ANDROGEN LEVELS IN BLOOD AND FOLLICULAR FLUID OF IVF PATIENTS WITH DIMINISHED OVARIAN RESERVE

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Androgen concentrations in follicular fluid samples collected from patients undergoing in vitro fertilization (IVF) may provide useful clinical indicators. This study aimed to analyze possible associations of the androgen levels in follicular fluid and blood plasma in patients with diminished ovarian reserve (POR) in IVF programs. Cross-sectional study with a parallel group design, conducted in 2019–2021, enrolled 300 patients with infertility, aged 18–42 years, applying for assisted reproduction involving IVF/intracytoplasmic sperm injection and embryo transfer. The androgen profiles of blood plasma and follicular fluid were determined by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Androgen concentrations in blood plasma and follicular fluid, particularly those of dehydroepiandrosterone (DHEA-S), androstenedione and total testosterone, significantly correlated. The results implicate androgen levels in blood plasma and follicular fluid as early markers of POR in patients with infertility.

Keywords: androgens, androgen deficiency, testosterone, dehydroepiandrosterone, androstenedione, reproductive age, infertility, assisted reproductive technologies, LC-MS/MS, follicular fluid

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Compliance with ethical standards: the study was approved by the ethical review board at the Kulakov National Medical Research Center for Obstetrics, Gynecology and Perinatology (protocol № 140 of 15 December 2014). The informed consent was submitted by all study participants.

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УРОВЕНЬ АНДРОГЕНОВ В КРОВИ И ФОЛЛИКУЛЯРНОЙ ЖИДКОСТИ У ЖЕНЩИН С БЕСПЛОДИЕМ И СНИЖЕННЫМ ОВАРИАЛЬНЫМ РЕЗЕРВОМ В ПРОГРАММАХ ВРТ

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Концентрация андрогенов в образцах фолликулярной жидкости у пациенток в программах вспомогательных репродуктивных технологий (ВРТ) может быть связана с процессами оогенеза и эмбриогенеза. Целью исследования было проанализировать связь уровня андрогенов в плазме крови и фолликулярной жидкости у пациенток с бесплодием и сниженным овариальным резервом яичников в программах ВРТ. Проведено одномоментное исследование в параллельных группах 300 пациенток 18–42 лет с бесплодием с 2019 по 2021 гг., обратившихся для проведения программы ЭКО/ИКСИ и ПЭ. Определяли андрогенный профиль в плазме крови и фолликулярной жидкости методом масс-спектрометрии жидкостной хроматографии и тандемной масс-спектрометрии (ВЭЖХ-МС/МС). Полученные результаты показали, что андрогены в плазме крови и фолликулярной жидкости, а именно уровни ДГЭА-С, андростендиона и общего тестостерона, могут быть ранними маркерами снижения овариального резерва (СОР) у женщин с бесплодием. Статистически значимая корреляционная связь между уровнями андрогенов в крови и фолликулярной жидкости свидетельствует об их вкладе в формирование снижения овариального резерва. Таким образом, при сниженном овариальном резерве выявлено снижение концентрации андрогенов в плазме крови и в фолликулярной жидкости, что свидетельствует о роли андрогенов в процессах фолликулогенеза, таких как тестостерон и андростендион.

Ключевые слова: андрогены, андрогенный дефицит, тестостерон, дегидроэпиандростерон, андростендион, репродуктивный возраст, бесплодие, ВРТ, ВЭЖХ-МС/МС, фолликулярная жидкость

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Poor ovarian reserve (POR) in patients with infertility has been associated with low rates of success in the assisted reproductive technology (ART) programs. The age-dependent and/or POR-related declines in androgen levels indirectly suggest their special relevance to folliculogenesis and successful ART outcomes.

Despite the overall diversity of androgens, only two of them, testosterone and dihydrotestosterone, exert high biological activity. The relationship between systemic and local androgen levels (as measured in plasma and follicular fluid, respectively) is an up-to-date prognostic factor of success in ART programs, especially in patients with DOR. Still, it is not clear which androgen and in which biological medium can be the highest prognostic value with regard to reproductive success in patients with DOR.

