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The menstrual cycle may not be limited to the endometrium but also may impact gut permeability

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Original Article

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Abstract

Objective: To examine associations between IgA responses to Gram-negative gut commensal bacteria and peri-menstrual symptoms and sex hormone levels during the menstrual cycle in women with and without premenstrual symptoms. *Methods:* Forty women aged 18–45 years completed the Daily Record of Severity of Problems (DRSP) during all 28 consecutive days of the menstrual cycle. We assayed, in plasma, IgA responses to six Gram-negative bacteria, that is, *Hafnei alvei, Pseudomonas aeruginosa, Morganella morganii, Klebsiella pneumoniae, Pseudomonas putida* and *Citobacter koseri*, progesterone and oestradiol at days 7, 14, 21 and 28 of the menstrual cycle. *Results:* Significant changes in Δ (actual − 1 week earlier) IgA to lipopolysaccharides (LPS) of the six Gram-negative bacteria during the menstrual cycle were observed with peak IgA levels at T4 (day 28) and lows at T1 or T2 (day 7 or 14). The ΔIgA changes in *H. alvei*, *M. Morganii, P. putida* during the menstrual cycle were significantly and positively associated with changes in the total DRSP score, and severity of physio-somatic, anxiety and breast-craving, but not depressive, symptoms. The changes in IgA responses to LPS were largely predicted by changes in progesterone and steady-state levels of progesterone averaged over the luteal phase. *Discussion:* Menstrual cycle-associated changes in IgA directed against LPS and by inference bacterial translocation may be driven by the effects of progesterone on transcellular, paracellular and vascular pathways (leaky gut) thereby contributing to the severity of physio-somatic and anxiety symptoms as well as fatigue, breast swelling and food cravings.

Significant outcomes

- During the menstrual cycle, there are highly significant changes in the load of gut commensal Gram-negative bacteria in serum with peaks at the end of the cycle.
- Increased load of gut commensal Gram-negative bacteria at the end of the menstrual cycle is associated with premenstrual symptoms including fatigue, physio-somatic and anxiety symptoms, breast swelling and food cravings.
- These changes may be driven by progesterone affecting transcellular, paracellular and vascular pathways.

Limitations

- It would have been even more interesting if we had measured the gut microbiome and stool assays including indicants of the transcellular, paracellular and vascular pathways.

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Introduction

Premenstrual syndrome (PMS) is defined as a constellation of physical, emotional and/or behavioural symptoms appearing during the luteal phase of the menstrual cycle and improving after the onset of menses (Deuster *et al.*, 1998; Dickerson *et al.*, 2003). However, there is no consensus definition for PMS and different diagnostic criteria have been proposed (see Table 1). The American College of Obstetricians and Gynecologists (ACOG) proposed that women with PMS must have at least one affective and one physical symptom appearing 5 days prior to menses for at least three menstrual cycles (American College of Obstetricians and

Table 1. Definition of four different diagnoses used in the current study to diagnose 'premenstrual' syndrome

Diagnostic label	Abbreviation	Definition
Premenstrual syndrome (American College of Obstetricians and Gynecologists)	ACOG	Subjects report one or more of the following affective and somatic symptoms at day −5 before menses in each of three prior menstrual cycles Affective somatic Depression breast tenderness Angry outbursts abdominal bloating Irritability headache Anxiety swelling of extremities Confusion Social withdrawal Symptoms relieved within 4 days after menses onset without recurrence until at least cycle day 13 Symptoms present in the absence of any pharmacologic therapy, hormone ingestion, or drug or alcohol use Symptoms occur reproducibly during two cycles of prospective recording Subjects suffer from identifiable dysfunction in social or economic performance
Premenstrual syndrome	PMS	PMS: subjects who scored ≥ 70 on the total DRSP score during day 24–28 of menstrual cycle, and in addition there is a difference of at least 30% in DRSP scores between premenstrual phase(late luteal phase day 24–28) and postmenstrual phase (mid-follicular day 6–10)
Peri-menstrual syndrome	PeriMS	Sum DRSP day 1+ day 2 + day 24 to 28 ≥ 307 (0.666 percentile value)
Menstrual cycle-associated symptoms	MCAS	Sum of all DRSP scores from day 1 to day 28 ≥ 1,050 (0.666 percentile value)

DRSP, daily record of severity of problems.

Gynecologists, 2014). Moreover, the symptoms must be relieved within 4 days after the onset of menses without recurrence until at least day 13 of the menstrual cycle (American College of Obstetricians and Gynecologists, 2014). Another gold standard method used to diagnose PMS includes measurement of the Daily Record of Severity of Problems (DRSP): women with a total DRSP score ≥70 on day −5 to −1 of menses and having at least a 30% difference between pre- and postmenstrual scores are diagnosed with PMS (Endicott *et al.*, 2006; Biggs & Demuth, 2011; Qiao *et al.*, 2012). In a recent study, two new case definitions were identified, namely 1) peri-menstrual syndrome (PeriMS), which refers to women with increasing DRSP ratings during the peri-menstrual period (day 1 + day 2 + day 24–28); and 2) menstrual cycle-associated symptoms (MCAS), which delineates women with increased DRSP ratings all over the menstrual cycle (Roomruangwong *et al.*, 2019). Furthermore, we verified that the diagnosis of PMS according to Biggs and Demuth (2011) as well as the diagnoses of PeriMS and MCAS, but not the ACOG-based PMS diagnosis, were externally validated by levels of the sex hormones oestradiol and progesterone (Roomruangwong *et al.*, 2019). In addition, a diagnosis of PMS according to Biggs and Demuth (2011) was only predicted by lower steady-state levels of progesterone in the luteal phase (Biggs & Demuth, 2011), while the PeriMS and MCAS diagnoses were significantly related to both sex hormones (Roomruangwong *et al.*, 2019). Lower steady-state levels of progesterone averaged over the luteal phase coupled with decreasing progesterone levels during the luteal phase also predicted changes in severity of the DRSP as well as alterations in severity of its four subdomains, namely a) depressive symptoms; b) fatigue and physio-somatic symptoms; c) increased appetite and craving combined with breast tenderness and swelling; and d) anxiety (Roomruangwong *et al.*, 2019). Therefore, we concluded that the diagnosis of PeriMS comprises the most accurate diagnostic criteria to describe changes in different symptoms dimensions in the periMS period and that the latter are at least in part mediated by sex hormones. Furthermore, it appeared that PeriMS is associated with a relative luteal phase deficiency or corpus luteum deficiency (Roomruanwong *et al.*, 2019).

