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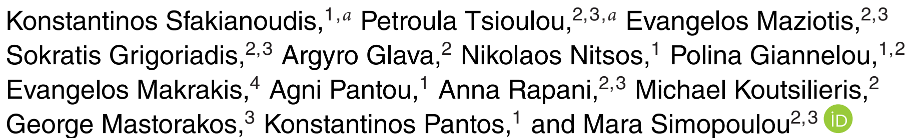
Investigating apoptotic, inflammatory, and growth markers in poor responders undergoing natural *in vitro* fertilization cycles: a pilot study



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This study investigates follicular fluid (FF) from patients with poor and normal ovarian response undergoing natural assisted reproductive technology cycles. We report about (1) cell-free DNA (cfDNA), which reflects apoptosis; (2) corticotropin-releasing hormone (CRH); (3) interleukin (IL)-15, which reflects inflammation; (4) granulocyte colony–stimulating factor (G-CSF); (5) vascular endothelial growth factor (VEGF); and (6) insulin-like growth factor I (IGF-I), which reflects follicular growth. Forty-four poor responders and 44 normal responders—according to the Bologna criteria—were recruited. FF samples were prepared for cfDNA quantification employing Q-PCR and for CRH, IL-15, G-CSF, VEGF, and IGF-I quantification employing ELISA. Statistically nonsignificant different levels of FF cfDNA, CRH, IL-15, VEGF, and IGF-I were observed. Interestingly, statistically significant higher G-CSF levels were observed in normal responders (302.48 ± 474.36 versus 200.10 ± 426.79 pg/mL, $P = 0.003$). Lower cfDNA integrity was observed in cycles resulting in clinical pregnancy for both groups (normal: 0.07 ± 0.04 versus 0.25 ± 0.17 ng/μL, $P < 0.001$; poor: 0.10 ± 0.06 versus 0.26 ± 0.12 ng/μL, $P < 0.001$). The results predominantly showcase similarities between normal and poor responders pertaining to inflammatory, apoptotic, and growth factors. This may be attributed to the employment of natural cycles in order to exclude controlled ovarian stimulation as a factor—indicating its detrimental effect. As G-CSF levels presented significantly higher in normal responders, its vital role in understanding a compromised ovarian response is highlighted.

Keywords: follicular fluid; biomolecules; folliculogenesis; natural IVF cycles; poor ovarian response

Introduction

Patients presenting with poor ovarian response (POR) are characterized by compromised folliculogenesis. Efficient management of POR still lacks consensus on an accepted treatment protocol,[1] which is mainly attributed to patient heterogeneity.[2] Understanding the pathophysiology and elucidating molecular mechanisms is imperative. Follicular fluid (FF), reflecting the oocyte's ambient microenvironment, is an excellent material for investigating noninvasive biomarkers.[3,4] The wide range of its components[5] plays a vital role in the dynamic process of ovarian folliculogenesis, from follicular and oocyte growth to oocyte maturation, ovulation, and

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atresia.[6] Following a thorough literature research, FF markers of heightened scientific interest were identified, specifically regarding their role in the process of folliculogenesis in POR patients. These included cell-free DNA (cfDNA), corticotropin-releasing hormone (CRH), interleukin (IL)-15, granulocyte colony–stimulating factor (G-CSF), vascular endothelial growth factor (VEGF), and insulin-like growth factor I (IGF-I).

From a molecular aspect, evaluating cfDNA levels in FF has been recently employed as a diagnostic tool in female infertility, particularly valuable for the assessment of ovarian function.[7] Cell-free DNA in FF corresponds to DNA fragments that are released owing to apoptotic or necrotic events involving granulosa cells or the oocyte itself.[8] Various studies have indicated a negative correlation of FF cfDNA levels with follicular size and oocyte maturation and yield, along with the number and quality of subsequent cleavage-stage embryos, following stimulated assisted reproductive technology (ART) cycles.[9–11]

From a hormonal perspective, CRH is the primary regulator of the hypothalamic–pituitary–adrenal axis, mediating stress-induced phenomena. Observations on animal models have showcased CRH production in peripheral organs, including the ovaries.[12,13] Numerous studies demonstrated that the presence of CRH may induce apoptosis of cumulus cells and oocytes,[14] exerting a negative impact on folliculogenesis, oocyte growth, maturation, ovulation, and steroid synthesis.[15–19]

Among the plethora of interleukins, IL-15 appears to be of paramount importance with respect to folliculogenesis and oocyte maturation.[20] In ART, higher levels of IL-15 in FF from infertile women were associated with small follicles and immature oocytes,[20] which were understandably associated with decreased clinical pregnancy (CP) rates.[21] Therefore, evaluation of FF IL-15 levels holds remarkable promise as a marker. In fact, it may predict poor prognosis in achieving CP,[22] especially if combined with G-CSF,[23] a hematopoietic growth factor involved in the production of granulocytes and macrophages.[24] The latter statement is supported by studies that reveal a positive correlation of G-CSF levels in FF with implantation outcome.[25,26] This is in accordance with recent findings that report an association of increased FF G-CSF levels with high probabilities of implantation and live birth rates.[27,28]

