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Menstruation distress is strongly associated with hormone-immune-metabolic biomarkers

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Abstract

Objective: To examine the associations between menstruation features and symptoms and hormone-immune-metabolic biomarkers

Methods: Forty-one women completed questionnaires assessing characteristic menstruation symptoms, duration of menstrual cycle and number of pads used/day and completed the Daily Record of Severity of Problems (DRSP) during the consecutive days of their menstrual cycle. Menses-related symptoms (MsRS) were computed from the sum of 10 pre- and post-menses symptoms and the menstruation blood and duration index (MBDI) was computed based on the daily number of pads and duration of menses. We assayed serum levels of various biomarkers at days 7, 14, 21, and 28 of the subjects' menstrual cycle.

Results: MBDI was significantly associated with a) MsRS including low abdominal cramps, and gastro-intestinal (GI) and pain symptoms (positively); b) plasma levels of haptoglobin (Hp), CCL5, insulin growth factor (IGF)-1, and plasminogen activator inhibitor (PAI)1 (all positively); and c) estradiol and paraoxonase (PON)1 arylesterase activity (both inversely). MsRS were significantly predicted by CCL5 and IGF-1 (both positively) and progesterone (inversely). Low-abdominal cramps, and gastro-intestinal and pain symptoms were associated with lower progesterone levels. The MBDI+MsRS score was significantly predicted by the cumulative effects of (in descending order of importance): Hp, IGF-1, PON1 arylesterase, estradiol and PAI.

Conclusion: Menstruation-related features including estimated blood loss, duration of menses, cramps, pain and GI symptoms are associated with hormone-immune-metabolic biomarkers, which mechanistically may explain those features. Women with an increased MBDI+MsRS index $\geq$ 0.666 percentile may be considered to have menstruation-related distress, including dysmenorrhea symptoms.

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Introduction

According to Sigmund Freud's psychoanalytical view, menstruation is related to cyclical nasal bleeding, and dysmenorrhea, which is caused by excessive masturbation, should be treated with nasal surgery [1]. Helen Deutsch, one of the most influential Western theorists of the 1930s, described menstruation as "*agitated periods during which previously repressed feelings are released*" [2], which would indicate that women are "*servants of the species*" and "*forever lost their wish for an imagined penis*" [2]. Another common psychiatric view was that losing blood in the menstrual cycle is equivalent to losing a baby and that "*menstruation is a crying to heaven in the mourning over a child*" [2, 3].

Nevertheless, the duration of a normal menstrual cycle is 28 days (range: 21-35 days) in adult women and 21-45 days in younger women [4]. At the beginning of the cycle (around day 0-5), increasing levels of estrogen in the follicular phase halt menstrual bleeding and thicken endometrium linings [5]. Ovarian follicles continue to develop under the influence of a complex interplay of hormones, and after several days, one follicle becomes dominant and will ovulate in the mid-cycle. After ovulation, the remains of the ovulated follicle turned into a "corpus luteum", becomes the primary source of progesterone production during the second half of the cycle. Progesterone prepares the uterine linings for potential implantation by changing it into a "secretory phase". If implantation does not occur within approximately 2 weeks, the corpus luteum will be degenerated, leading to a sharp decrease in both progesterone and estrogen levels, causing the uterus to shed its linings in the process of menstrual bleeding [5].

Menstruation may be accompanied by pain and other symptoms including nausea, vomiting, diarrhea, fatigue, low back pain, and irritability [6]. Dysmenorrhea, which is a sharp painful cramp in the lower abdomen [7, 8], is experienced by almost 90% of adolescent females

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and 25% of adult women [9]. A meaningful part of women also suffer from premenstrual syndrome (PMS) characterized by behavioral and physiosomatic symptoms appearing during the luteal phase of the menstrual cycle and ameliorating after the onset of menses [10, 11] and include a) depression (mood swings, sensitive to rejection, less interest, interference with hobbies and relationships), anxiety (hopelessness, anxious, insomnia, being overwhelmed), physiosomatic (concentration disturbances, lethargy, headache, muscle/joint pain) symptoms, and appetite and craving and breast tenderness and swelling [12]. Nevertheless, it has remained elusive whether there are associations between menses features, menses-related symptoms (MsRS) and PMS.

Recently we showed that PMS, as defined using a new case definition, namely menstrual-cycle-associated syndrome (MCAS), is caused by a relative corpus luteum insufficiency (insufficient progesterone production), which, in part may result from suboptimal pre-ovulatory follicular development (lowered estradiol production) while also immune-oxidative pathways are involved [12-14]. The latter include increased levels of a) uterus-associated chemokines such as chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-C motif) ligand 11 or eotaxin (CCL11), and regulated on activation, normal T cell expressed and secreted (CCL5); growth factors including epidermal growth factor (EGF), and lowered levels of paraoxonase (PON)1, an antioxidant enzyme that can hydrolyze lipid peroxides thereby protecting low and high-density lipoprotein (LDL-HDL) against oxidation [15] [16, 17].

Excessive or imbalanced amounts of prostanoids (including prostaglandins) and alterations in progesterone are pathophysiological factors of dysmenorrhea. Prostanoids released from the uterine endometrium during menstruation, lead to frequent uterus contractions with increased basal tone and increased active pressure [18, 19]. This uterine hypercontractility could lead to reduced uterine blood flow, which can finally induce ischemic pain in those women who have increased

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peripheral nerve hypersensitivity [18]. The production of prostaglandins is controlled by progesterone whereby a decrease in progesterone during the late luteal phase prior to menstruation causes prostaglandins levels to increase [19, 20].

Moreover, some previous studies showed that menstruation is associated with peripheral immune-inflammatory responses. Wander et al. (2008) found that menstruation is associated with a 17% increase in C-reactive protein (CRP), whereby a 10-fold increase in progesterone is associated with a 23% increase in CRP and a 10-fold increase in estrogen with a 29% decrease in CRP [21]. Haptoglobin (Hp), an acute phase response, is increased immediately after menses as compared with the luteal phase and is increased in uterine fluid during the secretory phase [14, 22]. Regulated upon activation, normal T cell expressed and secreted (RANTES or CCL5) is a chemokine attracting monocytes and activated T cells that is synthesized by stromal cells of both normal endometrium and endometriosis tissues [23]. Stromal cells of normal endometrium also express CCL5 mRNA transcripts and protein [23] and there are lymphoid aggregations [24] and leukocyte infiltrations during the luteal phase [25], suggesting that CCL5 has a role in the normal physiology of the endometrial immunological system [23].

