized that endometriosis occurs when viable endometrial cells are deposited on the peritoneum where they implant, develop and multiply (Baldi,*et al.*, 2008). This occurs as an effect of favourable environmental factors and an incompetent immune surveillance that is unable to clear the deposited fragments in the inappropriate sites. The fact that women with endometriosis, as opposed to healthy women, show

higher volumes of refluxed menstrual blood and endometriosis-tissue fragments gives much support to this theory. The viable cells are found in the ectopic sites and in effluent flow. These women are known also as having shorter cycles and long menstrual flow durations (Saffarieh & Sharami, 2020) (which are risk factors for endometriosis. In addition other predisposing factors include smoking, consumption of caffeine and alcohol and certain dietary lifestyle patterns (Saffarieh & Sharami, 2020; Parazzini, *et al*., 2017).

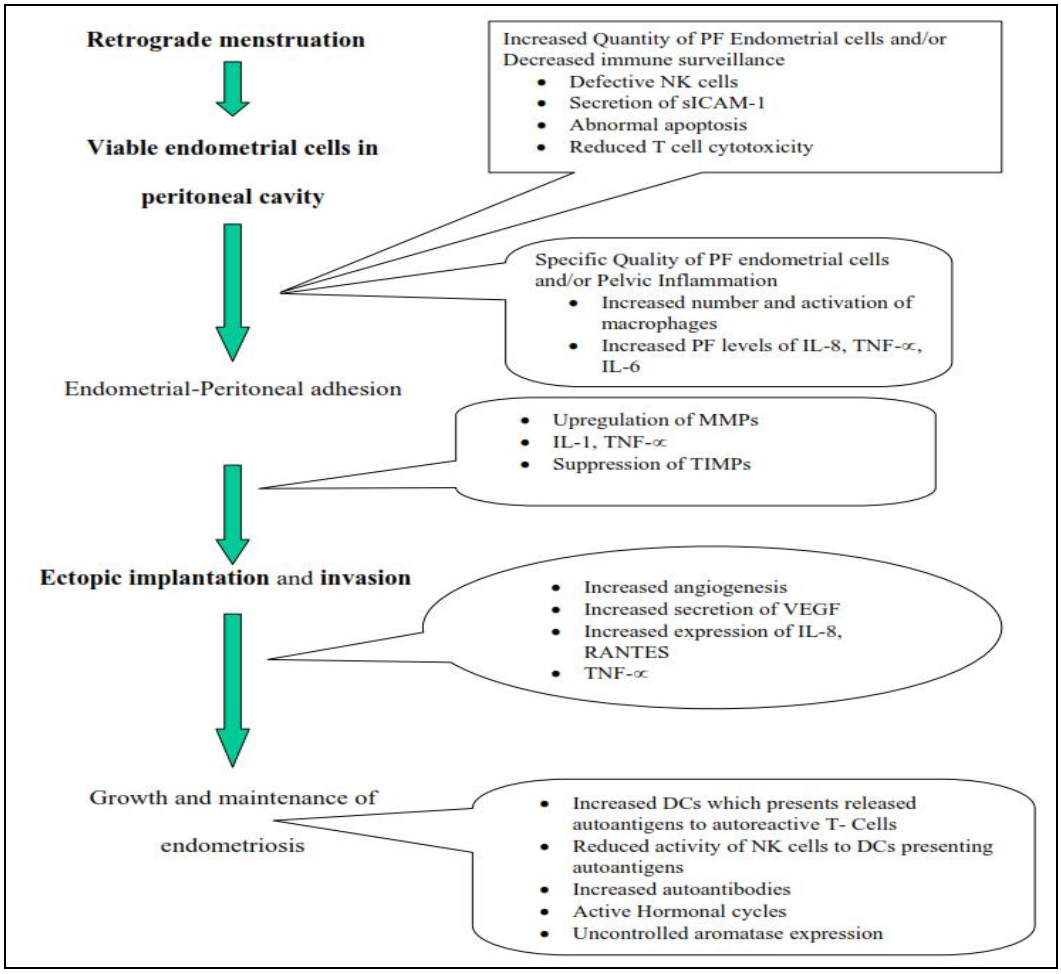


Figure **2.5: Diagrammatic representation of retrograde menstruation and development of endometriosis. (Kyama, *et al*., 2003)**

Increased menstrual flow volumes, as a result of experimental ligation of cervix in both women and baboons induces endometriosis a fact that adds support to this theory (Kyama, *et al.,* 2003 ; D'Hooghe, *et al.*, 1995). The only disagreement in this theory comes in that many women ranging between 76 and 90 % who experience reflux flow do not develop endometriosis. Sampson's theory also has failed to explain isolated occurrence of endometriosis in rare sites such as in lungs, nose or in men (Yovich, *et al*., 2020).

Another important theory in development of endometriosis is the coelomic metaplasia. Endometrium and peritoneum have same ancestral coelomic cells which are said to undergo metaplasia (transformation) from one type to another under the influence or trigger of inflammation. In the favourable peritoneal environment, endometriosis develops (Wellbery, 1999). Other factors such as hormonal infections or other environmental factors facilitate and enhance transformation of peritoneal cells into endometrial cells (Gruber & Mechsner, 2021). This hypothesis is supported by several facts. Thoracic, abdominal, and pelvic peritoneum and the mullerian ducts are said to be derived from the same cell lineage embryologically with the coelomic wall of the developing embryo. This explains the clinical appearance of endometriosis in men, in girls in adolescence prior to puberty and in thoracic cavity.

The embryonic rest theory hypothesizes that certain cells of Müllerian origin found in peritoneal cavity transform to endometrial tissue when induced by an appropriate stimuli. This theory explains the rare endometriosis in rectovaginal septum as well as in men (Bhat, et al., 2020).

The lympho-vascular metastasis theory accounts for the disease spread in the visceral organs such as the kidneys, lungs, brain, lymph nodes, extremities, the eyes and even in the abdominal walls. (Keichel, *et al*., 2011). This theory suggests that endometrial cells could disseminate and therefore spread to ectopic sites via lymphatic vessels through lymph and haematogenous (through blood) (Yovich, *et al*., 2020). The recent theory for

pathogenesis of endometriosis is stem cells theory. Bone marrow stem cells are said to have potential to seed endometrium as well as outside peritoneal cavity and trans-differentiate into functional endometrial cells that would implant under stimulation and proliferate into endometriosis (Hufnogel, *et al*., 2015; Figueira, *et al*., 2011).

In the immunological aspect of pathogenesis of endometriosis, a woman with an incompetent immune system and a genetic predisposition to developing endometriotic lesions, possibly in the setting of an uncharacterized environmental exposure, will very likely develop endometriosis (Roumandeh, *et al*., 2019). The ability of the immune system to survey, scavenge and clear the debris formed by the menstrual flow possibly by retrograde plays an important role in development and pathogenesis of endometriosis. The theory of altered immunosurveillance explains that a faulty immune system and an overwhelmed one fails to identify misplaced endometrial cells allowing them to implant and grow (Ioana & Razoan, 2013). The lesions formed escape immune surveillance and develop their own blood supply and thrive. The peritoneal environment subsequently is changed and is evident by increased peritoneal fluid, and activated macrophages. In women without endometriosis, activated macrophages release cytokines, growth factors and prostaglandins to remove tissue debris, red blood cells and misplaced endometrial cells. The elevated amounts of these indicate endometriosis Immune cell infiltration emerges as a major phenomenon in the pathogenesis of endometriosis (Lagana, *et al*., 2019). The increased population of activated macrophages, cytokines, and B-cells in both peritoneal fluid and peripheral blood is said to be important in development of endometriosis by promoting ectopic endometrial growth and releasing secretions of adhesion and or growth factors (Mehedintu, *et al*., 2014). Some hereditary factors, such as genetic alterations have been shown to predispose women towards endometriosis by influencing implantation of endometrial cells. There is a six times higher risk to endometriosis for women who are first degree relatives of women suffering severe endometriosis (Parasar, Ozcan, & Terry, 2017). The women who are not genetically

programmed to protect from the foreign endometrial cells develop endometriosis when there is right environment (Zondervan, et al., 2018).

