VOCATIONAL REGISTRATION PROGRAMME IN CERVICAL CYTOLOGY (VRPCC)

Workbook

2nd Edition 2016

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Co-authors

VOCATIONAL REGISTRATION PROGRAMME IN CERVICAL CYTOLOGY (VRPCC)
TRAINING WORKBOOK

INTRODUCTION to the Second Edition

The Vocational Registration Programme in Cervical Cytology was developed by the National Cervical Screening Programme (NCSP) in 2007 to provide a national competency-based foundation programme for Bachelor of Medical Laboratory Science (BMLSc) graduates beginning a career in cervical cytology. Successful completion of the programme is a mandatory requirement for all practitioners entering cytology for the first time. The VRPCC is funded by the NCSP and administered by the National Cervical Pathology Training Service (NCPTS).

The VRPCC Workbook includes educational material amply illustrated by case photomicrographs and provides a repository of practical information for the working cytologist. The Workbook sets out requirements for various assignments and screening slide sets and tests that are to be completed as part of the programme. A Logbook is provided with the Workbook so candidates can track their progress through the programme.

As authors of the second edition, we particularly wish to acknowledge Dr Harold Neal, creator of the VRPCC and author of the first edition of the VRPCC Workbook. His foresight and work to put the programme in place has left a legacy for New Zealand cytologists. The second edition would not have been possible without his previous work. The structure of the VRPCC workbook has largely been retained, with some rearrangement in the ordering of chapters. Harold has been invited to continue to contribute, as author of *Section 6: The History of Cytopathology*. We hope this arrangement will be retained in future editions.

The second edition became necessary because of the move to 100% liquid-based cytology (LBC) in New Zealand in 2009. All previous conventional slide cytology photomicrographs have been replaced with LBC photomicrographs which include both ThinPrep and SurePath examples. In 2009 New Zealand also introduced hrHPV testing in specific clinical circumstances and information relating to this has been included in this edition.

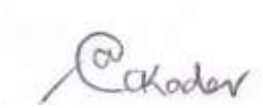
Others have contributed to the second edition also. Thanks go to Dr Fahimeh Rahnama for contributions to the hrHPV testing sections, Dr Diane Kenwright for reviewing Core topic 2.10: Invasive Squamous Cell Carcinoma and Christl Kirstein for contributions in relation to ThinPrep. We are grateful for assistance from both companies which support LBC services in New Zealand, BD (SurePath) and Hologic (ThinPrep) who provided technical information at various points. Cytology photomicrographs were taken by the NCPTS Training Team staff, using contributions of slides from New Zealand cytology laboratories. The original histology photographs taken by Harold Neal have almost all been retained in this edition.

We also wish to thank the three people who provided professional peer reviews of the chapters as the work proceeded: Dr Andrew Miller (Cytopathologist), Liz Pringle and Paul Spek

(Cytoscientists). Their comments were particularly helpful in shaping the final text and ensuring that the material included remained relevant to current cytology practice.

Final thanks go to Pauline Ward and Melinda Paterson (NCPTS Administrators) for assistance with formatting and proof reading, Southern Community Laboratories for managing the revision contract and the National Cervical Screening Programme for funding the project.

We hope that we have achieved our aim of providing an informative, engaging and stimulating workbook for all who use it and wish those commencing a career in cytology every success with their professional work in future years.



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CONTENTS

SECTION 2.0 MORPHOLOGY: CYTOPATHOLOGY and HISTOPATHOLOGY

TEXTBOOKS, REFERENCES and EDUCATIONAL MATERIAL

This workbook has been developed as a practical guide rather than a referenced text. A wide range of cytology textbooks and educational resources are available to VRPCC candidates through laboratory cytology departments. The following is a list of general reference texts and documents. Further references relating to specific topics are included at the end of the core topic sections.

- Boon M. 1991. *The Pap Smear.* Leydon: Coulomb Press.
- DeMay RM. 1996. *The Art and Science of Cytopathology* (Vol 1) Exfoliative Cytology. Chicago: ASCP Press.
- Geisinger KR, Stanley MW, Raab SS, et al. 2004. *Modern Cytopathology*. Philadelphia: Churchill Livingstone.
- Gray W, McKee G (eds). 2003. *Diagnostic Cytopathology* (2nd Ed). Philadelphia: Churchill Livingstone.
- Koss LG, Melamed MR (eds). 2006. *Koss' Diagnostic Cytology and its Histopathological Bases* (5th Ed., Vol 1). Philadelphia: Lippincott Williams &Wilkins.
- Meisels A, Morin C. 1997. *Cytopathology of the Uterus* (2nd Ed). Chicago: ASCP Press.
- NHSCSP (UK) 2000. *Cervical Cytopathology Education Workbook: Answerbook.* London: National Health Service Cervical Screening Programme (UK).
- Rubin E, Farber J (eds). 1999. *Pathology* (3rd Ed). Philadelphia: Lippincott Williams &Wilkins.
- Weid GL, Keebler CM, Koss, LG, et al. (eds). 1992. *Compendium on Diagnostic Cytology* (7th Ed). Chicago: Tutorials of Cytology, International Academy of Cytology.

