Endometrial BCL6 testing for the prediction of in vitro fertilization outcomes: a cohort study

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Objective: To evaluate endometrial BCL6 expression as a prognostic biomarker for IVF outcome in women with unexplained infertility (UI) before ET.
Design: Prospective cohort study.
Setting: University-associated infertility clinic.
Patient(s): Women with UI for >1 year.
Intervention(s): We studied women with UI who underwent testing for endometrial BCL6, in an LH-timed midluteal phase biopsy and completed an IVF cycle and ET.
Main Outcome Measure(s): Clinical pregnancy rate (PR) and live birth rate per transfer was compared for women positive or negative for BCL6 expression. An abnormal BCL6 result was defined by an histologic score (>1.4).
Result(s): Women with normal and abnormal BCL6 and those who conceived or not had similar characteristics. Women with low levels of BCL6 expression had a significantly higher clinical PR (11/17; 64.7%; 95% confidence interval [CI] 41.3–82.6) compared with women with abnormal (high) BCL6 expression (9/52; 17.3%; 95% CI 9.3–30.8). These results yield a relative risk of 0.267 (95% CI 0.13–0.53; $P=.0004$) for those with normal BCL6 expression, an absolute benefit of 47.4% (95% CI 22.5–72.0). Live birth rate was also significantly higher in women with low BCL6 expression (10/17; 58.8%; 95% CI 36.0–78.4) compared with women with abnormal BCL6 expression (6/52; 11.5%; 95% CI 5.4–23.0). The relative risk was 0.19 (95% CI 0.08–0.45; $P=.0002$), yielding an absolute benefit of 47.3% (95% CI 21.8–67.8).
Conclusion(s): Aberrant BCL6 expression (histologic score, >1.4) was strongly associated with poor reproductive outcomes in IVF cycles in women with UI. (Fertil Steril® 2017;108:1063–9. ©2017 by American Society for Reproductive Medicine.)
Key Words: Endometrium, BCL6, prognosis, IVF, pregnancy

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The first IVF pregnancy resulted in the birth of Louise Joy Brown. At present, nearly 40 years later, there are nearly 200,000 cycles performed annually in the United States alone (1), but success rates for live birth remains <50%. Even when preimplantation genetic screening-defined euploid embryos are transferred, nearly half failed to implant (2). This observation strongly suggests the presence of an undiagnosed endometrial deficit that contributes to IVF success or failure. Although studies have suggested that endometriosis does not alter IVF outcomes (3), the diagnosis of endometriosis is listed in only 3%–4% of cases in the most recent the Society for Assisted Reproductive Technology (SART) database (1), well below the expected prevalence in an infertile population. Endometriosis is likely present in ≤50% of infertile women (4) and ≤70% of patients with endometriosis will not have a live birth (5). Older studies have pointed to oocyte quality as a cause of IVF failure associated with endometriosis (6). A more recent, larger study, using fertilized sibling oocytes transferred into women with

Received July 24, 2017; revised August 25, 2017; accepted September 14, 2017; published online November 7, 2017.
L.D.A. has nothing to disclose. C.E.L. has nothing to disclose. B.S. has nothing to disclose. K.R.B. has nothing to disclose. R.S. has nothing to disclose. D.A.F. has nothing to disclose. P.B.M. has nothing to disclose. B.A.L. reports he is a scientific advisor for CiceroDX and holds Patent 9650676 for Leukocytes MicroRNAS for the use in diagnosis and treatment of endometriosis.
Supported by National Institute of Child Health and Human Development/National Institutes of Health R01 HD067721 and by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior: Grant 99999.003035/2015-08 (B.A.L.) and by CAPES/PROAP (R.F.S.).
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Fertility and Sterility® Vol. 108, No. 6, December 2017 0015-0282/$36.00

https://doi.org/10.1016/j.fertnstert.2017.09.017

and without endometriosis, demonstrated reduced implantation, clinical pregnancy rate (PR), ongoing PR, and live birth rate in women with endometriosis, in support of defects in endometrial receptivity as a cause of IVF failure (7). Given how common this disease is in the infertile population, the question remains whether endometriosis is a hidden cause of implantation failure in IVF (8).

