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ORIGINAL ARTICLE ***Reproductive endocrinology***

Endometrial stromal fibroblasts from women with polycystic ovary syndrome have impaired progesterone-mediated decidualization, aberrant cytokine profiles and promote enhanced immune cell migration *in vitro*

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STUDY QUESTION: Do endometrial stromal fibroblasts (eSF) in women with polycystic ovary syndrome (PCOS) (eSF_{pcos}) exhibit altered estrogen and/or progesterone (P_4) responses, which may explain some of the adverse reproductive outcomes and endometrial pathologies in these women?

SUMMARY ANSWER: *In vitro*, eSF from women with PCOS exhibit an aberrant decidualization response and concomitant changes in pro-inflammatory cytokine, chemokine and matrix metalloproteinase (MMP) release and immune cell chemoattraction. *In vivo* these aberrations may result in suboptimal implantation and predisposition to endometrial cancer.

WHAT IS KNOWN ALREADY: The endometrium in women with PCOS has several abnormalities including progesterone (P_4) resistance at the gene expression level, likely contributing to subfertility, pregnancy complications and increased endometrial cancer risk in PCOS women.

STUDY DESIGN, SIZE, DURATION: Prospective, university-based, case–control, *in vitro* study.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Cultures of eSF_{PCOS} ($n = 12$, Rotterdam and NIH criteria) and $eSF_{Control}$ (Ctrl) ($n = 6$, regular cycle length, no signs of hyperandrogenism) were treated with vehicle, estradiol (E_2, 10 nM) or E_2P_4 (10 nM/1 μM) for 14 days. Progesterone receptor (PGR) mRNA was assessed with quantitative real-time PCR (qRT–PCR) and eSF decidualization was confirmed by insulin-like growth factor-binding protein-1 (IGFBP-1) transcript and protein expression. Fractalkine (CX3CL1), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL) 6, 8 and 11, macrophage chemoattractant protein (MCP) 1 and 3, CCL5 (RANTES) and MMPs (MMP1, 2, 3, 7, 9, 10 and 12) were measured in conditioned media by Luminex multiplex assays, and chemotactic activity of the conditioned media was tested in a migration assay using CD14+ monocyte and CD4+ T-cell migration assay. Effects of IL-6 (0.02, 0.2, 2 or 20 ng/ml) or IL-8 (0.04, 0.4, 4, or 40 ng/ml) or combination (0.2 ng/ml IL-6 and 4.0 ng/ml IL-8) on 14-d decidualization were also tested. ANOVA with pre-planned contrasts was used for statistical analysis.

MAIN RESULTS AND THE ROLE OF CHANCE: Hormonal challenge with E_2P_4 to induce decidualization revealed two distinct subsets of eSF_{PCOS}. Eight eSF_{PCOS} (dPCOS) and all eSF_{Ctrl} (dCtrl) cultures showed a normal decidualization response to E_2P_4 as determined by morphology and IGFBP-1 secretion. However, 4 eSF_{PCOS} cultures showed blunted decidualization (ndPCOS) in morphological assessment and low IGFBP-1 levels even though all three groups exhibited normal estrogen-mediated increase in PGR expression. Interestingly dPCOS had decreased IL-6 and

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GM-SCF secretion compared with dCtrl, whereas the ndPCOS cultures showed increased IL-6 and 8, MCP1, RANTES and GM-CSF secretion at base-line and/or in response to E_2 or E_2P_4 compared with dCtrl and/or dPCOS. Furthermore, even though PGR expression was similar in all three groups, P_4 inhibition of MMP secretion was attenuated in ndPCOS resulting in higher MMP2 and 3 levels. The conditioned media from ndPCOS had increased chemoattractic activity compared with dCtrl and dPCOS media. Exogenously added IL-6 and/or 8 did not inhibit decidualization in eSF_{Ctrl} indicating that high levels of these cytokines in ndPCOS samples were not likely a cause for the aberrant decidualization.

LIMITATIONS, REASONS FOR CAUTION: This is an *in vitro* study with a small sample size, utilizing stromal cell cultures from proliferative and secretory phase endometrium. The effect of PCOS on endometrial epithelium, another major histoarchitectural cell compartment of the endometrium, was not evaluated and should be considered in future studies. Furthermore, results obtained should also be confirmed in a larger data set and with mid/late secretory phase *in vivo* samples and models.

WIDER IMPLICATIONS OF THE FINDINGS: The alterations seen in ndPCOS may contribute to endometrial dysfunction, subfertility and pregnancy complications in PCOS women. The results emphasize the importance of understanding immune responses related to the implantation process and normal endometrial homeostasis in women with PCOS.

STUDY FUNDING/COMPETING INTEREST(S): Sigrid Juselius Foundation, Academy of Finland, Finnish Medical Foundation, Orion-Farmos Research Foundation (to T.T.P.), the NIH *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) U54HD 055764-07 Specialized Cooperative Centers Program in Reproduction and Infertility Research (to L.C.G.), the NICHD the Ruth L. Kirschstein National Research Service Awards grant 1F32HD074423-03 (to J.C.C.). The authors have no competing interests.

Key words: PCOS / endometrium / decidualization / cytokines / MMP

Introduction

In humans decidualization is a progesterone (P_4) driven differentiation process essential to prepare the endometrium for successful embryo implantation and maintenance of pregnancy (Cha *et al.*, 2012). Decidualization occurs in the secretory phase when endometrium undergoes vast and complex cellular differentiation dependent on progesterone receptor (PGR) up-regulation by estradiol (E_2), activation of P_4 signaling and convergence of cyclic adenosine monophosphate (cAMP) signaling (Aghajanova *et al.*, 2009; Zhu *et al.*, 2014). Glandular secretory transformation occurs in response to P_4, and perivascular endometrial stromal fibroblasts (eSF) initiate decidualization throughout the endometrium that continues to progress if conception occurs (Cha *et al.*, 2012; Zhu *et al.*, 2014). Decidualized eSFs undergo morphological and functional changes with glycogen and lipid accumulation in the cytoplasm, providing a source of nutrition for the developing embryo prior to placental development (Cha *et al.*, 2012; Zhu *et al.*, 2014). In response to P_4-mediated activation of transcription factors forkhead box protein O1 (FOXO1) and homeobox A10 (HOXA10), decidualized eSFs produce insulin-like growth factor-binding protein 1 (IGFBP-1), commonly used as a marker of decidualization. IGFBP-1 restricts trophoblast invasion and endometrial growth, in part, by inhibiting insulin growth factor 1 (IGF-1) action (Irwin and Giudice, 1998).

Several growth factors, cytokines and matrix metalloproteinases (MMPs) play a role in the decidualization process to optimize the endometrial environment for implantation where balanced trophoblast invasion, outgrowth and vasculature establishment are achieved (Staun-Ram and Shalev, 2005). Several cytokines, e.g. IL-11, LIF, IL-6 and transient immune cells, are present during decidualization and participate in the communication between differentiated endometrium and the embryo (Kojima *et al.*, 1994; Cork *et al.*, 2001). In addition to cytokines, MMPs, a family of zinc-dependent proteases, are also highly expressed in decidua as they proteolyze the extracellular matrix, allowing tissue growth and remodeling in response to normal proliferative and differentiative signals (Godbole *et al.*, 2011). MMPs allow for histoarchitectural modifications necessary for embryo attachment and invasion where suppression of endometrial MMPs is necessary to maintain endometrial stability preventing excessive invasion of extravillus trophoblasts and abnormal placental development (Visse and Nagase, 2003; Licht *et al.*, 2007; Godbole *et al.*, 2011). In endometrium P_4 normally suppresses MMP expression directly, up-regulates tissue inhibitors of metalloproteinases (TIMPs) and modulates cytokine activity (Higuchi *et al.*, 1995; Bischof *et al.*, 1998; Wissink *et al.*, 1998)—all of which control MMP actions, although in P_4-resistant disorders, this suppression of MMPs is not observed.

