CHAPTER -3
MATERIALS AND METHODS

3.1 Study population and design

The study involved a hospital-based case-control study, a tertiary care centre where all Breast cancer cases were recurited from the Cancer Research Institute, Department of Radiotherapy, located at jolly grant, Dehradun, Uttarakhand, during the period from June 2020 to August 2023. This study included 110 freshly histopathologically diagnosed patients with breast cancer and age-matched 110 controls of 30-70 years of age. In addition, the institute's ethics committee reviewed the study and granted formal clearance (Appendix I). A written informed consent was acquired from the patients and controls after they were informed of the nature, methodology, and risks involved in the study. This study complied with all the provisions of the declaration of Helsinki (Appendix II).

As part of the estimation for the sample size, n-Master software considered matching case-control studies (1:1). Based on a proportion of 50 percent exposed controls with a significance level of 5 percent and a power of 90 percent to identify a two-fold increase in risk. In order to meet the requirement, at least one hundred ten discordant pairs were required in this study.

3.1.1 Study Design

As shown in Figure 8, the study design is illustrated in a graphical format.

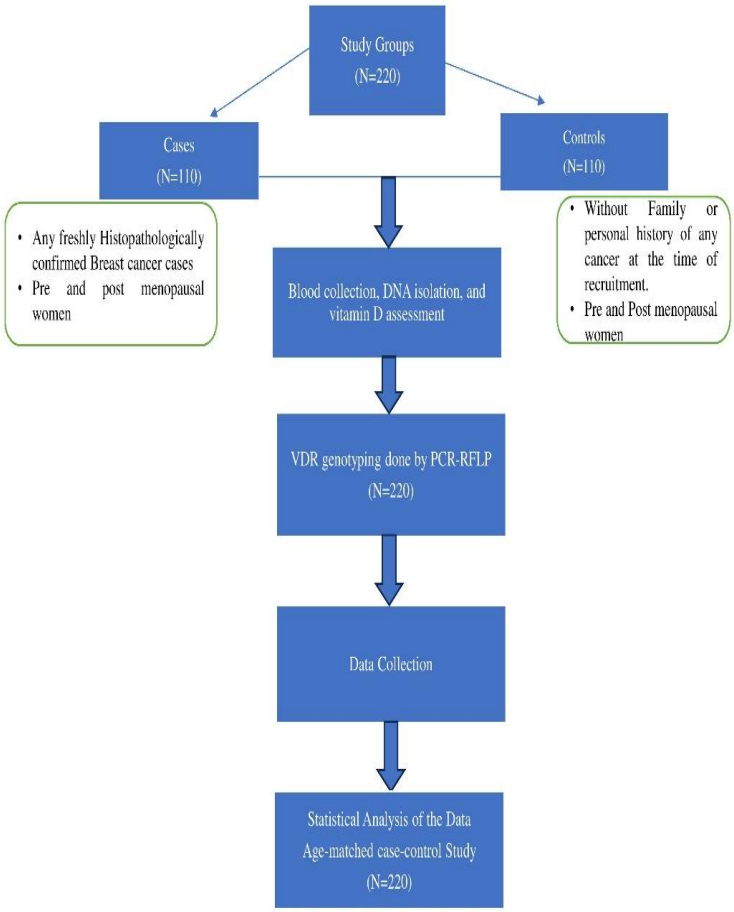

Figure 8: Diagram illustrating the research design and statistical analysis.

The study was designed to enroll 110 cases of breast cancer and 110 controls in an age-matched case-control study. Samples of blood from cases and controls were taken for the extraction of genomic DNA and Vitamin D assessment. A PCR-RFLP method was used to genotype distinct polymorphisms among the genes of interest. The statistical analysis of data was utilized to assess how VDR genetic polymorphic variation may promote breast cancer risk.

3.2 Selection Criteria

Patients were selected primarily based on the following inclusion and exclusion criteria.

3.2.1 Breast cancer patients' inclusion criteria

(a) Any freshly Histopathologically confirmed Breast cancer cases.

(b) Pre and post-menopausal women.

3.2.2 Breast cancer patients' exclusion criteria

(a) Patients who were under hormonal therapy.

(b) had a personal history of any other cancer.

(c) who were under the past six months of taking Vitamin D supplements.

(d) Lactating or pregnant Women.

3.2.3 Control Selection

The age-matched Controls, without family or personal history of any cancers, were randomly selected from women volunteers during the routine visit health checkup in the hospital following written informed consent. Blood samples were obtained from participants who agreed to take part.

