Does an increased body mass index affect endometrial gene expression patterns in infertile patients? A functional genomics analysis

Ioanna A. Comstock, M.D.,[a] Patricia Diaz-Gimeno, Ph.D.,[b] Sergio Cabanillas, M.D.,[b] Jose Bellver, M.D.,[b] Patricia Sebastian-Leon, Ph.D.,[b] Meera Shah, M.D.,[a] Amy Schutt, M.D.,[c] Cecilia T. Valdes, M.D.,[c] Maria Ruiz-Alonso, M.Sc.,[d] Diana Valbuena, M.D., Ph.D.,[d] Carlos Simon, M.D., Ph.D.,[a,b,c,d] and Ruth B. Lathi, M.D.[a]

[a] Stanford University Clinic for Reproductive Medicine, Sunnyvale, California; [b] Valencia University/Instituto Valenciano de Infertilidad, Valencia, Spain; [c] Baylor Family Fertility Center, Texas Children's Hospital Pavilion for Women, Houston, Texas; and [d] Igenomix, Valencia, Spain

Objective: To analyze the transcriptomic profile of endometrial gene alterations during the window of implantation in infertile obese patients.
Design: Multicenter, prospective, case–control study.
Setting: Three academic medical centers for reproductive medicine.
Patient(s): Infertile patients, stratified into body mass index (BMI) categories according to the World Health Organization guidelines, were included in the study.
Intervention(s): Endometrial samples were obtained from women undergoing standardized estrogen and P replacement cycles after 5 days of vaginal P supplementation.
Main Outcome Measure(s): To identify endometrial gene expression alterations that occur during the window of implantation in infertile obese patients as compared with infertile normal-weight controls using a microarray analysis.
Result(s): *XCL1*, *XCL2*, *HMHA1*, *S100A1*, *KLRC1*, *COTL1*, *COL16A1*, *KRT7*, and *MFAP5* are significantly dysregulated during the window of implantation in the receptive endometrium of obese patients. *COL16A1*, *COTL1*, *HMHA1*, *KRCL1*, *XCL1*, and *XCL2* were down-regulated and *KRT7*, *MFAP5*, and *S100A1* were up-regulated in the endometrium of obese patients. These genes are mainly involved in chemokine, cytokine, and immune system activity and in the structural extracellular matrix and protein-binding molecular functions.
Conclusion(s): Obesity is associated with significant endometrial transcriptomic differences as compared with non-obese subjects. Altered endometrial gene expression in obese patients may contribute to the lower implantation rates and increased miscarriage rates seen in obese infertile patients.
Clinical Trial Registration Number: NCT02205866. (Fertil Steril® 2017;107:740–8. ©2016 by American Society for Reproductive Medicine.)
Key Words: Endometrial gene expression, endometrial receptivity, infertility, metabolic syndrome, obesity

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The prevalence of maternal obesity has increased substantially over the last several decades and is a public health concern worldwide. The recent National Health and Nutrition Examination Survey found that among reproductive-age women in the United States approximately two-thirds are overweight (body mass index [BMI] $\geq$25 kg/m^2) or obese (BMI $\geq$30 kg/m^2), more than one-third (36%) are obese, and 8% have a BMI $\geq$40 kg/m^2 (1). This trend has serious implications for the general health of women as well as their reproductive potential.

Although many obese women conceive spontaneously, obesity adversely affects fertility. Obese women are three times more likely to suffer from anovulatory infertility than patients with a normal BMI (2). Even if the patients are ovulatory, the time to conception is

Received August 31, 2016; revised October 25, 2016; accepted November 9, 2016; published online December 2, 2016.
P.D.-G. has a patent pending on the ERA tool but has not received any royalty. I.A.C. has nothing to disclose. S.C. has nothing to disclose. J.B. has nothing to disclose. P.S.-L. has nothing to disclose. M.S. has nothing to disclose. A.S. has nothing to disclose. C.T.V. has nothing to disclose. M.R.-A. has nothing to disclose. D.V. has nothing to disclose. C.S. has nothing to disclose. R.B.L. has nothing to disclose.
I.A.C.'s present address is: 4435 Volta Place NW, Washington, DC 20007.
Reprint requests: Ioanna A. Comstock, M.D., Department of Obstetrics and Gynecology, Division of Reproductive Endocrinology and Infertility, Stanford University Medical Center, 1195 West Fremont Ave., Sunnyvale, California 94087 (E-mail: yanna.comstock@gmail.com).

Fertility and Sterility® Vol. 107, No. 3, March 2017 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2016.11.009

two-fold longer in overweight patients (3). Furthermore, obesity negatively impacts outcomes of assisted reproduction, with lower implantation and clinical pregnancy rates, higher miscarriage rates, and decreased live birth rates as compared with normal-weight women (4–7). However, it is unclear whether these negative pregnancy outcomes are due to factors affecting the endometrium and/or oocyte/embryo quality.

Perhaps the best human model for distinguishing the effect of an elevated BMI on oocyte/embryo quality from the endometrial factor is the oocyte donation model. A retrospective study investigated the effect of obesity on endometrial receptivity in 9,587 first-cycle recipients of non-obese donor oocytes (8). The authors reported a statistically significant decrease in implantation, clinical pregnancy, and ongoing pregnancy rates as BMI increased in the recipients. Although earlier studies had conflicting results (9, 10), more recent studies support a reduction of endometrial receptivity in obese recipients (11–13). Nevertheless, the molecular mechanism by which this occurs is still unknown.

