Diagnosis of polycystic ovary syndrome (PCOS): revisiting the threshold values of follicle count on ultrasound and of the serum AMH level for the definition of polycystic ovaries

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BACKGROUND: Polycystic ovarian morphology (PCOM) at ultrasound is currently used in the diagnosis of polycystic ovary syndrome (PCOS). We hypothesized that the previously proposed threshold value of 12 as an excessive number of follicles per ovary (FN) is no longer appropriate because of current technological developments. In this study, we have revisited the thresholds for FN and for the serum Anti-Müllerian hormone (AMH) level (a possible surrogate for FN) for the definition of PCOM.

METHODS: Clinical, hormonal and ultrasound data were consecutively recorded in 240 patients referred to our department between 2008 and 2010 for exploration of hyperandrogenism (HA), menstrual disorders and/or infertility.

RESULTS: According to only their symptoms, patients were grouped as: non-PCOS without HA and with ovulatory cycles (group 1, n = 105), presumption of PCOS with only HA or only oligo-anovulation (group 2, n = 73) and PCOS with HA and oligo-anovulation (group 3, n = 62). By cluster analysis using androgens, LH, FSH, AMH, FN and ovarian volume, group 1 appeared to be constituted of two homogeneous clusters, most likely a non-PCOM non-PCOS subgroup (n = 66) and a PCOM, non-PCOS (i.e. asymptomatic) subgroup (n = 39). Receiver operating characteristic curve analysis was applied to distinguish the non-PCOM non-PCO members of group 1 and to group 3. For FN and serum AMH respectively, the areas under the curve were 0.949 and 0.973 and the best compromise between sensitivity (81 and 92%) and specificity (92 and 97%) was obtained with a threshold values of 19 follicles and 35 pmol/l (5 ng/ml).

CONCLUSIONS: For the definition of PCOM, the former threshold of >12 for FN is no longer valid. A serum AMH >35 pmol/l (or >5 ng/ml) appears to be more sensitive and specific than a FN >19 and should be therefore included in the current diagnostic classifications for PCOS.

Key words: polycystic ovary syndrome / anti-Müllerian hormone / ovarian follicle / ultrasonography / diagnosis

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder, affecting up to 10% of women of reproductive age (Norman et al., 2007). Its prevalence varies according to the definition used and to the reference population (Diamanti-Kandarakis et al., 1999; Asuncion et al., 2000; Azziz et al., 2004).

The cardinal features of PCOS are hyperandrogenism (HA) and oligo-anovulation. The metabolic abnormalities often associated with this syndrome (obesity, insulin resistance, hyperinsulinemia and dyslipidemia) are not included in the definition of the syndrome because it is still unclear whether they are intrinsic to the disease or not (Moran and Teede, 2009). The current diagnostic classifications (The Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2004; Azziz et al., 2006) use HA, oligo-anovulation and polycystic ovarian morphology (PCOM) at ultrasound. Whether HA is a necessary criterion remains controversial. By allowing the diagnosis of PCOS with only two items out of the three (HA, oligo-anovulation and...
PCOM), the so-called Rotterdam classification includes patients without overt HA (Dewailly et al., 2006), which is still a disputed issue (Azziz, 2006; Franks, 2006).

We recently reported that this is only an apparent controversy, because in fact the presence of PCOM (defined largely by an excess of follicles < 10 mm at ovarian ultrasound (U/S) turned out, after principal component analysis, to be itself a sign of HA (Dewailly et al., 2010). The same was observed with a high serum level of Anti-Müllieran hormone (AMH) (Dewailly et al., 2010), a peptide produced by the granulosa cells (GC) of follicles, that is highly correlated to the excess of number of follicles (FN) in patients with PCOS (Pigny et al., 2003, 2006; Laven et al., 2004). We have therefore proposed a simplified classification for the diagnosis of PCOS: oligo-anovulation and HA should first be required. When one of these criteria is not present, FN or AMH could be used as a surrogate for HA or oligo-anovulation (Dewailly et al., 2010).

