SUMMARY AND CONCLUSIONS
The results obtained in the present study are summarized as follows

- Over a period of four years (2008-2012), two hundred and eighty eight clinically diagnosed and histologically confirmed breast cancer patients both pre and post menopausal women were selected for the present study. The age of the experimental subjects was between 29-74 years. The selected patients were categorized into the clinical stages of I, II, III, and IV. Healthy age matched (25-74 years) premenopausal (n = 36) and postmenopausal (n = 38) were selected from the patients attendants and hospital staff as control group in the present study.

- Out of 288 patients, 219 tumor biopsies were collected and the biopsies were categorized into I, II, and III clinical stages along with their respective non tumor biopsies (experimental control), in pre and post menopausal condition.

- Parameters studied include prognostic risk factors (age, anthropometric, reproductive and non reproductive), immunohistological markers (ER, PR, HER2/neu, p53, Bcl-2 and Ki-67), trace metals, pro- and antioxidant enzymes and metallothionein isoforms expression (MT-1F, MT-1E and MT-2A) in the pre and post menopausal breast cancer women.

- Age is an important risk factor in developing breast cancer. The breast cancer risk increases as the age advances. The maximum number of breast cancer cases was observed in 40-49 and 50-59 years old in all clinical stage patients. No significant change (t-test) was observed in average age of healthy subjects and the breast cancer patients of different clinical stages.

- Anthropometric factors such as height, weight and BMI were observed, no significant association was found between height and breast cancer. About 56-60 kgs body weight patients were shown 1-2 fold increased risk, whereas about 60kg or above body weight patients showed 2-3 fold risk (1st stage OR = 2.21, 2nd stage OR = 3.23, 3rd stage OR = 2.47, and 4th stage patients OR = 3.18) compared to less than 55kg body weight in all clinical stage patients. This indicates that the excess body weight is associated with increased incidence of breast cancer. Body weight might influence the growth of cancer cells through the promotion of hormones (E.g. estrogen) in the body.
The women having a BMI of 25-25.9 was showed two fold risk. Whereas, the women having a BMI of 30 and above was shown to be 2-3 fold risk when compared to women patients with BMI less than 24.5.

Non reproductive risk factors (NRFs) such as religion, abode, diet and family history were analyzed in all clinical stage patients. Among the three major religions – Hinduism, Islam and Christianity Muslims were found to be at an elevated risk of developing breast cancer (significant) followed by Christians compared with Hindus. In indicates that Muslims and Christians were shown higher risk compared to Hindu patients, may be due to consumption of high fat diet.

Women who residing in urban areas were shown to be at significant increased risk in selected cancer patients (1st stage OR = 1.76, (CI = 0.86- 3.58), 2nd stage OR = 1.29 (CI = 0.65-2.56), 3rd stage OR = 1.52, CI = 0.76-3.00) and 4th stage (OR = 1.72, CI = 0.84-3.52) compared to the women residing in rural areas.

The diet factor also plays an important role in breast cancer development. Non-vegetarians showed significantly increased risk compared to vegetarians in all clinical stage patients.

Reproductive factors (RFs) are major risk factors in the developing breast cancer, such as age at menarche, marital status and the age at the time of marriage, age at first childbirth, parity and null-parity, breast feeding and dieting of breast feeding, menopausal status and age at the time of menopause were analyzed.

No significant risk was observed among women whose age at menarche was 13, 14, 15 years and above. This indicates that the effect of early age at menarche on breast cancer risk may be mediated simply by the prolonged exposure of breast epithelium to estrogen produced by regular ovulatory cycle.

Women who had never-married were shown two fold risk compared to ever married women. Women who married after the age of 20 years were shown to be at two-fold increased risk in 2nd, 3rd and 4th stage breast cancer patients respectively (OR = 2.20, CI = 1.02-4.74; OR = 1.97, CI = 0.92-4.20 and OR = 2.11, CI = 0.98-4.55) compared to women who married at the age less than 20 years. No significant risk was found in women whose age at marriage was 20 years and above.
Age at first childbirth was observed to be an important risk factor for developing breast cancer. Compared to women whose age at first childbirth is under 20 years, the women whose age at first childbirth was 25 years or more had an increased breast cancer risk as observed in early and advanced stage patients (I, II and III, IV stages). Hence, there is increased risk of breast cancer in the women, who had delayed first child-birth.

