The role of immune and oxidative pathways in menstrual-cycle associated depressive, physio-somatic, breast and anxiety symptoms: modulation by sex hormones.

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Abstract

Objective: To examine whether 1) immune and nitro-oxidative stress (IO&NS) biomarkers are associated with premenstrual syndrome (PMS); and 2) changes in IO&NS biomarkers during the menstrual cycle (MC) are associated with PMS symptoms and plasma estradiol and progesterone.

Methods: Forty-one women completed the Daily Record of Severity of Problems (DRSP) rating scale during 28 consecutive days and MC Associated Syndrome (MCAS) was diagnosed when the summed DRSP score during the MC is > 0.666 percentile. We assayed plasma levels of complement C3 and C4, highly sensitive C-reactive protein (hsCRP), haptoglobin (Hp), advanced oxidation protein products (AOPP), lipid hydroperoxides (LOOH), nitric oxide metabolites (NOx), total radical-trapping antioxidant parameter (TRAP), sulfhydryl (-SH) groups and the activity of paraoxonase (PON)1 at days 7 (D7), 14 (D14), 21 (D21) and 28 (D28) of the MC.

Results: All biomarkers, except hsCRP, showed significant alterations during the MC. Arylesterase (AREase) was lowered at D28, while LOOH increased at D14 and C4 at D21 in women with MCAS. The total DRSP score was predicted by the combined effects of C4 (positively) and AREase and malondialdehyde (MDA) (both inversely associated). Progesterone lowered levels of LOOH, AOPP and C3 and estradiol lowered levels of Hp while both sex hormones increased 4-(chloromethyl)phenyl acetate (CMPA)ase and AREase activities and levels of -SH groups.

Conclusion: PMS/MCAS is not accompanied by a peripheral inflammatory response. Lowered MDA and antioxidant defenses and increased C4 may play a role in MC-associated symptoms while sex hormones may have a protective effect against oxidative stress toxicity.

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Introduction

Premenstrual syndrome (PMS) is a prevalent and multi-dimensional condition among reproductive-age women (50–80%) and is characterized by recurrent physical, emotional, and behavioral symptoms which develop the week before menses and disappear within a few days after menstruation (Ryu and Kim, 2015). The symptoms of PMS may interfere with women's psychosocial functioning and include depressed mood, irritability, anger, sleep problems, poor concentration, fatigue, bloating, food craving, breast discomfort, and muscle and abdominal cramps (Dennerstein et al., 2009). PMS is often comorbid with psychiatric disorders including anxiety disorders, major depressive disorder (MDD) (Uran et al., 2017) and bipolar disorder (BD), especially BD type II (Cirillo et al., 2012; de Carvalho et al., 2018).

One commonly used rating scale to measure severity of PMS symptoms is the Daily Record of Severity of Problems (DRSP) (Endicott et al., 2006), which was developed as a tool to screen for DSM-IV criteria for Premenstrual Dysphoric Disorder (PMDD) (Endicott et al., 2006). A cut-off value of > 50 on the first day of the menses may be used to screen for PMS (Biggs and Demuth, 2011; Endicott et al., 2006; Hofmeister and Bodden, 2016). A second case definition of PMS uses a DRSP score of at least 70 on day -5 to -1 of the cycle coupled with at least 30% difference between the premenstrual and postmenstrual score (Qiao et al., 2012). Another commonly used case definition for PMS is that of the American College of Obstetricians and Gynecologists (ACOG) (American College of Obstetricians and Gynecologists, 2014), namely at least one affective and physical symptom should be present 5 days prior to menses and symptoms should remit within 4 days after the onset of bleeding without recurrence until at least day 13 of the next cycle. This pattern should be present for at least 3 consecutive menstrual cycles, with significant dysfunctions in social, academic, or work

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performance during the symptomatic phase (American College of Obstetricians and Gynecologists, 2014).

Based on the inspection of the variations of daily measurements of the DRSP values across the menstrual cycle we developed 2 new case definitions for PMS: 1) Peri-Menstrual Syndrome (PeriMS) reflecting an increase in DRSP values during the "peri-menstrual period" and measured as sum of DRSP values at days 1, 2, 24, 25, 26, 27 and 28 $\geq$ 307 (0.666^{th} percentile of the distribution of the summed DRSP scores); and 2) Menstrual Cycle Associated Syndrome (MCAS) reflecting increased DRSP values all over the menstrual cycle coupled with exaggerated increases in the premenstrual period and which is diagnosed when the sum of all DRSP scores from day 1 through day 28 is $\geq$ 1.050 (Roomruangwong et al., 2019a). Moreover, in the latter study we also established the factor structure of the DRPS items and detected 4 interpretable dimensions namely 1) depressive dimension; 2) physio-somatic dimension, 3) eating & breast dimension which comprises symptoms of appetite, craving and breast tenderness/swelling, and 4) anxiety dimension.

In different biomarker studies we established that the case definition of MCAS was consistently externally validated by biomarkers whereas the ACOG and PMS case definitions were most often not associated with the same biomarkers (Roomruangwong et al., 2019c). For example, we observed that the levels of progesterone and estradiol were significantly lower in women with MCAS as compared with women without MCAS (Roomruangwong et al., 2019a). Moreover, alterations in the levels of these sex hormones during the menstrual cycle were significantly and inversely associated with changes in the DRSP scores during the menstrual cycle (Roomruangwong et al., 2019a).