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in reproductive aged women, affecting around 10% of this population and characterized as menstrual dysfunction, anovulatory infertility, hyperandrogenism and insulin resistance (Amsterdam ESHRE/ASRM-Sponsored 3rd PCOS Consensus Workshop Group 2012). In PCOS impaired follicle maturation and consequent anovulation cause a chronic P_4-deficient state that affects the endometrial milieu. Furthermore, even with ovulation (and thus P_4 acting on endometrium), the PCOS endometrium has several abnormalities including altered steroid receptor and glucose transporter 4 (GLUT4) expression as well as P_4-resistance (Apparao *et al.*, 2002; Villavicencio *et al.*, 2006; Savaris *et al.*, 2011; Carvajal *et al.*, 2013). Moreover, we as well as others have recently reported an inflammatory milieu in PCOS endometrium with an increased inflammatory profile in the proliferative phase and decreased uterine natural killer cell influx in late secretory phase. This is likely to contribute to subfertility, increased prevalence of pregnancy complications and endometrial cancer in these women (Matteo *et al.*, 2010; Haoula *et al.*, 2012; Piltonen *et al.*, 2013; Barry *et al.*, 2014; Naver *et al.*, 2014)

Since P_4-regulation of endometrium during the secretory phase involves a complex network of differentiative cues with growth and pro-inflammatory factors and MMPs, we hypothesized that eSF from women with PCOS may show aberrant P_4-responsiveness during *in vitro* decidualization and concomitant altered production of pro-inflammatory cytokines and MMPs.

Materials and Methods

Study subjects and tissues

Endometrial tissue biopsies were obtained through the National Institute of Health (NIH)/University of California, San Francisco (UCSF), Human Endometrial Tissue and DNA Bank in accordance with the guidelines of the Declaration of Helsinki. Informed consent was obtained from all participants in the UCSF Center for Reproductive Health, and the study was approved by the UCSF Committee on Human Research. For the cytokine challenge, tissue samples were acquired from the Department of Obstetrics and Gynecology, University of Oulu, Finland. The tissue collection was approved by the hospital ethics committee and informed consent was obtained from all participants. Endometrial biopsies (Pipelle, Cooper Surgical, Shelton, CT) were collected, and eSF were isolated from 12 women with PCOS fulfilling both Rotterdam and NIH criteria and 11 control women (Table I). All PCOS subjects had normal 17-hydroxyprogesterone, prolactin and thyroid hormone levels. Control samples were obtained from healthy volunteers and women undergoing benign gynecological surgery. All controls reported menstrual cycles with regular interval (25–35 days) and no indication of having PCOS. Only one control subject was a smoker. None of the subjects was exposed to hormonal medications for at least 3 months prior to tissue sampling and were confirmed not pregnant during the time of study participation. The clinical summary of the study participants and tissue samples is shown in Table I.

Primary eSF cultures and decidualization experiments

eSF isolation and culture

Endometrial samples were digested with collagenase (6.4 mg/ml collagenase type I and 100 U/ml hyaluronidase in Hanks Buffered Salt Solution with Ca^{2+} and Mg^{2+}) following filtration using an optimized protocol as previously described (Aghajanova *et al.*, 2009; Chen *et al.*, 2013). Stromal cell cultures we established as reported earlier with purity validated by vimentin and e-cadherin immunohistochemistry (Chen *et al.*, 2013). Cells were cultured in growth medium (phenol red-free medium of 3:1 high-glucose Dulbecco's Modified Eagle's Medium [DMEM]/MCDB-105 (a fibroblast-lineage supplement medium containing trace elements and amino acids), 0.676 mM sodium pyruvate, 10% (v/v) charcoal-stripped fetal bovine serum (FBS), 1% (w/v) penicillin–streptomycin mix, 50 μg/ml gentamycin, 5 μg/ml insulin] which was renewed approximately every 2–3 days for up to the third passage and thereafter the cells were used in experiments.

Decidualization

One hundred thousand eSFs were plated into 12-well plates in duplicate and cultured in growth medium. When confluent, cells were incubated for 24 h in low-serum medium (3:1 high-glucose DMEM/MCDB-105, 0.75 mM sodium pyruvate, 50 μg/ml gentamycin, 2% FBS) prior to hormone treatment. For decidualization, cultures of eSF_{PCOS} ($n = 12$) and eSF_{Ctrl} ($n = 6$) were treated with 0.1% ethanol vehicle (veh), estradiol (E_2,10 nM) or E_2P_4 (10 nM/1 μM) in low-serum medium for 14 days, with feeding every other day as previously described (Aghajanova *et al.*, 2009). At day 14 conditioned media were collected and stored at −80°C, and cells were trypsinized, counted (TC20 Automated Cell Counter, Bio-Rad, to adjust the cell counts for cytokine and MMP measurements) and stored at −80 °C for RNA isolation.

Morphological assessment

Cell morphology was assessed at every media change using inverted phase contrast light microscopy. At day 14, prior to cell harvesting all wells were imaged (Zeiss Thornwood, NY) and the morphological changes of eSF were estimated by two different observers. Decidualized eSF cultures were classified based on cell shape as decidualized (spindle-like) or non-decidualized (polygonal/cobblestone).

RNA isolation, cDNA synthesis and quantitative real-time polymerase chain reaction

Total RNA was isolated from cultured eSF (Nucleospin RNA purification kit, Machery Nagel, Bethlehem, PA) following the manufacturer's protocol, including DNAse treatment. Purity of all RNA samples was confirmed (Nanodrop, Wilmington, DE) and cDNA were synthesized utilizing the Taqman Preamp Master mix (Life Technologies, Grand Island, NY), utilizing a 14-cycle enrichment protocol, as previously described (Piltonen *et al.*, 2013).

To determine the hormonally-regulated gene expression in eSF in different study groups, PGR (a measure of estrogen responsiveness) and IGFBP-1 (a measure of progesterone responsiveness) mRNA expression was measured using 20 ng cDNA, and 1 μM for each primer pair. Amplification was performed using the Stratagene MX3005P (Agilent, Santa Clara, CA) Thermocycler with variables previously described (Aghajanova *et al.*, 2009). Dissociation curves for both target and housekeeping genes were utilized to ensure the absence of primer dimers and other non-specific amplification. Primers were designed by Fluidigm and optimized for quantitative real-time polymerase chain reaction (qRT–PCR) following the Fluidigm Biomark guidelines on mRNA amplification, including primer amplification efficiency, amplicon size and appropriate dissociation temperatures governing mRNA amplification. The amplification conditions were compliant with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (Johnson *et al.*, 2014). All target gene expressions were normalized to YWHAZ, a highly conserved gene in PCOS endometrium (Sadek *et al.*, 2012), coding the 14-3-3 signal transduction protein. No significant changes in YWHAZ expression were observed across dCtrl, dPCOS, ndPCOS, suggesting high stability in expression across treatment groups. The comparative (delta-delta) Ct method was used to measure relative gene expression (ABI User bulletin 2).

Protein measurements

ELISA

IGFBP-1 in conditioned media was measured using ELISA (Alpha Diagnostics, San Antonio, TX). Samples were assayed in duplicate and both standard curve and pre-measured IGFBP-1 recombinant protein controls were run in each experiment. Levels of IGFBP-1 for each sample were normalized to total cell count for each sample.