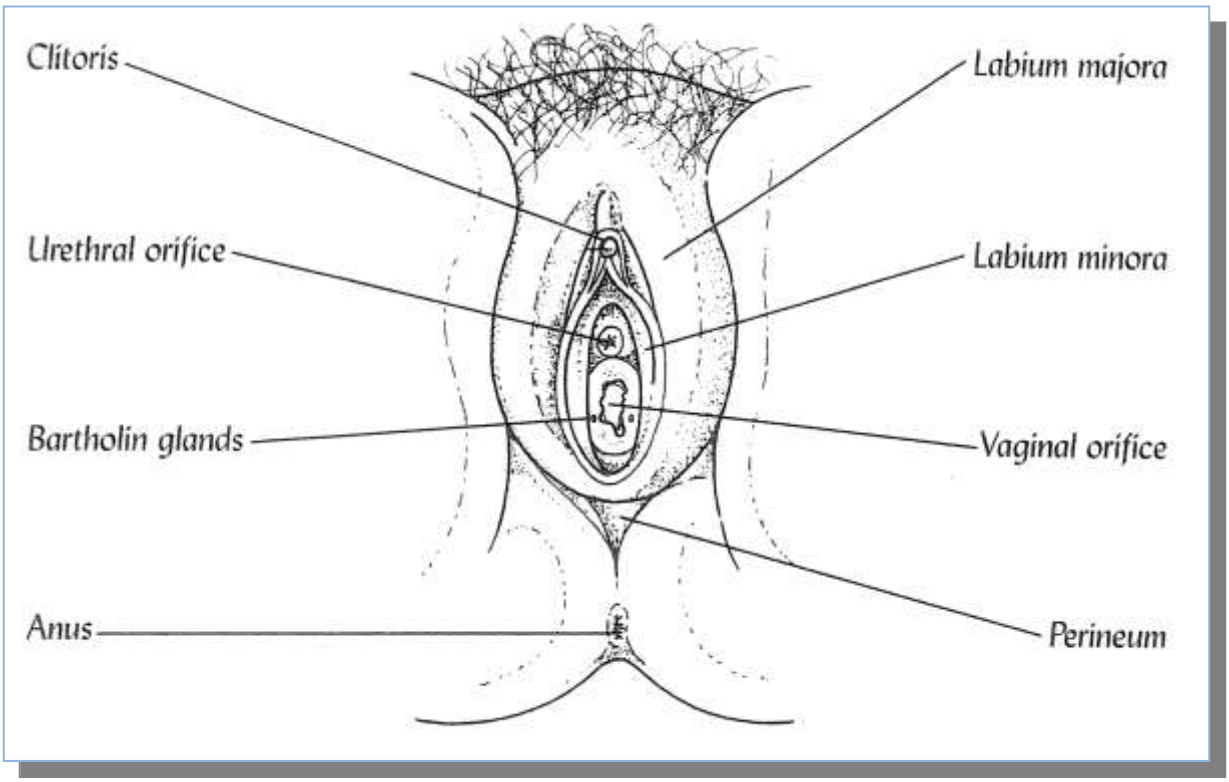
SECTION 1.0
SAMPLING, CYTOPREPARATION AND TECHNICAL CONSIDERATIONS

1.1 CORE TOPIC: ANATOMY AND PHYSIOLOGY

1.1.1 Introduction

The aim of this section is to provide basic but essential knowledge about the anatomy and physiology of the female genital tract. This will enable the practitioner to provide relevant information to sample takers and to appreciate the requirements for ensuring a good quality liquid based cytology (LBC) sample.

