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Branched-chain amino acids and specific phosphatidylinositols are plasma metabolite pairs associated with menstrual pain severity

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Menstrual pain affects women's quality of life and productivity, yet objective molecular markers for its severity have not been established owing to the variability in blood levels and chemical properties of potential markers such as plasma steroid hormones, lipid mediators, and hydrophilic metabolites. To address this, we conducted a metabolomics study using five analytical methods to identify biomarkers that differentiate menstrual pain severity. This study included 20 women, divided into mild (*N* = 12) and severe (*N* = 8) pain groups based on their numerical pain rating scale. We developed pretreatment procedures that allowed all analyses from only 100 µL of finger-prick blood collected across the menstrual cycle. Among the 692 quantified metabolites, branched-chain amino acids and specific phosphatidylinositol (PI), especially PI(36:2), were identified as potential biomarkers. Furthermore, the ratio of PI(36:2) to each BCAA or total BCAA effectively discriminated between the severity levels of menstrual pain. These ratios correlated positively with NPRS, indicating high accuracy in pain assessment. This study highlights the potential of small molecular markers to objectively assess menstrual pain severity, aiding evidence-based support and intervention.

Menstruation is a physiological process characterised by cyclic, monthly vaginal bleeding in women[1]. Severe menstrual pain, or dysmenorrhea, has a significant impact on the quality of life and work productivity of young women. Primary dysmenorrhea occurs in the absence of gynaecological conditions such as endometriosis or uterine fibroids, indicating a normal ovulatory cycle without underlying pelvic pathology[2]. Despite the recognised importance of individual variability in the severity of menstrual pain, no objective molecular markers have been established. This lack of biomarkers hinders the assessment and comparison of pain severity in women, often delaying gynaecological consultation and increasing the risk of gynaecological disorders.

Low molecular weight metabolites in the blood can capture phenotypic variations that genes or transcripts alone cannot, making them promising candidates for menstrual pain biomarkers[3,4]. Prostaglandins (PGs) are well-recognised low-molecular-weight bioactive molecules implicated in the onset and severity of dysmenorrhea[5]. Elevated levels of PGF2α cause spastic contractions of the uterine muscle, reducing blood flow and causing ischaemia, the primary mechanism behind menstrual pain[6]. In addition, metabolites associated with oxytocin, glucocorticoids, and amino acids are involved in the pathogenesis of menstrual pain[5–7]. Therefore, monitoring the levels of these bioactive molecules or their metabolites in the blood may objectively be used as markers or predictors of menstrual pain severity.

Several studies have investigated small molecule biomarkers for primary dysmenorrhea. Akdemir et al. identified asymmetric dimethylarginine as a potential biomarker using serum samples from Turkish women[8]. Ocktariyana et al. reported malondialdehyde in the plasma of Indonesian women as a biomarker reflecting the pain phenotype[9]. Fang et al. proposed 10 metabolites, including steroid hormones such as progesterone and androstenedione, as potential markers in the urine of Chinese women during the luteal phase. They also identified 13 metabolites, including amino acids, as candidates in urine collected during the menstrual period[10,11]. Despite these advances, inconsistencies remain in the classes of metabolites measured and the timing of sample collection during different phases of the menstrual cycle. This highlights the need for non-targeted

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analysis covering hydrophilic metabolomics, and lipidomics and targeted measurements of prostaglandins and steroid hormones throughout the menstrual cycle.

To address these gaps, our study used five different analytical methods to evaluate small molecule metabolites with a wide variety of chemical and physical properties and concentration ranges comprehensively. In addition, we developed a pretreatment method using only 100 µL of finger-prick blood to minimise the sample collection burden and allow evaluation across the menstrual cycle, including the follicular, luteal, and menstrual phases. These methodological advances enabled us to identify a molecular marker index that accurately assesses menstrual pain by analysing metabolites that differ in both blood concentration and physical properties.

Results

Demographic characteristics of participants and study design

Twenty healthy women with regular menstrual cycles (25–38 days, with a variation of ≤ 6 days) and menstrual periods lasting 3–7 days were enrolled in the study. Recent gynaecological examinations confirmed the absence of any disorders or abnormalities (Supplementary Table 1). The mean age was 31 ± 4.8 years, chosen to represent the demographic group most affected by menstrual pain among Japanese women[12]. Participants had a mean body mass index of 20.0 ± 1.84; while none were classified as obese, three were classified as underweight (Supplementary Table 2). Most participants reported pain localised to the uterus, including the lower abdomen and lower back (Supplementary Table 2). Based on their numerical pain rating scale (NPRS) scores, participants were divided into two groups: severe pain group (NPRS ≥ 4, $n = 8$) and mild pain group (NPRS < 4, $n = 12$) (Fig. 1a and Supplementary Table 2).

A notable strength of this study is the use of five different analytical methods to quantify both water-soluble and organic solvent-soluble small molecules over a broad concentration range. This comprehensive profiling was achieved using only approximately 100 µL of finger-prick blood, allowing the detection of 692 metabolites. These included 23 steroid hormones, 85 lipid mediators, 429 abundant lipids, and 155 hydrophilic metabolites (Fig. 1b). The specific metabolites analysed using each method are detailed in (Supplementary Table 3).

Severity of menstrual pain, not menstrual cycle, alters global plasma metabolite profile

Progesterone peaks during the luteal phase, whereas estrogen levels are lowest during the menstrual phase[13]. Consistent with this, our cohort had significantly higher levels of progesterone and 17-hydroxyprogesterone (17-OHP) during the luteal phase than in other phases (Fig. 2a). In addition, 17β-estradiol and estrone levels were significantly higher in the luteal phase than in the follicular phase. Although there was no statistically significant difference in 17β-estradiol levels between the follicular and luteal phases, estrone levels differed significantly (Fig. 2a). These results confirm that the sample collection accurately captured the three specific menstrual cycle phases in this population.

