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## METABÓLITOS DO ÓXIDO NÍTRICO SALIVAR COMO BIOMARCADOR PARA RECORRÊNCIA EM PACIENTES OBESOS COM CÂNCER DE MAMA

FRANCISCO BELTRÃO – PR (FEVEREIRO/2025)

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Área de concentração: Ciências da Saúde.

Orientador(a): Dra. Aedra Carla Bufalo Kawassaki

Co-orientador(a): Dra. Carolina Panis

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## FOLHA DE APROVAÇÃO

## PAULA RIZZATTI ANTONIOLLI

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Essa dissertação foi julgada adequada para obtenção do título de Mestre em Ciências Aplicadas à Saúde e aprovada em sua forma final pelo(a) Orientador(a) e pela Banca Examinadora.

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## Metabólitos do óxido nítrico salivar como biomarcador para recorrência em pacientes obesos com câncer de mama Resumo

O câncer de mama (CM) é a neoplasia mais comum diagnosticada em mulheres em todo o mundo. Métodos considerados menos invasivos e capazes de fornecer detecção precoce estão sendo estudados, como o uso da saliva. Nesse contexto, este estudo avaliou os níveis de metabólitos de óxido nítrico (NOx), um biomarcador de estresse oxidativo amplamente ligado ao desenvolvimento e progressão da doença, em amostras de saliva de mulheres com CM (n = 129) e um grupo controle saudável (n = 200). Além disso, determinamos a relação entre os níveis de NOx e os parâmetros clinicopatológicos. Os níveis salivares de NOx foram determinados pelo método cádmio-cobre-Griess. Em relação à categorização clinicopatológica (idade ao diagnóstico; menopausa ao diagnóstico; índice de massa corporal (IMC); histológico; receptor de estrogênio; receptor de progesterona; receptor HER2; índice de proliferação celular - Ki-67; grau histológico; coágulos; metástase linfonodal: metástase à distância: quimiorresistência; recorrência) do grupo CM, não foram encontradas diferenças significativas. Posteriormente, o IMC das pacientes com câncer de mama foi avaliado em relação aos demais parâmetros, sendo encontrada diferença significativa no parâmetro de recidiva para mulheres com excesso de peso, com valor de p de 0,035. A saliva ainda não é utilizada no diagnóstico e monitoramento de pacientes com câncer de mama, portanto estes resultados fornecem informações para novos estudos que analisem o NOx salivar para sua potencial aplicação como biomarcador.

**Palavras-chave:** câncer de mama, óxido nítrico, estresse oxidativo, saliva, biomarcadores.

## Salivary Nitric Oxide Metabolites as a Biomarker for Recurrence in Obese Patients with Breast Cancer

#### Abstract

Breast cancer (BC) is the most common neoplasia diagnosed in women worldwide. Methods that are considered less invasive and may be able to provide early detection are being studied, such as the use of saliva. In this context, this study evaluated the levels of nitric oxide metabolites (NOx), a biomarker of oxidative stress widely linked to disease development and progression, in saliva samples of women with BC (n = 129) and a control healthy group (n = 200). Also, we determined the relationship between NOx levels and clinicopathological parameters. Salivary NOx levels were determined by the cadmium-copper-Griess method. In relation to the clinicopathological categorization (age at diagnosis; menopause at diagnosis; body mass index (BMI); histological; estrogen receptor; progesterone receptor; HER2 receptor; cell proliferation index - Ki-67; histological grade; clots; lymph node metastasis; distant metastasis; chemoresistance; recurrence) of the BC group, no significant differences were found. Subsequently, the BMI of the patients with breast cancer was assessed in relation to the other parameters, and a significant difference was found in the recurrence parameter for overweight women, with a p-value of 0.035. Saliva is not yet used in the diagnosis and monitoring of patients with breast cancer, so these results provide information for new studies that analyze salivary NOx for its potential application as a biomarker.

Keywords: breast cancer; nitric oxide; oxidative stress; saliva; biomarkers.

## **ARTIGO CIENTÍFICO 01**

Salivary Nitric Oxide Metabolites as a Biomarker for Recurrence in Obese Patients with Breast Cancer.