The advancement of transcriptomics microarrays has provided a way to identify differential gene expression patterns within the endometrium. Bellver et al. (14) used a microarray to assess endometrial gene expression during the window of implantation (WOI) in natural cycles of ovulatory normal-weight and obese subjects and controlled stimulated cycles with recombinant FSH in obese patients with polycystic ovary syndrome (PCOS). After examining 28 endometrial samples, they found that obese women had a more dysregulated gene expression pattern than normal-weight controls. Furthermore, this gene dysregulation was exaggerated when obesity was associated with PCOS. However, this study did not control for the impact of ovarian dysfunction and its effects on the endometrium.

The molecular and histopathologic effect of an elevated BMI on the endometrium has yet to be fully elucidated. Insulin has been implicated in the regulation of endometrial development, metabolism, and receptivity (15, 16). Therefore, insulin resistance commonly exhibited by obese women may impart a negative influence on implantation and subsequent pregnancy. An abundance of GLUT4 glucose transporter in human adipocytes is highly correlated with insulin sensitivity. Rosenbaum et al. (17) found that obese women with normal glucose tolerance had a 40% decrease in the expression of GLUT4 in adipocyte membranes when compared with lean controls, suggesting tissue insulin resistance. A similar GLUT4 reduction has been shown in the endometrium of obese normoinsulinemic women with PCOS (18). Although its exact effect on endometrial receptivity is unclear, endometrial insulin resistance may potentially be one mechanism that negatively impacts fertility in obese patients.

The purpose of this study was to analyze the transcriptomic profile of endometrial gene alterations during the WOI in obese patients using a clinically validated microarray, the Endometrial Receptivity Array (ERA), and to determine whether these alterations were adversely affected by the presence of metabolic syndrome. The ERA is a customized microarray measuring relative expression of 238 genes that conformed to the transcriptomic signature of human endometrial receptivity (19). Gene expression is linked to a computational predictor identifying the personalized WOI in each patient and classifying the endometrial sample as receptive or nonreceptive, being pre- or postreceptive.

MATERIALS AND METHODS

Study Design

This was a multicenter, prospective, case–control study performed at the Stanford University Clinic for Reproductive Medicine (Palo Alto, CA), Valencia University/Instituto Valenciano de Infertilidad (Valencia, Spain), and the Baylor Family Fertility Center, Texas Children's Hospital Pavilion for Women (Houston, TX). It was approved by the institutional review boards of all participating sites and was registered at ClinicalTrials.gov (NCT02205866). Written, informed consent was obtained from all participants.

The use of historical cohort samples for the transcriptomics analysis was approved by the Ethics Committee of the Instituto Valenciano de Infertilidad, Valencia, Spain (1401-FIVI-002-CS).

Patients

Infertile women aged 21–45 years with a normal uterus (on two-dimensional/three-dimensional ultrasound and/or hysteroscopy) and the presence of at least one ovary were invited to participate in the study. Exclusion criteria were the presence of submucosal fibroids or polyps, intramural fibroids >4 cm, stage 3 or 4 endometriosis, or an unligated hydrosalpinx. Oocyte donors and women with a history of recurrent implantation failure (three or more unsuccessful embryo transfers) or recurrent pregnancy loss (two or more biochemical/clinical losses) were also excluded from the study.

Height and weight were measured on each patient upon enrollment. Patients were then grouped according to the World Health Organization obesity classification system (20). Normal-weight patients were defined as those having a BMI 18.5–24.9 kg/m^2, overweight as a BMI 25–29.9 kg/m^2, and obese as a BMI $\geq$30 kg/m^2 (class I, 30.0–34.9 kg/m^2; class II, 35.0–39.5 kg/m^2; class III, $\geq$40.0 kg/m^2).

Other parameters that were collected were waist-to-hip ratio, tubal patency, total motile sperm count, infertility diagnosis (including PCOS), TSH, PRL, and the presence of metabolic syndrome. The Rotterdam criteria were used to diagnose PCOS and required patients to exhibit two of the following three signs/symptoms: oligo- or anovulation, clinical and/or biochemical signs of hyperandrogenism, and sonographic evidence of polycystic ovaries (21). Metabolic laboratory tests were only obtained in patients who were at risk for having metabolic dysfunction, such as overweight/obese patients and those with a PCOS diagnosis, regardless of weight. Metabolic syndrome was defined by the presence of at least three of the following five conditions in women: waist circumference $\geq$88 cm, blood pressure $\geq$130/85 mm Hg, fasting blood glucose $\geq$100 mg/dL, triglycerides $\geq$150 mg/dL, high-density lipoprotein cholesterol <50 mg/dL (22). If patients were being treated for glucose intolerance with metformin before study enrollment, they were asked to discontinue this medication and resume it only after their study participation was completed.

Endometrial Preparation and Sampling

All participants underwent endometrial preparation with a hormone replacement cycle. By using this model, many ovarian factors affecting endometrial receptivity can be minimized. Each woman underwent estrogen (E) priming with either E_2 oral tablets (6 mg/d) or patches (E_2 hemihydrate 9.6-mg patch every 48 hours) beginning cycle day 2 or 3 of a spontaneous or induced menstrual bleed. This regimen was continued for at least 10 days, after which endometrial pattern and thickness were evaluated with transvaginal ultrasonography. When the endometrium had a trilaminar appearance and the thickness was $\geq$7 mm, vaginal P supplementation with micronized P (400 mg twice daily) was initiated. An endometrial biopsy was performed after 10 doses (or 5 days) of P supplementation. A schematic of this hormone replacement cycle and timing of the endometrial biopsy is shown in Supplemental Figure 1 (available online). The endometrial biopsy specimens were placed into a cryotube containing RNAlater (Qiagen) for ERA transcriptomic analysis.

RNA Isolation and Microarray Hybridization

Ribonucleic acid was extracted from the endometrium, assessed, and hybridized according to ERA technology as previously described (19). All samples were unidentified and sent to the same laboratory for ERA analysis.

