

# Protein oxidation in breast microenvironment: Nipple aspirate fluid collected from breast cancer women contains increased protein carbonyl concentration

Ferdinando Mannello\*, Gaetana A. Tonti and Virginia Medda

*Department of Biomolecular Sciences, Section of Clinical Biochemistry, University of Urbino, Urbino, Italy*

**Abstract.** *Background:* Protein carbonyl levels are the most frequently used biomarker of protein oxidation in several human diseases, including cancer. Breast cancer, a worldwide disease with increasing incidence, develops from ductal/lobular epithelium from which nipple aspirate fluid can be collected and analysed to assess tissue metabolic activity. Our aims were to perform an exploratory investigation on the protein carbonyl accumulation in breast secretions from healthy and cancer patients and its correlation with lipid peroxidation markers.

*Methods:* Protein carbonyls were determined by ELISA in 288 Nipple Aspirate Fluids (NAF) from Control, Pre-malignant and Cancer patients.

*Results:* Significantly higher protein carbonyl concentration was found in NAF from breast cancer (BC) patients compared to Control subjects. Cancer patients accumulated in NAF significantly higher levels of carbonyls in post-menopausal condition. A significant inverse relationship between carbonyls and 8-F<sub>2α</sub>-isoprostanes in NAF was found in Cancer patients. NAF levels of protein carbonyls are significantly higher in women with pre-malignant conditions than in healthy subjects.

*Conclusion:* Our results support the hypothesis that oxidative stress in breast microenvironment plays a role in breast cancer; measurement of protein and lipid oxidative products in NAF may improve the identification of women at increased breast cancer risk.

**Keywords:** Nipple aspirate fluid, oxidative stress, protein carbonyl, breast cancer

## 1. Introduction

Oxidative stress is characterized by the imbalance between the activity of antioxidant enzymes (e.g., superoxide dismutase, catalase and glutathione peroxidase) and the concentration of reactive oxygen species (ROS) (e.g., superoxide, hydrogen peroxide and hydroxyl radical), resulting in the damage of lipids, carbohydrates, proteins and nucleic acids [1,30]. In particular, the oxidative damage to proteins brings to the formation of protein–protein cross-linked derivatives or oxidation of aminoacid side-chains, through structural changes of protein backbone and/or pep-

ptide bond cleavage, finally resulting in loss of physiological functions [38]. Although ROS are generated during normal cellular metabolism with possible cancer protective functions [35,37,42], ROS-induced DNA/lipid/protein damages play important roles during the development and progression of several human pathological processes [14], including cancer [18,43]. Carbonyl groups are formed during the oxidation of protein side chains, resulting in chemically stable products which serve as useful markers for assessing oxidative stress *in vivo* [38]; their accumulation in biological fluids has been observed in several physio-pathological processes [4,10]. In particular, plasma protein carbonyl levels were significantly higher in breast cancer patients than in healthy women [34,41], suggesting that enhanced protein carbonyl concentrations are significantly associated with increased breast cancer risk [34].

\*Corresponding author: Ferdinando Mannello, Dipartimento Scienze Biomolecolari, Sezione di Biochimica Clinica, Facoltà di Scienze e Tecnologie, Università “Carlo Bo” di Urbino, 61029 Urbino (PU), Italy. Tel.: +39 0722 351479; Fax: +39 0722 322370; E-mail: ferdinando.mannello@uniurb.it.

Breast cancer (BC) is the third most common worldwide malignancy among women, and is continuously increasing in incidence [16]. The high frequency of human BC emphasizes the need to understand the mechanisms involved in breast tumorigenesis [11]. Because BC develops from ductal and lobular epithelium, a method to assess the metabolic activity within the mammary gland is the analysis of Nipple Aspirate Fluid (NAF), which represents the mirror of the breast tissue microenvironment [32,36]. In fact, the adult non-pregnant, non-lactating breast secretes NAF into the breast ductal system which can be easily and non-invasively obtained through nipple aspiration [11]. NAF consists of secreted proteins and cells sloughed from ductal and lobular epithelium [31] and contains several biomarkers [21,22,24] that are useful research tools in epidemiological, molecular and clinical studies [3,9,15].

In previous studies, a relationship between NAF levels of isoprostanes and breast cancer risk has been observed, suggesting that lipid peroxidation products may be involved in BC etiology [5,17,26]. To complete the oxidative stress profile in the breast microenvironment, in the present study we investigated the degree of protein oxidation, measured as the level of protein carbonyls in non-invasively collected NAF. To improve the identification of women at increased breast cancer risk, the protein oxidation marker was also correlated to lipid peroxidation biomarkers in order to achieve their potential utility in BC risk stratification.

## 2. Materials and methods

### 2.1. Subjects and sample processing

The present work was carried out in accordance with the ethical standards of the Helsinki Declaration of 1975 (as revised in 1983) and after the approval of the Ethics Committee of University "Carlo Bo" of Urbino (protocol 18/CE). All subjects analyzed signed informed consent prior to enrollment. Five hundred and eighty women were recruited and enrolled as unselected consecutive patient populations attending the Center of Senology of Pesaro-Urbino (January 1997–May 2008) for the routine breast screening belonging to the "Progetto Donna" program. We excluded 40 patients reporting pregnancy within 3 years before the study or who were medically treated during the previous year, in order to avoid the influence on protein carbonyl formation by lactation status or involvement

of drugs in mammary secretory function. Several clinical variables were taken into consideration in the remaining 540 women (e.g., menopausal status, fruits and vegetables intake, use of either birth control pill or hormone replacement therapy and cigarette smoking status). Among these women, we selected three hundred and twenty women who presented more homogeneous characteristics; in fact, on the basis of literature evidence, the age of menarche, fruit and vegetable intake, use of hormones and cigarette smoking habit may significantly affect the protein carbonyl formation and accumulation [20,40]; for this reason, we finally selected 320 women (including Control and Cancer groups) who did not show significant differences among these variables in order to evaluate protein carbonyl concentrations not linked to life habits, but mainly related to breast diseases (Table 1). Moreover, to avoid the known effect of women's age on protein carbonyl formation and the possible bias due to the different age between controls and breast cancer women, we performed statistical analyses of protein carbonyls after the age-adjustment. Demographic data for analyzed subjects are reported in Table 1. From the three-hundred and twenty women, we were not able to collect NAF sample in 32 women from Control group (90% success percentage of total nipple fluid aspiration), whereas we successfully collected NAFs from all women of Pre-malignant and Cancer groups. All NAF samples from the remaining 288 non-lactating women (age range 31–67 years) were aspirated non-invasively from one breast only using the Sartorius breast pump [21,36]. Two hundred and sixteen fluids were collected from healthy women without evidence of cancer in the

