6. SUMMARY AND CONCLUSIONS

In the present study of “Evaluation of ER-α gene (ESR1) polymorphism in breast cancer patients” 200 patients and 100 controls were studied from Department of Radiotherapy, Pt. B.D. Sharma University of Health Sciences Rohtak. The percentage frequency of breast cancer was 21% in 2013, 20% in 2014, 18% in 2015, 23% in 2016 and 22% in 2017. In present study, majority of the cases belonged to eight districts i.e. Rohtak, Jind, Bhiwani, Sonipat, Jhajjar, Hissar, Karnal and Panipat. The highest incidence of breast cancer was noted from Rohtak (16%) district and frequency was low in other districts.

The incidence of breast cancer was greater in rural areas (66%) as compared to urban areas (34%). The mean age of onset of breast cancer was 49.47±10.43. Maximum number of cases (34%) were found in 50-59 age group and maximum number of controls (37%) were in <40 years age group. About 5% breast cancer cases were found with family history of breast cancer.

Various risk factors were divided in two categories (reproductive and anthropometric) to highlight their role in breast cancer occurrence. The information was collected for breast cancer cases and control groups. A strong association was noted between reproductive risk factors and breast cancer risk. The present study also focused on breast cancer risk factors among pre-menopausal and post-menopausal women and provides evidence that breast cancer risk factors are different at menopausal stage.

Age was reported as stronger risk factor for estrogen receptor positive breast cancer as compare to estrogen receptor negative breast cancer. In premenopausal breast cancer cases with unmarried status, menarche before the age of thirteen years, >29 years age at first birth and less number of child births were found to be significantly associated with breast cancer risk. An inverse association was observed for null parity and postmenopausal breast cancer. Late age at first full term pregnancy was found significant in both subgroups (premenopausal and postmenopausal) of breast cancer cases (p<0.05).

In this study, it was observed that breast feeding plays a protective role in premenopausal women. Abortion history and age at first abortion were not found to be significantly associated with breast cancer in both premenopausal and postmenopausal women. The use of birth controlling pills, cigarette or tobacco smoke and alcohol
consumption was not found to be significantly associated with breast cancer risk. In the present study, positive correlation of breast cancer was observed for height, weight and body mass index (BMI) in postmenopausal women. The tall and obese postmenopausal women were at high risk of breast cancer than short and low weight women.

Breast cancer comprises of distinct biological subtypes having varied spectrum of clinical, pathological with different prognostic and therapeutic implications. Present study reveals the significant difference between the hormone receptors of breast cancer having clinical and pathological variables. Among various clinicopathological factors, grade I (59%) tumors were more common followed by grade II. Tumor size showed maximum occurrence of 2-4.9cm (45%) and a very less number of patients were found with tumor size above 5cm.

In this study, majority of the patients (55%) were found with <4 positive node metastasis. The infiltrate ductal carcinoma type (93%) was found more in this study as compared to lobular type of carcinoma (7%). Majority of patients (45%) were having NPI score 3.4 to 5.4 (moderate prognosis). It has been reported that different characteristics of breast cancer are important for clinical management and their incorporation into the NPI score could significantly improve in decision making difficulty in medicine in breast cancer patients.

In menopausal status of women, it was reported that tumor grade and tumor size showed statistical significant values. Tumor grade I and II were observed maximally in the premenopausal breast cancer women but not statistically significant. Tumor grade III was noted to be significantly confined to the postmenopausal breast cancer women (p=0.0015). Majority of postmenopausal women noted to have tumor size <2-2.9 cm. Tumor size >5 cm was found in majority of premenopausal breast cancer women and showed statistically significant association (p=0.0067).

Maximum postmenopausal women had <4 positive lymph nodes for metastasis. On the other hand premenopausal breast cancer women had >4 positive lymph nodes for metastasis. Cancer stage and NPI score showed varied results in both pre and postmenopausal breast cancer women. Majority of pre- menopausal and post-menopausal women were observed to possess positive status of estrogen receptor, progesterone receptor and Her2/neu receptor. However receptors of estrogen, progesterone and
Her2/neu did not show any significant association with menopausal status of breast cancer women.