Transcriptomics Analyses

Standardized WOI transcriptomics analysis with the ERA was performed as described previously (19). Data were normalized using extra quantile normalization to avoid batch effects in gene distribution (23).

All the statistical analyses and file processing were implemented in R statistical software version 3.2.0 (24). The exploratory analysis was performed using principal component analysis with the R PRcomp function. A concentration ellipse using the factoextra R package (25) with a confidence interval of 99% was implemented for analyzing sample distribution and detection of outliers (Supplemental Fig. 1B).

The limma R package (26) from Bioconductor was used for statistical analyses (i.e., *t* test, two-way analysis of variance) to identify endometrial gene expression patterns associated with ERA results of receptivity, with BMI, and a combination of both variables. An adjusted P value $<.05$ was considered statistically significant. An adjusted P value using the Benjamini-Hochberg false discovery rate (FDR) (27) was calculated for conducting multiple comparisons in a microarray analysis. The statistical power of each comparison was calculated using SizePower R library (28).

The functional annotation of biomarkers was conducted using BioMart Ensembl Gene 84 version for Gene Ontology (29). Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation was performed with the KEGG mapper tool from the KEGG pathways database (30). The functional relationship between genes was analyzed using network modeling of overlapping gene ontologies and KEGG terms to identify the functional meaning of these obesity biomarkers in reproductive physiology. Network relationships between genes and their functions were visualized using Cytoscape (31). Coexpression relationships between these genes from external databases were analyzed using GeneMania (32).

Outcomes

The primary outcome measure of the study was to identify endometrial gene expression alterations that occur during the WOI in infertile obese patients using a microarray analysis as compared with infertile normal-weight controls, and to determine whether these alterations were adversely affected by the presence of metabolic syndrome.

RESULTS

Endometrial samples from a total of 91 infertile women were collected prospectively and included in this analysis. An additional two patients were enrolled but did not complete study participation and were therefore excluded. The patients were grouped according to their BMI: normal-weight (NW) (BMI 19–24.9 kg/m^2) (n = 11), overweight (OW) (BMI 25–29.9 kg/m^2) (n = 13), obese class I (O) (BMI 30–34.9 kg/m^2) (n = 40), and obese class II and III (MO) (BMI $\geq$35 kg/m^2) (n = 27). Another classification for BMI categorized the samples into two major groups: obese (OB) (BMI $\geq$30 kg/m^2) and non-obese (NOB) (BMI <30 kg/m^2). The presence of metabolic syndrome was evaluated for in a subgroup of obese patients (n = 26).

Figure 1 summarizes the demographics of the prospectively recruited patients. The four groups were similar in age, but BMI was statistically significantly different between the groups because this criterion was used for population stratification (Fig. 1A). There was a statistically significant difference in the incidence of a PCOS diagnosis among the various groups (P=.04); however, this difference was not observed when we compared the overall NOB vs. OB groups (16.7% vs. 14.9%, respectively). The presence of metabolic syndrome was examined in a subgroup of obese patients, and there was no difference in the incidence of this disorder between class I (O) and class II and III (MO) patients.

Endometrial biopsies performed during the expected WOI were classified by the ERA as receptive or nonreceptive. The indicated proportions of receptive vs. nonreceptive patients within each BMI group are shown in Figure 1A. There was a trend for a higher incidence of a nonreceptive ERA result as BMI increased; however, this did not reach statistical significance.

The demographics of the obese patients who underwent metabolic testing are shown in Figure 1B. Obese women with metabolic syndrome were similar in age, BMI, and PCOS incidence as compared with those without metabolic syndrome. Expectedly, the patients with metabolic syndrome had a significantly higher rate of hyperglycemia, hypertriglyceridemia, and hypoalphalipoproteinemia (high-density lipoprotein deficiency). Of the 11 patients with metabolic syndrome, 6 were being treated with metformin before study enrollment. These patients, however, were instructed to discontinue metformin during the duration of study participation to minimize the influence of treatment on endometrial

FIGURE 1

A

	PROSPECTIVELY RECRUITED PATIENTS								
	Overall	NW	OW	O	MO	TEST	NOB	OB	TEST2
Total	91	11	13	40	27	N / A	24	67	N / A
Age (Yrs)	36.955 [35.914, 37.995]	33.9 [30.356, 37.444]	37.692 [35.136, 40.249]	38.103 [36.577, 39.628]	36.038 [33.981, 38.096]	0.06	36.043 [33.947, 38.14]	37.277 [36.055, 38.499]	0.3
BMI	32.409 [31.069, 33.749]	21.393 [20.225, 22.56]	27.158 [26.176, 28.139]	32.365 [31.869, 32.862]	39.49 [37.718, 41.263]	4.3E-032 ***	24.515 [23.096, 25.935]	35.237 [34.1, 36.374]	6.7E-017 *
PCOS diagnosis	15.4% (14/91)	36.4% (4/11)	0% (0/13)	10% (4/40)	22.2% (6/27)	0.04 *	16.7% (4/24)	14.9% (10/67)	1
Metabolic Syndrome	42.3% (11/26)	N / A% (0/0)	N / A% (0/0)	30.8% (4/13)	53.8% (7/13)	0.43	N / A% (0/0)	42.3% (11/26)	1
Receptive ERA	76.9% (70/91)	90.9% (10/11)	92.3% (12/13)	77.5% (31/40)	63% (17/27)	0.15	91.7% (22/24)	71.6% (48/67)	0.05

