ANOGENITAL DISTANCE AND ENDOMETRIOSIS: RESULTS OF A CASE-CONTROL STUDY

Running title: Anogenital distance and endometriosis

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ABSTRACT

AGD is the distance measured from the anus to the genital tubercle. Recent evidence suggests that a shorter AGD, a sensitive biomarker of the prenatal hormonal environment, could be associated with higher endometriosis risk. However, studies investigating AGD in affected women are scanty. We have set up a case-control study recruiting nulliparous women (aged 18-40 years) with endometriosis between 2017 and 2018. Cases were 90 women with a surgical or with a current nonsurgical diagnosis of endometriosis (n = 45 deep infiltrating endometriosis (DIE), and n = 45 ovarian endometrioma (OMA)). Controls were 45 asymptomatic women referring for periodical gynaecological care and without a previous diagnosis of endometriosis. They were matched to cases for age and BMI. For each woman, two measures were obtained using a digital caliper: AGD_{AC}, from the clitoral surface to the upper verge of the anus, and AGD_{AF}, from the posterior fourchette to the upper verge of the anus. Each distance was derived from the mean of six measurements acquired from two different gynaecologists. The mean±SD AGD_{AC} in women with DIE, OMA and without a diagnosis of endometriosis was 76.0±12.1, 76.1±11.1, and 77.8±11.4 mm, respectively (p = 0.55). The mean±SD AGD_{AF} in women with DIE, OMA and without a diagnosis of endometriosis was 22.8±5.0, 21.7±9.0, and 23.7±7.8 mm, respectively (p = 0.38). Our study failed to find an association between AGD and the presence of endometriosis. AGD does not seem to represent a reliable indicator of the presence of endometriosis to be used in clinical practice.

Keywords: endometriosis; anogenital distance; AGD; deep endometriosis; endometrioma.

INTRODUCTION

Endometriosis is a disease characterized by the presence of endometrium-like epithelium and/or stroma outside the endometrium and myometrium, usually with an associated inflammatory process [1]. Endometriosis affects about 5% of women of reproductive age [2]. Endometriosis lesions could be schematically subdivided into peritoneal/superficial implants, ovarian endometriotic cyst/endometrioma, deep endometriosis, and extra-abdominal localizations [1]. In the majority of the patients, symptoms can be managed via pharmacological inhibition of ovulation and menstruation [3]; however, in some cases, a surgical approach should be considered, in particular in women with deep infiltrating forms [4]. The exact etiopathogenetic origin of the disease is still to be defined. The most widely accepted hypothesis is represented by the retrograde menstruation theory, characterized by the backward flux of menstrual debris that contains viable endometrial cells through the fallopian tubes into the pelvic cavity [5]. However, some authors suggest an intrauterine origin of the disease [6, 7]. The potential role of early-life influences, such as intrauterine hormonal environmental exposure to oestrogens and endocrine disruptors, are receiving growing consideration as a risk factor for endometriosis in adult life [8–17]. In addition, accumulating evidence suggests that immune cells, adhesion molecules, extracellular matrix metalloproteinase, and pro-inflammatory cytokines activate/alter the peritoneal microenvironment, creating the conditions for differentiation, adhesion, proliferation and survival of ectopic endometrial cells [18, 19].

Anogenital distance (AGD) is a sexually dimorphic feature and represents the distance measured from the anus to the genital tubercle [12, 20] . There is considerable evidence in animal and human models that AGD represents a biomarker of the prenatal hormonal environment [21, 22]. Prenatal exposure to androgens results in a longer AGD, whereas a prenatal estrogenic environment in a shorter one. In addition, observational studies suggest that AGD is a biomarker of prenatal exposure to endocrine disruptors [23, 24] and androgens during the development of the

reproductive system [25, 26]. Recent evidence suggests that a shorter AGD could be associated with higher endometriosis risk, particularly with deep infiltrating forms [12, 27–32] (Table 1). Hypothetically, a shorter AGD, reflecting a more estrogenic intrauterine hormonal milieu, could represent an indicator of the presence of endometriosis. However, studies investigating AGD in affected women are scanty. Therefore, the potential association between AGD and endometriosis remains to be clarified. In this light, we have set up a case-control study to compare AGD measurements between women with and without a diagnosis of endometriosis.