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Menstruation is an immune-inflammatory process which is strongly modulated by sex hormones (Evans and Salamonsen, 2012; Finn, 1986). During the late luteal phase, progesterone, which has anti-inflammatory properties, declines leading to a sequence of local inter-cellular inflammatory interactions within the endometrium (Evans and Salamonsen, 2012). Moreover, loss of progesterone is accompanied by decreased defenses against reactive oxygen species (ROS) leading to increased oxidative stress (Sugino et al., 1996) which subsequently results in an increased release of nuclear factor (NF)-κB and increased synthesis of pro-inflammatory prostaglandins, cytokines, chemokines and matrix metalloproteinases (MMP) (Gloire et al., 2006; Sugino et al., 2004). This process results in leukocyte recruitment and increased levels of degradative enzymes and MMP activators which together with a hypoxic environment lead to tissue breakdown and menstrual bleeding (Gloire et al., 2006; Sugino et al., 2004). After endometrial shedding, growth factors coupled with the microenvironmentally-induced changes in macrophages and neutrophils from pro- to anti-inflammatory phenotypes lead to re-epithelialization and restoration of the endometrial tissue integrity (Evans and Salamonsen, 2012).

There is increasing interest whether PMS or premenstrual symptoms are associated with exaggerated inflammatory responses (Bertone-Johnson, 2016; Gold et al., 2016; Graziottin and Zanello, 2015). In women of reproductive age, plasma and endometrial levels of inflammatory mediators including C-reactive protein (CRP), interleukin (IL)-6, IL-1β, and tumor necrosis factor-α (TNF- α) are increased after ovulation and peak during menstruation (Berbic et al., 2014). CRP levels are positively associated with PMS symptom severity, with strongest associations with mood and pain symptoms (Puder et al., 2006). A study on 277 young women found that the severity of both emotional and physical symptoms was positively associated with

the levels of IL-2, IL-4, IL-10, IL-12, and interferon-gamma (IFN-γ) while the levels of IL-12 and IFN-γ were more than twice as high in women with PMS than in those without (Bertone-Johnson et al., 2014). We observed that plasma levels of chemokines, including CCL2 (C-C motif ligand 2), CCL5 (C-C motif ligand 5 or RANTES) and CCL11 (C-C motif ligand 11 or eotaxin) were significantly increased in women with MCAS (Roomruangwong et al., 2019c). Moreover, IgA immune responses to lipopolysaccharides (LPS) of commensal gut Gram-negative bacteria were highly significantly associated with changes in the DRSP score whereby peaks in the IgA responses occurred at the end of the cycle when also the severity of MCAS symptoms peaked (Roomruangwong et al., 2019b).

All in all, it appears that sex hormones and immune biomarkers could have a significant role in PMS/MCAS. Moreover, activated immune-inflammatory pathways are associated with increased oxidative and nitrosative stress (O&NS) (Maes et al., 2011; Moylan et al., 2014) while depressive, physio-somatic and anxiety symptoms are accompanied by increased O&NS (Gerwyn and Maes, 2017; Maes et al., 2018; Maes et al., 2019b; Maes et al., 2019c; Morris et al., 2016). Duvan et al. (2011) found significantly increased levels of lipid hydroperoxides (LOOH) and lowered total antioxidant capacity (TAC) on day 21 of the menstrual cycle in women with PMS as compared with controls, whereas there were no significant differences in malondialdehyde (MDA), protein carbonyl (PC), and sulfhydryl or thiol (-SH) groups at day 3 or day 21 of the menstrual cycle (Duvan et al., 2011). Incebiyik et al. (Incebiyik et al., 2015) found a trend towards higher serum total antioxidant status (TOS), oxidative stress index (OSI), LOOH and –SH levels in PMS as compared to controls using a single time blood sample drawing between day 21 and 28 of the menstrual cycle. However, to the best of our knowledge, there are

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no data on those O&NS biomarkers in PMS/MCAS and whether changes in those biomarkers during the menstrual cycle are associated with symptom DRSP subdomains.

Hence, the aims of this study were to examine a) whether immune and O&NS (IO&NS) biomarkers are associated with different case definition of PMS; and b) whether changes in these IO&NS biomarkers during the menstrual cycle are associated with changes in the severity of PMS/MCAS symptoms, estradiol and progesterone. In accordance with the immune-inflammatory hypothesis of PMS and the knowledge that inflammation is accompanied by O&NS we would expect to find that PMS/MCAS is accompanied by increased levels of acute phase proteins (CRP and Hp) and complement factors (C3, C4) as well as O&NS markers including LOOH, MDA, advanced oxidation protein products (AOPP) and nitric oxide metabolites (NOx) and by lowered levels of antioxidants including paraoxonase 1 (PON1) activity, total radical trapping antioxidant parameter (TRAP) and -SH groups.