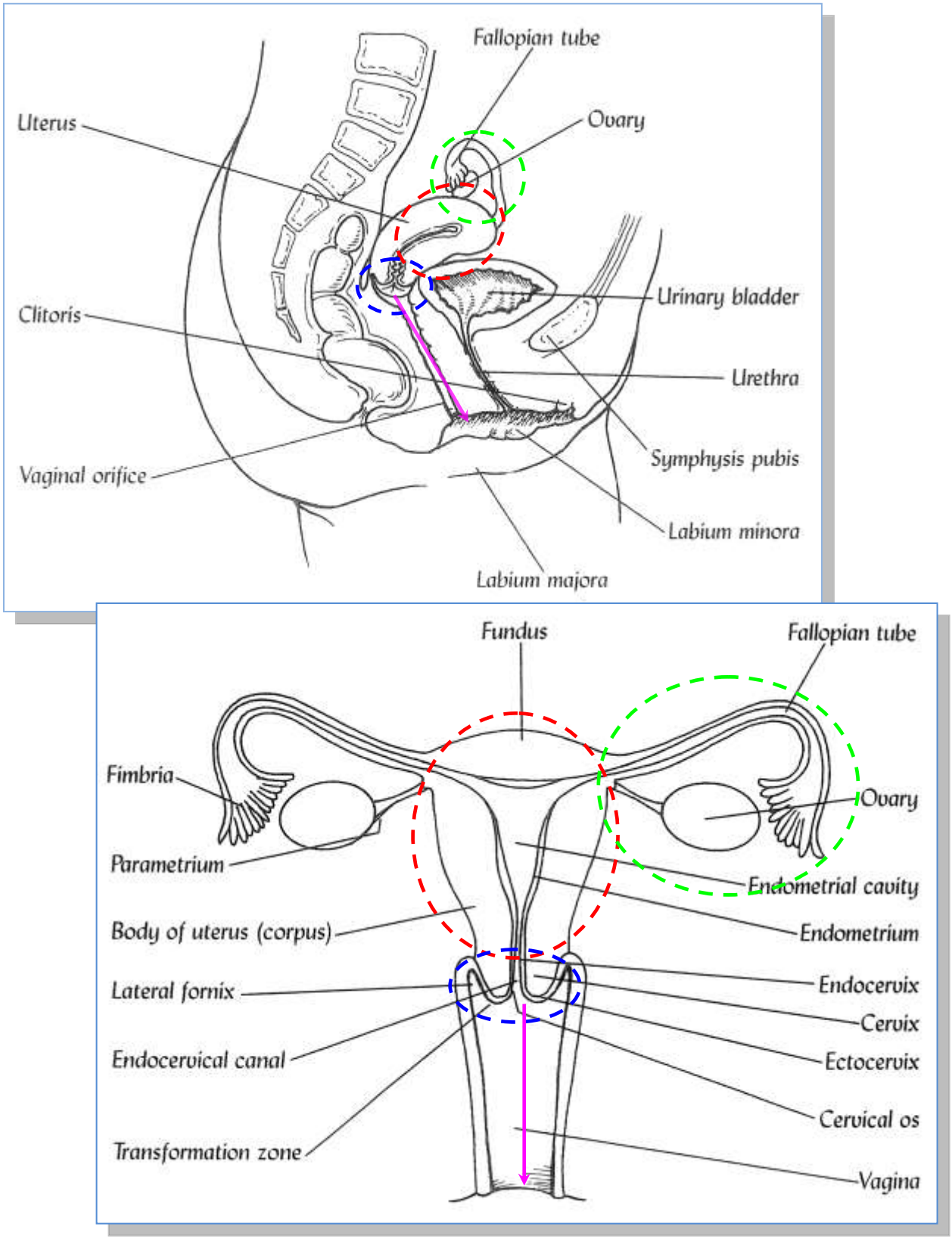
1.1.2 Anatomy of the female genital tract



The diagram above and the next two diagrams (pg 2) show the anatomy of the female external genitalia and the female genital tract. It is important for the cytoscientist/cytotechnician to have a good understanding of these anatomical locations in order to:

- Appreciate the sites that the cells come from
- Be able to converse with colleagues
- Offer advice to sample takers
- Relate pathological conditions
- Appreciate the sensitivity of the procedure and the anxiety women may experience during sampling of the cervix
- Develop cultural awareness

(Diagrams adapted from NHS Cervical Cytopathology Education Workbook).



Upper – Anatomical location of the female genital tract (sagittal view)
Lower – Internal organs of the female genital tract (anterior view)
Key: Vagina – pink arrow: Cervix/endocervix – blue circle;
Uterus – red circle; Fallopian tube and ovary – green circle.

