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Cytokine Levels and Symptoms Among Women with Irritable Bowel Syndrome: Considering the Role of Hormonal Contraceptive Use

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Abstract

Background: Young to middle-aged women are more likely than men to be diagnosed with irritable bowel syndrome (IBS). Immune dysfunction may be present in IBS, however, few studies have tested whether hormonal contraceptive use is linked to inflammatory markers. The purpose of this study was to compare cytokine levels between women (ages 18–45) with and without IBS and with and without hormonal contraceptive use and to examine the relationships of cytokine levels to IBS gastrointestinal (GI) and non-GI symptoms within those using and not using hormonal contraceptives. **Methods:** Seventy-three women with IBS and 47 healthy control women completed questionnaires (demographics, hormonal contraceptive use) and kept a 28-day symptom diary. Fasting plasma and LPS-stimulated pro-inflammatory (IL-1β, IL-6, IL-12p40, IL-12p70, IL-8, and TNF-α) and anti-inflammatory (IL-10) cytokines were assayed. **Results:** No differences were found in plasma or stimulated cytokine levels between IBS and control women. Levels of IL-1β ($p = 0.04$) and TNF-α ($p = 0.02$) were higher among women who did not use hormonal contraceptives compared to women who used hormonal contraceptives. Among women with IBS, significant correlations were found between daily psychological distress and plasma IL-10, IL-12p70, IL-1β, IL-6, and IL-8 cytokine levels. **Conclusions:** These results suggest that hormonal contraceptive use might reduce IL-1β and TNF-α cytokine levels in women with IBS. The impact of hormonal contraceptive use on innate immune activation among women with IBS requires further research.

Keywords

irritable bowel syndrome, cytokines, contraception, hormonal contraception, symptoms

Irritable bowel syndrome (IBS) is a functional gastrointestinal (GI) disorder that is characterized by abdominal pain and alterations in bowel habits (Drossman, 2016). IBS is diagnosed based on GI symptoms and can be categorized into subtypes based on predominant bowel habits including: diarrhea (IBS-D), constipation (IBS-C), or mixed bowel habits (IBS-M). Estimates of the prevalence of IBS range from 7% to 17% of adults in the U.S. (Lovell & Ford, 2012), making it a significant health concern for many people. IBS is diagnosed more often in women than men (Kim & Kim, 2018). In the U.S., expected healthcare costs were 37% higher for individuals with IBS compared to those without IBS (Poulsen et al., 2019). There is increasing acknowledgment that IBS is a heterogeneous condition with multiple factors contributing to its pathogenesis and clinical phenotypes. Immune system dysfunction including elevations in pro-inflammatory cytokines and decreases in anti-inflammatory cytokines has been suggested as one etiologic factor, at least for a subgroup of patients with IBS (Martin-Vinas & Quigley, 2016; Schmulson et al., 2012).

The immune system is likely important since inflammation can influence the function of smooth muscle and nerves, leading to dysmotility and pain (Collins et al., 2001; Cremon et al., 2009). Several studies indicate that low grade GI inflammation is related to abdominal pain among adult and pediatric IBS

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patients (Cremon et al., 2009; Liebregts et al., 2007; Shulman et al., 2008, 2014). Cytokine levels, measured in the serum, plasma, or in response to a standardized stimulus, have been examined in IBS groups (Bashashati, Moradi, & Sarosiek, 2017; Hua et al., 2011; Lazaridis & Germanidis, 2018; Liebregts et al., 2007). Elevations in cytokine levels indicate systemic inflammation and may contribute to IBS symptoms through the gut-brain axis (Bashashati et al., 2012; Choghakhori et al., 2017). The literature reveals inconsistencies as to whether pro- and/or anti-inflammatory cytokines are altered in IBS patients. A 2017 meta-analyses (n = 4 studies) found higher levels of pro-inflammatory cytokine IL-6 in persons with IBS (Standardized Mean Difference: 2.40 [95% CI: 0.53–4.28]; $p = 0.01$) relative to healthy control cohorts (Bashashati et al., 2017). An earlier (2014) systematic review focused on TNF-α and IL-10 (Bashashati et al., 2014). Examination of six studies measuring TNF-α found levels to be significantly higher in women with IBS as compared to men and control women. However, no consistent differences were found in circulating IL-10 levels between IBS and healthy controls (Bashashati et al., 2014). Understanding the potential role of immune dysfunction in relation to symptom phenotypes is still lacking in IBS (Lazaridis & Germanidis, 2018).