### NOx, Recurrence and Obese Patients with Breast Cancer.

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#### Abstract

Breast cancer (BC) is the most common neoplasia diagnosed in women worldwide. Methods that are considered less invasive and may be able to provide early detection are being studied, such as the use of saliva. In this context, this study evaluated the levels of nitric oxide metabolites (NOx), a biomarker of oxidative stress widely linked to disease development and progression, in saliva samples of women with BC (n = 129) and a control healthy group (n = 200). Also, we determined the relationship between NOx levels and clinicopathological parameters. Salivary NOx levels were determined by the cadmium-copper-Griess method. In relation to the clinicopathological categorization (age at diagnosis; menopause at diagnosis; body mass index (BMI); histological; estrogen receptor; progesterone receptor; HER2 receptor; cell proliferation index - Ki-67; histological grade; clots; lymph node metastasis; distant metastasis; chemoresistance; recurrence) of the BC group, no significant differences were found. Subsequently, the BMI of the patients with breast cancer was assessed in relation to the other parameters, and a significant difference was found in the recurrence parameter for overweight women, with a p-value of 0.035. Saliva is not yet used in the diagnosis and monitoring of patients with breast cancer, so these results provide information for new studies that analyze salivary NOx for its potential application as a biomarker.

Keywords: breast cancer; nitric oxide; oxidative stress; saliva; biomarkers.

#### 1. INTRODUCTION

Breast cancer is a malignant neoplasia characterized by the disorderly multiplication of abnormal cells in the mammary glands, developing a malignant

tumor that can invade other tissues and organs. It is a heterogeneous disease related to genetic and environmental factors, predominantly affecting women and being the most common cancer in this population both in Brazil and globally<sup>1</sup>.

Breast cancer can be caused by genetic mutations, which are mainly related to tumor suppressor genes (BRCA1 and BRCA2) in about 10% of cases. However, most of the cases are linked to environmental and intrinsic factors such as age, late menopause, along with extrinsic factors such as obesity and hormone use, are some of the primary agents that promote the development of the disease<sup>2</sup>.

Various technologies allow for the diagnosis of breast cancer at early stages, including mammograms, magnetic resonance imaging with contrast, and biomarker detection through plasma analysis. However, other less invasive methods, which could possibly offer early detection of the disease, are under study, such as the use of saliva. However, there is still a lack of scientific validation for the applicability of this method<sup>2</sup>.

Saliva can be an alternative to blood and other fluids, being highlighted particularly for being a non-invasive method that has already been proven in other studies for detecting specific biomarkers present in breast cancer, such as c-erbB-2 (erb) and cancer antigen 15-3 (CA15-3)<sup>3,4</sup>. On the other hand, similar to the study by Vacario et al., which, despite analyzing the presence of oxidative stress markers (nitric oxide metabolites – NOx) in the plasma of BC patients, noted that NOx levels were increased in various clinicopathological parameters<sup>5</sup>. However, there are not many reports on whether changes in salivary nitric oxide (NO) levels occur in this disease or if they can be detected.

Thus, this study aimed to assess potential changes in NOx levels in the saliva of women with breast cancer and relate them to important clinicopathological parameters in the prognosis and treatment of the disease.

#### 2. MATERIALS AND METHODS

2.1 Study design and sample obtention

This research was conducted as a retrospective, cross-sectional observational study carried out at the Health Sciences Center (CCS) of the Francisco Beltrão Campus – UNIOESTE - Paraná. It involved information and materials collected from patients hospitalized for investigation of the presence of breast cancer at the CEONC Hospital in Francisco Beltrão, from 2015 to 2022. This project was approved by the Research Ethics Committee with Human Beings, under the CAAE 35524814.4.0000.0107. All participants were informed about the research and signed the Free and Informed Consent Term (TCLE).

Saliva samples were collected in separate plastic tubes through natural, unstimulated salivation. Prior to collection, all participants observed a fasting period of at least eight hours. The samples were gathered simultaneously and preserved in a freezer at -20°C until analysis.

The following inclusion criteria were considered for the selection of this material: volunteer patients who arrived at CEONC with abnormalities in breast imaging tests (BIRADS 4 and 5) and who underwent surgical biopsy; female patients only; patients with breast cancer who had clinical-pathological data collected from medical records and medical history; and patients who had collected and sufficient saliva samples for conducting the test.