B

	OBESE PATIENTS METABOLIC SYNDROME TESTING			
	Overall	nonMS	MS	TEST
Total	26	15	11	N / A
Age (Yrs)	37.154 [34.884, 39.423]	37 [34.239, 39.761]	37.364 [32.905, 41.823]	0.87
BMI	36.708 [34.179, 39.236]	35.12 [32.402, 37.838]	38.873 [33.877, 43.869]	0.13
PCOS diagnosis	30.8% (8/26)	33.3% (5/15)	27.3% (3/11)	1
Waist circumference>88cm	100% (26/26)	100% (15/15)	100% (11/11)	1
BP>130/85 mm Hg	46.2% (12/26)	33.3% (5/15)	63.6% (7/11)	0.22
Fasting Blood Glucose>100mg/dL	23.1% (6/26)	0% (0/15)	54.5% (6/11)	0.002 *
Triglycerides>150mg/dL	38.5% (10/26)	13.3% (2/15)	72.7% (8/11)	0.004 *
HDL-C < 50mg/dL	50% (13/26)	20% (3/15)	90.9% (10/11)	0.001 *
Receptive ERA	57.7% (15/26)	60% (9/15)	54.5% (6/11)	1

Demographics for prospectively recruited study participants. (**A**) Characteristics of study participants within each BMI category. (**B**) Demographics of a subset of obese patients who underwent metabolic dysfunction testing. BMI categories: NW = BMI 19–24.9 kg/m^2; OW = BMI 25–29.9 kg/m^2; O = BMI 30–34.9 kg/m^2; MO = BMI ≥35 kg/m^2; NOB = BMI <30 kg/m^2; OB = BMI ≥30 kg/m^2. Test refers to the statistical test performed. In age and BMI variables, a *t* test comparing population mean was performed. In PCOS diagnosis, metabolic syndrome, and receptive ERA proportions, a Fisher exact test was performed. *P<.05 denotes statistical significance.

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gene expression patterns. Metabolic dysfunction did not affect the incidence of a nonreceptive ERA result.

Transcriptomics Analysis

Given the underrepresentation of NW and OW samples with a nonreceptive ERA result (only 1 per group), 18 nonreceptive historical cohort controls (10 NW, 8 OW) from the ERA database were included in the transcriptomics analysis for the study. These historical controls were obtained using the same inclusion/exclusion criteria as the prospectively recruited subjects and had endometrial biopsies during hormone replacement cycles that exactly mimicked those of the prospectively recruited patients.

Endometrial samples from 109 infertile women (91 prospectively recruited subjects and 18 historical controls) were included in the transcriptomics analysis. After outliers were removed for technical reasons (Supplemental Fig. 2A), the final transcriptomics analysis was carried out on a total of 102 endometrial samples. The demographics of the final cohort are shown in Supplemental Table 1. The populations were homogeneous and comparable, with similar ages, incidence of PCOS diagnosis, and presence of metabolic syndrome. All patients, prospectively recruited and historical controls, achieved an endometrial lining thickness ≥7 mm and a trilaminar appearance within 10–12 days of E replacement. Of 102 patients, 59 (57.8%) received the oral E replacement regimen, and 43 (42.2%) received the E patch regimen. The final cohort received the exact same vaginal P supplementation (400 mg twice daily for 5 days) before the endometrial biopsy. A principal component analysis showed a sample distribution related to endometrial receptivity (Supplemental Fig. 2B). A 99% confidence interval for receptive and nonreceptive samples indicates that the distribution of historical cohort samples is within the same distribution of the prospectively collected samples.

Obesity Biomarkers for Endometrial Receptivity

A differential expression analysis was performed to identify potential biomarkers of the effect of obesity on endometrial transcriptomics as an independent variable using different statistical approaches. Three separate analyses were performed: [1] analyzing all the samples within each BMI category independent of receptivity result; [2] analyzing samples within each BMI category that had a receptive ERA profile; and [3] analyzing samples within each BMI category that had a nonreceptive ERA profile. Interestingly, nine genes were significantly differentially expressed in the receptive endometrium of the obese (OB) (BMI ≥30 kg/m^2) vs. the non-obese (NOB) (BMI <30 kg/m^2) population (FDR <0.05) and were therefore selected as obesity biomarkers (Fig. 2). There was no statistically significant differential gene expression between BMI categories in endometrial samples that were nonreceptive.

COL16A1, *COTL1*, *HMHA1*, *KRCL1*, *XCL1*, and *XCL2* were down-regulated in the receptive endometrium of obese patients (OB) as compared with the receptive non-obese group (NOB). This down-regulation was amplified when comparing the extreme populations of class II and III obese (MO) (BMI ≥35 kg/m^2) vs. normal-weight (NW) (BMI <25 kg/m^2) (R_MOvsNW in Fig. 2A). *KRT7*, *MFAP5*, and *S100A1* were

FIGURE 2

A

	MSvsNOB		OBvsNOB		R_OBvsNW		R_OBvsNOB		MOvsOW		MOvsNW		R_MOvsNW		ANOVA BMI
GENE NAME	FC	FDR	FC	FDR	FC	FDR	FC	FDR	FC	FDR	FC	FDR	FC	FDR	FDR
COL16A1	-1.31	0.869	-1.297	0.12617	-1.458	0.2209	-1.2539	0.3391	-1.355	0.2862	-1.766	0.0029	-1.715	0.0472	0.338
COTL1	-1.48	0.028	-1.246	0.02679	-1.392	0.0656	-1.3101	0.0613	-1.353	0.0651	-1.387	0.0304	-1.5454	0.0377	0.022
HMHA1	-1.41	0.0027	-1.207	0.02679	-1.159	0.3875	-1.2264	0.0697	-1.409	0.0011	-1.307	0.0304	-1.2633	0.1432	0.004
KRT7	1.02	0.9615	1.361	0.06451	1.861	0.0283	1.5148	0.0613	1.1754	0.9942	-1.428	0.1468	1.8219	0.0524	0.228
KLRC1	-1.45	0.0323	-1.160	0.26922	-1.221	0.3047	-1.1954	0.2830	-1.248	0.2599	-1.248	0.1468	-1.3119	0.1559	0.135
MFAP5	-1.08	0.956	1.191	0.49137	1.713	0.0283	1.2809	0.3249	1.0240	0.9942	1.316	0.316	1.8706	0.0377	0.888
S100A1	1.04	0.956	1.312	0.0268	1.420	0.1764	1.3350	0.1019	1.244	0.7565	1.327	0.1468	1.4076	0.1569	0.125
XCL1	-1.33	0.0846	-1.208	0.0268	-1.337	0.0283	-1.3205	0.00675	-1.248	0.1595	-1.216	0.1468	-1.3646	0.0787	0.010
XCL2	-1.71	0.0283	-1.359	0.0244	-1.542	0.0283	-1.5309	0.00675	-1.514	0.0345	-1.474	0.0473	-1.7084	0.0377	0.0028

