Study Title	A prospective, observational and multicentric study to evaluate the performance and clinical utility of EndoID ELISA Kit to determine circulating synuclein gamma (SNCG) and B-cell lymphoma-6 (BCL6) levels as potential biomarkers for endometriosis"
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SYNOPSIS

Assessment of Synuclein Gamma (SNCG) and B-Cell Lymphoma 6 (BCL-6) as Potential Non-invasive Biomarkers for Diagnosing Endometriosis

At present, the diagnosis of endometriosis can only be definitively made by laparoscopy and biopsy of endometrial lesions for histologic confirmation. Since these are invasive procedures and usually performed at the late stages, there is an urgent need to identify non-invasive biomarkers to effectively indicate endometriosis especially at the early stages for better clinical management.

The present study is aimed to assess the levels of Synuclein gamma (SNCG) and B-Cell Lymphoma 6 (BCL-6) proteins and evaluate their diagnostic potential as non-invasive biomarkers for differentiating between endometriosis, non-endometriosis, and healthy individuals.

INTRODUCTION

Background:

Endometriosis is a benign inflammatory disease defined by the presence of endometrial glands and stroma in areas outside the uterus[1]. It is estimated to affect 5%–15% of women of reproductive age [2] and is even more prevalent among women with chronic pelvic pain and infertility [3]. Endometriotic implants may be located anywhere within the pelvis, including the ovaries, pelvic peritoneum, rectovaginal septum, uterosacral ligaments, and cul-de sac. The pathophysiology of endometriosis still remains unclear. The etiologic mechanism most widely accepted is retrograde menstruation resulting in tubal backflow of endometrial cells into the pelvis during menses [4]. Over time, these cells may adhere to peritoneal surfaces, proliferate, and progressively grow. The amount, duration, and frequency of pelvic exposure to menstrual bleeding may also determine the severity of endometriosis [5]. At present, the diagnosis of endometriosis can only be definitively made by laparoscopy and biopsy of endometrial lesions for histologic confirmation. Since these are invasive procedures and usually performed at the late stages, there is an urgent need to identify non-invasive biomarkers to effectively indicate endometriosis especially at the early stages for better clinical management.

Rational:

Many genes and proteins have been proposed as biomarkers for diagnosis and follow up of endometriosis [6, 7], but none used at the present time is sensitive and specific enough either to diagnose endometriosis nor to determine the effectiveness of treatment of endometriosis.

Synuclein gamma (SNCG), a member of the synuclein family of neuronal proteins, is involved in cellular proliferation by interacting with the mitotic checkpoint kinase BubR1, and inhibiting its regulatory role on cellular proliferation [8]. During the last decade, several studies have demonstrated that SNCG is correlated with poor outcome of breast cancer, ovarian cancer, colon cancer and pancreatic cancer. Interestingly, SNCG has previously been shown to have elevated expression ranging from 5- to 53-fold in ovarian endothelial cells of endometriosis lesions compared with eutopic endometrium from the same individual [9]. Similarly, B-Cell Lymphoma 6 (BCL6) protein is encoded by the BCL6 oncogene and is a zinc finger transcription factor that regulates the transcription of signal transducer and activators of transcription (STAT)-dependent IL-4 responses of B-cells [10]. Previously, BCL6 was shown to express in the endometrial epithelial cells and B-cells in lymph node germinal centres [11,12]. In addition, over-expression of BCL6 has been reported in endometrial biopsies and has been linked to the progesterone resistance and pathogenesis of endometriosis [13]. Together, both of these proteins (SNCG and BCL6) have been linked to endometriosis. However, their diagnostic/prognostic utility by non-invasive means such as measurement in peripheral blood and menstrual fluid of patients with endometriosis remains to be investigated.

Hypothesis:

We hypothesize that SNCG and BCL6 concentrations are altered in patients with endometriosis and measurements of circulating SNCG and BCL6 levels in serum/menstrual blood could be used for diagnosis and/or prognosis of endometriosis.

