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Alterations in xenobiotic-metabolizing enzyme activities across menstrual cycle in healthy volunteers

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FOOTNOTES

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Altered xenobiotic metabolic enzymes across menstrual cycle

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d) Nonstandard abbreviations: CYP1A2 (cytochrome P450 1A2); CYP2A6 (cytochrome P450 2A6); XO (xanthine oxidase); NAT2 (N-acetyltransferase-2); CMR (Caffeine metabolic ratio); EFP (Early Follicular Phase); LFP (Late Follicular Phase); LP (Luteal Phase); LH (Luteinizing Hormone); FSH (Follicle-Stimulating Hormone); AST (aspartate aminotransferase); ALT (alanine aminotransferase); ALP (alkaline phosphatase); γ-GT (gamma-glutamyl transpeptidase)

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ABSTRACT

The purpose of the study was to determine whether the *in vivo* activities of drug-metabolizing enzymes CYP1A2 and CYP2A6, Xanthine Oxidase (XO) and N-acetyltransferase-2 (NAT2) vary across menstrual cycle. Forty-two healthy women were studied at Early-Follicular (EFP: 2^{nd}-4^{th}day), Late-Follicular (LFP: 10^{th}-12^{th}day) and Luteal (LP: 19^{th}-25^{th}day) phases of a single menstrual cycle at which blood and urine samples were collected. Spot urine samples obtained 6h following 200mg caffeine administration were used to determine Caffeine Metabolite Ratios (CMRs); blood samples were used to determine CYP1A2*1F (rs762551) and CYP1A2*1C (rs2069514) polymorphisms and the hormonal profile (estradiol, progesterone, Luteinizing (LH) and Follicle-Stimulating (FSH) hormones) at EFP, LFP and LP. CMRs and hormone variations were analyzed at three levels (EFP, LFP, LP) using One-Way Repeated Measures ANOVA. CYP1A2 activity was lower and those of CYP2A6 and NAT2 were higher at LFP compared to EFP and LP. Enzyme alterations were significant in volunteers (n=21) whose hormonal profiles at EFP, LFP and LP corresponded to expected levels, but not in volunteers (n=15) with presumed early or late sampling around LFP. No significant difference was detected in any enzyme activity in presumed anovulatory volunteers (n=6). The reduction of CYP1A2 activity at LFP was not associated with smoking or CYP1A2*1F polymorphism. XO and NAT2 (fast-acetylators) activities remained unaltered. It is suggested that drug-metabolizing enzyme activities are altered across menstrual cycle. Selection of appropriate sampling periods verified by hormonal assessment and identification of anovulatory cycles are decisive factors in disclosing altered enzyme activity across menstrual cycle.

INTRODUCTION

Hormonal fluctuations during menstrual cycle have been postulated to align with disease exacerbation as in catamenial epilepsy (Herzog, 2008) and perimenstrual asthma (Graziottin and Serafini, 2016). These fluctuations may also influence the treatment outcome by modifying drug pharmacokinetics and/or pharmacodynamics. Enhanced metabolism has been reported for phenytoin during menstruation (Shavit *et al.*, 1984), methaqualone during ovulation (Wilson *et al.*, 1982), omeprazole during both menstruation and the luteal phase (Nazir *et al.*, 2015) and methylprednisolone during the luteal phase (Lew *et al.*, 1993); reduced metabolism has been reported for theophylline (Bruguerolle *et al.*, 1990) and paracetamol during the luteal phase of menstrual cycle (Wójcicki *et al.*, 1979).

The influence exerted by reproductive hormones on xenobiotic-metabolizing enzymes is supported by studies showing that Cytochrome P450 (CYP) 1A2 activity is inhibited by estrogen-containing oral contraceptives (OCs) (Granfors *et al.*, 2005), estrogen replacement therapy (Pollock *et al.*, 1999; O'Connell *et al.*, 2006) and pregnancy (Vistisen *et al.*, 1992; Tsutsumi *et al.*, 2001; Tracy *et al.*, 2005); CYP2A6 activity is enhanced in pregnancy (Dempsey *et al.*, 2002), or during the use of estrogen-only OCs (Benowitz *et al.*, 2006), whereas, the effect of pregnancy (Tsutsumi *et al.*, 2001), or estrogen therapy (Shelepova *et al.*, 2005; O'Connell *et al.*, 2006) on N-acetyltransferase-2 (NAT2) and Xanthine Oxidase (XO) activity has been poorly explored. With respect to the activity of drug-metabolizing enzymes across menstrual cycle, available evidence is sparse and conflicting as both reduced (Bruguerolle *et al.*, 1990; Lane *et al.*, 1992; Nagata *et al.*, 1997) and no effect (Kashuba *et al.*, 1998; Zaigler *et al.*, 2000; Hukkanen *et al.*, 2005) have been reported. These conflicting results are most likely due to suboptimal study designs based on small numbers of women, lack of characterization of menstrual cycle phases through assessment of hormonal concentrations in plasma and, most importantly, lack of sampling at the time of the highest hormonal fluctuation as are those

occurring between EFP and LFP (Fig. 1). In fact, due to the retrospective nature of menstrual cycle protocols and the variability of a normal menstrual cycle, there is an inherent difficulty of menstrual cycle studies to identify the precise timing of peak and trough hormone levels that may influence the activity of drug-metabolizing enzymes, unlike studies on OCs, Hormonal Replacement Therapy (HRT) or pregnancy in which sampling occurs at presumed steady-state hormone levels.

The activity of drug-metabolizing enzymes, including CYP1A2, CYP2A6, XO and NAT2 has long been assessed through molar ratios of the different caffeine metabolites (Fig.2; Asprodini *et al.*, 1998; Begas *et al.*, 2007; Hakooz, 2009). Human CYP1A2 has the highest catalytic activity in the 2-hydroxylation of estradiol (Yamazaki *et al.*, 1998) and it is responsible for the metabolism of many clinically used drugs (Faber *et al.*, 2005). CYP2A6 catalyses the biotransformation of nicotine and drugs such as valproic acid; XO oxidizes endogenous purines and pyrimidines and metabolizes drugs such as thiopurines and methylxanthines. Similarly, NAT2 is involved in the acetylation of many drugs (Evans, 1989).

The aim of the present study was to examine the activity of CYP1A2, CYP2A6, XO and NAT2 enzymes in three sampling phases of the menstrual cycle (EFP, LFP and LP) in healthy women using caffeine as a metabolic probe. The menstrual status of each volunteer was verified upon evaluation of the hormonal profile of estradiol, progesterone, LH and FSH concentrations in blood. Moreover, the effect of genotype on the phenotypic expression of CYP1A2 across menstrual cycle was investigated by examining CYP1A2 CMRs in relation to the two most common CYP1A2 single nucleotide polymorphisms CYP1A2*1C and CYP1A21*F.

MATERIALS AND METHODS

Subjects

The population study consisted of 42 (25 non-smokers, 17 smokers) apparently healthy female volunteers with regular menstrual cycles. Their health status was based on medical history and physical examination and was confirmed by routine laboratory tests (aspartate aminotransferase, (AST); alanine aminotransferase, (ALT); alkaline phosphatase, (ALP); gamma-glutamyl transpeptidase, (γ-GT); serum creatinine; serum urea; Table 1). Exclusion criteria were the use of OCs (or any hormonal birth control method), pregnancy, menstrual cycle irregularities, medications known to induce or inhibit the activity of the enzymes of interest and consumption of alcohol. A form regarding demographic and lifestyle data (age, weight, height, regularity and duration of menstrual cycle, chronic diseases, medication intake, smoking and alcohol consumption habits, occupation and exposure to xenobiotics) was completed for all participants. Detailed description of inclusion/exclusion criteria, the study protocol and data collection is provided in a supplemental file (Supplemental Methods).

The study was carried out in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Larissa University Hospital, Greece (1862015/24227). Written informed consent was obtained from all subjects before entering the study.

Study protocol

Blood and spot urine samples were collected during a single menstrual cycle at days corresponding to three sampling phases: EFP (2^{nd}-4^{th}day), LFP (10^{th}-12^{th}day) and LP (19^{th}-25^{th}day) (Fig. 1a); expected hormone levels at EFP, LFP and LP were defined according to reference concentration ranges reported in the literature (Reed and Carr, 2000).

Volunteers were asked to abstain from methylxanthine-containing food or beverages for at least 24h and to refrain from cruciferous and apiaceous vegetables (Kall *et al.*, 1996; Lampe *et al.*, 2000; Peterson *et al.*, 2009), grapefruit juice (Fuhr *et al.*, 1993) and broiled meat (Kall *et*

al., 1996; Perera *et al.*, 2012) for 48h before the caffeine test. On the day of the test, volunteers were administered a 200mg caffeine capsule. Spot urine samples were collected 6h later in containers preloaded with 200μL 6N HCl (Fig. 1a).

