The uterine-chemokine-brain axis: menstrual cycle-associated symptoms (MCAS) are in part mediated by CCL2, CCL5, CCL11, CXCL8 and CXCL10

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Abstract

Objective: To examine associations between chemokines and menstrual cycle associated symptoms (MCAS).

Methods: Forty-one women completed the Daily Record of Severity of Problems (DRSP) rating scale during 28 consecutive days of the menstrual cycle. MCAS is diagnosed when the total daily DRSP score during the menstrual cycle is > 0.666 percentile. We assayed plasma CCL2, CCL5, CCL11, CXCL8, CXCL10, EGF, IGF-1, and PAI-1 at days 7, 14, 21 and 28 of the menstrual cycle.

Results: CCL2, CCL5, CCL11 and EGF are significantly higher in women with MCAS than in those without. Increased CCL2, CXCL10, CXCL8, CCL11 and CCL5 levels are significantly associated with DRSP scores while CCL2 is the most significant predictor explaining 39.6% of the variance. The sum of the neurotoxic chemokines CCL2, CCL11 and CCL5 is significantly associated with the DRSP score and depression, physiosomatic, breast-craving and anxiety symptoms. The impact of chemokines on MCAS symptoms may differ between consecutive weeks of the menstrual cycle with CCL2 being the most important predictor of increased DRSP levels during the first two weeks, and CXCL10 or a combination of CCL2, CCL11 and CCL5 being the best predictors during week 3 and 4, respectively.

Discussion: The novel case definition “MCAS” is externally validated by increased levels of uterus-associated chemokines and EGF. Those chemokines are involved in MCAS and are regulated by sex hormones and modulate endometrium functions and brain neuro-immune responses, which may underpin MCAS symptoms. As such, uterine-related chemokines may link the uterus with brain functions via a putative uterine-chemokine-brain axis.

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Introduction

Premenstrual syndrome (PMS) comprises both physical (e.g cramps, bloating, breast tenderness, headache, and fatigue) and emotional/behavioral (e.g. depression, anxiety, anger, irritability and mood lability) symptoms, which appear during the luteal phase of the menstrual cycle and remit shortly after the onset of menses. [1,2] The prevalence of PMS ranges from 12% in France through 98% in Iran, with an overall prevalence of 47.8% (95% CI: 32.6-62.9). PMS is common among adolescent girls and young women (58.1 to 92.3%) [3,4] and is a disabling condition associated with substantial functional impairment comparable to that observed in dysthymia. [5] PMS may lead to impaired work productivity [6,7] and decreased health-related quality of life,[8] and interfere with marital relationships, [9] family/homemaking functions, [10] hobbies and social activities. [11] Moreover, PMS also appears to be a risk factor of perinatal depression.[12-15]

Different case definitions of PMS have been used in different settings, e.g. the PMS case definition according to the American College of Obstetricians and Gynecologists (ACOG), [16] which considers PMS when one or more affective and physical symptoms are present 5 days prior to menses with symptomatic improvement within 4 days after the onset of menses and without recurrence until at least day 13 of the next cycle; additionally, this pattern should be present for at least 3 consecutive menstrual cycles and being accompanied by significant dysfunctions in social, academic, or work performance during the symptomatic phase. [16] The Daily Record of Severity of Problems (DRSP), which is a self-rating questionnaire which was developed as a tool to screen for DSM-IV criteria for Premenstrual Dysphoric Disorder (PMDD), may be used to acurately measure the severity of menstrual symptoms. [17] One case definition of PMS is based on a DRSP score $\geq$ 70 on day -5 to -1 of the cycle coupled with a difference of at least 30% between the premenstrual (day -5 to -1) and postmenstrual (day 6-10) score. [17-19] Recently, we have developed two new case

definitions of PMS-related conditions based on the DRSP scores during the menstrual cycle namely 1) peri-menstrual syndrome (PeriMS), defined as a total DRSP score on days 1 + day 2 + day 24 to 28 > 0.666 percentile; and 2) menstrual-cycle associated symptoms (MCAS) when the total DRSP score summed up over all days of the cycle is higher than the 0.666 percentile. [20] Furthermore, we observed that menstrual-cycle symptoms consisted of 4 symptomatic subdomains namely 1) depressive subdomain; 2) physiosomatic subdomain; 3) breast swelling & food craving subdomain; and 4) anxiety subdomain. [20] These symptom domains are reminiscent of the phenomenology of major depression, chronic fatigue syndrome, fibromyalgia, and anxiety disorders as well as craving behaviors.

Our new case definitions of PeriMS and MCAS were externally validated by biomarkers including sex hormones and IgA responses to Gram-negative bacteria, which both show significant variations during the menstrual cycle. [20,21] The case definitions of PeriMS and MCAS were significantly associated with steady-state levels of these sex hormones while the DRSP total score and its subdomains were associated with cyclic variations in the sex hormones indicating that both PeriMS and MCAS are associated with a relative corpus luteum insufficiency resulting from a suboptimal pre-ovulatory follicular development. [20] Moreover, IgA responses to gut-commensal Gram-negative microbiota showed peak levels at day 28 and lows at day 7 and day 14 and were highly significantly associated with the DRSP scores indicating that a cyclic pattern in immune responses is involved in PeriMS and MCAS. [20,21] It is important to stress that all these associations could not be detected when using the ACOG diagnosis, indicating that menstrual cycle problems should be assessed using the case definitions of PeriMS/MCAS as well as the cyclic variations in DRSP during the menstrual cycle.

