Chronic Cigarette Smoking Alters Circulating Sex Hormones and Neuroactive Steroids in Premenopausal Women

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Summary

Chronic smoking alters the circulating levels of sex hormones and possibly also the neuroactive steroids. However, the data available is limited. Therefore, a broad spectrum of free and conjugated steroids and related substances was quantified by GC-MS and RIA in premenopausal smokers and in age-matched (38.9±7.3 years of age) non-smokers in the follicular (FP) and luteal phases (LP) of menstrual cycle (10 non-smokers and 10 smokers, in the FP, and 10 non-smokers and 8 smokers in the LP). Smokers in both phases of the menstrual cycle showed higher levels of conjugated 17-hydroxypregnenolone, 5α-dihydroprogesterone, conjugated isopregnanolone, conjugated 5α-pregnane-3β,20α-diol, conjugated androstenediol, androstenedione, testosterone, free testosterone, conjugated 5α-androstane-3α/β,17β-diols, and higher free testosterone index. In the FP, the smokers exhibited higher levels of conjugated pregnenolone, progesterone, conjugated pregnanolone, lutropin, and a higher lutropin/follitropin ratio, but lower levels of cortisol, allopregnanolone, and pregnanolone. In the LP, the smokers exhibited higher levels of free and conjugated 20α-dihydropregnenolone, free and conjugated dehydroepiandrosterone, free androstenediol, 5α-dihydro-testosterone, free and conjugated androsterone, free and conjugated epiandrosterone, free and conjugated etiocholanolone, 7α/β-hydroxy-dehydroepiandrosterone isomers, and follitropin but lower levels of estradiol and sex hormone binding globulin (SHBG) and lower values of the lutropin/follitropin ratio. In conclusion, chronic cigarette smoking augments serum androgens and their 5α/β-reduced metabolites (including GABAergic substances) but suppresses the levels of estradiol in the LP and SHBG and may induce hyperandrogenism in female smokers. The female smokers had pronouncedly increased serum progestogens but paradoxically suppressed levels of their GABA-ergic metabolites. Further investigation is needed concerning these effects.

Key words

Smoking • Menstrual cycle • Luteal phase • Follicular phase • Sex hormones • Neuroactive steroids

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Introduction

Cigarette smoking is one of the most serious substance abuse problems. A variety of human and animal studies have shown that nicotine can induce changes in female hormonal balance (Kapoor and Jones 2005, Tutka 2001, Tziomalos and Charsoulis 2004, Windham *et al.* 2005). Besides for the common

reproductive functions in women (Kapoor and Jones 2005, Windham *et al.* 2005), such as the menstrual cycle, fertility, gravidity and the health of the fetus, chronic smoking may also influence the activities of the pituitary-adrenal axis and central nervous system (Rohleder and Kirschbaum 2006). The effects of chronic smoking on cognition (Durazzo and Meyerhoff 2007, Hill *et al.* 2003, Richards *et al.* 2003, Sabia *et al.* 2008), mood (Bertone-Johnson *et al.* 2008), and severity of drug addiction (Sofuoglu *et al.* 2009, Sofuoglu *et al.* 2007) have been reported. It is generally accepted that the chemicals in tobacco smoke alter endocrine function in women, perhaps at the level of the ovaries, which in turn influences the release of pituitary hormones. More than 30 carcinogenic chemicals, including nicotine, cotinine cadmium, and polyaromatic hydrocarbons, are present in tobacco smoke; many of them are fat-soluble and resistant to metabolism (Mlynarcikova *et al.* 2005, Mukherjee *et al.* 2006, Shiverick and Salafia 1999). This endocrine disruption likely contributes to reported associations of smoking with adverse reproductive outcomes, including menstrual dysfunction (Windham *et al.* 1999), infertility, and earlier menopause. Studies on the effects of smoking on female sex hormones have concentrated mainly on estrogens and progesterone in the follicular phase of the menstrual cycle (Lucero *et al.* 2001, Zumoff *et al.* 1990).

Mood and well-being are strongly affected by neurotransmitters and corresponding receptors in brain structures. The effect of smoking on the concentration of gamma-aminobutyric acid (GABA) in the brain has been reported (Epperson *et al.* 2005). Short-term abstinence had no significant effect on cortical GABA concentrations in either men or women. There was, however, a significant effect of sex, diagnosis (smoker/non-smoker), and the menstrual cycle phase on cortical GABA levels. The female smokers experienced a significant reduction in cortical GABA levels during the follicular phase but no cyclicity in GABA levels across the menstrual cycle. Alternatively, cortical GABA levels were similar in smoking and nonsmoking men. The aforementioned data indicate possible alterations in the biosynthesis and/or catabolism of neuroactive steroids in premenopausal smokers. However, only little attention was paid to neurosteroids (known as potent modulators of type A GABA receptors ($GABA_A$-r) and NMDA receptors (NMDA-r)) in these subjects. Therefore, we attempted to establish whether the pattern of circulating neuroactive steroids, their precursors and metabolites (Figures 1 and 2), differs between premenopausal smokers and age-matched non-smokers in the follicular (FP) and luteal phases (LP) of the menstrual cycle.

Patients and Methods

A broad spectrum of C_{19} and C_{21}-steroid metabolites and related substances was determined by GC-MS and RIA in four groups of smokers and in age-matched non-smokers in the follicular (FP) and luteal phases (LP) of the menstrual cycle. The first group consisted of premenopausal chronic smokers in the FP (n=10), while the second group involved the age- and phase of the menstrual cycle-matched non-smokers (n=10). The third group contained premenopausal chronic smokers in the LP (n=8) and the fourth group included the age and phase of the menstrual cycle-matched non-smokers (n=10). The patients were smokers who were interested in cessation and visited The Center for Tobacco Dependence, First Faculty of Medicine and General University Hospital, Prague. The controls had no history of smoking.

The study included women with regular menstrual cycles lasting 28 days. This inclusion criterion was fulfilled in both controls and patients. The blood in both groups was collected the 3^{rd}-5^{th} day of the menstrual cycle in the FP and the 22^{nd}-24^{th} day in the LP. The women, in whom the hormonal parameters did not accord with the phase of the menstrual cycle, were additionally excluded from the study. For the correct determination of the phase of the menstrual cycle, we have used the patient's self report and we also checked their serum hormonal levels. The ranges within the 3^{rd} and the 5^{th} day and within the 22^{nd} and 24^{th} day of the menstrual cycle were selected due to organizational reasons. In both patients and controls, we strived to complete the blood withdrawal as closest to the 3^{rd} and 22^{nd} days of the menstrual cycle as possible.

