The Journal of Clinical Endocrinology & Metabolism, 2020, Vol. XX, No. XX, 1–18
doi:10.1210/clinem/dgaa880
Clinical Research Article

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Description of the Follicular Fluid Cytokine and Hormone Profiles in Human Physiological Natural Cycles

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Abbreviations: A, androstenedione; E1, estrone; E2, estradiol; FF, follicular fluid; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; hCG, human chorionic gonadotropin; ICSI, intracytoplasmic sperm injection; IFN-γ, interferon-γ; IGF, insulin-like growth factor; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; IP-10, IFN-γ-induced protein 10; IVF, in vitro fertilization; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LIF, leukemia inhibitory factor; MCP-1, monocyte chemoattractant protein 1; MIP-1β, macrophage inflammatory protein 1 beta; OTC, ovarian tissue cryopreservation; P, progesterone; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T cell expressed and secreted; T, testosterone; TGF, transforming growth factor; TNFα, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

Received: 22 May 2020; Editorial Decision: 23 November 2020; First Published Online: 28 November 2020; Corrected and Typeset: 22 December 2020.

Abstract

Purpose: Exogenous gonadotrophins administration during in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) cycles could significantly alter the endogenous follicular regulation system and could influence oocyte quality. The analysis of the follicular fluid (FF) cytokine and hormone profiles in physiological natural cycles is crucial to appreciate the role of FF milieu on follicle development. So far, the FF cytokine profile has been analyzed only in controlled ovarian stimulation cycles and in modified natural cycles. Our study defines, in physiological natural cycles, the cytokine and hormone profiles of individual FF aspirated from antral follicles.

Methods: A total of 203 FFs obtained from 83 women with regular menstrual cycles undergoing ovarian tissue cryopreservation were analyzed: 115 FFs from Group 1 (10 to 29 years of age) and 88 FFs from Group 2 (30 to 40 years of age). In individual FF, 27 cytokines were measured with xMAP technology, and progesterone, estrone,

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

estradiol, testosterone, androstenedione concentrations were determined by liquid chromatography–tandem mass spectrometry.

Results: FF hormone profiles were not different in follicular and luteal phase, suggesting that FF hormones are regulated independently of the endogenous gonadotrophins—possibly because 74% of the punctured follicles, which were ≤6 mm, did not require cyclic pituitary function. The follicle size was influenced not only by the FF cytokine profile but also by the FF hormone profile, both of which are dependent on age.

Main Conclusions: In physiological natural cycles, FF hormones seems to be regulated independently of the endogenous gonadotropins. Age influences FF hormone and cytokine profiles and the compelling relationship between FF hormones and FF cytokines could influence the follicle development.

Key Words: cytokines, follicular fluid, hormones, ovary, physiological natural cycles

Human ovarian folliculogenesis is a complex process regulated locally by hormones (gonadotrophins) and by factors present in the follicular fluid (FF). Any dysfunction of hormones and FF factors may result in abnormal folliculogenesis (1). The FF factors include cytokines, such as epidermal growth factors, fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), neurotrophin growth factors, transforming growth factors-β (TGF-β), and vascular endothelial growth factors (VEGFs) (1). Many other cytokines, such as interleukin (IL)-6 (2), IL-1β (3), IL-12 (4), and IL-8 (5), are also found in FF.

Cytokines could play a key role in ovarian physiology by regulating the microenvironment that supports each phase of follicle maturation (6-9). Indeed, some cytokines, including basic fibroblast growth factor (β-FGF) (10), bone morphogenic protein 4 (BMP4) (11), leukemia inhibitory factor (LIF) (12), and activin (13), are involved in primordial follicle activation and follicle growth. Other cytokines, such as the anti-Müllerian hormone, a TGF-β family member, inhibit follicle recruitment (14). Other cytokines such as inhibins stimulate ovarian hormone production. The inhibins produced by the dominant follicle stimulate androgen production in the theca cells required for estradiol synthesis (13, 15). IL-6, produced by the granulosa cells of the preovulatory follicle in response to gonadotrophins, is considered to be an intraovarian regulatory factor related to steroidogenesis (16), and cooperates with IL-8 to promote follicle rupture and neovascolarization in the corpus luteum (8, 17). In addition, VEGF seems to be crucial for the angiogenesis of the peri-ovulatory follicle and for the early corpus luteum (18).

Cytokines also play a pivotal role in reproductive success, creating an immune-permissive and embryotrophic environment that supports fertilization, early embryo development, blastocyst implantation, and fetal growth (19-21). Cytokines, including growth differentiation factor-9 (22), granulocyte colony-stimulating factor (G-CSF) (23-26), inhibin B (27), and LIF (28), seem to influence the embryo quality, implantation, and pregnancy rate.

Therefore, considering the crucial role of cytokines on reproductive physiology, an alteration of their balance could be critical for follicle development, ovulation, and corpus luteum formation, as well as for embryo implantation and pregnancy maintenance (28).

The exogenous gonadotrophins and the supraphysiological levels of estradiol during the controlled ovarian stimulation, changing the concentrations of various cytokines, directly and indirectly influence, through the intermediation of cumulus oophorus and granulosa cells, the embryo quality, the implantation rate, and the perinatal outcome (29-31). In a recent study (8) designed to clarify the effects of controlled ovarian stimulation on the FF cytokine profile by comparing stimulated in vitro fertilization (IVF) cycles with modified natural cycles (which are not physiological natural cycles), the authors reported that FF LIF, and FF stromal cell-derived factor 1α were significantly higher in modified natural cycles with addition of human chorionic gonadotropin (hCG) than in stimulated cycles. By contrast, other authors (32) reported that serum but not FF cytokine levels were increased in stimulated cycles versus modified natural cycles.

The analysis of FF cytokines and hormones in the physiological natural cycles without hCG administration is essential to understand the role of FF cytokines and FF hormones on the follicle development and on the pathogenesis of reproductive diseases and to refine the stimulation protocols in the optimization of the intrafollicular environment during oocyte maturation. In fact, hCG that stimulates oocyte maturation alter the cytokine and hormone profile in the antral milieu (33).

Here we determined the FF levels of 27 cytokines and 5 hormones (progesterone [P], androstenedione [A], testosterone [T], estradiol [E2], and estrone [E1]) in a large cohort of young women with normal ovulatory cycle, who

underwent an ovarian tissue cryopreservation (OTC) for nongynecological malignancies, in order to (i) evaluate the possible changes in FF cytokine and hormone profiles in follicular and luteal phase; (ii) evaluate the FF cytokine profile based on age of the woman; (iii) establish the possible relationship between FF cytokine and hormone profiles and the follicle size; and (iv) determine the possible relationship between FF cytokines and hormones in physiological natural cycles.

Materials and Methods

Subjects and follicular fluid collection

From January 2015 to February 2016, 83 female patients (age 10-40 years, mean age ± SD: 27 ± 8 years) suffering from nongynecological malignancies were enrolled at the Gynecology and Physiopathology of Human Reproduction Unit of S.Orsola-Malpighi Hospital, University of Bologna to perform OTC (34). In order to preserve ovarian function and fertility, OTC procedure was performed as previously reported (35). Our study and OTC procedure were approved by the Ethical Committee of the S. Orsola-Malpighi Hospital, Bologna (74/2001/O). Women received verbal and written information about the aim of the research and signed the informed consent (for minors, informed consent was signed by the parents). The inclusion criteria for enrollment were: no previous cytotoxic treatment, no previous pelvic surgery, presence of regular menstrual cycle (26-32 days), no perimenopausal condition as confirmed by the measurement of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels, no abnormality in ovarian morphology, no evidence of endocrine/metabolic or systemic diseases, no medication or hormonal therapy in the previous 3 months, negativity for infectious tests, and no smokers.

Before the OTC procedure, an accurate medical history describing the anamnestic and clinical characteristics; serum hormone determination (FSH, LH, prolactin, E2, P, T); and biometric measurement of the ovaries (antral follicle count, right and left ovarian volume), regardless of the day of the menstrual cycle, were performed.