1.1.3 Physiology of the female genital tract

Before studying the cytological changes seen at the various stages of the menstrual cycle and over the lifetime of a woman, it is helpful to have an understanding of the various female hormones and their effects. Hormonal status also influences the approach to taking cervical cytology samples and the interpretation of cellular content of the sample, as there is a direct association between oestrogen and progesterone levels and the positioning of the squamo-columnar junction (SCJ). The location of the SCJ will influence the device or devices the sample taker uses to adequately sample the ectocervix and endocervix in order to provide the cytoscientist/cytotechnician with an adequate and representative sample. Poor sampling can result in pre-cancerous abnormalities of the cervix being missed, reducing the success and efficiency of the National Cervical Screening Programme in reducing the incidence and mortality of invasive disease. One of the roles of the cytoscientist/cytotechnician is to recognise the reasons that result in an unsatisfactory sample in order to provide accurate and constructive advice back to the sample taker.

Hormone production

The genital tract is morphologically complete at birth but does not function until the hypothalamus has matured, as the production of female hormones is stimulated and regulated by the central nervous system.

The hypothalamus and pituitary gland are located under the thalamus at the base of the brain. The hypothalamus secretes "releasing-factors" which are transferred to the anterior lobe of the pituitary via a closed circuit blood supply. The anterior pituitary gland is responsible for the release of follicle stimulating hormone (FSH) and luteinising hormone (LH). FSH and LH both have a Molecular Weight of 35,000. They are very similar in chemical composition, with an identical α chain and slight differences in amino acid structure of the β chains. FSH and LH have specific effects on the gonads.

The hypothalamus controls the release of hormones from the anterior pituitary gland via the hypothalamic-hypophyseal portal system, the closed circuit blood supply. The anterior lobe of the pituitary gland produces 7 hormones including FSH, LH, prolactin and growth hormone. Cells in the hypothalamus produce hormone releasing substances which affect the production of FSH and LH from the pituitary as follows:

Neuroendocrine cells produce FSH-releasing and LH-releasing factors
↓
The releasing factors enter the portal circulation
↓
Stimulates the production of FSH and LH in the anterior lobe of the pituitary
↓
FSH/LH is released into the general circulation

Profile of hormone release

The relationship between the hypothalamus and anterior pituitary gland is responsible for the release of LH and FSH, which in turn has an effect on the gonads. This leads to production of the hormones oestrogen and progesterone. These two latter hormones then have a negative feedback effect on the hypothalamus to regulate the production and release of FSH and LH.

This regulatory cycle is responsible for the changes in the ovary and endometrium that occur during the menstrual cycle.

During the menstrual cycle, LH shows the "tonic" pattern of release that is required for gamete development and maintenance of reproductive tissue, until mid-cycle. Just prior to mid-cycle, LH shows a "surge" pattern with a very dramatic increase in release. This release corresponds to ovulation. LH release drops dramatically after ovulation. FSH shows less fluctuation than LH over the course of the cycle.

If enough FSH/LH is secreted, the ovaries will be stimulated causing the follicles to ripen and begin secretion of oestrogen and progesterone. FSH causes ripening of the primary follicle and oestrogen production. The sudden peak of LH at mid-cycle is necessary for ovulation to occur. Following the FSH/LH peak at ovulation, the gonad will produce progesterone as well as oestrogen.

1.1.4 Hormonal development with age

Childhood

Sex hormones in girls (and boys) show little variation until about the 8^{th} - 9^{th} year of age. At this stage the hypothalamus in girls begins to secrete releasing factors periodically, which results in the ovaries producing oestrogen.

Puberty

Sexual maturity is achieved at puberty. In girls this begins at about the age of 10 and is completed by about the 16th year. At around the 10^{th} year a dramatic growth spurt begins in girls. Breast development starts during years 10 -11 followed by pubic hair (11-13 years). The first uterine bleed occurs between years 11 and 13 and it is this first bleed that is referred to as the menarche. Both oestrogen and other growth regulators influence growth in girls. The uterine bleeding (menstruation) which occurs more or less periodically after menarche is usually anovulatory at first. Girls are not sexually mature until their ovaries are producing oocytes (ovulation) as well as hormones.

Sexual Maturity

Sexual maturity is defined as the ability to bear children. This begins with the first biphasic cycle (FSH/LH) and ends when ovulation ceases (about a 30 year span). The essential feature of sexual maturity is the ability to ovulate, releasing eggs. If the oocyte is fertilised by spermatozoa following ovulation and implantation is successful, pregnancy will follow but if this does not occur, the system will revert to the next menstrual cycle.