B

GENE	Description	FC
COL16A1	Collagen, type XVI, alpha 1 [Source:HGNC Symbol;Acc:HGNC:2193]	Down
COTL1	Coactosin-like F-actin binding protein 1 [Source:HGNC Symbol;Acc:HGNC:18304]	Down
HMHA1	Histocompatibility (minor) HA-1 [Source:HGNC Symbol;Acc:HGNC:17102]	Down
KRT7	Keratin 7, type II [Source:HGNC Symbol;Acc:HGNC:6445]	Up
KLRC1	killer cell lectin-like receptor subfamily C, member 1 [Source:HGNC Symbol;Acc:HGNC:6374]	Down
MFAP5	Microfibrillar associated protein 5 [Source:HGNC Symbol;Acc:HGNC:29673]	Up
S100A1	S100 calcium binding protein A1 [Source:HGNC Symbol;Acc:HGNC:10486]	Up
XCL1	Chemokine (C motif) ligand 1 [Source:HGNC Symbol;Acc:HGNC:10645]	Down
XCL2	Chemokine (C motif) ligand 2 [Source:HGNC Symbol;Acc:HGNC:10646]	Down

Obesity biomarkers for endometrial receptivity. (**A**) Table of the nine genes that were identified as proposed biomarkers from all statistical analyses performed in the differential expression analysis and their differential gene expression patterns when comparing various groups of samples. Gray color is highlighting statistically significant adjusted *P* values. (**B**) List of gene descriptions of the obesity biomarkers and their overall fold change (FC). Fold change is summarized as up- or down-regulation. BMI categories: NW = BMI 19–24.9 kg/m^2; OW = BMI 25–29.9 kg/m^2; O = BMI 30–34.9 kg/m^2; MO = BMI ≥35 kg/m^2; NOB = BMI <30 kg/m^2; OB = BMI ≥30 kg/m^2.

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up-regulated in the receptive endometrium of obese patients as compared with the non-obese group (R_OBvsNOB in Fig. 2A).

The presence of metabolic syndrome in a subgroup of obese patients is shown to further affect endometrial gene expression in four obesity biomarkers. In receptive endometria, there is a statistically significant increased down-regulation of *COTL1*, *HMHA1*, *KLRC1*, and *XCL2* (Fig. 2A) when comparing the non-obese population (NOB) vs. obese patients with metabolic syndrome (MSvsNOB in Fig. 2A).

To better illustrate the pattern of differential gene expression of the obesity biomarkers within the endometrium, a gene expression analysis along increasing BMI in receptive and nonreceptive samples was performed (Fig. 3). The genes that were found to be up-regulated in the endometrium of obese patients (*KRT7*, *MFAP5*, *S100A1*) (Fig. 2B) had similar expression in the normal-weight group (NW) regardless of whether the endometrium was receptive or nonreceptive. However, the level of gene expression in the receptive endometrium increased as BMI increased, and these changes become apparent even in the overweight population (OW) (Fig. 3A). Furthermore, the level of gene expression of *COTL1*, *HMHA1*, *XCL2*, *XCL1*, and *KLRC1* decreased with increasing BMI in receptive samples and ultimately had respective levels of gene expression similar to those of nonreceptive normal-weight controls (Fig. 3A). This down-regulation of the aforementioned genes was exaggerated in the receptive endometrium of patients with metabolic syndrome as compared with non-obese patients (NOB = NW+OW) and obese patients without metabolic syndrome (Fig. 3B).

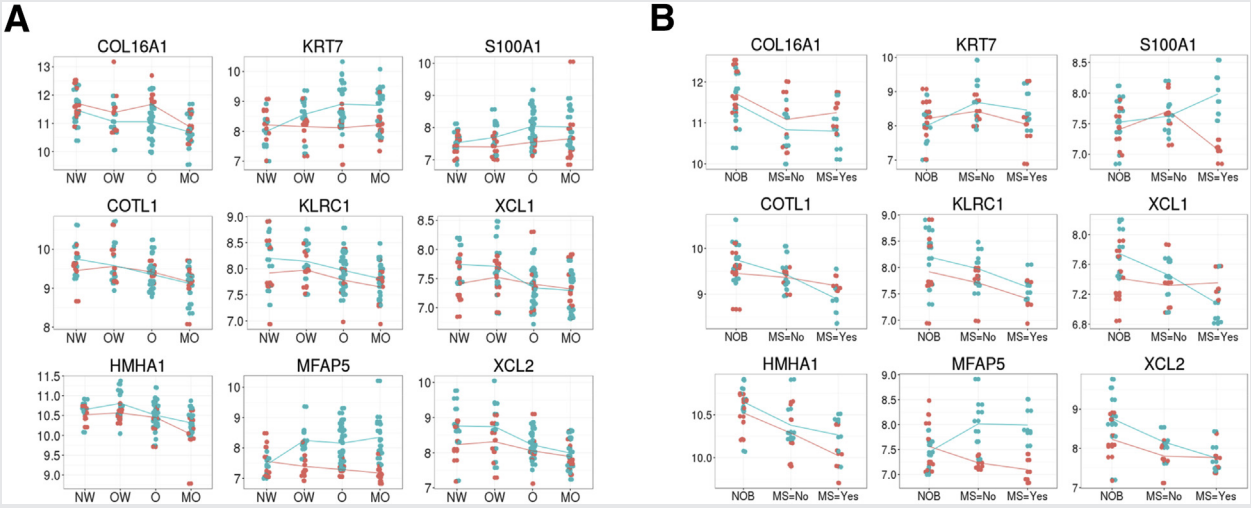
To ensure that we were accurately describing the differential gene expression patterns shown in our study, a power analysis was performed and is shown in Supplemental Table 2. With the sample size of 102 subjects in the transcriptomics analysis, we were powered to detect a 99.9% difference in gene expression as it relates to increasing BMI and a 79.8% difference between the gene expression patterns in obese patients with and without metabolic syndrome.