Moreover, some results suggest that the menstruation may be associated with metabolic hormonal biomarkers. Fore example, endometrial plasminogen activator inhibitor type 1 (PAI-1) activity is increased in women with menorrhagia (menstrual blood loss > 80 ml/cycle) during the late secretory phase and menstruation [26]. The insulin-like growth factor (IGF) autocrine/paracrine system may play a role in sex hormones-mediated endometrial differentiation [27] and is implicated as an important regulator of pre-implantation and placental development [28] and fetal development [29]. Nevertheless, there are no data whether menstruation features or dysmenorrhea-like symptoms are associated with the sex hormones progesterone and estradiol, the

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uterus-associated chemokines (e.g. CCL2, CCL5 and CCL11), EGF, hormonal-metabolic biomarkers including PAI and IGF-1, and antioxidant enzymes as well.

Hence, this study was carried out to delineate a) the association between menstrual features, MsRS and PMS, and b) their associations with hormonal-immune-metabolic biomarkers.

Subjects and Methods

Participants

Forty-one female participants aged 18-45 years were recruited by verbal announcements at the King Chulalongkorn Memorial Hospital during the period April-May 2018, including 21 with subjective complaints of PMS and 20 without such complaints. Inclusion criteria were: 1) women aged 18-45 years; 2) being able to read and write the Thai language; 3) having a regular menstrual cycle with a cycle length of 27-30 days during the past years; 4) able to complete the self-ratings for all consecutive days of the menstrual cycle; and 5) able to have 4 blood samples drawn at day 7 (T1), day 14 (T2), day 21 (T3) and day 28 (T4) of the menstrual cycle. Subjects are excluded if they 1) have a lifetime history of any psychiatric illness; 2) have 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) are currently pregnant, lactating or using hormonal contraceptive agents; and 4) 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

 doi:10.20944/preprints202003.0416.v1

All participants were evaluated by an experienced psychiatrist specialized in women's health (CR) to exclude women with medical and/or psychiatric conditions before obtaining informed consent. The participants were requested to complete socio-demographic and clinical data questionnaires, and a detailed menstrual history was obtained including age of menarche and regularity of the menstrual cycle. We also obtained data on amounts of pads used per day during the menses and duration of menses. Based on those data we computed an index reflecting menstruation blood loss and duration as z score of pads/day + z score of duration of menses, labeled as menstruation blood & duration index (MBDI). We also dichotomized the study group into those with higher versus lower MBDI values using a 0.666 percentile threshold value. We also measured 10 common MsRS (self-rated yes/no) before menses (Pre-Ms) and after menses (Post-Ms), namely: acne, gastro-intestinal upset, diarrhea, constipation, bloating, back pain, muscle ache, dizziness, headache, and lower abdominal cramps. The sum of these 10 symptoms both at pre-Ms and post-Ms as well as their sum was used as an index of MsRS. As such alterations in the repeated measurements of MsRS from pre-Ms to post-Ms reflect changes in MsRS during the menses. We also computed MsRS subscores namely gastro-intestinal (GI) or irritable-bowel-like (IBS) symptoms as sum of gastro-intestinal upset, diarrhea, constipation, and bloating, and pain symptoms as sum of back pain, muscle ache, and headache. Consequently, we computed a composite score reflecting menses bleeding and symptoms changes during the menses as: z score of pads/day + z score duration of menses + z score MsRS (pre-Ms and post-Ms), labeled as MBDI+MsRS. Additionally, all participants completed the Daily Record of Severity of Problems (DRSP) during the consecutive days of their menstrual cycle starting on day 1 of menstrual bleeding. The DRSP is a self-report instrument consisting of 21 items plus 3 functional impairment items commonly used to assess PMS [30]. Each item is rated from 1 to 6 (1 = not at all, 2 =

 doi:10.20944/preprints202003.0416.v1

minimal, 3 = mild, 4 = moderate, 5 = severe, 6 = extreme). The DRSP has been used to measure the "presence" and "severity" of PMS-related symptoms and can be used to screen for a DSM-IV diagnosis of premenstrual dysphoric disorder (PMDD) [31].

Based on the DRSP score, we made different diagnosis of PMS using three case definitions [12]: a) The diagnosis of PMS is 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 [30-32]. b) The "PeriMS" case definition was made when the sum of DRSP scores during the peri-menstrual period (days 1 and 2 + 24 to 28) > 0.666 percentile [12]. c) The case definition "MCAS" was used when the total daily DRSP score during the menstrual cycle > 0.666 percentile [12].

Assays

Fasting blood was sampled at 8.00 a.m. at day 7 (D7), Day 14 (D14), day 21 (D21), and day 28 (D28) of the subjects' menstrual cycle to assay estradiol, progesterone, C-reactive protein (CRP), haptoglobin (Hp), chemokines and PON1 status. These 4 time points reflect different phases of the menstrual cycle, namely D7: the mid-follicular phase with increasing estrogen levels; D14: mid-cycle phase around ovulation with declining estrogen levels; D21: mid-luteal phase with peak progesterone levels; and D28: end of the cycle and start of menses with the sex-hormones levels reaching their nadir [12, 33-35]. For an immunoassay of estradiol and progesterone, we used in vitro quantitative determination using Cobas® 601 with a competition principle as described previously [36]. 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 intra-assay CV value is 2.7%.

 doi:10.20944/preprints202003.0416.v1

Serum haptoglobin was measured using immunoturbidimetry on the Architect C series analyzer (Abbott Diagnostics, Abbott Park, IL, USA). Human serpin E1/PAI-1 and human IGF-I were assessed 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 CCL-2/MCP-1, CXCL10/IP-10, IL-8/CXCL8, CCL-11/eotaxin, EGF, and CCL-5/RANTES 50 µl of serum (1:2 dilution in calibrator diluent) was mixed with 50 µl of microparticle cocktail containing those the same factors (R&D Systems, Inc, Minneapolis, MN, USA) 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.). The assay of PON1 status was explained previously [37], 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 (Sigma, USA), which is an alternative to the use of the toxic paraoxon. PON1 activity was determined by the rate of hydrolysis of phenyl acetate under low salt condition (AREase). Analysis were conducted in a microplate reader (EnSpire, Perkin Elmer, USA) [38]. Although the PON1 Q192R genotypes were assayed,

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those data yielded non-significant results and as such the data are not presented here [37]. The intra-assay coefficients of variation were <10% for all analytes. In the present study, we also used the sum of the biomarkers obtained at the 4 time points during the menstrual cycle [14]

Statistics

We used analysis of contingency tables ($\chi 2$ test) and analysis of variance (ANOVA) to ascertain associations among categorical variables and differences in scale variables, respectively. Multiple regression analysis was used to delineate the best biomarker combination predicting various dysmenorrhea indices. Multivariate GLM analysis was employed to examine the associations between a set of biomarkers and MBDI subgroups. Consequently, univariate GLM analysis was used to examine the differences in each of the biomarkers used in the multivariate GLM analysis between both MBDI classes. Generalized estimating equation (GEE), repeated measures, was used to ascertain the effects of time and MBDI classes on symptom profiles. GEE, repeated measurements, was also employed to assess the associations between MBDI+MsRS and biomarkers. Principal Component Analysis was used as a feature reduction method. Binary logistic regression analysis was employed to delineate the best prediction of women with a MBDI+MsRS score $\geq$ 0.666 percentile. 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.

