ANALYSIS OF THE EFFICACY OF SALIVARY TOTAL ANTIOXIDANT CAPACITY AS A POTENTIAL BIOMARKER FOR THE ASSESSMENT OF RISK AND PROGNOSIS OF BREAST CANCER IN PRE- AND POST-MENOPAUSAL WOMEN- A COMPARATIVE STUDY

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Abstract

Background – Breast cancer is a disease that affects women at an alarming rate, especially in the Indian subcontinent regardless of age, education level, or socioeconomic standing. Saliva is a fluid that is enriched in tumour markers in cancer patients including those types of cancer which are located outside the oral cavity, thus supporting the idea that saliva is a potential novel liquid biopsy. Miller et al. (1993) developed the TAC (Total Antioxidant Capacity) test to measure the total antioxidant status. The main benefit is that it allows you to measure not only the antioxidant capacity of a single chemical, but also the antioxidant capacity of all antioxidants in a biological sample, which makes it a novel and appropriate method with potential to be used in cancer diagnostics.

Objective - To evaluate the efficacy of salivary total antioxidant capacity as a potential biomarker for the risk assessment and prognosis of Breast Cancer in pre- and post-menopausal women

Methodology – In this study, 40 female participants were involved. They were majorly divided upon their menopausal status as Pre- and Post-menopausal women and were then sub-divided into 10 groups with 5 under each major group. There were 4 participants in each group. Among the 40, 32 were Breast cancer patients who were divided based upon their histopathological grade and menopausal status and 8 were healthy controls and were divided based upon their menopausal status. The samples were recruited from Department of Oncology, SRM Medical College, Kattankulathur and the analysis was done in the Department of Oral and Maxillofacial Pathology and Microbiology, SRM Dental College, Ramapuram. After informing the participants regarding the study, consent form was obtained from the patient. Detailed history was taken and histopathological grading were assessed before obtaining unstimulatory salivary samples from each patient. The samples were transported using an icebox within 2 hours. The samples were centrifuged and the resultant supernatants were separated, aliquoted, and kept in a -80°C freezer until analysis. Salivary TAC was measured using ELISA – FRAP [Enzyme linked Immunosorbent Assay – Ferric Reducing Antioxidant Power Assay] analysis.Shapiro-Wilk test was used to determine the samples' normality, and the results showed that the samples were normal. This led to the implementation of parametric statistical tests – independent student t tests and one-way ANOVA [Analysis of Variance] using SPSS [Statistical Package for Social Sciences] software version 22.

Results - On comparison within the Premenopausal group and within Postmenopausal group showed statistical significance respectively. The comparison among Pre-menopausal vs Post-menopausal women between subgroups showed no statistical significance.Salivary TAC levels were seen in the pre- and post-menopausal groups, respectively, at various histological cancer stages decreased with each grade of malignancy—Grade I >Grade II > Grade III and increased in the treatment group.

Conclusion – Salivary TAC levels can be used as a prognostic marker for Breast Cancer especially to predict whether the disease gets worsened or improved during course of treatment.

Keywords: Breast Cancer, Salivary Total Antioxidant Capacity, saliva, ELISA -FRAP

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1. Introduction

With the incidence and mortality of cancer increasing at an alarming rate around the world, with developing nations leading the way, Breast cancer (CA) is a malady that especially has adisturbing rate of occurrence in women, particularly in the Indian subcontinent. According to statistics, there are annually between 527,624 and 1,671,149 new instances of cervical and breast cancer, respectively. Every year, India adds roughly 122,844 instances of cervical cancer and 144,937 cases of breast cancer to this [Pati et.al.,2013]. It affects women irrespective of their age, educational or socio-economic status.Malignancy detection at an advanced stage is associated with a high fatality rate in India [Gupta et.al.,2015; Pati et.al.,2013]. One of the key reasons for late detection is a lack of understanding of signs and symptoms, which is exacerbated by burdensome referral pathways for diagnosis, a lack of suitable regional treatment centres, incomplete treatment due to costly out-of-pocket expenses, and many socioeconomic, geographical, and cultural barriers connected [Ali et.al., 2008; Sathwara et.al.,2017].It is this lacuna that the current study is attempting to fill by using a simple, non-invasive method of risk and prognosis prediction of breast cancer which would encourage the women to undergo frequent periodic testing without any discomfort.Thus, the quest for an entirely non-invasive, painless method of prognostic approach paved the way for this study which proposes to use the saliva of the affected patients as the non-invasive prognostic aid.