To investigate whether plasma metabolic profiles vary across these phases, we performed multivariate analyses, including principal component analysis (PCA; Supplementary Fig. 1) and partial least squares discriminant analysis (PLS-DA; Fig. 2b). The score plots showed no clear separation of clusters, suggesting that there is no global change in plasma metabolite composition across the menstrual cycle. This suggests that only a limited subset of bioactive molecules responds to menstrual cycle fluctuations, whereas most small molecules in the blood remain unchanged.

Next, we used PLS-DA to examine whether the global plasma metabolite composition differed between the severe and mild pain groups, defined using an NPRS threshold of 4. Interestingly, the PLS-DA score plots revealed significant differences between the two groups at each cycle phase (Fig. 2c). The most pronounced cluster separation was observed during the menstrual phase, prompting us to focus subsequent analyses on metabolite profiles specific to this period.

Plasma abundant lipids and hydrophilic metabolites as indicators of menstrual pain severity

To determine which class of metabolites most effectively discriminates between pain severity levels, we performed PLS-DA on the profiles of steroid hormones, lipid mediators, abundant lipids, and hydrophilic metabolites during the menstrual phase. As shown by the clear separation between the mild and severe menstrual pain groups in the PLS-DA score plot (Fig. 3a), the profiles of abundant lipids (phospholipids, triacyl glycerides, and cholesterol esters, etc.) and hydrophilic metabolites effectively discriminated between the severity levels of menstrual pain. The profiles of steroid hormones and lipid mediators did not show a clear separation between groups, suggesting that most bioactive lipids were not correlated with pain severity.

To further identify specific metabolites contributing to this distinction, we generated a volcano plot (Fig. 3b). Among the abundant lipids, phosphatidylinositol (PI) (36:2), PI(38:3), PI(38:4), and diacylglycerol (DAG) (34:3) were significant with a fold change > 1.2 and $p < 0.015$. Among the hydrophilic metabolites, levels of branched-chain amino acids (BCAAs) and 4-hydroxyproline were significantly different, with a fold change > 1.2 and $p < 0.015$. These results suggested that levels of these eight metabolites could be used to help identify the severity of menstrual pain.

Ratio of PI(36:2) to BCAAs most effectively distinguishes between severe and mild pain groups

To evaluate the efficacy of the eight metabolites identified as potential biomarkers for severe menstrual pain, we performed receiver operating characteristic (ROC) analysis. All eight metabolites showed high discriminatory power, with an area under the curve (AUC) of 0.8 or higher (Fig. 4a). PI(36:2) had the highest AUC (0.906), followed by valine (0.865), isoleucine (0.865), and leucine (0.865). Plasma levels of these four metabolites significantly differed between the severe and mild pain groups: PI(36:2) increased 1.42-fold, while leucine,