Table 1

Demographic data for women analyzed in the present study ( $n = 320$ )

NAF samples	Control ( $n = 248$ )	Cancer ( $n = 72$ )*	<i>p</i> value
No. (% detectable)	216 (87.1)	72 (100)	<0.001
Median age (range)	42 (31–57)	52 (47–67)	<0.0001
Pre-menopausal (%)	108 (43.9)	17 (26.6)	<0.0001
Use of BCP (%)	51 (20.7)	2 (3.1)	<0.0001
Age of menarche ( $\pm$ SD)	12 ( $\pm$ 1.5)	11 ( $\pm$ 1.3)	0.88
Parity ( $\pm$ SD)	2.4 ( $\pm$ 1.3)	2.1 ( $\pm$ 1.4)	0.08
F&V intake (no. cup/week)	10 ( $\pm$ 5)	9 ( $\pm$ 4)	0.17
Use of HRT	Non-users	Non-users	
Cigarette smoking status	Never	Never	

NAF: nipple aspirate fluid; F&V: fruits and vegetables intake; BCP: birth control pills; HRT: hormone replacement therapy. \* = The cancer group also includes 14 women from whom NAF was collected before breast cancer developed.

analyzed breast (Control subjects). Fifty-eight NAFs were obtained from Cancer patients, who had at the time of NAF collection biopsy proven confirmed ductal carcinoma *in situ* (DCIS,  $n = 37$ ) and invasive breast cancer (IBC,  $n = 21$ ). NAFs were collected prior to biopsy from women who underwent an invasive procedure (needle or surgical biopsy and mastectomy). For women with breast cancer, all specimens were collected before definitive treatment. Clinico-pathological characteristics (e.g., disease stage, tumour size, nodal status, or distant disease spread) were determined according to the American Joint Committee on Cancer TNM staging system for breast cancer [39]. Moreover, we also collected NAF samples from fourteen women who developed BC within five years (histologically categorized at the time of NAF collection as affected by proliferative pre-malignant breast diseases such as hyperplasia with atypia, papilloma with atypia) (Table 1). NAF fluid in the form of droplets was non-invasively collected in calibrated capillary tubes, the volume recorded and the ends sealed with clay. The median volume of NAF collected was 15  $\mu$ l (range 5–400  $\mu$ l), with no statistically significant difference between NAF volume and presence of cancer (data not shown). The high variability of NAF volume may be related to complex mechanisms of active secretion by ductal cells, passive filtration from plasma, and physiologic fluid reabsorption [21,32]. Without pooling, after collection NAF specimens were immediately snap-frozen at  $-80^{\circ}\text{C}$  until use. NAFs were centrifuged at 15,000g for 15 min at  $4^{\circ}\text{C}$ , and the supernatants analyzed. To avoid possible interference of the age differences between cases and controls, we performed the age-adjustment based on the gamma distribution [12]. NAF samples were firstly diluted and then analyzed for total protein and then for protein carbonyl content. All samples were assayed at least in duplicate.

## 2.2. Protein determination and carbonyl ELISA assay

Total protein concentrations were determined by commercially available protein assay kit using Bicinchoninic method (Pierce), and the samples were diluted with phosphate-buffered saline solution (PBS) to a final protein concentration of 4 mg/ml. The levels of NAF carbonyl groups were assessed using non-competitive ELISA, according to the original method previously described [27], as adapted/improved in [34]. Briefly, the oxidized protein standards were prepared by incubation of bovine serum albumin (BSA) (50 g/l) with 0.73 M  $\text{H}_2\text{O}_2$  and 0.42 mM  $\text{Fe}^{2+}$

in PBS pH 7.4 for 1 hour at  $37^{\circ}\text{C}$ . The reaction was stopped with 40  $\mu\text{M}$  butylated hydroxytoluene. The carbonyl content of the oxidized BSA standard was measured spectrophotometrically [19]. It was then diluted with native (unoxidized) BSA in PBS pH 7.4 to give a final carbonyl content of 2.0 nM/mg protein and protein concentration of 4 mg/ml. After the derivatization of the sample with 2,4-dinitrophenylhydrazine (DNPH) generating a stable 2,4-nitrophenylhydrazone product (DNP), the plate was coated with NAF sample (containing 1  $\mu\text{g}$  protein) and incubated overnight at  $4^{\circ}\text{C}$  in the dark. The plate was washed with PBS containing 0.05% Tween-20 and blocked with 0.1% BSA in PBS for 1.5 hours. After another washing step, biotinylated primary anti-DNP antibody (Molecular Probes) (diluted 1:1500 v:v with PBS containing 0.1% BSA and 0.1% Tween-20) was added and the plate was incubated at  $37^{\circ}\text{C}$  for 1 hour. Another washing was followed by adding the streptavidin horse-radish peroxidase conjugate (Amersham) (diluted 1:4000 v:v in PBS containing 0.1% BSA and Tween-20), and incubation at room temperature for 1 hour. Colour was developed by adding the tetramethyl benzidine substrate (Sigma). The reaction was stopped with  $\text{H}_2\text{SO}_4$  after 20 min incubation in the dark; the absorbance was measured with a microplate reader at 450 nm (BGM LabTech). NAF protein carbonyl concentration was expressed as nM/mg protein. Each sample was at least analysed in duplicate. The intra-assay and inter-assay coefficient of variability for the repeated analyses was 8.2% and 8.8%, respectively. To exclude NAF “matrix” artefacts caused by interference substances (e.g., lipids, hormones, etc.), we serially diluted randomly selected samples, reanalyzing them for the response linearity.

## 2.3. Statistical analyses

The demographic characteristics of patients and controls were compared using the Student's *t*-test. In view of the non-Gaussian distribution of data for protein and carbonyl levels, the differences between Control and Cancer NAFs, and among breast cancer patients with different clinico-pathological features were compared using non-parametric Man-Whitney rank *U*-test. The data are presented as median and range (minimum and maximum), and carbonyl levels were expressed as nM/mg protein. The association studies were estimated using the non-parametric Spearman correlation coefficients method. The age-adjustment was based on the gamma distribution [12].