The majority of clinical chemistry tests available today are based on outdated technology, and these tests are neither sensitive nor specific for any given illness, and conventional indicators only grow dramatically after a large degree of disease impairment. As a result, more sensitive disease markers are desperately needed, especially for early illness identification; extremely sensitive and specific biomarkers as primary indicators are far more beneficial [Yu et.al.,2006; Wang et.al.,2012].The proposed study intends to combine the advantage of the non-invasive painless saliva coupled with a highly sensitive biomarker for breast CA - salivary Total Antioxidant Capacity (TAC) aiming to evaluate the relationship between the salivary TAC and the pathology of breast cancer.

TAC is a measure of the number of free radicals sc avenged by a test solution and is used as a sign of s aliva's total antioxidant capacity [Battino et.al.,2002].Hence, analysis of the antioxidants will provide an excellent means to evaluate the disease status of an individual, thus serving as a potent diagnostic and prognostic biomarker.

2. Materials and Methods

This work was approved by the Institutional Review Board and the SRM University Ethical Committee. Study samples were recruited from Department of Oncology, SRM Medical College Hospital and Research Centre, Kattankulathur.

In this study, 40 patients were enrolled and were divided into 10 groups. A total of 32 patients had breast cancer, with four in each of the following groups: Grade I, Grade II, Grade III, completed or receiving therapy respectively in pre-menopausal and post-menopausal women. Of the remaining 8 patients were healthy controls [4 premenopausal and 4 postmenopausal].

The Inclusion criteria for the healthy controls were based on menopausal status – Pre- and Post-menopausal and for the study group menopausal status of the patient, Breast cancer diagnosis and Histopathological grading were important. For the groups V and X if the patient had completed their treatment, the treatment must have been completed two months back or the patient must have been in the last phase of their treatment.

The exclusion criterion for the healthy controls was that the subjects with medically diagnosed disease or condition and for the study group subjects with any other co-morbidity and patients with breast cancer showing oral metastasis.

Subjects were enrolled in the investigation and allocated to the appropriate group after obtaining their medical history, age, menstrual status, histopathological findings (only in the study group), current stage of therapy and informed consent. The study population consisted exclusively of patients who had been graded and histopathologically verified.

Before the research began, the aim was conveyed to each patient, and signed informed consent was obtained.Saliva samples were obtained from chosen participants relevant to the groups for further analysis.

Method of Collection of Salivary Sample and Storage

After instructing the individuals to rinse their mouths with water, unstimulated saliva was obtained. A single, sterile salivary uricup was provided to every subject and they expelled 5 ml of saliva into it without any stimuli. The participants were told to incline their heads forward and expectorate their saliva into the container. The samples were subsequently labelled, packed in an ice box, and transferred from SRM Medical

College in Kattankulathur to the Department of Oral and Maxillofacial Pathology and Microbiology at SRM Dental College in Ramapuram within 2 hours. The samples were then transferred to 15ml centrifuge tubes and were centrifuged to remove cell debris. The centrifuge was done at 5000 rpm for 10 minutes at - 4°C.The resultant supernatants were separated, aliquoted, and kept in a -80°C freezer until Salivary TAC was measured using ELISA [Enzyme Linked Immunosorbent Assay]. Each sample was limited to a single freeze-thaw cycle.

Investigational Product Management

To avoid mix up of the samples – Each sample was labelled with the patient's number and date of collection.

