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Does Serum Testosterone Levels in Postmenopausal Women Have An Effect on Developing Carcinoma Breast: A Case Control Study

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Abstract: *Large prospective cohort studies have shown that high levels of oestrogen in postmenopausal women increases mammary proliferation and is associated with an increased risk of developing breast carcinoma. However, there is conflicting evidence between the association of circulating androgen levels and breast carcinoma. Some studies show elevated levels of androgens (and oestrogens) to be associated with increased risk of breast cancer. Others show that androgens act with protective patterns. Finally, there are some studies that observe no association between serum concentrations of androgens and breast cancer risk.We aim to investigate whether the serum testosterone levels of postmenopausal women diagnosed to have carcinoma breast are lower than that of the women who do not manifest this diagnosis. This was a single centre case-control study conducted from January 2017 to June 2018. One-point blood samples were collected and tested for serum testosterone levels by Chemiluminescence method. Data was analysed with a confidence interval of 95% and power of study at 80%. A total of 118 patients were included in the study, 59 postmenopausal women newly diagnosed to have carcinoma breast and 59 control subjects. Ages of the study population ranged from 45 to 85 years, the mean age of patients in the case and control groups were 55.15 ± 8.4 years and 61.59 ± 8.7 years (p = 0.00). Serum testosterone levels in the case group were significantly lower by nearly 50 percent when compared to the control populations, mean levels were 0.079ng/ml in cases and 0.159ng/ml in the control group (p = 0.009).*

We conclude that higher testosterone levels may adopt a protective role by reducing the proliferative effects of oestrogens on the mammary tissue and by possibly inhibiting the development of carcinogenesis in mammary tissue.

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I. Introduction

Carcinoma breast has become the most common cancer amongst the female population, and accounts for almost 25% of all cancers globally. In India it is the leading cancer among women, accounting for 14% of all cancers in women. It carries an incidence of 25.8 per 100,000 women, and a mortality of 12.7 per 100,000 women. (1) In addition, India continues to have a low survival rate for women with carcinoma breast, with only 66.1% of the women diagnosed with the disease between 2010 and 2014 surviving. Thus, there is a need to better understand the disease and to identify risk factors that can help in its early detection and prevention. Worldwide, an estimated 10% of breast cancers is genetic or due to an inherited DNA mutation. The majority of cases are due to lifestyle factors, events related to reproductive life and factors that modify endogenous sex hormone levels. (2) Large prospective cohort studies have shown that high levels of oestrogen in postmenopausal women increases mammary proliferation and is associated with an increased risk of developing carcinoma breast. However, the correlation between circulating androgen levels and carcinoma breast has been debatable. Oestrogens stimulate breast development, whereas androgens such as testosterone inhibit breast development. (3) It is this balance between the stimulatory effects of oestrogen and the inhibitory effects of androgens that is critical in regulating mammary cell proliferation, both in normal and cancer tissues. (4) Androgen receptors are found, in abundance, in normal mammary epithelium as well as in a majority of carcinoma breast specimens. They are found with oestrogen and progesterone receptors within the epithelial cells. It is this co-expression of oestrogen and androgens that suggests that the effects of these hormone on breast tissue proliferation are integrated. (4) Studies have shown that there is a negative association between breast cell proliferation and levels of free testosterone in both pre- and postmenopausal women. It has also been demonstrated in animal models and cell cultures that androgens exert an anti-proliferative effect on the breast. However, the relationship between endogenous testosterone levels and carcinoma breast is still unclear, with most studies and experimental data providing conflicting results. (2)

Dimitrakakis et al. [(2)] have shown that endogenous bioavailable testosterone levels are lower in women with breast cancer as compared to relevant control women by using saliva as a sample source. Whilst taking 20ng/dl as the lower normal value of testosterone, there were more breast cancer patients with levels below normal values. They concluded that salivary testosterone levels are significantly lower in breast cancer patients compared to controls, and these differences are profound postmenopausal.