In all instances, significance was indicated if the two-side  $p$  value was  $<0.05$ . All the data were analyzed with Prism software version 3 (Graph-Pad).

### 3. Results

Three hundred and twenty women that did not show significant differences among life style habits (such as age of menarche, fruit and vegetable intake, use of hormone therapy and cigarette smoking habit, known variables influencing protein carbonyl formation [19,20,27,40]) were recruited for the analyses of NAF protein and carbonyl concentrations (Table 1). A significant difference was observed for the median age between Control and Cancer patients ( $p < 0.0001$ ). In both groups most subjects were in post-menopausal status. In 288 out of 320 women, we successfully obtained NAF specimens, which have been analysed for the protein carbonyl study.

Starting from the recent evidence that protein carbonyl levels circulating in plasma showed a significant association with breast cancer risk [34], for the first time we report detectable levels of protein carbonyls in NAF (a biological fluid, representing the breast tissue microenvironment, secreted by epithelial/stromal cells lining human breast ducts and lobules in healthy and diseased conditions). Due to the known wide variation in NAF protein content from sample to sample [21,31], protein carbonyl concentration in NAF was expressed as nM/mg protein. In all NAF samples ( $n = 288$ ) we detected appreciable levels of protein carbonyls (median 0.439 nM/mg, range 0.081–2.41 nM/mg). As reported in Table 2, a significant different content of protein carbonyls was found among NAF collected from Control ( $n = 216$ ) and Cancer ( $n = 58$ ) patients ( $p < 0.0001$ ). Moreover, in additional 14 cancer patients (histologically categorized at the time of NAF collection as affected by proliferative pre-cancerous breast diseases who within five years developed BC) (Pre-malignant subjects) we found protein carbonyl levels significantly higher than Control women (1.09 vs. 0.368 nM/mg protein, respectively) and significantly lower respect to Cancer women ( $p < 0.0001$ ) (Fig. 1, Table 2). Cancer patients showed the highest levels of protein carbonyls ( $n = 58$ ; median 2.07 nM/mg protein, range 1.01–2.41 nM/mg), statistically different from Control and Pre-malignant women (Table 2, Fig. 1). Among the 58 Cancer patients, no statistically significant difference was found between DCIS and IBC ( $p = 0.391$ ) (Table 2). No significant correla-

Table 2

NAF samples	Protein carbonyls (nM/mg prot)	$p$ value
Total ( $n = 288$ )	0.439 (0.081–2.410)	
Control ( $n = 216$ )	0.368 (0.081–0.792)	<i>a, b</i>
Pre-menopause ( $n = 108$ )	0.387 (0.081–0.792)	<i>c</i>
Post-menopause ( $n = 108$ )	0.332 (0.081–0.776)	
Pre-malignant/Cancer ( $n = 14$ )	1.090 (1.010–1.490)	<i>d</i>
Cancer ( $n = 58$ )	2.070 (1.010–2.410)	
Pre-menopause ( $n = 15$ )	1.280 (1.010–1.460)	<i>e, f</i>
Post-menopause ( $n = 43$ )	2.140 (1.410–2.410)	<i>g</i>
DCIS ( $n = 37$ )	2.000 (1.010–2.360)	<i>h</i>
IBC ( $n = 21$ )	2.090 (1.010–2.410)	

NAF: nipple aspirate fluid; *a*: control vs. Pre-malignant/Cancer,  $p < 0.0001$ ; *b*: Control vs. Cancer,  $p < 0.0001$ ; *c*: Control, pre-menopause vs. post-menopause,  $p = 0.115$ ; *d*: Pre-malignant/Cancer vs. Cancer,  $p < 0.0001$ ; *e*: Cancer, pre-menopause vs. post-menopause,  $p < 0.0001$ ; *f*: Cancer pre-menopause vs. Control pre-menopause,  $p < 0.0001$ ; *g*: Cancer post-menopause vs. Control post-menopause,  $p < 0.0001$ ; *h*: ductal carcinoma *in situ* (DCIS) vs. invasive breast cancer (IBC),  $p = 0.391$ .

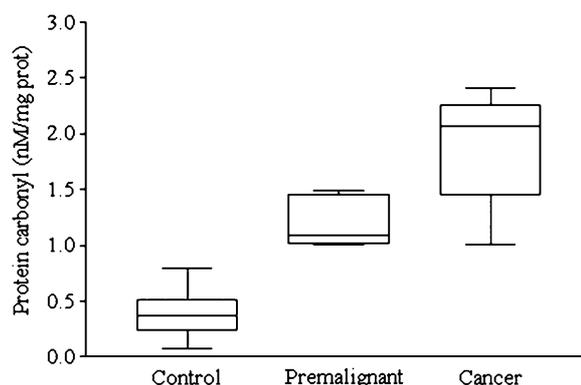


Fig. 1. Median levels of protein carbonyls in NAF collected from Control subjects ( $n = 216$ ), from patients histologically categorized at the time of NAF collection as affected by pre-malignant conditions (e.g., hyperplasia with atypia, papilloma with atypia) who within five years developed BC (Pre-malignant,  $n = 14$ ), and from patients who had biopsy proven confirmed ductal carcinoma *in situ* and invasive breast cancer ( $n = 58$ ). (Control vs. Pre-malignant,  $p < 0.0001$ ; Control vs. Cancer,  $p < 0.0001$ ; Pre-malignant vs. Cancer,  $p < 0.0001$ .)

tion was found between carbonyl median levels and clinico-pathological features; no difference in protein carbonyl concentrations among subgroups of primary tumour sizes, tumour stages, lymph node status and distant disease spread was also found (data not shown).

