ORIGINAL ARTICLE: REPRODUCTIVE SCIENCE

Elevated levels of adrenomedullin in eutopic endometrium and plasma from women with endometriosis

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Objective: To test adrenomedullin (*Adm*, ADM) as a downstream target of signal transducer and activator of transcription 3 (STAT3) in endometrial cells and to test midregional proadrenomedullin (MR-proADM) as a biomarker of endometriosis.
Design: Cross-sectional analysis of *Adm* expression in eutopic endometrium and of MR-proADM in plasma from women with and without endometriosis; and prospective study of MR-proADM levels in women with endometriosis undergoing surgical resection of ectopic lesions.
Setting: Academic medical centers.
Patient(s): Fifteen patients with endometriosis and 11 healthy control subjects who donated eutopic endometrial biopsies; and 28 patients with endometriosis and 19 healthy control subjects who donated plasma for MR-proADM analysis.
Intervention(s): None.
Main Outcome Measure(s): *Adm* mRNA levels according to quantitative real-time polymerase chain reaction after activation of STAT3 by interleukin-6 (IL-6) in Ishikawa cells; immunohistochemistry for ADM in eutopic endometrial biopsies from women with endometriosis compared with healthy donors; and MR-proADM levels measured by commercial immunoassay in plasma from healthy women and women with endometriosis who subsequently underwent surgical resection of ectopic lesions.
Result(s): Activation of STAT3 by IL-6 up-regulated *Adm* mRNA expression in Ishikawa cells. ADM protein levels were elevated in the eutopic endometrium of women with endometriosis. MR-proADM concentrations were higher in women with endometriosis but were not correlated with disease stage, corrected by surgery, or predictive of fertility outcome.
Conclusion(s): MR-proADM may be able to serve as a biomarker of endometriosis, but it is unknown whether elevated MR-proADM levels are secondary to the estrogenic and inflammatory properties of endometriosis or an inciting pathogenic factor. (Fertil Steril® 2018;■:■–■. ©2018 by American Society for Reproductive Medicine.)
Key Words: Adrenomedullin, endometriosis, midregional proadrenomedullin

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Endometriosis is a common gynecologic disease characterized by the presence of endometrial tissue outside of the eutopic endometrium, commonly in the peritoneum of the pelvis and in pelvic organs, and often causing scarring and pain (1, 2). Although endometriosis is also strongly associated with subfertility and infertility, the mechanisms underlying fertility problems in women with stage 1–2 disease is a subject of debate (3, 4). The nonspecific nature of endometriosis symptoms makes the disease difficult to diagnose, and confident diagnosis usually requires visualization of ectopic lesions during surgical exploration. Endometriosis is equally difficult to treat; hormonal therapies and surgical excision of ectopic lesions are the mainstays of treatment but are not always effective (5, 6). These diagnostic and therapeutic challenges have fueled interest in identifying biomarkers and signaling pathways associated with endometriosis (7).

Recently, Kim et al. demonstrated aberrant activation of signal transducer and activator of transcription 3 (STAT3) in the eutopic endometrium of women with endometriosis (8). Other studies consistently point to

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endometriosis-associated factors that affect STAT3 activation: the cytokine interleukin 6 (IL-6), which activates STAT3 via the IL-6 receptor, is elevated in the peritoneal fluid of women with endometriosis (9–11); miR120, which targets STAT3, is elevated in endometriotic cyst stromal cells (12); and protein inhibitor of activated STAT3 and dual-specificity phosphatase 2, negative regulators of STAT3, are down-regulated in endometriosis (13, 14). Kim et al. also found hypoxia inducible factor 1A (HIF1A), which is stabilized by STAT3, to be elevated in the eutopic endometrium of women with endometriosis. Taken together, these data strongly implicate the IL-6–STAT3–HIF1A pathway in the pathophysiology of endometriosis (8).

Both STAT3 and HIF1A have been previously identified as regulators of the versatile peptide hormone adrenomedullin (*Adm*, ADM) (15–18). ADM is expressed in the female reproductive system and has been associated with female reproductive physiology, including embryo implantation and placentation, and pathophysiology, including subfertility and complications of pregnancy, such as preeclampsia (19, 20). In endometriosis, ADM is higher in intrafollicular fluid and negatively associated with oocyte maturity and embryo quality in women with endometriosis, underscoring a potential link between ADM, endometriosis, and fertility (21). Collectively, these data imply that ADM may be able to serve as a biomarker of endometriosis.