Methods

Participants

Forty-one female participants were recruited by verbal announcements at King Chulalongkorn Memorial Hospital during the period April-May 2018 with 21 having subjective complaints of PMS and 20 without such complaints. Inclusion criteria were: 1) non-pregnant women aged 18-45 years; 2) having a regular menstrual cycle with a cycle length of 27-30 days during the past years; 3) being able to read and write in Thai; 4) willing to have 4 blood samples drawn at day 7 (D7), day 14 (D14), day 21 (D21) and day 28 (D28) of the menstrual cycle; and 5) being able to complete the DRPS ratings for all consecutive days of one menstrual cycle. We excluded 1) women with a lifetime history of psychiatric illness (including major depression,

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bipolar disorder, schizophrenia, generalized anxiety disorder, and obsessive-compulsive disorder); 2) women with a history of medical illness, including diabetes type 1, and (auto)immune-inflammatory disorders including rheumatoid arthritis, inflammatory bowel disease, psoriasis, multiple sclerosis, and stroke; and 3) women who currently use any psychotropic medications or hormonal preparations. This 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.

Clinical assessments

All participants were requested to complete a questionnaire with demographic and clinical data (including age, education, menstrual history, body weight and length, a history of substance use and lifestyle variables), and all subjects were evaluated by an experienced psychiatrist before recruitment in the study to exclude medical and/or psychiatric conditions. After informed consent, all participants were requested to complete the Daily Record of Severity of Problems (DRSP) during the consecutive days of their menstrual cycle starting on day 1 of menses. The DRSP is a self-report instrument consisting of 21 items plus 3 functional impairment items commonly used to assess PMS (Endicott et al., 2006). Each item is rated from 1 to 6 (1 = not at all, 2 = minimal, 3 = mild, 4 = moderate, 5 = severe, 6 = extreme). The DRSP has been used to measure the “presence” and “severity” of premenstrual symptoms and can be used to screen for a DSM-IV diagnosis of premenstrual dysphoric disorder (PMDD) (Biggs and Demuth, 2011). The diagnosis of ACOG was made according to ACOG criteria (American College of Obstetricians and Gynecologists, 2014) and the diagnosis of PMS is made when the

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total DRSP score was ≥ 70 on day -5 to -1 of menses and when there was a 30% difference between premenstrual and postmenstrual scores (Biggs and Demuth, 2011; Endicott et al., 2006; Qiao et al., 2012). Additionally, all participants were categorized into PeriMS when the sum of the DRSP scores during the peri-menstrual period (days 1 and 2 + 24 to 28) > 0.666 percentile; and into MCAS when the total daily DRSP score during the menstrual cycle was > 0.666 percentile (Roomruangwong et al., 2019a).

We also computed scores of the four subdomains of the DRSP, namely 1) depressive dimension, which comprises depression, mood swings, sensitivity to rejection, angry-irritable, more conflicts, less interest, out of control, and interference with hobbies and relationships; 2) physio-somatic symptoms comprising concentration disturbances, lethargy, sleepiness, headache, muscle/joint pain and lowered productivity; 3) breast & craving symptoms including changes in appetite and craving, and breast tenderness and swelling; and 4) anxiety symptoms including hopelessness, anxious, lethargy, insomnia, being overwhelmed, and muscle-joint pain. (Roomruangwong et al., 2019a)

Assays

Blood for the assay of IO&NS biomarkers was sampled at 8.00 a.m. after an overnight fast at 4 different time points during the menstrual cycle and blood was immediately centrifuged and the serum aliquoted and stored at −80 °C until thawed for assays. We collected blood samples on day 7 (D7), day 14 (D14), day 21 (D21), and day 28 (D28) of the subject's menstrual cycle. D7 represents the mid-follicular phase when estrogen levels are rising. D14 represents mid-cycle phase when ovulation occurs. D21 represents the mid-luteal phase when progesterone

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levels reach their peak values. D28 represents the end of the cycle when all hormones levels decline to their baseline levels (Messinis et al., 2014; Mihm et al., 2011; Owen, 1975).

The IO&NS biomarkers included AOPP, LOOH, NOx, TRAP, -SH groups and the activity of PON-1. AOPP was quantified in a microplate reader (EnSpire, Perkin Elmer, USA) at a wavelength of 340 nm (Hanasand et al., 2012) and is expressed in mM of equivalent chloramine T. LOOH was quantified by chemiluminescence in a Glomax Luminometer (TD 20/20), in the dark, at 30 °C for 60 min (Gonzalez Flecha et al., 1991; Panis et al., 2012) and the results are expressed in relative light units (RLU). NOx was assessed in a microplate reader (EnSpire®, Perkin Elmer, USA) at a wavelength of 545 nm by measuring the concentration of nitrite and nitrate (Navarro-Gonzálvez et al., 1998) and results are expressed as µM. TRAP was evaluated in a microplate reader (Victor X-3, Perkin Elmer, USA) and results are expressed in µM trolox (Repetto et al., 1996). -SH groups were evaluated in a microplate reader (EnSpire®, Perkin Elmer, USA) at a wavelength of 412 nm and results are expressed in µM (Hu, 1994; Taylan and Resmi, 2010). The methods to assay PON1 enzymatic activities were explained previously (Matsumoto et al., 2019), namely "to stratify individuals in the functional genotypes of the PON1 Q192R polymorphism (QQ, QR, and RR), the substrates used were phenyl acetate (PA, Sigma, USA) under high salt condition and 4-(chloromethyl)phenyl acetate (CMPA, Sigma, USA), which is an alternative to the use of the toxic paraoxon. PON1 activities were determined by the rate of hydrolysis of CMPA (CMPAase, which is influenced by the PON1 Q192R polymorphism) as well as by the rate hydrolysis of phenyl acetate under low salt condition (AREase, which is less influenced by the PON1 Q192R polymorphism). Analysis were conducted in a microplate reader (EnSpire, Perkin Elmer, USA) (Richter et al., 2008). Although the PON1 Q192R genotypes were assayed, those data yielded non-significant results and as such