MATERIALS AND METHODS

This case-control study was performed in an academic hospital, the Fondazione Ca' Granda-Ospedale Maggiore Policlinico, which includes a tertiary referral centre for the study and management of endometriosis. Participants were recruited from June 2017 to October 2018, and were consecutively evaluated for inclusion in the study. Only nulliparous Italian women of Caucasian origin aged between 18 and 40 years were deemed eligible. Cases were women with a surgical diagnosis of endometriosis in the previous 24 months or with a current nonsurgical diagnosis of endometriosis. Nonsurgical diagnoses were based on previously published criteria [33–36]. Specifically, the nonsurgical diagnosis was based on physical examination, direct visual inspection of the posterior fornix and biopsy of vaginal lesions in those with rectovaginal endometriosis, and on ultrasonographic criteria in patients with ovarian endometriomas, rectovaginal endometriosis, and full-thickness bladder detrusor nodules. Magnetic resonance imaging (MRI) was performed in selected situations. Affected women were subcategorized into two groups, namely, deep infiltrating endometriosis and ovarian endometrioma. The former included women with rectovaginal plaques, bladder detrusor nodules, bowel lesions, intrinsic ureteral endometriosis, and deep endometriosis infiltrating the pouch of Douglas and parametria. The rectovaginal space is defined as the area behind the pouch of Douglas, enclosed anteriorly by the uterus and the posterior vaginal wall, posteriorly by the rectum, and laterally by the uterosacral and

the Mackenrodt ligament [1]; therefore, rectovaginal plaques include nodules infiltrating this anatomical space. Women with both deep infiltrating endometriosis and endometrioma were included in the group of deep infiltrating endometriosis. In the same period, women attending our outpatient clinics for periodic well-woman visits, contraception, and cervical cancer screening program, and without a previous clinical or surgical diagnosis of endometriosis were enrolled as the control group. Endometriosis was excluded based on gynaecological history, pelvic transvaginal ultrasound, gynaecological bimanual examination, and visual inspection of the posterior vaginal fornix. Exclusion criteria included pregnancy state, presence of genitourinary prolapse, or current/previous oncological treatments.

Cases and controls were matched for age (+/- 1 year) and body mass index (BMI) (four categories: underweight BMI, <18.5 Kg/m^2; normal weight BMI, 18.5-25 Kg/m^2; overweight BMI, 25-30 Kg/m^2; obese BMI, > 30 Kg/m^2). Every match triplet included a woman with endometriomas, a woman with of deep infiltrating endometriosis and a control.

In women who agreed to participate, two female gynaecologists, unaware of the patient's gynaecological condition, measured AGD using a digital caliper (Borletti CDJB15 150 mm Digital Caliper). During measurements, women were laid down in lithotomy position with thigs at 45° to the examination table. For each woman, two different measures were performed: AGD_{AC}, from the anterior clitoral surface to the upper verge of the anus, and AGD_{AF}, from the posterior fourchette to the upper verge of the anus (Fig. 1). Each distance was derived from the mean of six measurements (measures were made in triplicate by each of the two gynaecologists). In addition, data were collected on standardized forms, including demographic information and clinical characteristics.

The local Institutional Review Board (Comitato di Etica Milano Area B) approved the study (approval no. 261_2017bis). All patients provided written consent before enrolment.

Data were archived using Excel 2003 (Microsoft Corporation, Redmond, Washington) and exported in SPSS 18.0 (SPSS, Inc, Chicago, Illinois) for statistical analysis.

