

# Protein carbonyl products, malondialdehyde, glutathione and vitamins C/E of breast cancer patients subjected to chemotherapy

Produtos carbonílicos proteicos, malondialdeído, glutathione e vitaminas C/E de pacientes com câncer de mama submetidas à quimioterapia

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## ABSTRACT

**Introduction:** Various endogenous and exogenous processes contribute to the production of oxidative stress, of which anticancer drugs may be one. This study aimed at evaluating the effect of breast cancer chemotherapy on oxidative stress. **Material and Methods:** Oxidative markers and antioxidant defense molecules were monitored in 59 women undergoing a year of treatment for breast cancer. **Results:** During the treatment, the levels of vitamin C and glutathione decreased, while both malondialdehyde and protein carbonyl products increased. Vitamin E levels were affected to a smaller extent. The patients were grouped by principal component analysis using their oxidative stress profiles according to the time of the chemotherapy and 95.9% of the total variance was explained by the first three principal components. **Conclusion:** The oxidative stress profile of the study population was modified extensively during one year of exposure to antineoplastic drugs.

**Keywords:** Oxidative stress; Biomarkers; Breast diseases.

## RESUMO

**Introdução:** Vários processos endógenos e exógenos contribuem para a produção de estresse oxidativo, dos quais os fármacos antineoplásicos podem ser um deles. Este estudo teve como objetivo avaliar o efeito da quimioterapia do câncer de mama sobre o estresse oxidativo. **Material e Métodos:** Marcadores oxidativos e moléculas de defesa antioxidante foram monitorados em 59 mulheres submetidas a um ano de tratamento para câncer de mama. **Resultados:** Durante o tratamento, os níveis de vitamina C e glutathione diminuíram, enquanto o malondialdeído e os produtos proteicos carbonílicos aumentaram. Os níveis de vitamina E foram afetados em menor grau. As pacientes foram agrupadas por análise de componentes principais usando seus perfis de estresse oxidativo de acordo com o tempo da quimioterapia e 95,9% da variância total foi explicada pelos três primeiros componentes principais. **Conclusão:** O perfil de estresse oxidativo da população estudada foi amplamente modificado, durante um ano de exposição aos antineoplásicos.

**Descritores:** Estresse oxidativo; Biomarcadores; Doenças da mama.

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## INTRODUCTION

On a global scale, breast cancer is the most common cancer in women, representing 24.2% of total cancer cases in this population. In Brazil, breast cancer is the second most prevalent in women (21% of total), after only of non-melanoma skin cancer (29.5% of total cancer) and there are 66.280 new cases of breast cancer registered each year.<sup>(1,2)</sup> Due to the variability of breast cancer, there are a huge number of therapeutic protocols available,<sup>(3,4)</sup> and the absence of selectivity between tumoral and non-tumoral cells represents a problem, which may result in damage to non-tumoral cells.<sup>(5-7)</sup>

Free radicals are reactive molecules present in pathological and physiological conditions and their concentration may be increased by exposure to toxic agents such as antineoplastic drugs.<sup>(5)</sup> The production of reactive oxygen and nitrogen species occurs during the treatment process with such drugs as doxorubicin,<sup>(8)</sup> taxanes,<sup>(9)</sup> and alkylating agents.<sup>(10)</sup> The tissues have endogenous molecules and enzymes that work to produce the neutralization of these reactive species. Vitamin E, vitamin C, and glutathione are among the most important low molecular weight defense systems.<sup>(11)</sup>

Vitamin E ( $\alpha$ -tocopherol) and vitamin C (ascorbic acid) work together in free radical defense. Vitamin E reacts with lipid hydroperoxyl radicals to yield a tocopheryl radical, which is resonance stabilized and has lower reactivity than lipid hydroperoxyl radicals. Subsequently, the tocopheryl radical may be regenerated back to  $\alpha$ -tocopherol by vitamin C.<sup>(12)</sup> The role of these vitamins in disease has been extensively studied: in high doses, vitamin C depresses cancer stem cells;<sup>(13)</sup> low serum vitamin C has been linked with peripheral arterial disease in smokers<sup>(14)</sup> and vitamin E reduces the oxidative stress in rats treated with cisplatin.<sup>(15)</sup> Glutathione is a tripeptide with a cysteine thiol group which can oxidize to disulfide. This oxidation is able to neutralize various reactive species, which cause oxidative damage in cellular structures.<sup>(16)</sup> The modulation of glutathione levels has a role in tumor initiation, progression, and anticancer drug resistance.<sup>(17,18)</sup>