The role of follicular fluid (FF) as a biological medium surrounding the oocyte, participating in metabolic processes within the follicle, and ultimately the medium for the conclusive steps in oogenesis, is a major focus of clinical research on both hypo- and hyperandrogenic conditions [1–3]. The

endocrine environment, particularly the anti-Müllerian hormone concentration (AMH), in FF has been associated with the yields of mature follicles and implantation potential of the embryos [4–6]. These biological phenomena depend on complex dynamic interactions between the oocyte and its microenvironment composed of FF and other structural components of the follicle.

At an advanced reproductive age, serum levels of both AMH and testosterone decrease concomitantly with the decline in folliculogenesis [7]. Several studies addressed hormonal profiles in blood serum and FF with regard to patient's age in natural IVF cycles. A decrease in FF testosterone levels was observed in patients aged 40–45 years compared with 20–25-year-olds [8].

Comparative measurements of testosterone in serum and FF may have certain diagnostic value as well. For instance, the age-related decrease in serum testosterone levels can be unaccompanied by a similar decrease in FF testosterone levels specifically in patients with poor response in natural cycle [9]. Obviously, more comprehensive efforts are needed to validate the possible correlation of endocrine status between blood and FF for clinical significance in patients with infertility and POR.

Previous studies used immunochemical methods to measure steroid levels in FF. Such methods have a major disadvantage of low specificity due to the cross-reactions with structurally related compounds [10–12]. The methods of liquid chromatography (LC) and mass-spectrometry (MS) are more specific [13] and allow simultaneous quantitative measurements of several steroids [14, 15].

This study aimed to assess for possible correlations of blood and FF androgen levels with POR in IVF programs.

METHODS

The cross-sectional study with a parallel group design enrolled 300 reproductive age patients with infertility, aged 18–42 years, applying for ART program at FSBI «National Medical Research Medical Center For Obstetrics, Gynecology and Perinatology named after Academician V. I. Kulakov» Ministry Of Healthcare of the Russian Federation in 2019–2021. Blood samples were collected from all patients on day 2–3 of menstrual cycle. Inclusion criteria: age 18–42 years; a history of infertility; written informed consent for the study. Exclusion criteria: surgical menopause; hysterectomy; adrenal failure; hormone-producing tumors; obesity (BMI ≥ 30 kg/m^2); body mass deficiency (BMI ≤ 18 kg/m^2); AIDS and other immunodeficiency, immunoinflammatory rheumatic diseases, immunomodulatory therapy, glucocorticoids, oncological diseases.

The patients were assigned into groups depending on age: group 1 — 149 young reproductive age (18–35 year-olds), group 2 — 151 patients of advanced reproductive age (35–42 year-olds).

In accordance with the Patient-Oriented Strategies Encompassing Individualized Oocyte Number (POSEIDON) the patients were assigned into subgroups depending on ovarian reserve: subgroup 0 — normal ovarian reserve (76 early reproductive age pts and 69 advances reproductive age pts; anti-Müllerian hormone (AMH) ≥ 1.2 ng/mL, antral follicle count (AFC) ≥ 5), subgroup 1 — POR (73 young reproductive age pts and 82 advanced reproductive age pts; AMH < 1.2 ng/mL, AFC < 5). The block-scheme is given in Fig. 1.

Individual histories collected for the study included age; mother's age at menopause; a history of internal genital surgeries/traumas; a history of inflammations/infections genital diseases; a history of endometriosis/adhesive diseases; a history of IVF cycles (number); obstetric anamnesis.

Folliculogenesis was monitored by ultrasound scans. The IVF program was carried out in accordance with the standard, using a gonadotropin-releasing hormone (GnRH) antagonist-based protocol with urinary or recombinant gonadotropins, or a combination of both, as inducers of folliculogenesis. The daily doses of gonadotropins were determined individually depending on the ovarian reserve status, but not less than 225 IU a day; human chorionic gonadotropin was used as an ovulation trigger. Biological samples (blood, follicular fluid from the first follicle) were collected upon transvaginal ovarian puncture.

