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Context: Members of the Transforming-Growth-Factor-β (TGF-β) family have been implicated in aberrant follicle development in women with polycystic ovaries (PCO).
Objective: Are there quantitative differences in the concentrations of TGF-β family members in fluid from small antral follicles (hSAF) from women with or without PCO?
Design & Setting: Follicle fluids (FF) were collected from 4-11 mm hSAF obtained from women undergoing ovarian tissue cryopreservation for fertility preservation.
Patients: FFs from 16 women with PCO (FF=93) and 33 women without PCO (FF=92).
Main outcome measures: Intrafollicular concentrations of Growth-Differentiation-Factor-9 (GDF9), Anti-Müllerian-Hormone (AMH), inhibin-A and -B, total inhibin, activin-A, -B and -AB, follistatin, follistatin-like-3, estradiol, and testosterone.
Results: Activin-B concentrations are reported for the first time in hSAF and concentrations were 10 times higher than activin-A and -AB. Activin-B showed significant associations to other growth factors. Concentrations of inhibin-A and -B were significantly lower in FF from women with PCO, especially in hSAF below 8 mm in diameter. AMH concentrations did not differ between the groups in hSAF below 8 mm, however, AMH remained high in hSAF above 8 mm in PCO but decreased in non-PCO women. Estradiol was significantly lower in FF from women with PCO and showed significant associations with AMH. Concentrations of

GDF9 are reported for the first time showing significantly higher concentrations in PCO FF of follicles above 6 mm.

Conclusions: Altered concentrations of TGF-β family members in hSAF from women with PCO highlight altered growth factor signaling as a potential mechanism for follicle growth arrest.

Concentrations of ten TGF-β family members in small antral follicle fluids from women with or without PCO highlight endocrinological characteristics that may be related to aberrant follicle growth.

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting 5-10% of women of reproductive age, and is the major cause of anovulatory infertility, hyperandrogenism and irregular menstrual bleeding (1-4). One characteristic feature of PCOS is anovulation/oligo amenorrhea with arrested growth of small antral follicles (hSAF) around 3–11 mm in diameter (5,6). The specific causes for follicle growth arrest in women with PCOS remain unclear and is most likely multifactorial (6,7). The increased LH/FSH ratio frequently observed in women with PCOS is believed to reflect an increased drive for theca cell derived androgen via LH stimulation combined with a reduced FSH drive, which impairs antral follicle development. The ovarian androgen production in theca cells has been shown to be highly upregulated by the synergistic action of LH and granulosa cell secreted growth factors, especially Transforming-Growth-Factor-β (TGF-β) family members like inhibins as well as insulin like growth factors (IGFs), while activins counteract this upregulation (8,9). The secretion of these growth factors from granulosa cells is governed by FSH and inhibin-B and various isoforms of FSH may regulate inhibin-B differently (10,11). Acidic FSH isoforms are specifically secreted when concentrations of estrogens are low in the beginning of the follicular phase and have been reported to favor inhibin-B synthesis (10-13). Regulation of ovarian steroidogenesis and follicular growth and development of hSAFs is highly complex most likely involving a multitude of regulatory steps that are independently controlled (14). Another TGF-β family member that affects the follicular steroid output is AMH, which has been suggested to be a negative regulator of estrogen synthesis in hSAFs (15). AMH is present in very high intrafollicular concentrations in hSAFs (i.e. a thousand times higher than in circulation) (16,17) and some studies have suggested that AMH production per follicle is augmented in women with PCOS (18-20).

It is well recognized that members of the TGF-β family affect follicular and ovarian function and several studies have implicated that some of the differences between women with or without PCOS could be ascribed aberrant regulation of one or more TGF-β family members (21-26).

The aim of the current study was to measure concentrations of TGF-β growth factors in 4-11 mm hSAF's obtained from women with or without polycystic ovaries (PCO) by analyzing 185 follicle fluid (FF) samples and measuring concentrations of AMH, inhibin-B, inhibin-A, total inhibin, activin-A, activin-B, activin-AB, follistatin, and follistatin-like 3 (FTSL-3), including a new highly sensitive assay for human Growth-Differentiation-Factor-9 (GDF9).

Material and Methods

Study population

Donated FFs were obtained from unstimulated ovaries of 49 women undergoing ovarian tissue cryopreservation for fertility preservation at the Laboratory of Reproductive Biology, University Hospital of Copenhagen, Denmark, from year 2011-2018. The procedure involves excision of one entire ovary which is laparoscopically removed from patients at a random day during their natural menstrual cycle and before initiation of gonadotoxic treatment (27,28).

Only the cortical tissue is suitable to be frozen for fertility preservation, and the surplus medullary tissue, including hSAF, was donated by patients giving written consent after orally conveyed information. The use of surplus tissue for research has been approved by the Ethical committee of the Capital Region, Denmark (H-2-2011-044).

