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Research Article

Effect of Maternal Age on Androstenedione and Pregnenolone Levels and Relationship with Retrieved Oocytes in Iraqi Women

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ABSTRACT

Follicular fluid (FF) entails a substantial number of steroids produced by granulosa cells (GCs) and theca cells, which are essential for developing oocytes. Nevertheless, it remains unclear how endogenous steroids in the FF change with advancing maternal age, which can affect oocyte maturation, subsequent embryo growth, and pregnancy outcomes after *in vitro* fertilization (IVF). Therefore, the aims of the present study are: (1) to examine the impact of maternal age on steroid (androstenedione A and pregnenolone P) levels in FF. (2) to test their association with IVF outcomes over age. (3) to assess whether these steroids are capable of forecasting pregnancy after IVF treatment. Forty women aged 36 to 47 are classified as the older group, and forty women aged 25 to 35 are classified as the younger group. The FF samples were drawn when the oocytes were picked up and steroidized. Steroid (A and P) levels were quantified using a Fa/Fs sandwich enzyme-linked immunosorbent assay (ELISA). A significant decline in the two selected steroid levels was observed in FF from the older women compared with those from the younger women ($P<0.05$). Steroid levels were positively associated with retrieved oocytes over time ($P<0.05$). Still, they did not achieve statistical significance with cleaved embryos ($P>0.05$) and did not have a predictive effect on pregnancy. Older women may have declined steroids and perturbed granulosa/theca cells' steroidogenesis, which subsequently perturbs oocytes and ovarian function, proposing a pathophysiology for the reduction in female reproductive capacity.

1. Introduction

Ovarian aging is a recognized factor that diminishes reproductive capacity in women (Z. Zhu *et al.* 2023). As age advances, there is a gradual decline in both the quality and quantity of oocytes, impacting embryo quality, pregnancy rates, and live birth probability, leading to worsening outcomes of *in vitro* fertilization (IVF) (Trawick *et al*. 2021; Adebayo *et al*. 2023; Havrljenko *et al*. 2023). Ovarian follicles produce the following androgens: androstenedione (A), testosterone (T), and dehydroepiandrosterone (DHEA), which are essential for the regular menstrual cycle and command the dynamic fluctuations that occur during ovarian steroidogenesis (Melmed *et al*. 2015; Astapova *et al*. 2019; Franks 2021). In this pathway, cholesterol, an initial precursor, is converted to the rate-limiting pregnenolone (P). Thereafter, via the Δfour pathways, P is transformed

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into progesterone (P4) by the enzymatic activity of steroidogenic 3βHSD. In theca cells, the steroidogenic CYP17A1 converts P to 17-hydroxypregnenolone, which is subsequently converted to DHEA, triggering the synthesis of A, the major ovarian androgen, via 3βHSD1. In granulosa cells (GCs), the CYP19A1 accelerates the aromatization of androgens (A & T) into estrogens (Strauss III 2019).

Follicular fluid (FF), the milieu for oocyte growth, entails a substantial number of steroids produced by GCs and theca cells in response to gonadotropins, which are essential for developing oocytes (Walters *et al*. 2019; Yang *et al*. 2021). Overall, Androgen deficiency in aging women has been linked to diminished sexual function (Davison and Davis 2011). An investigation revealed that circulating androgen levels declined in healthy women aged 18 to 75, predominantly during their reproductive years (Davison *et al*. 2005). Klein and colleagues state that women with advanced reproductive age typically have diminished levels of androgens (T and A) in their (FF) (Klein *et al*. 1996). Although Garzia *et al*. (2022) demonstrated that A may be crucial for optimal follicular growth, they did not detect significant variations in the baseline A level of women under and above 35 years of age. In mammals, *in vitro* investigations have demonstrated that micromolar doses of A combined with follicle-stimulating hormone (FSH) enhance follicular survival, oocyte growth, and embryo developmental competence (Spears *et al*. 1998) (Gervásio *et al*. 2014) (Zhao *et al*. 2023). However, clinical findings remain controversial. Some researchers report no association between follicular androgens (A, T, DHEA) and fertilization rates (Wiweko *et al.* 2016), while others link FF levels of P, P4, A, T, and estradiol (E2) to live birth rates (Kushnir *et al.* 2016). These conflicting results challenge our ability to determine how FF androgen levels contribute to age-related reproductive decline and IVF outcomes. The existence of P in FF has been demonstrated in both animal and human investigations (Costermans *et al*. 2019b; Yu *et al*. 2021; Téteau *et al*. 2022), and P levels have been shown to correlate with follicle size during the mid-follicular phase in mammals (Costermans *et al*. 2019b). Despite its biological relevance, the age-related variation of endogenous FF steroids—especially A and P—and their implications for oocyte maturation, embryo development, and pregnancy outcomes are insufficiently understood. Thus, a gap remains regarding how maternal age influences FF A and P steroid levels and whether these steroids can predict IVF outcomes.