For sample size calculation, we took as a reference the anogenital distance's mean and SD (anus to posterior fourchette) identified in the largest published study (27 ± 6 mm) [12], and considered biologically relevant, demonstrating a mean reduction of at least 4 mm. Therefore, we considered a 1:1 ratio between case and controls and 0.05 and 0.20 as acceptable type I and II errors, respectively. We also considered a sample size that would allow two comparisons, between patients with endometriomas and controls and between those with deep infiltrating endometriosis and controls; thus, we decided to consider 0.025 as type I error (Bonferroni correction 0.05/2). On these bases, the calculated sample size was a total of 135 patients, divided into three groups of 45 patients each (45 patients with ovarian endometriosis, 45 patients with DIE and 45 controls).

Continuous variables were compared using univariate analysis of variance (GLM - general linear model). Age and BMI were tested as covariates; BMI was included because AGD is an anthropometry-dependent measure. All tests were two-tailed at 0.05 significance level.

RESULTS

Recruitment continued until the pre-planned number of participants was reached (deep infiltrating endometriosis (DIE) $n = 45$; ovarian endometrioma (OMA) $n = 45$; controls $n = 45$). The DIE group included 43 patients with rectovaginal endometriotic plaques, one with full-thickness bladder detrusor nodules, and one with intrinsic ureteral endometriosis. In the endometriosis group, 53 participants (59%) had a surgical diagnosis of the disease with histologic verification. No women, neither in the group of cases nor in the controls, had a concomitant diagnosis of polycystic ovary syndrome. Cases and controls had similar demographic and clinical characteristics, as shown in Table 2.

The analysis on the means of six measures taken by two different operators was conducted by general linear model, with age and BMI tested as covariates. The main results are shown in Table 3. We found no significant associations between AGD_{AC} or AGD_{AF} and the presence of either ovarian or deep-infiltrating endometriosis.

To assess the quality of the measurements, the correlation between measures taken by the two operators (using the mean of the triplicate) for both AGD_{AC} and AGD_{AF} were tested; they were found to be highly correlated [Pearson's correlation (r) = 0.9, $p<0.001$]. In addition, to rule out possible confounders related to inter-operator variation, we considered ratios between the means of the three measures taken by each operator and repeated all the analyses excluding ratios smaller than 0.9 and higher than 1.1. Measures of AGD_{AC} excluded due to high inter-operator variability were a total of 16 (eight patients were excluded from the ovarian endometriosis group, three from the DIE group, and five from the control group). Measures of AGD_{AF} excluded due to high inter-operator variability were a total of 36 (13 patients were excluded from the ovarian endometriosis group, 16 from DIE group, and seven from the control group). Overall, results were very similar, and relations between AGD_{AC} or AGD_{AF} and endometriosis did not emerge (data not shown).

We then dichotomized values of AGD_{AC} and AGD_{AF} using previously found cut-offs [28] for endometriomas (AGD_{AC} = 67.6 mm; AGD_{AF} = 25.4 mm) and DIE (AGD_{AC} = 77.9 mm; AGD_{AF} = 20.9 mm). Odds ratios (ORs) adjusted for age and BMI were calculated for the two groups of women with the disease compared to controls. For endometriomas' ORs of AGD_{AC} and AGD_{AF} were 1.2 (95%CI: 0.4-3.4) and 1.5 (95%CI: 0.5-4.0), respectively; for deep-infiltrating endometriosis' ORs of AGD_{AC} and AGD_{AF} were 1.1 (95%CI: 0.5- 2.7) and 0.8 (95%CI: 0.4-2.0), respectively.

Finally, ROC curves were generated to assess the ability of AGD_{AC} and AGD_{AF} to predict the presence of endometriomas or deep peritoneal endometriosis, and AUCs (area under curve) were examined. For endometriomas, AUCs of AGD_{AC} and AGD_{AF} were 0.55 (95%CI: 0.43- 0.67) and 0.60 (95%CI: 0.48-0.72), respectively (Figure 2, *upper panel*); for deep-infiltrating endometriosis, AUCs of AGD_{AC} and AGD_{AF} were 0.54 (95%CI: 0.42-0.66) and 0.49 (95%CI: 0.37 – 0.61) respectively (Figure 2, *lower panel*).

DISCUSSION

We did not observe an association between AGD, neither AGD_{AC} nor AGD_{AF}, and the presence of endometriosis. The present results do not support the use of these measurements to screen advanced endometriosis and/or to predict the risk of developing ovarian or deep disease forms in the future.

