


# Studies on CYP3A activity during the menstrual cycle as measured by urinary $6\beta$ -hydroxycortisol/cortisol

Helena Bergström<sup>1</sup>  | Anna Lindahl<sup>2,3</sup> | Anna Warnqvist<sup>4</sup> | Ulf Diczfalusy<sup>2,3</sup> | Lena Ekström<sup>5</sup> | Linda Björkhem-Bergman<sup>1,6</sup>

<sup>1</sup>Department of Neurobiology, Care Sciences and Society (NVS), Division of Clinical Geriatrics, Karolinska Institutet, Huddinge, Sweden

<sup>2</sup>Department of Laboratory Medicine, Division of Clinical Chemistry, Karolinska Institutet, Stockholm, Sweden

<sup>3</sup>Department of Clinical Chemistry, Karolinska University Laboratory, Karolinska University Hospital, Stockholm, Sweden

<sup>4</sup>Department of Environmental Medicine, Division of Biostatistics, Karolinska Institutet, Stockholm, Sweden

<sup>5</sup>Department of Laboratory Medicine, Division of Clinical Pharmacology, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden

<sup>6</sup>Department of Palliative Medicine, Stockholms Sjukhem, Stockholm, Sweden

## Correspondence

Helena Bergström, Department of Neurobiology, Care Sciences and Society (NVS), Division of Clinical Geriatrics, Karolinska Institutet, Blickagången 16, Neo floor 7, SE-141 83 Huddinge, Sweden. Email: helena.bergstrom.1@ki.se

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## Abstract

The  $6\beta$ -OH-cortisol/cortisol ratio ( $6\beta$ -OHC/C) in urine is an endogenous marker of drug-metabolizing enzyme cytochrome P450 3A (CYP3A). The primary aim of this single center, prospective, non-interventional cohort study, was to investigate the variability of  $6\beta$ -OHC/C during the menstrual cycle. In addition, possible associations between the CYP3A activity and sex hormones, gut microbiota metabolite trimethylamine-N-Oxide (TMAO) and microRNA-27b, respectively, were investigated. Serum and urinary samples from healthy, regularly menstruating women followed for two menstrual cycles were analyzed. Twenty-six complete menstrual cycles including follicular, ovulatory, and luteal phase were defined based on hormone analyses in serum.  $6\beta$ -OHC/C were analyzed in urine and sex hormones, TMAO and miRNA-27b were analyzed in serum at the same time points.  $6\beta$ -OHC/C did not vary between the follicular, ovulatory, or luteal phases. There was a difference in the relative miRNA-27b expression between the follicular and ovulatory phase ( $p = .03$ ). A significant association was found between  $6\beta$ -OHC/C and progesterone during the follicular ( $p = .005$ ) and ovulatory ( $p = .01$ ) phases ( $n = 26$  for each phase). In addition, a significant association was found between the ratio and TMAO during the ovulatory ( $p = .02$ ) and luteal ( $p = .002$ ) phases.  $6\beta$ -OHC/C and gut microbiota TMAO were significantly associated ( $p = .003$ ) when evaluating all values, for all phases ( $n = 78$ ). Interestingly, the finding of an association between  $6\beta$ -OHC/C in urine and levels of TMAO in serum suggest that gut microbiota may affect CYP3A activity.

## KEYWORDS

$6\beta$ -hydroxycortisol/cortisol, CYP3A activity, menstrual cycle, miRNAs, TMAO

**Abbreviations:**  $6\beta$ -OHC/C,  $6\beta$ -hydroxycortisol to cortisol; ABP, Athlete Biological Passport; CYP3A, cytochrome P450 3A4; FSH, follicular stimulating hormone; LH, luteinizing hormone; miRNA, microRNA; TMAO, trimethylamine-N-Oxide.

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## 1 | INTRODUCTION

Cytochrome P450 3A (CYP3A) is the major drug-metabolizing enzyme in the liver and the gut, metabolizing more than 50% of all drugs. CYP3A also participate in the metabolism of endogenous steroids and xenobiotics. Factors like age, ethnicity, diet, inflammatory disease, and alcohol intake influence the CYP3A activity.<sup>1-3</sup> In addition, the expression of CYP3A is tissue- and sex specific, and it has been shown that women have higher levels of CYP3A mRNA and protein in the liver compared to men.<sup>4,5</sup>

Phenotyping of CYP3A activity using midazolam clearance in plasma is the golden standard for studies using an exogenous marker.<sup>6</sup> However, there is a currently a lack of consensus concerning sex differences in CYP3A activity using exogenous probes.<sup>7</sup>

The menstrual cycle variability of midazolam has been explored in three studies. However, while one study of 1-hydroxylation of midazolam showed an increase during the follicular phase, two other studies of midazolam clearance did not demonstrate any differences between phases.<sup>8-10</sup> Regarding investigations of potential menstrual cycle variability in endogenous markers of CYP3A, studies are sparse and the results are difficult to interpret due to methodological issues.<sup>9-14</sup> One of the endogenous markers used to detect CYP3A activity is the 6 $\beta$ -OH-cortisol/cortisol (6 $\beta$ -OHC/C) ratio in urine since cortisol is a substrate for CYP3A.<sup>15</sup> The advantage with 6 $\beta$ -OHC/C is its short half-life that can detect rapid changes in CYP3A activity.<sup>16</sup> In contrast, another endogenous marker, 4 $\beta$ -OH-cholesterol/cholesterol ratio (4 $\beta$ -OHC/C) measured in plasma, has a long half-life of more than 2 weeks and reflects CYP3A activity over longer time period.<sup>17,18</sup> Thus, this marker is less suitable for detecting short-term changes in CYP3A activity.

In one study exploring menstrual cycle variability of 6 $\beta$ -OHC/C, cycle phase was determined by a single sample of progesterone in blood<sup>19</sup> and in other studies on patient narrative or first day of bleeding.<sup>20-23</sup> At the same time, in a study of plasma 6 $\beta$ -OHC/C in 31 women, menstrual cycle phase was not mentioned.<sup>24</sup> Finally, few studies have evaluated urine 6 $\beta$ -OHC/C variability in the basal state, as most studies were performed during induction or inhibition of CYP3A.<sup>17,25</sup> Thus, the question of variability of 6 $\beta$ -OHC/C during the menstrual cycle phases remains unanswered.

Intestinal microbes, the microbiota, functions like an endocrine organ producing bioactive amines, that may affect host physiology.<sup>26</sup> The most well-known representative, is the metabolite trimethylamine-N-Oxide (TMAO), that has been shown to be strongly associated with cardiovascular disease.<sup>27</sup> In addition, it has been suggested that microbiota influence the CYP3A activity in the gut<sup>28</sup> and the liver.<sup>29</sup> Microbiota may affect the homeostasis between primary and secondary bile acids, and in this manner, nuclear receptors regulating CYP3A expression are affected.<sup>28,29</sup> To our knowledge, the association between TMAO and CYP3A activity assessed as 6 $\beta$ -OHC/C has not been studied before.

MicroRNAs are small, noncoding RNAs that, among other things, have been shown to modulate the expression of drug-metabolizing enzymes.<sup>1</sup> Micro RNA-27b (miRNA-27b) has been suggested to

negatively regulate CYP3A mRNA- and protein expression in vitro.<sup>30</sup> This finding has been confirmed in a small clinical study where miRNA-27b levels in serum correlated with CYP3A activity in serum.<sup>31</sup> Furthermore, this association was also found in the liver, however, not in the same individuals. To our knowledge, possible variation of miRNA-27b has not been studied during menstrual cycle before.

6 $\beta$ -OHC/C in urine is not yet approved for formal drug-drug interaction (DDI) studies.<sup>32</sup> However, with the aim of increasing the number of DDI studies performed in vulnerable populations like fertile women of childbearing age, there is a need for more information on this endogenous marker.

Thus, the primary objective of this pilot study was to test the hypothesis that there is a variability of CYP3A activity during the menstrual cycle that could be detected by 6 $\beta$ -OHC/C using repeated single-spot urine samples during two menstrual cycles in healthy volunteers. The secondary objectives were to study possible factors that may affect the CYP3A activity during the menstrual cycle including levels of sex hormones, the microbe metabolite TMAO and miRNA-27b.