In the laboratory, the diameter of antral follicles in each resected ovary was measured with a sterile ruler by the same operator, individual FFs were gently aspirated from the follicular antrum, the volume of FF (in µL) was evaluated. For each patient, each follicle and the corresponding FF was uniquely numbered. Each individually aspirated FF was frozen at –80°C for subsequent cytokine and hormone analysis. We prepared a filing system to store information enabling the tracing of FF cytokine and hormone contents, the tracing of the corresponding follicle diameter, and the tracing of the menstrual cycle phase of the patient.

FF cytokine determination

The concentration of the 27 cytokines was measured in each FF sample with the xMAP technology (bead-based multiplex immunoassay) in the Laboratory of Immunology of the Department of Experimental and Clinical Medicine, University of Florence, Italy.

The multiplex bead–based immunoassay, which enables measurement of multiple analytes simultaneously in individual FF samples, was described elsewhere (23). Briefly, the bead-based multiplex sandwich immunoassay and Bioplex 200 (Bio-Rad Laboratories, Hercules, CA, USA) were used to measure the concentrations of the following cytokines, chemokines, and growth factors: IL-1β, IL 1 receptor antagonist (IL-1Ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), G-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF), VEGF, platelet-derived growth factor (PDGF), FGF, interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), regulated on activation, normal T cell expressed and secreted (RANTES), eotaxin, macrophage inflammatory protein 1 alpha (MIP-1α), and macrophage inflammatory protein 1 beta (MIP-1β). Fifty µL of each individual FF sample were added to 50µL of antibody-conjugated beads directed against the cytokines/chemokines/growth factors listed above (Bio-Rad) in a 96-well filter plate (Bio-Rad). After a 30-minute incubation, the plate was washed and 25 µL of biotinylated anti-cytokine antibody solution were added to each well before another 30-minute incubation. The plate was then washed and 50 µL of streptavidin-conjugated phycoerithrin were added to each well. After a final wash, each well was resuspended with 125 µL of assay buffer (Bio-Rad) and analyzed by Bioplex 200 (Luminex 100 or 200 Flow Cytometry Analyzer System, RRID:SCR_018025).

FF hormone determination

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used for the evaluation of steroid hormones P, A, T, E2, and E1. Their internal standards (Progesterone-2H3, Androstenedione-13C3, Testosterone-2H3, Estradiol-2H3 and Estrone-2H4) were purchased from Sigma-Aldrich and stored at 1000 µg/mL in methanol. Water and methanol ULC-MS grade, formic acid, ammonium formate, and ammonium fluoride were purchased from Sigma-Aldrich.

An AB Sciex QTrap 6500 equipped with an Electrospray Ionization source coupled with an Agilent two-dimensional HPLC, high-performance liquid chromatography system, consisting of a binary and a isocratic pump were used for the analysis. P, A, and T were analyzed in positive ion mode while E2 and E1 in negative mode in 2 different runs. The mass spectrometry conditions were reported in Table 1. In this table, the registered transitions were reported for each steroid. Two transitions were applied, Quant for quantification and Qual as qualifier transition. IS indicated the internal standard transition, M1 the precursor ion and M2 the product ion. For each transition, dwell time and Collision Energy (CE) were reported. Briefly, FF samples (5µL) were diluted with the internal standard solution (100 µL, in methanol), centrifuged and the samples were diluted to 1000 µL with water. 10 µL were injected. A two-dimensional chromatography was performed, loading the sample on a Phenomenex Luna 5-µm C18 20 × 2 mm column in isocratic conditions (water:methanol 95:5 [v/v]) for 4 minutes and successively carrying out the analytical separation in gradient mode using a Phenomenex Luna 3-µm C18 50 × 2 mm for the positive ion mode acquisition (androgen analysis), or Phenomenex Luna 3-µm PFP (2) 50 × 2 mm for the negative ion mode acquisition (estrogen analysis). Both columns were maintained at 40 °C. For the analysis in positive ion mode, the mobile phase consisted of water and methanol with 10mM of formic acid and 5mM ammonium acetate buffer added. The separation in negative ion mode was performed using water with 0.2mM ammonium fluoride and methanol added.

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS, Inc, Evanston, IL). Due to the nonparametric distribution, all comparisons of cytokine or hormones concentrations in FF were performed with the Wilcoxon test. Correlations were determined using Pearson test. The Pearson correlation coefficient R and the *P* value were calculated. The Bonferroni method for correcting the significance level for multiple comparisons was applied. A *P* value of < 0.016 was considered as statistically significant.

Results

Characteristics of patients and punctured follicles

All 83 enrolled women had regular ovulatory menstrual cycles, were nulliparous, and had ovarian parameters that were all within reference values.

The age of 30 years appears to be associated with a definite start of age-related fertility decline in nulliparous women. In fact, (i) women seem to be definitely fertile through 29 years, whereas from 30 to 35 years, there may be a progressive decrease of fertility that can be more marked after 35 years in nulliparous women; and (ii) a significant decrease in fertility occurred gradually and relentlessly from age 28 onward (36, 37). Therefore, the whole cohort of women (mean age ± SD 27 ± 8 years; range, 10-40 years) was divided into 2 homogeneous groups based on age: 47 women were under 30 years (mean age ± SD: 21.4 ± 5 years; range, 10-29 years)

Table 1. Recorded Transitions for Hormones

Compound	M1 m/z precursor ion	M2 m/z product ion	Dwell time (msec)	Collision energy (volts)
Testosterone Quant (quantification)	289	109	50	30
Testosterone Qual (transition)	289	97	50	27
Testosterone IS (Internal Standard transition)	292	97	50	27
Androstenedione Quant (quatification)	287	97	50	27
Androstenedione Quant (quatification)	287	109	50	30
Androstenedione IS (Internal Standard transition)	290	100	50	30
Progesterone Quant (quantification)	315	97	50	25
Progesterone Qual (transition)	315	109	50	30
Progesterone IS (Internal Standard transition)	318	100	50	25
17 - OH Prog Quant (quantification)	331	109	50	30
17 - OH Prog Qual (transition)	331	97	50	30
17 - OH Prog IS (Internal Standard transition)	334	100	50	30
Estradiol Quant (quantification)	271	183	100	- 55
Estradiol Qual (transition)	271	145	100	- 55
Estradiol IS (Internal Standard transition)	274	145	100	- 50
Estrone Quant (quantification)	269	183	100	- 50
Estrone Qual (transition)	269	145	100	- 50
Estrone IS (Internal Standard transition)	273	147	100	- 50

(Group 1) and 36 women were over 30 years of age (mean age ± SD: 34.2 ± 3 years; range, 30-40 years) (Group 2). A total of 203 FF were aspirated and analyzed: 115 and 88 FF from Group 1 and 2, respectively. The period of time during the menstrual cycle in which the follicles were punctured was at day 16.6 ± 9 (thus from 2 to 34 days), the number of follicles (thus the number of FF) retrieved per patient was 2.5 ± 1.2 (from 2 to 5), and the mean follicular size was 5.7 ± 3 mm (from 2 to 24 mm) (Table 2).

The menstrual cycle phase of the patient was identified only for 169 follicles out of the 203 punctured follicles. Fifty-three follicles were punctured during the follicular phase and 116 follicles were punctured during the luteal phase of the menstrual cycle (Table 2). The great majority of the punctured follicles, showed a diameter ≤6 mm. In fact, 125 out of 169 (74%) punctured ovarian follicles were small follicles ≤6 mm (from 2 to 6 mm), whereas only 44 (26%) showed a size beyond 6 mm (from 7 to 24 mm) (Table 3). Thirty-three out of the 125 follicles that were ≤6 mm (19%) were punctured in the follicular phase and 92/125 (55%) in the luteal phase, while 20/44 follicles >6 mm (12%) were aspirated in the follicular phase and 24 (14%) in the luteal phase (Table 3).