The menstrual cycle

The menstrual cycle is divided into different phases depending on ovarian activity and its effects. The classic cycle from day one of menstruation through to the day prior to the commencement of the next cycle is 28 days, but this varies from woman to woman as does the exact onset of the different cyclic phases. The menstrual cycle can be detailed as follows: (exact terminology may vary from author to author).

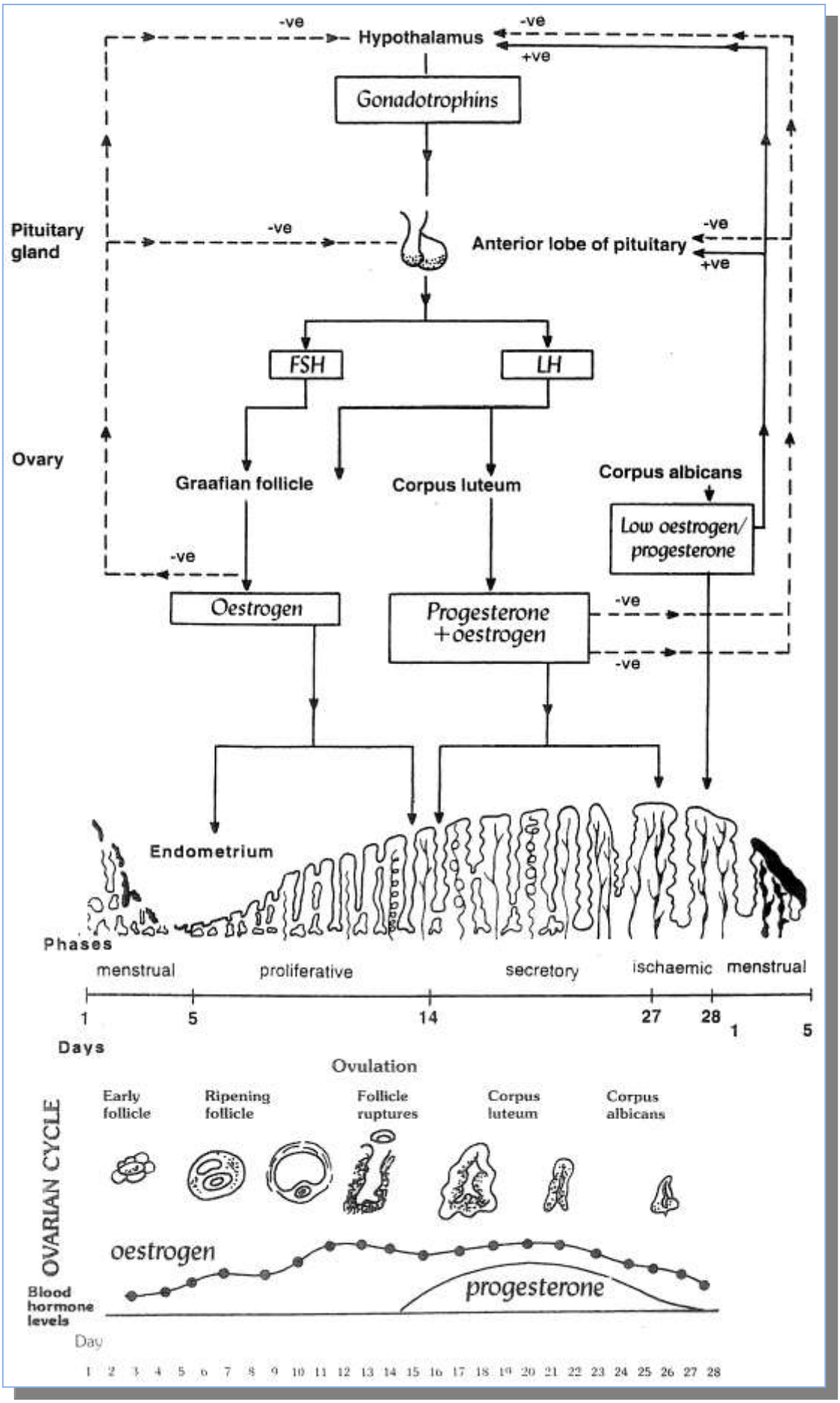
Phase	Menses		Follicular (Proliferative)	Pre-Ovulation	Ovulation	Luteal (Secretory)		Menses
Day	1	4	8	12	14	20	24	28

Following menstruation FSH/LH is gradually released leading to ripening of a follicle in the ovary, called the follicular phase. Oestrogen production increases.

