

## RESEARCH COMMUNICATION

**Evaluation of Plasma Non-enzymatic Antioxidants in Breast Cancer Etiology**

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**Abstract**

**Objective:** Oxidative stress has emerged as a major etiological factor for breast cancer. Diet derived antioxidants play an important role against oxidative stress and the aim of the present study was to examine roles of non-enzymatic antioxidants in breast cancer in India. **Methods:** Plasma non-enzymatic antioxidants; beta-carotene, vitamin A, vitamin E and vitamin C were analyzed spectrophotometrically from 70 healthy female controls, 30 patients with benign breast diseases (BBD) and 125 untreated breast cancer patients (BCPT). **Results:** Plasma vitamin C levels were significantly lower in patients with BBD as compared to the controls ( $p=0.043$ ). Plasma beta-carotene, vitamin E and vitamin C levels were significantly lower in BCPT as compared to the controls ( $p=0.0001$ ,  $p=0.040$  and  $p=0.0001$ , respectively). Plasma vitamin A levels were significantly higher in patients with BBD and BCPT as compared to the controls ( $p=0.0001$  and  $p=0.0001$ ; respectively) and in BCPT as compared to patients with BBD ( $p=0.030$ ). ROC curve analysis revealed that plasma beta-carotene and vitamin A could significantly discriminate between controls and patients with BBD ( $p=0.016$  and  $p=0.000$ ; respectively). Plasma beta-carotene, vitamin A, vitamin E and vitamin C could significantly discriminate between controls and BCPT ( $p=0.000$ ,  $p=0.000$ ,  $p=0.001$  and  $p=0.001$ , respectively). Plasma vitamin E levels could significantly discriminate between patients with BBD and BCPT ( $p=0.055$ ). Odds ratio analysis revealed that, increasing levels of plasma beta-carotene, vitamin E and vitamin C were significantly associated with decreased risk of breast cancer ( $p=0.0001$ ,  $p=0.003$ , and  $p=0.0001$ ; respectively), whereas, increased risk was linked to plasma vitamin A ( $p=0.001$ ). **Conclusions:** The trends of the current study provide interesting clues to the etiology of breast cancer and suggest significance of interplay of non-enzymatic antioxidants in breast cancer. Further in-depth study is warranted to elucidate role of these antioxidants as a preventive measure.

**Key Words:** Breast cancer - antioxidants - vitamins - beta-carotene

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**Introduction**

Breast cancer is the leading female malignancy with varying morbidity and mortality globally. In India, increasing trend for carcinoma of breast is observed in females during the last few years (Bagchi 2008; Yeole 2008, ). The major influences on breast cancer risk appear to be certain reproductive factors, body size/obesity, alcohol, physical activity, exogenous hormones (oral contraceptives, hormone replacement therapy), and, possibly, diet. However, the varying rates of breast cancer signify that environment and lifestyle factors are important culprit for breast cancer (Parkin 2005). Evidence indicates that oxidative stress (OS) resulting from a little imbalance between oxidants and antioxidants has strong linkage with breast cancer risk (Ambrosone 2000). OS contributes to multistep carcinogenesis by several distinct mechanisms including direct damage to DNA, alterations in intracellular signal transduction pathway leading to

abnormal cellular growth and forcing damaged or initiated cells to undergo progression (Klaunig and Kamendulis, 2004). Cells are endowed with an antioxidative defense system, consisting of a variety of enzymatic and non-enzymatic antioxidants to combat oxidative insults thereby protecting cellular macromolecules from detrimental effects of OS.

Among these antioxidants, dietary antioxidants (e.g. b-carotene, vitamin A, vitamin E and vitamin C) have been associated with altered cancer risk. Antioxidative vitamins have a number of biological activities such as immune stimulation, inhibition of nitrosamine formation and alterations of metabolic activation of carcinogens (Ray and Husain 2002). It is documented that, vitamin A and b-carotene have several potentially anticarcinogenic properties in mammary cancer cell lines including effects on proliferation, differentiation and apoptosis (Prakash et al, 2000). Vitamin E is the major chain breaking antioxidant, which inhibits carcinogenesis primarily