Sex hormones, including estrogen, have a potential modulating effect on inflammation and symptomatology in IBS as well as other chronic pain conditions (Cremon et al., 2009; Mulak et al., 2014; Ribeiro-Dasilva et al., 2017). In 48 patients with IBS, mucosal immune activation was higher in women than men, and mast cell infiltration was associated with greater GI symptoms in women with IBS (Cremon et al., 2009). In our previous study (Heitkemper et al., 2003), women with IBS taking oral contraceptives showed reduced monthly abdominal pain symptoms compared to women with IBS not taking oral contraceptives. Many review articles on sex and gender differences in IBS hypothesize a potential role of hormonal contraceptives (Bharadwaj et al., 2015; Kim & Kim, 2018); however, few studies have sought to examine this issue. Thus, the relationship between hormonal contraceptive use and cytokine levels in women with IBS remains under investigated. In a meta-analysis of cytokines in IBS (Bashashati et al., 2014), only one study controlled for menstrual cycle phase (Chang et al., 2012) even though women were the predominant sex examined in the IBS cohorts. There is a gap in our understanding whether exogenous hormonal use contributes to study differences in cytokine levels and ultimately discrepancies in the literature.

Increased levels of pro-inflammatory cytokines and decreased levels of anti-inflammatory cytokines have been associated with increased IBS symptoms (Dinan et al., 2008; Hua et al., 2011; Rana et al., 2012). However, Bennett and colleagues, when examining subgroups of "immune-active" and "immune-normal" individuals with IBS based on levels of unstimulated IL-1ß, IL-6, IL-8, IL-10, and TNF-α, found no differences based on bowel predominant subgroups and no associations with IBS symptoms (Bennet et al., 2018). Psychologic symptoms, such as anxiety, depression, and somatization, have also been associated with increased pro-inflammatory cytokine levels among individuals with IBS as well as the general population (Bennet et al., 2016; Himmerich et al., 2019). Yet, much of the previous research has relied on retrospective symptom measures and has not considered the potential impact of hormonal contraception on the relationship between cytokines and symptoms.

Therefore, the primary aims of our study were to compare cytokine levels, plasma and stimulated and incubated whole blood between women with IBS and healthy control women and to determine if differences exist based on hormonal contraceptive use. We hypothesized that a subgroup of women with IBS would have elevated pro-inflammatory cytokine levels compared to healthy control women. Our secondary exploratory aim was to examine the potential correlation between cytokines and psychological distress symptoms with and without hormonal contraceptive use in the IBS group. For the current study, pro-inflammatory cytokines (IL-12p40, IL-12p70, IL-1β, IL-6, IL-8, and TNF-α) and anti-inflammatory cytokine (IL-10) were included.

Methods

Design and Participants

This comparative study involved women with IBS and without IBS (i.e., healthy controls [HCs]). Patients with IBS were balanced to HCs with respect to age and race. Participants were recruited from healthcare providers or self-referral from community advertisements.

All women were between 18 and 45 years old, had natural menstrual cycles or used hormonal contraceptives, and read and wrote in English. Women ages 18–45 were selected because these women would experience menstrual cycles and because the prevalence of IBS is greater among individuals younger than 50. Both IBS and HCs subjects were excluded if they had: 1) current or recent (within the last 2 months) GI bacterial or viral infection; 2) a history of inflammatory bowel disease; 3) medical history of abdominal surgery with central incision; 4) first degree relative with colorectal cancer before age 60, or first degree relative with inflammatory bowel disease; 5) poorly controlled mental health disorder (e.g., history of psychosis, bipolar disorder, drug or alcohol abuse); 6) regular medication use (at least 4 days a week) for IBS including antibiotics within the past 2 months; 7) women who were pregnant, breast feeding, or planning to get pregnant in the next 2 months; and 8) anyone with a "red flag" symptom (e.g., unintended weight loss $\geq$ 10 pounds, blood in stool, anemia).

For the IBS group, patients had a diagnosis of IBS for at least 6 months that was made by a healthcare provider and met the Rome III research criteria for IBS. Based on the Rome III criteria, participants were categorized into IBS-C, IBS-D, or IBS-M subtypes (Longstreth et al., 2006). For HCs, participants were included if they did not report any current moderate to severe disease, disorder, or syndrome. Individuals were excluded if they had a prior history or current IBS-like symptoms.