Table 1: Mean Levels of hormone measurements by group and according to menopausal status[(2)]

T	Controls (SD)	Cases (SD)	p-value
Pre	34.4 (16.1)	31.4 (15.3)	0.234
Post	31.0 (17.6)	25.1 (12.6)	0.002
Overall	32.2 (17.5)	27.2 (13.9)	<0.001

In a separate study, Dimitrakakis et al. [(5)] conducted a retrospective, observational study that followed 508 postmenopausal women receiving testosterone in addition to usual hormone therapy.They found that the incidence of breast cancer in testosterone users was substantially less than in women receiving oestrogen/progestin in the WHI study and in the Million-woman study. And in a randomized, double-blind, placebo-controlled study by Hofling et al. [(6)], testosterone use was found to inhibit exogenous oestrogen-induced breast tissue proliferation in 99 postmenopausal women (P<0.001). Farhat et al. [(7)] who conducted a study on the relationship between sex hormone levels and ER receptor status in breast cancers, found similar results. Their study showed that in postmenopausal women who were not taking hormone therapy, higher serum testosterone levels were associated with lower risk of ER-negative breast cancers.

Sieri et al. [(8)] who conducted a study on the association between serum sex hormone levels and breast cancer risk in postmenopausal women, provide confounding evidence. Through several lines of research, they showed that high levels of circulating testosterone were directly associated with breast cancer risk; and that high total testosterone levels were significantly associated with increased risk of ER-positive cancers, irrespective or Progesterone Receptor status. These are in consensus with findings by T Key, P Appleby, I Barnes and G Reeves who reanalysed nine prospective studies.

Lubet et al. [(9)] in a study on dimethylbenza(a)anthracene (DMBA)-induced mammary carcinoma in rats, reported that Dehydroepiandrosterone(DHEA) had an inhibitory effect on the development of mammary carcinoma. They showed that rats treated with implants of DHEA progressively inhibited the development of DMBA-induced mammary carcinoma.

A possible explanation for the increased levels of androgens in postmenopausal women with breast cancer is because of the adrenal hormone Androstenediol, also known as Hermaphrodol. This hormone is a weak Oestrogen Receptor agonist. In the presence of high oestrogen levels in premenopausal women androstenediol could exhibit anti-oestrogenic effects; whilst in the hypo-oestrogenic postmenopausal women the agonistic effect may predominate. [(4)] The aim of this study was to investigate whether the serum testosterone levels of postmenopausal women diagnosed to have carcinoma breast are lower than that of the women who have not been diagnosed to have carcinoma breast at our institute.

II. Material And Methods

This was a case-control study carried out on 118 patients admitted in the Department of General Surgery at Justice K S Hegde Charitable Hospital, Mangalore between January 2017 and June 2018.

Study Design: Case control study.

Study Setting: Justice K S Hegde Charitable Hospital, Derlakatte, Mangalore.

Study Period: From January 2017 to June 2018

Sample Size: Using EPI INFO STATCAL software with a Confidence Interval of 95% and a Power of 80% a sample size of 59 case subjects (postmenopausal women diagnosed to have Ca. Breast) and 59 control subjects (postmenopausal women without Ca. Breast) has been determined to be statistically significant. [(2)(8)]

Subjects & selection method: The case study subjects were defined as postmenopausal women who have been diagnosed to have carcinoma breast, and have not received treatment in any form (i.e., surgery, chemotherapy, radiotherapy). Control study subjects were defined as postmenopausal women who have no prior diagnosis of carcinoma breast or any other malignancy.

Definition of a Postmenopausal woman:

Study subjects will be considered to be postmenopausal by the following:

- All women above 55 years will be assumed to be postmenopausal
- Women between 45-55 years will be classified as postmenopausal if the plasma follicular stimulating hormone (FSH) level was greater than 50IU/L

- Women who reported having bilateral oophorectomy and have plasma FSH concentrations greater than 50IU/L

Inclusion Criteria for Case Subjects:
- Women over the age of 35 years who are determined to be postmenopausal by the above criteria.
- Carcinoma breast cases admitted in the Department of General Surgery at Justice K S Hegde Charitable Hospital, Mangalore.
- Patients who give consent and are willing to participate in the study.

