DOI: 10.7860/JCDR/2021/45836.14478

Review Article

Obstetrics and Gynaecology Section

Diagnostic Efficacy of DNA Ploidy in Liquid Based Cervical Cytology using DNA Cytometry

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ABSTRACT

Worldwide cervical cancer is the fourth most common cancer in women and high incidence is reported from India. Liquid Based Cytology (LBC) provides good morphology for detection of cellular abnormalities. We, therefore, reviewed diagnostic efficacy of conventional Pap staining, flow cytometry and Human Papilloma Virus (HPV) testing in cervical pre cancer and cancer. Narrative review of cervical pre cancer and cancer candidate biomarkers including Pap staining, HPV and flow cytometry from cervical cytology fluids, is based on a detailed review of the literature. Based on the so far conducted studies, a promising conclusion can be drawn, that cytometry when coupled with HPV DNA typing or the conventional cytology gives better results as compared to that of conventional cytology or DNA cytometry alone. Liquid cytology provides a good and stable source of cervical cells to carry out ploidy studies using DNA cytometry. The procedure should be used in conjunction with LBC and HPV detection.

Keywords: Flow cytometry, High grade squamous intraepithelial lesion, Human papilloma virus, Low grade squamous intraepithelial lesion, Squamous cell carcinoma

INTRODUCTION

LBC has been shown to be more effective than the conventional Pap smear for screening of cervical cancer by significantly improving detection of low-grade and high-grade squamous intraepithelial lesions and a significant improvement in specimen adequacy [1]. The greatest advantage of LBC is that ancillary techniques like HPV testing and DNA ploidy can be performed on the remainder left over sample in the vial. DNA ploidy has been effectively performed for diagnostic and prognostic applications of cervical, ovarian and endometrial cancer. Image cytometry is the most commonly used cytometry technique, others include laser scanning cytometry and flow cytometry. Researchers have utilised liquid based preparation for measurement of DNA content of cervical epithelial cells as it provides satisfactory monolayer for DNA measurement. For flow Cytometric DNA analysis, cells in suspension are required which may be prepared from cervical biopsies or LBC sample. Cell cycle analysis on LBC samples provides useful information for selecting women with chance of developing lesion (Aneuploidy or High S phase fraction) [2-4]. This article provides a review of various Cytometric researches aimed at studying DNA ploidy in cervical cytology samples and to evaluate whether LBC proves to be a suitable sample type for ploidy studies.

Cell Cycle and DNA Ploidy

DNA content of a cell is an essential tool to monitor cell proliferation, cell cycle and DNA ploidy. Cell division undergoes through various phases which form the cell cycle with different amount of DNA content in each phase. Before the cell division starts, the cell remains in a resting phase, known as the G0 phase. As soon as the cell receives signal, the cell starts proliferating and enters G1 phase. In this phase, the cells are diploid and the chromosome number is 2N. The cell then enters S phase which is called the synthesis phase and where the DNA replicates. Replication leads to tetraploidy which contains double the amount of DNA content. This is followed by G2 phase when cell prepares for division and enters the mitosis M phase. The cell in a cell cycle has to overcome two checkpoints G1/S and G2/M. At these checkpoints the cells are checked for DNA damage. These checkpoints prevent the cell to enter into S and M phase, respectively until the damage is repaired. In normal steady state conditions and in low grade/ early lesions, 85% cells are in G0/G1 phase and 15% are in G2/M phase. This anomaly can serve as an efficient diagnostic tool to detect cancer in cells at an early stage [5].

Burden of Cervical Cancer

In women, cancer of the cervix is the 4th most common cancer with 528,000 new cases and 266,000 deaths in 2012. This accounted for 7.5% of all female cancer deaths in 2012. Ninety percent of deaths due to cervical cancer occur in developing regions. The occurrence and mortality due to cervical cancer is highest in Africa and Melanesia [6].

It was estimated in 2015 that every year 122,844 women are diagnosed with cancer of cervix and 67,477 deaths are contributed by cervical cancer in India. It is 2nd most common cancer in females of reproductive age group. In general population, 5% women are expected to harbour HPV-16/18 infection, and most of the invasive cervical cancers (83.1%) are attributed to HPV-16/18 [7].