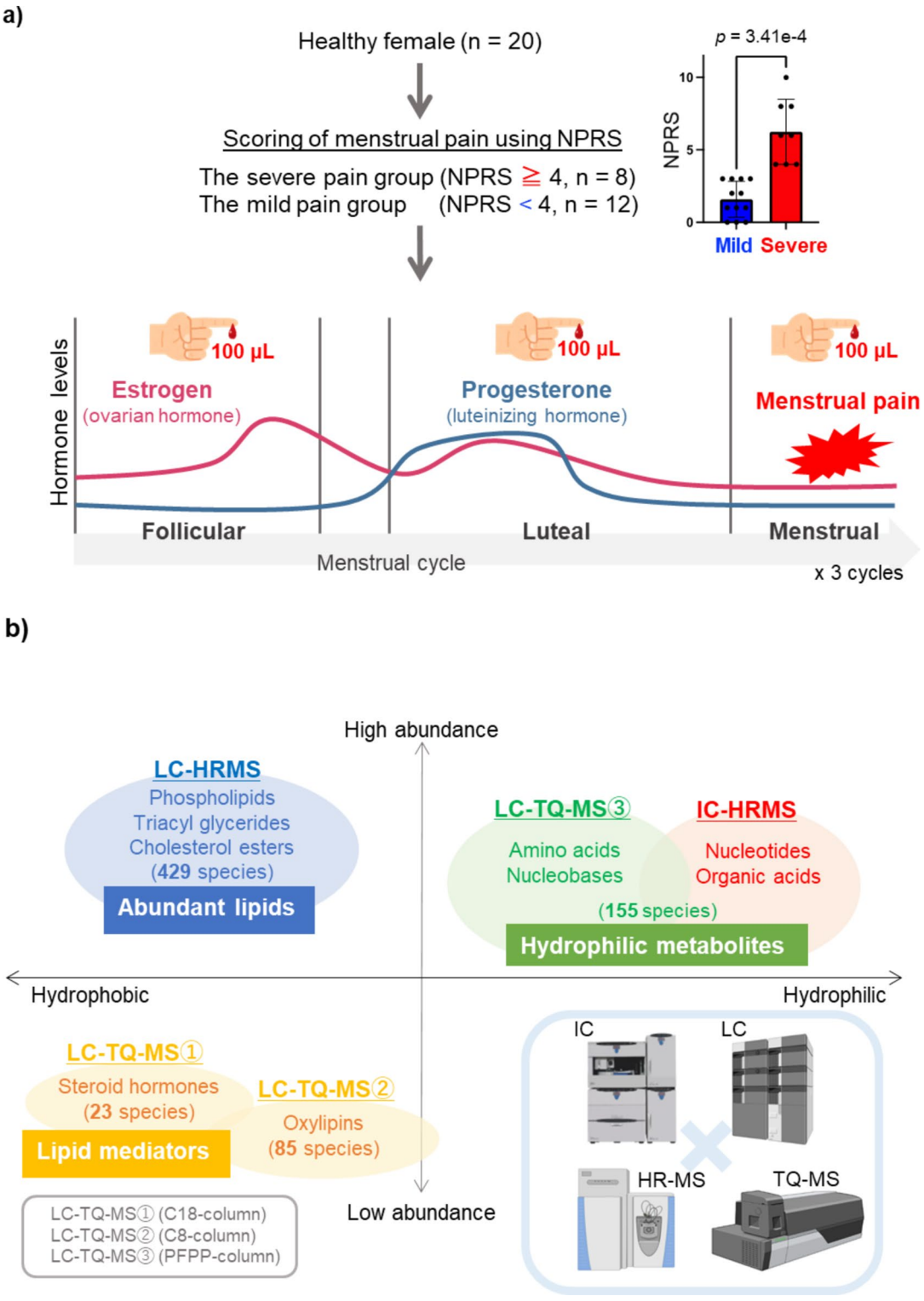

Fig. 1. Clinical study design and comprehensive analytical methods used. (**a**) Study design used to identify plasma markers that differentiate menstrual pain severity. Participants with numerical pain rating scale (NPRS) score of 4 or more were classified as having severe pain, whereas those with NPRS score of less than 4 were classified as having mild pain. NPRS scores (mean ± SD) for severe and mild pain groups are presented, with statistical significance determined using Welch's t-test (unpaired, two-tailed) (p-value indicated). Plasma samples were from finger-prick blood collected during follicular, luteal, and menstrual phases. (**b**) Summary of steroid hormones, lipid mediators, abundant lipids, and hydrophilic metabolites measured from plasma samples. A total of 692 metabolites were detected in approximately 100 µL of finger-prick blood, including 23 steroid hormone metabolites, 85 lipid mediator metabolites, 429 abundant lipids, and 155 hydrophilic metabolites.

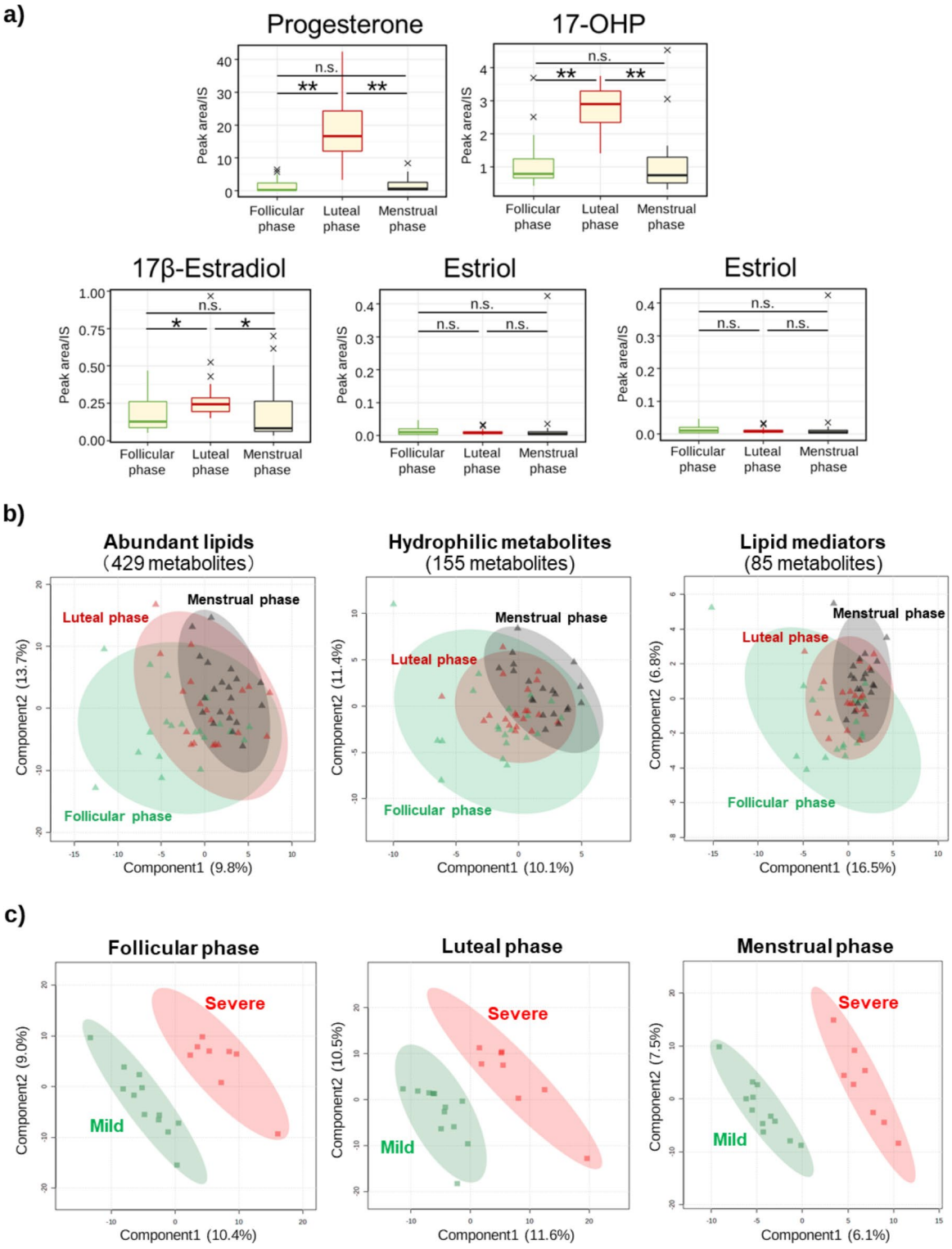

Fig. 2. Menstrual pain severity, not menstrual cycle, alters global plasma metabolite profile (**a**) Changes in hormone levels during menstrual cycle. Plasma levels of progesterone, 17-hydroxyprogesterone (17-OHP), and estrogens (estrone, 17β-estradiol, and estriol) were analysed. Progesterone and 17-OHP were elevated during luteal phase, whereas oestrogen levels were lower during menstrual phase, confirming validity of sample collection. Statistical significance was determined using Steel–Dwass test: $^{**}p < 0.01$, $^{*}p < 0.05$. (**b**) PLS-DA score plots for steroid hormones, lipid mediators, abundant lipids, and hydrophilic metabolites in plasma across menstrual cycle. No significant differences were found between three phases. Details of cross-validation and VIP scores are shown in (Supplementary Fig. 2 and Supplementary Table 4). (**c**) PLS-DA score plots showing plasma metabolite levels in each menstrual cycle phase. Clear separation between severe and mild pain groups was observed in all phases. Details of PLS-DA cross-validation and VIP scores are shown in (Supplementary Fig. 3 and Supplementary Table 5).