Concentrations of hormones (testosterone, dehydroepiandrosterone (DHEA), dehydroepiandrosterone-sulfate (DHEA-S), androstenedione, dihydrotestosterone, progesterone, hydroxyprogesterone, pregnenolone, hydroxypregnenolone and cortisol) were quantified by tandem mass-spectrometry using Steroid Hormones in Serum LC-MS/MS Analysis Kit (JASEM; Türkiye) as analytical standards. The kit contained four calibrating mixtures of 16 steroid hormones, two-level quality control samples and internal standards. The mobile phases A and B contained 0.01% formic acid in Milli-Q water and acetonitrile, respectively. Methyl tert-butyl ether (MTBE, ≥ 99.5%, HPLC grade, Fisher Chemical; USA), methanol (99.9%, HPLC Basic, Scharlau; Spain), acetonitrile (99.9%, HPLC Gradient grade, Fisher Chemical; USA) and formic acid

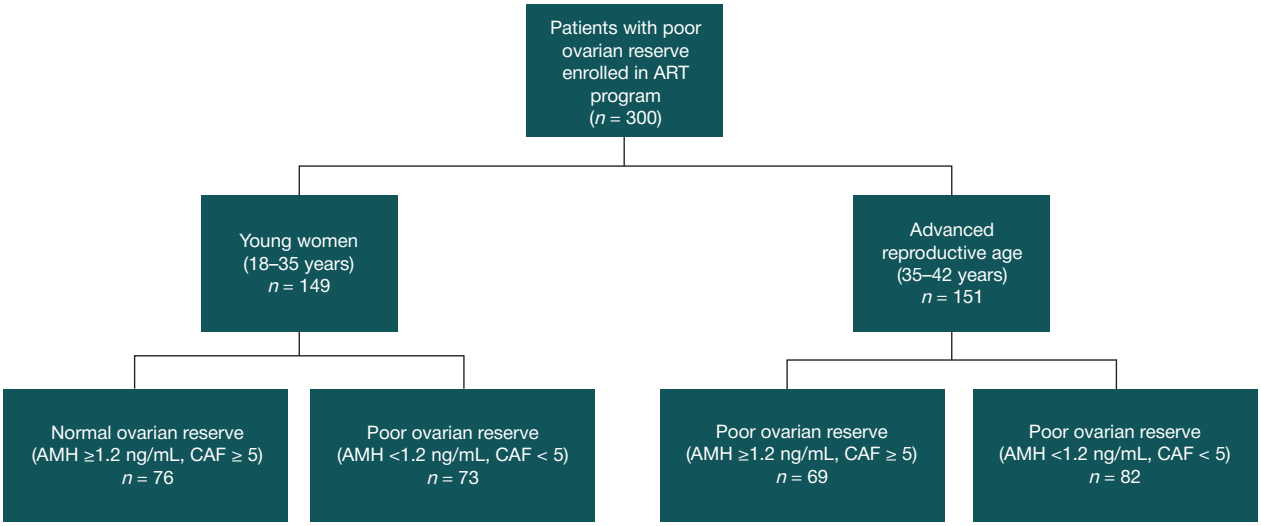

Fig. 1. Block-scheme of the study

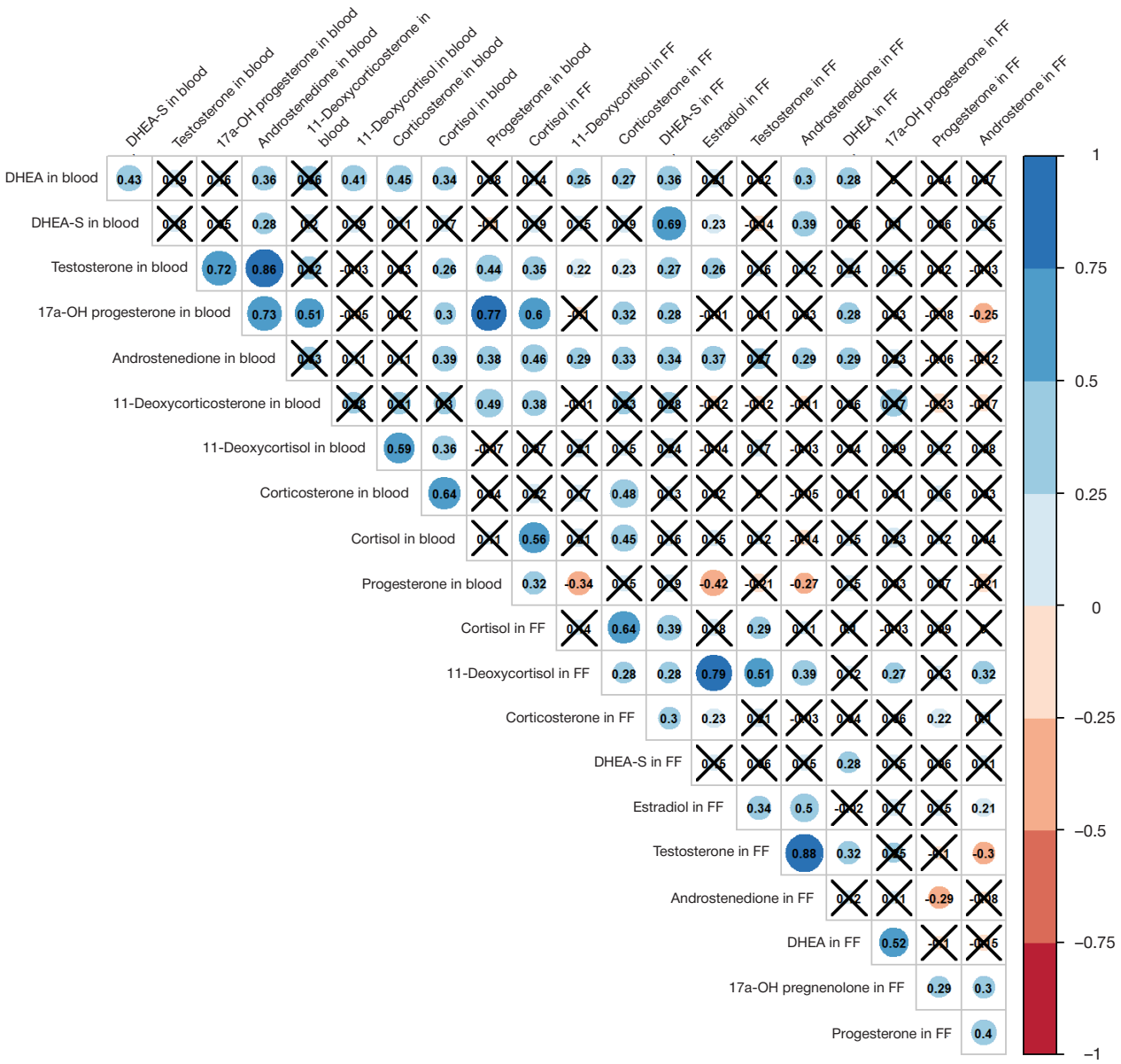

Fig. 2. Correlation coefficients for hormone concentrations in plasma vs follicular fluid as measured by LC-MS/MS in patients with infertility and diminished ovarian reserve

(98%, Sigma-Aldrich; USA) were used in phase preparation and sample preparation procedures. The hormones were separated in a 100 mm Poroshel 120 EC-C18 column (Agilent; USA) with 2.1 mm inner diameter and 2.7 μm particle size.