Another extensively examined growth factor is VEGF, which is actively secreted into FF by granulosa cells.[29–32] Increased levels of VEGF were observed in FF originating from *in vitro* fertilization (IVF) patients with low (or even no) response to controlled ovarian stimulation (COS).[33–36] Contradictory data indicate both a positive[37,38] and a negative association of high levels of FF VEGF with the number and size of follicles, along with the number of mature oocytes.[33–35,39–42]

The IGF-I system plays a key role in folliculogenesis.[43] A positive correlation of IGF-I levels in FF with mature oocytes[44–46] was found, establishing its contribution to oocyte maturation. Patients with low ovarian response were observed to have lower mean levels of FF IGF-I compared with normal or high ovarian response patients.[47–49] Interestingly, other studies provide discordant results. Indeed, increased FF IGF-I levels were observed in women with low ovarian response,[46] while Hammadeh *et al.* indicated that FF IGF-I levels were similar between low and high responders.[50] Notably, the majority of studies involve stimulated cycles.[47,51–53] However, following the comparison of stimulated versus natural ART cycles, similar IGF levels in FF were reported.[46,49]

Each of the aforementioned markers appears to be correlated with folliculogenesis, which is a compromised process in POR patients.[54] The considerable heterogeneity reported among studies leads to confounding data, which prompted the design of this study. The rationale is to explore apoptotic, inflammatory, and growth factors that are specifically related to folliculogenesis, in an effort to delineate causative relationships in POR. It is widely acknowledged that COS may independently exert a detrimental effect, serving as a potential bias when one aims to study folliculogenesis in the context of ART.[55] The decision to recruit a strictly defined population uniquely undergoing natural cycles—hence excluding COS as a factor in the equation—is of added value, rendering this study timely and essential. This study aims to evaluate in an all-inclusive fashion the levels of cfDNA, CRH, IL-15, C-GSF, VEGF, and IGF-I in FF samples from normal and poor responders during fresh natural ART cycles, thus excluding COS as a factor from the equation, along with revealing possible associations with embryology and clinical outcomes.

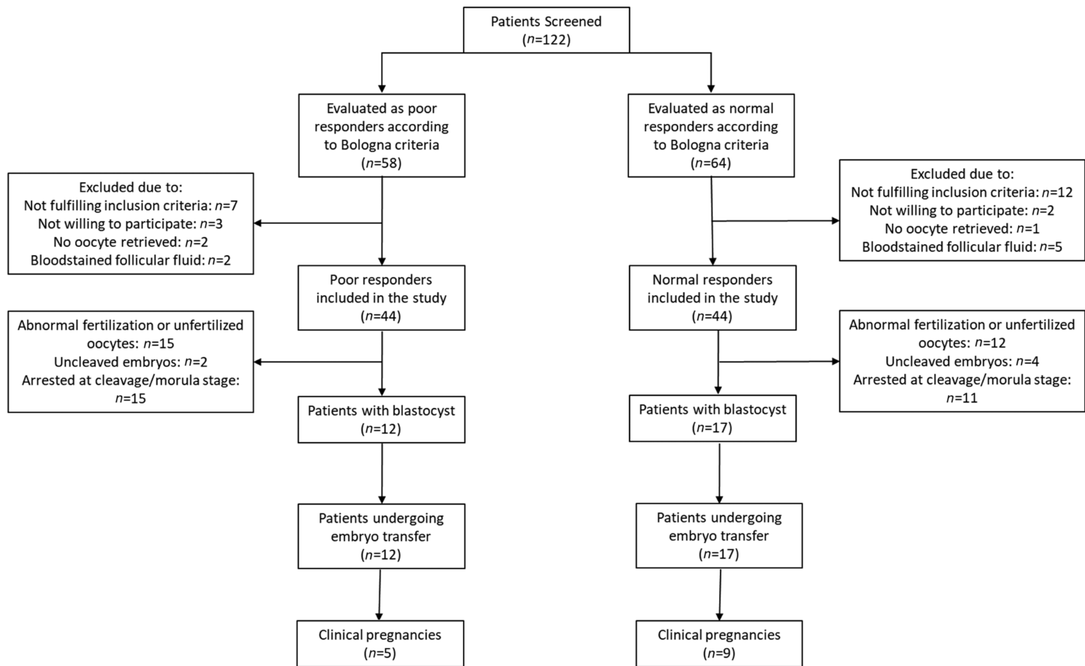

Figure 1. A detailed work flowchart outlining the design of the study.