Recently, evidence indicates that increased translocation of Gram-negative gut commensal bacteria may play a pathophysiological role in major depression (Maes *et al.*, 2008, 2012, 2013a; Martin-Subero *et al.*, 2016; Slyepchenko *et al.*, 2016, 2017), fatigue and physio-somatic symptoms (Maes *et al.*, 2007, 2013b, 2014; Maes & Leunis, 2008), anxiety/stress (Gareau *et al.*, 2008; Galley & Bailey, 2014; Keightley *et al.*, 2015; Roomruangwong *et al.*, 2017a; Sgambato *et al.*, 2017) and postpartum depression (Roomruangwong *et al.*, 2017b, 2018). However, it remains unclear whether bacterial translocation of Gram-negative bacteria could play a role in PMS or PeriMS and its four symptom domains.

This hypothesis is conceivable since sex hormones may modulate gut permeability (Edwards *et al.*, 2017). Furthermore, studies in pregnancy and postpartum, which are periods of dramatic changes in sex hormonal state, have reported altered gut functions and bacterial composition (Brantsaeter *et al.*, 2011; Koren *et al.*, 2012). These hormonal changes may affect gut contractility thereby increasing gut transit time (Mayer *et al.*, 2014), which may constitute an adaptive response to allow a better absorption of nutrients during pregnancy (Edwards *et al.*, 2017). Furthermore, pregnancy is accompanied by decreased gut permeability and a lowered bacterial translocation as indicated by significantly decreased IgA responses to Gram-negative bacteria, suggesting that pregnancy (with relatively high levels of oestrogen and progesterone) could attenuate bacterial translocation (Roomruangwong *et al.*, 2017a,b). Another study found an

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increased susceptibility to *Listeria monocytogenes* infection during pregnancy leading to adverse obstetrics outcomes including preterm delivery or stillbirth, which were partly modulated by elevated oestrogen and progesterone levels (Garcia-Gomez *et al.*, 2013). In patients with irritable bowel syndrome , sex hormones may affect peripheral and central regulatory processes of the brain–gut axis, leading to alterations in visceral sensitivity, intestinal barrier function and immune activation of intestinal mucosa (Mulak *et al.*, 2014). Cyclical changes of ovarian hormones during the menstrual cycle can arguably modulate gastrointestinal (GI) functions including small intestinal transit, gastric emptying and mucosal blood flow (Heitkemper *et al.*, 2003; Longstreth *et al.*, 2006). Lowered levels of ovarian hormone levels during menses are associated with exacerbations of GI symptoms including abdominal discomfort, bowel habit changes and bloating (Whitehead *et al.*, 1990; Moore *et al.*, 1998; Mulak & Taché, 2010). However, there are no data whether changes in sex hormones during the menstrual cycle are associated with increased bacterial translocation.

Hence, the current study was carried out to examine whether increasing plasma IgA levels to lipopolysaccharides (LPS) of Gram-negative bacteria during the menstrual cycle could be associated with the pathophysiology of PMS or PeriMS and whether those associations could be related to alterations in sex hormones during the menstrual cycle.

Methods

Participants

Forty female participants aged 18–45 years were recruited by word of mouth at the King Chulalongkorn Memorial Hospital during the period of April–May 2018, including 20 women with subjective complaints of PMS and 20 women without such complaints. Participants comprised hospital's staffs or friends/relatives of hospital's staffs and women accompanying patients to the hospital. Inclusion criteria were: 1) women aged 18–45 years; 2) having a regular menstrual cycle with a cycle length of 27–30 days during the past year; 3) being able to read and write in Thai; 4) willing to have four blood samples drawn at day 7 (T1), day 14 (T2), day 21 (T3) and day 28 (T4) of the menstrual cycle; and 5) able to complete the DRPS ratings for all consecutive days of the menstrual cycle. Exclusion criteria for both groups were: 1) those with a lifetime history of psychiatric illness (including major depression, bipolar disorder, schizophrenia and obsessive compulsive disorder); 2) those with a history of medical illness, including type 1 diabetes and autoimmune/immune-inflammatory disorders (including rheumatoid arthritis, inflammatory bowel disease, psoriasis and multiple sclerosis); 3) pregnant women or women who are currently using hormonal contraceptive agents; and 4) women who are currently using any psychotropic medications. The study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB No.611/60, COA No. 1111/2017). Written informed consent was obtained from all participants prior to the study. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