Patients were categorized into two groups: women diagnosed with (n = 129) or without (n = 200) breast cancer, with the latter serving as our control group. For all participants, clinical-pathological data from medical records were collected and saliva samples were used to analyze nitric oxide metabolite (NOx) levels. The study design is represented in Figure 1.



### Figure 1 - Design of the study

Each clinicopathological variable studied was categorized into the following groups: age at diagnosis ( $\leq$  50 years or > 50 years); menopause at diagnosis (present or absent); body mass index (BMI) (eutrophic  $\leq$  24.9 kg/m<sup>2</sup>, overweight 25 kg/m<sup>2</sup> to 29.9 kg/m<sup>2</sup>, or obese >30 kg/m<sup>2</sup>); histological subtype (Luminal A, Luminal B, Triple-negative, and HER2); estrogen receptor (positive or negative); progesterone receptor (positive or negative); HER2 receptor (positive or negative); cell proliferation index - Ki-67 (<14% or  $\geq$  14%); histological grade (1, 2, 3); emboli (present or absent); lymph node metastasis (present or absent); sites of metastasis (present or absent); chemoresistance (yes or no); recurrence (yes or no).

2.2 Measurement of NOx levels

The determination of NOx concentration in the saliva samples was performed using the technique described by Mezoni et al. (2025) The method is based on the reduction of nitrate to nitrite mediated by redox reactions between the nitrate in the sample and the cadmium-copper system of the reagents, followed by diazotization and colorimetric detection of the azo compound formed by the addition of Griess. The absorbance measured at 550 nm in a microplate spectrophotometer was used, and the plasma nitric oxide levels were expressed in micromolar (µM) of nitrite<sup>6</sup>.

#### 2.3 Statistical Analysis

The clinicopathological variables are presented as absolute values (n) and frequency. NOx results were initially evaluated by checking the normality of the data distribution using the Shapiro-Wilk test. Since the data showed a non-normal distribution, the Mann-Whitney test, for non-parametric data, was used to compare two groups, and when there were more than two groups, the One-Way ANOVA test was applied or the Kruskal-Wallis test for non-parametric data. The NOx values are presented as mean, standard deviation, median, minimum, and maximum. The values of the variables that appear as not computed (NC) represent samples with fewer than 5 participants, which were not considered in the statistical analysis. The JASP software, version 18.3 r, was used for data analysis, and p < 0.05 was considered significant.

#### 3. RESULTS

The study analyzed a total of 329 saliva samples, with 129 from patients diagnosed with breast cancer and 200 from women without breast cancer. Initially, the patients were divided into two groups (Breast Cancer and Controls) and their

salivary NOx results were compared. The breast cancer group presented a mean of  $51.37 \pm 52.15$  and a median of 36.109 (16.543 - 400.022), while the control group showed a mean of  $51.02 \pm 37.36$  and a median of 38.500 (15.674 - 243.283), with a p-value of 0.304 (Figure 2).



Figure 2 - Salivary nitric oxide metabolites (NOx) levels in patients with breast cancer or control. NOx values expressed per micromolar ( $\mu$ M), mean ± standard deviation, p= 0.304.

In relation to the salivary NOx levels according to the clinicopathological categorization of the breast cancer group, no significant differences were found. The results are shown in Table 1.

The patients with breast cancer had a higher frequency of women over 50 years old, postmenopausal, and with a predominance of overweight. Regarding the tumor characteristics, it was found that most of the tumors were of the Luminal subtypes, with positive estrogen and progesterone receptors, high cell proliferation rate, histological grade II, absence of emboli, absence of metastasis in lymph nodes, absence of chemoresistance, and absence of recurrence.