In clinical routine, there are numerous markers available for oxidative stress assessment. The two most studied of these are protein carbonyl products.<sup>(19,20)</sup> and malondialdehyde.<sup>(21)</sup> Their concomitant evaluation allows for the assessment of oxidative damage in proteins by protein carbonyl,<sup>(19,20)</sup> while malondialdehyde evaluates the damage on cellular lipids.<sup>(21)</sup>

Protein oxidation is involved in the regulation of physiological events as well as in the minimization of tissue damage, having a key role in the pathophysiology of diseases and aging. It is commonly recognized that no carbonyl groups are a natural part of proteins. However, oxidants may lead to the oxidative cleavage of protein bonds, yielding carbonyl products that are able to be quantified by their derivatization.<sup>(20)</sup> The lipid peroxidation also affects the cell membrane lipid bilayer structure and function. Malondialdehyde is an aliphatic di-aldehyde that exists in equilibrium with its enol form and is the end-product of polyunsaturated lipid oxidation.

Due to the presence of an aldehyde free group, this compound is highly reactive and able to produce stable adducts with nucleic acids and proteins.<sup>(21)</sup>

It should be highlighted that this is the first study of the long-term effects of breast cancer treatment on oxidative stress profiles. The data available in the literature make measurements only before and some hours after cancer chemotherapy,<sup>(22-25)</sup> or when the monitoring focuses for a higher time a small number of different stress oxidative parameters was measured.<sup>(26)</sup> An understanding of this, is vital when assessing the effect of the drugs on such patients and in the development of therapeutic schemes aimed at avoiding the possible progress of oxidative stress induced pathologies. With specific emphasis on these aspects, this study aimed to evaluate the long-term effects of breast cancer chemotherapy on oxidative stress markers and defense systems against oxidative stress.

## METHODS

### Study population

The data was collected from an oncology chemotherapy center located in Erechim, RS – Brazil between 2013 and 2015. Fifty-nine women with diagnosed breast cancer were included in the investigation, prior to treatment. In concordance with study protocol, informed consent was obtained from each participant. The participants completed a questionnaire identifying socio-demographical data and risk factors. The study protocol was approved by the ethics committee of URI-Erechim under the number 01902612.8.0000.5351.

### Sample preparation

Blood was obtained by venipuncture, in a clot activator gel tube to obtain serum, and a tube with heparin to obtain plasma. Tubes were centrifuged at 3000rpm, and serum/plasma samples separated and stored at -20°C for further analysis.

### Biochemical analysis

Malondialdehyde and vitamin C were determined by HPLC following the methodology of Karatepe (2004).<sup>(27)</sup> One hundred microliters of 0.1M perchloric acid and 1ml of distilled water were added to a 100ml aliquot portion of plasma. Acid was then added in order to precipitate proteins and release the malondialdehyde, which was bound to the protein amino groups and other amino compounds whilst still maintaining the stability of the ascorbic acid. The samples were centrifuged at 4.500rpm for 5 min and used for HPLC analysis. The mobile phase was monobasic potassium phosphate (pH 3.6, 30 mM): methanol 82.5:17.5 (v/v) while the flow rate was 1.2mL/min. A C18 reverse phase column was used and the chromatograms were monitored at 250nm for the two analytes. The concentrations were calculated according to external standardization.

The formation of carbonyl groups in plasma, a parameter of oxidative damage to proteins, was measured based on the reaction of these groups with 2,4-dinitrophenylhydrazine as derivatizing agent.<sup>(28)</sup> The absorption of the product was measured in a spectrophotometer at 370nm. Results were expressed as nmol carbonyl/mg protein.

Vitamin E levels were determined using the Hansen and Warwick (1969)<sup>(29)</sup> method with modifications. In a cuvette tube, 140µL Milli-Q water (Millipore, Bedford, MA, U.S.) was added to 20µL of butylated hydroxytoluene 10mM, 140µL of the sample and 2.1mL of ethanol solution (66%). The mixture was then vortex-mixed for 10 seconds and 3.5mL of n-hexane was added and mixed for 1min, followed by centrifugation at  $1.800 \times g$  for 10min. 3mL of superior phase were then transferred to fluorimeter cuvettes and vitamin E was measured:  $\lambda_{ex}$  = 295nm and  $\lambda_{em}$  = 340nm. Calibration curves with  $\alpha$ -tocopherol were used to determine the concentration, following the same procedure used for the samples.