According to the menopausal status, in Control women no difference in protein carbonyl levels was

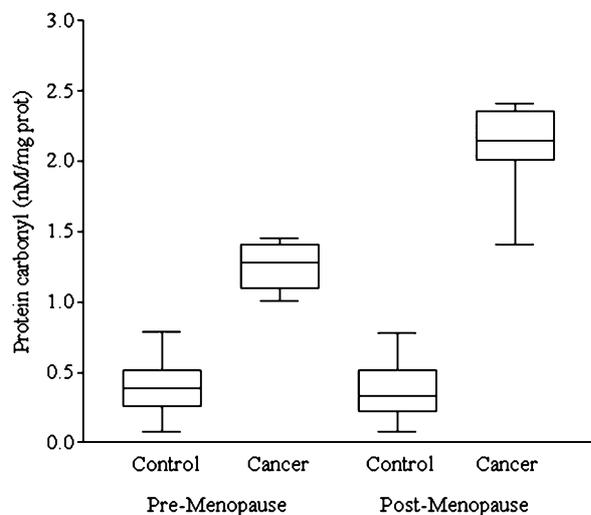


Fig. 2. Median levels of protein carbonyls in NAF collected from Control and Cancer women in pre-menopause ( $n = 108$  and  $n = 15$ , respectively) and in post-menopause ( $n = 108$  and  $n = 43$ , respectively). (Control pre-menopause vs. post-menopause,  $p = 0.115$ ; Cancer pre-menopause vs. post-menopause,  $p < 0.0001$ ; Control pre-menopause vs. Cancer pre-menopause,  $p < 0.0001$ ; Control post-menopause vs. Cancer post-menopause,  $p < 0.0001$ .)

found between pre- and post-menopause ( $p = 0.115$ ) (Table 2); NAF collected from Cancer patients contained significant different protein carbonyl levels between pre- and post-menopausal status ( $p < 0.0001$ ) (Fig. 2). Among Cancer patients in post-menopausal status ( $n = 43$ ), NAF collected from patients affected by IBC ( $n = 14$ ) contained significantly higher levels of protein carbonyl than those with DCIS ( $n = 29$ ) (2.36 vs. 2.13 nM/mg protein, respectively;  $p = 0.019$ ), whereas no significant difference between IBC ( $n = 7$ ) and DCIS ( $n = 8$ ) was found in Cancer patients in pre-menopause (1.33 vs. 1.26 nM/mg protein, respectively;  $p = 0.536$ ) (data not shown). Although efforts were undertaken to obtain NAF samples from breast cancer patients and controls with similar age range, protein carbonyl levels did not change substantially after the age-adjustment approach showing that the significant increase of protein carbonyls in breast cancer NAF is the mirror of oxidative stress in breast microenvironment and not due to the difference in the age of the cases vs. the controls (data not shown).

To assess whether BC risk is related to markers of both protein oxidation and lipid peroxidation, we compared protein carbonyl and 8-F<sub>2α</sub>-isoprostane levels in NAF (analysed in our previous study [26]; these NAF samples were a subset of the samples evaluated for protein carbonyls in the present study)

(Fig. 3). It is important to preliminarily underline that the 8-series isoprostanes represent an early marker of lipid peroxidation whereas the 15-series isoprostanes are prostaglandin-like compounds obtained after prolonged oxidation by free radicals of esterified arachidonic acid [29]. As shown in Fig. 3(a), between Control and Cancer women a significant difference of both protein and lipid oxidative markers was found ( $p < 0.0001$ ). Interestingly, while mean levels of 8-F<sub>2α</sub>-isoprostanes were significantly higher in Control than in Cancer NAF samples (18743 vs. 1803 pg/ml, respectively;  $p < 0.0001$ ), mean protein carbonyl levels showed an opposite behaviour, with higher concentration in Cancer respect to Control NAF specimens (1.980 vs. 0.384 nM/mg prot;  $p < 0.0001$ ). Moreover, within Control and Cancer groups a significant negative correlation was also found: in fact, in NAF samples collected from Cancer patients the levels of protein carbonyls showed an inverse correlation with the concentrations of 8-isoprostanes ( $r^2 = 0.322$ ,  $p = 0.0005$ ) (Fig. 3(b)). On the other hand, in Control women lower levels of NAF protein carbonyls were inversely related to higher concentrations of 8-series isoprostanes ( $r^2 = 0.445$ ,  $p < 0.001$ ) (data not shown).

#### 4. Discussion

Although several protein oxidation products have been previously identified in plasma of women with breast diseases [34,41], as well as in human milk [28], to the best of our knowledge our study is the first report of the identification of protein carbonyls (a major index of protein oxidation and oxidative stress [10,18,38]) in NAF samples, non-invasively collected from healthy women and patients affected by breast diseases, included proven breast cancer. According to the constitutive presence of low levels of protein carbonyls in milk samples during physiological lactation [8,28], our results in NAF (a breast fluid secreted from non-lactating women) suggest that protein oxidation naturally occurs in the breast microenvironment, and that enhanced protein carbonyl formation/accumulation in breast cancer may be generated by increased levels of reactive oxygen species and oxidative stress [18,43]. Although previous studies demonstrated that cigarette smoking, diet rich in fat and fruit/vegetable, alcohol consumption and physical activity may strongly influence plasma protein carbonyl levels [7,10,14], the plasma levels of protein carbonyls show a significant positive association with breast cancer risk [34] and a strong correla-

