The human ovarian follicular fluid level of interleukin-8 is associated with follicular size and patient age

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Objective: To investigate the relationship between interleukin-8 (IL-8) in the human ovarian follicle and follicular size, patient age, and fertility factors in IVF cycles.
Design: Prospective study.
Setting: University hospital research laboratory and infertility clinic.
Patient(s): Women undergoing IVF with oocyte retrieval.
Intervention(s): Follicular fluid (FF) aspiration, oocyte isolation, FF storage, and experimental studies.
Main Outcome Measure(s): Quantization of IL-8 by ELISAs and protein microarray; high-performance liquid chromatography (HPLC) followed by ELISA and Western blotting to evaluate α_2-macroglobulin (α_2M) bound IL-8; association of IL -8 to follicular size, patient age, and IVF outcomes.
Result(s): Samples of FF from 63 patients contained an average of 629.59 pg/mL of IL-8 with 50%–70% bound to α_2M. Large follicles contained higher levels of IL-8 than small follicles (937.34 vs. 86.97 pg/mL). The IL-8 concentration in the large follicles of women of young age was higher than that of older reproductive age women (1,373.61 vs. 673.29 pg/mL). There were no statistically significant associations found between IL-8 concentration and other IVF cycle factors or pregnancy outcome.
Conclusion(s): Our findings indicate that IL-8 is present in FF, both in its free and α_2M-bound state, and its concentration is correlated with follicular size and patient age. (Fertil Steril® 2010;93:537–43. ©2010 by American Society for Reproductive Medicine.)

Key Words: Cytokines, interleukin-8 (IL-8), follicular fluid, follicular size, α_2-macroglobulin

The ovary is the site of extensive cellular activity involving follicular development, ovulation, and corpus luteum (CL) formation. The immune system, including leukocytes and cytokines, plays a vital role in the physiology of these ovarian processes (1–3). As modulators of the immune system, cytokines are produced by virtually all cells in the human body and perform an array of functions including cell growth, differentiation, chemotaxis, recruitment of other cytokines, and angiogenesis. Their involvement in both physiologic and pathologic states in the female reproductive system has been an area of extensive interest and research. Cytokines have been proposed to regulate monthly ovarian processes, including promotion of ovarian follicular growth, leukocyte infiltration and activation necessary for ovulation, and tissue remodeling during luteinization and luteolysis (1).

Interleukin-8 (IL-8), an angiogenic, proinflammatory, growth-promoting cytokine, has been implicated in the pathogenesis of inflammation (4). IL-8 acts on the family of receptors known as CXCR (chemokine, CXC motif receptor), which inhibit adenylyl cyclase activity and are known to be important in the attraction of neutrophils, acute inflammation, and angiogenesis. IL-8 is produced by macrophages and T lymphocytes and, since it was first described in ovarian tissue in 1996, has been shown to be produced by multiple ovarian cell types including granulosa cells (GC), theca cells, and other stromal cells (5–9). IL-8 is thought to be important in the developing follicle for the inflammatory events that occur at the time of ovulation and luteolysis (6, 8, 10).

Previous studies have demonstrated the presence of IL-8 within human ovarian follicular fluid (FF) of both IVF patients (6, 11–15) and normally cycling patients (8, 12). Most of these previous studies have measured cytokine levels in pooled FF samples without taking into account that the levels detected may vary based on the size of the follicle. Some studies have noted a correlation of

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IL-8 with follicular size (6, 9, 11); however, these studies are limited by the inclusion of follicles of only large or mature size and small sample size. In addition, few investigators have sought to correlate IL-8 with IVF cycle characteristics or outcomes (13–15) and no previous reports investigated a relationship between IL-8 concentration and patient age.

Studies of the human airway indicate that α_2-macroglobulin (α_2M) may be an important mediator of IL-8 action through its binding of the protein (16–19); however, there are no reports of α_2M in the human ovary.

This study was designed to determine the presence of α_2M-bound IL-8 within human ovarian FF and measure the concentration of IL-8 to determine its correlation with a wide range of follicular size. Exploration of a relationship between IL-8 concentration and baseline characteristics of the patient or IVF cycle factors and outcomes was also sought. These associations were sought to explore the role of IL-8 as an intrafollicular marker in the developing human ovarian follicle.