Clinical assessments

All participants were requested to complete a demographic and clinical data questionnaire, that is, menstrual information, age, education, height, weight, a history of substance use and life style, and they were evaluated by an experienced psychiatrist before enrolment in the study to rule out other medical and/or psychiatric conditions. All participants completed the DRSP during all consecutive days of their menstrual cycle starting on day 1 of menses to assess the severity of PMS symptoms. The DRSP consists of 21 items + 3 functional impairment items commonly used to assess PMS (Endicott *et al.*, 2006). All items are rated from 1 to 6 (1 = not at all, 2 = minimal, 3 = mild, 4 = moderate, 5 = severe, 6 = extreme). The DRSP is a self-report instrument that rates both the 'presence' and 'severity' of premenstrual symptoms and that can be used to reliably screen for a DSM-IV diagnosis of premenstrual dysphoric disorder (Biggs & Demuth, 2011). The presence of PMS was considered when the total DRSP score was ≥70 on day −5 to −1 of menses and when there was a 30% difference between premenstrual (day −5 to −1) and postmenstrual (day 6–10) scores (Endicott *et al.*, 2006; Biggs & Demuth, 2011; Qiao *et al.*, 2012). In addition, participants were also categorised in those who had PeriMS with increased DRSP ratings during the perimenstrual period (day 1+ day 2 + day 24–28) and MCAS (Roomruangwong *et al.*, 2019). We also computed scores of the four subdomains of the DRSP, namely a) depressive dimension; b) physio-somatic component; c) increased appetite and craving combined with breast tenderness and swelling; and d) anxiety dimension (Roomruangwong *et al.*, 2019).

Assays

In all women, we sampled fasting blood at 8.00 a.m. at T1, T2, T3 and T4 for the assay of IgA directed to Gram-negative bacteria, oestradiol and progesterone. We described in detail elsewhere the assay to detect IgA antibodies directed to Gram-negative bacteria (Roomruangwong *et al.*, 2017a). Briefly, LPS derived from Gram-negative bacteria were assayed, namely *Hafnia alvei, Klebsiella pneumonia, Morganella morganii, Pseudomonas aeruginosa, Citrobacter koseri* and *Pseudomonas putida*. Polystyrene 96-well plates (NUNC) were coated with 200 µl solution containing bacterial components at 4 µg/ml in 0.05 M carbonate buffer at pH 9.6. Well plates were incubated at 4°C for 16 h under agitation. Then, we added 200 µl blocking solution (PBS, Tween 20 0.05%, 5 g/l BSA) for 1 h and placed at 37°C. Following two washes with PBS, plates were filled up with 100 µl of sera diluted at 1 : 1000 in the blocking buffer A (PBS, 0.05% Tween 20, 2.5 g/l BSA) and incubated at 37°C for 105 min. After three washes with PBS-0.05% Tween 20, plates were incubated at 37°C for 1 h with peroxidase-labelled anti-human IgA secondary antibodies diluted, respectively, at 1 : 15 000 and 1 : 10 000 in the blocking buffer (PBS, 0.05% Tween 20, 2.5 g/l BSA). Afterwards, plates were washed three times with PBS-0.05% Tween 20 and incubated with the detection solution for 10 min in the dark. Chromogen detection solution (tetramethylbenzedine) was used for the peroxidase assay at 16.6 ml per liter in 0.11 M sodium acetate trihydrate buffer (pH 5.5) containing 0.01% H_2O_2. The reaction was stopped with 25 µl 2-N HCl. After addition of stop solution (H_2SO_4 or HCl), the obtained, proportional absorbance in the tested sample (compared to established concentration of respective antibodies), was measured at 450 nm with one alpha of correction at 660 nm.

The methods to assay both sex hormones were also described in detail previously (Roomruangwong *et al.*, 2019). In brief, we used an immunoassay for the quantitative determination of estradiol and progesterone using Cobas® 601. For estradiol, the two steps of assay included: 1) first incubation: incubating the sample

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Table 2. Demographic and clinical data of women with and without PMS

Variables	No PMS	PMS	F/χ^2	df	p
Age (years)	29.8 (7.3)	32.3 (6.9)	1.22	1/39	0.276
Education (years)	16.2 (1.2)	15.8 (1.6)	1.15	1/39	0.290
Age menarche (years)	13.0 (1.3)	12.6 (1.2)	0.92	1/39	0.345
Length cycle (days)	28.0 (1.9)	27.3 (5.3)	0.28	1/39	0.601
Duration menses (days)	4.5 (1.3)	4.9 (1.6)	0.68	1/39	0.416
BMI (kg/m^2)	21.8 (3.6)	22.5 (3.8)	0.43	1/39	0.416
DRSP (sum of all items during 28 days)	878.4 (210.5)	974.5 (204.0)	2.15	1/39	0.151

PMS, premenstrual syndrome; BMI, body mass index; DRSP, daily record of severity of problems.
All results are shown as mean (SD).
PMS: diagnosis according to the criteria of the American College of Obstetricians and Gynecologists.

(25 µl) with two estradiol-specific biotinylated antibodies, immune complexes are formed, the amount of which is dependent upon the analyte concentration in the sample; 2) second incubation: after addition of streptavidin-coated microparticles and an estradiol derivative labelled with a ruthenium complex, the still-vacant sites of the biotinylated antibodies become occupied, with formation of an antibody–hapten complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin, and the reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Precision was determined using Elecsys reagents, samples and controls in a protocol (EP5-A2) of the Clinical and Laboratory Standards Institute (CLSI): two runs per day in duplicate each for 21 days ($n = 84$) with the intra-assay CV value of 1.2%. For progesterone, the two steps of assay included: 1) first incubation: incubating the sample (20 µL) with a progesterone-specific biotinylated antibody, immunocomplexes are formed, the amount of which is dependent upon the analyte concentration in the sample; 2) second incubation: after addition of streptavidin-coated microparticles and an progesterone derivative labelled with a ruthenium complex, the still-vacant sites of the biotinylated antibodies become occupied, with formation of an antibody–hapten complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin, and the reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are also removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results are determined via a calibration curve which is instrument specifically generated by two-point calibration and a master curve provided via the reagent barcode. Precision was also determined using Elecsys reagents, samples and controls in a protocol (EP5-A2) of the CLSI as in estradiol: two runs per day in duplicate each for 21 days ($n = 84$) with an intra-assay CV value of 2.3%.

