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Polycystic Ovarian Morphology in Normocyclic Non-Hyperandrogenic Adolescents

--Manuscript Draft--

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Abstract:	Structured Abstract: Objective: To evaluate whether an increased stromal area/total area (S/A) ratio could add useful information to label ovarian morphology in normocyclic non-hyperandrogenic female adolescents. Design: Cross-sectional population-based study. Setting: High school female students aged 14-18 years in Cagliari, Italy. Patients: 257 normocyclic non-hyperandrogenic female adolescents. Intervention(s): Clinical examination and medical history, blood sampling and pelvic ultrasound (US). Main Outcome Measure(s): Postmenarchal years, total testosterone (tT) and delta-4-androstenedione (A), ovarian volume, follicular number per section, follicular distribution, and S/A ratio measured by pelvic US. Results. Subjects were categorized into three groups: i) normal ovarian morphology (NOM; n=154); ii) polycystic ovarian morphology (PCOM) with S/A ratio ≤0.3 (PCOM-NS; n=70); and iii) PCOM with S/A ratio >0.3(PCOM-IS; n=33). The NOM group had more postmenarchal years than the PCOM-NS and PCOM-IS groups, and lower A and tT than the PCOM-IS group. The PCOM-NS group had fewer postmenarchal years and lower A than the PCOM-IS group. Interestingly, unlike NOM and PCOM-NS, whose prevalence significantly increased or decreased, respectively, with the three phases of postmenarchal age (1-3 years vs. 3-4 years vs. > 5 years), the prevalence of PCOM–IS remained constant among the three phases (10% vs 16% vs 15%, p NS). Conclusions. This study demostrates that PCOM can be a transient morphological condition during adolescence, whereas high S/A ratio is a stable US morphological alteration present since early postmenarchal years.

Point by point Rebuttal

Thank- you for your suggestion on our errors!

Reviewer #1: The authors have responded satisfactorily to queries. Just a couple of questions the need to be addressed prior to acceptance:

1. IN the introduction: "not be a reliable indicator in very young subjects". Would they mind changing this to "not be a reliable indicator in adolescents."?

The sentence was modified

2. in the text of the article, Under subjects: "Figure 1 shows a flow chart of the study". Under Figure legends the flow chart is figure 2. please clarify.

3. in the text of the article, under pelvic ultrasound "Figure 1 shows how the ovarian stroma....." However, in the text earlier, they noted that Figure 1 is the flow chart. Please carefully review the figures in the text and the figure legends to make sure correct.

The Fig 1 is the ovary image, The figure 2 is the flow Chart. In the text the fig citation for flow chart was wrong! Thank-you

4. "Body mass index (BMI) was calculated as the ratio between weight (in kilograms) and height (in meters)," should this be meters squared?

A Correct BMI definition was done

Running Title: Polycystic Ovarian Morphology in Adolescence

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Capsule: Polycystic ovarian morphology can be a physiological step in early postmenarchal age. At variance, the increased ovarian stroma seems to be a precocious and stable ultrasound morphological alteration.

Keywords:

Polycystic ovarian morphology, stroma/total area ratio, adolescence, ovarian morphology

Disclosure statement: The authors have nothing to disclose.

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Capsule: Polycystic ovarian morphology is a physiological step in early postmenarchal girls. In contrast, the increased ovarian stroma seems to be a precocious and stable ultrasound morphological alteration.

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Abstract:

Objective: To understand whether polycystic ovarian morphology (PCOM) represents a transient phase and whether an increased stroma could help to characterize the phenotype of the ovary in adolescence.

Methods: Cross-sectional population-based study on high-school students in Cagliari, Italy. The study population consisted of 257 normocyclic non-hyperandrogenic girls selected from a sample of 600 healthy volunteers recruited from 2012 to 2016. Clinical examination, medical history, blood sampling, and pelvic ultrasound (US) were performed.

Postmenarchal years, and body mass index (BMI) were estimated. Follicle stimulating hormone (FSH), Luteinizing hormone (LH), 17ßEstradiol (E2), total testosterone (tT), delta-4-androstenedione (A) and 17-hydroxyprogesterone (17-OHP) were measured. Ovarian volume, follicular number per section (FNPS), and S/A ratio were measured by pelvic US.

Results. Following the Rotterdam guidelines for US PCOS diagnosis and setting the normal S/A ratio at ≤0.3, subjects were categorized into three groups: i) normal ovarian morphology (NOM; n=154, 60%); ii) polycystic ovarian morphology (PCOM) with normal S/A ratio (PCOM-NS; n=70, 27%); and iii) PCOM with increased S/A ratio (PCOM-IS; n=33, 13%). The NOM group had more postmenarchal years and a lower LH than both the PCOM groups, and lower A and tT than the PCOM-IS group. The PCOM-NS group had fewer postmenarchal years and lower A than the PCOM-IS group. Interestingly, unlike NOM and PCOM-NS, the prevalence of PCOM–IS remained constant among the three phases of postmenarchal age (10% vs 16% vs 15%, p NS).

Conclusions. This study demonstrates that PCOM can be a transient condition, whereas a high S/A ratio is a stable US alteration present from early postmenarchal years.

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Introduction.

Polycystic ovarian morphology (PCOM), which is one of the diagnostic criteria of polycystic ovary syndrome (PCOS) (1-2), is frequently found in adolescence but often disappears over time (3-4). This positive evolution of the ovarian morphology can be the consequence of spontaneous PCOM remission or, more probably, of a misinterpretation of ovarian morphology during adolescence. In fact, ovarian size is larger during adolescence than in adulthood, and decreases with age. A high ovarian volume, which is suggested as a suitable parameter in the diagnosis of PCOM in adulthood (5-6) may therefore not be a reliable indicator adolescents~~in very young subjects~~. The number of follicles may also seem high during adolescence (7-8) because the threshold to determine the normal follicular count in adults may be misleading when applied to young girls (9). Moreover, transvaginal (TV) ultrasound (US) often cannot be performed in young subjects, however a follicular count may be difficult to perform in transabdominal (TA) US, thus giving misleading results.

It is therefore still under debate whether PCOM in young girls, defined using the ovarian volume and follicle number, should be considered as a true PCOM morphology or as a variant of normality (10,11). This issue needs to be clarified to improve the labelling of normal or pathologic ovaries during adolescence and youth.

New US markers are being investigated that are more likely to correctly label the ovarian morphology. Of these, the stromal/ovarian area (S/A) ratio has been suggested as a good US marker for PCOS.

The S/A ratio > 0.3 seems to be a reliable marker for hyperandrogenism (12) and a good parameter to differentiate normoandrogenic PCOM from hyperandrogenic PCOM and, therefore, PCOS (13-15). However, although the S/A ratio was recently demonstrated to have the best US performance for diagnosing PCOS, particularly when associated with the follicle number count per ovary (FNPO) (16-17), it has not yet been officially included in the US diagnostic criteria for PCOS.

This cross-sectional study was designed to investigate: i) the frequency of PCOM in healthy adolescent girls with different postmenarchal age ranges, in order to better understand whether PCOM represents a physiological and transient phase in normocyclic non-hyperandrogenic adolescents; and ii) whether a high S/A ratio could add useful information to the ovarian volume and follicle number in attributing the right label to the ovarian morphology.

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Materials and Methods

Subjects

The study group was made up of 600 untreated healthy female volunteers ~~aged between~~aged between 14-18 years who were prospectively recruited from 2012 to 2016 using a health survey carried out at their school. After an initial selection (regular menstrual cycles after menarche and willingness to undergo pelvic US), 482 subjects were studied at the center for Gynecological Diseases in Childhood and Adolescence of the 'Policlinico Universitario Duilio Casula', Monserrato (University of Cagliari, Italy). Fifty-five subjects were excluded due to invalid pelvic US for technical reasons (lack of full bladder in TA US, not well visualized ovaries in TA US, lack of compliance in TV US), and five subjects were excluded because they had ovarian cysts (> 3 cm diameter).