At day 12 (pre-ovulation) there is the sudden increase of FSH/LH secretion leading to ovulation about 48 hours later, at day 14 (mid-cycle). The oocyte is released, and the remaining cells of the follicle secrete progesterone. The follicle is then called the corpus luteum. The FSH/LH peak drops dramatically and the ovary produces both oestrogen and progesterone. This phase lasts to the onset of menstruation and is called the luteal phase. The endometrium no longer proliferates but instead becomes secretory and produces glycogen which is the primary nutrient for a potential implanted ovum.

The combined oestrogen/progesterone levels inhibit the production of hypothalamic FSH/LH releasing factors thereby reducing FSH/LH production. This leads to a decrease in ovarian activity and a drop in oestrogen/progesterone levels. The endometrium degenerates, leading to bleeding i.e. menstruation. The cycle then repeats itself.

The diagram below (pg 6) combines hormone production and release with their effects on the ovary and endometrium for a normal 28-day menstrual cycle (*adapted from NHS Cervical Cytopathology Education Workbook).*


-ve
Hypothalamus
-ve
+ve
Gonadotrophins
Pituitary gland
-ve
Anterior lobe of pituitary
-ve
+ve
FSH
LH
Ovary
Graafian follicle
Corpus luteum
Corpus albicans
Low oestrogen/ progesterone
-ve
Oestrogen
Progesterone +oestrogen
-ve
-ve
Endometrium
Phases
menstrual
proliferative
secretory
ischaemic
menstrual
1
5
14
27
28
1
5
Days
Ovulation
OVARIAN CYCLE
Early follicle
Ripening follicle
Follicle ruptures
Corpus luteum
Corpus albicans
oestrogen
progesterone
Blood hormone levels
Day
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28

Pregnancy

The biphasic cycle is suspended during pregnancy and a prolonged extension of the secretory phase follows under the influence of progesterone. This increase in progesterone is associated with loss of the biphasic cyclical pattern and is maintained by the placenta during pregnancy. Following delivery, lactation begins and the hormone prolactin is secreted by the pituitary gland. While breast feeding is occuring, the biphasic cycle will be suppressed and occur less frequently than in a normal menstrual cycle. When the placenta is removed after delivery, there is a sudden reduction in hormone levels that may lead to post-natal depression.

Climacteric

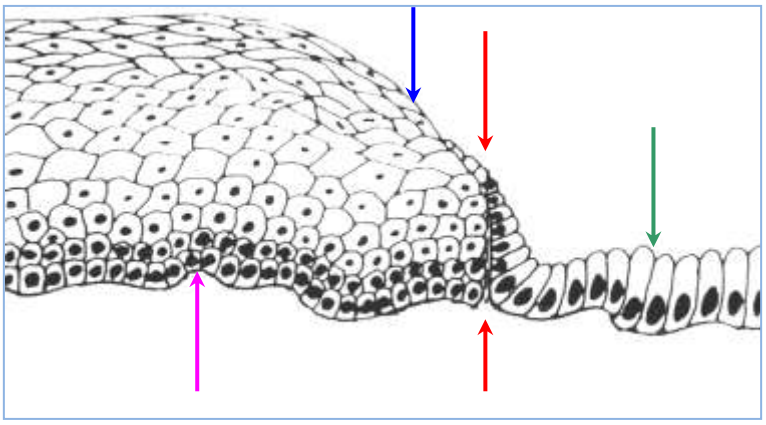
This describes the change from sexual maturity to menopause. Gonadal function decreases and biphasic cycles usually alternate with anovulatory cycles. Oestrogenic activity gradually decreases which induces bleeding. This early phase is called pre-menopause. Ovarian endocrine function gradually decreases until bleeding no longer occurs. The end of menstruation is called the menopause, after which a woman is described as being post-menopausal.

Old Age

A new equilibrium is obtained during old age. Ovarian function barely exists. The adrenal cortex takes over some ovarian function by producing small quantities of oestrogen. The genitalia atrophy and senile vaginitis commonly occurs as a result of oestrogen deficiency. Dryness and itching of the vagina and vulva result.