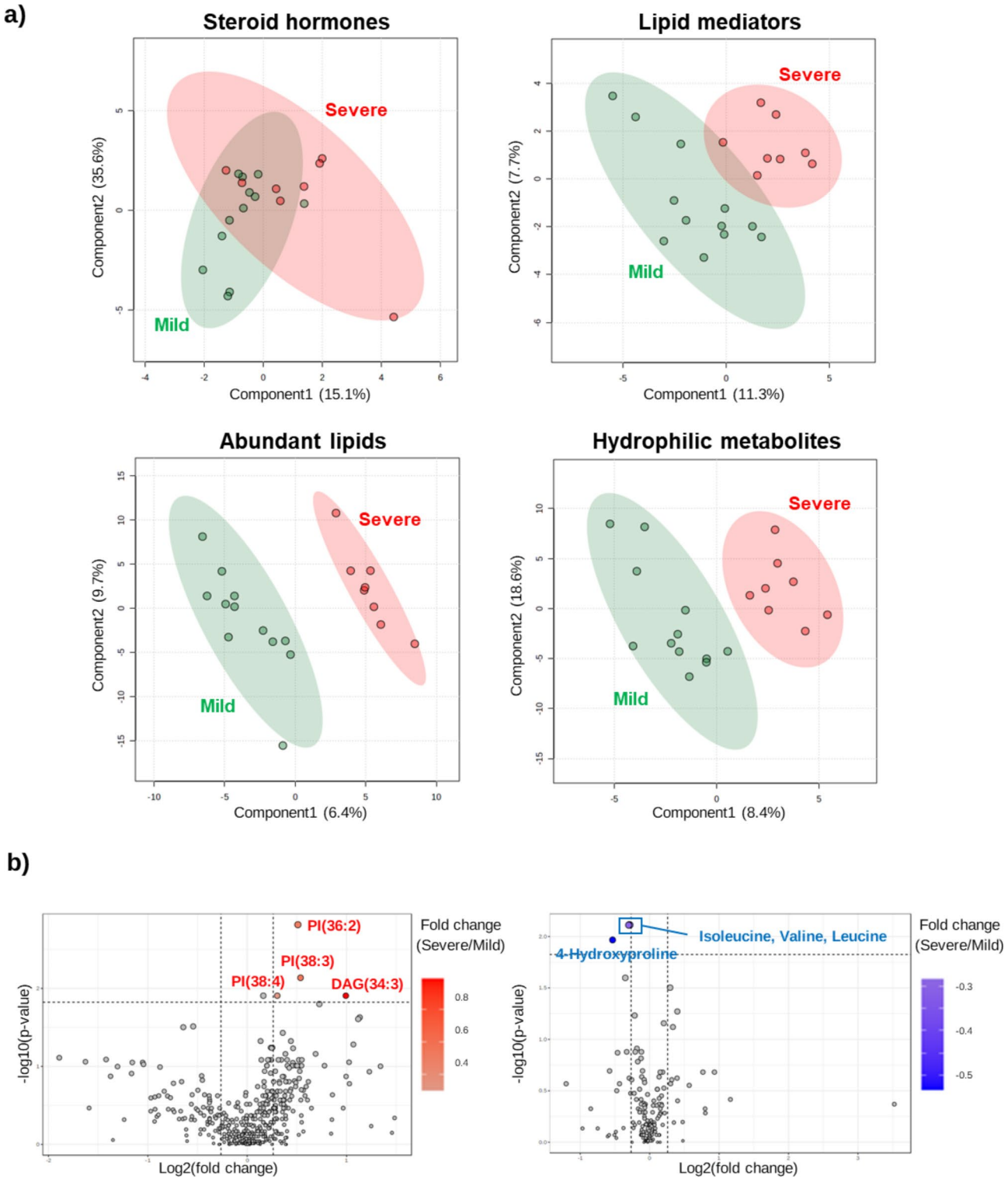

Fig. 3. Plasma lipid and hydrophilic metabolite profiles during menstrual phase as indicators of menstrual pain severity. (**a**) PLS-DA score plot of plasma levels of steroid hormones, lipid mediators, abundant lipids, and hydrophilic metabolites during menstrual phase. For steroid hormones and lipid mediators, no clear separation was observed between severe and mild pain groups. In contrast, abundant lipids and hydrophilic metabolites clearly separated into distinct clusters. Details of PLS-DA cross-validation and VIP scores are shown in (Supplementary Fig. 4 and Supplementary Table 6). (**b**) Volcano plot showing relative metabolite levels in the severe pain group compared to those in the mild pain group. Red and blue dots represent significant increases or decreases with fold changes > 1.2 and $p < 0.015$. Statistical significance was determined using Wilcoxon rank-sum test. *PI(36:2)* phosphatidylinositol (36:2), *PI(38:3)* phosphatidylinositol (38:3), *PI(38:4)* phosphatidylinositol (38:4), *DAG(34:3)* diacylglycerol (34:3).