In practice, this classification can be used only if we have specific thresholds of AMH and FN, beyond which these two parameters can be considered as markers of PCOM. Threshold values have been proposed previously (Balen et al., 2003; Pigny et al., 2006) but their validity is questionable in the view of current technological developments. For instance, the FN threshold proposed in 2003 (Jonard et al., 2003) (i.e. 12 follicles per ovary) leads to a major but artificial increase in the prevalence of PCOM in normal populations (especially in women aged < 30 years) when using new U/S equipment (Duijkers and Klippping, 2009; Johnstone et al., 2010; Kristensen et al., 2010). This lead some authors to conclude recently that PCOM has no pathological significance (Johnstone et al., 2010), while others recommended revisiting the threshold for FN (Kristensen et al., 2010).

There is indeed an urgent need to revisit these markers, but setting thresholds to define PCOM is particularly difficult. This is mainly due to the high incidence of asymptomatic women with PCOM on U/S, whose prevalence in the literature varies from 10 to 30% depending on the equipment and the definitions used (reviewed in Johnstone et al., 2010). In many studies, these women were included in the control groups, limiting the power of statistical procedures, such as receiver operating characteristic (ROC) analysis (Zweig and Campbell, 1993), used to study the sensitivity (Se) and specificity (Spe) of markers of PCOM. In this study, we have tried to circumvent this difficulty by a preliminary step using cluster analysis (Hartigan, 1985). Our hypothesis was that asymptomatic patients with PCOM could constitute a mathematically homogenous group among the control patients. If this were true, it would be possible to isolate and then to exclude those women from the control group. This would allow subsequent definition of new diagnostic thresholds for FN and serum AMH that could be used with acceptable Se and Spe for the detection of PCOM.

Materials and Methods

Patients

Data were obtained from a database including clinical, hormonal and U/S features that were consecutively recorded between 2008 and 2010 from patients referred to our department. These patients were referred for exploration of HA, menstrual disorders and/or infertility due to male factor and/or tubal abnormality. Women with unexplained infertility or endometriosis were excluded. Clinical, hormonal and U/S examinations were performed in the early follicular phase, between Day 2 and 5 of the menstrual cycle. In patients with menstrual disorders, the last menstrual period was either spontaneous or induced by the administration of dydrogesterone (10 mg/day for 7 days). This study was approved by the Institutional Review Board of the University Hospital of Lille. All patients gave their informed consent before inclusion in this study.

Exclusion criteria were the following: age < 18 or more than 35 years, suspicion of low ovarian reserve (FSH > 12 IU/l), hyperprolactinaemia (serum prolactin > 20 ng/ml on two subsequent determinations) or non-classic 21-hydroxylase deficiency [basal 17-hydroxyprogesterone (17-OHP) > 5 ng/ml and/or post-adrenocorticotrophic hormone-stimulated value > 12 ng/ml]. Ovarian or adrenal tumours were excluded on the basis of serum total testosterone (TT) or dehydroepiandrosterone sulphate (DHEA-S) levels lower than 1.5 ng/ml or 15 µmol/l, respectively. Any patient with criteria for hypothalamic amenorrhoea was also excluded. Furthermore, any patient with at least one follicle with a diameter > 9 mm at U/S or a serum estradiol (E2) level above 80 pg/ml was excluded from the study.

Investigations

During the medical examination, patients were specifically asked about their menstrual history. Oligomenorrhoea was defined as an average cycle length of more than 35 days and included women with frank amenorrhoea. Clinical HA was defined by the presence of hirsutism (modified Ferriman–Gallwey score over 6) and/or acne located in more than two areas. Hyperandrogenemia was defined as a serum TT level > 0.5 ng/ml and/or a serum androstenedione level (A) > 2.02 ng/ml, as previously reported (Dewailly et al., 2006).