1.1.5 The Transformation Zone

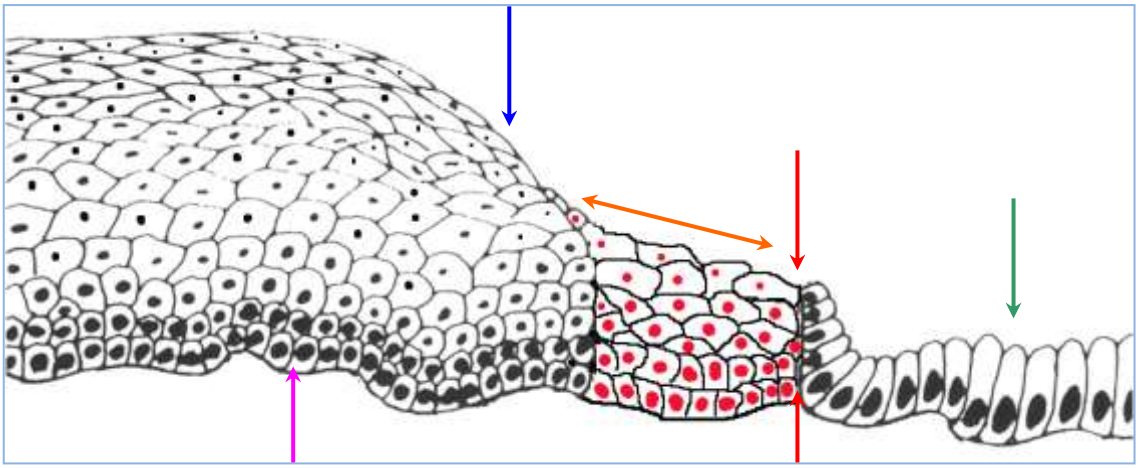
The *transformation zone (TZ)* in the cervix is very important because the epithelial cells (*squamous metaplastic cells)* from the TZ are the cells that are the most susceptible to carcinogens and most likely to undergo neoplastic/malignant transformation. Squamous metaplasia development and morphology is detailed in Section 2.1.5.



Abrupt transition between original squamous epithelium (blue arrow) and endocervical epithelium (green arrow) is termed the SCJ (red arrow). The basement membrane is indicated by the pink arrow.

Before puberty the ectocervix appears pink and is covered by stratified squamous epithelium. In contrast, the endocervical canal and crypts have a surface of mucus-secreting ciliated columnar epithelium. The abrupt transition between the squamous and endocervical epithelium is termed the squamo-columnar junction (SCJ) and prior to puberty, it is located within the endocervical canal.

At puberty with the onset of menstruation (and during the first pregnancy) an increase in growth results in an increase in the volume of the cervix, which leads to eversion of the columnar epithelium, called ectopy/ectropion. The columnar epithelium now lies on the part of the cervix open to the vagina, the ectocervix. This area is at first, covered by a single layer of endocervical cells and appears red. The redness is due to the vessels and connective tissue being very near to the surface with only a single layer of overlying epithelial cells.



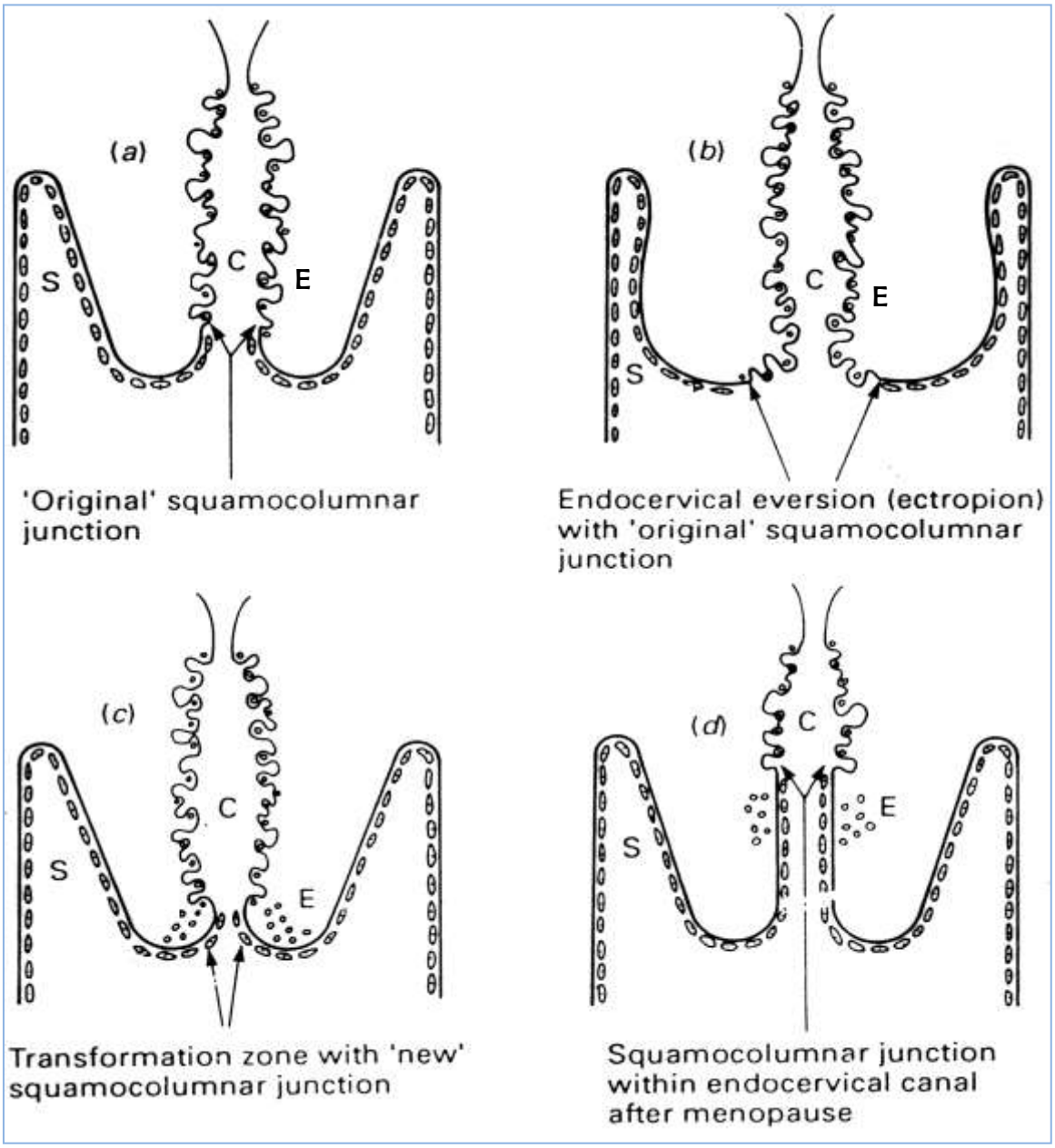
The development of squamous metaplastic cells (red nuclei) forming the transformation zone (orange arrow). The SCJ (red arrow) is where the metaplastic epithelium meets the endocervical epithelium. Endocervical cells (green arrow), original squamous epithelium (blue arrow), and the basement membrane (pink arrow) are marked.