For ethical reasons, the design of the study does not allow sampling the same women in both cycles, even if the multiple cycles sampling in the same two groups (smokers vs. non-smokers) would be optimal and would increase the validity of our findings. Unfortunately, it was impossible to harmonize the patient's preference of the day, when the woman decided to cease smoking, with her menstrual cycle. Therefore, only those smokers who were within the aforementioned ranges for FP or LP on the beginning of the smoking cessation were included in the study.

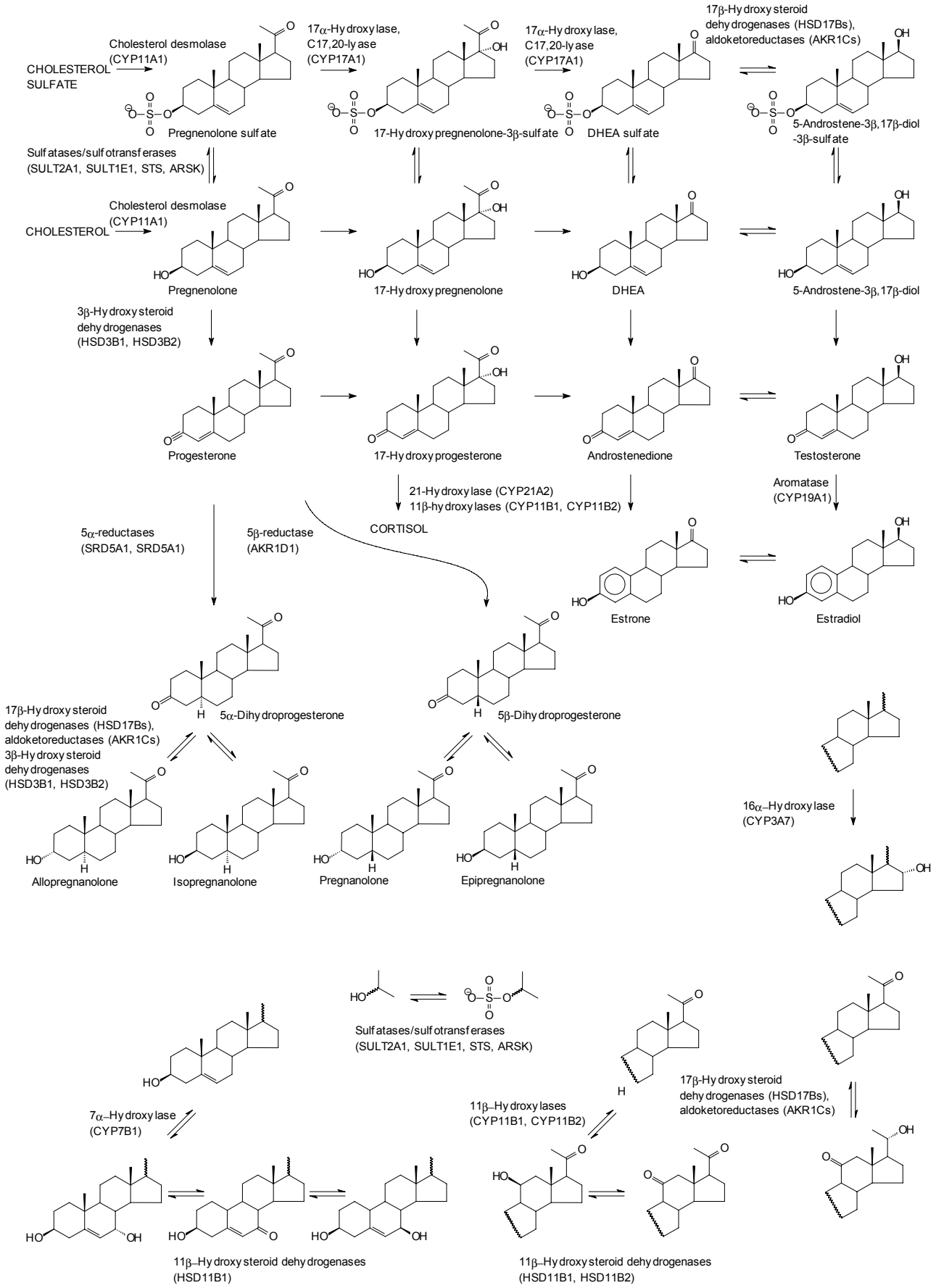

Fig. 1. Simplified scheme of steroidogenesis.