Inclusion Criteria for Control Subjects:
- Women over the age of 35 years who are determined to be postmenopausal by the above criteria.
- Women who have no prior diagnosis of any malignancy.
- Women who are willing to give consent and partake in the study.

Exclusion Criteria:
- Women who have used postmenopausal hormone therapy (PHT) in the past 3 months.
- Women with any adrenal tumors or other primary neoplastic lesions.
- Women who are not willing to give consent for the study.

Data Collection: One-point bloodsamples were collected from both cases and control. Blood samples were tested for Testosterone and FSH levels by Chemiluminescence method, using Testosterone and FSH Immunoassay kits.

Statistical Analysis: The data acquired from the case and control groups, during the study period, will be subject to the following analysis:
- The descriptive statistics of both the case and control groups will be documented.
- The comparison of serum testosterone between the case and control groups will be done using the Student t-test.
- The association between the categorical variables will be tested by using the ANOVA Test.
- A P value <0.05 will be considered as statistically significant.

Ethical Considerations: There were no specific ethical concerns encountered during the study period. The study was approved and ethical clearance was granted by the Institutional Ethics Committee of K. S. Hegde Medical Academy.

III. Results

A total of 118 patients were included in the study cohort, 59 cases and 59 controls. Descriptive statistics such as mean, standard deviation and percentages were calculated. Inferential statistics like, independent sample t-test, one-way ANOVA were computed using SPSS version 20 (IBM SPASS statistics, IBM Corp., released 2011).

The study population of the two groups was first analysed by age.

TABLE 3: DISTRIBUTION OF THE SUBJECTS BY AGE

Age	Number of Cases	Number of Controls
45 – 54 years	31	11
55 – 64 years	21	28
65 – 74 years	4	14
>75 years	3	6
Total	**59**	**59**

TABLE 4: DISTRIBUTION OF THE SUBJECTS BASED ON MEAN AGE

	N	Minimum	Maximum	Mean	Std. Deviation
Cases	59	45	80	55.15	± 8.4
Controls	59	46	85	61.59	± 8.7

Between the cases and controls, the ages of the study population ranged from 45 to 85 years of age. The largest number of patients, in the cases group, was seen in the age group of 45 to 54 years, 31 patients (Table 3); whereas in the control group, the largest number of patients was seen between 55 – 64 years of age. The mean distribution of age amongst the case population was 55.15 ± 8.4 years of age, and in the control population it was 61.59 ± 8.7 years of age (Table 4; Fig. 6).

After applying the independent sample t-test between the cases and controls, the difference in ages between the two groups was found to be statistically significant, $p = 0.00$ (Table 11).

TABLE 5: DISTRIBUTION OF CASES BASED ON HISTOPATHOLOGY

		No. of Cases	Percentage
CASES	Invasive Carcinoma Breast	15	25.4
	Invasive Lobular Carcinoma	16	27.11
	Invasive Mammary Carcinoma	28	47.4
	Total	**59**	**100.0**

On analysis of the control subjects and the histopathological type of carcinoma breast, it was found that a majority of the patients were diagnosed to have Invasive Mammary Carcinoma, 28 patients (47.4%), followed by Invasive Lobular Carcinoma, 16 patients (27.11%), and Invasive Carcinoma Breast, 15 patients (25.4%) (Table 5; Fig. 7).

Where available, the immunohistochemistry of the case cohort was analysed. ER, PR and HER-2/neu receptor status was available for 45 out of the 59 patients.

TABLE 6: DISTRIBUTION OF THE CASES BASED ON ER

Receptor Status	No. of Cases	Percent
Positive	27	45.8
Negative	18	30.5
Not Available	14	23.7
Total	**59**	**100.0**

Based on the ER status, 27 patients were ER Positive (45.8%), 18 patients were ER Negative (30.5%) (Table 6; Fig. 8).

TABLE 7: DISTRIBUTION OF CASES BASED ON PR

Receptor Status	No. of Cases	Percent
Positive	21	35.6
Negative	24	40.7
Not Available	14	23.7
Total	**59**	**100.0**

Based on PR status, 24 patients (40.7%) showed PR Negative status, while 21 patients (35.6%) showed PR Positive status (Table 7; Fig. 9).