## 2 | MATERIALS AND METHODS

### 2.1 | Description of study population and study design

The power calculation of 30 subjects in this study, was based on the number of subjects needed to show an association between menstrual cycle phases and urinary epitestosterone used in the Athlete Biological Passport (ABP). As data on intraindividual variability of 6 $\beta$ -OHC/C for CYP3A in women have been inconsistent, an accurate power calculation based on this endpoint was not feasible. Most previous studies on menstrual cycle variability using midazolam as a probe, have included seven to at the most 16 subjects, and there are no studies on 6 $\beta$ -OHC/C and menstrual cycle variability, thus 30 seemed an appropriate number.<sup>9,10,12,13</sup> This evaluation was strengthened by the longitudinal study design, with repeated measurements for 6–8 weeks in the same subjects.

Unfortunately, we were not able to recruit more than 19 subjects, and as two subjects (11 and 19) withdrew due to lack of time to participate, the final number of subjects was 17. The study was conducted at the outpatient clinic at the Department of Clinical Pharmacology, Karolinska Hospital, Stockholm, Sweden. Clinical trial registration was not performed as this was a small, observational study.

Thus, in this single center, prospective, non-interventional pilot study, 17 healthy regularly menstruating women aged 18–45 years, were followed during two consecutive menstrual cycles. The mean age was 33.2 years, BMI 21.6, bleeding 4.9 days, and cycle length 26.9 days. In addition, the mean number of blood samples were 7.3. Further information on study cohort demographics and a figure of study design, can be found in the articles describing the study

results for the ABP by Mullen et al. and Schulze et al.<sup>33,34</sup> Results on paired lipid and TMAO analyses can be found in an article by Bergström et al.<sup>35</sup>

Morning urine spot samples were collected three times weekly and blood samples were collected once a week. Urine samples to be analyzed for steroids included in the ABP, were treated separately. The aim was that urine samples to be analyzed for  $6\beta$ -OHC/C in our part of the study, were samples collected on the same day as blood sampling of hormones once a week. Menstrual cycle phase for each blood sample was determined using an algorithm.<sup>36</sup> A selection of six urinary samples for each subject, two for each menstrual cycle phase (follicular, ovulatory, and luteal), were to be analyzed for  $6\beta$ -OH-cortisol and cortisol. Additionally, for two subjects (subject 5 and 19) with long menstrual cycles based on narrative, all urine samples collected three times a week, were to be analyzed for  $6\beta$ -OHC/C to evaluate the intraindividual variability with a higher number of samples. Blood samples were taken once a week also for these subjects.

Serum samples for miRNA extraction were selected to represent one menstrual cycle including the three phases per subject. Finally, urine and serum samples were either analyzed the same day as sampling, or frozen and stored at  $-70$  until analysis.

## 2.2 | Analysis of sex hormones, safety parameters, and TMAO

Estrogen, progesterone, follicle stimulating hormone (FSH), luteinizing hormone (LH), and routine safety parameters were analyzed in the subjects. Methods for the analyses are described in the cited articles.<sup>33-35</sup> Analysis of TMAO in human plasma using LC-MS/MS, was performed at the Swedish Metabolomic Centre in Umeå. Validation of the method is described in the article by Missiliadis et al.<sup>37</sup>

## 2.3 | Analysis of cortisol and $6\beta$ -hydroxycortisol using LC-MS/MS

$6\beta$ -hydroxycortisol, cortisol, and [ $^2\text{H}_4$ ]cortisol were acquired from Sigma-Aldrich Sweden AB, Stockholm, Sweden. [ $^2\text{H}_4$ ] $6\beta$ -hydroxycortisol was purchased from Santa Cruz Biotechnology, Dallas, United States.

$6\beta$ -hydroxycortisol and cortisol in urine were quantified according to a previously described and validated method.<sup>38</sup> In brief, sample preparation was performed using a Hamilton Microlab STARlet automated liquid handler (Hamilton Company). Urine, quality control samples, and calibrators were diluted 1:1 v/v with water containing internal standards ([ $^2\text{H}_4$ ] $6\beta$ -hydroxycortisol, 1  $\mu\text{mol/L}$ , [ $^2\text{H}_4$ ]cortisol, 0.4  $\mu\text{mol/L}$ ). Samples (200  $\mu\text{l}$ ) were then applied to an Oasis HLB  $\mu\text{Elution}$  96-well solid phase extraction plate (Waters). Following a wash with 200  $\mu\text{l}$  5% methanol in water, analytes were eluted with 100  $\mu\text{l}$  acetonitrile/isopropanol 2:3 v/v followed by dilution with water (400  $\mu\text{l}$ ). Levels of  $6\beta$ -hydroxycortisol and cortisol were

determined using a Xevo TQ MS with an electrospray ionization source, interfaced with an Acquity Classic UPLC system (Waters). Samples (5  $\mu\text{l}$ ) were separated on an Acquity UPLC BEH Shield RP18 (1.7  $\mu\text{m}$ , 2.1  $\times$  150 mm) column (Waters) held at 55°C, using a linear gradient with 0.2% formic acid (mobile phase A) and acetonitrile (mobile phase B). The gradient starting conditions were 5% B at a flow rate of 0.4 ml/min, which were ramped up to 50% B over a period of 6 min and then increased to 95% B over 0.5 min. 95% B was then maintained for 1 min before re-equilibration at 5% B for 3 min. The total run time was 12 min per sample. MS/MS data were acquired in negative mode for the detection of  $6\beta$ -hydroxycortisol, and positive mode for the detection of cortisol, in the same run. Two MRM transitions, one quantifier-, and one qualifier transition, were set up for each analyte: 347  $\rightarrow$  313 and 347  $\rightarrow$  125 for  $6\beta$ -hydroxycortisol and 363  $\rightarrow$  121 and 363  $\rightarrow$  97 for cortisol. Peak integration, calibration, and quantification was performed using the software TargetLynx (Waters).

## 2.4 | Analysis of miRNA-27b

Micro RNA was extracted from serum samples using miRNAs Serum/Plasma Advanced Kit (Qiagen). The microRNA samples were then subject to cDNA conversion using Thermo Fisher cDNA TaqMan™ Advanced miRNA cDNA Synthesis Kit and the cDNA were diluted in accordance with the protocol. The diluted cDNA was used as template in premade TaqMan assays targeting miR27b (assay IDs 478270\_mir, Life Technology) using TaqMan™ Fast Advanced Master Mix. The real-time PCR were conducted on StepOne. To control for unwanted sources of variation, the relative miRNA expression was calculated with the ddCT formula<sup>39</sup> using miRNA-26b expression (assay ID 478418-mir) as a control as recommended by the provider. This method has previously been described and evaluated by the authors.<sup>31</sup>

## 2.5 | Statistical analysis

D'Agostino and Pearson normality test was conducted to test for Gaussian distribution of data and arithmetic means. Median, maximum, minimum, and interquartile range (IQR) were calculated for all variables. None of the variables had Gaussian distribution.

As some subjects had  $6\beta$ -OHC/C values from two menstrual cycles and some only one, it was necessary to use a statistical analysis considering the intraindividual correlations caused by repeated measurements. Linear regression using clustered robust covariance estimator was therefore used in all linear regression analyses. To evaluate if  $6\beta$ -OHC/C differed between the three menstrual phases using linear regression, data were subset to include two phases at the time. Possible associations between urine  $6\beta$ -OHC/C and estrogen, progesterone, follicle stimulating hormone (FSH), luteinizing hormone (LH), and TMAO were also assessed using linear regression.

The interindividual differences in  $6\beta$ -OHC/C were evaluated using Kruskal–Wallis test. For the intraindividual variability, coefficient of variation (CV), IQR, and median in the ratio in each subject were calculated. For all subjects, mean ratio and CV were calculated.