Luminex multiplex assays

A custom multiplex Luminex kit (EMD Millipore, Billerica, MA) was utilized to assay the quantity of cytokines and MMPs secreted into the conditioned media. Targeted cytokines included fractalkine (CX3CL1), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL), 8, 11, monocyte chemoattractant protein 1 (MCP-1; CCL2) and 3 (CCL7), and Regulated upon Activation, Normal T-cell Expressed, and Secreted Chemokine (c-c motif) ligand 5 (CCL5; RANTES). In addition, MMP 1, 2, 3, 7, 9, 10 and 12 were also measured. All protocols were based on the manufacturer's specifications and performed as previously described (Chen *et al.*, 2013). All experiments were conducted in duplicate. To determine inter-assay variability, several samples were run on two or more kits and showed an inter-assay variability <5%.

Migration assays

Immune cells utilized for migration assays were isolated from buffy coats purchased from the Stanford Blood Bank (Palo Alto, CA). Buffy coats were

Table I Clinical characteristics of the study subjects.

ID	Group	Age	BMI	Histology	PCOS	Diagnosis	Procedure	Medication	Smoking	Ethnicity
C1[a]	Ctrl	37	32.36	PE	No	Undesired fertility	Pipelle, tubal ligation	None	No	Asian
C2[a]	Ctrl	39	24.31	PE	No	Undesired fertility	Pipelle, tubal ligation	None	No	Asian
C3[a]	Ctrl	41	24	SE	No	Ovarian cyst	Pipelle, cystectomy	None	No	Caucasian
C4[a]	Ctrl	43	31.22	PE	No	Menorrhagia, ut. fibroids, adenomyosis	Pipelle, hysterectomy	None	No	Black
C5[a]	Ctrl	24	18.8	SE	No	Volunteer	Pipelle	None	No	Asian
C6[a]	Ctrl	32	24.9	PE	No	Volunteer	Pipelle	None	No	Caucasian
C7[b]	Ctrl	48	25.06	PE	No	Fibroids, rectocele	Pipelle, hysterectomy, kolporafia posterior	None	No	Caucasian
C8[b]	Ctrl	29	21.48	SE	No	Cystocele, descensus uteri	Pipelle, hysterectomy, kolporafia anterior	None	No	Caucasian
C9[b]	Ctrl	42	28.7	PE	No	Abdominal pain	Pipelle, diagnostic laparoscopy	Omepratzol	Yes	Caucasian
C10[b]	Ctrl	43	32	SE	No	Rectocele	Pipelle, kolporafia posterior	Lamictal, melatonin, mirtazapin	No	Caucasian
C11[b]	Ctrl	41	25.6	PE	No	Volunteer	Pipelle	Seloken	No	Caucasian
dPC1[a]	PCOS	29	41.15	PE	HA, A, PCO	PCOS, reflux, sleep apnea, IGT	Pipelle	Metformin	No	Caucasian
dPC2[a]	PCOS	23	18.21	SE	Hirsutism, OA, PCO	PCOS	Pipelle	None	No	Middle eastern
dPC3[a]	PCOS	40	39.2	SE	HA, OA, PCO	PCOS	Pipelle	None	No	Caucasian
dPC4[a]	PCOS	28	21.8	Interval	HA, OA, PCO	PCOS	Pipelle	None	No	More than one
dPC5[a]	PCOS	26	22.24	SE	HA, acne, OA, PCO	PCOS	Pipelle	None	No	Caucasian
dPC6[a]	PCOS	27	33.6	PE	HA, hirsutism, OA, PCO	PCOS, IGT	Pipelle	Metformin	No	N/A
dPC7[a]	PCOS	25	24.3	PE	HA, acne, OA, PCO	PCOS	Pipelle	None	No	More than one
dPC8[a]	PCOS	36	33	PE	HA, OA, PCO	PCOS	Pipelle	Salbutamol inh.	No	Asian
ndPC1[a]	PCOS	30	26.26	PE	HA, hirsutism, acne, A, PCO	PCOS, Factor V Leiden heterozygote	Pipelle	None	No	Caucasian
ndPC2[a]	PCOS	25	30.37	PE	HA, acne, A, PCO	PCOS	Pipelle	None	No	Caucasian
ndPC3[a]	PCOS	38	39.76	PE	HA, hirsutism, acne, OA, PCO	PCOS, migrane, IGT	Pipelle	None	No	Black
ndPC4[a]	PCOS	33	34.8	N/A	HA, hirsutism, OA, PCO	PCOS	Pipelle	None	No	Hispanic

PE, proliferative phase; S, secretory phase; HA, biochemical hyperandrogenism; OA, oligo-amenorrhea; A, amenorrhea; PCO, polycystic ovaries; IGT, impaired glucose tolerance in OGTT; N/A, not acquired.
[a]Samples used in the primary decidualization study.
[b]Samples used in cytokine challenge test.

processed by Ficoll-density gradient centrifugation to separate peripheral blood mononuclear cells from red blood cells, granulocytes and platelets. Monocytes were positively selected with $CD14^+$ and T-cells with $CD4^+$ specific microbeads (Miltenyi Biotec, Auburn, CA). Both $CD14^+$ monocytes and $CD14^-/CD4^+$ T cells were allowed to equilibrate in Roswell Park Memorial Institute medium (RPMI) 1640 with 10% FBS overnight. Cells were then treated with serum-free media for an additional 12 h before initiation of migration experiments.

$CD14^+$

$CD14^+$ monocyte migration was conducted using the Millipore QCM 5 μm migration assay (EMD Millipore). Attached monocytes in culture were detached using Accutase (EMD Millipore), pelleted and resuspended in PBS with 1% BSA, and 10^5 cells were added to the upper migration chamber. Then, 500 μl of low-serum medium with E_2P_4 (same concentrations as utilized for the decidualization experiment) were added to the bottom chamber as negative controls. Standard curves were established with medium containing 2% FBS, a known chemoattractant for monocytes and T cells, and 6 h was determined as the equilibrium period during which maximal migratory activity occurred. Conditioned media pooled from eSF cultures (dCtrl, dPCOS and ndPCOS) treated with or without hormones were added to the lower chamber. Cells that migrated across the membrane and adhered to the opposite side were stained with the provided staining solution and counted. Four random non-overlapping fields at ×200 were counted and averaged. Experiments were conducted in triplicate and repeated twice.

$CD4^+$

$CD4^+$ T cell migration was conducted using the QCM 3 μm non-adherent migration assay (EMD Millipore), designed to measure cell migration of non-adherent cells, including T cells. As with monocyte experiments, cells were treated with low-serum media prior to the experiment. 10^5 cells were added to the upper migration chamber while E_2P_4-treated conditioned media from different eSF cultures (dCtrl, dPCOS and ndPCOS) were added to the lower chamber. After 6 h migrated cells were collected in the bottom chamber. The provided WST reagent was added to measure the metabolic activity of migrated T cells to provide a quantification of T-cell migration (Chen *et al.*, 2014). After an additional 2 h for viability signal development, 50 μl of the WST medium containing cells were measured for optical density at 450 nm.

Cytokine challenge during decidualization

During the cytokine challenge 0.02, 0.2, 2 or 20 ng/ml IL-6 or 0.04, 0.4, 4, or 40 ng/ml of IL-8 or combination of 0.2 ng/ml IL-6 and 4.0 ng/ml IL-8 were added to E_2P_4 treatments during 14-d decidualization experiments in eSF_{Ctrl}, as described above. Morphology was assessed as in the primary decidualization study, and condition media were collected, stored, and the cells were counted in each well, as described above. WST assays were performed to confirm the non-toxicity of the cytokine concentrations added (data not shown).