In the present study, positive expression of ER was found to be 74%, maximum quick score was counted 2+ve (20.8%) and minimum score was counted 4 +ve and 5+ve (9.9%). The positive expression of PR was found to be 76%, maximum frequency of PR positivity was counted 2+ve (22.3%) and minimum score was counted 4 +ve (88.8%). 75% patients were found to be positive for the expression of Her2/neu and maximum quick score was counted as 2+ve (38.5%).

This study confirms that receptor expression of ER found to be significantly associated with tumor grade and tumor size, NPI score and Her2/neu receptor status. However, no association of ER expressions was observed with lymph node metastasis status, cancer stage, histological type and progesterone receptor expression of breast cancer. These findings could have clinical importance in breast cancer treatment.

The genotype distribution of five ESR1 SNPs (rs138724640, rs2234693, rs9340799, rs373404617 and rs2077647) was examined in breast cancer cases and controls to study the contribution of ESR1 SNPs in disease progression. In rs138724640 (Taq1) maximum cases and controls were in heterozygous condition. In case of rs2234693 (PvuII) SNP, the CC genotype frequency was found to be lower in breast cancer cases but in control group frequency of TT genotype was lower. Majority of participants of both groups were reported to be heterozygous. The C allele was found to be more prevalent in both cases and controls of rs138724640 & rs2234693.

The GG genotype frequency was found lower for ESR1 rs9340799 (XbaI) SNP in breast cancer cases and control group. AG frequency higher in both groups of patients and controls for ESR1 rs9340799 (XbaI) SNP. The frequency of AA genotype was found to be lower in breast cancer cases and GG genotype in controls for ESR1 SNPs rs373404617 (BstUI). Maximum number of breast cancer cases and controls were heterozygous. A-allele was more prevalent in breast cancer cases and controls of rs9340799. G-allele was found to be more in breast cancer cases of rs373404617. In case of rs2077647 (SfaNI) majority of cases were found with CT genotype, whereas TT genotype was in maximum number in the control group. T-allele provides the protective effect against the breast cancer.
The genotype analysis showed no significant difference among cases and controls for rs138724640 and rs373404617 (p=0.4015 and p=0.8422 respectively). SNP rs2077647 showed only borderline significance (p=0.0777). Whereas SNP rs2234693 and rs9340799 showed significant association with breast cancer (p=0.0111 and p=0.0002 respectively).

AGTC haplotype of SNPs rs2234693 and rs9340799 showed statistically significant association with breast cancer risk (Chi square=22.3; DF=8; p=0.0004); whereas GGTT haplotype showed significant protection from breast cancer (p=0.0201). The other haplotypes showed no significant association with breast cancer risk. Two SNPs rs2234693 and rs9340799 were found to be in linkage disequilibrium ($r^2$ =0.60735, D=0.173453, D’=1).

The genotypes study of the different ESR1 SNPs in association with personal history, lifestyle parameters revealed that rs138724640 was associated with weight, lactation duration and marital status. Lesser age at menarche and marital status played a role in onset of breast cancer and was found to significantly associated with rs2234693 (p=0.0231 and p=0.0048 respectively).

In rs9340799 and rs373404617 SNPs, lactation duration, weight and marital status showed association with breast cancer (p=0.0422 and p=0.0565 respectively). Lesser age at first child birth and first abortion was found to show the protective effect against breast cancer in rs2077647 (p=0.0421 and p=0.0311 respectively). Marital status showed statistical significance among breast cancer cases and controls (p=0.0090 and p=0.0533 respectively).

Association of clinicopathological factors of breast cancer cases and ESR1 SNPs (rs138724640, rs2234693, rs9340799, rs373404617 and rs2077647) genotypes was studied. Lymph nodes <4 positive status for metastasis was found to be associated with TC genotypes of SNP rs138724640 and rs2234693. TT genotype for rs138724640 and rs2234693 showed >4 positive lymph node status for metastasis (p=0.0217 and p=<0.0001 respectively). Histological type showed significant association with TT genotype of rs138724640 (p=0.0436). Breast cancer cases with AA genotype of ESR1 SNP rs373404617 had positive receptor status for progesterone.