3.3 Data Collection

The data collected were from all eligible participants using a well-drafted, peer-reviewed detailed proforma (Appendix III and IV). These included demographic parameters, including age and menopausal status, co-morbidities, breast cancer history in first-degree relatives, and Body mass index[273], weight, and height were recorded as anthropometrics. The prognostic features of breast cancer tumors were determined accordingly as the size and type of the tumor, the histological stage, the grade, an evaluation of the lymphatic nodes within the axilla, and the expression of hormonal receptors. The pertinent clinical data were retrieved from our online database having a unique hospital ID-UHID. The tumor morphology were classified according to criteria; Elstons and Ellis based on architecture features, measures of nuclear differentiation, and mitotic index[274] based on the 7th edition of stage of breast

cancer accordingly to TNM developed by the cancer joint committee of the American Medical Association (AJCC). The Modified Bloom-Richardson (MBR) grading score were also considered for prognostic significance[275]. In addition, hormone receptor status was assessed, it comprises estrogen receptors (+ve/-ve), progesterone receptors (+ve/-ve), human epidermal growth factor receptor2 (HER2) (+ve/-ve), and triple-negative stature (ER-ve, PR-ve, and HER2-ve). The molecular phenotypes were categorized based on surrogate definitions of the St. Gallen consensus 2013 as LuminalA type (ER & PR (+ve) & HER2 (-ve) & Ki-67 (< 14%), HER2-positive luminalB type (ER (+ve) & HER2 (+ve) & Any Ki-67 or Any PR) and HER2-negative luminal B type (ER (+ve) & HER2 (-) & Ki-67 (≥ 14%) or PR (< 20%)[276].

3.4 Sample Collection and DNA Extraction

After reviewing the inclusion and exclusion criteria and obtaining informed consent, Whole blood samples (3ml) were collected in tubes containing ethylenediamine tetraacetic acid (EDTA) as an anticoagulant. DNA isolation was done manually from fresh and frozen Blood samples[277] and by kit method (Himedia, catalog no. MB505). The purity and yield of DNA don't vary much for downstream applications. Samples were stored at -20^0 C for further use.

3.4.1 Assessment of serum levels of vitamin D

The Serum 25-hydroxy Vitamin D levels was assessed for all eligible cases and controls using the chemiluminescent immunoassay (CIA) method (Immunotechs a Beckman Coulter Company, France) by the trained Laboratory technicians. Serum 25-OHvitD levels were classified as Sufficient/Normal (75-250 nmol/l), Insufficient (50-<75 nmol/l), and Deficienct (< 50 nmol/l)[278]. Results were expressed in nmol/l.

3.4.2 Chemicals and Reagents

The chemicals used in this method are standard chemicals found at major suppliers, Himedia Chemicals, for this study. These include Ammonium chloride (NH_4Cl), Sodium bicarbonate ($NaHCO_3$), Ethylene diamine tetra acetic acid (EDTA), Tris-Cl, Magnesium chloride ($MgCl_2$), Triton X-100, 2- mercaptoethanol, Sodium dodecyl sulfate (SDS), Sodium chloride (NaCl), Sodium hydroxide (NaOH), Tris Base, Glacial acetic acid, Chloroform: Isoamyl alcohol (24:1), Isopropanol, Ethanol, 100bp DNA Ladder M (MBT130), Elution Buffer (10mM Tris-Cl, pH8.5), Agarose (MB002-500G), Ethidium bromide (10mg/ml), and 6X gel Loading dye (ML015).

3.4.3 Reagents Preparation

In the following section, the reagents were prepared.

Table 5: RBC Lysis Buffer, denoted as Lysis buffer R (10X) 100ml, contains

Chemicals	Amount
Ammonium Chloride NH_4Cl (1.54M)	8.26 gms
Ammonium Bicarbonate $NaHCO_3$ (0.14M)	1.19gms
Ethylene diamine tetra acetic acid EDTA (0.5M)	0.37gms

pH was adjusted to 8, and the solution was converted to the 1X working solution.

Table 6: Nucleic acid Lysis Buffer, denoted as Lysis buffer N (10X) 100ml, contains

Chemicals	Amount
Tris-Cl (50 mM)	0.60gms
$MgCl_2$ (50 mM)	0.11 gms
NaCl (150mM)	0.87gms
0.1% Triton X-100	100µl

pH was adjusted to 8, and the solution was converted to the 1X working solution.

Table 7: TAE Buffer (50X) 250ml, contains

Chemicals	Amount
Tris-Base	60.5gms
Glacial Acetic acid	14.2 ml
Ethylene diamine tetra acetic acid EDTA (0.5M) pH 8	25 ml

The solution was converted to the 1X working solution.