MBDI and socio-demographic and clinical data

Table 1 shows the socio-demographic and clinical data of the 41 women in the present study divided into those with an increased MBDI versus a lower MBDI index (dichotomized using

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a 0.666 percentile threshold value). There were no significant differences in age, years of education, income, BMI, marital status, age at menarche and length of the menstrual cycle between both study groups. In addition, there were no significant associations between the MBDI index and different diagnosis of PMS namely the MCAS, PeriPMS and PMS case definitions. The total DRSP score obtained by daily measurements all over the cycle was not significantly different between women with and without an increased MBDI score and there was no significant association between the MBDI and the DRSP score (r=0.257, p=0.105).

MBDI and MsRS

Duration of menses was significantly associated with daily number of pads (r=0.329, p=0.036) and MsRS (r=0.366, p=0.019). PCA shows that a first PC extracted from number of pads, duration of menses and MsRS explained 54.7% of the variance and that all three variables loaded highly on this first PC (all > 0.701). Furthermore, there was a strong association between this PC and the composite score MsRS (r=0.999, p<0.001) indicating that the latter score reflects the three manifestations of menstruation-associated features/symptoms.

Table 2 shows the differences in MsRS symptoms between women with and without an increased MBDI score. IBS and pain symptoms and the total MsRS were significantly higher in women with an increased MBDI score. Pre-Ms (p=0.023) and post-Ms (p=0.001) MsRS scores were significantly higher in women with an increased MBDI score as compared with those with a lower score. **Figure 1** shows the significant correlation between the MBDI and the MsRS scores (r=0.430, p<0.01). Women with low abdomen cramps showed a significantly increased duration of menses (F=6.13, df=2/38, p=0.005) and increased GI symptoms (F=4.43, df=2/38, p=0.019).

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There was a significant association between the MsRS and the total DRSP score (r=0.405, p=0.009).

Biomarkers of MBDI

We examined the associations between the MBDI score (dichotomized using a 0.666 percentile threshold value) and the biomarkers using multivariate and univariate GLM analysis. We found - using multivariate GLM analysis – that there was a highly significant association between MBDI and biomarkers with an effect size of 0.519, including CCL5, PAI, IGF-1, Hp and estradiol (**Table 3).** Univariate GLM analyses showed that CCL5, PAI, IGF-1, and Hp were significantly increased in women with a high MBDI score, while estradiol was significantly lowered in women with a high MBDI score. The most significant associations (based on effect size) was (in decreasing order of importance): Hp, estradiol, PAI, IGF-1 and CCL5. There were no significant differences in the other chemokines, EGF, hsCRP, progesterone and PON1 arylesterase activity.

We also examined the best biomarker predictors of MBDI/MsRS using automatic stepwise multiple regression analyses with biomarkers as explanatory variables while allowing for the effects of age, age at menarche, education and BMI. **Table 4**, regression #1 shows that 23.0% of the variance in number of pads/day was explained by Hp (positively associated) and PON1 arylesterase activity (inversely associated). Regression #2 shows that 27.4% of the variance in duration of menses was explained by the regression on Hp and IGF-1. Model #3 shows that 38.6% of the variance in the MBDI score was predicted by the cumulative effects of 3 biomarkers, namely Hp, IGF-1 (both positively associated) and PON1 arylesterase activity (inversely associated). **Figure 2** shows the significant correlation (partial regression obtained by regression #2) between

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the MBDI and Hp levels (r=0.513, p<0.001). MsRS was significantly associated with CCL5 and IGF-1 (both positively associated), which explained 23.3% of the variance (see Table 4, regression #4). MBDI+MsRS (regression #5) was to a large extent (44.7% of the variance) explained by 3 biomarkers, namely Hp, IGF-1 (both positively) and PON1 arylesterase activity (inversely associated). **Figure 3** shows the partial regression between the MBDI+MsRS score and IGF-1 (r=0.417, p=0.008).

We have also examined the associations between the MsRS indices at pre- and post-Ms conditions and the biomarkers using GEE, repeated measures. **Table 5**, GEE shows that GI symptoms were associated with CCL5 and IGF-1; pain symptoms and low abdominal cramps with progesterone (inversely); and MsRS with progesterone, IGF-1 and Hp (GEE #1-4). GEE #5 shows that a combination of 5 biomarkers significantly predicted the MBDI+MsRS index, namely in descending order of importance Hp, IGF-1, PON1 arylesterase, estradiol and PAI.

Finally, we have performed binary logistic regression analysis to delineate the best prediction of women with a MBDI+MsRS score ≥ 0.666 percentile versus those with a lower score (using the random oversampling approach with multiple copies of the minority class to obtain a balanced split between both classes). **Table 6** shows that increased IGF-1 and Hp and lowered estradiol best predicted an MBDI+MsRS score ≥ 0.666 percentile with a Nagelkerke value of 0.506. The DRSP score was not significant in this regression.

Discussion

The first major finding of this study is that numbers of pads used during menses and duration of menses are associated with MsRS including low abdominal cramps, and GI and pain symptoms. These results extend those of a previous paper reporting that women who perceive their

 doi:10.20944/preprints202003.0416.v1

menstrual bleeding as “heavy” or “very heavy” tend to experience severe or very severe menstrual pain [39]. The average amount of menstrual blood flow during the menses is between 10-80 mL and the average duration of menstrual bleeding is approximately 4–5 days with shedding of the superficial stratum functionalis (epithelial, stromal and vascular) of the endometrium [40]. Using the MBDI composite score at a threshold value ≥ 0.666 percentile delineates a subgroup of women with increased duration of menses (mean ±SD = 6.00 ±1.37 days) and increased blood loss as estimated by the number of pads used.

We also observed that women with low abdominal cramps had a significantly higher duration of menses and more severe GI symptoms. Painful menstruation (dysmenorrhea) is a common condition among menstruating women and is commonly accompanied by other symptoms including nausea, vomiting, diarrhea, loss of appetite, headache, back pain, and general aching [41]. Worsening of GI symptoms, i.e. abdominal pain, bloating and diarrhea, has been reported during menses among women with IBS [42, 43]. Nevertheless, our data show that cramps and GI symptoms partially overlap indicating they have a common pathophysiology. All in all, the composite score MBDI+MsRS using a threshold value of ≥ 0.666 percentile delineates a subgroup of women with more menstruation distress as indicated by longer duration of menses, greater estimated blood loss and more cramps, GI and pain symptoms. In contrast, dysmenorrhea indicates painful menstruation with low abdominal cramps but does consider other menstruation distress features as delineated in the current study.