2. 5 Diagnosis of endometriosis

Endometriosis, being a hormonal dependent disease (estrogen) usually becomes apparent in the reproductive years when the lesions are stimulated by ovarian hormones ((Chantalat, *et al.,* 2020; Mehedintu, *et al*., 2014). Often the disease is asymptomatic and is only suspected when pelvic pains (often mistaken for menstrual pains), dysmenorrhea (painful heavy bleeding) and infertility (failure to conceive) appear (Soumekh & Nagler, 2014; Aragon & Lessey, 2017). Misdiagnosis of endometriosis brings a delay between the onset of symptoms and a diagnosis for as long as 8-11 years (Vodolazkala, *et al*., 2012). Lack of noninvasive diagnostic test contributes to this delay. The symptoms of endometriosis are wide and differ from one woman to another and are also shared in characteristics with other conditions such as pelvic inflammatory disease (PID) and irritable bowel syndrome (Aragon & Lessey, Irritable Bowel Syndrome and Endometriosis: Twins in Disguise, 2017). The only reliable method which is also the gold standard test for conclusive diagnosis remains laparoscopic pelvic examination and is always followed by a histological confirmation (May, *et al*., 2010). Other noninvasive diagnostic procedures available are the transvaginal ultrasound (TVS), which lacks the resolution power to identify adhesions and peritoneal implants and the magnetic resonance imaging, MRI which is a highly sensitive test for detection of endometriosis though expensive and not affordable by many. (Berker & Seval, 2015). In addition, immunologic, genetic, serum and plasma biomarkers have been used for diagnosis of endometriosis.

A good diagnostic test is one that will detect endometriosis at its mild and minimum stages or even on the onset of the disease (Fassbender, *et al*., 2013). The test should be non-invasive and affordable to all. It should reduce the time taken for a diagnosis. Such a test has high sensitivity and specificity (Marcos dos Reis, *et al*., 2017).

Several diagnostic tests have been researched on and are shedding light into a good diagnostic test. A study on proteomic profiling of eutopic and ectopic endometrial tissues found some molecular changes in endometriosis (Irungu, *et al*., 2019). This will improve on development of a diagnostic test.

Ellett.*et al.* (2015) reported that endometrial nerve fibrers which were found in women both with and without endometriosis failed as a diagnostic tool. It had been reported that there was a small density of nerve fibres in endometrium which had been thought of as specific to endometriosis.

Magnetic resonance imaging and transvaginal ultra sound have been used for diagnosis of endometriosis with TVU providing a more accurate picture of endometriosis than MRI (Gutierrez, et al., 2019).

2.6 Biomarkers as used in Diagnosis

Several biomarkers have been sought to measure disease activity and monitor progression of treatment (Mihalyi, *et al*., 2005). The most commonly used are serum and plasma biomarkers. This is because blood allows repeated investigations and is easily obtained. Glycoproteins mostly investigated include follistatin (which was found increased in endometriosis), Glycodelin-TNFα and IL-6, and CA-125 (May, *et al*., 2010). Others include the immunological serum cytokines, (IL-1β, IL-6, IL-8, IL-17, 1L-21, RANTES, IFN-gamma, MCP-1, MIF, CRP and TNF alpha) matrix metalloproteinases (MMP), adhesion molecules and markers of angiogenesis (May, *et al*., 2011). CA-125, CA 19-9, C-reactive Protein (CRP) have been investigated as a combined panel with cytokines IL-6, IL-8 and TNFα. This panel was found to be able to diagnose endometriosis in minimum to mild stage (Fassbender, *et al*., 2015; Mihalyi, *et al*., 2010). Studies have shown that it's difficult for a single biomarker to have specificity and sensitivity enough to be an effective biomarker (Ahn *et al*., 2017).

In addition to the above markers endometrial tissue, menstrual and uterine fluids have been investigated. Urine and saliva too have been investigated for biomarkers. There has not been any that can reliably be used for diagnosis due to low specificity and sensitivity. Urine samples are easy to access and have a non-complex composition. Several investigations for various diseases have been carried out. A creatinine soluble fms-like tyrosine kinase was found significantly raised in urine of patients with endometriosis. (Cho, *et al*., 2007). Cytokeratin-19 (CK19), also found in urine samples was relatively raised (Tokushige, *et al.,* 2011).

The critical properties required for a biomarker to be appreciated for diagnosis of endometriosis are its high sensitivity and specificity. In addition the biomarker ought to be simple and noninvasive. It is important that it must be reproducible (Burney, 2014).

2.6.1 T-Plastin: Role in endometriosis

T-plastin, a cytoplasmic protein regulating actin assembly and cellular motility**,** was found up-regulated in the secretory phase endometrium from women with minimal to mild endometriosis compared with controls (Giudice, *et al*., 2012; Kyama, *et al.,* 2011). T-plastin protein functions in the formation of actin bundles that are required for cell locomotion and maintenance of the cellular architecture. It was not clear whether T-plastin is present in peripheral blood, because there was scarce data in the literature showing the concentration in serum or plasma (Kyama, *et al.,* 2011).

2.6.2 Transgelin and its role in endometriosis

Transgelin which is a 22- to 23-kDa protein was reported upregulated in endometriosis lesions when compared with normal peritoneum (Kyama, *et al*., 2006). It is said to play a possible role of smooth muscle actin– binding protein in the development of endometriotic lesions. However transgelin expression is down regulated in many cell lines and therefore can be an early and sensitive marker for the onset of transformation. In women with endometriosis, smooth muscle cells are present around endometriotic

lesions and are absent in unaffected peritoneal sites and eutopic endometrium. A study has also found transgelin increased in endometriotic sites and believed to form a component that contributes to formation of podosomes. Podosomes are actin based structures that are capable of altering pathway for establishment of endometriosis. Transgelin is possibly related to development and establishment of endometriosis. It was found upregulated in endometriosis suggesting it can be used as a biomarker (Hidalgo *et al*., 2011).

2.6.3 Cancer Antigen 125 (CA-125) as a serum biomarker in diagnosis of endometriosis

Cancer Antigen 125 (CA-125) has been reported as the most well investigated serum biomarker for ovarian cancer and endometriosis (Vodolazkala, *et a*l., 2012; Hsu, *et al*., 2010; May, *et al.*, 2010). It is produced by endometrial and mesothelial cells and exudes into circulation via the endothelial lining of capillaries in response to inflammation (Gupta *et al*., 2006). However, CA-125 (a glycoprotein of high molecular weight) whose levels in the peripheral blood are shown to be elevated lacks diagnostic power as a single biomarker of endometriosis (Dochez, et al., 2019). Not one single serum biomarker has been found to be specific for diagnosis of endometriosis and therefore a panel of them may be successful.

