Chapter 3
Material & Methods

3.1 Background

- For early detection of patients for cervical cancer and study the symptoms, we have screened 498 women for cervical cancer during our study period i.e., 16 months in G. G. G. Hospital, Jamnagar, and G. T. Sheth Hospital, Rajkot.
- Participants' informed permission was obtained along with Institutional Ethics Committee (IEC) approval. Confidentiality was maintained for each subject in every aspect as per IEC norms. Participants who were not willing were excluded from the study. IEC approval and format of 'Informed Consent form' is attached in annexure. Figure 3.1 shows the workflow of the research work

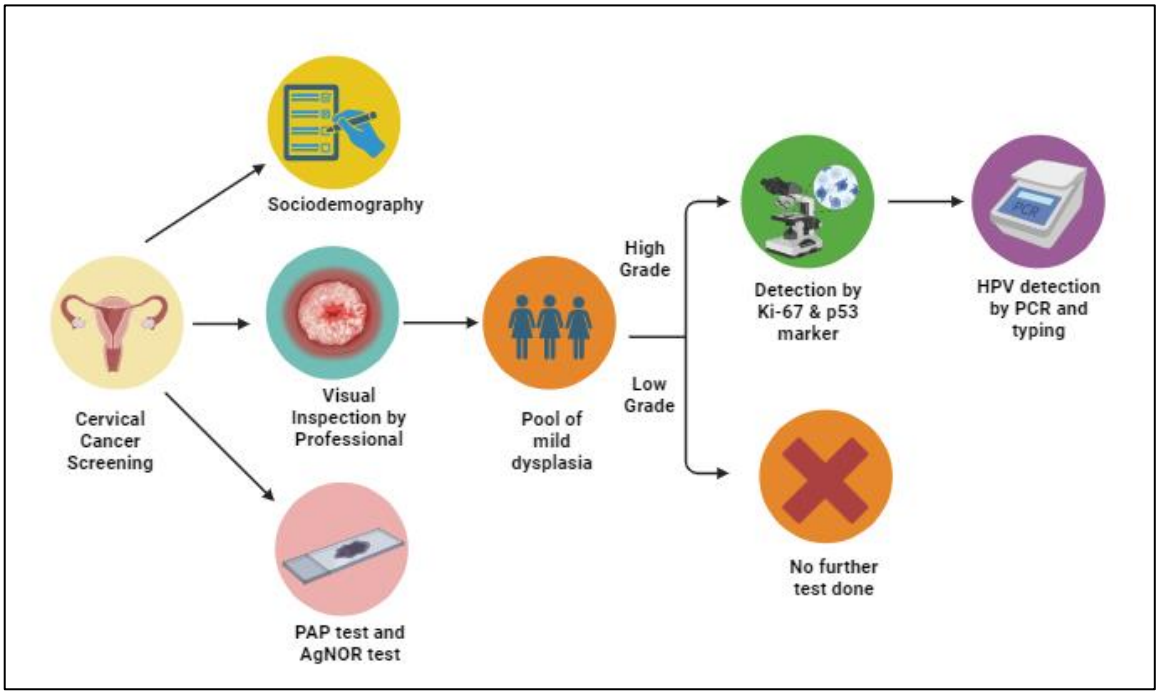

Figure 3.1: Workflow of the research

3.2 Sample collection

- The cervical smears for the study were collected from the Gynecology department of GG Government Hospital, Jamnagar & G T Sheth Cancer Hospital, Rajkot.
- The relevant clinical and personal history regarding age, parity, marital status, detailed menstrual history, etc. were taken at the time of screening.
- Papanicolaou smear test was performed to diagnose the cervical malignancy which was

carried out by the cytopathology department. Results for the same were collected for the study purposes.

- The cervical smears were collected in pairs, one for the specialized AgNOR staining and one for HPV detection by PCR.
- DNA typing was done at Multi-disciplinary Research Unit, Shri M. P. Shah Government Medical College, Jamnagar.

3.3 Socio Demographic data

- Complete assessment was done by taking personal and clinical history and the study variables are mentioned in table 3.1.