Nulli-parous women had a two-fold risk of developing breast cancer compared to parous women. Whereas, women who have not breast fed the children were shown at higher breast cancer risk compared to those who have breastfed. Women who breastfed their children for more than 24 months showed lower risk for development of breast cancer over women who breastfed for less than six months.

Post menopausal women were shown to be at higher risk (ORs 1.19, 1.32, 1.33, and 1.38) compared to premenopausal breast cancer patients. Women who attained menopause at 50 years of age or above showed twofold increased risk (I, II and IV stage) ORs = 2.9, OR = 2.68 and OR = 2.42 respectively). Whereas, more than one-fold (OR = 1.74) increased risk was observed in 2nd stage women patients compared to women who attained menopause at less than 50 years of age. This indicates that early age menarche and late age menopause increased the breast cancer risk. Parity, breastfeeding and late age at menopause may be implying that the longer exposure to sex hormones (estrogen and progesterone) or imbalance during the reproductive years.

From this finding, the selected risk factors in the present study may serve as prognostic markers for the early detection and assessment of breast cancer.

The main clinical and histopathological features of the tumors are analyzed. Tumor staging was done by according to the TNM classification system. The number of cases found in stage 0, 33% (36) in stage I, 34% (38) in stage II and 33% (36) in stage III. The tumor size in 28 breast cancer biopsies showed (25.4%) less than 2 cm in diameter, 48 biopsies (43.6%) were in between 2 and 5 cm and 34 biopsies were (30.9%) 5 cm or above. The breast cancer biopsies were categorized into three grades viz., low grade tumors (31.8% - GI), intermediate grade (41.8% - GII) and high grade (26.4% - GIII) tumors.
Ninety six (87.3%) patient’s biopsies were identified as auxiliary node positive and 14 (12.7%) as node negative. It indicates that the risk of auxiliary lymph node metastasis increases as tumor size increases and it suggests that nodal metastasis is an indicative of tumor chronology.

One of the interesting and important findings of this study indicated that the response of hormone receptors and tumor specific markers in patients with respect to classical prognostic markers, menopausal status and stage of breast cancer provided valuable information pertaining to the use of hormonal markers and tumor specific markers as prognostic and predictable variation in the management of breast cancer.

The ER positive expression is found in 66.4% of cases (73), while 33.6% of cases (37) had a negative expression. Whereas, ER status is significantly associated with lymph node involvement ($\chi^2 = 6.488$, p = 0.039) and also, significant correlation was observed to tumor grade and ER ($\chi^2 = 7.31$, p = 0.026). No statistical association was found between ER expression and tumor size, menopausal status and clinical stage. This indicates that the estrogen receptor is closely associated with the development of tumors, particularly in breast carcinomas, because ER-positive tumors use the steroid hormone estradiol or estrogen as their major stimulator of tumor growth and progression.

The PR positive expression is found in 78 of cases (70.9%), while 32 of patients (29.1%) had a negative expression. A significant association was found with tumour size ($\chi^2 = 11.13$, p = 0.003), lymph node involvement ($\chi^2 = 8.011$, p = 0.018), tumour grade ($\chi^2 = 9.62$, p = 0.008) and PR expression. No statistical correlation was found between menopausal status, clinical stage and PR expression. From this finding, both hormonal receptors (ER and PR) are closely associated to breast cancer and these may be important prognostic and predictive markers in the treatment decision i.e., Tamoxifen.