The total glutathione was determined in 500µg of protein. Samples were suspended in 0.1% Triton-X and 0.6% sulfosalicylic acid in a 0.1M potassium phosphate buffer, plus 5mM ethylenediaminetetraacetic acid, pH 7.5. Subsequently, the mixture was sonicated in three cycles of sonication/ice for 20s, followed by two freeze/thaw cycles, and centrifuged at  $6.500 \times g$ . The supernatant (100µL) was placed in potassium phosphate buffer, with 100µM 5,5'-dithiobis (2- nitrobenzoic acid) and 0.1units/mL glutathione reductase and incubated for 30 seconds. The reaction was started by the addition of 50µM  $\beta$ -NADPH and monitored in kinetic mode for 5min at 412nm in a spectrophotometer UV-VIS (Shimadzu RF-5301PC, Japan).<sup>(30)</sup>

The lipidic profile was evaluated using commercial kits for total cholesterol (colorimetric enzymatic assay), HDL cholesterol (colorimetric test by precipitation in phosphotungstic acid and magnesium chloride) and triglycerides (colorimetric enzymatic test). LDL cholesterol was calculated using Friedewald formula. C-reactive protein was determined by immunoturbidimetry methodology.

### Statistical analysis

The data normality was evaluated by the Shapiro-Wilk test and the values of oxidative stress markers were expressed as mean  $\pm$  standard deviation and compared by non-parametric ANOVA (Kruskal-Wallis test) followed by Dunn's test in GraphPad Prism 6.0. A value of  $p < 0.05$  was considered as statistically significant. The correlation among the variables was assessed by correlogram construction and Pearson coefficient calculations in a R environment by using the corrplot package. The principal component analysis was performed in Minitab 19 software aiming to group the patients according to time of chemotherapy.

## RESULTS

Fifty-nine patients with an average age of  $57.84 \pm 11.85$  (37-84) were included in the study population. The socio-demographical data of these participants are described in Table 1 and data linked with risk factors and tumor features in Table 2. The majority of the study patients showed breast cancer with tumor stages II (42.37%) or III (50.87%).

**Table 1.** Socio-demographic profile of study population.

Age (years)		57.84 $\pm$ 11.85 (37-84)
Ethnic group	Caucasian	88.14 % (N = 52)
	Others	11.86% (N = 7)
Years of schooling	0	5.08% (N = 3)
	1-3	20.34 % (N = 12)
	4-7	49.16% (N = 29)
	8-11	20.34% (N = 12)
	> 12	5,08% (N = 3)
Number of children	0	11.86% (N = 7)
	1	10.17% (N = 6)
	2	32.21% (N = 19)
	3	22.04% (N = 13)
	4	8.47% (N = 5)
	> 4	15.25% (N = 9)

**Table 2.** Risk factors of patients and biochemical characteristics of tumors.

Breastfeeding	Yes	77.97% (N = 46)
	No	22.03% (N = 13)
Oral contraceptive use	Yes	76.27% (N = 45)
	No	23.73% (N = 14)
Time of contraceptive use (years)		9.10 $\pm$ 8.64 (0-30)
First reproductive cycle (age)		12.90 $\pm$ 1.48 (10-17)
Menopause	Yes	76.27% (N = 45)
	No	23.73% (N = 14)
Cases of cancer in family	0	23.73% (N = 14)
	1	52.54% (N = 31)
	2	20.34% (N = 12)
	> 3	3.39% (N = 2)
Smoke	Yes	18.64% (N = 11)
	No	81.36% (N = 48)
Ethanol	Yes	93.22% (N = 55)
	No	6.78% (N = 4)
Hormone replacement therapy	Yes	20.33% (N = 12)
	No	79.67% (N = 47)
Tumor stage	I	3.38% (N = 2)
	II	42.37% (N = 25)
	III	50.87% (N = 30)
	IV	3.38% (N = 2)
KI67 (%)		20.13 $\pm$ 14.12 (5-75)
Estrogen receptor	Positive	89.84% (N = 53)
	Negative	10.16% (N = 6)
Progesterone receptor	Positive	76.28% (N = 45)
	Negative	23.72% (N = 14)
HER-2	0 or + (negative)	47.46% (N = 28)
	++	44.07% (N = 26)
	+++	8.47% (N = 5)