- **10% SDS.** 2.5 gms of sodium dodecyl sulfate was constituted in 25ml autoclaved deionized water.
- **NaCl (6Molar).** 8.7 gms of NaCl was constituted in 25 ml of autoclaved deionized water.
- **Ethylene diamine tetra acetic acid EDTA (0.5M) pH 8 in 100 ml**

For a 100 ml stock solution of 0.5 M EDTA, weighed out 18.6 gms of EDTA (MW=372.2). Initially dissolved in 80 ml distilled water, stirred with the rod, and observed the pH with a pH meter. The pH was adjusted by adding approximately two gms of NaOH and, once fully dissolved, adding up to the final solution 100ml using distilled water. The solution was autoclaved and stored at 4^0 C for further use.

3.4.4 Methodology for manual DNA extraction

- **Step1.** A whole Blood sample of 500µl from the vacutainer was transferred to the Eppendorf tube. The serum was carefully aspirated from the sample by centrifuging at 2500 rpm for three minutes at 4°C.
- **Step2.** Approximately 1ml of Lysis buffer R (1X) was added to the pellet, mixed gently with periodic inversions, and kept for 5 min at room temperature.
- **Step3.** Centrifuged the cell mixture for 5 min at 5000 rpm and discarded the Supernatant carefully. Repeated steps 2 and 3 if the pellet was not white.
- **Step4.** Approximately 500µl of Lysis Buffer N (1X) was added to the white pellet obtained from the previous steps, mixed gently with a wide bore pipette, followed by adding 50 µl of 2% 2- mercaptoethanol and 50µl of 10% SDS. The mixture was then incubated at 55-60°C for 30 min.
- **Step5.** Upon completion of incubation, 200µl of 6 Molar of NaCl was added, vortexed vigorously, and centrifugation was done at 8000 rpm for 5 minutes.
- **Step6.** The supernatant was at that time taken in a clean Eppendorf tube and added an equal volume of Chloroform: Isoamyl alcohol (24:1). Mix well by gentle inversions, afterward centrifugation for 12,000 rpm for one minute.
- **Step7.** Removed the Aqueous phase (DNA present) carefully without disturbing the base layer and transferred it to a clean Eppendorf tube containing an equal volume of chilled Isopropanol. Shaking the tube for a few seconds resulted in fine white threads appearing in the solution.
- **Step8.** Transferred the floating precipitate into a clean Eppendorf tube, washed with chilled 70% ethanol to remove any salts trapped with DNA, and centrifuged at 13,000 rpm for 3 min.

- **Step9.** Drained the tube and evaporated the Ethanol completely. The pellet was allowed to dry at 37°C.
- **Step10.** Added sufficient (1X) TAE solution and dissolved the precipitate by light fingertip vibration. Do not vortex to avoid mechanical breakage of DNA. A solution of DNA was then stored at -20°C for further use.

3.4.5 Genomic DNA: qualitative and quantitative assessment

Using 0.8% agarose gels (10mg/ml of ethidium bromide) and 1X TAE buffer, the DNA quality of the isolated genomic DNA was measured by agarose gel electrophoresis. A total of 8µl of genomic DNA was loaded into the wells after being mixed with 6X loading dye. The agarose gel was visualized using a UVP GelDoc-It® Imager documentation system (Analytikjena). Quantifying genomic DNA was performed by spectrophotometric analysis using a UV-1800 spectrophotometer (SHIMADZU). The amount of DNA per microliter was measured in nanograms concerning the dilution factor. At 260nm, one absorbance unit (1OD) equals 50ng/ml of dsDNA. As a result of the OD260/OD280 ratio, DNA purity was estimated, and a ratio of 1.7 to 1.8 was obtained. In the PCR reaction, 50-150ng of genomic DNA per microliter was used.

3.5 Genotyping analysis

The Genotyping analysis of VDR polymorphisms, FokI (rs 2228570), BsmI (rs 1544410), and ApaI (rs 7975232), was examined by PCR-RFLP analysis.

3.5.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was one of the innovations that changed molecular science, affecting dozens of biological subdisciplines. PCR was conceptualized by Keppe and colleagues in 1971, but it was not until 1985 that Kary

Mullis described and experimentally verified the complete process at Cetus Corporation. This technique amplifies a specific region of DNA or gene sequence.

There are three steps involved in thermocycling amplification. Double-stranded DNA is first denatured, then annealed with both forward and reverse primers after being separated for a short time. After that, the dNTPs present in the PCR mix allow the two strands of DNA to extend, creating complementary stretches of new DNA. The sequence of interest is amplified exponentially during 34 cycles of denaturation, annealing, and extension[279].