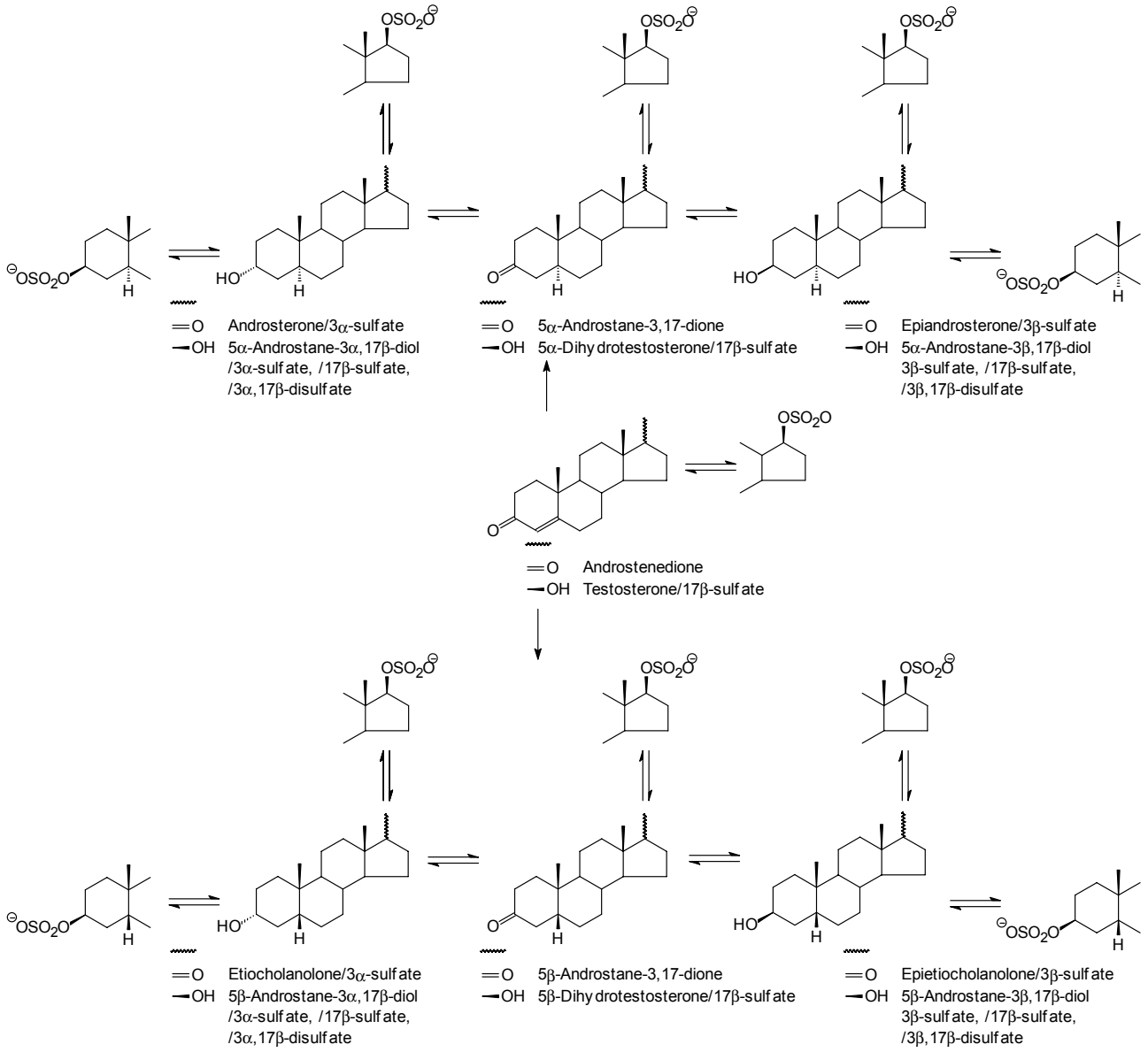

Fig. 2. Simplified scheme of the biosynthesis of 5α/β-reduced C19 steroids.

Neither the control nor patients used hormonal contraception at least 6 months before the trial. They did not use anti-inflammatory drugs, antiepileptic drugs, antidepressants, or medication influencing the steroidogenesis. The women did not suffer from chronic disease or endocrinopathy. The age and BMI of the participants and the smoking behaviors of the smokers are illustrated in Table 1. The BMI of the controls and smokers did not differ significantly and was within the normal limit. The exclusion criteria for both groups were endocrine disease of any type, the use of hormonal contraception, and irregular menstruation.

A peripheral blood sample (10 ml) was collected from the cubital vein, either in the FP or LP. The phase of the cycle was checked by the determination of circulating gonadotropins, estradiol, and progesterone. A progesterone serum level over 6 nmol/l was selected as the inclusion criterion for the LP (Young 1987). Cooled plastic tubes were used for blood sampling. The serum was obtained after centrifugation for 5 minutes at 2000 g at 4 °C. The serum samples were stored at −20 °C until analyzed.

The Ethics Committee of the Institute of Endocrinology approved the study, and all the patients and volunteers signed informed consent forms before participating in the study.

Table 1. Comparison of anthropometric characteristics and indices of smoking in the sera of premenopausal female non-smokers and sex- and age-matched smokers (the data are shown as medians with quartiles in the parentheses).

Variable	FOLLICULAR PHASE		LUTEAL PHASE		ANCOVA
	Non-smokers	Smokers	Non-smokers	Smokers	followed by LSD multiple
	CF	SF	CL	SL	comparisons
Age	39.5 (31.8, 44.1)	39.7 (32.2, 48.1)	35.3 (31.7, 44.1)	38.7 (35, 43.5)	NS
BMI	21.3 (20.9, 23.9)	22.3 (20.9, 24.3)	21.3 (20.9, 23.9)	20.4 (19.4, 21.9)	NS
Cigarette consumption/day	0 (0, 0)	22.5 (20, 25)	0 (0, 0)	25 (20, 25)	-----
First experience with cigarette	-----	15.5 (15, 17.8)	-----	16 (14.8, 18.3)	-----
Start of smoking	-----	19 (17, 21)	-----	19 (18, 21.3)	-----
Number of attempts to cease smoking	-----	2.5 (2, 4.5)	-----	2.5 (1.75, 3)	-----
FTND	-----	5 (2, 7.25)	-----	6.5 (2.75, 8)	-----
CDS5	-----	18 (16.5, 20.3)	-----	18.5 (17.8, 21.3)	-----
CDS12	-----	49.5 (40.8, 55.5)	-----	52.5 (44.5, 57)	-----

NS=not significant; CF=controls in the follicular phase; CL=controls in the luteal phase; SF=smokers in the follicular phase; SL=smokers in the luteal phase

Steroids and chemicals

Steroid standards were purchased from Steraloids (Wilton, NH, USA), and the solvents for the extraction and HPLC of an analytical grade were from Merck (Darmstadt, Germany). The derivatization agent Sylon BFT was from Supelco (Bellefonte, PA, USA) and the derivatization agent methoxylamine hydrochloride, as well as the remaining chemicals and solvents were from Sigma-Aldrich (St. Louis, MO, USA).

Instruments

The GC-MS system GCMS-QP2010 Plus was supplied by Shimadzu (Kyoto, Japan). The system consisted of a gas chromatograph equipped with automatic flow control, an AOC-20s auto-sampler, and a quadrupole electron-impact detector with an adjustable electron voltage of 10-195 V. A capillary column with a medium polarity RESTEK Rxi (diameter 0.25 mm, length 15 m, film thickness 0.1 μm) was used for the analyses.

Steroid analysis

Pregnenolone, conjugated pregnenolone, progesterone, 5α-dihydroprogesterone, allopregnanolone, conjugated allopregnanolone, isopregnanolone, conjugated isopregnanolone, pregnanolone, conjugated pregnanolone, conjugated epipregnanolone, 20α-dihydro-pregnenolone, conjugated 20α-dihydropregnenolone, 20α-dihydroprogesterone, conjugated 5α-pregnane-3β,20α-diol, conjugated 5β-pregnane-3α,20α-diol, 16α-hydroxy-pregnenolone, dehydroepiandrosterone, conjugated dehydroepiandrosterone, androstenediol, conjugated androstenediol, androstenedione, testosterone, 5α-dihydrotestosterone, androsterone, conjugated androsterone, epiandrosterone, conjugated epiandro-sterone, etiocholanolone, conjugated etiocholanolone, conjugated epietiocholanolone, conjugated 5α-androstane-3α,17β-diol, conjugated 5α-androstane-3β,17β-diol, 7α-hydroxy-dehydroepiandrosterone, 7β-hydroxy-dehydroepiandrosterone, 5-androstene-3β,7α,17β-triol, and 5-androstene-3β,7β,17β-triol, measured using the GC-MS method that was published in detail elsewhere (Hill *et al.* 2009). The conjugated steroids were measured after hydrolysis according to Dehennin *et al.* (Dehennin *et al.* 1996).