As the individuals contributed with only one value for each phase for miRNA-27b, the variability of miRNA between the menstrual phases was evaluated with Wilcoxon sign-rank test. The association between  $6\beta$ -OHC/C and miRNA ( $n = 27$ ) was assessed with Spearman's rank-order test, for all values only and not for each phase, due to the small sample size.

Analyzes were performed in Stata 15 (2017 Stata Statistical Software: StataCorp LP). A value of  $p < .05$  was considered statistically significant.

### 3 | RESULTS

To confirm known variations in sex hormones and gonadotropins during the menstrual cycle, levels of estrogen and progesterone as well as FSH and LH were controlled (Figure 1). For estrogen, there were significant differences between all phases, whereas

progesterone was significantly higher in the luteal phase while FSH was low. As expected, there was a peak in LH during the ovulatory phase.<sup>35</sup>

#### 3.1 | $6\beta$ -OH-cortisol/cortisol samples

Thirteen subjects (subject 5 and 19 included) contributed with six urinary samples of  $6\beta$ -OHC/C values and four subjects had three, that is one cycle. Thus, the total number of urinary samples of  $6\beta$ -OHC/C was  $n = 90$  for evaluating intra- and interindividual variability. For the evaluation of intraindividual variability with additional samples, subject 5 contributed with 23 urine samples and 19 with 20 samples.

A total of 78 urine samples could be matched with blood samples analyzed for sex hormones and TMAO. Eleven subjects had six  $6\beta$ -OHC/C urinary samples, with matching blood samples ( $n = 66$ ), while three subjects had three urine samples with matching blood samples ( $n = 12$ ). The reason for this discordance, was failure of blood sampling capturing all three phases or that there were urine samples from one menstrual cycle only, that were analyzed. In addition, analyzed urine samples that were taken  $>2$  days before or after the blood sampling,

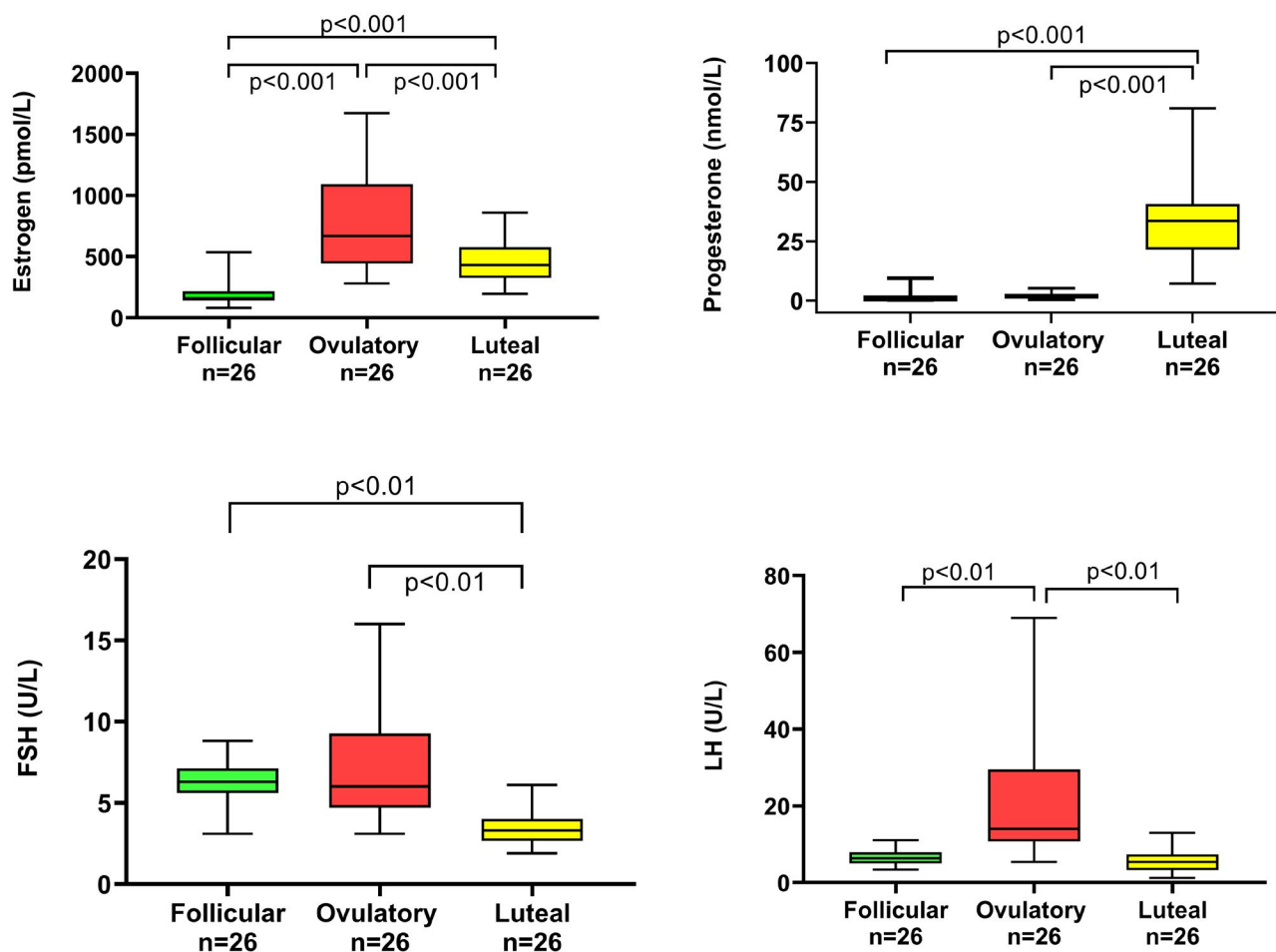


FIGURE 1 Variations in estrogen, progesterone, follicle stimulating hormone (FSH), and luteinizing hormone (LH) during 26 menstrual cycles from 17 healthy volunteers ( $n = 78$ )

were excluded. The selection of what urine samples to analyze were based on what menstrual phase the subject most likely would be in, based on patient narrative on menstrual cycle and visits.

Thus, altogether 78 urine and blood samples from 26 menstrual cycles ( $78/3 = 26$ ), were used to evaluate differences in  $6\beta$ -OHC/C over the menstrual phases and the associations between  $6\beta$ -OHC/C and sex hormones and TMAO, respectively.

### 3.2 | $6\beta$ -OH-cortisol/cortisol variability

There were significant interindividual differences in the ratio ( $n = 90$ ,  $p = .002^{**}$ ), as evaluated by Kruskal-Wallis test. The intraindividual variability of  $6\beta$ -OHC/C was substantial (Figure 2), with mean coefficient of variation (CV) 42%, mean ratio 12 and interquartile range (IQR) 7.43 to 15.17 for all subjects ( $n = 90$ ) (Table S1). For subject 5 and 19, the median in  $6\beta$ -OHC/C was 16.93 and 14.63, respectively (Figure S2). CV in the ratio was 33% for subject 5, and 51% for subject 19.

There were no significant differences in  $6\beta$ -OHC/C ( $n = 78$ ) between the follicular, ovulatory, and luteal phases (Figure 3) and no significant difference in the CV between the menstrual cycle phases.

### 3.3 | $6\beta$ -OH-cortisol/cortisol and associations

Comparing the relationship between  $6\beta$ -OHC/C and sex hormones and gonadotropins during the menstrual phases ( $n = 26$  for each phase), there was a significant association between the ratio and progesterone during the follicular ( $p = .005$ ) and ovulatory ( $p = .01$ ) phase (Figure 4).