3.5.2 Materials

Himedia Chemicals is a major supplier of the chemicals used in this study. Hi-Chrom PCR Master mix (MTBT089), Molecular biology grade water (M064-500ML), Forward and Reverse primers (Sigma-Aldrich), 96 Well Thermal Cycler Veriti (Thermo Fisher Scientific), PCR Tubes (200 µl and 500 µl), and micropipettes.

3.5.3 Standard Procedure

Hi-Chrom PCR master mix was thawed at room temperature and spun briefly to collect the material at the bottom of the tube. Further, the reaction mixture was prepared on ice as follows.

Table 8: Reagents for making a 25 µl PCR reaction mix

Sr. No.	**Components**	**Amount added**
1.	Hi-Chrom PCR master mix, 2X	12.5 µl (1X)
2.	Forward Primer,10 pmol/ µl	1 µl
3.	Reverse Primer, 10 pmol/ µl	1 µl
4.	Template DNA	1 µl (50ng)
5.	Molecular Biology Grade Water	9.5 µl

As soon as the entire content had been finely mixed, the tubes of PCR loaded with the master mix was placed in a thermocycler, and the reaction of PCR was launched. According to generalized scenarios, the denaturation procedure was performed initially at 94^0 C for 3 mins, following 34 cycles of cyclic denaturation at 94^0 C for 1mins, annealing for 50 sec at a suitable temperature, extension at 72^0 C for 1 min (Time is determined by the product's length) and one final cycle of final extensions at 72^0 C for 8 mins and final hold at 4^0 C. The primers were taken from the previous studies and verified through primer BLAST (NCBI). All PCR reactions were conducted using the primers and the annealing temperature listed in the following Table 9.

Table 9: The details of the primer sequences and their annealing temperatures.

VDR gene (References)	**Primer sequence**	**Annealing Temperature**
FokI (T/C)[170] rs 2228570	*forward: 5'-GAT GCC AGC TGG CCC TGG CAC TG-3'* *reverse: 5'-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3'*	71□C
ApaI(C/A)[176] rs 7975232	*forward: 5'-CAG AGC ATG GAC AGG GAG CAA G-3'* *reverse: 5'-CGG CAG CGG ATG TAC GTC TGC AG-3'*	71□C
BsmI(A/G)[280] rs1544410	*forward:5'CAACAAGACTACAAGTACCGCGTCAGTGA 3'* *reverse: 5' AACCAGCGGGAAGAGGTCAAGGG 3'*	68 □C

The PCR products (10 µl) and the 100bp marker (3 µl) were loaded into the wells and then run on 2% agarose gel electrophoresis at 90 volts for one hour and genotyped using RFLP.

3.5.4 Restriction fragment length polymorphism (RFLP)

DNA restriction enzymes, also known as restriction endonucleases, recognize short and specific sequences of DNA (often palindromic). In addition to cleaving double-stranded DNA (dsDNA), they also cleave DNA adjacent to or within their recognition sequences. The optimal activity of each restriction enzyme depends on specific requirements. The ideal conditions for storage and assay favor the highest level of enzyme activity and fidelity. Several factors influence enzyme activity and stability, including temperature, pH, enzyme cofactor(s), salt composition, and ionic strength. The restriction enzymes were obtained from New England Biolabs, USA (FokI and BsmI) and Promega Company (ApaI).

3.5.5 Setting up a Restriction Enzyme Digestion

In accordance with the manufacturer's protocol, an enzyme digest was performed on PCR-amplified products using corresponding restriction enzymes. The restriction enzyme digestion was thoroughly mixed before assembly, then centrifuged briefly to collect the bottom contents, and the respective reaction mixtures were performed on ice as follows.

Table 10: Restriction Enzyme protocol for FokI and BsmI

Sr. No.	**Components**	**20 µl Reaction**
1.	Molecular Biology Grade Water	12 µl
2.	10X NEBuffer	2.5 µl
3.	PCR Template	5 µl
4.	Restriction Enzyme (10U/ µl)	0.5 µl

Mixed the reaction gently in a 200 µl autoclaved PCR tube by pipetting up and down and microfuged briefly. Incubated at 37^0C and 65^0C for 2 hrs for FokI and BsmI-RFLP.