Cervical Cancer Screening and Diagnosis

Invasive squamous carcinoma of the cervix is the result of pre-invasive lesions known as Cervical Intraepithelial Neoplasia (CIN). In histology CIN is graded as mild dysplasia (CIN 1), moderate dysplasia (CIN 2) and severe dysplasia (CIN 3). Out of these CIN 1 and 2 may regress but CIN 3 progresses to the invasive carcinoma [8,9]. The Bethesda system has improved the reporting and classified it as Negative for intraepithelial lesion or malignancy (NILM), Atypical Squamous Cells of Undetermined Significance (ASC-US), Low grade Squamous Intraepithelial Lesions (LSIL), High Grade SIL (HSIL) and Invasive Carcinoma [10].

Cervical cancer is caused by the HPV infection which induces CIN lesions in the cervix [11-13]. Dysregulated viral oncogene expression caused by integration of viral oncogene in affected cells results in chromosomal instability, aneuploidization and progression of the disease [14]. Epigenetic changes and interference of the viral oncogene in the normal cell cycle may also lead to variation in nuclear DNA content [12,15]. There is evidence that chromosomal instability and aneuploidisation precede and favour high risk HPV genome [14]. Studies have shown that the variation in the ploidy content indicates invasive carcinoma or prospective neoplastic development in cervical

dysplasia [16,17]. However, there is no technique which can predict cervical dysplasia clinically with high sensitivity.

Pap test plays important role in screening of cervical carcinoma; however its sensitivity and specificity is limited. It has been reported by Sulik SM et al., (2001) that LBC is more sensitive (90%; 95% CI 77-96%) compared to conventional cytology (79%; 95% CI: 59-91%) for CIN 2 or more severe lesions [18]. LBC has been found to be equivalent or superior to conventional cytology for CIN diagnosis. False positive rate of pre-malignant and malignant lesions by Pap test is approximately 30% and false-negative rate lies between 6-55% [19-23].

Analysis of cervical biopsies has shown that women who develop LSIL have a probability to develop moderate to severe CIN [13]. To diagnose and prevent cervical malignancy a number of diagnostic techniques have been developed. One such technique for the assessment of DNA ploidy to detect cervical dysplasia is DNA cytometry. DNA ploidy has been identified as a prognostic factor for estimation of risk of progression of cervical lesions to invasive cervical carcinoma [24-29]. Aneuploidy aids in identification of dysplasia and provides a predictive value for malignant transformation [30]. Cytometric techniques provide additional information for identification of dysplasia and neoplasia beyond morphology.

Methodology for DNA Ploidy Estimation and Interpretation of Results

DNA image cytometry: Several researchers have utilised the method described below with minor variations to estimate DNA ploidy in LBC. After preparing a second monolayer from the remaining LBC sample, slides are air dried and fixed in buffered formalin for 30 minutes. Following 1-hour acid hydrolysis (5N hydrochloric acid) at room temperature staining with Feulgen (Thionin) is carried out. For calibration of each staining procedure calibration slides are added. Image cytometry is then performed using ploidy measurement software on image cytometer. By and large the interpretation of DNA histogram is similar in all studies with recognition of diploid, polyploid, aneuploid peaks and S Phase fraction. Some researchers have suggested minor modifications in interpretation which are as follows: Auer GU et al., (1980) presented the DNA ploidy value as a "c" for chromosome [31]. The DNA cytometry histogram was classified as normal or suspect; normal corresponding to diploid with low proliferation fraction and polyploid (diploid + tetraploid) histograms without any cells exceeding 5c. All other histograms with any of these were regarded as suspect and patients with suspect results underwent colposcopy.

1. Any cells with DNA content >5c
2. Diploid cells with >10% cells in proliferation fraction
3. Aneuploid cell population

Study of Bollmann M et al., 2006, suggested interpretation of the DNA histogram which is as follows:

1. Diploid as DNA peak between 1.8c and 2.2c.
2. Minimum of 2 stem lines with DNA peaks between 1.8c-2.2c and 3.6-4.4c or around 8c and 16c to be read as polypoid.
3. DNA peaks beyond "diploid" or "Polyploid" peaks and/or presence of single cells with DNA content >9c to be read as aneuploid [32].

Further variation in the histogram interpretation was suggested by Guillaud M et al., 2006 [33]. They defined DNA aneuploidy as a function of three parameters:

1. Total number of counted cells on a slide;
2. A DNA ploidy index, beyond which a cell is called aneuploid; and
3. A cut-off value presenting the number of cells, beyond which a specimen is called aneuploid.