MATERIALS AND METHODS

Subjects

Follicular fluid samples were collected from women undergoing IVF with controlled ovarian hyperstimulation (COH) at Boston IVF (Waltham, Massachusetts). Patient and cycle characteristics were recorded including: age, gravity, parity, cycle day 3 FSH, body mass index (BMI), cycle number, cycle length, peak E_2 level, total units of gonadotropin stimulation, embryology data (detailed later), pregnancy rate (PR), and outcome. Pregnancy was defined as fetal heartbeat visualized by transvaginal ultrasound (TVS) approximately 4 weeks after embryo transfer. Couples with male factor infertility underwent IVF cycles including intracytoplasmic sperm injection (ICSI) using standard protocol and World Health Organization (WHO) criteria.

Written consent was obtained from each patient at the time of oocyte retrieval. The study protocol was approved by the Beth Israel Deaconess Medical Center Institutional Review Board, Boston, MA.

In Vitro Fertilization Cycle Protocol

Patients underwent standard COH as described elsewhere (20). Briefly, multiple follicular development was achieved with exogenous gonadotropins monitored by TVS measurements and serum E_2 concentrations. Human chorionic gonadotropin (10,000 IU or recombinant hCG 250 μg) was administered SC to complete follicular maturation approximately 36 hours before vaginal oocyte retrieval.

Oocytes were assessed for maturity and inseminated or underwent ICSI. Normal fertilization was confirmed the following day by the presence of two pronuclei (2PN) and two polar bodies. Data on the number of oocytes retrieved and normally fertilized were recorded in the database.

In general, embryo transfer took place 3 days after oocyte retrieval. The number of embryos transferred followed national guidelines by patient age (21) with some variation according to individual patient needs.

Follicular Fluid Aspiration

Individual follicles were measured before aspiration in two dimensions by TVS. One to three large follicles (defined as a mean diameter of $\geq$14 mm) and at least three small follicles (defined as a mean diameter of $\leq$12 mm or less) were identified for each patient. The follicles were aspirated with a 16-gauge single lumen needle and each follicle was emptied completely into polystyrene round bottom tubes (BD Falcon #352057; BD Biosciences, Boston, MA) that did not contain any heparin or media. Follicular fluid from large and small follicles was kept separate during the entire procedure and blood-stained fluid was discarded. A detailed record was kept of follicular diameters and number of follicles aspirated.

Follicular fluid was placed into sterile containers after identification and removal of the oocyte. The FF was taken immediately to the laboratory where centrifugation at 3,000 $\times$ *g* took place for 20 minutes to eliminate cells and cellular debris. The cleared supernatant was separated, aliquoted, and placed immediately on dry ice for transport to the laboratory of Beth Israel Deaconess Medical Center. Samples were stored at -80°C until experimental studies were performed.

To minimize differences due to antibody avidity, epitope availability, and recognition we applied two different enzyme-linked immunoassays (ELISAs) and a microarray based on different IL-8-specific antibodies to measure IL-8 content.

Immunoassays

The IL-8 concentration was measured using sandwich ELISAs and SearchLight Proteome Arrays (Pierce Biotechnology, Woburn, MA). The array is a quantitative multiplexed sandwich ELISA containing highly specific capture antibodies on a 96-well plate. The bound proteins were detected with the addition of a biotinylated detection antibody, streptavidin-horseradish peroxidase, and a chemiluminescent substrate. The plates were then immediately imaged with the SearchLight imaging system. Follicular fluid samples were undiluted for this analysis and run in duplicate. The lower limit of detection for IL-8 was 0.4 pg/mL and no cross-reactivity was observed. The precision of this analysis was approximately 14% intra-assay and 10% interassay.

Confirmatory ELISA (R&D Systems, Minneapolis, MN) was performed for IL-8 in duplicate. Follicular fluid aspirates were diluted 1:2 in phosphate-buffered solution (PBS) for these analyses. The intra-assay and interassay coefficients of variation were less than 10% with a limit of detection of 3.5 pg/mL. According to the manufacturer there was no measurable cross-reactivity with other known cytokines.

Protein Microarray

Follicular fluid samples were analyzed using human protein microarray technology (Allied Biotech, Inc., Ijamsville, MD). The samples were labeled with a fluorescent substrate and added to the microarray chip that contained cytokine-specific antibodies. A streptavidin-Cy5 conjugate was used for assay detection. The intensity of the fluorescence was read by an optical device and computerized image analysis was performed to achieve final concentration results. The assay was done in quadruplicate with positive and negative controls spotted on each microarray. The lower limit of sensitivity was 1.0 pg/mL with a cross-reactivity of less than 5%. The coefficients of variation for intra-assay was 8%, interarray was 12%, and interslide was 16%.

