Systemic inflammation induced by microRNAs: Endometriosis Derived Alterations in Circulating microRNA 125b-5p and Let7b-5p regulate Macrophage Cytokine Production

Sepide E. Nematian, Ramanaiah Mamillapalli, Trisha S. Kadakia, Masoumeh Majidi Zolbin, Sarah Moustafa and Hugh S. Taylor

The Journal of Clinical Endocrinology & Metabolism
Endocrine Society

Submitted: May 25, 2017
Accepted: October 03, 2017
First Online: October 12, 2017

The Journal of Clinical Endocrinology & Metabolism; Copyright 2017 DOI: 10.1210/jc.2017-01199

Systemic inflammation induced by microRNAs: Endometriosis Derived Alterations in Circulating microRNA 125b-5p and Let7b-5p regulate Macrophage Cytokine Production

Sepide E. Nematian [1], Ramanaiah Mamillapalli [1], Trisha S. Kadakia [1,2], Masoumeh Majidi Zolbin [1], Sarah Moustafa[1] and Hugh S. Taylor [1]

[1]*Obstetrics, Gynecology and Reproductive Sciences, Yale School of Medicine, New Haven, CT*

[2]*Obstetrics & Gynecology, Mount Sinai Beth Israel Medical Center, New York, NY*

Received 25 May 2017. Accepted 03 October 2017.

Context. Endometriosis is characterized by aberrant inflammation. We previously reported increased levels of miRNA 125b-5p and decreased levels of miRNA let 7b-5p in serum of patients with endometriosis.
Objective. Determine the regulatory function of microRNAs 125b-5p and Let 7b-5p on production of pro-inflammatory cytokines in endometriosis
Design. Case-control study
Setting. University hospital
Patients. Women with (20) and without (26) endometriosis; human U937 macrophage cell line
Intervention. Sera were collected from surgically diagnosed patients and differentiated U937 cells that were transfected with microRNAs 125b-5p and Let 7b-5p mimics and inhibitor.
Main Outcome Measures. ELISA for TNF-α, IL-6, IL-8, and IL-1β levels and qRT-PCR for expression of microRNAs 125b-5p and Let 7b-5p in sera of patients with and without endometriosis. Transfected macrophages were evaluated for expression of inflammatory cytokines, intracellular production and secretion of these cytokines.
Results. We noted significant elevation of TNF-α, IL-1β and IL-6, significant up-regulation of microRNA 125b and significant down-regulation of Let-7b in sera of endometriosis patients versus control. There was a positive correlation between miR 125b levels and TNF-α, IL-1β, and IL-6 and a negative correlation between miR Let7b levels and TNF-α in sera of patients with endometriosis. Transfection experiments showed a significant up-regulation of TNF-α, IL-1β, IL-6, and IL-8 in macrophages transfected with miRNA 125b mimic or let7b inhibitor. The secreted cytokine protein levels and intracellular imaging studies closely correlate with the mRNA changes.
Conclusions. Endometriosis-derived miRNAs regulate macrophage cytokine production that contributes to inflammation associated with this condition.

We studied the regulatory role of circulating miR-125b and let7b in endometriosis and found that they induce the production of pro-inflammatory cytokines in endometriosis.

INTRODUCTION

Endometriosis is a common, inflammatory disease characterized by the growth of ectopic endometrial tissue outside the uterus [1,2]. It affects 5-15% of reproductive age women, and is present in as many as 30–50% of patients with infertility and/or pain [1,3–5]. Additionally, endometriosis has significant social and psychological effects, with 63% of women reporting serious negative impact on their quality of life [6,7]. Societal costs for the treatment of endometriosis exceed $22 billion in the USA [8]. Owing to the complexity of its development and symptomatology, this disease has been widely studied however its systemic effects are still

poorly understood [9,10]. An area of great significance in endometriosis is the increase in inflammatory activity and its implications in the pathogenesis of this condition [2,11].

The role of immune system and its response to endometriosis has been studied extensively. The endometriosis-associated inflammatory reaction has been described in previous studies demonstrating the relative increase in the number and sensitivity to activation of macrophages and concurrent increase in inflammatory cytokines [12–15]. Cytokines are distinct proteins that perform a fundamental role in controlling different cellular functions such as proliferation, chemotaxis, adhesion, morphological changes and angiogenesis [13,16]. They play a crucial role in cell-to-cell communication within the immune system [9]. Alterations in this immunological process is both local and systemic and the disruption in this dynamic interaction contributes to the progression of the condition [10,17,18].

Several cytokines, have been associated with the evolution of endometriosis [19]. TNF-α has potent inflammatory, cytotoxic and angiogenic potential[20]. IL-6 promotes endometrial cell growth and is a potent stimulator of the vascular endothelial growth factor gene[21]. IL-8 stimulates cell proliferation and has a role in endometriosis as an autocrine regulator of endometrial cell growth [22]. It has likewise been implicated as a prominent cytokine involved in angiogenesis [23]. IL-1β is a major pro-inflammatory cytokine secreted by macrophages that plays a vital role in endometriosis pathogenesis by orchestrating interaction between the microenvironment of the peritoneal cavity[24]. Several studies have confirmed the increased concentrations of IL-1β, IL-6 and TNF-α in women with endometriosis compared to controls [14,25,26]. Consistent with that finding, peritoneal macrophages secrete more IL-8, IL-10 and TNF-α in patients with endometriosis compared to healthy control [25,27]. The mechanism by which endometriosis leads to systemic inflammation and macrophage alteration is still poorly characterized.