Our data do not align with previous findings [12, 27–29] (Table 1). Indeed, an association between shorter AGD_{AF} and endometriosis, particularly deep infiltrating forms, was identified in a Spanish case-control study conducted on 219 women (endometriosis $n = 114$; controls $n = 105$) [12]. The same group subsequently reported some more in-depth analyses of their series [27, 28]. A French prospective cohort study [29] conducted on 168 women (endometriosis $n = 98$; controls $n = 70$) confirmed an association between a shorter AGD and the presence of the disease and these findings were also later confirmed by the same group using magnetic resonance imaging to measure AGD on the same cohort of patients [32]. Finally, in 2020 Peters *et al.* [31] evaluated AGD and the 2D:4D digit ratio in 172 women (endometriosis $n = 43$; Mayer-Rokitansky-Kuster-Hauser syndrome $n = 43$; polycystic ovarian syndrome (PCOS) $n = 43$; controls $n = 43$). Even after adjustment for BMI and age, the authors observed an increased AGD_{AC} in women with PCOS and a decreased AGD_{AC} in women with endometriosis. The digit ratio did not differ between groups.

Explaining this inconsistency is challenging. We hypothesize that selection criteria may have played a role. Of relevance here, we designed the study specifically to prevent confounders. We selected exclusively nulliparous women and matched cases and controls for age and BMI. Even if the evidence is inconsistent, it seems reasonable to infer a possible impact of all these variables on AGD_{AC} and AGD_{AF} [12, 37, 38]. Of note, in the study by Sánchez-Ferrer *et al*. [28], the frequency of previous vaginal deliveries significantly differed between women with and without endometriosis, being 41% and 21%, respectively. Age also significantly differed, as the mean age of women with the disease was seven years older. Similarly, in Crestani *et al's*. [29] study, the two study groups significantly differed in terms of age at recruitment and percentage of vaginal deliveries.

Our findings are unexpected. In fact, the rationale supporting the idea that endometriosis may develop as a consequence of endocrine exposures occurring during ~~the~~ intrauterine life is intriguing. As AGD is a surrogate marker of such an exposure, its measure could be used as a predictor of endometriosis in case the intrauterine pathogenic hypothesis will be confirmed. On the other hand, it should be emphasized that our findings cannot be used to rule out an intrauterine origin of endometriosis. Of note, a recent prospective cohort study on 205 neonates [39] suggested that AGD in humans is fixed in early gestation (likely during the masculinization programming window (MPW), at 8-14 weeks of gestation) and is unaffected by androgen levels thereafter. Thus, AGD may act as a biomarker of intrauterine androgens action in early gestation, but only for a limited time period. Thus, it could be speculated that during this limited MPW intrauterine hormonal environment may not have a role as a risk factor for endometriosis in adulthood, but it cannot be excluded that exposures in other gestational periods could be detrimental. Hypothetically, pathogenic intrauterine noxae (i.e. endocrine disruptors, hormonal environment) may have a role in the development the disease in different gestational time windows compared to those that have an impact on AGD. In addition, it should also be underlined that the intrauterine endocrine factors that modulate AGD could differ from those that could influence endometriosis development.

The present study has some limitations. Firstly, selection and measurement biases need to be considered. However, the decision to perform six measurements for each AGD from two different gynaecologists who were blinded to women conditions should have reduced measurement variability. In addition, limiting the recruitment to nulliparous women should have limited any bias related to changes in external genital anatomy after vaginal delivery and episiotomy. Moreover, Domenici *et al*. [40] demonstrated that AGD in post-menopausal women was significantly shorter than AGD in pre-menopausal women, as a consequence of vulvovaginal atrophy.