The DNA ploidy index was determined within the range of 2c-9c. The aneuploid cells were determined in the range of 1-50 cells. The sensitivity and specificity was calculated by combining the above definitions to find the best diagnostic accuracy. In a number of studies, 2c DNA content is defined as a diploid cell, 4c as tetraploid cell and 5c as a cut off for aneuploid cell, however Bollmann R et al., Bollmann R et al., and Lorenzeto M et al., suggest 9c [34-36]. Number of cells with DNA exceeding beyond 5c is frequently called the 5c-exceeding rate (5cER) [37].

Flow cytometry for DNA ploidy estimation: Researchers have analysed DNA ploidy by flow cytometry in various solid tumours and LBC samples [38-44]. Single cell suspension was prepared by mechanical or enzymatic disaggregation of the tissue followed by staining with Propidium Iodide (PI) containing Ribonuclease (RNase) for 30-60 minutes at 4°C before analysing on flow cytometer. Gates were set up on FL2W versus FL2A dot plot to exclude doublets and aggregates. FL2A area signals were then used to generate single parameter DNA histograms. Usually two major peaks are observed; one peak is labelled as diploid and another one as an aneuploid (if present). A sample with single G0/G1 peak is defined as diploid, while a sample with two distinct G0/G1 peaks is considered as DNA aneuploid. DNA Index (DI) for aneuploid cells is obtained by dividing the mean channel number of the aneuploid G0/G1 peak by the mean channel number of the diploid G0/G1 peak. For diploid cells DI corresponds to 1 while DI≠1.0 defines aneuploidy. Coefficient of Variation (CV) of G0/G1peak is used to check the quality of DNA histogram. Some studies have also used ModFit software for analysis of DNA histogram.

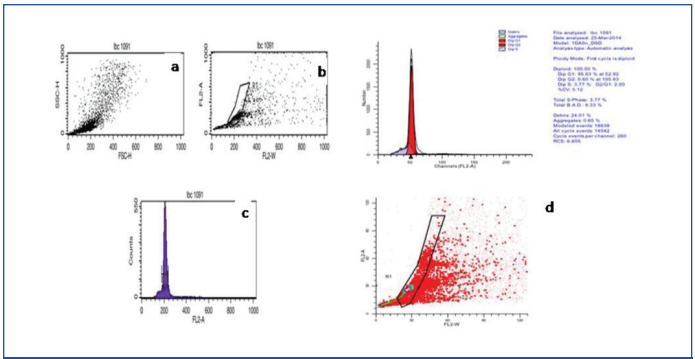
Authors have successfully standardised flow cytometry to assess DNA ploidy in LBC samples of cervical pre cancer and cancer. Cytologically confirmed cases of LSIL, HSIL and SCC along with cases negative for intraepithelial lesion or malignancies (NILM) were used as control for DNA ploidy analysis. Briefly, LBC samples were centrifuged to obtain a cell pellet and washed with equal volume of Phosphate buffer saline (PBS, pH-7.4). Cells were stained with Telford reagent and processed as per Mishra S et al., [45]. Stained cells were acquired using flow cytometer and dot plot and histograms as shown in [Table/Fig-1a,b,2a,b]. Diploid samples were identified by the presence of single G0/G1 peak [Table/Fig-1c,d], while aneuploidy was defined when DI≠1.0 [Table/Fig-2c,d].

Laser Scanning Cytometry

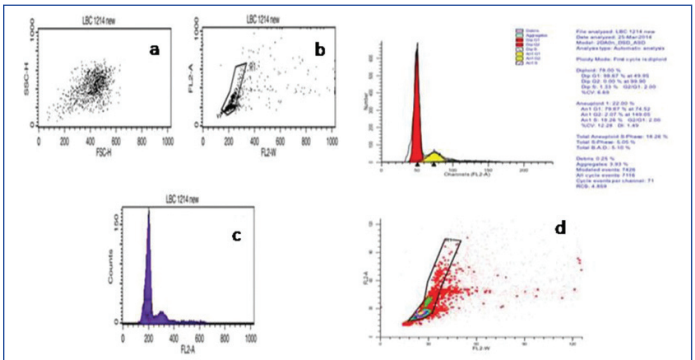
This technique uses the 2nd monolayer slide stained with Propidium iodide and RNase for 1 hour at 37°C. After the incubation slides were mounted in glycerol and covered with glass. Using the laser scanning cytometer, at least 10,000 cells were measured and diploid and aneuploid cells were defined as per the first peak intensity of DNA histogram containing normal leucocytes. The Coefficients Of Variation (CV) were reported in a range between 4.0 and 7.5. Cells with elevated DNA content as stained by PI (>5c and >9c) were individually evaluated. Haroske G et al., defines, isolated cells with non-superficial cell morphology and a DNA content of greater than 9c as "Rare cells" with abnormally high DNA content [30].