Materials and methods

Study population

The present pilot study prospectively enrolled a total of 88 patients during the period of September 2018 to July 2019. The study group included 44 poor responders according to the Bologna criteria,[56] while 44 normal responders served as the control group. Written informed consent was obtained from each participant. Prospective participants were provided information regarding the study by their treating physicians. Women who agreed to participate were provided with the informed consent form and were invited to thoroughly read and comprehend the nature, aim, and design of the study. Each patient who opted to participate in the present study signed the informed consent form. Women who opted not to participate and did not sign the informed consent form were not enrolled in the study. In order to quantify the levels of the biomolecules of interest, FF samples were collected in the ovulatory phase during the oocyte retrieval procedure following ovulation triggering. In an attempt to eliminate the potential impact of the COS protocol on FF quality, only fresh natural cycles were included. Intracytoplasmic sperm injection (ICSI) was performed for all cases, enabling the evaluation of oocyte maturation status. Moreover, only patients with a body mass index (BMI) ranging from 18.5 to 29.9 were included. Patients who were diagnosed with polycystic ovary syndrome, pelvic inflammatory diseases, endometriosis, sexually transmitted diseases, or autoimmune and neoplastic diseases were excluded from the study. Furthermore, cases that were diagnosed with any additional infertility etiology were excluded. Smoking, abnormal BMI, and excessively high physical activity levels were exclusion criteria on the grounds of being a potential source of cfDNA.[57] Finally, cases presenting with bloodstained FF samples were excluded. A detailed work flowchart outlining the design of the study is presented in Figure 1. The study protocol (152-29/08/18) was approved by the Hospital Ethics Board in accordance with the Helsinki Declaration.

Natural ICSI cycle protocol

The baseline hormonal profile for each patient was assessed during the basic infertility investigation. Thus, serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), anti-Müllerian hormone (AMH), and prolactin were evaluated on day 3 of the previous menstrual cycle, while

progesterone was evaluated on day 21, via the chemiluminescent microparticle immunoassay (Roche Diagnostics GmbH, Mannheim, Germany).

Regular evaluations of follicular growth via transvaginal ultrasonography, along with serum estradiol (E_2) levels, were performed on each patient starting from day 8 until the detection of the dominant follicle, which was defined by a diameter of ≥18 mm, coupled with serum E_2 levels >150 picograms per milliliter. Ovulation was triggered via an intramuscular injection of 6500 IU of human chorionic gonadotropin (hCG). Following 36 h, ultrasound-coupled transvaginal follicular aspiration was performed under mild anesthesia. Denudation of cumulus–oocyte complexes was performed at 38 h after hCG through mechanical and enzymatic removal of cumulus cells (Sage In-Vitro Fertilization, Inc., Cooper Surgical, Trumbull, CT). All denuded oocytes were assessed with respect to maturity, and they were classified as mature when in metaphase II (MII; featuring one polar body) or immature when in metaphase I (MI; not featuring a polar body). Oocytes at the germinal vesicle stage (GV) were classified as immature. All MII oocytes were subjected to ICSI 40 h after hCG administration. Sixteen to eighteen hours after ICSI, the subsequent zygotes were evaluated using the inverted microscope and classified as follows. Normally fertilized oocytes presented with two pronuclei (PN) and a second polar body. Abnormally fertilized oocytes presented with one PN or three PN, while unfertilized oocytes featured no PN. Another classification would describe a lysed oocyte. Normally fertilized oocytes were placed into an embryo culture dish with continuous single culture medium (Fujifilm Irvine Scientific Inc., Santa Ana, CA) under mineral oil and were cultured at 37 °C, 6% CO_2, 5% O_2, and 89% N_2 in a humidified incubator until the blastocyst stage. Blastocyst-stage embryo quality was assessed on the basis of Gardner's scoring system.[58] In particular, blastocysts that were graded as 4AA, 5AA, or 6AA were considered as top quality. All other grades were considered as non-top-quality blastocysts. Successful blastocyst formation—which was employed as an outcome measure for this study—refers to cycles featuring an embryo that successfully reached the blastocyst stage while being of top or non-top quality.

Five days following the oocyte retrieval procedure, fresh blastocyst-stage embryo transfer was performed. All embryo transfer procedures in this study involved the transfer of a single blastocyst. CP was assessed via ultrasound monitoring following the confirmation of fetal heartbeat detection 4 weeks following the embryo transfer procedure.

FF sample preparation

Following the ovum pick-up procedure, the remaining FF sample for each patient was collected into round-bottom Falcon tubes and immediately centrifuged at 1000 × *g* for 15 min in order to remove any histologic remnants. Aliquots of each sample were prepared, and only those that were destined for cfDNA quantification were filtered using a 0.45-μm filter to eliminate cell debris. All FF aliquots were immediately stored at −80 °C.

Cell-free DNA extraction and quantification

Cell-free DNA extraction was performed on the filtered FF aliquots according to the protocol described by Umetani and colleagues.[59] The levels of cfDNA were measured via real-time polymerase chain reaction (RT-PCR) for human Alu repeats (Custom DNA Oligos, Eurofins, Genomics, Austria) using two primer sets that generate a 115-bp amplicon (ALU115 primers) and a 247-bp amplicon (ALU247 primers). It has been reported that ALU115 amplifies both short cfDNA fragments originating from apoptosis and long fragments originating from necrosis, in contrast with ALU247, which amplifies only long cfDNA fragments.[60] Thus, DNA integrity was assessed employing the ratio of Q247/Q115. If the cfDNA integrity is below 0.5, cfDNA is attributed mainly to apoptotic events, otherwise it is attributed mainly to necrotic events.