Undiagnosed endometriosis was the focus of a study by Littman et al. (9) who studied women with unexplained IVF failure. At laparoscopy, most were found to have endometriosis, and many conceived naturally without IVF, once the disease was identified and treated. Such studies recognize a need to identify less invasive, nonsurgical methods to predict endometriosis before starting IVF, especially in women with unexplained infertility (UI).

We recently reported that most women with UI overexpress the protein BCL6, a new biomarker for the presence of endometriosis (10). BCL-6 is a proto-oncogene and transcriptional repressor that contributes to cell cycle control and differentiation, as well as apoptosis inhibition (11, 12). The overexpression has been associated with increased cellular proliferation (13) and BCL6 is stabilized by STAT3 activation and stimulates cytokine expression, including interleukin-1, interleukin-6, and interleukin-18 (14–16). We recently showed that endometrial BCL6 pairs with a histone deacetylase sirtuin-1 (SIRT1), which is also aberrantly expressed in response to activated KRAS in endometriosis (17). Together, SIRT1/BCL6 complex binds to and inactivates key regulators of P action in the endometrium such as *Gli* in women with endometriosis. Progesterone is essential for establishment of pregnancy and BCL6 appears to be a central cause of P resistance.

Aberrant BCL6 expression histologic score (HSCORE) >1.4 cutoff has a high sensitivity and specificity for the diagnosis of all stages of endometriosis (10). The objective of this study was to use BCL6 as a surrogate biomarker for endometriosis and to determine whether aberrant BCL6 predicts IVF outcome in a population of women with otherwise unexplained difficulty conceiving.

MATERIALS AND METHODS

Study Design/Setting

This cohort study was conducted (recruitment, exposure, follow-up, and data collection) between January 1, 2008 and December 31, 2016, at the Fertility Center of the Carolinas in Greenville Health System, South Carolina. Institutional Review Board was approved by the Committee for the Protection of Human Subjects (GHS #00013885).

Participants

Ovulatory women with healthy male partners with ≥ 1 year of infertility were invited for this cohort study. To be included each woman was required to have regular cyclic menses (25–32 days apart), partners with normal sperm parameters according to the World Health Organization (18), and at least one patent fallopian tube. All patients underwent an LH-timed endometrial biopsy 7–10 days after ovulation, performed within 6 months before IVF. Only fresh IVF cycles with ET were included, and none received surgical or medical suppression of endometriosis before their IVF cycle. Exclusion included the discovery of significant fibroids (>4 cm), male factor infertility, endometritis on endometrial biopsy, or lack of adequate tissue for analysis on the biopsy result.

Endometrial Biopsies

Endometrial biopsy was performed in all participants using a pipelle device (Cooper Surgical), 7–10 days after a urinary LH surge. Endometrial biopsies were placed in 10% buffered formalin and transported to the Pathology Laboratory for paraffin embedding, sectioning, and immunostaining. The menstrual cycle stage was determined according to Noyes et al. (19).

Immunohistochemistry

Immunohistochemistry was performed on an automated system by a certified pathologist (Pathology Associates, GHS, Greenville, South Carolina) using the Bond immunostainer platform (Leica Biosystems). Sections of endometrium from archived blocks were stained for BCL6, using an automated system, with clone LN22 as primary antibody (Leica Biosystems), as previously described (10). Lymph nodes served as a positive external control. Adequacy of the endometrium sample was a requirement for inclusion in this study.

BCL6 expression was assigned an HSCORE, which ranged from 0–4. The HSCORE was calculated using the following equation: $\text{HSCORE} = \sum \text{Pi}(\text{i}+1)/100$, where i = intensity of staining with a value of 1, 2, or 3 (weak, moderate, or strong, respectively) and Pi is the percentage of stained epithelial cells for each intensity, varying from 0–100%, as previously described (20). All HSCOREs were assigned in a blinded fashion without knowledge of the clinical history or outcome.