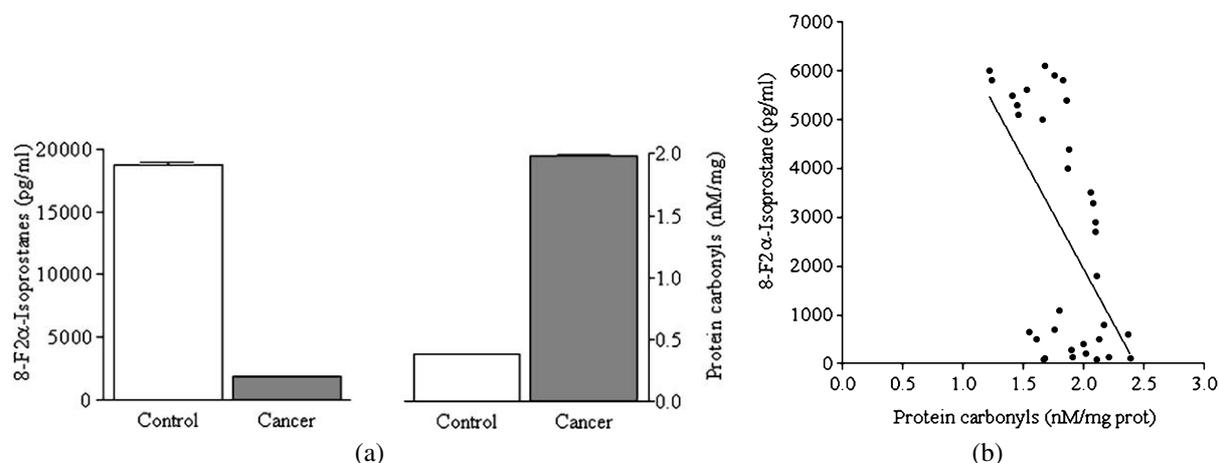


Fig. 3. Relationship between the levels of 8-F<sub>2α</sub>-isoprostanes and protein carbonyls in NAF collected from Control and Cancer women. In the same subset of women analysed in our previous study for NAF 8-F<sub>2α</sub>-isoprostanes [25], we evaluated also NAF concentrations of protein carbonyls (Control  $n = 113$ , Cancer  $n = 34$ ). (a) Comparative representation of the opposite behaviours of protein and lipid oxidative biomarkers in the same subset of NAFs (8-F<sub>2α</sub>-isoprostanes: 18743 vs. 1803 pg/ml, Control vs. Cancer respectively,  $p < 0.0001$  – Protein carbonyls: 0.384 vs. 1.980 nM/mg protein, Control vs. Cancer respectively,  $p < 0.0001$ ). (b) Linear regression analysis of NAF 8-F<sub>2α</sub>-isoprostane and protein carbonyl levels in the same subset of Cancer women (slope  $-4503 \pm 1155$ ,  $r^2 = 0.322$ ,  $p = 0.0005$ ).

tion with progressive breast diseases [41]. Our results in selected NAF samples (without significant differences between parity, fruit and vegetable intake, cigarette smoking status and hormone replacement therapy; see Table 1) support the hypothesis that the possible source of protein carbonyls in NAF is not related to passive filtration from plasma but seems to derive from the oxidative stress pathways within breast tissue, making NAF a useful mirror of metabolic alterations in breast microenvironment [21,31,36]. In this respect, mounting evidences have demonstrated the reliability of NAF to directly assess intraductal metabolic pathways in order to improve the identification of women at increased breast cancer risk [11,22,25]; accordingly, we have evaluated protein and lipid oxidation status directly in NAF, without comparison to plasma and urine which have been excellently studied in previous reports [33,34,41].

We found the lowest levels of protein carbonyls in NAF collected from healthy women without any evidence of cancer in the analysed breast, consistent with the low levels of carbonyls detected during the physiologic condition of human lactation [8,28]. On the other hand, our findings of increased protein carbonyl levels in NAF collected from Cancer patients are consistent with the findings demonstrating that enhanced protein oxidation may occur in cancer [4,18,37,42,43]. Moreover, the high levels of protein carbonyls detected in NAF samples from women histologically categorized at the time of NAF collection as affected by pro-

liferative precancerous breast diseases (e.g., hyperplasia with atypia, papilloma with atypia), who within five years developed BC (Pre-malignant), provide evidence that carbonyls (as index of oxidative stress) may be produced/secreted by highly metabolizing apocrine cells lining breast ducts [21,32]. Previous studies suggest that protein carbonyl levels in plasma, tissues and human cell lines may be related to tumour progression [7,10,18,34,41]; although we do not find a difference between protein carbonyl concentrations and tumour stage in our series of NAF samples, our findings in women with pre-malignant breast diseases who progressed to cancer are consistent with previous reports [18,34,41]. In this respect, on the basis of the results obtained in plasma of breast cancer patients, protein carbonyl determination may be useful tool to evaluate tumour stages and their progression even though plasma levels may be a surrogate biomarker showing elevated concentrations due to several pathological conditions; on the other hand, protein carbonyls in NAF may represent a more specific marker to evaluate protein oxidation occurring in breast tissue microenvironment, allowing an early indication of oxidative stress metabolic pathway within the breast duct during physio-pathological processes. In our opinion, measurement of protein carbonyls in NAF may help to early identify women at increased risk of BC, especially when plasma carbonyl levels are not significantly altered and so not fully reliable and discriminant.

Although the increase in breast cancer risk was similar for younger and older women, literature data describe increased plasma levels of protein carbonyls in older women [34]. Accordingly, we found in Cancer NAF a significant increase of protein carbonyls in post-menopausal patients respect to subjects in pre-menopause (Fig. 2, Table 2). These observations are in agreement with the evidence that the processes of aging and cancer are accompanied by increasing levels of oxidative damage, including protein oxidation [4,18,42]. On the other hand, in Control women no differences were found in NAF between pre- and post-menopause (Fig. 2, Table 2), suggesting that either decreased protein oxidation/oxidative stress or a better antioxidant defence system may be present in breast microenvironment [7,10,43].

To assess whether breast cancer risk is related to increased levels of both protein oxidation and lipid peroxidation markers, we compared NAF protein carbonyl levels with those of early and late lipid peroxidation biomarkers found previously in NAF (8- and 15-series isoprostanes) [5,17,26]. Membrane lipids and polyunsaturated fatty acids are esterified, hydrolysed and released into extracellular fluid through phospholipase A<sub>2</sub> activity releasing arachidonate [2]. Isoprostanes are prostaglandin-like compounds produced primarily from esterified arachidonic acid in tissues by non-enzymatic reaction catalysed *in vivo* by ROS/free radicals [6], playing a crucial role in breast cancer initiation [13]. The most abundant isoprostanes are analogous to prostaglandin F<sub>2α</sub>, and have been categorized with a prefix (*n*-series), which identifies the location of the hydroxyl group in the side-chain. Among these, the 8-series F<sub>2α</sub>-isoprostanes are recognized as early peroxidation products whereas the 15-series F<sub>2α</sub>-isoprostanes have been characterized as late peroxidative compounds [2]; for these reasons, the 15-series are the most abundant isoprostane class in both plasma and urine [6].