Relationship between FF hormones and FF cytokines and the menstrual cycle phase in natural physiological cycles

We compared the levels of hormones in the FF derived from follicles punctured in the follicular phase and luteal phase of physiological natural cycles. There was no statistically significant difference between hormone levels in the FF aspirated during the follicular phase and hormone levels in those aspirated during the luteal phase (Table 4). Accordingly, the levels of the FF hormones did not correlate with the follicular and luteal phase in the physiological natural cycles (data not shown). The absence of correlation between the levels of FF hormones and the cycle phases could be due to the fact that the great majority of the punctured follicles, showed a diameter ≤6 mm (38) (Table 3). Moreover, as FF hormones, FF cytokines levels were not correlated with the cycle phases in physiological natural cycles (data not shown).

Table 2. Characteristics of the Patients

		Mean ± SD	Range
Patient characteristics	Cycle day of the patient (days)	16.6 ± 9	2-34 days
Follicle characteristics	Number of follicles/patients	2.5 ± 1.25	2-5
	Follicular size (mm)	5.7 ± 3	2-24 mm
Number follicles punctured in the cycle phase	Proliferative phase	53	NA
	Luteal phase	116	NA

NA= not applicable

Table 3. Characteristics of the Follicles

Follicle size (mm)	Number of punctured follicles (%)	Number of follicle in follicular phase (%)	Number of follicle in luteal phase (%)
≤6 mm	125 (74%)	33 (19%)	92 (55%)
>6 mm	44 (26%)	20 (12%)	24 (14%)

Table 4. Levels of FF Hormones in the Follicular and Luteal Cycle Phases of Physiological Natural Cycles

FF Hormones	FF hormone levels: median/ (IQR)		*P* value
	follicular phase (pg/ml)	luteal phase (pg/ml)	
FF P	46/ (41-68)	48/ (40-63)	NS
FF E1	11/ (3-39)	7/ (3-18)	NS
FF E2	10/ (7-59)	68/ (15-240)	NS
FF T	27/ (12-42)	17/ (11-39)	NS
FF A	983/ (649-1271)	870/ (355-1508)	NS

The levels of hormones in the FF derived from follicles punctured in the follicular phase and luteal phase of physiological natural cycles were compared. Values of P, E1, E2, T and A are presented as median (IQR, 25th-75th percentile).
Abbreviation: NS, not significant.

FF cytokine profile based on women's ages in physiological natural cycles

The level of 27 cytokines was measured in the FFs of 2 groups of nulliparous women (Group 1 and Group 2), in order to establish the FF cytokine profile associated with age-related fertility (Group 1) and age-related fertility decline (Group 2) in natural physiological cycle. All the 27 cytokines were detected in the FF of the whole group of women as well as in the FF of Group 1 and 2, except IL-17, which was never detected in any of the women as reported in Table 5. The levels of the different cytokines in the FF varied; some were strongly expressed, other were slightly expressed. Interestingly, IL-5 was slightly present in the FF of Group 1, whereas was never detected in FF in Group 2, unlike the other 25 cytokines that were found in the FF of the whole group of women.

The FFs of women of Group 1 showed IL-1Ra, IL-5, IL-8, eotaxin, and RANTES levels significantly higher than those of Group 2. Conversely, IP-10 levels were significantly lower in Group 1 than in Group 2. The other 21 cytokines measured in FF (IL-1β, IL-2, IL-4, IL-6, IL7, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ, G-CSF, GM-CSF, VEGF, MCP-1, PDGF, FGF, TNF-α, MIP-1α, and MIP-1β) were not statistically different in the 2 groups.

The whole group of nulliparous women was also divided into 2 other groups based on age in order to establish the FF cytokine profile associated with age-related fertility (Group 1a) (including surely fertile women up to 29 years and women from 30 years to 34 with a progressive decrease of fertility) and age-related manifest fertility decline (≥35 years) (Group 2a). Sixty-five women were under 35 years (Group 1a) and 18 women were over 35 years of age (Group 2a). A total of 203 FF were aspirated and analyzed: 154 and 49 FF from the Group 1a and 2a, respectively.

The level of 27 cytokines was measured in the FFs of Group 1a and Group 2a (Table 6). As was reported

Table 5. Cytokine Profile Based on Woman Age (<30 Years and ≥ 30 Years) in Physiological Natural Cycles

FF Cytokine (pg/ml)	Whole group Mean ± Standard Deviation (a)	Group 1 Mean ± Standard Deviation (b)	Group 2 Mean ± Standard Deviation (c)	*P* value (a) vs (b)	*P* value (a) vs (c)	*P* value (b) vs (c)
IL-1Ra	349 ± 84.11	528 ± 141	116 ± 52	NS	0.009	4 × 10-5
IL-5	0.21 ± 0.08	0.36 ± 0.1	0 ± 0	NS	0.002	5×10^{-5}
IL-6	21.34 ± 6.37	14 ± 1.9	31 ± 15	NS	NS	NS
IL-8	186 ± 43.8	252 ± 65	101 ± 54	0.012	0.003	2×10^{-5}
EOTAXIN	3757 ± 2407	6530 ± 4190	38 ± 3.8	0.006	0.001	2×10^{-6}
IFN-γ	34.72 ± 3.95	36 ± 4.5	33 ± 7	NS	NS	NS
IP-10	9233 ± 3295	7738 ± 1120	11162 ± 7428	0.005	0.001	1×10^{-6}
PDGF	14.96 ± 4.65	19 ± 6	9.71 ± 7.3	NS	NS	NS
RANTES	241 ± 137	423 ± 241	4 ± 1.5	NS	NS	0.008
FGF	10.71 ± 2.33	9 ± 2.7	13 ± 4	NS	NS	NS
TNF-α	1.81 ± 0.3	2.25 ± 0.43	1.23 ± 0.41	NS	NS	NS
IL-1β	0.62 ± 0.09	0.62 ± 0.008	0.62 ± 0.18	NS	NS	NS
IL-2	0.75 ± 0.134	0.59 ± 0.08	0.96 ± 0.29	NS	NS	NS
IL-4	0.6 ± 0.13	0.61 ± 0.17	0.58 ± 0.20	NS	NS	NS
IL-7	7.08 ± 1.64	8.5 ± 2.6	5.12 ± 1.67	NS	NS	NS
IL-9	4.59 ± 1.18	6.7 ± 2	1.72 ± 0.39	NS	NS	NS
IL-10	1.54 ± 0.25	1.71 ± 0.34	1.29 ± 0.35	NS	NS	NS
IL-12	3.98 ± 0.59	3.55 ± 0.67	4.52 ± 1.02	NS	NS	NS
IL-13	1.72 ± 0.3	2 ± 0.45	1.34 ± 0.34	NS	NS	NS
IL-15	2.41 ± 0.21	2.52 ± 0.27	2.25 ± 0.33	NS	NS	NS
IL-17	0 ± 0	0 ± 0	0 ± 0	NS	NS	NS
G-CSF	4.88 ± 1.5	4.65 ± 2	5.17 ± 2.30	NS	NS	NS
GM-CSF	0.012 ± 0.012	0.02 ± 0.02	0 ± 0	NS	NS	NS
MCP-1	197 ± 54	248 ± 86	129 ± 54	NS	NS	NS
MIP-1α	1.59 ± 0.34	1.80 ± 0.42	1.32 ± 0.55	NS	NS	NS
MIP-1β	11.8 ± 1.66	13.42 ± 2.2	9.58 ± 2.52	NS	NS	NS
VEGF	370 ± 133	104 ± 18	722 ± 304	NS	NS	NS

The levels of the 27 cytokines were measured with multiplex bead-based assays in FF obtained from the whole group of women, women <30 years (Group 1) and women ≥30 years (Group 2). The mean values ± standard deviation (mean ± SD) of the 27 FF cytokine were calculated in the 3 groups of women. The Bonferroni method for correcting the significance level for multiple comparisons was applied.
Abbreviation: NS, not significant.

for women <30 years (Group 1) and women ≥30 years (Group 2) all the 27 cytokines were detected in the FF of Group 1a (<35 years) and 2a (≥35 years), except IL-17, which was never detected in any of the women. As was seen in Table 5 for Group 1 and Group 2, IL-5 was slightly present in the FF of Group 1a, whereas it was never detected in FF of Group 2a (Table 6). As was observed in Group 1 and Group 2 (Table 5), the FF IL-1RA and FF IL-5 levels were significantly higher in Group 1a than those of Group 2a and IP-10 levels were significantly lower in Group 1a than those of Group 2a (Table 6). Contrary to what was observed in Group 1 and Group 2 (Table 5), IL-8, eotaxin and RANTES did not increase in Group 1a compared with Group 2a (Table 6). Thus, after 35 years where a marked fertility decline is observed, the number of cytokines that were associated with age-related fertility (<30 years) decreased in the follicular milieu.