Evaluation of Ploidy as a Diagnostic Procedure in Cervical Cancer

DNA ploidy measurement has been established as a prognostic factor and to be of prognostic significance in ovarian and endometrial cancer though in cervical cancer there are conflicting results [46-52]. In few studies, flow cytometric analysis of DNA ploidy in CIN and invasive cervical has been reported to have prognostic significance for estimation of disease progression into more advanced lesion [38]. The published researches aimed to study the value of DNA ploidy by image cytometry as well as flow cytometry on LBC and solid tissue are summarised in the [Table/Fig-3]. According to most of the studies, HPV typing and DNA ploidy measurement helps in the identification of cytologic dysplasia. LBC has proven to be suitable and useful tool for performing DNA ploidy.



[Table/Fig-1]: Shows the acquisition of cervical epithelial cells on flow cytometer, stained with Telford Reagent. (a-c) Shows acquisition of stained cells on FSC vs. SSC, FL2-A vs. FL2-W and FL2-A vs. Count on Cell Quest Pro software (B.D Biosciences, Singapore). (d) Shows the analysis of acquired FCS file on ModFit LT 3.2 (Verity Software House). Based on ModFit analysis case was found to be Diploid with single G0/G1 peak. Histogram statistics showed on top right.
Image courtesy Mishra et al. [45]



[Table/Fig-2]: Shows an Aneuploid case of HSIL on cytomorphology acquired on flow cytometer, stained with Telford Reagent. (a-c) Shows acquisition of stained cells on FSC vs. SSC, FL2-A vs. FL2-W and FL2-A vs. Count on Cell Quest Pro software (B.D Biosciences, Singapore). (d) Shows the analysis of acquired FCS file on ModFit LT 3.2 (Verity Software House). Based on ModFit analysis case was found to be Aneuploid on appearance of second G0/G1 population to the right of first G0/G1 peak with DNA index of 1.49.
Image courtesy Mishra et al. [45]

DISCUSSION

DNA ploidy has proved to be an effective tool in detecting high grade neoplastic lesions which helps in the early screening of cancer. Compared to conventional cytology, DNA Cytometry has better sensitivity and specificity. Among the various kinds of cytometry, image cytometry has been widely used and has given positive results in detecting neoplastic lesions. Although flow cytometry is a common modality for studying DNA ploidy in cell suspension viz., blood cells and body fluids, there are only few studies available for assessment of DNA ploidy by Flow Cytometry in LBC samples [38,39].

[Table/Fig-3] suggests that LBC sample is suitable enough to study DNA ploidy and other ancillary techniques. In a Study by Saxena M et al., sensitivity and specificity for diploid G0/G1 to discriminate the cases from controls was 96.77% and 100%, however total S phase and aneuploidy revealed 100% sensitivity [39]. In contrast to this, Singh M et al., reported aneuploidy in 51.31% mild, 77.77% moderate and 91.66% severe cases. In ASCUS, aneuploidy was found in 14.03% cases and interstingly, in 8.69% of controls [38]. Authors further suggested that cases which were found aneuploid should be followed-up for developing advanced grade lesion.

When both cytometry and conventional cytology tests are considered in combination, the figures rise up to 100% and 91.8%, respectively. Though these additional tests improve the sensitivity and specificity, it increases the cost. DNA ploidy analysis appears to be an attractive technology for established programs [33].

[Table/Fig-4] represents the diagnostic efficacies of various techniques used for the diagnosis of pre cancer/cancer in a cervical sample and suggests a diagnostic algorithm for cervical cancer screening. The LBC is much sensitive and specific as compared to the conventional cytology. Depending on the grade of intraepithelial neoplasia, further workup on HPV testing or DNA ploidy can be carried out. As seen in the [Table/Fig-3] sensitivity of HPV testing is high whereas the specificity of DNA ploidy is high, hence as proposed by various authors DNA cytometry when used in association with HPV testing or conventional cytology gives a better sensitivity and specificity [34-37,53]. Apart from these techniques, some other techniques have also been successfully tried on LBC samples and they are immunocytology using $p16^{INK4a}$ marker and HPV E6/E7 mRNA detection [54]. They can be used along with the other techniques to increase the diagnostic accuracy [55-58].