For each 96-well PCR plate, a 100× reaction mixture was prepared by adding 800 μL nano-filtration H_2O, 1000 μL SYBR Green I Master Mix (Kapa Biosystems), and 50 μL of 0.25 μM forward and 50 μL of 0.25 μM reverse primers (either ALU115 or ALU247). In each well, 1 μL of each proteinase K–digested FF sample was added to 9 μL of reaction mixture (final volume = 10 μL), and then a film was used to carefully cover the loaded PCR plate. A negative control and two intracontrol samples were added to each PCR plate. All measurements were conducted in quadruplicate. The concentrations of FF cfDNA were calculated on the basis of a standard curve derived from successive dilutions (10 ng–0.01 pg) of prepared genomic DNA obtained from the peripheral blood sample of a healthy volunteer. The

detection limit of the method was 0.01 picogram. Following RT-PCR, electrophoresis was performed employing a 2% agarose gel to verify the results.

CRH, IL-15, G-CSF, VEGF, and IGF-I quantification

The concentration of CRH in FF was measured via an enzyme-linked immunosorbent assay (competitive ELISA) technique, employing the Human CRH ELISA Kit (Elabscience Biotechnology Co., Ltd). The concentration of IL-15 in FF was measured via a solid-phase sandwich ELISA technique employing the Human IL-15 Quantikine ELISA Kit (R&D Systems). The concentration of G-CSF in FF was measured via a sandwich ELISA technique employing the Human G-CSF ELISA Kit (Origene). The concentration of VEGF in FF was measured via a solid-phase sandwich ELISA technique employing the Human VEGF ELISA Kit (Origene). The concentration of IGF-I in FF was measured via a solid-phase competitive ELISA technique employing the IGF1 ELISA Kit (Origene).

Statistical analysis

Statistical analysis was performed using the R statistical programming language, through the RStudio interpreter (Boston, MA). Spearman's correlation coefficient was employed to evaluate possible associations. The normality of the distribution was examined via the Shapiro–Wilk's test. The distribution of most parameters was not normal, and thus the Wilcoxon rank-sum (Mann–Whitney U) test was preferred to examine potential differences between groups. In the cases where the distributions of both groups were normal, Student's *t*-test was preferred. Power analysis was conducted in order to calculate the sample size, with a 0.05 level of significance and a power of 90%. Power analysis indicated that 88 patients should be included in the study, equally distributed between the two groups.

Results

A total of 88 women were included in the present study. Forty-four of them were classified as poor responders according to the Bologna criteria, and the remaining 44 were normal responders. The mean age and serum baseline hormonal levels, along with E_2 levels on the day of hCG administration, are provided in Table 1.

In normal responders, a positive correlation between CRH and IL-15 was established ($P <$ 0.001), as well as a positive correlation between G-CSF and progesterone ($P = 0.03$). A negative correlation between E_2 and cfDNA integrity was also observed ($P = 0.005$). In poor responders, a positive correlation was established between VEGF and ALU 115 ($P = 0.006$), as well as ALU 247 ($P = 0.01$). A negative correlation was established between VEGF and FSH ($P = 0.02$). IGF-I was negatively correlated with AMH ($P = 0.04$) and CRH ($P = 0.008$). A positive correlation between CRH and IL-15 was also observed ($P = 0.005$). The correlations between hormonal markers and patient characteristics are presented in Table 2 for normal responders and Table 3 for poor responders.

Table 1. Mean values of the hormone levels of the patients

	Normal responders (control group) Mean ± SD	Poor responders (study group) Mean ± SD
Age (years)	40.59 ± 3.59	41.80 ± 3.28
FSH[a] (mIU/mL)	9.34 ± 2.99	11.17 ± 2.80
LH[a] (mIU/mL)	7.28 ± 3.86	8.69 ± 4.66
AMH[a] (ng/mL)	2.93 ± 1.97	0.56 ± 0.43
Prolactin[a] (ng/mL)	17.11 ± 9.32	16.86 ± 8.09
Progesterone[b] (ng/mL)	16.88 ± 6.90	16.03 ± 5.11
Estradiol[c] (pg/mL)	205.43 ± 63.69	204.82 ± 79.07

[a] Hormone assessment performed on day 3 of the previous menstrual cycle.
[b] Hormone assessment performed on day 21 of the previous menstrual cycle.
[c] Hormone assessment performed on the hCG trigger day before oocyte retrieval.
FSH, follicle-stimulating hormone; LH, luteinizing hormone; AMH, anti-Müllerian hormone; SD, standard deviation; hCG, human chorionic gonadotropin.

