

Original Article

Regular cigarette smoking influences the transsulfuration pathway, endothelial function, and inflammation biomarkers in a sex-gender specific manner in healthy young humans

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Abstract: Cigarette smoking (CS) is the primary cause of preventable morbidity and mortality. Abundant clinical evidence suggests that CS is more harmful to women; however, the mechanisms responsible for these differences are not yet known. CS alters endothelial function, the redox state, inflammation, and global DNA methylation, which is associated with one-carbon metabolism and the transsulfuration pathway. However, it is not known whether the previously identified alterations are sex-gender related. Healthy adult men and oral contraceptive-free women with regular menstrual cycles were enrolled; women were examined during the follicular phase. Men had higher plasma levels of uric acid, total bilirubin, homocysteine, glutamylcysteine, total glutathione, cysteinylglycine; had more monocytes and released more TNF-alpha from human monocytes derived macrophages (hMDMs), but they had fewer platelets and lower levels of DNA methylation, and their hMDMs released less TNF-alpha after LPS stimulation. MDA, taurine, cysteine, arginine, ADMA, and SDMA were not different. CS decreased global DNA methylation more in women and increased the platelet, monocyte, and lymphocyte counts and the homocysteine, arginine, and ADMA levels only in women, whereas increased the neutrophil and eosinophil counts only in men. Additionally, CS reduced the sex-gender differences in total bilirubin, basal and LPS-induced TNF-alpha release, total glutathione, and glutamylcysteine, leaving unchanged cysteinylglycine, taurine, SDMA, MDA, and cysteine. These data suggest that cardiovascular risk factors seem to come earlier in young healthy female smokers than in young healthy male smokers, supporting the greater alarmism regarding the effects of CS in women and providing a basis for understanding the sex-gender differences. These results also suggest that cessation programs targeting women are needed.

Keywords: Cigarette smoking, sex-gender differences, ADMA, transsulfuration pathway, global DNA methylation

Introduction

The effects of cigarette smoking (CS) on health are well known, with CS being the main cause of preventable morbidity and mortality [1]. In the US population, CS-related death is the leading cause of preventable death in women [2]. CS greatly increases a woman's relatively risk of myocardial infarction [3] and cardiovascular outcomes [2], especially in women who use oral contraceptives (OC) [4]. In addition, it has been calculated that the risk of myocardial

infarction due to CS is greater in women than in men [5]. Despite the substantial declines in the prevalence of smoking, at least among adults in the Western world, the prevalence of smoking is increasing among girls [6], and smoking cessation is more frequent among men than women [7].

The underlying mechanisms by which CS might be more harmful to smoker women are still unclear. CS can affect many functions that can have prognostic implications for subsequent

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cardiovascular diseases (CVDs), such as the production of reactive oxygen species [8], the induction of inflammatory processes [9], and alterations in endothelial function [10] and global DNA methylation [11, 12], which is associated with the one-carbon metabolism pathway. Homocysteine plays a central role in this pathway, in which it is converted into methionine or cleared by the transsulfuration pathway [13]. However, it is scanty known whether the previously identified mechanisms are influenced by sex-gender.

The term “sex-gender” reflects both physiological distinctions between each sex, dictated by genetics and biology, and environmental and social influences (e.g., diet, lifestyle, social status, and cultural influences) [14].

Therefore, this study aimed to investigate the previously identified mechanisms in young healthy adult smokers and non-smokers. We examined the endothelial function by evaluating an endogenous inhibitor of NO synthesis, asymmetric dimethylarginine (ADMA) [10]; the precursor of NO synthesis L-arginine; and symmetric dimethyl arginine (SDMA), which seems to favour inflammation [15]. We also measured the total glutathione, glutamylcysteine, cysteinylglycine, and cysteine levels together with the level of homocysteine, which has a role in the transsulfuration pathway [13].

The levels of lipid peroxidation, which is an oxidative stress indicator; the hydrophilic antioxidant uric acid [16]; and total bilirubin [17] were evaluated because they have different correlations with CVD; the first is inversely correlated with CVD, and the second is directly associated with the risk of CVD [18, 19]. The platelets, white blood cell (WBC) and subtype counts are considered markers of inflammation [8], as is TNF-alpha release from human monocytes-derived macrophages (hMDMs). Cytokine release was evaluated under basal conditions and after stimulation with lipopolysaccharide (LPS), an endotoxin produced by gram-negative bacteria that binds Toll-like receptor 4 [20] and is also present in tobacco [21].

Materials and methods

Ethics statement

The study was approved by the local ethical committee of Azienda Ospedaliero-Universitaria

of Sassari. Informed consent was obtained, and blood sampling was performed during a voluntary blood donation. Women were asked to donate blood during the follicular phase, and all participants were informed that an aliquot of blood would be kept for the study. Blood chemistry analyses were performed as a scheduled service for blood donors.

Population

A total of 83 healthy adult men and 85 healthy adult women with regular menstrual cycles (28 days) aged between 18 and 40 years were enrolled during the period from July 2007 to November 2010 at the Servizio di Diagnosi e Cura di Endocrinologia, Azienda Ospedaliero-Universitaria, Sassari, and at Diabetologia Aziendale ASL 2 Olbia, San Giovanni di Dio Hospital. The subjects were free of kidney, liver, heart, and endocrine diseases and had not had an infectious disease for at least two months prior to the study. None of the subjects were on long-term medications, including OC for women. All women were analysed during the follicular phase (1-10 days) and had not used OC for at least three months to ensure an appropriate wash-out period. A regular smoker was defined as a subject who smoked at least one cigarette per day at baseline.