Statistics

We used analysis of contingency tables (χ^2 test) and analysis of variance (ANOVA) to assess associations between categorical variables and differences in continuous variables between diagnostic groups, respectively. Generalised estimating equation (GEE) analysis, repeated measures, was used to check effects of time, diagnosis and time × diagnosis interaction on the IgA levels, while adjusting for age, cycle length, age of menarche and duration of menses. Using GEE analyses, repeated measurements, we also examined the relationships among the IgA levels to Gram-negative bacteria and either the DRSP values over time (T1, T2, T3 and T4) or changes in sex hormones during the menstrual cycle. Furthermore, we used a distributed lag model to predict the DRPS values over time (dependent variable) by lagged (1 week) values of the IgA responses to Gram-negative bacteria and we computed the ΔIgA responses as current IgA values − lagged IgA values obtained 1 week earlier, which denotes the changes in IgA values the last week before blood sampling. We also use steady-state hormonal levels, namely the sum of the z scores of the progesterone hormone levels at T2, T3 and T4 ($zT2 + T3 + T4$). Tests were two-tailed and a p-value of 0.05 was considered for statistical significance. All statistical analyses were performed using IBM SPSS windows version 25.

Results

Demographic and clinical data

Table 2 shows the demographic and clinical data in participants with and without PMS. There were no significant differences in age, years of education, age of menarche, cycle length, duration of menses, total DRSP scores and BMI between groups.

Table 3 shows the DRSP score and subscores at the four different time points, T1, T2, T3 and T4. Thus, there were highly significant variations in those scores all over the menstrual cycle with higher total DRSP and physio-somatic scores at T4 compared to the other time points, and higher at T1 compared to T2 and T3, while T3 showed higher scores than T2. In addition, depression scores were higher at T4 than at T2 and T3, at T1 than at T2, while there were no differences between T2 and T3. Breast-craving and anxiety symptoms were higher at T4 than at T1, T2 and T3, while lowest scores were detected at T2.

Menstrual cycle-associated changes in IgA levels to Gram-negative bacteria

In Table 4, we examine the effects of time on IgA and ΔIgA (i.e. actual value − value 1 week earlier) responses to the Gram-negative bacteria. The data were analysed using GEE analysis considering effects of time, time × PMS diagnosis (according to the four definitions) and PMS diagnosis, while adjusting for age, cycle length, age of

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Table 3. Measurements of DRSP and subdomains, and plasma levels of oestradiol and progesterone during the menstrual cycle

Variables	T1	T2	T3	T4	Wald χ^2	df	*p*
DRSP total score (daily values)	31.2 (1.8)2,4	27.4 (0.8)1,3,4	30.9 (1.4)2,4	39.8 (3.5)1,2,3	31.02	3	<0.001
Depression score	11.7 (0.7)2	10.2 (0.3)1,4	11.1 (0.6)4	14.5 (1.4)2,3	22.95	3	<0.001
Fatigue and physio-somatic symptoms	8.0 (0.5)2,4	7.0 (0.3)1,3,4	8.0 (0.5)2,4	10.4 (1.0)1,2,3	28.26	3	<0.001
Breast and craving score	5.2 (0.4)4	4.6 (0.2)3,4	5.8 (0.4)2,4	7.4 (0.7)1,2,3	28.35	3	<0.001
Anxiety score	6.5 (0.5)4	5.9 (0.3)4	6.4 (0.4)4	7.8 (0.6)1,2,3	17.58	3	<0.001
Oestradiol (pmol/l)	289.5 (43.9)2,3	606.4 (75.2)1,4	597.8 (44.2)1,4	281.9 (25.9)2,3	68.16	3	<0.001
Progesterone (nmol/l)	0.54 (0.05)2,3,4	3.55 (0.95)1,3,4	34.65 (4.22)1,2,4	11.45 (2.03)1,2,3	102.39	3	<0.001

DRSP, daily record of severity of problems.

Table 4. Results of GEE analysis, that is, effects of time on the ΔIgA responses directed against LPS of six Gram-negative bacteria as dependent variables

Variables	T1	T2	T3	T4	Wald χ^2	df	*p*
Δ *Citrobacter koseri*	−0.579 (0.149)3,4	−0.295 (0.141)3,4	0.250 (0.137)1,2	0.624 (0.129)1,2	33.83	3	<0.001
Δ *Pseudomonas putida*	−0.291 (0.193)4	−0.339 (0.109)3,4	0.023 (0.111)2,4	0.628 (0.152)1,2,3	30.71	3	<0.001
Δ *Klebsiella pneumoniae*	−0.223 (0.127)4	−0.373 (0.174)4	0.175 (0.159)	0.422 (0.133)1,2	13.88	3	0.003
Δ *Hafnia alvei*	−0.351 (0.130)4	−0.576 (0.128)3,4	−0.059 (0.099)2,4	0.987 (0.143)1,2,3	57.47	3	<0.001
Δ *Pseudomonas aeruginosa*	−0.218 (0.145)4	−0.342 (0.171)4	−0.118 (0.120)4	0.596 (0.149)1,2,3	17.26	3	0.001
Δ *Morganella morganii*	−0.260 (0.152)4	−0.366 (0.133)4	−0.293 (0.110)4	0.936 (0.135)1,2,3	61.88	3	<0.001
Sum of all Δ values	−1.922 (0.711)4	−2.293 (0.671)3,4	−0.013 (0.606)2,4	3.960 (0.757)1,2,3	34.09	3	<0.001

GEE, generalised estimating equation.
Results are shown as mean (±SE) and as *z* scores.
Δ: computed as actual value − values 1 week earlier.