Chemicals

Caffeine metabolites 1,7-dimethylxanthine (17X), 1,7-dimethyluric acid (17U) and 1-methyluric acid (1U) were purchased from Sigma-Aldrich, Germany; 1-methylxanthine (1X) was purchased from TCI, Belgium and 137X from Fluka, Switzerland. 5-acetylamino-6-formylamino-3-methyluracil (AFMU) was kindly provided by Professor Wolfgang Pfleiderer (Konstanz University). Chloroform and isopropanol were purchased from Chemlab, Belgium, and Honeywell Research Chemicals, Germany, respectively.

Sample analysis

Urinary caffeine metabolites were quantified by Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) as previously described (Begas *et al.*, 2007). Urine samples were acidified to pH=3.5 to ensure AFMU stability (Wong *et al.*, 2002); 1ml aliquots were stored at −20°C until analysis. Caffeine metabolites were isolated from urine samples by liquid-liquid extraction using chloroform/isopropanol. Calibration curves for caffeine metabolites in urine were linear at concentrations 10-500μM with R^2>0.99. Inter-day precision was 90.38-97.70% and accuracy was 94.04-105.65% at concentrations 25, 150 and 400μM (n=10). The low limit of quantitation for metabolites was 5μM and the limit of detection 0.08, 0.03, 0.09, 0.10 and 0.17μM for AFMU, 1U, 1X, 17U and 17X, respectively. *In vivo* evaluation of enzyme activities was assessed using the metabolic ratios of caffeine, CMRs, as follows: CYP1A2: (AFMU+1U+1X)/17U, CYP2A6: 17U/(17U+17X), XO: 1U/(1X+1U) and NAT2: AFMU/(AFMU+1U+1X) (Begas *et al.*, 2007).

Estradiol, progesterone, LH and FSH concentrations were determined by electrochemiluminescence immunoassay (ECLIA; Cobas e 411 analyzer). According to the

manufacturer, the precision of the assays for estradiol, progesterone, LH and FSH were 2.5-11.9%, 3.7-5.5%, 1.9-5.2% and 2.9-5.3%, respectively. Venous blood samples were collected, centrifuged and stored at -20°C until analysis.

CYP1A2 genotype analysis

Peripheral blood was collected from each subject and genomic DNA was extracted using Invitrogen Purelink genomic DNA mini kit (Invitrogen Corp, USA). Genotyping was performed by the polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP). 4μl of DNA were added in each tube containing 1X buffer, 1.6mM dNTPs, 2mM $MgCl_2$, 400nM of each primer and 1.5U of Taq DNA polymerase (Fermentas Inc., Hanover, MD) in a total volume of 50μl. Digestion was performed by BslI and PspOMI restriction enzymes for rs2069514 (-3860G>A) and rs762551 (-163C>A) SNPs, respectively. (PCR conditions and specific primers' sequences are shown in Table 2). PCR and digestion products were evaluated by agarose gel electrophoresis under ethidium bromide staining.

Statistical analysis

Fluctuations in hormone (estradiol, progesterone, LH, FSH) concentrations and CMRs were compared at three levels (EFP, LFP, LP) using One-Way Repeated Measures Analysis of Variance (ANOVA). CYP1A2 CMRs were compared between smokers and non-smokers within the three sampling phases using Mixed Repeated Measures ANOVA. Specifically, CYP1A2 CMRs were compared across EFP, LFP and LP within subjects using as control factors the A/A and C/A genotypes and the non-smoking/smoking habits of the subjects; repeated contrasts were used to test for significant differences among sampling phases. The levels of the factor genotype were reduced to two, as genotype C/C was encountered in only one subject. Results are reported as estimated marginal means for each level with 95% CIs and as parameter estimates for subgroups (mean values±SE). CYP2A6, XO and NAT2 CMRs were compared within the three sampling phases using One-Way Repeated Measures ANOVA.

Multiple pairwise comparisons in ANOVA were P-corrected using Bonferroni adjustment. Statistical difference between two independent groups was examined by T-test. All statistical analyses were performed using SPSS v24 software (IBM, USA). *P* values <0.05 were considered statistically significant.

RESULTS

Forty-two women met the inclusion criteria and were enrolled in the study; demographic characteristics and biochemical data of the participants are presented in Table 1. No subject reported adverse effects after the caffeine test.

Hormone levels during menstrual cycle

Estradiol, progesterone, LH and FSH blood concentrations were measured at the EFP, LFP and LP (Supplemental Table 1; Fig. 1A: grey-shaded blocks). Upon completion of the study protocol, the concentration profile of all hormones was compared to the profile of reference concentrations reported in the literature (Reed and Carr, 2000) (depicted with solid red, dashed purple, dotted grey and dashed cyan lines, respectively, within grey blocks in Fig. 1A). In twenty-one volunteers (21/42, 50%) serum hormonal concentrations determined at EFP, LFP and LP corresponded to expected hormone levels (Group 1; red solid line within grey block, Fig. 1A), such that the concentrations of estradiol and LH peaked at LFP in relation to EFP and LP (Table 3; Fig. 2). In fifteen volunteers (15/42, 35.7%) estradiol and LH levels at LFP did not concur with the expected levels presumably due to earlier or later sampling relative to hormone concentration peaks at LFP (Group 2). Six volunteers (6/42, 14.3%) were considered to have anovulatory menstrual cycles (Group 3) as their serum progesterone level, measured at LP, was <2.5ng/ml (Prior *et al.*, 2015) (Table 3; Fig. 2). Conversely, serum progesterone concentration was higher in LP compared to EFP and LFP in Groups 1 and 2. FSH levels at LP were significantly lower compared to EFP and LFP in Groups 1 and 2. Group 3 did not exhibit significant differences in any hormone concentration among EFP, LFP and LP.

In vivo activity of CYP1A2 during menstrual cycle

The effect of genotype and smoking on the phenotypic expression of CYP1A2 during menstrual cycle was examined by comparing CYP1A2 CMRs at EFP, LFP and LP among different genotypes in non-smoking and smoking volunteers.

The frequencies of CYP1A2*1F (rs762551) polymorphism (-163 C>A) within the C/A, A/A and C/C genotypes were 24/42 (57.1%), 17/42 (40.5%) and 1/42 (2.4%), respectively. These frequencies did not differ significantly from the values predicted by the Hardy-Weinberg equilibrium model (chi square test, p=0.084). The G>A polymorphism in -3860 position (CYP1A2*1C rs2069514) was not detected, in accordance to other Caucasian populations (Dobrinas *et al.*, 2011). CYP1A2 CMRs, examined at EFP (n=42), were similar between C/A and A/A genotypes in non-smokers; conversely, smokers homozygous for the A allele exhibited a trend for higher CMRs compared to heterozygous C/A suggesting that the A/A genotype confers higher CYP1A2 inducibility (Fig. 3*b1, b2*), as has been previously reported (Sachse *et al.*, 1999; Gunes *et al.*, 2009; but see Dobrinas *et al.*, 2011).

In Group 1 (genotypes C/A and A/A, n=20), CYP1A2 CMR was significantly lower (23%) at LFP compared to EFP and LP; no significant difference was detected between EFP and LP suggesting that CMR recovers at LP to baseline EFP level (Table 3; Fig. 3a). CYP1A2 CMR was 13.6% and 24.3% lower at LFP compared to EFP in non-smokers and smokers, respectively. CYP1A2 CMR was increased (7.3% and 9.9%) and remained almost the same (1.1% and 2.4% reduction) in four non-smokers at LFP compared to EFP; the rest exhibited 18.5% decrease (range: 5.7-32.0%). Correspondingly, the metabolic ratio was increased by 26.3% and remained almost the same (1.1% reduction) in two smokers at LFP compared to EFP; the rest of the smokers exhibited 27.7% decrease (range: 5.6-52.9%) indicating large inter-individual variation.

Group 2 exhibited reduced CYP1A2 CMR compared to LP, but not EFP, probably due to slight shift in sampling, either earlier or later, relative to estradiol peak at LFP (see

discussion). No significant difference was detected in CYP1A2 CMR between EFP and LP suggesting that CMR in LP recovers to baseline EFP level.

Group 3 exhibited non-significant difference in CYP1A2 CMRs among EFP: 3.92 (95% CI: 3.03-4.80), LFP: 4.12 (95% CI: 3.00-5.24) and LP: 3.51 (95% CI: 2.81-4.22) (Table 3).

In all groups neither genotype nor smoking exhibited a significant interaction with sampling phase.

CYP1A2 CMRs at EFP (n=42) were significantly lower in non-smokers compared to smokers confirming the inducing effect of smoking on CYP1A2 activity.