Luteinizing hormone (LH) and follicle stimulating hormone (FSH) were estimated by IRMA kits from Immunotech (France). Sex hormone binding globulin (SHBG) and estradiol were assessed using IRMA kits from Orion (Finland). Cortisol was measured by the RIA kit from Orion (Finland). 17-hydroxy-progesterone was measured using the RIA kit from Immunotech (France). The immunoassays were processed on the automatic analyzer Stratec (France). Free and conjugated 17-hydroxypregnenolone was measured using our previously published methods (Hill *et al.* 1999, Vcelakova *et al.* 2007). The levels of free

testosterone were calculated using the method reported elsewhere (Vermeulen *et al.* 1999).

Statistical data analysis

The ANOVA model consisting of smoking status (smokers vs. non-smokers), phase of the menstrual cycle (follicular- vs. luteal phase) as the main factors, and smoking status × phase of the menstrual cycle interaction was used for the simultaneous evaluation of the effects of smoking and phase of the menstrual. Least significant difference multiple comparisons (LSD) followed the ANCOVA testing.

Due to the complexity of our results, which exhibit strong correlations between individual variables; the ANOVA testing was followed by multivariate regression with the reduction of dimensionality (bidirectional orthogonal projections to a latent structure, OPLS).

The OPLS method enables the prediction of the variable average daily consumption of cigarettes constituting the vector **Y** from variables constituting the matrix **X**. The predictivity of individual variables for the model may be simply expressed as a correlation of the variable with the common predictive component. The predictive component extracts the variability from **X**, which is shared between **X** and **Y** from the variability within the **X**, which is separated into the orthogonal components.

The OPLS method (Trygg *et al.* 2007, Trygg and Wold 2002) is effective in coping with the problem of severe multicollinearity within the matrix **X**. The OPLS enabled us to detect the variables with high predictivity for the description of relationships between **X** and **Y**. The OPLS model may be expressed as follows:

$$\mathbf{X} = \mathbf{T}_p \mathbf{P}_p^{\,t} + \mathbf{T}_0 \mathbf{P}_0^{\,t} + \mathbf{E}$$

$$\mathbf{Y} = \mathbf{T}_p \mathbf{P}_p^{\,t} + \mathbf{F}$$

where **X** is the matrix with *l* independent variables and *i* subjects, **Y** is the vector of dependent variable and *i* subjects. $\mathbf{T}_p$ and $\mathbf{T}_0$ represent the matrices of the component scores from the predictive and orthogonal components, respectively extracted from **X.** $\mathbf{P}_p$ and $\mathbf{P}_0$ represent the matrices of component loadings from the predictive and orthogonal component, respectively extracted from **X**. **E** and **F** are error terms.

We tested the relevance of individual variables for the model using a criterion Variable Importance (VIP). Only the variables that showed significance for the relevant predictive components were included in the model.

Most of the data showed skewed data distribution and heteroscedasticity. To attain the Gaussian distribution and constant variance in the data and residuals, the original data were transformed by a power transformation for correct statistical testing. For each variable, we have used the parameter providing maximum agreement with the Gaussian distribution as evaluated using linear regression between theoretical percentiles of the Gaussian distribution and actual percentiles for individual transformed variables. The statistical software Statgraphics Centurion version XV from Statpoint Inc. (Herndon Virginia, USA) was used for simultaneous data transformation, ANOVA testing, and LSD multiple comparisons. The same software was used for the determination of optimal data transformations before the subsequent multivariate regression. The multivariate regression was accomplished using statistical software SIMCA-P+ Version 12.0.0.0 from Umetrics AB (Umeå, Sweden). This software also allowed for the detection of multivariate non-homogeneities using Hotelling's T^2 statistics and to check the multivariate normal distribution and homoscedasticity using the multivariate normal probability plot for residuals.

Results

The characteristics of smoking behavior for both groups of patients are illustrated in Table 1. We found no significant differences in age or BMI between the groups of study participants.

The concentrations of serum LH, steroids, and steroid polar conjugates and SHBG exhibited significant differences between the smokers and non-smokers in the FP and/or LP (Tables 2 and 3). Smokers in both phases of the menstrual cycle showed higher levels of conjugated 17-hydroxypregnenolone, 5α-dihydroprogesterone, conjugated isopregnanolone, conjugated 5α-pregnane-3β,20α-diol, conjugated androstenediol, androstenedione, testosterone, free testosterone, conjugated 5α-androstane-3α/β,17β-diols, and higher free testosterone index. In the FP, the smokers exhibited higher levels of conjugated pregnenolone, progesterone, conjugated pregnanolone, lutropin, and a higher lutropin/follitropin ratio, but lower levels of cortisol, allopregnanolone, and pregnanolone. In the LP, the smokers exhibited higher levels of free and conjugated 20α-dihydropregnenolone, free and conjugated dehydroepiandrosterone, free androstenediol, 5α-dihydro-testosterone, free and conjugated androsterone,

Table 2. Comparison of the levels of C21 steroids (nmol/l), and gonadotropins (U/l) in the sera of premenopausal female non-smokers and sex- and age-matched smokers in follicular and luteal phases of menstrual cycle (the data are shown as medians with quartiles in the parentheses).