MicroRNAs (miRNAs) are short, 18-22 nucleotide size, non-coding ribonucleic acids that act as post-transcriptional modulators of gene expression [28–31]. Circulating miRNAs are of interest because they are stable allowing for potential use as reproducible noninvasive biomarkers for various pathologies[32–34]. MiRNAs are involved in the pathogenesis of endometriosis [33–35] and several reports including our own have demonstrated the differential expression of microRNAs in the serum of patients with endometriosis. [32,36,37] Of particular interest are two miRNAs, 125b-5p which was found to be highly up-regulated [36] and Let-7b-5p which was significantly down-regulated [32,35] in patients with severe endometriosis. Changes in circulating miRNAs can regulate gene expression in cells remote from the location and cell type in which they are produced. This form of extensive cell-cell communication may serve as a mechanism by which endometriosis alters cytokine production in macrophages systemically. To investigate the potential role of these two miRNAs in the immunological response, particularly endometriosis associated inflammation, we aimed to determine the levels of pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin 1B (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8) in macrophages with enforced overexpression or repression of these miRNAs. We expect that overexpression of miRNA 125b and inhibition of Let 7b will induce expression and subsequent production and secretion of these inflammatory cytokines. In addition, this study investigates the serum pro-inflammatory cytokine profile in patients with endometriosis compared to healthy controls. This allowed us to correlate the effects on macrophages with the cytokine milieu in diseased patients.

MATERIALS AND METHODS

Cell Culture

The human myelo-monocytic cell line, U937 was a generous gift from Robert Mean, Department of Pathology, Yale School of Medicine. Cells were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 IU/ml penicillin, 50 µg/ml streptomycin) and maintained at 37°C in 5% CO2-atmosphere, media was changed every 48-72 hrs. Differentiation to mature macrophages was carried out by treating with Phorbol-12-myristate-13-acetate (PMA) at 100 ng/ml of the cell suspension (1 x10^6 cells/ml). After 48 hours, adherent cells (macrophages) were washed twice with ice-cold Phosphate Buffered Saline solution (PBS) and allowed to grow for 48 hours in fresh growth medium. In vitro experiments with U937 cell line were carried out using sub-culture passage 3.

Transfection of microRNAs

Transfection was carried out in 24 hr old macrophages in a 6-well plate (2×10^5 cells/well) without antibiotics using Lipofectamine ™ RNAiMAX (Invitrogen) according to the manufacturer's protocol. Briefly, the transfection medium contains 50 nmol microRNA and 3.5 µl Lipofectamine ™ RNAiMAX in a volume of 500 µl of Opti-MEM® (Gibco) separately, without serum and allowed to sit for 5 minutes at room temperature, then combined, mixed gently and incubated at room temperature for 20 min before adding to the well with cells and cultured overnight. Transfection medium was then replaced with fresh growth medium containing 10% FBS and antibiotics. Each transfection condition was carried out under sterile conditions with respective controls in duplicate wells. The transfected macrophages were processed for experiments after 48 hr post-transfection.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from 48 hr post-transfected macrophages using TRIzol® reagent (Invitrogen) as described in the manual. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green (Bio-Rad) and optimized in the MyiQ Single Color Real-Time PCR Detection System (Bio-Rad). The specificity of the amplified transcript and absence of primer-dimers was confirmed by a melting curve analysis. All products yielded the predicted melting temperature. Gene expression was normalized to the expression of human β-actin for each sample. Relative mRNA expression for each gene was calculated using the comparative cycle threshold (Ct) method, also known as the $2^{-\Delta\Delta C(T)}$ method [38]. All experiments were carried out in triplicate and nuclease-free water was used as a negative control replacing the cDNA template. Primers were obtained from the W. M. Keck Oligonucleotide Synthesis Facility (Yale University).

Primer sequences for all the genes are

TNF-α forward, CACCATGAGCACTGAAAGCA, reverse GCTCTTGATGGCAGAGAGGAG; IL-1β forward, TTCGAGGCACAAGGCACAAC, reverse, CTGGAAGGAGCACTTCATCTGT; IL-6 forward ACCCCCAGGAGAAGATTCCA, reverse, GTCTTCCCCCACACCAAGTT; IL-8 forward, TCTGTGTGAAGGTGCAGTTTTG, reverse, GGGGTGGAAAGGTTTGGAGT; beta-actin forward GAAGATCAAGATCATTGCTC, reverse AACGCAACTAAGTCATAGTC.

Macrophages Stimulation by LPS and Cytokine Protein Measurements by ELISA

Transfected macrophages were stimulated with lipopolysaccharide (LPS) 48 hr post-transfection (Sigma, St Louis, MO) at different concentrations and durations (10, 100 and 1000 ng/ml for 6, 12 and 24 hours) and conditioned media was collected and stored in aliquots at -80°C until use for the determination of secreted cytokine proteins by Enzyme-Linked Immunosorbent Assay kit

(ELISA) (Affymetrix) according to the manufacturer's instructions. Optical density was read in a microplate reader at 450nm and a standard curve was subsequently generated to extrapolate cytokine concentration in the samples. Kit sensitivity was approximately 4pg/ml for TNF-α, 2pg/ml for IL-1β, IL-6 and IL-8 with standard curve ranges of 4-500, 2–150, 2-200 and 2-250 pg/ml respectively.