Biochemical tests and blood cell counts

Fasting venous blood samples (8 and 10 am) were obtained and used for laboratory assessments of the selected biomarkers. Blood was collected using the appropriate anticoagulant, and the plasma was aliquoted and used within 1 month after storage at -80°C to measure the cysteine, homocysteine, total glutathione, cysteinylglycine, glutamylcysteine, arginine, ADMA and SDMA levels and to measure the MDA level as described in Campesi et al. [22]. The platelet, WBC and WBC subset counts were performed using whole blood aliquots. WBCs were also used to determine the degree of global DNA methylation as described in Sotgia et al [23].

Isolation of human monocytes and differentiation into hMDMs

Monocytes were isolated from aliquots of blood (30 ml) withdrawn from healthy volunteers according to Campesi et al. [22]. Purified mono-

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Table 1. Age, body weight and height of the enrolled subjects

	M (N = 55)	W (N = 53)	SM (N = 28)	SW (N = 32)
Age (years)	28 (18-39)	27 (20-40)	25.5 (19-37)	26 (18-40)
Weight (kg)	73 (55-105)	53 (38-70)*	66.5 (51-95)	55 (44-78) [†]
Height (m)	1.74 (1.62-1.90)	1.60 (1.50-1.74)*	1.73 (1.63-1.85)	1.62 (1.50-1.80) [†]

The data are expressed as medians and ranges. All women were analysed during the follicular phase. M = male non-smokers; W = female non-smokers; SM = male smokers; SW = female smokers. *P < 0.001 versus M; [†]P < 0.001 versus SM; N = sample size.

cytes were obtained by adhesion; non-adherent cells (mainly lymphocytes) were removed by gentle washing with PBS. hMDMs were differentiated by culturing the isolated monocytes for 8-10 days in a 5% CO₂ incubator at 37 °C in RPMI 1640 supplemented with 20% foetal bovine serum, 1% antibiotic-antimycotic, and 10 mM HEPES. hMDMs were prepared and characterised as described in Campesi et al [22].

Quantification of tumour necrosis factor- α release from hMDMs

hMDMs (4 x 10⁴/cm²) were incubated in a 96-well plate for 24 h in the absence or presence of 100 ng/ml LPS (Sigma Aldrich, Milano, Italia). The supernatants were then collected and stored at -80 °C until analysis of the tumour necrosis factor- α release using a commercial ELISA kit (human TNF- α /TNFSF1A DuoSet ELISA kit, R & D Systems, Milano, Italy) following the manufacturer's instructions. All samples were assayed in duplicate.

Statistical analysis

Statistical analysis was performed by comparing men with women as a function of smoking status. This analysis was performed using the Family-Wise Error Rate (FWER) approach, and the values were corrected for multiple-hypothesis testing using Bonferroni's correction (if the probability of type I error is set at α and m tests are performed, each test is controlled at the level $\alpha^* = \alpha/m$; Bonferroni, 1936). This correction guarantees that the probability of a false positive is at most equal to α [24].

The distribution of the samples was assessed using the Kolmogorov-Smirnov and Shapiro tests. The strength of the association between pairs of variables was measured using the Pearson Product Moment correlation coefficient

when the data were normally distributed and with the Spearman Product Moment correlation coefficient when the data had a non-Gaussian distribution. These analyses were performed using SigmaStat software.

Results

Age, body weight, and height

A total of 83 men and invece di based on si potrebbe mettere upon 85 women were enrolled and stratified based on smoking status; 28 males and 32 females smoked, and 55 males and 53 females did not smoke. As shown in **Table 1**, all groups were well matched for age, but as expected, men were heavier and taller than women independent of CS.

Evaluation of endothelial function

Notably, in healthy young adults, CS affected the levels of ADMA (an endogenous inhibitor of NOS) and L-arginine (the precursor of NO) in a sex-gender specific manner. Specifically, ADMA was not different between non-smokers but it was more elevated (approximately 20%) in female smokers than in males smokers, and consequently, the ADMA/SDMA ratio differed only between men and women who smoke (**Figure 1**). The L-arginine level did not differ between non-smokers, but it was more elevated in female smokers than in female non-smokers. Finally, the SDMA level and the ADMA/arginine ratio did not sexually diverge either in smokers or non-smokers (**Figure 1**).

Plasma thiols involved in the methylation and transsulfuration processes.

The homocysteine level was affected by sex-gender, being lower in female non-smokers than in male non-smokers, and by CS, being