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the data are not presented here. Nevertheless, the genotypes were added as covariates in the different analyses (Matsumoto et al., 2019). The intra-assay coefficients of variation were <10% for all O&NS analytes.

Assays of serum levels of C3 and C4 were assayed using the Binding Site SPAPLUS® turbidimetric analyzer. The assay of soluble antigen concentrations by turbidimetric methods involves the reaction with specific antiserum to form insoluble complexes. Concentrations are automatically calculated by reference to a calibration curve. The intra-assay CV value is 1.7% for C3 and 1.9% for C4. The assay of hsCRP was performed using CardioPhase® hsCRP, which is a diagnostic reagent for the quantitative determination of hsCRP in human serum by means of particle enhanced immunonephelometry using BN* Systems. The principle of the assay is that polystyrene particles coated with monoclonal antibodies specific to human CRP are aggregated when mixed with samples containing CRP. The result is evaluated by comparison with a standard of known concentration. The intra-assay CV value is 2.7%. Serum haptoglobin was measured by immunoturbidimetry on the Architect C series analyzer (Abbott Diagnostics, Abbott Park, IL, USA) according to the manufacturer's instructions.

An immunoassay for the in vitro quantitative determination of estradiol and progesterone using Cobas® 601 with competition principle was used. The methods of the assay had been previously described in our previous published work (Roomruangwong et al., 2019b). As explained in our paper, we also used indices of steady state levels of those hormones which averaged the measurements over the menstrual cycle, namely the sum of the concentrations at D14 + D21 + D28 for progesterone (D14+D21+D28 progesterone) and the sum of the 4 measurements for estradiol (D7+D14+D21+D28 estradiol). In addition, we employed distributed lag models, which allow to predict the biomarkers by sex hormonal levels measured one week

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earlier as expressed as Δ values. The intra-assay CV value is 1.2% for oestradiol and 2.3% for progesterone.

Statistics

Analysis of contingency tables ($\chi 2$ test) and analysis of variance (ANOVA) were used to assess associations among nominal variables and differences in continuous variables between categories, respectively. Generalized estimating equation (GEE), repeated measures, was used to assess effects of time, MCAS diagnosis and the time X MCAS interaction on DRSP and various biomarker data. GEE, repeated measurements, was also used to delineate the associations between the biomarkers and the DRSP values measured at D8, D14, D21 and D28. We also examined distributed lag models, which allow to predict the DRSP scores by biomarkers measured one week earlier. Tests were 2-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.

Sociodemographic and clinical data

Table 1 shows the demographic data of the 41 women recruited to participate in the current study as published in Roomruangwong et al. (2019). There were no significant differences in age, education, income, age at menarche, and cycle length between women with and without MCAS. The duration of menses was somewhat longer in women with than without MCAS. **Table 2** shows the changes during the menstrual cycle in the total DRSP score and its

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subdomains. The DRSP score and all its subdomains peaked at D28, whereas lows were detected at D14.

Variations in IO&NS biomarkers during the menstrual cycle

GEE analysis, repeated measurements, was used to examine the effects of time, MCAS (and the other 3 case definitions) and MCAS x time on the IO&NS biomarkers. There were no significant effects of MCAS or any of the other case definitions on the biomarkers while a significant interaction pattern time X MCAS was established for AREase, LOOH and C4 values. **Table 3** shows that all biomarkers, except hsCRP, showed significant alterations during the menstrual cycle. TRAP values showed lows at D14 and D28. CMPAase activity shows lows at D7 while AREase activity was lower at D28 than at D21. There was also a variation in -SH groups with lows at D21 and in NOx with lows at D14. LOOH levels were increased at D14 while AOPP levels were higher at D7. MDA levels were significantly lower at D28 than at D7, D14 and D21. Hp, C3 and C4 showed peak levels at D7.

Table 4 describes the three significant two-way interaction between time and MCAS diagnosis. First, PON1 AREase was lower at D28 in women with MCAS as compared with D7 and D14 values in both subgroups and D28 values in women without MCAS. Second, LOOH values were higher in women with MCAS at D14 as compared with all women without MCAS. Third, C4 concentrations were significantly higher in women with MCAS at D21 as compared with at all other values.

