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Does telomerase activity have an effect on infertility in patients with endometriosis?

Running headline: Telomerase activity in endometriosis

Nigar Sofiyeva, MD, MSc candidate[1,3], Seda Ekizoglu, Ph.D.[2], Altay Gezer, MD[1], Handan Yilmaz, MD[1,4], Tugba Kolomuc Gayretli, MD[1], Nur Buyru, Ph.D.[2], Engin Oral, MD[1]

[1] Department of Obstetrics and Gynecology, Istanbul University, Cerrahpasa Medical Faculty, Istanbul, Turkey

[2] Department of Medical Biology, Istanbul University, Cerrahpasa Medical Faculty, Istanbul, Turkey

[3] Department of Obstetrics, Gynecology and Reproductive Sciences, Yale University, School of Medicine, New Haven, Connecticut, USA

[4] Department of Obstetrics and Gynecology, Hakkari State Hospital, Hakkari, Turkey

Corresponding author: Nigar Sofiyeva, MD, MSc candidate

Address: Department of Obstetrics, Gynecology, and Reproductive Sciences, Yale School of Medicine, 310 Cedar Street, New Haven, CT, 06510, USA

Telephone: +12036458589

e-mail: nigarsofiyeva@mail.com

Abstract

Objective:

This study aimed to investigate the role of telomerase activity in the development of endometriosis-related infertility by evaluation of the serum telomerase in eutopic and ectopic endometrial tissue.

Study Design:

Eutopic endometrium, cystic wall/ovarian cortex, and venous blood were assessed in forty-seven patients. The following groups of patients were identified: females with endometriosis requiring surgical intervention and healthy control females. Patients with histopathologically confirmed endometriosis were further subdivided in the infertile (n=14) and fertile (n=17) groups. Patients who underwent hysterectomy and oophorectomy for benign gynecological conditions were enrolled in the healthy control group (n=16). Telomerase activity was evaluated with three-group, endometriosis-based and fertility-based designs. Analyses were performed regardless the menstrual cycle phase (Phase G), in proliferative (Phase P) (n=22) and secretory phases (Phase S) (n=25).

Telomeric Repeat Amplification Protocol PCR was applied for telomerase activity assessment.

All statistical analyses were performed with STATA 14.2, GraphPad Prisma 7.01.

Results:

In analyses of the eutopic endometrium, with three-group design, a significant difference was not found in Phase G and P (p=0.58 and p=0.33, respectively). However, a statistical difference was shown in Phase S (p=0.008). A significant difference was not established in Phase G, P and S of endometriosis-based design (p=0.35, p=1.0, p=0.13, respectively). No difference was detected in Phase G and P of fertility-based design (p=0.66 and p=0.14, respectively), whereas in secretory phase difference was approved (p=0,049).

Telomerase activity was not established in ectopic endometrium and in serum assessment.

Conclusions:

Telomerase activity is useless as a biomarker in peripheric blood analysis. The absence of activity in cystic wall approves the high differentiation of endometriosis tissue, what is the possible reason of low malignancy risk. The high rate of telomerase activity in the eutopic endometrium of the infertile group may be considered as a cause of endometriosis-related infertility.

Keywords: Endometriosis, infertility, telomerase activity

Manuscript

Introduction:

Endometriosis is a presence of extrauterine endometrial glandular and stromal cells leading to the chronic, inflammatory reaction (1). About 25-50% of the cases of female infertility is due to endometriosis, whereas only 35-50% of the women with endometriosis are infertile (2, 3). Despite a vast amount of the published studies, the actual cause of endometriosis-related infertility still remains a controversy.

Lack of the 3'end replication in most of the somatic eukaryotic cells causes progressive DNA shortening with each replication (4). A telomere is a repeated short nucleotide sequence (GGGTTA) located at the end of the chromosome. The linear structure of chromosome is maintained by this nucleoprotein cap, what prevents an end-to-end fusion and rearrangement (5, 6). Telomerase is an RNA-dependent DNA polymerase that synthesizes telomeric DNA sequences, and, thereby, prevents the chromosomal loss.

Telomerase activity is very low or undetectable in the majority of the somatic cells (7). Endometrial cells are unique with cyclic telomerase activity which occurs in response to sex steroids (8, 9). Telomerase activity was found

to be enhanced in eutopic endometrial tissue either in proliferative or secretory phases in patients with endometriosis compared to healthy females (7, 10).

This study aimed to investigate the role of telomerase activity in the development of endometriosis-related infertility by evaluation of the serum, eutopic and ectopic endometrial tissue.