The distribution of ESR1 SNP genotypes according to menopausal status showed the association of postmenopausal women with high risk of breast cancer with
TT genotype of rs2234693 (OR, 0.3157; 95% CI, 0.1771 to 0.5629). The genotype TC and CC showed statistical significance in premenopausal women (OR, 1.8974; 95% CI, 1.0416 to 3.4566; OR, 3.2436; 95% CI, 1.3676 to 7.6929 respectively). TC genotype observed as protective genotype similar to a study (Ramalhinho et al., 2013).

In premenopausal breast cancer cases, GG genotype of rs373404617 SNP was found to be statistically significant (OR, 2.8519; 95% CI, 1.5943 to 5.1012). The CC genotype of rs2077647 SNP was found to be associated with breast cancer risk in postmenopausal breast cancer cases (OR, 0.3750; 95% CI, 0.1992 to 0.7059).

ER α gene GT dinucleotide repeat is highly polymorphic and found to be associated with risk of breast cancer in the present study. (GT)15 repeats were relatively common in breast cancer cases. The inverse correlation was observed for the presence of the (GT)16 and (GT)17 repeats and breast cancer risk. The present study revealed (GT)15/(GT)15 allele association with breast cancer risk. (GT)15 repeats were more confined to breast cancer cases at premenopausal stage.

Hemoglobin concentration was noted in breast cancer cases and was found to be in range of 9-14g/dl. The mean of hemoglobin level was 12g/dl ±1.45 before the start of chemotherapy and 10.9g/dl ±1.54 during the course of chemotherapy. Total leukocyte count was found with 8000-10,000 cells/µl in majority of cases before the start of chemotherapy. It was observed that majority of cases had total leukocyte count ranged from 6,000-10,000cells/µl during the different courses of chemotherapy (C1-C5). Majority of breast cancer cases were found with 3-4 lakhs/ml before chemotherapy treatment. Platelet count was found to increase as the treatment proceeds from first chemotherapy to fifth course chemotherapy which is indicative of tumor progression.

Differential leukocyte count observed 5-6×10⁹/l neutrophils in maximum number of cases before the start of courses of chemotherapy. During the chemotherapy, neutrophils count was found to be 4-6×10⁹/l among maximum breast cancer cases. Total lymphocyte count was noted as 3.0 to 3.5×10⁹/l in majority of cases before chemotherapy. A decreased pattern of number of lymphocytes was noted during the course of chemotherapy which is associated with tumor progression. Eosinophils were found to be 0.3 to 0.4×10⁹/l and monocytes count was 0.6-0.7×10⁹/l in maximum
number of cases before the chemotherapy. However, eosinophils and monocytes did not show any significant variation during the different courses of chemotherapy.

In biochemical tests liver and kidney function tests were used to monitor disease progression and possible side effects of medication used in chemotherapy. The level of blood urea nitrogen (BUN) was observed to be more than normal range (7-20 mg/dl) during the different courses of chemotherapy. The mean value of blood urea nitrogen was found to be 32.58±19.7 before the start of chemotherapy. The mean value of creatinine was noted to be 1.05±0.59 before the start of treatment and was within normal reference range (0.6-1.1 mg/dl) during the chemotherapy treatment. No variation was observed in blood urea nitrogen and creatinine level during the different course of chemotherapy.

The uric acid level was found to be higher than the normal reference range (2.4-6.0 mg/dl) during the different courses of chemotherapy treatment. The mean value of SGOT and SGPT was noted to be 27.3±4.02 and 27.9±10.24 respectively before the start of chemotherapy treatment. An increasing level of SGOT and SGPT enzyme was reported as the courses of chemotherapy proceeds indicating improper liver functioning. The alkaline phosphatase level was within in normal range (44-147 IU/L), but slightly increased at fifth cycle of chemotherapy.

The mean value of direct and total bilirubin was 0.13±0.11 and 0.30±1.3 respectively before the starting of the chemotherapy treatment. The direct and total bilirubin level increased as treatment proceeded from first course to fifth course chemotherapy. In the present study, a decreased level of total serum protein and albumin was observed in breast cancer cases undergoing chemotherapy treatment. The mean value of total serum protein and albumin was observed to be 3.5±0.07 and 8.11±0.5 respectively before chemotherapy.