Associations between DRSP ratings and biomarkers during the menstrual cycle

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In order to examine the associations between DRSP rating scores and biomarkers during the menstrual cycle we performed GEE analysis, repeated measures, with the DRSP scores as dependent variables and the biomarkers as explanatory variables. **Table 5** shows that the DRSP total score and the depression subscore were best predicted by the combined effects of the lagged C4 values (positively associated) and PON1 AREase and MDA (both inversely associated). The physio-somatic domain score was best predicted by the combined effects of C4 lagged values (positively) and MDA (inversely associated). The breast-craving domain score was significantly associated with MDA values only while the anxiety domain score was positively associated with LOOH and C4 lagged values.

Associations between the biomarkers and sex hormones during the menstrual cycle

In order to examine the associations between the biomarkers and sex hormones during the menstrual cycle we performed GEE analysis, repeated measures, with the biomarkers as dependent variables and the sex hormones as explanatory variables. **Table 6** shows that PON1 CMPAase activity was positively associated with estradiol levels, while AREase activity was positively associated with steady state levels in progesterone (averaged over D14, D21 and D28) and Δ changes in progesterone levels. The -SH groups were significantly and positively associated with increasing progesterone levels. AOPP levels were significantly and inversely predicted by steady state progesterone levels and increases in Δ estradiol levels during the previous week. LOOH was significantly and inversely associated with progesterone levels. Hp levels were significantly and inversely associated with the steady state estradiol levels while C3 was significantly and inversely associated with progesterone steady state levels. There were no significant associations between the sex hormones and either hsCRP, C4 or MDA.

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Discussion

The first major finding of this study is that there are no pathological increases in the inflammatory markers hsCRP and Hp during the pre-menstrual period and that the PMS/MCAS case definitions are not associated with pathologically increased levels of hsCRP (> 5 pg/mL) and Hp (> 150 mg/mL) (LaGow, 2007; Pagana and Pagana, 2014). As such, there is no evidence that PMS/MCAS and the menstrual period are accompanied by a peripheral inflammatory response. These findings suggest that the inflammatory processes in endometrial bleeding, ovulation and remodeling during the cycle (Berbic et al., 2014) are not accompanied by peripheral signs of inflammation and, therefore, that the blood-uterine barrier (McRae, 1988) is not more permeable in PMS/MCAS or during the peri-menstrual period. These negative findings also show that our previous results on increased chemokines in MCAS (Roomruangwong et al., 2019c) do not necessary indicate that PMS/MCAS is an inflammatory condition.

Nevertheless, we found significant (albeit not in the inflammatory range) alterations in Hp, C3 and C4 (but not hsCRP) levels during the menstrual cycle with peak levels immediately after the menses while MCAS is associated with elevated C4 levels at day 21. A previous report showed higher hsCRP levels in the early follicular phase between day 1 to 4 of the menstrual cycle (Puder et al., 2006). Interestingly, C3 and C4 genes are up-regulated on day 19-21 of the menstrual cycle among women with a history of implantation failure (Huang et al., 2017). C3 is primarily synthesized by the liver, but is also produced by other hematologic cells and human endometrium (Hasty et al., 1994; Isaacson et al., 1991; Sayegh et al., 1996). Endometrial stromal and glandular cells express the C3 gene, and C3 expression is more prominent in secretory endometrium (endometrium during luteal phase of the menstrual cycle) than in proliferative

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endometrium (Sayegh et al., 1996). One mechanism explaining our findings is that the inflammatory process of menstrual bleeding may lead to modest but significant increases in plasma C3, C4 and Hp through spill-over to the peripheral blood. Although in our study no significant alterations in hsCRP during the cycle could be detected, another study found that peak CRP values were established in the early follicular phase between day 1 to 4 of the menstrual cycle (Puder et al., 2006).

The second major finding of our study is that in PMS/MCAS or in the pre-menstrual period there are no signs of pathologically increased nitro-oxidative stress biomarkers as established in major depression or GAD (Maes et al., 2018; Maes et al., 2019b). Our findings are in agreement with those of a preliminary study, which could not detect changes in lipid peroxidation in PMS (Kalia et al., 2001). Our present findings of the current study contrast with our a priori hypothesis and the findings that major and bipolar depression and GAD are frequently associated with very high levels of the same O&NS biomarkers as those measured here (Maes et al., 2018; Maes et al., 2019b). Nevertheless, we detected that AOPP levels showed peak values after the menses and lows just before the menses yielding a highly significant difference of 1.17 SDs between both time points. In contrast to our a priori hypothesis, MDA levels were significantly higher during the first three weeks of the cycle as compared with day 28 while there was no significant association with PMS/MCAS. Two previous studies found no significant differences in MDA levels between PMS patients and controls (Balat et al., 2007; Duvan et al., 2011).

Our results suggest that increased indicants of protein oxidation (AOPP levels) and aldehyde formation (MDA levels) may accompany the post-menses increments in C3, C4 and Hp whereby increased levels of MDA may remain increased during three consecutive weeks to

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decrease one week before the menses. This may be important as increased MDA expression may induce IgM-mediated responses, which have anti-inflammatory and housekeeping properties by clearing cell debris (Maes et al., 2019a; Roomruangwong et al., 2018). This latter mechanisms may play a role during endometrial bleeding and the restoration of the endometrial integrity after the menses (Evans and Salamonsen, 2012). Moreover, we found that LOOH levels are significantly increased at day 14 in women with MCAS as compared with non-MCAS women indicating a modest increase in lipid peroxidation around ovulation in MCAS women only. These results extend those of previous data that LOOH levels are increased in PMS subjects at day 21 of the menstrual cycle (Duvan et al., 2011).