Statistics

R-commander (2012) was used to determine differences in IGFBP-1 levels in conditioned media, using ANOVA with Tukey's posthoc analysis in both decidualization and cytokine challenge studies. To determine the effects of hormones and disease on patterns of cytokine and MMP secretion by eSF, data were analyzed using preconceived orthogonal contrasts with pairwise comparisons of specific experimental groups with the Statistical Analysis System software (SAS, 2011) (Chen *et al.*, 2011). Pairwise contrasts included the effects of hormones (veh versus E_2, veh versus E_2P_4 and E_2 versus E_2P_4) and the effects of disease [veh or E_2 or E_2P_4 for dCtrl versus respective treatment groups in PCOS (dPCOS and ndPCOS)]. Pairwise contrasts were also utilized to determine the effects of the conditioned media on inducing $CD14^+$ monocyte and $CD4^+$ T cell migration.

Results

Clinical characteristics

Participants in the ndPCOS group tended to be more obese than the dCtrl, although this did not reach statistical significance by ANOVA. The mean age across dCtrl, dPCOS and ndPCOS groups was comparable. In the dPCOS group, two patients were on Metformin therapy. These subjects were obese and were tested for impaired glucose tolerance in OGTT (Table I).

Impaired decidualization in eSF_{PCOS}

Morphology

All cell cultures were monitored throughout the decidualization experiment for morphological changes, and the images were captured from all cultures and treatments at day 14 prior harvesting. According to the morphological assessment none of the vehicle and E_2 cultures showed any sign of change in morphology at day 14 (Fig. 1A). However, in E_2P_4 treated wells all six control samples and eight PCOS samples showed distinct morphological changes from typical cobblestone eSF morphology into spindle-like cells beginning from day 10 (data shown at day 14, Fig. 1A). Alternatively, four PCOS samples showed no change in morphology suggesting compromised decidualization (Fig. 1A).

qRT–PCR analysis of hormone-responsive genes

To determine estradiol (E_2) responsiveness of cells, PGR expression was measured during E_2 treatment. The results showed significantly increased (3-fold, $P < 0.05$) expression of PGR transcripts in dCtrl, dPCOS and ndPCOS during E_2 treatment compared with vehicle. Expression of PGR did not differ statistically significantly among the three groups, indicating that PCOS did not affect E_2-induced PGR expression (although on average the relative expression of PGR was lower for ndPCOS in veh and E_2P_4 treated groups compared with dCtrl or dPCOS; F) (Fig. 1B). To validate the morphological findings and to confirm whether the decidualization process was successful or impaired, IGFBP-1 mRNA was also measured after vehicle or E_2P_4 treatment. The data showed that the IGFBP-1 mRNA expression in response to E_2P_4 in ndPCOS was significantly decreased ($P < 0.05$) compared with both dCtrl and dPCOS (Fig. 1B).

IGFBP-1

Decidualization was also assessed by measuring secreted IGFBP-1 protein (ELISA) into conditioned media. For vehicle and E_2 treated cells no IGFBP-1 was detected, as expected (data not shown). In contrast, all six control samples treated with E_2P_4 showed high IGFBP-1 concentration (mean ± SD; 118.94 ± 4.12 ng/10^5 cells) validating the morphological findings (Fig. 1C). The IGFBP-1 concentration in E_2P_4 treated morphologically decidualized PCOS samples (dPCOS) was equivalent to the dCtrl samples (101.58 ± 4.65 ng/10^5 cells, $P > 0.05$) (Fig 1C). As expected, PCOS samples that showed aberrant decidualization morphology, also had concomitantly low IGFBP-1 secretion (26.67 ± 13.74 ng/10^5 cells, $P < 0.05$) compared with dCtrl and

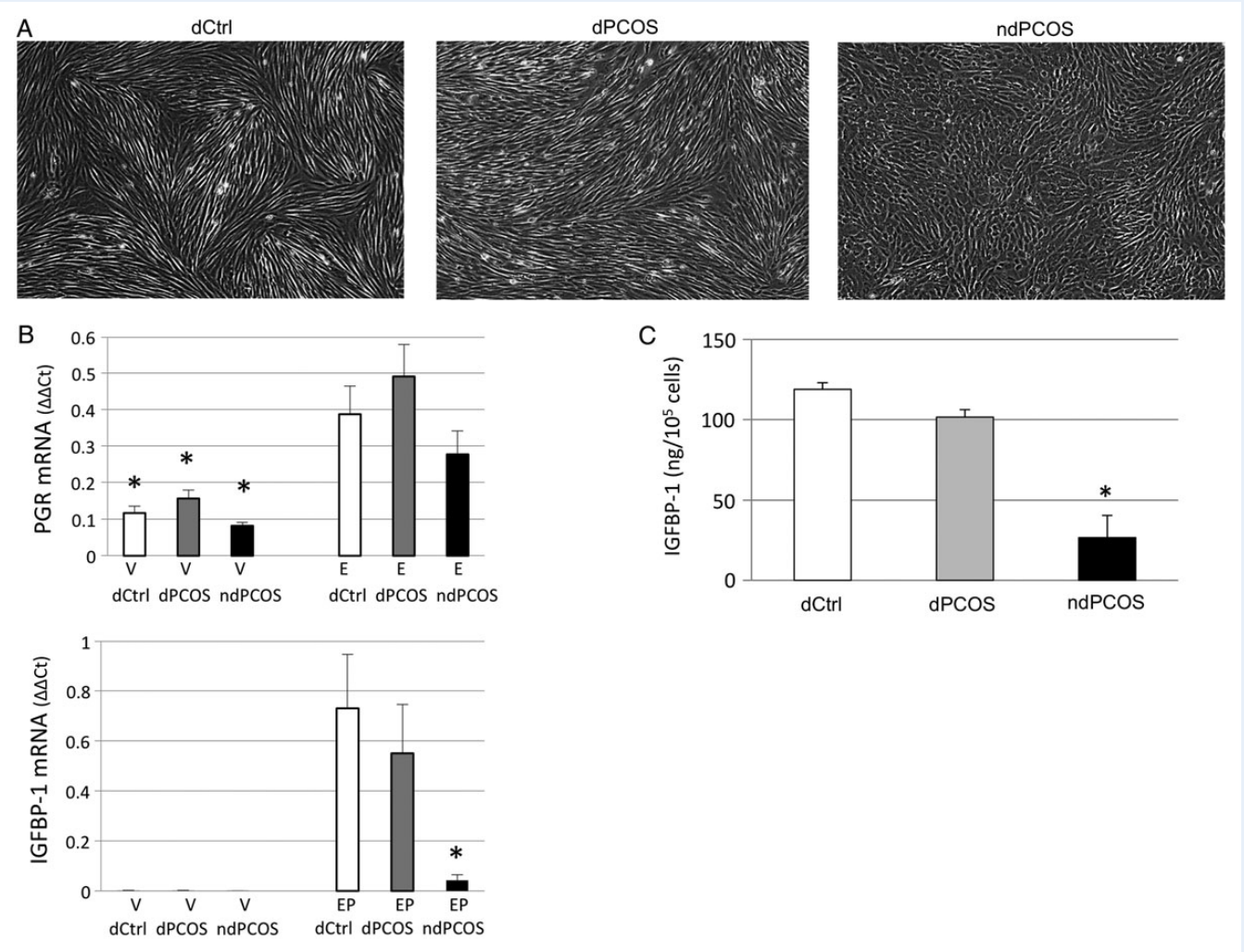

Figure 1 (**A**) Morphological assessment of eSF after 14-days treatment with 10 nM estradiol + 1 μM progesterone (E_2P_4). eSF from controls (dCtrl) and 8/12 women with PCOS (dPCOS) decidualized but eSF from 4/12 women with PCOS did not (ndPCOS). (**B**) PGR and insulin-like growth factor binding protein 1 (IGFBP-1) mRNA expression after vehicle (V) or steroid hormone (10 nM E_2 or E_2P_4) treatment. PGR expression was comparable between the study groups whereas ndPCOS presented with decreased IGFBP-1 mRNA expression. (**C**) IGFBP-1 response was also decreased at the protein level (ELISA) in ndPCOS compared with other study groups. Only cells treated with E_2P_4 are shown. No IGFBP-1 was detected after incubation with V or E_2.

dPCOS, and the difference remained after correcting for cell counts (Fig. 1C).