In vivo activities of CYP2A6, XO and NAT2 during menstrual cycle

Previous studies have shown that smoking does not affect CYP2A6 (Nowell *et al.*, 2002; Begas *et al.*, 2007), XO (Chung *et al.*, 2000; Aklillu *et al.*, 2003; Benowitz *et al.*, 2003; Begas *et al.*, 2007) and NAT2 activity (Benowitz *et al.*, 2003; Begas *et al.*, 2007). Therefore, smoking was not included as a factor in the analysis of CYP2A6, XO and NAT2 CMRs among menstrual sampling phases.

In Group 1, CYP2A6 CMRs were significantly higher at LFP compared to EFP and LP with no significant difference between EFP and LP, suggesting that CMR at LP recovers to baseline EFP level. Similarly, Group 2 exhibited higher CYP2A6 activity at LFP compared to EFP and LP, although statistical significance was reached only between LFP and LP. Group 3 exhibited CYP2A6 CMRs that did not differ significantly among EFP, LFP and LP. XO CMRs did not differ among the three sampling phases of the menstrual cycle in all groups studied (Table 3; Fig. 4).

Volunteers were classified as slow- (n=27/42, 64.3%) and fast- (n=15/42, 35.7%) acetylators according to the antimode 0.25 (Begas *et al.*, 2007). Slow-acetylators in Group 1 exhibited significantly higher NAT2 CMRs at LFP compared to EFP and LP. No significant

difference was detected in NAT2 CMRs of fast-acetylators in Group 1 and slow- and fast-acetylators in Groups 2 and 3 among menstrual sampling phases (Table 3; Fig. 4).

DISCUSSION

The present study is the first to show that CYP1A2 CMR is significantly reduced at LFP compared to EFP and recovers at LP; the reduction at LFP is most prominent when sampling coincides with the estradiol peak and less prominent when it occurs earlier or later relative to LFP estradiol peak. The reduction of CYP1A2 activity at LFP is not associated with smoking or CYP1A2*1F (rs762551) polymorphism. CYP1A2 CMR is not altered in presumed anovulatory cycles.

The effect of menstrual cycle on CYP1A2 activity has been disputed over the past decades by several investigators (Fig. 1). Studies reporting no effect of menstrual cycle on CYP1A2 activity base their conclusion upon comparisons between two sampling phases, namely, early follicular (EFP) and luteal phase (LP); indeed, no effect of menstrual cycle on CYP1A2 activity on caffeine CMR (Kashuba *et al.*, 1998), or caffeine clearance (Lane *et al.*, 1992) was found between EFP and LP. Studies reporting altered CYP1A2 activity across menstrual cycle base their conclusion upon comparisons among three sampling phases, namely, EFP, LFP and LP. Nagata and coworkers (Nagata *et al.*, 1997) and Kamimori and coworkers (Kamimori *et al.*, 1999) reported that theophylline and caffeine metabolism, respectively, is reduced in the ovulatory (LFP) compared to the menstrual (EFP) and luteal phases (LP); they also reported that there is no difference in CYP1A2 activity between EFP and LP. Therefore, the dispute among studies on the effect of menstrual cycle on CYP1A2 activity is only apparent since all studies, including the present one, agree that CYP1A2 activity is unaltered when comparisons are made between EFP and LP. Consequently, it is possible that alterations of CYP1A2 activity across menstrual cycle are unraveled only in those experimental protocols that employ sampling at the late follicular phase, when steep alterations in estradiol levels occur, namely, either at the peak (present study, Group 1; Kamimori *et al.*, 1999), or around the peak of estradiol concentration (present study, Group 2; Nagata *et al.*, 1997; Kamimori *et al.*, 1999). This

hypothesis is supported by a recent report showing that, in ovulatory cycles, the most prominent increase in serum caffeine concentration is observed between early- (day 7) and mid-follicular (day 12) phases with a gradual recovery to baseline concentration during the luteal phase (Schliep *et al.*, 2016). It is noteworthy that hormonal assessment throughout sampling facilitates the identification of anovulatory cycles, which are characterized by unaltered CYP1A2 activity (present study; Zaigler *et al.*, 2000), and when included in the sample may contribute to underestimation of results (Kamimori *et al.*, 1999). Overall, the present study provides evidence that selection of appropriate sampling phases, verification of menstrual phases by hormonal assessment and identification of anovulatory cycles in study protocols may be decisive factors in disclosing an influence of the menstrual cycle on CYP1A2 activity.

Reduced CYP1A2 activity, with varied magnitude of reduction, has been reported following oral contraceptive use (20-55%; Vistisen *et al.*, 1992; Granfors *et al.*, 2005), estrogen replacement therapy in postmenopausal women (30%; Pollock *et al.*, 1999; O'Connell *et al.*, 2006) and in pregnancy (30-65%; Vistisen *et al.*, 1992; Tsutsumi *et al.*, 2001; Tracy *et al.*, 2005). Notably, women experience diverse patterns of exposure to estradiol in different conditions: sustained, yet low, estradiol concentration following exogenous administration of OCs (up to 50pg/ml; Reape *et al.*, 2008) and HRT (up to 150pg/ml; Pollock *et al.*, 1999; O'Connell *et al.*, 2006), long-lasting incremental estradiol concentration reaching drastic levels during pregnancy (up to 6137pg/ml; Abbassi-Ghanavati *et al.*, 2009), steep alteration in estradiol concentration occurring acutely during the menstrual cycle (up to 250pg/ml at LFP; present study; Hambridge *et al.*, 2013). Whether these different conditions share a common mechanism in reducing CYP1A2 activity, or whether estradiol is the only key player responsible for this reduction, remains largely unknown. Nevertheless, as these studies have used caffeine as a metabolic probe, it is conceivable that the reduction in CYP1A2 activity could be attributed to pharmacologic interaction between estradiol and caffeine, as they are

both substrates for CYP, although experimental evidence from *in vitro* studies has shown that estradiol is only a weak competitive inhibitor of CYP1A2 (Karjalainen *et al.*, 2008; Chang *et al.*, 2009). An alternative candidate mechanism could be transcriptional down-regulation of CYP1A gene, as has been shown previously for CYP1A1 and CYP2E1 (Beischlag and Perdew, 2005; Konstandi *et al.*, 2013).

The higher CYP2A6 activity observed at LFP compared to EFP and LP is not surprising as previous reports have associated enhanced CYP2A6 activity with conditions of increased estrogen levels such as pregnancy (Dempsey *et al.*, 2002), or the use of estrogen-only OCs (Benowitz *et al.*, 2006). Furthermore, estradiol has been shown to increase the expression of CYP2A6 in human hepatocytes *in vitro* (Choi *et al.*, 2013). Similar pharmacokinetics of nicotine, a substrate of CYP2A6, between the mid-follicular and the mid-luteal phase has been reported, although the authors acknowledged the possibility of altered CYP2A6 activity during other phases, such as the ovulatory or menstrual (Hukkanen *et al.*, 2005).

The metabolic ratio for XO activity remained unaltered across menstrual cycle. Only a few studies are available to date reporting lack of effect of the menstrual cycle on XO activity (Lane *et al.*, 1992; Kashuba *et al.*, 1998). Despite the paucity in the literature regarding the influence of menstrual cycle phases on XO activity, indirect data from studies on pregnant women (Tsutsumi *et al.*, 2001), women taking OCs (Shelepova *et al.*, 2005), or gender related studies (Vistisen *et al.*, 1992; Chung *et al.*, 2000; Nowell *et al.*, 2002; Aklillu *et al.*, 2003; Begas *et al.*, 2007) indicate lack of any effect of estrogens on the activity of XO.

The frequency distribution of metabolic ratios for slow (27/42, 64.3%) and fast (15/42, 35.7%) acetylators in the present study was in agreement with previously reported for the Greek population (Asprodini *et al.*, 1998; Begas *et al.*, 2007). The metabolic ratio for NAT2 activity in slow-acetylators was significantly higher at LFP compared to EFP and LP, whereas, fast-acetylators exhibited similar NAT2 activities among the three sampling phases. The lack of

difference in slow-acetylators between EFP and LP agrees with an earlier study reporting lack of difference between the follicular and the luteal phase in NAT2 activity (Kashuba *et al.*, 1998). Studies on pregnant women (Vistisen *et al.*, 1992; Tsutsumi *et al.*, 2001), or women on estrogen therapy (Shelepova *et al.*, 2005; O'Connell *et al.*, 2006) report no alteration of NAT2 activity in the presence of estrogens; these studies, however, base their conclusion on a limited number of subjects and, as expected, with low power for distinction between fast- and slow-acetylators.