Patient diagnoses included breast cancer (n=20), lymphoma (n=11), sarcoma (n=5), brain cancer (n=3), colorectal cancer (n=2), cervical cancer (n=1), leukemia (n=1), ovarian cancer (n=1), medulloblastoma (n=1), aplastic anemia (n=1), sickle cell anemia (n=1), black fan-diamond syndrome (n=1), and sclerosis (n=1).

Collection of follicle fluids from small antral follicles

Individual hSAF visible from the outside of the ovary and within the medullary tissue were aspirated with a 23G needle attached to a syringe (Figure 1A+B). The number of aspirated FF was, however, not comparable with an antral follicle count (AFC) identified by ultrasonography, as only visible and accessible antral follicles larger than 3 mm were aspirated. Thus, the number of aspirated FFs was in all cases only a fraction of the actual AFC and could only be used as an indicative parameter of the number of AFC. The diameter of the follicles was calculated based on the aspirated volume assuming a spherical follicle. Aspirated FFs were centrifuged at 400 x *g* for 3-5 min to separate FF and granulosa cells. The FFs was transferred to a clean 1.5 ml Eppendorf tube and snap frozen in liquid nitrogen, whereas granulosa cells were washed in 500 µl PBS, centrifuged at 400 x *g* for 3-5 min, and snap frozen.

Hormone measurements

A new line of commercially available hypersensitive immunoassays from Ansh Lab, Texas, USA were used to detect intrafollicular concentrations of GDF9 (AL-179), picoAMH (AL-124), inhibin-A (AL-123), inhibin-B (AL-107), total inhibin (alpha subunit, AL-134), activin-A (AL-110), activin-B (AL-150) and activin AB (AL-153), follistatin (AL-117) and FTSL-3 (AL-152). All FF samples were diluted 1:5 in the GDF9 calibrator A prior to measuring in GDF9 ELISA. Samples were diluted 1:200 in 10mM phosphate buffer saline with 1 % BSA prior to assaying activin-A, activin-B, activin-AB, inhibin-A, follistatin and FSTL-3. Samples were diluted 1:2000 (2 step dilution, first dilution at 1 :200 followed by a 1:10 dilution in 10mM phosphate buffer saline with 1 % BSA) and assayed for inhibin-B, total inhibin, and picoAMH. Analytical assay characteristics are described in Table 1.

Intrafollicular concentrations of estradiol and testosterone were measured using commercially available ELISA assays (NovaTec Immundiagnostica, Dietzenbach, Germany, DNOV003 and DNOV002, respectively) according to the manufacturer's instructions. The intra- and inter-assay coefficients of variation (CV) for the 17 beta-Estradiol assays were <9% and <10%, respectively, and the lowest standard had a concentration of 20 pg/ml. The intra- and inter-assay CV for the testosterone assays were <7% and <8%, respectively, and the lowest standard had a concentration of 0.2 ng/ml. Estradiol and testosterone were only analyzed in a subset of FFs (n=93; 47 non-PCO and 46 PCO) due to a lack of material.

Follicle fluids were collected at various times during the menstrual cycle which could potentially affect hormone measurements, however, previous studies have shown that intrafollicular levels of steroid hormones in hSAF remain constant throughout the menstrual cycle (29).

Blood samples were collected a few days before ovary excision and serum levels of AMH, FSH, LH, estradiol, progesterone and testosterone were measured as clinical routine samples by the clinical biochemical departments at the University hospitals of Copenhagen, Odense and Aarhus.

Statistics

A linear mixed effect model was used with random intercept for each woman, a linear effect of follicle diameter and case (PCO/non-PCO) as well as the interaction between follicle diameter and case was set as fixed effects. Tests of statistical significance was carried out using Wald tests with a significance level of 0.05. Statistical calculations for the linear mixed effect model were performed in R version 3.4.3. Spearman correlations in between growth factors in the non-PCO and PCO groups, as well as, ANOVA and T-tests for comparison of concentrations of selected growth factors and follicle groups were performed using GraphPad Prism 8.

Results

Patient demographics and clinical information for non-PCO and PCO

In connection with fertility preservation there was rarely enough clinical information to make a secure diagnosis of PCOS, but women were defined as having PCO when the ovarian volume exceeded 10 ml (measured by weighing the ovary prior to processing for fertility preservation (30)), ovarian PCO appearance with a high number of hSAFs located in the periphery of the ovary and aspiration of 7 or more FFs. The morphological findings of PCO were supported by hormone profiles including serum AMH, FSH, LH, LH/FSH ratio, estradiol, progesterone and testosterone (Table 2). Sixteen women were included in the PCO group and 33 women without PCO-like characteristics were matched according to age and follicle size (Table 2). The two groups of patients were selected according to the specific phenotypes and do not reflect the prevalence of PCO-like phenotypes within the entire cohort of patients having ovarian tissue cryopreserved. No clinical information regarding menstrual cycle or BMI was available.