	n (frequency)	NOx (µM)	p value
Age at diagnosis			
≤ 50 years	51 (40.8%)	36.54 (16.76 – 400.02) 48.53±55.04	0.806
> 50 years	74 (59.2%)	35.13 (16.54 – 273.50) 53.79±51.68	
Menopause at diagnosis			
Presence	71 (60.1%)	36.76 (16.54 – 273.50) 55.93±52.39	0.477
Absence	47 (39.8)	36.32 (16.70 – 400.02) 48.50±57.02	
Body mass index (BMI) (kg/m²)			
Eutrophic	19 (11.04%)	35.02 (18.71 – 159.80) 43.87±30.99	
Overweight	41(23.84%)	38.50 (17.84 – 400.02) 55.91±65.28	0.360
Obese	31 (18.2%)	34.80 (16.54 – 105.23) 40.27±22.39	
Molecular subtype			
Luminal A	34 (29.82%)	32.08 (18.71 – 400.02) 48.25±65.01	
Luminal B	47 (41.22%)	40.02 (16.54 – 273.50) 60.90±56.53	0.101
Triple-negative (TN)	21 (18.42%)	32.19 (17.84 – 67.84) 36.79±14.49	
HER2 -amplified	12 (10.52%)	37.41 (23.50 – 140.45) 51.29±36.15	
Estrogen receptor			
Positive	89 (76.31%)	36.10 (16.54 – 400.02) 55.85±60.39	0.749
Negative	27 (23.68%)	36.32 (17.84 – 140.45) 43.67±27.14	

Table 1 – Frequencies of clinicopathological variables and salivary levels of NOx ( $\mu$ M) among breast cancer patients participating in the study.

Progesterone receptor

Positive	59 (55.66%)	35.67 (16.54 – 400.02) 63.57±72.03	0.000
Negative	47 (44.33%)	33.71 (16.76 – 123.06) 38.69±18.31	0.223
Ki-67 proliferation index			
≤ 14%	38 (34.54%)	32.63 (18.17 – 400.02) 51.18±67.18	0.094
> 14%	72 (65.45%)	38.60 (16.54 – 273.50) 56.01±49.14	0.004
Histological grade			
Grade I	26 (25.5%)	31.21 (16.76 – 400.02) 49.97±73.10	
Grade II	63 (61.76%)	37.41 (16.54 – 273.50) 56.59±51.73	0.219
Grade III	13 (12.74%)	38.50 (23.28 – 222.19) 50.72±52.41	
Clots			
Presence	12 (10.43%)	33.71 (16.76 – 123.06) 46.58±33.46	0.589
Absence	103 (89.56%)	35.67 (16.54 – 400.02) 52.37±56.59	
Lymph nodes metastasis			
Presence	27 (28.42%)	40.02 (16.54 – 400.02) 67.26±81.42	0.475
Absence	68 (71.58%)	33.71 (17.84 – 273.50) 46.79±42.11	
Distant metastasis			
Presence	33 (41.77%)	35.67 (17.84 – 186.76) 60.80±74.82	0 426
Absence	46 (58.22%)	32.41 (16.54 – 400.02) 38.36±24.87	0.120
Chemoresistance			
Presence	19 (20.87%)	29.37 (21.54 – 400.02) 72.16±99.16	0.777
Absence	72 (79.12%)	33.60 (16.54 – 186.76) 42.74±27.80	

Recurrence			
Yes	9 (9.47%)	32,63 (21.54 – 105.23) 38.93±25.94	0.431
Νο	86 (86.72%)	34,91 (16.54 – 222.19) 44.63±33.91	

The statistical calculations presented were calculated using the Mann Whitney Test, for comparing two independent groups and Kruskal-Wallis, for comparing three independent groups. Results presented as frequency, median (minimum-maximum), mean±standard deviation. HER2 = human epidermal growth 2 receptor.

In a second analysis, when comparing salivary NOx levels in breast cancer patients categorized according to BMI, it was observed that in the eutrophic group, there was a predominance of women under 50 years old, postmenopausal, with luminal tumors, positive estrogen receptor, high cell proliferation rate, histological grade I, with no emboli, no metastasis in lymph nodes, and no chemoresistance. In the overweight group, there was a predominance of women under 50 years old, with luminal tumors, positive estrogen and progesterone receptors, high cell proliferation rate, histological grade II, with no emboli, no metastasis in lymph nodes, no chemoresistance, and no metastasis sites. The obese group showed a predominance of women over 50 years old, with luminal tumors, positive estrogen and progesterone receptors, high cell proliferation rate, histological grade II, with no

In relation to the salivary NOx levels according to the clinicopathological categorization of the breast cancer / BMI groups, no significant differences were found. For the variables presented in Table 2.