During the sample preparation procedure, a mixture of 460 μL sample with 25 μL internal standard was double-extracted with 1 mL MTBE followed by retrieval of the supernatant (800 μL) in a clean Eppendorf tube for drying under flowing nitrogen gas at 50 °C. The dry residue was dissolved in 100 μL of 50% MeOH and transferred into a vial with an insert for LC-MS/MS measurements.

The samples were run in a LC-MS/MS system consisting of an AB Sciex QTRAP 5500 quadrupole mass-spectrometry detector with an electrospray ionization source and an Agilent 1260 Infinity liquid chromatograph (Agilent; USA) equipped with a high-pressure pump, column thermostat and an automated sampling unit for 108 vials.

Statistical analysis

Statistical analysis was carried out using scripts in R [16]. Correlation analysis was used to determine a possible relationship between variables. Statistical significance of correlations for analytical measurements was assessed using a nonparametric Spearman's test. In cases of missing numerical values, the correlation coefficient was calculated for all available complete pairs of measurements for each pair of variables individually. Statistical significance of correlations for the questionnaire data was assessed by nonparametric Kendall's test. The quantitative data were described by medians (Me) with lower and upper quartiles in a Me (Q_1; Q_3) format.

RESULTS

The mean age of participants was 37.3 ± 2.4 years. The body mass index (BMI) tended to increase with age: in advanced reproductive age patients with POR, it reached 24.6 ± 5.4 though remained statistically similar to BMI in other groups.

The reproductive histories typically included unsuccessful IVF cycles/cryo protocols, which ended in a negative result at the stage of hormone pregnancy test. The infertility duration increased significantly with age and ovarian reserve decrease, amounting to 7.2 ± 2.4 years in the group of advanced reproductive age patients with POR, 65.4% of whom had