Prior to sample Collection - Rinsing mouth with water to remove food residue and waiting at least 10 minutes after rinsing to avoid sample dilution before collecting saliva.

To prevent contamination of saliva samples, the following steps was employed

1. Only single-use materials were used for sample collection/transfer to prevent possible contamination between research subjects.
2. While collecting and transferring the samples gloves was worn to avoid touching collection device materials and samples.
3. The swab or brushes was not touched with fingers.

Investigational Product Accountability

The investigational products were solely used only for the purpose of this study. Post examination the remaining saliva samples were discarded.

Method of estimation of Salivary Total Antioxidant Capacity

Total Antioxidant Capacity for salivary samples in Breast cancer patients was measured using Total Antioxidant Capacity (T-AOC) Colorimetric Assay Kit using FRAP[Ferric Reducing Antioxidant Power Assay] method in ELISAkit from Elabscience.

Detection Principle

Fe3+-TPZ [Ferric tripyridyltriazine] can be reduced by antioxidants and produce blue Fe2+-TPTZ [ferrous-tripyridyltriazine] under acid condition. The antioxidant capacity of sample can be calculated by detection of the absorbance value at 593nm.

Prior to usage, all reagents and samples were brought to room temperature.

Reagent Preparation

The needed amount of FRAP working solution was prepared according to the ratio of reagent 1: reagent 2: reagent 3 = 10:1:1. The reagents were mixed fully and stored with shading light. Fresh solutions were prepared just before use. Then 100nM of$FeSO_4$[Ferrous sulfate]was prepared by weighing 27.8mg of reagent 4 accurately and dissolving it in 1 mL of double distilled water. The solution has to be freshly prepared prior to use. The standard was prepared by diluting 100mmol/L $FeSO_4$ solution with distilled water to a serial concentration which were: 0,0.3,0.6,0.9,1.2, 1.8mmol/L.

Assay Procedure

All reagents and samples were brought to room temperature before use.The samples were thawed before the assay. All the reagents were mixed thoroughly by gently swirling before pipetting and to avoid foaming. The measurement of the samples was done by taking 5 µl of standard solutionwith different concentrations to the standard wells and 5µl of samples were added to sample wells. 180µl of FRAP working solution was added to standard as well as sample wells. They were then incubated at 37°C for 3-5 min, then OD [Optical Density] values of each well were measured with microplate reader at 593nm.

Statistical analysis

Comparisons were made both within and between the groups.The data were statistically analyzed using SPSS [Statistical Package for Social Sciences] software version 22.For each group, descriptive statistics like mean, standard deviation were determined for all parameters (Refer Table 1).Shapiro-Wilk test was used to determine the samples' normality, and the results showed that the samples were normal. This led to the implementation of parametric statistical tests.Statistics were compared between the pre- and post-menopausal groups using independent student t tests, respectively.One-way ANOVA [Analysis of Variance]
was applied to analyse data within the groups.

4. Results

On comparison within the Premenopausal group it showed statistical significance (Table 2). Similarly, on comparison within Postmenopausal group also showed statistical significance (Table 3).

The table4 shows the mean ± SD [Standard Deviation] and result of independent sample t-test of TAC levels between the sub-groups[Premenopausal Control vs Postmenopausal Control, Premenopausal Grade I Vs Postmenopausal Grade I, Premenopausal Grade II

vs Postmenopausal Grade II, Premenopausal Grade III vs Postmenopausal Grade III, Premenopausal undergoing/completed treatment vs Postmenopausal undergoing/completed treatment]. And the result of comparison among Pre-menopausal vs Post-menopausal women between subgroups showed no statistical significance