Similarly, an association was shown for  $6\beta$ -OHC/C and TMAO during the follicular ( $p = .063$  ns), ovulatory ( $p = .023$ ), and luteal ( $p = .002$ ) phase (Figure 5). We have previously shown that there was no menstrual cycle variability in TMAO.<sup>35</sup>

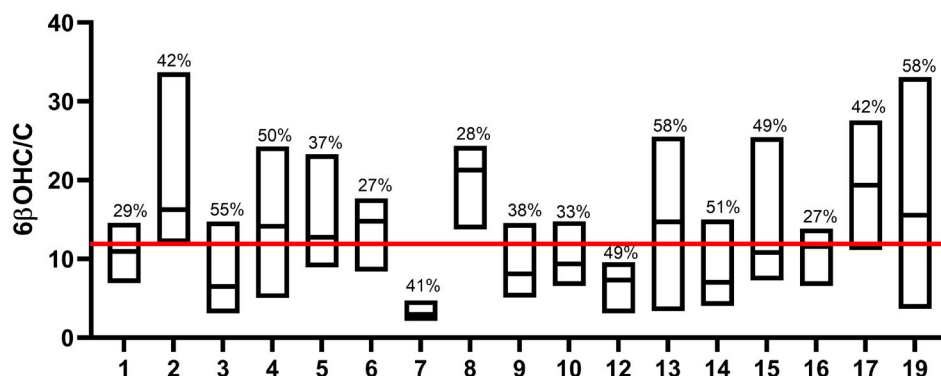
Furthermore, there were no significant associations between  $6\beta$ -OHC/C and estrogen, progesterone or FSH when comparing all values, all phases ( $n = 78$ ). For  $6\beta$ -OHC/C and LH, a border significant association ( $p = .055$ ) was found (Figure S1). In addition, there was a significant association ( $p = .003$ ) between  $6\beta$ -OHC/C and TMAO for all values, all phases (Figure 6).

### 3.4 | miRNA-27b variability and association

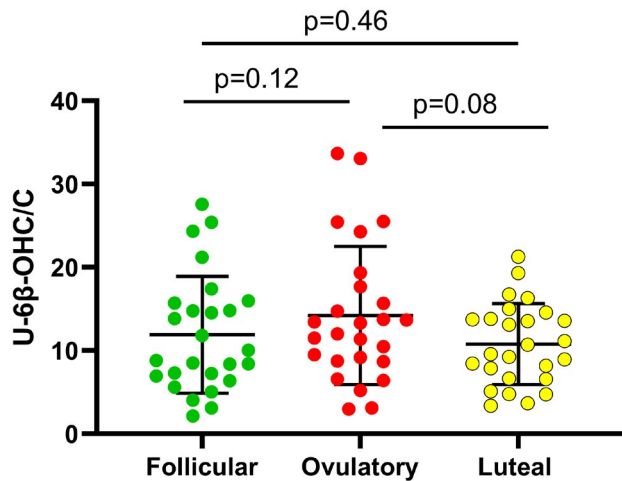
The aim was to extract miRNA from one complete menstrual cycle including the three phases from all 17 subjects ( $n = 51$ ). However, miRNA-27b was only successfully extracted from 39 samples. For four subjects there were not enough serum samples available, and for three participants only two phases were present, and therefore excluded from the statistical analysis. Thus, nine complete menstrual cycles from nine subjects ( $n = 27$ ) were included in the final analysis. There was a significant difference ( $p = .03$ ) between the follicular and ovulatory phase in the relative miRNA-27b expression (Figure 7). When evaluating the association between  $6\beta$ -OHC/C and miRNA-b27 ( $n = 27$ ) all phases, there was no correlation ( $p = .51$ ). Finally, there was no correlation between miRNA27b and all values, all phases of estrogen ( $p = .96$ ), progesterone ( $p = .96$ ), FSH ( $p = .34$ ), LH ( $p = .92$ ), or TMAO ( $p = .67$ ). The median CV for miRNA-27b was 74.9% in the follicular phase, 105.5% in the ovulatory, and 172.6% in the luteal phase.

## 4 | DISCUSSION

In this pilot study, no significant menstrual cycle variability in the constitutive urinary metabolic ratio of  $6\beta$ -OHC/C was found. The intra- and interindividual variability was in line with previous findings in women.<sup>20,22</sup> There was a significant difference in the relative expression of miRNA-27b between the follicular and ovulatory phase. When evaluating one menstrual phase at the time, there was



**FIGURE 2** The inter- and intraindividual variations in urine  $6\beta$ -OH-cortisol/cortisol ( $6\beta$ -OHC/C) during two menstrual cycles in 17 healthy volunteers ( $n = 90$ ). The interindividual difference in  $6\beta$ -OHC/C was statistically significant ( $p = .002^{*}$ ). Subject 1,2,3,4,5,6,9,10,13,14,15,16, and 19 ( $n = 13$ ) had six analyzed urine samples, while 7,8,12, and 17 ( $n = 4$ ) had three analyzed urine samples. The figure shows the median, interquartile range (IQR), and coefficient of variation (CV) of  $6\beta$ -OHC/C for each subject. The red line shows the mean (12) in  $6\beta$ -OHC/C for all subjects ( $n = 90$ ). \*Tested by Kruskal-Wallis test



6β-OHC/C	
Menstrual phase	Median (IQR)
Follicular n=26	59% (6.82-15.79)
Ovulation n=26	58% (8.73-18.11)
Luteal n=26	45% (6.64-14.02)

**FIGURE 3** Variations in 6β-OH-cortisol/cortisol (6β-OHC/C) during the three phases of the menstrual cycle including 26 menstrual cycles from 17 healthy volunteers ( $n = 78$ ). The coefficient of variations (CV) in 6β-OHC/C during the menstrual cycle phases are shown in the table

a significant association between 6β-OHC/C and progesterone during the follicular and ovulatory phase. Similarly, an association between the ratio and TMAO during the ovulatory and luteal phase was shown. When analyzing all values, from all phases, there was a significant association between 6β-OHC/C and microbiota TMAO and a border significant association with LH.

Compared to previous studies on 6β-OHC/C during the menstrual cycle, the three different menstrual phases are more exactly defined in this study, based on hormone levels in the circulation. In addition, this is the first study evaluating how CYP3A activity in the basal state may be associated with the microbe metabolite TMAO and to miRNA27-b during the menstrual cycle.

#### 4.1 | Differences in 6β-OHC/C related to sex- and menstrual cycle phases

Although it has been suggested that estrogen regulate CYP3A expression in the liver,<sup>40,41</sup> investigations of sex difference in CYP3A activity measured as 6β-OHC/C in urine have shown diverging results.<sup>25</sup> While one study found the ratio to be higher in men,<sup>19</sup> another study found the ratio to be higher in women.<sup>42</sup> However, many studies of 6β-OHC/C have been performed in males only.<sup>43-45</sup>

With regard to effects of the menstrual cycle on the 6β-OHC/C in women, this has been considered in five studies.<sup>19-23</sup>

However, in one study, 4 of 10 subjects were using oral contraceptives or hormone replacement therapy,<sup>22</sup> and in the remaining studies there were no significant menstrual cycle fluctuations. The strength of our study, compared to the previous studies, is the use of urine sampling for two complete menstrual cycles, in parallel with repeated hormone measurements in serum, in the same individuals ( $n = 17$ ). In this manner, the determination of the different phases of the menstrual cycle was made more accurate than in previous studies.

In addition, disparities in methods analyzing cortisol and 6β-OH-cortisol makes it hard to compare values from different studies. Indeed, methods have varied from radioimmune- or enzyme immunoassay (RIA), to HPLC-UV, LC-MS/MS, and GC-MS/MS<sup>25,38,46</sup> Also, both single-spot sample and 24-hour urine collection has been used. Critics argue that the circadian rhythm of cortisol is not captured using spot sampling<sup>47</sup> while others argue that a single morning sample adequately reflect the diurnal variability.<sup>16,22</sup> Finally, the 6β-OHC/C varies with age and sex in the investigated population.<sup>48</sup>