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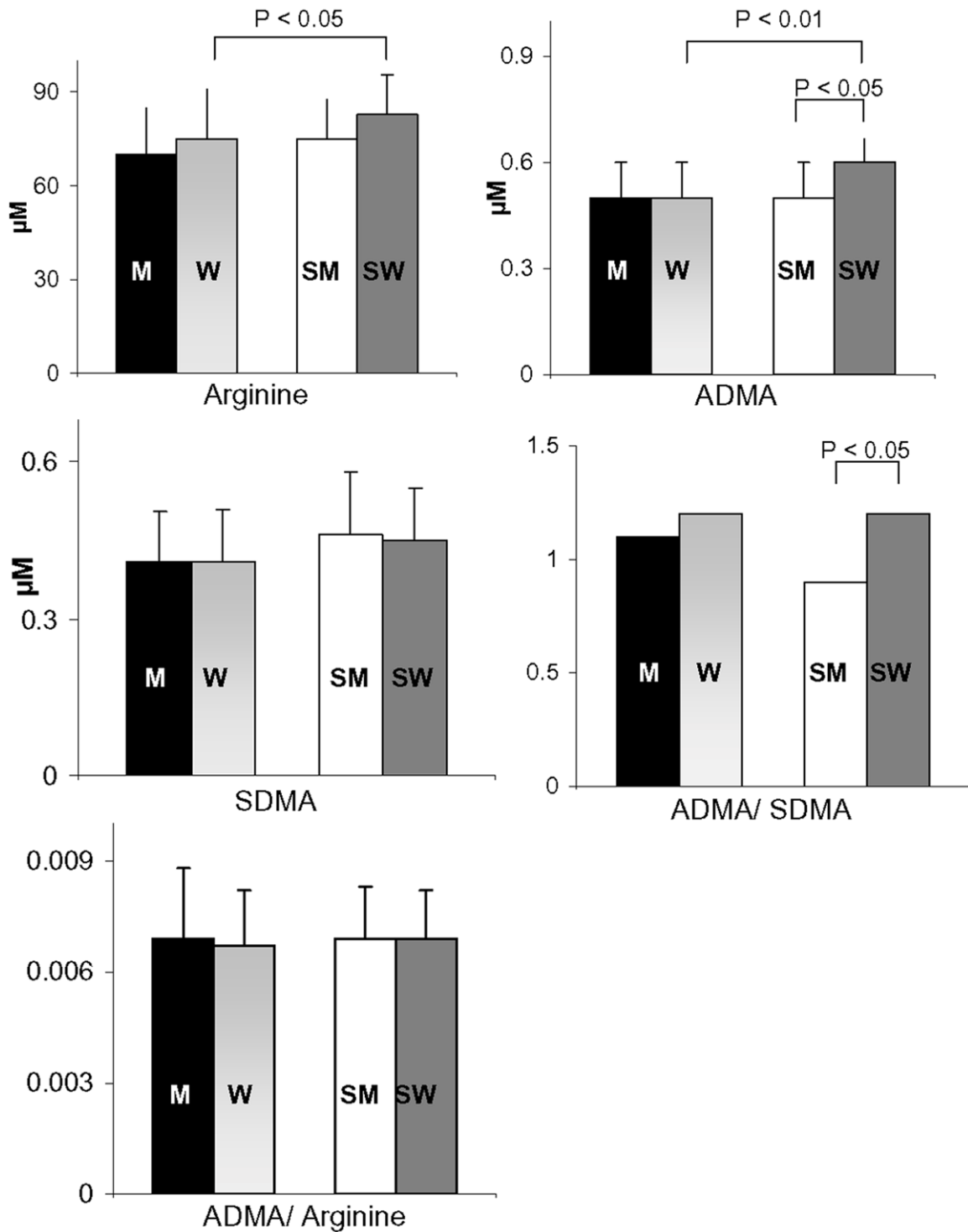


Figure 1. Arginine, ADMA, SDMA, ADMA/SDMA and ADMA/arginine levels in male non-smokers (M; N = 36), female non-smokers (W; N = 44), male smokers (SM; N = 21), and female smokers (SW; N = 26). All women were analysed during the follicular phase. The data are expressed as the means \pm std. dev. or as median values. Bars represent the statistical differences.

elevated only in female smokers (**Figure 2**). The plasma levels of total glutathione, its immediate precursor glutamylcysteine, and cysteinylg-

lycine (the degradation product of glutathione) diverged in non-smokers, being significantly lower in women than in men (-13.5%, -15%, and