5. Discussion

Breast cancer accounted for one-fourth of all female cancer cases diagnosed worldwide in 2018, totalling 2.1 million cases [Valko et.al 2006]. It is still difficult to identify at-risk individuals who do not have obvious warning risk indicators.Human cancer formation is a complicated process involving cellular and molecular alterations driven by a variety of endogenous and external stimuli.Cells must, however, repair the damages when they are damaged or injured. In that case, the cell may go through dedifferentiation to develop into a primitive stage and repair the tissue [Ramadoss R et.al., 2021]. The development of cancer is known to be triggered by oxidative DNA damage [Valko et.al., 2004]. The balance between pro-oxidant/antioxidant reactions in living things is disrupted during oxidative stress, which is brought on by metabolic processes that need oxygen [Jayasekharan V. P., et al., 2014].Oxidative Stress may have an effect on several stages of cancer development [Jomova and Valko, 2011]. In the initiation stage, it can modify the DNA [Deoxyribonucleic acid] sequence; in the promotion stage, it may boost cell division and decrease apoptosis; and in the progression stage, it can encourage further DNA changes [Reuter et.al.,2010].A recognized paradigm of pathogenesis for premalignant oral diseases, according to Nandakumar et al., 2020, is oxidative stress (PMODs). Additionally, they cited a past research by Volkova et al., 2012that focused on identifying oxidative stress markers in leukoplakia, erythroplakia, and lichen planus. They came to the conclusion that a lack of antioxidant status and an increase in lipo-peroxidation were the established causes of PMODs.

Total antioxidant capacity has the benefit of assessing all known and undiscovered antioxidants while taking their synergistic interactions into account, allowing for an objective assessment of the antioxidant potential in a given sample [Ghiselli et.al., 2000]. Enzymes like catalase, glutathione peroxidase, and superoxide dismutase are measured, as well as macromolecules such as albumin, ferritin, ceruloplasmin and tiny molecules like ascorbic acid, reduced glutathione, α-tocopherol,uric acid, β- carotene, bilirubin were all measured using TAC.[Bartosz, 2003]. An effort to repair the tissue damage brought on by the antioxidant species' depletion may result in a lower TAC level [Gupta et.al., 2012]. On the other side, a higher number of antioxidants may be seen as a preventive step to shield the bodily cells from the disease's attack [Ray et.al., 2000].

Saliva as an alternative tool to enhance medical evaluations has drawn increasing attention during the past several years [Streckfus et.al., 2005; Porto-Mascarenhaset.al.,2017]. In this regard, saliva sampling is the best option for the diagnosis of breast cancer because it is non-invasive, safe and reasonably easy to carry out. This makes it feasible to utilize saliva samples to monitor a patient's clinical status and identify potential systemic disorders since the study of salivary biomarkers gives additional benefits that represent the physiological circumstances of the body [Liu et.al., 2012].

All of these criteria convinced us that salivary total antioxidant would make the best biomarker for our investigation. In the assessment of other malignancies, such as head and neck cancer, brain tumours, salivary TAC have been explored. But the overall effectiveness in the context of breast cancer is yet unknown.

As seen in the Chart 1, the Salivary TAC levels were lower in the Premenopausal control group and the Salivary TAC levels are greater in postmenopausal women, as per Chart 2. This is accordance with the study done by Limberaki et. al., According to this study, greater serum antioxidant capacity was seen in middle-aged and elderly patients compared to younger subjects. The findings revealed that oxidative stress in young individuals is independent of their present stress (the majority of whom were dentistry students) and may be explained by their food and daily routine. Young individuals, and students in particular, are under biological and mental stress. High cortisol levels may result from this, which is another factor that might contribute to the decline of antioxidants.Elderly individuals eat healthier than younger people. Although the exact origin of this notion is unknown, it is hypothesized that these middle-aged and older participants' good health and dietary habits may contribute to their well-balanced antioxidant capacity [Limberaki et.al., 2012].

Salivary Total Antioxidant levels also decrease with each cancer grade in breast cancer patients while increased in those who are received treatment or at the conclusion of their course of treatment in both pre and postmenopausal group [Chart 1 and 2].Increased blood levels of total antioxidant status are linked to lower risks of breast cancer, according to a research by Ching S et al.,2002 which is

consistent with the findings of the present investigation.

