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Disturbed estrogen and progesterone action in ovarian endometriosis

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Summary

Endometriosis is a very common disease in pre-menopausal women, where defective metabolism of steroid hormones plays an important role in its development and promotion. In the present study, we have examined the expression of 11 estrogen and progesterone metabolizing enzymes and their corresponding receptors in samples of ovarian endometriomas and control endometrium. Expression analysis revealed significant up-regulation of enzymes involved in estradiol formation (aromatase, sulfatase and all reductive 17β-hydroxysteroid dehydrogenases) and in progesterone inactivation (AKR1C1 and AKR1C3). Among the estrogen and progesterone receptors, ERα was down-regulated, ERβ was up-regulated, and there was no significant difference in expression of progesterone receptors A and B (PRAB). Our data indicate that several enzymes of estrogen and progesterone metabolism are aberrantly expressed in endometriosis, which can lead to increased local levels of mitogenic estradiol and decreased levels of protective progesterone. Changes in estrogen receptor expression suggest that estradiol may also act via non-estrogen-receptor-mediated pathways, while expression of progesterone receptors still needs further investigation.

Keywords: endometriosis, 17β-hydroxysteroid dehydrogenases, AKR1C1, AKR1C3, estrogen and progesterone metabolizing enzymes, estrogen receptors, progesterone receptors

Introduction

Endometriosis is a complex disease that is defined as the presence of endometrial glands and stroma outside the uterine cavity and musculature (Olive and Schwarz, 1993). It is most commonly diagnosed in women of reproductive age (Berkley et al., 2005). There are three different types of endometriosis, namely ovarian endometriosis, peritoneal endometriosis and deep endometriotic nodules, which represent three different entities with different pathogenesis (Nap et al., 2004). Pathophisiological mechanisms have been extensively studied on animal models, but due to species-specific metabolism and reproductive physiology they still cannot replace studies on human samples (Grümmer 2006).

In general, endometrial implants behave like normal endometrium in their response to hormones, where estradiol (E2) stimulates proliferation and progesterone (P) promotes differentiation (Olive and Schwartz, 1993). In premenopausal women the ovaries are the primary source of E2, but E2 can also be produced in peripheral tissue from inactive precursors (Labrie et al., 2000). Locally, in endometriotic tissue, E2 can be synthesised in two ways: by the aromatase pathway, which includes conversion of ovarian or adrenal androstenedione to the weakly estrogenic estron (E1); this is further converted to active E2 (Bulun et al., 1999, Bulun et al., 2000, Bulun et al., 2001) by 17β-hydroxysteroid dehydrogenases (17β-HDSs) types 1, 7 and 12. The reverse reaction is catalyzed by the oxidative 17β-HSDs types 2, 4 and 8 (Luu-The 2001, Penning 2003, Midnich et al., 2004, Vikho et al., 2004, Luu-The et al., 2006).

The use of aromatase inhibitors for the treatment of endometriosis has been successful, as this is the rate-limiting step in local E2 production (Bulun et al., 2001, Patwardhan et al., 2008). The other pathway, the so called sulfatase pathway, includes sulfatase and sulfotransferase. Sulfatase converts estrogen sulfate to E1, which should be activated by the reductive 17β-HSDs, while sulfotransferase inactivates E1 and E2, forming their sulfates. The production of estrogens from circulating levels of estrogen sulfate is another important alternative or complementary pathway to ovarian steroidogenesis.

P action can also be regulated at the pre-receptor level. Locally P can be converted to the less active 20α-hydroxyprogesterone (20α-OHP) and/or to 5α-reduced metabolites (Pollow et al., 1975). The major human reductive 20α-HSDs are aldo-keto reductases AKR1C1 and AKR1C3, with the latter also acting as a reductive 17β-HSD and potentially also involved in E2 synthesis (Penning 2000).