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The immune system plays an important role in female reproductive functions and PMS as well. [22,23] In reproductive age women, plasma and endometrial levels of inflammatory compounds including C-reactive protein (CRP), interleukin (IL)-6, IL-1β, and tumor necrosis factor-α (TNF-α) are increased after ovulation and show peak values during menstruation. [24] CRP levels are positively associated with mood, behavior, pain and physical symptom severity during the menstrual cycle. [25,26] IL-2, IL-4, IL-10, IL-12, and interferon-gamma (IFN-γ) are also associated with emotional and physical symptom in women with PMS [27] whereby levels of IL-12 and IFN-γ are more than twice as high in PMS as compared with controls. Nevertheless, there are no studies whether changes in chemokine levels are associated with menstrual cycle symptoms.

Chemokines are a family of small chemoattractant cytokines which are classified into 4 main subfamilies, namely CXC, CC, CX3C, and XC. [28] They may exert pro-inflammatory activities, including recruiting leukocytes, monocytes, and neutrophils and guiding their migration towards infectious sites thereby protecting the body from invasion of pathogens. [29,30] Examples are CCL2 or chemokine (C-C motif) ligand 2; CXCL8 or chemokine (C-X-C motif) ligand 8 or IL-8, CCL11 or chemokine (C-C motif) ligand 11 or eotaxin; CXCL10 or interferon-γ inducible protein 10 (IP10); and CCL5 (RANTES) or regulated on activation, normal T cell expressed and secreted. [29] Chemokines also function as chemoattractants to recruit cells to locations where tissue damage occurs thereby exerting regulatory functions by promoting wound healing or maintaining tissue homeostasis. [28] Chemokines are expressed by cells of the uterine endometrium [31] including CCL2, CCL5 and their receptors CXCR4, CCR2, CCR5, which are involved in embryonic implantation while increased expression of CCL2 and CCR2, CCL5 and CCR5 are associated with unexplained recurrent spontaneous abortion. [32] Two growth factors play a role in the endometrium and neuronal cells as well. The first is epidermal growth factor (EGF), [33] which binds to EGF

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receptors resulting in cellular proliferation, differentiation, and survival. [34] The second is insulin-like growth factor (IGF), which regulates neurogenesis, myelination, neuroprotection and synaptogenesis after neuronal damage. [35] EGF and IGF-1 are found in the human uterine endometrium and regulate endometrial proliferation and differentiation [36] and mediate endometrial-trophoblast interactions leading to successful pregnancy. [37,38] Another protein that is involved in the menstrual cycle is plasminogen activator inhibitor-1 (PAI-1), which functions as the principal inhibitor of fibrinolysis. [39] Decreased progesterone during the late luteal phase may alter PAI-1 expression to create a pro-hemorrhagic and fibrinolytic milieu, and prepare for menstrual bleeding. [40,41] However, no studies have examined EGF, IGF-1, and PAI-1 in association with the above-mentioned chemokines in women with menstrual cycle symptoms.

Hence, the current study was conducted to investigate variations in plasma chemokines (CCL2, CCL5, CCL11, CXCL8, and CXCL10), EGF, IGF-1, and PAI-1 throughout the menstrual cycle as well as their associations with changes in DRSP and its subdomains and the PeriMS/MCAS and ACOG case definitions.

Methods

Participants

We recruited 41 female participants aged 18-45 years by verbal announcements at the King Chulalongkorn Memorial Hospital during the period April-May 2018. The subjects included 21 women with subjective complaints of PMS and 20 women without such complaints. 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 years; 3) being able to read and write in Thai; 4) willing to have 4 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. Excluded were 1) women with a lifetime history of psychiatric illness (including major depression, bipolar disorder, schizophrenia, and obsessive-compulsive disorder); 2) women with a history of medical illness, including diabetes type 1, and autoimmune/immune-inflammatory disorders including rheumatoid arthritis, inflammatory bowel disease, psoriasis and multiple sclerosis; 3) pregnant women or women using hormonal contraceptive agents; and 4) women who currently use 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.

Clinical assessments

All participants were requested to complete a demographic and clinical data questionnaire, including menstrual history, age, education, height, weight, a history of substance use and lifestyle, and they were evaluated by an experienced psychiatrist before enrollment in the study to exclude those with medical and/or psychiatric conditions. After informed consent, all participants were assigned 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 [17]. 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 reliably to screen for a DSM-IV diagnosis of premenstrual dysphoric disorder (PMDD) [18]. The diagnosis of PMS is also made 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. [17-19] All participants were categorized using the "PeriMS" case definition, namely sum of DRSP scores during the peri-menstrual period (days 1 and 2 + 24 to 28) > 0.666 percentile; and MCAS with a total daily DRSP score during the menstrual cycle > 0.666 percentile [20]. We also computed scores of the four subdomains of the DRSP, namely a) 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) physiosomatic 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. [20]