Materials and methods:

This study was approved by Ethical Committee of Istanbul University, informed consent was obtained from participants.

A case-control study was conducted between July 2014-March 2016. From a total 94 initially enrolled women, forty-seven patients were eligible for the final analyses. Most of them (38/94, 34.7%) were excluded due to the lack of eutopic endometrial tissue and 11.7% of participants were ineligible based on the histopathological assessment (11/94) (Fig 1).

The following groups of patients were identified: females with endometriosis requiring surgical intervention (31 patients) and healthy females (16 patients). Patients with histopathologically confirmed endometriosis were further subdivided in the Group IE (infertile endometriosis, n=14) and Group FE (fertile endometriosis, n=17). Patients who underwent hysterectomy and oophorectomy for benign gynecological conditions were enrolled in the Group HC (healthy control, n=16). Besides that, analyses were performed by menstrual cycle phases in each group and a number of patients were approximately equal in proliferative (n=22) and secretory phases (n=25).

Infertility was defined according to the guidelines set forth by the American Society of Reproductive Medicine Committee Opinion (11). The patient was classified as fertile if she has a history of at least one spontaneous pregnancy. Patients were eligible for the study if there was a histopathological confirmation of endometrioma in the study group, or a histopathological exclusion of endometriosis, adenomyosis in the control group after exclusion of the malignancy in both groups. Exclusion criteria were virginity of the patients and refusal for endometrial sampling.

In endometriosis group, ectopic endometrial tissue was obtained as a 2-cm line from the specimen immediately after cystectomy. Eutopic endometrium was sampled with vacuum curettage using Pipelle cannula (Pipelle de cornier®, Prodimed, France) at the same session with cystectomy. In control group, ovarian cortex and endometrial tissue were obtained from specimen immediately after hysterectomy and oophorectomy. Fresh tissue

samples were frozen properly at -80ºC. Peripheral venous blood samples were collected into ethylenediaminetetraacetic acid-treated vacutainer tubes (Greine Bio-One, Kremsmünster, Austria) immediately after venous catheterization.

Telomerase activity assay: Telomerase activity was determined using the TeloTAGGG Telomerase PCR ELISA Kit (Roche Diagnostics, Mannheim, Germany). For the protein extraction, frozen tissue samples were homogenized in 200µl ice-cold lysis buffer provided in the kit and incubated on ice for 30 min. The lysate was then centrifuged at 16000g for 20 min at 4°C and the supernatant was collected.

For the isolation of serum from whole blood, the blood was left undisturbed at room temperature for 30 min after sampling. The clot was then removed by centrifuging at 2000g for 10 min.

The protein concentrations were measured using the DC Protein Assay (Bio-Rad, CA, USA).

TRAP (Telomeric Repeat Amplification Protocol) Reaction: TRAP reaction was performed in a final volume of 50µl containing 25µl reaction mixture provided in the kit, 50µg of total protein and sterile water using the GeneAmp PCR System 9700 (Applied Biosystems, CA, USA). The TRAP reaction condition included a primer elongation step of 30 min at 25°C, a telomerase inactivation step of 5 min at 94°C, 30 cycles of amplification at 94°C for 30 s, 50°C for 30 s, 72°C for 90 s and a final step at 72°C for 10 min.

Detection by ELISA: Hybridization and detection steps were performed according to the manufacturer's instructions. The absorbance of each sample was measured at 450 nm (with a reference wavelength of 690 nm) using the Multiskan EX ELISA microplate photometer (Thermo Scientific, MA, USA). Negative control prepared with heat-treatment of the cell extract for 10 min at 85°C prior to the TRAP reaction was used for the interpretation of the results. The absorbance of the negative control was subtracted from those of the samples. Samples were regarded as telomerase-positive if the difference in absorbance (ΔA) was higher than 0.2.

Statistical analysis: Telomerase activity was assessed with three designs: Design A – a three-group method (IE vs. FE vs. HC), Design B – an endometriosis-based method (Group EP (endometriotic patients) vs. HC) and Design C – a fertility-based method (IE vs. FE). In addition to these designs, analyses were performed based on menstrual cycle phases: regardless the phase (Phase G), in proliferative (Phase P) and secretory phases (Phase S).

The sample distribution was assessed with Shapiro-Wilk test and the central tendency was reported in mean with standard deviation or median with interquartile range. Telomerase activity between the groups was assessed with Fisher's Exact test. A two-sided unpaired t-test, one-way ANOVA, Mann-Whitney U, Fischer's exact test were applied to compare demographic and clinical data. Multiple comparisons were evaluated with Bonferroni correlation and Dunn's test when it was suitable. Logistic regression was used to adjust for covariates and reported with odds ratios (OR) with 95% confidence interval. A p value less than 0.05 was accepted as a statistical significance.