Immunocytochemistry

Transfected macrophages in culture chambers were fixed by incubation in 100% chilled methanol at room temperature for 5 minutes then washed three times with ice-cold Phosphate Buffered Saline (PBS). Permeabilization was carried out by incubation in PBS containing 0.25% Triton X-100 and subsequently washed with PBS three time for 5 minutes. Blocking was done with the use of 1% Bovine Serum Albumin (BSA) in PBST (PBS + 0.1% Tween 20) for 30 minutes. Immunostaining was done by incubation in the diluted primary antibody in 1% BSA in PBST in a humidified chamber overnight at 4C. Primary antibody (TNF-α, IL-1β, IL-6, IL-8; Santa Cruz Biotechnology, Inc) use and dilution for all cytokines were based on the antibody data sheet recommendations. Slides were washed and incubated with the secondary antibody in 1% BSA in the dark at room temperature for 1 hr. The secondary antibody solution was decanted and slides were washed three times with PBS for 5 minutes in the dark. Counterstaining for 1 minute with DAPI was done and slides were mounted with a coverslip and a drop of mounting media.

Study Population

Institutional Review Board (IRB) approval was obtained from Yale School of Medicine (New Haven, Connecticut) for using human samples. Written informed consent was obtained from subjects admitted to the hospital and undergoing laparoscopy or laparotomy for suspected benign indications such as pelvic masses, pelvic pain, infertility and endometriosis. Inclusion criteria included women who were aged 18-49 years. Exclusion criteria included post-menopausal patients, hyperplasia or polyps, malignancy, autoimmune disease and cardiovascular disease. All stages of endometriosis as well as untreated and treated subjects were included to provide a full spectrum of disease resulting in varied microRNA levels. The total number of women from whom serum was collected was forty nine (49). Among 49 women, three were excluded due to an unexpected co-morbidity, while forty six (46) were included in the study and divided into two groups as follows: The endometriosis group has 20 women with surgically diagnosed and histologically verified endometriosis and control group has 26 women, who were visually verified to be free from endometriosis during the surgery. The phase of the menstrual cycle was determined based on the patient's menstrual history and last menstrual period.

Cytokine Measurements in Human Serum from Endometriosis Patients by ELISA

Whole blood samples were collected in a sterile condition, kept at RT for 30 min. and the serum was obtained by centrifuging the blood clot at 2000 × g for 15 minutes at 4°C. The serum was immediately aliquoted for storage at -80°C until needed for ELISA avoiding freeze-thaw cycles. Quantification of cytokine levels in individual samples was done twice each in duplicate wells by ELISA (Affymetrix, Santa Clara, CA) following the manufacturer's protocol.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) for miRNAs in Sera

Total microRNA was extracted from 200 μl of serum sample using the miRNeasy mini Kit (Qiagen) and reverse transcribed using TaqMan Advanced miRNA cDNA synthesis Kit (Thermofisher) according manufacturer's specifications. MicroRNA levels were quantified with qRT-PCR using SYBR Green (Bio-Rad) and optimized in the MyiQ Single Color Real-Time

PCR Detection System (Bio-Rad). Gene expression was normalized to the expression of human U6 small nuclear RNA to determine relative miRNA expression for each sample. Relative expression was calculated using the comparative cycle threshold (Ct) method, also known as the $2^{-\Delta\Delta C(T)}$ method [38]. All determinations were done twice each using triplicate wells. Primers for miRNAs and the U6 genes were obtained from the W. M. Keck Oligonucleotide Synthesis Facility (Yale University). Primer sequences are as follows:

MiR-125b-5p forward UCCCUGAGACCCUAACUUGUGA;

MiR-let-7b-5p forward, TGAGGTAGTAGGTTGTGTGGTT;

U6 forward, CTCGCTTCGGCAGCACA.

Statistical Analysis

Analysis was performed using GraphPad Prism software version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA) using Mann-Whitney U test. For correlation statistics between the relative expression of microRNAs and pro-inflammatory cytokine levels in serum, we made use of the Pearson correlation coefficient. Differences were considered as statistically significant for P-values <0.05.

RESULTS

Increased Serum Levels of Pro-Inflammatory Cytokines in Women with Endometriosis

We first examined the pro-inflammatory cytokine levels in women with histologically confirmed endometriosis and a comparison of serum cytokine concentrations between women with endometriosis and disease-free controls. The mean age (+/-SD) of the women with endometriosis was 35.35 +/- 8.66 years which was not significantly different from the mean age of patients in the control group 33.05 +/- 7.5 years (P>0.05). The mean body mass index (BMI) (+/-SD) did not differ between groups (endometriosis group: 29.55 +/- 8.76; control group: 26.66 +/- 5.59). Supplemental Table 1 shows a summary of patient characteristics including phase of the menstrual cycle and stage of endometriosis. As shown in Figure 1, serum TNF-α ($p<0.001$), IL-1β ($p<0.001$), and IL-6 ($p<0.001$) levels were significantly higher in the endometriosis group than in the control group, and IL-8 levels showed a trend towards increased secretion. Supplemental Table 2 shows the mean concentration of cytokines based on the phase of the menstrual cycle. There was no significant difference in cytokine levels between phases in each subject group (ie: control proliferative phase versus secretory phase; endometriosis proliferative phase versus secretory phase). The same significant difference in cytokine concentration of TNF-α, IL-1β, and IL-6 between control and endometriosis groups matched by phase of menstrual cycle were observed (ie: control proliferative phase versus endometriosis proliferative phase). We subsequently measured the levels of endometriosis-derived miRNA in the serum samples. MicroRNA 125b was significantly elevated in serum of patients with endometriosis compared to controls ($p=0.002$) while microRNA let 7b showed a significant down-regulation ($p<0.001$) as shown in Figure 2.