TABLE 8: DISTRIBUTION OF CASES BASED ON HER-2/neu

Receptor Status	No. of Cases	Percent
Positive	8	13.6
Equivocal	5	8.5
Negative	32	54.2
Not Available	14	23.7
Total	**59**	**100.0**

Based on HER-2/neu receptor status, 32 patients (54.2%) were HER-2/neu positive, 8 patients (13.6%) were HER-2/neu negative and the remaining 5 (8.5%) patients showed an equivocal result (Table 8; Fig. 10).

Upon comparing the testosterone levels between the case and control populations it was found that the testosterone levels were higher in the controls (0.159 ± 0.215), than the cases (0.079 ± 0.089) (Table 9; Fig. 11). After applying the independent sample t-test between the cases and controls, the difference in testosterone levels was found to be statistically significant, $p = 0.009$ (Table 10).

TABLE 9: DISTRIBUTION OF THE SUBJECTS BASED ON MEAN TESTOSTERONE LEVELS (ng/mL)

	N	Minimum	Maximum	Mean	Std. Deviation
Cases	59	0.025	0.41	0.079	0.089
Controls	59	0.025	0.89	0.159	0.215

TABLE 10: COMPARISON OF AGE, TESTOSTERONE (CASES AND CONTROLS) USING INDEPENDENT SAMPLE t-TEST

	Mean difference between cases and controls	t value	p value
Age	-6.44	-4.06	0.00*
Testosterone	-0.80	-2.6	0.009*

*significant

A sub analysis of the testosterone values amongst the control group was done using one-way ANOVA, and the values were compared.

TABLE 11: COMPARSION OF TESTOSTERONE VALUES (ng/mL) BETWEEN DIFFERENT HISTOPATHOLOGICAL SUBTYPES, USING ONE-WAY ANOVA

	N	Minimum	Maximum	Mean	Std. Deviation	F value	p value
Invasive Ca. Breast	15	0.025	0.234	0.062	0.070	2.32	0.10
Invasive Lobular Ca.	16	0.025	0.419	0.119	0.124		
Invasive Mammary Ca.	28	0.025	0.283	0.065	0.069		

When comparing the testosterone levels between the different histopathological types of carcinoma breast, it was found that serum testosterone levels were higher in patients with invasive lobular carcinoma (0.119 ± 0.124ng/mL) when compared to invasive mammary carcinoma (0.065 ± 0.069ng/mL), and invasive carcinoma breast (0.062 ± 0.07). However, the differences in serum testosterone levels was not found to be significant, *p value = 0.10* (Table 11; Fig. 12).

To analyse the testosterone levels based on the ER, PR and HER-2/neu receptor status of the case cohort, the patients were first sub grouped based on their receptor status (Table 13). It showed that the largest number of patients in the case group were of ER and PR positive and HER-2/neu negative receptor status (16 patients, 38.1%); while PR positive with ER and HER-2/neu negative receptor status had the fewest number of patients (2 patients, 4.8%) (Table 12).

TABLE 12: DISTRIBUTION OF CASES BASED ON DIFFERENT ER, PR, HER-2/NEU SUBGROUPS

	No. of Cases	Percent
ER Positive (PR, HER2/neu Negative)	8	19.0
PR Positive (ER, HER2/neu Negative)	2	4.8
ER, PR Positive (HER2/neu Negative)	16	38.1
ER, PR Negative (HER2/neu Negative)	8	19.0
HER2/neu Positive (+/- ER, PR Positive)	8	19.0

Using one-way ANOVA, the serum testosterone levels were compared between these subgroups. It showed that serum testosterone levels were highest within the HER-2/neu positive subset (0.13 ± 0.17ng/mL), and the lowest testosterone levels were observed within the ER positive, PR and HER-2/neu negative, subset (0.05 ± 0.05ng/mL) and ER, PR negative, and HER-2/neu negative, subset (0.05 ± 0.04ng/mL). The differences in the testosterone values, when analysed, were found to have no statistical significance, *p = 0.34* (Table 13; Fig.13).