Statistical analyses were performed with STATA 14.2 (StataCorp LP, Texas, USA) and GraphPad Prisma7.01 (GraphPad Software, California, USA).

Results:

Telomerase activity was assessed as a primary outcome. In a comparison of all three groups in Phase G and P significant difference was not detected (*p=0.58* and *p=0.32*, respectively), however, in Phase S the difference was established (*p=0.008*). In endometriosis-based comparison (EP vs. HC) a significant difference was not found in any of the phases (*p=1.0, p=0.13* and *p= 0.35*, respectively). In assessment with fertility-based method (IE vs. FE) difference was not detected in Phase G and P (*p=0.66 and p=0.14*, respectively), whereas in secretory phase marginally significant difference was established (*p=0,049*) (Table 1 and Fig 2).

In a comparison of demographic parameters, age and body mass index (BMI) was found different between groups (*p<0.001 and p=0.003*, respectively). In the assessment of laboratory findings, a significant difference was revealed between groups' CA-125, CA 19-9 values (*p<0.001 and p=0.003*, respectively) (Table 2).

In post hoc analysis with Bonferroni correction, BMI of the Group IE was established statistically lower than Group FE and HC (*p=0.01* and *p=0.006*, respectively). Dunn's multiple comparisons established CA 125 of Group HC lower than Group IE and Group FE (*p=0.001* and p<*0.001*, respectively) and CA 19-9 in Group HC was established lower than Group IE (*p=0.001*) (Table 3).

Fertility criterion gave rise to the age difference between the Groups IE and FE. Hysterectomy and oophorectomy indication for the control group was the main reason of high age in group HC. Logistic regression was applied to adjust for the age difference. No significant difference was found in study groups after adjustment (Group IE *p=0.43*, Group FE *p=0.65*, and Group HC *p=0.97)* (Table 4).

Comment:

Certain molecular features such as angiogenesis, tissue invasion, and metastasis make endometriosis a borderline benign condition with malignant behavior (12, 13). Etiology of endometriosis-related infertility remains debatable despite the proposed theories as tubal obstruction, distorted pelvic anatomy with abnormalities in utero tubal transport, impaired hormonal and cellular, endocrinological and ovulatory functions, impaired implantation, oocyte and embryo quality (14).

Our hypothesis stems from the fact that endometriosis originates mainly from the endometrial epithelial cells, which has a unique cyclic telomerase activity. It raises the possibility of the association between telomerase activity and endometriosis. There is limited scientific evidence about this aspect of endometriosis. It varies based on the study design (animal vs. human model (15)), phase of menstrual cycle (10, 16-18), comparison of endometriosis vs. healthy groups (17, 18), infertile (16), infertile vs. fertile groups (13), with (13, 16, 19, 20) or without ectopic endometriosis assessment (10, 17, 18) (Appendix 1). In our study, we focused on telomerase activity of eutopic, ectopic endometrium and serum of infertile, fertile endometriosis, and healthy controls.

This study comes with the inherent limitations of the small sample size due to the refusal of consent for endometrial sampling particularly among virgin patients and infertile females who had concerns for the endometrial invasive procedure which were the 80% (38/47) of analyzed patients. Furthermore, in order to comply with ethical standards, we enrolled patients who underwent hysterectomy and salpingo-oophorectomy for benign gynecological conditions in the control group. This fact led to an apparently anticipated difference between groups' age. A previous study by Kyo et al. (7) comparing telomerase activity in eutopic endometrium of pre- and postmenopausal patients found an activity even in postmenopausal patients. Therefore, we did not expect a statistically meaningful impact of the age on telomerase activity. This assumption was statistically confirmed after adjustment by logistical regression, where we found that age does not affect telomerase activity.

The lower BMI was observed among infertile patients with endometriosis, which was an anticipated difference. According to the large prospective cohort conducted among more than 100,000 female nurses, endometriosis presents 39% less in patients with BMI> 40kg/m^2. Also, an additional analysis in the same population

stratified by fertility status demonstrated a 55% lower risk in obese infertile women with a BMI>35kg/m^2 (21). Our results were similar to the one reports in this trial with low BMI in infertile endometriosis group women.

Hapanagama et al. (17) assessed the telomerase activity in the eutopic endometrium and found a high activity in endometriosis group as compared to the healthy controls. To address this issue, we also compared endometriosis patients regardless of the fertility status and healthy controls. The phase of the menstrual cycle had no significant implications (Fig 2, Design B, Phase G, P, and S).