Our study bears limitations including the assessment of CYP1A2 activity over a single menstrual cycle, the use of an indirect method for detecting ovulation and the lack of verification of caffeine abstinence by baseline sample analysis. Furthermore, despite the consent of participants to comply with the study protocol, the outpatient setting of our study precluded any rigid control over lifestyle factors influencing the activity of the enzymes studied, thus, contributing to both intra- and inter-individual variation. The considerable variability in altered CYP1A2 metabolic ratios in LFP ranging from +26.3 to -52.9%, in combination with the marked overlap of metabolic ratio values between non-smokers and smokers renders the prediction of CYP1A2 activity alteration among individuals difficult.

Variations in xenobiotic-metabolizing enzyme activity across menstrual cycle may pose challenges in women in terms of drug efficacy and toxicity, adverse reactions and potential drug-drug interactions, a major concern in medicine for both clinicians and patients. In addition to drug metabolism, women may be susceptible to variations in xenobiotic transformation of several carcinogenic and pre-carcinogenic compounds, such as nitrosamines, aflatoxins and polycyclic aromatic hydrocarbons (PAHs). The results of the present study suggest that sex-related physiological factors may be an important variable in xenobiotic metabolism.

In conclusion, the present study provides evidence for significant alterations in drug-metabolizing *in vivo* enzyme activities across menstrual cycle. Although the clinical impact of

the present data remains to be determined, our study provides a better understanding on pharmacokinetic alterations during menstrual cycle and forms a basis for future clinical investigations and optimisation of drug therapy in women.

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AUTHORSHIP CONTRIBUTIONS

E. Asprodini and V. Tsiokou contributed equally to this work

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FOOTNOTES

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E. Asprodini and V. Tsiokou contributed equally to this work

FIGURE LEGENDS

Figure 1. Sampling phases in experimental protocols studying enzyme activity during menstrual cycle. **(A)** Schematic diagram of the experimental protocol followed in the present study across one menstrual cycle. Samples were collected at three sampling phases designated by the grey shaded areas: EFP: 2^{nd}-3^{rd}, LFP: 10^{th}-12^{th} and LP at 19^{th}-25^{th} day post-onset of menses. Blood and urine samples were collected at each phase 6h after the caffeine test, following 24h abstinence from caffeine-containing foods and beverages (box preceding coffee cup). Reference levels of estradiol, progesterone, LH and FSH during menstrual cycle are shown in different line types. (**B)** Literature review of sample collection phases determining xanthine pharmacokinetics across menstrual cycle. Reduced CYP1A2 activity is reported in studies which include LFP sampling in their experimental protocol and compare EFP and LP to LFP. Conversely, no effect in enzyme activity is reported in studies which are designed to make comparisons between Follicular and Luteal Phases.

Figure 2. Metabolic pathways involved in caffeine metabolism in humans. The major pathway in the metabolism of caffeine is catalyzed by CYP1A2 and it involves the N-1, N-3 and N-7 demethylations of caffeine (137X) to form theobromine (3, 7-dimethyl xanthine, 37X), paraxanthine (1,7-dimethylxanthine, 17X) and theophylline (1,3-dimethylxanthine, 13X), respectively (shown in blue), accounting for about 80, 11 and 4% of caffeine metabolism (heavy arrows; Lelo et al., 1986; Gu et al., 1992). Dimethyl-xanthines are N-demethylated to the corresponding monomethylxanthine, 1-methylxanthine (1X), 3-methylxanthine (3X) and 7-methylxanthine (7X). Caffeine and xanthines are hydroxylated into their corresponding uric acids 1,3,7-trimethyluric acid (137U), 1,3-dimethyluric acid (13U), 1,7-dimethyluric acid (17U), 3,7-dimethyluric acid (37U), 1-methyluric acid (1U), 3-methyluric acid (3U) and 7-methyluric acid (7U). CYP2A6 catalyzes the conversion of paraxanthine to 17U. The

polymorphic enzyme N-acetyltransferase, NAT2, catalyzes the C8–N9 bond scission and the acetylation of paraxanthine to produce 5-acetylamino-6-formylamino-3-methyluracil (AFMU) which is then converted non-enzymatically into 5-acetylamino-6-amino-3-methyluracil (AAMU) in urine. Xanthine oxidase (XO) is responsible for the conversion of 1X into 1U. Metabolites, enzymes and metabolic molar ratios used as indices of enzyme activities in the present study are shown in red. Dashed arrows indicate minor metabolic pathways.

Figure 3. Hormone levels during menstrual cycle. Individual data **(A)** and mean estimates (±SE) (**B)** of estradiol (E2), progesterone (PRG), LH and FSH in 42 healthy volunteers (Group 1: n=21; Group 2: n=15; Group 3: n=6) at the three sampling phases (EFP, LFP and LP). Asterisk (*) denotes that EFP is different from LFP and LP; cross (†) denotes that LP is different from EFP and LFP. CMRs were compared across sampling phases at three levels (EFP, LFP, LP) using One-Way Repeated Measures ANOVA.

Figure 4. CYP1A2 *in vivo* indices during menstrual cycle. **(A)** Individual data (upper graphs) and mean estimates (±SE, lower graphs) of CYP1A2 CMRs in non-smoking (straight lines) and smoking (dashed lines) volunteers measured in the three groups of volunteers (Group 1: n=21; Group 2: n=15; Group 3: n=6) at EFP, LFP and LP. Thick solid lines in lower graphs represent overall (non-smoking and smoking) mean CYP1A2 CMR estimates (error bars denote 95% CIs). The asterisk (*) denotes that the overall CYP1A2 CMR value in Group 1 is significantly reduced at LFP compared to EFP ($p=0.002$) and LP ($p<0.001$) (One-Way Repeated Measures ANOVA). The paragraph sign (§) denotes that overall CYP1A2 CMR value in Group 1 is reduced at LFP compared to EFP ($p=0.056$) and LP ($p=0.003$) (One-Way Repeated Measures ANOVA). Group 3 did not exhibit any significant difference in CYP1A2 CMRs among the three sampling phases. **(B)** PCR amplification products of genomic DNAs were extracted from

peripheral blood (***b1***, left); M: 100 bp DNA ladder, NG: negative sample. PCR products were digested by PspOMI and PCR-RFLP of CYP1A2*1F (rs762551) polymorphism were subjected to agarose gel electrophoresis (***b1***, right); A/A genotype: positions 1,2,3,5,7,8, C/A genotype: positions 4, 6, 9. ***b2:*** Scatter plot of CYP1A2 CMRs, considered at EFP, of all volunteers (n=42) stratified by genotype and smoking. Mean CYP1A2 CMR values (horizontal lines) are higher in smokers compared to non-smokers ($p<0.001$; T-test). Symbols for genotype are as follows: non-smokers: diamond C/C (n=1), filled circles C/A (n=15), open circles A/A (n=10); smokers: filled triangles C/A (n=9), open triangles A/A (n=7). ***b3*:** Mean CYP1A2 CMR estimates (±SE) at EFP, LFP and LP in Group 1 (n=21). Neither genotype nor smoking exhibited any significant interaction with sampling phase; straight lines: non-smokers (n=15), dashed lines: smokers (n=6).

Figure 5. CYP2A6, XO and NAT2 *in vivo* indices during menstrual cycle. Individual data **(A)** and mean estimates (±SE) (**B)** of CMRs measured in the three groups of volunteers (Group 1: n=21; Group 2: n=15; Group 3: n=6) at EFP, LFP and LP. Asterisk (*) denotes that CYP2A6 CMR at LFP is significantly higher compared to EFP ($p=0.002$) and LP ($p=0.009$) (One-Way Repeated Measures ANOVA). Similarly, NAT2 CMR (slow-acetylators) at LFP is significantly higher compared to EFP ($p=0.002$) and LP ($p=0.002$) (One-Way Repeated Measures ANOVA).

Table 1. Demographic characteristics and biochemical data of the participants (n=42; non-smokers n=26, smokers n=16).

		ALL		Non-smokers		Smokers		
Variable		**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	**Reference value**
Age (years)		30.5	18-45	30	18-45	32	18-44	
Weight (kg)		63	46-95	62	46-95	63	53-90	
Body mass index (BMI, kg/m^2)		22.4	17.5-40.6	21.9	17.5-	22.8	18.6-33.9	
Duration of menstrual cycle (days)		30	27-33	28	27-30	28	28-35	
Sampling days	EFP	3	2-4	3	2-4	3	2-4	
	LFP	11	10-12	11	10-12	11	10-12	
	LP	22	19-25	22	19-25	22	20-24	
AST (IU/L)		15.0	9.0-32.0	15.0	10.0-	18.0	9.0-32.0	5.0-32.0
ALT (IU/L)		11.0	7.0-40.0	11.0	7.0-	11.5	7.0-28.0	5.0-41.0
ALP (IU/L)		57.5	39.0-84.0	55.0	35.0-	63.5	40.0-78.0	40.0-129.0
γ-GT (IU/L)		11.0	7.0-23.0	9.0	7.0-	12.5	8.0-23.0	5.0-39.0
Serum creatinine (mg/dl)		0.70	0.49-0.97	0.70	0.56-	0.68	0.49-0.87	0.50-0.90
Serum urea (mg/dl)		22.0	14.0-43.0	26.0	14.0-	21.5	14.0-27.0	15.0-50.0

Table 2. Primers and PCR conditions used for CYP1A2 genotyping.

