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Higher lipid peroxidation in former-smokers vs. never-smokers – study in postmenopausal women

Dorota Sagan [1], Jan Stepniak [1], Adam Gesing [1], Andrzej Lewinski [2,3], Malgorzata Karbownik-Lewinska [1,3]

1 Department of Oncological Endocrinology, Medical University of Lodz, Lodz, Poland
2 Department of Endocrinology and Metabolic Diseases, Medical University of Lodz, Lodz, Poland
3 Polish Mother's Memorial Hospital – Research Institute, Lodz, Poland

Correspondence to: Prof. Malgorzata Karbownik-Lewinska, MD., PhD.
Department of Oncological Endocrinology, Medical University of Lodz
7/9 Zeligowski St., 90-752 Lodz, Poland.
tel/fax: +48 42 639 31 21 (22); e-mail: MKarbownik@hotmail.com

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Abstract

OBJECTIVES: One of the most spectacular exogenous prooxidative agents is cigarette smoking, constituting a well documented risk factor for several diseases. In turn it is suggested that hormone replacement therapy (HRT) in postmenopausal women can contribute to oxidative status. The aim of the study was to evaluate the level of oxidative damage to membrane lipids in blood serum collected from never-smokers and former-smokers. The study was performed in postmenopausal women, who were or were not HRT users.

METHODS: Ninety (90) female volunteers, aged from 46 to 67 years, were enrolled. Two major groups were considered, i.e. never-smokers (n=44) and former-smokers (n=46), which were additionally subgrouped to HRT users (HRT+) and HRT non-users (HRT–). Anthropometric parameters related to obesity were also calculated. The main groups were well matched at baseline in terms of age. The level of malondialdehyde+4-hydroxyalkenals (MDA+4-HDA), as the index of LPO, was measured spectrophotometrically.

RESULTS: The level of LPO was higher in former-smokers than in never-smokers, regardless of HRT use. The level of LPO did constitute the only independent factor associated with past smoking in the entire examined group, as well as after stratification to HRT users and HRT non-users. LPO level was not associated with HRT treatment. No positive correlations were found between LPO level and anthropometric parameters.

CONCLUSION: Past smoking is independently associated with the increased damage to membrane lipids regardless of the use of HRT in postmenopausal women. Smoking cessation is not always associated with complete reversion of excessive oxidative damage to all biological macromolecules.

ORIGINAL ARTICLE

INTRODUCTION

Oxidative reactions, producing reactive oxygen species (ROS), free radicals included, occur at certain level in all biological structures and they are indispensable for numerous physiological processes. However, when ROS are in excess, they are harmful, as they can damage all biological macromolecules, such as DNA, lipids and proteins. In consequence, the increased oxidative stress may potentially contribute to different disorders. The increased oxidative damage to macromolecules may result from both exogenous agents or endogenous disturbances, both documented in clinical and experimental studies (Karbownik *et al.* 2001; Karbownik and Lewinski 2003; Karbownik-Lewinska *et al.* 2008; 2012a; 2012b; Lewandowski *et al.* 2014; Milczarek *et al.* 2013; Modra *et al.* 2013; Stepniak *et al.* 2013; Szokalska *et al.* 2015; Stara *et al.* 2014; Szychta *et al.* 2014).

One of the most spectacular exogenous prooxidative agents is cigarette smoking. Smoking is a well documented risk factor for several diseases, such as for example lung cancer (Goldkorn *et al.* 2014), chronic obstructive pulmonary disease (Domei *et al.* 2014), cardiovascular disease (Siasos *et al.* 2014) and many others, with oxidative stress being deeply involved in the induction and progression of these diseases. Additionally, it is postulated that women are more sensitive to smoking, especially with respect to cardiovascular diseases (Kralova Lesna *et al.* 2012).

In agreement with the above, the increased oxidative stress was well documented in smokers (Hakim *et al.* 2012; Haswell *et al.* 2014). However, in most comparative studies two groups of subjects were usually taken into account, i.e. current-smokers versus never-smokers or current-smokers versus former-smokers. Only in few studies never-smokers were compared with former-smokers with reference to oxidative stress (e.g. Haswell *et al.* 2014).