The mean age of women with PCO (26 ±6 years (±SD); range 16-34 years) and without (26 ±5 years; range 15-34 years) was similar. Ovarian volume between the two groups was significantly different with non-PCO and PCO ovaries having a mean volume of 6.8 ±2.0 ml (range 2.9-9.5 ml) and 14.4 ±3.2 ml (range 10.0-20.9), respectively (Table 2). Median number of FFs collected per woman was also significantly different between the two groups with a median of 5 FF collected in the non-PCO women (range 1-7) and 10 FF in PCO women (range 7-14). Furthermore, ovarian volume and serum AMH concentrations were strongly correlated to the number of collected FF samples per woman (Figure 1C+D).

Clinical hormone data showed that PCO women had significantly higher serum AMH concentrations (42 ±5.8 pmol/L) compared to non-PCO women (15 ±1.5 pmol/L) (Table 2). Serum FSH concentrations did not differ between the two groups, but LH concentrations were significantly higher in the PCO women (10.7 ±1.8 IU/L) compared to non-PCO women (5.6 ±0.7 IU/L), and the ratio between LH/FSH was significantly elevated in PCO women (Table 2). Serum concentrations of estradiol, progesterone, and testosterone did not differ between the two groups (data not shown).

Sample details

In total, 93 FF samples were collected from women with PCO and 92 FF samples from women without PCO. The diameters of analyzed hSAF included in this study ranged from 4.6-10.7 mm (Figure 1E). Mean follicle size was similar in both non-PCO samples (6.3 ±1.3 mm) and PCO samples (6.3 ±1.3 mm) (Figure 1F). The number of follicles included per woman ranged between 1-7 in the non-PCO group with a median of 2 follicles analyzed per woman and 2-12 in the PCO group with a median of 5 follicles analyzed per woman (Table 2).

Overall, intrafollicular concentrations in all 185 samples showed a significant positive association for inhibin-A ($P<0.0001$), total inhibin ($P<0.05$), and estradiol ($P<0.001$) according to increasing follicle size, and a significant negative association for activin-B ($P<0.0001$), follistatin ($P<0.0001$), GDF-9 ($P<0.0001$), and AMH ($P<0.05$) according to

increasing follicle size (Figure 1G-O). Inhibin-B (P=0.068), activin-AB (P=0.105) and testosterone (P=0.746) did not show statistically significant associations with increasing follicle size.

Intrafollicular concentrations of inhibins, activins, and follistatin in non-PCO/PCO

Overall, intrafollicular concentrations of inhibin-B were high with a mean concentration of 178 ±15 ng/ml (±SEM) in all samples, whereas inhibin-A concentrations were considerably lower with a mean concentration of 9.5 ±1 ng/ml (±SEM) (Table 3), with the notion that statistical comparisons were not appropriate as different assays may not be directly comparable. Total inhibin concentrations were on average 37 ±3 ng/ml when all samples were included. Concentrations of both inhibin-B, inhibin-A and total inhibin were significantly lower in FF from women with PCO as compared to non-PCO women (Figure 2A+B; Table 3). The low concentrations of inhibin-B, inhibin-A, and total inhibin were especially pronounced in hSAF < 7 mm in diameter, corresponding to follicles just before selection of the dominant follicle (Figure 3A+B). Moreover, intrafollicular concentrations of activin-B were high with a mean concentration of 155 ±7 ng/ml in all samples, whereas activin-AB concentrations were considerably lower with a mean concentration of 8.9 ±0.4 ng/ml (Table 3). Overall, concentrations of follistatin were high in both non-PCO and PCO with a mean concentration of 686 ±27 ng/ml (±SEM) in all samples (Figure 2E; Table 3). Intrafollicular concentrations of activin-B, activin-AB, and follistatin did not differ between PCO and non-PCO (Figure 2C-E; Figure 3C; Table 3).

There were highly significant associations between all three inhibins (inhibin-B, inhibin-A, total inhibin) and activin-B in FF from non-PCO women, but non-significant associations in women with PCO (Table 4). In contrast, non-significant or weaker associations were found between inhibins and activin-AB in FF from non-PCO women, whereas strong significant associations were found in PCO women (Table 4). Follistatin showed significant, strong associations with activin-B and activin-AB in both PCO and non-PCO women, but the associations between follistatin and the inhibins switched from significant negative associations in non-PCO women to positive or non-significant associations in PCO women (Table 4).

Concentrations of activin-A were below the detection limit of the assay (i.e. 10.4 ng/ml) in all FF samples except for 10, and FTSL-3 was only detectable in 18 and 17 FF samples from the non-PCO and PCO group, respectively, while the remaining samples were below the detection limit of the assay (61 ng/ml). Detectable levels of FTSL-3 were distributed across all follicle diameters in a similar way for both non-PCO and PCO samples. FSTL-3 concentrations did not differ between the groups (96 ±10 ng/ml in non-PCO and 92 ±8 ng/ml in PCO; Table 3).