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through its antioxidant activity (Helliwell, 1994). Vitamin C acts as antioxidant by scavenging physiologically relevant reactive oxygen species. Vitamin C can also regenerate other small molecule antioxidants, such as vitamin E, glutathione, urate and b-carotene from their respective radical species (Padayatty et al, 2003). Various investigators have reported importance of dietary intake of these antioxidants and their role as anticarcinogenic agents in various malignancies (Divisi et al, 2006; Jenab et al, 2006; Weinstein et al, 2007). However, very few studies have explored circulating non-enzymatic antioxidants in breast cancer etiology (Simon et al, 2000; Wu et al, 2000; Ray and Husain, 2001; Ching et al, 2002; Sato et al, 2002; Tamimi et al, 2005; Sharhar et al, 2008).

Thus, a special attention is needed for these potential protective factors and to their levels in breast cancer. Therefore, aim of the present study was to analyze plasma levels of non-enzymatic antioxidant status in breast cancer patients (BCPT), patients with benign breast diseases (BBD) and healthy female individuals to determine their role in the etiology of breast cancer.

## Materials and Methods

### Subjects

Women referred for management of biopsy confirmed breast cancer at the out patients department of The Gujarat Cancer & Research Institute (GCRI) were recruited for the study. Eligible cases were aged between 25 to 82 years. Breast cancer cases ineligible for the study were as follows: pregnant women, women with multivitamin supplementation, women with lumpectomy done before, women who received alternative treatment and women having major illness in recent past. One hundred twenty five cases after diagnosis of breast cancer but before initiation of any treatment were enrolled and their clinical details were collected (Table 1). The majority (83.2%) had invasive ductal carcinomas. TNM staging was conducted with grouping into early (stages I and II) and advanced (Stage III and IV). Lymph node (LN) involvement was also assessed. Seventy healthy female controls and 30 patients with BBD were also included in the study. Informed consent was obtained from all the subjects. Fasting blood samples were also collected from the subjects in heparinized vacutettes.

### Plasma Analysis

The separated plasma was stored at light tight conditions at -20°C until analysis. The entire analyses of b-carotene, vitamin A and E were carried out in dim red light. Beta-carotene and vitamin A (Dugan et al, 1976), vitamin E (Baker and Frank, 1968) and vitamin C (Roe, 1961) were estimated by highly sensitive spectrophotometric methods.

### Statistical Analysis

Statistical analysis of the data was carried out with SPSS statistical software (version 10). Student's 't' test was used to compare mean levels. Receiver operating characteristic (ROC) curves were constructed to determine the discriminating efficacy of plasma non-enzymatic

**Table 1. Clinical Details for Breast Cancer Patients**

Mean Age (Range)	45 (25-82) Years
Mean BMI (Range)	22.30 (11.5-36.2)
Menopausal status	Pre-menopausal 44 Post-menopausal 73 Peri-menopausal 8
Histopathology:	Ductal Carcinoma In situ (DCIS) 4 Invasive ductal carcinoma (IDC) 104 Infiltrating DC (INFDC) 4 Infiltrating lobular carcinoma (ILC) 1 Others 12
Stage of the disease ( TNM)	Stage I 3 Stage II 57 Early (Stages I+II) 60 Stage III 61 Stage IV 4 Advanced (III+IV) 65
Lymph node involvement	Yes 52 No 41

antioxidant levels among the three categories of the subjects. Odds ratios (ORs) were calculated by logistic regression with 95% CI. Pearson's correlation analysis was carried out to find out the correlation of plasma non-enzymatic antioxidants in BCPT. A significant effect of variable was judged with a 'p' value  $\leq 0.05$ .

## Results

### Plasma levels of non-enzymatic antioxidants in controls, patients with BBD and breast cancer patients

Comparison of plasma non-enzymatic antioxidants between controls patients with BBD and BCPT is illustrated in Table 2. Mean levels of plasma beta-carotene were significantly lower in BCPT as compared to the controls and patients with BBD ( $p=0.0001$  and  $p=0.034$ ; respectively). Plasma vitamin A levels were significantly higher in patients with BBD and BCPT as compared to the controls ( $p=0.0001$  and  $p=0.0001$ ; respectively) and the levels were also elevated in BCPT as compared to patients with BBD ( $p=0.030$ ). Plasma vitamin E levels were significantly lower in BCPT ( $p=0.04$ ) as compared to the controls. Plasma vitamin C levels were significantly lower in patients with BBD and BCPT as compared to the controls ( $p=0.043$  and  $p=0.0001$ ; respectively) and also lower in BCPT as compared to patients with BBD ( $p=0.045$ ).