Variables

The following variables were analyzed: age, body mass index (BMI), peak E_2, days of stimulation, number of oocytes retrieved, fertilization rate, number of embryos transferred, clinical pregnancy rate (PR), live birth rate (LBR) and median values of BCL6 expression as a continuous variable. Positive and negative BCL6 expressions were based on HSCORE results (normal ≤ 1.4; overexpressed >1.4). Clinical PR was defined as a pregnancy documented by ultrasound that shows a gestational sac in the uterus with a cardiac activity. A positive and increasing hCG with an early loss without evidence of an intrauterine sac on ultrasound (biochemical pregnancy) was not counted as a pregnancy.

Data Sources/Measurement

Data from the variables were obtained from the SART database and medical records. They were analyzed as mean ($\pm$SD) or as median (range), depending whether they had passed the normality test for normal distribution. BCL6 positivity was judged as abnormal if the HSCORE was >1.4, as

TABLE 1

Characteristics of the sample population based on outcome (clinical pregnancy rate).

Characteristic	Pregnant (n = 20)	Not pregnant (n = 49)	*P* value
Age (y), mean ± SD	36.3 ± 3.2	34.5 ± 3.9	.05[a]
BMI (kg/m^2), median (range)	24.3 (18.6–44.6)	23.9 (17.9–36.0)	.8[b]
BCL6 expression, median (range)	0.9 (0–4)	2.1 (0.5–4.0)	.01[b]
Cycle characteristic			
Stimulation (d), mean ± SD	10.4 ± 1.9	10.4 ± 1.8	.9[a]
Peak E_2 (pg/mL), median (range)	1,387 (341–5,000)	1,945 (477–5,000)	.7[b]
Oocyte retrieved (n), median (range)	14 (2–35)	11 (3–56)	.3
Oocytes fertilized (n), median (range)	7 (1–24)	6 (1–21)	.4
Embryos transferred (n), median (range)	2 (1–4)	2 (1–3)	.2[b]

Note: BMI = body mass index.
[a] Student's *t* test.
[b] Mann-Whitney *U* test.

Almquist. Endometrial BCL6 and IVF outcome. Fertil Steril 2017.

defined by receiving operating characteristic curve analysis for the diagnosis of endometriosis (10).

Bias

Two researchers (B.A.L., K.R.B.) verified the electronic records (SART database) independently to reduce bias. The biopsies were read by a single blinded pathologist without knowledge of IVF outcome. The pregnancy tests and ultrasound results were performed without knowledge of the BCL6 results.

Study Size

Calculation of sample size as a prognostic factor for pregnancy was performed according to the literature (21). All calculations considered an alpha error of 5% and a power of 80%. We expected that 80% of patients will have an abnormal overexpression of BCL6. We considered a clinically significant relative risk (i.e., $\leq$0.2), as suggested in the literature (22), for those who have normal expression of BCL6. With these values, it would expect that at least 19 cases of pregnancy in the cohort, with a mean follow-up of 6 months, a cumulative PR of 60% and 20% in those with normal and abnormal BCL6, respectively. It would be necessary to have at least 65 subjects in the cohort, 52 in the group with abnormal BCL6 expression and 13 in the group with normal BCL6.

Comparing the proportion of BCL6 between the two groups, we expected that aberrant BCL6 expression would be present in 80% of the population, and 20% would have a normal (low) expression of BCL6 (10). With this in mind, sample size was calculated according to the literature (23) and it was verified that at least 14 patients (7 normal and 7 overexpressed BCL6) were required to have a 80% chance of detecting, significant at the 5% level, an overexpression of BCL6 in 80% of the nonpregnant group, whereas BCL6 would be overexpressed in 20% in the normal (pregnant) group.