Altered cytokine secretion in eSF_{PCOS}

IL-6 and 8, 11, RANTES, MCP-1 and 3, GM-CSF and Fractalkine were measured in the conditioned media (Supplementary data, Table SI).

IL-6 and 8

The basal IL-6 secretion decreased in response to E_2 and/or E_2P_4 administration (Fig. 2A and Supplementary data, Table SI) within dCtrl (E_2P_4, $P = 0.03$) and ndPCOS groups (E_2 and E_2P_4, $P < 0.001$), but not in dPCOS. Interestingly, basal IL-6 secretion was lower in dPCOS compared with dCtrl ($P = 0.03$); whereas, basal IL-6 secretion was increased in ndPCOS versus dCtrl ($P < 0.0001$). Furthermore the steroid hormone response of IL-6 was blunted in ndPCOS as E_2 and E_2P_4 treated cells presented with increased IL-6 secretion compared with dCtrl and dPCOS (E_2 and E_2P_4, $P < 0.01$) (Fig. 2A, Supplementary data, Table SI). Basal IL-8 levels were comparable between the study groups; however, the E_2P_4 treated IL-8 levels were higher in ndPCOS than in dCtrl and dPCOS ($P < 0.05$, $P < 0.01$, Fig. 2B).

MCP-1, RANTES and GM-CSF

Basal MCP-1 secretion was increased in ndPCOS versus dPCOS ($P < 0.01$, Fig. 2C, Supplementary data, Table SI) and a similar trend was observed compared with dCtrl ($P = 0.06$). Treatment with E_2 and E_2P_4 decreased MCP-1 secretion in ndPCOS ($P < 0.05$, $P < 0.01$). A similar trend was also observed in dCtrl and dPCOS (Fig. 2C). Similar to IL-6, basal ndPCOS RANTES secretion was increased versus dCtrl and dPCOS (both $P < 0.0001$, Supplementary data, Table SI). In dPCOS and ndPCOS RANTES secretion was induced by P_4 compared

with basal and/or E_2 treatment ($P < 0.05$), and a similar trend was also observed in the dCtrl group although the increase did not reach statistical significance (Fig. 2D). Moreover, the E_2P_4-treated ndPCOS samples had increased RANTES levels compared with dCtrl ($P < 0.01$) (Fig. 2D). Interestingly basal GM-CSF levels were decreased in dPCOS compared with dCtrl (Fig. 2E). E_2 treatment decreased basal GM-CSF levels in dCtrl ($P \leq 0.01$), but not in the PCOS groups. E_2P_2 treatment decreased GM-CSF levels in dCtrl compared with basal levels; however, this decrease was not observed in dPCOS and there was only a trend in ndPCOS. On the other hand the E_2P_4 treatment resulted in GM-CSF levels that were statistically comparable among the groups (Fig 2E).

No statistically significant differences were observed in IL-11, MCP-3 and fractalkine levels among the study groups (Supplementary data, Table SI).

Impaired decidualization results in high secreted MMPs by ndPCOS

Matrix metalloproteinase 1, 2, 3, 7, 9, 10 and 12 were measured in the condition media (Supplementary data, Table SII).

MMP2 and 3

Basal MMP2 levels were higher in ndPCOS compared with dCtrl and dPCOS (Fig. 3A). Interestingly, regulation of MMP2 by E_2 differed in all three groups: E_2 treatment did not alter, increased and decreased MMP2 levels in dCtrl, dPCOS, and ndPCOS, respectively (Fig. 3A). Overall, in E_2-treated cells, MMP2 levels were increased in PCOS samples compared with dCtrl. E_2P_4 treatment increased MMP2 levels in all groups, and the levels were higher in the ndPCOS group versus dCtrl ($P < 0.001$, Fig. 3A). MMP3 levels did not differ between dCtrl and dPCOS. However, basal MMP3 levels were decreased by E_2P_4 treatment only in the dCtrl group; whereas, levels were increased in all treatment groups in ndPCOS versus dCtrl (Fig. 3B)

No statistically significant differences were found in secreted levels of MMP1, 7, 10 and 12 between the study or treatment groups. MMP9 was not detected.

Impaired decidualization promotes immune cell migration in PCOS

To test whether increased cytokines secreted by ndPCOS affected the migration potential of different immune cells, $CD14^+$ monocytes and $CD4^+$ T-cells were exposed to pooled dCtrl, dPCOS or ndPCOS condition media (Fig. 4). Both cell types showed increased migratory potential in a presence of ndPCOS media compared with the media of dCtrl or dPCOS, consistent with high chemotactic cytokine concentrations (IL-8, MCP-1 and RANTES) in ndPCOS.

IL-6 or IL-8 did not inhibit decidualization

As IL-6 and IL-8 levels were increased in ndPCOS samples during decidualization compared with dCtrl and dPCOS, we investigated whether IL-6 and/or 8 could inhibit decidualization. All control stromal cell samples showed typical decidualization morphology after 14-d E_2P_4 + cytokine challenge test regardless of concentration or cytokine(s) used. IGFBP-1 in condition media was assessed, and all samples had comparable IGFBP-1 concentrations to decidualizing control eSF (data not shown).

Discussion

To our knowledge, this is the first *in vitro* study showing impaired endometrial differentiation in response to steroid hormones, in women with PCOS. These novel data report decidualization failure in a subset of eSFs obtained from PCOS endometrium with concomitant altered cytokine and MMP profiles. We postulate that these abnormalities may contribute to endometrial pathologies common in PCOS women, i.e. subfertility, pre-eclampsia and endometrial cancer.

Steroid hormone response of eSF_{PCOS}

The present data revealed a subpopulation of women with PCOS whose eSFs exhibited a blunted decidualization response to E_2P_4 treatment. Interestingly, the non-decidualizing phenotype was not related to any specific clinical characteristics of the patients. Although, all ndPCOS samples were from the proliferative phase and thus may reflect their hormonal (P_4) response profile. The altered E_2P_4 response was also unrelated to PGR expression, as all study groups (dCtrl, dPCOS and ndPCOS) exhibited a robust increase in PGR gene expression in response to E_2 even though expression of decidualization marker IGFBP-1 was compromised in PCOS samples. Thus, it is possible that blunted P_4 response in eSF_{PCOS} is rooted elsewhere. DNA methylation or microRNAs have been implicated in the governing of hormonal-responsive genes, including P_4 mediated pathways (Lam *et al.*, 2012; Houshdaran *et al.*, 2014), and in this context warrant further investigation.