In turn, one of the most important endogenous prooxidative agents is aging (Edrey *et al.* 2014), which is of particular importance in females due to hormonal postmenopausal changes. It is suggested that the depletion of estrogen in postmenopausal period can cause the enhanced oxidative stress in addition to known typical symptoms (Sánchez-Rodríguez *et al.* 2012).

Hormone replacement therapy (HRT) with the use of estrogen only or in a combined form (i.e. estrogen plus progestin) has been recommended in some postmenopausal women, in whom potential benefits justify potential risks (Sassarini *et al.* 2015). It is postulated that HRT can contribute to oxidative status in postmenopausal women. It was found recently that HRT with estrogen plus progestin had increased antioxidant enzymes and total antioxidant capacity in postmenopausal women (Unfer *et al.* 2015).

Different indices of oxidative damage to macromolecules can be measured in humans and in experimental models. One of the most frequently evaluated is lipid peroxidation (LPO), which results from oxidative damage to membrane lipids (Karbownik *et al.* 2001; Karbownik and Lewinski 2003; Karbownik-Lewinska *et al.* 2008; 2012a; 2012b; Lewandowski *et al.* 2014; Milczarek *et al.* 2013; Stepniak *et al.* 2013; Szokalska *et al.* 2015; Szychta *et al.* 2014).

Concerning studies in humans, the indices of oxidative stress are usually measured in blood serum/plasma (Karbownik-Lewinska *et al.* 2008; 2012a; 2012b; Lewandowski *et al.* 2014; Szychta *et al.* 2014).

The aim of the study was to evaluate the level of oxidative damage to membrane lipids in blood serum collected from never-smokers and from former-smokers. The study was performed in postmenopausal women, who were or were not HRT users.

MATERIALS AND METHODS

The procedures, used in the study, were approved by the Ethical Committee of the Medical University of Lodz (the approval number RNN/205/09/KE), and fully informed, written consent was obtained from the participants. The results presented in this paper constitute a part of the Ph.D. thesis of the first author.

A questionnaire was polled in a group of female participants being clients of the beauty parlour. The questionnaire contained questions related to major risk factors for civilization-related diseases (dietary history, physical activity, alcohol consumption, cigarette smoking, weight gain, contraceptive pills, hormone replacement therapy or other therapeutic agents using, ionizing radiation exposing, vaccinations, medical history of patient and family history).

Exclusion criteria constituted: cigarette smoking in the last 12 months, alcohol consumption, exposure to ionizing radiation or to any other potential prooxidative agent, and any diagnosed/undiagnosed acute or chronic disease.

Finally, ninety (90) adult female volunteers, aged from 46 to 67 years, were enrolled in the study. Two major groups were considered, i.e. never-smokers (n=44) and former-smokers (n=46), which were additionally subgrouped to HRT users (HRT+) and HRT non-users (HRT–). Therefore, 4 subgroups were considered: never-smokers (HRT+) (n=24), never-smokers (HRT–) (n=20), former-smokers (HRT+) (n=18), and former-smokers (HRT–) (n=28) (Table 1). The studied main groups were well matched at baseline in terms of age (no statistically significant differences between never-smokers and former-smokers were found when evaluated by a Student's unpaired *t* test) (Table 1). However, HRT users were younger that HRT non-users (from both main groups of never- and former smokers) (Table 1).

Body mass and body height were measured to calculate BMI, and waist circumference and hip circumference were measured to calculate waist/hip ratio (WHR). Concerning BMI, no statistical differences were found

between never-smokers and former-smokers, as well as between HRT users and HRT non-users among former-smokers. In the group of never-smokers, HRT non-users had higher BMI than HRT users (Table 1).

Blood samples were collected after an overnight fast. After collection, blood (1 ml) was centrifuged (3 000 × *g*, 10 min, 4°C) in order to obtain serum, and stored at –80 °C until assay.