The second major finding of our study is that the composite MBDI score was associated with lowered estradiol. The shedding of the ‘old’ stratum functionalis usually takes 1–2 days, whereas menstrual bleeding still continues during the proliferation and repair of the surface epithelium which may take several days. The menstruation period is followed by regeneration of

 doi:10.20944/preprints202003.0416.v1

stratum functionalis cells under the influence of estrogen [44]. After ovulation, as the production of progesterone by the corpus luteum increases, the endometrium changes into a secretory phase whereby spiral arterioles grow within the stratum functionalis and acquire muscles. Estradiol and progesterone withdrawal due to corpus luteum demise triggers the initiation of menstrual bleeding [45] by induction of stromal shrinkage and spiral arteriolar vasoconstriction, leading to relative hypoxia in the functionalis layer and influx of leucocytes and immune cells [46, 47]. Thus, our findings suggest that women with lowered estradiol are more likely to have more blood loss and a longer duration of menses due to reduced or postponed proliferation, repair and regeneration of the surface epithelium.

The third major finding of our study is that MsRS including cramps and pain symptoms were significantly and inversely associated with plasma progesterone levels. These data are in agreement with the knowledge that that progesterone levels control prostaglandin production and that a decrease in progesterone is inversely associated with increased prostaglandins levels [19, 20]. Increased endometrial production of the prostaglandins $PGF_{2\alpha}$ and PGE_2 cause increased myometrial contractility with lower abdominal cramps, as well as GI symptoms including nausea, vomiting, and diarrhea [48-50]. In women with IBS, increased release of prostaglandins by the endometrium worsens GI symptoms during menses [43,51]. Blocking the synthesis of prostaglandins with cyclooxygenase inhibitors lowers prostaglandin levels in menstrual fluid thereby significantly decreasing dysmenorrhea symptoms [52]. Interestingly, combined treatments with estrogen and progestin in oral contraceptive pills are widely used to treat dysmenorrhea [53] by limiting endometrial growth and reducing the amount of endometrial tissue available for prostaglandin production [54]. Prostaglandins secreted by disintegrating cells during menstruation as a consequence of lowering progesterone levels cause an increase in prostaglandins, which

 doi:10.20944/preprints202003.0416.v1

promote uterine contractions and vasoconstriction [20,55]. Therefore, our findings suggest that women with lowered progesterone levels are more likely to display MsRS and menstrual distress.

The fourth finding of this study is that while MsRS symptoms were correlated with the DRSP scores measured all over the cycle, there were no significant associations between menstruation distress and PMS as assessed with different case definitions including MCAS. In some but not all studies there is a high comorbidity between dysmenorrhea and PMS [56-58]. For example, 91.5% of young women with dysmenorrhea have PMS and 85% of young women with PMS have dysmenorrhea, whereas in other studies the comorbidity between dysmenorrhea and PMS symptoms is 17.4% indicating that dysmenorrhea may occur in women with and without PMS [56-58]. It is interesting to note that both dysmenorrhea and PMS cause a significantly decreased health-related quality of life [59]. Therefore, our findings may indicate that while menstrual cycle-related symptoms and MsRS show a moderate overlap, there are no associations between the more extreme expressions of both concepts, namely MCAS versus menstruation distress. This may indicate differences in the pathways underpinning MCAS and menstruation distress.

The fifth major finding of this study is that the MBDI and MBDI+MsRS scores are strongly associated with Hp, but not CRP, and IGF-1, PAI, CCL5 and PON1 arylesterase, but not CCL2, CXCL-8, CXCL-10 and EGF, which are biomarkers of MCAS. Therefore, menstruation distress (Hp, IGF-1, PAI) and MCAS (CCL2, CXCL-10, CXCL-8 and EDF) are externally validated by different sets of biomarkers supporting that both menstruation distress and MCAS are different concepts, although lowered levels of estradiol and progesterone and increased CCL5 are shared biomarkers which may explain the partial overlap between both concepts.

 doi:10.20944/preprints202003.0416.v1

CCL5, which is associated with both MCAS and menstruation distress, is a chemokine that is synthesized by stromal cells of normal and endometriosis tissues [23] and plays a role in endometrial immunological physiology and endometrial disease [23] For example, in endometriosis and deep infiltrating endometriosis, CCL5 is correlated with the clinical stages of disease and with dysmenorrhea [23, 60]. Additionally, in stromal cells of adinomyosis, a condition in which the endometrium invades through the muscle wall of the uterus, CCL5 expression is increased as compared with eutopic endometrium, and the elevated CCL5 levels in eutopic endometrium are significantly correlated with higher severity of dysmenorrhea [61].

Also increased levels of IGF-1, PAI and EGF as well as lowered PON1 activity may explain in part MsRS and menstruation distress. Increased endometrial PAI-1 is associated with menorrhagia as defined by menstrual blood loss > 80 ml/cycle in the late secretory phase and menstruation [26]. PAI-1 functions as the principal inhibitor of tissue plasminogen activator and urokinase, which lead to the physiological breakdown of blood clots. Platelet aggregation, thrombus formation, and fibrin deposition are mechanism responsible for cessation of menstrual bleeding [62]. Women with menorrhagia have looser plasma fibrin clots formation and shorter clot lysis time, which is associated with lower platelet counts, tissue plasminogen activator antigen, and PAI-1 antigen [63]. Importantly, increased PAI-1 expression in endometriotic tissues is significantly correlated with increased dysmenorrhea [64]. PAI is an inflammatory marker, which is induced by pro-inflammatory cytokines [65] and affects gastric emptying and food intake and may induce hyperphagia [66, 67]. As such, the positive associations of PAI-1 with MBDI may be explained by the inflammatory response during menstruation which is accompanied by increased PAI-1 regulating fibrinolysis.

 doi:10.20944/preprints202003.0416.v1

Hp is significantly higher at the end of the menstruation as compared with the luteal phase [14] and is increased in uterine fluid during the secretory phase [22]. Hp is an acute phase reactant and is elevated in endometritis and inflammation of the uterine endometrium [68]. Hp displays high affinity for free hemoglobin (Hb), released from red blood cells, and the formed Hp-Hb complex is removed from the body by the reticuloendothelial system (mostly by the spleen) thereby lowering oxidative stress and inflammation as well [69,70]. As such, increased levels of Hp at the end of menses may be a compensatory regulatory mechanism during the inflammatory response during menstruation attenuating overzealous inflammatory and oxidative responses.