Between the two groups, no statistically significant difference was observed regarding the levels of IL-15, CRH, IGF-1, or VEGF. The levels of G-CSF were statistically significantly higher in normal responders when compared with the poor responders (302.48 ± 474.36 versus 200.10 ± 426.79 pg/mL, $P = 0.003$). A nonstatistically significant difference was observed between the groups regarding cfDNA concentration or cfDNA integrity. The mean levels of inflammatory, growth, and apoptotic factors are presented in Table 4.

Embryology data regarding the normal and poor responders are presented in Table 5. Seventeen women from normal responders and 12 from

Table 2. Spearman's rho for the associations between the hormonal markers and patient characteristics in normal responders

	CRH	IL-15	G-CSF	VEGF	IGF-1
CRH	n/a	0.603	−0.010	−0.022	0.288
IL-15	0.603	n/a	−0.115	0.114	0.251
G-CSF	−0.010	−0.115	n/a	−0.001	0.179
VEGF	−0.022	0.114	−0.001	n/a	0.007
IGF-1	0.288	0.251	0.179	0.007	n/a
FSH	−0.161	−0.056	−0.028	0.022	0.139
LH	0.054	−0.002	−0.201	0.255	−0.187
Prolactin	−0.295	−0.008	0.089	0.307	−0.119
Progesterone	−0.252	−0.157	0.388	0.101	0.289
E_2	0.077	0.198	0.133	0.164	0.037
AMH	0.123	0.194	−0.215	0.267	0.074
Age	−0.051	0.092	0.013	−0.001	0.262
ALU 115	0.157	−0.040	0.215	0.128	0.129
ALU 247	−0.046	−0.130	0.196	−0.057	−0.057
cfDNA integrity	−0.251	−0.011	0.016	−0.251	−0.116

CRH, corticotropin-releasing hormone; IL-15, interleukin-15; G-CSF, granulocyte colony–stimulating factor; VEGF, vascular endothelial growth factor; IGF-I, insulin-like growth factor I; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E_2, estradiol; AMH, anti-Müllerian hormone; cfDNA, cell-free DNA.

poor responders underwent single-blastocyst fresh embryo transfers. Cycles featuring an embryo that successfully reached the blastocyst stage presented with lower cfDNA levels in normal responders (0.38 ± 0.26 versus 0.82 ± 0.66 ng/μL, $P = 0.03$). Cycles with top-quality blastocysts presented with lower cfDNA levels and cfDNA integrity levels in normal responders (0.37 ± 0.24 versus 0.96 ± 0.44 ng/μL, $P = 0.005$; 0.07 ± 0.04 versus 0.12 ± 0.04, $P = 0.02$; respectively) when compared with cycles with non-top-quality blastocysts. Cycles that resulted in CP presented with lower cfDNA integrity

Table 3. Spearman's rho for the associations between the hormonal markers and patient characteristics in poor responders

	CRH	IL-15	G-CSF	VEGF	IGF-1
CRH	n/a	0.359	0.180	−0.151	−0.294
IL-15	0.359	n/a	−0.188	0.122	0.040
G-CSF	0.180	−0.188	n/a	0.098	−0.035
VEGF	−0.151	0.122	0.098	n/a	0.024
IGF-1	−0.294	0.040	−0.035	0.024	n/a
FSH	0.209	0.204	−0.304	−0.349	−0.110
LH	0.132	0.238	−0.195	−0.142	−0.020
Prolactin	0.073	−0.016	−0.282	−0.047	−0.014
Progesterone	−0.111	−0.145	0.008	0.091	0.021
E_2	0.009	−0.265	0.129	−0.221	−0.066
AMH	−0.173	−0.027	−0.025	−0.002	−0.322
Age	0.287	0.151	−0.114	0.028	0.159
ALU 115	−0.146	−0.124	−0.007	0.595	0.286
ALU 247	−0.136	−0.033	−0.017	0.406	0.196
cfDNA integrity	0.092	0.047	0.076	−0.405	−0.221

CRH, corticotropin-releasing hormone; IL-15, interleukin-15; G-CSF, granulocyte colony–stimulating factor; VEGF, vascular endothelial growth factor; IGF-I, insulin-like growth factor I; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E_2, estradiol; AMH, anti-Müllerian hormone; cfDNA, cell-free DNA.

Table 4. Descriptive statistics of the levels of biomolecules assessed in FF regarding the normal and poor responder groups

Levels of examined biomolecules in FF	Normal responders (control group) Mean ± SD	Poor responders (study group) Mean ± SD	*P* value
CRH (pg/mL)	22.54 ± 56.09	29.75 ± 66.14	NS
IL-15 (pg/mL)	6.21 ± 2.18	6.42 ± 3.51	NS
G-CSF (pg/mL)	302.48 ± 474.36	200.10 ± 426.79	0.003
VEGF (pg/mL)	1000.45 ± 377.81	1023.87 ± 365.71	NS
IGF-I (ng/mL)	34.45 ± 17.46	37.74 ± 16.51	NS
cfDNA (ng/μL)	0.64 ± 0.56	0.78 ± 0.72	NS
cfDNA integrity	0.22 ± 0.17	0.24 ± 0.13	NS

FF, follicular fluid; CRH, corticotropin-releasing hormone; IL-15, interleukin-15; G-CSF, granulocyte colony–stimulating factor; VEGF, vascular endothelial growth factor; IGF-I, insulin-like growth factor I; cfDNA, cell-free DNA; SD, standard deviation; NS, not significant.

