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

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**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% women in their reproductive ages are affected by the disease globally. Currently, diagnostic methods are laparoscopy with histological confirmation and non-invasive such as ultrasound and the quantification of CA-125 biomarker in serum. 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, 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 in secretory phase of endometrium respectively. 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 identify whether T-plastin, Transgelin and CA-125 are potential biomarkers in early diagnosis of endometriosis using animal 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 prospective, case-control study, ten female baboons (*Papio anubis*, 9-15 kg each) of proven fertility that have had at least one menstruation during captivity, were induced by intra-pelvic injection of menstrual endometrium on day 1 or 2 of menstruation 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 at $p<0.005$. T-Plastin and Transgelin had insignificant p values in both peripheral blood and peritoneal fluid while CA-125 showed $p=0.0003$ in peripheral blood and $p=0.0279$ in peritoneal fluid. 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 unreliable for use as biomarkers in endometriosis. CA-125 levels in peritoneal fluid had an AUC of 0.7900 indicating it can be used as potential biomarkers for endometriosis.
**Key words:** Endometriosis, biomarkers, T-Plastin, Transgelin, CA-125, ELISA, Olive Baboons.

## 1    Introduction

Endometriosis is a common benign, chronic and multifactorial complex gynecological disease which is estrogen dependent (Sinaii *et al*., 2008; D'Hooghe, 1997). It is characterized by chronic pelvic pain, dysmenorrhea and infertility and defined by presence of endometrial stroma and glands outside the uterine cavity (Nisoile & Donnez, 1997; Farquhar, 2001). Clinically endometriosis presents in three varying phenotypes; peritoneal endometriosis, ovarian endometriotic cyst and deep infiltrating endometriosis (D'Hooghe *et al*., 2003). Ten percent of women in the reproductive age globally are reported to suffer from endometriosis (Olive & Schwartz, 1993; Cramer &

Wilson, 1987; Giudice & Kao, 2004), while 40-60% of women with infertility and chronic pelvic pain have endometriosis (Vermeuleri *et al.,* 2014). According to American Society of Reproductive Medicine (ASRM, 1996) report on classification and staging of endometriosis, there are four classes/stages of endometriosis, minimum, mild, moderate and severe (1-4 stages). There is no correlation between the symptoms and the stage of the disease (Mehedintu *et al*., 2014). Despite the studies done on endometriosis, its etiology remains unclear (Baldi *et al*., 2008). Several theories have been hypothesized; the most popular and well accepted being retrograde menstruation in which endometrium fragments are refluxed via fallopian tubes into the pelvis (Sampson, 1927). Inflammatory factors such as IL-1, IL-2, IL-6, IL-8, and TNF alpha (expressed by macrophages) are associated to developmental of endometriosis (Ioana & Razoan, 2013). A defective immunosurveillance against endometrial cells deposited on the peritoneal cavity due to a dysfunctional immune system in women allows development of endometriosis (Leboric *et al*., 2001). Due to lack of a non-invasive diagnostic test, there is usually a delay of 8-11 years (Fassbender *et al*., 2015; Fauconnier & Charpron, 2000). Endometriosis is sometimes asymptomatic (Rawson, 1991). Studies have shown that levels of Transgelin increase in lesions in endometriosis (Kyama *et al*., 2006), while T-Plastin is upregulated in the secretory phase in endometriosis (Kyama *et al*., 2011). CA125 elevated in endometriosis is used as a serum marker in endometriosis (Chen *et al*., 1998; Barbati *et al*., 1994). The gold standard for diagnosis is laparoscopy followed by histological confirmation (Kennedy *et al*., 2005). A simple, low-cost, non-invasive, fast, accurate and reliable diagnostic test will solve the delay and give light to treatment at early stages of the disease.

In this study, ten female baboons were induced with endometriosis. Peripheral blood and peritoneal fluid samples were collected at three stages, prior to induction, twenty six days after induction and upon full development of endometriosis. Enzyme Linked Immunosorbent Assay (ELISA) was used to evaluate the antigen levels. The study sought to determine the T-plastin, Transgelin and CA125 antigen levels in peripheral blood and peritoneal fluid using ten baboons induced with endometriosis and evaluate their variability as potential biomarkers for the detection of the onset of endometriosis.