menarche and duration of menses. There were highly significant effects of time on the six IgA and ΔIgA levels to Gram-negative bacteria. Table 4 shows differences in ΔIgA responses to the six Gram-negative bacteria at the four different time points of the menstrual cycle. Peak ΔIgA levels for all Gram-negative bacteria were detected at T4. The lowest ΔIgA responses were detected at T1 (for *C. koseri*) or T2 (for all other bacteria). The ΔIgA responses were significantly higher at T4 than at T1, T2 or T3 for *P. putida, H. Alvei, P. aeruginosa* and *M. morganii* and significantly higher at T4 than T1 and T2 for *C. koseri* and *Klebsiella pneumoniae*. There were no significant differences between any of the ΔIgA values between T1 and T2. The ΔIgA values at T3 occupied an intermediate position with values which were often significantly different from T2 and T4. Fig. 1 shows the mean ΔIgA values (in *z* scores) across the four time points. As an index of the overall LPS load, we computed a *z* unit-weighted composite score, namely the sum of all *z* ΔIgA values. Table 4 shows that there were highly significant differences in this overall index with significantly higher values at T4 than the other three time points while the values were higher at T3 than T2 and no differences between T1 and T2 could be established. GEE analyses showed that the effects of time on IgA directed to Gram-negative bacteria were highly significant and that peak levels were obtained at T4 with lows at T2 or T3 (not significantly different) while IgA levels to LPS at T1 occupied an intermediate position. There were no significant effects of diagnosis (using the four diagnostic criteria) or the interaction term time × diagnosis on the IgA or ΔIgA to LPS of Gram-negative bacteria. GEE analyses showed that there were significant and positive effects of age on the ΔIgA levels to *H. alvei* ($W = 17.87$, df = 1, $p < 0.001$), *K. pneumoniae* ($W = 4.51$, df = 1, $p = 0.034$) and *P. aeruginosa* ($W = 5.46$, df = 1, $p = 0.019$). There were also significant and positive effects of cycle length on ΔIgA to *H. alvei* ($W = 5.71$, df = 1, $p = 0.017$) and *P. putida* ($W = 7.60$, df = 1, $p = 0.006$).

Prediction of DRSP symptoms by IgA response to Gram-negative bacteria

Table 5 shows the associations between total DRSP and subdomain scores (as dependent variables) and changes in IgA responses to Gram-negative bacteria during the menstrual cycle (explanatory variables). We used GEE analysis, repeated measures, to analyse these associations and entered the actual measurements of IgA responses as well as the Δ responses in the analyses. We detected that the changes in DRSP were significantly associated with the Δ (but not actual) IgA levels of *H. alvei, M. morganii* or *P. putida*. We also found significant associations between changes in the severity of fatigue and physio-somatic and breast-craving symptoms with the ΔIgA levels to LPS of the same three bacteria, while changes in ΔIgA responses to *C. koseri* also predicted breast-craving symptoms. Changes in anxiety symptoms were predicted by ΔIgA responses to *H. alvei*.

In addition, we have carried out a second series of GEE analysis whereby we entered ΔIgA responses to LPS together with the lagged progesterone values and the *z*T2 + T3 + T4 progesterone scores as explanatory variables (Roomruangwong *et al.*, 2019). Table 5 shows that after considering the effects of both progesterone values, the effects of ΔIgA values on the DRSP and anxiety scores were no longer significant. Nevertheless, the effects of ΔIgA responses to *P. putida* on physio-somatic and breast-craving symptoms remained significant. The oestradiol values were not significant in these GEE analyses and the effects of the IgA levels to different bacteria remained significant after introducing oestradiol data.

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Table 5. Results of GEE analysis with the DRSP score and subdomains as dependent variables

Dependent variables	Explanatory variables	Wald χ^2	df	*p*
DRSP total score	Δ *Hafnia alvei*	6.51	1	0.011
	Δ *Morganella morganii*	4.76	1	0.029
	Δ *Pseudomonas putida*	4.00	1	0.046
Fatigue and physio-somatic symptoms	Δ *H. alvei*	4.88	1	0.027
	Δ *M. morganii*	5.05	1	0.025
	Δ *P. putida*	8.84	1	0.003
Breast and craving symptoms	Δ *H. alvei*	11.53	1	0.001
	Δ *M. morganii*	7.81	1	0.005
	Δ *P. putida*	10.40	1	0.001
	Δ *Citrobacter koseri*	4.52	1	0.033
Anxiety symptoms	Δ *H. alvei*	8.40	1	0.004
DRSP total score	Δ *H. alvei*	0.59	1	0.442
	Lag progesterone	7.87	1	0.005
	Progesterone T2 + T3 + T4	21.02	1	<0.001
Fatigue and physio-somatic symptoms	Δ *P. putida*	5.41	1	0.020
	Lag progesterone	6.85	1	0.009
	Progesterone T2 + T3 + T4	8.87	1	0.003
Breast and craving symptoms	Δ *H. alvei*	3.68	1	0.055
	Lag progesterone	5.16	1	0.023
	Progesterone T2 + T3 + T4	7.33	1	0.007
	Δ *P. putida*	5.73	1	0.017
	Lag progesterone	6.98	1	0.008
	Progesterone T2 + T3 + T4	7.71	1	0.005
Anxiety symptoms	Δ *H. alvei*	1.24	1	0.266
	Lag progesterone	5.93	1	0.015
	Progesterone T2 + T3 + T4	6.26	1	0.012

GEE, generalised estimating equation; DRSP, daily record of severity of problems.
Lag progesterone: 1 week lagged values.
Δ: computed as actual value − values of 1 week earlier.