OBJECTIVES

Objectives:

The aim of this study is to measure the SNCG and BCL6 concentrations in peripheral and menstrual fluid from endometriosis patients compared to age-matched healthy subjects or in patients after the treatment of endometriosis. We have the following specific objectives:

1. Identification of endometriosis and control groups and sample collection: Eligible patients with active endometriosis (n=100) confirmed through standard clinical diagnosis will be recruited along with age matched healthy control subjects (n=50). Peripheral blood and menstrual fluid samples will be collected from both of these groups. In addition, the endometriosis group will be followed post treatment (either medically or surgically) and their blood and menstrual fluids samples will be collected at the 3rd and 6th month safter treatment. These collected samples will be processed for serum/supernatant isolation and stored at -80 C until further analysis.

2. Analysis of circulatory SNCG and BCL6 levels using ELISA method: SNCG and BCL6 levels in serum/menstrual supernatant will be quantitated using specific ELISA kits and will be compared for significant differences in patients with endometriosis from control subjects as well as their changes before and after the treatment in endometriosis group.

Expected Outcome and significance:

We expect that SNCG and BCL6 concentrations in patients with endometriosis would be significantly higher compared to age-matched healthy subjects. In addition, we expect that SNCG and BCL6 concentrations may also show differences in samples of endometriosis patients treated with suppression medication or after surgery. This study aims to validate their diagnostic/prognostic potential of SNCG and BCL6 concentrations as non-invasive biomarkers for endometriosis.

STUDY DESIGN

This study will focus on measuring circulatory SNCG and BCL6 concentrations in patients with endometriosis compared to age-matched healthy subjects without endometriosis. Patients with confirmed endometriosis, and the healthy control group will be recruited at TRIO Fertility following informed consent. Peripheral blood samples and menstrual samples will be collected from both groups and serum/supernatant from these samples will be used for measuring the baseline differences of SNCG and BCL6 levels. In addition, post-treatment changes in these proteins will also be measured in samples collected from endometriosis patients at the 3rd and 6th month following endometriosis suppression with dienogest, Lupron depot and addback, or Myfembree, or after laparoscopic surgery with excision or vaporization of endometriosis lesions. Analysis of circulatory SNCG/BCL6 levels will be performed using SNCG and BCL6 specific enzyme linked immunosorbent assays (ELISA), respectively, and will be compared for significant differences between the endometriosis and the control group, and pre and post treatment in endometriosis group.

Methodology for Objective 1: Sample Size: To demonstrate a difference between the control group (estimated 5% positive SNCG/BCL6) and the study group (estimated 50% positive SNCG/BCL6), with an alpha error of 5% and beta error of 90%, the required sample size would be 75. After considering cases lost to follow-up and other technical problems, we estimate that the appropriate sample size will be 50 endometriosis and 50 controls (total 100 participants). Sample size was estimated using sample size and power analysis software (PASS-16).

Recruitment: Subjects will be identified by the investigators and eligible patients with the following inclusion criteria will be informed of the purpose of the study and what study participation will involve. Subjects who express initial interest will be provided with detailed verbal and written information (Consent form). The following criteria will be used for recruiting the patients.

1. **Criteria for endometriosis group (N=50):**

Inclusion Criteria:

- Women of reproductive age group (18Y-45Y).

- Women with active endometriosis diagnosed by ultrasound/MRI/laparoscopy.

Exclusion Criteria :

- Women <18Y and >45Y.
- Women with endometriosis during stimulation with gonadotropins.
- Current or prior history of uterine, ovarian, colon, pancreatic, breast or any other type of cancer.
- Any prior history of chronic illness.
- Pregnant women.

2. Criteria for control group (N=50):

Inclusion Criteria:

- Women of reproductive age group (18Y-45Y).
- Women without endometriosis and any other gynaecological problem.

Exclusion Criteria

- Women <18Y and >45Y
- Prior history of any disease including cancer or chronic illness.
- Pregnant women.