The abundance and distribution of estrogen receptors (ERs) and progesterone receptors (PRs) will determine whether E2 or P will have a particular effect (Pearce and Jordan, 2004). There are two distinct ERs: ERα and ERβ, where ERα has a proliferative effect and ERβ acts as a repressor of ERα. Also PRs exist in two isoforms, as products of the same gene; where PRA lacks 164 amino acids from the N-terminal end of PRB (Oehler et al., 2000). PRB acts as transcriptional activator of progesterone target genes, while PRA has dual role which is cell and promoter specific. It can act as transcriptional factor or as repressor of PRB function (Vegeto et al., 1993).

In the present study, we have examined the mRNA levels of 11 estrogen- and progesterone-metabolizing enzymes (17β-HSD types 1, 2, 4, 7, 8 and 12, sulfatase, sulfotransferase, aromatase, AKR1C3 and AKR1C1) and steroid receptors (ERα, ERβ and PRAB) in specimens of ovarian endometriosis and control endometrium.

Materials and methods

Tissue samples

A total of 34 samples were collected: 24 of ovarian endometriomas and 10 of control endometrium, from women with *Uterus Myomatosus* or *Myoma uterii* (Table 1, 2). The study was approved by the National Medical Etics Committee of the Republic of Slovenia. RNA extraction, cDNA synthesis, real-time PCR and statistical analyses were performed as previousley reported (Šmuc et al., 2007a, Šmuc et al., 2007b). Expression analysis of 17β-HSD types 4, 8 and 12, AKR1C1 and AKR1C3 was performed on the 24 samples, and for another 9 genes on 16 samples of ovarian endometriomas.

Western blotting

Aliquots from the protein fractions of ovarian endometriomas (isolated from the same samples as for RNA) were separated by SDS PAGE. The proteins were transferred from the gels to membranes and incubated with 5% non-fat milk to avoid non-specifical binding. The membranes were then incubated overnight with primary antibodies: mouse anti-AKR1C3 (1:1000, Sigma-Aldrich, USA), mouse anti-PR-B (1:500, Santa Cruz, USA). The next day, the membranes were incubated with the secondary antibodies (anti-mouse peroxidase conjugated, dillutions 1:3000 and 1:10000, Jackson ImmunoResearch Laboratories Inc., USA) for 1 h. Supersignal West Pico Chemiluminiscence Substrate (Pierce Biotechnology) was used for the detection of the bound antibodies, according to the manufacturer instructions.

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples containing ovarian endometriomas were dewaxed in xylene and rehydrated. The sections were incubated in H_2O_2 to block endogenous peroxidase. After antigen retrieval in sodium citrate buffer, sections were incubated with anti-AKR1C3 (1:500, Sigma-Aldrich, USA), anti-ERα (1:20, DakoCytomation, Denmark), or anti-PR-B (1:50, Santa Cruz, USA) antibodies. The peroxidase-antiperoxidase complex with DAB as substrate was used to detect bound antibodies.

Results and discussion

Aromatase is a key enzyme in local estrogen production (Noble et al., 1996) and aromatase inhibitors have proven to be beneficial for the treatment of endometriosis in some patients (Amsterdam et al., 2005). We saw that aromatase transcript levels were significantly higher ($p < 0.001$) in the endometriosis group, compared to normal (Fig. 1I) (Šmuc et al., 2007b), confirming previously published data (Noble et al., 1996, Bulun et al., 2005). The aromatase pathway also includes 17β-HSDs, which convert E1 to the highly active E2, and *vice versa*. In endometriotic tissue, all of the reductive 17β-HSDs were up-regulated. Although Zeitoun and colleagues (1998) have previously reported no differences in steady-state 17β-HSD type 1 mRNA between eutopic endometrial and endometriotic samples in different phases of the cycle, our published data (Šmuc et al., 2007a, Šmuc et al., 2007b) show higher levels of type 1 transcript in ovarian endometriomas (Fig. 1A) ($p < 0.001$). Also, Dassen and colleagues (2007) recently reported up-regulation of 17β-HSD type 1 in deep infiltrating endometriosis. We have also shown higher levels of 17β-HSD type 7 in endometriotic tissue compared to normal (Šmuc et al, 2007a, Šmuc et al., 2007b) (Fig. 1B) ($p < 0.001$). The expression of 17β-HSD type 12 has not yet been examined either in normal or diseased endometrium; here, we detected 17β-HSD type 12 mRNA in ovarian endometriosis and in normal endometrium tissue samples, and for the first time, we show that also 17β-HSD type 12 is up-regulated in the diseased endometrium (Fig. 1C) ($p = 0.0016$).