Therefore, it is important to confirm whether T-plastin, Transgelin and CA-125, are potential biomarkers in early diagnosis of endometriosis using animal model for endometriosis.

2. 7 Management of endometriosis

Two approaches are available for management of endometriosis, the medical (pharmacological) management and surgery. Both the approaches aim at reducing pain, increase fertility, suppresses and delay recurrence and progression of disease for a longer time (Parasar, *et al.*, 2017; Wellbery, 1999). Medical approach utilizes the principal of

reducing inflammation as well as suppressing ovarian cycles and therefore inhibiting the effect of estrogens.

Hormonal suppression drugs include the progestin, danazole, gonadotropin releasing hormone (GNRH) analogues and other oral contraceptives (NICE, 2017; Wellbery, 1999). Non-steroidal anti-inflammatory drugs are also widely used to treat chronic pain.

Surgery management attempts either to remove only the identified endometriotic lesion or complete removal of pelvic organ (Dunselman, *et al*., 2014). While this may promote disease recurrence, it may as well reduce the risk of progression of disease to severe or future ovarian cancer (Sourial, *et al.,* 2014). Surgery too increases chances of fertility (Parasar, *et al.*, 2017).

This study sought to investigate the use of the three parameters :T-Plastin,Transgelin and CA-125 as potential biomarkers that could be used in non invasive diagnosis of endometriosis especially in early and initial stages. The baboon model being a suitable experimental model due to its genetic proximity to human beings gives results that can be comfortably used in human beings diagnosis. This will give way to reducing time and period taken for diagnosis of endometriosis,reduce the cost related with endometriosis and also improve the quality of life of women.

CHAPTER THREE

MATERIAL AND METHODS

3.1 Study site

The study site was Institute of Primate Research (IPR), Karen where the animals were maintained. Laboratory analysis was carried out from the Reproductive Health Laboratory of IPR and Biochemistry department of Kenyatta National Hospital.

3.2 Study design

The research was experimental design study.

3.3. Experimental Animals

Ten female baboons (*Papio anubis*), each weighing 9-15 kg each and of proven fertility were selected for the study. The animals were housed indoors in a well-lit environment, as natural as possible and were fed with commercial pellets and substitute of vegetables and fruits.They had at least one menstruation during captivity. All the animals were obtained from the Institute of Primate Research (IPR) in Karen, and had previously be caught from wild forests. Procedures and care for handling the animals were approved by the Institutional Animal Care and Use Committee (Appendix I).

Included were all the animals screened clean without any condition of disease. All animals also had to have at least one menstrual cycle. Animals with any clinical disorders or unclear menstrual cycle were excluded.

Figure 3.1: Olive Baboons from IPR-Karen, Kenya

(**www.Primateresearch.org, Feb** 15, 2018)

3.4. Experimental Design

The animals were allowed interactions with each other. They were all screened and after establishing that the animals had normal pelvic through laparoscopy screening, the 10 baboons were induced for endometriosis by means of intrapelvic injection of menstrual endometrium on day 1 or 2 of menstruation. This is laparoscopy 2, cycle 2, after the 1st one of screening (D'Hooghe, *et al*., 1995).The first samples of peripheral blood and peritoneal fluid, i.e. prior to induction were collected at this point. These were referred to as comparison group or pre induction (control) samples. This was followed by staging laparoscopy which is laparoscopy 3, cycle 3. The primary outcome was the detection of endometriosis implants (number, size, surface area and volume). The second sampling was done at this time. Samples of peripheral blood and peritoneal fluid twenty five days after induction were collected. Secondary outcomes were the examination of type of lesion (typical, subtle red plus white, suspicious). Second post induction sampling was done and peripheral blood and peritoneal fluid collected after disease had been established or developed for at least fifty days. Some biopsies were collected for histological confirmation of macroscopic endometriosis and immunohistochemical evaluation. Peripheral blood and peritoneal fluid samples were used for T-plastin, transgelin, and CA-125 levels evaluation or determination.

All the ten baboons were included for the study since they were screened clean from any other gynaecological condition.

3.5 Sample size

A total of ten adult female baboons was used. The baboons are expensive animals and so a limited number was arrived at for use in this study. The sample size was determined based on the 3Rs principle which emphasizes replacement, reduction and refinement of animals (Russel & Burch, 1959). Other researchers also have done similar work and used ten animals and while others have used three (Zondervan, *et al*., 2018; Fassbender, *et al*., 2015). The animals were divided into two groups of fives, the first group was induced for endometriosis between 14th Sep 2015 and 25th Sep 2015 while the second group was induced between 9th Sep 2017 and 21st Feb 2018. All animals had blood samples and peritoneal fluid samples collected three times, first sampling done before induction and twice after induction and development of disease.

3.6 Induction of baboons with endometriosis

Olive baboons are reported to have spontaneous endometriosis. Induction of endometriosis was performed in ten baboons with a normal pelvis on the first or second day of menstruation (Story & Kennedy, 2004). Induction at a later time was not performed as the induction would have been less successful, since most of the endometrial tissue has disappeared after day 2 of menstruation. Endometrial tissue was extracted from each baboon by transcervical curettage, and fragmented through an 18-gauge needle creating a menstrual "paste" as has been described before (Chai *et al*., 2007; D'Hooghe, *et al.,* 1995). A standardized amount of the resulting paste (1000 mg) of tissue paste was then seeded by inoculation onto various peritoneal sites (uterosacral ligaments, uterovesical folds, posterior uterine peritoneum, cul-de-sac, broad ligament and ovary) as described before (D'Hooghe, *et al*., 1995). The disease developed for fifty days during which time the animals were monitored.

3.7 Sample collection

3.7.1 Preparation of Animals for Sample Collection

The animals were monitored for any infections and pain where antibiotics and pain killers were used to manage any eventuality. The monitoring was carried out for three months before induction of endometriosis. All surgical procedures to the animals were done under anesthesia. First samples of both peripheral blood and peritoneal fluid were collected during laparoscopic examination just before induction. Twenty five days after induction and development of endometriosis, the second sampling was done and thirty days later the third and final sampling was done. In total three samples were collected from each animal, the first sample prior to induction being referred to as the comparison group (control) and the next two being samples of post induction samples , (diseased baboons).

3.7.2 Collection of Peripheral Blood (PB)

Four BD Vacutainer K2E 4ml tube of venous blood was collected from each baboon and labeled (PB was processed within 30 minutes). Following collection, the blood was gently inverted several times (approximately 7-10 times) to prevent the formation of blood clot. The peripheral blood samples were then centrifuged at 3000 rpm for 10 minutes at 4^0C. Then 500µl aliquots of plasma were prepared into labeled tubes and stored at –80ºC until time of use. Each baboon had a name tag and code (Appendix 11). Plasma samples were labelled by the animal code, (Pb) for peripheral blood and date of collection representing whether it was prior to induction, first sampling after induction (after 25 days) or second sampling after induction (after fifty days).