Correlation between expression levels of microRNAs and serum levels of cytokines in patients with endometriosis

While we have established the increase in amount of pro-inflammatory cytokines as well as determined the relative expression of endometriosis derived miRs 125b and let7b in serum of patients with endometriosis compared to control, we next evaluated the correlation between the microRNA levels and cytokine concentration. Figure 3(a) shows the Pearson correlation scatter plot illustrating a positive correlation between expression levels of miR 125b-5p and

concentration of TNF-α (p<0.001, r=0.759), IL-1β (p<0.001, r=0.761), and IL-6 (p<0.001, r=0.961) in serum of patients with endometriosis. Interleukin-6 showing the highest positive correlation. On the other hand, Figure 3(b) shows the negative correlation between expression levels of miR Let7b and concentration of TNF-α (p=0.017, r= -0.53) in serum of patients with endometriosis.

Increased Expression of Pro-inflammatory Cytokines in Macrophages Transfected with miR-125b Mimic and Let-7b Inhibitors

We have previously demonstrated a significant increase in circulating miR-125b and decrease in miR-let-7b in women with endometriosis [35]. Here we investigated changes in the relative expression of cytokines resulting from overexpression of miRNAs miR-125b and miR-let-7b in macrophages. As shown in Figure 4, qRT-PCR was used to demonstrate that there was a statistically significant increase in mRNA expression of cytokines TNF-α (2.66 fold, p<0.001) IL-1β (1.86 fold, p=0.0019), IL-6 (1.51 fold, p=<0.001), and IL8 (1.70 fold, p=0.0078) in macrophages transfected with miR-125b-5p mimic. Transfection with miR-let7b mimic caused significant decrease in expression of TNF-α (0.58 fold difference, p<0.001), IL-8 (0.66 fold difference, p=0.0019), and a trend toward down-regulation for IL-1B (0.88 fold difference) and IL-6 (0.74 fold difference). While microRNA125 is increased, Let 7b levels are decreased in women with endometriosis; we therefore specifically examined the effect of inhibiting this microRNA. The miR-let7b inhibitor transfection caused a significant increase in expression of TNF-α (1.5 fold, p=0.002), IL-1β (2.94 fold, p=0.002), IL-6 (1.59 fold, p=0.006), and IL-8 (6.19 fold, p=0.008).

To confirm the intracellular changes in cytokine protein production, we then proceeded with immunostaining of the transfected macrophages. Fixed cells were visualized under a fluorescence microscope. Figure 5 gives the representative merged confocal images of the different cytokines in the three-miRNA experimental groups and their corresponding scramble (control) sets. Macrophages transfected with miR-125b and Let7b inhibitor showed significantly greater proportion of highly fluorescent cells for all four cytokines TNF-α, IL-1β IL-6 and IL-8. In contrast, miRNA-Let 7b transfected macrophages did not display any significant difference from the controls.

LPS Increased the Cytokine Secretion in Macrophages Transfected with miR-125b Mimic and Let7b Inhibitor

Multiple cytokines are secreted from activated macrophages [39]. Therefore, we stimulated the 48 hr post-transfected macrophages with LPS in varying doses and time intervals in order to know if translation of cytokines and subsequent secretion reflect mRNA changes. LPS dose and timing of maximal cytokine secretion of macrophages vary across literature [25,40–43] but optimal time point was set at 24 hours following a dose of 100ng/ml for this study. Figure 6 shows ELISA results from conditioned media of transfected macrophages treated with 100ng/ml LPS for 24 hours. A significant increase in TNF-α (p=0.004), IL-1β (p=0.016), IL-6 (p=0.029) and IL-8 (p=0.016) protein secretion in macrophages transfected with miR 125b. In contrast, the macrophages transfected with miR-Let7b showed a significant decrease in protein secretion of TNF-α (p=0.0095), IL-1β (p=0.016), and IL-6 (p=0.029). Figure 6 also shows a significant increase in secretion of all these cytokines in macrophages transfected with miR-Let 7b inhibitor TNF-α (p=0.016), IL-1β (p=0.029), IL-6 (p=0.016) and IL-8 (p=0.004).

DISCUSSION

Endometriosis has been associated with inflammation and increased inflammatory cytokine production. Here we confirm an elevation of several inflammatory cytokines in the circulation of women with endometriosis [14,44] Endometriosis associated inflammation is thought to be mediated in part by macrophages; increased macrophage activity is reflected by a rise in number and activation potential resulting in an increase in secretion of cytokines and chemokines [45]. However, it is not clear how endometriosis leads to the increased systemic inflammation. We demonstrate here that alterations in circulating microRNAs are one mechanism by which endometriosis causes immune dysfunction and inflammation. As these microRNAs are altered in the circulation of women with the disease, they can influence the cytokine expression of macrophages systemically, including areas remote from endometriosis.

Alterations of circulating microRNA in endometriosis furthers the concept that endometriosis is a systemic disease. We have previously demonstrated system effects of endometriosis remote from the site of lesions. While peritoneal endometriosis affects the uterus and endometrial receptivity, we have demonstrated that endometriosis placed under the skin of mice far from the uterus still affects the eutopic endometrium [46]. Similarly, endometriosis can affect liver metabolism leading to weight loss[47]. Further endometriosis alters stem cell trafficking, leading to stem cell deficiency in the uterus and infertility [48,49]. All of these examples demonstrate that endometriosis is not a disease where the effects are localized to the pelvis. The ability to systemically alter macrophage function and induce an inflammatory state adds to the list of widespread, whole body effects of endometriosis. Endometriosis should be considered a disease with multi organ involvement. Alterations in circulating microRNAs clearly induce inflammation and may well contribute substantially to the systemic manifestations of the disease.