Telomerase activity fluctuates with the menstrual cycle phases as a result of estrogen-progesterone changes (8, 9). Kim et al. (10) investigated the telomerase activity in different phases of the menstrual cycle in the eutopic endometrial tissue of endometriosis patients and healthy controls. The highest activity was shown in the late proliferative and early secretory phases of the cycle. Zafrakas et al. (13) detected the increased activity in endometriosis group's eutopic endometrium tissue investigated in the proliferative phase. Based on these results, we evaluated telomerase activity in regards to different menstrual phases. Similar to the previously published studies, in our trial, 60.3% of activity detected patients were in the proliferative phase. As a result of comparison of patients in proliferative phase, there was no a significant difference in telomerase activity between groups in all three designs (Fig 2. Design A/Phase P, Design B/Phase P, Design C/Phase P).

Hapangama et al. (17) showed a high telomerase activity in mid- and late luteal phases of endometriosis patients comparing to the healthy women. Kim et al. (10) established significantly higher activity in secretory phase of endometriosis group; moreover, telomerase activity in nine patients from both groups evaluated in luteal phase was undetectable. According to the results of Mafra et al. (16), high telomerase activity was reported in secretory phase patients with infertile endometriosis compared to the fertile control women. Our results were in congruence with the previously reported data. In other words, telomerase activity was established in none of the patients in secretory phase of the control group, whereas in endometriosis group, half of activity detected participants were in the secretory phase. Significant difference was observed in the secretory phase of three-group design, while the null hypothesis failed to reject in endometriosis-based design (Fig 2.Design A/Phase S and Design B/Phase S, respectively). In fertility-based design, our results showed marginal significance in secretory phase (Design C/Phase S), however, this is in line with

previous findings reported in the literature (13), and we speculate that it would be validated with the larger sample size.

Mafra et al. (16) assessed the ectopic endometrial tissue obtained from peritoneal lesions of infertile endometriosis patients. According to their results, telomerase activity was not detected in ectopic endometrial cells in all but one patient. According to the study of Zafrakas et al. (13) in which ovarian ectopic endometrium was assessed, no activity was reported in endometriosis tissue. In our study telomerase activity was not observed either in ectopic endometrial samples obtained from the cystic wall of endometriosis group or correspondingly in ovarian tissue of healthy control group. Considering the highly-differentiated feature of endometriosis, the absence of telomerase activity confirmes the loss of stem cell properties in these cells. This finding supports the hypothesis of minimal or no malignancy risk of endometrioma (13).

The literature on plasma telomerase activity leads to the different conclusions. According to Chen et al. (22) results, telomerase activity was observed in 28% of plasma samples obtained from breast cancer patients, whereas about 94% of patients had it in tumor samples. El-Mazny et al. (23) evaluated the serum telomerase activity as a tumor marker for hepatocellular carcinoma and they established 77.14% sensitivity, 100% specificity of telomerase activity as an oncomarker. Based on this review, we aimed to evaluate of telomerase activity in peripheric venous blood serum as a potential biomarker for endometriosis. However, telomerase activity was not detected in any of the patients in our study. This fact should be explained with the benign character of endometriosis and undetectably low levels of endometriotic cells in peripheric blood in comparison to malignant conditions.

Assessment of telomerase activity in ectopic, eutopic endometrial tissues and serum in patients with endometriosis and/or infertility presence in different menstrual phases increases the strengths of our study. Small sample size was the main limitation of the trial and with a large number of participants, the power and significance might be increased.

Overall, for eutopic endometrial telomerase activity, the significant difference was revealed only in secretory phase. The difference founded in a three-group design was reassessed with the endometriosis-based method which demonstrated no significant difference in the telomerase activity between the Group EP and HC. Whereas in the

evaluation of fertility-based groups significant difference was approved. Based on the previous studies, telomerase activity reaches undetectably low levels in secretory phase in healthy women and activity detected in secretory phase is associated with the presence of endometriosis (10). Hence, we may conclude that high telomerase activity in infertile endometriosis patients may be a cause for endometriosis-related infertility that is independent of age.