3.7.3 Collection of Peritoneal Fluid (PF)

Peritoneal fluid was aspirated using a 20cc syringe attached to a needle which was inserted through the skin of the abdomen. This procedure was done using laparoscopy. The syringe was also attached to a labeled tube for collection. Peritoneal fluid (PF) was

processed within one hour. Following PF collection, the volume was measured and color appearance recorded. The PF sample was then centrifuged at 1500rpm for 10 minutes. 500µl aliquots of PF supernatant were prepared into labeled tubes and stored at –80ºC until use (D'Hooghe , *et.al.,* 2001). In event the peritoneal fluid was inadequate for collection, a lavage sample was collected, where a little amount of pbs was introduced into the peritoneal cavity and sucked back to the collection tube.

3.7.4 Enzyme Linked Immunosorbent Assay (ELISA)

Enzyme linked immunosorbent assays (ELISA) are assays involving antigen bound onto an inert surface, usually a 96 well plate, and an antibody and enzyme molecules conjugated to specific antibody or antigen. The reaction yields a colored product which is read using an ELISA reader. The intensity of the color depends on the amount of antigen or antibody in the sample in use. There are different types of ELISA techniques including Indirect, direct and sandwich abcam. (2018).

T-Plastin, Transgelin and CA-125 were evaluated in blood and peritoneal fluid samples before and after induction of endometriosis using sandwich ELISA. Protocols used were as provided by manufacturer and supplier of the antibodies.

Levels of T-Plastin were evaluated using Human Plastin 3 (PLS3) ELISA kit (My BioSource, USA, Cat. No. MBS9312912). Briefly, the serum and peritoneal fluid samples were brought to room temperature (18°-25°C) prior to starting the assay procedure. Fifty microliters of the samples and sample diluent were added to each well of the 96 well ELISA plate, in duplicate. Horse radish Peroxidase conjugate was added to each well at a quantity of 100µl. The plates were covered and incubated for 60 minutes at a temperature of 37ºC. The plates were then washed four times with wash buffer. Chromogen solutions were added and the plates were placed in the dark for 15minutes at 37ºC to allow color formation. Fifty microliters of Stop solution was added to the wells. There was color change from blue to yellow and the Optical Density was

read at 430nm using the ELISA reader (biotek Elx 808) within fifteen minutes after adding stop solution.

Transgelin levels were evaluated using the Transgelin (TAGLN) ELISA Kit (online.com antibodies, Germany, Cat. No.ABIN 1567594). Serum and peritoneal fluid samples were brought to room temperature prior to running the assay. They were centrifuged after thawing and thoroughly mixed. One hundred microliters of the sample was added to the wells and incubated for 90 minutes at 37^{o}C. This assay was run in duplicates. Biotinylated detection antibody was added into each well and incubated for an hour at 37^{o}C before washing thrice with a wash buffer (350 µl). One hundred microliters of Horse radish Peroxidase (HRP) conjugate was added into each well and incubated for 30 minutes at 37^{o}C. The plates were washed with 350 microliters of buffer for five times before a 90 microliters substrate solution was added, incubated for 15 minutes and the reaction stopped with 50 microliters of stop solution, (usually an acid). Optical density measurements were determined using Biotek Elx 808 ELISA machine and a micro plate reader set at 450nm.

Cancer Antigen 125 (CA-125) levels in blood and peritoneal fluid was measured using the Roche Cobas 6000 analyzer (Roche Diagnostics, Mannheim, Germany). Serum and peritoneal fluid samples were brought to room temperature. One milliliter (1mL) of each sample was transferred into the vials which were fed into the machine. Results of CA-125 were measured in units per milliliter (U/mL).

3.8 Ethical consideration

The experimental protocols carried out in this study underwent review by the Institutional Science and Ethics Committee (ISERC) based at IPR, Kenya. Animals were treated according to standard guidelines for the use and care of laboratory animals (Ref: IRC/04/15).

3.9 Statistical Analysis

Data was recorded in laboratory work book and MS excel work sheets. It was analyzed using non-parametric test, Kruskal Wallis and Mann Whitney U. The statistical level of significance was set at $p \leq 0.05$.

Evaluation of the diagnostic performance of the tested biomarkers was performed by calculating the area under curve (AUC) of Receiver Operating Characteristics (ROC) curves. Each of the AUC estimated at 95% confidence interval (95% CI) and $P < 0.05$ were considered statistically significant. An AUC of 0.5 and below indicated the test was positive 50 % of the time. The higher the AUC, the better the value was considered to be. An excellent model has AUC near to the 1. A poor model was considered when AUC was below 0.5 and neared 0. This was done using the GraphPad Prism software version 7.00 for Windows. The antigens that recorded higher AUC nearing 1 were considered to be better biomarkers for diagnosis of endometriosis than those that had an AUC of 0.5 or around that.

CHAPTER FOUR

RESULTS

4.1 Introduction

Baboons have been used as the model for study of endometriosis. In order to carry out investigations for antigens, T-Plastin, Transgelin and CA-125 in serum and peritoneal fluid with a possibility of them being a potential diagnostic biomarker, the disease endometriosis was induced in ten female baboons and monitored for fifty days.

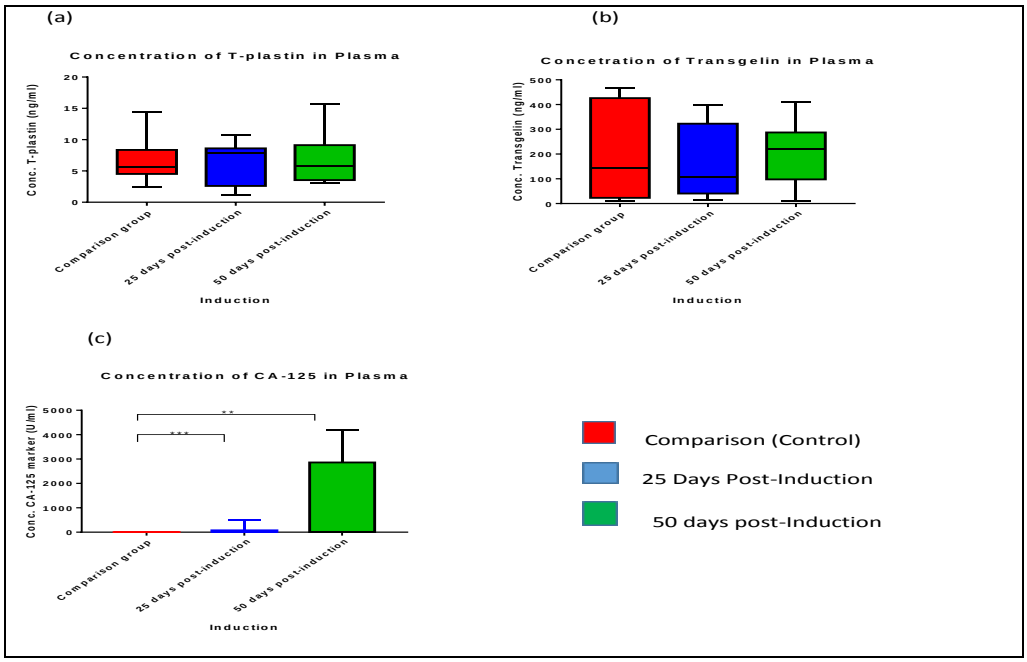
Through video laparoscopy endometriosis developed in the ten baboons with lesions and ascites similar to those of women with endometriosis.