Procedures

The University of Washington Human Subjects Review Committee approved the study. After providing verbal consent, potential participants were screened for initial eligibility over the phone. If participants met inclusion criteria and were interested in participating, consent was obtained. Participants completed baseline questionnaires and a brief 2-week GI symptom diary (symptoms included abdominal pain, constipation, diarrhea, and a chart to assess stool consistency) before their first visit. At the first visit, the 2-week diary was reviewed to ensure that the IBS participants met the abdominal pain inclusion criteria of at least 2 days of abdominal pain per week. Women who were menstruating (naturally or as a withdrawal from hormones) were instructed to start their daily symptom diary on the first day of menstrual bleeding. Women using intrauterine hormone devices were instructed to start their daily symptom diary within the next two weeks. All women were asked to continue the diary for 28 days. Days 5–10 after starting the daily symptom diary (which corresponds with the follicular phase of the menstrual cycle for women who were menstruating), participants had a morning fasting blood draw for cytokines. By selecting the follicular phase days 5–10, we aimed capture the time in which estrogen was increasing and to avoid symptoms that may be due to menses.

Measurements

Demographic characteristics. Demographic variables included age, race and ethnicity, marital status, highest formal education, work, and annual income.

Hormonal contraceptive use. Women self-reported any hormonal contraceptive medications on the medication history as well as in the daily diary

Daily symptoms. In a 28-day symptom diary (Han et al., 2018; Jarrett et al., 2009, 2016), participants rated individual symptoms on a Likert-scale from not present (0) to very severe (4). Each individual symptom is the mean severity rating over the 28 days, for the days that were completed. Symptoms included: abdominal pain, bloating, gas, distention, diarrhea, constipation, anxiety, depression, panic, and stressed. Subscales were created by taking the average of each individual symptom: 1) Daily GI Distress included the average of the individual symptoms of gas, bloating, and distention and 2) Daily Psychological Distress included the average of individual symptoms: anxiety, depressed, panic, and stressed. The construct validity has been established in previous IBS studies (Han et al., 2019, 2020). The Daily Psychological Distress scale was significantly correlated with the retrospective Hospital Anxiety and Depression subscales.

Hospital Anxiety and Depression Scale (HADS). This scale includes 7 anxiety and 7 depression items, each with a 4-point rating scale (0 [not at all] to 3 [most of the time]). It is a validated and reliable screening tool to measure current anxiety and depression symptoms in the general population (Bjelland et al., 2002; Zigmond & Snaith, 1983).

Somatization. The Patient Health Questionnaire (PHQ-15) is a validated measure which assesses somatic symptom severity (Kroenke et al., 2002). The measure includes 15 somatic symptoms (three GI symptoms). The summary scale is calculated with (PHQ-15) and without (PHQ-12) GI symptoms.

Cytokines. For plasma cytokines, blood was collected in an EDTA tube. Following centrifugation, the upper plasma portion was removed and frozen in 1 mL aliquots. For analysis of cytokine production *ex vivo* (stimulated cytokines), blood was collected in a Heparin tube. A 0.5 mL portion of the heparinized whole blood was diluted 1:1 into RPMI 1640 media containing 1 ng/mL of lipopolysaccharide (LPS), followed by incubation for four hours at 37°C. After incubation, the treated blood was centrifuged and the top layer was frozen in 150 μL aliquots, in conditions identical to the unstimulated plasma samples. Both extracts were stored at −70 °C until assayed. The specific cytokines (IL-10, IL-12p40, IL-12p70, IL-1β, IL-6, IL-8, and TNF-α), were quantified using the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel (MilliporeSigma, Burlington, MA USA) and detected using Luminex xMAP. The lower detection limit for IL-10 and IL-12p40 was 3.2. The lower detection limit for IL-12p70, IL-1β, IL-6, IL-8, and TNF-α was 0.64. Measurements found below the detection limit were set as equal to the detection limit. Since plasma cytokines are often below detection limits, LPS-stimulated cytokines were examined in order to characterize immune dysregulation (Decker et al., 2017).

Statistical Analysis

SPSS Version 19.1 was used for data analysis. Participants were included in the analysis if they had cytokine and daily symptom diary data. Participant demographics were described using counts and percentages for categorical variables and means and standard deviations for continuous variables. Demographics and symptoms were compared between IBS and HC participants. Tabular associations between categorical variables were assessed with χ^2, and ANOVA models were used for continuous variables.