Functional Gene Significance in Reproductive Physiology

Gene ontology was used to investigate the biologic processes, molecular functions, cellular components, and KEGG pathways for each of the nine endometrial biomarkers. Gene descriptions and functional annotations are summarized in Supplemental Table 3.

When examining the gene ontology molecular functions, all nine differentially expressed genes in the receptive endometrium of obese patients were involved in protein binding,

FIGURE 3



Obesity biomarkers gene expression patterns in receptive and nonreceptive samples of increasing BMI. Points represent the median value of gene expression for each gene (log2) in the *y* axis for receptive (*green*) and nonreceptive (*red*) populations; in the *x* axis are the various BMI groups. (**A**) The *x* axis represents the BMI categories: NW = BMI 19–24.9 kg/m^2; OW = BMI 25–29.9 kg/m^2; O = BMI 30–34.9 kg/m^2; MO = BMI ≥35 kg/m^2. (**B**) The *x* axis represents the non-obese patients (NOB; BMI <30 kg/m^2), obese patients without metabolic syndrome (MS=NO), and obese patients with metabolic syndrome (MS=YES).

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and *XCL1* and *XCL2* were additionally involved in chemokine activity. We discovered that the main biologic processes of the biomarkers were involved in immune response, chemotaxis, signal transduction, and extracellular matrix organization. The cellular components of these genes were located within the intracellular region, within the plasma membrane, and within the extracellular region, indicating that they encompass a complete cellular component pathway for signal transduction to occur.

Only three genes (*KRCL1*, *XCL1*, and *XCL2*) were noted in KEGG pathways database. We used this database to characterize the relationship between these biomarkers and obesity. Supplemental Tables 4 and 5 summarize the four main pathways that involve our biomarkers and their interaction with gene pathways related to four KEGG classifications—metabolism, endocrine system, endocrine and metabolic diseases, and obesity-related diseases. The network model proposed in Figure 4A illustrates the complex links between different signaling cascades involving three of our endometrial obesity biomarkers and genes involved in metabolism, endocrine function, metabolic diseases, and reproduction. A strong association between the three biomarkers and KEGG pathways involved in obesity-related diseases was identified and is shown in Figure 4B. In particular, the chemokine signaling pathway genes (*XCL1* and *XCL2*) were strongly overlapping with genes related to type 2 diabetes mellitus and insulin secretion.

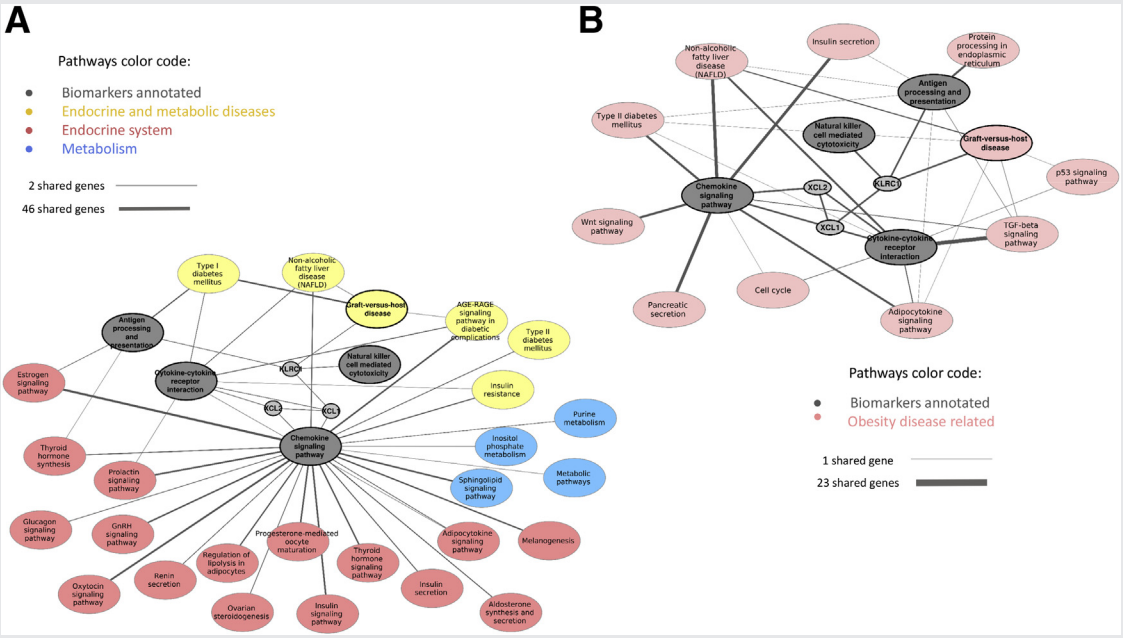
DISCUSSION

Obesity impairs fecundity by negatively impacting various aspects of the female reproductive tract, including the endometrium. This study of an infertile population demonstrates that obesity is associated with significant endometrial transcriptomic differences during the WOI of receptive endometria when compared with non-obese subjects. Our findings have identified a particular subset of genes implicated in this endometrial alteration. We hypothesize that this endometrial gene expression alteration is a significant contributor to poorer reproductive outcomes in infertile overweight/obese women.

