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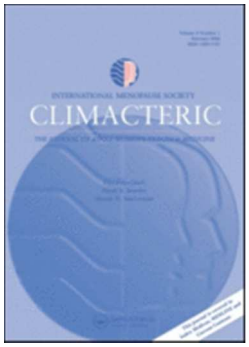

Relation Between Oxidative Stress and Climacteric Symptoms in Early Postmenopausal Women.

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Keywords:	free radicals, antioxidant, hot-flush, lipid, menopause, pulsatility index, carotid artery

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Relation Between Oxidative Stress and Climacteric Symptoms in Early Postmenopausal Women.

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Short Title: Flush Oxidative Stress

Key Words: free radicals; antioxidant; lipid; hot-flush; menopause; pulsatility index; carotid artery

Abstract

Objectives: To evaluate the relation between climacteric symptoms or other risk factors for cardiovascular disease and oxidative status of postmenopausal women.

Methods: Cross-sectional investigation performed at the outpatient service for the menopause at University hospital, on 50 apparently healthy women in physiological post-menopause. Whole blood free oxygen radicals (FORT), free oxygen radical defence (FORD) age, months since menopause, weight, body mass index (BMI), waist girth, waist to hip ratio (WHR), estradiol, lipids, glucose, insulin, insulin resistance (glucose/insulin and HOMA-IR), and fibrinogen were evaluated. The Greene's scale with its subscales was used to evaluate climacteric symptoms. The pulsatility index (PI), an index of downstream blood flow resistance, was determined for both the internal carotid and the brachial artery.

Results: WHR ($r=0.540$; $p<0.0001$), estradiol ($r=0.548$; $p<0.0004$) and waist ($r=0.345$; $p<0.02$) were independently related to blood FORT. Score of the Greene's vasomotor sub-scale was the only parameter independently related to blood FORD ($r=0.55$; $p<0.0001$). FORT was not related to artery PI, while FORD was negatively related to the PI of both internal carotid ($r=549$; $p<0.0001$) and brachial ($r=0.484$; $p<0.0001$) artery.

Discussion: In postmenopausal women, abdominal adiposity and hypoestrogenism increase oxidative stress. Climacteric symptoms, particularly vasomotor symptoms, markedly reduce antioxidant defences. Lower antioxidant defences are associated with higher resistance to blood flow of great arteries. In women early after the menopause, visceral fat, hypo-estrogenism, and climacteric symptoms may increase the risk for cardiovascular disease.

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1. Introduction

Oxidative stress, consequent to excessive production of free oxygen radicals or impaired oxygen defence, is linked to diseases such as metabolic syndrome, diabetes, obesity, chronic sub-clinical inflammation, and cardiovascular disease [1-9]. The role played by the menopause on oxidative stress is less clear, with either increases [10,11] or decreases [12,13] being described. Visceral adiposity [14,15], often occurring in postmenopausal women [16-18], seems to be associated with an increased oxidative status. Similarly, one study reported that oxidative stress is increased by the presence of hot flushes [9]. Hot flushes and climacteric symptoms have been related to insulin resistance, lipid abnormalities or adiposity [20-24], all parameters that may impact on oxidative balance. Whether climacteric complaints indeed relate, independently, to oxidative stress, is presently unclear and it was herein investigated. The impact of oxidative status on vessel compliance was also evaluated.

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2. **Methods**

In this cross-sectional investigation women in post-menopause were recruited at the outpatient centre for the menopause and post-menopausal osteoporosis of our University Hospital. The local ethics committee had previously approved the study and at enrolment each woman signed a consent form.

Out of 320 consecutive peri-post-menopausal women screened in a 6-month period, 50 patients were recruited and gave their informed consent to participate into the study. Number of subjects was selected on the basis of previous studies performed with the same procedures [7-9,25]. All women were Caucasian and in physiological menopause for at least 12 months and with no major chronic disease. None was receiving hormone therapy, medicine for the control of glucose or lipid levels, anti-hypertensive, nutritional supplements or vitamins possibly interfering with oxidative stress.