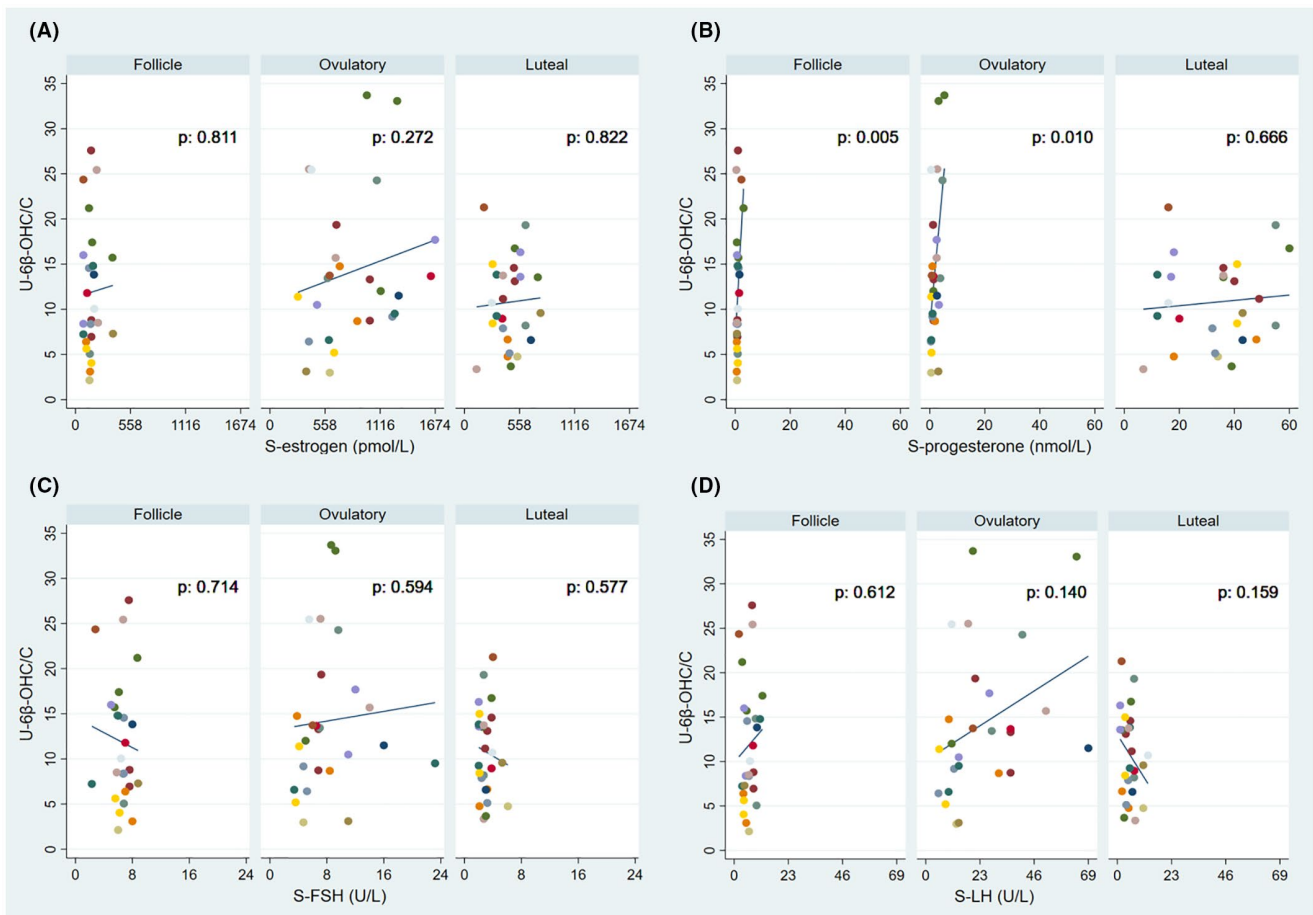
#### 4.2 | 6β-OHC/C and progesterone and LH

Enzyme CYP3A is involved in the 6β-hydroxylation of both cortisol and progesterone, so the association between 6β-OHC/C and progesterone in our study, is not unexpected.<sup>49</sup> Also, LH stimulates the secretion of progesterone and vice versa, and as such they are interrelated.<sup>50</sup> The association between 6β-OHC/C and progesterone was significant during the follicular and ovulatory phases. Progesterone is at its lowest during these phases, but levels rise sharply after the short (12-24 h) ovulation. The lack of an association between the ratio and progesterone during the luteal phase, could be due to changes in CYP3A enzyme activity or to changes in 6β-OHC/C unrelated to CYP3A. In vitro data suggest that progesterone may affect the active, binding site of CYP3A though allosteric modification, and in this manner could change the substrate preference of CYP3A during the luteal phase.<sup>51</sup>

#### 4.3 | 6β-OHC/C and microbiota TMAO

In our study, the association between 6β-OHC/C and TMAO reached significance for the ovulatory and luteal phases, when estrogen is higher compared to the follicular phase (Figure 1). In addition, high levels of TMAO were associated with decreased levels of 6β-OHC/C, indicating decreased CYP3A activity.

The association between 6β-OHC/C and TMAO, suggest that microbiota may affect drug metabolism.<sup>29,52,53</sup> In fact, a reduction in the levels of CYP3A mRNA and protein in the liver was found in a study of germ-free mice lacking intestinal microbiota, when compared to specific pathogen-free mice.<sup>54</sup> Interestingly, sex differences in the effects of the microbiome on mRNA expression of liver CYP3A have been found in mice.<sup>55</sup>



**FIGURE 4** Linear regression analysis between  $6\beta$ -OH-cortisol/cortisol ( $6\beta$ -OHC/C) and (A) estrogen, (B) progesterone, (C) follicle stimulating hormone (FSH), and (D) luteinizing hormone (LH) during the follicular, ovulatory, and luteal phases of the menstrual cycle in 17 healthy women. Each subject is represented by a color, but as 11 subjects contributed with two complete cycles (six  $6\beta$ -OHC/C values) and four subjects with one cycle (three values), some colors are shown twice in each phase. A total of 78 values  $6\beta$ -OHC/C together represent 26 complete menstrual cycles ( $78/3 = 26$ )

The formation of TMAO has been shown to be dependent on estrogen levels.<sup>56</sup> Still, we have previously shown that the levels of TMAO did not vary significantly during the different menstrual phases.<sup>35</sup> Moreover, there was no significant association between TMAO and estrogen, for all phases<sup>35</sup> or during the three phases (unpublished data). Nonetheless, the phase-specific association between TMAO and  $6\beta$ -OHC/C suggest that high levels of estrogen may be involved in the possible effect TMAO has on CYP3A activity.

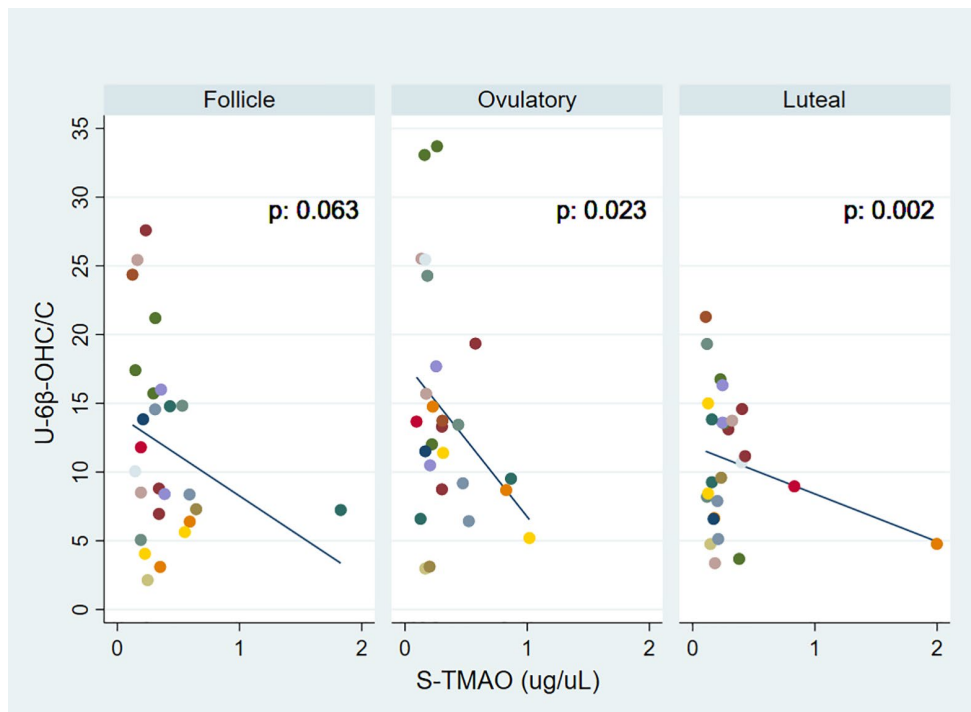
Another possible mechanism, is that secondary bile acids produced by microbiota, act as signaling molecules by activating nuclear receptor Pregnane X receptor (PXR), a known regulator of CYP3A expression in the liver.<sup>53,57</sup> Interestingly, bile acids also activate Farnesoid X receptor (FXR), that regulate levels of TMAO.<sup>57,58</sup>

