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The Association of Sonographic Evidence of Adenomyosis with Severe Endometriosis and with Gene Expression in Eutopic Endometrium

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Human Research and Ethics Committee approval for this project was obtained from the hospital's Research and Ethics Committee (Projects 11-24 and 16-43).

Precis: Sonographic evidence of adenomyosis in patients undergoing surgery for investigation of suspected endometriosis is associated with severe endometriosis

Abstract

Study objective: To examine the presence of sonographic evidence of adenomyosis (SEOA) in patients undergoing laparoscopic surgery for investigation of endometriosis and to assess if there is an association between SEOA and endometriosis severity. Using gene expression analysis, we also aimed to determine if gene expression in eutopic endometrium differed in patients with and without adenomyosis.

Design: A prospective study (Canadian Task Force classification II-2).

Setting: A tertiary medical center.

Patients: Reproductive-age women who underwent laparoscopic surgery after presenting to a pelvic-pain focused gynecology clinic.

Interventions: Endometrial tissue, detailed patient questionnaires, pathology and surgical notes were collected. Sonographic data from tertiary ultrasounds performed up to 12 months before surgery was retrospectively added (n=234; researchers blinded to surgical and pathological findings). Gene array data from endometrial biopsies (n=41) was used to analyze differential gene expression; patients were divided into two groups according to presence or absence of SEOA.

Measurements and Main Results: Of 588 patients recruited, 234 (40%) had an available pelvic scan and were included in this study. The average age of included

women was 30.6 years, with 35% having SEOA. Patients with SEOA were 5.4 years older (p=0.02). There was no significant difference in rates of endometriosis between groups, however, patients with SEOA were more likely to have stage IV endometriosis (41% vs 9.8%, p<0.001). Patients with SEOA were also more likely to have other markers of severe endometriosis such as endometriomas and deep infiltrating endometriosis (p<0.001). No significant difference was observed in endometrial gene expression between adenomyosis cases and controls after adjusting for menstrual cycle phases and presence/absence of endometriosis.

Conclusion: Sonographic features of adenomyosis may be included as a component of the clinical assessment when attempting to predict the presence of severe endometriosis. No differences in gene expression were observed. Further research is needed to characterize uterine adenomyosis and to explore molecular pathways involved in its pathogenesis.

Key words: Adenomyosis; Ultrasound; Gene expression; Severe endometriosis.

Introduction

The most common cause of secondary dysmenorrhoea is endometriosis, followed by adenomyosis [1]. In adenomyosis, endometrial glands and stroma are present within the myometrium. It is most prevalent among women aged 35-50 years [2] and often presents with heavy and painful menstrual bleeding [3].

Diagnosis of adenomyosis has traditionally been made via histopathology after hysterectomy [4]. A systematic review found that magnetic resonance imaging (MRI) has a sensitivity and specificity of up to 77 and 89 percent, respectively [5]. However, MRI is a high-cost modality and its availability is limited [6]. Recent studies show that the sensitivity and specificity of trans-vaginal ultrasound are as high as 92% and 88%, respectively [7, 8].

Some have speculated that endometriosis and adenomyosis share a common pathophysiology of tissue injury and repair [9]. Microarray analysis and studies of genetic polymorphisms have also been able to shed light on common pathways of those diseases [10-12]. Previous studies have shown high prevalence of adenomyosis in women undergoing surgery for endometriosis [13-15] but have not specifically assessed the association of adenomyosis and endometriosis severity.

The aim of this study was to examine the presence of sonographic characteristics consistent with adenomyosis in a cohort of patients having exploratory surgery for possible endometriosis and to assess if there is an association between the severity of endometriosis and sonographic signs of adenomyosis. Using gene array analysis, we also aimed to explore whether gene expression in eutopic endometrium in patients with adenomyosis differs from that in patients without adenomyosis.

Materials and Methods

Women of reproductive age attending a tertiary gynecology clinic and booked for laparoscopic surgery to investigate potential endometriosis that was suspected on clinical grounds, were recruited between May 2012 and May 2016. The vast majority of these patients presented because of menstrual and/or pelvic pain. Women were recruited as part of a larger study examining the genetic basis for pathophysiology and stratification of endometriosis. Ethical approval for the study was obtained from the Royal Women's Hospital Human Research Ethics Committee (Projects 11-24 and 16-43). Informed consent was obtained from all participants.