Strong positive membrane staining of the HER-2/neu was observed in 69 (62.7%) tumors, while no membrane staining in 41 (37.3%) tumor cases. Whereas, HER2/neu status is significantly associated with tumor grade. This suggests that the over expression of HER-2/neu patients may be eligible for immunotherapy with trastuzumab (Herceptin).
p53 positive expression is found in 61 tumors (55.5%), while 49 (44.5%) tumors had a negative expression. A statistically significant association was found between lymph node status ($\chi^2 = 7.9, P = 0.019$), tumor grade ($\chi^2 = 11.04, P = 0.003$), clinical stage ($\chi^2 = 8.42, P = 0.015$) and positive expression of p53. No statistical correlation found between tumor size, menopausal status and p53 expression tumors. This indicates, the p53 protein accumulation would be a reasonable prognostic marker of more aggressive carcinoma and it is most useful in the routine diagnostic evaluation of breast tumors.

Bcl-2 expression is positive in 75 (68.2%) and negative in 35 (31.8%) tumors. No statistical correlation was found between lymph node status, menopausal status, clinical stage and Bcl-2 expression. Whereas, a statistically significant correlation was observed between tumor size ($\chi^2 = 17.53, P = 0.0001$), tumor grade ($\chi^2 = 10.62, P = 0.004$) and Bcl-2 expression. From this finding, the Bcl-2 gene positivity increases with increasing tumor size and Bcl-2 expression indicates its anti-apoptotic influence on cell proliferation.

Ki-67 expression was found in 77 (70%) tumors, while the proliferation index is low 33 (30%) cases. A statistically significant association was observed between tumor size ($\chi^2 = 13.44, P = 0.001$), tumor grade ($\chi^2 = 11.3, P = 0.001$) and positive expression of Ki-67. No statistical correlation was found between lymph node status, menopausal status, clinical stage and Ki-67 positive expression tumors. This indicates that the Ki-67 is closely associated to breast cancer and it is an indicator of cell proliferation activity.

The present finding suggests that the markers viz. ER, PR, HER-2/neu, p53, Bcl-2 and Ki-67 are closely related to the breast cancer and may be strongly advocated among the population to prevent the incidence of the miserable disease and better treatment.

The trace metals play a crucial role in breast carcinogenesis. A large accumulation of trace metals such as Pb, Cd, Cr, Ni, Zn, Fe, Cu, and Zn were observed in the breast tumor tissues. Whereas, selenium was significantly decreased in pre- and post-menopausal breast tumor tissues over to non tumor (control) during different clinical stages. From this finding, it indicates that the low Se content may play an important role in breast carcinogenesis and it may be possible to monitor the prognosis of breast tumors using Se as a biomarker.
Pb, Cd, Cr, and Ni were significantly increased (p< 0.05) in breast tumor tissues compared to non tumor breast tissue. This indicates that the possible association between the exposure to environmental Pb and risk of breast cancer, given the known impact of lead on human health. The environmental exposure to lead is toxicologically significant to generate ROS, leading to oxidative damage, and/or may be the direct participation of lead in free radical reactions, which may increase risk of breast tumors.

Cadmium, chromium and nickel have been recognized as mutagens and carcinogens because of their ability to inhibit the repair of damaged DNA. Cadmium was also involved in the activation of cell proliferation, differentiation, blockage of apoptosis, or activation of transcription factors. Whereas, increased content of nickel may be involved in the induction of oxidative DNA damage and inhibit the repair mechanism of damaged DNA. The accumulation of chromium may indicate that the stimulation of free radical production, which induces oxidative DNA damage and it leads to carcinogenesis.

The Fe and Mn content was significantly increased (p<0.05) in pre and postmenopausal breast tumors than non tumors in different clinical stages. Fe plays an important role in the regulation of cell growth and differentiation, therefore it may appear to implicate in the process of carcinogenesis. In addition, increased content of Mn in breast tumors may be closely related to malignant growth process.

Cu and Zn were significantly increased (p< 0.05) in breast tumor tissues compared to non tumor breast tissue. It indicates that the copper increased content in the breast cancer tissue possibly promote breast cancer through angiogenesis and oxidative DNA damage. Whereas, Zn in breast tumors may be closely related to malignant growth process, since zinc is required for cell proliferation and tumor growth.