Quantitative Variables

Age (years-old and months), BMI, and peak E_2 were analyzed as continuous variables. Days of stimulation, number of oocytes retrieved, fertilized oocytes, and number of embryos

TABLE 2

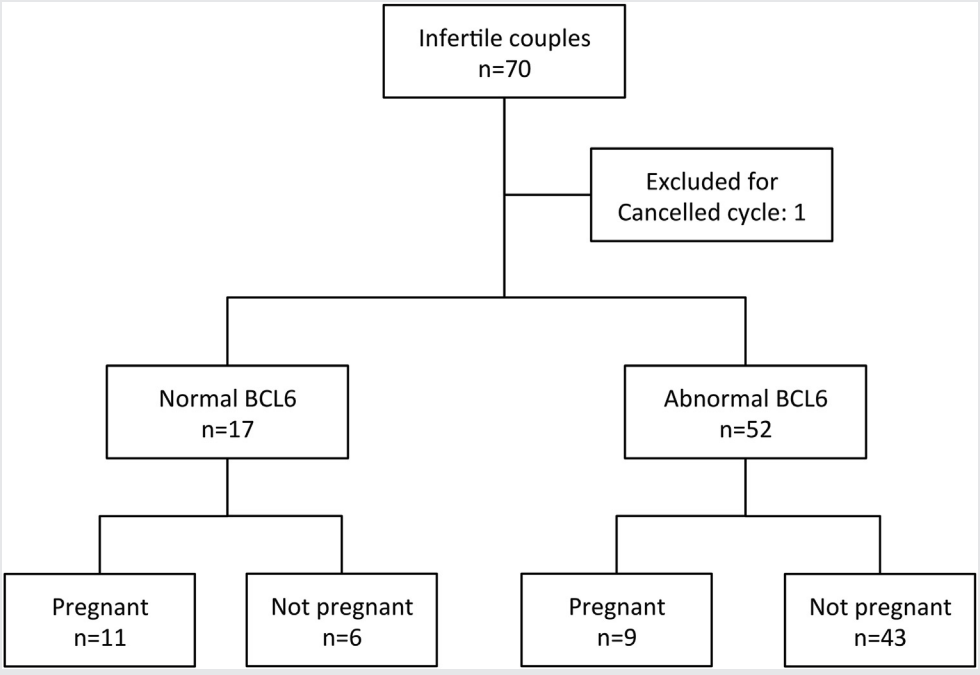
Characteristics of the sample population based on BCL6 expression.

Characteristic	Normal (n = 17)		Abnormal (n = 52)	*P* value
Age (y),mean ± SD	35.6 ± 3.1		34.8 ± 3.9	.05[a]
BMI (kg/m^2),median (range)	24.8 (18.6–36.4)		23.6 (17.9–44.6)	.9[b]
Clinical pregnancyrate, n (%)	11 (64.7)		9 (17.3)	.0004[c]
Normal BCL6, relative risk (95% CI)		0.26 (0.13–0.53)		
Live birth rate,n (%)	10 (58.8)		6 (11.5)	.0002[c]
Normal BCL6, relative risk (95% CI)		0.19 (0.08–0.45)		
Cycle characteristic				
Stimulation (d), mean ± SD	10.4 ± 2.1		10.4 ± 1.7	.9[a]
Peak E_2 (pg/mL), median (range)	1,331 (341–3,096)		1,650 (477–5,000)	.3[b]
Oocyte retrieved (n), median (range)	11 (2–35)		12.5 (2–56)	.8[b]
Oocytes fertilized (n), median (range)	6 (1–24)		7 (1–21)	.6[b]
Embryos transferred (n), median (range)	2 (1–3)		2 (1–4)	.9[b]

Note: BMI = body mass index; CI = confidence interval.
[a] Student's *t* test.
[b] Mann-Whitney *U* test.
[c] Fisher's exact test.