Relationship between FF cytokines and follicle size in natural physiological cycles

The correlation of FF cytokines with follicle size in the whole group of women and in Groups 1 and 2 is shown in Table 7. Cytokines were measured in 203 FFs obtained from the whole group of women, 115 FFs from Group 1 and 88 FFs from Group 2. FF IL-10 was correlated with follicle size in the whole group of women, Group 1, and Group 2, and therefore the levels of FF IL-10 were correlated with the size of the follicle independent of women's ages. Conversely, FF IL-2, IL-7, IL-12, IL-15, IFN-γ, and VEGF were correlated

Table 6. Cytokine Profile Based on Women's Age (<35 Years Versus ≥35 Years) in Physiological Natural Cycles

FF Cytokine (pg/mL)	Whole group mean ± SD (a)	Group 1a mean ± SD (b)	Group 2a mean ± SD (c)	*P* value (a) vs (b)	*P* value (a) vs (c)	*P* value (b) vs (c)
IL-1Ra	349 ± 84.11	439 ± 107	65 ± 25	NS	NS	0.002
IL-5	0.21 ± 0.08	0.3 ± 0.1	0 ± 0	NS	NS	0.008
IL-6	21.34 ± 6.37	14 ± 1.7	45 ± 26	NS	NS	NS
IL-8	186 ± 43.8	193 ± 49	166 ± 97	NS	NS	NS
EOTAXIN	3757 ± 2407	4937 ± 3168	44 ± 5	NS	NS	NS
IFN-γ	34.72 ± 3.95	33 ± 4	41 ± 11	NS	NS	NS
IP-10	9233 ± 3295	6170 ± 870	18740 ± 13262	NS	0.004	0.0002
PDGF	14.96 ± 4.65	14 ± 4	16 ± 13	NS	NS	NS
RANTES	241 ± 137	315 ± 179	4 ± 2	NS	NS	NS
FGF	10.71 ± 2.33	11 ± 3	9 ± 4	NS	NS	NS
TNF-α	1.81 ± 0.3	2 ± 0.4	1.5 ± 0.5	NS	NS	NS
IL-1β	0.62 ± 0.09	0.65 ± 0.12	0.5 ± 0.1	NS	NS	NS
IL-2	0.75 ± 0.134	0.6 ± 0.07	1.2 ± 0.5	NS	NS	NS
IL-4	0.6 ± 0.13	0.6 ± 0.14	0.6 ± 0.3	NS	NS	NS
IL-7	7.08 ± 1.64	7 ± 2	6.5 ± 3	NS	NS	NS
IL-9	4.59 ± 1.18	5.4 ± 1.5	2 ± 0.6	NS	NS	NS
IL-10	1.54 ± 0.25	1.7 ± 0.3	1 ± 0.4	NS	NS	NS
IL-12	3.98 ± 0.59	4 ± 0.7	3 ± 1	NS	NS	NS
IL-13	1.72 ± 0.3	2 ± 0.4	1 ± 0.3	NS	NS	NS
IL-15	2.41 ± 0.21	2.5 ± 0.3	1.9 ± 0.24	NS	NS	NS
IL-17	0 ± 0	0 ± 0	0 ± 0	NS	NS	NS
G-CSF	4.88 ± 1.5	6 ± 2	2 ± 0.5	NS	NS	NS
GM-CSF	0.012 ± 0.012	0.01 ± 0.01	0 ± 0	NS	NS	NS
MCP-1	197 ± 54	198 ± 65	195 ± 95	NS	NS	NS
MIP-1α	1.59 ± 0.34	1.43 ± 0.3	2 ± 1	NS	NS	NS
MIP-1β	11.8 ± 1.66	11 ± 2	13 ± 4	NS	NS	NS
VEGF	370 ± 133	333 ± 143	485 ± 318	NS	NS	NS

The levels of the 27 cytokines were measured with multiplex bead-based assays in FF obtained from the whole group of women, women <35 years (Group 1a) and women ≥35 years (Group 2a). The mean values ± standard deviation (mean ± SD) of the 27 FF cytokine were calculated in the 3 groups of women. The Bonferroni method for correcting the significance level for multiple comparisons was applied.

Abbreviations: FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; IGF, insulin-like growth factor; IL, interleukin; IP-10, IFN-γ-induced protein 10; MCP-1, monocyte chemoattractant protein 1; MIP-1, macrophage inflammatory protein 1; NS, not significant; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T cell expressed and secreted; SD, standard deviation; VEGF, vascular endothelial growth factor.

Table 7. Correlation Between FF Cytokines and Follicle Size in Physiological Natural Cycles

FF cytokine (pg/ml)	Follicle size correlation coefficient R	Whole group *P* value	Follicle size correlation coefficient R	Group 1 *P* value	Follicle size correlation coefficient R	Group 2 *P* value
IL-2	0.2	0.002	0.4	1×10^{-5}	0.1	NS
IL-4	0.07	NS	0.2	NS	-0.05	NS
IL-6	0.4	1×10^{-6}	0.02	NS	0.4	0.001
IL-7	0.1	NS	0.3	4×10^{-4}	-0.07	NS
IL-10	0.2	0.012	0.3	0.001	0.3	0.015
IL-12	0.3	2×10^{-5}	0.5	4×10^{-3}	0.3	NS
IL-15	0.2	0.01	0.4	3×10^{-4}	0.09	NS
IFN-γ	0.3	2×10^{-4}	0.3	3×10^{-5}	0.2	NS
VEGF	0.2	0.005	0.2	3×10^{-6}	0.2	NS
IL-1β	-0.2	NS	0.08	NS	-0.06	NS
IL-1Ra	-0.1	NS	-0.1	NS	-0.1	NS
IL-5	0.03	NS	0.1	NS	Not calculable	
IL-8	-0.08	NS	-0.06	NS	-0.08	NS
IL-9	0.007	NS	0.04	NS	- 0.008	NS
IL-13	-0.05	NS	-0.05	NS	-0.05	NS
IL-17	Not calculable	NS	Not calculable			
Eotaxin	-0.06	NS	-0.04	NS	0.2	NS
FGF	-0.08	NS	0.006	NS	-0.2	NS
G-CSF	-0.07	NS	-0.01	NS	-0.1	NS
GM-CSF	-0.04	NS	-0.05	NS	Not calculable	
IP-10	-0.09	NS	-0.01	NS	-0.1	NS
MCP1	-0.07	NS	-0.06	NS	-0.08	NS
MIP1α	-0.1	NS	-0.1	NS	-0.2	NS
MIP1β	-0.07	NS	-0.05	NS	-0.08	NS
PDGF	-0.02	NS	0.07	NS	-0.07	NS
RANTES	- 0.05	NS	-0.07	NS	-0.2	NS
TNF-α	0.1	NS	0.2	NS	0.2	NS

The levels of the 27 cytokines were measured with multiplex bead-based assays in FF in physiological natural cycles. The diameter of the antral follicles (from which FFs derived) was measured in the resected ovaries. The correlation between the 27 FF cytokines and the follicle size cycles were determined using Pearson test. The Bonferroni method for correcting the significance level for multiple comparisons was applied.