All pre-menopausal women were eligible to participate in this study; women were included regardless of current pharmacological/hormone therapies, menstrual cycle irregularities, fertility status or ethnicity. Detailed patient questionnaires, past and present clinical histories, menstrual cycle information, pathology findings and surgical

notes were collected prospectively and recorded for each participant. All surgeries were performed by surgeons from the Endometriosis and Pelvic Pain Unit at our institution who all specialize in laparoscopic surgery, with emphasis on endometriosis surgery. Endometriosis was diagnosed following surgical visualisation and histopathological confirmation of disease when a histopathological sample was available. At the end of each surgery, a detailed operative form was completed by the involved surgeon. This form recorded sites in the pelvis where endometriosis was visualised; absence or presence and location of deep infiltrating endometriosis (DIE); the revised American Fertility Society (rAFS) classification of endometriosis (1997) and the Endometriosis Fertility Index (EFI). Disease severity was staged according to the rAFS score: Stage I Minimal (score 1 - 5); Stage II Mild (score 6 - 15); Stage III Moderate (score 16 - 40) and Stage IV Severe (score >40).

Ultrasound data, particularly sonographic evidence of adenomyosis, was added retrospectively, however, the reporting doctor was blinded to the surgical and pathological findings and used a standardised reporting methodology. Patients were only included in this study if they had an ultrasound scan performed in the imaging department of the tertiary hospital in the 12 months prior to surgery. For the purpose of this study, stored images of these ultrasound scans were reviewed by a single doctor (DN) certified in Obstetrical and Gynaecological Ultrasound by the Royal Australian and New Zealand College of Obstetricians and Gynaecologists and who specialises in complex gynecological ultrasound. Each patient was assessed for various gynecological conditions including but not limited to uterine fibroids, markers of superficial or deep endometriosis, adnexal pathology and adenomyosis. Routine measurements of the uterus were also recorded. Each scan was evaluated for the following sonographic features associated with adenomyosis: echogenic sub-endometrial lines (known also as venetian blind shadowing), myometrial cysts, heterogenous myometrium and thickening or asymmetry of the myometrial wall (Figure

1) [16]. As there is no consensus regarding imaging criteria for diagnosis of adenomyosis [7], sonographic diagnosis of adenomyosis was made if one or more of these features were present.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (version 24, IBM Corporation, NY, USA). Patient demographics and characteristics were analysed by unpaired t test and Chi-square tests. Absolute, relative frequency (%) and Chi-square tests were used to describe the qualitative variables. A *P*-value of less than 0.05 was considered statistically significant for all analyses.

RNA extraction and gene array analysis

Gene array data which had been undertaken as part of a study examining gene regulation in human endometrium [17] was available for a subset of patients (n=41) and was examined for the purpose of this study. This cohort of patients was free from exogenous hormone treatment in the three months prior to surgery. Individual endometrial tissue samples were split and either stored in RNA*later* (Life Technologies, Grand Island, NY, USA) at -80°C until RNA extraction, or formalin fixed and processed routinely for histological assessment. Endometrial cycle stage of samples included in this analysis were: Early Proliferative (EP)=3, Mid-Proliferative (MP)=18, Early Secretory (ES)=4, Mid-Secretory (MS)=10 and Late Secretory (LS)=6.

Total RNA was extracted from homogenized endometrial tissues (30 controls, 10 adenomyosis cases and 1 unknown) using RNA lysis solution (RLT buffer) and AllPrep DNA/RNA mini kit according to the manufacturer's instructions (QIAGEN, Valencia, CA). RNA integrity was assessed with the Agilent Bioanlayzer 2100 (Agilent Technologies, Santa Clara, CA) with all samples having high-quality RNA (RNA Integrity Number (RIN) > 8), and concentrations were determined using the NanoDropND-6000.

Gene expression array methods were previously described by us [19]. Briefly, total RNA was amplified and converted to biotinylated cRNA using the Ambion Illumina TotalPrep RNA amplification kit (Ambion). Expression profiles were generated by hybridising cRNA to Illumina Human HT-12 v4.0 Beadchips (as per manufacturer's instructions, Illumina Inc, San Diego, USA). Samples were scanned using an Illumina iScan Reader. Gene expression normalisation procedures are also detailed in Fung et al. and included pre-processing using Illumina GenomeStudio software (Illumina) [17]. Pre-processed transcript levels were transformed to normalise across individuals, chips and genes.