The production of IGF-1, a neurotrophin, is stimulated by a pulsatile secretion of growth hormone (GH) and its major activity is to stimulate cellular growth and differentiation [71]. The IGF autocrine/paracrine system plays a key role in sex hormone-mediated endometrial differentiation [27] and IGF-1 is an important regulator of pre-implantation, placental [28], and fetal development [29]. IGF-1 mRNA is expressed by the subepithelial stroma of the endometrium (in dairy cows) and plays a role in human tissue regeneration and activates wound healing, cell proliferation and collagen synthesis, indicating that IGF-1 promotes uterine stroma and epithelium proliferation during uterine involution [72]. IGF-1 also affects the gastro-intestinal tract including by increasing mucosal mass [73] and may enhance pain by modulating calcium channels [74]. As such, increased IGF-1 may play a key role in menstrual repair mechanisms involving proliferation and remodeling [72], but could increase pain sensation explaining the strong association of IGF-1 and IBS-like symptoms and MsRS. Finally, lowered PON-1 arylesterase activity may lead to increased oxidative stress and inflammatory responses [75] and therefore, women with lowered PON1 activity are more likely to have exaggerated inflammatory responses during menstruation [75]. Moreover, lower PON1 activity is associated with increased translocation of Gram-negative

 doi:10.20944/preprints202003.0416.v1

bacteria, which play a role in the pathophysiology of increased DRSP ratings at the end of the menstrual cycle [37]. Interestingly, PON1 plays a role in human fertility and reduced PON1 activity is associated with early pregnancy failure [76]. Moreover, the PON1 Q192R polymorphism is a risk factor of uterine leiomyoma [77].

Conclusions

Figure 4 summarizes the features of MsRS and menstruation distress and shows the differences between the latter and MCAS. The new composite score MBDI is strongly associated with lowered estradiol levels, while MsRS and GI and pain symptoms are associated with lowered progesterone levels. Both indices are significantly associated with biomarkers which play a role in MsRS, namely Hp, IGF-1, PAI, CCL-5 and PON1 arylesterase activity. Menstruation distress and MCAS are externally validated by different clinical features and biomarkers, namely menstruation distress by indices of increased blood loss, longer duration of menses, cramps, GI and pain symptoms, increased IGF-1, PAI, and Hp; and MCAS by increased depressive, anxiety, and physiosomatic symptoms as well as breast swelling and appetite changes, increased CCL2, CCL5, CXCL-8, CXCL-10 and EGF. Increased CCL5 and lowered levels of sex hormones are shared biomarkers underpinning both menstruation distress and MCAS. These findings show that symptoms during the menstrual cycle can best be conceptualized as: a) MsRS and menstruation distress when MBDI+MsRS $\geq$ 0.666 percentile; and b) MCAS computed as sum of total DRSP scores from day 1 through day 28 at a threshold of $\geq$ 0.666th percentile.

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Authorships

CR and MM made the design of the study. CR recruited and screened the participants. MM performed statistical analyses. SS, AKM, APM, LOS, JVLP, EGM, and DSB performed analyses. All authors agreed upon the final version of the paper.

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, clinical and biomarker data of 41 women divided according to their menstruation bleeding & duration index (MBDI).

Variables	MBDI <0.666 (n=27) $	MBDI ≥ 0.666 (n=14) $	F/ X^2	df	*p*
Age (years)	31.4 (7.1)	31.1 (7.3)	0.02	1/39	0.900
Years of education	15.8 (1.7)	16.2 (0.6)	0.71	1/39	0.403
Income (Baht/month)	83640 (94211)	88214 (57365)	0.03	1/37	0.870
BMI (Kg/m^2)	21.8 (3.8)	22.9 (3.4)	0.83	1/39	0.368
Age menarche (years)	13.0 (1.1)	12.4 (1.4)	2.60	1/39	0.115
Length cycle (days)	27.0 (5.0)	28.8 (1.6)	1.77	1/39	0.191
Peri-MS (No/Yes)	15/ 12	7/ 7	0.11	1	0.735
MCAS (No/Yes)	20/ 7	7/ 7	2.38	1	0.123
PMS (No/Yes)	15/ 4	11/ 11	3.68	1	0.055
Single / divorced	19/ 8	10/ 4	0.01	1	0.944
Pads / day	2.89 (0.85)	4.54 (1.71)	20.79	1/39	<0.001
Duration of menses (days)	4.01 (0.99)	6.00 (1.37)	59.38	1/39	<0.001
Total DRSP score	904.8 (211.7)	992.1 (200.5)	1.62	1/39	0.210

All results are shown as mean (±SD). All results of ANOVA (F) or analysis of contingency variables (X^2)
$ MBDI is estimated as z number of pads/day + z duration menses (in days); a high MBDI score is defined as MBDI ≥ 0.666 z score, and low when MBDI < 0.666 z score; BMI = body mass index,
Peri-MS = Peri-menstrual syndrome as computed by sum of DRSP scores during the peri-menstrual period (days 1 and 2 + 24 to 28) > 0.666 percentile, MCAS = Menstrual cycle-associated syndrome as computed by a total daily DRSP score during the menstrual cycle > 0.666 percentile, DRSP = Daily Record of Severity of Problems.

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Table 2 Association between menstruation bleeding & duration index (MBDI) and menses-related symptoms (MsRS)

Symptom	Pre-Ms symptoms		Post-Ms symptoms		Time		MBDI (median split)	
	MBDI < 0.666 (n=27)	MBDI ≥ 0.666 (n=14)	MBDI < 0.666 (n=27)	MBDI ≥ 0.666 (n=14)	Wald X^2	*p*	Wald X^2	*p*
Cramps	14/ 13	4/ 10	15/ 12	4/ 10	0.11	0.739	2.85	0.092
IBS symptoms	1.30 (0.56)	2.21 (0.31)	1.37 (0.22)	2.43 (0.33)	0.77	0.380	7.15	0.007
Pain symptoms	1.86 (0.16)	2.07 (0.26)	1.52 (0.18)	2.21 (0.29)	2.31	0.129	4.44	0.035
Total MsRS	4.63 (0.37)	6.21 (0.56)	4.07 (0.35)	6.50 (0.66)	0.34	0.559	8.88	0.003

All results of GEE analysis, repeated measures, with pre-menses (pre-Ms) and post-menses (post-Ms) symptoms as dependent variables and time and MBDI as explanatory variables. MBDI is computed as z score of number of pads / days + z score of duration of menses (in days); a high MBDI score is defined as MBDI ≥ 0.666 z score, and low when MBDI < 0.666 z score.

MsRS: menses-related symptoms computed as sum of acne, gastro-intestinal upset, diarrhea, constipation, bloating, back pain, muscle ache, dizziness, headache, and lower abdominal cramps (cramps). IBS: symptoms reminiscent of irritable bowel syndrome computed as sum of gastro-intestinal upset, diarrhea, constipation, and bloating; and Pain symptoms: pain symptoms including back pain, muscle ache, and headache.