Intrafollicular concentrations of GDF9 and AMH in non-PCO/PCO

Overall, intrafollicular concentrations of AMH were high with a mean concentration of 979 ±56 ng/ml (±SEM) in all samples (Table 3). GDF9 concentrations were considerably lower with a mean concentration of 3.7 ±0.2 ng/ml (Table 3). Intrafollicular concentrations of GDF9 did not differ between PCO and non-PCO when data were plotted continuously in relation to follicle diameter (Figure 2F; Table 3). However, when follicles were grouped according to ranges of diameter, a significant higher concentration of GDF9 was found in PCO FF compared to non-PCO FF from 7-8 mm hSAF (Figure 3D). GDF9 concentrations associated significantly to concentrations of activin-B and follistatin, irrespective of whether the woman had PCO or not (Table 4).

Interestingly, intrafollicular concentrations of AMH did not differ between PCO and non-PCO (Figure 2G; Table 3). However, in hSAF larger than 8 mm AMH concentrations were significantly higher in PCO FF compared to non-PCO FF (Figure 3E). Thus, intrafollicular

AMH concentrations remained high in 9-11 mm follicles from women with PCO, corresponding to the diameter at which selection of the dominant follicle normally takes place, which was in contrast to decreasing AMH concentrations found in the non-PCO ovaries. AMH concentrations showed significant negative associations to all three inhibins but significant positive associations to concentrations of activin-B and follistatin, irrespective of whether the woman had PCO or not (Table 4).

Intrafollicular concentrations of steroid hormones in non-PCO/PCO

The mean concentrations of estradiol and testosterone were 176 ±48 ng/ml (±SEM) and 110 ±10 ng/ml, respectively, in all FFs samples (Table 3). Intrafollicular concentrations of estradiol were significantly lower in FF from women with PCO compared to non-PCO women (Table 3; Figure 4A). The low concentrations of estradiol were especially pronounced in hSAF > 9 mm (Figure 4B). Testosterone concentrations did not differ between PCO and non-PCO (Table 3; Figure 4C+D). Concentrations of estradiol showed significant negative associations with AMH in both PCO (R = - 0.53; P = 0.0001) and non-PCO women (R = - 0.37; P = 0.009). In contrast, no associations were found between testosterone and AMH.

Discussion

This study is the first to characterize hSAFs from women with or without PCO measuring quantitative differences of ten TGF-β family members in individual FFs. We report for the first-time quantitative concentrations of activin-B and oocyte specific GDF9 in hSAFs. Surprisingly, activin-B was the major activin present in FF from both non-PCO and PCO women, being present in concentrations at least one order of magnitude higher than that of activin-AB and activin-A. Furthermore, concentrations of inhibin-B, inhibin-A and total inhibin were significantly reduced in women with PCO compared to the non-PCO group, especially in follicles with a diameter below 7 mm, thus confirming and extending previous studies (21-25). Follistatin showed strong associations with activin-B and activin-AB, but more surprisingly, the associations between follistatin and the inhibins shifted from being significantly negative associated in the non-PCO group, to being significantly positive associated or non-significant in the PCO group.

It is interesting that concentrations of AMH did not differ in FF between women with or without PCO when follicles with a diameter of less than 9 mm were considered, but in larger follicles AMH concentrations were higher in FF from women with PCO, although the number of follicles with diameters exceeding 8 mm was relatively small. This was reflected in FF concentrations of estradiol, which remained significantly lower in the PCO group as compared to the non-PCO group.

Collectively, these data are not easily explained but highlight that women with PCO have an aberrant regulation of the inhibin-activin-follistatin axis as well as AMH, which potentially may reflect some of the underlying mechanisms characterizing follicle growth arrest in the PCO condition.

Previously, inhibins have been measured in both circulation and in FF from non-PCOS and PCOS women (21-25;31). These independent studies found, depending on the available assays, a significantly attenuated inhibin production in women with PCO as compared to non-PCO, which the current study confirms and extends. It is well established that FSH stimulates granulosa cells to produce inhibins. However, the concentrations of FSH in the two groups of women in the present study were similar and the FSH concentrations alone were unable to explain the difference in inhibin production. One difference between the two groups of women may relate to follicle stimulating hormone receptor (FSHR) expression. Intrafollicular concentrations of inhibin-B from non-PCO women show significant positive association with FSHR expression in the corresponding granulosa cells, thus lower concentrations of inhibins

associate with lower expression of FSHR (14). Simultaneously, inhibin-B in these follicles also demonstrate significant positive associations with intrafollicular concentrations of androstenedione and testosterone (14). As androgens have been shown to induce and associate to FSHR expression on granulosa cells in primates and humans (16,32-35) a reduced drive for FSHR expression may cause less inhibin to be produced.

Part of the reduced inhibin production may also relate to the observation that granulosa cells from a subset of follicles in women with PCOS develop responsiveness to LH earlier than those from non-PCOS women (36-38). The early LH responsiveness and the increased concentrations of LH may affect granulosa cells in women with PCOS by androgen receptor (AR) downregulation and thereby further reduce AR induced FSHR expression and FSH responsiveness (Owens et al., unpublished data. Revised manuscript submitted to JCEM in June 2019).