Study Variable	**Categories**
Personal Data	Age at the time of screening, Age of marriage, Residence, Community, Occupation
Menstrual history	Menarche
Labour history	Mode of delivery, Place of delivery
Obstetric History	Gravidity, Parity, Living, Abortion, Tubal ligation
Symptoms	Vaginal discharge, Irregular menses, Foul smell, burning micturition, Itching, Abdominal pain, White discharge

Table 3.1. Description of the study variables

3.4 Visual Inspection by Professional

- Visual inspection was carried out by professionals using 3-5% Acetic Acid Stain, without any breach in ethical norms and data was observed and collected by us. Bimanual pelvic examination and sterile speculum examination. In this gynecologic examination, the doctor checked for any unusual changes in the patient's cervix, uterus, vagina, ovaries, and other nearby organs. To start, the doctor looked for any changes to the vulva outside the body and then, using an instrument called a speculum to keep the vaginal walls open, the doctor looked inside the vagina to visualize the cervix. If some of the nearby organs were not visible during this examination, the doctor inserts 2 fingers of 1 hand inside the vagina while the other hand gently presses

on the lower abdomen to feel the uterus and ovaries.

3.5 AgNOR staining procedure

- AgNOR staining can be widely used on different type of specimens as described by Ploton et al (Ploton et al., 1986). In our study, AgNOR staining was carried out by using Ogunsola et al protocol (Ogunsola & Antia, 2018). The materials and procedure were used for the silver colloid staining silver nitrate method for AgNOR protein sites. Formalin-fixed smear slides were used for staining.

3.5.1 Preparation of stock solution

1) 50% silver nitrate solution: 50 g of silver nitrate was dissolved in 100 ml of distilled water.

2) Gelatin Solution: 2 g of Gelatin was added in 1 ml of Formic acid and made up the volume by distilled water up to 100 ml.

3.5.2 Preparation of a working solution

- Two parts of 50% silver nitrate solution and one part of gelatin solution were mixed immediately before use and the number of slides to be stained determines the amount of working solution to be used. The working solution was kept for 45 minutes at room temperature in the dark before use.

3.5.3 Staining Procedure

- The first wash was given by xylene and then dipped into alcohol. Slides were then cleaned with distilled water and left to dry naturally. Slides were once more cleaned with distilled water for one minute. Allowed it to air dry and placed under the microscope for observation. AgNORs which appeared as black dots under an oil immersion lens were counted. AgNOR sites appeared as intranuclear black dots and the Background appeared as reddish or Pale Yellow (Garg, Raj, & Chandra, 2013). The results were in 4 categories i.e., Normal cervix, Low squamous intraepithelial lesion (LSIL), High squamous intraepithelial lesion (HSIL), and Cancer positive. Normal superficial squamous epithelial cells of the cervix that on average had 1 AgNOR per cell nucleus was taken as a control. Structures and configuration of AgNOR are well described in figure 3.2.

3.5.4 AgNOR counting

- The low-grade PIN, high-grade PIN, and atypical adenomatous hyperplasia foci in

AgNOR-stained sections are counted, along with the corresponding foci in H&E-stained sections. AgNORs can be seen as intranuclear black spots. They are counted in a specific foci in 100 nuclei. Based on size, each dot was categorised as small, medium, or giant. A small dot is one that is just discernible but not tiny. Dots that were three times or larger than a small one was categorised as medium, while those that were five times or more were categorised as giant. By calculating the average and counting the number of dots in each focus, the mean AgNOR count was recorded (Meenakshisundaram K, 2017).

- AgNOR scores were determined by multiplying the number of medium dots by three, the number of large dots by five, and the number of small dots by one. Then, the three factors were added up to determine the average. Next, basal cells and luminal cells were counted independently in each foci using the AgNOR method. AgNOR scores were computed using a comparable method for both basal and luminal cells, and the outcomes were tallied (Meenakshisundaram K, 2017).