ANOVA was used to compare cytokine levels between IBS and HCs. A 2 × 2 factorial ANOVA compared cytokine levels based on group (IBS vs. HCs) and by hormonal contraceptive use. Since cytokine levels did differ across plates, all analyses of cytokines controlled for plate. To assist in interpretability, median and interquartile range (IQR) of cytokine levels are presented. However, due to the distribution of the cytokine data, the formal analyses and reported *p*-values are based on the log of the cytokine values. The stimulated and incubated cytokines which, based on literature, were expected to be elevated after 4 hours incubation are presented (Jansky et al., 2003). These cytokines include: IL-6, IL-8, and TNF-α. Among women with IBS, the relationship between cytokines and symptoms were assessed using partial Pearson's correlation coefficient, controlling for plate. In this exploratory work,

analyses are not adjusted for multiple testing/multiple comparisons, and the results should be interpreted cautiously.

Results

Demographic Characteristics

One-hundred and twenty women (IBS = 73; HCs = 47) had data for both cytokines and symptoms and were included in the analysis. The mean age of the women was 28.3 years (Table 1).

Table 1. Demographic Characteristics of Women with and without Irritable Bowel Syndrome.

	IBS (n = 73)	Control (n = 47)	
Continuous variables	*M (SD)*	*M (SD)*	*p-value*[a]
Age	29.0 (8.3)	27.3 (5.6)	0.200
BMI	23.5 (4.6)	23.3 (3.1)	0.766
Categorical variables	*n (%)*	*n (%)*	
Race, white	62 (89.9%)	36 (80.0%)	0.139
Not Hispanic or Latino	67 (91.8%)	42 (89.4%)	0.654
Married/partnered	28 (38.4%)	18 (38.3%)	0.995
Education, college or greater	52 (71.2%)	37 (78.7%)	0.360
Annual Income, >$60,000	14 (31.8%)	10 (30.3%)	0.887
Hormone Contraceptive Use	28 (38.4%)	18 (38.3%)	0.995
Job			0.309
Student	28 (39.7%)	25 (53.2%)	
Professional-Managerial	23 (31.5%)	13 (27.7%)	
Other	21 (28.8%)	9 (19.1%)	
IBS Subgroups			
IBS-Constipation	17 (23.3%)		
IBS-Diarrhea	37 (50.7%)		
IBS-Mixed	19 (26.0%)		

Note. IBS = Irritable Bowel Syndrome; BMI = Body Mass Index. [a]Comparison between IBS and controls.

The majority were white (82%) with a college education or greater (74%). For IBS bowel subgroups, 51% ($n = 37$) had IBS-D, 26% ($n = 19$) had IBS-M, and 23% ($n = 17$) had IBS-C. There were no significant differences between women with IBS and HCs based on demographic characteristics.

Hormonal contraceptive use was similar between groups with 38% of HCs ($n = 18$) and 38% ($n = 28$) of women with IBS using hormonal contraceptives. Among HC women who used hormonal contraceptives, 72% reported oral contraceptive use ($n = 13$), 17% reported using hormonal IUDs ($n = 3$), and 11% reported other types of hormonal contraceptives ($n = 2$). Among women with IBS who used hormonal contraceptives, 82% reported oral contraceptives use ($n = 23$) and 18% reported using hormonal IUDs ($n = 5$). Women with IBS using hormonal contraceptives had significantly lower daily abdominal pain ($p = 0.034$), somatization ($p = 0.013$), and depression ($p = 0.014$) compared to women with IBS who were not using hormonal contraceptives (Table 2).

Plasma Cytokines Between IBS and HCs

Plasma and LPS-stimulated levels of pro- and anti-inflammatory plasma cytokines are shown in Table 3. Overall, there were no statistically significant differences between IBS and HC groups in plasma or LPS-stimulated cytokine levels controlling for plate.

Cytokine Levels by Hormonal Contraceptive Use

After controlling for plate and main effect of group, levels of plasma IL-1β ($p = 0.04$) and TNF-α ($p = 0.02$) were higher among women who did not use hormonal contraceptives compared to women who used hormonal contraceptives (Table 4). This difference is mostly due to women with IBS who do not

Table 2. Symptom Characteristics of Women with and without Irritable Bowel Syndrome (IBS) by Hormonal Contraceptive Use.