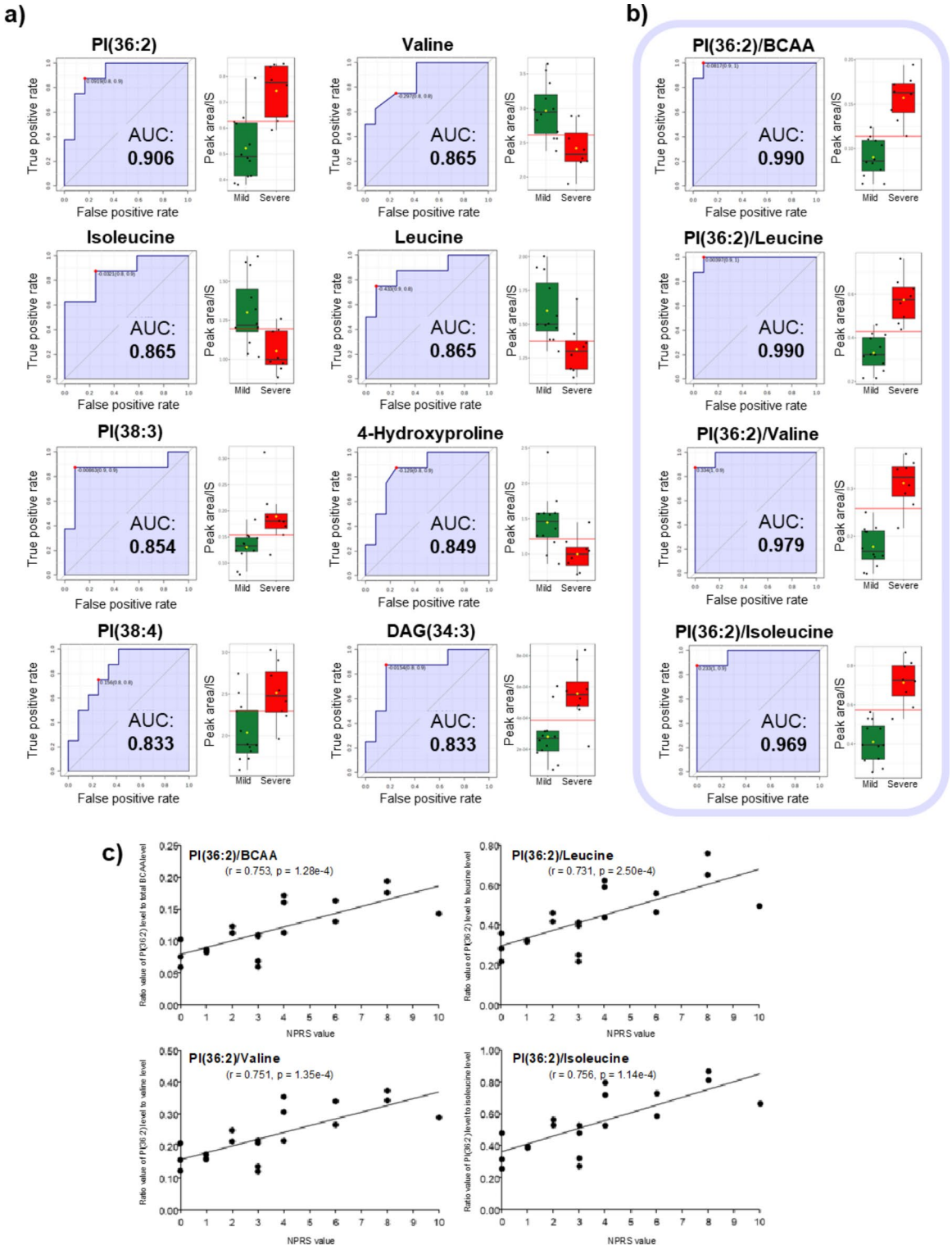

Fig. 4. PI(36:2) to BCAA ratios effectively discriminated between severe and mild pain groups. (**a**) ROC analysis and box-and-whisker plots for plasma markers discriminating between severe and mild pain groups. AUC values for each metabolite are shown within ROC curves. In box plots, median is represented by centre line, 75th percentile by top edge, and 25th percentile by bottom edge. Whiskers extend to maximum and minimum values. PI(36:2) had highest AUC (0.906), followed by BCAAs (valine, isoleucine, and leucine, all with AUCs of 0.865). (**b**) ROC analysis and box-and-whisker plots of ratios of PI(36:2) to each BCAA or total BCAA. AUCs for PI(36:2)/BCAA, PI(36:2)/leucine, PI(36:2)/valine, and PI(36:2)/isoleucine were 0.990, 0.979, 0.969, and 0.969, respectively, indicating higher discrimination than PI(36:2) alone. (**c**) Pearson correlation analysis between ratios of PI(36:2) to each BCAA or total BCAA during menstrual phase and NPRS scores. Correlation coefficient (r) and p-value are given for each ratio.

	AUC value		
	Follicular phase	Luteal phase	Menstrual phase
PI(36:2)/BCAA	0.854	0.927	0.990
PI(36:2)/Leucine	0.885	0.958	0.990
PI(36:2)/Valine	0.833	0.927	0.979
PI(36:2)/Isoleucine	0.823	0.906	0.969

Table 1. AUC values of the ratio of PI(36:2) to each BCAA or total BCAA.

	Pearson correlation coefficient					
	First menstrual cycle			Additional two different menstrual cycles		
	Follicular phase	Luteal phase	Menstrual phase	Follicular phase	Luteal phase	Menstrual phase
PI(36:2)/BCAA	0.687**	0.593*	0.753**	0.318*	0.291†	0.318*
PI(36:2)/Leucine	0.701**	0.635**	0.731**	0.353*	0.337*	0.328*
PI(36:2)/Valine	0.704**	0.591**	0.751**	0.310†	0.273†	0.323*
PI(36:2)/Isoleucine	0.582**	0.502**	0.756**	0.278†	0.254	0.281†

Table 2. Pearson correlation coefficients were calculated to determine correlation between the ratios of PI(36:2) to each BCAA or total BCAA and NPRS. (**$p < 0.01$, *$p < 0.05$, †$p < 0.10$)

isoleucine, and valine decreased to approximately 0.8-fold, indicating an inverse correlation between PI(36:2) and BCAAs.