Mid-regional pro-adrenomedullin (MR-proADM) is a by-product of post-translational processing of preproADM peptide and is a more stable analyte than the mature ADM peptide (22). In the past decade, many groups have found prognostic value for MR-proADM plasma concentrations as a biomarker of heart failure (23), community-acquired pneumonia (24), and sepsis (25), among other diseases. In reproduction, MR-proADM has been tested as a biomarker of gestational diabetes and preeclampsia (26, 27). Here, we test the hypothesis that MR-proADM, as a surrogate for ADM potentially downstream of the IL-6–STAT3 axis, can serve as a biomarker of endometriosis.

MATERIALS AND METHODS

Study Design and Human Subjects

The study was approved by the Institutional Review Boards of Greenville Health System and the University of North Carolina at Chapel Hill. Informed consent was obtained from all study participants, who were 18–45 years of age and had not used hormonal therapies or an intrauterine device in the 3 months preceding biopsy or plasma collection. Eutopic endometrial biopsies were collected from healthy donor women and women with endometriosis in both proliferative and secretory phases at the time of surgery at Greenville Health System and the University of North Carolina. Plasma samples for analysis of MR-proADM concentrations were collected from healthy women and from women with endometriosis in both proliferative and secretory phases at Greenville Health System and the University of North Carolina. Patients who wished to conceive were followed expectantly after surgery for up to 6 months and pregnancies recorded. Pregnancy was defined as a visible gestational sac with cardiac activity on ultrasound and referral for obstetrical care. The clinical characteristics of women from whom plasma was collected are presented in Table 1. MR-proADM concentrations were measured in undiluted plasma with the use of a commercial assay (Brahms MR-proADM Kryptor; Phadia Immunology Reference Laboratory).

Immunohistochemistry

Five-micrometer sections of paraffin-embedded endometrial biopsies were deparaffinized and hydrated. After antigen retrieval in 10 mmol/L citric acid/0.05% Tween-20 (pH 6.0), endogenous peroxidase activity was quenched by means of 3% hydrogen peroxide in phosphate-buffered saline solution (PBS). Tissues were permeabilized with the use of PBS/0.1% Triton X-100 (PBST) and then blocked in 10% normal goat serum/1% bovine serum albumin in PBST. Tissues were incubated in anti-ADM primary antibody (1:200; Abcam ab69117) in block overnight at room temperature. The following day, slides were washed and incubated in biotinylated goat antirabbit antibody (1:250, Jackson Immunoresearch) for 1 hour. Avidin-biotin complexes (Vectastain Elite ABC Kit; Vector Laboratories) were added to tissues for 30 minutes, and then diaminobenzidine (DAB Peroxidase [HRP] Substrate Kit; Vector Laboratories) was added for 2 minutes. Slides were rinsed with tap water, counterstained with hematoxylin (Vector Laboratories) for 20 seconds, and then rinsed with tap water again. Tissues were dehydrated and then coverslipped with the use of DPX mountant (VWR). Slides were imaged on a Zeiss AxioImager with ProgRes Capturepro software (Jenoptik). Staining intensity was determined by a blinded observer (K.E.Q.) and graded on a scale of 0 (no staining) to 4 (strong staining).

Cell Culture, Western Blot, and Quantitative Real-Time Polymerase Chain Reaction

Ishikawa cells were cultured in DMEM/F12 (Gibco) + 10% fetal bovine serum (FBS) + 1× penicillin/streptomycin (Gibco) in a 37°C incubator containing 5% CO_2. For Western blot analysis, Ishikawa cells were grown to confluency in 10-cm dishes and treated with a vehicle control or 1, 10, or 100 ng/mL human IL-6 (R&D Systems) for 15 minutes. Cells

TABLE 1

Clinical characteristics of study participants.

Characteristic	Control (n = 19)	Endometriosis (n = 28)
Age, y	26.2 ± 4.4 (20–33)	32.9 ± 4.9 (23–41)
BMI, kg/m^2	22.4 ± 2.9 (18.3–28.2)	23.7 ± 4.7 (18.7–42.5)
Gravidity at biopsy	0 (0–3)	0 (0–4)
Race		
White	9	26
Black	7	0
Asian	3	1
Multiple	0	1

Note: Age and body mass index (BMI) are presented as mean ± standard deviation (range). Gravidity is presented as median (range).