After blood sampling analysis, a further 120 subjects were excluded as they were found to have hyperprolactinemia, defined as prolactin (Prl) levels >20 ng/ml (> 69.8 nmol/l), or biochemical hyperandrogenism, defined as total testosterone (tT) >0.7 mg/ml (>2.43 nmol/L), and/or delta-4-androstenedione (A) >3.5 ng/ml (>12.2 nmol/L). We also excluded subjects with 17-hydroxyprogesterone (17OHP) >1.4 ng/ml, (> 4.45 nmol/L), given the possibility of having non-classic congenital adrenal hyperplasia due to 21 hydroxylase deficiency. After physical examination, a further 45 subjects were excluded because of hirsutism. The final sample was therefore composed of 257 young females.

Figure 2 ~~1~~ shows a flow chart of the study.

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Ethical approval

Informed consent was obtained from each subject, and, in the case of minors, informed consent was also obtained from parents. The study protocol was approved by the local ethics committee.

Study protocol

All subjects who were enrolled in the study were healthy, had regular menstrual cycles after menarche, had no hirsutism or hyperandrogenemia, and had never had hormonal treatment. Regular menstrual cycles were defined as a menstrual cycle interval of 21 to 35 days for at least three consecutive months. Hirsutism was excluded when the modified Ferriman-Gallway (mFG) score was < 8 (18).

The US examination was performed in the early morning, in the early-mid follicular phase (from days 5 to 8) of the menstrual cycle. During the same morning, those subjects who had a valid US analysis had their blood sampled and were clinically examined by a trained

physician. All subjects underwent blood sampling under fasting conditions, blood was immediately centrifugated, and serum was stored at -20°C until assayed. At clinical examination, information on family history and medical history was collected, including age of menarche, menstrual cycle characteristics, presence or absence of dysmenorrhea, estimation of acne and hirsutism, as well as measuring body weight, height and waist and hip circumferences. Pelvic US, clinical examination and blood sampling were performed by the same specialist medical doctor. Four medical doctors were involved in the study.

Pelvic ultrasound

Pelvic US examination was conducted using a high-performance ultrasound machine (Voluson I, General Electric, Milwaukee, WI, USA). A convex TA probe of 3.5-5 MHz or a 5–9 MHz frequency TV probe was used for TA or TV pelvic US, respectively. TV approach was attempted in subjects who were sexually active; 173 out of 257 subjects analyzed, i.e. 67.3% of the entire population, had a TA US. The inter-individual variation among the four operators, which was calculated on the first 30 subjects included in the study, did not exceed 6% in any of the parameters analyzed. Ovarian volume was calculated with two-dimensional (2D) US using the formula for the prolate ellipsoid: π/6 x (D1 x D2 x D3) where D represents the length, width and thickness of the ovary, respectively. In accordance with the Rotterdam criteria (5), a threshold equal to or above $10cm^3$ was considered significant for the definition of PCOM.

The follicle number per single cross-section (FNPS) and the follicle distribution pattern were also evaluated in the ovarian maximal plain section. In accordance with the Rotterdam criteria (5), a threshold for FNPS equal to or above 12 was considered significant for the definition of PCOM. For the evaluation of these parameters, a detailed high-resolution scan of an ovarian section image was used to count the follicular number. The stromal area was assessed by outlining the outer profile of the stroma in the maximum plain section of the ovary with a caliper. In addition, the total area of the ovary was evaluated by tracing the outline of the ovary with a caliper. The S/A ratio was obtained by dividing these two parameters (12,19). **Figure 1** shows how the ovarian stroma and area were measured. We measured both ovaries in each subject and, for the TV pelvic US, we used data obtained from the mean of the right and left ovaries. In contrast, for the TA pelvic US, we used data obtained from the right ovary, since the left ovary may not be visualized as well due to the presence of rectum ampulla. Consensus statements indicate that a unilateral evaluation is sufficient to meet the definition of PCOM (6, 17-18) . Recently Yarret, Lujan et al demonstrated that the PCOM assessment in a single ovary does not impact the FNPS in

PCOS. However, to the best of our knowledge, similar data do not exist for S/A detection (19).

Clinical Measurements

Hirsutism was evaluated using the mFG score (18). Acne was assessed using the Cremoncini system (23). Dysmenorrhea was evaluated using the visual analogue scale (VAS) (24). Body mass index (BMI) was calculated as the ratio between weight (in kilograms) and height (in meters squared), and waist-hip ratio (WHR) as the ratio between waist and hip circumferences.

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Hormonal assays included follicle stimulating hormones (FSHs), luteinizing hormones (LHs), 17ß estradiol (E2), tT, A, 17-OHP and Prl.

Serum E2, FSH, LH, tT were measured by chemiluminescence immunoassay Immulite, (SIEMENS Products Corporation, Los Angeles, CA).

Radioimmunoassay (RIA) was used to measure serum A, Prl and 17-OHP (Diagnostic System Laboratories, Inc. DSL, Webster-TX). The intra-assay and inter-assay coefficients of variation at low (level 1), medium (level 2) and high (level 3) concentrations for each of the analytes were as follows: FSH 4.9 % and 4.1% for level 1, 3.2% and 4.1% for level 2, and 3.1% and 7.9% for level 3 respectively; LH: 13.0% and 23.9% for level 1, 3.6% and 6.7% for level 2, 6.0%, and 7.1% for level 3 respectively; tT: 16.3% and 24.3% for level 1, 15.2% and 15.6% for level 2, and 5.1% and 7.2% for 3, respectively; E2: 11.0% and 2.0% for level 1, 2.7% and 2.0% for 2 and 2.6% and 0.9% for 3, respectively; A: for level 1 were 4.5% and 9.0%, respectively; for level 2, 3.7% and 5.9%, respectively; Prl: 5.1% and 5.8% for level 1, 3.9% and 4.8% for level 2, and 3.4% and 5.4% for level 3, respectively; 17OHP: <7% and 1.0% for level 1, < 7% and 14.9% for level 2; and <7% and 8.7% for level 3, respectively.

Measurement of A cross-reacted principally with androsterone (0.58%) and with T(0.24%); tT cross reacted principally with 5 alfa-dihydrotestosterone (2%) and with A (0.6%); 17OHP cross reacted with 17OH pregnenolone (4.1%), progesterone (1.3%), prednisone (0.23%); LH cross reacted principally with HCG (0.2%); and Prl cross reacted with the other hormones <0.5%.

The lower limit of detection for each of the analytes in our laboratory was: FSH and LH:0.1 mIU/ml; tT: 0.5 nmol/L; A: 0.3 ng/ml; 17OHP: 0.3 ng/ ml; E2: 10 pg/ml.; Prl 0.35 ng/ml.

Cut-off values were determined in-house and had been previously used as reference for normality (12,15,28).

Statistical analysis

All statistical analyses were performed using SPSS statistical software (SPSS, Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD), or frequency (%). The results were analyzed using an Anova test with Bonferroni correction when three groups were compared. A P value <0.05 was considered as statistically significant. Quantitative variables expressed as frequencies were analyzed by $\chi 2$ tests.

Results

Following the Rotterdam Consensus guidelines for US diagnosis in PCOS and the measurement of S/A (5)**,** the 257 subjects included in the study were categorized into three groups: i) Normal Ovarian Morphology (NOM) when less than 12 follicles <10mm of diameter were counted in FNPS measured in the larger longitudinal ovarian cross-section, and the ovarian volume was <10 cm^3, or when a dominant follicle (>10mm) was identified (n=154; 60% of the cohort); ii) Polycystic Ovarian Morphology with Normal Stroma (PCOM-NS) when at least 12 follicles <10mm of diameter were counted in FNPS or the ovarian volume ≥ 10cm^3 was measured in the absence of increased stroma in the same section (S/A < 0.3) (n=70; 27% of the cohort); and iii) Polycystic Ovarian Morphology with Increased Stroma (PCOM-IS) when at least 12 follicles <10mm of diameter were counted in FNPS or the ovarian volume ≥ 10cm^3 was measured in the presence of increased stroma in the same section (S/A > 0.3) (n=33; 13% of the cohort) (**Figure 2**).