To enhance discrimination accuracy, we calculated the ratios of PI(36:2) to each BCAA or the total BCAA level. Subsequent ROC analysis revealed even higher accuracy, with AUC values of 0.990 for PI(36:2)/BCAA, 0.979 for PI(36:2)/leucine, and 0.969 for both PI(36:2)/valine and PI(36:2)/isoleucine. Furthermore, AUC values for the ratio of PI(38:3) or PI(38:4) to each BCAA or the total BCAA level were calculated. The AUC value for PI(38:4)/leucine was 0.979, which was less than 0.990 for PI(36:2)/BCAA or PI(36:2)/leucine (Supplementary Fig. 5). Therefore, the pairs of PI(36:2) and BCAA were demonstrated to exhibit the most effective discrimination performance (Fig. 4b). Additionally, these ratios showed high discrimination accuracy across all menstrual cycle phases, with a significant positive linear correlation with NPRS (Fig. 4c and Table 1). Notably, the AUC exceeded 0.8 in both the follicular and luteal phases, indicating consistently high discrimination performance (Table 1). These findings suggested that these ratios can predict and monitor menstrual pain severity regardless of the menstrual cycle phases. Furthermore, their correlation with NPRS supported their use for objective pain assessment (Table 2).

As demonstrated in Fig. 3, the PLS-DA models exhibited clear discrimination between the severe and mild pain groups. However, the Q2 values of these models were so low that we could not dismiss the possibility that these models were overfitting (Supplementary Table 5). Consequently, we examined the robustness of these metabolite pairs identified in this model as discriminative markers in data sets other than those utilized for model construction. To substantiate this assertion, blood samples were collected from the same participants over additional two different menstrual cycles We tested whether the correlation between the ratio of PI (36:2) to each BCAA or total BCAA and NPRS persisted in the follicular, luteal, and menstrual phases over additional two different menstrual cycles. The analysis revealed consistent positive correlations between PI(36:2)/BCAA, PI(36:2)/Leucine, PI(36:2)/Valine, and PI(36:2)/Isoleucine and NPRS in each phase of additional two different menstrual cycles (Table 2). Among them, PI(36:2)/Leucine demonstrated a significant positive correlation with NPRS in all three phases of the additional two different menstrual cycles. These findings suggested that these ratios can serve as reliable markers for distinguishing the severity of menstrual pain at various phases of the menstrual cycle.

Discussion

Menstrual pain has a significant impact on women's quality of life and ability to work, with considerable individual variability in its severity. However, there is currently no objective method to discriminate between different levels of menstrual pain using metabolite biomarkers. This study aimed to identify small molecular markers that reflect the severity of menstrual pain using plasma metabolite analysis.

We identified several potential biomarker metabolites, including BCAAs (isoleucine, valine, and leucine) and 4-hydroxyproline among hydrophilic metabolites and PI(36:2), PI(38:3), PI(38:4) and DAG(34:3) among abundant lipids. Significant differences in the levels of these metabolites were observed between the severe and mild pain groups during the menstrual phase (Fig. 3). Furthermore, ROC analysis and correlation with the NPRS showed that the ratio of PI(36:2) to each BCAA or total BCAAs effectively discriminated between the severity level of menstrual pain, regardless of phases of the menstrual cycle (Fig. 4; Table 1). These ratios were also consistently and positively correlated with NPRS over the two different menstrual cycles (Table 1),

suggesting that these ratios are reliable markers for differentiating between the severity level of menstrual pain at various menstrual cycle stages.

BCAA (leucine, isoleucine, and valine) levels were significantly lower in the severe pain group than in the mild pain group. BCAAs are essential for muscle protein synthesis, and their blood levels correlate with muscle mass and strength[14,15]. Higher blood levels of BCAAs are associated with greater muscle strength and mass[16,17]. In addition, during periods of exercise or fasting, muscles release BCAAs into the bloodstream, which over time can cause muscle breakdown and reduce total body muscle mass[18]. This suggests that individuals in the severe pain group may have reduced muscle mass compared to those in the mild pain group and that severe menstrual pain may create a metabolic environment conducive to muscle breakdown.

4-Hydroxyproline was also significantly reduced in the severe pain group. This amino acid is mainly found in collagen and is released into the blood during collagen breakdown and resynthesis in bone remodelling and repair[19,20]. It serves as a biomarker for increased bone resorption in conditions such as osteoporosis or connective tissue breakdown in osteoarthritis[21]. These findings suggested that the severe pain group may have increased bone resorption and lower bone mass than the mild pain group.

A significant proportion of the body consists of muscle, bone, and connective tissue, with muscle accounting for approximately 40% of body weight, bone 15%, and connective tissue (e.g. skin, tendons, and ligaments) less than 10%[22–24]. BCAAs and 4-hydroxyproline levels are significantly higher in collagen-rich tissues, including muscle, bone, and connective tissue[25,26]. The observed changes in these metabolites may reflect disrupted metabolic turnover in these tissues due to the severity of menstrual pain. However, as muscle and bone mass were not measured in this study, future research should include these parameters to further elucidate the relationships between tissue composition and menstrual pain severity.

The levels of PI(36:2), PI(38:3), PI(38:4), and DAG(34:3) were significantly increased in the severe pain group compared to those in the mild pain group. The total level of PI was also higher in the severe pain group (Supplementary Fig. 6). PI is synthesised in the Golgi apparatus and endoplasmic reticulum and is released into the circulation via cellular secretion and degradation. However, the mechanisms of PI synthesis, release, and transport remain unclear. Metabolic abnormalities of PI are associated with several diseases, including cancer and diabetes[27–30], suggesting its potential as a biomarker for pathological conditions[31,32]. To better understand the biological implications of PI fluctuations, including their role in menstrual pain, further research is needed into the mechanisms of PI synthesis, release, and transport and physiological significance of different PI molecular forms.

This study has certain limitations. In this study, the ratio of PI(36:2) to each BCAA or total BCAA effectively discriminated between the severity of menstrual pain. However, further research is necessary to elucidate the potential interactions and biological relationships between the numerator and denominator of these ratios in blood, in order to justify their use for providing an evidence-based framework for pain diagnosis and management. Moreover, when the false discovery rate correction was performed on the data set for the first menstrual cycle utilized for PLS-DA model construction (Figs. 2 and 3), statistical significance was lost. The small sample size and single-centre design may have affected the generalisability of the findings. In addition, a lack of data on muscle and bone mass, which are crucial for understanding the underlying mechanisms, limited our ability to draw causal inferences. Nevertheless, we hypothesise that using a larger sample size and more diverse cohorts, including data on muscle and bone composition, could overcome the limitations of the current study and provide a clearer understanding of the causal relationships.