4.2 Levels of T-plastin, Transgelin and CA-125 biomarkers in plasma

A total of ten baboons induced with endometriosis were evaluated for T plastin, Transgelin and CA-125 concentrations in plasma. There was a slight increase in levels of T-plastin 25 days after induction, however this was not significant ($p=0.8633$). 30 more days after second sampling, the levels increased slightly, however this was also not significant ($p=0.9999$). As in Figure 4.1 there was an upward trend of T-Plastin in peripheral blood of animals with induced endometriosis.

Transgelin showed insignificant increase in levels after first post-induction sampling in plasma compared to the comparison group, (control). There was slight increase in Transgelin in serum concentration 50 days after induction. However this difference was not significant (Figure 4.1 b).

The concentrations of CA-125 in plasma showed significant increase 25 days after induction ($p=0.0003$). Figure 4.1 c shows that there was also a significant rise in concentration of CA-125 in serum 50 days after induction ($p=0.0014$).

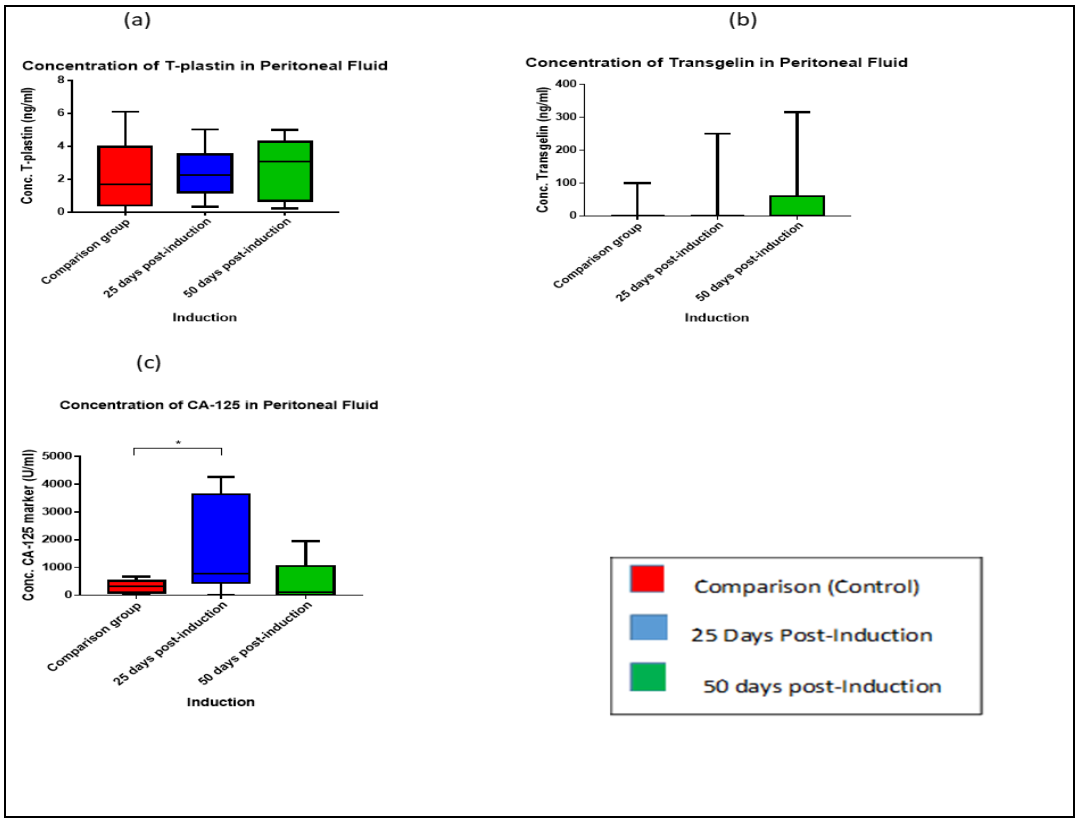


Values were considered significant when p≤0.05 and assigned **p=0.0014, ***p=0.0003.

Figure 4.1 a, b and c: Concentrations of T-plastin, Transgelin and CA-125 in peripheral blood,

4.3 Levels of T-Plastin, Transgelin, and CA-125 in peritoneal fluid.

After induction of endometriosis the concentrations of T-plastin, Transgelin and CA-125 in peritoneal fluid were evaluated, 25 days after induction and 50 days after induction. Figure 4.2a shows the concentration of T-plastin in peritoneal fluid increased slightly between pre induction and post induction measurements. The increase however was not significant (p=0.4463). Transgelin did not show significant rise in concentration between pre and the post induction readings in peritoneal fluid (p=0.1192) as shown in figure 4.2b. The results however showed that CA-125 concentrations were significantly higher in peritoneal fluid especially between comparison group and 25days post induction reading (p=0.0279). The concentration between pre induction and 50 days post induction however was insignificant, (p=0.9682) as shown in fig 4.2c.



,*Values were considered significant when $p \leq 0.05$ and assigned *p=0.0279.

Figure 4.2 a, b, and c: Concentrations of T-Plastin, Transgelin and CA-125 in peritoneal fluid.

4.4 Receiver operating Characteristics (ROC) Analysis for T-plastin, Transgelin and CA-125

Analysis determining the diagnostic performance of the biomarkers in plasma using the receiver operating characteristics (ROC) showed T-plastin-AUC=0.5 with 95% confidence interval (CI) of 0.2275-0.7727, p>0.999, Transgelin, AUC=0.505 with 95% CI 0.2455-0.764, p=0.9698, CA-125 has AUC=0.6100, 95%CI 0.395-0.8200, p value=0.3331. The highest AUC of 0.79 was found in CA-125 as shown in figure 4.3 a, b, c.

(a) (b) (c)

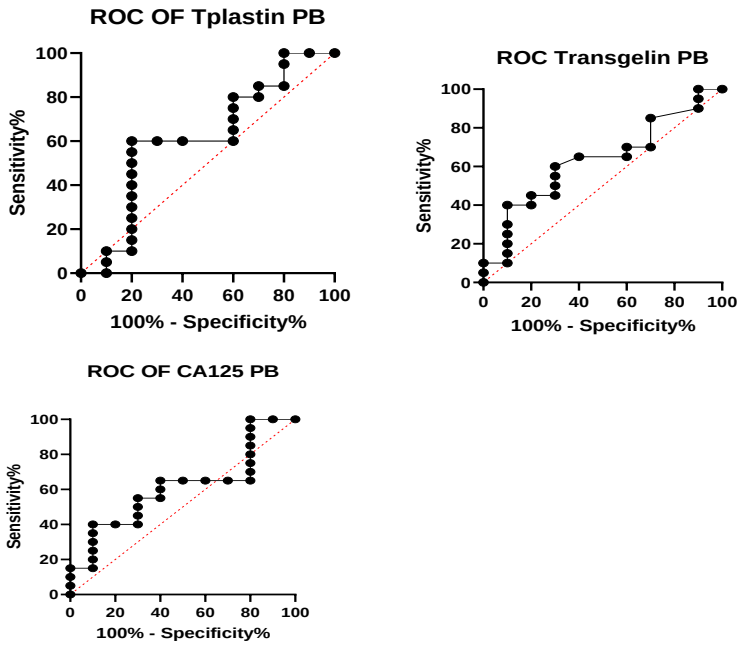


Figure 4.3 a, b, and c: Receiver operating characteristics (ROC) curves of T-plastin, Transgelin and CA-125 respectively in plasma.