	Women with IBS			Healthy Controls		
	Hormone Contraceptive Use (n = 28)	No Hormone Contraceptive Use (n = 45)		Hormone Contraceptive Use (n = 18)	No Hormone Contraceptive Use (n = 29)	
	M (SD)	*M (SD)*	*p-value*[a]	*M (SD)*	*M (SD)*	*p-value*[b]
Daily GI Symptoms						
Abdominal Pain	0.98 (0.45)	1.27 (0.61)	0.03	0.11 (0.13)	0.14 (0.17)	0.47
Bloating	0.92 (0.71)	0.98 (0.63)	0.70	0.18 (0.27)	0.19 (0.26)	0.89
Constipation	0.83 (0.73)	0.64 (0.61)	0.23	0.07 (0.10)	0.09 (0.13)	0.55
Diarrhea	0.54 (0.41)	0.39 (0.38)	0.11	0.06 (0.08)	0.05 (0.06)	0.83
Non-GI Symptoms						
Daily Psychological Distress[c]	0.60 (0.46)	0.77 (0.51)	0.14	0.20 (0.28)	0.25 (0.24)	0.45
Somatization (PHQ-12)[d]	6.08 (3.68)	8.26 (3.47)	0.01	2.48 (1.49)	2.88 (2.08)	0.49
Anxiety (HADS)	9.57 (3.58)	10.20 (4.3)	0.52	4.89 (2.97)	5.38 (3.26)	0.61
Depression (HADS)	3.53 (2.75)	5.35 (3.16)	0.01	1.06 (1.64)	1.69 (1.65)	0.15

Note. GI = gastrointestinal; HADS = hospital anxiety and depression scale; IBS = irritable bowel syndrome; PHQ = patient health questionnaire. [a]Comparison of hormonal contraceptive use among IBS group only. [b]Comparison of hormonal contraceptive use among HC group only. [c]Daily Psychological Distress includes the average daily symptom diary items of anxiety, depression, panic, and stress. [d]The PHQ-12 is the patient health questionnaire excluding three GI specific questions.

use hormone contraceptives having higher levels of IL-1β and TNF-α than the other three groups. However, the interaction between group and hormone contraceptives was not statistically significant (Table 4).

Relationship between Cytokines and Symptoms Among Women with IBS

Among women with IBS, significant correlations were found between daily psychological distress and plasma IL-10, IL-12p70, IL-1β, IL-6, and IL-8 cytokine levels (Table 5). There were no significant correlations between cytokines and GI symptoms. When examining the relationship between cytokines and symptoms separately for those with and without hormonal contraceptive use, a few significant correlations were found in one group but not the other. There was a trend, mostly non-significant, for HADS anxiety to be negatively correlated with most of the cytokines among those using hormonal contraceptives but positively correlated among those not using hormonal contraceptives.

Table 3. Plasma Cytokines (Plasma and LPS-Stimulated) in Women With and Without Irritable Bowel Syndrome (IBS).

	IBS (n = 73)	Control (n = 47)	
Cytokine (pg/ml)	*Median [IQR]*	*Median [IQR]*	*p-value*[a,b]
Plasma			
IL-10	19.0 [9.4, 32.9]	16.1 [5.6, 27.9]	0.18
IL-12p40	74.7 [35.5, 113.5]	48.2 [21.5, 89.0]	0.17
IL-12p70	14.3 [6.5, 22.3]	10.9 [3.4, 24.4]	0.27
IL-1β	5.9 [3.1, 8.9]	4.0 [1.5, 7.6]	0.12
IL-6	3.9 [2.1, 6.6]	3.2 [0.9, 6.0]	0.26
IL-8	3.4 [2.1, 5.4]	2.6 [0.9, 4.4]	0.62
TNF-α	14.4 [7.8, 24.6]	12.5 [6.0, 22.5]	0.23
LPS-Stimulated PBMCs			
IL-6	179.9 [12.5, 1124.3]	192.9 [33.2, 1650.2]	0.36
IL-8	351.1 [120.9, 1176.3]	471.1 [144.7, 1245.4]	0.16
TNF-α	805.7 [203.2, 2267.4]	569.8 [315.0, 631.0]	0.30

Note. Values are median and IQR [25%, 75%] on the original scale. IBS = irritable bowel syndrome; IL = interleukin; LPS = lipopolysaccharide; PBMC = peripheral blood mononuclear cell; TNF = tumor necrosis factor. [a]Controlling for plate. [b]*p*-value from ANCOVA based on log values.