Table 1 shows the anthropometric, clinical, hormonal and sonographic features of the study groups. Age was significantly lower in the NOM and PCOM-NS groups with respect to PCOM-IS, whereas subjects in the NOM group had significantly more postmenarchal years than those in the PCOM-NS and PCOM-IS groups, but significantly fewer years were observed in PCOM-NS with respect to PCOM-IS. The three groups had similar menarche ages, BMI and WHR, as well as a similar prevalence of acne and dysmenorrhea.

FSH did not differ among the three groups and E_2 circulating levels were similarly low in the three groups, thus confirming that the subjects were in the early follicular phase of the menstrual cycle. On the other hand, LH levels were significantly lower in NOM than in PCOM-NS and PCOM-IS.

Interestingly, NOM had lower levels of both A and Ttot with respect to PCOM-IS. On the other hand, PCOM-NS had lower levels of A, but higher levels of 17OH-P than PCOM-IS.

The US data showed a lower ovarian volume in NOM than in PCOM-NS, and lower FNPS in NOM than in both PCOM-NS and PCOM-IS and, interestingly, a different follicular distribution in PCOM-IS (subcortical) compared to NOM and PCOM-NS groups (diffuse).

The 60 subjects excluded after US examination due to ovarian cysts, or to technical reasons, presented similar clinical and anthropometric parameters to the 257 subjects who were included in the study group (data not shown).

We also analysed the prevalence of NOM, PCOM-NS, and PCOM-IS in three different phases of postmenarchal age: early phase (1-3 postmenarchal years, 60 subjects); intermediate phase (4-5 years, 142 subjects); late phase (>5 years, 55 subjects) (**Figure 3**). Interestingly, the prevalence of NOM was observed in 34% of subjects in the early phase, and increased to 65% in the intermediate phase, and to 77% in the late phase ($p<0.05$ among the three phases). In contrast, the PCOM-NS pattern had the highest prevalence in the early phase (57%), then significantly decreased to 20% in the intermediate phase, and reached 7% in the late phase ($p<0.01$ among the three phases). In contrast, the prevalence of PCOM-IS was not significantly different between the three phases (10% vs 16% vs 15%, p NS).

Finally, we explored the influence of BMI in the US pattern, estimating the prevalence of NOM, PCOM-NS and PCOM-IS in three categories of BMI (<20, 20-24, and >24 kg/m^2) (**Figure 4**). We found no difference in the frequency of US ovarian patterns in the three BMI categories.

Discussion

The main result of this cross-sectional study is that increased ovarian stroma, i.e. S/A > 0.3, when combined with high ovarian volume or high FNPS, seemed to be a reliable US morphological alteration from early postmenarchal age in normocyclic non-hyperandrogenic adolescents. In fact, unlike the morphological patterns of NOM and PCOM-NS, whose prevalence increased or decreased significantly moving away from the menarche age to a prevalence of 77% and 7% after 5 years, respectively, PCOM-IS maintained a constant prevalence through the different phases of postmenarchal age (10, 16 and 15% at 1-3 years, 3-5 years, and above 5 years, respectively).

In addition, although only healthy normoandrogenic girls were included in this study, the PCOM-IS group had higher levels of A than the NOM and PCOM-NS groups, and higher levels of tT than the NOM group, and thus a more androgenic pattern profile.

Finally, the PCOM-IS group had a different follicle distribution than the NOM and PCOM-NS groups. In fact, it was characterized by a subcortical follicle distribution, whereas the other two morphological patterns were characterized by a diffuse follicle distribution.

If confirmed in larger and prospective studies, these results could add an important US criterion to differentiate between transient and permanent ovarian morphology alterations in the very early gynecological years.

The criteria currently used to differentiate between normal ovarian morphology and PCOM in adolescents, particularly during early adolescence, are constantly changing and are consequently misleading (7,25-27). Moreover, the US criteria currently used to define PCOS, i.e. ovarian size >10 cm^3 or FNPS >12, can be misleading in adolescence.

This has led to postponing the diagnosis of PCOM until the eighth year of menarche (6). This means that a diagnosis of PCOS may be unnecessarily delayed along with the start of a specific therapy in subjects who might have benefitted from early-onset therapy. Some data demonstrate, in fact, that the precocious treatment of insulin resistance and/or of hyperandrogenism may decrease hepato-visceral adiposity, and thus attenuate the development of oligo-anovulation or of metabolic complications such as diabetes (25).

There have been proposals to increase the threshold of ovarian size (7) or of the FNPS - however, there is still no agreement on which cut-off to use to differentiate between normality and pathology.

Recent guidelines (6,20**)**, largely based on two studies in adults, recommend modifying the US diagnostic features for PCOM by increasing the threshold of the number of follicles to 25 per ovary (FNPO) - scanned using a TV transducer probe with a frequency of >8 MHz - and counting follicles with a specific software application. Unfortunately, it is difficult to introduce such diagnostic criteria in clinical practice because TV transducer probes are not commonly available and there are time-related difficulties in using the software. In addition, the diagnostic features of PCOM suggested above are not advisable for a TA scan, which is frequently needed during adolescence.

The ovarian stroma could be measured either using TV or TA, or in 2D or in 3D, and also via transrectal ultrasound scanning (25-27). The analysis in 2D does not require special software or probes, and thus can be exploited using standard technologies in 2-3 min (12,31). In addition, the increased S/A ratio has a low inter-operator variability, and provides great sensitivity (96%) and specificity (86%) for diagnosing PCOS. Battaglia and Sun (28,29) identified exactly the same S/A cut-off in 3D and transrectal US studies (28-29). S/A combined with FNPO was recently found to have the best US PCOS diagnostic performance, whereas the FNPO is the best single marker (16). An ovarian stroma evaluation could therefore help to improve US performance in adolescent subjects in order to diagnose PCOS at early ages, but also to differentiate between the permanent and transient form of PCOM.

PCOM can be a transient morphological condition during adolescence due to a transitory alteration of the hypothalamic-pituitary axis leading to an increase in LH levels. A similar morphological pattern of the ovary but with normal-low LH levels may be observed during functional hypothalamic amenorrhea, due to nutritional disorders, stress, or low body weight, which are frequent in adolescence. In all these cases the stroma is normal, thus these conditions can be identified using the PCOM-NS morphology, previously known as the multifollicular ovary-MFO (32).

Accordingly, our PCOM-NS population, which was characterized by normal menses (an inclusion criteria of the study), and by increased LH levels and a similar BMI to the NOM group, can be considered as being a transient morphological adolescent variant of PCOM. This conclusion is supported by the extremely low prevalence of PCOM-NS in the late phase of postmenarchal age. Our study suggests that those adolescents with PCOM-IS should be followed closely until adulthood.

In conclusion, this study confirms the importance of including the S/A ratio in the US ovarian evaluation in order to define the US normality in adolescent subjects. Further studies are necessary to demonstrate whether adolescents with PCOM-IS are at risk of developing PCOS.

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Legend to figures.

Figure 1. Practical example of how to measure ovarian stroma and area.

Figure 2. Flowchart of participation in the study and stratification.