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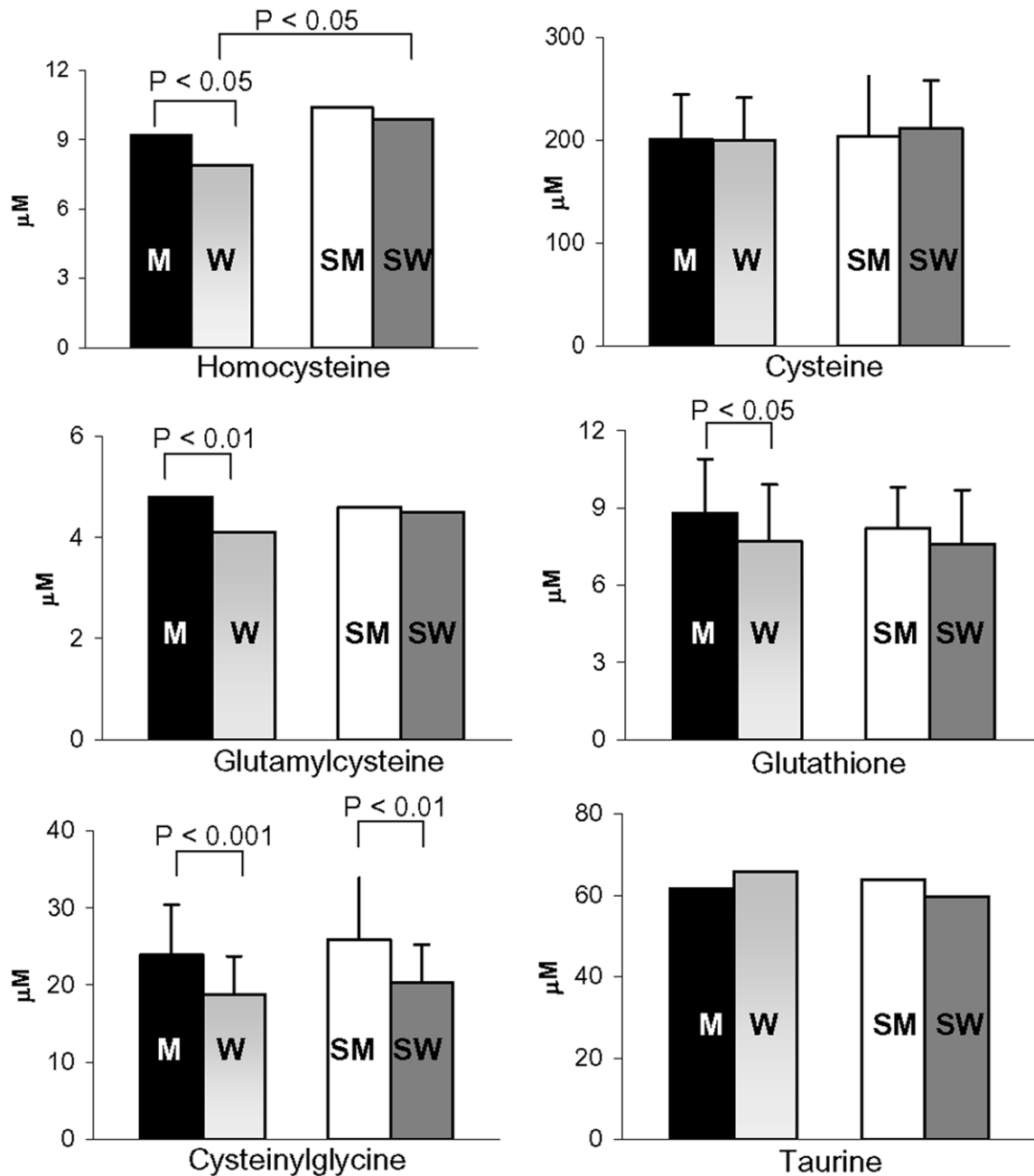


Figure 2. Effect of regular CS on the plasma anti-oxidant and pro-oxidant levels in male non-smokers (M; N = 36), female non-smokers (W; N = 44), male smokers (SM; N = 21), and female smokers (SW; N = 26). All women were analysed during the follicular phase. The data are expressed as the means \pm std. dev. or as median values. Bars represent the statistical differences.

-22%, respectively). Notably, these differences, with the exception of that for cysteinylglycine, were not observed in smokers (**Figure 2**). Finally, the levels of cysteine and taurine, one of the end products of cysteine metabolism and an endogenous scavenger of hypochlorous acid [25], did not diverge between men and women and were not affected by CS (**Figure 2**).

Global DNA methylation

DNA methylation alters the gene expression pattern in cells and plays a crucial role in the development of many diseases, including cardiovascular diseases [12, 26]. In non-smokers, the level of global DNA methylation was significantly higher in women than in men (**Figure 3**).

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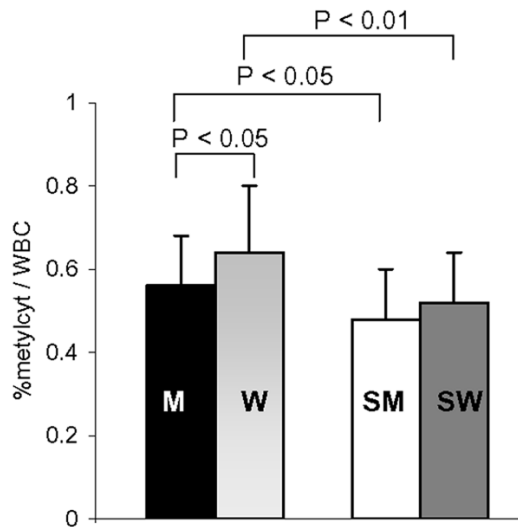


Figure 3. Effect of smoking on global DNA methylation in WBCs from male non-smokers (M; N = 37), female non-smokers (W; N = 45), male smokers (SM; N = 21), and female smokers (SW; N = 26). All women were analysed during the follicular phase. The data are expressed as the mean \pm std. dev. Bars represent the statistical differences.

CS reduced global DNA methylation in both men and women; however, the reduction was greater in women than in men (**Figure 3**).

Effect of regular CS and sex-gender on the redox state and inflammatory parameters

Plasma lipid peroxidation, uric acid, and total bilirubin levels: The MDA level did not differ between men and women and was not affected by CS (data not shown), whereas in non-smokers, the total bilirubin and uric acid levels were significantly lower in women than in men. CS reduced the magnitude of the sex-gender difference observed for the total bilirubin level, but it did not significantly affect the uric acid level (**Figure 4**).

Platelet, WBC and WBC subset counts: As shown in **Figure 5**, all values were within the normal range for non-smokers and smokers. As expected, the platelet count and monocyte counts were higher in women and in men (**Figure 5**), whereas the WBC, neutrophil, eosinophil, basophil, and lymphocyte counts did not differ between men and women. Notably, CS significantly increased the platelet count only in women and affected the WBC count and the counts of all WBC subtypes (**Figure 5**) except basophils. CS elevated the WBC count in both men and women. However, neutrophils and

eosinophils were significantly increased only in men, whereas lymphocytes and monocytes were elevated only in women.