For oxidative 17β-HSDs, Zeitoun et al. (1998) have reported an absence of 17β-HSD type 2 mRNA transcripts in extra-ovarian endometriotic tissue with Northern blotting; however, our more sensitive study saw this transcript in both the control and the endometriosis group, although we did not detect any significant changes in the expression levels between these groups (Šmuc et al., 2007a, Šmuc et al., 2007b) ($p > 0.05$) (Fig. 1D). In contrast, Matsuzaki et al. (2006) and Dassen et al. (2007) have shown lower expression of 17β-HSD type 2 in deep infiltrating endometriotic tissue. There have been no reports of decreased levels of 17β-HSD type 2 in ovarian endometriosis. For 17β-HSD types 4 and 8, we did not find significant differences in their expression levels (Fig. 1E, F), although Dassen and colleagues (2007) have reported a significant down-regulation of 17β-HSD type 4 in deep infiltrating endometriosis. However, at least to our knowledge, we are the first to show expression of 17β-HSD type 8 in normal and ectopic endometrium.

For the sulfatase pathway, we saw that the sulfatase mRNA levels were significantely higher in samples of endometriosis, as compared to disease-free tissue (Šmuc et al., 2007a, Šmuc et al., 2007b) ($p < 0.001$) (Fig. 1G). The sulfotransferase mRNA was not detected in 4 samples of endometriotic tissue, and the differences between the medians for normal tissue *versus* endometriosis tissue were not statistically significant (Šmuc et al., 2007a, Šmuc et al., 2007b) (Fig. 1H). Hudelist et al. (2007) also reported no changes in sulfotransferase levels between ectopic and eutopic endometrial tissues when using immunohistochemical staining.

Among the receptors we studied, only ERs were significantly differentially expressed. The levels of ERα were much lower (Šmuc et al., 2007a, Šmuc et al., 2007b) ($p = 0.036$) (Fig. 3A), and the levels of ERβ mRNA were much higher, in endometriosis than in normal tissue (Šmuc et al., 2007a, Šmuc et al., 2007b) ($p = 0.001$) (Fig. 3B). Our immunohistochemical study showed ERα in the nucleus of glandular cells in 9 samples, nuclear staining was also seen in stromal cells in 6 samples out of 12 investigated

(Fig. 4B). The published data for the expression of ERs in endometriosis are rather contradictory. Matsuzaki et al. (2001) reported no ERβ expression in 17% of ovarian endometriosis samples by TaqMan RT-PCR, while Fujimoto et al. (1999) showed significantly higher expression of ERβ in ovarian endometriosis compared to normal endometrium. Recently, Bukulmez et al. (2008) reported a significantly elevated ratio of ERβ/ERα mRNA in endometriomas, compared with eutopic endometrium, in agreement with our data. They concluded that this is due to chronic inflammation in endometrial lesions (Bukulmez et al., 2008). We would suggest that in ovarian endometriomas, E2 may act via non-ER-mediated pathways, although this needs further investigation.