Assays

Human Serpin E1/PAI-1 and human IGF-I assays were performed by using a quantitative sandwich enzyme immunoassay technique (R&D Systems, Inc, Minneapolis, MN, USA). Briefly, 50 ul of serum was added in anti-human Serpin E1 or anti-human IGF-I coated wells. Samples for human IGF-I needed an additional pretreatment step to release IGF-I from binding proteins. After conjugate, substrate and stop solution steps, the plates were read at 450 nm. The sensitivity (Minimum Detectable Dose) of Human Serpin E1 and Human IGF-I were 014-0.142 (0.059) and 0.007-0.056 (0.026) ng/mL, respectively. For CCL2/MCP-1, CXCL10/IP-10, IL-8/CXCL8, CCL11/eotaxin, EGF, and CCL5/RANTES 50 µl of serum (1:2 dilution in calibrator diluent) was mixed with 50 µl of microparticle cocktail containing CCL2/MCP-1, CXCL10/IP-10, IL-8/CXCL8, CCL11/eotaxin, EGF, CCL5/RANTES (R&D Systems, Inc, Minneapolis, MN, USA)

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per well of a 96-well plate provided by manufacturer and incubated for 2 hours at room temperature on a shaker at 800 rpm. The mixture was then washed 3 times with wash buffer and 50 µl diluted Biotin Antibody cocktail was added and then incubated for 1 hour. Wells were washed 3 times before another 50 µl of diluted Streptavidin-PE was added and further incubated for 30 minutes. Finally, wells were washed 3 times and 100 µl of wash buffer was added and left at room temperature for 2 minutes before being read with Bio-Plex® 200 System (Bio-Rad Laboratories, Inc.).

Statistics

Analysis of variance (ANOVA) and analysis of contingency tables ($\chi 2$ test) were employed to check differences in continuous variables between categories and associations among nominal variables, respectively. Generalized estimating equation (GEE), repeated measures, was employed to assess effects of time, MCAS diagnosis and the time X MCAS interaction on DRSP and biomarker data, while adjusting for age, duration of menses, cycle length, and age of menarche. GEE, repeated measurements, was also used to delineate the associations between the biomarkers and the DRSP values measured at T1, T2, T3 and T4. Mutiple regression analysis was employed to assess the best biomarker predictors of the DRSP scores. 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.

1. Demographic data

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Table 1 shows the socio-demographic data of the 41 women divided into those with and without MCAS. There were no significant differences in age, education, family income, age at menarche, cycle length, or regularity of the menses between both groups. Duration of menses was somewhat greater in women with MCAS than in those without. The sum of the DRSP score during the 28 days of the menstrual cycle was significantly higher in those with MCAS than in women without MCAS.

2. Association of MCAS with the DRSP score and its subdomains

Figure 1 shows the daily measurements of the DRSP scores in women with and without MCAS. GEE analysis showed a significant effect of time (Wald=7.06.28, df=27, $p<0.001$), MCAS (Wald=181.42, df=1, $p<0.001$) and a significant time X MCAS interaction (Wald=256.81, df=27, $p<0.001$). The interaction indicates a greater increase in the DRSP scores the last week of the menstrual cycle in MCAS women as compared to women without MCAS. In order to further examine this interaction we have carried out GEE analyses, repeated measurements, performed on the values obtained at day 7, 14, 21 and 28 (same days we measured the biomarkers). **Table 2**, regression 1 shows the mean values obtained in women with and without MCAS on these 4-time points while **Table 3** shows the outcomes of this GEE analysis and additionally the mean daily DRSP measurements between both diagnostic groups. As shown in Tables 2 and 3 there were significant higher DRSP values at all time points in MCAS women while there was an additional increase in the DRSP score at day 28 in women with MCAS.

Consequently, we performed GEE analyses on the 4 subdomains of the DRSP. Table 2 shows that the depression scores were higher at all time points in MCAs women than in those without MCAS. There was again a significant interaction pattern between time X MCAS with

much higher means daily depression scores at day 28 in women with MCAS as compared to those without MCAS. The same profile was observed for physio-somatic symptoms and breast craving symptoms, with significant effects of time, MCAS and the time X MCAS interaction. There was no significant interaction between time and MCAS for anxiety scores. Nevertheless, all anxiety scores were significantly higher in women with MCAS than in those without MACA and this at all time points. In addition, the anxiety scores were significantly elevated in MCAS women at day 28 as compared with day 14.

Associations between biomarkers and MCAS diagnosis

Table 4 shows the measurements of the biomarkers in women with and without MCAS, the raw values as well as their z scores. GEE analyses showed that CCL2, CCL11, EGF, RANTES, and the z sumTOX were significantly higher in women with than without MCAS. The differences were greatest for CCL2 (1.06 SDs), RANTES (0.765 SDs) and the z sumTOX (0.93 SDs). We also examined the differences in the biomarkers among the three other case definitions (ACOG, PMS, and PeriMS) but could not find any associations.

Associations between biomarkers and DRSP scores

Table 5 shows the correlations between the DRSP total score and its subdomains and the biomarkers. Toward this end, we performed GEE analyses, repeated measurements, with the clinical severity scores at days 7, 14, 21 and 28 as dependent variables and the biomarkers measured at days 7, 14, 21 and 28 as explanatory variables. Those analyses were performed with and without inclusion of the time factor and those analyses yielded similar results. We found that CCL2 values and the z sumTOX score were significantly associated with the DRSP total score and

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the depression, physio-somatic, breast-craving and anxiety subdomain scores. IP10 was significantly and positively correlated with the DRSP total score and the physio-somatic score. CXCL8 and RANTES were both significantly associated with the DRSP total score, and the physio-somatic, breast-craving and anxiety subdomain scores.