To avoid biasing our results with genes not expressed at certain samples, analyses were restricted to genes expressed in ≥90% of samples, leaving 15,226 probes, mapping to 12,329 unique RefSeq genes. We performed the differential expression analysis between adenomyosis cases and controls using the *eBayes* method, which is implemented in the limma package. The resulting p-values were corrected for multiple testing to control the false discovery rate (FDR) using the Benjamini-Hochberg method. We selected probes with a fold change > 1.5 (corresponding to a 1.5 standard deviations) and a study-wide FDR <0.05 as differentially expressed.

Results

During the research period, 588 patients were recruited to the larger study. Of these, 234 patients (43%) had a suitable pelvic ultrasound scan available and were included in the current study. Patient demographics and characteristics from these 234 patients are presented in Table 1. SEOA was detected in 35% of women included in this study. 69.2% of patients were found to have endometriosis at surgery (of whom 37.7% also had SEOA). Of the patients who did not have endometriosis at the time of surgery,

29.2% had SEOA. In addition, 25% of patients who did not have endometriosis at the time of surgery had adhesions; of these, SEOA was only noted in 16.7% of patients prior to surgery. No etiology for pelvic pain was found in 51.4% of those patients without endometriosis (15.8% of the entire cohort). Ten patients had a hysterectomy at the time of laparoscopy. In one of these 10 patients, SEOA was not observed prior to surgery and this was confirmed by histopathology. The remaining nine patients were all diagnosed with SEOA prior to surgery, of whom eight were confirmed by histopathology.

The average age of women included in this study was 30.6 years. Patients with SEOA were 5.4 years older than patients with no SEOA ($p=0.02$). Patients with SEOA were more likely to be parous. A history of 2 or more previous deliveries was noted in 30% and 9.8% of patients with and without SEOA, respectively ($p<0.001$). BMI, ancestry, smoking status and hormone use were not statistically different between women with and without SEOA.

As expected, the majority of women in this study cohort reported a current or past experience of severe dysmenorrhea (94.8%). The age at which women had their first experience of period pain did not differ between those with/without SEOA. Rates of a current or past history of dyspareunia were significantly higher in patients with SEOA.

The most prevalent sonographic features noted in women with SEOA were heterogenous myometrium and thickened posterior wall (Figure 2). In contrast, thickened anterior wall was encountered less often in this cohort of women. Thickened posterior wall was significantly associated with moderate to severe (stage 3-4) endometriosis ($p=0.02$). Myometrial cysts, thickened anterior wall and thickened posterior wall were all significantly associated with dyspareunia ($p=0.006$-0.02). Thickened posterior wall combined with heterogenous myometrium, found in 15.9% of cases, was the most common combination of sonographic features. Thickened

posterior wall combined with linear striation and thickened posterior wall combined with myometrial cysts were found in 12.2% and 11% of cases, respectively.

Uterine pathologies were more common in women with SEOA (Table 3). Uterine volume was 60% larger in women with adenomyosis as compared to women without SEOA ($p<0.001$). Fibroids were found in ultrasounds of 15.8% of the patients. Fibroids, in particular intramural fibroids, were more likely to be found in patients with SEOA ($p=0.01$).

Table 2 presents further associations between adenomyosis and endometriosis, and endometriosis severity. As expected in this cohort of patients attending a tertiary gynaecology clinic, both adenomyosis and non-adenomyosis groups demonstrated high, yet not significantly different, rates of surgically proven endometriosis. Patients without SEOA were more likely to have minimal (stage 1) endometriosis and patients with SEOA were more likely to have severe (stage 4) endometriosis ($p<0.001$). In addition to the rAFS classification, presence/absence of DIE was recorded as an alternative method of defining disease severity. Patients with SEOA were also more likely to have DIE as well as other markers of severe disease such as endometriomas and bilateral endometriomas ($p<0.001$).

Adenomyosis case/control gene expression analysis

We analysed adenomyosis cases and controls for differences in the mean expression of genes/probes expressed in 90% of samples. After correction for effects of stage of the menstrual cycle and / or presence/absence of endometriosis, and multiple testing with Benjamini-Hochberg FDR < 0.05, there were no genes with significantly different gene expression between adenomyosis cases and controls.

Discussion

We demonstrated a significant association between SEOA and surgically-proven severe endometriosis. As a large proportion of women attending our pelvic pain clinic have sonographic signs of adenomyosis (35%), we suggest that women presenting to a pelvic pain clinic would benefit from an ultrasound prior to surgery for investigation of adenomyosis. Our finding of high rates of SEOA should also encourage doctors to specifically discuss adenomyosis and management options with patients. Some of those treatments, including the levonorgestrel-releasing intra-uterine system that could be inserted during laparoscopy, may ultimately reduce the later need for hysterectomy [18, 19].

