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Autoantibodies common in patients with gastrointestinal diseases are not found in patients with endometriosis: A cross-sectional study

Malin Ek[a,*], Bodil Roth[a], Lil Valentin[b], Johanna Nordengren[b], Bodil Ohlsson[a]

[a] Department of Clinical Sciences, Division of Internal Medicine, Skåne University Hospital, Lund University, Malmö, Sweden
[b] Department of Obstetrics and Gynecology, Lund University, Skåne University Hospital, Malmö, Sweden

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ABSTRACT

Objectives: Gastrointestinal symptoms are common in endometriosis, but the mechanisms behind these symptoms are yet poorly understood. Associations between endometriosis and irritable bowel syndrome (IBS), celiac disease, and various autoimmune diseases have been reported. These diseases express characteristic autoantibodies. The aim of the current study was to investigate autoantibodies against gonadotropin-releasing hormone 1 (GnRH1) and luteinizing hormone (LH) and their receptors, tenascin-C, matrix metalloproteinase-9, deamidated gliadin peptide, and tissue transglutaminase in a cohort of women with endometriosis, compared to controls and women with IBS or enteric dysmotility.
Study design: One hundred seventy-two women with laparoscopy-verified endometriosis completed questionnaires regarding socio-demographics, lifestyle habits, medical history, and gastrointestinal symptoms, and sera were analyzed with ELISA for the abovementioned antibodies. Healthy female blood donors (N = 100) served as controls, and women with IBS or enteric dysmotility (N = 29) were used for comparison.
Results: A non-significantly higher prevalence of IgM antibodies directed at tenascin-C (7.6% vs. 2.0%; p = 0.06) was the only observed difference in autoantibody levels in endometriosis compared to controls. Antibody presence was not associated with any clinical parameters. Patients with IBS or enteric dysmotility expressed higher levels of IgM antibodies against GnRH1 compared to both patients with endometriosis (p = 0.004) and healthy controls (p = 0.002), and higher levels of tenascin-C antibodies compared to healthy controls (17.2% vs. 2.0%; p = 0.006).
Conclusions: Women with endometriosis do not express higher prevalence of autoantibodies found to be characteristic in other patient groups with gastrointestinal symptoms.

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Introduction

Gastrointestinal (GI) symptoms similar to those of irritable bowel syndrome (IBS) are common in endometriosis and have been reported to occur almost as frequently as gynecological symptoms [1–3]. The mechanisms behind these symptoms have not yet been elucidated [4]. However, endometriosis has been reported to be associated with both celiac disease (CD) [5] and inflammatory bowel disease (IBD) [6].

Gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) regulate the reproductive cycle [7], but GnRH and LH receptors (LH-R) are also present in the enteric nervous system (ENS) [8]. IgM antibodies against GnRH1 and its receptor (GnRH-R) have been observed in patients with IBS and enteric dysmotility (ED) [9], suggesting enteric neuropathy as a causal mechanism. Sporadic cases of severe GI symptoms associated with IgM antibodies against GnRH1 have been reported after treatment with GnRH analogs [8], and patients treated with GnRH analogs in the current cohort experienced aggravated abdominal pain [2,3].

Tenascin is an extracellular matrix glycoprotein involved in cell differentiation, proliferation, and migration [10]. Elevated expression of tenascin has been reported in endometriosis [11,12] and is hypothesized to be linked to development of the disease. Tenascin-C (TN-C) is a member of the tenascin family. There is a sparse expression in humans [13], but TN-C has been identified in the human endometrium [14], and serum levels are increased in ulcerative colitis (UC) [15].

Matrix metalloproteinases (MMPs) are a family of endopeptidases, involved in degrading extracellular matrix (ECM) [16]. TN-C can be cleaved by MMPs, which may affect TN-C function [13]. Increased levels of MMP-9 have been reported in ectopic

* Corresponding author at: Department of Clinical Sciences, Skåne University Hospital, Jan Waldenström gata 15, floor 5, S-205 02, Malmö, Sweden.
E-mail address: Malin.Ek@med.lu.se (M. Ek).