Nevertheless, induction of luteinizing hormone receptor (LHR) on granulosa cells is to a large extent FSH driven (39) and it appears difficult to explain the early appearance of LHR on granulosa cells in PCO women compared to non-PCO women, given that FSH concentrations remain similar. However, the pituitary released FSH isoform distribution may play a role. The released pituitary FSH isoform distribution is governed by circulating concentrations of estradiol with more acidic isoforms released when concentrations of estradiol are relatively low (40,41). Some studies have found that peripheral concentrations of estradiol are increased in women with PCOS (42) while others have failed to demonstrate an increase (43), but an augmented biological active fraction of estradiol is present in circulation reflecting a reduction in concentrations of sex hormone-binding globulin (SHBG) in women with PCOS (44). Thus, it may be hypothesized that women with PCO/PCOS may have increased concentrations of biologically active estradiol, that will induce a higher drive for release of less acidic FSH isoforms, which in turn are likely to induce LHR more effectively.

One additional aspect may also relate to the FSH isoform distribution because, in rats, acidic FSH isoforms are more effective in inducing inhibin gene expression compared to the less acidic FSH isoforms (10,11), which, if applicable to humans, may attenuate inhibin secretion as observed in the present study.

These observations further strengthen the notion that aberrant TGF-β signaling plays an integral part in the mechanism of arrested follicle development, in the face of normal serum FSH concentrations, in women with PCO/PCOS.

To our knowledge, this study is the first to measure all three types of activins simultaneously in hSAFs from women with or without PCO. Surprisingly, our data demonstrates that activin-B is by far the most abundant activin present, with concentrations of activin-AB being more than one order of magnitude lower and concentrations of activin-A undetectable (i.e. below 10ng/ml) in most samples. Activin-B demonstrated significant associations to all other members of the growth factors measured. The functional significance of these findings is not yet clarified. Using different ELISAs for detection of activin-A, other studies found concentrations of activin-A in FF from hSAFs to be in the same range as in the present study (i.e. below or around 10 ng/ml) (22,45,46), confirming that activin-B and activin-AB are likely to be present in much higher concentrations in hSAFs. One previous study found that sheep small antral follicles contained high concentrations of activin-B, which decreased as the follicle size increased (47) similar to what was observed in the present study. Both activin-A and -B suppressed sheep theca cell androgen production *in vitro*, an effect blocked by inhibins (47).

The total concentration of follistatin was around four times higher than that of activins collectively and confirm that the biological activity of activins within hSAFs appear to be tightly controlled and attenuated by follistatin rather than FSTL-3 which was only detectable

in a small fraction of the samples. This is confirming an earlier study (47), which, however, only measured activin-A and follistatin.

No overall differences between non-PCO and PCO women in intrafollicular concentrations of AMH from hSAF was observed. However, non-PCO women showed a strong decline of AMH in parallel to increasing follicle diameter especially beyond 8 mm as previously described (16,48,49). In contrast, PCO-women maintained high concentrations of AMH in follicles with a diameter beyond 8 mm confirming and extending a previous study (50). AMH has been shown to down-regulate aromatase expression in granulosa cells *in vitro* (51,52), and several studies have shown a strong inverse correlation between intrafollicular concentrations of AMH and estradiol in hSAFs (16,48,49). Interestingly, the present study found significantly reduced concentrations of estradiol in FFs from women with PCO compared to the non-PCO group in follicles exceeding 8 mm in diameter highlighting potential differences in AMH regulation in the two groups. It has been suggested that estradiol acting via estrogen receptor β on granulosa cells may be the main mechanism for AMH downregulation in humans (53). Thus, in normal women, the FSH induced aromatase expression and estradiol synthesis can override the negative effects from AMH and cause AMH down-regulation in follicles selected for further development beyond a diameter of 8 mm. This may be a result of sufficient inhibin production that via increased local androgen production and AR expression augments FSHR expression and render the follicle capable of responding with aromatase expression, which in turn down regulates AMH and further stimulates aromatase activity. In women with PCO one or more of these regulatory loops are not providing a normal and appropriate response leading to an insufficient drive for follicular growth.

This is the first paper to present quantitative measurements of GDF9 in FF, a hormone exclusively secreted by the oocyte. This is obviously a highly sensitive assay that allows detection of this oocyte secreted factor from a cell with a diameter of around 110 µm being diluted in a follicle with a volume of 50 – 100 µl. This sensitive assay now, for the first time, allows a detailed study of how the oocyte and growth factors interact in small antral follicles. Although no significant differences in GDF9 concentrations were observed in women with or without PCO, strong positive associations to concentrations of activin-B, follistatin, and to a lesser extent to AMH were found. This could potentially reflect novel signaling pathways active within the follicle.