Ours is the first study to specifically assess the association of SEOA and the severity of endometriosis in patients referred to a tertiary-level surgical clinic with an endometriosis and pelvic pain focus. Earlier work which aimed to investigate the prevalence of adenomyosis in a large population attending a general gynecological clinic, demonstrated a 21% rate of SEOA [15]. Unlike our study, pain was the primary indication for scanning in only 25% of patients. The authors reported an association of endometriosis with adenomyosis however, they did not elaborate on the clinical or surgical severity of endometriosis and its association with adenomyosis.

This study has focused on the utility of SEOA, rather than on histopathological confirmed adenomyosis. The most prevalent sonographic features of adenomyosis in our study were posterior uterine wall thickening and heterogenous myometrium, followed by sub-endometrial linear striation. These features have been shown to be strongly linked to histologically-proven adenomyosis [8]. Previous studies suggest a positive correlation between the number of sonographic features of adenomyosis and severity of menstrual pain and that could be used to establish a classification system of adenomyosis severity [20]. We have also observed associations between specific sonographic features of adenomyosis and clinical traits (i.e. dyspareunia,

endometriosis and uterine fibroids), lending further support to the concept of utilising sonographic features to improve the predictive capacity of adenomyosis and associated co-morbidities.

A positive correlation between SEOA and severe endometriosis was evident through comparison with rAFS scores as well as via markers of severe disease such as deep infiltrating endometriosis and endometriomas [21]. Since there is no good clinical correlation between symptoms and endometriosis severity [22], identification of preoperative markers could potentially play an important role in predicting disease severity and planning management. While deep endometriosis lesions can be identified by skilled sonographers, this is largely limited to rectal endometriosis [23, 24], thus there is a need for additional sonographic traits that can be used in a wider range of clinical situations. Relative to endometriosis, sonographic features of adenomyosis are easier to identify and hence are useful and accessible pre-surgical predictors of endometriosis severity.

While adenomyosis is common in women aged over 40 years and previous reports suggest that the condition is less common in younger patients [25], our data highlights that SEOA is not uncommon in younger women presenting for tertiary-level care. Various studies have speculated that molecular pathways are altered with adenomyosis [26-30]. For instance, differential gene and long non-coding RNA expression was reported in eutopic endometrium from women with/without adenomyosis [29, 30]; it was hypothesised that these endometrial abnormalities may predispose to disruption of the myometrial interface and subsequent formation of adenomyosis. However, we found no significant differences in gene expression between patients with or without adenomyosis, which may reflect our sample size, the stringency of our protocols to minimise type I statistical errors relative to other studies, and/or the extent of disease progression and presence of comorbidities in this cohort.

In combination, however, the variations among studies highlight the need for focused research on the molecular pathways influenced by adenomyosis in eutopic and adenomyotic endometrium.

In addition to endometriosis, studies have suggested shared molecular and genetic mechanisms of adenomyosis and other uterine and pelvic pathologies such as uterine fibroids [31-33]. This is supported by the significant associations found in our study between SEOA, increased uterine volume and uterine fibroids. While an increased uterine volume is not considered pathological, this is a feature that could relatively easily be measured and it may be that the combination of pain and increased uterine volume should prompt referral to a tertiary level scan.

Patients in our study were recruited from a surgical clinic focused on patients referred due to pelvic pain. Further studies are needed to explore whether the associations shown in our study are limited to patients with SEOA and pain or can be generalised to all patients with adenomyosis. We have analysed all patients who met the inclusion criteria within the study time frame. However, further prospective studies designed specifically to assess the association of SEOA, formal diagnosis of adenomyosis, gene/protein expression patterns and severe endometriosis are needed. One of the other limitations of the study is the retrospective analysis of stored ultrasound images for diagnosis of SEOA, rather than real-time imaging. This analysis relied on the operator having recorded images of focal features of adenomyosis if present, and performing a dynamic evaluation. As the patients assessed in this study are patients who have attended our endometriosis and pelvic pain clinic, all were thoroughly assessed for presence of adenomyosis and multiple good-quality images were available for each patient. It should again be noted that we have assessed ultrasound evidence of adenomyosis and not histologically confirmed adenomyosis.