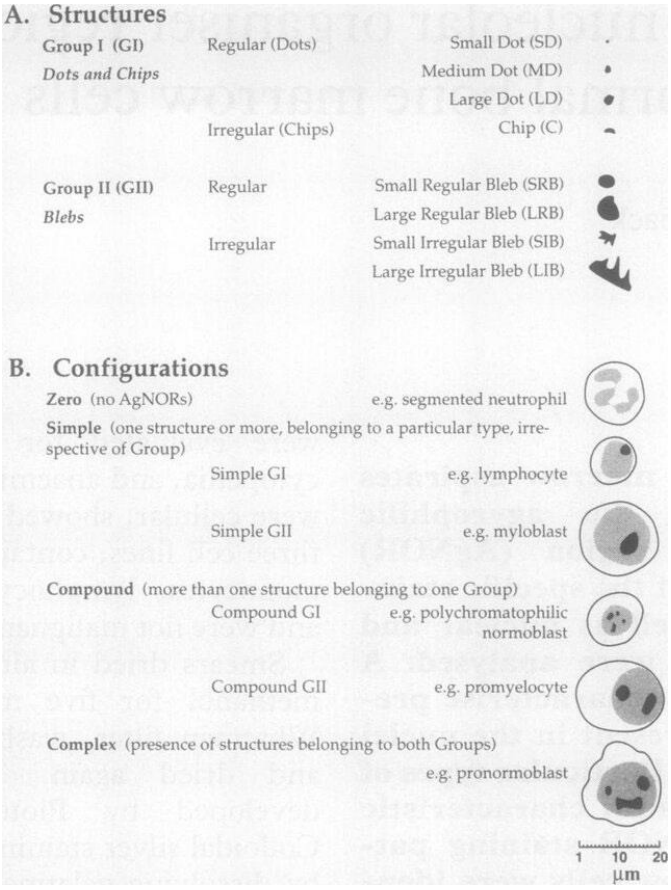

Figure 3.2: AgNOR structures and configurations *(Nikicicz & Norback, 1990)*

3.6 Markers p-53 and ki-67 evaluation

- Immunohistochemistry (IHC) was performed on cervical smear sample to differentiate high grade and malignancy.
- Horse-peroxidase method was used for a panel of two antibodies i.e., p53 (Biogenex, BP53-12) and Ki-67 (Biogenex, BGX-297).
- For antigen retrieval, citrate buffer of pH - 6.0 using microwave power 10 for 6 minutes x 3 cycles were done (applicable for both antibodies). Counterstain done with Haematoxylin.
- All the slides were examined. To ensure the internal quality control of immunohistochemistry methods was carried out as part of an established and approved quality assurance system to ensure the dependability of the experimental investigation.
- Cells were noted as positive when they showed nuclear/ cytoplasmic immunoreactivity (brown precipitate).
- Only the neoplastic region of each tissue section was evaluated. To evaluate the marker positivity, at least 1000 cells per case were counted.
- Quantification of the results was made by determining the nuclear positivity (number of cells marked by the antibody divided by the number of cells counted per sample). Positivity was nuclear for p53, Ki-67.
- The staining intensity was also assessed based on the category as mild, moderate, and high or grade I to III. This is mainly based on subjective assessment.

3.6.1 Interpretation of p-53 and ki-67

- Every slide was scrutinized using light microscopy. It was believed that strong nuclear staining indicated p53 and Ki67 positivity. In order to calculate the percentage of cell positivity, 100 cells were assessed in representative high-power fields. The percentage of stained cells determined the grade of nuclear staining. Grade 0 samples for p53 have no positivity (negative), Grade I sample have mild expression (less than 10% of cells show positivity), Grade II samples have moderate expression (between 11% and 50% of cells show positivity), and Grade III samples have intense expression (more than 50% of cells show positivity). When the Ki67 index was less than 5%, between 5 and 30%, and

more than 30%, Grade-1+, 2+, and 3+ were assigned, accordingly (Mishra RK, 2016).