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Table 3 Association between menstruation bleeding and duration index (MBDI) and biomarkers

Biomarker	MBDI < 0.666	MBDI ≥ 0.666	F	df	*p*	Partial Eta squared
Multivariate GLM	-	-	7.13	5/ 33	<0.001	0.519
Univariate GLM*						
CCL5 (z score)	-0.205 (0.179)	0.395 (0.243)	3.99	1/37	0.053	0.097
CCL5 (pg/mL)	183358 (44564)	266889 (19655)				
PAI (z score)	-0.245 (0.183)	0.473 (0.255)	5.19	1/37	0.029	0.123
PAI (ng/mL)	54.7 (2.4)	66.1 (4.6)				
IGF-1 (z score)	-0.177 (0.143)	0.341 (0.199)	4.42	1/37	0.042	0.107
IGF-1 (ng/mL)	652.8 (46.4)	769.9 (59.8)				
Hp (z score)	-0.296 (0.158)	0.571 (0.221)	10.06	1/37	0.003	0.214
Hp (mg/dL)	327.4 (21.7)	471.9 (45.3)				
Estradiol (z score)	0.252 (0.185)	-0.486 (0.258)	5.36	1/37	0.026	0.126
Estradiol (pmole/L)	1967.153.1	1418.7 140.5				
CCL2 (pg/mL)	1031.7 (95.3)	1214.1 (111.7)	1.41	1/38	0.243	-
CXCL-10 (pg/mL)	340.5 (24.4)	362.2 (173.6)	0.21	1/38	0.650	-
CXCL-8 (pg/mL)	433.0 (185.6)	433.6 (168.2)	0.00	1/38	0.998	-
CCL11 (pg/mL)	578.8 (37.9)	622.6 (52.6)	0.46	1/38	0.501	-
EGF (pg/mL)	1696.8 (101.5)	1968.9 (165.9)	2.19	1/38	0.148	-
hsCRP (mg/L)	6.2 (9.6)	9.9 (10.5)	1.16	1/37	0.289	-
Progesterone (nmole/L)	54.1 (5.6)	48.7 (7.3)	0.35	1/38	0.560	-
PON1 AREase (U/L)	837.3 (42.2)	773.3 (55.5)	0.83	1/38	0.370	-

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MBDI is computed as z score of number of pads / days + z score of duration of menses (in days). These data are dichotomized according to 0.666 percentile values.

All results of GLM analysis with age and body mass index as covariates. * Results of multivariate GLM analysis with Regulated upon activation, normal T cell expressed and secreted (RANTES or CCL5), Plasminogen activator inhibitor (PAI), Insulin-like growth factor 1 (IGF-1), haptoglobin (Hb) and estradiol as dependent variables.

EGF: epidermal growth factor, hsCRP: high sensitive C-reactive protein, PON1 AREase: paraoxonase 1 arylesterase activity

*Results of univariate GLM analysis performed on each of the biomarkers separately. Shown are the model-generated estimated marginal mean values (SE) and their z scores.

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Table 4 Prediction of menstruation bleeding and duration index (MBDI) and menses-related symptoms (MsRS) using biomarkers as exploratory variables

Dependent variables	Explanatory variables	β	*t*	*P*	F_{model}	df	*p*	R^2
#1. Number of pads/day	**Model**				5.66	1/38	0.007	0.230
	Hp	0.464	3.07	0.004				
	PON1 AREase	-0.336	-2.23	0.032				
#2. Duration menses in days	**Model**				7.17	2/38	0.002	0.274
	Hp	0.384	2.78	0.008				
	IGF-1	0.373	2.70	0.010				
#3. MBDI	**Model**				7.74	3/37	<0.001	0.386
	Hp	0.571	4.18	<0.001				
	IGF-1	0.286	2.18	0.035				
	PON1 AREase	-0.324	-2.37	0.023				
#4. MsRS	**Model**				5.77	2/38	0.006	0.233
	CCL5	0.380	2.68	0.011				
	IGF-1	0.302	2.13	0.040				
#5. MBDI+MsRS	**Model**				9.96	3/37	<0.001	0.447
	Hp	0.593	4.62	<0.001				
	IGF-1	0.341	2.79	0.008				
	PON1 AREase	-0.315	-2.46	0.019				

All results of multiple regression analysis with menses features as dependent variables and biomarkers as explanatory variables.

MBDI: computed as z score of number of pads/day + z score of duration of menses (in days); MsRS: computed as sum of acne, gastro-intestinal upset, diarrhea, constipation, bloating, back pain, muscle ache, dizziness, headache, and lower abdominal cramps; MBDI+MsRS: computed as z score of MBDI + z score of MsRS.

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Table 5 Associations between Menstrual Bleeding & Duration Index (MBDI), menses-related symptoms (MsRS) and biomarkers

Dependent variables	Exploratory variables	B	SE	Wald	df	*p*
#1. IBS	CCL5	0.331	0.1114	8.81	1	0.003
	IGF-1	0.324	0.1210	7.17	1	0.007
#2. PAIN	Progesterone	-0.311	0.1067	8.50	1	0.004
#3. Cramps	Progesterone	-0.605	0.2702	5.017	1	0.025
#4. MsRS	Progesterone	-0.304	0.0994	9.38	1	0.002
	IGF-1	0.327	0.1236	7.01	1	0.008
	Hp	0.292	0.1267	5.29	1	0.021
#5. MBDI+MsRS	Hp	0.491	0.1618	9.21	1	0.002
	IGF-1	0.374	0.1036	13.02	1	<0.001
	Estradiol	-0.242	0.0928	6.79	1	0.009
	PON1	-0.271	0.0809	11.24	1	0.001
	PAI	0.180	0.0865	4.34	1	0.037

All results of GEE analysis, repeated measures, with dependent variables assessed at the end of the menstrual cycle and 7 days later and biomarkers (assayed at the same time points) as explanatory variables while allowing for the effects of age, education and body mass index (all non-significant).

IBS: symptoms reminiscent of irritable bowel syndrome computed as sum of gastro-intestinal upset, diarrhea, constipation, and bloating; Pain: pain symptoms including back pain, muscle ache, and headache; Cramps: low abdominal cramps; MsRS: menses-related symptoms computed as sum of acne, gastro-intestinal upset, diarrhea, constipation, bloating, back pain, muscle ache, dizziness, headache, and lower abdominal cramps. MBDI: Menstrual Bleeding & Duration Index computed as z score of number of pads/day + z score of duration of menses (in days). MBDI+MsRS: sum of z score of pads/day + z score of duration of menses + z score MsRS. Hp = Haptoglobin, IGF-1= Insulin-like growth factor 1, PON1 = Paraoxonase 1 arylesterase, PAI = Plasminogen activator inhibitor.

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Table 6. Results of binary logistic regression analysis with an increased menstrual bleeding and duration index (MBDI) + menses-related symptoms (MsRS) composite score ≥ 0.666 percentile as dependent variable and lower scores as reference group.

Explanatory variables	B	SE	Wald	p	Odds ratio	95% CI interval
IGF-1	1.021	0.431	5.60	0.018	2.78	1.19-6.46
Hp	1.406	0.520	7.30	0.007	4.08	1.47-11.31
Estradiol	-1.139	0.426	7.15	0.008	0.32	0.14-0.74

CI: confidence intervals; IGF-1= Insulin-like growth factor 1, Hp = Haptoglobin

MBDI+MsRS: sum of z score of pads/day + z score of duration of menses + z score MsRS (menses-related symptoms computed as sum of acne, gastro-intestinal upset, diarrhea, constipation, bloating, back pain, muscle ache, dizziness, headache, and lower abdominal cramps).