Accordingly, our study aims: (1) to examine the impact of maternal age on steroid (A and P) levels in FF. (2) to test their association with IVF outcomes over age. (3) to assess whether these steroids are capable of forecasting pregnancy after IVF treatment.

2.2. Materials and Methods

2.1. Woman Subjects

Eighty women undergoing their first IVF cycle at the Reproductive Medical Center of Fertility and IVF at the Kamal AL-Samarai Hospital in Baghdad/ Iraq, were prospectively recruited from June 2023 to January 2024.

Inclusion criteria represented women who: (1) have tubal factor infertility, which includes women who underwent the removal of the fallopian tube (s) due to ectopic pregnancy and proximal tubal obstruction, (2) are aged between 25 and 47 years, (3) are receiving gonadotropin-releasing hormone agonist protocol (GnRHa), and (4) exhibit normal sex hormone levels and typical menstrual cycles. All women underwent a gynaecological examination, hormonal assessment, and transvaginal ultrasound. In tubal infertility cases, laparoscopy and hysterosalpingography as screening techniques were conducted by specialists following worldwide guidelines (Ott *et al*. 2020).

The exclusion criteria involved women with the following issues: (1) pelvic endometriosis, (2) pelvic inflammatory disease, (3) adenomyosis, (4) ovarian malignancy, (5) polycystic ovary syndrome, (6) poor ovarian response, (7) thyroid disorders, (8) hyperprolactinemia, (9) cardiovascular disease, and (10) diabetes mellitus, were excluded. Women in this study were separated into two groups based on maternal age. The younger cohort consisted of 40 women aged 25 to 35 years, and the older cohort comprised 40 women aged 36 to 47 years. This classification was established based on the clinical category of maternal aging as delineated by the American College of Obstetricians and Gynecologists (ACOG 2022). The Al-Iraqia University Ethics Committee (Approval number FM.SA.433) approved all the procedures. Consent was obtained in written form from each woman. A group of embryologists and gynaecologists collaborated on all aspects of the research (ovarian stimulation protocol, oocyte retrieval procedures, IVF, and oocyte and embryo evaluation).

2.2. Ovarian Stimulation Protocol, Oocyte Aspiration, and Outcomes

Every woman in the study received pituitary suppression with a long GnRHa protocol. The standard protocol involved subcutaneous injection of a GnRH analogue (GnRHa, Decapeptyl® 0.1mg/1mL; Ferring, Germany) once daily, starting in the mid-luteal phase and continuing until the day of human chorionic gonadotropin (hCG) administration. The administration of recombinant FSH (rFSH, Gonal-F®150 IU /day; Merk Serono, Switzerland) achieved ovarian stimulation. The first response scan was performed on the sixth day after stimulation and was assessed by consecutive transvaginal ultrasonography and measurement of serum E2 levels. A steady increase in serum E2 levels and three or more follicles measuring 18 mm or larger were required to administer recombinant hCG (rhCG, Ovitrelle® 250 micrograms/0.5 mL; Merck Serono, Italy). Oocytes were collected through a needle-guided aspiration via the transvaginal ultrasound 34 to 36 hours following an hCG injection. After retrieval, to assist the luteal phase, 200 milligrams of vaginal micronized progesterone were administered. The quantity of oocytes retrieved was the primary outcome of the research. Further, early embryo growth and transfer were recorded and analyzed. On days 2 and 3 of embryo development, 2 to 3 embryos were transferred. Biochemical pregnancy was confirmed by the serum βhCG test (12) days after embryo transfer.