Prolactin, LH, FSH, EZ, OHP, DHEA-S, A and TT levels were measured by immunoasays as described previously (Dewailly et al., 2006). Serum AMH levels were assessed using the second-generation enzyme immunoassay AMH-EIA (ref A16507) provided by Beckman Coulter Immunotech (Villepinte, France), as described previously (Catteau-Jonard et al., 2007).

For every patient, U/S examination was performed with a Voluson E8 Expert (General Electric Systems, VELIZY, France) with a 5–9 MHz transvaginal transducer. U/S measurements were taken in real-time, according to as standardized protocol. The highest possible magnification was used to examine the ovaries. After determination of the longest medial axis of the ovary, the length and thickness were measured and the ovarian volume (OV) was calculated as described previously (Jonard et al., 2003). For each ovary, the total number of all visible follicles smaller than 10 mm in diameter was counted by slow and continuous scanning of the entire ovary, from one margin to the other in longitudinal cross-section. Every operator was asked to count any follicle that can now be detected with the new equipment (Fig. 1) without using any lower cut-off value. For the OV and the FN, the data used for statistical analysis were the mean of recorded values for the left and right ovaries. We excluded from the analysis patients in whom transvaginal ultrasonography was not possible (due to virginity or patient refusal) and those with a history of ovarian surgery.

Statistical methods

All statistical analyses were performed using SPSS and SAS software. Statistical significance between mean values was attributed to two-tailed P < 0.05. The results are expressed as median with 5th and 95th percentiles. Comparisons between groups were performed using both Kruskal–Wallis test and a non-parametric analysis of variance (ANOVA) after rank transformation using the methodology suggested by Conover and Iman (1981). The Bonferroni correction was applied for multiple comparisons in post hoc tests. Significant relationships between serum AMH, FN
and age were evaluated by the non-parametric Spearman correlation coefficient.

ROC curves were constructed to examine the diagnostic test performance, i.e. the ability to discriminate between groups (Zweig and Campbell, 1993). Se (y-axis) against 1-Spe (x-axis) was plotted at each threshold level, and the area under the curve (AUC) was computed by the non-parametric Wilcoxon test. The AUC represents the probability of correctly identifying controls and patients with PCOS. A value of 0.5 means that the result is no better than chance.

In order to test our hypothesis that asymptomatic patients with PCOM constitute a mathematically homogenous group among the control patients, we analysed the homogeneity of the control group using a cluster analysis. The variables that are considered as markers of PCOM according to the available literature (reviewed in Johnstone et al., 2010), i.e TT, A, LH, AMH, OV and FN were included in the analysis. The age of patients was also included since it may confound some variables.

Cluster analysis is a statistical multivariate classification procedure used to classify patients in different groups or clusters according to different profiles (Hartigan, 1985). These clusters are not defined a priori and are such that individuals in a given cluster are close to each other in the sense of a similar measure and individuals in different clusters tend to be dissimilar. The cluster analysis was based on the k-means method. In this method, the similarity between individuals is measured using the usual euclidian distance. The homogeneity of clusters was assessed by the squared correlation ratio ($R^2$) which is the ratio of the between-cluster variation and the total variation computed from all the variables. The graphical representation of the $R^2$ values against the number of clusters was used to choose the most appropriate number of cluster. In addition, the $R^2$ of each variable was computed in order to determine the most important variables in the identification of clusters.

### Results

According to their symptoms, the 240 patients included in this study were divided into three groups: group 1 ($n = 105$) including women...
without HA (clinical or biological) and with regular menses (non-PCOS group), group 2 (n = 73) including women with only HA or only oligo-anovulation (presumption of PCOS) and group 3 (n = 62) including women with HA and oligo-anovulation, i.e. patients with genuine PCOS as defined by the current classifications (The Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2004; Azziz et al., 2006). U/S data were not used in this classification.

Group 1 was subjected to cluster analysis using age, TT, A, LH, AMH, OV and FN as classifying variables. Since the models with three or four clusters were not accompanied by a significant increase of total $R^2$ by comparison with the model with two clusters, we considered that two subgroups (1A and 1B) were generated by the analysis, comprising 66 and 39 women, respectively. The critical parameters for this classification were primarily the serum AMH level and then the FN and OV, with $R^2$ values of 0.72, 0.31 and 0.22, respectively. All other variables, including age, had $R^2$ values <0.05.