SNP	Primer name	Sequence 5'→ 3'	PCR Program	$[Mg^{2+}]$ (mM)	PCR product size (bp)	Restriction enzyme	Incubation Temperature	Genotype patterns
rs2069514 (-3860 G→A)	Fw3Rv2	GCTACACATGATC GAGCTATAC CAGGTCTCTTCAC TGTAAAGTTA	[94^{0}C 10min] &35x [94^{0}C-30s 56^{0}C-30s 72^{0}C 1min]	2.0	568	BslI	55^{0}C	G/G:343-132-93 A/A:475-93 G/A:
rs762551 (-163 C→A)	Fw1Rv1	TGAGGCTCCTTTC CAGCTCTCA AGAAGCTCTGTGG CCGAGAAGG	[94^{0}C 10min] & 35x [94^{0}C-30s 60^{0}C-30s 72^{0}C 1min]	2.5	265	PspOMI	37^{0}C	C/C:211-54 A/A:265 C/A:

Table 3. Serum hormone concentrations and enzyme CMR values in all groups studied. For each sampling phase, measured values are reported as parameter estimates (mean values±SE) and model results as estimated marginal means with 95% CIs (in parentheses).

			Group 1 (n=21)				*Group 2 (n=15)*				*Group 3 (n=6)*		
Parameter measured			**EFP**	**LFP**	**LP**		**EFP**	**LFP**	**LP**		**EFP**	**LFP**	**LP**
E2	(pg/ml)		51.67±4.70	248.29±20.10*	121.67±11.77†		40.60±5.63	69.93±7.95	148.67±25.96†		29.67±10.66	49.63±10.83	49.67±8.60
PRG	(ng/ml)		0.55±0.07	0.69±0.09	12.18±1.49†		0.60±0.09	0.81±0.20	12.70±1.85†		0.50±0.10	1.13±0.66	0.98±0.26
LH	(mIU/ml)		5.30±0.40	17.25±3.76*	3.92±0.45		5.00±0.48	6.84±0.63	6.28±1.16		4.03±0.51	6.38±0.92	6.53±1.04
FSH	(mIU/ml)		7.71±0.45	7.64±1.31	3.17±0.26†		7.40±0.67	6.18±0.61	3.78±0.39†		6.82±1.44	5.58±0.54	5.17±0.98
CYP1A2	**ALL**	**n=20&**	4.53	3.49*	4.24	**n=15**	4.31	3.77§	4.35	**n=6**	3.92	4.12	3.51
			(3.93-5.13)	(3.09-3.88)*	(3.80-4.68)		(3.36-5.25)	(3.16-4.43)§	(3.72-4.97)		(3.03-4.80)	(3.00-5.24)	(2.81-4.22)
	non-smokers	***n=14&***	3.02±0.17	2.61±0.17	3.08±0.21	***n=8***	3.52±0.53	2.95±0.32	3.14±0.34	***n=3***	3.31±0.33	3.10±0.55	3.18±0.32
	*CYP1A2*1F C/A*	*n=7*	2.86±0.26	2.46±0.14	2.84±0.15	*n=5*	4.05±0.76	3.23±0.48	3.33±0.53	*n=3*	3.31±0.33	3.10±0.55	3.18±0.32
	A/A	*n=7*	3.17±021	2.76±0.32	3.31±0.40	*n=3*	2.63±0.21	2.49±0.12	2.83±0.19				
	C/C	*n=1*	1.79	1.92	1.75								
	smokers	***n=6***	5.71±0.83	4.32±0.35	5.04±0.57	***n=7***	5.18±0.59	4.90±0.48	5.79±0.40	***n=3***	3.79±0.92	4.11±1.05	3.38±0.62
	*CYP1A2*1F C/A*	*n=2*	7.06±2.22	4.47±0.10	6.50±0.99	*n=5*	5.05±0.84	5.19±0.63	6.03±0.53	*n=2*	2.91±0.49	3.06±0.15	2.78±0.23
	A/A	*n=4*	5.04±0.67	4.24±0.55	4.31±0.35	*n=2*	5.49±0.26	4.18±0.10	5.20±0.45	*n=1*	5.54	6.20	4.59
CYP2A6	**ALL**	**n=21**	0.60±0.027	0.70±0.029*	0.63±0.028	**n=15**	0.62±0.033	0.67±0.028‡	0.56±0.030	**n=6**	0.57±0.047	0.65±0.034	0.68±0.045
XO	**ALL**	**n=21**	0.52±0.013	0.52±0.012	0.51±0.010	**n=15**	0.52±0.013	0.54±0.013	0.51±0.014	**n=6**	0.52±0.025	0.52±0.012	0.52±0.028
NAT2	**slow**	**n=11**	0.074±0.007	0.090±0.006*	0.073±0.006	**n=11**	0.082±0.010	0.081±0.008	0.078±0.008	**n=5**	0.100±0.010	0.094±0.004	0.088±0.010
	fast	**n=10**	0.36±0.016	0.38±0.019	0.36±0.014	**n=4**	0.41±0.031	0.38±0.025	0.37±0.029	**n=1**	0.41	0.37	0.40

[&]C/C has been excluded from the model

*LFP different from EFP $p<0.01$ and LP $p<0.01$

†LP different from EFP and LFP $p<0.01$

§LFP different from LP $p<0.01$ (but not from EFP $p=0.056$)