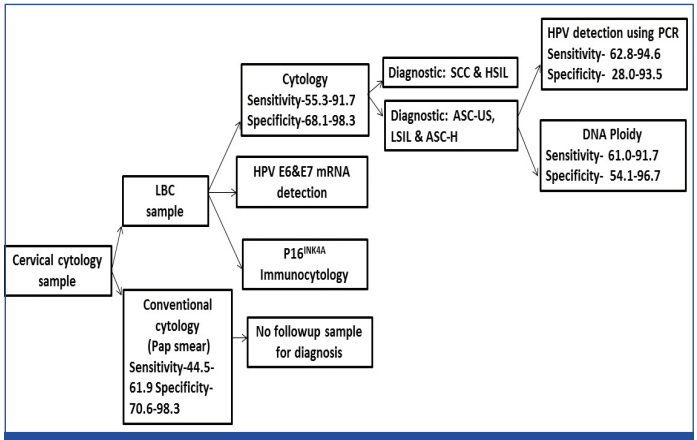
CONCLUSION(S)

Cytometry when coupled with HPV DNA typing or the conventional cytology gives better results as compared to that of conventional cytology or DNA cytometry alone. Thus, LBC media provide a good and stable source of cervical cells to carry out ploidy studies using DNA Cytometry. The procedure should be used in conjunction with LBC and HPV detection. Liquid based preparation allows to measure DNA content of cervical epithelial cells that provides more accurate and sensitive result which can alternatively serve as a marker for early stage diagnosis. Use of LBC sample for measurement of DNA content of cervical epithelial cells in the form of aneuploidy or high S

S. No.	Author (years)	Objective of the study	Number of cases	Result	Conclusion
1.	Lorenzato M et al. (2002) [36]	To study the usefulness of DNA ploidy measurement on LBC smears showing conflicting results between cytology and HR-HPV typing using Image Cytometry	Total 7944 cases out which 984 underwent ploidy	Normal DNA profile predicted clearance of HPV with sensitivity 81.5%, specificity 45.4%, PPV 69% and NPV 62.4% In persistent HR-HPV infection suspected DNA profile PPV increased from 10.8% to 22.7%, for HSIL detection sensitivity was 95.2%	Cytometry should be complemented with HR-HPV test to select women with a high risk for developing a histologic lesion.
2.	Bollmann R et al. (2003) [35]	To determine HPV typing and DNA ploidy of squamous intraepithelial lesions in LBC samples using Laser Scanning Cytometry	112 SIL cases	Out of 112 cases, 110 (98.2%) were HPV+, out of these 95 (84.8%) were HR-HPV+ and 46 out of 95 (48.4%) presented aneuploid squamous cells with >9c DNA content.	Complex analysis of cervical lesions from LBC samples is highly informative HPV typing and DNA ploidy measurement helps in the objectivation of cytologic atypia and both can be performed efficiently from the same LBC sample.
3.	Shirata NK et al. (2003) [55]	To evaluate nuclear DNA content of cervical lesions in LBC specimens using Static Image Cytometry	Total 47 samples out of which CIN1;n=25, CIN2;n=5, CIN3;n=2 and chronic cervicitis=15	Chronic cervicitis All diploid CIN1 44% diploid, 12% tetraploid, 32% aneuploid, 12% polyploid CIN2 60% diploid, 40% aneuploid CIN3 100% aneuploid	LBC proved to be suitable and highly useful for DNA analysis. Discrimination could be made between CIN3 and CIN1,2 but not between CIN1 and CIN2
4.	Guillaud M et al. (2006) [33]	To compare DNA ploidy with HPV-testing and conventional cervical cytology as a primary screening test for HSIL and cancer using Image Cytometry	1555 patients	Cytology Sensitivity 54% Specificity 93% PPV 41% NPV 92% HPV Testing Sensitivity 91% Specificity 80% PPV 70% NPV 90% DNA ploidy Sensitivity 61% Specificity 91% PPV 59% NPV 93%	DNA ploidy shows comparable sensitivity, specificity, PPV and NPV values to conventional cytology and HCII DNA ploidy is semi-automated and can be performed in less than 8 hours.