### Plasma levels of non-enzymatic antioxidants relative to stage of disease and LN involvement

Table 3 depicts comparison of plasma levels of non-enzymatic antioxidants in patients with early and advanced stage of disease as well as patients without LN involvement and with LN involvement. Mean levels of plasma vitamin A were significantly lower in patients with advanced stage disease ( $p=0.05$ ). Vitamin E levels were significantly lower ( $p=0.033$ ) in patients with LN involvement.

### ROC Curve Analysis

Figure 1a/b/c documents ROC curve analysis of plasma beta-carotene, vitamin E and vitamin C between

$p=0.000$ ) between controls and breast cancer patients.

*Assessment of risk : odds ratio analysis for non-enzymatic antioxidants*

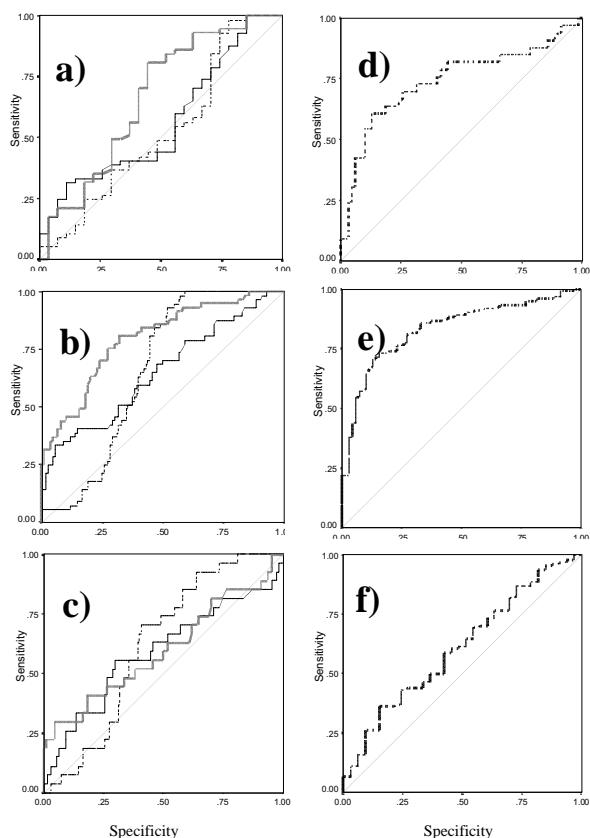
The OR for non-enzymatic antioxidants was calculated for breast cancer risk. The data obtained by comparison of the highest with the lowest quartiles were as follows: plasma b-carotene, 0.10 (C.I. 0.04-0.27); plasma vitamin A, 62.09 (C.I. 13.18-292); plasma vitamin E, 0.04 (C.I. 0.00-0.36); and plasma vitamin C, 0.17 (C.I. 0.07-0.42). The OR analysis revealed that higher quartile with higher levels of plasma vitamin A were significantly associated with increased risk of breast cancer ( $p=0.0001$ ). In contrast, higher quartiles with increasing levels of plasma b-carotene, vitamin E and vitamin C were significantly associated with decreased risk of breast cancer (Figure-2a). The lipid adjusted OR for lipid soluble vitamins shows the similar results: plasma b-carotene, 0.126 (C.I. 0.04-0.37); plasma vitamin A, 62.95 (C.I. 12.76-310.36) plasma vitamin E, 0.05 (C.I. 0.006-0.41) (Figure-2b).