SPECIMEN COLLECTION:

Details of sample collection: Peripheral blood (10 mL) will be drawn by a research assistant into a plain blood collection vial (VACUTAINER), allowed to clot and centrifuged to separate serum that will be stored in 1 ml aliquots at -80 degrees. For menstrual blood, a sample cup (DIVA reusable menstrual cup) will be provided and the subjects will be requested to collect an aliquot (approx. 5ml) of menstrual blood (at 1st or 2nd day of the cycle) at their home/during visit to TRIO. If collected at home, they will be requested to inform us at the earliest and sample will be picked up by a designated personal from their home on the same day (for local cases only). Following treatment in the endometriosis group, peripheral blood samples and menstrual blood will be collected during their visit at 3 and 6 months post treatment. Samples of both peripheral and menstrual blood will be processed by clotting at room temperature for 15-30 min followed by centrifugation at 1,000-2,000 x g for 10 minutes in a refrigerated centrifuge for serum/supernatant separation. Samples will be stored in 1 ml aliquots at -80 degrees till batch analysis of SNCG/BCL6 proteins by ELISA.

Methodology for objective 2:

PRODUCT DESCRIPTION

EndoID ELISA kit is an Enzyme-linked immunosorbent assay (ELISA) is used to analyze the supernatants of serum and menstrual fluid samples from both groups to measure SNCG and BCL6 levels using a Human SNCG Pre-Coated ELISA Kit and Human BCL6 Pre-Coated ELISA Kit and following the protocol provided in the kit.

Briefly, this sandwich ELISA employs an antibody specific for Anti-human -BCL6/-SNCG that is coated on a 96-well plate. Pure standards (25ng/ml to 0.195 ng/ml for BCL6, and 25 ng/ml to 0.39 ng/ml for SNCG) or equal volumes of test samples (i.e. undiluted, 1:2 or 1:10 dilutions) were pipetted into the wells separately and incubated at 37◦C for 60 min in triplicate. Upon binding of the respective BCL6 or SNCGs in the sample to the immobilized antibody, the wells were washed and incubated further with anti-human-BCL6/-SNCG antibody for 60 min at 37◦C. Upon completion of incubation, the wells were washed again to drain the primary antibody and detection antibody. An HRP conjugate was added to each well and incubated for the next 60 min at 37◦C. The wells were again washed and incubated with conjugate working solution for 60 min at 37◦C. Finally, after washing, a TMB substrate solution was added to the wells and allowed to develop a blue color in the next 5 min. Subsequently, a stop solution was added that changed the color from blue to yellow, and the intensity of the color was measured at 450 nm using an ELISA plate reader (BioTeK Instrument Inc.). The absorbance values of the samples are extrapolated using standard curves, and the concentrations of BCL6 and SNCG will be calculated and plotted. Each measurement will be repeated independently at least 2-3 times.

TEST PROCEDURES

TEST PROTOCOL FOR ENDOID ELISA KIT (DUAL BIOMARKER – SNCG & BCL6)

(A) REAGENT PREPARATION

(1) Preparation of 1 X Wash buffer: Prepare 1 X Wash buffer by diluting 20X wash buffer in distilled water. Diluted wash buffer may be stored at 4°C, however we recommend preparing fresh 1X wash buffer for each experiment.

(Example: add 10 ml of 20X Wash buffer in 190 ml of sterile water).

(2) Preparation of Sample Diluent Buffer:

(i) BCL-6: 2% BSA, 0.1% proclin in 1X TBST is used as the sample diluent buffer**.**

Example: 0.4gm of BSA, 20µl of proclin to be used for 20ml of 1X TBST.

(ii) SNCG: 0.1% PVP,0.1% gelatin,0.1% proclin in 1X TBST which is provided in the kit is used as sample diluent buffer.