Basal and LPS-induced release of TNF-alpha from hMDMs: The basal level of TNF-alpha released from hMDMs was significantly higher for male non-smokers than female non-smokers (**Figure 6**). This difference was not observed for smokers. LPS dramatically increased the release of TNF-alpha from hMDMs obtained from all cohorts (**Figure 6**). Notably, the level of LPS-induced TNF-alpha release was higher in female non-smokers than male non-smokers, whereas there was no statistically significant difference observed between men and women who smoke.

Analysis of correlations

In female non-smokers, age did not correlate with any of the studied parameters, whereas in female smokers, age was negatively associated with the lymphocyte ($r = -0.47$; $P = 0.007$) and platelet counts ($r = -0.34$; $P = 0.04$) and the cysteinylglycine ($r = -0.53$; $P = 0.006$), cysteine ($r = -0.50$; $P = 0.01$) and arginine levels ($r = -0.41$; $P = 0.03$). Age was also positively associated with the levels of taurine ($r = 0.46$; $P = 0.04$) and DNA methylation ($r = 0.40$; $P = 0.04$) in female smokers. Even fewer correlations were found for men. Age was positively associated with the basophil count ($r = 0.27$; $P = 0.04$) and the MDA level ($r = 0.46$; $P = 0.04$) in non-smokers and in smokers, respectively.

In addition, body weight was related with few parameters. In female non-smokers, weight was positively correlated with the WBC count ($r = 0.28$; $P = 0.04$) and negatively correlated with the total bilirubin level ($r = -0.36$; $P = 0.008$), whereas in female smokers, was correlated only with the ADMA/arginine ratio ($r = 0.40$; $P = 0.04$). Finally, in male non-smokers, weight was negatively correlated with the arginine ($r = -0.35$; $P = 0.04$) and cysteinylglycine levels ($r = -0.37$; $P = 0.03$), whereas in male smokers, weight was correlated only with the uric acid level ($r = 0.45$; $P = 0.01$).

In male and female non-smokers, the ADMA level was positively correlated with the arginine level ($r = 0.55$; $P < 0.001$ and $r = 0.42$; $P = 0.004$ respectively, the SDMA level ($r = 0.60$; $P < 0.001$ in men and $r = 0.49$; $P < 0.001$ in women), the ADMA/SDMA ratio ($r = 0.55$; $P <$

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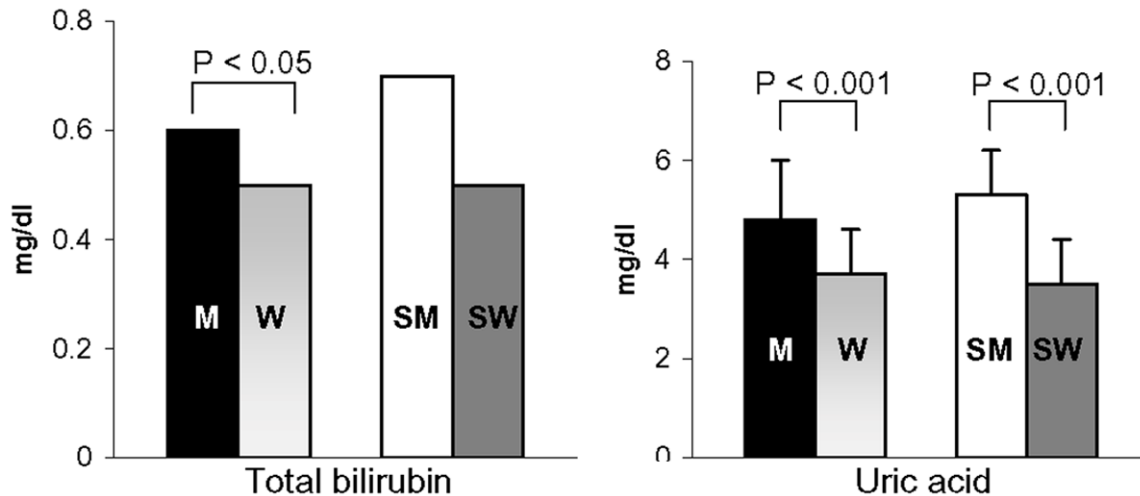


Figure 4. Effect of regular CS on the plasma bilirubin and uric acid levels in male non-smokers (M; N = 55), female non-smokers (W; N = 53), male smokers (SM; N = 28), and female smokers (SW; N = 32). All women were analysed during the follicular phase. The data are expressed as the means \pm std. dev. or as median values. Bars represent the statistical differences.

0.001 in men and $r = 0.41$; $P = 0.006$ in women), and the ADMA/arginine ratio ($r = 0.60$; $P < 0.001$ in men and $r = 0.53$; $P < 0.001$ in women) and negatively associated with the taurine ($r = -0.48$; $P = 0.003$ in men and $r = -0.30$; $P = 0.05$ in women) and MDA levels ($r = -0.52$; $P = 0.002$ in men and $r = -0.65$; $P < 0.001$ in women). In addition, in male non-smokers, the ADMA level was positively correlated with the cysteinylglycine level ($r = 0.38$; $P = 0.02$) and negatively associated with the monocyte ($r = -0.34$; $P = 0.04$), whereas in female non-smokers, ADMA was inversely correlated with the neutrophil count ($r = -0.31$; $P = 0.04$).