2.3. Collection and Handling of Follicular Fluids

In each woman, the FFs were collected from aspirated ovarian follicles when the oocytes were picked up, transferred to the laboratory, and centrifuged at 3000×g for 10 minutes. The supernatant was gathered and preserved at -80°C until examination. Only follicles aspirated free from blood contamination and including an oocyte were considered for examination.

2.4. Steroid Analysis in Follicular Fluids

Steroid (A and P) levels were quantified in 80 FF samples using the human androstenedione (A) and pregnenolone (P) Kits (Sun Long Biotech, Co., LTD, China) by sandwich enzyme-linked immunosorbent assay (ELISA). Intra- and Inter-assay precision CV% of each A and P is <10 and <12. The FF samples were diluted (1:5) to determine A and P concentrations. The concentrations of A and P in FF were expressed in pg/mL and then converted to ng/mL by dividing by 1000.

2.5. Statistical Analysis

The Statistical Package for the Social Sciences (IBM SPSS® software, version 29; SPSS Inc., USA) was employed to analyze the data. The non-normal distribution of every continuous variable was detected using the Shapiro-Wilk and Kolmogorov-Smirnov tests. Consequently, the variables were reported as median values with interquartile range (IQR) and then compared using the Mann-Whitney U test. The Spearman correlation test was used to assess the association between variables using the Spearman rank correlation coefficient (r). Further, for IVF outcome prediction, the area under the curve (AUC) with a 95% confidence interval (CI) was calculated using the receiver operating characteristic (ROC) curve. An asterisk (*) denoted statistical significance when the P-value was less than 0.05.

3. Results

3.1. General Characteristics and IVF Cycle Outcomes

Table 1 records the general characteristics and IVF outcomes of the 40 younger women (25-35 years) and the 40 older women (36-47 years). Regarding body mass index (BMI) and duration of infertility, there were no significant variations between the two groups. The number of oocytes retrieved and cleaved embryos, which indicate IVF cycle outcomes, was significantly lower in older women than in younger women.

Table 1. General characteristics and IVF cycle outcomes of the younger and older women

	Younger group (N=40)	Older group (N=40)	*P* value
Age	29 (25-35)	40 (36-47)	0.00
BMI (kg/m^2)	24 (22-27)	24.5 (22-29)	0.43
Duration of infertility (years)	4.40 (2.50-6.00)	4.70 (3.00-7.00)	0.12
Number of oocytes retrieved	12 (6-14)	6 (5-12)	0.00
Number of cleaved embryos	6 (2-6)	3 (2-5)	0.00

3.2. Steroids of Follicular Fluids among Younger and Older Women

The FFs were collected and analyzed from women of younger and older ages. Medians and ranges of the selected steroids (A and P) in the FFs and P values are displayed in Figure1A trend to a significant decline of the two selected steroids (A and P) levels was observed in FFs corresponding to the older women compared with the younger ones.

3.3. Spearman Correlation between Maternal Age, Steroid Levels, and IVF Cycle Outcomes

First, we did Spearman rank correlation tests to elucidate further maternal age's effects on FF steroid level and IVF cycle outcomes; accordingly, our analysis indicated that maternal age significantly and inversely correlated with retrieved oocyte number, cleaved embryo number, A level, and P level, among all the participants (Figure 2A). Besides, to explore the potential impact of age-dependently declined steroids on IVF cycle outcomes, we conducted correlation tests to examine their relationship with the number of oocytes retrieved and cleaved embryos. Levels of steroids, including A and P, were positively associated with decreased retrieved oocytes over age (Figure 2B). Furthermore, the steroid levels followed the same trend as cleaved embryo numbers but did not reach statistical significance (Figure 2C).