The increased content of trace metals were observed in pre- and postmenopausal breast tumors stage III, stage II and followed by stage I compared to their respective non tumor counterparts, whereas selenium content are decreased in pre and postmenopausal breast tumors in stage dependent manner from stage I to III.

Trace metals content is strongly associated with tumors in premenopausal than in postmenopausal breast cancer women. This indicates that the premenopausal breast
tumors tend to be more aggressive than those that develop after menopause. In addition, the increased content of all trace metals are probably due to the increased cell activity in malignant tissue and active enzyme systems requiring more nutrients and oxygen, which consequently increases the blood supply.

- Oxidative stress plays a significant role in the cancer development and disease progression. A significantly increased lipid peroxidation was observed in pre- and postmenopausal breast cancer tumors than non tumors during all clinical stages. It suggests that augmented lipid peroxidation may be attributed to an overproduction of reactive oxygen species (ROS). Higher levels of ROS might be involved in the damage of protein, RNA, DNA and exert diverse cellular and molecular effects including mutagenicity, cytotoxicity and carcinogenicity.

- To deal with the overproduction of ROS, the cells are equipped with antioxidant detoxifying mechanisms to compensate against oxidative stress which include antioxidant such as reduced glutathione (GSH) and antioxidant enzymes such as SOD, CAT, GST, GPx, and GR.

- The activity levels of antioxidant enzymes SOD and CAT were increased significantly in pre- and post-menopausal breast tumors compared to non tumor controls. It indicates that SOD is the essential antioxidant enzyme that protects the cell against deleterious effects of superoxide anion. Higher activity levels of SOD may be a stress adaptive response for the increased conversion of highly reactive superoxide to less reactive molecular O₂ and hydrogen peroxide. Catalase, in turn protects the cell against H₂O₂ generated by various reactions.

- Increased GPx activity levels were observed in breast tumors over the non tumors in all clinical stage patients. It indicates that the GPx might be reducing lipid or non-lipid hydro-peroxides by oxidizing reduced glutathione (GSSH) to oxidized glutathione (GSSG). In addition, the increased activity of GR was observed in the pre and post menopausal malignant breast tumors, it may be an attempt to convert more GSSG to GSH in order to raise GSH content and should ordinarily indicate an increase in GSH concentration.
The activity levels of GST were increased in breast tumors relative to non tumors. It indicates that the higher levels of GST may promptly detoxify anticancer agents, thereby preventing their cytotoxic action and oxidative stress.

Reduced glutathione content was increased significantly (p<0.05) in pre and postmenopausal breast tumors compared to non tumor of breast in different clinical stages. It indicates that the increased GSH content may in conjunction with GPx protects the cells against a wide variety of xenobiotics, ROS and other toxic compounds.

In the present study, the levels of lipid peroxidation (Lpx) and antioxidant enzymes (SOD, CAT, GST, GPx, GR and GSH) were increased significantly in stage III, stage II followed by stage I breast tumors compared to their relative non tumor counterparts. It suggests that the increased lipid peroxidation and antioxidant enzymes represent potential biomarkers for the assessment of breast cancer.

No statistically significant changes were observed in the expression levels of GAPDH in non tumors of breast (0.82%, 0.86% and 0.85) and tumor of pre- and post-menopausal women (0.82, 0.91 and 0.84) at different clinical stages (I, II and III) of breast cancer patients.

Metallothionein proteins play very important role in homeostasis of essential metals (Cu and Zn), detoxification of heavy metals (Hg, Cd, Cu etc.), and in the antioxidation of ROS species which induce DNA damage.

The metallothionein isoforms viz., MT-1F, MT-1E and MT-2A expression levels were detected by semi-quantitative PCR technique in pre and post menopausal breast cancer tumors and relative non tumors in term of mRNA levels during different clinical stages.