The ERA was used to determine endometrial receptivity during the WOI in this study. By comparing only samples that were determined to be receptive by the ERA, we can look specifically at a cohort of samples that are presumed as "optimal" within the WOI. Despite this normalization, we are still able to detect significant differences in gene expression among the various BMI categories. This transcriptomics analysis shows that there is a down-regulation of particular biomarkers included in the ERA (*COTL1*, *HMHA1*, *XCL2*, *XCL1*, and *KLRC1*), with levels of expression in receptive obese samples that are similar to those of nonreceptive normal-weight controls (Fig. 3A). This down-regulation was even more pronounced in obese patients with evidence of metabolic syndrome, despite nearly half of the patients with metabolic syndrome (6 of 11) having had some prior treatment with metformin (Fig. 3B).

We propose that these identified endometrial gene alterations may adversely affect the WOI in obese patients. The biologic processes of several of these biomarkers relate to the immune response, which has been implicated in embryo implantation in prior studies (33, 34). However, large prospective studies are needed to determine whether these alterations in gene expression can ultimately lead to a higher incidence of a nonreceptive endometrium and decreased implantation rates as BMI increases.

FIGURE 4



Pathway network modeling between biomarker genes, obesity, and endometrial receptivity. Network model of three obesity biomarkers (*KLRC1*, *XCL1*, and *XCL2*) and their interaction with gene pathways related to four KEGG classifications—metabolism, endocrine system, endocrine and metabolic diseases, and obesity-related diseases. *Gray* nodes are KEGG pathways, and edge thickness is related to the number of genes that are shared between two pathways. (**A**) Relationship between pathways related to endocrine system (*red*), endocrine and metabolism diseases (*yellow*), and metabolism (*blue*) and the obesity biomarkers. (**B**) Relationship between obesity diseases associated pathways (*pink*) and the obesity biomarkers.

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Additionally, genes that are up-regulated (*MFAP5*, *KRT7*, and *S100A1*) in overweight/obese receptive endometrial samples are related to extracellular structural and calcium-binding matrix functions. Endometrial stromal cells undergo decidualization, which involves significant changes in the extracellular matrix and cytoskeletal organization to regulate placental trophoblastic invasion (35). These gene alterations seen in our obese population could, therefore, indirectly be affecting the proper gene expression necessary for normal decidualization.

Our analysis of the functional meaning of these genes in reproductive physiology suggests that these endometrial gene alterations may represent the adverse effect of obesity and its associated metabolic dysfunction on the endometrium. The target biomarkers, as shown in the Supplemental Tables 3–5 and in Figure 4, have been implicated in other target tissues that are clearly affected by obesity and the presence of metabolic syndrome. For example, *XCL1* and *XCL2* are involved in the positive regulation of extracellular signal-regulated kinases (ERKs) 1 and 2 pathways. The ERK pathways, a subfamily of the mitogen-activated protein kinases (MAPKs), can be activated by many different stimuli, including growth factors and cytokines, and are involved in essential cellular processes, such as proliferation and differentiation (36). The ERK pathway has been shown to be activated by adipogenic stimuli, such as insulin, leading to adipocyte hypertrophy, recruitment of new adipocytes through differentiation, and development of insulin resistance in obesity (37). In a murine model, ERK1 knockout mice given a high-fat diet were shown to be resistant to diet-induced obesity and protected from developing insulin resistance (38).

Regarding the endometrium, extra-villous trophoblast invasion is essential for normal placentation and fetal growth. Epidermal growth factor plays a role in the migration and invasion of trophoblasts into the endometrium via activation of MAPK/ERK pathways (39). In our study, the ERK signal transduction was found to be down-regulated during the WOI in obese patients. This, perhaps, represents one possible mechanism of decreased implantation rates, higher miscarriage rates, and poorer obstetric outcomes associated with obesity.

The present prospective study was performed in a very well defined study population undergoing endometrial preparation with a standardized hormone replacement cycle. This allowed for us to minimize the effect of many non-uterine factors on endometrial receptivity, particularly ovarian dysfunction, which is often encountered in an obese population. The ERA test was used to evaluate the endometrial transcriptomic profile because of its accuracy and validity in examining differential gene expression during the WOI (19, 40, 41). A power analysis demonstrated that we included enough samples to effectively describe the difference in gene expression patterns during the WOI among the various BMI groups in our transcriptomics analysis.

Certain limitations of this study, however, should be considered. A larger number of obese individuals screened for metabolic syndrome would provide a more accurate representation of the effect of metabolic dysfunction on endometrial gene expression. Additionally, a greater number of subjects in all BMI categories would have better powered the study to examine whether the alteration of our proposed biomarkers are in fact leading to a displaced window of implantation as BMI increases. Furthermore, the endometrium of obese patients may not respond similarly to a standardized dosage of vaginal P as that of normal-weight individuals. This potential inadequate absorption of vaginal P may also be contributing to the observed trend in a higher incidence of a nonreceptive endometrium as BMI increased. Finally, clinical outcomes of fertility treatment were not assessed but would certainly contribute to our understanding of the role of the endometrium in infertile obese patients.