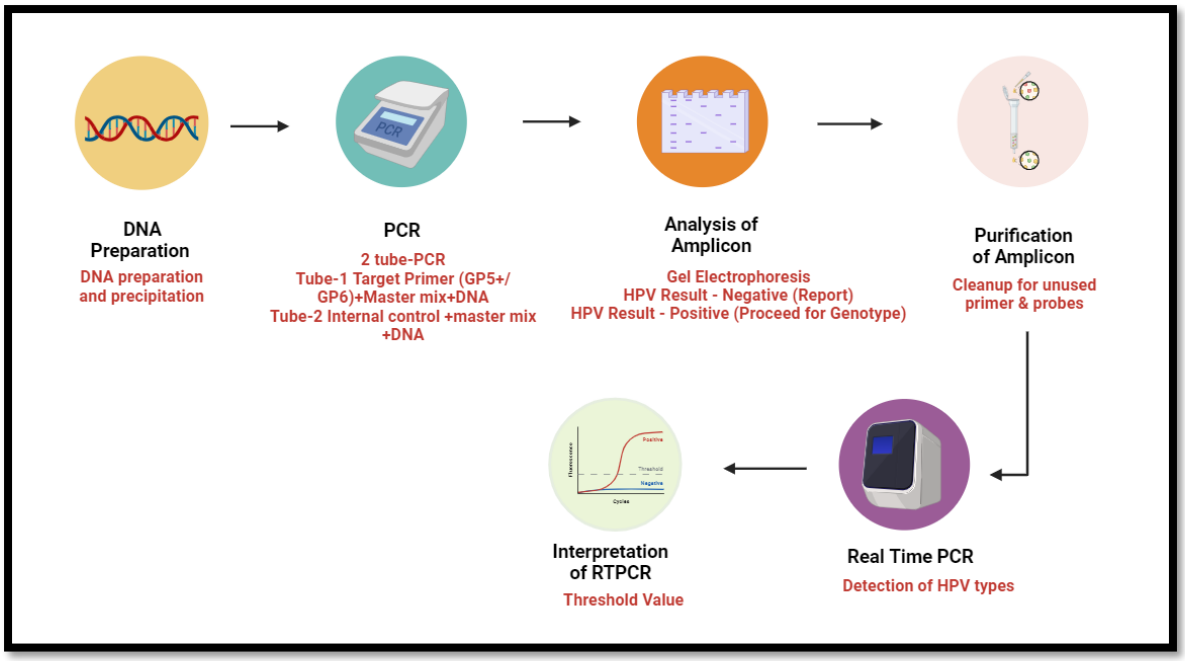

Figure 3.3: Work flow of HPV detection and HPV typing

3.7 DNA isolation

- DNA isolation was done by Phenol Chloroform method (Gopalkrishna, Francis, Sharma, & Das, 1992; Moberg, Gustavsson, & Gyllensten, 2003; Payan et al., 2007). Cervical smears were collected, and stored at room temperature in a slide container. The smear was scraped in PBS solution which was 2.5 ml. The method involves mainly two steps: 1. Washing in Tris-Triton buffer (TTB) and 2. Proteinase K digestion in Tris-EDTA buffer (TEB).

3.7.1 Washing in Tris-Triton buffer (TTB)

- 2 ml PBS solution containing scraped cervical cells was taken in a 2.2 ml Eppendorf tube and centrifuged at 10,000 rpm for 5 min. The cell pellet was washed once in 1 ml cold PBS and twice in 1 ml chilled TTB containing (10 mM Tris-HCl (pH 8. O), 10 mM MgC12, 300 mM sucrose, and 0.8% Triton X100). The pellet was collected by centrifuge at 10,000 rpm for 5 min.

3.7.2 Proteinase K digestion in Tris-EDTA buffer (TEB)

- The pellet was rewashed at maximum rpm for 5 min in 0.5 ml cold TE buffer containing (10 mM Tris-HCl, pH 8.0, 10 mM EDTA and 10 mM NaCl). Additional washings were given if the detergent used was not removed completely. Finally, the

pellet was resuspended in 200 µl TE buffer supplemented with 20mg/ml proteinase K, and incubated at 65°C water bath for 2.5 h. The tubes were vigorously shaken every 15-20 min to allow uniform lysis of the pellet and efficient extraction of DNA. When the solution became transparent it was assumed that the extraction was completed.