Variable	FOLLICULAR PHASE		LUTEAL PHASE		ANCOVA followed by LSD multiple comparisons
	Non-smokers CF	Smokers SF	Non-smokers CL	Smokers SL	
Pregnenolone	1.42 (1.07, 2)	1.79 (1.14, 2.3)	2.19 (1.92, 2.64)	3.79 (2.49, 5.16)	M*
Conjugated pregnenolone	63.8 (39.5, 103)	118 (92.6, 148)	64.2 (41.6, 96.3)	109 (104, 129)	S**, CF<SF
17-Hydroxy-pregnenolone	2.48 (1.52, 3.74)	4.4 (1.75, 7.74)	4.75 (3.15, 8.94)	2.1 (0.478, 5.1)	NS
Conjugated 17-hydroxy-pregnenolone	12 (7.79, 13.9)	33.9 (25.9, 37.1)	5.6 (3.99, 7.45)	41.7 (28.8, 47.8)	S***, S×M*, CF<SF, CL<SL
Progesterone	0.519 (0.405, 0.79)	2.76 (1.08, 3.32)	18.5 (14.7, 24.8)	74.9 (35.3, 140)	S*, M***, CF<SF
17-Hydroxy-progesterone	1.3 (1.16, 1.64)	1.31 (0.993, 2.87)	6.41 (4.96, 7.75)	6.1 (3.94, 7.75)	M***
Cortisol	396 (336, 445)	261 (225, 306)	430 (390, 466)	348 (300, 370)	S*, M*
5α-Dihydroprogesterone	0.534 (0.434, 0.597)	0.86 (0.532, 1.37)	0.895 (0.748, 1.08)	5.84 (1.95, 9.22)	S***, M***, CF<SF, CL<SL
Allopregnanolone	0.208 (0.156, 0.449)	0.0997 (0.0847, 0.208)	0.506 (0.453, 0.792)	1.51 (1, 2.58)	M***, S×M*, CF>SF
Conjugated allopregnanolone	5.09 (2.65, 11.3)	5.38 (3.79, 11.5)	20.8 (15.8, 27.8)	36.1 (21.7, 57.1)	M***
Isopregnanolone	0.118 (0.0811, 0.195)	0.142 (0.106, 0.2)	0.362 (0.279, 0.465)	0.77 (0.478, 1.5)	M***
Conjugated isopregnanolone	5.25 (3.92, 7.11)	11.5 (8.76, 15.9)	13.4 (10.9, 15.6)	32.6 (13.4, 44.6)	S***, M***, CF<SF, CL<SL
Pregnanolone	0.13 (0.095, 0.301)	0.068 (0.0449, 0.0853)	0.219 (0.147, 0.279)	0.824 (0.405, 1.17)	M***, S×M**, CF>SF
Conjugated pregnanolone	6.1 (4.74, 9.08)	12.1 (10.8, 21.9)	18.7 (16.3, 24)	36 (25.7, 54.8)	S**, M***, CF<SF
Conjugated epipregnanolone	1.99 (1.43, 2.43)	1.71 (0.934, 2.81)	3.98 (2.85, 5.1)	6.32 (3.89, 21.3)	M*
20α-Dihydropregnenolone	1.3 (0.505, 1.56)	1.05 (0.661, 1.5)	0.891 (0.605, 1.28)	2.76 (2, 3.15)	S*, M*, S×M**, CL<SL
Conjugated 20α-dihydropregnenolone	215 (122, 430)	284 (233, 355)	186 (139, 284)	330 (310, 365)	S*, CL<SL
20α-Dihydroprogesterone	0.63 (0.575, 0.818)	0.718 (0.409, 0.941)	4.7 (3.32, 5.72)	8.3 (5.86, 15.9)	M***
Conjugated 5α-pregnane-3β,20α-diol	21 (14.1, 32.4)	63.1 (29.4, 74.2)	95.9 (81, 115)	330 (197, 418)	S**, M***, CF<SF, CL<SL
Conjugated 5β-pregnane-3α,20α-diol	38.9 (28.6, 69.7)	33 (19.3, 42.8)	137 (114, 165)	191 (100, 232)	M***
16α-Hydroxy-pregnenolone	0.26 (0.189, 0.34)	0.334 (0.181, 0.486)	0.249 (0.176, 0.383)	0.424 (0.275, 0.485)	NS
Lutropin	1.75 (1.37, 3.6)	5.83 (5.34, 11.2)	4.76 (1.73, 9.56)	3.35 (1.87, 9.17)	S*, S×M*, CF>SF
Follitropin	5 (2.32, 7.83)	8.21 (6.3, 9.17)	3.53 (1.86, 5.55)	6.59 (4.46, 7.65)	S*, CL<SL
Lutropin/follitropin	0.503 (0.409, 0.575)	1.17 (0.632, 1.93)	1.68 (0.492, 2.64)	0.599 (0.425, 1.15)	S×M**, CF<SF, CL>SL

* p<0.05, ** p<0.01, *** p<0.001; S=smoking status; M=phase of the menstrual cycle; S×M=interaction smoking status × phase of the menstrual cycle; CF=controls in the follicular phase; CL=controls in the luteal phase; SF=smokers in the follicular phase; SL=smokers in the luteal phase; only significant differences (p<0.05) between controls and smokers in individual phases of menstrual cycle are show; NS=not significant

Table 3. Comparison of the levels of C19 steroids, estradiol, SHBG (nmol/l) and related indices (U/l) in the sera of premenopausal female non-smokers and sex- and age-matched smokers in follicular and luteal phases of menstrual cycle (the data are shown as medians with quartiles in the parentheses).