It is a limitation of the current study that PCO women did not receive a complete diagnosis of PCOS according to the Rotterdam criteria (54,55), but these data were not recorded, nor required in management of these women for whom the priority was fertility preservation However, patients selected to represent the PCO group had an average ovarian volume of 14 ml, a high number of small antral follicles collected, high circulating concentrations of AMH and LH, and a high LH/FSH ratio, which collectively indicate a potential PCO-like phenotype. Further, the present study is unable to address whether these TGF-β family members are mechanistically related to follicle arrest in PCO/PCOS or secondary to an, as yet unknown, overriding defect. Finally, only a small number of hSAF larger than 8 mm in diameter were available for analysis and potential variations in serum hormone measurements in between the hospitals biochemical departments could not be accounted for.

In conclusion, the current study showed that TGF-β family members are present in very high concentrations within hSAF and appeared to be involved in the highly complex and intricate regulation of follicular growth and development, especially around the time of follicular selection at a diameter of around 7-8 mm. The current data suggest that, in particular, the interactions in the activin-follistatin-inhibin axis may be central to explaining the aberrant follicle development seen in women with PCO. The reduced Inhibin output may,

via androgens, cause less FSHR expression, leading to a reduced aromatase expression and estradiol output which result in insufficient downregulation of AMH, that in turn attenuate estradiol production. Thus, TGF-β members and sex-steroids may potentially interact especially with inhibins and AMH in orchestrating para- and autocrine signaling in small antral follicles especially around follicular selection at a diameter of 7-11 mm.

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The authors have nothing to disclose.

DATA AVAILABILITY
The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.;

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Figure 1: Collection of follicle fluids (FFs) from small antral follicles and distribution of analyzed FFs. (A) An ovary cut into half. Arrows point to small antral follicles within the medullary part of the ovary. (B) Follicle fluids were aspirated using a 23G needle from the outside of the ovary or within the medulla. Ovarian volume (C) and serum AMH levels (D) in relation to the number of collected FF from hSAF per woman. (E) Number of FFs analyzed according to follicle diameter (mm). (F) Distribution of follicle sizes between PCO and non-PCO. The mean ±SD follicle size of PCO and non-PCO samples was the same (6.3 ±1.3 mm). (G-O) Intrafollicular levels of inhibin-B, inhibin-A, activin-B, activin-AB, follistatin, GDF9, AMH, estradiol and testosterone in small antral follicles grouped by follicle diameter. Number of FFs analyzed according to follicle diameters; 4-6mm (n=130), 7-8mm (n=46), 9-11mm (n=9). Significant differences between groups are marked with different letters. $P < 0.05$ was considered significant. Values are mean ± SEM.

Figure 2: Scatter plots showing intrafollicular levels of TGF-β family members in relation to follicle diameters in PCO and non-PCO. (A) Inhibin-B. (B) Inhibin-A. (C) Activin-B. (D) Activin-AB. (E) Follistatin. (F) GDF9. (G) AMH. Dotted lines depict linear regression analysis. Black lines: Non-PCO; orange lines: PCO.

Figure 3: Intrafollicular levels of inhibin-B, inhibin-A, activin-B, GDF9 and AMH in small antral follicles grouped by follicle diameter and non-PCO/PCO. (A) Inhibin-B. (B) Inhibin-A. (C) Activin-B. (D) GDF9. (E) AMH. Number of FFs analyzed according to follicle diameters (non-PCO/PCO); 4-6mm (n=66/64), 7-8mm (n=21/25), 9-11mm (n=5/4). Significant differences between groups are marked with different letters. $P < 0.05$ was considered significant. Values are mean ± SEM.

Figure 4: Intrafollicular levels of estradiol and testosterone in small antral follicles grouped by follicle diameter and non-PCO/PCO. (A) Scatter plot of estradiol concentrations. (B) Estradiol according to follicle groups. (C) Scatter plot of testosterone concentrations. (D) Testosterone according to follicle groups. Number of FFs analyzed according to follicle diameters (non-PCO/PCO); 4-6mm (n=28(non-PCO)/29(PCO)), 7-8mm (n=12/13), 9-11mm (n=5/4). Significant differences between groups are marked with different letters. $P < 0.05$ was considered significant. Values are mean ± SEM.

Table 1: Analytical characteristics of the ELISAs.