Table 5. Embryology data for the normal and poor responders

	Normal responders (control group)	Poor responders (study group)
U/S follicle count	73	69
Oocytes retrieved	52	43
MII	48	36
MI	2	3
GV	1	1
Abnormal	1	3
2PN zygotes	29	23
1PN zygotes	9	6
3PN zygotes	3	3
Lysed	5	0
Not fertilized	2	4
Blastocyst formation	17	12
Top-quality	9	6
Non-top-quality	8	6
Clinical pregnancy	9	5

U/S, ultrasound; MII, metaphase II; MI, metaphase I; GV, germinal vehicle; PN, pronucleus/-i; SD, standard deviation.

compared with the cycles not leading to CP, both in the normal and the poor responders (normal: 0.07 ± 0.04 versus 0.25 ± 0.17, $P < 0.001$; poor: 0.10 ± 0.06 versus 0.26 ± 0.12, $P < 0.001$; respectively).

Discussion

The present pilot study focused on prospectively evaluating the levels of cfDNA (serving as an apoptotic marker), CRH and IL-15 (serving as inflammatory markers), and G-CSF, VEGF, and IGF-I (selected as growth factors), aiming to investigate the folliculogenesis process. It is of paramount importance to investigate the role of the identified biomolecules that are implicated in the pathophysiology of POR. Their levels are known to be negatively or positively associated with the process of follicullogenesis, follicular growth, and subsequent oocyte maturation and quality.[6] Assessing their levels in FF from poor and normal responders during natural ART cycles allows exploring the pathophysiology of POR.

Our results demonstrated slightly higher, albeit not statistically significant, FF cfDNA levels in poor responders in comparison with normal responders. Published data report higher FF cfDNA levels with respect to low ovarian response following ovarian stimulation.[10,11] However, the fact that we employed strictly natural ART cycles excludes the possibility that a factor is COS, which is known to exert a deleterious effect on the microenvironment of the oocyte, which serves as a potential source of cfDNA. Indeed, a positive association has been reported between the long or high-dosage COS protocol with higher cfDNA levels, independently of employing an antagonist or an agonist protocol.[11] Even though implementation COS in IVF treatment boosts the oocyte yield, this comes at the cost of obtaining immature oocytes of lower competence.[55] That study highlighted an altered gene expression in cumulus cells stemming from donor oocytes following stimulated cycles, in contrast to

unstimulated ones, highlighting the detrimental impact of stimulation on the meiotic and ovulation events.[55] Another animal model study observed that noncustomized high-dose gonadotropin protocols could induce apoptosis of cumulus cells, negatively affecting oocyte nuclear maturation.[61] Apart from the lack of COS, another contributing factor accounting for the nonstatistically significant different levels of cfDNA could be the heterogeneous nature of the group of poor responders. Hitherto, various well-described criteria have been proposed in an attempt to inclusively define this distinct IVF patient group that is characterized by decreased ovarian response to COS.[56,62] However, poor responders appear with high heterogeneity in the context of ART. This is clear especially in light of the overlap with regard to the pathophysiology entailed in non-poor responder patients presenting with ovarian insufficiency or those of advanced maternal age, both conditions entailing a lower follicular pool that is usually of compromised quality.[63] Numerous other mechanisms other than age[64] could be implicated in the clinical manifestations of POR, ranging from ethnicity[65] and various genetic factors, such as polymorphisms of certain hormonal receptors,[66] to various environmental factors.[67] The aforementioned add another level of complexity that still challenges IVF clinicians with regard to the definition, stratification, and efficient management of poor responders while addressing infertility.[1]

Data provided here further indicate a nonstatistically significant difference regarding the levels of CRH and IL-15 in the FF between the two groups, albeit presenting higher in the poor responder group. It has been established that the aforementioned biomolecules could serve as ovarian proinflammatory cytokines, playing an important autocrine/paracrine role in the aseptic inflammatory process of ovulation.[12,21] Current published data have revealed the negative impact of CRH on mouse preantral follicles,[15] along with the negative impact of IL-15 on oocyte maturation when studying modified natural cycles.[20,22] Given that we included strictly natural ART cycles, the process of ovulation was not subject to any intervention for either group. This serves as a major equalizer and a common denominator between the two groups. Along with the study's limited sample size, it may be a factor contributing toward failing to reach a statistically significant level of difference between the groups.