In the present study, we could not find that key antioxidant biomarkers such as TRAP, PON1 CMPAase activity and -SH groups were either associated with PMS/MCAS or with the pre-menstrual period. This contrasts with our a priori hypothesis and with findings of Duvan et al. (Duvan et al., 2011) who established decreased plasma antioxidant capacity in subjects with PMS at day 21 of the menstrual cycle. Our negative findings concur with those of a preliminary study, which could not detect changes in antioxidant status in PMS (Kalia et al., 2001). Nevertheless, we observed lowered PON1 AREase activity at day 28 in women with MCAS. A previous study found no significant differences in PON1 (presumably CMPAase) activity between PMS and controls, although PON1(presumably CMPAAse) was higher in the luteal phase in controls as compared with PMS (Ozcan et al., 2017). Lowered PON1 AREase activity is reported in depression, bipolar disorder and GAD although these disorders are also accompanied by lowered PON1 CMPAase, TRAP and -SH groups (Maes et al., 2018; Moreira et al., 2019). PON1 activity protects against oxidation of lipoproteins thereby providing protection against cardiovascular diseases and metabolic syndrome (de la Iglesia et al., 2014).

The third major finding of this study is that the alterations in the DRSP score during the cycle are inversely associated with changes in PON1 AREase activity and MDA levels and positively with changes in C4 concentrations. Moreover, the changes in depressive, physio-somatic, breast-craving and anxiety symptoms during the menstrual cycle are predicted by different combinations of these biomarkers and LOOH. Thus, it appears that small increases in the inflammatory mediator C4 (but not CRP or Hp) and lowered antioxidant defenses (PON1 AREase) are associated with increases in MCAS symptoms during the menstrual cycle. Increased C4 levels are reported in depression (Maes et al., 1997) while PON1 enzyme activity is frequently decreased in depression (Bortolasci et al., 2014; de Melo et al., 2017 ; Moreira et al., 2017), especially in severe cases (Oglodek, 2017). An association between severity of PMS symptoms and inflammatory markers was established by various authors (Bertone-Johnson, 2016; Gold et al., 2016; Puder et al., 2006) who reported that the severity of PMS symptoms was positively associated with levels of CRP, IL-2, IL-4, IL-10, IL-12, and IFN-γ.

Contrary to our priori hypothesis, we found that lower MDA levels predicted DRSP severity and most symptom domains (all except anxiety). This contrasts findings in depression and GAD, which are accompanied by highly increased MDA levels (Maes et al., 2018; Maes et al., 2019b). As discussed above, increased expression of MDA elicits anti-inflammatory IgM-mediated immune responses (Maes et al., 2019a; Roomruangwong et al., 2018) and, as such, lowered MDA levels at the end of the menstrual cycle may be accompanied by less clearing of necrotic cells and slower healing of the endometrium thereby producing more symptoms by for example increasing uterine chemokine levels (Roomruangwong et al., 2019c).

The fourth major finding of this study is that changes in sex hormone levels during the menstrual cycle and their steady state levels averaged over the cycle appear to regulate the levels

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of key antioxidants and OS toxicity biomarkers. Thus, we found that progesterone was accompanied by lowered LOOH, AOPP and C3, and estradiol with lowered levels of Hp, while both sex hormones increased -SH groups and both PON1 CMPAase and AREase activities. These results suggest a protective effect of both sex hormones against OS toxicity in part by enhancing antioxidant defenses. These findings extend the knowledge that estrogen and progesterone have protective effects against oxidative stress (Tang et al., 1996; Yagi, 1997). In animal studies, progesterone treatment significantly decreases markers of neuroinflammation and oxidative stress (Webster et al., 2015). In an animal model of sepsis, progesterone improved sepsis by reducing the levels of inflammatory cytokines (IL-6 and TNF-α) while restoring antioxidant enzyme activities (Aksoy et al., 2014). Estrogens attenuate oxidative stress by preventing the generation ROS and by scavenging ROS in the myocardium and in the vasculature (Arias-Loza et al., 2013). Ovariectomized female animals display an increased production by NADPH oxidase in systemic and cerebral arteries, which is consistent with the concept that estrogens normally suppress the oxidative stress (Miller et al., 2007; Tsuda et al., 2005).

A first limitation of the current study is that we employed a case-control design which does not allow to establish causal associations. Second, we recruited a relatively small study sample (n=41), although the power of the repeated measurement analyses was adequate (>0.8) (Roomruangwong et al., 2019a). Third, it would have been more interesting if we had assayed all biomarkers on a daily basis and performed group spectral analysis to examine common rhythms in biomarkers and DRSP scores including using distributed lag models.