As shown in Table I and Fig. 2, group 1B differed from group 1A by a significantly higher mean rank of AMH, FN and OV, while mean ranks of TT, A, LH and FSH were similar. Group 1B differed from group 2 by lower mean ranks of TT and A, while mean ranks of AMH, FN, OV, FSH and LH were similar. Compared with group 3, group 1B had significantly lower mean ranks of LH, TT, A, AMH, FN and OV (Table I).

Considering that group 1B represented asymptomatic women with PCOM, it was excluded from the data and we then performed a ROC curve analysis using a population gathering groups 1A and 3 (non-PCOM non-PCOS controls and PCOS women, respectively).

A highly significant correlation was observed between the values of AMH and FN in the entire study population (Fig. 3).

### Table II Results of ROC analysis using a population consisting of groups 1A and 3 (non-PCOM non-PCOS women and those with PCOS, respectively).

<table>
<thead>
<tr>
<th></th>
<th>AUC (95% CI)</th>
<th>Threshold</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicle number</td>
<td>0.949</td>
<td>17</td>
<td>87</td>
<td>83</td>
</tr>
<tr>
<td>(0.915–0.982)</td>
<td>19</td>
<td>81</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>(0.915–0.982)</td>
<td>21</td>
<td>78</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>Ovarian volume</td>
<td>0.923</td>
<td>7 ml</td>
<td>87</td>
<td>89</td>
</tr>
<tr>
<td>(0.874–0.973)</td>
<td>8 ml</td>
<td>75</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>(0.874–0.973)</td>
<td>9 ml</td>
<td>63</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>(0.874–0.973)</td>
<td>10 ml</td>
<td>50</td>
<td>99.5</td>
<td></td>
</tr>
<tr>
<td>Serum AMH</td>
<td>0.973</td>
<td>30 pmol/l</td>
<td>92</td>
<td>82</td>
</tr>
<tr>
<td>(0.947–0.998)</td>
<td>35 pmol/l</td>
<td>92</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>(0.947–0.998)</td>
<td>40 pmol/l</td>
<td>85</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

AUC, area under the ROC curve; CI, confidence interval. Bold values indicate best compromise between sensitivity and specificity.
(r = 0.771, P < 0.0001). Both FN and serum AMH were negatively correlated to age, with modest, although significant, R values (−0.235 and −0.207 respectively, P < 0.0001 for both). When the earlier ROC analysis was restricted to the non-PCOM non-PCOS patients who were aged <30, the AUC for AMH was 0.979, with the best compromise at 37 pmol/l (Spe = 100%, Se = 95%) and the AUC for FN was 0.960, with the best compromise at 20 (Spe = 94%, Se = 84%).

**Discussion**

So far, no single marker other than FN at U/S has been able to separate accurately women with normal ovaries from those with PCOM among a group of asymptomatic women. Consequently, it might seem impossible to exclude the former from a control group without using a given threshold for FN. However, as a whole, women having PCOM differ from those with normal ovaries by having slightly higher mean serum androgens (Adams et al., 2004; Mortensen et al., 2009) or AMH levels (Johnstone et al., 2010), although this was not significant in all studies (reviewed in Johnstone et al., 2010).

By analysing the profiles of the control patients according to the markers of PCOS without using any pre-determined threshold, our cluster analysis has been able to isolate a homogenous subgroup of women within our initial control group. We can confidently assert that these asymptomatic women represented a population with PCOM, since the most relevant variables used by the analysis were first the serum AMH and then the FN and OV. Indeed, an increased serum AMH level has been recently reported in normal women with PCOM (Johnstone et al., 2010) as being stronger than that obtained when using FN with older U/S machines (Pigny et al., 2003; Laven et al., 2004). This is in keeping with the immuno-cytobehavioural (ICB) data in humans showing that AMH is maximally expressed in GC from follicles measuring 1–4 mm (Weenen et al., 2004).