# Table 2 – Salivary levels of NOx in breast cancer patients according to BMI and prognostic parameters.

	Eutrophic (n=19)	p value	Overweight (n=41)	p value	Obese (n=31)	p value
Age at diagnosis						
≤ 50 years	n=11 37.63 (18.71 64.80) 38.97±15.33	O.860	n=25 40.23 (17.84- 400.02) 59.20±75.29	1.000	n=12 30.45 (16.76- 67.84) 34±17	0.282
> 50 years	n=7 35.02 (24.15- 159.80) 53.74±47.89		n=14 37.41 (22.63- 222.19) 53.42±50.33		n=19 36.76 (16.54- 105.23) 44.41±25.72	
Menopause at diagnosis						
Presence	n=12 38.60 (24.15- 159.80) 49.46±37.17	0.673	n=7 34.80 (21.54- 222.19) 51.32±46.55	0.681	n=7 35.78 (16.54- 105.23) 44.52±26.46	0.568
Absence	n=6 33.82 (26.54- 60.23) 36.90±12.45		n=7 42.41 (17.84- 400.02) 61.90±79.64		n=7 33.28 (16.76- 67.84) 35.80±14.46	
Molecular subtype						
Luminal A	n=6 27.19 (18.71- 28.71) 26.07±3.78	0.082	n=11 33.50 (20.23- 400.02) 71.68±109.84		n=1 NC	
Luminal B	n=5 45.45 (24.15- 159.80) 65.06±54.05	0.002	n=9 44.80 (17.84- 80.23) 45.48±18.98	0 727	n=17 34.80 (16.54- 91.76) 41.74±22.50	0 262
Triple-negative	n=2 NC		n=7 33.06 (22.63- 66.10) 38.12±14.29	0.727	n=6 24.58 (22.63- 67.84) 34.26±18.18	0.000
HER2-amplified	n=0 NC		n=5 38.50 (31.54- 140.45) 59.45±45.81		n=2 NC	
Estrogen receptor						
Positive	n=13 NC	NC	n=24 42.30 (17.84- 400.02)	0.699	n=22 36.87 (16.54- 105.23)	0.263

			64.±2581.89		43.75±24.11	
Negative	n=2 NC		n=12 37.41 (22.63- 140.45) 47.01±31.54		n=6 24.58 (22.63- 67.84) 34.26±18.18	
Progesterone receptor						
Positive	n=8 29.69 (24.15- 159.80) 48.44±45.60	0.916	n=18 44.69 (17.84- 400.02) 75.23±94.93	0 356	n=15 34.80 (16.54- 105.23) 43.70±25.32	0 736
Negative	n=8 33.93 (18.71- 64.80) 39.72±17.62	0.010	n=15 36.32 (22.63- 68.06) 39.55±13.60	0.000	n=11 38.06 (16.76- 67.84) 36.20±14.31	0.100
Ki-67						
≤ 14%	n=6 28.28 (18.71- 64.80) 32.55±16.24	0.097	n=15 40.23 (20.23- 400.02) 76.52±101.99	0.904	n=2 NC	NC
> 14%	n=9 42.19 (24.15- 159.80) 53.79±41.44	0.087	n=20 39.26 (17.84- 140.45) 46.08±26.63	0.094	n=23 NC	NC
Histological grade						
Grade I	n=8 29.04 (18.71- 64.80) 37.25±17.40	0 558	n=7 40.23 (28.71- 400.02) 97.10±134.99		n=4 NC	
Grade II	n=5 30.02 (25.89- 159.80) 59.63±57.09	0.000	n=20 38.17 (17.84- 140.45) 44.41±26.66	0.514	n=18 NC	NC
Grade III	n=2 NC		n=5 44.58 (31.54- 222.19) 77.45±81.21		n=1 NC	
Clots						
Presence	n=2 NC	NC	n=2 NC	NC	n=6 24.47 (16.76- 91.76)	0.305