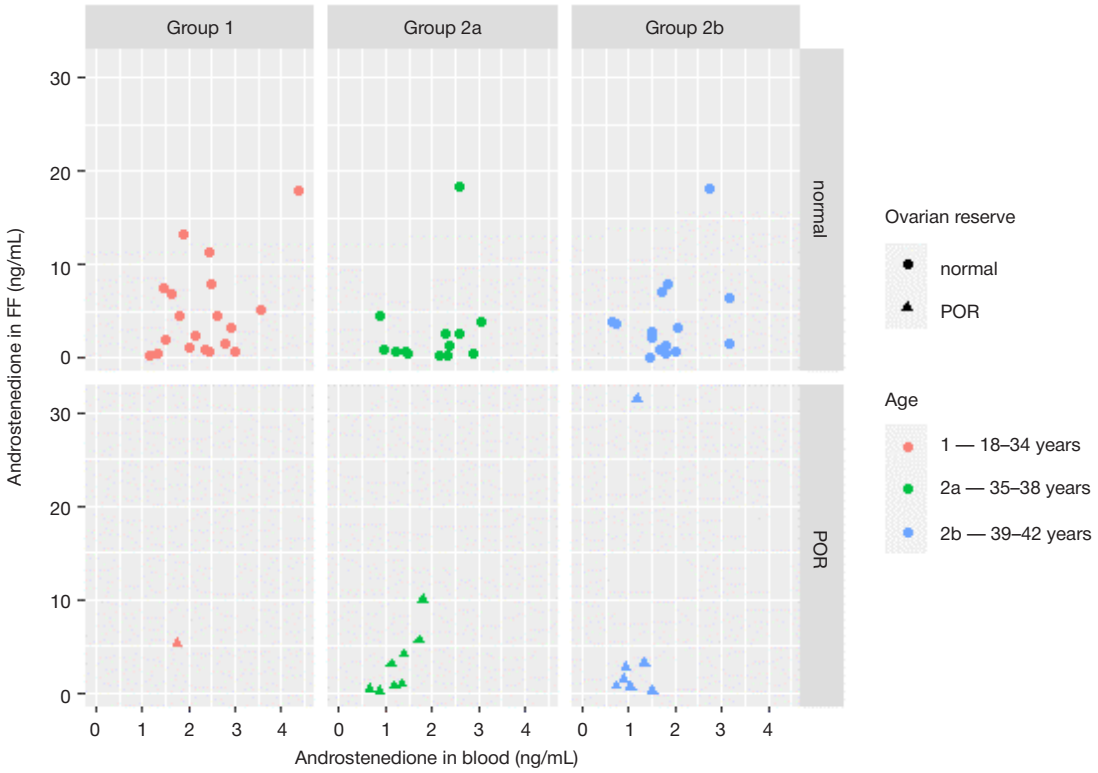

Fig. 3. The concentrations of androstenedione in follicular fluid (FF) and blood plasma per groups: younger than 35 years (group 1), aged 35–38 years (group 2a) and older than 39 years (group 2b), with ovarian reserves diminished (subgroup 1) or normal (subgroup 0)

primary infertility. The ART cycle number showed a similar tendency amounting to 2.3 ± 0.9 in the advanced reproductive age patients with POR.

The analytical data on hormone concentrations in blood plasma and follicular fluid collected on the day of oocyte aspiration were subject to correlation analysis. For many of the studied hormones (DHEA-S, total testosterone, 17-OH progesterone, androstenedione), concentrations in plasma and follicular fluid correlated (Fig. 2). Blood vs FF levels of the same hormone revealed weak association for androstenedione (correlation coefficient 0.29) and moderate association for DHEA-S (correlation coefficient 0.69). Strong correlations between androstenedione and testosterone levels in blood (correlation coefficient 0.86) and FF (correlation coefficient 0.88) confirm the pathogenetic link between POR and androgen levels as dependent\connected factors of folliculogenesis.

Concentrations of androstenedione and DHEA-S in blood and FF were comparatively analyzed in patients of different age groups with regard to ovarian reserve. For a detailed assessment of age-dependent dynamics in ovarian function by measuring DHEA-S and androstenedione in blood and FF, patients of group 2 (35–42 years) were stratified as 35–38-year-olds (subgroup 2a) and 39–42-year-olds (subgroup 2b).

Comparative analysis of blood and FF concentrations revealed a POR-related decrease for androstenedione (Fig. 3) and DHEA-S (Fig. 4) in both biological media. A strong agreement between blood and FF levels of DHEA-S across the groups (Fig. 5) adds to its prospective clinical value as a marker of androgenic activity and androstenedione precursor.

DISCUSSION

This study involved comparative measurement of androgens as estrogen precursors using the sensitive and highly specific LC-MS/MS method [9, 15] in blood and FF samples collected during IVF cycles. The results indicate significant association of POR with 'ovarian androgen deficiency' defined as selective decrease in androgen output from thecal cells. Such effects can be regarded as early signs of the age-related androgen deficiency. We also revealed a correlation between androgen levels in blood and FF, the latter being the essential medium for oocyte growth and maturation. A decrease in concentration of androstenedione as a precursor for testosterone in FF was significant, as well as a similar decrease for DHEA-S as a precursor for androstenedione and a marker of androgenic activity in FF. Moreover, FF concentrations positively correlated with blood levels for each of these hormones.

The known ovarian reserve markers, notably AMH and AFC, often misrepresent the real clinical picture and embryological parameters in both early and late reproductive age patients. These markers only loosely correlate with the mature oocyte numbers, fertilization efficacy and blastulation frequency [15, 17, 18], as well as with androgen levels and their receptor concentrations and activities. FF, which constitutes the microenvironment for oocyte growth and maturation, becomes available as a biological sample during follicular puncture in IVF programs. FF provides a useful substrate for biochemical tests gaining information on the course of oogenesis.

For instance, in patients with polycystic ovarian syndrome (PCOS) have higher testosterone concentrations in mature follicles compared to women with normal ovarian reserve, whereas ovarian stimulation in such patients only slightly enhances the FF testosterone levels, as compared with natural cycles [10].

In a prospective cohort study of endogenous steroid concentrations determined by liquid chromatography and mass spectrometry in the follicular fluid, a significant androgen in the follicular fluid was identified, which was androstenedione [9].