*Correlation of antioxidants in breast cancer patients*

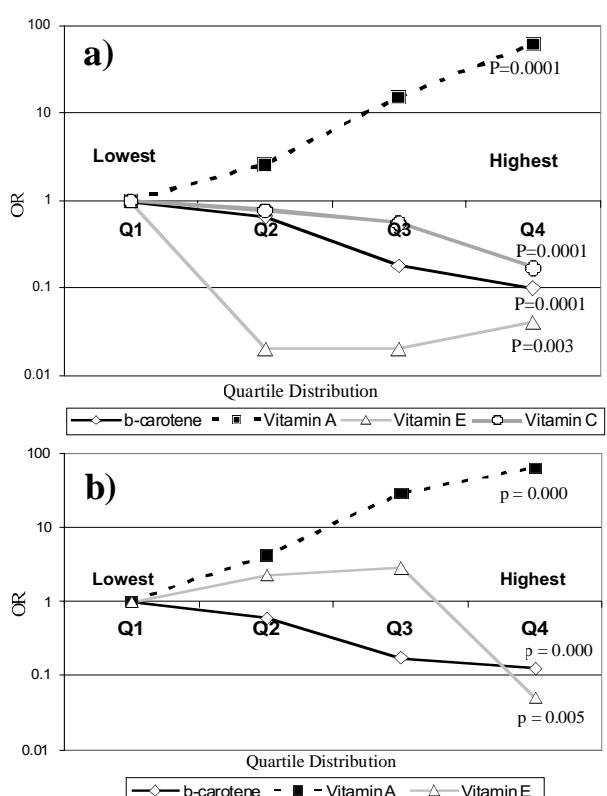
The correlation of plasma non-enzymatic antioxidants is shown in Table 4. Pearson's correlation documented that the alterations in plasma b-carotene levels showed significant positive association with plasma vitamin E ( $p=0.028$ ) and significant negative association with plasma vitamin A ( $p=0.000$ ). Alterations in plasma vitamin A levels showed significant positive association with plasma vitamin C ( $p=0.000$ ) and significant negative association with plasma vitamin E ( $p=0.000$ ) and

Parameter	b-Carotene	Vitamin A	Vitamin E	Vitamin C
Controls	2.34±0.14	0.53±0.04	15.1±1.22	3.54±0.36
BBD	1.87±0.24	1.06±0.13	14.1±1.54	2.57±0.30
Cancer	1.32±0.05	1.41±0.08	12.6±1.03	1.90±0.10

different groups. Plasma b-carotene could significantly discriminate between controls and patients with BBD (AUC=0.663,  $P=0.016$ ) (Figure 1a). It was also found that mean levels of plasma b-carotene (AUC=0.795,  $p=0.00$ ), vitamin E (AUC=0.656,  $p=0.001$ ) and vitamin C (AUC=0.652,  $p=0.001$ ) could significantly distinguish between controls and BCPT (Figure-1b). The mean plasma vitamin E (AUC=0.619,  $p=0.055$ ) could significantly discriminate between patients with BBD and BCPT (Figure -1c). Figure 1d/e/f shows ROC curves for plasma vitamin A between controls and patients with BBD, BCPT, and BBD and BCPT. It revealed that ROC curve for mean levels of plasma vitamin A (AUC=0.744,  $p=0.000$ ) could significantly discriminate between controls and patients with BBD as well as (AUC=0.834,



**Figure-1. ROC curves for nonenzymatic antioxidants.**  
a) beta-carotene; b) vitamin C; c) vitamin E; d) vitamin A with BBD; e) vitamin A with BCPT; f) vitamin A with BBD + BCPT



**Figure 2. Odds Ratio Analysis for Plasma Non-enzymatic Antioxidants. a) Non-adjusted; b) Lipid-adjusted**

**Table 4. Pearson's Correlation Coefficients (R) Between Plasma Non-enzymatic Antioxidants**

Parameter	Vitamin C		Vitamin E		Vitamin A	
	R	P value	R	P value	R	P value
b-carotene	0.030	NS	0.208	0.028	-0.040	<0.001
Vitamin A	0.325	<0.001	-0.468	<0.001		
Vitamin E	-0.28	0.002				

NS, Not significant

**Table 5. Multivariate Analysis between Non-enzymatic Antioxidants and Clinicopathological Parameters**

Parameters	b-Carotene		Vitamin A		Vitamin E		Vitamin C	
	F	P	F	P	F	P	F	P
Age	0.35	0.55	0.36	0.55	0.23	0.62	0.31	0.57
Menopausal	0.07	0.78	1.17	0.28	0.01	0.89	1.23	0.27
LN+	0.51	0.47	0.76	0.38	4.26	0.04	0.002	0.97
Early/adv.	0.43	0.51	3.99	<b>0.05</b>	0.20	0.65	0.20	0.65
BR score	0.77	0.38	0.02	0.87	5.98	<b>0.02</b>	2.90	<b>0.09</b>
N Grade	0.65	0.42	0.62	0.43	0.02	0.87	5.01	0.03

BR, Bloom-Richardson; N, nuclear

alterations in plasma vitamin E showed significant negative associated with vitamin C in BCPT ( $P = 0.002$ ).