Our data also show disturbed P metabolism in ovarian endometriomas. As with our previously report on up-regulation of AKR1C3 (Šmuc et al., 2007a), here we confirmed up-regulation of AKR1C3 in a larger group of 24 patients ($p < 0.01$) (Fig. 2B). Also AKR1C1, which is the major 20α-hydroxysteroid dehydrogenase was up-regulated ($p<0.001$) (Fig. 2A). AKR1C3 was also detected at the protein level in 13 samples out of 15 investigated (Fig. 2C). With immunohistochemical staining, AKR1C3 was detected in glandular epithelial cells in 12 of 14 samples (Fig. 4A). We are thus here the first to show expression of AKR1C1 and AKR1C3 in endometriosis. These data show increased conversion of P into the biologically less active 20α-OHP, which has a lower affinity towards PRs, and which could be another way of enhancing E2 effects. The data on expression of PRs are also rather controversial: Misao et al. (1999) have reported significantly higher levels of PRB mRNA in ovarian endometriosis samples compared to the normal endometrium. Attia et al. (2000) have reported that PRB was not expressed in non-ovarian endometriosis, while Bukulmez et al. (2008) detected three isoforms PRC, PRB and PRA in ovarian endometriomas. In contrast, our results showed no significant differences in mRNA levels of PRAB (Fig. 3C), with the protein levels of PRB also not significantly different between the control and endometriosis groups ($p > 0.05$), although we detected PRB in 13 out of 16 endometriosis samples (Fig. 3D). Immunohistochemistry revealed staining of PRB in nuclei of stromal and glandular cells in 12 out of 12 samples (Fig. 4C). PRA and PRB activation of gene transcription may have an important role in development of endometriosis. Since PRA dominantly acts as repressor of PRB, the ratio could be crucial (Misao et al., 1999). Unchanged PRAB expression observed in our study may thus mask differences in the PRB/PRA ratio, which may affect progesterone action in ovarian endometriomas. Expression analysis of PRA is currently in progress, which will help us to obtain insights into the roles of the PR isoforms in ovarian endometriosis.

Conclusions

Our gene expression analysis has revealed up-regulation of enzymes that are involved in E2 activation and P inactivation, suggesting that the E2 and P actions are disturbed in ovarian endometriomas. Decreased expression of ERα and increased expression of ERβ indicate that the E2 formed locally may act also via non-ER-mediated pathways. The unchanged expression of PRAB suggests that pre-receptor regulatory enzymes have the most important roles in diminished P action, while the PRB/PRA expression ratio may also affect P action.

The majority of our gene expression analysis data are in agreement with previously reported results. Some differences observed may be explained by different type of endometriosis studied and different study design. Due to ethical reasons we examined expression in ovarian endometriomas compared to eutopic endometrium of non-endometriosis patients. In addition to genes, which have already been examined in endometriosis, we were the first to show expression of 17β-HSD type 8, AKR1C1 and AKR1C3 in ectopic endometrium.

Acknowledgements

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Table 1. Patients details: endometriosis group

Sample	Age	Diagnosis	Phase
13	33	Endometriosis ovarii	proliferative
14	24	Endometriosis ovarii	proliferative
15	33	Endometriosis ovarii	late proliferative/early secretory
16	30	Endometriosis ovarii	late proliferative/early secretory
18	44	Endometriosis ovarii	proliferative
19	27	Endometriosis ovarii	early secretory
20	30	Endometriosis ovarii	late proliferative/early secretory
21	34	Endometriosis ovarii	proliferative
22	32	Endometriosis ovarii	secretory
23	27	Endometriosis ovarii	secretory
24	36	Endometriosis ovarii	proliferative
25	33	Endometriosis ovarii	proliferative
26	28	Endometriosis ovarii	late proliferative/early secretory
27	40	Endometriosis ovarii	proliferative
28	29	Endometriosis ovarii	secretory
29	39	Endometriosis ovarii	late proliferative/early secretory
30	31	Endometriosis ovarii	*
31	28	Endometriosis ovarii	secretory
32	37	Endometriosis ovarii	*
36	47	Endometriosis ovarii	proliferative
37	28	Endometriosis ovarii	proliferative
41	37	Endometriosis ovarii	secretory
43	26	Endometriosis ovarii	*
45	25	Endometriosis ovarii	proliferative