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Conflict of Interests Statement

Conflict of Interest, financial interest, and disclosures for Nigar Sofiyeva - None

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Conflict of Interest, financial interest, and disclosures for Nur Buyru – None

Conflict of Interest, financial interest, and disclosures for Engin Oral – None

References:

1. Kennedy S, Bergqvist A, Chapron C, D'Hooghe T, Dunselman G, Greb R, et al. ESHRE guideline for the diagnosis and treatment of endometriosis. Human reproduction. 2005;20(10):2698-704.
2. Giudice LC, Kao LC. Endometriosis. Lancet (London, England). 2004;364(9447):1789-99.
3. Bulletti C, Coccia ME, Battistoni S, Borini A. Endometriosis and infertility. Journal of assisted reproduction and genetics. 2010;27(8):441-7.
4. Olovnikov AM. Telomeres, telomerase, and aging: origin of the theory. Experimental gerontology. 1996;31(4):443-8.
5. Blackburn EH. Switching and signaling at the telomere. Cell. 2001;106(6):661-73.
6. Cong YS, Wright WE, Shay JW. Human telomerase and its regulation. Microbiology and molecular biology reviews : MMBR. 2002;66(3):407-25, table of contents.
7. Kyo S, Takakura M, Kohama T, Inoue M. Telomerase activity in human endometrium. Cancer research. 1997;57(4):610-4.
8. Tanaka M, Kyo S, Takakura M, Kanaya T, Sagawa T, Yamashita K, et al. Expression of telomerase activity in human endometrium is localized to epithelial glandular cells and regulated in a menstrual phase-dependent manner correlated with cell proliferation. The American journal of pathology. 1998;153(6):1985-91.
9. Williams CD, Boggess JF, LaMarque LR, Meyer WR, Murray MJ, Fritz MA, et al. A prospective, randomized study of endometrial telomerase during the menstrual cycle. The Journal of clinical endocrinology and metabolism. 2001;86(8):3912-7.
10. Kim CM, Oh YJ, Cho SH, Chung DJ, Hwang JY, Park KH, et al. Increased telomerase activity and human telomerase reverse transcriptase mRNA expression in the endometrium of patients with endometriosis. Human reproduction (Oxford, England). 2007;22(3):843-9.
11. Definitions of infertility and recurrent pregnancy loss: a committee opinion. Fertility and sterility. 2013;99(1):63.
12. Varma R, Rollason T, Gupta JK, Maher ER. Endometriosis and the neoplastic process. Reproduction (Cambridge, England). 2004;127(3):293-304.
13. Zafrakas M, Tarlatzis BC, Streichert T, Pournaropoulos F, Wolfle U, Smeets SJ, et al. Genome-wide microarray gene expression, array-CGH analysis, and telomerase activity in advanced ovarian endometriosis: a high degree of differentiation rather than malignant potential. International journal of molecular medicine. 2008;21(3):335-44.
14. Endometriosis and infertility: a committee opinion. Fertility and sterility. 2012;98(3):591-8.
15. Hapangama DK, Turner MA, Drury J, Heathcote L, Afshar Y, Mavrogianis PA, et al. Aberrant expression of regulators of cell-fate found in eutopic endometrium is found in matched ectopic endometrium among women and in a baboon model of endometriosis. Human reproduction (Oxford, England). 2010;25(11):2840-50.
16. Mafra FA, Christofolini DM, Cavalcanti V, Vilarino FL, Andre GM, Kato P, et al. Aberrant telomerase expression in the endometrium of infertile women with deep endometriosis. Arch Med Res. 2014;45(1):31-5.
17. Hapangama DK, Turner MA, Drury JA, Quenby S, Saretzki G, Martin-Ruiz C, et al. Endometriosis is associated with aberrant endometrial expression of telomerase and increased telomere length. Human reproduction (Oxford, England). 2008;23(7):1511-9.
18. Chang HJ, Lee JH, Hwang KJ, Kim MR, Yoo JH. Peroxisome proliferator-activated receptor gamma agonist suppresses human telomerase reverse transcriptase expression and aromatase activity in eutopic