Figure 3. Prevalence of NOM, PCOM-NS and PCOM-IS in relation to postmenarchal age, divided into three phases 1-3 years (60 subjects); 4-5 years (142 subjects); >5 years (55 subjects)

Figure 4. Prevalence of NOM, PCOM-NS and PCOM-IS in relation to BMI, divided into three categories (<20, 20-24, and >24 kg/m^2)

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Running Title: Polycystic Ovarian Morphology in Adolescence

Title: Polycystic Ovarian Morphology in Normocyclic Non-Hyperandrogenic Adolescents

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Capsule: Polycystic ovarian morphology is a physiological step in early postmenarchal girls. In contrast, the increased ovarian stroma seems to be a precocious and stable ultrasound morphological alteration.

Keywords:
Polycystic ovarian morphology, stroma/total area ratio, adolescence, ovarian morphology

Disclosure statement: The authors have nothing to disclose.

Abstract:

Objective: To understand whether polycystic ovarian morphology (PCOM) represents a transient phase and whether an increased stroma could help to characterize the phenotype of the ovary in adolescence.

Methods: Cross-sectional population-based study on high-school students in Cagliari, Italy. The study population consisted of 257 normocyclic non-hyperandrogenic girls selected from a sample of 600 healthy volunteers recruited from 2012 to 2016. Clinical examination, medical history, blood sampling, and pelvic ultrasound (US) were performed.

Postmenarchal years, and body mass index (BMI) were estimated. Follicle stimulating hormone (FSH), Luteinizing hormone (LH), 17ßEstradiol (E2), total testosterone (tT), delta-4-androstenedione (A) and 17-hydroxyprogesterone (17-OHP) were measured. Ovarian volume, follicular number per section (FNPS), and S/A ratio were measured by pelvic US.

Results. Following the Rotterdam guidelines for US PCOS diagnosis and setting the normal S/A ratio at ≤0.3, subjects were categorized into three groups: i) normal ovarian morphology (NOM; n=154, 60%); ii) polycystic ovarian morphology (PCOM) with normal S/A ratio (PCOM-NS; n=70, 27%); and iii) PCOM with increased S/A ratio (PCOM-IS; n=33, 13%). The NOM group had more postmenarchal years and a lower LH than both the PCOM groups, and lower A and tT than the PCOM-IS group. The PCOM-NS group had fewer postmenarchal years and lower A than the PCOM-IS group. Interestingly, unlike NOM and PCOM-NS, the prevalence of PCOM–IS remained constant among the three phases of postmenarchal age (10% vs 16% vs 15%, p NS).

Conclusions. This study demonstrates that PCOM can be a transient condition, whereas a high S/A ratio is a stable US alteration present from early postmenarchal years.

Introduction.

Polycystic ovarian morphology (PCOM), which is one of the diagnostic criteria of polycystic ovary syndrome (PCOS) (1-2), is frequently found in adolescence but often disappears over time (3-4). This positive evolution of the ovarian morphology can be the consequence of spontaneous PCOM remission or, more probably, of a misinterpretation of ovarian morphology during adolescence. In fact, ovarian size is larger during adolescence than in adulthood, and decreases with age. A high ovarian volume, which is suggested as a suitable parameter in the diagnosis of PCOM in adulthood (5-6) may therefore not be a reliable indicator adolescents. The number of follicles may also seem high during adolescence (7-8) because the threshold to determine the normal follicular count in adults may be misleading when applied to young girls (9). Moreover, transvaginal (TV) ultrasound (US) often cannot be performed in young subjects, however a follicular count may be difficult to perform in transabdominal (TA) US, thus giving misleading results.

It is therefore still under debate whether PCOM in young girls, defined using the ovarian volume and follicle number, should be considered as a true PCOM morphology or as a variant of normality (10,11). This issue needs to be clarified to improve the labelling of normal or pathologic ovaries during adolescence and youth.

New US markers are being investigated that are more likely to correctly label the ovarian morphology. Of these, the stromal/ovarian area (S/A) ratio has been suggested as a good US marker for PCOS.

The S/A ratio > 0.3 seems to be a reliable marker for hyperandrogenism (12) and a good parameter to differentiate normoandrogenic PCOM from hyperandrogenic PCOM and, therefore, PCOS (13-15). However, although the S/A ratio was recently demonstrated to have the best US performance for diagnosing PCOS, particularly when associated with the follicle number count per ovary (FNPO) (16-17), it has not yet been officially included in the US diagnostic criteria for PCOS.

This cross-sectional study was designed to investigate: i) the frequency of PCOM in healthy adolescent girls with different postmenarchal age ranges, in order to better understand whether PCOM represents a physiological and transient phase in normocyclic non-hyperandrogenic adolescents; and ii) whether a high S/A ratio could add useful information to the ovarian volume and follicle number in attributing the right label to the ovarian morphology.

Materials and Methods

Subjects

The study group was made up of 600 untreated healthy female volunteers aged between 14-18 years who were prospectively recruited from 2012 to 2016 using a health survey carried out at their school. After an initial selection (regular menstrual cycles after menarche and willingness to undergo pelvic US), 482 subjects were studied at the center for Gynecological Diseases in Childhood and Adolescence of the 'Policlinico Universitario Duilio Casula', Monserrato (University of Cagliari, Italy). Fifty-five subjects were excluded due to invalid pelvic US for technical reasons (lack of full bladder in TA US, not well visualized ovaries in TA US, lack of compliance in TV US), and five subjects were excluded because they had ovarian cysts (> 3 cm diameter).

After blood sampling analysis, a further 120 subjects were excluded as they were found to have hyperprolactinemia, defined as prolactin (Prl) levels >20 ng/ml (> 69.8 nmol/l), or biochemical hyperandrogenism, defined as total testosterone (tT) >0.7 mg/ml (>2.43 nmol/L), and/or delta-4-androstenedione (A) >3.5 ng/ml (>12.2 nmol/L). We also excluded subjects with 17-hydroxyprogesterone (17OHP) >1.4 ng/ml, (> 4.45 nmol/L), given the possibility of having non-classic congenital adrenal hyperplasia due to 21 hydroxylase deficiency. After physical examination, a further 45 subjects were excluded because of hirsutism. The final sample was therefore composed of 257 young females.

Figure 2 shows a flow chart of the study.

Ethical approval

Informed consent was obtained from each subject, and, in the case of minors, informed consent was also obtained from parents. The study protocol was approved by the local ethics committee.

Study protocol

All subjects who were enrolled in the study were healthy, had regular menstrual cycles after menarche, had no hirsutism or hyperandrogenemia, and had never had hormonal treatment. Regular menstrual cycles were defined as a menstrual cycle interval of 21 to 35 days for at least three consecutive months. Hirsutism was excluded when the modified Ferriman-Gallway (mFG) score was < 8 (18).

The US examination was performed in the early morning, in the early-mid follicular phase (from days 5 to 8) of the menstrual cycle. During the same morning, those subjects who had a valid US analysis had their blood sampled and were clinically examined by a trained

physician. All subjects underwent blood sampling under fasting conditions, blood was immediately centrifugated, and serum was stored at -20°C until assayed. At clinical examination, information on family history and medical history was collected, including age of menarche, menstrual cycle characteristics, presence or absence of dysmenorrhea, estimation of acne and hirsutism, as well as measuring body weight, height and waist and hip circumferences. Pelvic US, clinical examination and blood sampling were performed by the same specialist medical doctor. Four medical doctors were involved in the study.

Pelvic ultrasound

Pelvic US examination was conducted using a high-performance ultrasound machine (Voluson I, General Electric, Milwaukee, WI, USA). A convex TA probe of 3.5-5 MHz or a 5–9 MHz frequency TV probe was used for TA or TV pelvic US, respectively. TV approach was attempted in subjects who were sexually active; 173 out of 257 subjects analyzed, i.e. 67.3% of the entire population, had a TA US. The inter-individual variation among the four operators, which was calculated on the first 30 subjects included in the study, did not exceed 6% in any of the parameters analyzed. Ovarian volume was calculated with two-dimensional (2D) US using the formula for the prolate ellipsoid: π/6 x (D1 x D2 x D3) where D represents the length, width and thickness of the ovary, respectively. In accordance with the Rotterdam criteria (5), a threshold equal to or above $10cm^3$ was considered significant for the definition of PCOM.