#### *Multivariate analysis of non-enzymatic antioxidants to evaluate their association with clinicopathological features*

Multivariate analysis was performed to evaluate possible correlation of non-enzymatic antioxidants with clinicopathological features of breast cancer patients. The clinicopathological parameters included age, menopausal status, lymphnode involvement, early and advanced stage of the disease, BR score and nuclear grade of the tumor. The correlation of non-enzymatic antioxidants with clinicopathological features is documented in Table 5. Multivariate analysis revealed that alterations in vitamin E levels were significantly associated with lymph node involvement ( $F=4.26$ ,  $p=0.042$ ) and BR score ( $F=5.98$ ,  $p=0.01$ ). Vitamin C levels were significantly associated with tumor nuclear grade ( $F=5.01$ ,  $p=0.03$ ) and vitamin A levels with disease stage ( $F=3.99$ ,  $p=0.05$ ).

## Discussion

Assessment of serum/plasma levels of vitamins is important as, evaluation of serum/plasma micronutrient status is more reliable and biologically meaningful approach than dietary estimation of nutrition. Plasma levels reflect the dietary intake, absorption, utilization and other metabolic aspects including depletion of serum/plasma and tissue nutrients due to oxidative stress (Handelmann et al, 1996). Further, measurement of plasma non-enzymatic antioxidants b-carotene, vitamin E and vitamin C are considered as valid ways to measure exposure of dietary antioxidants and also called 'Nutritional Biomarkers' (Mayne, 2003). The biomarker serves as an integrated measure of metabolism of the nutrient of interest and can be used as a measure of internal dose, which is an indication of the amount of nutrients available to the tissues after absorption and metabolism (Potischman and Freudenheim, 2003). Thus, the advantage

of the present study of non-enzymatic antioxidant is that it did not rely on dietary recall or records but on direct measurement of plasma levels of antioxidants as it may give a more accurate approximation of the amount available to the target tissue than intake estimates.

In the present study, lower levels of plasma b-carotene was observed which may be mainly due to the ability of b-carotene to quench the singlet oxygen radical. b-carotene can act as an intracellular redox agent that behaves as protector against free radicals and can promote free radical formation (Palloza et al, 2002). Plasma vitamin A levels were significantly higher in BCPT and patients with BBD as compared to controls. The higher levels of retinol may suggest abrupt retinoic acid signaling. Cellular retinol binding proteins and nuclear retinoic acid receptors are mediators of retinoid action and it is reported that CRBP and RAR-b are down regulated in breast cancer (Kuppumabatti et al, 2000; Yang et al, 2002). The higher levels may also be due to consequence of deficient retinol storage possibly due to the disabled genes such as CRBP-1 or LRAT which are required for retinol storage function and thus unable to store and metabolize retinol appropriately and compromises RAR activity leading to loss of cell differentiation and tumor progression (Farias et al, 2005). However, the lower levels of vitamin A in patients with advanced stage of the disease may be due to large requirement of retinoic acid needed for the tumor cells in effort to become fully differentiated cells, which is obtained by higher conversion of retinol to retinoic acid leading to depleted levels of retinol in patients with advanced stage of disease. Lower levels of plasma vitamin C and vitamin E were observed in the patients with BBD and BCPT as compared to the controls and plasma vitamin E levels were significantly lower in BCPT with LN involvement as compared to patients without LN involvement. The lower levels of vitamin E may be in response to prevent oxidation of poly unsaturated fatty acid (PUFA) against ROS attack. Vitamin E is the major chain breaking antioxidant and it is recycled from a-tocopheroyl radical to a-tocopherol by vitamin C. Thus vitamin C plays an important role in recycling of a-tocopheroyl radical and thus indirectly involved in neutralization of ROS. Vitamin C also spares glutathione (GSH) and together with vitamin E prevent oxidation of GSH and regeneration of both, vitamin E and vitamin C requires GSH. (Kumaraguruparan, 2002). We have also found significantly lower levels of thiol in patients with BBD and BCPT as compared to controls [unpublished data] and the deficiency of plasma GSH may also be responsible for low levels of these antioxidants in patients with BBD and BCPT. Significant lower levels of plasma non-enzymatic antioxidants in the current study strongly suggest higher OS in the breast cancer patients. Analysis of plasma enzymatic antioxidants in this group of subjects showed altered levels of enzymatic antioxidants, higher lipid peroxidation and low thiol levels [unpublished data] strengthen the fact of higher OS.