High-Pressure Liquid Chromatography

It is known that in the human airway α_2M interacts with IL-8 modulating its activity (16–19); therefore, we probed for the presence of free and α_2M-bound IL-8. Individual FF samples were fractionated by size exclusion chromatography on Superose 6 column and SMART high-performance liquid chromatography (HPLC) (Pharmacia, Uppsala, Sweden), as previously described (22). Individual fractions were next analyzed by Western blot or ELISA in duplicate for the presence and content of α_2M–IL-8 complexes and free IL-8 using protein-specific antibodies. The figure of Western blot results includes the gel portion corresponding to the protein-specific immunologic reactions. The protein content was evaluated by pixel counting of the specific immunoreactive bands with ImageJ software (NIH, Bethesda, MD). ELISA was performed on the fractions to evaluate the concentration of IL-8 in the samples as described above.

Statistical Analysis

Statistical analyses were preformed using SPSS software (Version 14.0; SPSS, Chicago, IL). Concentrations are reported as means ± SD. Nonparametric methods were used for analysis including the Kruskal-Wallis test and the Mann-Whitney *U* test. Correlation was assessed with Spearman's correlation coefficients. Two-sided *P* values $<.05$ were considered to be statistically significant.

The IL-8 concentration in large and small follicles was analyzed separately and compared. Large follicle results were further analyzed to look for differences with patient characteristics, IVF cycle parameters, and cycle outcome. Patients were divided into tertiles and the young reproductive age group ($\leq$35 years) was compared with the old reproductive age group ($\geq$40 years). Association with IL-8 concentration was also analyzed with patients dichotomized into two groups based on means: body mass index (BMI) ($\leq$24, $\geq$25), total gonadotropin dosage ($\leq$2,699 units, $\geq$2,700 units), peak E_2 level ($\leq$1,099 pg/mL, $\geq$1,100 pg/mL), number of eggs retrieved ($\leq$9, $\geq$10), fertilization rate ($\leq$66%, $\geq$67%), and pregnancy (Y, N).

Because our samples contained fluid pooled from multiple follicles, we performed a subanalysis of IL-8 concentration in large follicles that had *all* measurements more than 14 mm and small follicles that had *all* measurements less than 12 mm.

RESULTS

Patients

The baseline patient characteristics of our cohort of 63 patients are reported in Table 1. The average patient age was 36.05 years with a range of 23–43 years. Our patients had an average cycle day 3 follicle-stimulating hormone (FSH) level of 6.3 mIU/L and a mean BMI of 24.5.

Cycle characteristics are also reported in Table 1. A gonadotropin dose range of 835–8,400 IU (mean 2,854 IU) was observed and male factor infertility was diagnosed in 40.5% of our population.

Our population had an average of 10.2 oocytes retrieved, 62.4% fertilization rate, and 2.4 embryos transferred. The pregnancy rate for our cohort was 37.8%.

Follicular Fluid IL-8 Concentration

IL-8 was found in follicular samples from 0–17,277.66 pg/mL with an average of 629.59 pg/mL.

Follicular Fluid IL-8 Concentration and Follicle Size

Figure 1 depicts the average IL-8 concentration in large versus small follicles. Large follicles were an average of 17.0 mm in diameter, whereas the small follicles were 9.5 mm. Large follicles contained an average of 937.34 ± 293.52 pg/mL of IL-8, whereas small follicles contained 86.97 ± 24.29 pg/mL (P=.002).

To evaluate the effect of pooled fluid on our results we limited our analysis by strict size criteria, as described above. The mean concentration of IL-8 in the large follicles increased to 1,300.99 ± 490.13 pg/mL, whereas the IL-8 concentration in the small follicles decreased to 72.12 ± 34.63 pg/mL (P=.012).

Follicular Fluid IL-8 Concentration and Patient Age

Figure 2 depicts the IL-8 concentration in large follicles in women of young and old reproductive age. Average concentration in young women ($\leq$35 years) was 1,373.61 pg/mL ± 332.80 pg/mL and in women 40 years and older the IL-8 concentration was 673.29 pg/mL ± 113.08 pg/mL (P=.041). Correlation between age and IL-8 concentration in large follicles was -0.256 with a *P* value of .037.