Consequently, we have examined the associations between the sums of the daily DRSP scores during the four consecutive weeks of the menstrual cycle and the biomarkers. The sums were computed as the sum of the DRSP scores on days 1, 2, 3, 4, 5, 6 + 7 (denoted as sumDRSP days 1-7) while the first time point of the week was used as explanatory variable. For example the biomarkers measured at day 1 were used as predictors of the sumDRSP days 1-7. **Table 5** shows the outcome of a GEE analysis, repeated measures, which examined the association between the sum of the DRSP scores during the four consecutive weeks and the biomarkers at the beginning of the same weeks. The results showed that three chemokines, namely CCL2, CCL10, and CCL5, significantly predicted the weekly DRSP scores during the menstrual cycle.

In order to examine the effects of the chemokines on the sumDRSP days 1-7, 8-14, 15-21 versus 22-28 scores we have performed multiple regression analyses with the sumDRSP scores as dependent variables and the biomarkers at the first day of the week and the delta changes in the biomarkers during the same week as explanatory variables. **Table 6** shows that 41.5% of the variance in the sumDRSP days 1-7 was significantly predicted by CCL2 measured (day 1), while 37.4% of the variance in the sumDRSP days 8-14 was predicted by CCL2 (day 8) and the Δ change in CCL2 day 8-14. The variance in the sumDRSP days 15-21 (11.2% of the variance) was best predicted by CCL10 at day 15 while sumDRSP days 22-28 was best predicted by z sumNOX. Finally, we found that 39.6% of the variance in the DRSP score averaged over the cycle was significantly associated with the average values of CCL2.

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Discussion

The first major finding of this study is that CCL2, CCL5, CCL11 and EGF are significantly higher in women with MCAS than in women without MCAS and that there are no differences in those biomarkers among women with or without a diagnosis of PeriMS, ACOG or PMS. As such, the case definition MCAS is externally validated by chemokines and EGF. Previously, we reported that the case definitions of MCAS and PeriMS, but not ACOG, were externally validated by levels of progesterone and estradiol. [20] Based on these data it appears that menstrual cycle-related problems can best be expressed using both the MCAS and PeriMS case definitions.

We found that overall severity of MCAS and the different symptom domains display significant variations during the menstrual cycle but only in women with MCAS, whereas the same measurements are very stable in women without MCAS. As such, the case definition MCAS reflects increased total DRSP, depression, physiosomatic, physical, and anxiety scores during all weeks of the menstrual cycle although it also captures increased severity the week prior to the menses. Using the same data set, we previously detected significant changes during the menstrual cycle in sex hormones and IgA responses to gut commensal bacteria, whereas this study shows that there is no menstrual cycle variation in chemokines, EGF, IGF-1 or PAI-1. Previously, it was shown that not only CXCL8 and CCL5, but also IL-6, IL-10, IL-15, IFN-α, and granulocyte colony-stimulating factor (G-CSF) varied during the menstrual cycle and that the levels of CXCL8, IL-1β, and IL-6 were 1.5-3 times higher during menses.[42] During the luteal phase, cell-mediated immunity may be suppressed, whereas humoral immunity is promoted to prepare for a potential embryo implantation in case pregnancy occurs.[43] Progesterone may stimulate the development of T0-cells into T helper (Th)-2 cells and the synthesis of IL-4 and IL-5 thereby exerting regulatory

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activities.[44] Also, estrogen may suppress cell-mediated immunity and attenuate the production of IFN-γ, a Th-1 cytokine,[45] while lower estradiol and progesterone concentrations are associated with increased CXCL8, IL-1β, and IL-6 levels.[42]

The second major finding of our study is that increased steady-state levels of CCL2, CXCL10, CXCL8, CCL11 and CCL5 (averaged over the menstrual cycle) were significantly associated with increased severity and that CCL2 was the most significant predictor of the DRSP, explaining 39.6% of its variance while the steady-state levels of the total DRSP score were best predicted by a combination of increased CCL2, CCL5, and CXCL10. Moreover, the impact of chemokines on the symptoms may differ between the periods of the menstrual cycle with CCL2 being the most important predictor of increased DRSP levels during the first two weeks, whereas CXCL10 and a combination of CCL2, CCL11 and CCL5 are the best predictors of the DRSP score during weeks 3 and 4, respectively. All in all, such results indicate that immune activation (as indicated by increased chemokine levels) may impact depressive, physiosomatic, physical (including craving and breast swelling) and anxiety symptoms during the menstrual cycle, either by direct effects of CCL2, CCL11, CCL5, CXCL10 and/or CXCL8 or indirect effects through downstream or upstream mechanisms. Therefore, it is important to discuss our results with respect to previous findings in major depression, chronic fatigue syndrome and fibromyalgia (all characterized by physiosomatic symptoms), anxiety disorders and changes in appetite or craving behaviors.