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endometrium [16] and in peritoneal and follicular fluid in endometriosis [17]. Mucosal expression and serum levels of MMP-9 are elevated in patients with IBD, and the levels are associated with the disease activity [18]. Duodenal mononuclear cells in CD express a basal pattern of MMP dominated by MMP-9 and MMP-12 [19].

Thus, endometriosis has been linked to increased levels of tenascin and MMP-9 [11,12,14,16,17], tissue factors that are associated with GI symptoms [15,18,19]. GnRH analogs are used to treat endometriosis, and GnRH and LH have been linked to the function of the GI tract [8,9]. However, no previous studies have investigated the connection among endometriosis, GI symptoms, and autoantibodies against these factors.

Our overall goal is to explore the underlying pathophysiology of GI symptoms in endometriosis and to be able to separate endometriosis-related GI symptoms and IBS, because these conditions demand quite different treatments. The specific aim of the present study was to investigate the prevalence of autoantibodies against GnRH1, GnRH-R, LH, LH-R, TN-C, MMP-9, deamidated gliadin peptide (DGP), and tissue transglutaminase (tTG) in a cohort of 172 women with endometriosis, compared to controls and women with IBS/ED.

Material and methods

Subjects

Endometriosis patients were recruited from the Department of Gynecology at Skåne University Hospital, Malmö, Sweden. Patients were identified by using the International Statistical Classification of Diseases and Related Health Problems, ICD-10, N 80. The recruitment process was conducted between March 2013 and July 2014, and between September 2016 and March 2017. The inclusion criterion was a definitive diagnosis of endometriosis confirmed by laparoscopy. Exclusion criteria were: living too far from the geographical area of the hospital, an uncertain diagnosis, multiple comorbidities of severe somatic or mental illnesses, IBD, or current pregnancy.

The control group for analysis of antibodies directed at GnRH1, GnRH-R, LH, LH-R, TN-C, and MMP-9 consisted of 100 healthy female blood donors, median age 42.5 (30.0–53.0) years.

Samples from women with IBS or ED (N = 29), median age 34.0 (25.5–51.5) years, were used for comparison of antibody expression against GnRH1, GnRH-R, TN-C and MMP-9. The group consisted of IBS with mixed bowel habits (IBS-M) (N = 11), constipation-predominant IBS (IBS-C) (N = 6), diarrhea-predominant IBS (IBS-D) (N = 2), and ED (N = 10) [9].

Study design

Patients were interviewed and completed a study questionnaire and *the Visual Analogue Scale for Irritable Bowel Syndrome* (VAS-IBS). Medical journals were scrutinized. Blood samples were drawn and sera were immediately separated (1500 G) for 15 min at room temperature (RT) and kept frozen at −20 °C. Sera were analyzed for antibodies against GnRH1, GnRH-R, LH, LH-R, TN-C, MMP-9, DGP, and tTG. Female blood donors served as controls, except when analyzing antibodies against DGP and tTG, for which control values were provided by the manufacturer. Samples from women with IBS/ED were used for comparison in analyses of antibodies against GnRH1, GnRH-R, TN-C, and MMP-9.

Questionnaires

The study questionnaire consisted of questions regarding sociodemographics, lifestyle habits, medical history, and pharmacological treatment. VAS-IBS is a validated questionnaire used to investigate the GI symptoms of abdominal pain, diarrhea, constipation, bloating and flatulence, vomiting and nausea, psychological well-being, and intestinal symptoms' influence on daily life. The items are measured on a scale from 0 mm to 100 mm, where 100 mm represents very severe symptoms and 0 mm a lack of symptoms. The values are inverted from the original format [20].

Immunological analyses

Analyses of antibodies against GnRH1, GnRH-R, LH, and LH-R were conducted by in-house enzyme-linked immunosorbent assays (ELISAs) as previously described [9,21].