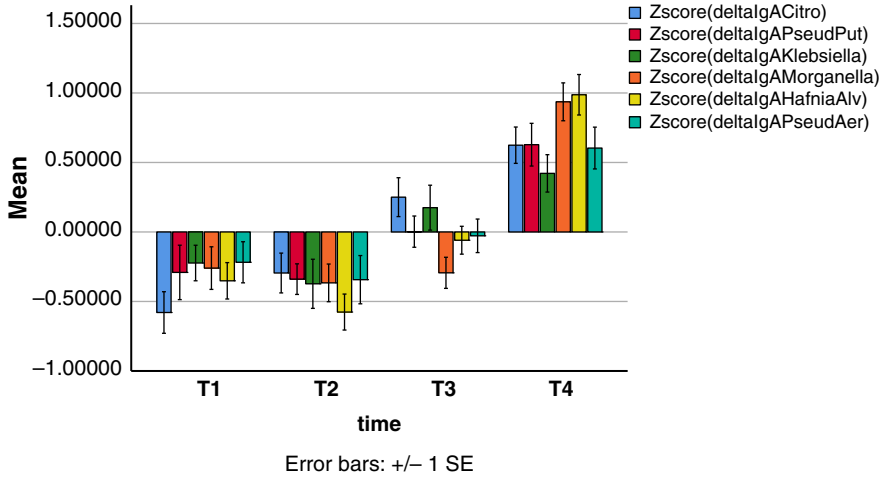

Fig. 1.

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Table 6. Results of GEE analysis with the IgA directed to LPS of six Gram-negative bacteria as dependent variables

Dependent variables	Explanatory variables	Wald χ^2	df	*p*
Δ *Hafnia alvei*	Lag progesterone	27.55	1	<0.001
	Progesterone T2 + T3 + T4	30.97	1	<0.001
	Δ Progesterone	8.41	1	0.004
Δ *Morganella morganii*	Lag progesterone	31.82	1	<0.001
	Progesterone T2 + T3 + T4	37.57	1	<0.001
Δ *Pseudomonas putida*	Lag progesterone	22.40	1	<0.001
	Progesterone T2 + T3 + T4	11.28	1	0.001
	Δ Progesterone	7.50	1	0.006
Δ *Citobacter koseri*	Lag progesterone	17.15	1	<0.001
	Progesterone T2 + T3 + T4	15.78	1	<0.001
	Δ Progesterone	9.50	1	0.002
Δ *Pseudomonas aeruginosa*	Lag progesterone	10.58	1	0.001
	Progesterone T2 + T3 + T4	8.23	1	0.004
	Δ Progesterone	6.22	1	0.013
Δ *Klebsiella pneumoniae*	Lag progesterone	13.05	1	<0.001
	Progesterone T2 + T3 + T4	10.96	1	0.001

GEE, generalised estimating equation.
Lag progesterone: 1 week lagged values.
Δ: computed as actual value − values 1 week earlier.

Associations between IgA responses to Gram-negative bacteria and sex hormones

In Table 6, we examine the effects of progesterone (explanatory variables) on the ΔIgA levels to Gram-negative bacteria (dependent variables). We used three different progesterone levels, namely the lagged progesterone values, the Δ changes and the steady-state progesterone values averaged over the second part of the cycle (zT2 + T3 + T4). The Δ changes in *H. alvei, P. putida, C. koseri* and *P. aeruginosa* were significantly associated with the lagged progesterone values (positively), the Δ changes in progesterone (positively) and zT2 + T3 + T4 (negatively). The ΔIgA responses to *M. morganii* and *K. pneumoniae* were significantly associated with the lagged progesterone data (again positively) and zT2 + T3 + T4 (again negatively). In addition, another z composite score denoting the ratio between steady-state progesterone/steady-state oestradiol values (computed as z(zT1 + zT2 + zT3 + zT4) progesterone − z(zT1 + zT2 + zT3 + zT4) oestradiol values) was significantly associated (inversely) with the ΔIgA data and could be used instead of the zT1 + T2 +T3 progesterone scores shown in Table 6 (same significance levels).

Also, the IgA response to LPS of Gram-negative bacteria was significantly associated with the lagged progesterone data but the effects of progesterone were markedly less as compared with the ΔIgA data. Thus, the lagged progesterone levels were significantly associated with the IgA levels to LPS of *C. koseri* ($W = 5.23$, df = 1, $p = 0.022$), *P. putida* ($W = 10.16$, df = 1, $p = 0.001$), *K. pneumonia* ($W = 4.36$, df = 1, $p = 0.037$) and *M. morganii* ($W = 4.64$, df = 1, $p = 0.031$), but not *H. alvei* or *P. aeruginosa*.

Discussion

The first major finding of this study is that there are highly significant changes in the six IgA levels to Gram-negative bacteria during the menstrual cycle. Overall, peak changes in IgA levels to LPS of all bacteria were observed at T4 (day 28) with lows at T1 (day 7) or T2 (day 14). These results indicate that women exhibit common rhythms in IgA responses to LPS during the menstrual cycle and by inference that changes in LPS load in the plasma and, consequently, in bacterial translocation may ensue during the menstrual cycle. Phrased differently, our findings indicate increased LPS load at the end of the menstrual cycle with a corresponding reduction in LPS load of potentially harmful pathogens after menstruation. In this regard, Profet hypothesised that menstruation may help to clean the vaginal tract of pathogens (Profet, 1993), although in 1993 there was no evidence for elevated pathogen load before menstruation.

To the best of our knowledge, there are no previous studies suggesting significant menstrual cycle-associated rhythms in LPS load. Previously, no dysfunctions in gut permeability were observed during the menstrual cycle in normal women using the lactulose/mannitol test, a less sensitive test to assess leaky gut (Torella *et al.*, 2007). Nevertheless, one study demonstrated a relationship between gut microbiota and an irregular menstrual cycle as indicated by a relative *Prevotella*-enriched microbiome, but lower *Bacteroidales* S24-7, *Clostridiales, Ruminococcus* and *Lachnospiraceae* (Sasaki *et al.*, 2019). *Prevotella* is associated with increased gut permeability since it may degrade mucin (Brown *et al.*, 2011), whereas *Clostridiales, Ruminococcus* and *Lachnospiraceae* are butyrate-producing bacteria, which play a role in maintaining gut homeostasis (Hamer *et al.*, 2008; Pryde *et al.*, 2002) through providing energy sources to intestinal epithelial cells and producing anti-inflammatory effects (Inan *et al.*, 2000). Moreover, decreased mucin production may lead to a micro-inflammatory environment which may be associated with ovulatory disorders (Sasaki *et al.*, 2019) as indicated by recent findings that inflammation may exert a detrimental effect on ovarian follicle growth and ovulation (Boots & Jungheim, 2015).