To our knowledge, this is the first study to evaluate lipid mediators, steroid hormones, lipids, and hydrophilic metabolites comprehensively and to identify metabolite ratios with high accuracy in discriminating between menstrual pain severity levels.

Materials and methods

Subjects and study design

This study was conducted under the approval of the Lion Corporation Clinical Review Committee (Clinical Review No. 329). Twenty healthy female employees of our company, aged 25–39 years, were enrolled. The reason for not recruiting general participants was to ensure that blood samples were collected during the three phases of the menstrual cycle (follicular, luteal, and menstrual phases). It was anticipated that it would be difficult to ensure that general participants would be able to visit the study site during each phase based on basal body temperature levels. In this study, we explained to the participants the prohibitions and restrictions on the use of medications, foods, and supplements, such as hormonal contraceptives, which could affect the evaluation from the study start date until the end of the study and ensured their compliance before collecting the specimens. Details of the recruitment conditions, and prohibitions and restrictions for participants in this study are provided in the Supplementary Information (Supplementary Table 1). The participants provided finger-prick blood during the follicular, luteal, and menstrual phases of their menstrual cycle. Basal thermometers were distributed to the participants, and the timing of blood collection was based on the changes in the values. In addition, to minimise the effect of the circadian rhythm of metabolites[33,34], blood collection time was between 9 and 11 am. Participant demographics were recorded using a questionnaire, namely, age, BMI, and regions of menstrual pain. Details of recorded participant information are provided in (Supplementary Table 2).

Phenotyping of menstrual pain

The severity of menstrual pain in each participant was assessed using the NPRS[35], and participants were grouped into two phenotypes[36,37]: NPRS < 4 as the mild pain group ($N = 12$) and NPRS ≧ 4 as the severe pain group ($N = 8$) (Supplementary Table 2). During the study period, participants were instructed to abstain from non-steroidal anti-inflammatory drugs and alcohol the day before sample collection to minimise potential effects on phenotypes. Details of the restrictions imposed on participants are provided in (Supplementary Table 1).

Collection of finger-prick blood

The participants pricked their fingertips with a lancet needle (21G) and collected blood in a collection container (EDTA-2 K) at the designated sample collection site at the Lion Corporation. The blood was mixed by inverting the collection container. We received collection containers through a blind curtain to ensure the privacy of the participants and obtained the plasma using centrifugation (4 °C, 800 × *g*, 5 min)[38]. The collected plasma was divided into a 30-µL aliquot for the measurement of steroid hormones and lipid mediators and a 5 µL aliquot for the measurement of lipids and hydrophilic metabolites. It was then frozen in liquid nitrogen and stored at −80 °C until analysis.

Sample preparation for metabolomic study

For the measurement of steroid hormone and lipid mediator levels, plasma (30 µL) was supplemented with methanol (MeOH) (340 µL) containing 0.1% v/v formic acid and a mixed solution of internal standards (10 µL) (IS; Supplementary Table 7) and mixed thoroughly, followed by centrifugation (15,300 × *g*, 10 min, 4 °C). The supernatant was diluted with 0.1% v/v formic acid-water (1 mL) and loaded onto a preconditioned solid phase extraction 96-well plate (Strata-X, 10 mg, 2 mL, Phenomenex Inc., Torrance, CA, USA). It was then washed with 0.1% v/v formic acid-water (1 mL) and then with 0.1% v/v formic acid/15% v/v ethanol (1 mL). Lipids were eluted with MeOH (200 µL) and then with 2-propanol (200 µL). The eluent was dried with nitrogen gas and reconstituted in MeOH (40 µL).

For the measurement of abundant lipids and hydrophilic metabolites, MeOH (382 µL) containing IS (Supplementary Table 8) (for measurement of hydrophilic metabolites) and chloroform ($CHCl_3$; 35 µL) containing IS (Supplementary Table 9) (for measurement of abundant lipids) were added to plasma (5 µL) and mixed thoroughly. $CHCl_3$ (383 µL) and water (155 µL) were added and mixed thoroughly, followed by centrifugation (10,000 × *g*, 4 °C, 3 min). The aqueous layer was used for the measurement of hydrophilic metabolites, and the organic layer for abundant lipids. For abundant lipids, the total amount was dried at 4 °C using a centrifugal concentrator. $CHCl_3$/MeOH (1:2, v/v; 200 µL) and water (13 µL) were added, mixed thoroughly, and incubated at approximately 25 °C for 5 min. Centrifugation (9,100 × *g*, 5 min, 4 °C) was performed, and the entire supernatant was dried at 4 °C using a centrifugal concentrator and reconstituted in $CHCl_3$/MeOH (1:1, v/v; 35 µL). For hydrophilic metabolites, the aqueous layer (300 µL) was filtered through a 5-kDa cut-off filter, dried at 30 °C using a centrifugal concentrator, and reconstituted with $CHCl_3$/MeOH (1:1, v/v; 50 µL).

LC-QqQ for steroid hormones

The LC/MS system consisted of two LC-30AD pumps, an SIL-30AC auto-sampler, a CTO-20 A column oven, a CBM-20 A system controller, and a triple quadrupole mass spectrometer LCMS-8060 (Shimadzu Corp., Kyoto, Japan). A core-shell column (Kinetex Biphenyl, 2.1 × 150 mm, 2.6 µm, Phenomenex Inc., Torrance, CA, USA) was used for chromatographic separation. For mobile phases A and B, 0.15 mM ammonium fluoride in water and MeOH were used, respectively. The flow rate was set at 0.4 mL/min. The column oven temperature was set at 30 °C. The gradient of mobile phase B concentration was programmed as 50% (0 min) – 60% (3 min) – 90% (8 min) – 95% (10 min) – 95% (12 min) – 50% (12.1 min) – 50% (17 min). The sample injection volume was 10 µL, co-injected with 15 µL of water. The MS parameters were set as follows: nitrogen gas used as a nebuliser and drying gas were set at 3 L/min and 10 L/min, respectively. Argon gas (purity, > 99.9995%) was used for collision-induced dissociation. Heat block and desolvation line temperatures were set at 500 °C and 150 °C, respectively. Scheduled multiple reaction monitoring (SRM) transitions set in the method with continuous ionisation polarity switching are shown in (Supplementary Table 10).