Conclusions

In this current study, there is no evidence that PMS/MCAS and the menstrual period are accompanied by a peripheral inflammatory response or pathologically increased nitro-oxidative stress. However, small increases in the inflammatory mediator C4 (but not hsCRP or Hp) and lowered antioxidant defenses (PON1 AREase) are associated with increases in PMS/MCAS symptoms during the menstrual cycle. Moreover, sex hormones (both estradiol and progesterone) may have a protective effect against oxidative stress and a stimulatory effect on antioxidant defenses.

Authorships

CR and MM made the design of the study. CR recruited and screened the participants. MM performed statistical analyses. AKM, APM, LOS, JVLP, EGM, SS, and DSB performed analyses. AFC 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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Conflict of interest

The authors have no conflict of interest with any commercial or other association in connection with the submitted article.

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Table 1 Socio-demographic data of 41 women with and without the menstrual cycle-associated syndrome (MCAS) (Roomruangwong et al., 2019).

Variables	No MCAS	MCAS	F/*X*2/ψ	df	p
Age (years)	31.4 (6.5)	30.9 (8.2)	0.05	1/39	0.828
Education (years)	16.0 (1.0)	15.9 (2.1)	0.09	1/39	0.766
Single / married	22/5	7/7	4.41	1	0.036
Family income (baht/month)	94,440 (95,885)	68,928 (48,369)	0.86	1/37	0.359
Age menarche (years)	12.7 (1.1)	13.0 (1.5)	0.67	1/39	0.419
Length cycle (days)	27.8 (2.2)	27.1 (6.7)	0.23	1/39	0.633
Duration menses (days)	4.3 (1.3)	5.4 (1.5)	5.99	1/39	0.019

All results are shown as mean (±SD)

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Table 2 Variations in the DRSP (Daily Record of Severity of Problems) score and subdomain scores during the menstrual cycle.

Variables	Day 7 [A]	Day 14 [B]	Day 21 [C]	Day 28 [D]	Time effects		
					Wald	df	p
Total DRPS score	32.8 (2.1) [B,D]	28.5 (0.9) [A,C,D]	32.4 (1.5) [B,D]	42.7 (3.7) [A,B,C]	32.09	3	<0.001
Depression score	12.3 (0.8) [B]	10.6 (0.3) [A,D]	11.8 (0.7) [D]	16.2 (1.5) [B,C]	23.08	3	<0.001
Physio-somatic score	8.4 (0.5) [B,D]	7.3 (0.3) [A,C,D]	8.5 (0.5) [B,D]	11.4 (1.0) [A,B,C]	35.09	3	<0.001
Breast-craving score	5.5 (0.4) [D]	4.6 (0.2) [C,D]	5.9 (0.3) [B,D]	8.1 (0.7) [A,B,C]	34.64	3	<0.001
Anxiety score	6.9 (0.6) [D]	6.3 (0.4) [D]	6.8 (0.5) [D]	8.6 (0.7) [A,B,C]	16.19	3	0.001

All results are shown as mean (SE). Day 7, 14, 21 and 28: days of the subject's menstrual cycle

A,B,C,D: pairwise comparisons between the 4 time points

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Table 3 Variations in immune and nitro-oxidative stress biomarkers during the menstrual cycle: effects of GEE analysis, repeated measures

Variables	Day 7 [A]	Day 14 [B]	Day 21 [C]	Day 28 [D]	Time effects		
					Wald	df	p
TRAP (µmol trolox)	1005.5 (19.2) [B,C]	903.8 (17.8) [A,C]	990.2 (17.3) [B,D]	923.8 (16.9) [A,D]	31.67	3	<0.001
CMPAase (U/mL)	30.8 (1.3) [B,C.D]	33.2 (1.5) [A]	35.2 (1.8) [A]	33.9 (1.4) [A]	14.41	3	0.002
AREase (U/mL)	202.5 (10.4)	200.8 (10.1)	216.0 (9.8) [D]	195.6 (8.3) [C]	8.62	3	0.035
-SH group (µmol/L)	327.6 (9.5) [C]	331.7 (7.5) [C]	300.7 (9.4) [A,B]	325.1 (8.8)	8.29	3	0.040
NOx (µmol/L)	5.61 (0.52)	4.77 (0.56) [C,D]	6.61 (0.92) [B]	5.52 (0.30) [B]	10.63	3	0.014
AOPP (µmol/L/eq. Cloramin T)	75.1 (6.2) [D]	71.3 (7.0) [D]	69.8 (5.1)	61.3 (4.4) [A,B]	12.07	3	0.007
LOOH (URL)	1328 (103)	1629 (172) [C]	1196 (103) [B]	1615 (240)	7.99	3	0.046
MDA (µM)	1.17 (0.04) [D]	1.07 (0.05) [D]	1.17 (0.05) [D]	0.80 (0.04) [A,B,C]	46.00	3	<0.001
hsCRP (mg/L)	1.57 (0.31)	1.76 (0.34)	1.53 (0.40)	2.66 (1.14)	2.71	3	0.438
Haptoglobin (mg/dL)	101.3 (6.8) [C,D]	94.7 (5.9)	92.9 (6.2) A	89.0 (5.9) A	11.05	3	0.011
C3 (mg/dL)	120.5 (4.2) [B,D]	113.5 (3.9) [A]	117.1 (3.5) [D]	113.2 (3.4) [A,C]	18.05	3	<0.001
C4 (mg/dL)	26.1 (1.3) [B,D]	24.5 (1.2) [A]	25.4 (1.2)	24.8 (1.3) [A]	10.85	3	0.013