Almquist. Endometrial BCL6 and IVF outcome. Fertil Steril 2017.

FIGURE 1



Flow diagram of the study.

Almquist. Endometrial BCL6 and IVF outcome. Fertil Steril 2017.

transferred were analyzed as nominal variables. Clinical PR and LBR were analyzed as percentages, whereas positive or negative BCL6 were analyzed as categorical variable. The BCL6 expression (normal/abnormal) was compared with the outcome of clinical PR (pregnant, nonpregnant) or LBR. Groups were divided into normal and aberrant BCL6 because they were considered as prognostic factors for clinical PR and LBR.

Statistical Methods

Fisher's exact test, relative risk, and 95% confidence interval (CI) were used for comparisons of categorical data. Parametric data were compared between groups using Student's *t* test if data had a Gaussian distribution. Gaussian distribution was verified by D'Agostino & Pearson omnibus normality test. Mann-Whitney *U* test was used if Gaussian distribution was not present. Statistical analysis was performed with GraphPad Prism, version 6.00 for Mac, (GraphPad Software).

RESULTS

Participants and Descriptive Data

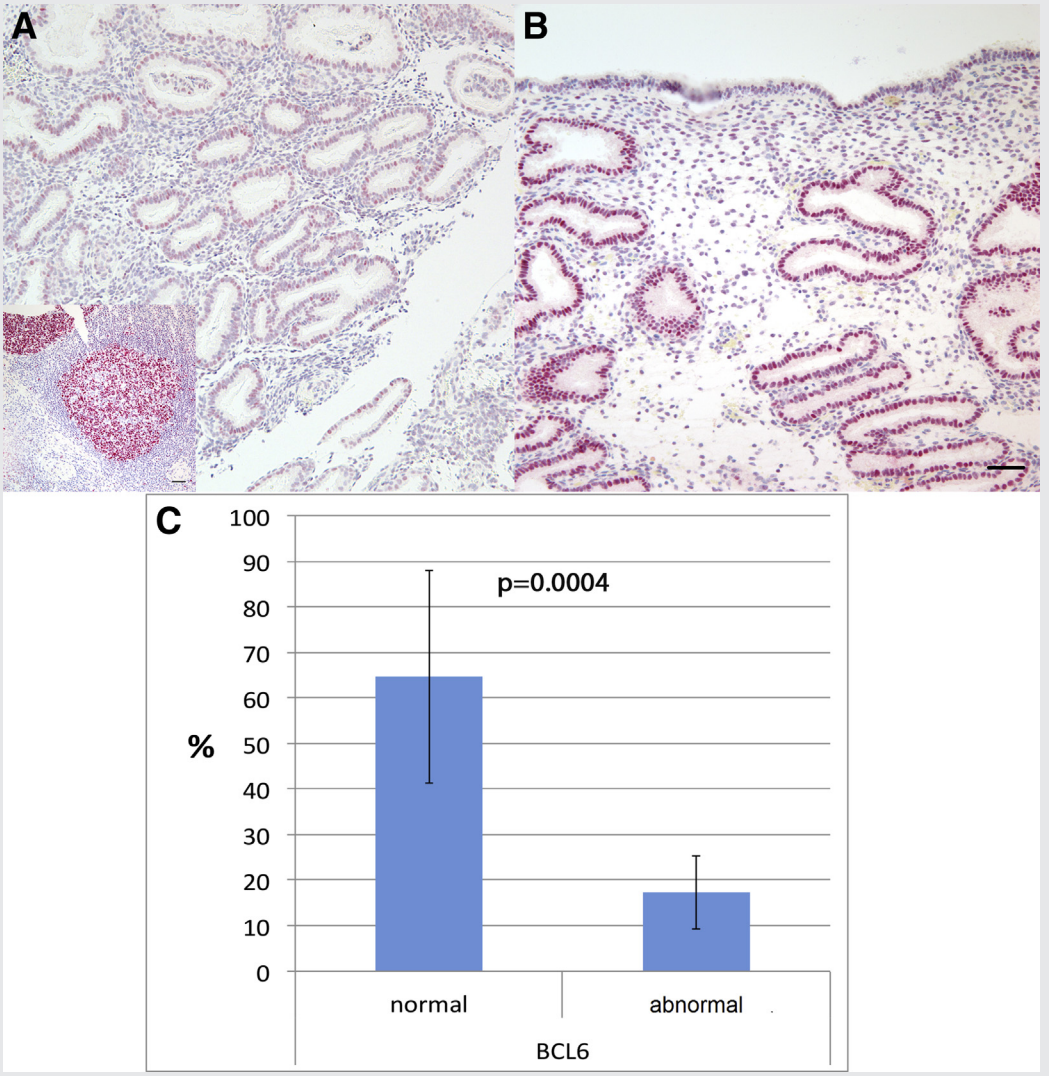
A total of 70 patients met the inclusion criteria and 69 completed a fresh IVF cycle with follow-up and these patients were analyzed. Age, BMI, days of stimulation, peak E_2, median number of oocyte retrieved, fertilized, and transferred were similar between pregnant (n = 20) and not pregnant (n = 49) groups (Table 1). Results were also examined based on BCL6 expression (Table 2 and Fig. 1).