TABLE 13: COMPARSION OF TESTOSTERONE VALUES (ng/mL) BETWEEN ER, PR, HER-2/NEU SUBGROUPS, USING ONE-WAY ANOVA

	N	Minimum	Maximum	Mean	Std. Deviation	F value	p value
ER Positive (PR, HER2/neu Negative)	8	0.03	0.16	0.05	0.05	1.14	0.34
PR Positive (ER, HER2/neu Negative)	2	0.11	0.13	0.12	0.02		
ER, PR Positive (HER2/neu Negative)	16	0.03	0.23	0.10	0.08		
ER, PR Negative (HER2/neu Negative)	8	0.03	0.14	0.05	0.04		
HER2/neu Positive (+/- ER, PR Positive)	8	0.03	0.42	0.13	0.17		

IV. Discussion

Breast cancer has complex aetiologies, comprising of a heterogeneous group of tumours that differ in clinical behaviours, response to therapy and outcome. However, several studies have established that the presence of endogenous sex steroids, clearly influences the progression of this disease.

The data of our study supports the hypothesis that serum testosterone levels will be reduced in postmenopausal women diagnosed with carcinoma breast, as compared to postmenopausal women of the normal population.

Our study has found a significant association between lowered endogenous serum testosterone levels in postmenopausal patients manifesting breast cancer as compared with controls. Testosterone levels were significantly higher in controls (0.159 0.215 ng/ml) as compared to cases (0.079 0.089 ng/ml). Independent sample t-test applied between cases and controls showed a significant difference, $p = 0.009$.

Independent sample t-test was applied between cases and controls for age, to ascertain that women in the two groups (cases and controls) matched the inclusion criteria - also showed statistically significant difference for age, $p = 0.00$.

The data on the association between androgen levels and risk of breast cancer is conflicting. However, our study is indicative of support of the "androgen-protection deficiency" hypothesis in breast cancer patients. These results can be explained by the established fact that estradiol is the final stimulator of breast epithelium proliferation and aromatase inhibitors facilitate the conversion of testosterone to estradiol. Increased expression of the androgen producing enzyme 5α-reductase is also well documented in breast cancer tissue as it catalyses the conversion of testosterone into its stronger and non-aromatizable dihydroxytestosterone (DHT). [(10)] Testosterone and DHT are the primary androgens in women because they both have a high binding affinity for the AR. Given that the binding affinity for steroids bound bysex hormone binding globulin (SHBG) is the highest for DHT followed by testosterone, their consequent conversion, can explain the lowered total T levels in the serum. [(11)]

In a review of literature supporting the protective role of androgens, Secreto et al. [(12)] state peripheral tissues as the site where conversion of adrenal DHEA to oestrogen and most of the active androgens takes place. They report of increased breast cancer risks being attributed to the progressive decline in production of DHEA with age and thus implicating postmenopausal women. [(2)]

These data add support to studies by Dimitrakakis et al. [(2)], who have demonstrated that saliva testosterone levels are lower in patients with Ca breast.Their study used salivary testosterone as immune assays for the measurement of salivary testosterone are more sensitive and reliable even at lowered concentrations. [(13)] Salivary testosterone assays demonstrate a close correlation with free testosterone levels in the serum and studies show that testosterone in its free or bioavailable testosterone is a more accurate predictor of androgenic effects than its form in which it is bound to SHGB.

Dimitrakakis et al. [(3)], suggest that the anti-proliferative effects of androgens on mammary tissue may occur either indirectly, via down-regulation of other receptors like PRs, or directly, through breast AR stimulation.

A study by RA Lobo [(14)] indicated that oral oestrogen treatments increases the production of SHBG in the liver and via suppressing of the luteinizing hormone, which consequently reduces the level of bioavailable androgens and results in the inhibition of ovarian androgen production. Thus conventional HT could promote breast cancer by increasing oestrogen levels and simultaneously decreasing the effect of androgens.

In a separate study of 508 postmenopausal women Dimitrakakis, Jones and Bondy [(15)] observed a decreased incidence of breast cancer in women who received exogenous testosterone in addition to their normal HRT. They speculate "unopposed" oestrogenic effects could be counteracted by the addition of androgens to both OCP and HRT.