At any time during the therapeutic protocol, depending on the nature of the tumor, these patients made use of doxorubicin or a taxane. During the year in which the 59 patients were monitored, only 24 of them continued with the initial treatment protocol. Most of the therapeutic protocols involved the use of doxorubicin, cyclophosphamide and a taxane (docetaxel or paclitaxel). Where these drugs were not used, the alternatives included anastrozole (aromatase inhibitor), tamoxiphene (estrogen receptor antagonist) or trastuzumabe (monoclonal antibody).

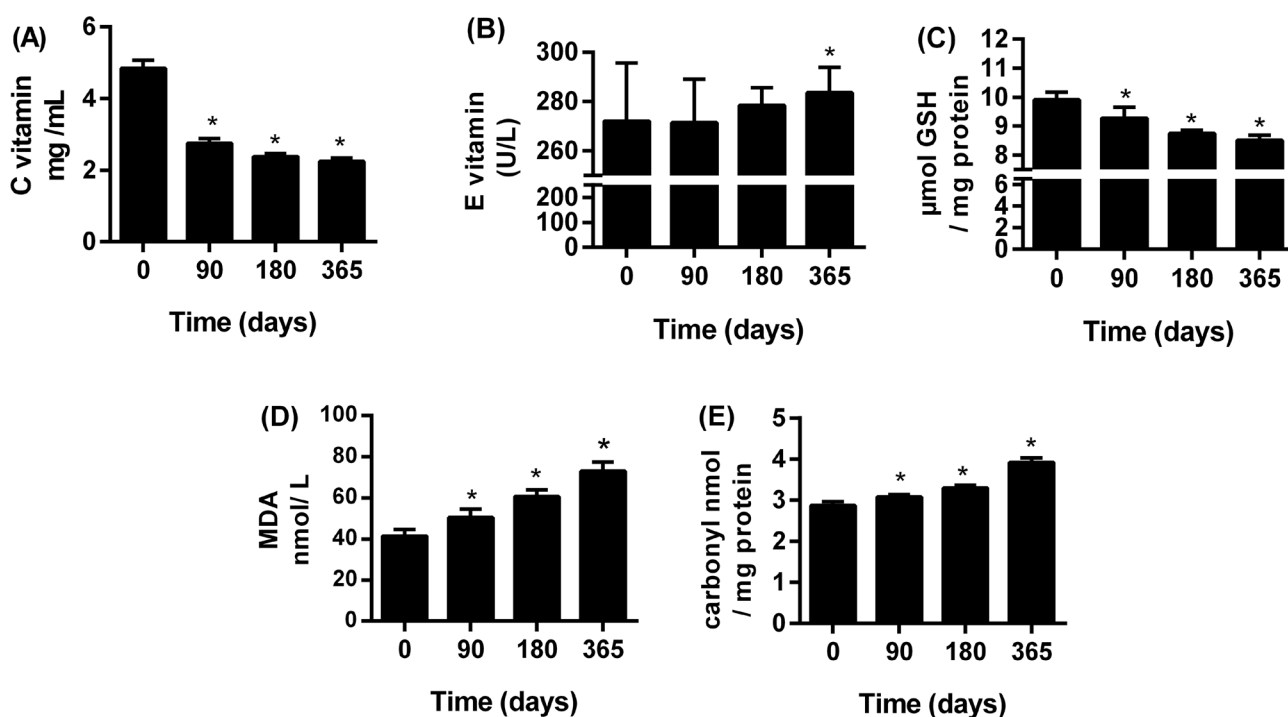
Vitamins and oxidative marker levels were intensively modified according to the time of chemotherapy (Figure 1). Vitamin C levels decreased drastically after the three first months of treatment and were maintained low, while the modifications in vitamin E levels were less expressive, differing only at the basal time of 365 days. The depletion in vitamin C levels was accomplished by decreasing the glutathione during the time of investigation.