Decidualization and concomitant endometrial IGFBP-1 production from differentiating stromal cells are essential for embryo attachment, and thus decreased IGFBP-1 may compromise normal implantation as it regulates IGF II bioavailability at the feto-maternal interface and restricts trophoblast invasion (Giudice *et al.*, 1993; Irwin *et al.*, 1993; Irwin *et al.*, 1999). In this study, the IGFBP-1 levels in dPCOS tended to be lower than in dCtrl, although this did not reach statistical significance. On the other hand, ndPCOS with failed decidualization, demonstrated morphologically as well as by low IGFBP-1 production, may explain implantation abnormalities in some women with PCOS (Gratton *et al.*, 2002; Palomba *et al.*, 2012). In addition to its role in embryo implantation, IGFBP-1 may play a crucial role in the pathogenesis of endometrial hyperplasia/cancer, known risks in women with PCOS, by inhibiting IGF-1 and E_2-stimulated epithelial proliferation (Rutanen *et al.*, 1988; Rajkumar *et al.*, 1996).

Cytokines and chemokines secreted by eSF_{PCOS}

Cytokines and chemokines are secreted by endometrial epithelial cells and stromal fibroblasts and by stationary and transient immune cells. They participate in the dialogue between maternal and embryonic tissues and also between epithelial and stromal compartments (Cha *et al.*, 2012; Chen *et al.*, 2013). During the window of implantation (WOI) and the decidualization process, cytokines including the glycoprotein 130 (gp130) family members IL-11, LIF, IL-6 facilitate implantation; whereas, the chemokines IL-8, MCP-1 and RANTES recruit leucocyte cohorts to the implantation site (Dimitriadis *et al.*, 2005). Disturbances in cytokine and chemokine expression result in absolute or partial implantation failure and abnormal placental formation in mice and humans (Cha *et al.*, 2012; Banerjee *et al.*, 2013).

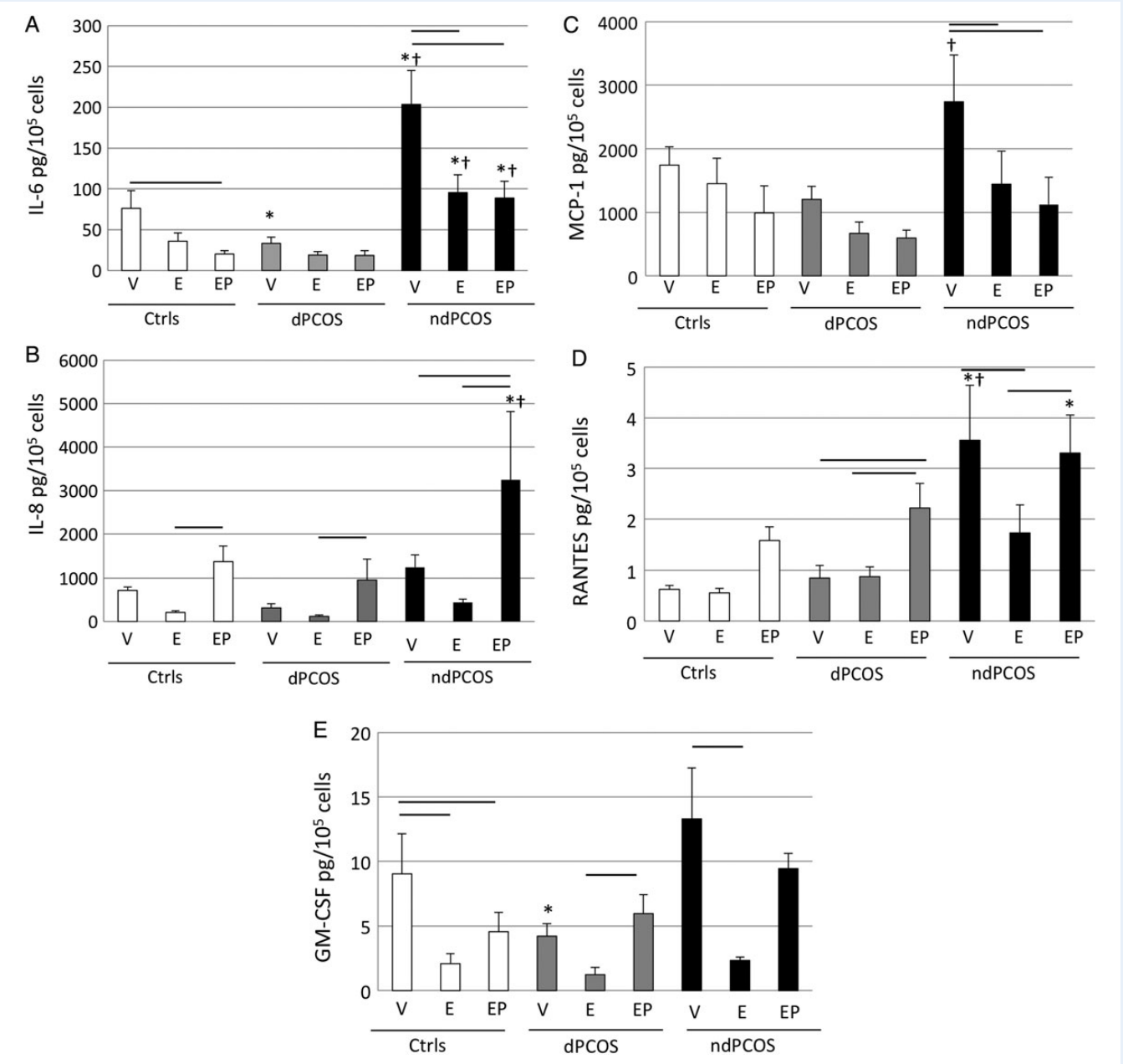

Figure 2 Multiplex protein analysis (Luminex) of different cytokines (pg/10^5 cells) after 14-days treatment with vehicle (V) or 10 nM estradiol (E_2), or 10 nM E_2 plus 1 μM progesterone (P_4). (**A**) Interleukin 6 (IL-6). (**B**) Interleukin 8 (IL-8). (**C**) Monocyte chemoattractant protein-1 (MCP-1). (**D**) Regulated on Activation, Normal T Cell Expressed (RANTES). (**E**) Granulocyte-macrophage colony-stimulating factor (GM-CSF). DCtrl, eSF from controls: dPCOS eSF from women with PCOS (8/12) that exhibit decidualization; ndPCOS, eSF from women with PCOS (4/12) that did not decidualize. The significant differences ($P < 0.05$) are marked as follows: − (line) between the different treatment groups within the study group (dCtrl or dPCOS or ndPCOS) and * (asterisk) compared with dCtrl and † (cross) compared with dPCOS in the same treatment group between different study groups.