With regard to growth factors, the majority of studies reported high FF VEGF levels in poor responders.[33–35] Our results indicated a nonstatistically significant difference in FF VEGF levels. This may be supported by studies reporting similar levels of FF VEGF when comparing COS and natural cycles.[30,68] Accounting for levels of pigment epithelium–derived factor (PEDF) and providing data on the PEDF-to-VEGF ratio could certainly provide further insight as PEDG appears to be more closely correlated with adequate folliculogenesis. Such an approach, which could possibly be employed by further studies, would be of added value. PEDF is antiangiogenic factor associated with oocyte quality,[69] follicular vascularity,[70] and oxidative stress in granulosa cells.[71] It has been reported that PEDF counteracts the effect of VEGF during stimulation cycles,[72] and, according to a recent study, PEDF gene knocked-out mice present with diminished ovarian reserve.[73] It appears that the PEDF-to-VEGF ratio may be more closely associated than either VEGF or PEDF alone with poor ovarian reserve and allow more conclusive conclusions to be drawn. As a point of interest and in an effort to provide direction for future research, investigating the possible role that the PEDF-to-VEGF ratio plays in folliculogenesis and oocyte competence may well be the way forward. It is widely known that patients of normal response and poor response are two distinct groups in the context of ART, presenting with high heterogeneity and discrepant profiles, mainly regarding oocyte yield and hormonal profile.[74] ART patients are classified as POR on the basis of their previous response to COS protocols, coupled with low AMH levels, along with their age.[56] One would expect to observe differences concerning the levels of growth factors contributing to folliculogenesis between normal and poor responders, especially taking into consideration the underlying pathophysiology of POR that leads to low oocyte yield, usually accompanied by poor maturity status.[1] An explanation for our data not presenting with differences between poor and normal responders, albeit such differences would be anticipated,[75] could be provided by the fact that poor response may indeed be a pathophysiology identified in the strict context of IVF treatment entailing COS. Therefore, by

removing COS from that equation, which being made possible by studying natural cycles, the differences between the two patient profiles are minimized. Nonetheless, at present, this stands as an extrapolation and further studies will be needed to confirm it. Besides the hypothesis that COS may be an underlying factor behind the greater differences between the two groups showcased in other studies, an additional theory can be proposed on the basis of the results of this study. It may be so that pathophysiology issues in both groups mainly pertain to the events preceding the ovulation process. A hypothesis could be that reaching the milestone of ovulation—irrespectively of being a normal or a poor responder—counteracts the respective folliculogenesis pathophysiology involved until that point. Nonetheless, lacking robust data enabled by basic research on the molecular mechanisms, these are pure assumptions.

Along the same line, our results indicated non-statistically significant levels of difference in FF IGF-I levels between normal and poor responders. These are in accordance with the study conducted by Hammadeh and colleagues that revealed similar levels between the two groups following COS.[50] Contrarily, numerous studies have reported higher FF IGF-I levels in normal responders,[47,48] focusing on the vital role of IGF-I produced by granulosa cells in follicle growth and maturation. To conclude, apart from the aforementioned extrapolations regarding the complicating factor of COS and the high heterogeneity of patients presenting with POR,[2] the reported differences could be also attributed to the diverse analytical techniques employed by the different studies, namely the performance of the outdated method of radioimmunoassay in contrast with the highly efficient method of ELISA, as well as the different ELISA kits that are commercially available.

Interestingly, our results demonstrated statistically significant higher FF G-CSF levels in normal responders compared with poor responders. These findings may highlight the pivotal role of this marker in normal ovarian function. The latter is supported by published data that highlights the role of the expression of G-CSF receptors by granulosa cells, which contributes to follicle development.[76,77] On top of that, a study conducted by Chimote and colleagues indicated that FF G-CSF levels appear to be a biomarker of paramount importance in predicting blastocyst formation, with almost 91% sensitivity and 73% specificity.[78] Furthermore, various studies have demonstrated a positive association of FF G-CSF levels with fertilization and pregnancy rates,[79] along with implantation rates.[26–28] Analyzing pregnancy outcome, our data show more pregnancies for normal responders, which had nine successful cases of CPs, in contrast with the poor responder group, which reported five. This further underscores that G-CSF may hold remarkable promise in predicting the ovarian response that goes hand in hand with a positive IVF outcome.

Notably, in both the normal and poor responders, the Q247/Q115 ratio (corresponding to cfDNA integrity) was <0.5. This indicates that certain apoptotic events may be more prevalent than necrotic ones with respect to the origin cfDNA in human FF, which agrees with the literature.[10] Additionally, a statistically significant negative association of serum E_2 levels and cfDNA integrity was documented for the normal responder group, which could be explained by the fact that higher levels of E_2 seem to contribute toward apoptotic events.[80] Indeed, a bovine model study reported that tumor necrosis factor alpha, which is widely known to be involved in necrosis, inhibits the production of E_2 in granulosa and theca cells of small follicles.[80] What is more, our results documented lower cfDNA integrity for the cycles that led to CP, when compared with the cycles that failed to lead to CP in both groups. Given the apoptotic nature of FF cfDNA in both groups, as mentioned above, this finding highlights the possible adverse effect of necrosis on CP outcome, which is also supported by the literature.[79] This further points out the potential role of cfDNA as a valuable biomarker for IVF outcome. Finally, in normal responders, our study reports lower cfDNA levels in cycles with successful blastocyst formation when compared with those featuring embryos of arrested development. Similarly, in normal responders, cycles with top-quality blastocysts presented with lower cfDNA levels and integrity compared with cycles featuring non-top-quality blastocysts. This difference in cfDNA levels between top- and non-top-quality blastocysts featuring cycles was not observed in poor responders. This may be attributed purely to sample size, as fewer poor responders presented with blastocysts, thus the sample size was smaller. These results are in accordance with the literature, as the number