The follicle number per single cross-section (FNPS) and the follicle distribution pattern were also evaluated in the ovarian maximal plain section. In accordance with the Rotterdam criteria (5), a threshold for FNPS equal to or above 12 was considered significant for the definition of PCOM. For the evaluation of these parameters, a detailed high-resolution scan of an ovarian section image was used to count the follicular number. The stromal area was assessed by outlining the outer profile of the stroma in the maximum plain section of the ovary with a caliper. In addition, the total area of the ovary was evaluated by tracing the outline of the ovary with a caliper. The S/A ratio was obtained by dividing these two parameters (12,19). **Figure 1** shows how the ovarian stroma and area were measured. We measured both ovaries in each subject and, for the TV pelvic US, we used data obtained from the mean of the right and left ovaries. In contrast, for the TA pelvic US, we used data obtained from the right ovary, since the left ovary may not be visualized as well due to the presence of rectum ampulla. Consensus statements indicate that a unilateral evaluation is sufficient to meet the definition of PCOM (6, 17-18) . Recently Yarret, Lujan et al demonstrated that the PCOM assessment in a single ovary does not impact the FNPS in

PCOS. However, to the best of our knowledge, similar data do not exist for S/A detection (19).

Clinical Measurements

Hirsutism was evaluated using the mFG score (18). Acne was assessed using the Cremoncini system (23). Dysmenorrhea was evaluated using the visual analogue scale (VAS) (24). Body mass index (BMI) was calculated as the ratio between weight (in kilograms) and height (in meters squared), and waist-hip ratio (WHR) as the ratio between waist and hip circumferences.

Hormonal assays included follicle stimulating hormones (FSHs), luteinizing hormones (LHs), 17ß estradiol (E2), tT, A, 17-OHP and Prl.

Serum E2, FSH, LH, tT were measured by chemiluminescence immunoassay Immulite, (SIEMENS Products Corporation, Los Angeles, CA).

Radioimmunoassay (RIA) was used to measure serum A, Prl and 17-OHP (Diagnostic System Laboratories, Inc. DSL, Webster-TX). The intra-assay and inter-assay coefficients of variation at low (level 1), medium (level 2) and high (level 3) concentrations for each of the analytes were as follows: FSH 4.9 % and 4.1% for level 1, 3.2% and 4.1% for level 2, and 3.1% and 7.9% for level 3 respectively; LH: 13.0% and 23.9% for level 1, 3.6% and 6.7% for level 2, 6.0%, and 7.1% for level 3 respectively; tT: 16.3% and 24.3% for level 1, 15.2% and 15.6% for level 2, and 5.1% and 7.2% for 3, respectively; E2: 11.0% and 2.0% for level 1, 2.7% and 2.0% for 2 and 2.6% and 0.9% for 3, respectively; A: for level 1 were 4.5% and 9.0%, respectively; for level 2, 3.7% and 5.9%, respectively; Prl: 5.1% and 5.8% for level 1, 3.9% and 4.8% for level 2, and 3.4% and 5.4% for level 3, respectively; 17OHP: <7% and 1.0% for level 1, < 7% and 14.9% for level 2; and <7% and 8.7% for level 3, respectively.

Measurement of A cross-reacted principally with androsterone (0.58%) and with T(0.24%); tT cross reacted principally with 5 alfa-dihydrotestosterone (2%) and with A (0.6%); 17OHP cross reacted with 17OH pregnenolone (4.1%), progesterone (1.3%), prednisone (0.23%); LH cross reacted principally with HCG (0.2%); and Prl cross reacted with the other hormones <0.5%.

The lower limit of detection for each of the analytes in our laboratory was: FSH and LH:0.1 mIU/ml; tT: 0.5 nmol/L; A: 0.3 ng/ml; 17OHP: 0.3 ng/ ml; E2: 10 pg/ml.; Prl 0.35 ng/ml.

Cut-off values were determined in-house and had been previously used as reference for normality (12,15,28).

Statistical analysis

All statistical analyses were performed using SPSS statistical software (SPSS, Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD), or frequency (%). The results were analyzed using an Anova test with Bonferroni correction when three groups were compared. A P value <0.05 was considered as statistically significant. Quantitative variables expressed as frequencies were analyzed by $\chi 2$ tests.

Results

Following the Rotterdam Consensus guidelines for US diagnosis in PCOS and the measurement of S/A (5)**,** the 257 subjects included in the study were categorized into three groups: i) Normal Ovarian Morphology (NOM) when less than 12 follicles <10mm of diameter were counted in FNPS measured in the larger longitudinal ovarian cross-section, and the ovarian volume was <10 cm^3, or when a dominant follicle (>10mm) was identified (n=154; 60% of the cohort); ii) Polycystic Ovarian Morphology with Normal Stroma (PCOM-NS) when at least 12 follicles <10mm of diameter were counted in FNPS or the ovarian volume ≥ 10cm^3 was measured in the absence of increased stroma in the same section (S/A < 0.3) (n=70; 27% of the cohort); and iii) Polycystic Ovarian Morphology with Increased Stroma (PCOM-IS) when at least 12 follicles <10mm of diameter were counted in FNPS or the ovarian volume ≥ 10cm^3 was measured in the presence of increased stroma in the same section (S/A > 0.3) (n=33; 13% of the cohort) (**Figure 2**).

Table 1 shows the anthropometric, clinical, hormonal and sonographic features of the study groups. Age was significantly lower in the NOM and PCOM-NS groups with respect to PCOM-IS, whereas subjects in the NOM group had significantly more postmenarchal years than those in the PCOM-NS and PCOM-IS groups, but significantly fewer years were observed in PCOM-NS with respect to PCOM-IS. The three groups had similar menarche ages, BMI and WHR, as well as a similar prevalence of acne and dysmenorrhea.

FSH did not differ among the three groups and E_2 circulating levels were similarly low in the three groups, thus confirming that the subjects were in the early follicular phase of the menstrual cycle. On the other hand, LH levels were significantly lower in NOM than in PCOM-NS and PCOM-IS.

Interestingly, NOM had lower levels of both A and Ttot with respect to PCOM-IS. On the other hand, PCOM-NS had lower levels of A, but higher levels of 17OH-P than PCOM-IS.

The US data showed a lower ovarian volume in NOM than in PCOM-NS, and lower FNPS in NOM than in both PCOM-NS and PCOM-IS and, interestingly, a different follicular distribution in PCOM-IS (subcortical) compared to NOM and PCOM-NS groups (diffuse).

The 60 subjects excluded after US examination due to ovarian cysts, or to technical reasons, presented similar clinical and anthropometric parameters to the 257 subjects who were included in the study group (data not shown).

We also analysed the prevalence of NOM, PCOM-NS, and PCOM-IS in three different phases of postmenarchal age: early phase (1-3 postmenarchal years, 60 subjects); intermediate phase (4-5 years, 142 subjects); late phase (>5 years, 55 subjects) (**Figure 3**). Interestingly, the prevalence of NOM was observed in 34% of subjects in the early phase, and increased to 65% in the intermediate phase, and to 77% in the late phase ($p<0.05$ among the three phases). In contrast, the PCOM-NS pattern had the highest prevalence in the early phase (57%), then significantly decreased to 20% in the intermediate phase, and reached 7% in the late phase ($p<0.01$ among the three phases). In contrast, the prevalence of PCOM-IS was not significantly different between the three phases (10% vs 16% vs 15%, p NS).

Finally, we explored the influence of BMI in the US pattern, estimating the prevalence of NOM, PCOM-NS and PCOM-IS in three categories of BMI (<20, 20-24, and >24 kg/m^2) (**Figure 4**). We found no difference in the frequency of US ovarian patterns in the three BMI categories.