In one study, focusing on intrafollicular concentrations of estradiol, progesterone, 17-hydroxyprogesterone, androstenedione and testosterone during periovulatory period, FF was collected on the day of the ovulation trigger injection

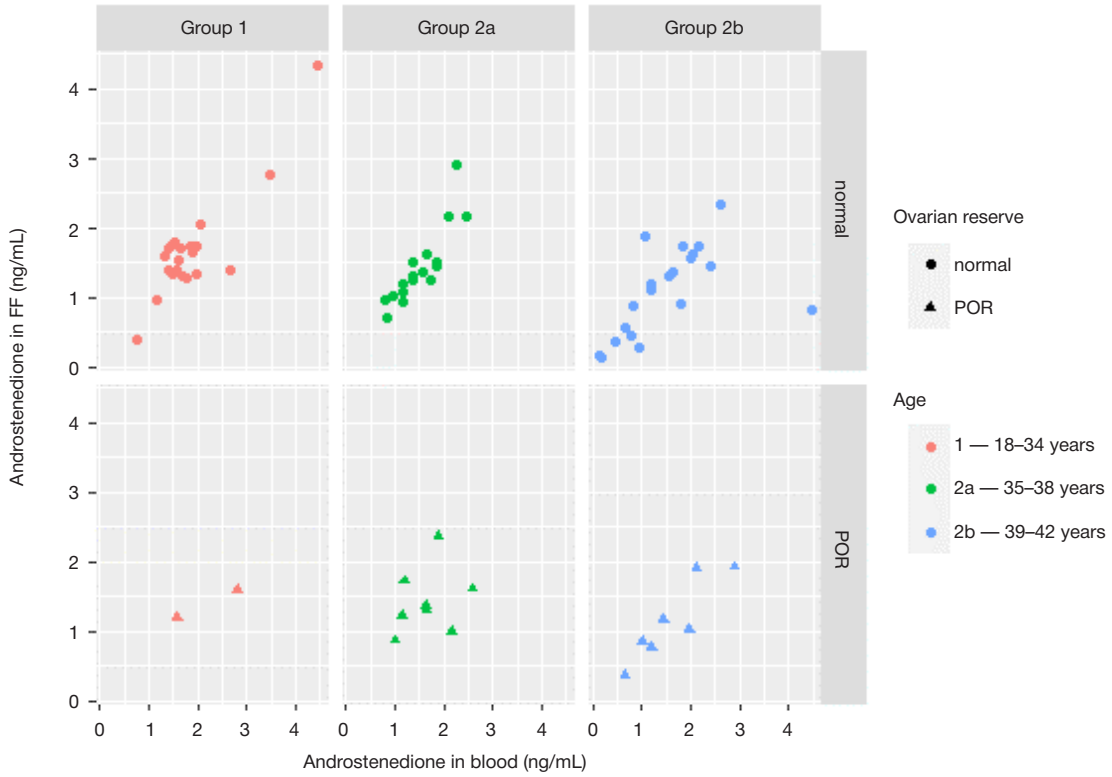

Fig. 4. The DHEA-S levels in follicular fluid (FF) and blood plasma per groups: younger than 35 years (group 1), aged 35–38 years (group 2a) and older than 39 years (group 2b), with poor ovarian reserves (subgroup 1) or normal (subgroup 0)

and then 12, 17, 32 and 36 h post-injection [17]. The analytical measurements revealed dynamic changes for estradiol and testosterone, whereas androstenedione levels in both blood and FF remained stable over the entire periovulatory period. Nevertheless, consistently with our findings, correlation analysis revealed a positive association between androstenedione and testosterone levels in FF. Considering that total blood levels of testosterone in women are low and their reliable measurement is problematic, the correlating levels of androstenedione and total testosterone in both plasma and FF implicate androstenedione as a candidate marker of POR in reproductive age women with infertility.

A statistically significant increase in FF androgen levels against the background of menotropins as ovarian stimulators has been reported as well [18]; however, our data provide no support for this association.