## 2    Materials and Methods

### 2.1    Study site

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

### 2.2    Study design and subjects

The research design was prospective, case-control study of ten female baboons (*Papio anubis*). The animals weighed approximately 9-15 kg and were of proven fertility, that is, they had at least one menstruation during captivity. All the animals were obtained from the Institute of Primate Research (IPR) in Karen. Care of the animals and all experimental procedures were approved by the Institutional Animal Care and Use Committee.

The animals were housed indoors in well-lit as natural as possible environment and were fed with commercial pellets and substitute of vegetables and fruits.

### 2.3    Induction of endometriosis

Induction of endometriosis was performed in ten baboons after establishment of a normal pelvis (screening laparoscopy 1) on the 1st or 2nd day of menstruation (screening laparoscopy 2, cycle 2), by intrapelvic injection (D'Hooghe *et al*., 1995) followed by staging laparoscopy (laparoscopy 3, cycle 3).

 Endometrial tissue was extracted from each baboon by transcervical curettage, and fragmented through an 18-gauge needle creating a menstrual "paste" as previously described (D'Hooghe *et al*., 1995; Chai *et al*., 2007). A standardized amount of the resulting paste (1000 mg) of tissue paste was then seeded 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).

### 2.4    Collection of Peripheral Blood (PB)

Four milliliters of venous blood was collected from each baboon. Blood collection was carried out prior to induction, 26th and 60th day post induction. . Following blood collection, it was gently inverted several times (approx.7-10 times) to prevent the formation of blood clot. The peripheral blood samples were then centrifuged at

3000rpm for 10 minutes at $4^0$C. 500µl aliquots of plasma was prepared into labeled tubes and stored at –80ºC until use.

## 2.5 Collection of Peritoneal Fluid

Peritoneal fluid was aspirated using a 20 cc syringe, this was done prior to induction, 25th and 60th day post induction. Following Peritoneal Fluid (PF) collection, the volume was measured and color appearance recorded. The PF samples were centrifuged at 1500rpm for 10 minutes. 500µl aliquots of PF supernatant were prepared into labeled tubes and stored at –80ºC until use.

## 2.6 Enzyme Linked Immunosorbent Assay

T-Plastin, Transgelin and CA-125 were evaluated on blood and peritoneal samples before and after induction of endometriosis using ELISA according to manufactures' instructions. 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 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 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. The Optical Density was read at 430nm using the ELISA reader (Biotek Elx 808).

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. A hundred microliters of the sample was added to the wells and incubated for ninety minutes at 37º C. Biotinylated detection antibody was then added into each well and incubated for an hour at 37ºC before washing thrice with a wash buffer (350 µl).100 µl of Horse radish Peroxidase conjugate was added into each well and incubated for thirty minutes at 37ºC. The plates were washed with 350 µl of buffer five times before a ninety microliter substrate solution was added, incubated for fifteen minutes and the reaction stopped with fifty microliters of stop solution. Optical density measurements were determined using Biotek Elx 808 ELISA machine and a micro plate reader set at 450nm.

One milliliter of each serum and peritoneal fluid samples were brought to room temperature. The vials containing the samples were fed into Cobas E601 automated analyzer of Cobas 6000 series (Roche Diagnostics) to determine the CA-125 levels.

## 2.7 Statistical Analysis

Data was analyzed using non parametric test, Mann Whitney. The statistical level of significance was set at $p \leq 0.05$. This was done using the GraphPad Prism software version 7.00 for Windows. Evaluation of the diagnostic performance of the tested biomarkers was performed by calculating the area under curve (AUC) of Receiver Operating Characteristics (ROC) curves. AUC of 0.5 and below indicated the test was positive 50% of the time.

## 2.8 Ethical Consideration

The experimental protocols carried out in this study underwent review by the Institutional Science and Ethics Committee (ISERC) based at IPR, Kenya.