Adenomyosis continues to be a poorly understood gynecological phenomenon. While its molecular and genetic mechanisms are not yet known, our findings demonstrate that sonographic evidence of adenomyosis is a highly important clinical entity for patients undergoing investigation for presence of endometriosis. As such, both early diagnosis and revealing the linkage between adenomyosis, endometriosis and other major causes of pelvic pain may have significant implications for patient care. Our findings might support the use routine pre-operative sonographic assessment of adenomyosis in women with pelvic pain. Further research is needed to characterise the complex association and predictive value of SEOA and to explore specific molecular and genetic pathways involved in its pathogenesis.

Precis: Sonographic evidence of adenomyosis in patients undergoing surgery for investigation of suspected endometriosis is associated with severe endometriosis

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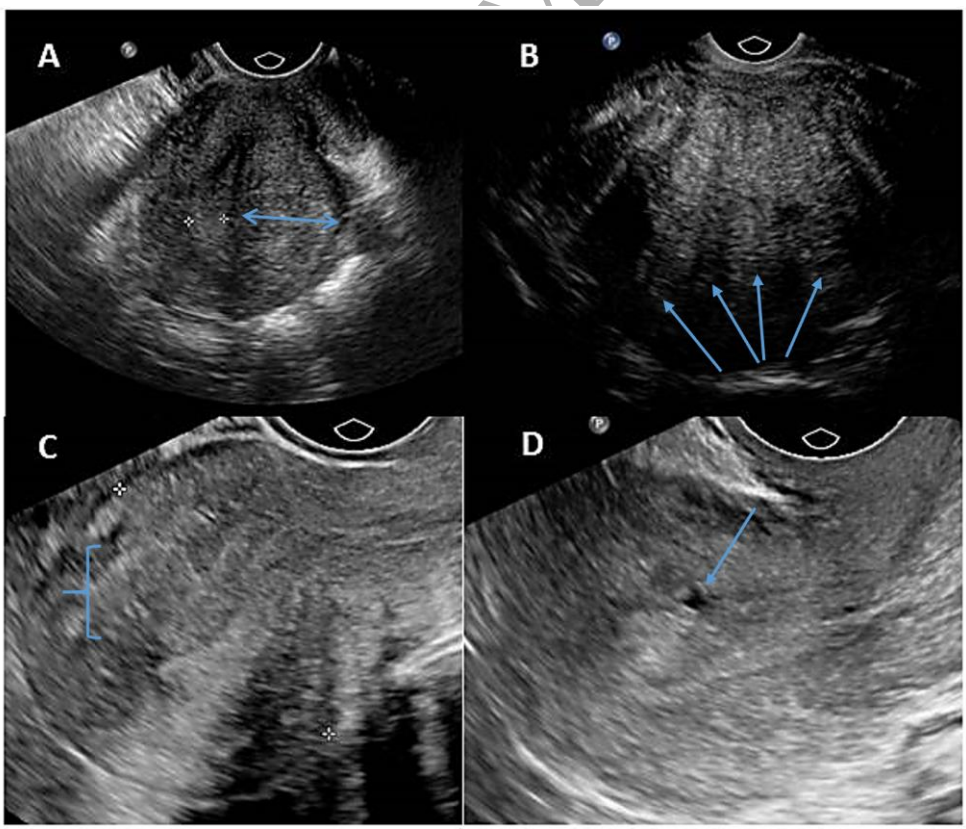

Figure 1. Sonographic Features of adenomyosis

A- Posterior wall thickening, B- Linear striations, C- Heterogeneous myometrium, D- Myometrial cysts