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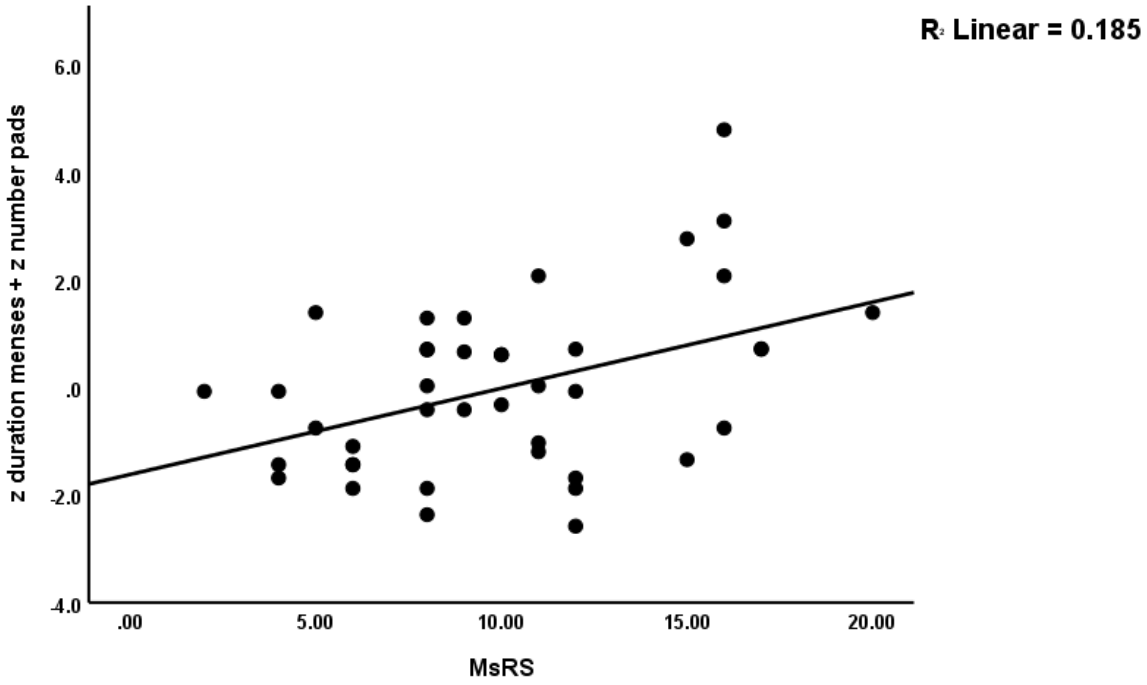

Figure 1 The significant correlation between the menstruation bleeding and duration index (computed as sum of z score of pads/day + z score of duration of menses) and menses-related symptoms (MsRS).

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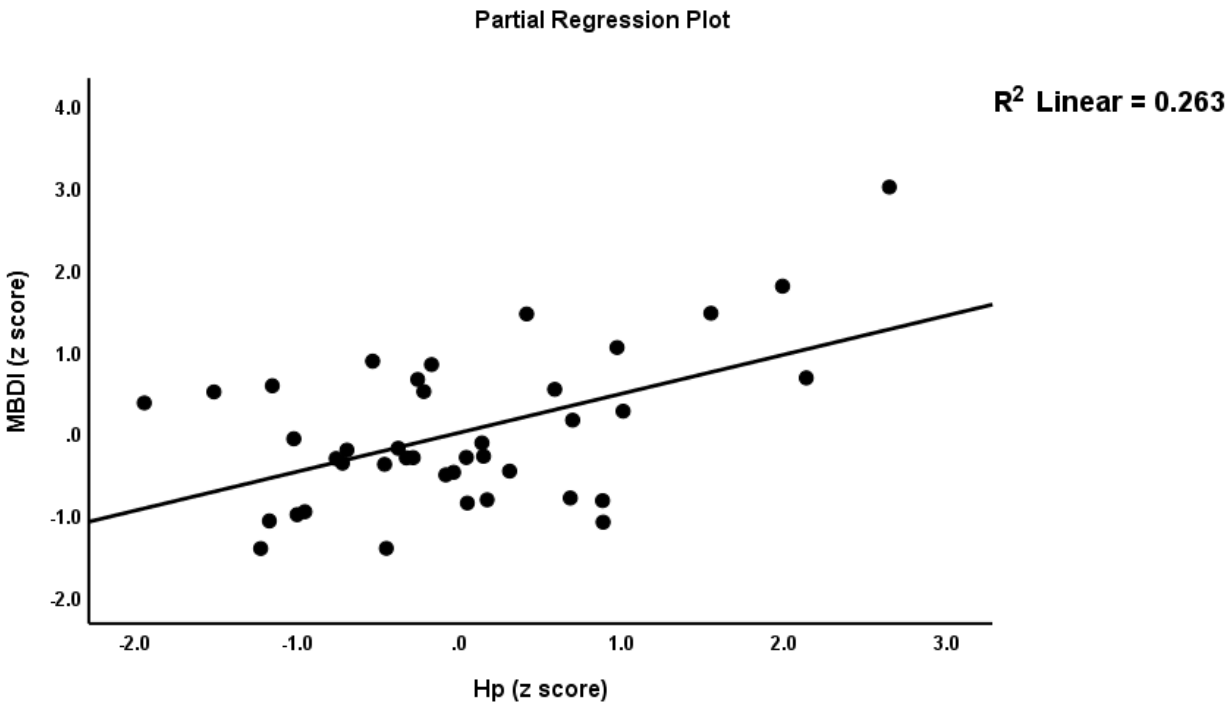

Figure 2 The significant partial regression of the menstruation bleeding and duration index (MBDI) on plasma haptoglobin (Hp) levels