Discussion

The main result of this cross-sectional study is that increased ovarian stroma, i.e. S/A > 0.3, when combined with high ovarian volume or high FNPS, seemed to be a reliable US morphological alteration from early postmenarchal age in normocyclic non-hyperandrogenic adolescents. In fact, unlike the morphological patterns of NOM and PCOM-NS, whose prevalence increased or decreased significantly moving away from the menarche age to a prevalence of 77% and 7% after 5 years, respectively, PCOM-IS maintained a constant prevalence through the different phases of postmenarchal age (10, 16 and 15% at 1-3 years, 3-5 years, and above 5 years, respectively).

In addition, although only healthy normoandrogenic girls were included in this study, the PCOM-IS group had higher levels of A than the NOM and PCOM-NS groups, and higher levels of tT than the NOM group, and thus a more androgenic pattern profile.

Finally, the PCOM-IS group had a different follicle distribution than the NOM and PCOM-NS groups. In fact, it was characterized by a subcortical follicle distribution, whereas the other two morphological patterns were characterized by a diffuse follicle distribution.

If confirmed in larger and prospective studies, these results could add an important US criterion to differentiate between transient and permanent ovarian morphology alterations in the very early gynecological years.

The criteria currently used to differentiate between normal ovarian morphology and PCOM in adolescents, particularly during early adolescence, are constantly changing and are consequently misleading (7,25-27). Moreover, the US criteria currently used to define PCOS, i.e. ovarian size >10 cm^3 or FNPS >12, can be misleading in adolescence.

This has led to postponing the diagnosis of PCOM until the eighth year of menarche (6). This means that a diagnosis of PCOS may be unnecessarily delayed along with the start of a specific therapy in subjects who might have benefitted from early-onset therapy. Some data demonstrate, in fact, that the precocious treatment of insulin resistance and/or of hyperandrogenism may decrease hepato-visceral adiposity, and thus attenuate the development of oligo-anovulation or of metabolic complications such as diabetes (25).

There have been proposals to increase the threshold of ovarian size (7) or of the FNPS - however, there is still no agreement on which cut-off to use to differentiate between normality and pathology.

Recent guidelines (6,20**)**, largely based on two studies in adults, recommend modifying the US diagnostic features for PCOM by increasing the threshold of the number of follicles to 25 per ovary (FNPO) - scanned using a TV transducer probe with a frequency of >8 MHz - and counting follicles with a specific software application. Unfortunately, it is difficult to introduce such diagnostic criteria in clinical practice because TV transducer probes are not commonly available and there are time-related difficulties in using the software. In addition, the diagnostic features of PCOM suggested above are not advisable for a TA scan, which is frequently needed during adolescence.

The ovarian stroma could be measured either using TV or TA, or in 2D or in 3D, and also via transrectal ultrasound scanning (25-27). The analysis in 2D does not require special software or probes, and thus can be exploited using standard technologies in 2-3 min (12,31). In addition, the increased S/A ratio has a low inter-operator variability, and provides great sensitivity (96%) and specificity (86%) for diagnosing PCOS. Battaglia and Sun (28,29) identified exactly the same S/A cut-off in 3D and transrectal US studies (28-29). S/A combined with FNPO was recently found to have the best US PCOS diagnostic performance, whereas the FNPO is the best single marker (16). An ovarian stroma evaluation could therefore help to improve US performance in adolescent subjects in order to diagnose PCOS at early ages, but also to differentiate between the permanent and transient form of PCOM.

PCOM can be a transient morphological condition during adolescence due to a transitory alteration of the hypothalamic-pituitary axis leading to an increase in LH levels. A similar morphological pattern of the ovary but with normal-low LH levels may be observed during functional hypothalamic amenorrhea, due to nutritional disorders, stress, or low body weight, which are frequent in adolescence. In all these cases the stroma is normal, thus these conditions can be identified using the PCOM-NS morphology, previously known as the multifollicular ovary-MFO (32)**.**

Accordingly, our PCOM-NS population, which was characterized by normal menses (an inclusion criteria of the study), and by increased LH levels and a similar BMI to the NOM group, can be considered as being a transient morphological adolescent variant of PCOM. This conclusion is supported by the extremely low prevalence of PCOM-NS in the late phase of postmenarchal age. Our study suggests that those adolescents with PCOM-IS should be followed closely until adulthood.

In conclusion, this study confirms the importance of including the S/A ratio in the US ovarian evaluation in order to define the US normality in adolescent subjects. Further studies are necessary to demonstrate whether adolescents with PCOM-IS are at risk of developing PCOS.

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Legend to figures.

Figure 1. Practical example of how to measure ovarian stroma and area.

Figure 2. Flowchart of participation in the study and stratification.

Figure 3. Prevalence of NOM, PCOM-NS and PCOM-IS in relation to postmenarchal age, divided into three phases 1-3 years (60 subjects); 4-5 years (142 subjects); >5 years (55 subjects)

Figure 4. Prevalence of NOM, PCOM-NS and PCOM-IS in relation to BMI, divided into three categories (<20, 20-24, and >24 kg/m^2)

CLINICAL FINDINGS in relation t

TABLE I: ANTHROPOMETRIC, CLINICAL, HORMONAL AND SONOGRAPHIC FEATURES IN RELATION WITH OVARIAN PATTERNS

Features/ Ovarian US pattern	NOM (154 patients)	PCOM-NS (70 patients)	PCOM-IS (33 patients)
Age (ys)	15.78 ± 1.62 [b]	14.90 ± 1.70 [c]	16.70 ± 1.75
Age at the menarche (ys)	12.04 ± 1.10	12.24 ± 1.05	12.10 ± 1.07
Years of postmenarchal life (ys)	4.78 ± 1.51 [a,b]	3.09 ± 1.65 [c]	4.63 ± 1.56
BMI (kg/m^2)	20.39 ± 2.98	20.80 ± 3.43	20.02 ± 1.84
WHR	0.74 ± 0.06	0.77 ± 0.08	0.74 ± 0.03
Acne (%)	29	28	29
Dismenorrhea (%)	37	42	49
FSH (mUI/mL)	5.65±1.63	6.20±2.5	5.49±1.6
LH (mUI/mL)	4.57±2.69 [a,b]	6.37±3.87	6.85±5.35
E2 (pmol/L)	112±70.1	86.2±37.3	100.5±67.7
A (nmol/L)	6.47±1.6 [b]	7.54±2.47 [c]	10.27±1.97
Ttot (nmol/L)	1.11±0.53 [b]	1.48±0.53	1.87±0.4
17OH-P (nmol/L)	3.77±1.08	4.59±1.28 [c]	3.64±0.56
Ovarian Volume (cm^3)	5.87±3.46 [a]	7.97±4.18	7.32±3.59
Follicular number per section (FNPS)	5.16±1.76 [a,b]	9.89±4.15	9.53±2.94
Follicle disposition	predominantly diffuse	diffuse	subcortical