and quality of blastocysts have previously been correlated with cfDNA levels.[11] However, it should be mentioned that no study hitherto has correlated blastocyst formation rate with FF cfDNA levels.

The current study attempts to simultaneously evaluate apoptotic, inflammatory, and growth markers specifically related to folliculogenesis during natural ART cycles in order to shed light on the underlying pathophysiology of low ovarian response. The small size of our study population could inevitably be considered as a limitation of this study, leading to the potential misinterpretation of nonstatistically significant differences. Indeed, observing differences pertaining to inflammatory and apoptotic events, as well as in growth factors, between the two groups of poor and normal responders was anticipated, as they present with entirely different profiles.[74] This is true regarding not simply the response to COS protocols, but more importantly regarding the integrity of ovarian functionality. Interestingly, the results shown here were mostly similar, highlighting that perhaps the projected difference in the pathophysiology involved may lie in events before ovulation. Perhaps reaching the ovulatory stage may be a milestone, serving to equalize subsequent events in both groups. More than that, studying these two groups outside the context of COS may play a critical role in the results provided here, highlighting its potential detrimental role, especially for the poor responders. Nonetheless, the statistically significant difference in FF G-CSF levels showcases its vital role in understanding the pathophysiology of POR. Further studies should focus on investigating the pathophysiology of the poor responder group by excluding COS as a factor through employing natural cycles, or perhaps even outside the context of IVF treatment.

To conclude, the fact that nonstatistically significant differences were observed in the levels of cfDNA, CRH, IL-15, VEGF, and IGF-I indicates that before reaching conclusions, caution should be exercised regarding the interpretation of our results. The small sample size of this pilot study could undoubtedly contribute to the lack of statistical significance for the results reported. Future studies are required in order to confirm these results by employing a larger scale evaluation of the levels of the aforementioned key biomolecules. Future studies should use a large, equally distributed number of patients that complies with strict inclusion and exclusion criteria, and a consistent and unbiased design. Despite the fact that these studies are observational, the design and the sample size are of paramount importance in order to reach reliable conclusions, similar to interventional studies. Furthermore, perhaps including serum data as well as reporting on additional valuable markers, such as PEDF and the respective PEDF/VEGF ratio, will allow for more robust conclusions.

The present study highlights the noteworthy involvement of G-CSF in the folliculogenesis process entailed in normal ovarian function. This observation renders G-CSF an excellent candidate for further investigation in delineating the pathophysiology of POR. Notably, our findings also revealed the potential promising role of cfDNA level as marker for IVF outcome, as shown by the association of cfDNA integrity with CP outcome. Both G-CSF and cfDNA could be significant markers for exploring the pathophysiology of ovarian response that still eludes our understanding. In this era of personalized medicine, appropriately identifying additional robust markers among the plethora of biomolecules found in FF is highly promising from both a research and a clinical perspective. Besides the aforementioned scientific perspective, IVF practitioners could benefit from a pallet of biomarkers that could serve as a noninvasive "add-on" tool while profiling the distinct category of patients presenting with POR. Precise categorization of these IVF patients could inevitably lead to their efficient IVF treatment. As a result, clinicians could benefit from further guidance regarding the decision-making process, aiming for a tailored management on the basis of the patient's profile. Undoubtedly, the integration of folliculogenesis biomarkers in the clinical practice for treating patients with compromised ovarian function merits further investigation and should be pursued further. However, it should be mentioned that FF biomarkers may only hold predictive value. The timing that FF analysis data become available means that the course of treatment has already been conducted for the present cycle. Hence, data originating from FF cannot be employed in the process of decision making to enhance the outcome by altering treatment of the patient. Its value lies in making associations and providing insights for the present cycle that may be useful for a future cycle. In order to develop a more clinically useful biomarker, future research should

aim toward the identification of the aforementioned markers in serum, as the latter may be of higher predictive value in some instances.[81] Serum and FF data perhaps used in a combinatorial fashion may contribute more substantially in identifying true biomarkers. From defining precise cutoff values that have clinical importance to accounting for the factors of sensitivity and specificity in predicting IVF outcome, we still have a long way to go. Conducting larger and well-designed prospective studies is imperative to strengthen our results and support the hypotheses made.

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Competing interests

The authors declare no competing interests.

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