‡LFP different from LP $p=0.027$

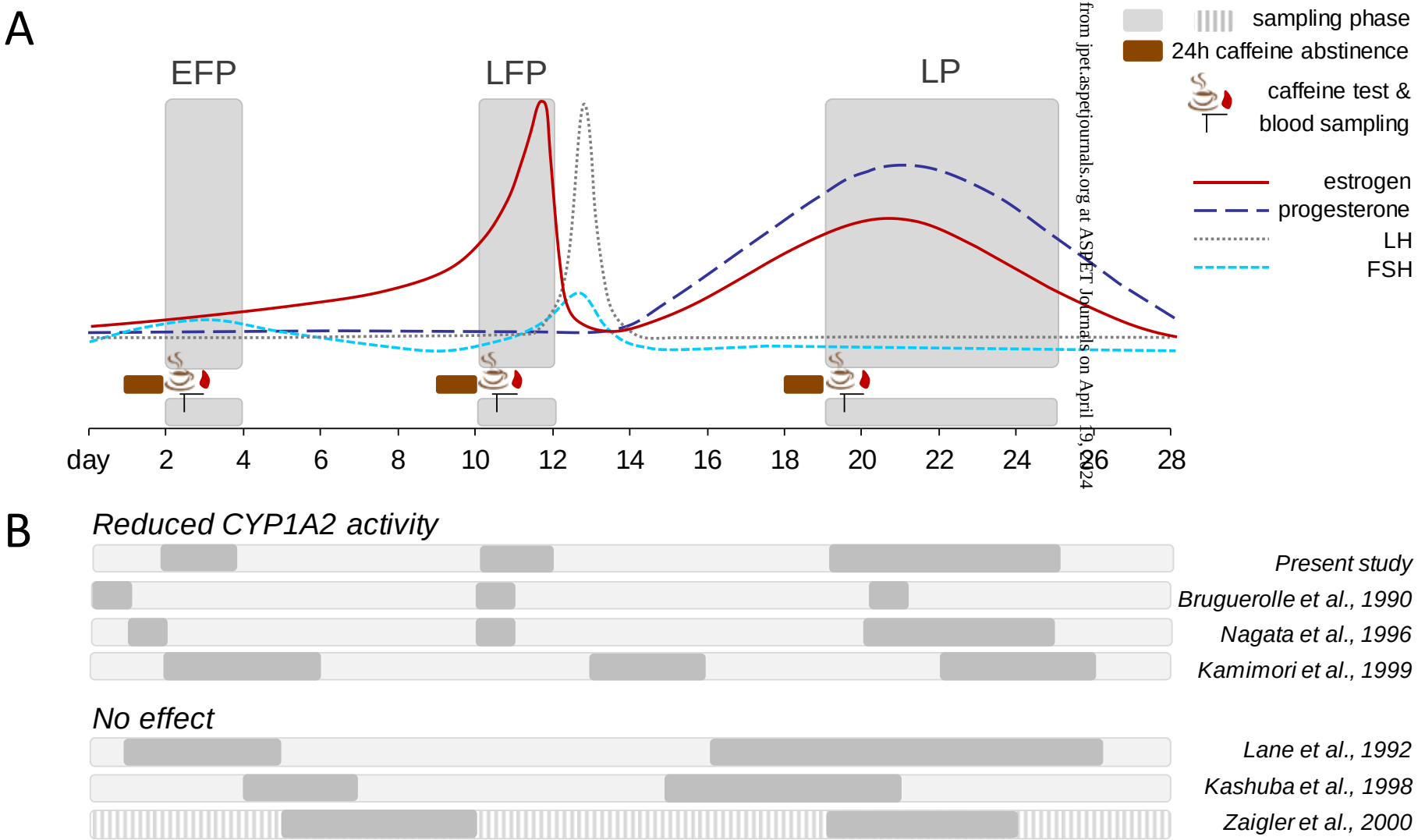

Figure 1

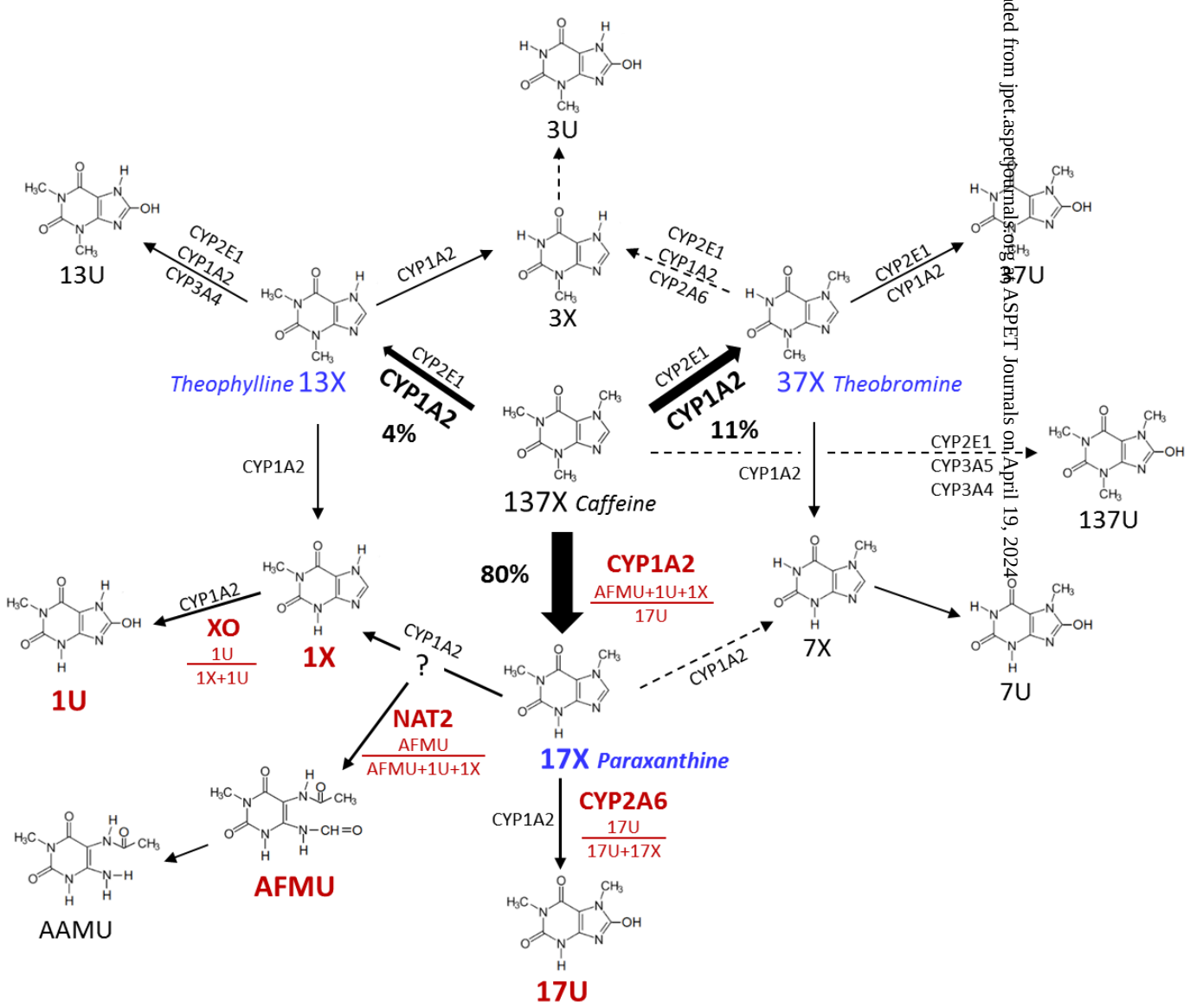

Figure 2

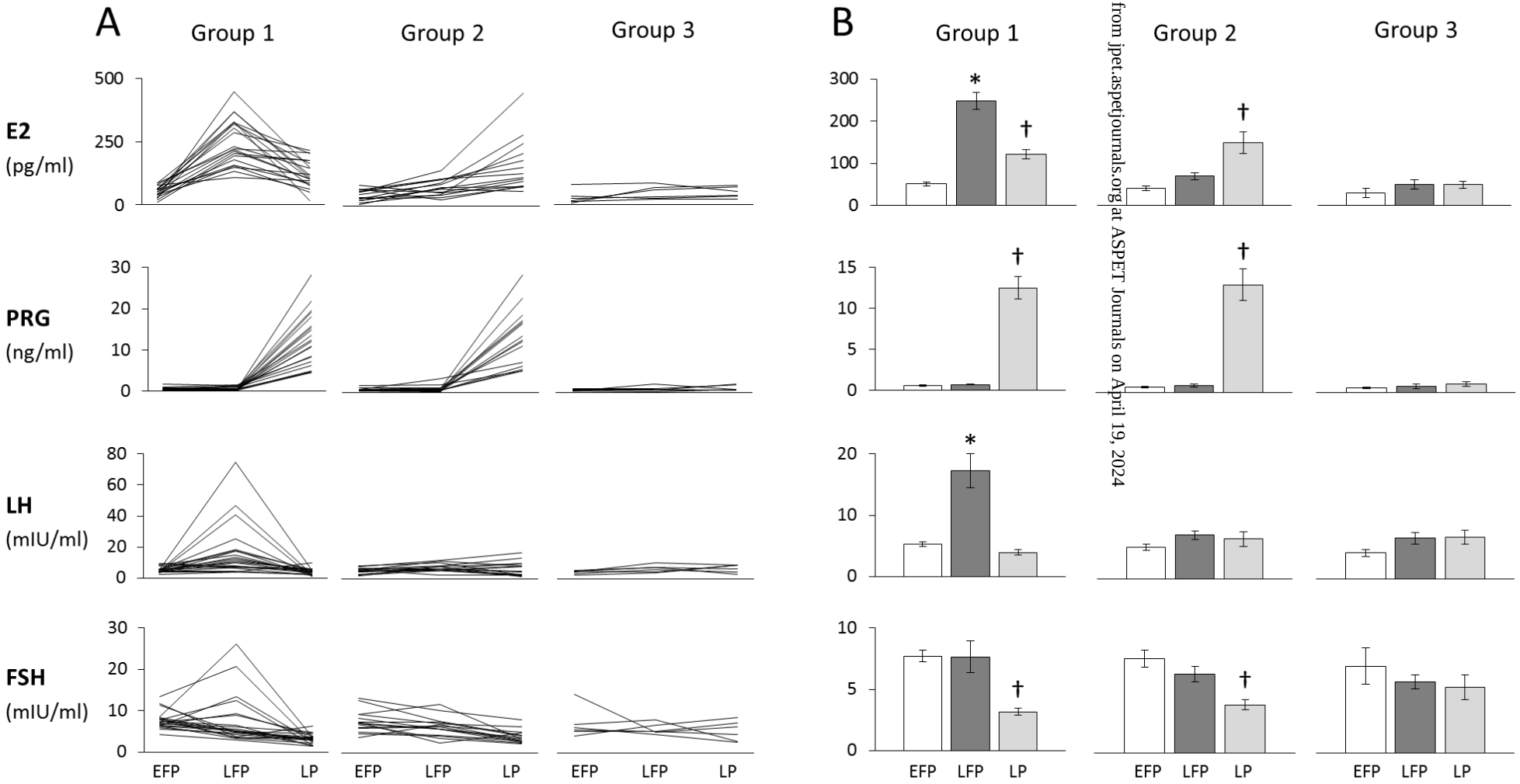

Figure 3

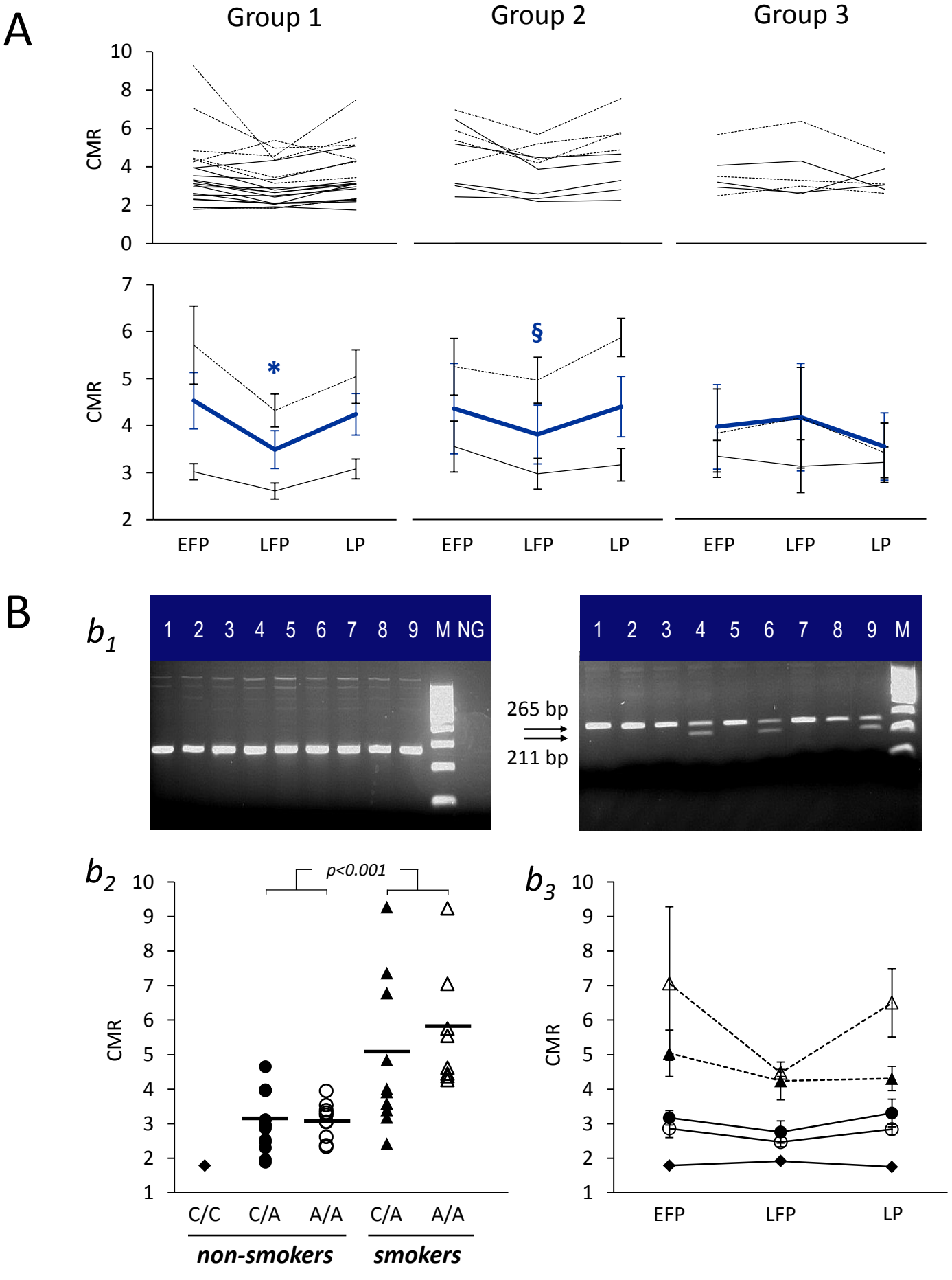
A
Group 1
Group 2
Group 3
CMR
EFP
LFP
LP
*
§
B
b1
1 2 3 4 5 6 7 8 9 M NG
1 2 3 4 5 6 7 8 9 M
265 bp
211 bp
b2
p<0.001
C/C
C/A
A/A
C/A
A/A
non-smokers