ELISA Reagents	Antibody Binding Region	Dynamic Range (pg/mL)	LoD (pg/mL)	Imprecision % CV @ (Measured Concentration)	Cross Reactant spiked at 50 ng/mL in Analyte free matrix unless otherwise stated (% Reactivity)
Inhibin A*	βA-(Capture)	5 -1200	5.4	6.2% (101.3 pg/mL)	Inhibin A (100%) Inhibin B (ND), Activin A (ND), Activin B (ND), Activin AB (ND), FST-315 (ND) and FSTL3 (ND)
AL-123	α-(Detection)			5.5% (344.8 pg/mL)	
Inhibin B*	βB-(Capture)	2 -1400	1.6	7.4% (68.9 pg/mL)	Inhibin B (100%), Inhibin A (ND), Activin A (ND), Activin B (ND), Activin AB (ND), AMH (ND), FST-315 (ND) and FSTL3 (ND)
AL-107	α-(Detection)			5.6% (99.4 pg/mL)	
Activin-A*	βA-Subunit	100-10,000	65.0	5.7% (673 pg/mL)	Activin A (100%), Activin A-FST complex (100%) Inhibin A (ND) Inhibin B (ND), Activin B (ND), Activin AB (2.3%), FST-315 (ND) and FSTL3 (ND).
AL-110	Mature			4.3% (2527 pg/mL)	
Total Inhibin	αC-(Capture)	10-500	1.5	4.5% (20.5 pg/mL)	Inhibin A (100% at 500 pg/mL) Inhibin B (30% at 500 pg/mL), Activin A (ND), Activin B (ND), Activin AB (ND), AMH (ND), FST-315 (ND) and FSTL3 (ND)
AL-134	αN-(Detection)			3.9% (69.8 pg/mL)	
Activin B	βB-Subunit	12 -1400	4.3	4.7% (51.8 pg/mL)	Activin B (100%), Inhibin A (ND), Inhibin B (ND), Activin A (ND), FST-315 (ND), FSTL-3 (ND), Activin AB (8.9% at 2 ng/mL)
AL-150	Mature			3.1% (225.5 pg/mL)	
Activin AB	βB-(Capture)	1.35-108	0.12	3.7% (22.2 pg/mL)	Activin AB (100%), Activin B (ND), Inhibin B (ND), FST-315 (ND), FSTl-3 (ND), Activin A (0.35%) and Inhibin A (0.17%)
AL-153	βA-(Detection)			5.3% (68.1 pg/mL)	
Follistatin	Fs3-(Capture)	600-20,000	183.0	6.3% (1122 pg/mL)	FST-315 (100%), Inhibin A (ND), Inhibin B (ND), Activin A (ND), Activin B (ND), Activin AB (ND) and FSTL-3 (ND)
AL-117	Fs1-(Detection)			3.9% (2693 pg/mL)	
FSTL3	Fs2 Domain	360-12,000	164.0	3.0% (1400 pg/mL)	FSTL-3 (100%), Inhibin A (ND), Inhibin B (ND), Activin A (ND), Activin B (ND), Activin AB (ND) and FST-315 (ND)
AL-152				3.2% (3700 pg/mL)	
GDF-9	Mature (Capture and Detection)	48-5800	3.0	3.6 % (492.2 pg/mL)	Mature GDF-9 (100%), GDF-9:BMP-15 hetero dimer (100 %), Mature BMP-15 (ND)
AL-176				2.1% (1335.0 pg/mL)	
picoAMH	N-Terminal (Capture)	1.3-1150	1.3	5.9% (14.2 pg/mL)	Pro + Mature AMH (100%), Mature AMH (0.2), Inhibin A (ND), Inhibin B (ND), Activin B (ND), FST-315 (ND), FSH (ND0, TSH (ND), LH (ND) and Estradiol (ND).
AL-124	Mature-(Detection)			5.3% (924.1 pg/mL)	
ND = Non-Detectable (< LoD)					

*The calibrators in the kit is traceable to WHO reference preparation. The traceability factor is reported as slope of observed WHO preparation w.r.t. known concentration when analyzed in the respective ELISAs. Inhibin A = 1.68 (WHO 91/624); Inhibin B = 0.4 (WHO 96/784, The WHO preparation is a mixture of Inhibin A, Inhibin B and Inhibin alpha); Activin A = 0.93 (WHO 91/626).

The cross reactants used in the analysis are commercially available unless otherwise specified. Inhibin A (AI035, AnshLabs), Inhibin B (BI043, AnshLabs), Activin A (338-AC/CF, R&D System), Activin B (659-AB/CF), Activin AB (1066-AB/CF), FSH (F4021, Sigma), TSH (T9265, Sigma), LH (L6420, Sigma), Estradiol (PHR1353, Sigma), FSTL-3 (BF017, AnshLabs), FST-315 (4889-FN, R&D System), Mature AMH (1737-MS/CF, R&D System), Pro+Mature AMH (BA047, Anshlabs), GDF-9 (BG016, AnshLabs), BMP-15 (BB022, Anshlabs), BMP15:GDF-9 Complex (BB023, AnshLabs).

Table 2: Patient demographics and clinical information.

	Non-PCO	PCO	P-value
Woman (N)	33	16	
Age in years (mean±SD) [range]	26.2 ±5.3 [16-34]	26.0 ±6.1 [15-34]	
Ovarian volume in mL (mean±SD) [range]	6.8 ±2.0 [2.9-9.5]	14.4 ± 3.2 [10.0-20.9]	*P < 0.001*
Median number of FF collected per woman [range]	4 [1-7]	10 [7-14]	*P < 0.001*
AMH in pmol/L (mean±SEM)	15.3 ± 1.5	42.2 ± 5.8	*P < 0.001*
FSH in IU/L (mean±SEM)	5.7 ± 0.5	5.3 ± 0.5	
LH in IU/L (mean±SEM)	5.6 ± 0.7	10.7 ± 1.8	*P < 0.01*