5.	Yu XR et al., (2011) [56]	To perform cell quantitative analysis of DNA ploidy in cervical cancer screening using Image Cytometry	776 women	Conventional Cytology Sensitivity 61.9% Specificity 98.3% DNA ploidy Sensitivity 83.6% Specificity 96.7%	Automated DNA cytometry may be a useful tool for cervical cancer screening in developed countries and has a competitive sensitivity and specificity compared to conventional cytology.
6.	Tong H et al., (2009) [57]	To perform DNA ploidy cytometry testing for cervical cancer screening in China using Image Cytometry	11,999 women for DNA cytometry testing and 11,994 women for cytologic testing	Diagnosis of cancer: DNA cytometry-40 Cytology-24 Cytometry Sensitivity 91.7% Specificity 54.1% Conventional Cytology Sensitivity 44.5% Specificity 70.6% Cytology and Cytometry Sensitivity 100% Specificity 91.8%	DNA cytometry is more beneficial in mass cervical cancer screening with greater sensitivity and positive predicted value than the conventional cytology testing in the developing countries.
7.	Li Z et al., (2010) [58]	To reduce the false-negative rates of population based cervical screening programs employing conventional cytology in combination with automated DNA Image cytometer	3603 women	Total diagnosis: 51 cases including, 27 CIN2, 16 CIN3 and 8 Invasive cancer cases. Cytology No. of Diagnosis 29 Sensitivity 56.8% Specificity 86.2% DNA Cytometry No. of Diagnosis 38 Sensitivity 74.5% Specificity 81.5% Cytology and Cytometry No. of Diagnosis 42 Sensitivity 82.4% Specificity 81.5%	Screening for high grade neoplastic lesions and cervical cancer by DNA Image cytometer or combination of conventional cytology and DNA Image cytometer is more sensitive than conventional cytology.
8.	Saxena M et al., (2010) [39]	Could addition of DNA content study using flow cytometry improves the detection of cervix cancer	Total of 100 including 38 normal and 62 cancer of cervix cases.	Fraction of Total S phase, Total Aneuploid and G2-M (Diploid) are significantly higher ($p < 0.01$); while G0-G1 (Diploid) and G0-G1 (Aneuploid) are significantly lower ($p < 0.01$) in cancer patients as compared to control. G0-G1 (Diploid) Sensitivity-96.77% Specificity-100% Total S phase or Aneuploid Sensitivity-100% Specificity 100%	G0-G1 (Diploid) may help in the diagnosis of carcinoma of the cervix which correlates well with histologically confirmed varied grading of cervical cancer as well as patient survival.
9.	Singh M et al., (2008) [38]	Study the DNA content by flow cytometry and to compare it with the cytological findings.	184 Cytologically diagnosed cases of mild (79),moderate (36), and severe (12) dysplasia along with 57 cases of ASCUS and 69 controls	Aneuploidy was found in 39/79 of mild, 28/36 of moderate, 11/12 of severe dysplasia, 8/57 of ASCUS and in 6/69 controls.	DNA flow cytometry can detect progressive lesions with the greatest possible sensitivity and specificity.
10.	Melsheimer P et al., (2004) [13]	DNA Aneuploidy and Integration of Human Papillomavirus Type 16 E6/E7 Oncogenes in Intraepithelial Neoplasia and Invasive Squamous Cell Carcinoma of the Cervix Uteri	Total 85 samples out of which CIN1/2 n=20, CIN3 n=50, Cacx=15	DNA aneuploidy CIN1/2= 4/20 CIN3= 16/50 Cacx= 12/15 HPV E6/E7 integration CIN1/2=1/20 CIN3=7/50 Cacx=12/15	Aneuploidization precedes integration of HR-HPV genomes in the progression of cervical dysplasia.
11.	Mishra S et al., (2017) [45]	Flow cytometric Analysis of DNA Ploidy in Liquid Based Cytology of Cervical Pre-cancer and Cancer	50 Cytologically diagnosed cases of Cervical cancer including 10 LSIL, 20 HSIL, 20 SCC and 31NILM cases as control	Mean diploid G1 values lowered significantly ($p<0.0$) while diploid S values were significantly ($p<0.01$) higher in both HSIL and SCC as compared to control	Diploid G1 and diploid S phase analysis do not appear to increase the overall sensitivity and specificity of detection.

[Table/Fig-3]: Summary of the studies assessing DNA Ploidy in LBC samples of cervical cytology and solid tissues.



[Table/Fig-4]: Suggested diagnostic algorithm for cervical cancer screening.

phase fraction provides an objective method to prognosticate and select women who may be developing lesions.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Jul 11, 2020
- Manual Googling: Oct 31, 2020
- iThenticate Software: Dec 28, 2020 (10%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was informed consent obtained from the subjects involved in the study? NA
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Jul 10, 2020**
Date of Peer Review: **Oct 06, 2020**
Date of Acceptance: **Nov 22, 2020**
Date of Publishing: **Feb 01, 2021**