LPO assay

The concentrations of malondialdehyde + 4-hydroxyalkenals (MDA+4-HDA), as the index of LPO, were measured in blood serum, using an LPO kit, purchased from Enzo Life Science (Farmingdale, NY). The serum (200 µl) was mixed with 650 µl of a methanol:acetonitrile (1:3, v/v) solution, containing a chromogenic reagent, N-methyl-2-phenylindole, and vortexed. After adding 150 µl of methanesulfonic acid (15.4 M), incubation was carried out at 45 °C for 40 min. The reaction between MDA+4-HDA and N-methyl-2-phenylindole yields a chromophore, which is spectrophotometrically measured at the absorbance of 586 nm, using a solution of 4-hydroxynonenal (10 mM) as the standard. The level of LPO was expressed as the amount of MDA+4-HDA (nmol) per 1 ml of serum.

Statistical analysis

The data were statistically analysed, using an unpaired Student's *t* test for two independent variables. The results are presented as means ± SEM. Univariate logistic regression analysis was used to determine which continuous variable might have determined smoking or determined no HRT treatment; in order to adjust for several risk factors, multivariate logistic regression analysis was performed with all the variables, found to be significant at the univariate analysis, entering in a single step. For the evaluation of correlation among particular parameters, Pearson's correlation coefficient was used. Statistical significance was determined at the level of $p<0.05$.

RESULTS

The level of LPO products in blood serum was evaluated in never-smokers or in former-smokers being HRT users (HRT+) or not being HRT users (HRT–) (Figure 1). The level of LPO was higher in former-smokers than in never-smokers regardless of HRT treatment (Figure 1A), as well as in both subgroups of HRT users and HRT non-users (Figure 2B). In the group of never-smokers, the level of LPO did not differ between HRT users and HRT non-users (Figure 1B). Similarly, in the group of former-smokers, the level of LPO did not differ between subjects obtaining HRT and not obtaining HRT (Figure 1B).

When Pearson's correlation coefficient was calculated between LPO level and such parameters as age, body mass, height, BMI, waist circumference, hip circumference and WHR, no positive correlations were found either in the entire group of participants or in subgroups of never- or former-smokers (Table 2). The statistically significant negative correlation between LPO level and hip circumference (Table 2) is probably incidental, as it is not in agreement with other results from the present study and from numerous other studies, and will not be discussed.

For the entire study group of subjects, as well as separately for subgroups obtaining HRT and not obtaining HRT, variables such as age, body mass, height, BMI, waist circumference, hip circumference and waist:hip ratio (WHR), and blood LPO level were submitted to a univariate and a multivariate logistic regression model. The purpose of the model was to determine which of those continuous variables might have been independently associated with past smoking (Table 3). The level of LPO did constitute the only independent factor associated with past smoking in the entire examined group,