LH/FSH ratio (mean±SEM)	1.0 ± 0.1	2.2 ± 0.3	*P < 0.001*
Follicle diameter (mm)	4.6-10.6	4.6-10.7	
Total no. of follicle fluids analyzed	92	93	
Median number of FF analyzed per woman [range]	2 [1-7]	5 [2-12]	
Diagnosis (N)	Breast cancer (15)	Breast cancer (5)	
	Lymphoma (7)	Lymphoma (4)	
	Sarcoma (1)	Sarcoma (4)	
	Brain cancer (3)	Cervical cancer (1)	
	Colorectal cancer (1)	Colorectal cancer (1)	
	Leukemia (1)	Others (1)	
	Ovarian cancer (1)		
	Others (4)		

Table 3: Intrafollicular levels of TGF-β family members and steroid hormones in hSAF from non-PCO and PCO.

	All samples (n=185)	**Non-PCO** n=92	**PCO** n=93	P-value
Inhibin-B (ng/ml)	178 ±15	253 ±27	108 ±10	*P = 0.002*
Inhibin-A (ng/ml)	9.5 ±1	13.6 ±1.5	5.8 ±0.6	*P = 0.005*
Total Inhibin (ng/ml)	37 ±3	51 ±5	23 ±2	*P = 0.002*
Activin-B (ng/ml)	155 ±7	163 ±10	147 ±9	NS
Activin-AB (ng/ml)	8.9 ±0.4	9.8 ±0.5	8.1 ±0.6	NS
Activin-A (ng/ml)	ND	ND	ND	
Follistatin (ng/ml)	686 ±27	732 ±36	643 ±41	NS
GDF9 (ng/ml)	3.7 ±0.2	3.4 ±0.3	4.0 ±0.2	NS
AMH (ng/ml)	979 ±56	901 ±88	1058 ±69	NS
FTSL-3* (ng/ml)	94 ±6	96 ±10	92 ±8	NS
Estradiol** (ng/ml)	176 ±48	219 ±90	58 ±17	*P = 0.02*
Testosterone** (ng/ml)	110 ±10	108 ±14	111 ±14	NS

Values are mean ±SEM. ND: not detectable. NS: Not significant. P values show significant differences between non-PCO and PCO. *) Only a total of 35 FF's (18 non-PCO and 17 PCO samples) showed detectable levels of FTSL-3. **) Only 93 FFs were analyzed (47 non-PCO and 46 PCO).

Table 4: Spearman correlations coefficients and *P* values between different TGF-β family members in fluid from small antral follicles of women with PCO (red) and non-PCO women (black)

	Activin-B	**Activin-AB**	**Follistatin**	**Inhibin-B**	**Inhibin-A**	**Total Inhibin**	**AMH**
Activin-AB	**R = 0.53; p<0.001**						
	R = 0.80; p<0.001						
Follistatin	**R = 0.88; p<0.001**	**R = 0.65; p<0.001**					
	R = 0.89; p<0.001	**R = 0.65; p<0.001**					
Inhibin-B	**R= – 0.44; p<0.001**	**R = – 0.22; p = 0.04**	**R= – 0.33; p =0.002**				
	R = 0.11; NS	**R = – 0.38; p<0.001**	**R = 0.23; p = 0.028**				
Inhibin-A	**R= – 0.45; p<0.001**	**R = 0.01; NS**	**R= – 0.26; p = 0.011**	**R = 0.91; p<0.001**			
	R = 0.03; NS	**R = 0.40; p<0.001**	**R = 0.18; NS**	**R = 0.94; p<0.001**			
Total Inhibin	**R= – 0.37; p<0.001**	**R = – 0.10; NS**	**R= – 0.20; p = 0.055**	**R = 0.97; p<0.001**	**R = 0.93; p<0.001**		
	R = 0.08; NS	**R = 0.30; p = 0.001**	**R= 0.25; p = 0.0162**	**R = 0.97; p<0.001**	**R = 0.93; p<0.001**		
AMH	**R = 0.58; p<0.001**	**R = – 0.07; NS**	**R = 0.42; p<0.001**	**R = – 0.44; p<0.001**	**R = – 0.52; p<0.001**	**R = – 0.40; p<0.001**	
	R = 0.26; p = 0.013	**R = – 0.03; NS**	**R= 0.21; p = 0.043**	**R= –0.33; P=0.0018**	**R = – 0.38; p<0.001**	**R= –0.32; p=0.0026**	
GDF-9	**R = 0.47; p<0.001**	**R = 0.08; NS**	**R = 0.44; p<0.001**	**R = – 0.06; NS**	**R = – 0.12; NS**	**R = 0.01; NS**	**R = 0.23; p = 0.026**
	R = 0.36; p<0.001	**R = 0.12; NS**	**R= 0.33; p = 0.0016**	**R = – 0.16; NS**	**R= – 0.26; p = 0.02**	**R = – 0.19; NS**	**R = 0.11; NS**