The receiver operating characteristics (ROC) for concentrations of T-plastin, Transgelin and CA-125 in peritoneal fluid showed an AUC of 0.6325 for T-Plastin has AUC=0.6125 with 95% CI 0.3242-0.9008, p=0.4239, Transgelin has AUC=0.685 with 95% CI 0.4422-0.9278 and a p value = 0.1620. CA-125 has AUC=0.7900 with 95% CI 0.5723-1.000 p=0.010) as shown in **fig**ure 4.4a, b, and c.

(a) (b) (c)

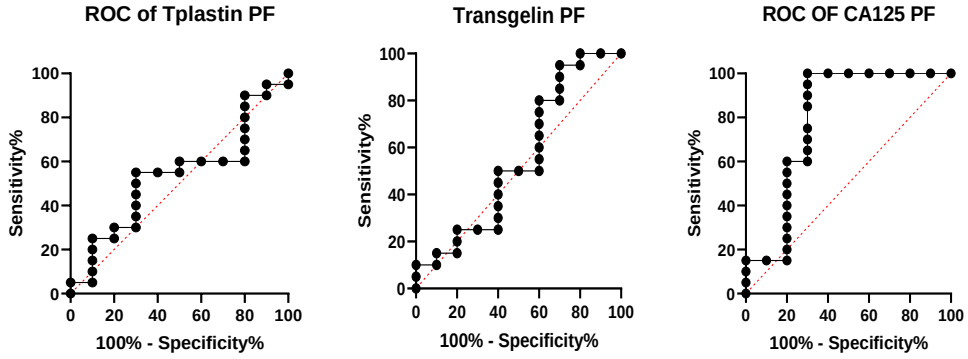


Figure 4.4 a, b and, c: Receiver operating characteristics (ROC) curves of T-plastin, Transgelin, and CA-125 in peritoneal fluid

CHAPTER FIVE

DISCUSSION

5.1 Induction of endometriosis in baboons.

The olive baboons easily developed endometriosis upon intra-pelvic induction which resembled spontaneous endometriosis found in women. This was similar to what was reported in an earlier study done on baboons being model for endometriosis (D'Hooghe, *et al*., 1995). The baboon model is useful for studying endometriosis since it's practically difficult to carry out long term objective tests in women (Hastings & Fazleabas, 2006). The baboons closely related to humans genetically and therefore give data that is easy to work with. Samples of blood and peritoneal fluid collected prior and post induction were analyzed for T-plastin, Transgelin and CA-125 concentrations by ELISA. The results indicated that concentrations of T-Plastin and Transgelin in serum were detected and measured but no significant increase was realized between the pre and the post induction samples during the two months of study. However slight upward trend was noted in serum levels of T-plastin between pre induction and first sampling 25 days after induction of endometriosis. There was a study done on endometrial biomarkers in women and which was able to diagnose minimum-mild endometriosis with upregulated Tplastin antigen concentrations (Kyama, *et al*., 2011).

5.2 Concentrations of T-plastin, Transgelin and CA-125 in Peripheral Blood

This study showed T-Plastin levels in peripheral blood to have a slight increase and a notable trend. The increase however was insignificant. In an earlier study by Kyama, *et.al*, 2011, T-plastin was found upregulated in endometrium of secretory phase in women who had minimum to mild endometriosis. T-plastin however was not quantified in plasma or serum of the women and data was unavailable to indicate concentrations. T-plastin has been associated with development of abnormal structures in pelvic peritoneum in endometriosis (Kyama, *et al*., 2011).

Transgelin appeared to have higher concentration in serum prior to induction and only slight increase was observed 25 days after induction. A study by (Hidalgo *et al.,* 2011) found that Transgelin is increased in endometriotic lesions compared with eutopic endometrium. The study suggested Transgelin may be associated with development and maintenance of endometriosis. Transgelin which is believed to be related to cell differentiation, cell migration and involved in tissue invasion was remarkably upregulated. A study by Tawfik, *et al*., 2014 and Kyama *et al*., 2006 indicated Transgelin to be a specific marker of smooth muscle differentiation and capable of being used as a useful marker in addition to other antigens. Significant rise in concentrations was expected in endometriosis. Due to its low sensitivity and specificity reported, Transgelin cannot be used as a single biomarker but may be considered together with a panel of others.

In peripheral blood, CA-125 was seen to have a significant rise ($p=0.0003$ and $p= 0.0014$) between pre induction, after 25 days induction and after 50 days of induction (diseased samples). This is in line with other studies that have established that CA-125 increases in initial stages of endometriosis but markedly increases in moderate to severe stages (Mol, *et al*., 1998; Muyldermans, *et al.,* 1995). Another study indicates CA-125 test has a specificity of 89% in detecting moderate to severe endometriosis (Child & Tan, 2001).

Cancer Antigen (CA-125) levels increase in serum in moderate to severe endometriosis (Bon, *et al*., 1995). The study explained that CA-125 released from endometrial cells can gain access to circulation and lymphatic system. CA-125 was significantly elevated in a study by Patton, *et al* ., (1986). Another study by Szubert, *et al*., 2012 had similar observations, CA-125 levels in patients with endometriosis in moderate to severe stages was significantly raised. Similar results of an increase in CA-125 levels have been found (Rokhgireh, *et al*.,). It has therefore been suggested in a more resent study that CA-125 can be used as a serum biomarker for endometriosis since it was found higly increased in women with endometriosis (Irungu, *et al*., 2019).

5.3 Concentrations of T-Plastin, Transgelin and CA-125 in Peritoneal Fluid

The study of T-plastin and Transgelin concentrations in peritoneal fluid did not reveal any significant difference between the control and endometriosis. The concentration of CA-125 however was seen to have significantly increased between the pre induction and disease in the peritoneal fluid. This is due to CA-125 and other proteins such as glycodelins being released into peritoneal cavity after secretion by the endometrial cells and finding their way into peritoneal cavity by retrograde menses (Kimber-Trojnar, et al., 2021).

A study in women showed peritoneal fluid CA-215 highest in disease than in controls (Amaral, *et al.,* 2006). This is a result of the presence of blood and eutopic endometrial tissue into the peritoneal cavity due to retrograde menses. A study has also suggested that CA-125 released from the endometrium can have access to the lymphatics and the circulation. CA-125 plays a role in development of endometriosis. Another study evaluating CA-125 concentrations in peritoneal fluid and serum of women with and without endometriosis found higher levels of CA-125 in PF of diseased women than in the non-diseased. CA-125 was sad to be a good biomarker for endometriosis helpful in daily clinical practice when endometriosis is suspected and for monitoring treatment or progress (Karimi-Zarch, *et al*., 2016).

5.4 ROC Curves Analysis of T-plastin, Transgelin and CA-125 in blood and peritoneal fluid

The area under curve (AUC) in receiver operating characteristics (ROC) that was used to determine diagnostic performance of T-plastin and Transgelin was low indicating the two biomarkers in serum cannot be effective or suitable test for endometriosis on their own until probably combined with others. The endometriosis established during the two months study could have been minimum-mild stages where antigens are detectable but not significantly established whether increased or not. A study trying to establish whether Transgelin is a novel biomarker to distinguish between smooth muscle tumors

and endometrial muscle tumors found transgelin completely absent in endometrial muscle tumors in women. However it was positively identified in smooth muscle tissues (Tawfik, *et al*., 2014). The AUC for serum CA-125 was slightly above baseline and therefore would be considered for use as a potential biomarker. The AUC for both T-plastin and Transgelin in the peritoneal fluid were slightly above 0.6 and would be considered for diagnostic tests combined with other strong biomarkers. However CA-125 showed a significantly high AUC in peritoneal fluid (0.7900) which indicates the diagnostic performance of CA-125 is high and together with other biomarkers can be used as a potential biomarker.