endometrial stromal cells from endometriosis. Clinical and experimental reproductive medicine. 2013;40(2):67-75.
19. Valentijn AJ, Saretzki G, Tempest N, Critchley HO, Hapangama DK. Human endometrial epithelial telomerase is important for epithelial proliferation and glandular formation with potential implications in endometriosis. Human reproduction (Oxford, England). 2015;30(12):2816-28.
20. Valentijn AJ, Palial K, Al-Lamee H, Tempest N, Drury J, Von Zglinicki T, et al. SSEA-1 isolates human endometrial basal glandular epithelial cells: phenotypic and functional characterization and implications in the pathogenesis of endometriosis. Human reproduction (Oxford, England). 2013;28(10):2695-708.
21. Shah DK, Correia KF, Vitonis AF, Missmer SA. Body size and endometriosis: results from 20 years of follow-up within the Nurses' Health Study II prospective cohort. Human reproduction (Oxford, England). 2013;28(7):1783-92.
22. Chen XQ, Bonnefoi H, Pelte MF, Lyautey J, Lederrey C, Movarekhi S, et al. Telomerase RNA as a detection marker in the serum of breast cancer patients. Clinical cancer research : an official journal of the American Association for Cancer Research. 2000;6(10):3823-6.
23. El-Mazny A, Sayed M, Sharaf S. Human telomerase reverse transcriptase messenger RNA (TERT mRNA) as a tumour marker for early detection of hepatocellular carcinoma. Arab journal of gastroenterology : the official publication of the Pan-Arab Association of Gastroenterology. 2014;15(2):68-71.

Fig 1

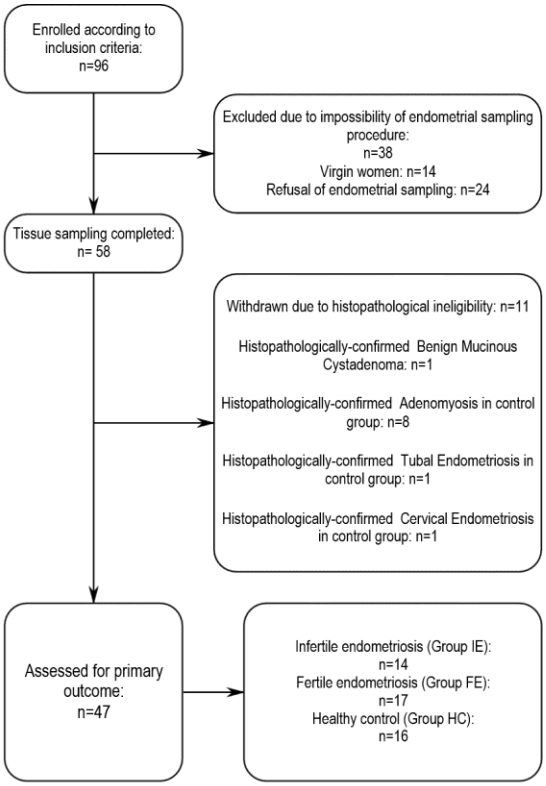

Fig 2

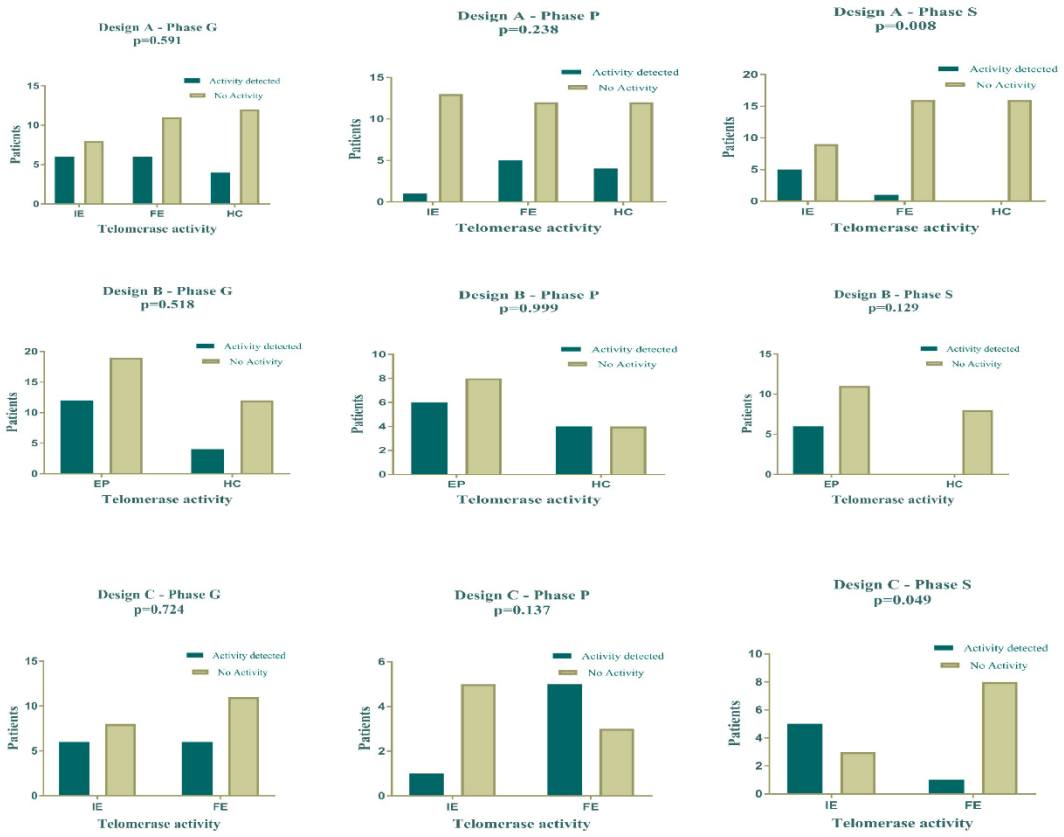

Table Legends:

Table 1. Comparison of telomerase activity by groups with different designs and in different phases.

Table 2. Demographic and clinical parameters of patients.

Table 3. Post-hoc analyses of multiple comparisons

Table 4. Telomerase activity among groups after adjustment by age

Appendix 1. Publications related to endometriosis and telomerase activity

Table 1. Comparison of telomerase activity by groups with different designs and in different phases.

Designs	**Definition of groups**	**Number of patients per groups n (%) (all)**		**Telomerase activity detection in different menstrual phases**		**p value***		
		Proliferative n (%)	**Secretory n (%)**	**Proliferative n (%)**	**Secretory n (%)**			
Three-group design (Design A)	IE vs.	14 (29.8%)	6 (12.8 %) 8 (17 %)	1 (2.1 %)	5 (10.6 %)	Phase G	Phase P	Phase S
	FE vs.	17 (36.2%)	8 (17 %) 9 (19.2 %)	5 (10.6 %)	1 (2.1 %)	p=0.59	p=0.24	***p=0.008***
	HC vs.	16 (34%)	8 (17 %) 8 (17 %)	4 (8.5 %)	0 (0%)			
Endometriosis-based desgin (Design B)	EP vs.	31 (66 %)	14 (29.8%) 17 (36.2%)	6 (12.8 %)	6 (12.8 %)	Phase G	Phase P	Phase S
	HC	16 (34%)	8 (17 %) 8 (17 %)	4 (8.5 %)	0 (0%)	p=0.52	p=0.99	p=0.13
Fertility-based design (Design C)	IE vs.	14 (29.8%)	6 (12.8%) 8 (17 %)	1 (2.1 %)	5 (10.6 %)	Phase G	Phase P	Phase S
	FE	17 (36.2%)	8 (17 %) 9 (19.2%)	5 (10.6 %)	1 (2.1 %)	p=0.72	p=0.14	***p=0.049***

*Fisher- Exact's test was applied for all comparisons.

IE- Infertile Endometriosis; FE- Fertile Endometriosis; HC- Healthy Controls

Phase G – regardless menstrual cycle phase; Phase P – proliferative phase; Phase S- secretory phase

Table 2. Demographic and clinical parameters of patients.

Parameters	**EP (n=31)**		**HC (n=16)**	**p value**
	IE (n=14)	**FE (n=17)**		
	Mean±SD or	**Mean±SD or**	**Mean±SD or**	**(p<0.05)**
	Median (IQR)	**Median (IQR)**	**Median (IQR)**	
	***event/total number**	***event/total number**	***event/total number**	
Age (years)	30.42 ±1.38	40.82±1.36	47.9±0.96	*0.000*[a]
Smoking status	2/14[*]	2/17[*]	4/16[*]	0.625[b]
Educational Status	4/14 college/higher[*] 8/14 high school[*] 2/14elementary/illiterate[*]	4/17 college/higher[*] 10/17 high school[*] 3/17 elementary/ illiterate[*]	2/16 college/higher[*] 8/16 high school[*] 6/16elementary/ illiterate[*]	0.578[b]
Marital status	14/14 married[*]	13/17 merried[*] 2/17 divorced[*] 2/17 widow[*]	13/16 married[*] 3/16 widow[*]	0.205[b]
Body Mass Index (BMI) (kg/m$^{2)}$	25.37±2.07	27.79 ±2.29	27.96±2.12	*0.003*[a]
CA 125 (U/mL)	70.86 (44.01-83.42)	73.53 (47.02-100.2)	19.8 (13.15 – 36.11)	*0.000*[c]
CA 15-3 (U/mL)	13.89 (10.53 – 24.37)	17.94 (16.07 – 23.68)	17.705 (14.59-25.77)	0.426[c]
CA 19-9 (U/mL)	21.39 (14.75 – 50.28)	18.99 (9.83-40.4)	7.36 (5.35-11.39)	*0.003*[c]
TSH (mIU/L)	2.4 (1.64-2.58)	2.46 (1.48 -3.38)	2.17 (1.51-3.09)	0.879[c]
WBC (mm^3)	7478.57 ±1666.95	7805.88 ± 2006.69	7393.75 ± 2092.67	0.814[a]
Lymphocyte (mm^3)	2385.71 ± 512.69	2341.17 ± 729.77	2050 ± 522.81	0.252[a]