Variable	FOLLICULAR PHASE		LUTEAL PHASE		ANCOVA followed by LSD multiple comparisons
	Non-smokers CF	Smokers SF	Non-smokers CL	Smokers SL	
Dehydroepiandrosterone	7.8 (5.93, 9.9)	12 (9, 19.2)	8.08 (6.59, 13.7)	23.8 (18.1, 27.5)	S**, M*, CL<SL
Conjugated dehydroepiandrosterone	1820 (1380, 2560)	2030 (1580, 2540)	786 (696, 1110)	1810 (1610, 2030)	S***, M*, S×M**, CL<SL
Androstenediol	1.02 (0.797, 1.16)	1.47 (0.851, 1.73)	0.718 (0.668, 0.985)	1.99 (1.59, 2.66)	S***, S×M*, CL<SL
Conjugated androstenediol	162 (57.5, 312)	480 (297, 601)	149 (117, 171)	492 (326, 1110)	S***, CF<SF, CL<SL
Androstenedione	3.45 (2.59, 3.86)	6.16 (4.72, 7.52)	3.27 (2.97, 4.97)	10.4 (8.59, 11.1)	S***, M**, S×M*, CF<SF, CL<SL
Testosterone	0.915 (0.734, 1.09)	1.8 (1.04, 2.18)	0.824 (0.717, 1.06)	1.65 (1.16, 3.29)	S***, CF<SF, CL<SL
Free testosterone index	1.04 (0.555, 1.43)	2.4 (1.79, 6.11)	1.23 (0.749, 1.82)	3.93 (2.11, 5.84)	S***, CF<SF, CL<SL
Free testosterone	7.95 (4.59, 10.8)	18.7 (12.3, 39.2)	8.97 (6.09, 12.2)	27.9 (13.6, 34.9)	S***, CF<SF, CL<SL
Estradiol	0.144 (0.084, 0.212)	0.203 (0.0885, 0.349)	0.37 (0.334, 0.761)	0.209 (0.183, 0.25)	M**, S×M*, CL>SL
5α-Dihydrotestosterone	0.18 (0.11, 0.261)	0.252 (0.219, 0.392)	0.226 (0.143, 0.347)	0.793 (0.54, 1.15)	S**, CL<SL
Androsterone	0.384 (0.265, 0.439)	0.283 (0.21, 0.521)	0.328 (0.232, 0.382)	0.799 (0.53, 1.01)	M***, S×M***, CL<SL
Conjugated androsterone	550 (392, 996)	832 (569, 1590)	220 (206, 278)	1220 (437, 1490)	S***, S×M**, CL<SL
Epiandrosterone	0.549 (0.377, 0.651)	0.437 (0.369, 0.834)	0.409 (0.32, 0.578)	1.1 (0.815, 1.2)	S*, S×M***, CL<SL
Conjugated epiandrosterone	226 (198, 276)	311 (283, 468)	115 (106, 126)	539 (343, 641)	S***, S×M***, CL<SL
Etiocholanolone	0.336 (0.267, 0.365)	0.154 (0.129, 0.322)	0.203 (0.166, 0.25)	0.495 (0.394, 0.665)	M***, S×M***, CF>SF, CL<SL
Conjugated etiocholanolone	82.3 (44.8, 94.5)	72 (45.7, 83.3)	33.9 (22.1, 35.6)	89.1 (65.4, 92.6)	S**, S×M***, CL<SL
Conjugated epietiocholanolone	14.4 (11.3, 20.4)	18.1 (9.59, 33.2)	6.13 (5.1, 6.72)	37.7 (28.3, 63.1)	S***, S×M***, CL<SL
Conjugated 5α-androstane-3α,17β-diol	3.07 (1.64, 4.04)	11.6 (8.17, 19.3)	3.31 (2.23, 3.6)	21.9 (14.9, 30.5)	S***, CF<SF, CL<SL
Conjugated 5α-androstane-3β,17β-diol	9.93 (3.92, 12.2)	37.8 (23.4, 72)	7.46 (6.95, 12.7)	68.6 (46.8, 118)	S***, M*, CF<SF, CL<SL
7α-Hydroxy-dehydroepiandrosterone	1.4 (1.27, 2.16)	2.26 (1.65, 2.59)	1.05 (0.884, 1.6)	2.2 (1.92, 2.68)	S***, S×M*, CL<SL
7β-Hydroxy-dehydroepiandrosterone	0.575 (0.434, 0.866)	0.453 (0.41, 0.75)	0.371 (0.324, 0.429)	0.78 (0.548, 1.41)	S×M***, CL<SL
5-Androstene-3β,7α,17β-triol	0.17 (0.115, 0.222)	0.122 (0.118, 0.139)	0.128 (0.0936, 0.176)	0.131 (0.0915, 0.153)	NS
5-Androstene-3β,7β,17β-triol	0.102 (0.0826, 0.13)	0.0928 (0.0774, 0.125)	0.0753 (0.056, 0.0946)	0.0995 (0.0801, 0.152)	NS
Sex hormone binding globulin	103 (61.6, 151)	59.7 (46.6, 77)	91.5 (50.4, 105)	42.8 (37.5, 74.9)	S*, M*, CL>SL

* p<0.05, ** p<0.01, *** p<0.001; S=smoking status; M=phase of the menstrual cycle; S×M=interaction smoking status × phase of the menstrual cycle; CF=controls in the follicular phase; CL=controls in the luteal phase; SF=smokers in the follicular phase; SL=smokers in the luteal phase; only significant differences (p<0.05) between controls and smokers in individual phases of menstrual cycle are show; NS=not significant

free and conjugated epiandrosterone, free and conjugated etiocholanolone, 7α/β-hydroxy-dehydroepiandrosterone isomers, and follitropin but lower levels of estradiol and sex SHBG and lower values of the lutropin/follitropin ratio.

The OPLS model simultaneously evaluated the relationships between the average daily consumption of cigarettes representing vector **Y** and the levels of steroids and related substances constituting matrix **X**. Using this approach, one predictive component was extracted. This predictive component separated the variability explaining the relationships between the average daily consumption of cigarettes and the levels of relevant analytes. To differentiate between the effects of the chronic smoking (expressed as the average daily consumption of cigarettes) in the FP and LP, the OPLS models were constructed separately for LP an FP. The results from the OPLS models generally agreed with the data from ANOVA.

Table 4. Relationships between chronic smoking (matrix **Y**) and relevant serum steroids and related substances in the follicular phase of the menstrual cycle in women of fertile age (matrix **X**) as simultaneously evaluated by multivariate regression with reduction of dimensionality using the method of orthogonal projections to latent structures (OPLS).

Variable		**Component loading**	**Component loading / 95 % CI**	**R** [a]
Y	*Cigarette consumption/day*	1.000	15.12	0.948 **
X	*Conjugated pregnenolone*	0.189	1.10	0.532 *
	Conjugated 17-hydroxy-pregnenolone	0.273	3.66	0.799 **
	Conjugated androstenediol	0.252	3.95	0.707 **
	Progesterone	0.209	1.38	0.588 *
	Pregnanolone	−0.210	−1.26	−0.590 *
	Conjugated pregnanolone	0.269	2.26	0.775 **
	Conjugated isopregnanolone	0.234	2.14	0.673 **
	Conjugated 5α-pregnane-3β,20α-diol	0.112	0.99	0.314
	Conjugated 5β-pregnane-3α,20α-diol	−0.131	−1.16	−0.369 *
	Androstenediol	0.186	2.18	0.522 **
	Androstenedione	0.243	1.76	0.682 **
	Testosterone	0.186	1.41	0.522 *
	Free testosterone index	0.190	2.25	0.586 **
	Free testosterone	0.190	2.20	0.586 **
	Conjugated epiandrosterone	0.133	1.12	0.373 *
	Etiocholanolone	−0.192	−1.54	−0.549 *
	Conjugated 5α-androstane-3α,17β-diol	0.264	3.48	0.741 **
	Conjugated 5α-androstane-3β,17β-diol	0.281	5.91	0.789 **
	Lutropin	0.298	3.38	0.835 **
	Follitropin	0.143	1.22	0.429 *
	Lutropin/follitropin	0.227	1.42	0.608 *
	Sex hormone binding globulin	−0.148	−1.40	−0.462 *
Variability explained by the predictive component		89.9 % (80.5 % after cross validation)		

[a] R=Component loading expressed as a correlation coefficient with the predictive component; * $p<0.05$, ** $p<0.01$; **X**=matrix **X**; **Y**=vector **Y**; CI=confidence interval

Table 5. Relationships between chronic smoking (matrix **Y**) and relevant serum steroids and related substances in the luteal phase of the menstrual cycle in women of fertile age (matrix **X**) as simultaneously evaluated by multivariate regression with reduction of dimensionality using the method of orthogonal projections to latent structures (OPLS).