The major limitation observed in this study was the development of endometriosis in the animals for the two months and measuring the antigens using ELISA. Different assays, such as Western blot, flow cytometry, immunohistochemistry, could be more sensitive and detect the increase in concentrations of these antigens in serum and peritoneal fluid.

5.5 Conclusion

This study revealed the presence of T Plastin, Transgelin and CA-125 in serum and peritoneal fluid. There was no significant increase in T plastin and Transgelin concentrations between the pre induction and post induction in serum and therefore the two would not be considered as reliable biomarker for endometriosis without other combinations. The levels of CA-125 increased in serum. This finding confirmed the earlier report that CA-125 can be used as a serum biomarker. However, the low diagnostic power established from the AUC values does not allow CA-125 to be used as a reliable individual biomarker. Thirdly this study revealed T-Plastin in peritoneal fluid very slightly increased. This was non-significant rise, and so was Transgelin. Transgelin however showed slight increase in peritoneal fluid. Levels of CA-125 showed a significant rise in peritoneal fluid. Evaluation of concentrations of the three antigens in blood and peritoneal fluid and determining their diagnostic performance indicates the

panel does not have the potential to be used as a diagnostic test or as potential biomarkers of endometriosis in early stages until more evaluation has been done.

5.6 Recommendations

Further research using different protocols to identify and quantify T-plastin, Transgelin and CA-125 in blood and peritoneal fluid in minimum to mild stages in endometriosis is well recommended. An evaluation of a panel of the three antigens T-plastin, Transgelin and CA-125 for detection of early endometriosis in different body fluid for instance urine is recommended.

A longer period of induction of endometriosis would also be recommended and a comparison on the levels of antigens between early and late endometriosis established.

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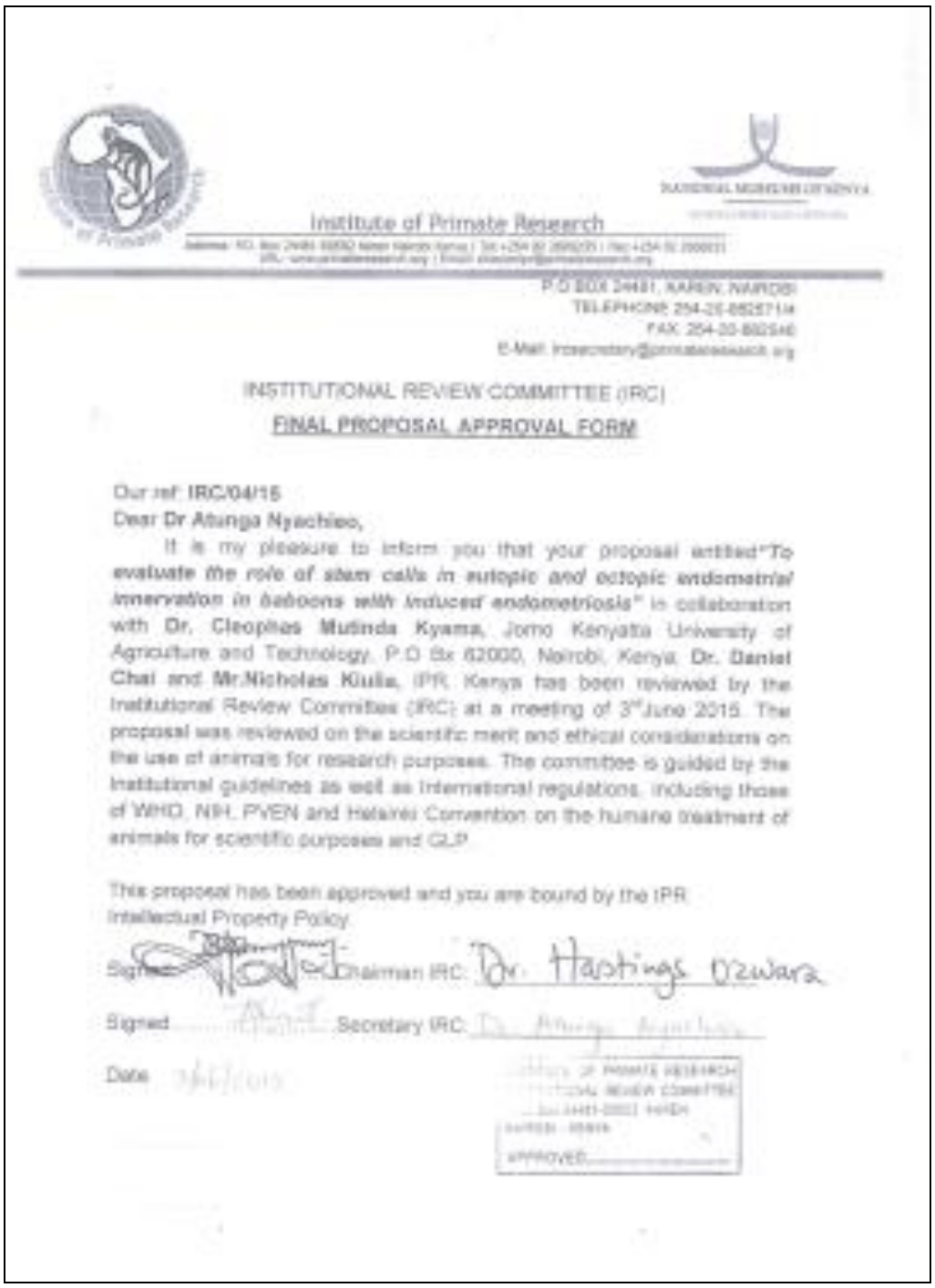
APPENDICES

Appendix I: Ethical Guidelines



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INSTITUTIONAL REVIEW COMMITTEE (IRC)

FINAL PROPOSAL APPROVAL FORM

Our ref: IRC/04/15

Dear Dr Atunga Nyachieo,

It is my pleasure to inform you that your proposal entitled "*To evaluate the role of stem cells in eutopic and ectopic endometrial innervation in baboons with induced endometriosis*" in collaboration with Dr. Cleophas Mutinda Kyama, Jomo Kenyatta University of Agriculture and Technology, P.O Bx 62000, Nairobi, Kenya, Dr. Daniel Chai and Mr.Nicholas Kiulia, IPR, Kenya has been reviewed by the Institutional Review Committee (IRC) at a meeting of 3rd June 2015. The proposal was reviewed on the scientific merit and ethical considerations on the use of animals for research purposes. The committee is guided by the Institutional guidelines as well as International regulations, including those of WHO, NIH, PVEN and Helsinki Convention on the humane treatment of animals for scientific purposes and GLP.

This proposal has been approved and you are bound by the IPR Intellectual Property Policy

Signed ..... Chairman IRC: Dr. Hastings Ozwara

Signed ..... Secretary IRC: .....

Date .....