Table 11: Restriction Enzyme protocol for ApaI

Sr. No.	Components	24 µl Reaction
1.	Molecular Biology Grade Water	16.3 µl
2.	Restriction Enzyme 10X Buffer	2 µl
3.	Acetylated BSA, 10 µg/ µl	0.2 µl
4.	PCR Template	5 µl
5.	Restriction Enzyme (10U/ µl)	0.5 µl

Mixed the reaction gently in a 200 µl autoclaved PCR tube by pipetting up and down and microfuged briefly. Incubated at 37 ^{0} C for 2 hrs.

3.5.6 Agarose Gel Electrophoresis

This study utilized electrophoresis to separate and visualize nucleic acids, especially PCR and RFLP products. DNA fragments are more easily separated in agarose gels with a higher percentage of agarose, while larger products are easier to resolve in gels with a lower agarose percentage[281].

A 2% agarose gel was made in 50 ml 1X TAE buffer in a 250 ml conical flask. A microwave oven was used to dissolve the agarose in the buffer. In the solution was added 2 µl Ethidium bromide (10mg/ml) and allowed to cool. The gel was poured into the specific trays after well-swirling the solution. Room temperature solidification was allowed for the comb to solidify in the slot allotted. The gel was placed in an electrophoresis chamber containing 1X TAE after solidification. The digested PCR products (20 µl) and 6X loading dye (4 µl) were mixed and loaded into agarose gel wells. As a marker, a 100bp DNA ladder (6 µl) was also loaded in one well and was

run at 90 volts for one hour. The digestion pattern observed for different PCR products was depicted in the table form below. To confirm the results of genotyping, a few random samples were sent for SNP sequencing at Biokart India Pvt. Ltd, Bangalore, to determine reproducibility, and the results obtained were complete concordance.

Table 12: The VDR gene SNPs with respective restriction enzymes

Sr. No.	VDR gene SNP	Amplified PCR Products (bp)	Restriction Enzymes (Manufacturer name)	RFLP- PCR Products (bp)
1.	FokI (rs 2228570)	272bp	FokI (NEB)	272bp, 198bp and 72bp
2.	BsmI (rs1544410)	825bp	BsmI (NEB)	825bp, 650bp, and 175bp.
3.	ApaI (rs 7975232)	352 bp	ApaI (Promega)	352bp, 216bp and 135 bp

The different sizes of DNA fragments obtained from PCR-RFLP were assigned for further data analysis. For FokI, BsmI, and ApaI, the alleles in lowercase (f, b, and t) signify a restriction site being present, in contrast, uppercase letters (F, B, and T) signify the lack of a restriction site. Similarly, the genotypes assigned for FokI, BsmI, and ApaI were FF, BB, and AA as Dominant Homozygotes, Ff, Bb, and Aa as Heterozygotes, and ff, bb, and aa as recessive Homozygotes.

3.6 Statistical Analysis

Data collection and entry were carried out in MS Excel. An array of statistical analyses was conducted with SPSS version 22 software. To assess whether or not the data sets differ from a normal distribution, the one-sample Kolmogorov-Smirnov test was conducted. Parametric tests was used to analyze normally distributed data, and data with non-normal distributions were analyzed employing non-parametric tests. Qualitative variables were described using descriptive statistics. For qualitative and categorical variables, frequency was calculated accompanied by percentages. A graphic illustration of the variables were shown to help a clear understanding of the results and the categorical data was analyzed implying the chi-square test for qualitative variables. An independent t-test was used for two groups and an ANOVA for more than two groups to compare mean differences.

The diversion from Hardy-Weinberg equilibrium (HWE) was tested for polymorphisms by examining the differences between genotype frequencies observed and those expected, utilizing the $\chi 2$ test. Women were classified based on their genotype, which included the alleles that are homozygous for minor alleles, heterozygous for major alleles, or both. They were also classified based on their carrier status, which included major allele homozygotes or minor allele carriers, for the corresponding polymorphisms. The $\chi 2$ test was implemented to determine the potential association between VDR polymorphisms and the risk of getting breast cancer. Additionally, it was used to determine if the frequencies of alleles varied from the expected Hardy- Weinberg equilibrium. The study generated odds ratios (ORs) and 95% confidence intervals (CIs) to assess the risk of cancerous breast

linked to a certain VDR genotypes. Logistic regression was carried out to examine the impact of variables on gene effects. It used statistical methods to find out the risk of breast cancer based on age, menopause state, and hormone receptor status. The odds ratios were principally based on considering the controls' ages at sampling and the diagnosis age for each case, as age at diagnosis is involved in breast cancer risk.

Considering, p-value <0.05, then the hypothesis can be considered statistically significant, and if p-value >0.05, then the hypothesis is said to be statistically insignificant.