- Isolated DNA's quantity and quality were checked by running on 0.8% agarose gel as well as by spectrophotometer. After validating the DNA isolation results, DNA was stored at -20^{o}c until PCR was performed (Gopalkrishna et al., 1992).

3.8 Polymerase chain reaction-based detection of Human papillomavirus

- Conventional single plex PCR was carried out using the following Forward and Reverse primer of the beta-globin gene (Amplicon size: 268 bp) with melting temperatures of 62oc and 60oc for forward and reverse primer respectively. This protocol was performed for the checking of DNA quality through beta globin primer.

- Beta globin primer: Forward: GAAGAGCCAAGGACAGGTAC, Reverse: CAACTTCATCCACGTTACACC

3.8.1 Master mix for first round PCR

- Master mix (12.5 µl) for the Conventional single plex PCR was prepared by using 5.9 µl sterile H2O, 1.3 µl of 10X PCR buffer, 1µl of 10 mM dNTP mix, 1 µl of 25 mM MgCl2, 0.3µl of 5 Units/µl* Taq DNA polymerase, 0.5µl of 20 pmol/µl forward and reverse primer and added 2 µl of crude DNA sample as Template DNA. Mix the all contents in PCR tubes in a PCR centrifuge for 30 sec to 60 sec at 5000rpm, to prevent bubbles (M. P. Singh, Gupta, Deepak, Kumar, & Ratho, 2017).

- The master mix for the cycle of PCR was subjected to 35 cycles of amplification in the Thermal Cycler; 5 min initial Denaturation at 94°C for the first cycle, 45 sec Denaturation at 94°C 35 cycle, 45 sec annealing at 58°C 35 cycle, 45 sec extensions at 72°C 35 cycle, 7 min final extension at 72°C and hold at 4°C. When the program was finished, PCR tubes were removed and stored at 4°C.

3.8.2 Agarose Gel Electrophoresis

- The PCR product was detected by loading aliquots of each sample (after well mixed with loading dye) into wells of 1.5% agarose gel. The standard 100 base pair DNA ladder was also run along with the sample to confirm the presence of the beta-globin gene.

3.9 HPV typing

- Human papillomavirus (HPV) is a group of more than 10 related viruses. Some types of HPV are considered high risk because they are associated with cancer. HPV tests detect the genetic material (DNA) of the virus. HPV cannot be cultured in vitro, and immunological tests are inadequate to determine the presence of HPV. PCR is a sensitive and specific molecular technique in which a diagnostic segment of Human papillomaviruses DNA is amplified and detected on a real time PCR platform with the help of fluorescent dyes specially used for this purpose.
- HPV typing was carried out by SARAGENE Human Papillomavirus RT-PCR kit (manufactured by CoSara Diagnostics Private Limited), which is a PCR-based assay for qualitative detection of HPV DNA of the 15 high-risk HPV genotypes, i.e., 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 67, & 68.
- Interpretation: In case of positive samples, the curve should cross the cycle threshold line prior to completion of PCR cycles while in negative samples the curve should fail to touch the cycle threshold line during the entire course of PCR. In all cases, the positive sample should cross the cycle threshold line while the negative one should remain below it. Note: All extracted DNA from clinical samples are independently tested for absence of PCR inhibition prior to reporting.
- Assay parameters: Analytical sensitivity: 100 genome copies/mL, Test specificity: 100%.

3.10 Statistical analysis

- Chi square test was performed to correlate menopausal symptoms and included in discussion section.
- Specificity and sensitivity of AgNOR, ki-67/p53 parameters among normal cervix, LSIL, HSIL, and cancer-positive were calculated using Microsoft Excel. Statistical tests like ANOVA were calculated using Microsoft Excel. Analysis of variance (ANOVA) divides the observed aggregate variability present in a data set into two categories: systematic variables and random factors. On the provided data set, the systematic components have a statistical impact, but the random factors do not. In a regression research, analysts employ the ANOVA test to ascertain the impact of independent factors on the dependent variable.

- The Formula for ANOVA is: $F = \frac{MST}{MSE}$

  where: F = ANOVA coefficient, MST = Mean sum of squares due to treatment, MSE = Mean sum of squares due to error.