smokers
b3

Figure 4

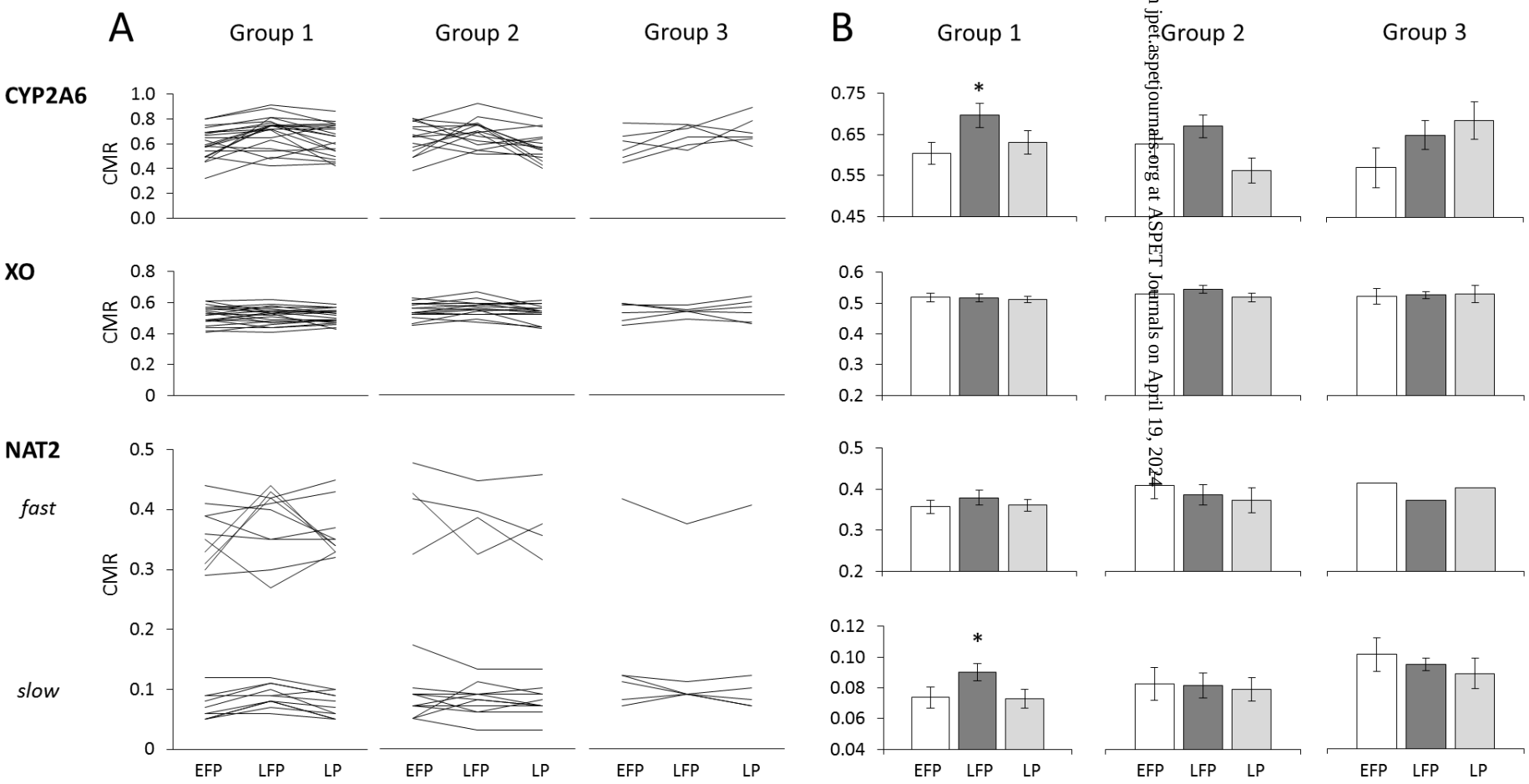

Figure 5

"*Alterations in xenobiotic-metabolizing enzyme activities across menstrual cycle in healthy volunteers*"

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Journal of Pharmacology and Experimental Therapeutics

Supplemental Methods: Methodology of the study design, visits and data collection.