IL-6

IL-6 is a multifunctional cytokine with a wide range of biological activities. In addition to epithelium IL-6 is expressed in endometrial stromal cells and in decidua and has an important role coordinating placental morphogenesis and trophoblast invasion (Lockwood *et al.*, 2008; Piltonen *et al.*, 2013). IL-6-deficient mice have reduced fertility and fewer implantation sites and *in vitro* IL-6 decreases mice embryo attachment and growth (Jovanovic and Vicovac, 2009; Prins *et al.*, 2012). Women experiencing recurrent miscarriage have decreased endometrial IL-6 levels, and IL-6 expression is elevated in decidua in women with pre-eclampsia (Demir *et al.*, 2009; Prins *et al.*, 2012). In this study eSF IL-6 secretion was decreased in response to E_2 and E_2P_4 in dCtrl and ndPCOS groups, demonstrating that eSF that fail to decidualize can respond in other ways to steroid hormones. Interestingly, in dPCOS basal IL-6 levels were decreased compared with controls, and they did not decrease in response to steroid hormones, similar to other groups, which may indicate

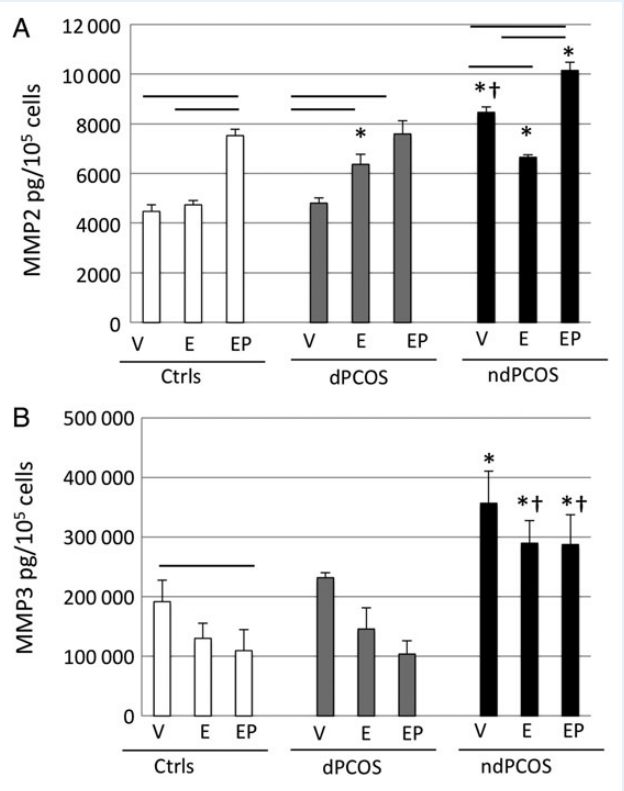

Figure 3 (**A**) MMP2 and (**B**) MMP3 multiplex protein concentration (pg/10^5 cells) after 14-days treatment with vehicle (V) or 10 nM estradiol (E_2), or 10 nM E_2 plus 1 μM progesterone (E_2P_4). DCtrl, eSF from controls; dPCOS, eSF from women with PCOS (8/12) that exhibit decidualization; ndPCOS, eSF from women with PCOS (4/12) that did not decidualize. The significant differences ($P < 0.05$) are marked as follows: – (line) between the different treatment groups within the study group (dCtrl or dPCOS or ndPCOS) and * (asterisk) compared with dCtrl and † (cross) compared with dPCOS in the same treatment group between different study groups.

cytokine secretion aberrancy also in the decidualized PCOS samples. On the other hand, ndPCOS with blunted decidualization presented with increased basal IL-6 secretion and in response to steroid hormones, versus dCtrl and dPCOS. We have recently shown that PCOS women with hyperandrogenism and oligo/amenorrhea have increased IL-6 secretion in proliferative phase endometrium compared with controls (Piltonen *et al.*, 2013). The data herein may imply that this inherent altered cytokine secretion persists later in secretory phase in the cycle, and if so, it may contribute to altered endometrial immune profile in these women (Piltonen *et al.*, 2013). As eSF communicate with endometrial epithelium and endometrial leukocytes, aberrant production of IL-6 could result in altered paracrine signaling throughout the endometrium, resulting in disrupted signaling in luminal epithelium and affecting the crosstalk at the maternal–fetal interface.

IL-8

IL-8 is a potent chemoattractant of migrating immune cells and is involved in implantation as well as in the pathogenesis of endometrial cancer (Caballero-Campo *et al.*, 2002; Ewington *et al.*, 2012). In this study P_4 increased IL-8 levels compared with E_2 treatment, although in ndPCOS IL-8 levels were increased compared with dCtrl and dPCOS. The increase in IL-8 may partly explain the chemoattractant profile of ndPCOS media in the migration assays and may relate to imbalanced leukocyte migration that has been suggested to be related to implantation failure (Tuckerman *et al.*, 2010; Ramos-Medina *et al.*, 2013).

As both IL-6 and 8 were increased in E_2P_4 treated ndPCOS samples, we also tested whether they were able to inhibit decidualization in eSF_{Ctrl}. After treatment, all samples showed high IGFBP-1 levels and typical morphology for decidualization, suggesting that the high cytokine levels are an adverse outcome of altered cell function in PCOS rather than a cause for the aberrant decidualization *per se*.

MCP-1 and RANTES

Basal levels of other chemoattractants (MCP-1 and RANTES) were also increased in ndPCOS compared with dCtrl and dPCOS. MCP-1 and RANTES are associated with monocyte and activated T-cell chemoattraction during implantation, and their actions are tightly linked to the inflammatory cascade and NFκB activity (Zhao *et al.*, 2002; Li *et al.*, 2011). Interestingly, up-regulation of RANTES and MCP-1 and activation of NFκB and MAPK signaling in first trimester decidua have been associated with pre-eclampsia, a condition with increased prevalence in PCOS (Li *et al.*, 2011). MCP-1 may also alter immune cell maturation. Previous studies have reported that Th1 cells (CD4 T-cells) are necessary during the implantation process to promote an immune-tolerant environment. High MCP-1 levels increase terminal differentiation of CD4T cells into Th2 helper cells, which theoretically should improve embryonic tolerance (He *et al.*, 2012). Also, RANTES modulates the immune balance. In *in vivo* studies in mice the placental RANTES expression is tightly regulated by P_4, and high levels lead to embryo resorption (Ramhorst *et al.*, 2007). That E_2P_4 treatment stimulated high levels of RANTES secreted by ndPCOS compared with dCtrl and dPCOS may imply impaired inhibitory P_4 action on RANTES regulation in ndPCOS. This finding is similar to that seen in endometriosis, a condition also presenting with P_4-resistance and subfertility (Hornung *et al.*, 2001; Wieser *et al.*, 2005).

GM-CSF

GM-CSF is a chemoattractant for migratory dendritic cells (DCs) and macrophages in endometrium and also promotes their maturation (Robertson *et al.*, 1999; Moldenhauer *et al.*, 2010). Interestingly, GM-CSF-null mice have reproductive defects with increased prevalence of fetal loss (Robertson *et al.*, 2000). That GM-CSF was down-regulated basally in dPCOS versus dCtrl may relate to poor endometrial receptivity and previous findings of altered DC migration in these women (Matteo *et al.*, 2010). However, as the hormone response patterns were similar in all groups, even though GM-CSF levels in ndPCOS tended to be higher compared with other groups, the clinical significance remains uncertain and warrants additional functional studies in PCOS endometrium. Altogether, high chemoattractant cytokine secretion in ndPCOS is consistent with our *in vitro* findings that ndPCOS condition media possess high chemoattractant properties for migratory immune cells.