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were lysed in a PBS + 10 nmol/L NaF + 2 mmol/L $Na_3(VO_4)$ + 2 mmol/L PMSF + protease inhibitor cocktail, and protein concentration in the lysates was determined with the use of a BCA Protein Assay Kit (Pierce). Twenty micrograms of protein per sample were loaded on a sodium dodecyl sulfate–polyacrylamide gel (Bio-Rad) and then transferred to a nitrocellulose membrane (GE Healthcare Life Sciences). Membranes were blocked in either 5% bovine serum albumin (Fisher Scientific) or 5% nonfat dry milk for 1 hour at room temperature and then incubated overnight at 4°C with primary antibody to phospho-STAT3 (1:2,000 in 5% BSA; Cell Signaling) or STAT3 (1:2,000 in 5% milk; Cell Signaling). Blots were washed three times in Tris-buffered saline solution/0.1% Tween-20 and then incubated for 2 hours at room temperature in secondary antibodies DyLight 680 goat antimouse (1:15,000; Thermo Scientific) and DyLight 680 goat antirabbit (1:15,000; Thermo Scientific). Membranes were then imaged on an Odyssey CLx (Li-Cor).

For gene expression analysis, Ishikawa cells were grown to near confluency in 10-cm dishes and then serum starved overnight in serum-free media. Cells were treated with 100 ng/mL IL-6 (R&D Systems) for 1 hour. RNA was collected and isolated with the use of Trizol (Thermo Fisher Scientific) according to the manufacturer's protocol. Complementary DNA was synthesized from 2,000 ng DNase-treated RNA with the use of M-MLV reverse transcriptase (Invitrogen). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the use of a human *Adm* Assay on Demand (Applied Biosystems; Hs00969450_g1) and human GAPDH primers and probe (Applied Biosystems; 4310884E) on a StepOne Plus (Applied Biosystems). qRT-PCR data were analyzed with the use of the $2^{-\Delta\Delta Ct}$ method.

Statistical Analyses

All statistical analyses were performed in Prism 5 (Graphpad Software). *Adm* gene expression in vehicle- and IL-6–treated Ishikawa cells, ADM staining intensity in endometrial biopsies from healthy women and women with endometriosis, and MR-proADM concentrations in plasma from healthy women and women with endometriosis were compared by means of unpaired *t* test. MR-proADM concentrations by menstrual cycle phase, stage of disease, surgical status, and fertility outcome were compared by means of one-way analysis of variance. Data was considered to be statistically significant if $P<.05$.

RESULTS

Activation of STAT3 by IL-6 Induces *Adm* Expression

Given elevated levels of IL-6 and phosphorylated STAT3 (pSTAT3) in peritoneal fluid and eutopic endometrium, respectively, of women with endometriosis (9–11), and previous evidence for STAT3 regulation of *Adm* expression (17, 18), we tested whether IL-6 could induce *Adm* expression in an endometrial cell line, Ishikawa cells. First, we treated Ishikawa cells with increasing doses of IL-6 to confirm that IL-6 induced phosphorylation of STAT3 in them. Indeed, we observed an IL-6 dose–dependent increase in pSTAT3 by Western blot (Fig. 1A). We then assessed whether IL-6–mediated phosphorylation of STAT3 induced *Adm* expression, finding that treating Ishikawa cells with 100 ng/mL IL-6 for 15 minutes up-regulated *Adm* gene expression approximately 1.4-fold according to qRT-PCR (Fig. 1B).