3.4. Prediction of Pregnancy from The Follicular fluid steroids (A and P) levels

The ability to predict pregnancy was evaluated using the ROC curve. Figure 3 shows that the AUC was 0.58 (95% CI: 0.44-0.72, P = 0.24) for A and 0.54 (95% CI: 0.40-0.68, P = 0.57) for P, indicating inadequate discriminatory power and no statistically meaningful predictive value ($p > 0.05$).

4. Discussion

This study conducted steroid profiling of two endogenous steroids (A and P) in FF samples obtained from women undergoing IVF treatment. Maternal age is a significant determinant of both the quantity and quality of oocytes (Moghadam *et al*. 2022). In turn, we analyzed the relationship between maternal age and steroid levels in FF. The study indicates an age-dependent disturbance in follicle metabolism, particularly in steroid-related lipid metabolism. Interestingly, significant relationships have been observed between the levels of the identified steroids and the number of oocytes, suggesting that these steroids may serve as potential biomarkers of oocyte maturation, particularly with age. The work presented here could improve our understanding of the steroid profile of human FF, particularly with respect to age. It may also contribute novel perspectives to endocrinology in aging and suggest future avenues for research on reproductive aging and ovarian function.

The ovary is a reproductive endocrine organ in women (Strauss III and FitzGerald 2019). Indeed, ovarian aging entails a progressive lowering in the quality and quantity of oocytes and follicles, leading to a decline in reproductive and endocrine functions with age (Moghadam *et al*. 2022; Wu *et al*. 2022). Likewise,

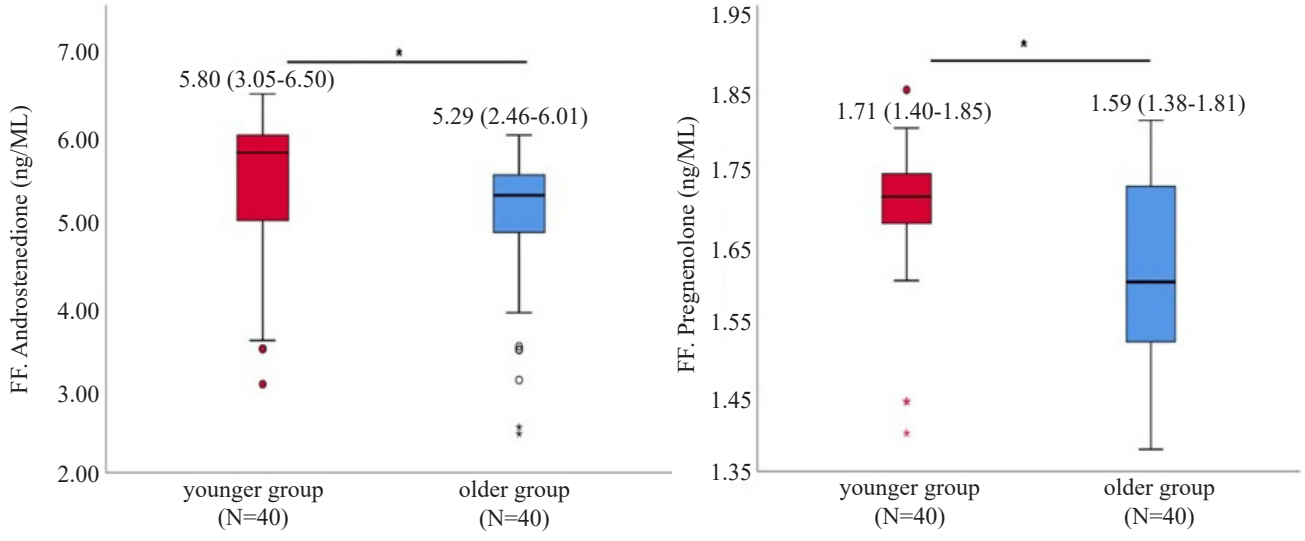

Figure 1. Box plots of the median (IQR) of the selected steroids FF levels in the older and the younger women groups (Mann-Whitney U test)