Visit 1: before entering the study:

V.T., M.D. (co-author) held an interview with the volunteers at the health examination station during which she explained the purpose of the study. During this visit, V.T.:

1. provided detailed description of the requirements of the study in terms of diet restrictions, methylxanthine abstinence and collection of biological samples during the month of the study
2. took a medical history about chronic diseases, medication intake and oral contraceptive or any other hormone-based birth control use
3. took a reproductive history on age at menarche, regularity and duration of menstrual cycle, duration of menstruation; *inclusion criteria:* only women with a self-reported menstrual cycle between 25 and 35 days for each menstrual cycle in the preceding 6 months without evidence of oligo-, poly- or amenorrhea or metromenorrhagia, as identified by screening questionnaire, were included; *exclusion criteria:* use of oral contraceptives or other hormone birth control method in the preceding 3 months prior to study enrollment, pregnancy, conditions known to affect menstrual cycle function (such as confirmed endometriosis of any stage, polycystic ovary syndrome), women with a history of chronic diseases (such as heart disease, inflammatory of autoimmune

disease, diabetes mellitus, thyroid disease or any other endocrine function), women on medications known to affect the activity of the enzymes of interest

4. asked about lifestyle characteristics: smoking, number and type of cigarettes, time of cigarette cessation if applicable, exposure to passive smoking, quantity and type of daily coffee intake, quantity and frequency of alcohol consumption (sporadic social alcohol consumption, i.e. 2-3 glasses of wine/week, was not considered as an exclusion criterion from the study; alcohol consumption was prohibited 48h prior to caffeine test)
5. collected demographic data on age and occupation; volunteers who were chronically exposed to fuels, solvents or xenobiotics such as pesticides or any other chemicals were excluded from the study
6. gathered the anthropometric data weight and height
7. performed a physical examination (vital signs, visual and physical exam)
8. collected a blood sample in order to examine routine biochemical indices (AST, ALT, ALP, γ-GT, creatinine, urea); an aliquot was used to perform DNA analysis for the detection of CYP1A2 polymorphisms
9. asked the volunteers to give their written consent in a form containing all information about the purpose of the study

Visit 2: upon entering the study:

A volunteer who met the inclusion criteria and the health requirements gathered from visit 1, was asked by V.T. to a second session during which menstrual cycle information was overviewed and the three subsequent visits (1st visit at EFP, 2nd visit at LFP and 3rd visit at LP) were planned using an algorithm accounting for each woman's self-reported cycle length. During this second meeting the volunteer was supplied with sterile urine-sample

containers; in addition, for reasons of convenience to the volunteer and protocol standardization, volunteers were supplied with caffeine gelatin-free capsules of a 200 mg standard dose prepared by a local pharmacist. All subjects were also handed a brochure with pertinent information for constant reference to the study protocol and the 48h diet restrictions (abstinence from cruciferous and apiaceous vegetables, grapefruit, broiled meat, alcohol consumption). The volunteers were instructed to consume limited amounts of the above-mentioned foods and drinks and, more importantly, to avoid changes in their diet during the month of the study; menu suggestions were included in order to facilitate the adherence of volunteers to the protocol.

Visits 3, 4, and 5: biological sample collection:

On the days of the caffeine test, V.T. collected urine and blood samples and inquired information about possible violations to the study protocol; the volunteers were, once again, instructed to adhere to the protocol and the required 48h diet and 24h methylxanthine restrictions. Blood samples were transferred to the Hospital Biochemistry Laboratory for determination of hormonal concentrations; an aliquot was transferred to the Department of Pathology for CYP1A2 genotyping. Urine samples were transferred to the Laboratory of Pharmacology, the pH was adjusted to 3.5 and aliquots were stored at -20^{0}C until sample pretreatment and chromatographic analysis.

Supplemental Table 1. Serum hormone concentrations determined in all participants of the study at the three sampling phases EFP, LFP and LP.

		Estradiol (pg/ml)			Progesterone (ng/ml)			LH (mIU/ml)			FSH (mIU/ml)		
	ID	**EFP**	**LFP**	**LP**	**EFP**	**LFP**	**LP**	**EFP**	**LFP**	**LP**	**EFP**	**LFP**	**LP**
Group 1													
non-smokers	GN	41	131	63	0.3	0.6	15.8	4.8	74.4	5.0	8.5	26.1	3.6
	GS	63	327	17	0.4	0.6	4.6	3.8	9.9	3.7	8.0	4.9	3.7
	TA	31	153	50	1.8	1.4	7.1	5.2	12.6	4.4	8.4	5.1	4.5
	KE	65	320	164	0.4	0.3	15.5	5.2	7.5	5.7	8.2	3.5	3.0
	NA	59	221	206	0.3	0.2	19.2	3.9	4.1	2.4	5.9	4.3	2.9
	SS	58	148	119	0.4	0.4	14.8	4.6	11.3	5.1	7.1	5.3	3.2
	SO	76	107	95	0.5	0.4	5.0	3.7	6.8	6.0	6.3	5.2	1.7
	SA	9	192	175	0.6	0.7	10.7	9.2	6.2	1.9	6.3	3.4	2.2
	SI	20	203	176	0.4	1.4	12.5	6.1	18.6	4.9	6.7	9.3	3.9
	SC	29	325	103	0.4	0.5	4.8	3.6	17.5	5.2	7.2	9.0	4.4
	TE	27	446	147	0.9	1.5	8.2	4.2	46.5	2.3	7.8	12.4	1.6
	TA	39	211	101	0.5	0.5	28.2	7.7	7.5	4.3	11.8	3.7	6.3
	TSA	51	302	74	0.3	0.3	13.5	8.4	15.1	2.2	11.3	4.8	3.4
	TP	42	369	118	0.4	0.7	12.0	5.4	18.1	1.5	7.5	6.6	1.5
	KT	61	284	216	0.8	0.9	19.5	5.4	10.6	6.0	5.6	4.6	3.2
smokers	GP	54	157	88	0.3	0.3	4.5	2.6	3.9	9.8	6.8	6.2	4.8
	KE	45	178	82	0.6	0.7	18.0	4.3	4.5	2.3	4.3	2.9	1.5
	LB	87	218	102	0.6	0.9	6.3	4.0	40.8	0.9	13.4	20.7	2.3
	PA	60	367	109	0.5	0.5	10.9	9.2	7.7	3.4	7.9	3.5	3.5
	PP	82	230	146	0.6	1.2	8.5	5.3	25.1	1.8	6.3	13.4	2.6
	TK	86	325	204	0.5	0.5	21.8	4.6	13.6	3.5	6.6	5.6	2.8
Group 2													
non-smokers	AE	61	52	103	0.4	0.4	5.1	7.5	11.5	16.3	7.2	7.3	3.9
	DA	21	65	54	0.3	0.1	18.2	5.8	2.2	2.4	7.2	2.3	4.7
	KS	55	98	174	0.7	0.6	11.9	8.2	9.9	2.2	8.1	5.6	3.3
	KA	60	101	122	0.4	0.4	6.2	2.0	5.2	1.9	4.8	4.2	2.8
	MS	5	71	74	0.3	0.3	12.3	4.2	6.0	4.7	12.9	10.0	7.7
	TF	19	67	108	1.1	1.0	10.9	5.1	10.8	8.1	5.8	6.2	2.7
	TV	29	33	73	0.5	0.4	5.4	1.6	7.8	9.3	3.6	6.6	2.9
	TA	12	43	236	0.7	1.0	17.0	2.9	6.3	2.4	9.0	11.4	3.2
smokers	DP	32	40	94	1.6	1.7	4.9	4.5	6.1	9.7	6.0	6.4	4.8
	ZE	64	24	76	0.3	0.9	27.7	5.3	7.5	1.3	12.3	7.4	4.1
	IK	44	81	198	0.5	0.5	13.2	6.2	7.9	13.0	9.0	6.9	6.1
	KS	79	49	76	0.6	3.2	7.0	6.7	5.0	2.9	4.5	3.9	2.3
	LE	51	134	428	0.7	0.8	16.6	4.5	5.2	7.7	7.1	5.6	2.5
	MV	52	103	145	0.5	0.5	16.2	6.0	6.0	4.3	6.8	5.5	3.5
	PP	25	88	269	0.4	0.3	22.2	4.5	5.2	8.0	6.7	3.4	2.2
Group 3													
non-smokers	DT	12	21	24	0.4	0.4	0.5	2.0	3.9	8.6	5.4	5.0	7.1
	MS	17	60	72	0.2	0.1	0.7	4.7	4.4	8.8	13.8	4.8	6.1
	TV	35	27	34	0.7	0.6	0.5	4.2	10.0	8.5	5.1	5.1	4.4

smokers	DK	27	33	39	0.8	0.8	1.7	4.9	7.3	6.2	6.0	4.3	2.5
	DX	79	87	53	0.3	2.0	0.6	3.1	5.6	4.4	4.0	6.5	8.3
	MM	8	69.8	76	0.6	0.5	1.9	5.3	7.1	2.7	6.6	7.8	2.6