a : NOM vs PCOM-NS [a] $p < 0.01$ with Bonferroni correction

b: NOM vs PCOM-IS [b] $p < 0.01$ with Bonferroni correction

c : PCOM-NS vs PCOM-IS [c] $p < 0.01$ with Bonferroni correction

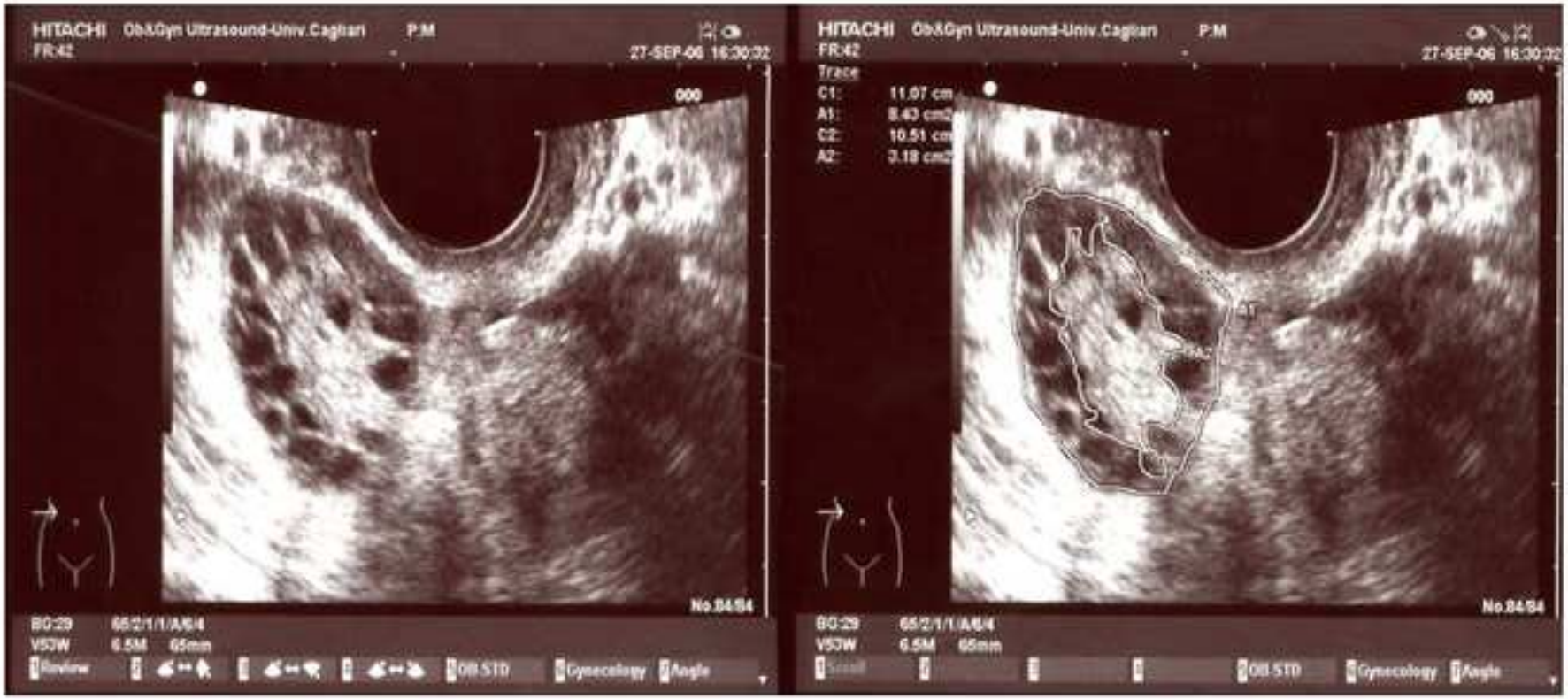
FSH= Follicle Stimulating Hormone; LH=Luteinising Hormone; BMI= Body Mass Index; WHR = Waist Hip Ratio;

E2= 17ßEstradiol; A=Androstenedione; Ttot= Testosterone Totale.17-OHP= 17-Idroxy Progestagen

Figure

Click here to access/download;Figure;Figure 1.jpg

Measurements of Ovarian Stroma(A2) / Area(A1)



Figure

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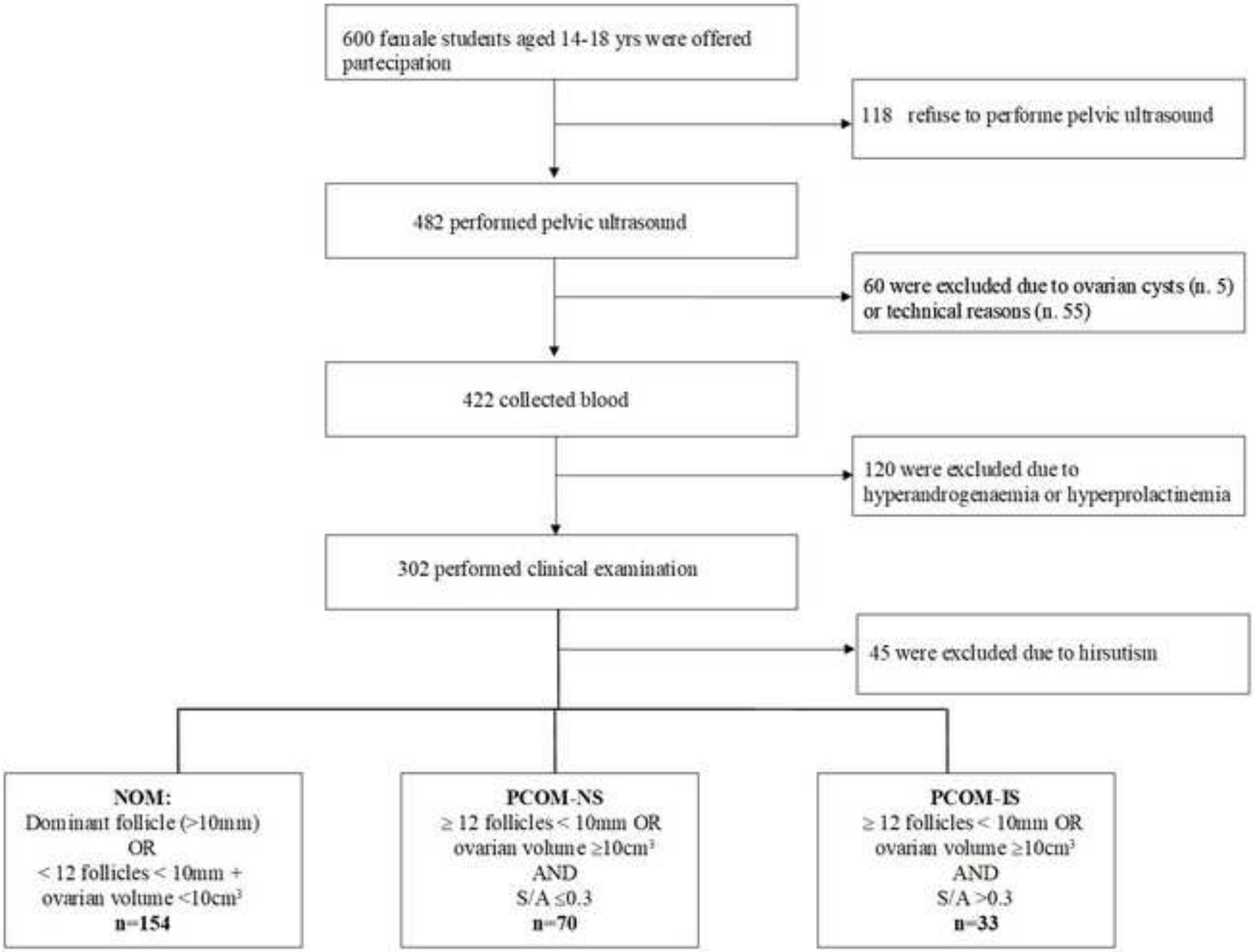