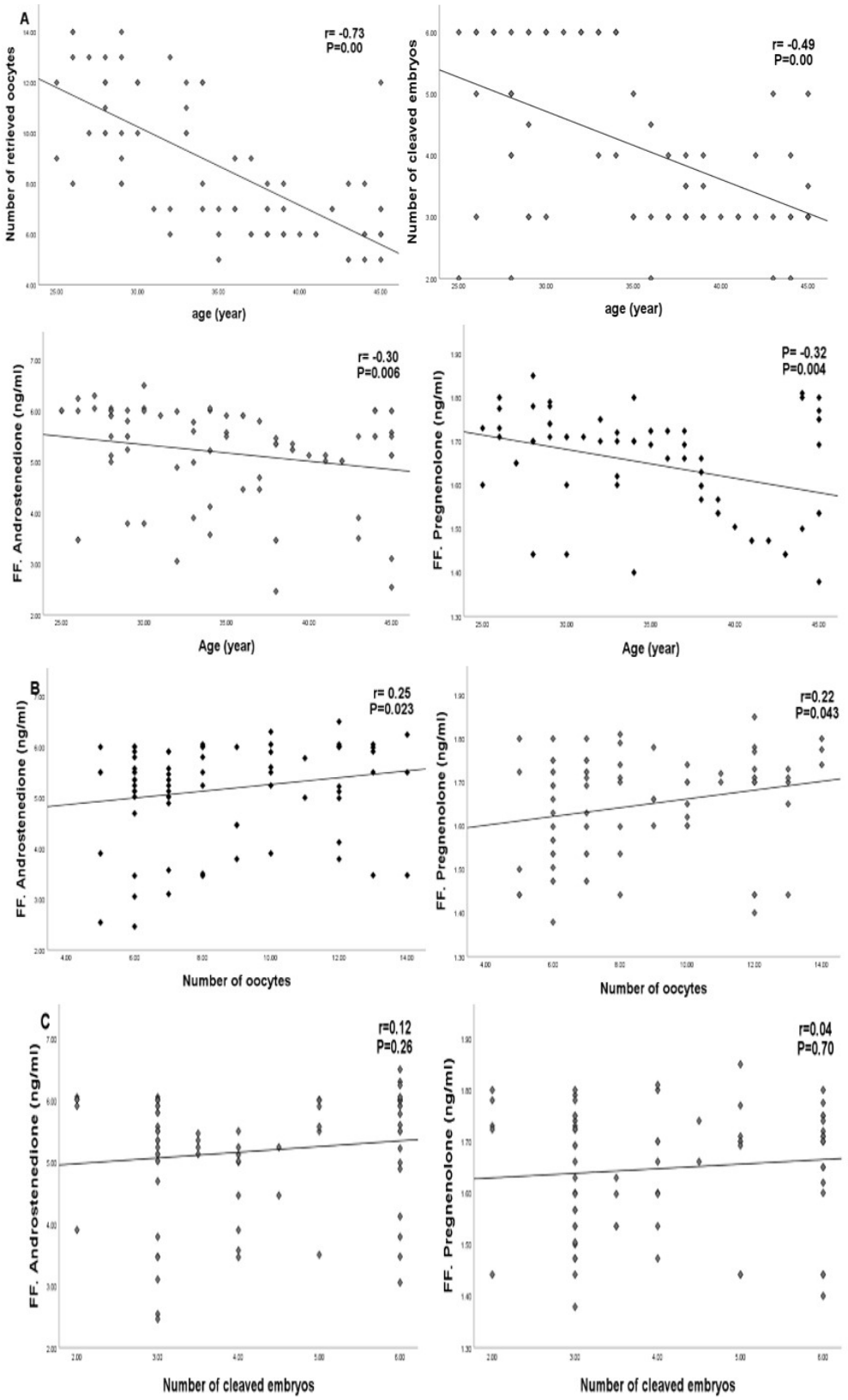

Figure 2. Relationship among maternal age, oocytes retrieved, and cleaved embryos with steroids (A and P). (A), Correlations between maternal age, steroids (A and P), and IVF cycle outcomes. (B), Correlations between steroids (A and P) and number of retrieved oocytes. (C), Correlations between steroids (A and P) and number of cleaved embryos