For each woman a medical history was retrieved and climacteric symptoms were evaluated. In addition the following parameters were collected: age, time since menopause, height (in meters) and weight (in kilograms), both obtained with women wearing light clothing and no shoes. Body mass index was calculated as the ratio of weight in kilograms to the square of height in meters (BMI; Kg/m^2). Waist and hip girths were measured on standing women along the horizontal plane at the level of the natural waist (narrowest abdominal circumference) and at the level of the hip (maximum extension of the buttocks). The waist/hip ratio (WHR) was calculated. A fasting blood sample was collected in the morning, at about 8.00 AM, for analysis of free oxygen radicals (FORT), free oxygen radical defence (FORD), estradiol, blood count, total cholesterol, HDL-cholesterol (HDL-C), triglycerides, glucose, insulin, and fibrinogen. Blood samples were collected into tubes placed on ice. An aliquot of blood was immediately used to evaluate FORT and FORD, while the other part was centrifuged. An aliquot of serum was immediately frozen at −25°C until and then used for insulin assay, and another aliquot immediately used for the other

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biochemical evaluations. In each woman the pulsatility index (PI) of the internal carotid artery and of the brachial artery [20], were evaluated in the afternoon, at least 4 hours after meal.

2.1. Climacteric Symptoms

The Greene's climacteric scale was used to evaluate climacteric symptoms [26]. The Greene's climacteric scale is composed of 21 items that evaluate vasomotor symptoms (two items), anxiety (six items), depression (five items), somatic symptoms (seven items), and sexuality (one item). All items have four options that range from not at all (0), a little (1), quite a bit (2), to extremely (3). The sum of items score is used to obtain the Greene climacteric scale score as a whole (range 0-63) or the scores of individual subscales for vasomotor symptoms (range, 0 to 6), anxiety (range, 0 to 18), depression (range, 0 to 15), somatic symptoms (range, 0 to 21) and sexuality (range, 0 to 3). Total score and subscales scores were used into the analysis.

2.2. Biochemical Evaluation

All analyses were performed in the same centralized laboratory at our University Hospital.

Reactive oxygen species were determined using the FORT test (FORM®, CR 2000, Callegari, Parma, Italy)[7-9], a colorimetric assay based on the ability of transition metals, such as iron, to catalyse the breakdown of hydroperoxides (ROOH) into derivative radicals, according to the Fenton reaction. When 20 µL of blood sample was dissolved in an acidic buffer, the hydroperoxides reacted with the transition metal ions liberated from the proteins in the acidic medium and were converted to alkoxy (RO·) and peroxy (ROO·) radicals. The radical species produced by the reaction interact with an additive (phenylenediamine derivative [2CrNH2]) that forms a coloured, fairly long-lived radical cation evaluable by spectrophotometer at 505 nm (linear kinetic-based reaction, 37°C). The intensity of the colour correlates directly to the

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quantity of radical compounds and to the hydroperoxides concentration and, consequently, to the oxidative status of the sample according to the Lambert-Beer law (Form CR 2000, Callegari).

All FORT reagents were stored at room temperature and ready to use without additional preparations. Results are expressed as FORT units, whereby 1 FORT unit corresponds to 0.26 mg/L H2O2. The intra-assay and inter-assay coefficients of variation of the method are 3.7% and 6.2%, respectively.

The FORD test [7] uses preformed stable and coloured radicals and determines the decrease in absorbance that is proportional to the blood antioxidant concentration of the sample according to the Lambert-Beer law. In the presence of an acidic buffer (pH = 5.2) and a suitable oxidant (FeCl3), the chromogen that contains 4-amino-N,N-diethylaniline sulphate forms a stable and coloured radical cation photometrically detectable at 505 nm. Antioxidant compounds in the sample reduce the radical cation of the chromogen, quenching the colour and producing a decolouration of the solution, which is proportional to their concentration. The absorbance values obtained for the samples are compared with a standard curve obtained using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a permeable cell derivative of vitamin E commonly used as an antioxidant.

All FORD reagents were stored at room temperature and ready to use without additional preparations. The intra-assay and inter-assay coefficients of variation are 4.2% and 6.6%, respectively.