An in-house ELISA was set up for analyses of IgM and IgG autoantibodies against TN-C. Microtiter plates (442404, Nunc, Roskilde, Denmark) were coated with recombinant TN-C (MBS1265425, Mybiosource, San Diego, CA, USA) in PBS-T, or in PBS-T only (to provide an internal blank), and incubated at 4 °C overnight. After incubation, the microtiter plates were washed three times with PBS-T and blocked with 1.0% BSA (A7030, Sigma, St. Louis, USA) in PBS-T. Dilutions of serum of 1:400, or rabbit anti-human TNC antibody (MBS5303408, Mybiosource) (in serial dilution to construct a standard curve) with BSA in PBS-T, were then added to the microtiter plates and incubated for 1.5 h at RT. The washing procedure was repeated, and deposition of antibodies against TN-C was detected using horseradish peroxidase (HRP)–conjugated IgG (P0214, DAKO, Glostrup, Denmark). A TMB peroxidase substrate system (2-C) (50-76-00 KPL, Careforde, Chicago, IL, USA) 1:1 was used to develop a color reaction.

Another in-house ELISA was set up for analysis of IgM-, IgG-, and IgA antibodies against MMP-9. Microtiter plates (442404, Nunc) were coated with a recombinant MMP-9 (Pierce RP-75655 lot no. QA 1951751, Thermo Scientific, Rockford, IL, USA) in PBS-T or in PBS-T only. After overnight incubation at 4 °C, the plates were washed three times with PBS-T and blocked with 0.5% BSA (A7030, Sigma) in PBS-T. Dilutions of serum of 1:1000 (IgM and IgG), 1:200 (IgA), or goat IgG anti-human MMP-9 antibody (ab38898, Abcam, Cambridge, MA, USA) in serial dilution (to construct a standard curve) with BSA in PBS-T, were then added to the plates in triplicate (two wells coated with MMP-9 and one well coated with PBS) and incubated for one hour at RT. The washing procedure was repeated, and deposition of autoantibodies against MMP-9 was detected using HRP-conjugated IgM-, IgG-, or IgA antibodies (P0214, P0216, and P0215, respectively, DAKO) or goat anti-rabbit IgG antibodies (P0448, DAKO) appropriately diluted in PBS-T. To develop a color reaction, a TMB peroxidase substrate system (2-C) (50-76-00, KPL) 1:1 was used.

The absorbance at 450 nm was measured after 30 min of incubation at RT. Antibody levels are presented as relative units (RU), and the concentration in each doublet is interpolated from the standard curve. The cut-off values to determine presence of antibodies was defined as RU > 97.5th percentile in the control group of 100 healthy female blood donors.

A combination of IgG anti-DGP and IgA anti-tTG showed very high specificity and a greater sensitivity than one single test in screening [22], why the Celiac Fusion™ (Immco Diagnostics Inc., Buffalo, NY, USA) solid-phase immunoassay was used according to the manufacturer's instructions [23]. Results are expressed as ELISA units/milliliter and reported as positive or negative (qualitative determination). According to the manufacturer (Immco), six of 112 healthy men and women (5.4%) are positive in this test.

Intra-assay and inter-assay coefficients of variance (CV) are shown in Supplementary Table 1.

Statistical methods

The SPSS for Windows (release 22.0; IBM) statistical software package was used to analyze data. As normality was rejected, the

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Table 1
Patient characteristics.

	Endometriosis N = 172
Age (years)	38.0 (32.0–43.0)
Disease duration (years)	11.0 (5.0–18.0)
Abdominal pain (mm)	40.0 (10.0–72.0)
Diarrhea (mm)	15.0 (0.0–55.0)
Constipation (mm)	28.0 (0.0–69.5)
Bloating and flatulence (mm)	55.0 (17.5–80.0)
Vomiting and nausea (mm)	9.0 (0.0–45.0)
Psychological well-being (mm)	30.0 (8.0–63.5)
Symptoms influence on daily life (mm)	40.0 (8.5–75.0)
Defecation urgency (n, %)	61 (35.5)
Sensation of incomplete evacuation (n, %)	95 (55.2)

On the inverted visual analogue scale for irritable bowel syndrome (VAS-IBS) (Ref No. 20), 0 mm represents absence of symptoms and 100 mm very severe symptoms. Values are given as median (interquartile range) and number (%).