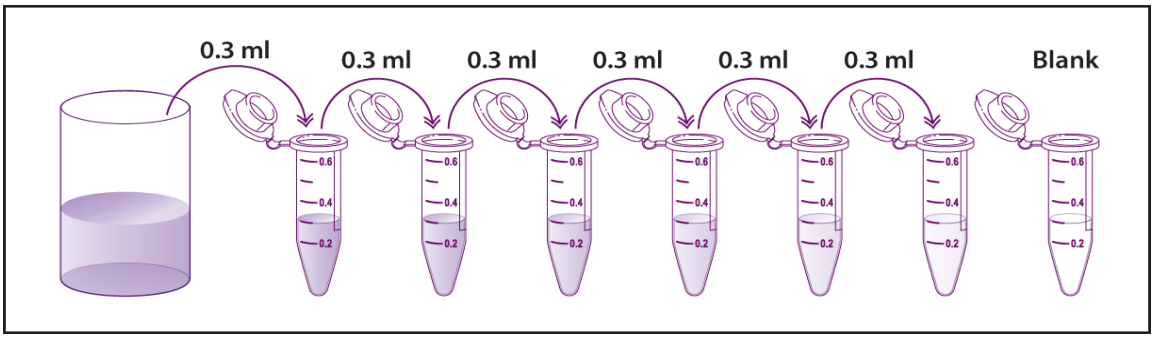
Example: 20mg of PVP, 20mg of gelatin, 20µl of proclin to be used for 20ml of 1X TBST.

(3) Reconstitution of the lyophilized Standard

Prepare the standard within 30 minutes before the start of the experiment. Use one tube for each experiment.

a. Quick spin down one vial of lyophilized standard ***(Do not dilute standard directly on the plate)***. Add 1ml of sample diluent buffer into one of the standard tube. Incubate at room temperature for 10 minutes. Mix thoroughly by continuous pipetting in the tube. Stock Standard concentration is 10ng/well. This solution can be stored in -20^0 C for one month.

b. Label 6 Eppendorf tubes with 5 ng/well, 2.5ng/well, 1.25 ng/well,0.62ng/well, 0.31ng/well, 0.15ng/well respectively. Add 0.3 ml of sample diluent buffer into each tube. Add 0.3 ml of stock standard (10ng/well) into 1st tube and mix thoroughly by continuous pipetting. Transfer 0.3 ml from 1st tube to 2nd tube and mix thoroughly by continuous pipetting. Transfer 0.3 ml from 2nd tube to 3rd tube mix thoroughly by continuous pipetting, and so on. Discard the extra 0.3ml of solution after mixing it in the final tube.



(4) Sample Preparation and storage: Test samples should be collected, analyze immediately or aliquot and store at -80°C for long term. Avoid multiple freeze-thaw cycles.

(5) Preparation of detection antibody working solution: Prepare within 30 minutes before the addition of detection antibody to the plate. Calculate total volume of working solution required. (100µl/well × number of wells).

Dilute detection antibody with sample diluent buffer at 1:100 and mix thoroughly by vortex.

(*Example:* add 1µl of detection antibody into 99 µl of sample diluent).

(6) Preparation of HRP-Conjugate working solution: Prepare within 30 minutes before the addition of HRP-Conjugate solution to the plate. Calculate total volume of working solution required. (100µl/well × number of wells).

Dilute HRP-Conjugate with sample diluent buffer at 1:100 and mix thoroughly by vortex.

(Example: add 1µl of detection antibody into 99 µl of sample diluent).

(7) Preparation of 1X TMB Substrate: Prepare 1X TMB substrate by diluting 20X TMB substrate in substrate diluent (1:20). Calculate total volume of working solution required

(100µl/well × number of wells). The 1X TMB substrate working solution should be prepared 10 minutes before adding to the wells.

(Example: add 10µl of 20X TMB substrate into 190 µl of substrate diluent).

(B) ASSAY PROCEDURE

(1) Wash plate once with 30 seconds incubation at room temperature and then tap the plate firmly on an adsorbent paper before adding standard, sample and blank into the well.

(2) Mark Standards, test sample and blank (control zero) wells on the pre-coated plate and then record their position. It is recommended to measure each standard and sample in duplicate. Add 0.1 ml of standard (10ng/well, 5 ng/well, 2.5ng/well, 1.25 ng/well, 0.62ng/well, 0.31ng/well, and 0.15ng/well) and test samples into respective wells. The sample diluent alone serves as the zero standards (0 ng/well).

(3) Add 0.1 ml of neat sera samples into test sample wells in duplicate. Cover the plate and incubate at room temperature for 1 hour.