Our study indicated that serum AMH level is a more reliable marker of PCOM than FN. First, cluster analysis showed that serum AMH appeared as the strongest parameter to isolate women with PCOM within the non-PCOS group. Second, in the population gathering non-PCOM non-PCOS women and those with PCOS, the area under the ROC curve (AUC) was better with serum AMH than with FN or OV, and this analysis yielded higher rates of Spe and Se (97 and 92%, respectively). These figures are also much better than in our previous study about AMH in 2006 (92 and 67%, respectively) (Pigny et al., 2006). This is explained by a much greater AUC in the present study (0.973 versus 0.851 respectively). In addition, the threshold proposed here is lower than that reported previously (35 versus 60 pmol/l, respectively) (Pigny et al., 2006). Rather than technical issues (the assay procedure did not change), the main reason for these discrepancies is probably the difference in the selection of the non-PCOS reference group. In 2006, using the U/S equipment and criteria of the time (Pigny et al., 2006), we probably failed excluding all asymptomatic women with PCOM from this group. Indeed, in the present study, U/S was less sensitive than AMH in detecting PCOM in the non-PCOS group and in patients with mild PCOS as
well. Therefore, our results in 2006 were presumably spoiled by a
greater overlap between the non-PCOM non-PCOS group and the
PCOS population. Conversely, our results with OV were similar to
those in our previous report (Jonard et al., 2005), with an optimal
threshold at 7 ml and similar Spe but with better Se (89 versus 91%
and 87 versus 67%, respectively). This indicates that the higher Se
threshold at 7 ml and similar Spe but with better Se (89 versus 91%
of the new U/S equipments has much less impacted this measure
than the FN and especially the AMH assay.

Knowing the negative effect of age on the FN and serum AMH
values, that we confirm here, although it was weak, some authors
have advocated adapting the thresholds to the patients’ age (Duijkers
and Klipping, 2009; Johnstone et al., 2010). Besides the inconvenience
of having to validate and then to memorize several thresholds, we
think that this adaptation is not crucial. Indeed, when our ROC anal-
ysis was restricted to non-PCOM non-PCOS women that were aged
<30, the data for both FN and serum AMH were very close to
that obtained in the whole population. Conversely, it must be empha-
sized that the criteria proposed here should not be applied to women
aged more than 35. Lastly, we must recognize that our control group
included exclusively patients referred to our clinic. It might therefore
be not fully representative of the general population. Further studies
are required to validate our data in other settings.

In conclusion, for the definition of PCOM and PCOS, serum AMH
appears as a sensitive and specific parameter that would probably be
easier to reproduce from one to another centre than the follicle
count, as the latter is highly dependent on the evolving quality of
the machines and/or the operator skill. When a single commercially
available AMH assay (Kumar et al., 2010) is universally used, it will
become urgent that an international consortium validates the
threshold for AMH proposed here to suggest the presence of
PCOM, i.e. 35 pmol/l (or 5 ng/ml). Since we have previously
reported that the serum AMH level is closely related to both the
markers of HA and ovulatory disturbance in PCOS (Dewailly et al.,
2010), the use of this parameter in the current classifications for
PCOS (The Rotterdam ESHRE/ASRM-Sponsored PCOS consensus
workshop group, 2004; Azziz et al., 2006) would allow simplification
of the diagnosis of PCOS, as proposed in Table III.

## Authors’ roles

D.D. participated in the study design, execution, analysis, manuscript
drafting and critical discussion. H.G. participated in the study
execution, analysis, manuscript drafting and critical discussion. E.P. par-
ticipated in the study design, execution and critical discussion. G.R.
participated in the study execution and critical discussion. M.L. partici-
pated in the study execution and critical discussion. P.P. participated in
the study execution and critical discussion. A.D. participated in the
study design, execution, analysis and critical discussion. S.C.-J. partici-
pated in the study design, execution, analysis, manuscript drafting and
critical discussion.

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