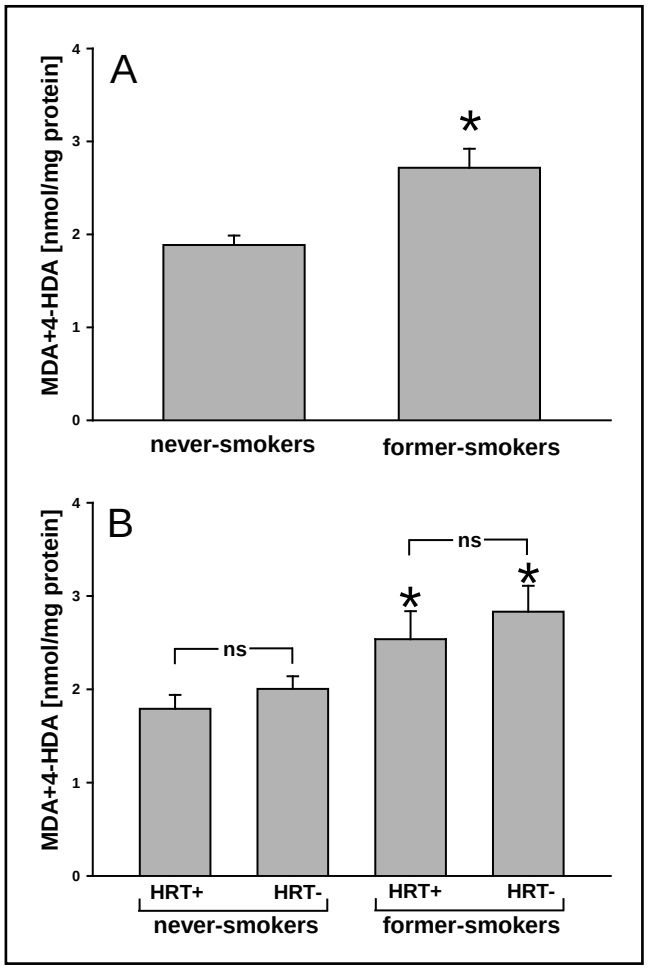

Fig. 1. Mean (± SEM) values of lipid peroxidation in never-smokers (n=44) and in former-smokers (n=46), stratified to HRT users (HRT+) (n=42) and HRT non-users (HRT-) (n=48). Statistical evaluation was performed by an unpaired Student's t-test. The level of LPO was expressed as the amount of MDA+4-HDA (nmol) per 1 ml of serum. *$p<0.05$ vs. never-smokers (A); *$p<0.05$ vs. never-smokers with the same HRT status (B).

Tab. 1. Basic characteristic of female patients.

	never-smokers n=44		former-smokers n=46		*p*-value
Age [years]	54.90±0.81		53.30±0.63		0.123
BMI [kg/m^2]	26.07±0.62		25.81±0.62		0.769
	HRT+ n=24	**HRT–** n=20	**HRT+** n=18	**HRT–** n=28	***p*-value**
Age [years]	51.62±0.75	58.85±0.99*	50.66±0.94	55.00±0.69*	<0.001 <0.001
BMI [kg/m^2]	24.73±0.78	27.69±0.89*	25.82±1.04	25.81±0.79	0.016 0.998

n – number of patients. Data are shown as mean ±SEM. *p<0.05 vs. HRT+ of the same main group. When two p values are given, the first one relates to never-smokers, and the second one relates to former-smokers.

Tab. 2. Correlations between LPO level and anthropometric parameters in all participants and after stratification to never-smokers and former-smokers.

	LPO [MDA + 4-HDA (nmol/ml)]		
	All	**Never-smokers**	**Former-smokers**
Age [years]	–0.114 *0.286*	0.060 *0.701*	–0.141 *0.349*
Body mass [kg]	–0.054 *0.612*	–0.238 *0.124*	0.064 *0.673*
Height [m]	–0.019 *0.858*	–0.201 *0.196*	0.152 *0.313*
BMI [kg/m^2]	–0.057 *0.594*	–0.164 *0.292*	–0.002 *0.988*
Waist circumference [cm]	–0.016 *0.878*	–0.299 *0.051*	0.115 *0.445*
Hip circumference [cm]	–0.071 *0.506*	–0.360 *0.018*	0.021 *0.888*
WHR	0.065 *0.542*	–0.162 *0.298*	0.192 *0.200*

Values in normal style present Pearson's correlation coefficient; values in italic present level of significance, with statistical significance accepted at the level of 0.05.

as well as in HRT users. Among HRT non-users, LPO level was positively associated with smoking at univariate regression model, but it lost its statistical significance after submitting to multivariate regression model (Table 3); in the subgroup of HRT non-users the only independent factor associated negatively with smoking was age (Table 3), which is without practical significance concerning the context discussed.

Similarly, the associated between the above variables, such as age, body mass, height, BMI, waist circumference, hip circumference, WHR and blood LPO level were submitted to a univariate and a multivariate logistic regression model to determine which of those continuous variables might be independently associated with no HRT treatment (Table 4). In this model, the level of LPO was not associated with HRT treatment in the entire examined group, as well as in subgroups of former- and never-smokers (Table 4). Age was positively associated with no HRT treatment (Table 4), which is in agreement with that that older patients were less frequently on HRT. The association of height with HRT treatment (Table 4) is probably incidental and will not be discussed in this paper.

DISCUSSION

In the present study we have found the association between oxidative damage to macromolecules and past smoking in postmenopausal women, regardless of HRT treatment.

First we will discuss the deleterious effects of smoking. Different biomarkers of oxidative damage can be measured in cigarette smokers but, which is of great importance, they do not measure identical aspects of oxidative stress and some of them reflect acute whereas others chronic oxidative stress (Seet *et al.* 2011). We documented in the present study that cessation of smoking for at least 1 year is still associated with the increased values of oxidative damage to membrane lipids. The increased oxidative damage to membrane lipids was documented by the higher absolute value of LPO in former-smokers of both subgroups, i.e. HRT users and HRT non-users. Additionally, it was confirmed in regression analysis, in which LPO did constitute the only independent determinant of past smoking in the entire examined group and after stratification to HRT users and HRT non-users. This finding suggests that LPO level is directly associated with smoking in the past.