Let-7 also targets the KRAS gene and its loss may permit initiation of the progression of ectopic lesions, and may represent a link between inflammation and the tumor like growth in endometriosis [32,50–52]. KRAS signaling leads to enhanced proliferation and invasion of cells including many tumors [51]. We have previously demonstrated a role for Let7 regulation of KRAS in endometriosis [52]. Similarly Let-7 also targets aromatase[53]. Let-7 may target multiple pathways that influence endometriosis and other processes in addition to the role described here.

The exact role of Let-7b in the regulation of macrophages is still an on-going area of research. A known target of importance is C/EBP-δ which is a transcription factor required for sustained responsiveness to TLR receptor signaling [54]. Our data clearly demonstrate that overexpression of miRNA Let 7b caused significant decrease in the expression of the pro-inflammatory genes whereas blocking of Let-7b caused a reciprocal increase in cytokine expression. These results are further confirmed by the data obtained for consistent protein pattern in intracellular protein production as well as secreted cytokine levels post-LPS stimulation in the respective transfection experiments. It is important to recognize that it is the collective effects of these miRNAs that determine the disease outcome. MicroRNAs each have multiple targets and it is likely that the inflammatory cytokines identified here represent important, but not exclusive, targets of these microRNAs.

The inhibition or stimulation of pro-inflammatory cytokines by these microRNAs identifies prospective novel targets for interventions in endometriosis. Restoration of normal peripheral microRNA signaling between cells and organs may well be a means to treat the systemic manifestations of this disease. MicroRNA mimics and inhibitor are exciting new therapeutics for endometriosis.

In summary, circulating miR-125b-5p and Let7b-5p function as regulators of the inflammatory response in endometriosis. They induce macrophage inflammatory cytokine production, defining a mechanism responsible for the systemic effects of this disease. The miRNAs with altered levels in endometriosis identified here have potential as both diagnostic tools and therapeutic targets.

Correspondence. Hugh S. Taylor, MD, Department of Obstetrics, Gynecology and Reproductive Sciences, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, USA, E-mail: hugh.taylor@yale.edu

Email of the Authors: Sepide E. Nematian: sepide.nematian@yale.edu, Ramanaiah Mamillapalli: ramana.mamillapalli@yale.edu, Trisha Kadakia: Trisha.Kadakia@mountsinai.org, Masoumeh Majidi Zolbin: Masoumeh.majidizolbin@gmail.com, Sarah Moustafa: sarah.moustafa@yale.edu

REFERENCES

1. Giudice LC. Endometriosis. N Engl J Med. 2010;362(25):2389-2398. doi:10.1056/NEJMcp1000274.
2. Bulun SE. Endometriosis. N Engl J Med. 2009;360(3):268-279. doi:10.1056/NEJMra0804690.
3. Donnez J, Binda MM, Donnez O, Dolmans MM. Oxidative stress in the pelvic cavity and its role in the pathogenesis of endometriosis. Fertil Steril. 2016;106(5):1011-1017. doi:10.1016/j.fertnstert.2016.07.1075.
4. Taylor HS, Osteen KG, Bruner-Tran KL, et al. Novel therapies targeting endometriosis. Reprod Sci. 2011;18(9):814-823. doi:10.1177/1933719111410713.
5. Sundheimer LW, Alford CE, Taylor RN, DeCherney AH. Endometriosis.; 2000. http://www.ncbi.nlm.nih.gov/pubmed/25905227.
6. Culley L, Law C, Hudson N, et al. The social and psychological impact of endometriosis on women's lives: A critical narrative review. Hum Reprod Update. 2013;19(6):625-639. doi:10.1093/humupd/dmt027.
7. Moradi M, Parker M, Sneddon A, Lopez V, Ellwood D. Impact of endometriosis on women's lives: a qualitative study. BMC Womens Health. 2014;14(1):123. doi:10.1186/1472-6874-14-123.
8. Simoens S, Hummelshoj L, D'Hooghe T. Endometriosis: Cost estimates and methodological perspective. Hum Reprod Update. 2007;13(4):395-404. doi:10.1093/humupd/dmm010.
9. Lebovic DI, Mueller MD, Taylor RN. 13 Immunobiology of endometriosis. Fertil Steril. 2001;75:1-10. http://www.ncbi.nlm.nih.gov/pubmed/11163805.
10. Nnoaham KE, Hummelshoj L, Webster P, et al. Impact of endometriosis on quality of life and work productivity: A multicenter study across ten countries. Fertil Steril. 2011;96(2). doi:10.1016/j.fertnstert.2011.05.090.
11. Gupta S, Goldberg JM, Aziz N, Goldberg E, Krajcir N, Agarwal A. Pathogenic mechanisms in endometriosis-associated infertility. Fertil Steril. 2008;90(2):247-257. doi:10.1016/j.fertnstert.2008.02.093.
12. Berkkanoglu M, Arici A. Immunology and endometriosis. Am J Reprod Immunol. 2003;50(1):48-59. doi:10.1034/j.1600-0897.2003.00042.x.
13. Capobianco A, Rovere-Querini P. Endometriosis, a disease of the macrophage. Front Immunol. 2013;4(JAN). doi:10.3389/fimmu.2013.00009.