Glucose was determined by the glucose oxidase method. Plasma total cholesterol and triglycerides were measured by enzymatic colorimetric methods (instrument Cobas c 501, Roche, Germany), while HDL-C was determined after precipitation with PEG 6000. HDL-C/T-cholesterol and HDL-C/Triglycerides were calculated as additional cardiovascular risk factors. Estradiol was measured in a single batch in all samples in duplicate by an ELISA kit (DRG Estradiol sensitive ELISA, DRG International, Springfield, NJ, USA). Intra- and Inter- assay of variation were 6.2% and 7.5%, respectively, and sensitivity was 1.4 pg/ml. Insulin levels were

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assayed in a single batch, in all samples (in duplicate) by an ELISA kit (DRG Instrument GmbH, Marburg, Germany). Intra-assay and inter-assay coefficients of variation were 2.6% and 6.0%, respectively, and sensitivity was 1.76 μU/ml. Insulin resistance was investigated by both the glucose/insulin ratio and the homeostatic model assessment for insulin resistance (HOMA-IR) which was calculated as [fasting glucose (mmol/l) * fasting insulin (IU/l)]/22.5.

2.3. Pulsatility Index

Each woman was requested to relax, supine, in a low-noise, low-light, constant-temperature (20–21°C) environment for the 30 min preceding the investigation. Blood flow velocity waveforms were evaluated by colour Doppler ultrasound with an ultrasound machine (Voluson E6, GE Medical Systems Italia SPA, Milano) following a procedure previously described [27]. The investigation was initiated when heart rate and systolic and diastolic blood pressure, recorded with an automatic device (Dinamap 845 XT, Critikon, Tampa, Florida) at 5 min intervals, varied by <5 beats per min and by <5 mmHg, respectively over two consecutive readings.

The PI, which is the ratio of the maximal Doppler shift in systole on the mean Doppler shift of the entire waveform, is thought to directly represent downstream vasomotor state, and it was measured, three times for each evaluation, from the blood flow velocity waveform. The mean of the three measurements was used for the statistical analysis. The internal carotid artery was sampled 1.5 cm above the common carotid artery, and the brachial artery was sampled proximal to the antecubital fossa by a 4-10.0 MHz linear-array transducer (11L-D H40432LN, GE Medical Systems Italia SPA, Milano). The same operator performed Doppler evaluations. The intra-operator coefficient of variation was <3.5%.

2.4. Statistical Analysis

Greene'scores, anamnestic (age, months since menopause), anthropometric (weight, BMI, waist, WHR), and biochemical parameters (estradiol, Total cholesterol, HDL-C, triglycerides, HDL-

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C/Total cholesterol, HDL-C/triglycerides, glucose, insulin, glucose/insulin, HOMA-IR) were related to FORT or FORD, by simple regression analyses. In order to identify indexes independently related to oxidative stress, parameters significantly related to FORT or FORD were entered into stepwise regression analysis with FORT or FORD as dependent variable. Simple regression analysis was used to relate FORT, FORD or all the other parameters to the PI of the internal carotid artery or of the brachial artery. The independent relation of FORT or FORD with artery PI was tested by stepwise regression analysis where Greene's scores, anamnestic, anthropometric or biochemical indexes, eventually related to that particular functional index, were included.

For all parameters a two-tail p value below 0.05 was considered as significant.

All numerical results are expressed as mean±standard deviation (SD).

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3. **Results**

General characteristics of the enrolled women are reported in Table 1 and Table 2.

3.1 Oxidative balance

FORT values were related, positively, to WHR (r=0.540; p=0.001), estradiol (r=0.545; p<0.0004), fibrinogen (r=0.538; p=0.001), and waist (r=0.345;p=0.02). By stepwise regression analysis (r=0.898; p=0.0001) only WHR, waist and estradiol remained independently related to FORT (Table 3), with WHR explaining 68.8%, waist 12.1% and estradiol 8.9% of FORT variation, respectively.

FORD values were related, negatively, to the Greene's score (0.408; p<0.004), to the Greene's sub-scores for depression (0.306; p<0.03), vasomotor symptoms (r=0.554; p<0.0001), and anxiety (r=0.371; p<0.01), and to Total cholesterol (r=0.23;p=0.05). A positive relation was observed between FORD and HDL-C/Total cholesterol (r=0.31;p<0.006). By stepwise regression analysis the Greene's sub-score for vasomotor symptoms remained the only variable independently related to FORD (Figure 1).