Abbreviations: FF, follicular fluid; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; IP-10, IFN-γ-induced protein 10; MCP-1, monocyte chemoattractant protein 1; MIP-1, macrophage inflammatory protein 1; NS, not significant; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T cell expressed and secreted; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

with the follicle size only in Group 1 and not in Group 2, whereas FF IL-6 was correlated with the follicle size only in Group 2 and not in Group 1. The FF levels of the other 19 cytokines (IL-1β, IL-1Ra, IL-4, IL-5, IL-8, IL-9, IL-13, IL-17, TNF-α, G-CSF, GM-CSF, PDGF, FGF, IP-10, MCP-1, RANTES, EOTAXIN, MIP-1α, and MIP-1β) were never correlated with the follicular size. Our results showed that age could influence the relationship between the follicle size and the levels of some cytokines in the FF.

Moreover, we investigated the cytokine profile of the FFs derived from small follicles (2-6 mm), from medium follicles (7-11 mm), from large follicles (12-17 mm), and from differentiated preovulatory follicles (18-24 mm). We found that the levels of IL-2, IL-4, IL-5, IL-10, IL-7, IL-9, IL-12, IL-15, G-CSF, IFN-γ, MCP1, and RANTES in the FF increased significantly with the increased size of the follicle, whereas the levels of FF PDGF and FF IP-10 decreased significantly with the increased size of the follicle (Fig. 1). The level of the other 11 cytokines (IL-1β, IL-1RA, IL-6, IL-8, IL-13, IL-17, GM-CSF, FGF, EOTAXIN, MIP-1α, and MIP-1β) in the FFs was independent of the follicle size.

Correlation between FF hormones and follicle size in natural physiological cycles

The correlation of FF P, T, E2, A, and E1 levels with the follicle size in the whole group of women (44 cases) and in Group 1 (24 cases) and Group 2 (20 cases) was shown in Table 8. Hormones were measured in 86 FFs obtained from 44 women of the whole group, 49 FFs from 24 women of Group 1, and 37 FFs from 20 women of Group 2.

P was correlated with the follicle size of the whole group of women, of Group 1, and 2. Therefore, P influenced the follicle size independent of the age of the woman. E2, E1, and A were correlated with the follicle size only in Group 1 and not Group 2. T was never correlated with the follicle size and therefore did not influence it.

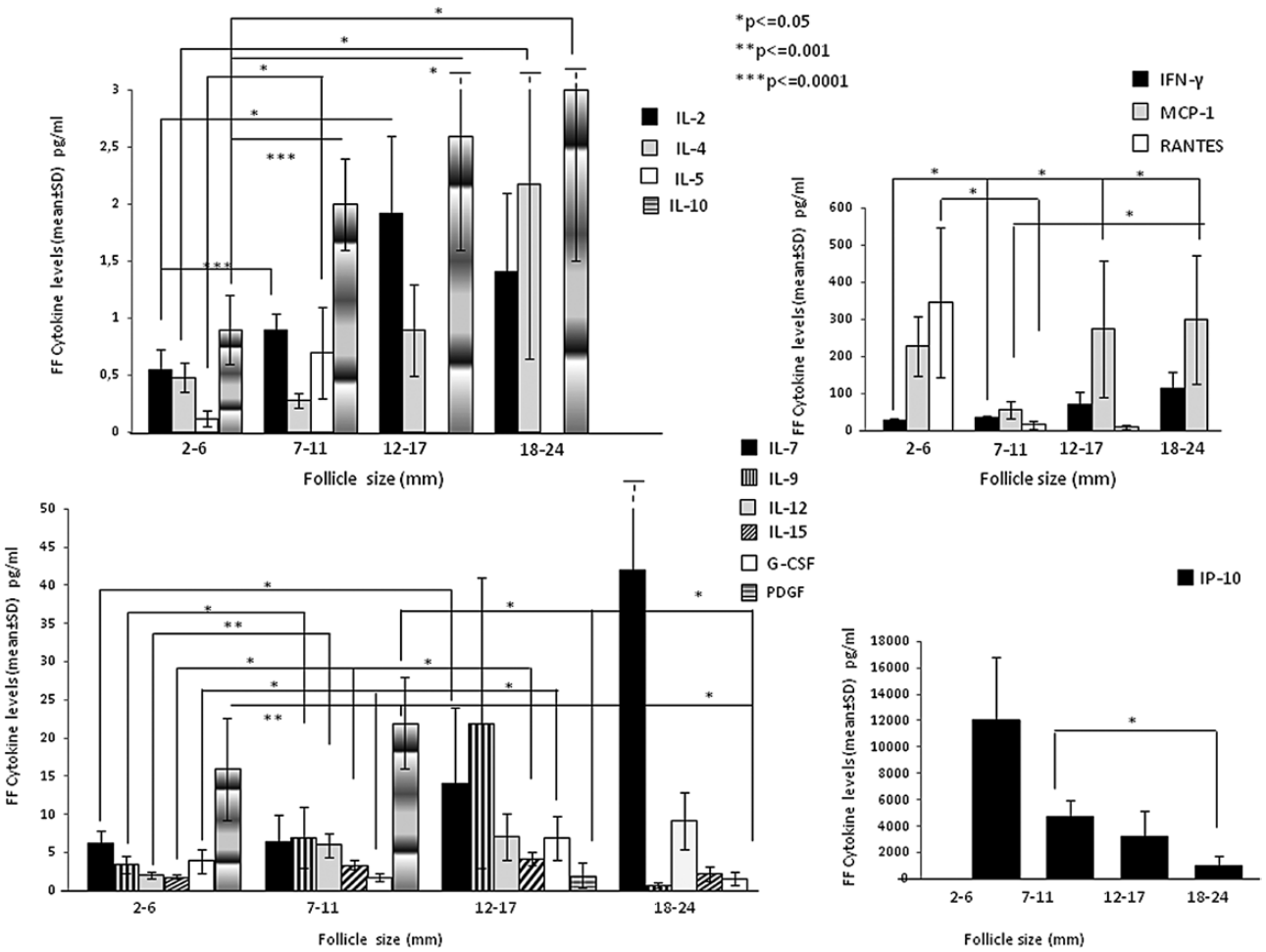

Figure 1. Cytokine profile based on follicle size in physiological natural cycles. The levels of the 27 cytokines were measured with multiplex bead-based assays in FF of small follicles (2-6 mm), of medium follicles (7-11 mm), of large follicles (12-17 mm) and of differentiated preovulatory follicles (18-24 mm) obtained from the whole group of women. The mean values ± standard deviation (Mean ± SD) of the 27 FF cytokine were calculated.

Table 8. Correlation Between FF Hormones and Follicle Size in Physiological Natural Cycles

Hormones (ng/ml)	Whole group	Group 1	Group 2
P	$P = 0.001$	$P = 1 \times 10^{-6}$	$P = 0.001$
T	NS	NS	NS
E2	NS	$P = 0.00005$	NS
A	NS	$P = 0.001$	NS
E1	NS	$P = 0.0005$	NS

The levels of Progesterone (P), Androstenedione (A), Testosterone (T), Estrone (E1) and Estradiol (E2) were measured with liquid chromatography coupled with tandem mass spectrometry (LC/MS-MS) in FF obtained from the whole group of women, women <30 years (Group 1) and women ≥30 years (Group 2). The diameter of the antral follicles (from which each FFs derived) was measured in the resected ovaries before ovarian tissue cryopreservation procedure. The correlations between the 5 hormones and the follicle size were determined using Pearson test. The Bonferroni method for correcting the significance level for multiple comparisons was applied.
Abbreviation: NS, not significant.

Table 9. Correlation Between FF Cytokines and FF Hormones in the Whole Group of Women With Physiological Natural Cycles

FF cytokine (pg/ml)	FF Hormones correlation coefficent (R), statistically significant *P* value (*P*)				
	P	A	T	E2	E1
G-CSF		$R = 0.375$ $P = 3 \times 10^{-4}$			
MIP-1β			$R = -0.279$ $P = 0.008$		
IL-12				$R = 0.620$ $P = 3 \times 10^{-5}$	$R = 0.692$ $P = 2 \times 10^{-5}$

The levels of the 27 cytokines were measured with multiplex bead-based assays in FF obtained from the whole group of women. The hormone levels were measured with LC/MS-MS. The correlation between the 27 FF cytokines and the 5 hormones in natural physiological cycles was determined using Pearson test. The Bonferroni method for correcting the significance level for multiple comparisons was applied.
Abbreviations: A, androstenedione; E1, estrone; E2, estradiol; FF, follicular fluid; G-CSF, granulocyte colony-stimulating factor; IL-12, interleukin-12; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MIP-1β, macrophage inflammatory protein 1 beta.