In female smokers, the ADMA level was positively correlated with the arginine level ($r = 0.57$; $P = 0.002$), the ADMA/SDMA ratio ($r = 0.63$; $P < 0.001$), the ADMA/arginine ratio ($r = 0.78$; $P < 0.001$), and the homocysteine level ($r = 0.45$; $P = 0.02$), whereas in male smokers, the ADMA level was positively correlated with the SDMA level ($r = 0.54$; $P = 0.04$) and negatively correlated with the monocyte count ($r = -0.45$; $P = 0.03$) and the levels of homocysteine ($r = -0.61$; $P = 0.003$), cysteine ($r = -0.44$; $P = 0.02$), glutamylcysteine ($r = -0.65$; $P = 0.001$), and MDA ($r = -0.50$; $P = 0.02$).

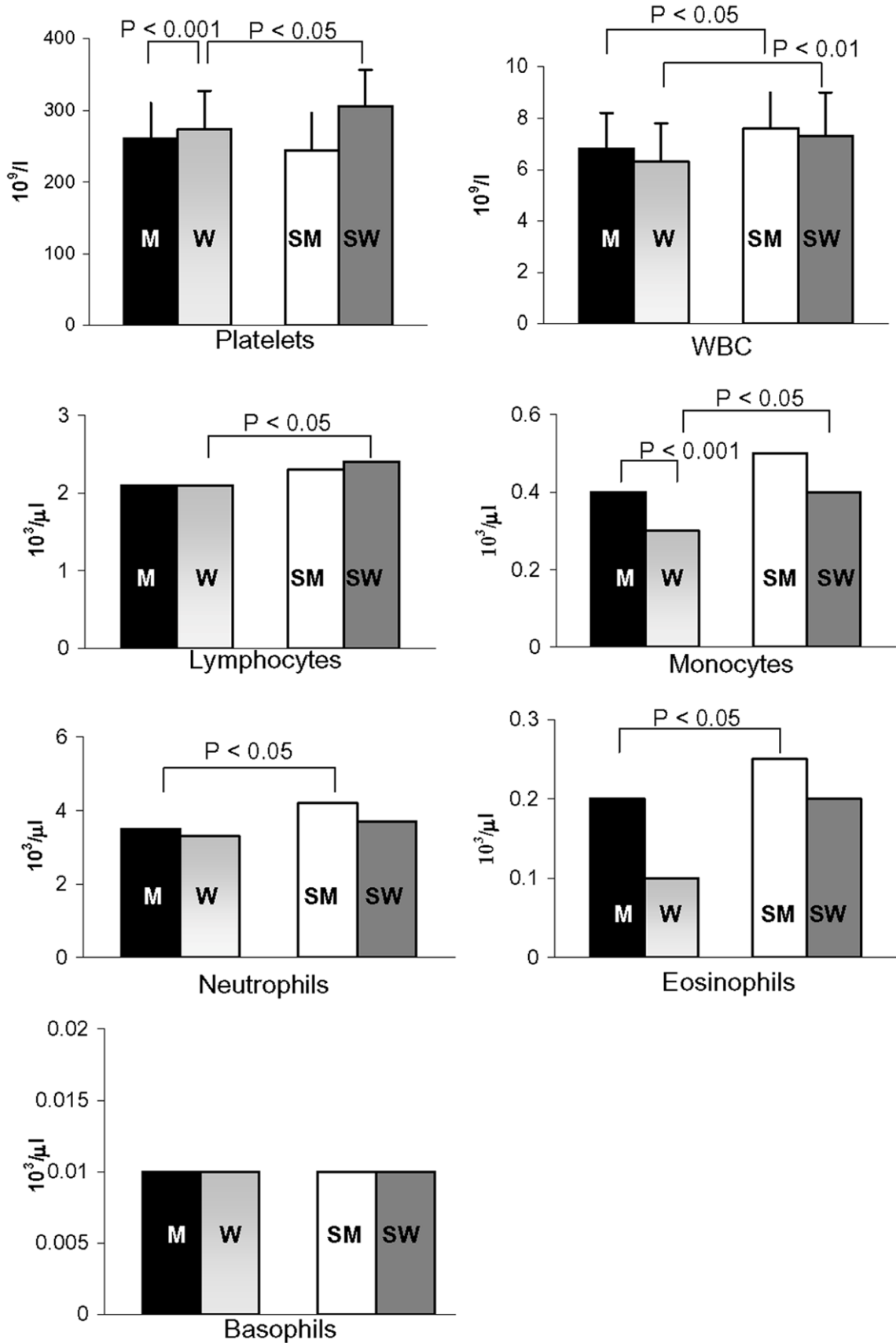
In non-smokers of both sexes, the SDMA level was positively associated with the arginine level ($r = 0.35$; $P = 0.04$ in men and $r = 0.39$; $P = 0.009$ in women). In addition, the SDMA level was negatively associated with the ADMA/

SDMA ratio in female non-smokers ($r = -0.56$; $P < 0.001$) and with the taurine level in male non-smokers ($r = -0.36$; $P = 0.03$). In male and female smokers, SDMA was negatively associated with the ADMA/SDMA level ($r = -0.48$; $P = 0.03$ and $r = -0.58$; $P = 0.002$ respectively). The SDMA level was also negatively associated with the cysteinylglycine and cysteine levels in female smokers ($r = -0.52$; $P = 0.007$ and $r = -0.44$; $P = 0.02$, respectively) and with the basophil count in male smokers ($r = -0.74$; $P < 0.001$).