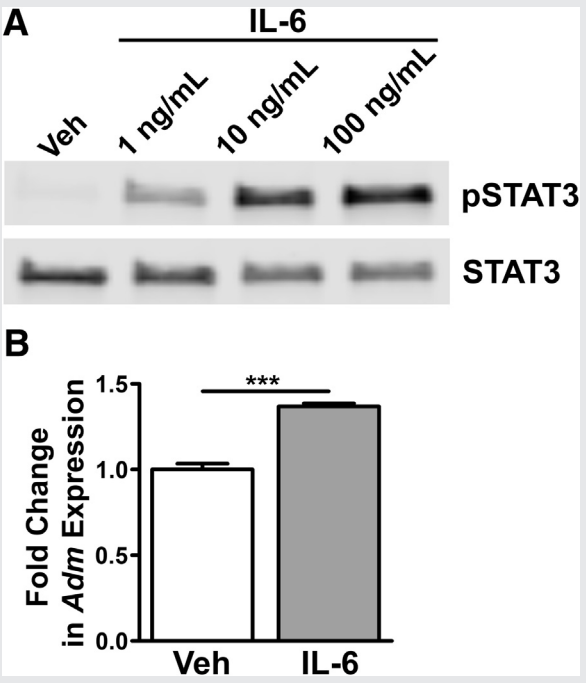
ADM Staining is Enhanced in Eutopic Endometrium of Women with Endometriosis

Considering the elevation of pSTAT3 in endometriotic endometrium coupled with our in vitro evidence for STAT3-mediated up-regulation of *Adm* expression in endometrial cells, we asked whether ADM is also up-regulated in the endometrium of women with endometriosis. Indeed, we found that ADM staining was significantly greater in luminal epithelium, glandular epithelium, and stroma of eutopic endometrium from women with endometriosis compared with healthy control subjects (Fig. 2).

MR-proADM Plasma Levels are Elevated in Women with Endometriosis

We then asked whether circulating plasma levels of MR-proADM, a stable precursor to the mature ADM peptide, are elevated in women with endometriosis. The clinical

FIGURE 1



Interleukin-6 (IL-6) activation of signal transducer and activator of transcription 3 (STAT3) up-regulates *Adm* (adrenomedullin) expression in Ishikawa cells. (**A**) Western blot analysis of pSTAT3 expression in Ishikawa cells after a 15-minute treatment of varying doses of IL-6. (**B**) qRT-PCR analysis of *Adm* expression in Ishikawa cells after a 15-minute treatment of 100 ng/mL IL-6. ***$P<.001$; unpaired *t* test. pSTAT3 = phosphorylated STAT3; Veh = vehicle.

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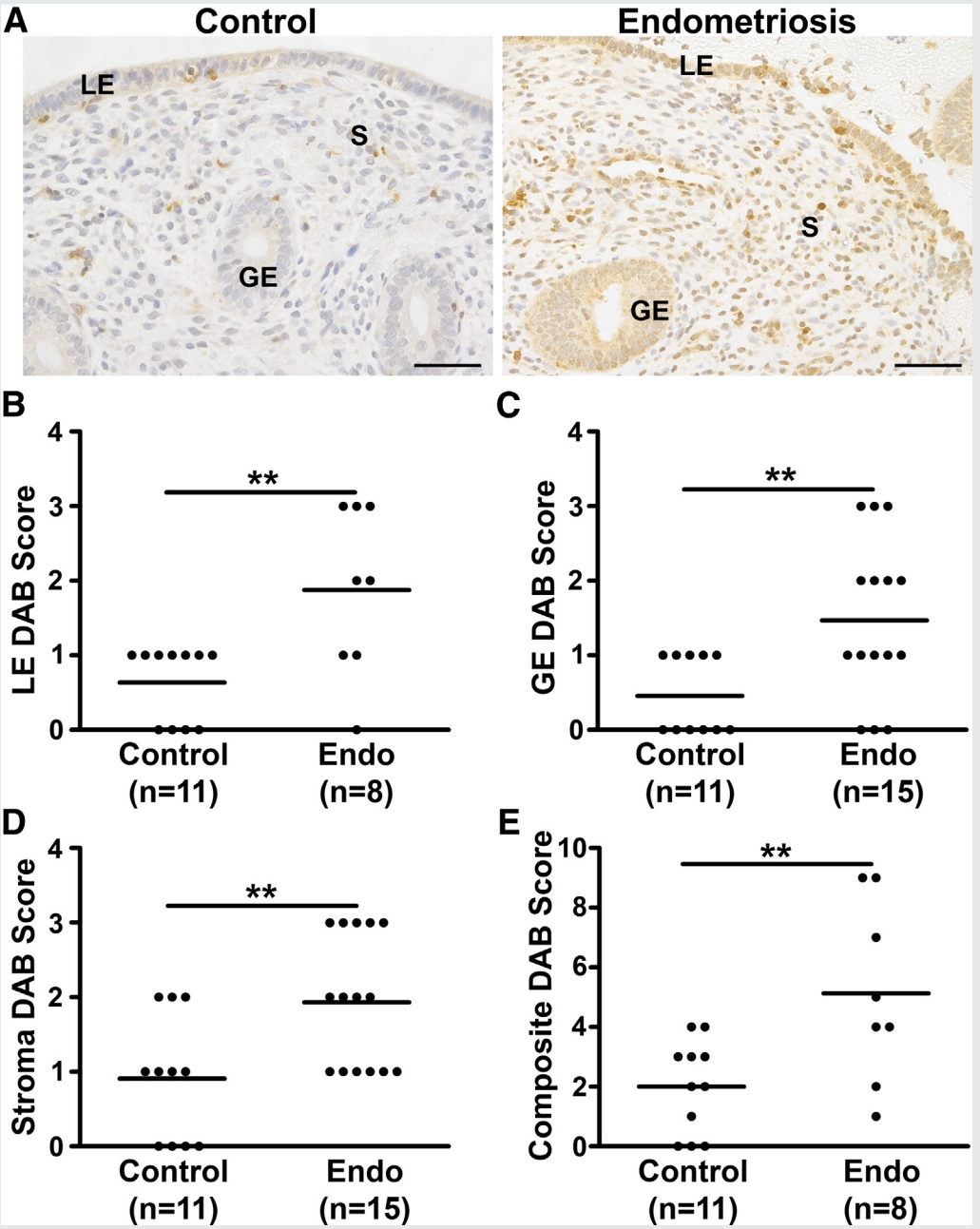
FIGURE 2