## 3 Results

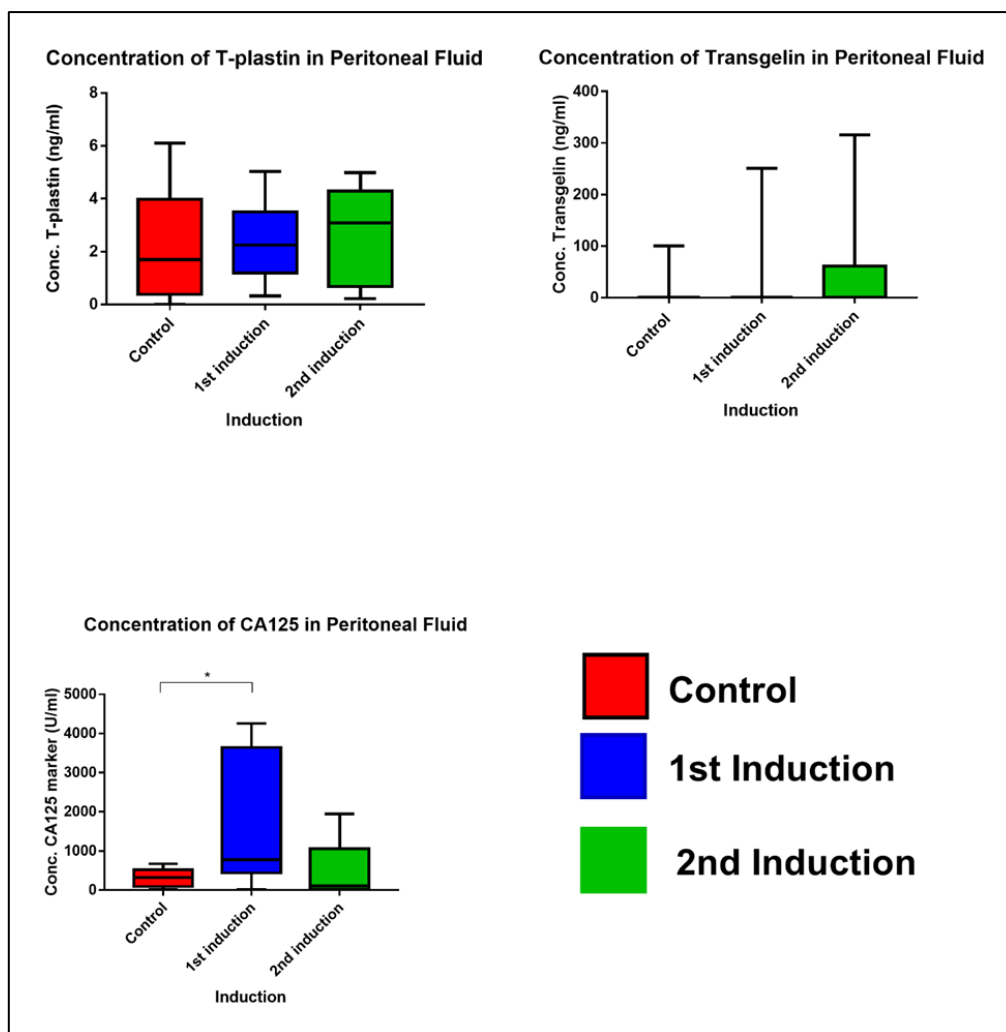### 3.1 Levels of T-plastin, Transgelin and CA125 biomarkers in serum