As for any case-control study, the choice of controls may be a cause of concern. In our study, we decided to enrol women without known endometriosis presenting to the gynaecological

unit for routine well-woman visit, contraception, and cervical cancer screening. Endometriosis was ruled out based on gynaecological and ultrasonographic examination, but we cannot exclude having accidentally included some cases among controls. However, the impact of this potential inexactness would be presumably modest, given the limited prevalence of asymptomatic endometriosis in the general population [2]. Moreover, misdiagnoses are more likely for early superficial peritoneal endometriosis, a condition of doubtful clinical relevance [41]. In addition, we decided to include also women without a surgical diagnosis of endometriosis. However, as recently suggested by Chapron *et al*. [42] the most appropriate and up-to-date diagnostic approach of endometriosis includes a combination of patient interview and clinical examination, followed by the execution of transvaginal ultrasonography or MRI. Therefore, diagnostic laparoscopy should no longer be used as the first-line approach in the diagnosis of endometriosis [42]. Moreover, in the last decade, the diagnostic accuracy of ultrasonography in detecting ovarian and deep infiltrating endometriosis has been repeatedly demonstrated to be highly reliable and reproducible [43–46]. In particular, according to a recent Cochrane meta-analysis, transvaginal ultrasound for OMA has a high sensitivity and specificity, respectively 93% (95%CI: 87-99%) and 96% (95%CI: 92-99%) [46].

Finally, as predictable, the percentage of women under hormonal treatments was significantly higher in the endometriosis group. We cannot exclude, albeit unlikely, that progestins and estrogen-progestins could have an impact on AGD. At now, no evidence is available regarding the potential impact of hormonal treatments on AGD length.

In conclusion, our study failed to find an association between AGD and a diagnosis of endometriosis. Therefore, in our experience, AGD does not seem to represent a reliable indicator of the presence of ovarian and deep disease. However, these findings should not be used to discard the theory of an intrauterine origin of endometriosis. Further evidence is needed to explore this pathogenic hypothesis.

DECLARATIONS

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Conflict of interest

Edgardo Somigliana reports personal fees from THERAMEX and MERCK-SERONO, and grants from FERRING and MERCK-SERONO. All the other authors declare that they have no conflicts of interest.

Ethics approval

The questionnaire and methodology for this study was approved by the local Institutional Review Board (Comitato di Etica Milano Area B). Ethics approval number: 261_2017bis.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Authors' contributions

L.B. contributed to the design of the study, recruitment of study participants, interpretation of the data, and drafted the manuscript; P.V. and E.S. contributed to the design of the study and critical revision of the manuscript; F.O. and D.D. contributed to the recruitment of study participants; E.S, G.S. contributed to the analysis and interpretation of the data. All authors reviewed and approved the final version of the manuscript.

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393 FIGURE LEGENDS

394 **Fig. 1** Landmarks for two measurements of anogenital distance (AGD): AGD-AC, from the anterior
395 clitoral surface to the upper verge of the anus (left) and AGD-AF, from the posterior fourchette to
396 the upper verge of the anus (right)

397 **Fig. 2** Receiver operating characteristics curves for anogenital distance (AGD) and presence of
398 endometriosis. In the upper panel, presence of endometriomas versus controls; in the lower panel,
399 presence of deep-infiltrating endometriosis versus controls

Table 1. Studies evaluating anogenital distance (AGD) in women with endometriosis.