No variation was observed in fasting blood sugar (FBS) level during different courses of chemotherapy. The mean value of FBS was reported as 96±25 before chemotherapy. The mean value of post-prandial glucose level was 110±25 before chemotherapy. Post-prandial glucose level was noted to increase during different courses of chemotherapy.

In present study, breast cancer cases were analysed for overall survival (OS) of ESR1 SNPs. The median follow-up time for the unavailable patients was 34 months.
The Cox regression method gives an estimate of the hazard ratio and its confidence interval. Mean survival for ER negative breast cancer cases was 36 months (SE 1.173; 95% CI 34.447 to 39.044) and median survival for both ER positive and ER negative was 43 months. Mean survival for ER positive breast cancer cases was 37 months (SE 1.059; 95% CI 34.855 to 39.004). No statistically significant association was reported for both groups (Log rank $\chi^2= 0.01634$, DF=1, $P = 0.8983$).

The mean survival for CC genotype of ESR1 SNP rs138724640 was 37 months (SE 1.287, 95% CI 34.413 to 39.460) whereas the median survival was 42 months (95% CI 34.000 to 43.000). The mean and median survival for TC genotype was 39 months (SE 1.289, 95% CI 35.508 to 40.561) and 43 months (95% CI 34.000 to 45.000) respectively. No statistically significant association was reported for all the genotypes of rs138724640 (Log rank $\chi^2= 1.4899$, DF=2, $P = 0.4748$).

The mean survival for CC and TC genotype of ESR1 SNP rs2234693 was 30 months (SE 1.285, 95% CI 26.864 to 31.900 and SE 1.083, 95% CI 27.483 to 31.727). Median survival was 30 months for both CC and TC genotype (95% CI 30.000 to 32.000 and 30.000 to 32.000 respectively). The mean survival was 27 months for TT genotype (SE 0.936, 95% CI 24.731 to 28.400) and median survival was 29 months (95% CI 25.000 to 30.000). Overall no statistically significant association was reported for all the genotypes (Log rank $\chi^2= -3.3929$, DF=2, $P = 0.1833$).

The mean survival for breast cancer cases with AG genotype of ESR1 SNP rs9340799 was 29 months (SE 1.320, 95% CI 26.057 to 31.232) and median survival was found as 27 months (95% CI 23.000 to 34.000). In breast cancer cases with GG genotype, mean and median survival was 27 months (SE 1.020, 95% CI 24.279 to 28.276; 95% CI 21.000 to 32.000 respectively). Mean survival for AA genotype was 28 months (SE 1.174 95% CI 25.552 to 30.152). The median survival for the patients having AA genotype was as 32 months (95% CI 23.000 to 33.000). The AG genotype was found to be significantly associated with good outcomes for breast cancer (Log rank $\chi^2= -18.1658$, DF=2, $p = 0.0351$).

In the present study, overall survival for breast cancer cases with AG genotype of ESR1 SNP rs373404617 was 34 months (SE 1.437, 95% CI 30.282 to 35.917). Median survival for AG genotype was 33 months (95% CI 31.000 to 43.000). In breast cancer cases with GG genotype, the mean survival was 32 months (SE 1.313, 95% CI
29.381 to 34.527) and median survival was 31 months (95% CI 31.000 to 34.000). Mean survival for AA genotype was 29 months (SE 1.231, 95% CI 26.536 to 31.360). The median survival for breast cancer cases having AA genotype was 31 months (95% CI 23.000 to 33.000). The AG genotype was significantly associated with good outcomes for breast cancer (Log rank $\chi^2$ 5.7632, DF=2, p= 0.0560).