					36.68±28.02	
Absence	n=11 NC		n=35 NC		n=25 35.67 (16.54- 105.23) 41.56±21.45	
Lymph nodes metastasis						
Presence	n=6 36.10 (25.89- 159.80) 36.68±28.02	0 755	n=10 50.13 (20.23- 400.02) 98.21±121.27	0 229	n=8 34.26 (16.54- 91.76) 37.27±24.10	0 196
Absence	n=25 32.19 (18.71- 60.23) 41.56±21.45	0.755	n=21 33.06 (21.54- 66.10) 38.81±12.19	0.220	n=15 42.41 (21.10- 105.23) 45.90±22.16	0.100
Distant metastasis						
Presence	n=7 30.02 (25.89- 159.80) 52.41±48.43	0.620	n=12 49.69 (20.23- 400.02) 88.59±112.06	0 211	n=10 34.26 (16.54- 91.76) 35.63±21.76	0 4 9 1
Absence	n=7 29.37 (18.71- 55.67) 29.37±33.74	0.020	n=13 33.06 (28.06- 57.84) 37.36±10.30	0.211	n=10 32.41 (22.63- 67.84) 36.60±13.89	0.401
Chemoresistance						
Presence	n=1 NC	NC	n=10 30.45 (21.54- 400.02) 71.37±116.23	0.210	n=7 25.89 (22.41- 91.76) 39.83±24.99	1 000
Absence	n=12 NC		n=22 42.41 (20.23- 140.45) 47.57±26.02	0.319	n=17 33.71 (16.54- 105.23) 40.08±24.27	1.000
Recurrence						
Yes	n=0 NC		n=5 28.71 (21.54- 40.02) 30.28±8.65	0.035	n=3 NC	
Νο	n=17 NC		n=27 44.58 (26.76- 222.19) 54.50±40.94	0.000	n=21 NC	

The statistical calculations presented were calculated using the Mann Whitney Test, for comparing two independent groups and Kruskal-Wallis, for comparing three independent groups. NC (not computed). BMI – Body mass index. Results presented as frequency, median (minimum-maximum), mean±standard deviation.

However, when the recurrence variable was analyzed, it was observed that in the overweight group, patients without recurrence ( $54.50\pm40.94$ ; 44.58, 26.76-222.19) had higher salivary NOx levels than those with recurrence ( $30.28\pm8.65$ ; 28.71, 21.54-40.02), with a p=0.035, as shown in Figure 3.



Figure 3 - Salivary NOx levels in overweight patients with breast cancer categorized according to recurrence. NOx values expressed as micromolar ( $\mu$ M), mean ± standard deviation, p= 0.035\*.

#### 4. DISCUSSION

In this study, a total of 329 saliva samples were collected, including 129 with a BC diagnosis and 200 control healthy group. When samples were compared in relation to NOx levels, there was no significant difference between women with BC and those in the control group, as well as when patients with BC were analyzed in relation to clinicopathological parameters. However, overweight women with BC, and without disease recurrence had higher NOX levels than overweight women with recurrence.

Studies show that concentrations of NOx in different body fluids, such as saliva, plasma, and breast milk, vary significantly, with higher levels found in saliva<sup>7</sup>. Evidence of NO secretion in saliva has existed for some time, as reported by Bodis (1993)<sup>8</sup>. Patients with oral mucosa diseases have higher salivary NO levels than healthy individuals, as well as patients with oral pre-cancer showing higher salivary nitrite levels than healthy volunteers<sup>9,10,11</sup>. Regarding breast cancer, the study by Bel'skaya et al. (2021) demonstrated that patients with this tumor or with benign breast pathologies had lower levels of NO metabolites in saliva than the control group<sup>12</sup>. In our study, no difference was observed between the control and BC groups; however, this result is similar to that of Bel'skaya et al. (2021)<sup>12</sup>, considering that our volunteers in the control group were women who had benign breast changes. Moreover, it should also be considered that at the time of saliva sample collection, the women participants were in the early stages of the disease, and Alagol et al. (1999) showed that the production of NO and its plasmatic metabolites increases, compared to controls, with BC progression, suggesting that these metabolites may be found at higher levels as the disease progresses<sup>13</sup>.

In a meta-analysis conducted by Koopaie et al. (2022), which included 14 studies analyzing various markers in saliva samples from BC patients, they concluded that this fluid shows promise for analyzing certain components and their relationship to the disease. Literature describes studies that address the association/relationship of changes in NO levels and its metabolites with prognostic parameters in BC, but the vast majority involve plasma and tumor tissue<sup>14</sup>.