Salivary TAC levels varied amongst the various groups with statistically significant increases and decreases. Studies done in the past focused serum TAC levels as an additional marker rather than just serum TAC in cancers alone except for few papers. Additionally, there are no papers in the literature that contrast healthy individuals' salivary total antioxidant capacity with various histopathological cancer grades and a therapy group. This gives our study the advantage of showing that the Salivary TAC might be employed as a possible prognostic marker in the case of treating breast cancer.

The findings of an independent T test that compared each group of premenopausal women to each group of postmenopausal women were statistically insignificant. There is a plethora of potential reasons for it. The first one may have had significant findings since our sample size in each group was little (n = 4), however when sample size could be extended to a larger size, there could be a chance. Age-related variables may be the following one. Age has a part in changes in salivary TAC levels, as can be observed above based on the research. Therefore, we were comparing two completely distinct age groups, although with a lower sample size this time. Both of which may have contributed to the possibility of an insignificant data.

Furthermore, similar changes in salivary TAC levels were seen in the pre- and post-menopausal groups, respectively, at various histological cancer stages. It decreased with each grade of malignancy—Grade I >Grade II > Grade III and increased in the treatment group. It has been demonstrated that salivary TAC levels decline as the disease worsens before rebounding once again in the last phase of treatment.

This might be used as an indicator to determine if the disease is getting worse or better. This confirms our fundamental theory, according to which, independent of the women's age or menopausal state, salivary TAC varies according to the various histological stages of breast cancer.

The outcomes of our study are consistent with those obtained utilising salivary TAC in various illnesses or tumours, according to some of the findings in the literature.An investigation by Bahar et al.,2007 revealed that patients with oral squamous cell carcinoma had lower salivary antioxidant capacity than controls. Despite the fact that antioxidants are the first line of defence against ROS [Reactive Oxygen Species] overproduction, Choromańska et al..2021 highlighted in their paper that the decreased levels of TAC, DPPH [2,2-diphenylpicrylhydrazyl] and FRAP clearly signal a reduced capacity to scavenge free radicals and, as a consequence, a lack of effective defence against oxidative stress in patients with adrenal tumours. Because of the compromised cellular metabolism that oxidative stress and damage may cause, these persons are more susceptible to them. In those with adrenal tumours, additional antioxidants may be taken into consideration. Omar ME and colleagues' research stated that the patients in their study have lower levels of oxidative stress and total blood antioxidant capacity. They asserted that oxidative stress has been linked to several anticancer drugs. It has been proposed that rise in oxidative stress may interfere with both processes, which might reduce the effectiveness of the therapy [Omar et.al., 2011].According to a study by Shetty et al., there was no statistically significant change between pre- and postoperative serum TAC levels despite the fact that the levels were significantly lower in oral cancer patients than they were in healthy individuals [Shetty et.al., 2015].

Limitations of our study

Even though the premenopausal and postmenopausal groups in this study did not vary statistically significantly from one another, the results may alter if the sample size is raised. Consequently, future study on the topic may make use of a larger population. Additionally, it would be more effective if the same groups were used to compare serum and salivary TAC levels.

We used FRAP analysis by ELISA to measure the levels of salivary TAC in our investigation. There are other methods that may be used, such the DPPH test. This test uses a stable 1,1-diphenyl-2-picrylhydrazyl free radical to measure the antiradical or radical scavenging activity [Gawron-Skarbek et.al., 2018]. Therefore, employing different approaches might lead to a difference in the levels. So, a comparison of all available techniques for estimating salivary total antioxidant capacity might be done. The oxygen radical absorbance capacity (ORAC) method is another method used by researchers to compare the efficiency of antioxidants to oxygen free radicals. The ability to evaluate compounds with delayed antioxidant effect as well as those without is one of this method's advantages[Wesolowski et.al., 2016].