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 30 days after induction, however this was not significant (p=0.8633). After 30 days the levels again increased slightly, however this was not significant (p=0.9999) (Fig 1). Transgelin showed insignificant increase in concentration after first post-induction. There was slight increase in concentration after the 60th post induction, however this difference was not significant. CA-125 concentrations in plasma showed significant increase 30 days after induction (p=0.0003).There was also a significant rise in concentration of CA-125 after the 60th day post -induction (p=0.0014).
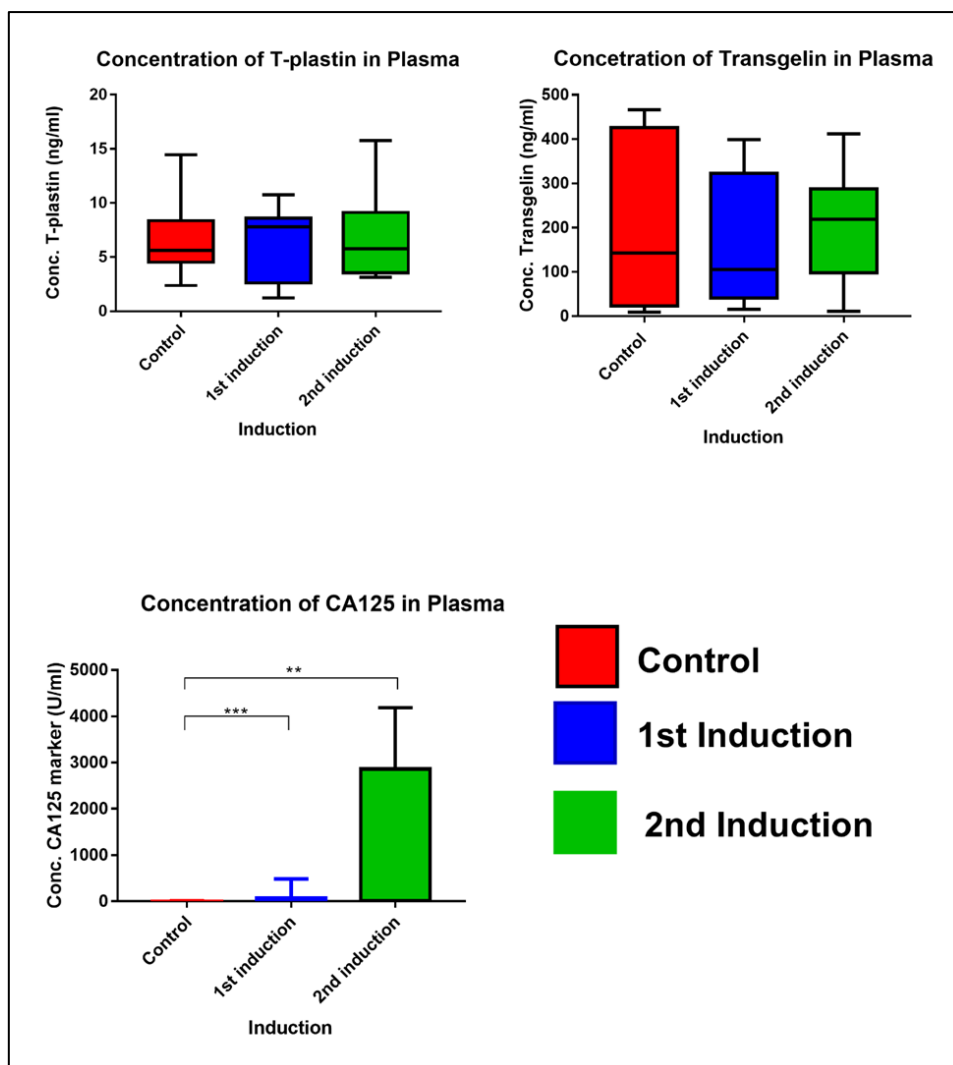
*Fig 1: Concentrations of T-plastin, Transgelin and CA125 Pre-induction, 1st post-induction and 2nd post-induction in peripheral blood in baboons induced with endometriosis.* Values were considered significant when p≤0.05 and assigned **p=0.0014, ***p=0.0003.

## 3.2    Levels of biomarkers in peritoneal fluid.

Ten baboons induced with endometriosis were evaluated for the concentrations of T-plastin, Transgelin and CA-125 in peritoneal fluid. Figure 2 shows the concentration of T-plastin observed with a slight increase between pre induction and both 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 with p=0.1192. The results also showed that CA125 concentrations were significantly higher in peritoneal fluid especially between pre induction and 1st post induction reading (p=0.0279). The concentration between pre induction and 2nd post induction however was insignificant, (p=0.9682).