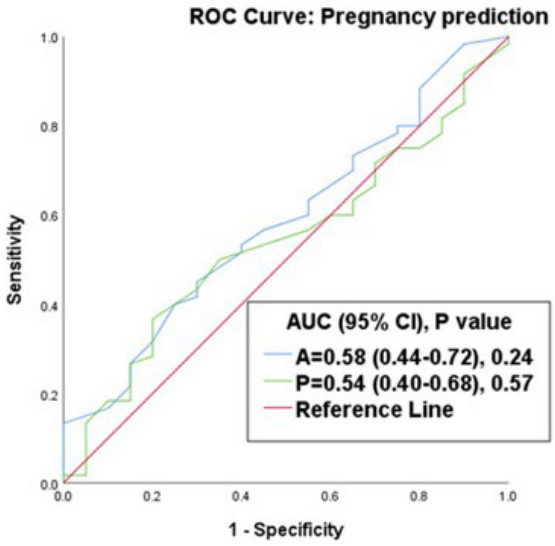

Figure 3. ROC curves of A and P levels from follicular fluids in the prediction of pregnancy after IVF cycle

oocyte quality deteriorates with age, particularly between the ages of 35 and 40 (Nikolaou and Seifer 2022). Overall, the metabolism of follicle hormones is closely linked to ovarian function and oocyte quality (Piccinni *et al*. 2021; Dai *et al*. 2024).

The present study observed age-dependent declines in A and P levels in FF. Hypothetically, preliminary mechanisms can be proposed to explain the reduction in A and P biosynthesis in theca cells in the context of ovarian aging. These mechanisms assume that: (1) A first possible mechanism might be that theca cell has less capacity to activate steroidogenesis, defective cholesterol trafficking inside mitochondria, and decreased cholesterol metabolism. (2) A second possible mechanism is increased precursors (A & P) consumption due to higher aromatase expression (3βHSD2,17βHSD3, and CYP19A1) with aging. (3) It is theoretically possible that a third mechanism, declining aromatase expression (CYP11A and 3βHSD1), occurs with ovarian aging. Nevertheless, there is currently insufficient evidence for these probabilities in the context of ovarian aging.

Indeed, cholesterol has been deemed crucial for female Fertility due to its significance as a substrate for steroid production in ovarian follicle cells (Huang *et al*. 2019). In agreement with our hypothesis, defective cholesterol trafficking and metabolism have been detected in females with diminished ovarian reserve, reducing steroid hormone synthesis in GCs (Bildik *et al*. 2022; Yang *et al*. 2022). Women with diminished ovarian reserve or who are older have experienced changes in the metabolic processes of their follicular cells and alterations in metabolites in FF and in P4 synthesis. The disturbed environment may be the reason for the deterioration in oocyte competence, which subsequently affects embryo development (Q. Zhu *et al*. 2023). In a previous study of older, regularly cycling women, GC CYP19A1 expression was higher, whereas FF A level was non-significantly lower than in younger women (Shaw *et al*. 2015). In the early follicular phase, a second study found that higher aromatase activity (lower A/E1 ratio) is associated with lower basal A levels and fewer follicles in older reproductive-aged women (Welt *et al*. 2006). The results of these studies support our hypothesis. It is worth noting that age-related variation in aromatase activity in the dominant follicle is FSH-dependent. During the dominant follicle maturation period, GC aromatase expression may be up-regulated by an epigenetic process brought about by exposure to greater FSH levels during the early follicular phase (DeManno *et al*. 1999). Nevertheless, the etiology and mechanism underlying the third one are unclear. However, the role of A and P in ovarian aging remains unclear.