Mann-Whitney U test was used to calculate differences in continuous variables between groups. Dichotomous variables were analyzed by using Fisher's exact test. Data is presented as median (interquartile range [IQR]) or numbers and percentages (n, %). $P < 0.05$ was considered statistically significant.

Ethical approval

This study was approved by the Ethics Review Board of Lund University, 2012/564 (09,102,012), 2016/56 (09,052,016) and 320-03 (06,102,003) and performed in accordance with the Declaration of Helsinki. All subjects gave their written, informed consent before inclusion.

Results

Subject characteristics

Patients with endometriosis, median age 38.0 (32.0–43.0) years, were included in two time periods. First, 307 women fulfilling the inclusion criterion were identified. Of those, 145 women declined to participate, 49 women had moved from the region, four women denied the diagnosis, and nine women were excluded because of an uncertain diagnosis, leaving 100 women included. Second, 266 women were identified. Of those, 162 women declined to participate, 23 women had moved from the region, and nine women had an uncertain diagnosis, leaving 72 women included.

In the cohort, 66 women (38.4%) had an isolated ovarian endometriosis, whereas 90 women (52.3%) had lesions in other anatomical localizations in the pelvic cavity, such as in the bowel, peritoneum, posterior vaginal wall, pouch of Douglas, rectovaginal septum, urine bladder, or uterosacral ligaments (with or without coexisting ovarian endometriosis). The remaining 12 women (7.0%) had lesions in other localizations, such as in the groin, rectus musculature, umbilicus, or surgical scars, and four women (2.3%) had lesions in unspecified locations. Disease duration was 11 [5–18] years.

Patients with endometriosis expressed several GI symptoms (Table 1). More than one-third of the patients had undergone serological screening for CD, without being diagnosed with CD. More than one-tenth of the patients perceived that gluten-containing foods worsened GI symptoms and therefore consumed a gluten free- or gluten-reduced diet.

In endometriosis, 46.5% were currently using systemic hormonal treatment, most commonly estrogen, including combined oral contraceptives (Table 2). Five women used an intrauterine device containing progestogen. The most common non-hormonal pharmacological treatments in both patient cohorts were antidepressant drugs, followed by nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids (Table 2).

Autoantibody measurements

All antibodies were first analyzed in the initial 100 women. The antibodies were further analyzed in the second cohort, when a tendency toward significant differences compared to controls was found. There was a non-significantly higher prevalence of TN-C in endometriosis patients than in controls (7.6% vs. 2.0%, $P = 0.06$). Patients with IBS/ED showed higher expression of IgM antibodies directed against GnRH1 than did either the endometriosis group or the controls ($P = 0.004$ vs. $P = 0.002$) and against TN-C compared to controls ($P = 0.006$). The remaining antibodies were found in low prevalence (Table 3). Among endometriosis, 32 patients (19.0%) expressed one or more of the abovementioned antibodies, compared to 19 controls (19.0%, $P = 1.00$) and 10 IBS/ED patients (34.4%, $P = 0.08$).

Patients with endometriosis and presence of TN-C antibodies ($N = 13$) did not differ in basal characteristics, disease duration, or GI symptoms compared to patients without any TN-C antibodies ($N = 155$) (Supplementary Table 2). However, patients with pelvic endometriosis ($N = 90$) (with or without ovarian endometriosis) had a tendency toward higher prevalence of TN-C antibodies than did women with isolated ovarian endometriosis (11.1% vs. 3.0%, $P = 0.06$).

Autoantibodies against DGP and tTG were found in low prevalence among endometriosis patients ($N = 4$; titer 6.5 (5.4–7.8) RU; $P = 0.75$).

Table 2
Pharmacological treatment in patients.