Reddy et al. (2024), when analyzing the presence of iNOS in the blood of BC patients, found that patients who had reduced iNOS levels had a better clinical response to treatment. This is because the authors confirmed a positive correlation between metastasis and increased NO levels in patients with metastatic tumors. Additionally, through in vitro tests using breast tissue samples, the increase in NO levels was also found when analyzing the chemotherapy resistance parameter, suggesting that iNOS inhibition may decrease chemotherapy resistance and improve treatment outcomes<sup>15</sup>.

Loibl et al. (2002) analyzed the presence of NOS in the blood and tissue of breast lesions in patients with BC and benign lesions, finding a positive response for NOS in the BC group and a negative response in the benign group. Furthermore, when analyzing parameters such as age and the presence of metastasis in lymph nodes, they observed an increase in NO levels in patients under 50 years old and a decrease in patients with negative lymph node metastasis<sup>16</sup>.

Ridnoour et al. (2024), for example, investigated the influence of NOS2/COX2 in estrogen receptor-negative breast tumors, showing that its elevation limits CD8+ T cell infiltration and is associated with metastatic regions and stem cells<sup>17</sup>, while Malik et al. (2024), in their study with 498 women, observed that elevated oxidant levels (MDA, ILs, MMPs, HSPs) and low antioxidant levels (SOD, GSH, CAT, vitamins A, C, and D) in patients suggest their role in the pathophysiology of câncer<sup>18</sup>. In study Ren et al. (2019) was observed that using an in vitro tumor progression model, NOx deprivation triggered the differentiation of myofibroblasts and epithelial-mesenchymal transition, contributing to increased collagen deposition and tumor development<sup>19</sup>. In another study by Ren et al. (2021), they demonstrated that tumor progression decreases basal NOx levels in tumor

tissue, likely due to reduced cofactor of NOx synthase enzyme, which favors the development of cancer cells<sup>20</sup>. Our results align with these studies, showing that NOx reduction is present in the recurrence group of patients, but it adds new information by evaluating this marker in saliva, a fluid currently undergoing increasing study.

It is known that oxidative stress is a key factor in the development of malignancies. Cancer development is a multi-stage process, and oxidative stress caused by the production of ROS in the breast can predispose individuals to BC<sup>21</sup>.

Silva et al. (2023), when evaluating the oxidative stress profile by measuring systemic levels of hydroperoxides and NOx, observed an increase in hydroperoxides in overweight patients with BC compared to the normal weight BC group. Thus, obesity, in addition to being a pro-inflammatory agent, plays a critical role in the development of BC, directly influencing the disease's prognosis<sup>22</sup>. Furthermore, Biglia et al. (2013) when assessing the BMI of women with BC, found no significant correlation with parameters such as Ki-67, tumor grade, and HER-2 receptor; however, when evaluating disease recurrence, the overweight group showed a higher index<sup>23</sup>.

On the other hand, in another study, a significant positive correlation was found between overweight/obesity patients and hormone receptor positivity. Most of those who had dysregulation of the antitumor immune response were overweight or obese at the time of diagnosis. The majority of these patients also had one or more affected lymph nodes, implying a higher likelihood of metastasis to distant organs, increasing the risk of disease recurrence and decreasing overall survival<sup>24</sup>.

At the time of diagnosis, obese patients are more likely to present highergrade tumors, larger tumors, and lymph node involvement compared to those with

a healthy BMI. Long-term follow-up studies have shown that obese breast cancer patients also develop metastatic disease more quickly, with a higher incidence of distant recurrence, compared to patients with a healthy BMI. Additionally, a retrospective analysis of 18,967 early-stage breast cancer patients found that obesity at the time of diagnosis was associated with a 46% increased risk of developing distant metastases within 10 years and a 38% higher risk of breast cancer mortality within 30 years, despite no correlation with locoregional recurrence<sup>25</sup>. A smaller retrospective study of 118 women who developed metastatic breast cancer suggested that obesity may influence the tropism of metastatic organs, with obese women presenting earlier spread to the lungs or liver<sup>26</sup>. Furthermore, obese women have a 34% higher risk of death from breast cancer<sup>27</sup>.

In our study, the BMI index, although not yielding a significant p-value (0.360) in the initial analysis, showed a high prevalence of overweight women (45.05%) and obese women (34.06%). Obese breast cancer patients, regardless of age or menopausal status, have significantly worse overall and breast cancer-specific survival compared to those with a healthy BMI<sup>25,27</sup>.