Outcome Data and Main Results

Subjects who were pregnant or not pregnant were similar in terms of age, BMI, peak E_2, days of stimulation, and embryo transferred (Table 1). The overall clinical PR in 69 cycles of IVF was 29% (95% CI 19.6–40.5). The HSCORE of >1.4 for BCL6 was more than the cutoff value in 52 of 69 patients (75.3%; 95% CI 64–84). Staining for BCL6 was predominantly localized in the nucleus (Fig. 2A and B). Normal BCL6 expression had low-level staining (≤1.4) (Fig. 2A). Abnormal samples exhibited aberrant expression of BCL6 (>1.4) (Fig. 2B). Lymph node was used as a positive control and exhibited high BCL6 expression (Fig. 2A, inset). Based on BCL6 results alone, women with normal BCL6 expression had a significantly higher clinical PR (11/17; 64.7%; 95% CI 41.3–82.6) compared with women with elevated BCL6 levels (9/52; 17.3%; 95% CI 9.3–30.8), as shown in Figure 2C. These results yield a relative risk of 0.267 (95% CI 0.13–0.53; *P*=.0004) for those with normal BCL6 expression; an absolute benefit of 47.4% (95% CI 22.5–72.2) was found. The LBR was also significantly higher in women with low BCL6 expression (10/17; 58.8; 95% CI 36.0–78.4) compared with women with abnormal BCL6 expression (6/52; 11.5%; 95% CI 5.4–23.0). The relative risk was 0.19 (95% CI 0.08–0.45; *P*=.0002), yielding an absolute benefit of 47.3% (95% CI 21.8–67.8).

In conclusions, abnormal expression of BCL6 (HSCORE, >1.4) was strongly associated with poor reproductive outcomes in IVF cycles. Our previous study reported that elevated expression of BCL6 is a validated biomarker for detection of endometrial inflammation and it is most commonly

FIGURE 2



Expression patterns of BCL6. (**A**) Normal, in phase endometrium; inset: lymph node (positive control). (**B**) Endometrium from patient with endometriosis who had IVF failure. Bars = 50 μm. (**C**) Percentage of pregnant women (bars = 95% confidence interval) according to BCL6 expression: normal ($\leq$1.4 HSCORE) or abnormal (overexpressed) of BCL6 (>1.4 HSCORE).