Finally, the activity in the immune system, has been shown to vary during the menstrual cycle and inflammation has been shown to down-regulate CYP expression in the liver.<sup>59,60</sup> From a theoretical point of view, activation of pro-inflammatory cytokines like TNF- $\alpha$  by TMAO, could modulate the activity of CYP3A, and thus explain the association.<sup>27,59</sup>

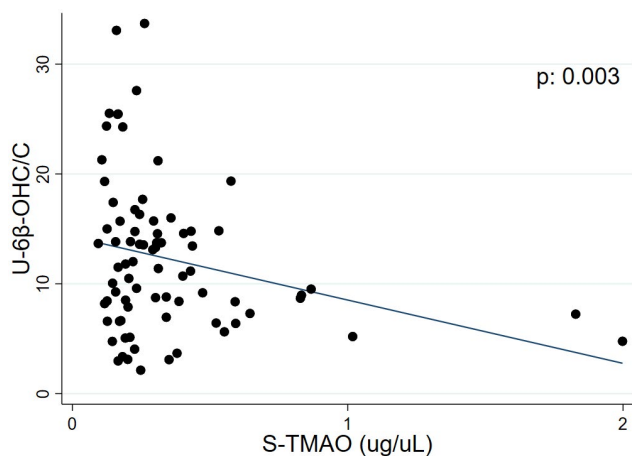
#### 4.4 | miRNA-27b

A significant difference in miRNA-26b between follicular and ovulatory menstrual phase was noted, and this finding should be investigated in larger studies.

In a recent study of 105 male patients with alprazolam treatment due to anxiety disorder, a significant association was found between  $6\beta$ -OHC/C and miRNA-27b.<sup>61</sup> However, there was no association between  $6\beta$ -OHC/C and miRNA-27b in this study. This may be due to differences between the sexes or to the different roles miRNAs may have in biological functions and diseases.<sup>62,63</sup> It is also possible that the sample size was too small to detect differences in this study cohort, that is, a type II error. The reproducibility of miRNA results may also be confounded by the choice of endogenous control.<sup>31</sup> Herein miR-26b was chosen as a control miRNA and was proven to be highly abundant in the serum with stable Ct values not dependent on the menstrual phases (data not shown).



**FIGURE 5** Linear regression analysis between  $6\beta$ -OH-cortisol/cortisol ( $6\beta$ -OHC/C) and the microbiota metabolite trimethylamine-N-Oxid (TMAO) during the follicular, ovulatory, and luteal phases of the menstrual cycle in 17 healthy women. Each subject is represented by a color, but as 11 subjects contributed with two complete cycles (six  $6\beta$ -OHC/C values) and four subjects with only one cycle (three values), some colors are shown twice in each phase. A total of 78 values  $6\beta$ -OHC/C together represent 26 complete menstrual cycles ( $78/3=26$ )



**FIGURE 6** Linear regression analysis between all values, for all phases of  $6\beta$ -OH-cortisol/cortisol ( $6\beta$ -OHC/C) and the microbiota metabolite trimethylamine-N-Oxid (TMAO) during the menstrual cycle in 17 healthy women

#### 4.5 | Implications of studies on menstrual cycle and CYP3A activity

Although a sample size of 17 individuals may appear small, most studies on menstrual cycle variability of CYP3A markers have small sample sizes, from four to an average of 20 individuals.<sup>7,11,13</sup> One reason for this, could be that studies involving administration of drugs causing inhibition or induction, in combination with phenotyping with midazolam iv and/or

orally, may be difficult to perform in vulnerable populations like women of child-bearing age, in elderly and in children.<sup>21,22</sup> Unfortunately, investigations have shown that women are still very much underrepresented in DDI studies, and it has been suggested that the use of endogenous markers of CYP3A could increase the number of women in DDI studies.<sup>32,64</sup>

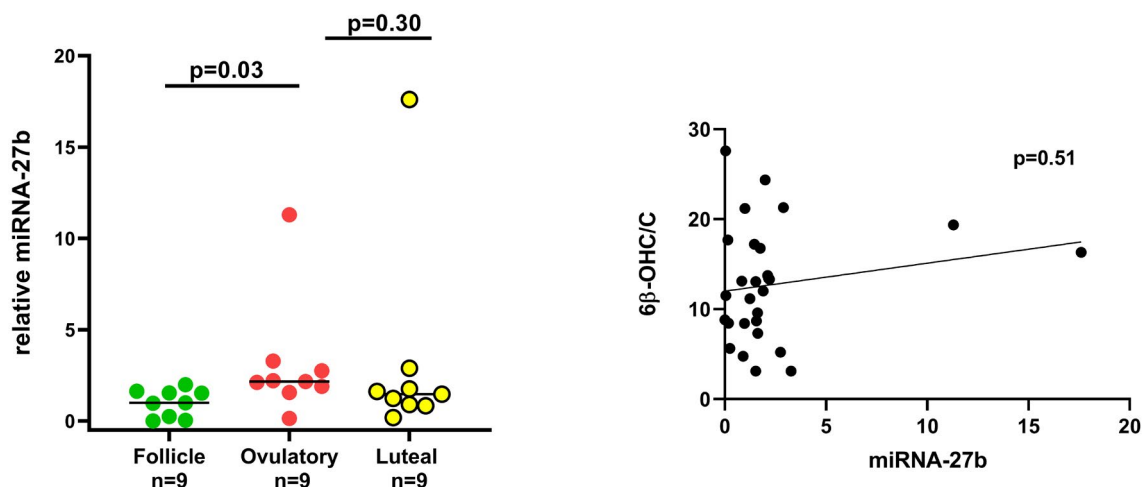
#### 4.6 | Limitations of this study

The limitations of our pilot study are several. As previously mentioned, the method of urine sampling may have had an impact on the results. In addition, due to the small sample size, we did not use genotyping to evaluate potential effects of genetic polymorphisms on our results. Finally, although there are data supporting that serum miRNA-27b may relate to hepatic miRNA-27b, this finding has yet to be confirmed in the same subjects.<sup>31</sup> However, obtaining liver tissue samples from healthy volunteers will most likely turn out to be a difficult task.

## 5 | CONCLUSION AND FUTURE DIRECTIONS

In conclusion, we did not find evidence supporting the hypothesis of a variability in CYP3A activity measured by  $6\beta$ -OHC/C, during the three menstrual cycle phases. Whether the association between  $6\beta$ -OHC/C and progesterone, will have any implications for the use of  $6\beta$ -OHC/C as a marker of CYP3A activity in fertile women—remains





miRNA27b	
Menstrual phase	Median CV (range)
Follicular n=9	74.90% (0.0001-1.99)
Ovulatory n=9	105.5% (0.1463-11.29)
Luteal n=9	172.6% (0.1856-17.60)

FIGURE 7 (A) Variations in miRNA-27b during the three phases of the menstrual cycles from nine healthy volunteers ( $n = 27$ ). (B) Correlation analysis between all values, for all phases of  $6\beta$ -OH-cortisol/cortisol ( $6\beta$ -OHC/C) and miRNA-27b ( $n = 27$ )

to be explored. Interestingly, the finding of an association between  $6\beta$ -OHC/C in urine and levels of TMAO in serum suggest that gut microbiota may affect CYP3A activity.

In the future, more thorough studies on how the gut microbiota may affect CYP3A activity in women and men are needed. It has recently been suggested that  $6\beta$ -OHC/C represents CYP3A activity in the liver only.<sup>44</sup> Thus, investigations of markers reflecting the CYP3A activity in the gut would also be of interest, and how this activity may be affected by gut microbiota as well as by sex hormones.