Discussion

Immune dysfunction has been hypothesized to be one factor that contributes, at least in a subset of persons with IBS, to the pathophysiology of GI symptoms in persons with IBS. We measured seven plasma cytokine levels and three stimulated and incubated whole blood to examine differences between women with IBS and healthy control women. Despite marked differences in IBS versus controls on symptoms, there were no statistically significant IBS versus control differences in plasma IL-10, IL-12p40, IL-12p70, IL-1β, TNF-α, IL-6, and IL-8 levels and 4-hour LPS-stimulated TNF-α, IL-6, and IL-8 levels. However, levels of IL-1β and TNF-α were higher among women who did not use hormonal contraceptives compared to women who used hormonal contraceptives. This relationship was mostly due to the IBS group. These preliminary findings point to a possible influence of hormonal contraception use on the innate immune plasma cytokine levels among women with IBS.

While immune dysfunction is considered either a potential risk for IBS development or a response to other pathophysiological alterations such as gut barrier disruption in IBS (Lazaridis & Germanidis, 2018), extant data regarding peripheral

Table 4. Plasma Cytokines (Plasma and LPS-Stimulated) by Hormone Contraceptive Use in Women With and Without IBS.

	Women with IBS		Healthy Controls				
Cytokine (pg/ml)	Hormone Contraceptive Use (n = 28) *Median [IQR]*	No Hormone Contraceptive Use (n = 45) *Median [IQR]*	Hormone Contraceptive Use (n = 18) *Median [IQR]*	No Hormone Contraceptive Use (n = 29) *Median [IQR]*	Main Effect Group *p-value*	Main Effect Hormone *p-value*	Interaction *p-value*
Plasma							
IL-10	16.8 [7.0, 27.3]	21.9 [10.2, 42.9]	17.7 [5.6, 27.6]	16.1 [5.6, 33.7]	0.13	0.11	0.82
IL-12 P40	65.0 [24.4, 111.7]	77.5 [43.6, 114.6]	38.2 [17.2, 69.8]	53.4 [19.6, 100.3]	0.16	0.27	0.89
IL-12 P70	11.6 [4.0, 18.6]	16.0 [8.2, 28.0]	9.2 [3.0, 25.2]	11.0 [3.5, 22.2]	0.19	0.22	0.88
IL-1β	3.6 [1.5, 7.1]	6.1 [3.8, 11.5]	4.2 [1.3, 7.7]	3.6 [1.9, 7.5]	0.07	0.04	0.57
IL-6	3.2 [1.3, 7.1]	4.8 [2.4, 6.4]	2.6 [0.6, 4.7]	3.3 [1.0, 6.6]	0.26	0.83	0.99
IL-8	2.9 [1.4, 6.5]	3.6 [2.3, 5.0]	2.4 [1.0, 3.8]	2.9 [0.9, 6.8]	0.54	0.90	0.44
TNF-α	8.3 [6.9, 18.8]	17.7 [11.0, 31.7]	12.4 [6.1, 21.9]	12.9 [5.5, 24.7]	0.16	0.02	0.84
LPS-Stimulated PBMCs							
IL-6	348.5 [4.2, 1207.0]	120.0 [14.9, 1095.2]	869.2 [58.3, 3125.3]	112.2 [28.6, 1142.9]	0.35	0.28	0.86
IL-8	280.3 [105.2, 1206.1]	390.3 [121.3, 1179.3]	765.8 [318.0, 1395.0]	336.5 [119.9, 1194.2]	0.16	0.91	0.68
TNF-α	789.3 [200.7, 1401.5]	896.0 [202.8, 2635.2]	641.5 [159.1, 5098.8]	569.8 [340.7, 2021.8]	0.30	0.85	0.53

Note. Values are median and IQR [25%, 75%] on the original scale. IBS = irritable bowel syndrome; IL = interleukin; LPS = lipopolysaccharide; PBMC = peripheral blood mononuclear cell; TNF = tumor necrosis factor. All *p*-values from ANOVA based on log values controlling for plate.

Table 5. Relationship of Plasma Cytokines to Symptoms in Women with Irritable Bowel Syndrome (IBS).