Source	***Country***	***Study design***	***Number of patients enrolled***	***AGD_{AF} and AGD_{AC} (mm)***	***Outcomes***
Mendiola *et al.*, 2016 [12] and Sanchez-Ferrer *et al.*, 2017 [28]	Spain	Case-control	219 (endometriosis *n* = 114; controls *n* = 105)	AGD_{AF}: Endometriosis: 23.5 ± 5.8 Controls: 27.3 ± 5.7 AGD_{AC}: Endometriosis: 73.8 ± 12.1 Controls: 75.7 ± 11.7	Shorter AGD was related to the presence of endometriosis. In particular, with regard to DIE, women with AGD_{AF} below the median, compared with those with AGD_{AF} above the median, were 41.6-times (95% CI 3.9–438; p = 0.002) more likely to have endometriosis.
Sanchez-Ferrer *et al.*, 2019 [27]	Spain	Case-control	150 (endometriosis *n* = 57; controls *n* = 93)	AGD_{AF}: Endometriosis: 22.8 ± 4.6 Controls: 27.2 ± 5.7 AGD_{AC}: Not evaluated	Women with endometriosis showed significantly shorter AGD_{AF} compared to controls (p < 0.001).
Crestani *et al.*, 2020 [29]	France	Prospective cohort study	168 (endometriosis *n* = 98; controls *n* = 70)	AGD_{AF}: Endometriosis: 21.5 ± 6.4 Controls: 32.3 ± 8.1 AGD_{AC}: Endometriosis: 100.9 ± 20.6 Controls: 83.8 ± 12.9	Surgically and histologically proven endometriosis is associated with a short AGD in women of reproductive age but not correlated either to the severity or to the location of the disease.
Peters *et al.*, 2020 [31]	Netherlands	Case-control	172 (endometriosis *n* = 43; PCOS *n* = 43; MRKH *n* = 43; controls *n* = 43)	AGD_{AF}: Endometriosis: 21.9 ± 6.2 PCOS 22.0 ± 5.8 MRKH 24.6 ± 6.2 Controls: 21.7 ± 6.2 AGD_{AC}: Endometriosis: 103.9 ± 12.6 PCOS 113.8 ± 16.9 MRKH 108.2 ± 11.3 Controls: 111.4 ± 13.7	The AGD_{AC} was significantly different between groups (p = 0.007), with a decreased AGD_{AC} in women with endometriosis.
Crestani *et al.*, 2021 [32]	France	Case-control	98* (endometriosis *n* = 67;	MRI-AGD_{AF}: Endometriosis: 13.3 ± 3.9	Surgically and histologically proven endometriosis was associated with a

			controls n = 31)	Controls: 21.2 ± 5.4 MRI-AGD$_{AC}$: Endometriosis: 40.4 ± 7.3 Controls: 51.1 ± 8.6	shorter MRI-AGD, especially MRI-AGD-$_{AF}$, in comparison with controls without the disease. No relation was found between MRI-AGD and the disease phenotype.

PCOS: polycystic ovary syndrome
MRKH: Mayer-Rokitansky-Kuster-Hauser
MRI: magnetic resonance imaging
* re-analysis of previously published data [29]; in this study AGD has been measured retrospectively by a senior radiologist with MRI

Table 2. Distribution of baseline demographic and clinical characteristics of women with deep infiltrating endometriosis (*n*=45), ovarian endometriomas (*n*=45), and controls (*n*=45).