In conclusion, obesity was shown to be associated with significant endometrial gene expression alteration during the optimal WOI, especially in patients with metabolic syndrome. As BMI increased, there was a higher incidence of a nonreceptive endometrium and higher fold changes of gene expression. This endometrial gene dysregulation possibly contributes to the increased risk of infertility, adverse pregnancy outcomes, and poor IVF outcomes seen in obese women. Because we observed a similar up-regulation of specific biomarkers (*MFAP5*, *KRT7*, and *S100A1*) in the overweight group as in those patients meeting obesity criteria, this may support the benefit of weight loss even in patients with borderline BMIs, although the effect of weight loss on endometrial receptivity is currently unknown. Additionally, the presence of metabolic syndrome in obese patients exaggerated this gene alteration and warrants further investigation. Further large, prospective studies are required to expand our current understanding of the molecular and pathophysiologic effects of obesity on endometrial function.

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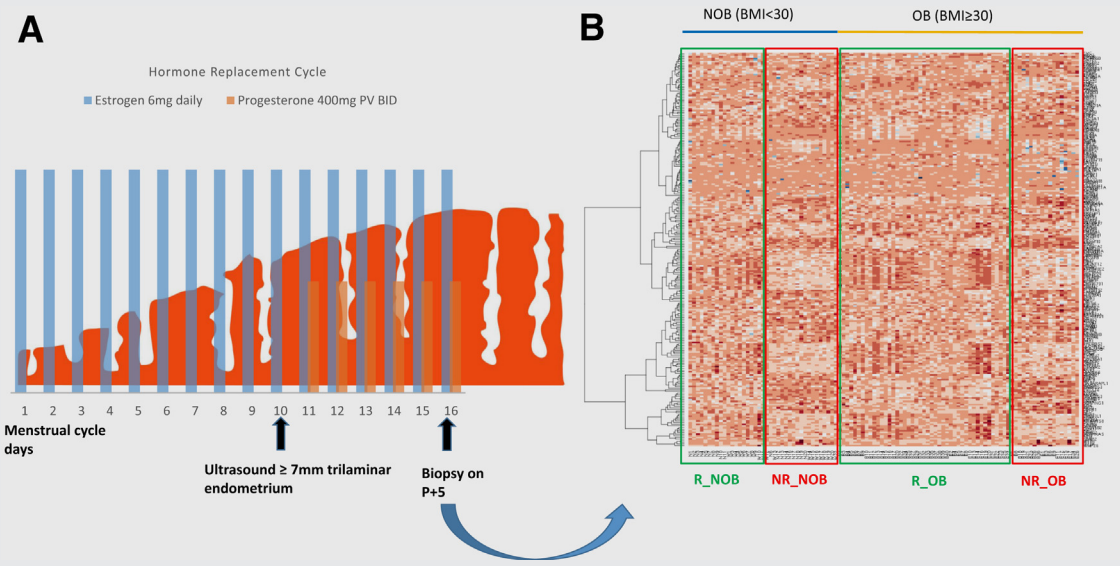
SUPPLEMENTAL FIGURE 1



Endometrial Receptivity Array hormone replacement cycle and transcriptomics analysis. (**A**) Timing of E and P administration and ERA biopsy for study participants. (**B**) Heatmap showing the gene expression in endometrial samples included in the final transcriptomics analysis. Non-obese samples (NOB) divided into receptive (R_NOB) or nonreceptive (NR_NOB) and obese samples (OB) divided into receptive (R_OB) and nonreceptive (NR_OB).

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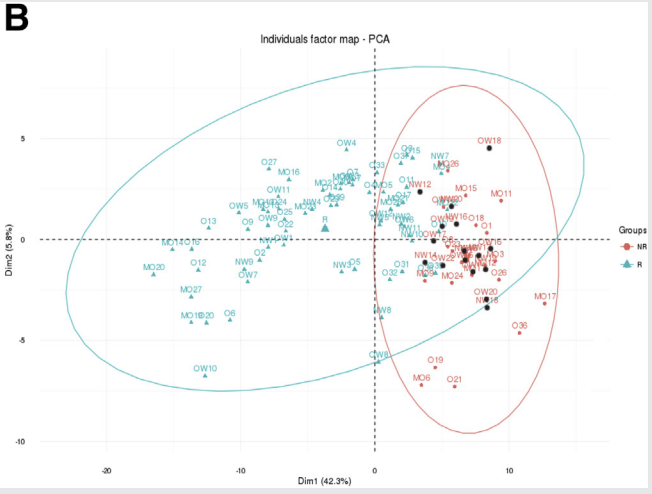
SUPPLEMENTAL FIGURE 2

A

N= 109			ERA	
			NR	R
BMI	OB	O	8 (1)	29 (2)
		MO	10	16 (1)
	NOB	NW	10 (1)	10
		OW	8 (1)	11 (1)

B



Transcriptomics analysis. (**A**) Eighteen nonreceptive non-obese historical controls were added to the transcriptomics analysis and are shown in *red*. A total of seven samples (indicated in parentheses) were detected as outliers and therefore excluded from the final transcriptomic analysis. (**B**) Principal component analysis (PCA) with sample distribution for all samples used in the transcriptomics analyisis in an ellipse concentration. The distribution of samples is related to ERA determination of receptivity. *Black dots* represent the 16 nonreceptive historical controls used in the final transcriptomics analysis (two historical controls detected as outliers). BMI categories: NW = BMI 19–24.9 kg/m^2; OW = BMI 25–29.9 kg/m^2; O = BMI 30–34.9 kg/m^2; MO = BMI ≥35 kg/m^2; NOB = BMI <30 kg/m^2; OB = BMI ≥30 kg/m^2.

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