In relation to the oxidative stress markers, the lipid peroxidation was determined on the basis of malondialdehyde levels, which increased significantly during the time of the cancer treatment. There was also a measurable rise in carbonyl protein relative to the time of treatment. In summary, the long-term effect of drugs against cancer contributes to a depletion of soluble antioxidant defense agents and an increase in oxidative stress markers associated with damage to proteins and lipids.

In addition, the lipidic profile (total, HDL and LDL cholesterol and triglycerides) and the C-reactive protein of patients were not significantly modified during the period of investigation.

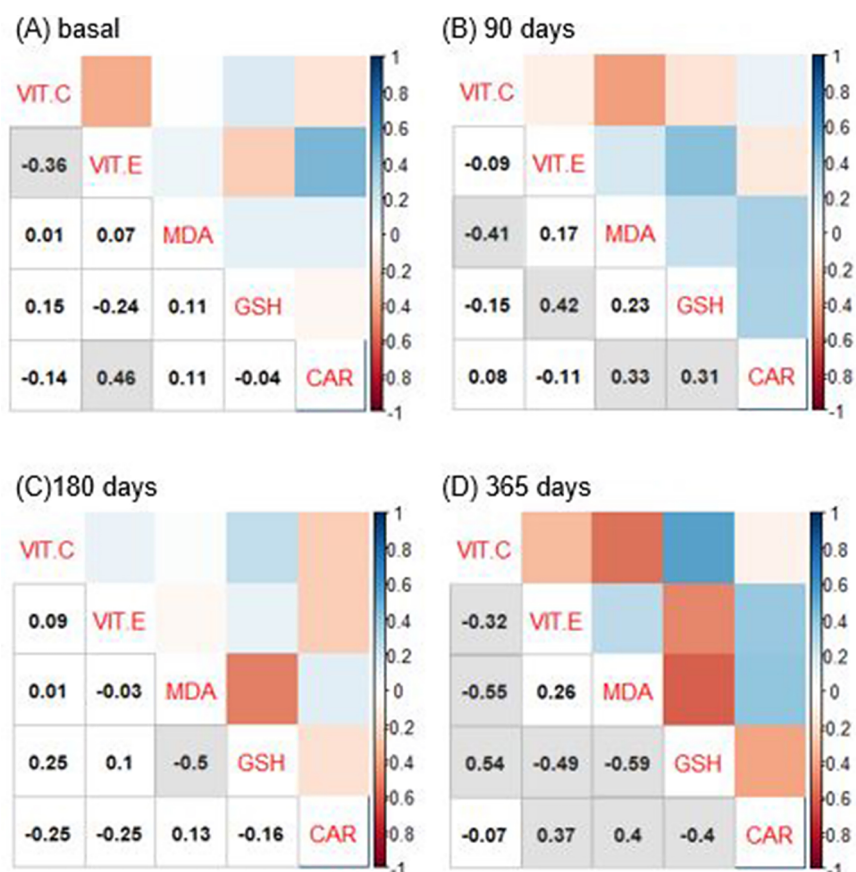
The correlation between the five oxidative stress parameters during the four different times of analysis was investigated by a correlogram construction in a R environment (Figure 2). The correlations became more intense toward the end of monitoring (Figure 2C). A significant negative correlation between vitamin C and vitamin E may be observed at base time and 365 days. Again, Figure 2 highlights significant negative correlation between malondialdehyde and glutathione at 180 and 365 days. At this time, the increase in malondialdehyde may be one of the reasons for glutathione depletion. In  $t=365$  days, a strong positive correlation may be noted between glutathione and vitamin C.

In order to understand the variability of oxidative stress markers in patients according to the time of chemotherapy, multivariate analysis by principal component analysis (PCA) was performed. The five original variables (vitamin E, vitamin C, glutathione, malondialdehyde, and carbonyl protein) were reduced in PC1 and PC2, which explained 89.5% of the total variance (Figure 3A). When the PC3 was added, 95.9% of the total variance was explicable (Figure 3B). This multivariate analysis facilitated the grouping of patients into four well-defined groups. The profile of oxidative stress markers for those patients at basal time showed them clustering to the left side in Figure 3A. The next groups were the patients at times 90, 180, and 365 days of treatment, respectively. The same number of groups, but with improvement in separation, was found when the first three components were used in the PCA analysis, and are shown in Figure 3B. Figure 3 shows that the lowest similarity was found between the patients at basal time and those at 365 days.