*Fig 2: Concentrations of T-Plastin, Transgelin and CA-125 pre-induction, 1st and 2nd post-induction in peritoneal fluid of baboons induced with endometriosis. Values were considered significant when p≤0.05 and assigned *p=0.0279*

## 3.3    Receiver operating Curves (ROC) Analysis

Analysis determining the diagnostic performance of the biomarkers in plasma using the receiver operating characteristics (ROC) showed T-plastin to have an area under curve AUC=0.5, followed by Transgelin that had AUC=0.505. CA125 had a markedly higher area under curve (AUC=0.79), as shown in figure 3.
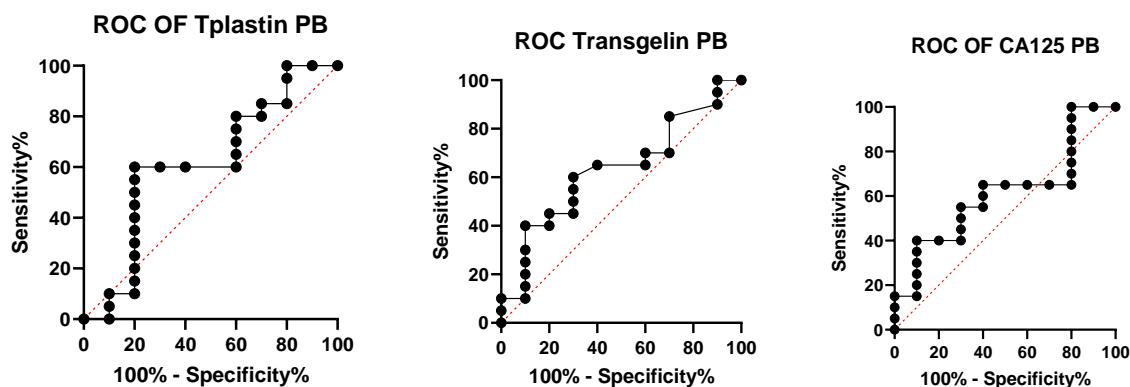
***Fig 3:*** *Receiver operating characteristics (ROC) curves of T-plastin, Transgelin and CA-125 in plasma. T-plastin-AUC=0.5 with 95% confidence interval (CI) of 0.2275-0.7727, p>0.999, Transgelin, AUC=0.505 with 95% CI 0.2455-0.764, p=0.9698, CA-125 has AUC=0.6100, 95%CI 0.395-0.8200, p value=0.3331*

The receiver operating characteristics (ROC) for concentrations of T-plastin, Transgelin and CA-125 in peritoneal fluid showed an area under curve AUC=0.6325 for T plastin, AUC=0.615 for Transgelin while CA125 had a markedly high area under curve, AUC = 0.7900, as shown in figure 4.
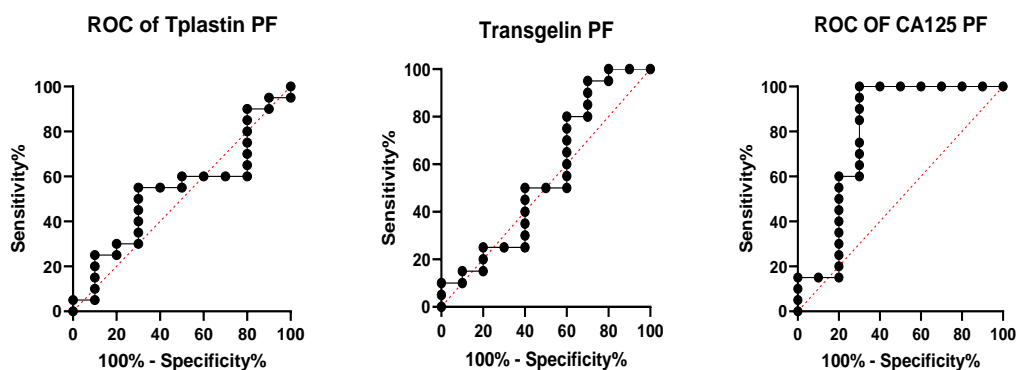