Figure

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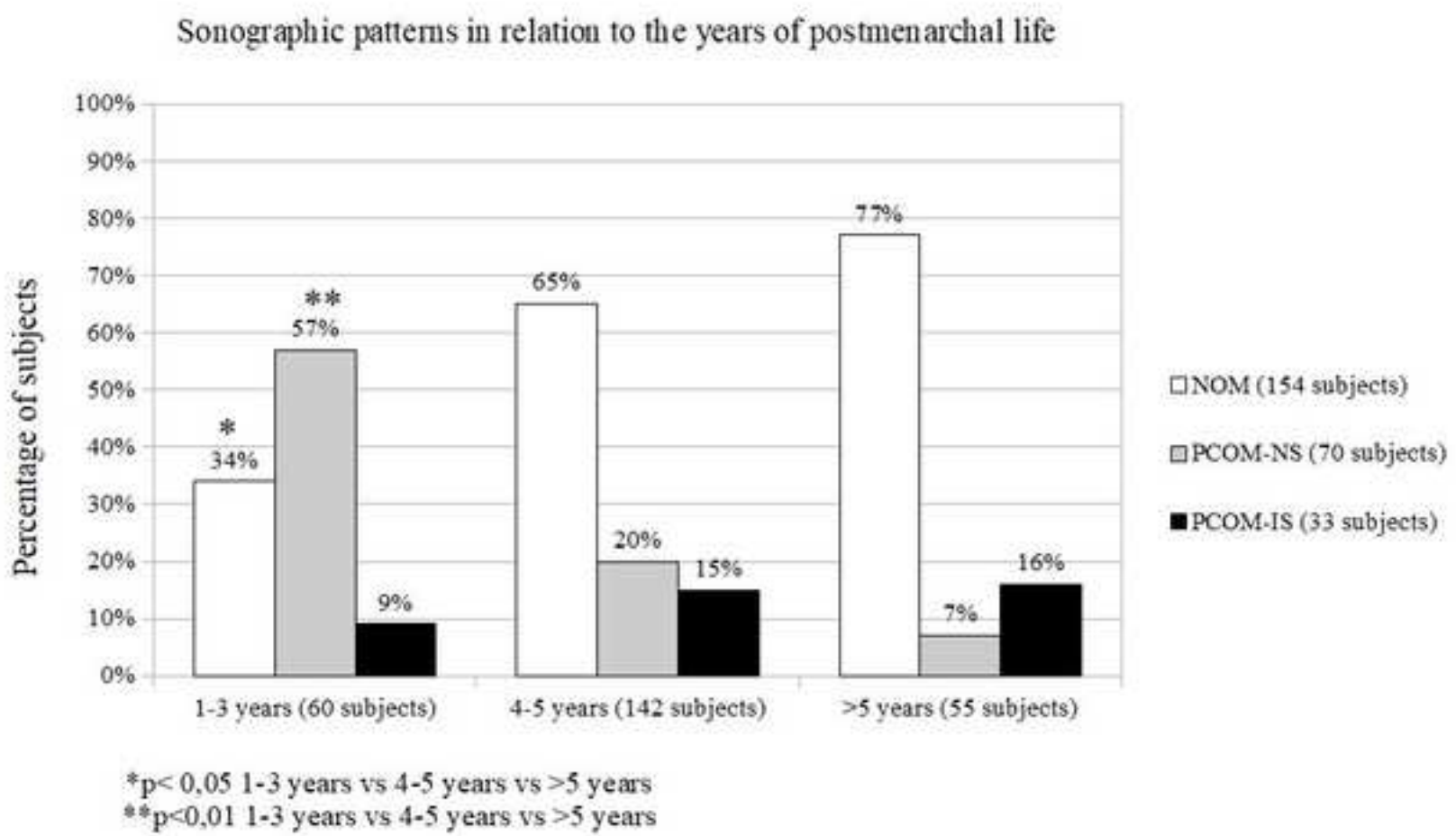
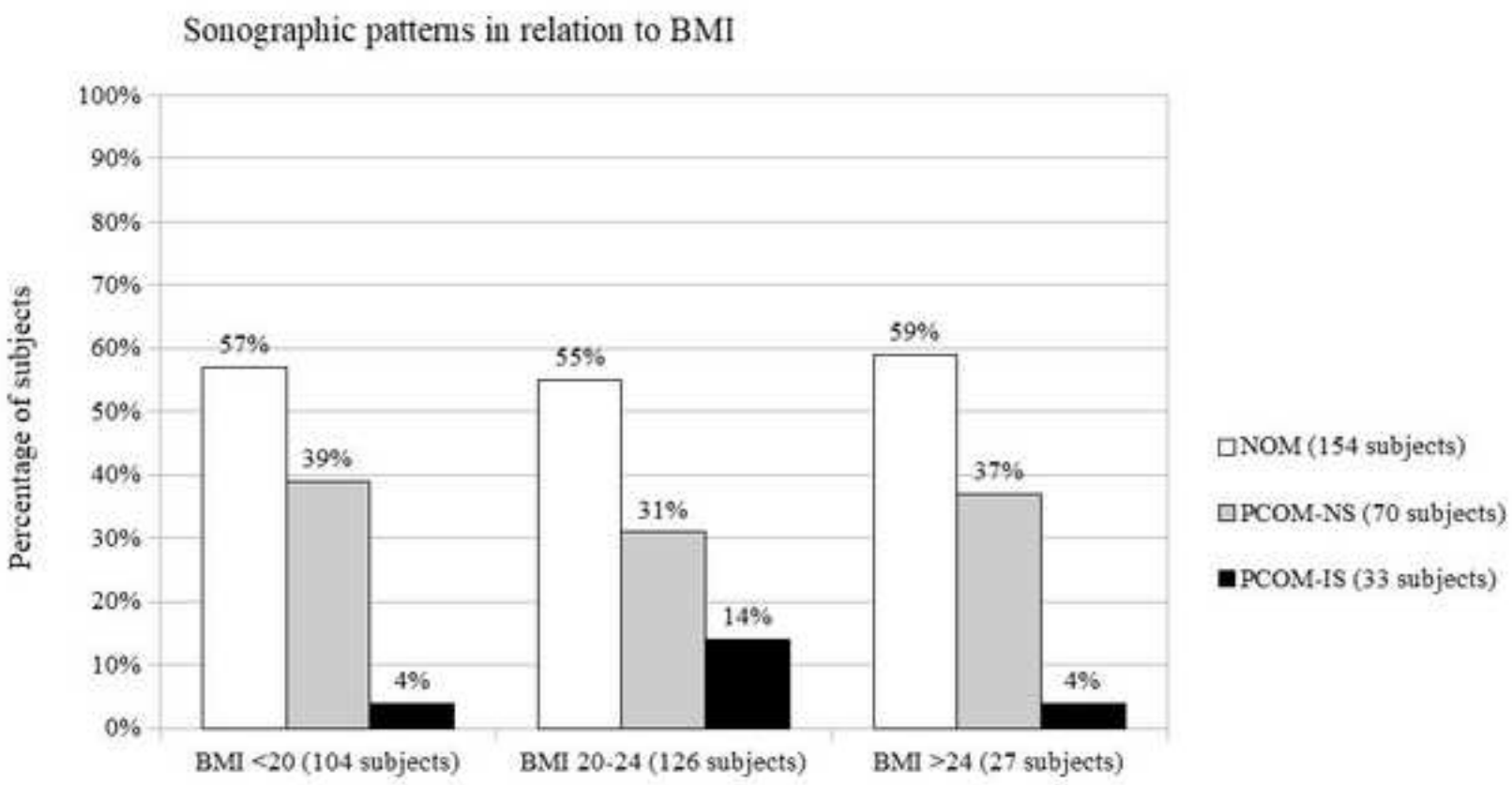


Figure



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17 January 2021

To whom it may concern

This is to declare that I have edited and proofread the English of the following paper:

Polycystic Ovarian Morphology in Normocyclic Non-Hyperandrogenic Adolescents

On behalf of:

Fulghesu et al.

My revision did not include the Bibliography. Subsequent to my revision, the authors may have made other changes or chosen not to implement some of the changes that I suggested. The correctness of the technical terms is also the responsibility of the authors.

The final version of the manuscript, as sent to the authors on **17 January 2021**, will be kept in our archives.

I have 30 years of experience of editing the English of scientific papers. I am also the author of *English for Writing Research Papers* published by Springer.

Please do not hesitate to contact me for any further information you may require:

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Best regards

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