These results indicated that the follicle size could depended not only on the FF cytokines but also on the FF hormone content, but above all, that with increasing age only P could influence the follicle size.

Correlation between FF cytokines and FF hormones in natural physiological cycles

As FF cytokines were not correlated with the cycle phases in physiological natural cycles after Bonferroni method correcting the significance level for multiple comparisons (data not shown), we evaluated if the FF cytokines levels were correlated with FF hormones levels (Tables 9 and 10).

The FF P, A, and T levels were evaluated in 96 FFs obtained from the whole group of women (50 cases), in 60 FFs from 33 women of Group 1, and in 36 FFs from 17 women of Group 2. FF E1 was evaluated in 40 FFs obtained from 27 women of the whole group (26 FFs from 18 women of Group 1 and 14 FFs from 9 women of Group 2), whereas FF E2 was evaluated in 32 FFs obtained from 24 women (23 FF from 17 women of Group 1 and 9 FF from 7 women of Group 2). The main correlations were shown in Tables 9 and 10.

In the whole group of women, FF G-CSF was positively correlated with A, and MIP-1β was negatively correlated with T. FF IL-12 was positively correlated with E2 and E1. No correlations were found between FF cytokines and P in the whole group of women (Table 9).

In Group 1, FF IL-2, IL-12, IL-10, IL-4, and IFN-γ were positively correlated with P, FF IL-15 and G-CSF were positively correlated with A, and FF G-CSF was positively correlated with T. In the same group, FF IL-2 and IL-12 were positively correlated with E2 and E1 (Table 10).

In Group 2,no correlation was found between FF cytokines and FF P, A, and T, respectively. FF IL-15, MCP-1, MIP-1α, MIP-1β, IL-1Ra, IL-8, IL-9, and IP-10 were positively correlated with E2 in Group 2. In addition, FF IL-12 and IL-7 were positively correlated with E1 in Group 2 (Table 10).

Discussion

Stimulation with exogenous gonadotrophins during in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) treatment could significantly interfere with the local follicular regulatory system, which in turn could affect oocyte quality. The studies dealing with IVF/ICSI natural cycle

Table 10. Correlation Between FF Cytokines and FF Hormones in Group 1 and Group 2 With Physiological Natural Cycles

FF Cytokine	FF Hormones correlation coefficent (R), statistically significant *P* value (P)									
	P		A		T		E2		E1	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
IL-2	R = 0.602 $P = 4 \times 10^{-6}$						R = 0.757 $P = 2 \times 10^{-5}$		R = 0.738 $P = 1 \times 10^{-4}$	
IL-15			R = 0.372 $P = 0.011$					R = 0.653 $P = 0.011$		
G-CSF			R = 0.525 $P = 2 \times 10^{-4}$		R = 0.403 $P = 0.005$					
MCP-1								R = 0.728 $P = 0.003$		
MIP-1α								R = 0.733 $P = 0.003$		
MIP-1β								R = 0.679 $P = 0.008$		
IL-12	R = 0.751 $P = 3 \times 10^{-10}$						R = 0.882 $P = 1 \times 10^{-8}$		R = 0.864 $P = 2 \times 10^{-7}$	R = 0.959 $P = 4 \times 10^{-5}$
IL-10	R = 0.538 $P = 1 \times 10^{-4}$									
IL-4	R = 0.410 $P = 0.001$									
IFN-γ	R = 0.397 $P = 0.004$									
IL-1Ra								R = 0.747 $P = 0.002$		
IL-8								R = 0.701 $P = 0.005$		
IL-9								R = 0.723 $P = 0.005$		
IP-10								R = 0.741 $P = 0.002$		
IL-7										R = 0.940 $P = 2 \times 10^{-4}$

The levels of the 27 cytokines were measured with multiplex bead-based assays in FF obtained from the women <30 years old (Group 1) and women ≥30 years old (Group 2). The levels of Progesterone (P), Androstenedione (A), Testosterone (T), Estrone (E1) and Estradiol (E2) were measured with liquid chromatography–tandem mass spectrometry (LC/MS-MS) in FF. The correlations between the 27 FF cytokines and the 5 hormones in natural physiological cycles were determined using Pearson test. The Bonferroni method for correcting the significance level for multiple comparisons was applied.

Abbreviations: FF, follicular fluid; G-CSF, granulocyte colony-stimulating factor; IFN-γ, interferon-γ; IL, interleukin; IP-10, IFN-γ-induced protein 10; MCP-1, monocyte chemoattractant protein 1; MIP-1, macrophage inflammatory protein 1.

actually report about modified natural cycles, in which ovulation is induced by the exogenous administration of hCG. These modified IVF/ICSI natural cycles are not fully equivalent to a physiological context since hCG influences the FF cytokine profile. Accordingly, it has been shown that FF LIF concentrations increased after hCG compared with before hCG treatment (39). Moreover, the fact that the cytokine profile of FFs obtained from stimulated cycles differs from the cytokine profile of FFs obtained from modified natural cycles (32) supports the hypothesis that conventional ovarian stimulation indirectly and heterogeneously influences the intrafollicular milieu, and therefore could affect the oocyte quality and the IVF/ICSI outcome.

Studies on physiological natural cycles are crucial to understand the function of FF cytokines and FF hormones on physiological follicle development and to improve the stimulation protocols. Our study is the first study that analyses 27 cytokines/chemokines and growth factors (IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ, TNF-α, G-CSF, GM-CSF, VEGF, PDGF, FGF, IP-10, MCP-1, RANTES, eotaxin, MIP-1α, and MIP-1β) and 5 hormones (P, A, T, E1, and E2) in the FF of natural physiological cycles and attempts to define the relationship between FF hormones and FF cytokines and to correlate these data with age, menstrual cycle phases and follicular size. In fact, at the moment scarce literature has focused on the relationship between a few of these cytokines in the FF of stimulated IVF cycles with only one particular feature (for example: the follicle size or the woman's age) (2, 40).

We found that in physiological natural cycles the FF cytokine profile varies on the basis of the age of the women. In fact, FF IL-1Ra, FF IL-5, FF IL-8, FF eotaxin, and FF RANTES increase, whereas FF IP-10 decreases in younger women (less than 30 years) compared with older women (more than 30 years). After 35 years, when a marked fertility decline is usually observed, the number of cytokines associated with age-related fertility in the follicular milieu decreases (indeed IL-8, eotaxin, and RANTES are no longer associated with age-related fertility), indicating that with increasing age the cytokine profile associated with age-related fertility probably suffers from depletion.

Very recently, Alhilali et al (41), measuring TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, GM-CSF, and IFN-γ in pooled FF of preovulatory follicles from patients undergoing ICSI, reported that elevated FF IL-5 levels are associated with poor oocyte quality and asserted that IL-5 could be a negative predictor to the pregnancy outcome in stimulated ICSI cycles. In physiological natural cycles we found that IL-5 is present at a very low level in individual FFs of the younger women but absent in the FFs of the older ones. These findings suggest that the oocyte quality of the enrolled younger women could be poor compared to the older women. Nevertheless, these results could also indicate that FF IL-5 measured in physiological natural cycles is not predictive for the outcome of pregnancy, as it appeared to be in stimulated ICSI cycles, since the cytokine profile of FFs obtained from stimulated cycles differs from the cytokine profile of FFs obtained from natural cycles (32), suggesting that conventional ovarian stimulation influences the intrafollicular milieu. The fact that the decreased G-CSF levels associated with aging in individual FF of stimulated IVF/ICSI cycles (23) is not found here, in individual FFs obtained from physiological natural cycles, supports the hypothesis that stimulation with exogenous gonadotrophins significantly affects the local follicular regulatory system.