Immunohistochemistry for adrenomedullin (ADM) in endometrial biopsies reveals elevated levels of ADM in women with endometriosis. (**A**) Representative images of immunohistochemistry for ADM in eutopic endometrial biopsies from women with endometriosis and from healthy control women. GE = glandular epithelium; LE = luminal epithelium; S = stroma. Semiquantitative analysis of ADM staining in (**B**) luminal epithelium (LE), (**C**) glandular epithelium (GE), and (**D**) stroma of endometrial biopsies. Diaminobenzidine (DAB) score reflects no (0), weak (1), moderate (2), or strong (3) staining. (**E**) Composite DAB score calculated as the sum of the compartmental DAB scores depicted in B–D. LE was not present in all endometrial biopsies from women with endometriosis, therefore LE DAB score and composite DAB score were unable to be calculated in all biopsies. Each dot represents an individual patient. $^{**}P<.01$; unpaired *t* test.

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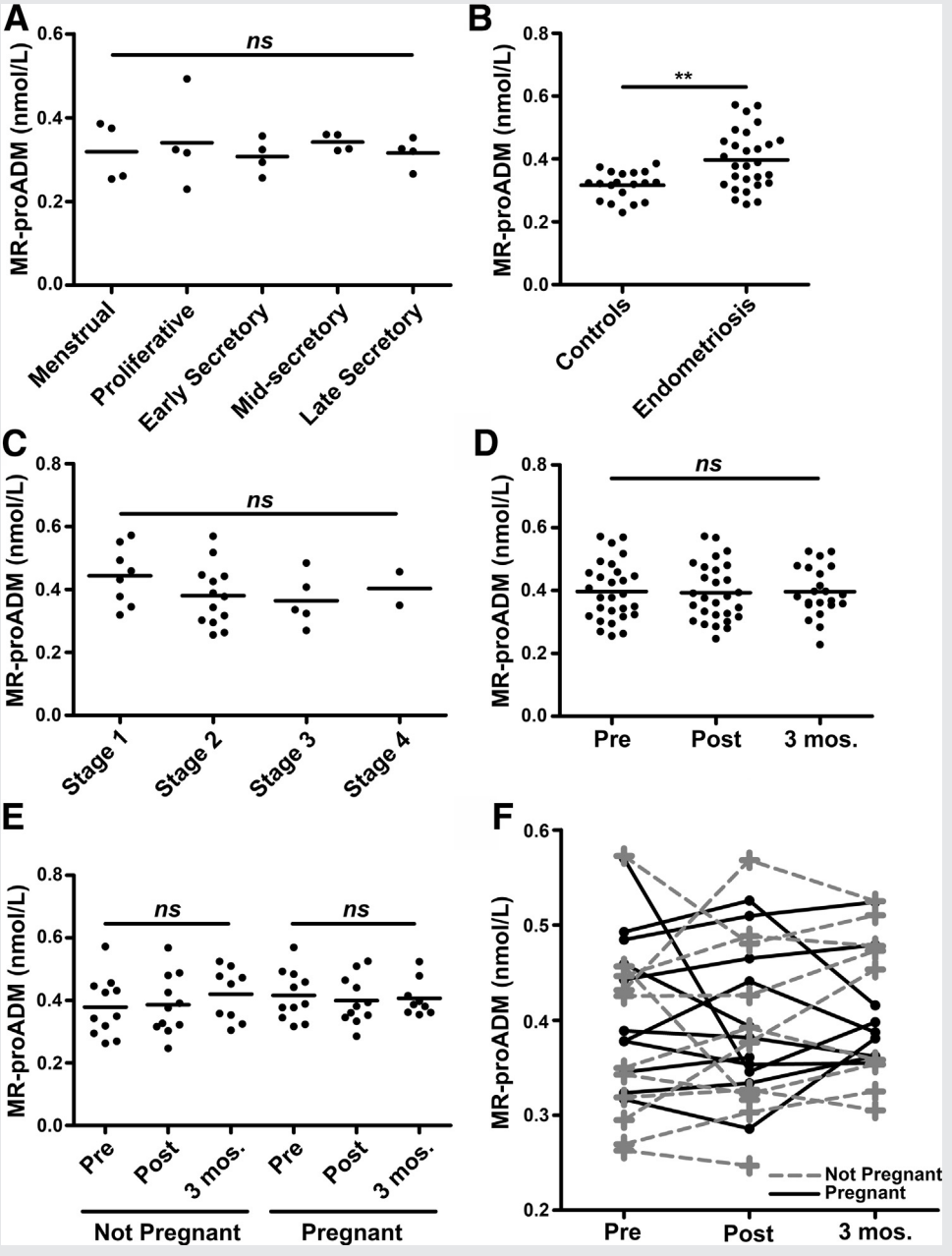
characteristics of healthy control subjects and women with endometriosis who donated plasma for MR-proADM analysis are presented in Table 1. First, we confirmed that MR-proADM levels are stable across all phases of the menstrual cycle in healthy women (Fig. 3A). MR-proADM levels averaged ~0.35 nmol/L, which is nearly equivalent to the mean MR-proADM concentration of 0.33 nmol/L across the general population (22). Subsequently, and consistent with higher levels of ADM in eutopic endometrium of women with endometriosis, we found that circulating plasma levels

FIGURE 3