A recent meta-analysis found increased plasma levels of CCL2, CCL11, and CXCL8 in depressed patients as compared with controls. [46] However, one study reported lowered plasma and CSF levels of CXCL8 among suicide attempters with anxiety as compared with healthy controls. [47] CCL2 is also increased in bipolar disorder (BD) as compared with major depression even when

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BD patients are in a remitted phase. [48] One study reported decreased CCL2 and CCL5 levels in patients with suicidal ideation and depression as compared with controls. [49] In chronic fatigue syndrome, increased levels of CCL11 and CXCL10 show a significant association with the severity of CFS [50] while in fibromyalgia, increased levels of CXCL8 and CCL2 are significantly correlated with changes in pain severity. [51] Nevertheless, there are also studies reporting lowered levels of CXCL8 in patients with fibromyalgia. [52]

There is increasing evidence that chemokines are involved in the central regulation of feeding behavior, catabolic processes and weight maintenance. [53,54,55] Moreover, chemokines exert their actions on hypothalamic nuclei, which control feeding behaviors and body weight. [53] Intracerebroventricular administration of chemokines (e.g. PF4/CXCL4) may suppress total daily food intake.[56] MIP-1a/CCL3 and MIP-1b/CCL4 directly injected in the ventromedial hypothalamus attenuate feeding behavior, while intracerebroventricular administration of CXCL8, CXCL10, CCL2 and CCL5 may decrease short-term (2 h) food intake. [57,58] CCL5 and CCL2 are increased in generalized anxiety disorder, [59] chronic stress [60] and post-traumatic stress disorder. [61] A study in healthy hospital workers, however, found that anxiety scores were inversely associated with lower levels of CCL2, CCL5, CCL11, and IL-6. [62] Chemokines may play a role in breast swelling because estradiol enhances macrophage influx with angiogenesis and vascularization through increased release of CCL2 and CCL5. [63]

Importantly, chemokines including CCL2, CXCL8, and CXCL10 may penetrate into the brain via the blood-brain barrier (BBB) through transendothelial transport and transcytosis, [64-66] while CCL11 also shows transport through brain microvascular endothelial cells (BMEC).[67,68] Moreover, CCL2 disrupts the BBB tight junctions thereby increasing the flow through the BBB, [69] and activates monocyte/macrophages, T lymphocytes, and dendritic cells [70] and, therefore, may

be accompanied by increased neurotoxic or behavioral effects of these cytokines. [71-73] CXCL8 (IL-8) has pleiotropic effects on the peripheral immune system including chemotactic effects on B and T lymphocytes, natural killer cells, dendritic cells and granulocytes. [70,74] Nevertheless, the chemokines, which are associated with MCAS symptoms, also exhibit intrinsic neuro-inflammatory, neurotoxic and neurocognitive effects.

In the nervous system, chemokines function as neuromodulators and regulate neuroinflammation. [75-77] In response to injuries, microglia, astrocytes, oligodendrocytes, and endothelial cells release chemokines, including CCL2, CXCL10 and CCL5, [78] and express chemokine receptors.[79,80] Neuroinflammatory responses entail transendothelial migration of immune cells across the BBB, leading to neuronal damage.[81] CCL2 may exert neurotoxic effects [82] through regulation of neuroinflammation by binding to CCR2, a mechanism which is involved in neuroinflammatory disorders such as stroke, Parkinson's disease, Alzheimer's disease, multiple sclerosis, and alcohol-induced neuropathy, [82] while CCL2 also activates dopamine thereby increasing excitability. [69] Increased levels of CXCL10 suggest that IFN-γ production is increased and, consequently, that the production of neurotoxic TRYCATs may be increased. [83] In Parkinson's and Alzheimer's disease, higher CXCL10 is associated with cognitive dysfunctions and increased progression of illness. [84] CCL5 plays a role in Parkinson’s Disease through effects on substantia nigra neurons with significant dopamine cell loss and associated locomotor deficits. [85] CCL11 significantly decreases neurogenesis thereby inducing cognitive deficits explaining that increased CCL11 levels are a major contributor to neurocognitive deficits in schizophrenia. [67]

The results of our study suggest that, in women with MCAS, increased levels of neurotoxic chemokines are insufficiently counterbalanced by increased levels of EGF, a protective growth factor (see Introduction). Increased levels of EGF in the CNS are the result of local synthesis (by

macrophages, glial cells and neurons) and increased uptake from the peripheral circulation, [86,87,88,89] and EGF receptors are expressed in the cerebral cortex, hippocampus, and anterior pituitary gland.[90-92] EGF acts as a neurotrophic and maintenance factor for CNS neurons [86] and this protein has neuromodulatory effects by inhibiting acetylcholine release from hippocampus and increasing dopamine uptake by mesencephalic neurons. [86] In the uterus, EGF controls the proliferation of the endometrium [93,94] by stimulating proliferation of various cell types, [95] and EGF receptors can be found in human endometrial cells. [93] EGF mediates estrogen-induced proliferation of uterine epithelial cells [96] and is regulated by estrogen and progesterone. [97] Interestingly, estrogen treatment can enhance the expression of EGF and EGF-receptors in the uterus, [98-100] suggesting that EGF may play an important role in estrogen-induced uterine growth.[98] Moreover, the activity of estrogens may be inhibited by an EGF-specific antibody and exposure to EGF alone mimics estrogen in the induction of uterine and vaginal growth and differentiation. [98]