MMPs

MMPs comprise a family of zinc-containing endopeptidases that degrade extracellular matrix, crucial for tissue growth and expansion and blood vessel development (Visse and Nagase 2003). MMPs are generally

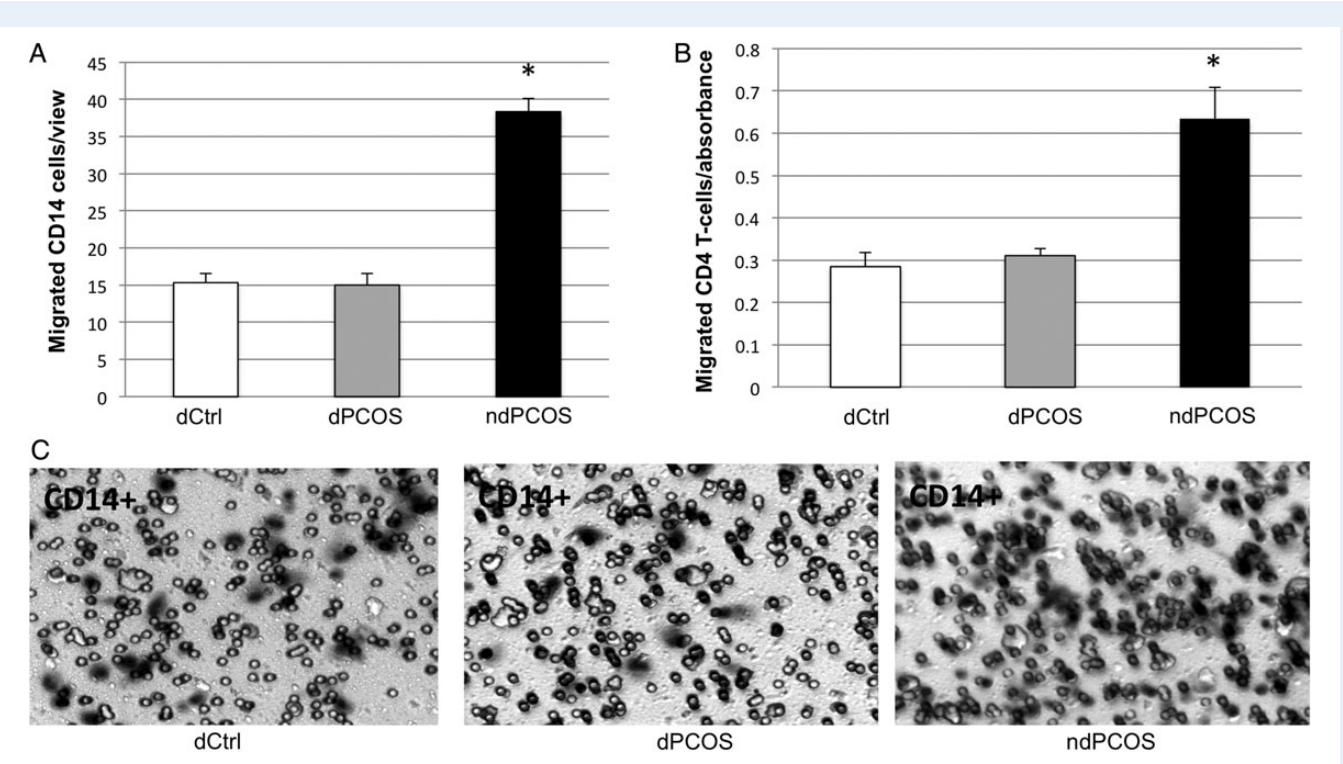

Figure 4 Migration response of (**A**) $CD14^+$ monocytes and (**B**) $CD4^+$ T-cells to conditioned media [14-d treatment with 10 nM estradiol (E_2) + 1 μM progesterone (E_2P_4)]. DCtrl, eSF from controls; dPCOS, eSF from women with PCOS (8/12) that exhibit decidualization; ndPCOS, eSF from women with PCOS (4/12) that did not decidualize. (**C**) Microsocpic analysis of $CD14^+$ monocyte migration in dCtr, dPCOS and ndPCOS.

inhibited by P_4 (Henriet *et al.*, 2002), and eSF-derived MMPs are up-regulated upon P_4 withdrawal, facilitating tissue shedding (Irwin *et al.*, 1996). Furthermore, epithelial MMP2 and 9 have been implicated in endometrial cancer pathogenesis (Karahan *et al.*, 2007). During the implantation process, MMPs, secreted by the epithelium, stroma and trophoblast cells, participate in this complex process (Cha *et al.*, 2012). Interestingly, endometrium in women with endometriosis has increased MMP2 and 3 expression, reflecting P_4-resistance (Chung *et al.*, 2002; Jana *et al.*, 2013), as in this study. Basal MMP2 and 3 levels were increased in ndPCOS but also in response to E_2 or E_2P_4 compared with dCtrl, consistent with a blunted response of P_4 despite normal PGR expression and suggesting a post-receptor defect. Increased MMP action has also been linked to pre-eclampsia and implantation abnormalities, supporting the inflammatory data presented herein (Lockwood *et al.*, 2014). Despite these compelling results, further studies with additional MMPs, as well as measurement of TIMPs warrant further investigation in PCOS endometrium to understand more fully how MMPs are involved in the PCOS-related endometrial abnormalities.

Limitations of this study

There are several limitations to our study. First, the sample size (especially in the ndPCOS group) was limited. The limitation of PCOS samples was partly due to the fact that only women fulfilling all Rotterdam criteria (and NIH criteria) (hyperandrogenism, oligo-amenorrhea, PCO) for PCOS were included. Furthermore, due to limited amount of samples, we had to include samples both from proliferative and secretory phase. However, we have previously demonstrated that by the third passage, the cycle phase from which eSF are derived does not influence their response to E_2 or P_4 (Aghajanova *et al.*, 2009). Also, in this study there were no signs of *in vivo* E_2 exposure (reflected in induced PGR gene expression) or E_2P_4 exposure (e.g. induced IGFBP-1 protein and transcripts), and the patterns of secreted proteins were similar among samples obtained in different cycle phases. As all experiments were conducted beyond passage 3, we included samples from different cycle phases.

Another limitation is that not all subjects were on Metformin. As Metformin has anti-inflammatory properties (Morin-Papunen *et al.*, 2003), it is possible that it could affect the cytokine response values. However, our statistical analysis indicated little variability of the dPCOS group average of cytokines with or without those two samples. Furthermore, after excluding these two samples no changes were observed with regard to the secreted proteins, *PGR* transcript expression (E_2 sensitivity) or *IGFBP-1* transcript expression (E_2P_4 sensitivity), suggesting that using our endpoints, the use of Metformin™ did not affect the readouts.

Other limitations include that our study focused on only one cell type even though previously, we have shown that freshly isolated endometrial epithelium of women with PCOS also exhibits a pro-inflammatory phenotype (Piltonen *et al.*, 2013). Moreover, as our previous study showed inflammatory crosstalk between stromal cells and epithelial cells (Chen *et al.*, 2013), future studies including several cell types should be undertaken. Lastly due to selection of the absolute anovulatory phenotype of PCOS patients in this study, this led to limited access of PCOS samples in the secretory phase (for the more common oligo-ovulatory PCOS phenotype), thus preventing more rigorous histological analysis under natural P_4 action *in vitro*.

Conclusion

In conclusion, the study provides novel *in vitro* data showing that a subset of women with PCOS have an aberrant decidualization response of their eSF to E_2 and P_4, with concomitant increased pro-inflammatory cytokine, chemokine and MMP release—creating a microenvironment conducive to recruiting migratory immune cells. These data support the idea that the endometrium of women with PCOS may present a compromised endometrial environment for implantation and also abnormal endometrial function, resulting in sub-optimal implantation, and predisposition to endometrial cancer.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

T.T.P.: Original study design, conducted sample collection, sample preparation and experiments, data analysis and drafted the manuscript, workload equal to J.C.C. J.C.C.: Improved study design, conducted sample preparation and experiments, data analysis and drafted the manuscript, workload equal to T.T.P. M.Kh.: Conducted sample preparation and experiments, data analysis and helped drafting the manuscript. M.Ka.: Conducted sample collection and preparation and helped drafting the manuscript. A.L.: Conducted sample preparation and histology readings and helped improving the manuscript. T.S.: Sample collection. N.T.: Sample collection. H.H.: Anthropometric clinical data query, helped improving the manuscript. J.C.I.: Histology readings and helped improving the manuscript. L.C.G. Principal investigator, study design, improved the manuscript.

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Conflict of interest

None declared.

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