All results are shown as mean (SE). Day 7, 14, 21 and 28: days of the subject's menstrual cycle

A,B,C,D: pairwise comparisons between the 4 time points

TRAP: total radical trapping antioxidant parameter; PON: paraoxonase; PON1 CMPAase: PON1 enzyme activity directed to 4-(chloromethyl)phenyl acetate; AREase: arylesterase; -SH: sulfhydryl groups; NOx: nitric oxide metabolites; AOPP: advanced oxidized protein products; LOOH: lipid hydroperoxides; MDA: malondialdehyde; hsCRP: high sensitivity CRP; C3, C4: complement factors C3 and C4.

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Table 4 Two-way interaction between time by diagnosis of menstrual cycle associated syndrome (MCAS): results of GEE analysis, repeated measures

Variables	Time	No MCAS	MCAS	MCAS x Time		
				Wald	df	p
PON1 AREase (U/mL)	Day 7	199.1 (12.8)	208.6 (17.6)	9.97	3	0.019
	Day 14	197.1 (9.1)	207.8 (23.2)			
	Day 21	220.6 (10.8)	207.3 (19.2)			
	Day 28	203.3 (9.5)	181.5 (15.4)			
LOOH (URL)	Day 7	1332 (131)	1320 (170)	11.00	3	0.012
	Day 14	1317 (129)	2209 (384)			
	Day 21	1033 (110)	1510 (295)			
	Day 28	1392 (211)	2032 (546)			
Complement C4 (mg/dL)	Day 7	25.2 (1.6)	27.6 (2.1)	8.24	3	0.041
	Day 14	23.2 (1.3)	26.8 (2.2)			
	Day 21	23.4 (1.3)	29.0 (2.1)			
	Day 28	24.3 (1.6)	25.7 (2.2)			

All results are shown as mean (SE)

Time effects: day 7, 14, 21 and 28: days of the subject's menstrual cycle

PON1 AREase: Paraoxonase-1 arylesterase; LOOH: lipid hydroperoxides

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Table 5 Results of GEE analysis, repeated measures, which examines the associations of time between the DRSP (Daily Record of Severity of Problems) total score and its subdomains and the immune and oxidative stress biomarkers

Dependent variables	Exploratory variables	B	SE	Wald	df	p
DRSP	PON1 AREase	-0.164	0.0686	5.72	1	0.017
	MDA	-0.159	0.0576	7.60	1	0.006
	C4	0.276	0.0812	11.59	1	0.001
Depression	C4	0.253	0.0763	10.99	1	0.001
	PON1 AREase	-0.190	0.0887	4.58	1	0.032
	MDA	-0.167	0.0495	11.44	1	0.001
Physio-somatic	C4	0.277	0.0965	8.73	1	0.003
	MDA	-0.134	0.0563	5.64	1	0.018
Breast-craving	MDA	-0.148	0.0677	4.76	1	0.029
Anxiety	LOOH	0.121	0.0588	4.23	1	0.040
	C4	0.338	0.1400	5.83	1	0.016

PON1 AREase: Paraoxonase-1 arylesterase; MDA: malondialdehyde; C4: Complement C4 (entered is the lagged value that is one week prior to DRSP scoring); LOOH: lipid hydroperoxides

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Table 6 Results of GEE analyses, repeated measures, with the immune-oxidative biomarkers as dependent variables and sex hormones as explanatory variables

Dependent variables	Exploratory variables	B	SE	Wald	df	p
PON1 CMPAase	Oestradiol	0.122	0.0551	4.88	1	0.027
PON1 AREase	Δ Progesterone	0.108	0.0324	11.05	1	0.00
	Progesterone D14+D21+D28	0.220	0.1122	3.84	1	0.050
-SH group	Δ Progesterone	0.103	0.0328	9.45	1	0.002
AOPP	Progesterone D14+D21+D28	-0.395	0.0446	8.96	1	0.003
	Δ Oestradiol	-0.113	0.0930	18.09	1	<0.001
LOOH	Progesterone	-0.198	0.0717	7.62	1	0.006
Haptoglobin	Oestradiol D7+D14+D21+D28	-0.115	0.0457	6.29	1	0.012
Complement C3	Progesterone D14+D21+D28	-0.499	0.1077	21.48	1	<0.001

PON: paraoxonase; PON1 CMPAase: PON1 enzyme activity directed to 4-(chloromethyl)phenyl acetate; AREase: arylesterase;

-SH: sulfhydryl groups; AOPP: advance oxidation protein products; LOOH: lipid hydroperoxides; Δ Progesterone and Δ Oestradiol:

changes in both hormones the week before blood sampling; Progesterone D14+D21+D28: summed progesterone values obtained

at day 7, 21 and 28; Oestradiol D7+D14+D21+D28: summed progesterone values obtained at day 7, 14, 21 and 28