* not determined

Table 2. Patients details: control group

Sample	Age	Diagnosis	Phase of menstrual cycle
1	42	Uterus myomatosus	non-proliferative
2	47	Myoma uteri	late secretory
3	43	Uterus myomatosus	early secretory
5	45	Uterus myomatosus	proliferative
6	50	Uterus myomatosus	early secretory
8	42	Myoma uteri	late secretory
9	47	Uterus myomatosus	proliferative
10	48	Myoma uteri	proliferative
11	43	Uterus myomatosus	early secretory
25	60	Myoma uteri	atrophic endometrium

Figure Legends

Figure 1. Relative expression levels (to cyclophilin A) of 17β-HSD types 1 (A), 7 (B), 12 (C), 2 (D), 4 (E), 8 (F), and of sulfatase (G), sulfotransferase (H) and aromatase (I).

Figure 2. Relative expression levels (to cyclophilin A) of AKR1C1 (A) and AKR1C3 (B). Western blotting for AKR1C3 (C) in protein fractions of endometriosis samples 14, 19, 22, 24, 25, 28. GAPDH was used as normalisation control.

Figure 3. Relative expression levels (to cyclophilin A) of ERα (A), ERβ (B) and PR (C). Western blotting for PRB (D) in protein fractions of endometriosis samples 13, 18, 19, 22, 26. GAPDH was used as normalisation control.

Figure 4. Immunohistochemistry for AKR1C3 (A), ERα (B), PRB (C) and the negative control (D) for samples 16, 24 and 30. Magnification: 15x