Follicular Fluid IL-8 Concentration and IVF Cycle Characteristics and Outcomes

There were no statistically significant differences found in the IL-8 concentration based on patient characteristics (other

TABLE 1

Baseline and IVF cycle characteristics of patients.

	Mean	Min–Max	SD
Age (y)	36.05	23–43	5.3
Gravity	1.3	0–6	1.5
Parity	0.3	0–2	0.5
CD 3 FSH (mIU/L)	6.3	3–12	2.0
BMI (kg/m^2)	24.5	19–34	3.6
Cycle number	2.4	1–5	1.6
Cycle length (d)	12.3	8–16	1.8
Male factor (%)	40.5	n/a	n/a
Peak E_2 level (pg/mL)	1,415.4	359–4,366	901.7
Total gonadotropin dosage (IU)	2,853.7	835–8,400	1,548.2
Number of oocytes retrieved	10.2	2–33	6.6
Fertilization rate (%)	62.4	0–100	20.0
Number of embryos transferred	2.4	0–6	1.4
Pregnancy rate (%)	37.8	n/a	n/a

CD 3 FSH = cycle day 3 FSH; BMI = body mass index.

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than age), IVF cycle parameters, or pregnancy. A nonsignificant trend was observed between high IL-8 in large follicles and the number of oocytes retrieved.

FIGURE 1

The interleukin-8 (IL-8) concentration in follicular fluid from human ovarian follicles of different size. The follicle size is shown on the x axis; large follicles are an average of 17 mm in diameter and small follicles are 9.5 mm in average diameter. The average IL-8 concentration determined by immunoassays and microarray is shown on the vertical axis. Large follicles contain 937.34 ± 293.52 pg/mL of IL-8, whereas small follicles contain 86.97 ± 24.29 pg/mL of IL-8 (P=.002).

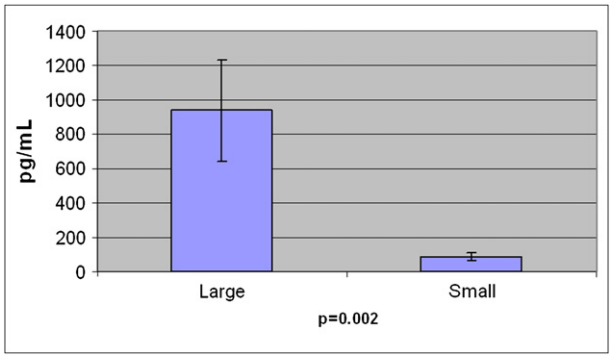

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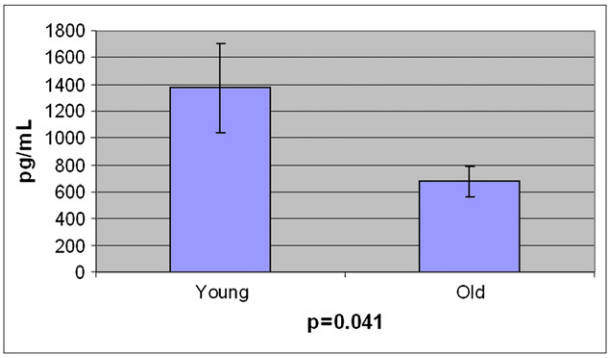
FIGURE 2

The interleukin-8 (IL-8) concentration in human ovarian follicles by patient age. The average IL-8 concentration of large follicles determined by immunoassays and microarray is shown on the vertical axis. The follicles of young patients (≤35 years old) contain 1,373.61 ± 332.80 pg/mL of IL-8, whereas the follicles of the older reproductive age patients (≥40 years old) contain 673.29 ± 113.09 pg/mL of IL-8 (P=.041).



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High Pressure Liquid Chromatography Results

Figure 3 depicts the results of HPLC fractionation of FF samples indicating the presence of free and α_2M-bound IL-8. Figure 3a shows the Western blot results for α_2M, indicating its presence in fractions 4 through 11. Figure 3b shows the graphic results of the IL-8 presence and concentration by ELISA, indicating a peak of free IL-8 in fraction 21 and α_2M-bound IL-8 in fractions 3 through 11. We estimate 50%–70% of IL-8 is bound to α_2M within the FF.