	Endometriosis N = 172	IBS or ED N = 29
Current hormonal treatment (n, %)	80 (46.5)	1 (3.4)
Estrogens	42 (24.4)	1 (3.4)
Progestogens	30 (17.4)	0 (0)
GnRH analogs	15 (8.7)	0 (0)
Other current pharmacological treatment (n, %)		
SSRIs & SNRIs	33 (19.2)	4 (13.8)
NSAIDs	33 (19.2)	0 (0)
Opioids	30 (17.4)	2 (6.9)
Paracetamol	28 (16.3)	0 (0)
Levothyroxine	19 (11.0)	0 (0)
Allergy and asthma medication	19 (11.0)	0 (0)
Laxatives and bulking agents	12 (7.0)	0 (0)
Hypertension medication	9 (5.2)	0 (0)
PPIs	6 (3.5)	7 (24.1)

ED = enteric dysmotility, GnRH = gonadotropin-releasing hormone, IBS = irritable bowel syndrome, NSAID = nonsteroidal anti-inflammatory drugs, PPI = proton pump inhibitors, SSRI = selective serotonin reuptake inhibitor, and SNRI = serotonin-norepinephrine reuptake inhibitor. Estrogens include use of combined oral contraceptives and pure estrogen. Values are given as numbers and percentages.

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Table 3
The prevalence of autoantibodies against gonadotropin-releasing hormone 1 (GnRH1); luteinizing hormone (LH); and their receptors (GnRH-R and LH-R), tenascin-C (TN-C), and matrix metalloproteinase-9 (MMP-9).

	Endo N = 100/172*	Controls N = 100	IBS or ED N = 29	Endo vs. controls, P-value	Endo vs. IBS/ED, P-value	IBS/ED vs. controls, P-value
Age (years)*	38.0 (32.0–43.0)	42.5 (30.0–53.0)	34.0 (25.5–51.5)	**0.006**	0.96	0.36
GnRH1 IgM n, (%)	3 (3.0)	2 (2.0)	6 (20.7)	1.00	**0.004**	**0.002**
GnRH1 IgG n, (%)	1 (1.0)	2 (2.0)	1 (3.4)	1.00	0.40	0.54
GnRH-R IgM n, (%)	4 (4.0)	2 (2.0)	3 (10.3)	0.68	0.19	0.08
GnRH-R IgG n, (%)	4 (4.0)	2 (2.0)	2 (6.9)	0.68	0.62	0.22
LH IgM n, (%)	3 (3.0)	2 (2.0)		1.00		
LH IgG n, (%)	2 (2.0)	2 (2.0)		1.00		
LH-R IgM n, (%)	2 (2.0)	2 (2.0)		1.00		
LH-R IgG n, (%)	0 (0)	2 (2.0)		0.50		
TN-C IgM* n, (%) Missing value = 4	13 (7.6)	2 (2.0)	5 (17.2)	0.06	0.15	**0.006**
TN-C IgG n, (%)	0 (0.0)	2 (2.0)		0.50		
MMP-9 IgA n, (%)	1 (1.0)	2 (2.0)		1.00		
MMP-9 IgM n, (%) Missing value = 1	1 (1.0)	2 (2.0)		1.00		
MMP-9 IgG* n, (%) Missing value = 5	10 (5.8)	2 (2.0)	1 (3.4)	0.22	1.00	0.54

* = 172 patients compared to 100 patients in other analyses. Endo = endometriosis, ED = enteric dysmotility, IBS = irritable bowel syndrome. Relative units >97.5th percentile in a cohort of 100 healthy female blood donors were considered as presence of antibodies. Values are given as median (interquartile range) or numbers (%). Fischer's exact test was used to calculate differences between groups. $P < 0.05$ was considered statistically significant.

Comments

The present study could not show significantly higher prevalence of the examined autoantibodies in endometriosis. High antibody prevalence suggests a model for molecular involvement in the pathophysiology, in addition to being a diagnostic tool. Of the 13 analyzed antibodies, only TN-C was elevated in a relatively low prevalence (7.6%), which disqualified it as a clinically useful biomarker. On the other hand, higher prevalence of GnRH1 and TN-C IgM antibodies were found in women with IBS/ED.

The pathophysiology of endometriosis and its related GI symptoms is not yet clearly elucidated. The theory of retrograde menstruation is widely accepted as an explanation for dissemination of endometrial cells, and altered immune activity and endometrial factors are discussed in relation to establishment of endometriosis lesions [24]. Recent studies highlight the importance of the role of direct cell–cell interactions in the endometrium [25] and non-coding RNAs [26] as possible pathophysiological mechanisms. There are obvious signs of an inflammatory reaction in endometriosis, either as a primary cause or as a secondary effect [27].