However, there are also some studies published showing that smoking cessation reverses oxidative damage to the level seen in non-smokers. For example, smoking cessation for 1 month only reduced the level of DNA double-strand breaks in peripheral mononuclear cells to the control level (Ishida *et al.* 2014). It is well known that different macromolecules are differently sensitive to prooxidative agents, with DNA being – on one hand – very sensitive, but – on the other hand – being rapidly repaired. In contrast, the damaged membranes are normally not repaired, which may result in persisted higher level of oxidative damage to their components. It was documented under experimental conditions that human brain cells loss their integrity (being dependent on membrane quality) along with the increased production of ROS in response to cigarette smoke extract/condensate treatment (Kim *et al.* 2015). Of importance is the finding that smoking changes significantly expression of genes, especially those involved in immune responsive pathway (Na *et al.* 2015), which may have an impact on long-lasting changes of different

Tab. 3. Univariate and multivariate logistic regression analysis of the univariate **past smoking** determinants (variables), performed in all examined subjects (n=90) and after stratification to HRT users (HRT+) (n=42) and HRT non-users (HRT–) (n=48).

Variable	All		HRT-				HRT+	
	Univariate regression		Univariate regression		Multivariate regression		Univariate regression	
	OR	95%CI	OR	95%CI	OR	95%CI	OR	95%CI
Age [years]	0.934 *0.125*	0.85–1.02	0.782 *0.007*	0.65–0.93	0.798 *0.018*	0.65–0.96	0.933 *0.417*	0.78–1.10
Body mass [kg]	0.984 *0.385*	0.94–1.03	0.947 *0.072*	0.89–1.00	–	–	1.013 *0.612*	0.96–1.06
Height [m]	0.003 *0.138*	1×10^{-5}–6.9	0.027 *0.571*	6×10^{-6}– 10689.99	–	–	0.008 *0.377*	10×10^{-6}– 460.284
BMI [kg/m^2]	0.985 *0.766*	0.88–1.09	0.892 *0.128*	0.76–1.03	–	–	1.070 *0.389*	0.91–1.25
Waist circumference [cm]	1.000 *0.996*	0.96–1.03	0.975 *0.349*	0.92–1.02	–	–	1.024 *0.463*	0.95–1.09
Hip circumference [cm]	1.002 *0.937*	0.95–1.05	0.982 *0.589*	0.91–1.04	–	–	1.018 *0.633*	0.94–1.10
WHR	1.094 *0.981*	0.00006– 1863.74	0.002 *0.253*	7×10^{-7}– 108.70	–	–	240.060 *0.358*	0.0014– 40612640
LPO level [MDA + 4-HDA (nmol/ml)]	2.211 *0.002*	1.32–3.68	2.139 *0.038*	1.02–4.47	1.753 *0.150*	0.79–3.85	2.171 *0.033*	1.04–4.52

OR, odds ratio; CI, confidence interval; the level of statistical significance is given in italic, with statistical significance accepted at the level of 0.05.

Tab. 4. Univariate and multivariate logistic regression analysis of the univariate **HRT non-use (HRT-)** determinants (variables), performed in all examined subjects (n=90) and after stratification to never-smokers (n=44) and former smokers (n=46).