Figure 1

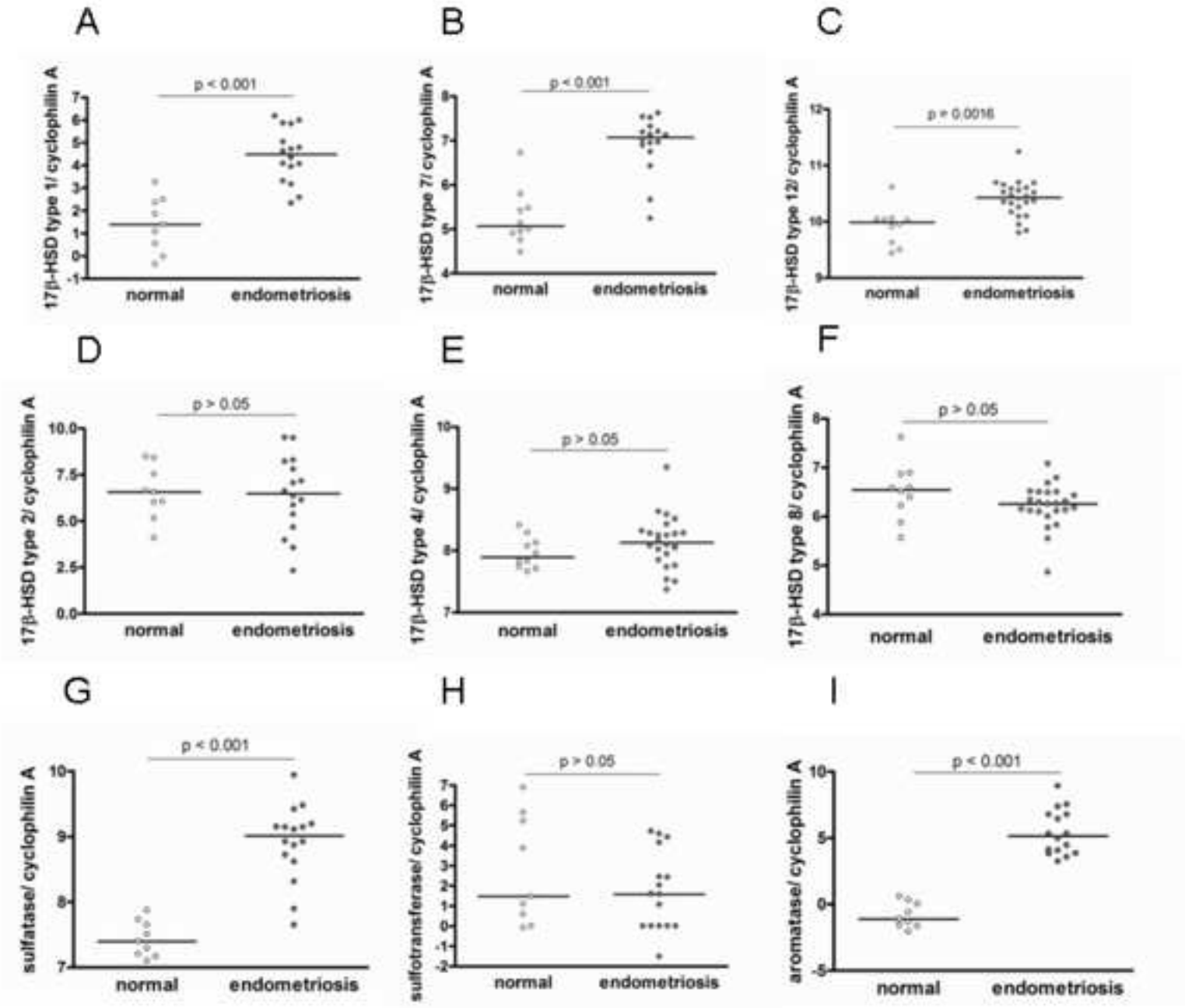

A
p < 0.001
17β-HSD type 1/ cyclophilin A
normal
endometriosis
B
p < 0.001
17β-HSD type 7/ cyclophilin A
normal
endometriosis
C
p = 0.0016
17β-HSD type 12/ cyclophilin A
normal
endometriosis
D
p > 0.05
17β-HSD type 2/ cyclophilin A
normal
endometriosis
E
p > 0.05
17β-HSD type 4/ cyclophilin A
normal
endometriosis
F
p > 0.05
17β-HSD type 8/ cyclophilin A
normal
endometriosis
G
p < 0.001
sulfatase/ cyclophilin A
normal
endometriosis
H
p > 0.05
sulfotransferase/ cyclophilin A
normal
endometriosis
I
p < 0.001
aromatase/ cyclophilin A
normal
endometriosis