In conclusion, our study shows that women with increased levels of CCL2, CCL5, CCL11, CXCL8, and CXCL10 are predisposed to suffer from increased depressive, anxiety, physiosomatic, and physical (breast swelling) symptoms and changes in appetite and food craving during the menstrual cycle. However, this is a case-control study and, therefore, causal inferences cannot be firmly established. These novel findings deserve indepencent replication. The chemokines and EGF measured herein show multiple intertwined associations with sex hormones and play a role in the endometrium, the menstrual cycle and in brain (immune) responsivity, which may mediate the symptoms of MCAS. As such, these chemokines and EGF may constitute links between the uterus and brain functions through the uterine-chemokine-brain (UCB) axis. Increased levels of those chemokines and EGF also externally validate MCAS, a new case definition of menstrual-related symptoms. The results show that a complete assessment of menstrual cycle-

related symptoms should consist of measuring daily DRSP ratings to examine the time variations in general symptoms and its subdomains and to make the diagnoses according to MCAS and PeriMS criteria.

Authorships

CR and MM made the design of the study. CR recruited and screened the participants. MM performed statistical analyses. SS 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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Figure 1. Measurements of the Daily Record of Severity of Problems (DRSP) rating scale during 28 consecutive days of the menstrual cycle in women with and without Menstrual-Cycle Related Symptoms (MCAS).

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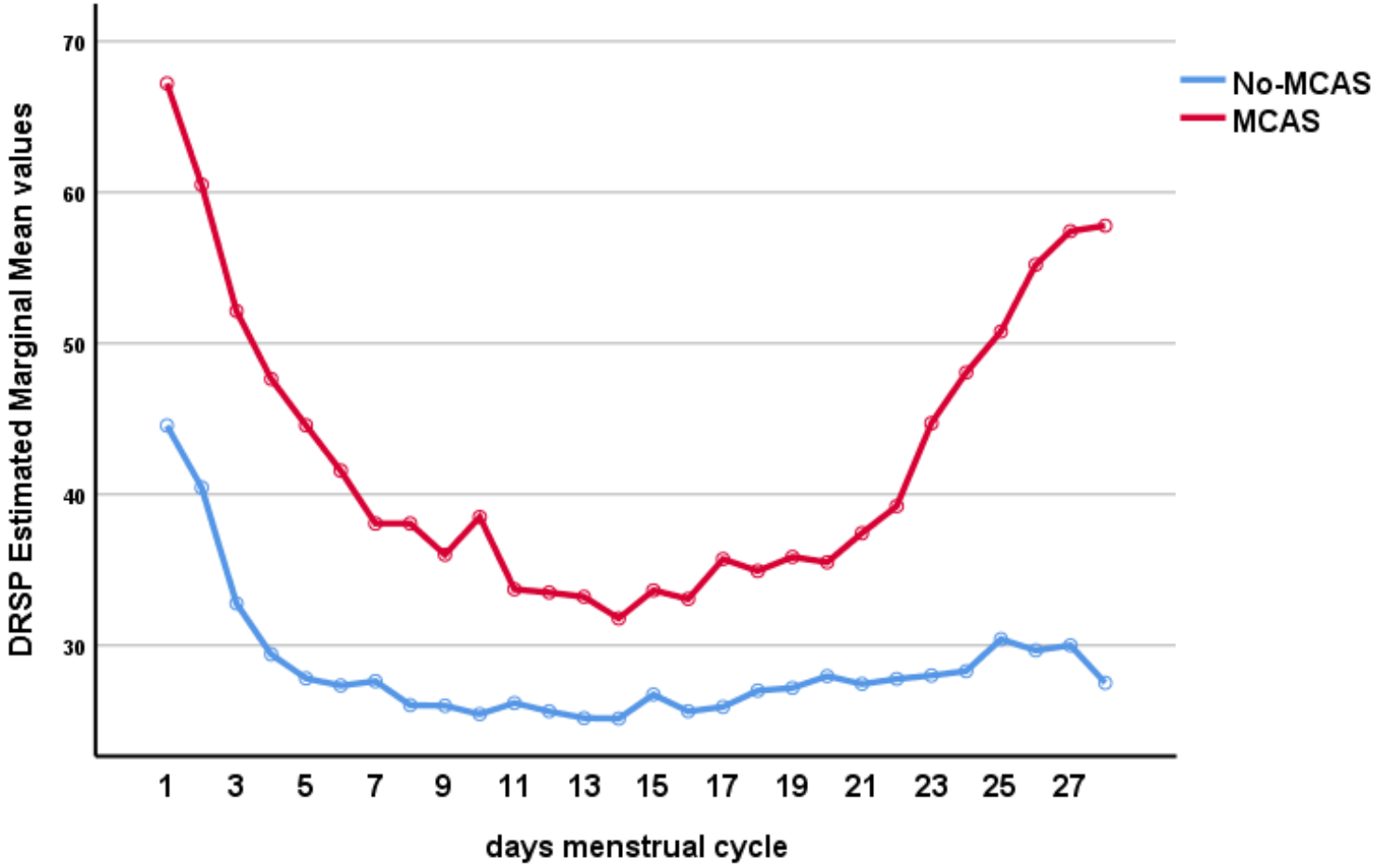

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Table 1 Socio-demographic and clinical data of 41 women divided into those with and without menstrual cycle-associated syndrome (MCAS)