Almquist. Endometrial BCL6 and IVF outcome. Fertil Steril 2017.

associated with endometriosis in women with UI (10). In the current study, we find that 75.3% of patients with UI tested positive for BCL6, similar to the previous report (10). This is the first article to examine BCL6 as a prognostic marker for IVF outcome and suggests that endometriosis may be commonly associated with IVF failure in UI. Aberrantly elevated BCL6 expression is analogous to what other investigators have reported for abnormal aromatase expression in women with IVF failure (24). Interestingly, BCL6 is associated with the similar inflammatory pathways involved in aromatase overexpression (25). Other alterations in endometrial receptivity have been described in endometriosis and UI, including reduced leukemia inhibitor factor (LIF) expression in UI (26–30) and IVF failure (31). The LIF is essential for the establishment of pregnancy, and defects in LIF expression have been associated with endometriosis and adenomyosis (32–35) and tubal disease with hydrosalpinx (36), similar to reports on aberrant BCL6 overexpression (10).

Abnormal BCL6 expression in the endometrium of women with UI is associated with an endometrial P resistance (37), which can help explain the association with poor IVF outcome. Progesterone is essential for the establishment of pregnancy, therefore reduction in P action would logically be associated with multiple down-stream changes in gene expression in the endometrium. In addition, inflammation is associated with P resistance and an immune-regulated impact on the endometrium (38, 39). BCL6 pairs with the histone deacetylase SIRT1, is centrally associated with epigenetic

alterations in endometrial gene expression associated with endometriosis, and likely has multiple effects on other key down-stream P-regulated genes (17).

The mean clinical PR published by SART for unknown factor for all ages (i.e., 27.1%) (1) is similar to the overall clinical PR of our study (20/69, 28.5%; 95% CI 19.0–41.3) found in our study. Thus, most women with UI were not successful at achieving pregnancy. Although overall IVF success rates have improved during the past 10 years, they remain <50% per cycle for most centers. There has been a reduction in the use of laparoscopy in infertile women (40) and the proportion of women in the SART database with endometriosis as their diagnosis has steadily decreased, whereas the proportion of women with unspecified diagnoses has increased (3). Furthermore, those women previously diagnosed have all had surgery for endometriosis and may no longer be representative of a larger proportion of women with undiagnosed (active) endometriosis.

This study has a few weaknesses. The prevalence of aberrant endometrial BCL6 was high in our study population (75.3%), raising concerns about the external validity. We previously reported, however, that BCL6 was elevated in 80% of women with UI (10), and our population reflects this diagnosis. The effect of endometrial scratching may have had benefit on implantation rates before IVF, although data on this topic are controversial (41, 42), but as scratching was performed in all subjects this should not be a confounder.

There are clear strengths in this study. This is a prospective cohort and subjects were recruited at a common point in their evaluation. Second, we used two clinically relevant outcomes (clinical PR and LBR). The follow-up for all patients was uniform and complete, using nonbiased assessment for pregnancy outcome. The pregnancy tests and ultrasound results were performed without knowledge of the biopsy results. The biopsies were read by a single gynecologic pathologist without knowledge of IVF outcome. We reported a 0.26 relative beneficial risk (Table 1) and this should call attention for other centers to try to reproduce these data (22), as an abnormal BCL6 expression in the UI population before IVF reduces the chance of having a successful IVF treatment in 74% of the population. The number of subjects with UI in our study was large enough and allowed us to perform a post-hoc power analysis for the association of BCL6 expression and pregnancy outcome. The significant difference found in those who did and did not get pregnant based on BCL6 immunohistochemical positivity had a power of 97.4%. We expect our data to have external validity, as our results are similar to those published at the SART database. Finally, the BCL6 test has been validated in women with UI and shown previously to be associated with both endometriosis and P resistance (10, 17), lending credence to the results obtained.

In conclusion, the aberrant expression of endometrial BCL6 is associated with poor reproductive outcomes in subsequent IVF cycles. As a biomarker for endometriosis, high levels of BCL6 expression in this cohort suggests that undiagnosed endometriosis may be a common factor that needs to be considered in women before undergoing IVF. More research is required to identify the factor(s) involved in implantation defects and to determine the best treatments before IVF treatment for women with abnormal BCL6 expression.

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