14. Wu M-Y, Ho H-N. The role of cytokines in endometriosis. Am J Reprod Immunol. 2003;49(5):285-296. doi:10.1034/j.1600-0897.2003.01207.x.
15. Sikora J, Mielczarek-Palacz A, Kondera-Anasz Z. Imbalance in Cytokines from Interleukin-1 Family - Role in Pathogenesis of Endometriosis. Am J Reprod Immunol. 2012;68(2):138-145. doi:10.1111/j.1600-0897.2012.01147.x.
16. Ponce C, Torres M, Galleguillos C, et al. Nuclear factor κB pathway and interleukin-6 are affected in eutopic endometrium of women with endometriosis. Reproduction. 2009;137(4):727-737. doi:10.1530/REP-08-0407.
17. Ilie I, Ilie R. Cytokines and Endometriosis - the Role of Immunological Alterations. Biotechnol Mol Biol Nanomedicine. 2013;1(2):8-19.
18. Harada T, Iwabe T, Terakawa N. Role of cytokines in endometriosis. Fertil Steril. 2001;76(1):1-10. doi:10.1016/S0015-0282(01)01816-7.
19. Burney RO, Giudice LC. Pathogenesis and pathophysiology of endometriosis. Fertil Steril. 2012;98(3):511-519. doi:10.1016/j.fertnstert.2012.06.029.
20. Bedaiwy MA, Falcone T, Sharma RK, et al. Prediction of endometriosis with serum and peritoneal fluid markers: a prospective controlled trial. Hum Reprod. 2002;17(2):426-431. doi:10.1016/S0015-0282(01)03024-2.
21. Mahnke JL, Dawood MY, Huang JC. Vascular endothelial growth factor and interleukin-6 in peritoneal fluid of women with endometriosis. Fertil Steril. 2000;73(1):166-170. http://www.ncbi.nlm.nih.gov/pubmed/10632434.
22. Arici A. Local cytokines in endometrial tissue: the role of interleukin-8 in the pathogenesis of endometriosis. Ann N Y Acad Sci. 2002;955:101-9, 396-406. http://www.ncbi.nlm.nih.gov/pubmed/11949939.
23. Calhaz-Jorge C, Costa AP, Santos MC, Palma-Carlos ML. Peritoneal fluid concentrations of interleukin-8 in patients with endometriosis depend on the severity of the disorder and are higher in the luteal phase. Hum Reprod. 2003;18(3):593-597. doi:10.1093/humrep/deg122.
24. Akoum A, Al-Akoum M, Lemay A, Maheux R, Leboeuf M. Imbalance in the peritoneal levels of interleukin 1 and its decoy inhibitory receptor type II in endometriosis women with infertility and pelvic pain. Fertil Steril. 2008;89(6):1618-1624. doi:10.1016/j.fertnstert.2007.06.019.
25. Rana N, Braun DP, House R, Gebel H, Rotman C, Dmowski WP. Basal and stimulated secretion of cytokines by peritoneal macrophages in women with endometriosis. Fertil Steril. 1996;65(5):925-930.
26. Martínez S, Garrido N, Coperias JL, et al. Serum interleukin-6 levels are elevated in women with minimal-mild endometriosis. Hum Reprod. 2007;22(3):836-842. doi:10.1093/humrep/del419.
27. Eisermann J, Gast MJ, Pineda J, Odem RR, Collins JL. Tumor necrosis factor in peritoneal fluid of women undergoing laparoscopic surgery. Fertil Steril. 1988;50(4):573-579.
28. Mar??-Alexandre J, S??nchez-Izquierdo D, Gilabert-Estell??s J, Barcel??-Molina M, Braza-Bo??ls A, Sandoval J. miRNAs regulation and its role as biomarkers in endometriosis. Int J Mol Sci. 2016;17(1). doi:10.3390/ijms17010093.
29. Kuokkanen S, Chen B, Ojalvo L, Benard L, Santoro N, Pollard JW. Genomic profiling of microRNAs and messenger RNAs reveals hormonal regulation in microRNA expression in human endometrium. Biol Reprod. 2010;82(4):791-801. doi:10.1095/biolreprod.109.081059.
30. Asirvatham AJ, Magner WJ, Tomasi TB. miRNA regulation of cytokine genes. Cytokine. 2009;45(2):58-69. doi:10.1016/j.cyto.2008.11.010.