Secondly, the immune characteristics of the female reproductive tract may share some similarities with those of the gut

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(Shacklett & Greenblatt, 2011). There are significant differences in microbiota in the female reproductive tract between the phases of the menstrual cycle (Chen *et al*., 2017). For example, increased presentation of *Lactobacillus species*, *Sphingobium sp*., *Propionibacterium acnes* and *Pseudomonas sp*. during the proliferative (day 1–14) and secretory (day 15–28) phases, whereas *P. acnes* appeared to be more abundant during the secretory phase. Overall, the proliferative phase appeared to be associated with increased bacterial proliferation when compared to the secretory phase as indicated by higher pyrimidine and purine metabolism, aminoacyl-tRNA and peptidoglycan biosynthesis, whereas during secretory phase, porphyrin, arginine and proline metabolism were increased, as well as the degradation of benzoate, nitrotoluene and biosynthesis of siderophore. Studies in primates also found that vaginal microbial ecologies are highly affected by the menstrual cycle, especially during the estrous phase (Keane *et al*., 1997; Narushima *et al*., 1997; Gajer *et al*., 2012). In humans, high midcycle oestrogen levels are associated with increased *Lactobacillus* proliferation (Boskey *et al*., 1999, 2001), whereas increased mucosal secretions are associated with growth of *Candida* (Schwebke & Weiss, 2001). High levels of oestrogen and progesterone during midcycle are associated with higher stability of microbial communities (Gajer *et al*., 2012), whereas there is a lower prevalence, intensity and diversity of microbiota during menstruation (Stumpf *et al*., 2013).

The second major finding of our study is that there were significant associations between the Δ changes in the IgA responses to LPS and the DRSP scores and its subdomains. Thus, the Δ changes in *H. alvei*, *M. morganii* and *P. putida* were significantly associated with changes in the total DRSP scores, physio-somatic symptoms and breast-craving symptoms, while *H. alvei* was also associated with anxiety. As such, the Δ changes in IgA responses to LPS of Gram-negative bacteria are associated with all symptom domains of the DRSP, except depression. Our current findings extent those of previous studies indicating that IgA levels to Gram-negative bacteria are significantly correlated with physio-somatic symptoms in depression and CFS/ME (Maes *et al*., 2008; Maes & Leunis, 2008). Gut microbiota also influences the host's appetite and food intake by modulating nutrient sensing and appetite and satiety-regulating systems (Turnbaugh *et al*., 2006; Huang & Douglas, 2015; Leitao-Goncalves *et al*., 2017; van de Wouw & Schellekens, 2017). In animal studies, essential amino acids and the concerted action of the commensal bacteria *Acetobacter pomorum* and *Lactobacilli* significantly modulate food choice, especially towards amino acid-rich food (Leitao-Goncalves *et al*., 2017). Studies in patients with anorexia nervosa found significantly lower alpha (within-sample) diversity in taxa abundance between admission and after discharge from hospital when compared to healthy controls, while severity of depression, anxiety and eating problems were associated with the composition and diversity of the intestinal microbiota (Kleiman *et al*., 2015). There are also profound microbial perturbations in patients with anorexia nervosa with higher levels of mucin degraders and members of *Clostridium* clusters I, XI and XVIII and lowered levels of the butyrate-producing *Roseburia sp*., while in anorexia nervosa patients with restrictive and binge/purging subtypes distinct perturbations in microbial community compositions were observed (Mack *et al*., 2016).

Moreover, the associations found in our study between changes in IgA to LPS of Gram-negative bacteria and breast symptoms may be explained by possible effects of the gut microbiome on breast symptoms via the modulating effects of oestrogen. Plottel and Blaser (2011) proposed the 'estrobolome' as the aggregate of enteric bacterial genes whose products are capable of metabolising oestrogens (Plottel & Blaser, 2011). Under normal conditions, oestrogens and their metabolites are conjugated in the liver through glucuronidation or sulfonation to allow for biliary excretion (Zhu & Conney, 1998). Conjugated oestrogens are excreted in bile, urine and feces (Raftogianis *et al*., 2000). Nevertheless, approximately 65% of estradiol is recovered in bile, 10–15% is found in feces while a significant proportion of oestrogens is reabsorbed into the circulation (Sandberg & Slaunwhite, 1957; Adlercreutz & Martin, 1980; Adlercreutz & Jarvenpaa, 1982). This reabsorption of hepatically conjugated oestrogens is mediated by deconjugation processes by gut bacteria with β-glucuronidase activity such as the *Clostridium leptum* and *Clostridium coccoides* cluster, and the *Escherichia/Shigella* bacterial group (Gloux *et al*., 2011; Kwa *et al*., 2016; Fernandez & Reina-Perez, 2018). Thus, a deconjugating enzyme-enriched estrobolome could promote reabsorption of free oestrogens thereby increasing oestrogen levels, which may contribute to breast tissue changes (Kwa *et al*., 2016; Fernandez & Reina-Perez, 2018).