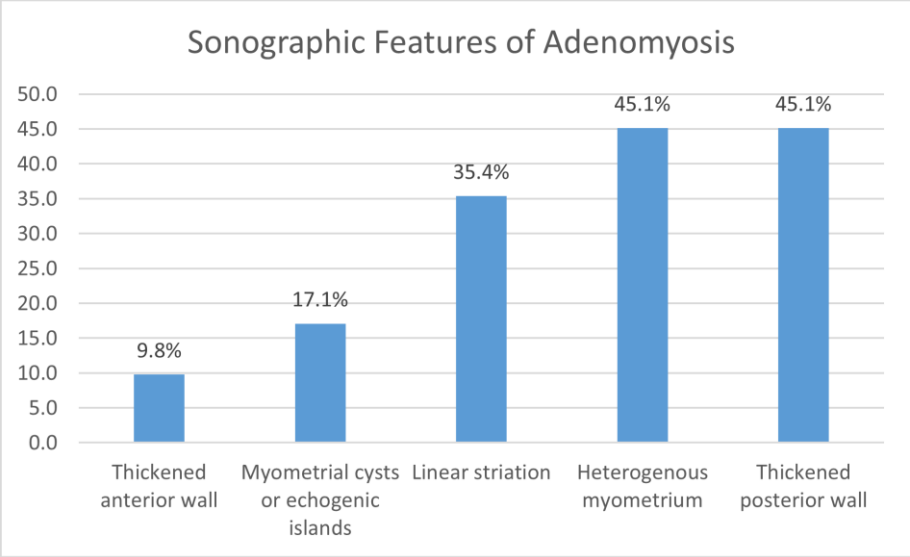

Figure 2. Distribution of sonographic features of adenomyosis

Rates of specific sonographic features of adenomyosis in patients presenting to surgery for investigation of endometriosis.

Table 1: Selected demographic and general characteristics of patients (n=234 unless otherwise stated)

		Total cohort	Adenomyosis	No Adenomyosis	P-value
Age (years, mean (SD))		30.6 (7)	34.1 (6.1)	28.7 (7.4)	<0.001
BMI (kg/M^2, mean (SD))		25.9 (6)	25.8 (5.6)	25.9 (6.2)	0.88
Ancestry (%) (n=223)	Europe	29.1	27.8	29.9	0.98
	Asia	9.4	8.9	9.7	
	America	2.2	2.5	2.1	
	Australia/ New Zealand	49.3	49.4	49.3	
	Middle-East/ Africa	9.9	11.4	9.0	
Smoking Status (%)	History of smoking	47.0	40.0	50.7	0.13

	Currently smoking (n=166)	44.6	45.3	44.2	1.00
Gravidity (%)	0	60.6	53.1	64.7	<0.001
	1	13.9	6.2	18.0	
	≥ 2	25.5	40.7	17.3	
Parity (%) (n=202)	0	68.3	51.4	77.3	<0.001
	1	14.9	18.6	12.9	
	≥ 2	16.8	30.0	9.8	
Hormone therapy (%) (n=175)		62.3	60.7	63.0	0.87
Has had experience of severe dysmenorrhea (%)		94.8	93.9	95.3	0.76
Has had experience of dyspareunia (%)		80.9	87.8	77.0	0.05
Has had experience of severe non-menstrual pelvic pain (%)		84.3	88.8	81.9	0.19
Intermenstrual vaginal bleeding (%) (n=216)		42.9	48.6	44.4	0.57
Age at menarche (years, mean (SD))			12.6 (1.7)	12.6 (1.7)	0.98
Infertility*		27.5	34.8	21.4	0.18
Family history of endometriosis*		25.4	28.9	24.7	0.52

* Out of women who have attempted to conceive (n=102)

Table 2. Association of adenomyosis with endometriosis severity

		Adenomyosis (n=82)	No Adenomyosis (n=152)	p- value
Endometriosis* (%[1], (n))		74.4 (61)	66.4 (101)	0.24
Endometriosis stage (%[2], (n))	Stage 1	27.9 (17)	60.8 (62)	<0.001
	Stage 2	13.1 (8)	13.7 (14)	
	Stage 3	18.0 (11)	15.7 (16)	
	Stage 4	41.0 (25)	9.8 (10)	
Endometriomas (%[1], (n))		39.0 (32)	15.1 (23)	<0.001
Bilateral Endometriomas (%[1], (n))		14.6 (12)	2.6 (4)	0.001
Deep Infiltrating Endometriosis (%[1], (n))		42.5 (34)	19.7 (30)	<0.001

* Surgically confirmed Endometriosis

[1]Percentage of total cohort

[2]Percentage of those with endometriosis

Table 3. Uterine imaging findings in patients with or without adenomyosis

		Total cohort	Adenomyosis	No Adenomyosis	p- value
Uterine volume (cm^3)		78.6 (69.0)	102.9 (78.0)	66.4 (60.7)	<0.001
Uterine position (%)	Anteverted	84.3	79.7	86.6	0.24
	Retroverted	15.7	20.3	13.4	
Fibroids (%)	All	15.8	23.2	11.8	0.04
	Submucosal	2.1	2.4	2.0	1.00
	Intramural	12.8	20.7	8.6	0.01
	Subserosal	3.4	3.7	3.3	1.00