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#### DISCLOSURE

The authors declare that they have no conflict of interest.

#### AUTHOR CONTRIBUTIONS

Linda Björkhem-Bergman, Lena Ekström, and Helena Bergström were responsible for study design, funding applications, application to Human Ethics Committee of Karolinska Institutet, Biobank of Sweden and Swedish Data Protection Authority. Recruiting and screening of subjects was performed by Linda Björkhem-Bergman as study physician and Lena Ekström. Ulf Diczfalusy and Anna Lindahl

were responsible for method and analysis of  $6\beta$ -OHC/C. Helena Bergström compiled data from laboratory reports and CRFs. Helena Bergström performed statistical analysis together with biostatistician Anna Warnqvist and Linda Björkhem-Bergman. All authors contributed to the manuscript. All authors read and approved the final manuscript.

#### ETHICS APPROVAL STATEMENT

The study was approved by the Human Ethics Committee of Karolinska Institutet (Dnr 2018/481-31/2 and Dnr 2020-00598). The study was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients prior to inclusion in the study.

#### DATA AVAILABILITY STATEMENT

As sample size is small, there is a risk for the individual privacy being compromised. De-identified data are therefore available from the corresponding author upon reasonable requests.

#### ORCID

Helena Bergström  <https://orcid.org/0000-0003-3079-0202>

#### REFERENCES

- Li Y, Meng Q, Yang M, et al. Current trends in drug metabolism and pharmacokinetics. *Acta Pharmaceutica Sinica B*. 2019;9(6):1113-1144.
- Guttman Y, Nudel A, Kerem Z. Polymorphism in cytochrome P450 3A4 is ethnicity related. *Front Genet*. 2019;10:224.

3. Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther.* 2013;138(1):103-141.
4. Wolbold R, Klein K, Burk O, et al. Sex is a major determinant of CYP3A4 expression in human liver. *Hepatology.* 2003;38(4):978-988.
5. Piccinato CA, Neme RM, Torres N, et al. Is cytochrome P450 3A4 regulated by menstrual cycle hormones in control endometrium and endometriosis? *Mol Cell Biochem.* 2017;427(1-2):81-89.
6. Magliocco G, Thomas A, Desmeules J, Daali Y. Phenotyping of human CYP450 enzymes by endobiotics: current knowledge and methodological approaches. *Clin Pharmacokinet.* 2019;58(11):1373-1391.
7. Chetty M, Mattison D, Rostami-Hodjegan A. Sex differences in the clearance of CYP3A4 substrates: exploring possible reasons for the substrate dependency and lack of consensus. *Curr Drug Metab.* 2012;13(6):778-786.
8. Zhu B, Liu Z-Q, Chen G-L, et al. The distribution and gender difference of CYP3A activity in Chinese subjects. *Br J Clin Pharmacol.* 2003;55(3):264-269.
9. Kharasch ED, Mautz D, Senn T, Lentz G, Cox K. Menstrual cycle variability in midazolam pharmacokinetics. *J Clin Pharmacol.* 1999;39(3):275-280.
10. Kashuba ADM, Bertino JS, Rocci ML, Kulawy RW, Beck DJ, Nafziger AN. Quantification of 3-month intraindividual variability and the influence of sex and menstrual cycle phase on CYP3A activity as measured by phenotyping with intravenous midazolam. *Clin Pharmacol Ther.* 1998;64(3):269-277.
11. Mitchell SC, Smith RL, Waring RH. The menstrual cycle and drug metabolism. *Curr Drug Metab.* 2009;10(5):499-507.
12. Nazir S, Iqbal Z, Ahmad L, Shah Y, Nasir F. Pharmacokinetics of omeprazole and its metabolites in three phases of menstrual cycle. *Eur J Drug Metab Pharmacokinet.* 2015;40(1):13-22.
13. Kirkwood C, Moore A, Hayes P, DeVane CL, Pelonero A. Influence of menstrual cycle and gender on alprazolam pharmacokinetics. *Clin Pharmacol Ther.* 1991;50(4):404-409.
14. Chen M, Ma L, Drusano GL, Bertino JS Jr, Nafziger AN. Sex differences in CYP3A activity using intravenous and oral midazolam. *Clin Pharmacol Ther.* 2006;80(5):531-538.
15. Ged C, Rouillon JM, Pichard L, et al. The increase in urinary excretion of 6 beta-hydroxycortisol as a marker of human hepatic cytochrome P450 IIIA induction. *Br J Clin Pharmacol.* 1989;28(4):373-387.
16. Lee C. Urinary 6 beta-hydroxycortisol in humans: analysis, biological variations, and reference ranges. *Clin Biochem.* 1995;28(1):49-54.
17. Björkhem-Bergman L, Bäckström T, Nylén H, et al. Comparison of endogenous 4 $\beta$ -hydroxycholesterol with midazolam as markers for CYP3A4 induction by rifampicin. *Drug Metab Dispos.* 2013;41(8):1488-1493.
18. Diczfalusy U, Nylén H, Elander P, Bertilsson L. 4beta-Hydroxycholesterol, an endogenous marker of CYP3A4/5 activity in humans. *Br J Clin Pharmacol.* 2011;71(2):183-189.
19. Lee J, Kim AH, Yi SJ, et al. Distribution of exogenous and endogenous CYP3A markers and related factors in healthy males and females. *AAPS J.* 2017;19(4):1196-1204.
20. Burstein AH, Reiss WG, Kantor E, Anderson GD. Cytochrome P450 3A4 activity in premenopausal and postmenopausal women, based on 6-beta-hydroxycortisol:cortisol ratios. *Pharmacotherapy.* 1998;18(6):1271-1276.
21. Shin KH, Ahn LY, Choi MH, et al. Urinary 6beta-hydroxycortisol/cortisol ratio most highly correlates with midazolam clearance under hepatic cyp3a inhibition and induction in females: a pharmacometabolomics approach. *AAPS J.* 2016;18(5):1254-1261.
22. Chen Z, Dunning LA, Anderson KE, Holtzman JL, Zheng W. Within-person variability of urinary 6 $\beta$ -hydroxycortisol to urinary ratios in Caucasian women. *Steroids.* 2004;69(1):67-70.
23. Lin Y, Anderson GD, Kantor E, Ojemann LM, Wilensky AJ. Differences in the urinary excretion of 6- $\beta$ -hydroxycortisol/cortisol between Asian and Caucasian women. *J Clin Pharmacol.* 1999;39(6):578-582.
24. Woolsey SJ, Beaton MD, Choi Y-H, et al. Relationships between endogenous plasma biomarkers of constitutive cytochrome P450 3A activity and single-time-point oral midazolam micro-dose phenotype in healthy subjects. *Basic Clin Pharmacol Toxicol.* 2016;118(4):284-291.
25. Galteau MM, Shamsa F. Urinary 6beta-hydroxycortisol: a validated test for evaluating drug induction or drug inhibition mediated through CYP3A in humans and in animals. *Eur J Clin Pharmacol.* 2003;59(10):713-733.
26. Tang WH, Kitai T, Hazen SL. Gut microbiota in cardiovascular health and disease. *Circ Res.* 2017;120(7):1183-1196.
27. Yang S, Li X, Yang F, et al. Gut microbiota-dependent marker TMAO in promoting cardiovascular disease: inflammation mechanism, clinical prognostic, and potential as a therapeutic target. *Front Pharmacol.* 2019;10:1-13.
28. Jarmusch AK, Vrbanac A, Momper JD, et al. Enhanced characterization of drug metabolism and the influence of the intestinal microbiome: a pharmacokinetic, microbiome, and untargeted metabolomics study. *Clin Transl Sci.* 2020;13(5):972-984.
29. Klaassen CD, Cui JY. Review: mechanisms of how the intestinal microbiota alters the effects of drugs and bile acids. *Drug Metab Dispos.* 2015;43(10):1505-1521.
30. Ingelman-Sundberg M, Sim SC, Gomez A, Rodriguez-Antona C. Influence of cytochrome P450 polymorphisms on drug therapies: pharmacogenetic, pharmacoeepigenetic and clinical aspects. *Pharmacol Ther.* 2007;116(3):496-526.
31. Ekström L, Skilving I, Ovesjö ML, et al. miRNA-27b levels are associated with CYP3A activity in vitro and in vivo. *Pharmacol Res Perspect.* 2015;3(6):e00192.
32. Tornio A, Filppula AM, Niemi M, Backman JT. Clinical studies on drug-drug interactions involving metabolism and transport: methodology, pitfalls, and interpretation. *Clin Pharmacol Ther.* 2019;105(6):1345-1361.
33. Mullen J, Baekken L, Bergström H, Björkhem Bergman L, Ericsson M, Ekström L. Fluctuations in hematological athlete biological passport biomarkers in relation to the menstrual cycle. *Drug Test Anal.* 2020;12(9):1229-1240.
34. Schulze J, Suominen T, Bergström H, Ericsson M, Björkhem Bergman L, Ekström L. Urinary steroid profile in relation to the menstrual cycle. *Drug Test Anal.* 2021;13(3):550-557.
35. Bergström H, Ekström L, Warnqvist A, Bergman P, Björkhem-Bergman L. Variations in biomarkers of dyslipidemia and dysbiosis during the menstrual cycle: a pilot study in healthy volunteers. *BMC Womens Health.* 2021;21(1):166.
36. Jewelewicz R, Dyrenfurth I, Ferin M, Bogumil J, Vande Wiele RL. Gonadotropin, estrogen and progesterone response to long term gonadotropin-releasing hormone infusion at various stages of the menstrual cycle. *J Clin Endocrinol Metab.* 1977;45(4):662-667.
37. Missailidis C, Hällqvist J, Qureshi AR, et al. Serum trimethylamine-N-oxide is strongly related to renal function and predicts outcome in chronic kidney disease. *PLoS One.* 2016;11(1):e0141738.
38. Marde Arrhen Y, Nylén H, Lovgren-Sandblom A, Kanebratt KP, Wide K, Diczfalusy U. A comparison of 4beta-hydroxycholesterol: cholesterol and 6beta-hydroxycortisol: cortisol as markers of CYP3A4 induction. *Br J Clin Pharmacol.* 2013;75(6):1536-1540.
39. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001;25(4):402-408.
40. Waxman DJ, Holloway MG. Sex differences in the expression of hepatic drug metabolizing enzymes. *Mol Pharmacol.* 2009;76(2):215-228.
41. Wang D, Lu R, Rempala G, Sadee W. Ligand-free estrogen receptor  $\alpha$  (ESR1) as master regulator for the expression of CYP3A4 and