In a previous study, we evaluated the arachidonate pathway in NAF samples in both Control and Cancer patients revealing that the group IIa secretory phospholipase A<sub>2</sub> (the key enzyme regulating the arachidonate release from cellular phospholipids) was significantly increased both in NAF and ductal cells from cancer-containing breast, and positively correlated with tumour stage, suggesting an involvement in breast cancer progression [23]. Moreover, the early lipid peroxidation product 8-series F<sub>2</sub>-isoprostane was identified in human milk and healthy NAF at concentrations significantly higher respect to plasma [26],

suggesting a possible role in normal breast physiology [13]. In NAF from Cancer patients we found significantly lower levels of 8-series F<sub>2</sub>-isoprostane than in NAF collected from healthy Control subjects [26], suggesting that the 8-series is physiologically produced in normal breast [23] and undergoes further peroxidation with the onset of breast cancer, leading to the formation of 15-series F<sub>2</sub>-isoprostanes [13,23]. In fact, some studies identified significantly higher levels of 15-series F<sub>2</sub>-isoprostane in NAF, as well as in plasma and urine, from breast cancer patients than in healthy women [5,17,33,34]. Moreover, a recent population-based case-control study demonstrated that women at increased risk of breast cancer showed increased levels of both urinary 15-series F<sub>2</sub>-isoprostane and plasma protein carbonyls, suggesting that elevation in these two oxidative stress biomarkers was associated with increased breast cancer risk and supporting the role of oxidative stress in breast cancer progression [33,34]. In the present study, we observed a significant inverse association between high levels of protein carbonyls and low concentrations of the early markers of lipid peroxidation (8-series F<sub>2</sub>-isoprostanes) in NAF from Cancer patients (Fig. 3). Our data are consistent with the hypothesis that in the breast tissue microenvironment during cancer condition may be present a long-lasting oxidative stress leading to the formation of protein oxidation and the end-stage of lipid peroxidation. In fact, being the levels of protein carbonyls in cancer tissue of post-menopausal women nearly twice as much as in cancer tissue of premenopausal women, it seems probably true that the older the woman the higher the level of protein oxidation associated with the switch to cancerous stage. Accordingly, NAF collected from Cancer patients contains high concentrations of 15-series F<sub>2</sub>-isoprostanes [5,17], low levels of 8-series F<sub>2</sub>-isoprostanes [26] and high content of protein carbonyls (present work) (Fig. 3), suggesting different oxidative profiles within breast tissue (Fig. 4). An enhanced oxidative profile found in NAF from patients with pre-malignant and cancer conditions (e.g., increased levels of protein carbonyls and 15-series F<sub>2</sub>-isoprostanes in combination with reduced amounts of 8-series F<sub>2</sub>-isoprostanes) may provide more relevant informations of oxidative status in breast tissues, helping to identify women at increased breast cancer risk.

Our study represents the first evidence of the protein carbonyl formation/accumulation within breast secretion from non-lactating women; the analysis of NAFs, fluid deriving from breast ductal cells, sheds light directly on the breast microenvironment and its early

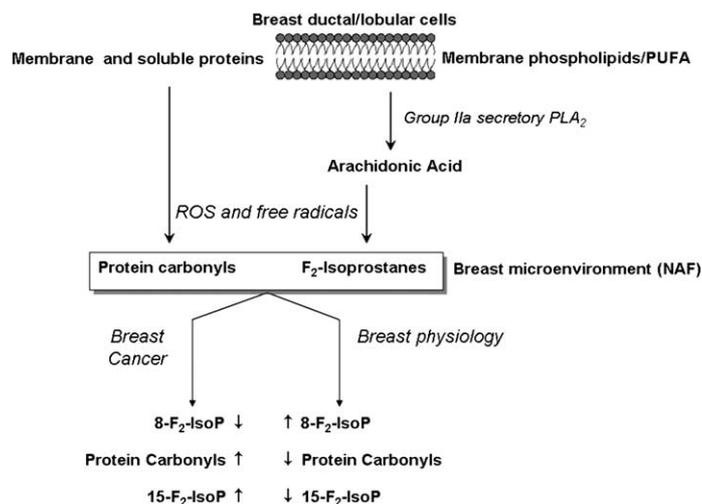


Fig. 4. Hypothetical scheme representing the involvement of protein and lipid oxidation biomarkers in breast physiology and cancer initiation/progression. Under physiological conditions, breast apocrine cells lining ducts and lobules release arachidonic acid from membrane phospholipids through the action of phospholipase A<sub>2</sub>. Both arachidonate and membrane/soluble proteins may be modified by reactive species of oxygen and free radicals, leading to the formation of protein carbonyls and F<sub>2</sub>-isoprostanes that may accumulate in the breast tissue microenvironment (as reflected in nipple aspirate fluid). At this point either of two pathways may occur: (1) in women without evidence of cancer and during breast physiological processes (such as lactation), high levels of 8-isoprostanes may be present in NAF; in the presence of normal/high levels of natural endogenous anti-oxidants, 8-isoprostanes are not oxidized to 15-isoprostanes (the end-stage of lipid peroxidation); in the same way, proteins are not significantly oxidized to carbonyls. (2) In breast cancer conditions, low levels of endogenous antioxidants and increased oxidative stress may enhance the further oxidation of 8-isoprostanes leading to significant accumulation of 15-isoprostanes, favouring oxidative modifications of proteins and carbonyl formation. (PUFA: polyunsaturated fatty acid; PLA<sub>2</sub>: phospholipase A<sub>2</sub>; ROS: reactive species of oxygen; NAF: nipple aspirate fluid; IsoP: isoprostanes.)

metabolic alterations in both healthy and diseased conditions [9,25]. On the other hand, our present study shows also some limitations: although NAF collection is considered a useful tool for breast cancer clinical, epidemiologic and molecular analyses [9,21,25,36], there may be the concern that NAF is not always practical, given that for a proportion of women this method is not very feasible [11] and NAF can be attempted but not obtained in all patients [3]. However, we have obtained NAF samples in all patients affected by pre-malignant diseases and breast cancer, suggesting that biochemical and morphological studies on NAF may be supportive tools for BC clinical research [3,9,25]. Another limitation may be the number of patients examined that, even though not too large as population based study, is however representative of a study focused to analyse the metabolic microenvironment of both healthy and diseased breast conditions. Although the strength of the study is the use of both a “high risk” population (i.e. women affected by pre-malignant breast diseases who subsequently developed BC) and biopsy proven cancer patients, a prospective study (possibly involving several breast cancer centres) using a wider number of NAF samples (such as a population-based study ensuring that many more