In accordance with the present study, Ito et al (1999) reported a significant inverse association between serum b-carotene and carotenoids and breast cancer risk. Toniolo et al (2001) showed that serum b-carotene levels were

lower and retinol levels were higher in BCPT as compared to controls and also documented that there was a progressive increase in the risk of breast cancer for decreasing serum concentration of b-carotene. Various studies have showed significantly lower levels of plasma vitamin E and vitamin C in patients with BBD and BCPT (Ray and Husain, 2001; Sharhar et al, 2008; Kumaraguruparan, 2002; Gerber et al, 1988; Thangaraju et al, 1994). Further, marked decrease in vitamin E and vitamin C levels was also observed in stage IV BCPT (Ray and Husain, 2001).

The OR in the current study also indicated that higher quartiles with increasing levels of b-carotene, vitamin E and vitamin C were significantly associated with reduction in breast cancer risk whereas, increasing quartiles with increasing levels of vitamin A were significantly associated with increased risk of breast cancer. Ching et al (2002) showed that increased quartiles of serum concentrations of vitamin A were significantly associated with reduction in breast cancer risk. Tamimi et al (2005) showed inverse relationship for b-carotene with breast cancer risk.

The ROC curve for plasma levels of b-carotene, vitamin E and vitamin C showed that plasma levels of b-carotene could significantly discriminate between controls and patients with BBD. Plasma b-carotene, vitamin E and vitamin C levels have good discriminatory efficacy between controls and breast cancer patients. Plasma levels of vitamin E could significantly discriminate between patients with BBD and breast cancer patients. The ROC curve for plasma levels of vitamin A showed that it could significantly discriminate between controls and patients with BBD as well as controls and breast cancer patients.

The results of Pearson's correlation coefficient in the current study showed significant negative association between plasma b-carotene and vitamin A as well as between plasma vitamin C and vitamin E. Vitamin A showed significant positive association with vitamin C and negative with vitamin E. It is known that redox buffering systems of antioxidants are all related in a stepwise, sequential recycling process for neutralization of free radicals. Thus antioxidants work in concert with each other by a series of redox reaction to quench free radicals (Drisko et al, 2003). In these regards the results of Pearson's correlation coefficient suggest that these antioxidants are not working in concert with each other in BCPT which is suggestive of disturbed oxidation reduction reactions to quench ROS and ultimately affect neutralization status of free radicals in breast cancer patients.

In conclusion, lower levels of plasma b-carotene, vitamin E and vitamin C indicate higher oxidative stress, which may be the cause of lipid peroxidation, DNA damage and mutations leading to higher risk of breast cancer. The altered levels of vitamin A in BCPT indicate abrupt vitamin A homeostasis suggesting possible role of CRBP and RARs in breast cancer and required in-depth analysis of retinol metabolism pathway in breast cancer. The trends of the current study provides interesting clues to the modifiable etiology of breast cancer and suggest that, increased intake of b-carotene, vitamin E and vitamin

C may inhibit the progression of malignant disease. In addition, this study of non-enzymatic antioxidants provides a scientific basis for the use of nutritional supplementation with antioxidants during further treatment of breast cancer. However, human population is heterogeneous and requirements for these antioxidants may differ among individuals suggesting baseline levels of these antioxidants is necessary. Thus, this study may help in the issues and challenges posed by the antioxidant conundrum in breast cancer, warrants more studies in this area and attention of the researchers and policy makers.

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