LC-QqQ for lipid mediators

This method was based on the ver. 2.0 package for lipid mediators provided by Shimadzu Corp. The LC/MS system was the same as those used for measuring the levels of steroid hormones. The analysis conditions were as previously described[39].

LC-HRMS for lipids

The LC/MS system consisted of an ACQUITY UPLC system I-Class (Waters Corp., Milford, MA, USA) equipped with a binary solvent delivery system, an auto-sampler, and a quadrupole-Orbitrap mass spectrometer Q-Exactive Plus (Thermo Fisher Scientific Inc., Waltham, MA, USA). The LC separation conditions were as previously described[40].The MS parameters fitted with a heated electrospray ionisation source were set as follows: spray voltage at + 3,000 V, sheath gas at 50, auxiliary gas at 20, sweep gas at 1, ion transfer tube temperature at 250 °C, and vaporiser temperature at 300 °C (positive) and 150 °C (negative). The ion transfer parameters were set as follows: mass range at "normal" and S-lens RF level at 45%. Scan parameters applied were as follows: acquisition time from 1 to 30 min, micro scan at "1", data type at "profile", and AGC target at 3×10^6. Data were acquired with dependent MS/MS acquisition in positive ionization mode (m/z 250–1200) and negative ionization mode (m/z 250–1200). Full scan spectra and fragment spectra used a resolution of 70,000 and 17,500, respectively.

IC-HRMS for anionic hydrophilic metabolites

The IC/MS system consisted of a Dionex ICS-500 + system (Thermo Fisher Scientific, Inc.) equipped with a Q-Exactive Plus. The analysis conditions were as previously described[41].

LC-QqQ for cationic hydrophilic metabolites

This method was referenced to the method package ver. 2.0 for primary metabolites provided by Shimadzu Corp., and SRM transitions for[15] N-labeled amino acids were added as additional internal standards. The LC/MS

system was the same as those used for measuring the levels of steroid hormones and lipid mediators. The analysis conditions used were as previously described[42]. The sample injection volumes were 0.3 μL for high-abundant metabolites and 3 μL for low-abundant metabolites.

Data processing and multivariate statistical analysis for metabolomic study

Peak detection and retention time (RT) correction for the LC-QqQ-MS/MS data (.lcd) were performed using LabSolutions software (ver. 5.109, Shimadzu Corp.). For some metabolites, the area values were corrected manually. The missing values were not specifically treated in any of the data analyses. The relative area (peak area/IS) values were calculated by dividing the obtained raw area values directly by those of the corresponding IS (Supplementary Tables 7, 8). Peak detection and RT correction for the LC or IC-HRMS/MS data (.RAW) were processed using Xcalibur software (ver. 4.2, Thermo Fisher Scientific, Inc.) and TraceFinder software (ver. 4.1, Thermo Fisher Scientific, Inc.). The identification of lipids was achieved by analysing the RT information for each lipid molecule predicted from the RT of the internal standard (Supplementary Table 9) and the accurate m/z values of the precursor and fragment ions that are characteristic of each lipid. In contrast, the identification of anionic hydrophilic metabolites was achieved by the analysis of RT information, which had been previously confirmed by our standard mixture, and the accurate m/z values of the precursor ions of each anionic hydrophilic metabolite. Peaks were automatically detected from scan spectra using our master method, which included information on the accurate *m/z* and predicted RT. The genesis algorithm was used for detection (nearest RT, minimum peak height (Signal-to-Noise; $S/N = 3$) and integration (smoothing point = 5, S/N threshold = 1, constrain peak width = no). The peak area/IS values were calculated by dividing the obtained raw area values directly by those of the corresponding IS (Supplementary Tables 8, 9). The missing values were not specifically treated in any of the data analyses. Multivariate statistical analysis was performed using MetaboAnalyst (ver. 5.0, McGill University, Canada). The data sets were auto-scaled (mean-centred and divided by the standard deviation of each variable). PCA, PLS-DA, volcano plot analysis, and ROC analysis were performed (Figs. 2, 3 and 4, and Supplementary Fig. 1). Significant differences were determined using Welch's t-test (unpaired, two-tailed) (Fig. 1a) performed by Microsoft Excel 2013, Steel-Dwass test performed by R (ver. 4.0.4) using the pSDCFlig function in the NSM3 library (ver. 1.16) (Fig. 2a), and Wilcoxon rank-sum test (Fig. 3b and Supplementary Fig. 6) performed by MetaboAnalyst. A p-value of < 0.05 was considered statistically significant. Pearson correlation analysis was also performed using Microsoft Excel 2013 (Fig. 4 and Table 1).

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

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Author contributions
A.S., K.Y., Y.I., and Y.K. conceived and designed the research. A.S. and K.Y. conducted experiments. A.S., Y.S., and K.Y. analysed data and interpreted the results. A.S. and Y.S. wrote the manuscript. All authors read and approved the final version of the manuscript. This research was supported by the Japan Agency for Medical Research and Development (AMED) under Grant Number JP24zf0127003, JP24zf0127007, JP24gm2010001, JPMJCR24T6 and JP24gm1210009 to Y.S..

Declarations

Competing interests
The authors declare no competing interests.

Ethical approval
This study was conducted in accordance with the Declaration of Helsinki 1964, and the study protocol was approved by the local ethics committee (Approval No. 329) at the Lion Corporation, Tokyo, Japan.

Informed consent
All participants in this study provided informed consent. All participants in this study are employees of the Lion Corporation. When obtaining consent to participate in this study, the authors explained that they could withdraw from this study at any time and for any reason, and that they would not be treated unfavourably if they withdrew their consent. Furthermore, a personal information manager was assigned separately from the authors to handle only the various types of anonymized data to fully protect the privacy of the participant.

Additional information
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