Variable		Component loading	Component loading / 95 % CI	R [a]
Y	*Cigarette consumption/day*	1.000	16.18	0.993 **
X	*Conjugated pregnenolone*	0.103	2.25	0.405 **
	Conjugated 17-hydroxy-pregnenolone	0.203	2.63	0.822 **
	Progesterone	0.149	1.71	0.607 **
	Cortisol	−0.141	−1.84	−0.583 **
	5α-Dihydroprogesterone	0.198	3.37	0.804 **
	Allopregnanolone	0.176	1.93	0.714 **
	Isopregnanolone	0.137	1.24	0.555 *
	Conjugated isopregnanolone	0.140	1.17	0.573 *
	Pregnanolone	0.169	2.45	0.687 **
	Conjugated pregnanolone	0.140	1.17	0.567 *
	20α-Dihydropregnenolone	0.175	3.76	0.703 **
	Conjugated 20α-dihydropregnenolone	0.141	1.97	0.565 **
	20α-Dihydroprogesterone	0.148	1.60	0.601 **
	Conjugated 5α-pregnane-3β,20α-diol	0.159	1.56	0.645 *
	16α-Hydroxy-pregnenolone	0.093	1.12	0.369 *
	Dehydroepiandrosterone	0.148	2.94	0.593 **
	Conjugated dehydroepiandrosterone	0.204	2.80	0.847 **
	Androstenediol	0.164	4.03	0.657 **
	Conjugated androstenediol	0.191	2.43	0.766 **
	Androstenedione	0.150	2.37	0.603 **
	Testosterone	0.135	1.67	0.541 **
	Free testosterone index	0.153	1.54	0.614 *
	Free testosterone	0.154	1.65	0.616 **
	Estradiol	−0.170	−2.03	−0.682 **
	5α-Dihydrotestosterone	0.164	1.94	0.663 **
	Androsterone	0.132	1.87	0.528 **
	Conjugated androsterone	0.169	11.69	0.728 **
	Epiandrosterone	0.147	2.46	0.590 **
	Conjugated epiandrosterone	0.203	7.06	0.873 **
	Etiocholanolone	0.150	2.91	0.604 **
	Conjugated etiocholanolone	0.181	2.11	0.761 **
	Conjugated epietiocholanolone	0.210	2.52	0.867 **
	Conjugated 5α-androstane-3α,17β-diol	0.207	3.36	0.882 **
	Conjugated 5α-androstane-3β,17β-diol	0.197	4.15	0.882 **
	7α-Hydroxy-dehydroepiandrosterone	0.164	2.90	0.657 **
	7β-Hydroxy-dehydroepiandrosterone	0.174	2.43	0.695 **
	5-Androstene-3β,7α,17β-triol	0.103	0.96	0.409
	Follitropin	0.122	1.12	0.524 *
Variability explained by the predictive component		98.6 % (89.4 % after cross validation)		

[a] R=Component loading expressed as a correlation coefficient with the predictive component; * $p<0.05$, ** $p<0.01$; **X**=matrix **X**; **Y**=vector **Y**; CI=confidence interval

In the FP (Table 4), the average daily consumption of cigarettes positively correlated with conjugated pregnenolone, conjugated 17-hydroxy-pregnenolone, conjugated androstenediol, progesterone, conjugated pregnanolone, conjugated isopregnanolone, conjugated 5α-pregnane-3β,20α-diol, androstenediol, androstenedione, testosterone, free testosterone index, free testosterone, conjugated epiandrosterone, conjugated 5α-androstane-3α,17β-diol, conjugated 5α-androstane-3β,17β-diol, lutropin, follitropin, and lutropin/follitropin ratio and negatively with pregnanolone, conjugated 5β-pregnane-3α,20α-diol, etiocholanolone, and sex hormone binding globulin.

In the LP (Table 5), the average daily consumption of cigarettes positively correlated with conjugated pregnenolone, conjugated 17-hydroxy-pregnenolone, progesterone, 5α-dihydroprogesterone, allopregnanolone, isopregnanolone, conjugated isopregnanolone, pregnanolone, conjugated pregnanolone, 20α-dihydropregnenolone, conjugated 20α-dihydropregnenolone, 20α-dihydroprogesterone, conjugated 5α-pregnane-3β,20α-diol, 16α-hydroxy-pregnenolone, dehydroepiandrosterone, conjugated dehydroepiandrosterone, androstenediol, conjugated androstenediol, androstenedione, testosterone, free testosterone index, free testosterone, 5α-dihydro-testosterone, androsterone, conjugated androsterone, epiandrosterone, conjugated epiandrosterone, etiocholanolone, conjugated etiocholanolone, conjugated epietiocholanolone, conjugated 5α-androstane-3α,17β-diol, conjugated 5α-androstane-3β,17β-diol, 7α-hydroxy-dehydroepiandrosterone, 7β-hydroxy-dehydroepiandro-sterone, 5-androstene-3β,7α,17β-triol, and follitropin but negatively with cortisol and estradiol.

Discussion

The present study is the first attempt to obtain a complex insight into the effects of chronic smoking on the steroid metabolome in premenopausal women. Some of the previous studies used a greater number of patients but were only focused on particular steroids. Concerning the steroid metabolites selected in the present study, besides for the common steroid hormones including their precursors and metabolites, we focused on the key metabolic steps, which are necessary for the synthesis of neuroactive and neuroprotective steroids. Although our study presents the most comprehensive analysis of serum steroid levels in female premenopausal smokers (both gonadal and adrenal) and it is the first study to look at both conjugated and unconjugated levels of steroids in these patients, due to technical reasons, the steroid inventory is still incomplete.

Most of other authors used immunoanalytical methods, which are inferior compared with GC-MS that was, for the most part, used in this study. On the other hand, the main limitation of this study is the relatively low number of subjects and consequently the low power of statistical testing. This means that there is a higher probability of falsely negative results, which may explain the small discrepancies between the results from multivariate regression and ANOVA.