Midregional proadrenomedullin (MR-proADM) plasma concentrations are elevated in women with endometriosis but not correlated with disease stage, surgical status, or pregnancy outcome. (**A**) MR-proADM concentrations in plasma from healthy women across all stages of the menstrual cycle. (**B**) MR-proADM concentrations in healthy control women and women with endometriosis. (**C**) MR-proADM concentrations in women with endometriosis binned by disease stage and severity. (**D**) MR-proADM concentrations in women with endometriosis before, immediately after, and 3 months after surgery. (**E**) MR-proADM concentrations in women with endometriosis before, immediately after, and 3 months after surgery, binned by those who became pregnant and those who did not. (**F**) Individual patient-level MR-proADM concentrations over time spanning the surgical period. ns = not significant; one-way analysis of variance.

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of MR-proADM were higher in women with endometriosis compared with healthy control women (Fig. 3B). However, MR-proADM levels did not vary by stage of disease (Fig. 3C).

We then asked whether the elevated levels of MR-proADM were corrected by surgical resection of ectopic endometrial lesions. Analyzing MR-proADM concentrations immediately after surgery and 3 months after surgery, we determined that elevated MR-proADM levels persisted through the postsurgical period (Fig. 3D). Finally, we assessed whether MR-proADM concentrations before, after, and 3 months after surgery correlated with whether participants were able to become pregnant. We did not find any difference

in MR-proADM levels between women with endometriosis who became pregnant and those who did not (Fig. 3E and 3F).