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
Regular / Irregular menses	25/1	12/2	0.189	-	0.232
Sum DRSP during one cycle	799.1 (80.3)	1195.9 (103.7)	183.99	1/39	<0.001

All results are shown as mean (±SD)

DRSP: Daily Record of Severity of Problems

Table 2. Measurements of the Daily Record of Severity of Problems (DRSP) total score and its four symptom domains during the menstrual cycle in women with and without Menstrual Cycle-Associated Syndrome (MCAS)

Symptoms	**MCAS**	**Day 7** [a]	**Day 14** [b]	**Day 21** [c]	**Day 28** [d]
DRSP	No MCAS	27.63 (1.28)	25.15 (0.34)	27.44 (0.99)	27.52 (1.90)
	MCAS	38.07 (3.97)*[d]	31.79 (1.71)*[d]	37.43 (2.74)*[d]	57.79 (7.39)*[a,b,c]
Depression	No MCAS	10.41 (0.56)	9.26 (0.15)	9.52 (0.34)	10.50 (0.43)
	MCAS	14.29 (1.48)*[d]	11.93 (0.61)*[d]	14.07 (1.29)*[d]	22.00 (3.02)*[a,b,c]
Physiosomatic	No MCAS	6.96 (0.36)	6.30 (0.11)	6.93 (0.38)	7.97 (0.75)
	MCAS	9.93 (1.01) *[d]	8.36 (0.65) *[d]	10.00 (0.92) *[d]	14.79 (1.85)*[a,b,c]
Breast-craving	No MCAS	4.78 (0.39)	4.48 (0.22)	5.67 (0.48)	5.69 (0.44)
	MCAS	6.14 (0.80) [d]	4.79 (0.27) [d]	6.07 (0.51) [d]	10.57 (1.39)*[a,b,c]
Anxiety	No MCAS	5.59 (0.31)	5.26 (0.12)	5.67 (0.20)	6.03 (0.25)
	MCAS	8.29 (1.09)*	7.29 (0.78)*[c]	7.93 (0.94)*	11.14 1.40)*[b]

All results are shown as mean (SE) of the ratings of the DRSP on days 7, 14, 21 and 28.

* Results of pairwise comparisons, namely no MCAS versus MCAS, at each time point.

a,b,c,d: results of pairwise comparisons among the time points

Table 3. Results of Generalized Estimating Equations (GEE), repeated measurements, with the Daily Record of Severity of Problems (DRSP) total score (and its four symptom domains) during the menstrual cycle as dependent variables and time and the Menstrual Cycle-Associated Syndrome (MCAS) as explanatory variables.

Variables	No MCAS	MCAS	Time		MCAS		Time x MCAS	
			Wald (df =3)	p	Wald (df =1)	p	Wald (df =3)	p
DRSP	26.94 (0.61)	41.27 (1.65)	26.21	<0.001	66.71	<0.001	10.73	0.022
Depression	9.92 (0.20)	15.57 (0.66)	21.41	<0.001	68.19	<0.001	9.20	0.027
Physiosomatic	7.04 (0.22)	10.77 (0.56)	37.90	<0.001	38.70	<0.001	9.62	0.022
Breast-craving	5.15 (0.22)	6.89 (0.32)	30.70	<0.001	19.73	<0.001	9.67	0.022
Anxiety	5.64 (0.12)	8.66 (0.74)	15.47	0.001	16.60	<0.001	5.28	0.153

All results of GEE analysis, repeated measurements, with the DRSP total score and its symptom dimensions as dependent variables (see Table 2 for measurements over time).

Table 4. Differences in biomarkers between women with and without menstrual cycle-associated syndrome (MCAS)

Biomarkers	Raw data		z values		Wald	df	p
	No MCAS	MCAS	No MCAS	MCAS			
CCL2 (pg/mL)	230.7 (10.9)	353.7 (33.2)	-0.362 (0.125)	0.696 (0.187)	21.66	1	<0.001
CXCL10 (pg/mL)	83.6 (5.9)	93.1 (11.4)	-0.054 (0.149)	0.088 (0.280)	0.19	1	0.661
CXCL8 (pg/mL)	69.1 (20.2)	182.3 (80.4)	-0.119 (0.122)	0.230 (0.164)	2.83	1	0.093
CCL11 (pg/mL)	137.0 (7.3)	172.4 (13.4)	-0.167 (0.158)	0.372 (0.221)	5.27	1	0.022
EGF (pg/mL)	412.3 (22.4)	509.6 (42.3)	-0.172 (0.135)	0.290 (0.147)	5.15	1	0.023
CCL5 (pg/mL)	43,693 (3,604)	70,276 (8,834)	-0.264 (0.115)	0.501 (0.170)	13.43	1	<0.001
PAI-1 (ng/mL)	14.5 (0.8)	15.1 (0.7)	-0.143 (0.217)	0.164 (0.162)	1.26	1	0.262
IGF-1 (ng/mL)	162.1 (11.8)	193.7 (13.8)	-0.157 (0.175)	0.291 (0.176)	3.48	1	0.062
z sumTOX	-	-	-0.324 (0.116)	0.603 (0.226)	13.34	1	<0.001

All results of Generalized Estimating Equations (GEE), repeated measurements, with the biomarkers as dependent variables and time and MCAS as explanatory variables. All time effects were non-significant.