(4) Remove the cover and discard samples and standard solution and tap the plate once firmly on an adsorbent paper to remove any remaining solution. Wash the plate 5 times with 280µl of 1X wash buffer in each well for each wash with 30 seconds of incubation at room temperature in between each wash before discarding the wash buffer. Tap the plate firmly on an adsorbent paper once in between each wash after discarding the wash buffer. After the final wash tap the plate firmly for 4-5 times on an adsorbent paper.

Note: *Do not let the wells completely dry any time.*

(5) Add 100µl of detection antibody working solution into the above wells (Standards, control and test samples).

(6) Cover the plate and incubate at room temperature on a shaker for 1 hour.

(7) Remove the cover and discard the solution in the wells, tap the plate once firmly on an adsorbent paper to remove any remaining solution. Repeat the washing step as described above.

(8) Add 100µl of conjugate working solution into the above wells (Standards, control and test samples).

(9) Seal plate with a cover and incubate at room temperature on a shaker for 1 hour.

(10) Remove the cover and discard the solution in the wells, tap the plate once firmly on an adsorbent paper to remove any remaining solution. Repeat the same washing step as described above.

(11) Add 100 µl of 1X TMB substrate into each well, cover the plate and incubate at room temperature in dark for 7 minutes. *(**Note**: This incubation time is for reference use only. The optimal time should be determined by end user)*. The shades of blue can be seen in the first 3-4 wells, only on most concentrated standards. Other wells show no obvious color.

(12) After 7 minutes add 50 µl of stop solution into each well and mix thoroughly. Color will change into yellow immediately.

(13) Read O.D. absorbance at 450 nm in a micro-plate reader immediately after adding the stop solution.

	Standard (Bcl-6) 1	Standard (Bcl-6) 2	3	4	5	6	Standard (SNCG) 7	Standard (SNCG) 8	9	10	11	12
A	**10ng/well**	**10ng/well**					**10ng/well**	**10ng/well**				
B	**5ng/well**	**5ng/well**					**5ng/well**	**5ng/well**				
C	**2.5ng/well**	**2.5ng/well**					**2.5ng/well**	**2.5ng/well**				
D	**1.25ng/well**	**1.25ng/well**					**1.25ng/well**	**1.25ng/well**				
E	**0.62ng/well**	**0.62ng/well**					**0.62ng/well**	**0.62ng/well**				
F	**0.31ng/well**	**0.31ng/well**					**0.31ng/well**	**0.31ng/well**				
G	**0.15ng/well**	**0.15ng/well**					**0.15ng/well**	**0.15ng/well**				
H	**0ng/well**	**0ng/well**					**0ng/well**	**0ng/well**				

DATA REPORTING

Report Format : Control Group

Sample ID	Age	BCL6 (Serum)		SNCG (Serum)		BCL6 (M.F.)		SNCG (M.F.)	
		Conc.1 (ng/ml)	Conc.2 (ng/ml)	Conc. (ng/ml)	Conc. (ng/ml)	Conc. (ng/ml)	Conc. (ng/ml)	Conc. (ng/ml)	Conc. (ng/ml)
CNT-1									
CNT-2									

Report Format : Active Group (Endometriosis)

Sample ID	Age	BCL6 (Serum)		SNCG (Serum)		BCL6 (M.F.)		SNCG (M.F.)	
		Conc.1 (ng/ml)	Conc.2 (ng/ml)	Conc. (ng/ml)	Conc. (ng/ml)	Conc. (ng/ml)	Conc. (ng/ml)	Conc. (ng/ml)	Conc. (ng/ml)
ENDOMT-1									
ENDOMT-2									

Summary and Conclusion

STATISTICAL ANALYSIS

Data will be compiled and presented in graphical forms using Prism Version 8 (Graph Pad,). Normality tests, outlier removal and univariate analyses will be 3 performed using SPSS (version 20) software. Student's t-test and Mann-Whitney U test will be used for group comparisons. The significance level will be set at $\alpha=0.05$ for all comparisons.

STUDY ADMINISTRATION AND RECORD RETENTION

The Study will be supervised by Dr. R. Casper, ("Principal Investigator") with assistance from a research assistant (Yasaman Sadeghi) and other colleagues at TRIO as required

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