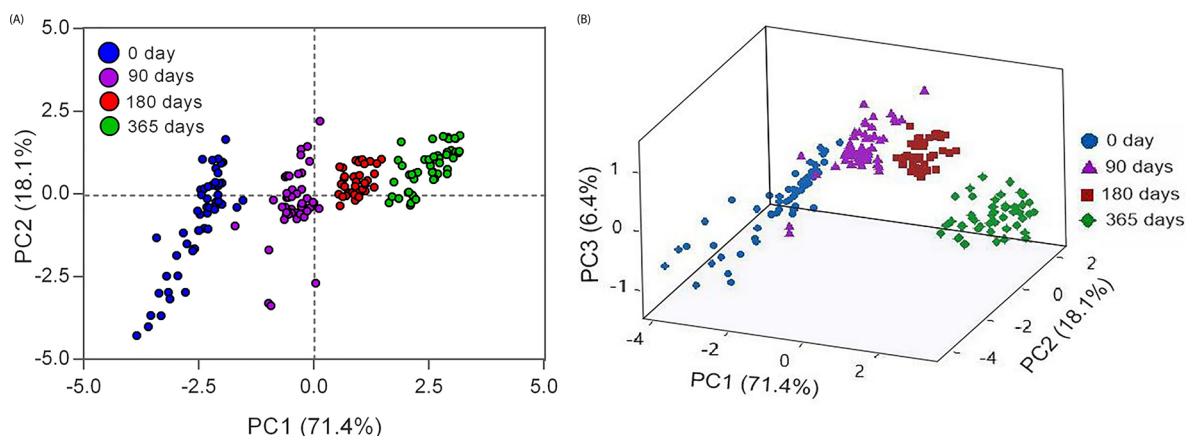


**Figure 1.** Vitamins, glutathione and oxidative marker levels in women undergoing chemotherapy treatment for breast cancer. \* $p < 0.05$  in relation to the basal level according to non-parametric ANOVA followed by Dunn's test. The data are shown as mean  $\pm$  standard-deviation.





**Figure 2.** Pairwise Pearson's correlation of the antioxidant markers considered in this study. The r-values highlighted in grey shown a significant correlation. VIT.C = Vitamin C; VIT.E = Vitamin E; MDA = Malondialdehyde; GSH = Glutathione; CAR = Protein carbonyl products.

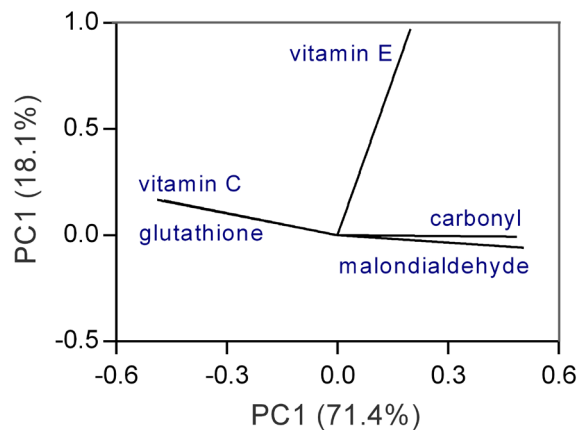


**Figure 3.** A. Distribution in the hyperspace of the scores of the first two components of patients according to their oxidative markers profile; B. Hyperspace of the scores of the first three components of the patients according to their oxidative markers.

The PCA analysis highlighted the change in the stress oxidative profile produced by chemotherapy. It should be noted that the level of similarity among the patients was higher in the data collected at 180 and 365 days.

The vector plot (Figure 4), was used to characterize the stress oxidative parameters and their relationship to patient clustering. PC1 was affected, to a large extent, by vitamin C, glutathione, carbonyl and malondialdehyde, while the vitamin E effect was important for the PC2 value, producing separation of groups along the y axis. High values of vitamin C and glutathione were the

most important factors in the production of negative values in PC1 and clustering of patients in group "0 day" was to the left. On the other hand, high values of malondialdehyde and protein carbonyl products contributed to positive PC1 values and consequent clustering of patients to the right of the plot. This plot also highlights the inverse correlation between the oxidative markers (malondialdehyde and protein carbonyl products) and the antioxidant defense systems (vitamin C and glutathione), which are placed in opposing regions on the graph described in Figure 4.