*Fig 4: Receiver operating characteristics (ROC) curves of T-plastin, Transgelin, and CA-125 in peritoneal fluid-Plastin-AUC=0.6125 with 95% CI 0.3242-0.9008, p=0.4239, Transgelin has AUC=0.685 with 95% CI 0.4422-0.9278 and a p value = 0.1620.CA125 AUC=0.7900 with 95% CI 0.5723-1.000 p=0.0107*

## 4    Discussion

The baboons developed endometriosis within the two month period after induction. Samples of blood and peritoneal fluid collected prior and post induction were analyzed for T-plastin, Transgelin and CA-125 concentrations both by ELISA and non-parametric analysis. The results indicate that concentrations of T-Plastin and Transgelin in serum were detected and measured but no significant increase was realized during the two months of study between the pre and the post induction samples. Slight increase was noted in T-plastin between pre induction and endometriosis group. Transgelin appeared to have higher concentration in serum prior to induction and only slight increase was observed 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. The area under curve (AUC) in receiver operating characteristics (ROC) that was used to determine diagnostic performance was quite low for the biomarkers (AUC=0.535, AUC=0.550) respectively indicating the two biomarkers in serum cannot be effective or suitable test for endometriosis. The endometriosis established during the two months study is likely to have been minimum-mild stages where antigens are detectable but not significantly increased. Serum CA-125 was seen to

have a significant rise (p=0.0003 and p= 0.0014) between pre induction, 1st post induction and 2nd post induction. It has been established that CA-125 increases in initial stages of endometriosis but markedly increases in moderate to severe stages (Muyldermans *et al.*, 1995; Mol *et al.*, 1998). Another study indicates CA-125 test has a specificity of 89% in detecting moderate to severe endometriosis (Child & Tan, 2001). T-Plastin which has been observed to regulate actin assembly and cellular motility was upregulated, however this has not been quantified in proteomics studies. T-plastin plays a major role in development of abnormal structures in pelvic peritoneum in endometriosis (Kyama *et al.*, 2011). However little information regarding presence of T-plastin in peripheral blood during the development of endometriosis is available.

 Transgelin which is believed to be related to cell differentiation, cell migration and involved in tissue invasion was remarkably upregulated in a study by Kyama *et al.*, 2006 and Tawfik *et al.*, 2014) 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.

CA-125 levels increase in serum in moderate to severe endometriosis, Bon *et al.*, 1995. The study indicates 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 by Giudice *et al.*, 1986.

The study of T-plastin and Transgelin concentrations in peritoneal fluid did not reveal any significant difference between the control and endometriosis. This is due to the mild –minimum stages of endometriosis in the two months it was developed. The concentration of CA-125 however was seen to have significantly increased between the pre induction and disease in the peritoneal fluid (P value< 0.005). 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.

In a study by Szubert *et al.*, 2012, serum CA-125 levels were significantly higher in endometriosis compared to controls. Peritoneal fluid CA-215 was also seen to be higher 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. Bon *et al.*, 1995 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.

The major limitation observed in this study was the animals developed endometriosis for two months only and therefore moderate to severe stages were not achieved. Different assays, such as Western blot, flow cytometry, immunohistochemistry, would be more sensitive and detect the increase in concentrations of these antigens in serum.

## 5    Conclusion

This study reveals 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 and therefore cannot be an accurate test or reliable biomarker for endometriosis.CA-125 levels increased both in serum and peritoneal fluid. However, the low diagnostic power does not allow CA-125 to be used as a reliable individual biomarker.

Further work using different protocols would be recommended to identify the tread of these antigens in the minimum to mild stages in endometriosis. Evaluation of the potential of combining the three antigens, CA-125, Transgelin and T-plastin to improve detection of endometriosis in early stages.

**Conflict of interest:** There is no conflict of interest among authors in this work

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