31. Bartel DP. MicroRNAs: Target Recognition and Regulatory Functions. Cell. 2009;136(2):215-233. doi:10.1016/j.cell.2009.01.002.
32. Cho S, Mutlu L, Grechukhina O, Taylor HS. Circulating microRNAs as potential biomarkers for endometriosis. Fertil Steril. 2015;103(5):1252-1260.e1. doi:10.1016/j.fertnstert.2015.02.013.
33. Ohlsson Teague EMC, Print CG, Hull ML. The role of microRNAs in endometriosis and associated reproductive conditions. Hum Reprod Update. 2009;16(2):142-165. doi:10.1093/humupd/dmp034.
34. Santamaria X, Taylor H. MicroRNA and gynecological reproductive diseases. Fertil Steril. 2014;101(6):1545-1551. doi:10.1016/j.fertnstert.2014.04.044.
35. Wang WT, Zhao YN, Han BW, Hong SJ, Chen YQ. Circulating microRNAs identified in a genome-wide serum microRNA expression analysis as noninvasive biomarkers for endometriosis. J Clin Endocrinol Metab. 2013;98(1):281-289. doi:10.1210/jc.2012-2415.
36. Cosar E, Mamillapalli R, Ersoy GS, Cho SY, Seifer B, Taylor HS. Serum microRNAs as diagnostic markers of endometriosis: a comprehensive array-based analysis. Fertil Steril. 2016;106(2):402-409. doi:10.1016/j.fertnstert.2016.04.013.
37. Suryawanshi S, Vlad AM, Lin HM, et al. Plasma MicroRNAs as novel biomarkers for endometriosis and endometriosis-associated ovarian cancer. Clin Cancer Res. 2013;19(5):1213-1224. doi:10.1158/1078-0432.CCR-12-2726.
38. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and. Methods. 2001;25:402-408. doi:10.1006/meth.2001.1262.
39. Sullivan KE, Cutilli J, Piliero LM, et al. Measurement of Cytokine Secretion, Intracellular Protein Expression, and mRNA in Resting and Stimulated Peripheral Blood Mononuclear Cells. Clin Vaccine Immunol. 2000;7(6):920-924. doi:10.1128/CDLI.7.6.920-924.2000.
40. Garrelds IM, Van Hal PTW, Haakmat RC, Hoogsteden HC, Saxena PR, Zijlstra FJ. Time Dependent Production of Cytokines and Eicosanoids by Human Monocytic Leukaemia U937 Cells; Effects of Glucocorticosteroids. Mediators Inflamm. 1999;8(4-5):229-235. doi:10.1080/09629359990397.
41. Naegelen I, Beaume N, Plançon S, Schenten V, Tschirhart EJ, Bréchard S. Regulation of Neutrophil Degranulation and Cytokine Secretion: A Novel Model Approach Based on Linear Fitting. J Immunol Res. 2015;2015. doi:10.1155/2015/817038.
42. Mosser DM, Zhang X. Activation of murine macrophages. Curr Protoc Immunol. 2008;Chapter 14:Unit 14.2. doi:10.1002/0471142735.im1402s83.
43. Braun DP, Gebel H, House R, Rana N, Dmowski NP. Spontaneous and induced synthesis of cytokines by peripheral blood monocytes in patients with endometriosis. Fertil Steril. 1996;65(6):1125-1129. http://www.ncbi.nlm.nih.gov/pubmed/8641484.
44. Arici A, Tazuke SI, Attar E, Kliman HJ, Olive DL. Interleukin-8 concentration in peritoneal fluid of patients with endometriosis and modulation of interleukin-8 expression in human mesothelial cells. Mol Hum Reprod. 1996;2(1):40-45. doi:10.1093/molehr/2.1.40.
45. Keenan JA, Chen TT, Chadwell NL, Torry DS, Caudle MR. IL-1 beta, TNF-alpha, and IL-2 in peritoneal fluid and macrophage-conditioned media of women with endometriosis. Am J Reprod Immunol. 1995;34(6):381-385. http://www.ncbi.nlm.nih.gov/pubmed/8607944.
46. Naqvi H, Mamillapalli R, Krikun G, Taylor HS. Endometriosis Located Proximal to or Remote From the Uterus Differentially Affects Uterine Gene Expression. Reprod Sci. 2016;23(2):186-191. doi:10.1177/1933719115613449.

47. Goetz LG, Mamillapalli R, Taylor HS. Low Body Mass Index in Endometriosis Is Promoted by Hepatic Metabolic Gene Dysregulation in Mice. Biol Reprod. 2016;95(6):115-115. doi:10.1095/biolreprod.116.142877.
48. Sakr S, Naqvi H, Komm B, Taylor HS. Endometriosis impairs bone marrow-derived stem cell recruitment to the uterus whereas bazedoxifene treatment leads to endometriosis regression and improved uterine stem cell engraftment. Endocrinology. 2014;155(4):1489-1497. doi:10.1210/en.2013-1977.
49. Santamaria X, Massasa EE, Taylor HS. Migration of cells from experimental endometriosis to the uterine endometrium. Endocrinology. 2012;153(11):5566-5574. doi:10.1210/en.2012-1202.
50. Chaudhuri A a, So AY-L, Sinha N, et al. MicroRNA-125b potentiates macrophage activation. J Immunol. 2011;187(10):5062-5068. doi:10.4049/jimmunol.1102001.
51. Roush S, Slack FJ. The let-7 family of microRNAs. Trends Cell Biol. 2008;18(10):505-516. doi:10.1016/j.tcb.2008.07.007.
52. Grechukhina O, Petracco R, Popkhadze S, et al. A polymorphism in a let-7 microRNA binding site of KRAS in women with endometriosis. EMBO Mol Med. 2012;4(3):206-217. doi:10.1002/emmm.201100200.
53. Cho SH, Mutlu L, Zhou Y, Taylor HS. Aromatase inhibitor regulates let-7 expression and let-7f induced cell migration in endometrial cells from women with endometriosis. Fertil Steril. 2016;106(3):673-680. doi:10.1016/j.fertnstert.2016.05.020.
54. Teng G gen, Wang W hong, Dai Y, Wang S jun, Chu Y xiang, Li J. Let-7b Is Involved in the Inflammation and Immune Responses Associated with Helicobacter pylori Infection by Targeting Toll-Like Receptor 4. PLoS One. 2013;8(2). doi:10.1371/journal.pone.0056709.

Figure 1. Quantification of cytokines by ELISA in serum from women with and without endometriosis. The control group was comprised of 26 subjects while the endometriosis group had 20 subjects. Determination of cytokine concentration for every serum sample was carried out twice in duplicate. Data points represent individual mean concentration and group mean +/- SD. Distribution of cytokine serum levels showed a statistically significant increase in the level of TNF-α, IL-1β and IL-6 in patients with endometriosis compared to control. *denotes statistical significance ($p < 0.05$) vs. control group.