In ESR1 SNP rs2077647 genotypes (CC, CT and TT) of breast cancer cases mean survival for CC and CT genotype was 35 months (SE 1.170, 95% CI 32.581 to 37.167 and SE 1.276, 95% CI 31.944 to 36.946). Median survival was 34 months for CC and 33 month for TC genotype (95% CI 33.000 to 35.000 and 32.000 to 42.000 respectively). The mean and median survival was 34 months for TT genotype (SE 1.180, 95% CI 31.194 to 35.821, 95% CI 32.000 to 35.021). No statistically significant association was reported for all the genotypes (Log rank $\chi^2$-0.9373, DF=2, p= 0.6258).
Conclusions

- Null parity and previous cancer history was found significantly associated with breast cancer. The Protective effect of ≤25 years of age at first full term pregnancy, more than 12 months of lactation duration and having 3+ babies was found for breast cancer cases.

- Tumor grade I, cancer stage III, tumor size 2-4.9 cm, <4 positive node status for metastasis, infiltrate ductal carcinoma histological type and NPI score ranging 3.4 to 5.4 was found in majority of the breast cancer cases.

- SNP rs2234693 and rs9340799 located on intron 1 were associated with breast cancer (p=0.0111, 0.0002 respectively). SNP rs2077647 located on exon 3 showed only borderline significance (p=0.0777).

- AGTC haplotype of SNPs rs2234693 and rs9340799 showed statistically significant association with breast cancer risk (chi square=22.3; DF=8; p=0.0004); whereas GGTT haplotype showed significant protection from breast cancer (p=0.0201).

- (GT)15/(GT)15 allele was associated with breast cancer risk (OR 7.0000; 95% CI, 1.3811 to 35.4791).

- Significant association of overall survival (OS) with AG genotype of ESR1 SNP rs9340799 and rs373404617 was observed in breast cancer cases (Log rank \(\chi^2\) 18.1658, DF=2, p= 0.0351; \(\chi^2\) 5.7632, DF=2, p= 0.0560 respectively).
Major outcomes of Study

- Breast carcinoma cases were frequently observed in postmenopausal age group with 49.47±10.43 mean age of onset of breast cancer.
- In the present study, null parity and previous cancer history was significantly associated with breast cancer. Protective effect of ≤25 years of age at first full term pregnancy, more than 12 months of lactation duration and having 3+ babies was observed for breast cancer cases. No significant association was observed for cigarette or tobacco smoke and alcohol consumption.
- Clinical parameters showed the presence of tumor grade I, cancer stage III, tumor size 2-4.9 cm, <4 positive node status for metastasis, infiltrate ductal carcinoma histological type and NPI score ranging 3.4 to 5.4 in majority of the breast cancer cases. The expression of estrogen receptor was correlated with tumor grade, tumor size, NPI and Her2/neu receptor expression.
- Genotypic frequency of ESR1 gene of breast cancer cases was found different from controls. Odd ratio analysis showed that SNP rs2234693 and rs9340799 located on intron 1 were associated with breast cancer (p=0.0111, 0.0002 respectively). SNP rs2077647 located on exon 3 showed only borderline significance (p=0.0777).
- The C-allele was found to be more prevalent in both breast cancer cases and controls of rs138724640 and rs2234693. A allele was more prevalent in breast cancer cases and it also found in controls of rs9340799 and rs373404617. T allele provided the protective effect against the breast cancer in rs2077647 SNP. The genotype analysis showed no statistically significant difference among cases and controls for rs138724640 and rs373404617 (p=0.4015 & p=0.8422 respectively).
- AGTC haplotype of SNPs rs2234693 and rs9340799 showed statistically significant association with breast cancer risk (chi square=22.3; DF=8; p=0.0004); whereas GGTT haplotype showed significant protection from breast cancer (p=0.0201).
- ER α gene GT dinucleotide repeat found to be associated with breast cancer risk in the present study. The present study linked (GT)15/(GT)15 allele association with breast cancer risk (OR 7.0000; 95% CI, 1.3811 to 35.4791) and mostly confined to premenopausal women.
• A decreased level of hemoglobin (Hb) concentration, total leukocyte and lymphocyte count was noted from first course of chemotherapy to fifth course of chemotherapy. Platelet count was increased as the treatment proceeds from first chemotherapy to fifth course chemotherapy. No statistically significant variation was observed for neutrophils, eosinophils and monocytes count during different chemotherapy courses.