cases and controls arise from a wide source of population) will be necessary for confirming if elevated levels of protein carbonyls may help to identify women at higher breast cancer risk. Finally, although efforts were undertaken to obtain NAF samples from breast cancer patients and controls with similar age range, protein carbonyl levels did not change substantially after the age-adjustment approach showing that the significant increase of protein carbonyls in breast cancer NAF is the mirror of oxidative stress in breast microenvironment and not due to the difference in the age of the cases vs. the controls. In a similar manner, to avoid that some variables may affect protein carbonyl production/accumulation in NAF fluids (such as the age of menarche, fruit and vegetable intake, use of hormones and cigarette smoking) [19,20,27,40], among all patients recruited we selected only those (320 out of 540 women; Table 1) who showed no differences among these demographic/habit style characteristics; our results suggest that the significant increase of protein carbonyl concentrations in NAF collected from BC respect to control women is mainly linked to the oxidative stress within the breast microenvironment in pre-malignant and cancerous conditions, and likely not to life style/habits [38].

All these results add further support to the hypothesis that oxidative stress may play a role in cancer etiology (including breast) [18,37], suggesting that measuring oxidative stress biomarkers in non-invasively collected NAF may provide direct and possible useful information in oxidative status of the breast microenvironment (Fig. 4), helping identify women at increased breast cancer risk [9]. Further biomolecular evaluations are in progress to provide insights on the balance between protein and lipid peroxidation and anti-oxidant molecules (e.g., catalase, superoxide dismutase, and glutathione reductase activities), clarifying the biological and cellular mechanisms of protein/lipid oxidative pathways occurring in human breast physiology and carcinogenesis.

### Acknowledgements

This work was supported by the Susan Love Research Foundation (Grant Award 2007 to FM), Pacific Palisades, CA, USA.

We would like to thank Dr. Maurizio Sebastiani (Senology Centre, AUSL 1 Pesaro, Italy) for NAF, and serum sample collection, and gathering patients' clinical data. We would also like to thank Mrs Eleonora Cencherle for English assistance.

### References

- [1] A. Azzi, Oxidative stress: a dead end or a laboratory hypothesis?, *Biochem. Biophys. Res. Commun.* **362** (2007), 230–232.
- [2] J. Balsinde, M.V. Winstead and E.A. Dennis, Phospholipase A<sub>2</sub> regulation of arachidonic acid mobilization, *FEBS Lett.* **531** (2002), 2–6.
- [3] K.A. Baltzell, M. Moghadassi, T. Rice, J.D. Sison and M. Wensch, Epithelial cells in nipple aspirate fluids and subsequent breast cancer risk: a historic prospective study, *BMC Cancer* **8** (2008), 75.
- [4] M.F. Beal, Oxidatively modified proteins in aging and disease, *Free Rad. Biol. Med.* **32** (2002), 797–803.
- [5] G. Chen and Z. Djuric, Detection of 2,6-cyclolycopene-1,5-diol in breast nipple aspirate fluids and plasma: a potential marker of oxidative stress, *Cancer Epidemiol. Biomarker Prev.* **11** (2002), 1592–1596.
- [6] D.J. Crankshaw and P.K. Rangachari, Isoprostanes: more than just mere markers, *Mol. Cell. Biochem.* **253** (2003), 125–130.
- [7] I. Dalle-Donne, R. Rossi, D. Giustarini, A. Milzani and R. Colombo, Protein carbonyl groups as biomarkers of oxidative stress, *Clin. Chim. Acta* **329** (2003), 23–38.
- [8] T.K. Dalsgaard, D. Otzen, J.H. Mielsen and L.B. Larsen, Changes in structures of milk proteins upon photo-oxidation, *J. Agric. Food Chem.* **55** (2007), 10968–10976.
- [9] R.S. Dua, C.M. Isacke and G.P. Gui, The intraductal approach to breast cancer biomarker discovery, *J. Clin. Oncol.* **24** (2006), 1209–1216.
- [10] E.M. Ellis, Reactive carbonyls and oxidative stress: potential for therapeutic intervention, *Pharmacol. Ther.* **115** (2007), 13–24.
- [11] C.J. Fabian, B.F. Kimler, M.S. Mayo and S.A. Khan, Breast-tissue sampling for risk assessment and prevention, *Endocr. Relat. Cancer* **12** (2005), 185–213.
- [12] M.P. Fay and E.J. Feuer, Confidence intervals for directly standardised rates: a method based on the gamma distribution, *Statist. Med.* **16** (1998), 791–801.
- [13] M. Gago-Dominguez, J.E. Castela, M.C. Picke, A. Sevanian and R.W. Haile, Role of lipid peroxidation in the epidemiology and prevention of breast cancer, *Cancer Epidemiol. Biomarkers Prev.* **14** (2005), 2829–2839.
- [14] F. Galli, M. Piroddi, C. Anneti, C. Aisa, E. Floridi and A. Floridi, Oxidative stress and reactive oxygen species, *Contrib. Nephrol.* **149** (2005), 240–260.
- [15] J.P. Jakupciak, A. Maggrah, S. Maragh, J. Maki, B. Reguly, K. Maki, R. Wittcock, K. Robinson, P.D. Wagner, R.E. Thayer, K. Gehman, T. Gehman, S. Srivastava, A. Ngom, G.D. Dakubo and R.L. Parr, Facile whole mitochondrial genome resequencing from nipple aspirate fluid using MytoChip v2.0, *BMC Cancer* **8** (2008), 95.
- [16] A. Jemal, R. Siegel, E. Ward, T. Murray, J. Xu and M.J. Thun, Cancer statistics, 2007, *CA Cancer J. Clin.* **57** (2007), 43–66.
- [17] I. Kato, G. Chen and Z. Djuric, Non-steroidal anti-inflammatory drug (NSAID) use and levels of a lipid oxidation marker in plasma and nipple aspirate fluids, *Breast Cancer Res. Treat.* **97** (2006), 145–148.
- [18] J.E. Klaunig and L.M. Kamendulis, The role of oxidative stress in carcinogenesis, *Ann. Rev. Pharmacol. Toxicol.* **44** (2004), 239–267.
- [19] R.L. Levine, D. Garland, C.N. Oliver, A. Amici, L. Climent, A. Lenz, B. Ahn, S. Shalteil and E.R. Stadtman, Determination of carbonyl content of oxidatively modified proteins, *Methods Enzymol.* **233** (1990), 464–478.
- [20] V.I. Lushchak, Free radical oxidation of protein and its relationship with functional state of organisms, *Biochemistry* **72** (2007), 809–827.
- [21] M. Malatesta, F. Mannello, G. Bianchi, M. Sebastiani and G. Gazzanelli, Biochemical and ultrastructural features of human milk and nipple aspirate fluids, *J. Clin. Lab. Anal.* **14** (2000), 330–335.
- [22] F. Mannello, L. Fabbri, E. Ciandrini and G.A. Tonti, Increased levels of erythropoietin in nipple aspirate fluid and in ductal cells from breast cancer patients, *Cell. Oncol.* **30** (2008), 51–61.
- [23] F. Mannello, W. Qin, W. Zhu, L. Fabbri, G.A. Tonti and E.R. Sauter, Nipple aspirate fluids from women with breast cancer contain increased levels of group IIa secretory phospholipase A<sub>2</sub>, *Breast Cancer Res. Treat.* **111** (2008), 209–218.
- [24] F. Mannello and M. Sebastiani, Zymographic analyses and measurement of matrix metalloproteinase-2 and -9 in nipple aspirate fluids, *Clin. Chem.* **49** (2003), 1546–1550.