3.2 Pulsatility Index

The internal carotid artery PI was related positively to the Greene's score (r=0.361; p<0.003), the Greene's sub-score for depression (r=0.276; p<0.025), somatization (r=0.484; p<0,0001), and vasomotor symptoms (r=0.477; p<0.0001), and negatively to FORD (r=0.587;p<0.0001). By stepwise regression analysis (r=0.655; p<0.0001), only FORD and the Greene's sub-score for somatization, remained independently related to the internal carotid artery PI. FORD explained 58.7% and the Greene's sub-score for somatization 6.8% of PI variance, respectively. The best relation between FORD and internal carotid artery PI was obtained by a second-degree polynomial regression (artery PI= 2.97-4.03x+2.09 x2; r=0.549; p=0.0001)(Figure 2).

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The brachial artery PI was related negatively only to FORD. The best relation between FORD and brachial artery PI was obtained by a second-degree polynomial regression (artery PI= 20.91-38.9x+21.8x2; r=0.484; p=0.0001)(Figure 2).

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4. **Discussion**

Present data show that in post-menopausal women FORT is mainly determined by visceral fat, indirectly evaluated by the measure of WHR or waist girth. This confirms previously published data in which visceral adiposity was evaluated also by DEXA (1,15,28). An antioxidant effect of estrogens was previously reported (11), and in our study, low levels of estradiol contribute to an increased oxidative stress. What is interesting is that in our post-menopausal women blood antioxidant defence are majorly determined by climacteric complaints. Among possible endocrine, metabolic and body parameters, it was only climacteric symptoms, which were capable to independently influence FORD. One group of researchers has previously shown that antioxidant defence are reduced in post-menopausal women with hot flushes (19). The present study implements that evidence by showing that climacteric symptoms, particularly vasomotor complaints, are linearly and negatively linked to FORD.

From all these data, it seems that clinical manifestations are more important than specific metabolic risk factors in determining oxidative status. Insulin resistance (1,5-7,28) or sub-chronic inflammation (1,8), herein exemplified by levels of fibrinogen (29), may indeed elevate free radicals. On the other hand, these metabolic modifications are often associated with increased visceral fat, and in our postmenopausal women it was visceral fat that was linearly related to FORT. Antioxidant defence can be increased by HDL-C lipoproteins that carry on their surface the antioxidant enzymes paraoxonase (1,28). Though not in all the studies climacteric complaint were associated to lipid abnormalities and atherosclerosis progression (30,31), in most of them, hot flushes were associated with decreased HDL-C levels (21-24). In our group of postmenopausal women it was climacteric complaint, and not HDL-C or other metabolic parameters, that was linearly related to the decrease of antioxidant defences.

Oxidative stress is a risk factor for cardiovascular diseases (1,3,4,9). Artery PI represents downstream resistance to blood flow, higher values being associated with higher resistance (27). In the internal carotid artery an increased PI is associated with hypertension, diabetes, lipid

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abnormalities, cerebral atherosclerosis and stroke [32-35]. In the present study we found that, among all those considered, the parameter majorly related to internal carotid artery PI is FORD, higher FORD values being associated with lower PI. The association appeared not only statistically but also clinically significant, high downstream resistance to blood flow (32-35) being reached in the internal carotid artery at low FORD values. The finding is in accordance with a previous study showing that in postmenopausal women the acute infusion of the antioxidant ascorbic acid decreases internal carotid artery PI, by 28% [36]. PI data of the internal carotid artery were replicated by brachial artery investigation, indicating a general positive association between FORD and a decreased resistance to blood flow of great arteries. Evaluation of major antioxidant and oxidative stress marker may have furnished a more appropriate appreciation of the oxidative stress levels that was herein evaluated by FORT and FORD. Furthermore these data cannot be expanded to women with chronic metabolic or cardiovascular disease, that were excluded from the study. Nevertheless, the data are rather strong in showing that in postmenopausal women oxidative stress is related to climacteric symptoms and visceral adiposity. The data furnish additional support for considering climacteric complaints a risk factor for cardiovascular disease.

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Conflict of Interest: No author declared any conflict of interest with the data reported on the manuscript

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Legends to Figures

Figure 1. Simple regression analysis between the score of the Greene's vasomotor sub-scale and blood free oxygen radical defence (FORD) of 50 healthy postmenopausal women.

Figure 2. Second-degree polynomial regression between blood free oxygen radical defence (FORD) and the pulsatility index of the internal carotid artery (upper graph) or brachial artery (lower graph) of 50 healthy postmenopausal women.