Yet another study focused on androgen concentrations in blood serum and FF in patients with poor ovarian response, assigned into four groups according to POSEIDON criteria. Despite the lack of significant differences in blood levels of testosterone, androstenedione and DHEA-S between patients with normal ovarian reserve (controls) and POSEIDON group 1, significantly decreased blood levels of hormones were revealed in POSEIDON group 3 compared with the controls. At that, FF concentrations of DHEA-S in group 3 were significantly lower, whereas FF concentrations of testosterone, androstenedione, estradiol and sex steroid-binding globulin (SSBG) were similar in

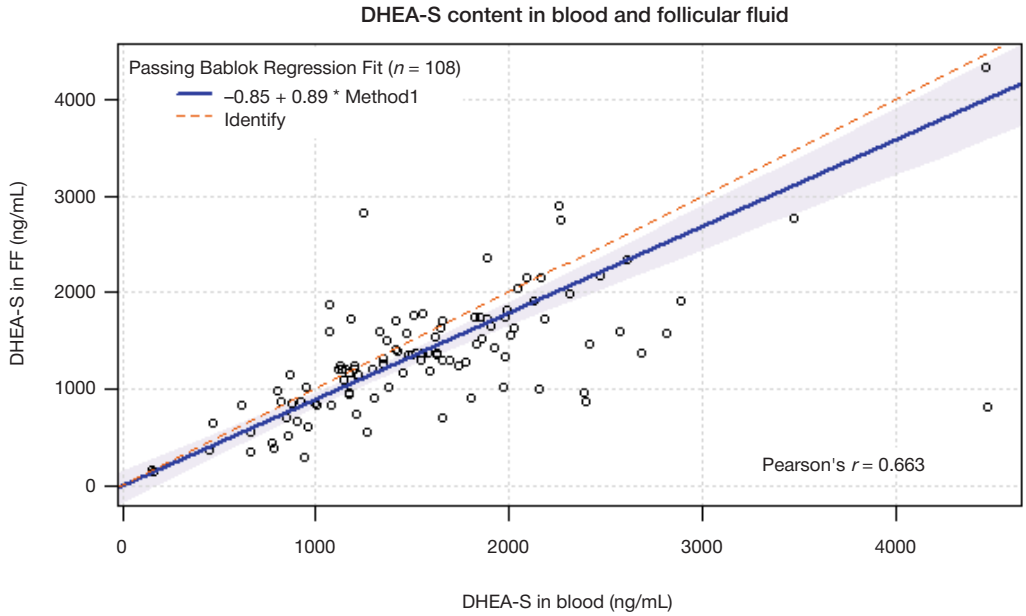

Fig. 5. The correlation and linear regression analyses of DHEA-S concentrations in follicular fluid (FF) vs blood plasma for all data points

all groups. In patients older than 35 years, serum testosterone levels were significantly lower independently of the ovarian reserve status (POSEIDON groups 2 and 4). The study also demonstrated a positive correlation between serum and FF concentrations for DHEA-S [19]. Our own data confirm the overall tendency of correlating hormone levels in blood and FF.

CONCLUSIONS

The presented data indicate that with a diminished ovarian reserve there is a selective decrease in androgen levels in the follicular fluid, which confirms the hypothesis about the contribution of androgens and the role of their deficiency. The findings of the correlation between total testosterone and androstenedione not only in the blood plasma but also in the follicular fluid indicate the diagnostic value of these androgens as early markers of androgen deficiency formation in women with infertility with decreased ovarian reserve. The use of LC-MS/MS to determine androgen levels in women with infertility in an IVF program may be predictive of the relationship between testosterone and androstenedione concentrations in plasma and follicular fluid as an oocyte microenvironment. The discussion about the possibility of predicting the outcomes of ART on the basis of androgen levels requires more in-depth research due to the growing clinical need to improve the effectiveness of IVF and to predict their outcomes at the stage of analysis of follicular fluid composition.

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