In male non-smokers, the total glutathione level was directly associated with the levels of cysteinylglycine and glutamylcysteine ($r = 0.48$; $P = 0.003$ and $r = 0.40$; $P = 0.02$, respectively), whereas in male smokers, the total glutathione level was directly associated with the glutamylcysteine level ($r = 0.73$; $P < 0.001$) and inversely associated with the arginine level ($r = -0.52$; $P = 0.02$). In female non-smokers, the total glutathione level was only positively associated with the glutamylcysteine level ($r = 0.40$; $P = 0.008$), whereas in female smokers, it was positively correlated with the levels of cysteine ($r = 0.55$; $P = 0.004$), homocysteine ($r = 0.63$; $P < 0.001$), and cysteinylglycine ($r = 0.48$; $P = 0.01$) and inversely correlated with the taurine level ($r = -0.52$; $P = 0.01$).

In all studied groups, global DNA methylation was negatively correlated with the WBC count ($r = -0.93$; $P < 0.001$ in male non-smokers, $r = -0.94$; $P < 0.001$ in female non-smokers, $r =$

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Figure 5. Effects of regular CS and sex-gender on blood cell counts in male non-smokers (M; N = 55), female non-smokers (W; N = 53), male smokers (SM; N = 28), and female smokers (SW; N = 32). All women were analysed during the follicular phase. The data are expressed as the means \pm std. dev. or as median values. Bars represent the statistical differences.

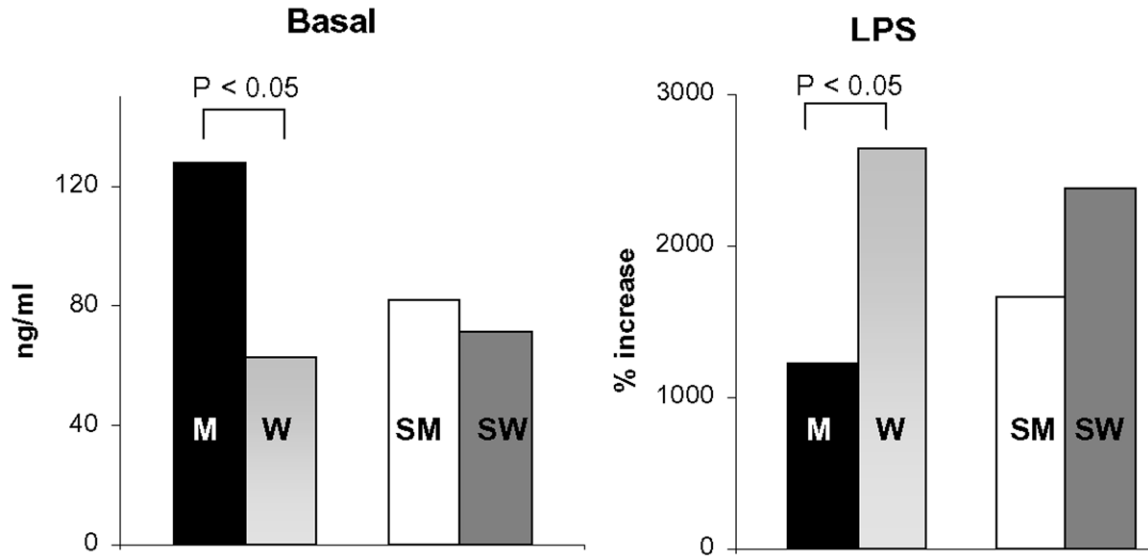


Figure 6. Basal and LPS-induced TNF- α release by hMDMs obtained from male non-smokers (M; N = 25), female non-smokers (W; N = 46), male smokers (SM; N = 24), and female smokers (SW; N = 25). All women were analysed during the follicular phase. The data are expressed as medians. Bars represent the statistical differences.

-0.93; $P < 0.001$ in male smokers and $r = -0.92$; $P < 0.001$ in female smokers), neutrophil count ($r = -0.79$; $P < 0.001$ in male non-smokers, $r = -0.85$; $P < 0.001$ in female non-smokers, $r = -0.68$; $P < 0.001$ in male smokers and $r = -0.72$; $P < 0.001$ in female smokers), and lymphocyte count ($r = -0.52$; $P < 0.001$ in male non-smokers, $r = -0.65$; $P < 0.001$ in female non-smokers, $r = -0.52$; $P = 0.02$ in male smokers and $r = -0.56$; $P = 0.003$ in female smokers). In male and female non-smokers, global DNA methylation was inversely correlated with the monocyte count ($r = -0.43$; $P = 0.008$ for men and $r = -0.56$; $P < 0.001$ for women), In female smokers, DNA methylation was negatively correlated with the taurine level ($r = -0.44$; $P = 0.03$) and the platelet count ($r = -0.49$; $P = 0.01$), whereas DNA methylation was inversely associated with the monocyte count only in male smokers ($r = -0.50$; $P = 0.02$).

Discussion

The major goal of this study was to determine whether regular CS affects inflammation and endothelial markers, global DNA methylation

and the transsulfuration pathway to better understand why smoking is more harmful to women than men. Female non-smokers had lower plasma levels of glutamylcysteine, cysteinylglycine, and total glutathione than male non-smokers, whereas the level of cysteine, the limiting amino acid in glutathione synthesis, was not sexually divergent. The previous results suggest that there are sexual dimorphisms in transsulfuration, which provides a catabolic route leading to sulphate [27]. It is important to note that, at least in rat liver, the key enzyme in glutathione synthesis is present at a lower level in females [22]. Notably, the levels of the sulphur-containing amino acids homocysteine, glutamylcysteine, total glutathione and cysteinylglycine were strongly influenced by CS, and these effects were sex-gender specific. In particular, the slowdown in the transsulfuration pathway was not observed in female smokers and became comparable to that in men, with the exception of the cysteinylglycine level, which remained reduced by approximately 22%. Further confirmation of the sex-gender differences in transsulfuration comes from the sex-gender differences

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observed in the correlations between sulphur-containing compounds. Specifically, the total glutathione level was positively correlated with the levels of both its precursor and its metabolite only in male non-smokers; the total glutathione level was correlated with only the level of its precursor in female smokers.