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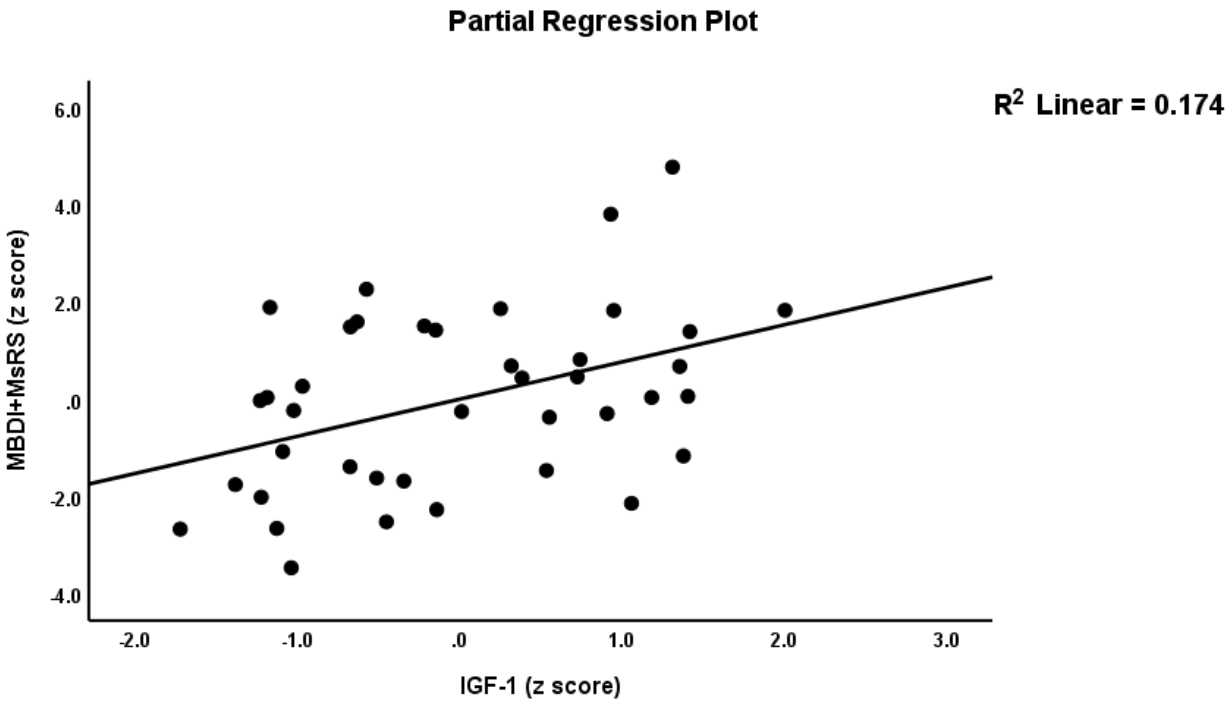

Figure 3 The significant partial regression of the menstruation bleeding and duration index (MBDI) + menstruation-related symptoms (MsRS) index on plasma insulin-like growth factor-1 (IGF-1).

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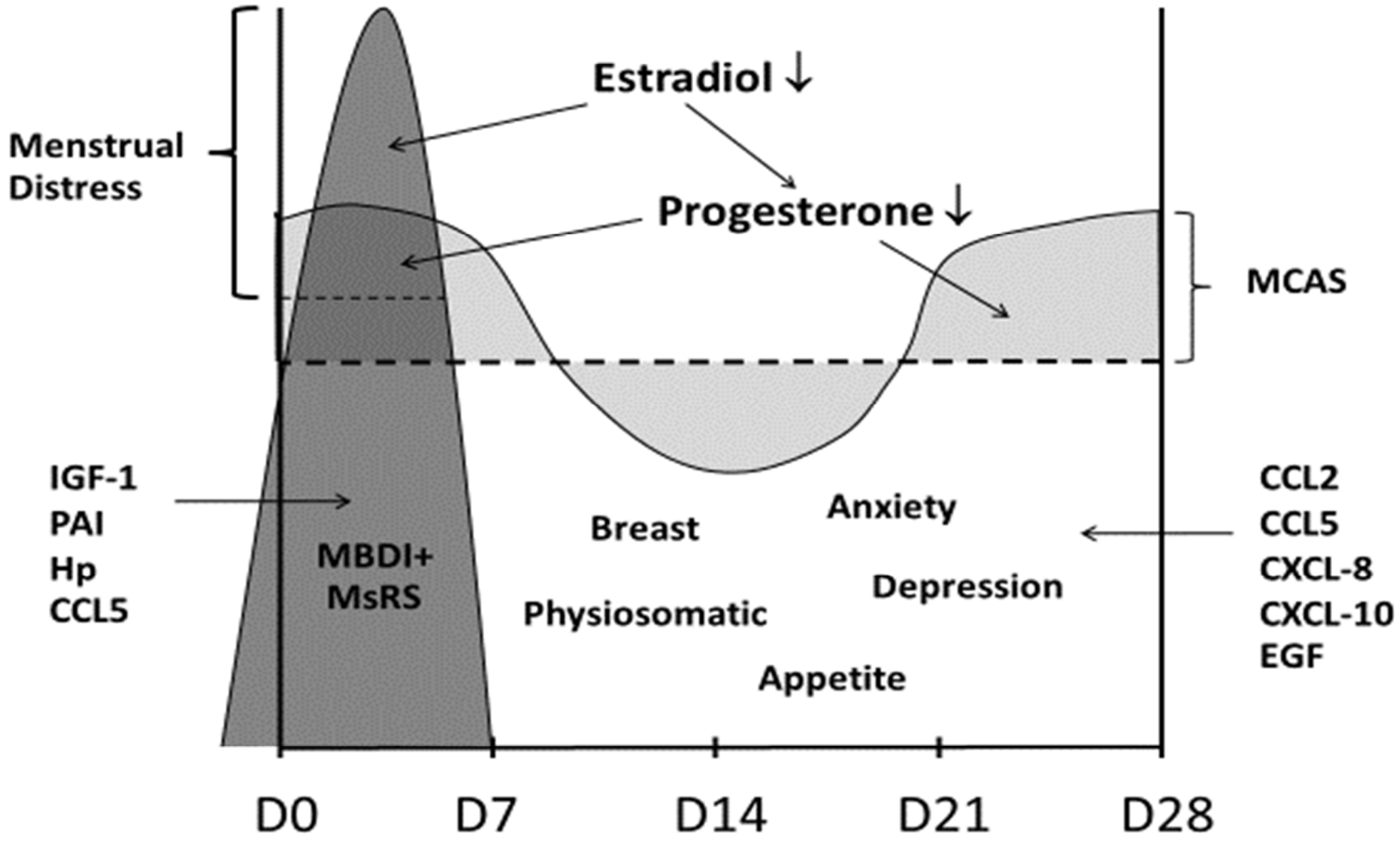

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Figure 4. The features of menstruation distress including the menstruation bleeding and duration index (MBDI) + menstruation-related symptoms (MsRS) in relation to menstrual cycle-associated symptoms and syndrome (MCAS). Menstruation distress is characterized by increased blood loss, longer duration of menses, cramps, gastro-intestinal and pain symptoms, and increased insulin-like growth factor-1 (IGF-1), plasminogen activator inhibitor (PAI), and haptoglobin (Hp). MCAS is characterized by depressive, anxiety, and physiosomatic symptoms as well as breast swelling and appetite changes, and increased CCL2, CCL5 (RANTES), CXCL-8 (IL-8), CXCL-10 (IP-10) and epidermal growth factor (EGF). Increased CCL5 and lowered levels of estradiol and progesterone are features of both MsRS/menstruation distress and MCAS.

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