Variable	All				Never-smokers				Former-smokers	
	Univariate regression		Multivariate regression		Univariate regression		Multivariate regression		Univariate regression	
	OR	95%CI	OR	95%CI	OR	95%CI	OR	95%CI	OR	95%CI
Age [years]	1.366 *0.000*	1.19–1.56	1.318 *0.000*	1.15–1.50	1.557 *0.001*	1.18– 2.04	1.438 *0.006*	1.09–1.88	1.347 *0.003*	1.10–1.64
Body mass [kg]	0.999 *0.949*	0.96–1.03	–	–	1.037 *0.222*	0.97– 1.10	–	–	0.975 *0.316*	0.92–1.02
Height [m]	0.000 *0.002*	1×10^{-10}– 0.0003	0.000 *0.046*	1×10^{-10}– 0.96	0.000 *0.019*	1×10^{-18}– 0.04	0.000 *0.200*	6×10^{-20}– 20413.55	0.000 *0.052*	8×10^{-10}– 1.53
BMI [kg/m^2]	1.088 *0.113*	0.97–1.20	–	–	1.215 *0.024*	1.02– 1.44	1.117 *0.853*	1.46–5.07	1.000 *0.998*	0.83–1.19
Waist circumference [cm]	1.035 *0.102*	0.99–1.08	–	–	1.062 *0.056*	0.99– 1.13	–	–	1.011 *0.709*	1.07–1.79
Hip circumference [cm]	1.029 *0.267*	0.97–1.08	–	–	1.062 *0.157*	0.97– 1.15	–	–	1.009 *0.791*	0.94–1.07
WHR	633.576 *0.102*	0.24– 16×10^5	–	–	187364.600 *0.040*	1.21– 24×10^9	5.891 0.841	1×10^{-6}– 3×10^7	2.557 *0.867*	6×10^{-6}– 2×10^5
LPO level [MDA + 4-HDA (nmol/ml)]	1.353 *0.128*	0.91–2.00	–	–	1.635 *0.301*	0.62– 4.26	–	–	1.175 *0.482*	0.73–1.86

OR, odds ratio; CI, confidence interval; the level of statistical significance is given in italic, with statistical significance accepted at the level of 0.05.

processes, oxidative processes included. As the method used in the present study measures LPO in all cellular membranes, with endoplasmic reticulum constituting the substantial part of them, it is worth mentioning that experimentally-induced exposure to smoke resulted in endoplasmic reticulum oxidative stress-mediated lipid accumulation (Kunchithapautham *et al.* 2014). It is not excluded that the last mechanism can have the leading role in persisted high LPO levels even after smoking cessation.

Although smoking cessation can be associated with reversion – at least to a certain extent – of cellular damage, long-term consequences are observed in some former smokers. It has been documented recently that, comparing to never-smokers, former heavy smokers have higher risk for heart failure and mortality, when evaluated at least 15 years after cessation of smoking (Ahmed *et al.* 2015). Additionally, it was found that ever-smokers (current- and former-smokers included) have increased risk for development of second primary lung cancer when compared to never-smokers (Boyle *et al.* 2015).

Concerning the use of HRT, it did not affect significantly the level of oxidative damage to membrane lipids in our study. Additionally, HRT non-users were older than HRT users, which theoretically could have contributed to higher oxidative damage in the former group, but it did not in the present study. In a similar clinical model, the level of lipid damage was not affected by combined HRT, although it was decreased by estrogen-only replacement therapy (Escalante Gomez & Quesada Mora 2013). In one of the earliest studies, oxidative stress was reduced in postmenopausal women with exercise training regardless of HRT use, although the baseline level of oxidative damage was higher in HRT non-users (Attipoe *et al.* 2008). Thus, there are some studies published suggesting the beneficial effects of HRT on oxidative processes in postmenopausal women. However, our findings do not confirm such beneficial effects of HRT and, at the same time, they do not support the universal use of HRT.

It is repeatedly documented, also by the authors of the present study (Karbownik-Lewinska *et al.* 2012a; 2012b; Szokalska *et al.* 2015), that overweight and obesity are associated with increased oxidative damage to membrane lipids and the extent, to which these macromolecules were oxidatively damaged, did depend on the degree of obesity with gradual significant increase in LPO serum level with increasing BMI (Karbownik-Lewinska *et al.* 2012b). However, in the present study no positive correlations between LPO and anthropometric parameters related to obesity were found, which can be explained by the influence of other factors, especially smoking, being probably stronger prooxidative agent than obesity. Additionally, LPO was measured in a specific group, i.e. in postmenopausal women, in whom hormonal changes may potentially affect oxidative processes.

Summing up, even smoking in the past is independently associated with the increased damage to membrane lipids regardless of the use of HRT in postmenopausal women. Smoking cessation is not always associated with complete reversion of excessive oxidative damage to all biological macromolecules.

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Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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