DISCUSSION

In this study, we have presented evidence for *Adm* as a STAT3 target in the uterus and investigated MR-proADM as a potential biomarker of endometriosis. Previous evidence for a STAT3-*Adm* axis is twofold: first, pSTAT3 and *Adm* levels are positively correlated in breast cancer (18); and second, activation of STAT3 by oncostatin M promotes *Adm* transcription in astroglioma cells (17). However, to our knowledge, the present study provides the first direct evidence for a STAT3-*Adm* axis in the uterus. This finding is not surprising, because pSTAT3 and ADM are colocalized in the uterus during early pregnancy (28, 29). Furthermore, reduction of STAT3 or ADM in the mouse uterus leads to problems with implantation, potentially due to problems with endometrial receptivity (28–31). It will be the subject of future studies to determine whether STAT3 is directly binding active sites in the *Adm* promoter in uterine cells or whether the STAT3-mediated stabilization of HIF1A induces *Adm* transcription. Furthermore, it will be pertinent to determine if this interaction is critical for reproductive physiology and pathophysiology and in the context of endometriosis.

We then asked whether ADM, like pSTAT3, was up-regulated in the eutopic endometrium of women with endometriosis, finding more ADM in both epithelial and stromal compartments of the uterus. The increased expression is consistent with previously demonstrated elevated pSTAT3 and HIF1A abundance in the eutopic endometrium of women with endometriosis, echoing the possibility of an indirect relationship between STAT3 and *Adm* via HIF1A (8). However, the expression of ADM in ectopic lesions remains unknown.

Because MR-proADM is a stable surrogate for ADM peptide in circulating plasma, we compared levels of MR-proADM in women with and without endometriosis. This is the first study, to our knowledge, to examine levels of MR-proADM across the menstrual cycle. Because the MR-proADM assay was developed barely a decade ago, reference intervals in different physiologic conditions and disease states are still emerging (32). Here, we contribute evidence for stable levels of MR-proADM across all stages of the menstrual cycle in healthy women.

Comparison of MR-proADM concentrations in women with and without endometriosis led to our primary finding of elevated MR-proADM in women with endometriosis. We posit several explanations for the finding: First, endometriosis is well understood to be an estrogenic disease, and *Adm* is transcriptionally regulated by estrogen (33, 34). Second, endometriosis is an inflammatory condition with consequences for fertility, and ADM is an antiinflammatory peptide commonly up-regulated in response to inflammation (35, 36). Endometriosis is also associated with an elevated risk of epithelial ovarian cancer, and ADM has been shown to promote angiogenesis in this subtype of ovarian cancer (37–39). Therefore, our finding of elevated MR-proADM in women with endometriosis is consistent with associations common to both endometriosis and ADM.

However, MR-proADM levels were not correlated with disease stage or corrected by surgical resection of ectopic lesions. Endometriosis staging is determined primarily by the location and extent of ectopic lesions, and surgical resection aims to remove these lesions. Therefore, if ectopic lesions were the primary source of circulating MR-proADM, we would expect MR-proADM levels to be positively correlated with disease stage and to be corrected by surgery. Although the expression of ADM in ectopic lesions was not examined in this study, together these data suggest that ectopic lesions are not the primary source of circulating MR-proADM.

MR-proADM levels were not predictive of fertility outcome in women with endometriosis, which is associated with subfertility or infertility, in women who underwent surgery. In this study, therefore, MR-proADM may not demonstrate a prognostic value in endometriosis that is equivalent to that observed by other groups in cardiovascular and pulmonary diseases. However, studies by our group and others demonstrate that levels of both maternal- and fetal-derived ADM are important for fertility and pregnancy (19, 20). It appears that ADM levels are carefully titrated in normal physiology; for example, whereas $Adm^{-/-}$ mice are embryonically lethal, $Adm^{hi/hi}$ mice, which overexpress ADM, develop hyperplastic hearts during development (40, 41). Therefore, it is becoming clear that concentrations of ADM at either extreme can be detrimental to normal physiology.

In summary, we present evidence for a STAT3-*Adm* interaction in vivo, for up-regulation of ADM in the eutopic endometrium of women with endometriosis, and for higher circulating levels of MR-proADM in endometriosis. Whether elevated MR-proADM in endometriosis is secondary to the estrogenic and inflammatory properties of endometriosis or an inciting pathogenic factor will be the subject of future study.

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