Our findings regarding the recurrence parameter and salivary NOx levels were evidenced in overweight women. Given the above about the role of excess weight on various clinical-pathological parameters and the diverse contradictory information regarding the role of NO and its metabolites on carcinogenesis, this leads us to suggest new studies that reveal the importance of this relationship and its consequences. Given that the application of saliva as a diagnostic method is not yet routinely used in breast cancer patient diagnosis, the expectation is that these findings will provide valuable information for healthcare professionals in the

treatment of these women, especially as a non-invasive and easy-to-apply option for early disease diagnosis and monitoring.

Early detection of breast cancer has the potential for less invasive treatments, such as smaller surgeries and reduced need for radiation or chemotherapy, leading to better survival rates. However, conventional screening methods, such as physical exams and mammography, have limitations in sensitivity and specificity. As a result, there is a growing need for innovative therapeutic approaches and reliable biomarkers that enable early, non-invasive breast cancer detection. Saliva, as a non-invasive detection method, could be a promising test for the early diagnosis of breast cancer, potentially helping to identify patients requiring closer monitoring and additional imaging exams while minimizing unnecessary biopsies, all in a cost-effective manner<sup>28,29</sup>.

However, the study faced several limitations, such as the sample size, especially when categorized by BMI, which hindered the analysis of several parameters. The volume of some saliva samples was also a factor preventing their use. At the same time, the study adopted a cross-sectional-observational design, limiting the acquisition of complete patient histories, as well as the lack of recorded data for the analyzed parameters.

#### 5. CONCLUSION

The analysis of salivary NOx, for has shown a potential link to cancer disease recurrence, which could represent a significant advancement in clinical practice. New research approaches should be pursued following studies like this, in the search for reliable biomarkers.

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## **ANEXOS** Anexo 1. Submissão

## Submission Confirmation

### Thank you for your submission

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#### Manuscript ID

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#### Title

Salivary Nitric Oxide Metabolites as a Biomarker for Disease Recurrence in Obese Patients with Breast Cancer.

#### Authors

Antoniolli, Paula Bellandi, Gabriela Almeida, Rafaella Vicenzi, Ricardo Berny, Maria Paula Przendziuk, Geisiane da Silva, Victor Rech, Daniel Panis, Carolina Kawassaki, Aedra

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20-Feb-2025

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## Anexo 2. Normas da Revista Eisntein

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Provide at least 5 and no more than 10 keywords, in English reflecting the content of the paper. Keywords must be based on the Medical Subject Headings (MeSH) of the National Library of Medicine, and available at <a href="https://www.ncbi.nlm.nih.gov/mesh/">https://www.ncbi.nlm.nih.gov/mesh/</a>

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## Acknowledgements

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#### Articles from electronic journals

Moniz MH, Low LK, Stout MJ. Intensive nurse home visiting program and adverse birth outcomes. JAMA. 2022;328(1):23-4.

Oliveira MM, Andrade KF, Lima GH, Rocha TC. Metformin versus glyburide in treatment and control of gestational diabetes mellitus: a systematic review with meta-analysis. einstein (São Paulo). 2022;20:eRW6155.

#### Books

Ritchie S. Science fictions: how fraud, bias, negligence, and hype undermine the search for trusth. New York: Metropolitan Books; 2020.

#### Chapters of books

Josephson CD, Strauss RG. Plasma transfusions. In: Behrman RE, Editor. Nelson textbook of pediatrics. 21st ed. Philadelphia (PA): Elsevier; c2020. p.2585-6.

#### Works presented in conferences

Rivarola E, Dimuro CA, Scandolo MC, Quintero Florez A. Design of gourmet menus high in fiber for diabetic patients of the French sanatorium: evaluation of the nutritional content, acceptability, organoleptic characteristics and glycemic control. Clinical Nutrition ESPEN. 2021;46:S690. [ESPEN 2021 Virtual Congress; 2021 Sep 9-14].

#### Thesis

Pinheiro LL. Avaliação da aorta torácica de brasileiros tabagistas por tomografia de tórax de baixa dose: diâmetros e prevalência de aneurismas [tese]. São Paulo: Faculdade Israelita de Ciências da Saúde Albert Einstein; 2021.

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