6. Conclusion

Thus, using all the data from our research, we propose

- ✓ Salivary TAC levels in the breast cancer group are impacted by the progression of the disease.

- ✓ Salivary TAC levels increase in the later stages or following the conclusion of cancer therapy.
- ✓ Salivary TAC levels in individuals with breast cancer are unaffected by age or menopausal status. However, age-related changes in salivary TAC levels may occur in the pre- and post-menopausal control groups.
- ✓ Salivary TAC can be used to assess the progression status and prognosis of the breast cancer.
- ✓ In future, point-of-care testing like self-use-home-kits can be developed (similar to pregnancy home kits) using salivary TAC-based diagnosis of breast cancer.

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3) Any conflict of interest - The authors declare no potential conflicts of interest.

4) How the ethical issue was handled (name the ethical committee that approved the research)- The study was approved by the Institutional Ethics Committee of SRM Dental College, Chennai, India.

5) Authors contribution

- SR contributed to the design of the experimental study. SR carried out the sample collection and experiments related to the study, participation in writing and reference gathering of paper, results and data interpretation, plagiarism checking.
- MN contributed to the design of the experimental study, writing Data interpretation, supervised the experiments and results. Also contributed to reference gathering, paper revision and article management.
- RK supervised experiments and results. Also contributed to paper revision.
- DMVM contributed in patient selection and collection of clinical data.
- RR contributed assistance in sample collection and data interpretation.

All authors reviewed and approved the final manuscript for submission.

6) Availability of data (if apply to your research) - Not applicable

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Table -1: Descriptive Statistics for Salivary TAC in Pre and Postmenopausal group

Groups			**Sample Size (n)**	**Age Group (years)**	**Sex [Females]**	**Salivary TAC**	
						Mean	**Standard Deviation**
Pre-	**Control**		4	26 - 27	4	3.073	0.561
	Breast	**Grade I**	4	38-50	4	3.633	0.781
		Grade II	4	41-48	4	3.820	0.337
		Grade III	4	43-50	4	2.732	0.892

Menopausal Group	**Cancer**	**Completed / Undergoing treatment**	4	30-48	4	5.346	2.027
	Total		**20**	**26-50**	**20**	**3.720**	**1.338**
Post-Menopausal Group	**Control**		4	51-60	4	3.966	0.925
	Breast Cancer	**Grade I**	4	60-75	4	4.070	0.496
		Grade II	4	54-72	4	3.431	0.821
		Grade III	4	56-65	4	2.334	1.299
		Completed/ Undergoing treatment	4	50-66	4	4.180	0.541
	Total		**20**	**51-75**	20	**3.596**	**1.0400**

[n- number, TAC- Total Antioxidant Capacity]

Table 2 - Statistical Analysis for Salivary TAC in Pre-menopausal group using ANOVA

	Pre – Menopausal Group		**F**	**P**	**Significance**
Between Groups		**Control**	3.414	0.036	**Significant**
	Breast Cancer	**Grade I**			
		Grade II			
		Grade III			
		Undergoing/ Completed Treatment			
Within groups		**Control**			
	Breast Cancer	**Grade I**			
		Grade II			
		Grade III			
		Undergoing/ Completed Treatment			

Table 3 – Statistical Analysis for Salivary TAC in Post-menopausal group using ANOVA

	Post – Menopausal Group		**F**	**P**	**Significance**
Between Groups		**Control**	3.091	0.048	**Significant**
	Breast Cancer	**Grade I**			
		Grade II			
		Grade III			
		Undergoing/ Completed Treatment			
Within groups		**Control**			
	Breast	**Grade I**			

	Cancer	**Grade II**			
		Grade III			
		Undergoing/ Completed Treatment			

Table 4 - Independent t-test for comparison of Salivary TAC levels among Pre- menopausal vs Post-menopausal Women