In the current study, we also found a significant association between the anxiety subdomain of the DRSP and increased LPS load in the plasma. These findings extent our previous results that increased IgA responses to *P. aeruginosa* at the end of term pregnancy are associated with anxiety 4–6 weeks after delivery (Roomruangwong *et al*., 2017a). It is plausible that the above associations between increasing LPS load and symptom domains including anxiety and physio-somatic symptoms may be explained by low-grade immune-inflammatory responses induced by LPS activation of the toll-like receptor-4 complex, a receptor of the innate immune system which upon activation causes release of reactive oxygen species, cytokines and nitric oxide (Lucas & Maes, 2013). This theory is corroborated by findings that increased root canal endotoxin in subjects with chronic apical periodontitis is associated with increased nitro-oxidative stress and depressive symptoms (Gomes *et al*., 2018). Moreover, repeated and intermittent administration of LPS may induce depressive-like behaviours in the rodent in association with increased microglial activation and increased levels of nuclear factor-kB, superoxide and cytokine production, lowered tryoptophan and increased neurotoxic tryptophan catabolites (Kubera *et al*., 2013; Rodrigues *et al*., 2018). Administration of LPS to humans not only induces the levels of pro- and anti-inflammatory cytokines, but also lowers mood, and induces anxiety and social disconnection (Eisenberger *et al*., 2010; Grigoleit *et al*., 2011). All in all, our findings may indicate that variations in LPS of Gram-negative bacteria during the menstrual cycle with peaks at the end of day 28 of the menstrual cycle could play a pathophysiological role in premenstrual and PeriMS symptoms.

The third major finding of our study is that many, but not all, associations between ΔIgA responses to LPS and symptom domains disappeared after introducing progesterone and changes in progesterone levels in the GEE analyses, although the effects of *P. putida* on physio-somatic symptoms and breast-craving remained significant. This may be explained as the increments in IgA responses to LPS are largely predicted by increasing progesterone levels coupled with lowered steady-state progesterone levels or a relative increase in oestradiol steady-state levels versus those of progesterone. Progesterone receptors are present in colon epithelial cells where they interact with progesterone and modulate the colonic transit time (Guarino *et al*., 2011). The colonic transit time is longer during the luteal phase (high progesterone) when compared to the follicular phase (low progesterone) (Wald *et al*., 1981; Jung *et al*., 2003). Progesterone also impairs smooth muscle contraction (Xiao *et al*., 2009; Li *et al*., 2012) and downregulates the barrier function of tight

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junctions which may contribute to cytoskeletal remodeling (Someya *et al.*, 2013) in uterine endometrium. Progesterone promotes endometrial remodeling via modifications of actin fibres architecture, which leads to cell membrane reshaping and movement (Pfaendtner *et al.*, 2010; Shortrede *et al.*, 2018; Svitkina, 2018). Moreover, progesterone controls actin polymerisation, branching and focal adhesion complex formation via membrane-organising extension spike protein and focal adhesion kinase (Sanchez *et al.*, 2013; Shortrede *et al.*, 2018). Adhesion assembly in uterine epithelial cells is regulated by progesterone while oestrogens concentrate talin and paxillin (Kaneko *et al.*, 2009). Progesterone also induces dickkopf homologue 1 (DKK1) and forkhead box O1 (FOXO1), resulting in inhibition of Wnt/β-catenin signalling in the human endometrium (Wang *et al.*, 2009). Moreover, oestrogens play a role in the tight junctions in the gut by decreasing zonula occludens 1 mRNA and protein expression thereby increasing gut permeability (Zhou *et al.*, 2017). Moreover, oestrogens increase mucin protection in intestinal epithelial cells thereby decreasing gut permeability (Diebel *et al.*, 2015). As such, increasing progesterone levels in the luteal phase may possibly affect the tight and adherens junctions of the paracellular pathway, the transcellular (talin) and the vascular barrier (catenin) pathways, which all protect against bacterial translocation (Maes *et al.*, 2019). Moreover, lowered steady-state levels of progesterone may be associated with upregulated progesterone receptors (Saracoglu *et al.*, 1985), which may increase sensitivity of, for example, colon muscle cells to progesterone (Cheng *et al.*, 2008). As a consequence, relatively small increments in progesterone coupled with upregulated progesterone receptors and relatively higher oestradiol steady-state levels could contribute to increased gut permeability and, in turn, bacterial translocation thereby stimulating IgA production 5–7 days later (Cerutti, 2008). As such, changes in progesterone during the menstrual cycle coupled with a relative corpus luteum insufficiency (Roomruangwong *et al.*, 2019) may drive menstrual cycle-associated increments in IgA responses to LPS and thus PMS/PeriMS symptoms. Nevertheless, no studies have examined the effects of sex hormones on the gut tight and adherens junctions and the gut vascular barrier.

The current findings should be interpreted within its limitations. First, it would have been even more interesting if we had measured the gut microbiome and stool assays including direct indicants of gut dysbiosis (Simeonova *et al.*, 2018). Second, we enrolled a relatively small sample to detect associations between the biomarkers and PMS or PeriMS classifications. Nevertheless, the strengths of the study are that we examined associations over time between biomarker measurements and clinical data during the menstrual cycle. Interestingly, while the repeated measurements in IgA responses were significantly associated with those in symptoms, no associations could be detected between LPS data and any of the diagnoses of PMS or PeriMS. This indicates that research in PMS or PeriMS should always examine the associations over time between biomarkers and affective, fatigue and physio-somatic symptoms because a diagnosis of PMS/PeriMS is a limited aspect of peri-menstrual symptoms that cannot capture those associations over time.

In conclusion, during the menstrual cycle there are significant changes in IgA responses to LPS of Gram-negative bacteria with peaks in the late luteal phase and lows from week 1 to ovulation. Increments in progesterone during the menstrual cycle superimposed on lowered steady-state progesterone levels during the cycle may drive those menstrual cycle-associated alterations in IgA responses to LPS thereby contributing to severity of peri-menstrual, physio-somatic, anxiety, food cravings and breast swelling symptoms.

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Author contributions. CR and MM made the design of the study. CR recruited and screened the participants. MM performed statistical analyses. MG performed analyses. AC contributed in a meaningful way to the intellectual content of this paper. All authors agreed upon the final version of the paper.

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