**Figure 4.** Vector-correlation plot between the variables examined.

## DISCUSSION

Due to the relation between oxidative stress and cancer treatment, it is natural for some questions to arise: 'what-if any-is' the influence of time of chemotherapy on oxidative stress markers. The investigations related to this question may be useful for define the role of antioxidant supplementation in cancer treating. In this context, a previous investigation identified the absence of changes in carbonyl protein products and malondialdehyde before, and 24 hours after, doxorubicin administration for breast cancer treating, suggesting that the changes reported here are associated with the chronic effect of antineoplastic drugs.<sup>(22)</sup> Despite of this, there are not studies reporting the effect of long-time breast treating on oxidative stress markers.

Recently, a strong inverse linear relationship between levels of malondialdehyde and glutathione ( $r=-0.947$ ) was found in blood samples of workers exposed to benzene.<sup>(31)</sup> In our results, this correlation also was significant at the 180 and 365 days. Another correlation present in the results reported here, was between vitamin C and glutathione that may be explained by the fact that glutathione and vitamin C mutually spare each other in any reaction between intracellular reactive species. An increase in ascorbate concentration may serve to spare glutathione and vice versa. In lymphocytes isolated from 240 healthy people, the correlation between these two main aqueous phase antioxidants produced a  $r$  value of 0.62.<sup>(32)</sup> The correlation found between vitamin C and vitamin E may be related to the role of vitamin C in regenerating vitamin E by its reduction.<sup>(12)</sup>

Another study helped to identify the role of oxidative stress in breast cancer progression. The cell proliferation index was negatively correlated with the plasmatic levels of non-enzymatic antioxidants (vitamin A:  $r=-0.52$ , vitamin C:  $r=-0.37$ , vitamin E:  $r=-0.56$ ) and the total antioxidant activity ( $r=-0.73$ ). These findings suggest that non-enzymatic antioxidants may be useful in the prediction of tumor growth and open the possibility of intervention with antioxidants.<sup>(33)</sup> In addition, the oxidative stress was modified according to different breast tumor stages, with total oxidant status and oxidant stress index gradually increasing as the disease progressed. At the same time, total antioxidant status diminished.<sup>(34)</sup>

According to clinical evidence investigating vitamin C supplementation in patients with breast cancer, a study reported that the intravenous administration of vitamin C in high doses produced a significant reduction of complaints induced by the disease and treatment. Vitamin C administration was shown to help with fatigue, depression, nausea, loss of appetite, sleep disorders, dizziness, and hemorrhagic diathesis.<sup>(35)</sup>

In this current investigation, the antineoplastic drugs mostly used in the therapeutic protocols are closely related to oxidative stress induction. The production and accumulation of reactive oxygen and reactive nitrogen species as a consequence of doxorubicin use are widely reported in the literature and associated with doxorubicin-induced cardiotoxicity.<sup>(8,36)</sup> Cyclophosphamide, an alkylating agent, produced a significant increase in the malondialdehyde content in the brain in rats treated with this drug.<sup>(10)</sup> The induction of apoptosis produced by the tubulin stabilizing agent, taxol, was associated with the generation of reactive oxygen species measuring 2',7'-dichlorofluorescein diacetate assay and glutathione depletion in chronic myelogenous leukemia K562 cells.<sup>(9)</sup>

Our study looked at the behavior of oxidative stress parameters during the chemotherapy targeting the breast cancer. Despite of the consistent correlations found among the measured parameter, some source of data variability, such as diet and lifestyle-related variables were not considered in our experimental design. Finally, our investigation was based on the experience of a single cancer treating service and the number of patients was limited. More studies involving larger samples are important aiming to confirm the results reported here.

## CONCLUSION

The chemotherapy used in the treatment of breast cancer had a significant effect on the oxidative stress profile of the study participants. The most radical modifications were the lowering in the vitamin C and glutathione levels and increasing the levels of malondialdehyde and protein carbonyl products. Understanding the long-term effects of pharmacological cancer treatment on oxidative balance is of immense importance when planning therapeutic schemes aimed at reducing collateral effects and preserving patient health.

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