Cyst size (cm)	9.14 ± 5.06	7.11 ± 3.21	-	0.328[d]
Cystic side	6/14 bilateral[*] 8/14 unilateral[*]	7/17 bilateral[*] 10/17 unilateral[*]	-	0.999[b]

[a] One-way ANOVA test was used; [b] Fisher's Exact test was used; [c] Kruskal-Wallis test was used; [d] Mann-Whitney U test was used.

IQR- interquartile range; BMI- Body Mass Index, CA 125, CA 15-3, CA 19-9 – Cancer antigen; TSH- thyroid stimulating hormone, WBC- White blood cells; IE – infertile endometriosis; FE – fertile endometriosis; HC – healthy control

Table 3. Post-hoc analyses of multiple comparisons

	Group IE vs. FE	**Group FE vs. HC**	**Group IE vs. HC**
Age [a]	*p=0.000*	*p=0.001*	*p=0.000*
BMI[a]	*p=0.010*	ND	*p=0.006*
CA-125[b]	ND	*p=0.000*	*p=0.001*
CA 19-9[b]	ND	ND	*p=0.001*

[a] Bonferroni correction was used; [b] Dunn's test was used

BMI – Body mass index; CA – cancer antigen; ND – no significant difference was found.

IE – Infertile Endometriosis; FE – Fertile Endometriosis; HC – Healthy Controls

Table 4. Telomerase activity among groups after adjustment by age

	Coefficient	**z value**	**p-value**	**Odds Ratio (OR)**	**95 % Confidence Interval (CI)**
Group IE	0.90	0.78	0.43	1.09	0.87 – 1.37
Group FE	-0.04	-0.46	0.65	0.96	0.79 – 1.15
Group HC	0.006	0.04	0.97	1.01	0.74 – 1.36

IE – Infertile Endometriosis; FE – Fertile Endometriosis; HC – Healthy Controls

Appendix 1. Publications related to endometriosis and telomerase activity

Study	Publication year	Study groups	Analyzed tissue	Phase of menstrual cycle	Result
Zafrakas (14)	2007	Infertile endometriosis	Eutopic and ectopic (ovarian) endometrium	Proliferative	Activity was found in all eutopic endometrium and no activity was reported in endometriosis tissue.
Kim (10)	2007	Endometriosis vs. without endometriosis	Eutopic endometrium	All phases	Significant difference was found a) comparison regardless the phases (higher in endometriosis group); b) in secretory phase higher in endometriosis group In late proliferative and early secretory phase higher in the endometriosis group (no significant difference)

Hapangama (17)	2008	Endometriosis vs. without endometriosis	Eutopic endometrium	Mid secretory	Weak or no activity in control group Increased activity in endometriosis group
Hapangama (16)	2010	Women with endometriosis vs. baboons after intraperitoneal menstrual endometrium inoculation vs. healthy baboons	Eutopic endometrium of baboons	Proliferative (n=2), mid-secretory (n=3), late-secretory (n=2) (Findings of women)	In none of the samples activity was detected
Chang (18)	2013	Endometriosis vs. without endometriosis	Cultured endometrial stem cells (ESCs) under PPARγ agonist (rosiglitazone) exposure obtained from endometrium	Mid- or late proliferative phase	Increased hTRET expression in cultured ESCs in endometriosis group, which was inhibited by rosiglitazone
Valentjin (20)	2013	Premenopausal and Postmenopausal without endometriosis and peritoneal endometriosis	Eutopic endometrium and ectopic endometrium from women with peritoneal lesions	Mixed	SSEA-I^+ (cell surface marker) cells showed higher activity than SSEA-I^- cells

Mafra (11)	2013	Infertile endometriosis vs fertile without endometriosis	Peritoneal lesions and eutopic endometrium	Late Secretory	Eutopic endometrium: endometriosis group showed the higher activity than control group Peritoneal lesions: All but one showed no activity
Valentjin (19)	2015	Peritoneal endometriosis (Group 4 patients)	Eutopic and ectopic endometrium	Not defined	Ectopic endometrium showed increased immunoreactivity for telomerase protein hTRET compared to matching eutopic endometrium