Age has an impact on FF IL-8 in physiological natural cycles. In fact, we showed that IL-8 levels are higher in the FF of younger women than in the FF of older women. In agreement with our data, Malizia et al (2010) (5) observed that in stimulated IVF cycles, FF IL-8 concentrations are higher in the large follicles of women of younger reproductive age than in those of women of older reproductive age. However, these authors showed that FF IL-8 is correlated with the follicular size, whereas in physiological natural cycles we found that FF IL-8 is not correlated with the follicular size. This difference between stimulated and physiological natural cycles could also suggest that conventional ovarian stimulation influences the follicular milieu, but in particular, for IL-8, the difference could be due to the administration of hCG used in stimulated cycles to induce the oocyte maturation. In fact, FF IL-8 concentrations are increased in post-hCG samples compared to pre-hCG samples (39). Accordingly, a role in the physiology of ovulation, such as timely follicular rupture, has been suggested for IL-8 (39).

IL-8, a chemokine also known as neutrophil chemotactic factor, is produced by macrophages along with IL-1, IL-6, and IL-12 (42); IL-8 induces the chemotaxis of primarily neutrophils as well as other granulocytes, causing their migration. It has been reported that just before ovulation in humans, the number of neutrophils increases markedly in the thecal layer of the dominant follicle, and the chemotactic activity of neutrophils increases in the stimulated IVF cycles (39). IL-8 could act to recruit neutrophils, but it is also known to be a potent promoter of angiogenesis and appears to play a role in the neovascularization of the corpus luteum (39).

Age also influences in physiological natural cycles FF Eotaxin, which is with RANTES the favorite candidates for recruiting eosinophils (43-45). We found that in physiological natural cycles, eotaxin levels are higher in the FF of younger than in older women. Recently, it has been reported that in early development eosinophils increase in

number in the bovine corpus luteum (46). Eosinophils have also been observed in ovine and porcine preovulatory follicles (47, 48). However, in human periovulatory structures only traces of eotaxin mRNA and no eotaxin secretion, even in stimulated granulosa cell cultures, independent of leukocyte levels, have been found (49). For the selective presence of eosinophils in human periovulatory structures, RANTES, but not eotaxin, seems to play an active role on the accumulation of these cells. Thus, the role of eotaxin on follicles of physiological natural cycles and its relationship with age remains unclear.

We also showed that in physiological natural cycles, IL-1RA levels are higher in the FF of younger than in older women. The IL-1 system consists of 3 structurally related polypeptides. The first 2 are IL-1α and IL-1β, each of which has a broad spectrum of beneficial and harmful biological actions, and the third, IL-1-receptor antagonist (IL-1Ra), inhibits the activities of IL-1. The IL-1 system seems to play a key role in the follicular preovulatory maturation and in the ovulatory process (50). Contrary to IL-1β, which is defined as a survival factor for ovarian follicle (51), and in fact, is able to suppress the spontaneous onset of apoptosis in cultured follicles (51) and is involved in ovulation process, oocyte maturation, and ovarian steroidogenesis (52-54), IL-1Ra blocks ovulation in vivo and in vitro (55). Our findings suggest that with aging there could be a minor effect of IL-1RA on ovulation and oocyte maturation induced by IL-1β.

Interestingly, aging seems to influence the relationship between follicle size and the FF cytokine profile. In fact, with increasing age, only FF IL-6 is correlated with follicle size, whereas in younger women, FF levels of IL-2, IL-7, IL-12, IL-15, IFN-γ, and VEGF are correlated with follicle size. The fact that IL-6, a proinflammatory cytokine (56, 57) already described in FF of stimulated IVF cycles (2, 58), has been considered a potent autocrine regulator of ovarian cumulus cell function, cumulus cell oocyte complex expansion, and oocyte competence (59), and a valuable marker in the evaluation of oocyte maturation (2), could explain that IL-6 is correlated with the follicular size.

VEGF, produced by macrophages and granulosa cells in the human ovulatory follicle (60), is known to be involved both in vasculogenesis (de novo formation of the embryonic circulatory system) and in angiogenesis (the growth of blood vessels from preexisting vascularization) (61). Contrary to FF IL-6, which is positively correlated with the follicle size in the older women in physiological natural cycles, we found that VEGF is positively correlated in the FF of physiological natural cycles with the follicle size of the younger women and not of the older ones. In stimulated IVF cycles, FF VEGF has been found to be correlated with the follicle size, ovarian volume, and antral follicle count (62, 63). However, the influence of the age of the woman on the relationship between FF VEGF and the follicle size has not been investigated. The influence of FF VEGF on the follicle size could be in part due to the key role showed by VEGF not only on the follicular development but also on the atresia in humans, rodents, and domestic animal species (64).

As for VEGF, we found that IL-15, known to be secreted by mononuclear phagocytes (65), is positively correlated in the FF of physiological natural cycles with the follicle size of the younger women and not of the older women. Nevertheless, in stimulated ICSI cycles the FF IL-15 concentrations are negatively correlated with the follicle size and with the maturity of the corresponding retrieved oocytes, suggesting that in stimulated ICSI cycles FF IL-15 could be a possible negative predictor for oocyte maturity (66). Again, we could hypothesize that stimulation with exogenous gonadotrophins could significantly perturb the intrafollicular cytokine network, impacting oocyte maturation/fertilization and embryo developmental competence (67).

IL-12 is another cytokine which is positively correlated in the FF of physiological natural cycles with the follicle size of the younger women and not of the older ones. In modified natural cycles increased concentrations of FF IL-12 has been found in follicles containing oocytes, suggesting an important role of IL-12 in reproduction (68). Accordingly, FF IL-12 is positively correlated with oocyte fertilization and embryo development in stimulated IVF cycles (69).

In physiological natural cycles, as for IL-12, VEGF, and IL-15, FF IFN-γ is positively correlated with the follicle size of the younger women and not of the older women. In contrast, very low levels of FF IFN-γ in spontaneously maturing large follicles have been found (70). IFN-γ, which is mainly produced by natural killer and natural killer T cells and CD4 Th1 and CD8 cytotoxic T lymphocyte effector T cells (71), is a key factor in adaptive immunity able to stimulate macrophages to eliminate intracellular pathogens (72). The fact that T cells have been detected in the ovarian follicles (70) suggests that T cells could be the source of IFN-γ in the FF. This hypothesis seems to be confirmed by the localization in large follicles of normal human ovary of few T helper cells, but numerous CD8+ cytotoxic T lymphocytes, in the theca, in the interstitial tissue, and in the follicular cavity (70). The IFN-γ positive cells are present in the preovulatory follicles only (70). Certainly, further studies are needed to investigate the possible role of the cytokine-producing immune cells on the follicular development.

Although in the physiological natural cycles, FF hormones (T, A, E1, and E2) seem to influence the FF cytokine profile, suggesting a compelling relationship between FF hormones and FF cytokines, the FF hormone and FF

cytokines levels seem to be not impacted by the menstrual cycle phases. Even if steroid hormone profiles in FF are different in hCG-modified natural IVF cycles compared with conventional gonadotrophin-stimulated IVF cycles (73), suggesting an alteration of the follicular metabolism induced by exogenous gonadotrophins, no significant difference of the FF E2, FF P, FF T, and FF A levels are found comparing the effect of the short and of the long protocol in stimulated IVF cycles (74). Thus, exogenous gonadotrophins could influence the FF steroid hormone profiles, but the type of protocol does not necessarily impact FF on steroid hormone profiles. Although serum steroid hormones are influenced by endogenous gonadotrophin stimulation, in physiological natural cycles it seems that endogenous gonadotrophin stimulation has no influence on the FF steroid hormone profile. The FF hormones in physiological natural cycles could possibly be regulated independently from endogenous gonadotrophin stimulation. However, the absence of correlation between the levels of FF hormones and the cycle phases could be explained by the fact that the great majority of the punctured follicles showed a diameter ≤6 mm. In fact, Westergaard et al (38) analyzed FF aspirates obtained during laparotomy from women with normal ovarian function and reported that although the pattern of steroid hormones in FF of healthy follicles is related to the stage of follicular development, small human ovarian follicles (diameter ≤6 mm) could develop and function independently of cyclic gonadotrophin secretion during the menstrual cycle, whereas development of follicles beyond 6 mm in diameter requires cyclic pituitary function. Thus, the absence of correlation between the levels of the FF hormones and the follicular or luteal phase in our study could be in part explained by the fact that 74% of the punctured follicles were ≤6 mm.