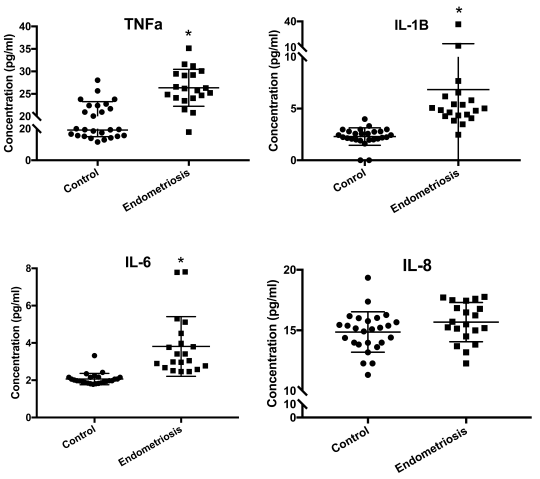
Figure 2. Expression levels of miRNA in serum of women with and without endometriosis by RT-qPCR. The control group was comprised of 26 subjects while the endometriosis group had 20 subjects. Experiments were carried out twice each time in triplicate. Data represents mean +/- SD. There is a statistically significant up-regulation of miR-125b and a significant down-regulation in patients with endometriosis compared to controls. *denotes statistical significance ($p < 0.05$) vs. control group.

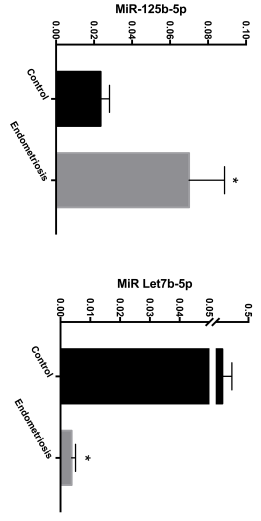
Figure 3 Pearson correlation scatter plots. (a) Showing positive correlation between expression levels of miR 125b-5p and TNF-α ($p<0.001$), IL-1β ($p<0.001$), and IL-6 ($p<0.001$). Interleukin-6 showed the highest positive correlation ($r= 0.961$). (b**)** Showing negative correlation between expression levels of miR Let7b-5p and TNF-α ($p=0.017$). Interleukin-1b and Interleukin-6 showed a trend towards a negative correlation. Pearson correlation coefficient (R) as shown for serum of subjects ($n=20$) with endometriosis.

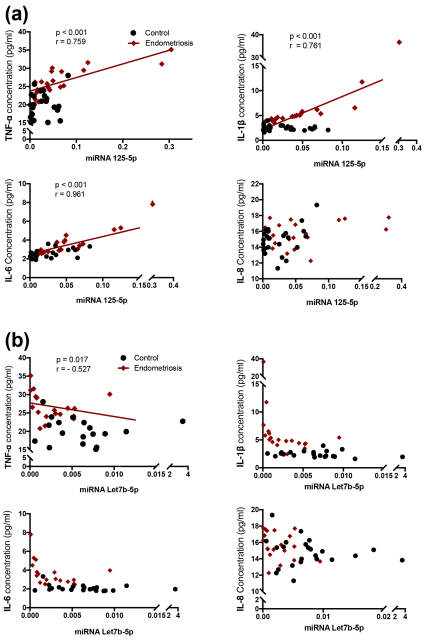
Figure 4. **Transfection of microRNAs in macrophages altered cytokine expression.** miR-125b is increased in the circulation of women with endometriosis while Let-7b is decreased. Cytokine mRNA expression in macrophages transfected with miR-125b and Let-7b mimic and inhibitor were assessed by qRT-PCR and presented as fold change relative to the control (scramble). Increased relative expression of TNF-α (2.66 fold, $p<0.001$) IL-1β (1.86 fold, $p=0.0019$), IL-6 (1.51 fold, $p<0.001$), and IL8 (1.70 fold, $p=0.0078$) in macrophages transfected with miRNA 125b-5p; decreased expression of TNF-α (0.58 fold, $p<0.001$), and IL-8 (0.66 fold, $p=0.0019$) in group transfected with miR Let-7b-5p; and increased expression of TNF-α (1.5 fold, $p=0.002$) IL-1β (2.94 fold, $p=0.002$), IL-6 (1.59 fold, $p=0.006$), and IL-8 (6.19 fold, $p=0.008$) in macrophages transfected with Let 7b-5p inhibitor. Bars represent the mean ± S.E. of three individual experiments, each performed in triplicate. *denotes statistical significance ($p < 0.05$) vs. control.

Figure 5. Confocal analysis of cytokine protein expression. Merged confocal images comparing different miRNAs mimic or inhibitor transfection In each image, blue represents cell nuclei stained with DAPI [4',6'-diamino-2-phenylindole]; for each row, red indicates TNF-α, purple indicates IL-1β, yellow indicates IL-6 and green indicates IL-8. Images are representative of three random fields in each slide, with n=4 in each group, done in two independent experiments. Scale bars, 100um. Macrophages transfected with miR-125b (elevated in endometriosis) and Let 7b (decreased in endometriosis) inhibitor showed a higher percentage of fluorescent cells for all four cytokines compared to their corresponding controls. Ratio of positive cells did not differ from the control in macrophages transfected with miR- Let 7b.

Figure 6. LPS stimulation induces cytokine secretion after microRNA alterations associated with endometriosis. LPS stimulation (100 ng/ml) of transfected macrophages showing protein levels determined by ELISA. TNF-α, IL-1β, IL-6 and IL-8 were significantly higher in conditioned media of cells transfected with microRNA 125b and Let 7b inhibitor compared to the controls. Bars represent the mean ± S.E. of three individual experiments, each performed in triplicate. Differences were considered as statistically significant for p-values <0.05. *denotes statistical significance between control mimic vs. microRNA mimic; $^{\#}$denotes statistical significance between control inhibitor vs. microRNA inhibitor.







ENDOCRINE SOCIETY ADVANCE ARTICLE: JCEM THE JOURNAL OF CLINICAL ENDOCRINOLOGY & METABOLISM