• The level of blood urea nitrogen and albumin protein was decreased during chemotherapy treatment. The increasing pattern of uric acid, SGOT, SGPT, alkaline phosphatase, total bilirubin, direct bilirubin and post-prandial blood glucose level was observed from first course of chemotherapy to fifth course of chemotherapy. However, no variations were observed in the level of creatinine, total serum protein and fasting blood sugar level during the chemotherapy treatment.

• Significant association of overall survival (OS) with AG genotype of rs9340799 was observed in breast cancer cases (Log rank $\chi^2$ 18.1658, DF=2, $p=0.0351$). In ESR1 SNP rs373404617, AG genotype was found to be significantly associated with breast cancer survival (Log rank $\chi^2$ 5.7632, DF=2, $p=0.0560$).
Significance of the study

- The present study has identified several risk factors associated with breast cancer so general awareness can be made in society to reduce the load of disease.
- The tumor grade III, cancer stage III, tumor size >5 cm, >4 positive lymph node status for metastasis and NPI score of >5.4 were found to be associated with poor prognosis of disease so these clinicopathological factors may help in defining the patients of high risk and low risk category.
- The present study has identified correlation of estrogen receptor expression with tumor grade, tumor size, NPI and Her2/neu receptor expression which will help in establishing their role in pathogenesis of disease.
- The examination of genotype frequency of ESR1 gene SNPs (rs138724640, rs2234693, rs9340799, rs373404617 and rs2077647) and GT gene polymorphism revealed their contribution in breast cancer progression. Molecular findings have an important significance in genetic counseling approaches.
- Association study of clinocopathological parameters and different risk factors along with ESR1 genotypes of different SNPs may be helpful in understanding the causes of breast cancer and to facilitate the development of effective prevention and therapy.
- The genotypes of ESR1SNP rs9340799 and rs373404617 were found to be the most important prognostic factor determining the 5-year breast cancer specific survival rate.
- In this study, AG genotype of both rs9340799 and rs373404617 SNPs was found to be significantly associated with breast cancer survival and are predictive of good outcome. Thus overall survival studies may be helpful in investigating whether progression or regression of the disease has occurred and also discriminate relapse from secondary malignancies which is essential for optimal management of breast cancer.
- The complete blood profile is a helpful diagnostic tool in the monitoring of disease, metastasis and different treatment strategies of breast cancer.
Future prospectus

The determination of hormonal status is an important primary assessment at the time of a breast cancer diagnosis. ESR1 genes polymorphism analysis will probably be indispensable for a rapid and objective assessment of the cell receptor and different intracellular characteristics.

The combination of ESR1 genes polymorphism with other clinical and pathological data will help to improve diagnosis and patient care. The quality control and standardization methods have recently been applied to the generation of polymorphic data that greatly improved their reliability. In future, researchers may reproduce and verify the data so as to give more conclusive clinical impact.

As recently reviewed and emphasized several fundamental questions remains to be answered, such as why or how the DNA becomes damaged. It could be genetic or environmental, or in most cases, a combination of the two. Our present understanding underlying the pathogenetic mechanisms and consequences of the unbalanced changes in established risk factors that are associated with breast cancer. There is much to be discovered about the clinical characteristics and associated correlation with ESR1 genes polymorphism.

Progress in the understanding and management of breast cancer can only be obtained if disease is thoroughly investigated, both clinically and with a series of molecular biological tools. This will make possible the identification of different prognostic factors and useful markers for the follow-up of patients with overall survival. Further studies should be aimed at genetic and hormonal relationship with clinical status using a large cohort. These results could help in setting risk factors and helpful in raising awareness about screening for high risk women so as to diagnose the disease at initial stages and thus reduces mortality.

Many studies have established the prognostic significance of peripheral blood in the monitoring of disease, metastasis and different treatment strategies of breast cancer. In addition to this there is a need of simple, accurate and combined prognostic index which should have independent value as assessed by multivariate analysis. Furthermore different therapy based validation strategies are required to improve the overall survival.
Prevention of the disease remains the most promising strategy for reducing both incidence and the mortality of breast cancer. Many epidemiological studies have established the development of lifestyle and medical approaches in cancer prevention. Future directions include genetic, proteomic and other molecular approaches for identifying pathways associated with breast cancer initiation and development and progress.