Moreover, we found a compelling relationship between FF steroid hormones and FF cytokines in the physiological natural cycles, in fact FF P, T, A, E1, and E2 seem to influence the FF cytokine profile. Our results seem to indicate that FF steroid hormones, and not serum steroid hormones, could directly affect the FF cytokine profile in the physiological natural cycles. Accordingly, very recently measuring the concentrations of 38 cytokines by multiplexed immunoassay in FF and serum of patients undergoing IVF treatment cycles, it has been shown that the levels of the serum cytokines (TGF-α, IL-12p70, IL-1Ra, IL-2, IL-9, IL-6, IL-7, GRO, eotaxin, VEGF, and MCP-1) are associated with the endocrine hormones in serum, but only 5 cytokines (EGF, eotaxin, sCD40L, TNF-β, MCP-1) in FF are found to be related to LH, E2, and P in serum (75). These findings obtained in stimulated IVF cycles confirm our results and suggest that very few cytokines in FF could be controlled by serum hormones and that the great majority of FF cytokines are controlled by FF hormones.

We also established that aging could influence the compelling relationship between FF steroid hormones and FF cytokines in the physiological natural cycles. Our results seem to indicate that FF estrogens, and in particular FF E2, are preferentially correlated with most of the FF cytokines in older women, whereas FF P and FF A, are correlated with FF cytokines preferentially in younger women (Table 10). It has been shown that aging is able to influence the FF hormone milieu in stimulated IVF cycles. Indeed, P level is lower, whereas E and T levels are higher in the FF of stimulated IVF cycles in younger women compared with older women (74). Moreover, the correlation of FF hormones with the follicle size showed that FF P influences the follicular size independently of the woman's age, while, FF E2, FF E1, and FF A impact follicle size in younger women only. These findings indicate that the follicle size depends on the FF cytokine profile controlled by FF hormone profile and aging. Only FF P could influence the follicle size in the older women, whereas FF T is not able to affect the follicular size at any age.

In addition, we found that FF IL-4 is correlated with FF P in younger women (Table 10). IL-4 is a key factor of adaptative immunity. IL-4 has many biological roles, including the B-cell class that switches to IgE, the differentiation of naïve T cells into Th2 cells and the elimination of helminths by activating eosinophils (76). IL-4 is mainly produced by Th2-type cells (77). Since T cells are present in the follicle (70) and in the cumulus oophorus (78, 79) and P stimulates IL-4 production by CD4+ T cells (80), we suggest that FF IL-4 could be produced by follicular T cells and in particular by cumulus oophorus T cells in response to FF P. In agreement with this hypothesis, high levels of P have been detected in the cumulus oophorus/oocyte complex (81) and IL-4 production by cumulus oophorus has been reported (78, 79).

A few years ago, we evaluated the cytokine profile in individual FF of stimulated ICSI cycles (23, 24) and of hCG-modified natural cycles (25). We found that FF G-CSF could be a noninvasive predictive biomarker of embryo implantation allowing the selection of the oocyte, and hence the embryo to be transferred in IVF/ICSI cycles. Here, we found that G-CSF is present in the FF of physiological cycles. This finding suggests that FF G-CSF is not induced by ovarian stimulation in conventional IVF/ICSI cycles or by hCG in modified natural cycles but is already present in physiological natural cycles. In addition, we found that in particular in the younger women the possible follicular regulators of FF G-CSF are follicular A and T.

Conclusions

We extensively investigated the FF cytokine and hormone profiles, which reflect the exchanges between the oocyte and its microenvironment, to determine the FF regulatory

system involved in physiological natural cycles. Our outcomes suggest that FF hormones could influence FF cytokines and that there is a compelling relationship between FF hormones and FF cytokines in physiological natural cycles. Further studies are needed, in particular to investigate the role of immune system cells present in the follicle and possible source of hormone controlled–cytokines in FF. Our findings could help to refine stimulation protocols in the context of optimizing the intrafollicular environment during oocyte maturation.

Acknowledgments

Financial Support: Fondazione Cassa di Risparmio di Firenze, project ID 9190.

Author Contributions: M.P.P. conceived and designed the study, analyzed all the data, supervised and wrote the manuscript. R.F., M.M., and R.V. participated in designing the study, revision of the manuscript, and recovery of FF from ovarian biopsies. R.P. and R.S. performed surgery and revised and edited the manuscript. L.L. performed multiplex bead-based assays and participated in the statistical analysis. F.L. and O.K. performed multiplex bead-based assays. G.D. and M.P. measured hormones in FF.

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Disclosures: MP.P. is an inventor on international patent number WO 2008/009705. R.V., R.F., R.P., M.M., R.S, G.D., M.P., L.L, F.L., and O.K. have nothing to declare.

Disclosure Summary: Cytokines seem to play a pivotal role in reproductive success, creating an immune-permissive and embryotrophic environment that supports fertilization, early embryo development, blastocyst implantation, and fetal growth. Accordingly, cytokines found in follicular fluid influence embryo quality, implantation, and pregnancy rate.

In IVF/ICSI cycles, the stimulation with exogenous gonadotrophins significantly interferes with local follicular regulatory system, which in turn influences oocyte quality. The fact that follicular fluid cytokine profile of stimulated IVF cycles differs from natural modified cycles supports the hypothesis that conventional ovarian stimulation indirectly and heterogeneously influences the intrafollicular milieu, and therefore affects the oocyte quality and the IVF/ICSI outcome. The studies dealing with IVF/ICSI natural cycle, actually report about modified natural cycles, in which ovulation is induced by the exogenous administration of hCG able to modulate follicular fluid cytokine content. Therefore, these IVF/ICSI natural cycles are not fully equivalent to a physiological context.

Studies on physiological natural cycles are crucial to understand the role of follicular fluid cytokines and follicular fluid hormones on the follicle development and to improve stimulation protocols. Our study is the first that tries to define in physiological natural cycles the cytokine and hormone profiles in individual follicular fluids gently aspirated from antral follicles of normal ovaries resected before Ovarian Tissue Cryopreservation. It is also the first study which analyzes 27 cytokines/chemokines and growth factors (IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ, TNFα, G-CSF, GM-CSF, VEGF, PDGF, FGF, IP-10, MCP-1, RANTES, EOTAXIN, MIP-1α, and MIP-1β) and 5 hormones (P, A, T, E1 and E2) in the follicular fluids of physiological natural cycles trying to define the relationship between follicular fluid hormones and cytokines and to correlate these data with age, menstrual cycle phases and follicular size.

Our results suggest that in physiological natural cycles there is a compelling relationship between follicular fluid cytokine profile and follicular fluid hormone profile, which are both age-dependent and both influence the follicle size. Follicular fluid hormone and cytokine levels are not different in the different phases of the menstrual cycle. The absence of correlation between the levels of the follicular fluid hormones and the follicular and luteal phase seems to indicate that hormones found in the follicular fluid of physiological natural cycles could be regulated independently of the endogenous gonadotropins, but could also be explained by the fact that 74% of the punctured follicles are ≤ 6mm and could therefore develop independently of cyclic gonadotrophin secretion. Follicular fluid cytokine and hormone profiles which are different in stimulated IVF/ICSI cycles and in physiological natural cycles, suggest that conventional ovarian stimulation could influence heterogeneously the intrafollicular milieu. Our findings could help to refine stimulation protocols in the context of optimizing the intrafollicular environment during oocyte maturation.

Data Availability: Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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