Women considered for in vitro fertilization (12% endometriosis) had a higher prevalence of IgM GnRH-R, IgG LH, and IgG LH-R autoantibodies than controls did [21]. Some women have developed GI dysmotility and GnRH antibodies, with almost total loss of GnRH-containing enteric neurons, following treatment with GnRH analogs [8]. Accordingly, women treated with GnRH analogs for endometriosis in the present cohort experienced aggravated GI symptoms [2,3]. However, no GnRH1 antibodies could be demonstrated, in contrast to patients with IBS and GI dysmotility [8,9]. GI symptoms in endometriosis may be caused by inflammatory activity [27,28], whereas GI symptoms in IBS/ED may depend on neurodegeneration [8,9]. Since IBS is a symptom-based disease [29], similar symptoms may be called IBS despite quite different etiologies.

Our previous studies have shown that the location of endometriosis lesions did not affect the degree of GI symptoms, but treatment with opioids promptly aggravated the symptoms [3]. Thus, reduction of opioid treatment should be the first choice of treatment, before evaluating GI symptoms further. Furthermore, the impaired psychological well-being strongly correlated to GI symptoms [3], which may be another etiologic factor in symptoms. An improvement of quality of life and sexual function in women with endometriosis and pelvic pain has been described in relation to treatment with a continuous regimen of combined oral contraceptives containing dienogest and ethinyl estradiol [30,31]. Studies have demonstrated an involvement of estrogen in maintaining mucosal barrier function of the GI tract and also in modulating intestinal inflammation in intestinal inflammatory diseases [32]. In IBS, a disease with female predominance, evidence suggests that estrogen and progesterone influence regulatory mechanisms of the brain–gut axis, affecting motility, permeability, and visceral hypersensitivity [33]. Thus, hormonal treatment may hypothetically have an impact on GI symptoms through its effect on the GI tract, alleviating endometriosis symptoms.

Tenascin is present in stromal cells in the human endometrium, with higher levels in endometrial implants than in normal endometrium [12]. Tenascin subtype C is expressed in a few connective tissues in human adults, underneath some epithelia and in certain stem cell niches [13], and is upregulated in inflammation [34]. TN-C is also expressed in human cancers [13], where it is associated with migration, angiogenesis, and cell proliferation, factors also associated with endometriosis [35]. TN-C has been identified in the human endometrium and is increased in the proliferative phase [14]. In addition, increased TN-C levels in IBD are associated with disease activity [15,36]. The present study is the first, to our knowledge, to analyze autoantibodies against TN-C in a pure endometriosis cohort. However, the slightly higher levels of IgM antibodies against TN-C did not show association with GI symptoms or disease characteristics.

MMP-9 contains a hemopexin domain previously recognized by autoantibodies found in endometriosis [37], not detected by the present ELISA. An increased risk of endometriosis in women with CD has been reported [5], and both conditions may present with primary infertility [38]. No increased prevalence of autoantibodies against DGP and tTG was found. A proportion of the patients were already excluding gluten from their diet or reducing gluten intake, which may render an absence of antibodies [22].

This study has several limitations. The controls were not age-matched, and endometriosis patients were significantly younger than controls. Antibodies may be more prevalent in older patients [39]. Therefore, the difference between patients and controls may be greater if age-matched controls are used. Position in the menstrual cycle was not known in the present study, a factor that could hypothetically affect results. However, almost half of the patients were using systemic hormonal treatment, affecting menstruation. The stage of endometriosis was not known, so this could not be evaluated in regard to the presence of antibodies.

GI symptoms in endometriosis cannot be explained by the presence of serum antibodies directed at GnRH1, GnRH-R,

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LH, LH-R, MMP-9, TN-C, DGP, or tTG. These results imply disparate mechanisms behind GI symptoms in endometriosis compared to other GI diseases. These findings need to be confirmed in further studies.

Conflicts of interests

The authors have no conflicts to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ejogrb.2019.05.040.

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