The increased expression levels of MT1F was observed in different tumor stages I, II and III in the breast tissue of pre-menopausal women tumor samples (10.4, 16.7 and 23.4, respectively) as compared to the expression levels of MT1F in the normal (non tumor) breast samples of pre-menopausal women. Whereas, the expression levels of MT1F were found to be increased in different tumor stages I, II and III in the breast tissue of post-menopausal women tumor samples (13.7, 9.17 and 16.2 respectively) as
compared to the expression levels of MT1F in the normal breast samples of post-menopausal women.

- The expression levels of MT1E were observed to be increased in different tumor stages I, II and III (19.3, 26.9 and 25.5 respectively) in the breast tissue of pre-menopausal women as compared to non tumor breast samples of pre-menopausal women. Whereas in the post-menopausal women breast tumor tissues, the expression levels of MT1E was observed enhanced in different tumor stages I, II and III (16.5, 25.9 and 24.8 respectively) as compared to the expression levels of MT1E in the normal breast samples.

- In pre-menopausal women tumor samples, the expression level of MT2A was observed elevated in different tumor stages I, II and III (17.2, 26.7 and 24.9 respectively) as compared to the expression levels of MT2A in the normal breast samples of pre-menopausal women. Whereas, the expression levels of MT2A were found enhanced in different tumor stages I, II and III (19.2, 16.7 and 14.2 respectively) in the breast tissue of post-menopausal women of tumor samples as compared to the expression levels of MT2A in the normal breast samples of post-menopausal women are. It indicates that the significant changes were observed in the expression levels of MT isoforms in the breast tissue of pre- and post-menopausal women at different stages of breast cancer.

- MT-1F isoform shows approximately 16 and 12 fold increase in mRNA expression in pre- and postmenopausal tumor tissues respectively over the non tumor tissues. Whereas, MT-1E shows approximately 21 and 22 fold elevated mRNA expression in tumor tissues respectively compared to relative non tumor tissues of pre and post menopausal women. The MT-2A isoform shows 16 and 22 fold elevated mRNA expression in pre- and postmenopausal breast tumors during all clinical stages respectively. In this study, as the tumor stage progressed from stage I to III, the expression levels of MT1F, MT1E and MT2A mRNA showed an increasing in pre and postmenopausal tumor tissues, except, the expression levels of MT2A mRNA shows a declining trend from 19.2% (stage I), 16.7% (stage II) to 14.4% (stage III) in the post-menopausal women.
It indicates that MT isoforms might be implicated in the malignant growth process. On the other hand, increased levels of MT in the breast cancer may be due to the increased accumulation of trace metals or to scavenge the free radicals induced by the metal toxicity. Hence, MTs are promising prognostic biomarkers in the prospective patient studies and may be validated to establish MT assays for use in clinical practice.

In the view of the above, the results of the present investigation suggests that the patient’s risk factors profile, tumor pathological characteristics, immunological markers, different trace metals content, antioxidant enzymes and metallothionein expression levels might be considered as the notable biomarkers in the assessment of breast cancer. The use of these biomarkers will be not only helpful in the prediction and prognosis of breast cancer, but also will be of use in the planning of individualized therapeutic regimen. However, these biomarkers before being used as diagnostic tools in the breast cancer diagnosis require further thorough in depth study and clinical validation.

The author is fully aware that further extensive studies are required to arrive at final definite conclusions, regarding the therapeutic approach in breast cancer of south India women. Speculations were made at many instances where the situation demanded, and of course, speculations are inevitable in any scientific study to pave the way for further investigation. Limitations in the availability of kits and chemicals have prevented the author from penetrating into the core of the project and henceforth he could only crawl over the periphery of the problem. For instance, the estimation of hormones, free radicals levels, and cloning procedures in metallothionein expression which characterize the breast cancer would have been more useful in understanding the overall spectrum of breast cancer. Due to constraints of funding, facilities, besides the time, the above experiments could not be carried over. Nevertheless, the author is hopeful to carry out these studies in detail in his post-doctoral pursuit.