	IL-10	IL-12 p40	IL-12 p70	IL-1β	IL-6	IL-8	TNF-α	Stim. IL-6	Stim. IL-8	Stim. TNF-α
Women with IBS (n = 73)										
Daily Abdominal Pain	.16	.01	.06	.16	.11	.12	.17	.03	.06	−.06
Daily GI Distress[a]	−.11	−.15	−.11	−.02	−.13	−.09	−.15	.09	.04	−.06
Daily Psychological Distress[b]	.27*	.20	.25*	.28*	.27*	.28*	.22	−.19	−.09	−.22
Somatization (PHQ-15)	.11	.11	.12	.14	.08	.16	.07	.05	.05	−.08
Somatization (PHQ-12)[c]	.07	.10	.12	.12	.04	.12	.03	.04	.09	−.05
Anxiety (HADS)	.16	.12	.05	.06	.10	.08	.05	−.07	.01	−.13
Depression (HADS)	.10	.14	.16	.18	.13	−.01	.18	−.10	.13	−.13
Women with IBS: Without Hormonal Contraception (n = 45)										
Daily Abdominal Pain	.29	.03	.22	.19	.21	.08	.29	.12	.20	.18
Daily GI Distress[a]	.07	−.15	.01	−.07	−.08	−.09	−.04	.20	.30	.22
Daily Psychological Distress[b]	.29	.17	.25	.26	.28	.23	.31*	-.14	.02	−.07
Somatization (PHQ-15)	.21	.28	.20	.19	.18	.34*	.19	.15	.15	.07
Somatization (PHQ-12)[c]	.13	.28	.20	.17	.14	.31*	.13	.15	.18	.07
Anxiety (HADS)	.31*	.27	.21	.24	.21	.28	.27	−.06	.04	−.12
Depression (HADS)	.17	.23	.12	.20	.21	.18	.13	−.08	−.01	−.13
Women with IBS: Hormonal Contraception (n = 28)										
Daily Abdominal Pain	−.22	-.18	−.39	−.17	−.15	.22	-.36	−.04	−.09	−.28
Daily GI Distress[a]	−.35	−.29	−.40	−.15	−.30	−.16	−.39	−.03	−.20	−.28
Daily Psychological Distress[b]	.17	.16	.11	.20	.17	.38	−.08	−.19	−.23	−.45*
Somatization (PHQ-15)	−.12	−.17	−.11	−.07	−.11	.03	−.32	.02	−.04	−.24
Somatization (PHQ-12)[c]	−.10	−.17	−.07	−.05	−.11	−.02	−.30	.01	.01	−.18
Anxiety (HADS)	−.14	−.11	−.18	−.20	-.16	−.04	−.46*	−.11	−.17	−.35
Depression (HADS)	−.12	−.08	.11	−.01	−.05	−.22	.08	−.03	.11	−.04

Note. * < 0.05 Controlling for plate. IL = interleukin; Stim. = stimulated cytokine; TNF = tumor necrosis factor; PHQ = patient health questionnaire; HADS = hospital anxiety and depression scale. [a]Daily GI Distress includes the average daily symptom diary items of gas, bloating, and distention. [b]Daily Psychological Distress includes the average daily symptom diary items of anxiety, depression, panic, and stress. [c]The PHQ-12 is the patient health questionnaire excluding three GI specific questions.

cytokines levels is inconsistent. Several investigators have noted increases in peripheral plasma pro-inflammatory cytokine levels in IBS when compared to healthy controls (Liebregts et al., 2007; Seyedmirzaee et al., 2016). Similar to our study, other groups have reported no differences in plasma cytokine levels when persons with IBS are compared to a healthy control group (Bashashati et al., 2014, 2017; Chang et al., 2012; Nasser et al., 2019; Rana et al., 2012). However, our results bring in question whether differences in the literature regarding cytokine levels may be due, at least, in part to failure to account for hormone contraception use or menstrual cycle phase among women of reproductive age.

In the current study, women with IBS not using hormonal contraception had higher pro-inflammatory cytokines (IL-1β and TNF-α) as compared to the other groups. The levels of cytokines in women with IBS using hormonal contraceptives showed little difference from healthy controls either using or not using hormonal contraceptives. Although there is ample evidence that estrogen and other sex steroids influence cytokine levels (Kovats, 2015; Zhang & Zein, 2019), there is sparse research related to hormonal contraception in studies of cytokine levels in IBS. One recent study among individuals with IBS reported significantly higher levels of IL-1β among females compared to males, although hormonal contraceptive use and menstrual cycle phase were not considered (Lee et al., 2020).