Notably, the levels of two molecules that affect endothelial function [28], homocysteine and ADMA, were increased by CS in women, whereas in men, CS elevated only the homocysteine level. Furthermore, this effect was weaker in men than in women. The increase in homocysteine was directly linked to the increase in ADMA, which occurred only in women, suggesting that CS also affects the demethylation process in a sex-gender-specific manner. In addition, these findings suggest that the complex interplay between homocysteine and methylated arginines [28] is disrupted by CS in a sex-gender-specific manner. Homocysteine and ADMA are risk factors for cardiovascular diseases and thus, CS is more dangerous for women than men considering that sex-gender differences in endothelial function may underlie sex-gender differences in CVD [29].

The published data on the effects of CS on plasma antioxidants are not consistent with studies indicating that the levels of these antioxidants are unchanged, decreased, or increased [30-35]. The observed variability might be the result of differences in the study populations, e.g., differences in age, hormonal status, and OC use, and these differences make it impossible to obtain a perfect overview of the effects of CS on the antioxidant status. In our study, the plasma uric acid levels were not affected by CS in either men or women. Notably, CS reduced the magnitude of the sex-gender disparity observed between male and female non-smokers with respect to the levels of total bilirubin, total glutathione, and glutamylcysteine.

Given the pro-inflammatory properties of CS, it is not surprising that it can stimulate the bone marrow, leading to increases in platelets and WBCs in the bloodstream. Because the platelet count is associated with vascular and non-vascular death [36] the observed increase in the platelet count in female smokers could be clinically relevant and could partially explain the more deleterious effect of smoking in women.

CS also had a sex-gender-divergent effect on the WBC and WBC subset counts. As previously shown [37], these counts were increased by CS with the exception of the lymphocyte count, and this increase is considered a predictor of CVD [38]. Notably, the counts of cells that are present during every phase of atherogenesis, such as monocytes [39], were elevated by CS only in women. It is important to note that an increase in the monocyte count is associated with an increase of 15% in the risk for heart disease [40]. Monocytes differentiate into macrophages in the vessels and can be a source of cytokines such as TNF-alpha [41], which promotes endothelial dysfunction [42]. Here, the basal level of TNF-alpha release in men was more than twice that in women, whereas the level of TNF-alpha release induced by LPS was typically greater in women than in men. Collectively, these results reveal that hMDMs orchestrate TNF-alpha release in a sex-gender-dependent manner. Once again, the literature data on the effects of CS on cytokine release are conflicting. Most previous studies used alveolar macrophages [43, 44] and did not report the donor's sex, with some exceptions [45, 46]. Previously, we demonstrated that TNF-alpha release, at least in women, depends on the hormonal status [22], with basal cytokine release being significantly greater from hMDMs derived from women that use OC than from hMDMs derived from non-users [22]. In contrast, LPS-induced release is more elevated for hMDMs obtained from women who did not use OC [22].

The global DNA methylation was lower in young men than in young women, suggesting that sex-gender needs to be taken into consideration in studies that assess global DNA methylation as an epigenetic marker for the risk of diseases. Some authors have reported that the level of global DNA methylation is higher in men than in women [47, 48], but these studies included elderly subjects and did not consider the menstrual cycle phase or the use of OC or hormone replacement therapy. These factors could explain the divergence because total DNA methylation is influenced by the use of OC [22], being inversely associated with the concentrations of sex hormones (estradiol, testosterone) in postmenopausal women with low serum folate levels [49]. In addition, global DNA methylation could be modified by numerous lifestyle-

related factors, such as the consumption of compounds with estrogenic activity [50]. In this study, CS reduced the level of global DNA methylation, especially in women. Some previous studies did not find an association between CS and global DNA methylation [51, 52], but the hormonal status was not reported even though these studies analysed women aged from 35 to 75 years [53] or elderly men [54]. We observed that global DNA methylation was positively associated with age only in female smokers. The sex-gender differences in global DNA methylation could have remarkable consequences because global DNA methylation correlates with atherosclerosis [26]. Indeed, it is known that CS is a risk factor for breast cancer [55] and that leukocyte DNA hypomethylation is independently associated with breast cancer development [53]. The described sex-gender differences suggest that sex-gender and smoking need to be taken into consideration when studying global DNA methylation and when the relationships between DNA methylation and health outcomes are being assessed.

In conclusion, the present study provides convincing evidence that regular CS induces significant alterations in DNA methylation, the platelet count, the WBC count, the WBC subset count, endothelial function, transsulfuration, and hMDM function. Notably, the majority of variations were observed in young adult women, providing a basis for understanding the greater alarm for women than men [56] and suggesting that cessation programs targeting young women are urgently needed. Among individuals 18-40 years of age, CS did not substantially affect risk factors in men. Instead, we observed significant changes in women at an age at which women are normally free from risk factors, and these changes could contribute to the onset of CVD in women. Finally, the results suggest that the normal range for healthy subjects should be determined according to sex-gender and smoking status.

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Disclosure of conflict of interest

None.

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