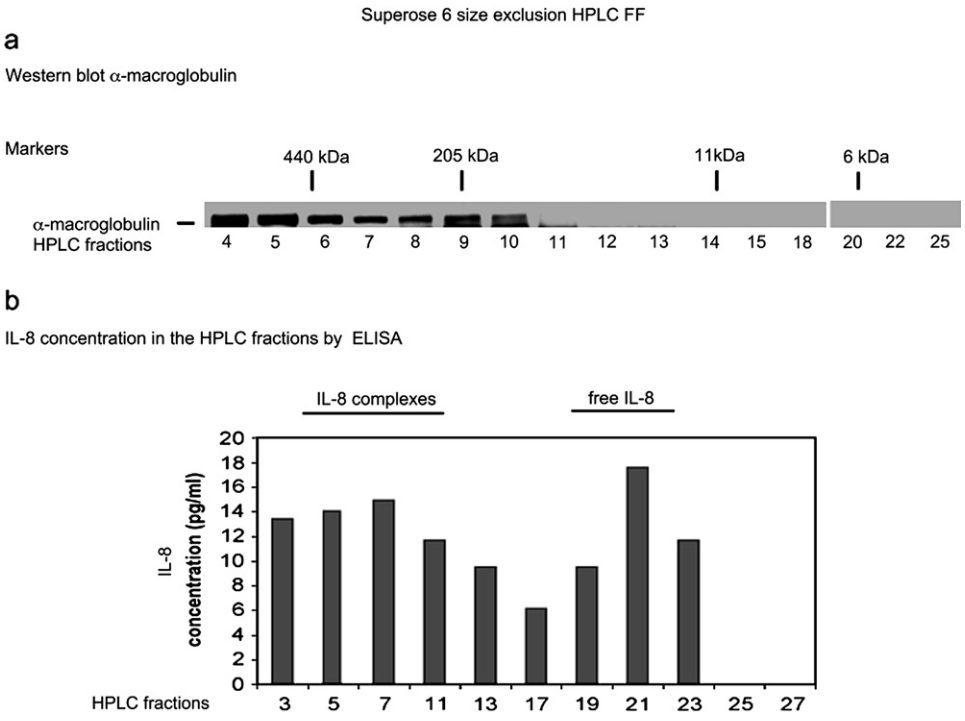
DISCUSSION

We found a follicular size-dependent difference in IL-8 concentration in human ovarian follicles obtained at the time of oocyte retrieval for IVF, highlighting the potential use of IL-8 as an intrafollicular marker of follicular maturity. In addition, the FF concentration of IL-8 in large follicles was decreased in older reproductive age women compared with younger women. We also found IL-8 in FF both in its free form and bound to a carrier protein, α_2M. The concentration of IL-8 in large follicles, however, was not found to be associated with other baseline patient characteristics, IVF cycle parameters, or pregnancy achievement.

We demonstrated IL-8 bound to α_2M in FF, which has not been previously reported in the human ovary. α_2M has been documented to be a carrier protein of IL-8 in the lung, facilitating the binding of IL-8 to neutrophils in an inflammatory setting (16–19). The precise function of free and α_2M-bound IL-8 in the human ovarian follicle remains a topic of further study. Yet, our finding suggests that IL-8 is present in FF for a specific and necessary function, as

FIGURE 3

High pressure liquid chromatography (HPLC) was applied to separate free interleukin-8 (IL-8) and IL-8 bound to α_2M. (**a**) Western blot with α_2M-specific antibody was used to identify α_2M within the HPLC fractions. The assay indicated α_2M presence in fractions 4 through 10. (**b**) The IL-8 concentration (in picograms per milliliter) determined by ELISA within the HPLC fractions. These results indicate that 50%–70% of total IL-8 is found in fractions together with α_2M. FF = follicular fluid.



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has been demonstrated in inflammatory processes in the human airway (16–19).

Our results of an IL-8 concentration gradient by follicle size further the reports previously published. The IL-8 was found to increase in late follicular follicles of normal menstrual cycles (6) and in the large follicles of four patients undergoing IVF (11). Fujii and colleagues (9) reported an increased IL-8 concentration related to follicular size, but included only 14 women and only large follicles. Our results strengthen the relationship of IL-8 with follicular size with the inclusion of nondominant (small) follicles and a larger sample size. The mean level of IL-8 we found within the follicles in our population is higher than that reported in some studies (9, 11), but comparable to other studies (6) and within the range reported by virtually all previous reports.