Given that approximately 25% of American women between the ages of 15 to 44 use some form of hormonal contraceptives, hormonal contraceptive use should be considered (Daniels & Abma, 2018). Women with IBS who did not use hormonal contraceptives reported increased abdominal pain, somatization, and depression compared to women with IBS who used hormonal contraceptives, similar to our previous study. The relationship between symptoms and hormonal contraceptives is likely complex. Some women use contraceptives to reduce dysmenorrhea or other pain-related conditions (Cooper & Mahdy, 2020). Furthermore, the literature has mixed reports of the relationship between hormonal contraceptives and depression. The mechanism that would account for the higher pro-inflammatory cytokine levels in a subgroup of women with IBS not using hormonal contraceptives is likely complex. Estrogen receptors are expressed on adaptive cells (T cells and B cells) as well as macrophage and neutrophil immune cells. Depending on factors such as dose, combination, and route of administration estrogen can have both anti- and pro-inflammatory effects (Cowie et al., 2019; Ribeiro-Dasilva et al., 2017; Straub, 2007). Cytokine samples were obtained during the follicular phase when estrogen levels are increasing. It is not known whether higher levels of these two cytokines in the non-hormone group would have been found at other points in the menstrual cycle when estrogen levels are lower such as menses. Differences between normal cycling and non-

hormonal contraceptive IBS groups may be due the cyclicity rather than the absolute level of estrogen across the menstrual cycle. Additional study with careful consideration of menstrual cycle phase or hormonal contraceptive use (dose, duration, route of administration) is warranted in studies that test the role of the innate immune system in IBS.

Overall, the current exploratory study found few statistically significant relationships between plasma cytokines and psychological distress symptoms among women with IBS. The lack of significant relationships could be due to small sample size, especially since many of the correlations have moderate strength. We were intrigued to discover a mostly non-significant trend for HADS anxiety to be negatively correlated with cytokines among those using hormonal contraceptives but positively correlated among those not using hormonal contraceptives. Whether this represents a physiological effect of constant, sustained levels of estrogen on central processing requires further study (Camilleri, 2020; Irwin, 2011; Jiang et al., 2019). In addition, the reasons for taking hormonal contraception may be an important consideration since some women may use contraceptives to reduce primary dysmenorrhea or other pain-related conditions. This exploratory finding underscores the importance of tracking hormonal contraceptive use in future studies.

This study has several limitations. The study recruited 120 participants (IBS = 73; HCs = 47). With usual assumptions (alpha = 0.05, two-tailed), this sample size would have 80% power for detecting between group differences with medium effect sizes (E.S. (d) = 0.53). Similarly, within the IBS group (n = 73) relationships with medium effect size (E.S. (r) = 0.33, percent of variance explained = 10%) should be discernable with 80% statistical power. While other studies reported a significantly higher LPS-stimulated levels of pro-inflammatory cytokines in patients with IBS (IL-1β, TNF-α and IL-6 (Liebregts et al., 2007)) and in an animal model relevant for IBS (IFN-γ and TNF-α (Clarke et al., 2009)) compared to HCs or non-stressed rats, we found no significant differences in the levels of LPS-cytokines between IBS and HCs. The lack of relationship could be based on the limited incubation period for stimulated cytokines. We only measured LPS-stimulated PMBC cytokine levels after four hours of LPS stimulation and as such were only able to test the release of cytokines previously shown to be increased during this period following stimulation. The current study included both oral pills as well as hormonal IUDs as hormonal contraceptives. Differences in active ingredients, dosage, and administration route of hormonal contraceptive methods could influence cytokines and symptoms. The sample, obtained using community-based recruitment methods, was predominantly young, white and well educated and thus the results may not be applicable to a more diverse IBS population.

In conclusion, this study highlights the need to consider the impact of hormonal contraceptive use on the measurement of cytokines and symptoms among women with IBS. Women with IBS did not differ from HCs based on plasma-stimulated and non-stimulated cytokine levels. However, women with IBS who did not use hormonal contraceptives had higher IL-1β and TNF-α cytokine levels than women who do use hormonal contraceptives and healthy controls. Understanding the role of sex as a biological variable must progress beyond male/female comparisons. Consideration of hormonal contraceptive use and menstrual cycle phase is needed to advance understanding of sex as a biological variable.

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