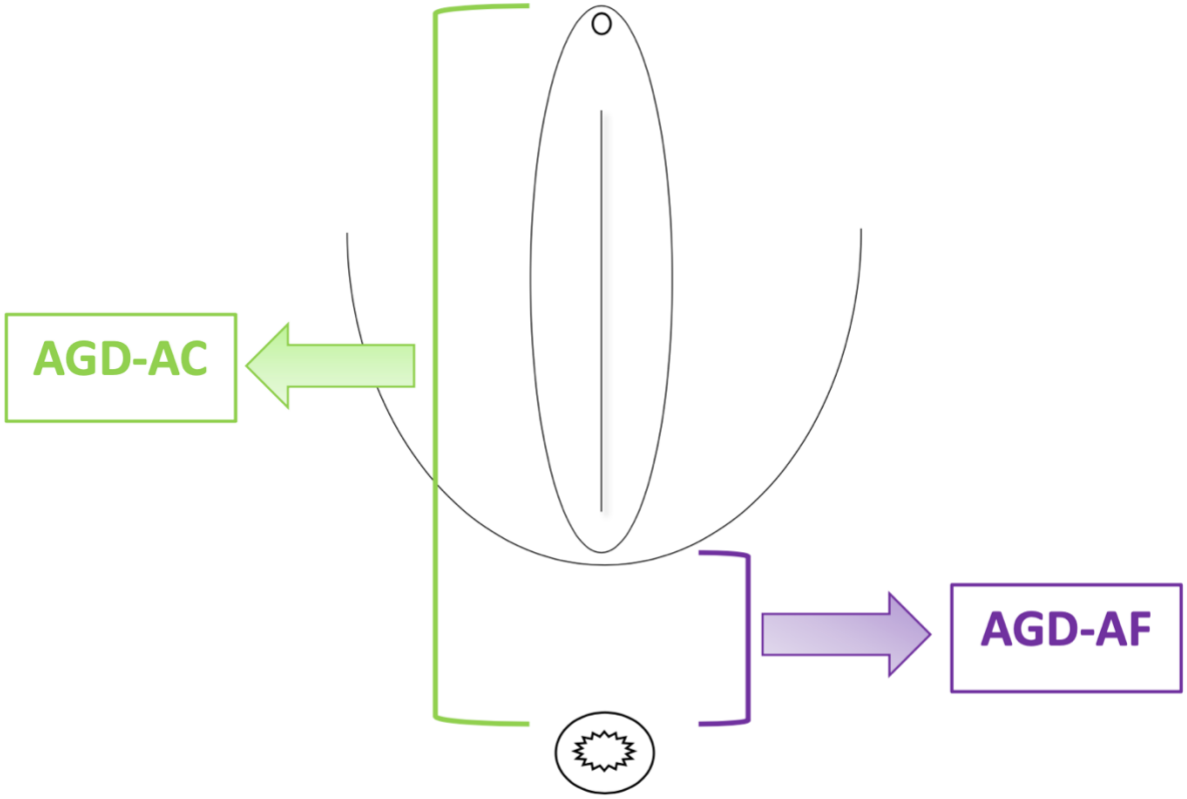
Characteristic	Deep infiltrating endometriosis (*n*=45)	Ovarian endometriomas (*n*=45)	Controls (*n*=45)
Age (years)	31.3 ± 5.0	31.3 ± 4.9	31.2 ± 4.9
BMI (Kg/m^2)	20.6 ± 2.3	21.2 ± 2.0	20.5 ± 2.0
Smoking	12 (27%)	12 (27%)	10 (22%)
Progestin or estro-progestin treatment	37 (82%)	29 (64%)	17 (38%)

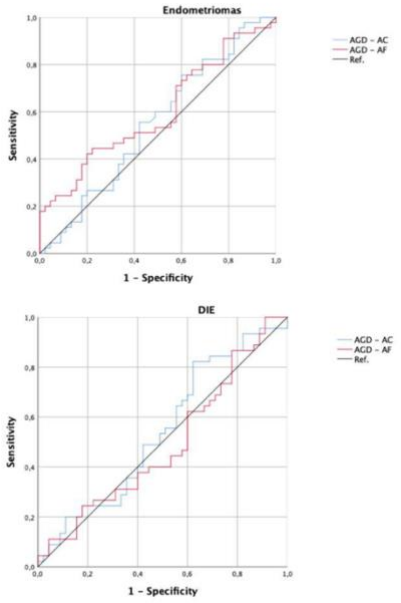
Data is reported as mean ± SD or number (percentage).
BMI = body mass index.

Table 3. Comparison of anogenital distances of women with deep infiltrating endometriosis (*n*=45), ovarian endometriomas (*n*=45), and controls (*n*=45).

Anogenital distance	Deep infiltrating endometriosis (*n*=45)	Ovarian endometriomas (*n*=45)	Controls (*n*=45)	*P*
AGD_{AC}	76.0 ± 12.1 (72.5-78.9)	76.1 ± 11.1 (72.9-79.3)	77.8 ± 11.4 (74.8-81.2)	NS
AGD_{AF}	22.8 ± 5.0 (20.6-25.1)	21.7 ± 9.0 (19.4-23.8)	23.7 ± 7.8 (21.6-26.0)	NS

Data is reported as mean ± SD and 95% confidence interval (CI).
AGD_{AC}: anus-clitoris distance.
AGD_{AF}: anus-fourchette distance.
NS: not significant


AGD-AC
AGD-AF


Endometriomas
Sensitivity
1 – Specificity
AGD – AC
AGD – AF
Ref.
DIE
Sensitivity
1 – Specificity
AGD – AC
AGD – AF
Ref.