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Table 1. Mean (±SD) characteristics and scores of climacteric symptoms (by the Greene scale) of 50 post-menopausal women enrolled into the study.

Age (yrs.)	55.5±4.3
Menopause (months)	69.6±32.3
Weight (kg)	65.2±12.1
BMI (m/kg2)	25.9±4.7
Waist (cm)	88.7±12.4
WHR	0.86±0.07
Greene	15.9±9.3
Greene anxiety	4.8±2.7
Greene depression	3.7±2.8
Greene somatization	3.6±3.2
Greene vasomotor	2.3±1.9
Greene sexuality	1.35±1.9

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Table 2. Mean (±SD) values of biochemical indexes and artery pulsatility indexes (PI) of 50 post-menopausal women enrolled into the study.

T-Cholesterol (mg/dl)	218.7±68.2
HDL-cholesterol (mg/dl)	63.8±9.7
Triglycerides (mg/dl)	116.2±53.3
Glucose (mg/dl)	88.2±26.8
Insulin (IU/l)	15.5±7.5
Glucose/insulin	9.6±5.4
HOMA-IR	3.3±1.8
Estradiol (pg/ml)	20.2±9.5
Fibrinogen	347.7±68.6
FORT (units)	356.4±111.8
FORD (mmol/l Trolox)	0.727±0.29
Internal Carotid artery PI	1.28±0.67
Brachial artery PI	6.11±5.36

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Table 3.
Significant Pearson's correlation coefficients (R), p values, and coefficients of regression (CR), with 95% confidence intervals (95% CI), of simple regression analyses performed between parameters related to FORT or FORD in 50 postmenopausal women.

FORT

	R	p	CR	95%CI
WHR*	0.540	0.0001	1067	585; 1550
Estradiol*	0.548	0.0004	-4.76	-7.24; -2.28
Waist*	0.345	0.02	3.25	0.68; 5.83
Fibrinogen	0.538	0.001	0.584	0.25; 0.910

FORD

	R	p	CR	95%CI
Greene vasomotor*	0.554	0.0001	-0.09	-0.13;-0.05
Greene	0.408	0.003	-0.015	-0.025;-0.005
Greene anxiety	0.371	0.008	-0.05	-0.08; -0.013
HDL-C/T-cholesterol	0.371	0.006	1.61	0.48; 2.75
Greene depression	0.307	0.03	-0.033	-0.06;-0.004
T-Cholesterol	0.233	0.05	-0.002	-0,004; -0,0001

* parameters independently related to FORT or FORD

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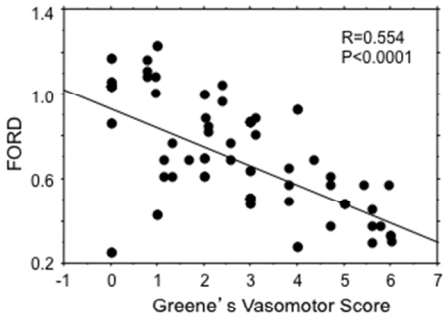

Figure 1. Simple regression analysis between the score of the Greene's vasomotor sub-scale and blood free oxygen radical defence (FORD) of 50 healthy postmenopausal women.
254x190mm (72 x 72 DPI)

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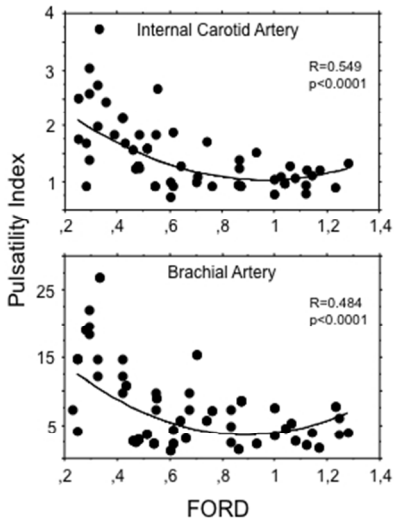

Figure 2. Second-degree polynomial regression between blood free oxygen radical defence (FORD) and the pulsatility index of the internal carotid artery (upper graph) or brachial artery (lower graph) of 50 healthy postmenopausal women.
254x190mm (72 x 72 DPI)

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