INSTITUTE OF PRIMATE RESEARCH
INSTITUTIONAL REVIEW COMMITTEE
APPROVED

Appendix II: Induction of endometriosis schedule

Date	ACTIVITY	Animal
14/9/15	Laparoscopy and	PAN 4181
15/9/15	1st screening	PAN 4177
15/9/15	Peritoneal fluid and Plasma collected	PAN 4136
16/9/15		PAN 4180
25/9/15		PAN 4178
Date		
12/10/15	Laparoscopy and	PAN 4181
12/10/15	2nd screening	PAN 4177
13/10/15	Peritoneal fluid and Plasma collected	PAN 4136
16/10/15		PAN 4180
22/10/15		PAN 4178
Date		
17/11/15	Laparoscopy	PAN 4136
17/11/15	and staging	PAN 4177
24/11/15	Peritoneal fluid and Plasma collected	PAN 4180
24/11/15		PAN 4181
1/12/15		PAN 4178
14/10/17	Laparoscopy and 1st	PAN 3888
10/11/17	Screening	PAN 3902
13/11/17	Peritoneal fluid and Plasma collected	PAN 4149
13/11/17		PAN 4156
28/1/18		PAN 4126
9/11/17	Laparoscopy and 2nd	PAN 3888
13/12/17	Screening	PAN 3902
14/12/17	Peritoneal fluid and Plasma collected	PAN 4149
14/12/17		PAN 4156
21/2/18		PAN 4126
13/12/17		PAN 3888
12/1/18	Laparoscopy	PAN 3902
16/1/18	and staging	PAN 4149
15/1/18	Peritoneal fluid and Plasma collected	PAN 4156
26/3/18		PAN 4126

Appendix III: T-Plastin

Assay Procedure

(1) Bring all reagents and samples to room temperature (18°C -25°C) naturally for 30 minutes before starting assay procedures.**DO NOT** use hot water baths to thaw samples or reagents. If necessary doing a low –speed centrifugation for one or two seconds to concentrate the standards to the bottom of the vials. The Micro Elisa Stripplate is detachable, detach unused strips from the plate frame, return them to the foil pouch with the desiccant pack, and reseal for preventing damps.

(2) Set Standard wells ,Sample Wells and Blank /Control wells ,add Standard 50µl to each Standard well, add sample 50µl to each sample well, add sample diluent 50µl each Sample well, add Sample Diluent 50µl to each Blank/Control well. It is recommended that all Standards, Samples and Sample Diluent be added in duplicate to the plate.

(3) Add 100µl of HRP –Conjugate reagent to each well, cover with a Closure Plate Membrane and incubate for 60 minutes at 37°C.

(4) Wash the plate four times.

(4.1) **Manual Washing**-Dump the incubation mixtures of the wells into sink or proper waste container. Using pipette or squirt bottle, fill each well completely with wash solution (1×), after about one minute‘s standing, invert and hit the plate onto absorbent papers or paper towels until no moisture appears. Repeat this procedure four times. **Note:** Hold the sides of the plate frame firmly when washing the plate to assure that all the strips remain securely in frame.

(4.2) **Automated Washing-** Aspirate all wells, then wash plates four times using wash Buffer (1×).Always adjust your washer to aspirate as much liquid as possible and set fill volume at 50µl/well/wash. After final wash, invert plate, and blot dry by hitting plate on to absorbent paper or paper towels until no moisture appears.

(4.3) Add chromogen solution A 50μl and Chromogen Solution B 50 μl to each well successively. Then protect from light to incubate for 15 minutes at 37°C.

(4.5) Add 50 μl Stop Solution to each well. The color in the wells should change from blue to yellow.

(4.6) Read the Optical Density (O.D.) at 450nm using ELISA reader within 15 minutes after adding Stop Solution. (Around five minutes is the best time).

(5) Calculation of Results

(5.1) Average the duplicate readings for each standard and sample to subtract average optical density of the Blank/Control (VB/C).

Standards (Concentration):	0	S1	SII	SIII	SIV	SV	SVI
Mean O.D.(450nm):	$V_{B/C}$	V_1	V_2	V_3	V_4	V_5	V_6
Adjusted:	0	V_1-$V_{B/C}$	V_2-$V_{B/C}$	V_3-$V_{B/C}$	V_4-$V_{B/C}$	V_5-$V_{B/C}$	V_6-$V_{B/C}$

(5.2) Using the professional curve fitting software to make a standard curve (usually most of the curves are linear, and a few curves are quadratic or cubic) and calculate the level of analyte.

Appendix IV: Transgelin ELISA Kit (TAGLN)

Assay Procedure

1. Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. All the reagents should be mixed thoroughly by gently swirling before pipetting, Avoid foaming. It's recommended that all samples and standards be assayed in duplicate.
2. Add Sample: Add 100µL of Standard, Blank, or Sample per well. The Blank well is added with Reference Standard and Sample Diluent. Solution are added to the bottom of micro ELISA plate well, avoid inside wall touching and foaming as possible. Mix gently. Cover the plate with sealer provided. Incubate for 90 minutes at 37°C.
3. Biotinylated Detection Ab: Remove the liquid of each well, don't wash. Immediately add 100µL of Biotinylated Detection Ab working solution to each well. Cover with the plate sealer. Gently tap the plate to ensure thorough mixing. Incubate for 1 hour at 37°C.

Wash: Aspirate each well and wash, repeating the process three times. Wash by filling each well with Wash Buffer (approximately 350µL) (a squirt bottle, multichannel pipette, manifold dispenser or automated washer are needed). Complete removal of liquid at each step is essential. After the last wash, remove remained Wash Buffer by aspirating or decanting. Invert the plate and pat it against thick clean absorbent paper.

HRP Conjugate: Add 100µL of HRP Conjugate working solution to each well. Cover with the Plate Sealer. Incubate for 30 minutes at 37°C.

Wash: Repeat the wash process for five times as conducted in step 4.

Substrate: Add 90μL of Substrate Solution to each well. Cover with a new Plate Sealer. Incubate for about 15 minutes at 37°C. Protect the plate from light. The reaction time can be shortened or extended according to the actual color change, but not more than 30 minutes. When apparent gradient appeared in standard wells, user should terminate the reaction.

Stop: Add 50μL of Stop Solution to each well. Then the color turns to yellow immediately. The order to add stop solution should be the same as the substrate solution.

OD measurement: Determine the optical density (OD value) of each well at once, using a microplate reader set to 450nm.User should open the micro plate reader in advance, pre heat the instrument, and set the testing parameters.

4. After experiment, put all the unused reagents back into the refrigerator according to the specified storage temperature respectively until their expiry.
5. CALCULATION OF RESULTS

   Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Create a standard curve by plotting the mean OD value for each standard on the y axis against the concentration on the x axis and draw a best fit curve through the points on the graph. It is recommended to use some professional software to do this calculation, such as curve expert 1.3 or 1.4. In the software interface, a best fitting equation of standard curve will be calculated using OD values and concentrations of standard sample. If samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it after appropriate dilution. The actual concentration is the calculated concentration multiplied dilution factor

Appendix V: Assay procedure for Cancer Antigen 125

Resuspension of the microparticles takes place automatically prior to use. Read in the test specific parameters via the reagent barcode. If in exceptional cases the barcode cannot be read enter the 15-digit sequence of numbers.

Bring the cooled reagents to approximately 20°C and place on the reagent disk (20°C) of the analyzer. Avoid foam formation. The system automatically regulates the temperature of the reagents and the opening/closing of the bottles.

Calculations

The analyzer automatically calculates the analyte concentration of each sample (either in U/ml, U/L or kU/L).