The decline in serum A of adrenal cortex origin with age has been described by others (Whitton and Baber 2024). There is little research in the scientific literature on declining A levels in FF of older patients who have undergone ovarian stimulation (Klein *et al*. 1996; Yokota *et al*. 2003; Welt *et al*. 2006). An old study by Klein *et al*. (1996) reported lower FF A and T levels, whereas higher FF E2 levels, FF P levels, E/androgen ratio, and early follicular phase FSH levels among older women compared with younger women. This study suggests that hormone changes with aging are reflexive of healthy ovarian follicles and that older women have better follicular growth. The explanation might be that the enhanced release of pituitary FSH and the better steroid profile in the FF could function as compensation procedures for the deteriorating quality of the aging oocyte (Klein *et al*. 1996; Yokota *et al*. 2003), reported that the older women had higher FF P4 levels and lower FF T, A, and E2 levels when compared with the younger. They speculated that younger women possess a more vital ability to initiate ovarian steroidogenesis and that aromatase activity in GCs is elevated. Therefore, GCs have a significant ability to aromatize androgens to E2, which is responsible for the production of high-quality oocytes and embryos (Lv *et al*. 2020; Al-Kady *et al*. 2021). Hence, the transient

increase in ovarian T and A might only impact younger women (Yokota *et al*. 2003).

The current study observed an age-dependent decrease in P levels in FF. With the cited studies in mind, compelling data support the hypothesis that luteinization may occur in our older group, leading to elevated P4 levels and reduced P and E2 levels in FF. Although others support this prospect (Gao *et al*. 2021), who did not assess the P level in FF, it requires further study. As age advanced, it has previously been demonstrated that the expression of GCs FSH receptor, CYP19A1, and 17βHSD declined, while the expression of LH receptor, CYP11A1, and P4 receptor was elevated. Interestingly, it has been shown that GCs exhibit reduced proliferation and accelerated programmed cell death with aging, suggesting that GCs may undergo age-related functional deterioration, resembling premature luteinization (Wu *et al*. 2015). As luteinization begins, GCs produce more P4 from P, suggesting increased consumption of the precursor (P) and biosynthesis of P4. Thus, LH may increase P4 availability and Δ5 3βHSD2 activity, thereby stimulating the Δ4 pathways and elevating P4 levels in FF. Further, the aromatizing activity of GCs declines, decreasing estrogen synthesis (Liu *et al*. 2021). These findings can be confirmed by decreased E2 synthesis in animals after LH injection and decreased 17βHSD3 activity (Katz and Armstrong 1976). Yet, no existing literature confirms lower P levels in FF with ovarian aging.

Surprisingly, in our study, age-dependent decreases in steroid hormones (A and P) were positively associated with the number of oocytes retrieved. Perhaps explanations for our result are that: (1) in the primordial and primary follicle, it is likely that insulin-like growth factor1 receptor (IGF1R), Insulin-like growth factor2 (IGF2), and binding protein-3 (IGFBP3) were observed to be expressed in GCs and oocytes, which are responsive to the androgen (A). However, this connection may impact early follicular activation, folliculogenesis, steroidogenesis, and GC proliferation (Costermans *et al*. 2019a; Afradiasbagharani *et al*. 2022). (2) Our findings support the idea that lowering follicular P availability may be the leading cause for the lowering of E2 precursors (P4, A, and T) (Craig *et al*. 2013), which subsequently disrupts oocyte numbers, quality, and IVF outcomes (Lv *et al*. 2020). Mechanistically, A can be aromatized into estrogens (Strauss III 2019), which are involved in developing connections between GCs and oocytes by increasing the number of GC transzonal projections with the oocytes. Precisely, GCs form gap junctions at the head of these projections, enabling the immediate delivery of substances essential for developing oocytes (Makita and Miyano 2014). These observations suggest that low follicular A and P levels limit the availability of some growth factors and estrogen's ability to support the development of oocytes. Though further study is still needed, linking impaired steroidogenesis to the oocytes and IVF outcomes is reasonable. Based on the aforementioned evidence, we hypothesize that A and P levels in FF may serve as biological markers of oocyte and ovarian function.