Figure 2

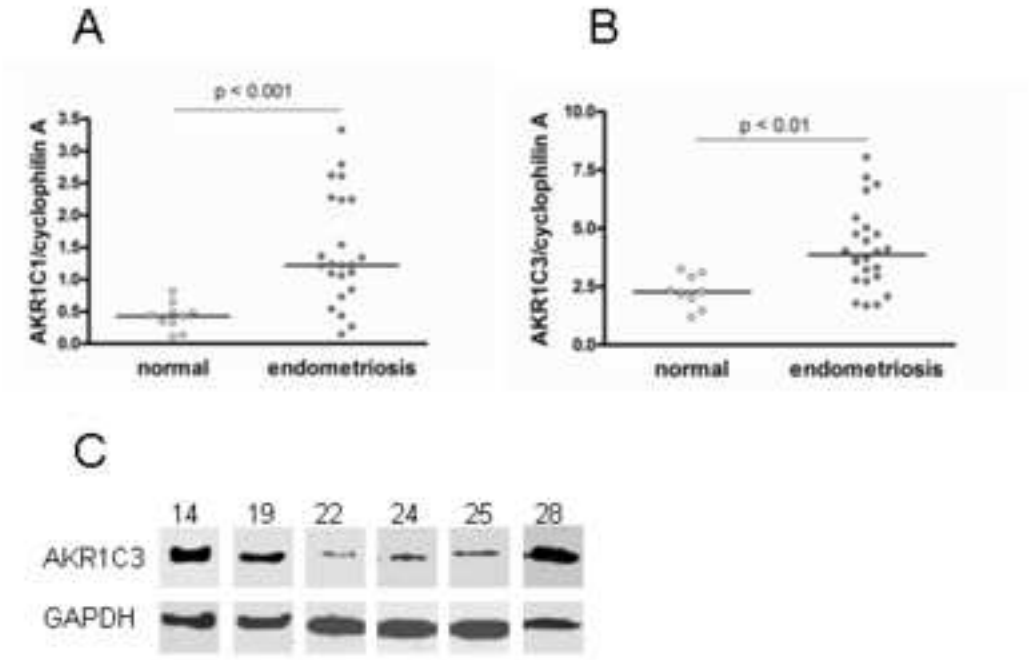
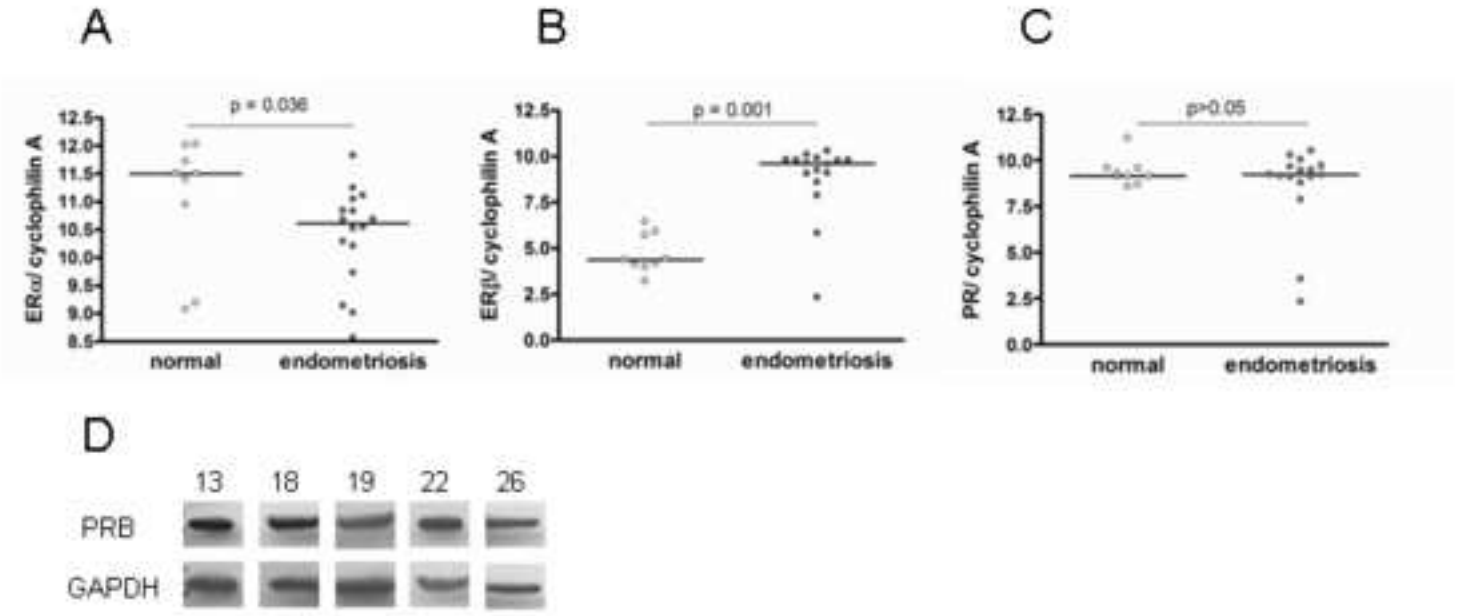

A
p < 0.001
AKR1C1/cyclophilin A
normal
endometriosis
B
p < 0.01
AKR1C3/cyclophilin A
normal
endometriosis
C
14
19
22
24
25
28
AKR1C3
GAPDH

Figure 3


A
B
C
p = 0.036
p = 0.001
p>0.05
ERα/ cyclophilin A
ERβ/ cyclophilin A
PR/ cyclophilin A
normal
endometriosis
D
13
18
19
22
26
PRB
GAPDH