CCL2: chemokine (C-C motif) ligand 2; IP10: interferon-γ inducible protein 10 (or CXCL10); CXCL8: chemokine (C-X-C motif) ligand 8 or interleukin-8; CCL11: chemokine (C-C motif) ligand 11 or eotaxin; EGF: epidermal growth factor; RANTES: regulated on activation, normal T cell expressed and secreted or chemokine (C-C motif) ligand 5 or CCL5; PAI-1: plasminogen activator inhibitor type 1; IGF-1: insulin-like growth factor; z sumTOX: sum toxic chemokines computed as zCCL-2 + zCCL-11 + zCCL5

Table 5. Associations between biomarkers and the Daily Record of Severity of Problems (DRSP) total score and its subdomains during the menstrual cycle in 41 women; results of Generalized Estimating Equations (GEE), repeated measurements.

Dependent variables	**Explanatory variables**	**B**	**SE**	Wald	**df**	**P**
DRSP	CCL2	0.291	0.051	32.75	1	<0.001
Depression	CCL2	0.277	0.063	18.89	1	<0.001
Physiosomatic	CCL2	0.269	0.058	21.88	1	<0.001
Breast	CCL2	0.190	0.069	7.51	1	0.006
Anxiety	CCL2	0.241	0.046	27.09	1	<0.001
DRSP	CXCL10	0.186	0.081	5.25	1	0.022
Physiosomatic	CXCL10	0.252	0.101	6.21	1	0.013
DRSP	CXCL8	0.143	0.057	6.23	1	0.013
Physiosomatic	CXCL8	0.177	0.059	8.89	1	0.003
Breast	CXCL8	0.145	0.072	4.02	1	0.045
Anxiety	CXCL8	0.117	0.048	6.07	1	0.014
DRSP	CCL5	0.190	0.062	9.47	1	0.002
Physiosomatic	CCL5	0.204	0.059	11.85	1	0.001
Breast	CCL5	0.164	0.064	6.29	1	0.012
Anxiety	CCL5	0.183	0.046	15.63	1	<0.001
DRSP	z sumTOX	0.256	0.057	20.12	1	<0.001
Depression	z sumTOX	0.208	0.070	8.87	1	0.003
Physiosomatic	z sumTOX	0.260	0.065	16.08	1	<0.001
Breast	z sumTOX	0.166	0.059	8.10	1	0.004
Anxiety	z sumTOX	0.232	0.068	11.76	1	0.001
sumDRSP 4 consecutive	CCL2	0.330	0.060	30.25	1	<0.001
weeks	CXCL10	0.181	0.060	9.33	1	0.002
	CCL5	0.237	0.096	6.06	1	0.014

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All results of Generalized Estimating Equations (GEE), repeated measurements, with the DRSP and subdomains as dependent variables and the biomarkers as explanatory variables. Entered are the 4 time points (days 8, 14, 21 and 28) with simultaneous and repeated measurements of the clinical and biomarker data.

CCL2: chemokine (C-C motif) ligand 2; CXCL10 or IP10: interferon-γ inducible protein 10; CXCL8: chemokine (C-X-C motif) ligand 8 or interleukin-8; CCL5 or RANTES: regulated on activation, normal T cell expressed and secreted or chemokine (C-C motif) ligand 5;

Z sumTOX: integrative index of toxic chemokines computed as zCCL2 + zCCL5 + zCCL11

Table 6. Associations between biomarkers and the mean total DRSP scores in 4 consecutive weeks of the menstrual cycle, results of GEE analyses

Dependent variables	Explanatory variables	β	t	p	F model	df	p	Partial eta squared
sumDRSP days 1-7	CCL2 day 1	0.644	5.06	<0.001	25.55	1/36	<0.001	0.415
sumDRSP days 8-14	CCL2 day 8	0.543	3.95	0.001	10.45	2/35	<0.001	0.374
	Δ CCL2 (day 8-14)	0.438	3.19	0.003				
sumDRSP days 15-21	CXCL10 day 15	0.334	2.16	0.038	4.65	1/37	0.038	0.112
sumDRSP days 22-28	Z sumNOX day 22	0.322	2.04	0.048	4.17	1/36	0.048	0.104
Total DRSP scores averaged over the menstrual cycle	CCL2 values averaged over the menstrual cycle	0.629	4.92	<0.001	24.25	1/37	<0.001	0.396

DRSP days (1-7- 8-14, 15-21, 22-28): mean DRSP scores averaged over 7 days during the 4 consecutive weeks of the menstrual cycle; GEE analyses are performed using the data of week 1, 2, 3 and 4 separately indicating the effects of the biomarkers on DRSP scores of each week.
DRSP 4 consecutive weeks: the mean DRSP values of the 4 consecutive weeks are entered in the analysis, indicating the effects of the biomarkers on DRSP scores during the 4 weeks of the menstrual cycle
CCL2: chemokine (C-C motif) ligand 2; CXCL10 or IP10: interferon-γ inducible protein 10; CCL5 or RANTES: regulated on activation, normal T cell expressed and secreted or chemokine (C-C motif) ligand 5. Z sumTOX: toxic chemokines computed as zCCL2 + zCCL11 + zCCL5