Variable			**T-test equality of Means**		
		N	**Mean**	**Standard Deviation**	**P**
Control	**Premenopausal**	4	3.07325	.561938	0.150
	Postmenopausal	4	3.96600	.925484	0.161
Grade I	**Premenopausal**	4	3.63325	.780641	0.381
	Postmenopausal	4	4.07000	.496257	0.388
Grade II	**Premenopausal**	4	3.81975	.336692	0.415
	Postmenopausal	4	3.43100	.821319	0.431
Grade III	**Premenopausal**	4	2.73200	.891940	0.632
	Postmenopausal	4	2.33425	1.298312	0.634
Undergoing / Completed treatment	**Premenopausal**	4	5.34575	2.026549	0.309
	Postmenopausal	4	4.17975	.541126	0.338

Chart 1 – Mean Salivary TAC levels in Pre-menopausal group

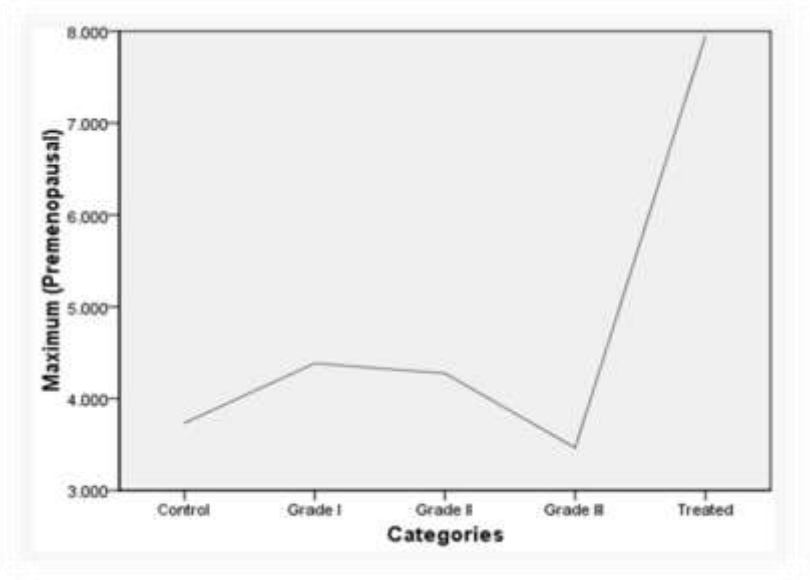
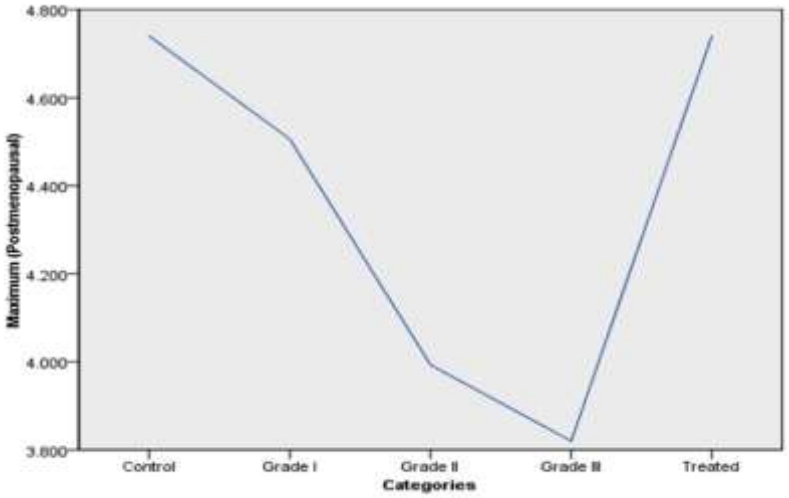


Chart 2 – Mean Salivary TAC levels in Post-menopausal group


4.800
4.600
4.400
4.200
4.000
3.800
Maximum (Postmenopausal)
Control
Grade I
Grade II
Grade III
Treated
Categories