The increase in IL-8 concentration within the follicle, as it increases in size, can either be accomplished through active transport of IL-8 into the FF or synthesis of IL-8 from within the human follicle. Reports have shown that the concentration of IL-8 is 14–30 times higher in FF than in serum (6, 11). Previous studies using cell culture and messenger RNA (mRNA) expression have demonstrated that GCs, theca cells, and stromal cells in the human ovary can secrete IL-8 (6–9). In the context of these reports, our findings support a synthesis pathway for IL-8 within the developing follicle.

The action of IL-8 within the follicle and the necessity of its presence are the next logical questions. IL-8 may serve many possible functions such as [1] stimulation of follicular cell proliferation as anti-IL-8 antibody treatment has been reported to inhibit cell proliferation (11); [2] angiogenesis with an increased concentration in preparation of corpus luteum formation (5, 23); and [3] a chemoattractant for neutrophil infiltration to prepare for ovarian rupture (6, 7, 11, 12). However, the actions of IL-8 do not appear to be endocrine mediated as are other ovarian functions, as no association between IL-8 and endocrine steroids has been reported (6, 9, 12, 23). We propose, like other investigators, that IL-8 is a necessary follicular constituent with important functions in the inflammatory processes related to ovulation. Our finding of decreased IL-8 in small follicles without the immanent need for ovulation supports this proposal.

The relationship of IL-8 concentration and reproductive age is interesting and, to our knowledge, has not been

reported previously. The only reports of differential cytokine concentration by patient age involve serum levels of other cytokines as markers of inflammation and possible risk factors for morbidity and mortality (24–26). The addition of leukocytes to in vitro perfused rat ovaries has been found to increase the rate of ovulation (27) and exogenous IL-8 has been shown to effect follicular maturation in rabbits (28) and rats (29) similar to the administration of LH or hCG. Given these animal models, higher IL-8 concentration within the human follicle could be seen in younger patients with greater numbers of oocytes to ovulate or who ovulate with greater efficiency. We did find a nonsignificant trend toward an increased IL-8 concentration in patients with higher numbers of oocytes retrieved. However, future studies are needed to decipher the exact IL-8 involvement in follicular maturation and reproductive aging.

Few studies have investigated a possible relationship of IL-8 with IVF outcome. No correlation was found by previous investigators (13–15) between IL-8 concentration and fertilization rates, PRs, or IVF/ICSI outcome. Our results concur with these studies. However, it is known that FF exerts chemotactic activity toward neutrophilic granulocytes and this activity is related to IVF outcome (30). IL-8 has been proposed as one factor involved in the chemotactic activity of FF and the lack of an association may be related to limitations in sample size of this and previous reports or to the multifactorial nature of pregnancy achievement with IVF.

A limitation of our study is that FF samples were pooled from several follicles and not directly linked to specific oocytes for correlation. We attempted to adjust for this with a subanalysis of only follicles meeting strict follicle size criteria where we observed a widening divergence in the follicular size dependent concentration of IL-8. However, we were not able to assess a direct correlation between IL-8 concentration and specific oocyte properties such as maturity, fertilization, or embryo development.

Our samples were obtained from patients undergoing a standard IVF protocol, raising the question of ovarian stimulation effects on IL-8 concentration. However, IL-8 concentration within FF appears to be unrelated to stimulation protocol (12, 14). Given the difficulty of obtaining human ovarian FF for study from naturally cycling patients, this is a common limitation of reports of any molecule within FF. Yet, previous studies have shown IL-8 in the FF of both stimulated and unstimulated women (12) with an increase in concentration around the time of ovulation in natural cycling women (6).

Cytokines have been associated with many conditions of the female reproductive tract including endometriosis, polycystic ovarian syndrome (PCOS), and ovarian hyperstimulation syndrome (23, 31, 32). None of our samples were obtained from women documented to have these conditions; however, we cannot eliminate the possibility of these or other unknown confounders affecting IL-8 concentration.

In conclusion, we report a follicular size-dependent concentration of IL-8 within human ovarian FF. Given the multiple sources of IL-8 production in and around the developing follicle, our results support a synthesis pathway for IL-8 within the follicle and the potential use of IL-8 as an intrafollicular marker of maturity. The IL-8 in the large follicle is likely necessary for the inflammatory events of ovulation. Neither our finding of α_2M-bound IL-8 in FF nor the association of IL-8 concentration in large follicles with patient age has been reported previously. With these results, further exploration is warranted to delineate the actions of free and bound IL-8 within the human ovary and its potential use as an intrafollicular maker.

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