- [25] F. Mannello, G.A. Tonti and F. Canestrari, Nutrients and nipple aspirate fluid composition: the breast microenvironment regulates protein expression and cancer etiology, *Genes Nutr.* **3** (2008), 77–85.
- [26] F. Mannello, G.A. Tonti, S. Pagliarani, S. Benedetti, F. Canestrari, W. Zhu, W. Qin and E.R. Sauter, The 8-epimer of prostaglandin  $F_{2\alpha}$ , a marker of lipid peroxidation and oxidative stress, is decreased in the nipple aspirate fluid of women with breast cancer, *Int. J. Cancer* **120** (2007), 1971–1976.
- [27] K. Marangon, S. Devaraj and I. Jalal, Measurement of protein carbonyls in plasma of smokers and in oxidized LDL by an ELISA, *Clin. Chem.* **45** (1999), 577–578.
- [28] A.E. Mitchell, A.D. Jones, R.S. Mercer and R.B. Rucker, Characterization of pyrroloquinoline quinone aminoacid derivatives by electrospray ionization mass spectrometry and detection in human milk, *Anal. Biochem.* **269** (1999), 317–325.
- [29] P. Montuschi, P.J. Barnes and L.J. Roberts II, Isoprostanes: markers and mediators of oxidative stress, *FASEB J.* **18** (2004), 1791–1800.
- [30] F.G. Ottaviano, D.E. Handy and J. Loscalzo, Redox regulation in the extracellular environment, *Circ. J.* **72** (2008), 1–16.
- [31] N.L. Petrakis, Physiologic, biochemical, and cytologic aspects of nipple aspirate fluid, *Breast Cancer Res. Treat.* **8** (1986), 7–19.
- [32] N.L. Petrakis, Studies on the epidemiology and natural history of benign breast disease and breast cancer using nipple aspirate fluid, *Cancer Epidemiol. Biomarkers Prev.* **2** (1993), 3–10.
- [33] P. Rossner Jr., M.D. Gammon, M.B. Terry, M. Agrawal, F.F. Zhang, S.L. Teitelbaum, S.M. Eng, S.K. Sagiv, M.M. Gaudet, A.I. Neugut and R.M. Santella, Relationship between urinary 15- $F_{2t}$ -isoprostane and 8-oxodeoxyguanosine levels and breast cancer risk, *Cancer Epidemiol. Biomarker Prev.* **15** (2006), 639–644.
- [34] P. Rossner Jr., M.B. Terry, M.D. Gammon, M. Agrawal, F.F. Zhang, J.S. Ferris, S.L. Teitelbaum, S.M. Eng, M.M. Gaudet, A.I. Neugut and R.M. Santella, Plasma protein carbonyl levels and breast cancer risk, *J. Cell. Mol. Med.* **11** (2007), 1138–1148.
- [35] R.I. Salganik, The benefits and hazards of antioxidants: controlling apoptosis and other protective mechanisms in cancer patients and the human population, *J. Am. Coll. Nutr.* **20** (2001), 473–475.
- [36] E.R. Sauter, E. Ross, M. Daly, A. Klein-Szanto, P.F. Engstrom, A. Sorling, J. Malick and H. Ehya, Nipple aspirate fluid: a promising non-invasive method to identify cellular markers of breast cancer risk, *Br. J. Cancer* **76** (1997), 494–501.
- [37] H.E. Seifried, Oxidative stress and antioxidants: a link to disease and prevention?, *J. Nutr. Biochem.* **18** (2007), 168–171.
- [38] E. Shacter, Quantification and significance of protein oxidation in biological samples, *Drug. Metab. Rev.* **32** (2000), 307–326.
- [39] S.E. Singletary and J.L. Connolly, Breast cancer staging: working with the sixth edition of the AJCC cancer staging manual, *CA Cancer J. Clin.* **56** (2006), 37–47.
- [40] E.R. Stadtman and R.L. Levine, Free radical-mediated oxidation of free aminoacids and aminoacid residues in proteins, *Amino Acids* **25** (2003), 207–218.
- [41] P. Tesarova, M. Kalousova, B. Trnkova, J.A. Soukupova, S. Argalasova, L. Petruzelka and T. Zima, Carbonyl and oxidative stress in patients with breast cancer: is there a relation to the stage of the disease?, *Neoplasma* **54** (2007), 219–224.
- [42] M. Valko, D. Leibfritz, J. Moncol, M.T. Cronin, M. Mazur and J. Telser, Free radicals and antioxidants in normal physiological functions and human disease, *Int. J. Biochem. Cell. Biol.* **39** (2007), 44–84.
- [43] M. Valko, C.J. Rhodes, J. Moncol, M. Izakovic and M. Mazur, Free radicals, metals and antioxidants in oxidative stress-induced cancer, *Chem. Biol. Interact.* **160** (2006), 1–40.



**Hindawi**  
Submit your manuscripts at  
<http://www.hindawi.com>