The first outcome of this study is the finding that chronic cigarette smoking (compared with permanent tobacco abstinence) increases the levels of serum androgens, their 5α/β-reduced metabolites (Tables 3, 4 and 5), and also the progestogen levels (progesterone, 5α-DHP) (Tables 2, 4 and 5) but significantly suppresses serum estradiol in the LP (Tables 3 and 5). These results are partly in accordance with some older reports (Cauley *et al.* 1989, Friedman *et al.* 1987, Khaw *et al.* 1988, Longcope and Johnston 1988).

Our results demonstrate decreased cortisol levels regardless of the menstrual phase (Table 2). This data is consistent with the report from Back *et al.* (2008) showing that women smokers evidenced a more blunted cortisol response compared to non-smoking women.

Although some authors found no difference for the testosterone levels between female smokers and non-smokers (Longcope and Johnston 1988), others observed increased levels of the steroid in premenopausal smokers (Friedman *et al.* 1987, Khaw *et al.* 1988, Sowers *et al.* 2001). Our data clearly demonstrate that premenopausal smokers have a tendency towards hyperandrogenism as documented by higher values of free testosterone index, elevated levels of total testosterone, free testosterone, and 5α-dihydrotestosterone, as well as by pronouncedly elevated levels of 5α-dihydrotestosterone metabolites, and conjugated 5α-androstane-3α/β,17β-diols. Although multiple comparisons for SHBG levels show no differences between premenopausal smokers and non-smokers in the FP, there is a difference in the LP and the effect of smoking in the ANCOVA model is significant, indicating consistently depressed SHBG regardless of the phase of the menstrual cycle (Table 3). This is consistent with elevated androgen levels. Estradiol levels in the LP are lower in the smokers. The aforesaid results are in agreement with a number of reports (Barbieri *et al.* 2005,

Cupisti *et al.* 2010, Jensen *et al.* 1985, Manjer *et al.* 2005, Michnovicz *et al.* 1986, Sterzik *et al.* 1996, Van Voorhis *et al.* 1996), but are contradictory to others (Key *et al.* 1991, Khaw *et al.* 1988, Thomas *et al.* 1993).

The consistently increased concentration of free and conjugated 5α/β-reduced androstane metabolites in LP and some of them in the FP (Table 3) may be a consequence of either augmented production rate of the precursors or a suppressed catabolism of the products. In contrast to our results, the measurements of metabolic clearance rate MCRs reported by Longcope and Johnson (Longcope and Johnston 1988) indicated that smoking does not alter the production and metabolism of androgens and estrogens in pre- and postmenopausal women. However, in the aforementioned study, those women who smoked had a lower BMI than non-smokers and their androgen and estrogen MCRs were lower, which probably resulted in higher plasma androgen concentrations (Longcope and Johnston 1988). In our study, smokers and non-smokers do not significantly differ in BMI.

Fertility problems in female smokers were established in many studies (Thomford and Mattison 1986, Freour *et al.* 2008). Increased levels of androgens could be one part of the problem.

Surprisingly, in contrast to the increased levels of progesterone and 5α-DHP in both phases of MC, the levels of GABA-ergic pregnanolone isomers allopregnanolone and pregnanolone are suppressed in the FP (Table 2). On the other hand, conjugated isopregnanolone and conjugated pregnanolone levels are higher in smokers than in non-smokers (Table 2), like most of the remaining steroid polar conjugates (Tables 2 and 3). This finding may indicate increased sulfotransferase activity in the patients than in the controls.

The well-being and health status in the women of reproductive age are generally associated with the phase of menstrual cycle and with the secretion of ovarian steroids. The results of Sufuoglu and colleagues who reported enhanced ratings of "bad effects," from nicotine and attenuation of the "drug liking" by progesterone indicate that neuroactive steroids might influence nicotine addiction (Sofuoglu *et al.* 2009). Neurocognitive functioning and mood in the LP may also be impaired due to changes in hypothalamic-pituitary-adrenal (HPA) axis function (Symonds *et al.* 2004). Our results show altered serum levels of various neuroactive and neuroprotective steroids (Tables 2-5). However, concerning the alterations in the serum levels of positive modulators of GABA-r, our results are not clear-cut. While allopregnanolone, pregnanolone, and etiocholanolone in the FP are suppressed in the patients (Table 1), the etiocholanolone and androsterone levels in the LP are increased in these subjects (Table 2). On the other hand, the negative modulators of GABA-r and/or positive modulators of NMDA-r in the smokers show an unambiguously increasing trend as documented by the higher levels of conjugated pregnenolone (Table 2), conjugated DHEA (Table 3), and conjugated pregnanolone isomers (Table 2).

The premenstrual syndrome is found 2times more often in female smokers than in non-smokers PMS (Bertone-Johnson *et al.* 2008). Allopregnanolone correlates with the development of premenstrual syndrome (Bicikova *et al.* 1998). The decrease in serum allopregnanolone in the FP and alteration between phases could be one of the causes of higher incidence of premenstrual syndrome in female smokers.

In the present study, most of the biochemical measures were strongly correlated. When using ANCOVA followed by LSD multiple comparisons, there are too many differences that either limit the validity of the message or that can hardly be conceptualized. Therefore, besides for the ANCOVA model we have used the multivariate regression with the reduction of dimensionality. The OPLS model simultaneously evaluated the relationships between the average daily consumption of cigarettes (matrix **Y**) and the strongly intercorrelated levels of steroids and related substances (matrix **X**). Tables 2-5 demonstrate that the results from ANCOVA testing and multivariate regression were consistent, even if the multivariate model was slightly less sensitive, possibly due to the low number of study participants.

In conclusion, the first outcome of the present study is the finding that chronic cigarette smoking (compared with permanent tobacco abstinence) augments serum androgens including some 5α/β-reduced androstane metabolites but suppresses estradiol levels in the LP, which may induce hyperandrogenism in the female smokers. The second outcome is the detection of increased serum progestogens but paradoxically suppressed levels of their GABA-ergic metabolites. The third outcome is the finding of lower cortisol in the patients supporting the concept of blunted cortisol response induced by chronic smoking as was recently suggested by Back *et al.* (2008). Although this study brings forth new data concerning the alterations in the steroid metabolome of chronic female smokers, further investigations are needed concerning these effects. In addition, the prospective changes or reinstatement of

neuroactive and neuroprotective steroids in women of fertile age after the cessation of smoking require further investigation.

Conflict of Interest

There is no conflict of interest.

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