- other cytochrome P450 enzymes in the human liver. *Mol Pharmacol*. 2019;96(4):430-440.
42. Lutz U, Bittner N, Ufer M, Lutz WK. Quantification of cortisol and 6 beta-hydroxycortisol in human urine by LC-MS/MS, and gender-specific evaluation of the metabolic ratio as biomarker of CYP3A activity. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2010;878(1):97-101.
  43. Lee J, Yoon SH, Yi S, et al. Quantitative prediction of hepatic CYP3A activity using endogenous markers in healthy subjects after administration of CYP3A inhibitors or inducers. *Drug Metab Pharmacokinet*. 2019;34(4):247-252.
  44. Lee S, Lee Y, Kim AH, et al. Urinary metabolic markers reflect on hepatic, not intestinal, CYP3A activity in healthy subjects. *Drug Metab Pharmacokinet*. 2020;36:100374.
  45. Shin KH, Choi MH, Lim KS, Yu KS, Jang JJ, Cho JY. Evaluation of endogenous metabolic markers of hepatic CYP3A activity using metabolic profiling and midazolam clearance. *Clin Pharmacol Ther*. 2013;94(5):601-609.
  46. Lee Y, Chae W, Yoon S, Chung J-Y, Cho J-Y. Development and validation of a method for the simultaneous quantification of endogenous steroids metabolized by CYP3A. *Transl Clin Pharmacol*. 2020;28(2):73-82.
  47. Ohno M, Yamaguchi I, Ito T, Saiki K, Yamamoto I, Azuma J. Circadian variation of the urinary 6beta-hydroxycortisol to cortisol ratio that would reflect hepatic CYP3A activity. *Eur J Clin Pharmacol*. 2000;55(11-12):861-865.
  48. Ackermann D, Groessel M, Puijck M, et al. Reference intervals for the urinary steroid metabolome: the impact of sex, age, day and night time on human adult steroidogenesis. *PLoS One*. 2019;14(3):e0214549.
  49. Niwa T, Okamoto A, Narita K, et al. Comparison of steroid hormone hydroxylation mediated by cytochrome P450 3A subfamilies. *Arch Biochem Biophys*. 2020;682:108283.
  50. Acconcia F, Marino M. *Principles of Endocrinology and Hormone Action- Steroid Hormones: Synthesis, Secretion and Transport*. Springer International Publishing AG; 2018.
  51. Ducharme J, Polic V, Auclair K. A covalently attached progesterone molecule outcompetes the binding of free progesterone at an allosteric site of cytochrome P450 3A4. *Bioconjug Chem*. 2019;30(6):1629-1635.
  52. Guthrie L, Kelly L. Bringing microbiome-drug interaction research into the clinic. *EBioMedicine*. 2019;44:708-715.
  53. Dempsey JL, Cui JY. Microbiome is a functional modifier of P450 drug metabolism. *Curr Pharmacol Rep*. 2019;5(6):481-490.
  54. Selwyn FP, Cui JY, Klaassen CD. RNA-Seq quantification of hepatic drug processing genes in germ-free mice. *Drug Metab Dispos*. 2015;43(10):1572-1580.
  55. Jourová L, Vavreckova M, Zemanova N, et al. Gut microbiome alters the activity of liver cytochromes P450 in mice with sex-dependent differences. *Front Pharmacol*. 2020;11:01303.
  56. Esposito T, Varriale B, D'Angelo R, Amato A, Sidoti A. Regulation of flavin-containing mono-oxygenase (Fmo3) gene expression by steroids in mice and humans. *Horm Mol Biol Clin Investig*. 2014;20(3):99-109.
  57. Pavek P, Pregnane X. Receptor (PXR)-mediated gene repression and cross-talk of PXR with other nuclear receptors via coactivator interactions. *Front Pharmacol*. 2016;7:1-16.
  58. Liu Y, Dai M. Trimethylamine N-oxide generated by the gut microbiota is associated with vascular inflammation: new insights into atherosclerosis. *Mediators Inflamm*. 2020;2020:4634172.
  59. Morgan ET, Skubic C, Lee CM, Cokan KB, Rozman D. Regulation of cytochrome P450 enzyme activity and expression by nitric oxide in the context of inflammatory disease. *Drug Metab Rev*. 2020;52(4):455-471.
  60. Oertelt-Prigione S. Immunology and the menstrual cycle. *Autoimmun Rev*. 2012;11(6-7):A486-A492.
  61. Zastrozhin MS, Skryabin VYU, Smirnov VV, et al. Effects of plasma concentration of micro-RNA Mir-27b and CYP3A4\*22 on equilibrium concentration of alprazolam in patients with anxiety disorders comorbid with alcohol use disorder. *Gene*. 2020;739:144513.
  62. Kabekodu SP, Shukla V, Varghese VK, D' Souza J, Chakrabarty S, Satyamoorthy K. Clustered miRNAs and their role in biological functions and diseases. *Biol Rev Camb Philos Soc*. 2018;93(4):1955-1986.
  63. Pérez-Cremades D, Mompeón A, Vidal-Gómez X, Hermenegildo C, Novella S. miRNA as a new regulatory mechanism of estrogen vascular action. *Int J Mol Sci*. 2018;19(2):473.
  64. Naidoo P, Chetty M. Progress in the consideration of possible sex differences in drug interaction studies. *Curr Drug Metab*. 2019;20(2):114-123.

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