Factors having impact on treatment options and prognosis for breast cancer include the immunohistochemical status of oestrogen receptors (ER), progesterone receptors (PR) and the human epidermal growth factor receptor 2 (HER-2/neu). Hence, an analysis of testosterone levels was done to investigate the associations between endogenous serum hormone testosterone levels with cancer subtypes.

Based on HER-2/neu, in cases, our study found that 32 (54.2%) had negative; 8(13.6%) had positive; 5(8.5%) had equivocal and it was not available for 14(23.7%) cases (Table 8).

The distribution of the hormonal receptor expression amongst the cases in this case-control study is in consensus with studies done by Ambriose et al. [(16)] in southern India, where our study was undertaken. They found "the hormonal receptor expression to be lower in the Indian population as compared to the West". In alignment with their findings, our study also found that a significant proportion of tumours with HER-2/neu overexpression also showed ER and PR positivity.

Furthermore, the trend of data from our study also aligns with that of research by Rajan et al. [(17)] who found 47.4% of postmenopausal patients to be ER positive whilst PR positivity was 34.7%. To conclude, ER and PR positivity was in 57% and 42% of cases in our study with the percent of ER positive being more than PR positive (Table 12).

Implications of this study: Postmenopausal hormone therapy, as well as, other oestrogen therapies such oral contraceptives are proven to increase the risk of carcinoma breast. This is because the normal oestrogen-androgen balance is disrupted and promotes the unopposed oestrogenic stimulation of mammary epithelial proliferation, thus potentially increasing the risk of developing breast cancer. This is due to the suppression of gonadotropins by exogenous oestrogen treatment, which causes suppression of ovarian steroidogenesis, resulting in decreased production of both oestrogen and androgens. Since only oestrogen is being supplemented in these hormone therapies, it's action on mammary epithelial cells is unopposed leading to proliferation of these cells and hence increasing the risk of the development of breast cancer. In addition, oral oestrogen supplementation stimulates hepatic production of SHBG which binds to testosterone with a high affinity, thus reducing its bioavailability. By these dual effects testosterone bioavailability is greatly reduced in women on HRT or taking OCP's. [(3)]

If androgens do indeed exhibit a protective action against oestrogen induced mammary proliferation, it's addition to oestrogen replacement therapies in physiological doses could protect the breast from unopposed oestrogenic effects.

It has also been shown that testosterone levels are a marker of hormone-dependent breast cancers and that the contemporary evaluation of ER status, AR expression, and circulating testosterone levels may identify different subsets of cancers whose growth may be influenced by androgens. [(12)]

V. Conclusion

From our data we conclude that this study supports our hypothesis. Serum testosterone levels were found to be significantly lower in postmenopausal women first diagnosed with carcinoma breast, when compared to postmenopausal women without carcinoma breast.

We can conclude that higher bioavailable testosterone may possibly reduce the proliferative effects of oestrogens on mammary tissue, and thus adopt a protective role by either inhibiting the development of carcinogenesis or tumour growth.

Limitation of Study: This study has a few limitations.

- Whilst this data adds to the literature that supports postmenopausal exogenous testosterone therapy administered to women who have low testosterone levels, no rapid, simple assay of testosterone levels has been shown to produce reliable results in women with low to normal serum testosterone levels.
- Hormone levels, such as testosterone, show substantial daily variability due to diurnal rhythm and can thus lead to inaccuracies during a study. As such our samples were all collected between 6AM and 9AM, to standardize sampling time.
- Most androgenic activity in women originates from the peripheral conversion of precursors such as DHEA into androgens within the cells or target tissues, as such this activity will not be detected by the measurement of circulating androgens.
- Most circulating testosterone is tightly bound to SHBG, while only the free hormone is bioactive; thus total testosterone levels vary widely based on genetic metabolic and endocrine influences.
- Finally, oestrogen is considered a strong risk factor for breast cancer. To draw any conclusions about an association between testosterone and breast cancer, a statistical method to adjust for the oestrogen effect must be employed when measuring serum testosterone.

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