Even more importantly, in the older age group, the decline in retrieved oocytes and in follicular A and P levels, along with the significant positive relationship between them, supports our hypothesis above: decreased cholesterol metabolism might be responsible for age-dependent decreases in follicular A and P levels. Our results could be explained by the fact that cholesterol homeostasis in oocytes is essential for ensuring their developmental potential. The existence of a cholesterol homeostasis system that regulates the movement between FF high-density lipoproteins (HDL) and oocytes has crucial consequences on female Fertility (Arias *et al*. 2022). The reduced number of retrieved oocytes in our study's older group could partly be explained by decreased steroid and cholesterol metabolism, which perturb cholesterol homeostasis. Nevertheless, whether this phenomenon results from causation or just an epiphenomenon remains uncertain. Although our results were intriguing, they require caution. Suppose these findings are substantiated with a larger sample size. In that case, a question might arise: Will the therapeutic role of A and P benefit older women, improving oocyte quality and ovarian function during controlled ovarian hyperstimulation? From this point of view, careful assessment is required to select the minimum effective dose of A and P.

Grupen *et al*. observed higher adult FF content in P4 and A than in prepubertal FF; differences in follicular environments led to greater adult oocyte capacitation in pigs (Grupen *et al*. 2003). In an animal study (Zhao *et al*. 2023), Zhao *et al*. observed that A and P4 supplementation during *in vitro* maturation significantly improved the maturation of pig oocytes and embryos. Furthermore, *in vitro* culture of bovine oocytes has confirmed that supplementation with E2 and A results in the attainment of oocyte competence and complete growth (Makita and Miyano 2014).

Although the positive relationship between retrieved oocytes and A basal serum level over age has been previously confirmed (Garzia *et al*. 2022), this relationship has not been confirmed in the FF until now. On the contrary, our study contradicts earlier studies by (Chabab *et al*. 1986; De Sutter *et al*. 1991), showing that elevated A levels in the related FF are associated with oocyte abnormality. It appears that A is a sign of the androgenic state of the immature follicle, which subsequently decreases the rate of oocyte cleavage and negatively affects metabolic processes in the fertilized oocyte. It has been confirmed that alterations in the pulse sensitivity would have a role in the final estrogen conversion and excess androgens (Summers *et al*. 2014). Recently, one study has documented the impact of P on follicle development and oocyte maturation in animals (Li *et al*. 2024). However, the literature has not explored the effects of maternal age on the association between human FF P levels and retrieved oocytes. Accordingly, our study hints at the possibility of using a new method to partially reverse the specific effects of ovarian aging for therapeutic purposes.

However, it is generally accepted that the endocrine environment within the follicle performs a crucial role in the embryo's development, which is influenced by several mechanisms, including metabolism and genetic variables (Huang and Zhou 2021; Fiscus *et al*. 2024). The absence of a significant association between the steroids FF level and cleaved embryos (Figure 2C) may reflect the scenario (Huang and Zhou 2021; Fiscus *et al*. 2024) or be secondary to our limited number of participants. Currently, IVF centers use biomarker tests to predict ovarian response and IVF cycle outcomes, relying on their own data analyses to assign cut-off levels (Guan *et al*. 2022). Accordingly, for pregnancy prediction after IVF cycles, our analysis (Figure 3) showed that A and P levels in FF perform poorly at predicting pregnancy.

Our research has both strengths and limitations. The strengths of our study are that the recorded women had the exact cause of infertility, and the same stimulation regimen of gonadotropin was utilized. The limitations are: a relatively small sample size; analysis of the steroids was performed by immunoassay rather than high-specificity, high-accuracy methods (Munar *et al*. 2022), and assessment levels of the two endogenous steroids instead of complete steroid profile in FF.

Conclusion

Our study provides novel evidence that age-related decline in these endogenous steroids may reflect disturbed granulosa/theca cells steroidogenesis. Our study proposes that steroidogenic disruption in these cells reduces the availability of key steroids, thereby impairing oocyte development and ovarian function. This proposed biochemical pathway links cellular steroidogenesis with decreased reproductive capacity in older females. As a result, these insights highlight the value of FF steroid profiling as an age-related marker of ovarian function and support its broader application in understanding ovarian aging. Moving forward, larger, well-controlled studies are needed to elucidate the mechanisms and to strengthen the clinical utility of these biomarkers in IVF practice.

Declaration of Computing Interest

The authors declare that they have no conflicts of interest.

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