This single layer of endocervical epithelium is now subjected to the effects of a lower (more acidic) vaginal pH and combined with the effects of hormonal changes, undergoes a metaplastic change of the epithelial cell type from endocervical to immature squamous metaplastic type, followed by subsequent maturation to mature squamous epithelium. The area of squamous metaplasia is called the transformation zone. The metaplastic epithelium provides a more robust and protective surface than the thin fragile endocervical epithelium.

Once fully mature, metaplastic squamous epithelium looks identical to the original squamous epithelium of the ectocervix. The transformation zone can be identified by endocervical glands in the underlying stroma, which do not undergo squamous metaplasia. The new SCJ in the surface epithelium is situated at the junction of the endocervical epithelium and the start of the squamous metaplastic epithelium (not between the squamous metaplastic and the original squamous epithelium). While these changes occur to some extent for all women, there can still be marked variation between different women in the location of the SCJ and the transformation zone at various stages of life. Epithelial changes during the menstrual cycle are minimal.

In the second and third decades the ectopic columnar epithelium continues to undergo metaplastic change which can be patchy, occurring at several different sites of the ectopic columnar epithelium, and at different times. The position of the SCJ changes again and eventually a confluent layer of mature metaplastic squamous epithelium is formed at or near the external os.

Finally at menopause the cervix begins to atrophy and the SCJ then starts to recede back up into the endocervical canal. When women are post-menopausal, the SCJ often lies well up the endocervical canal.



Changes to the surface epithelium and location of the SCJ during a woman's life.
a: Pre-puberty with the original SCJ between conventional squamous epithelium (S) and endocervical epithelium/glands (E) located up in the endocervical canal (C)
b: Puberty shows endocervical eversion and exposure to a lower pH
c: Transformation zone develops in the form of squamous metaplasia
d: Postmenopausal with the SCJ located high in the endocervical canal.

1.1.6 Obtaining a cervical/vaginal sample for cytology

Equipment

The following equipment is required when taking a cervical cytology sample.

- Vaginal speculum to insert into the vagina to allow the cervix to be visualised.
- The cervibroom is the recommended sampling device. It is used normally used alone for both SurePath and ThinPrep samples. A cytobrush may be used in combination with a broom for some patients.
- LBC vial – either Hologic ThinPrep® or BD SurePath®.
- Requisition Form
- Talc free gloves

Note: Both ThinPrep and SurePath manufacturers recommend that a broom device be used rather than a conventional spatula (see the manufacturer's instructions and recommendations for the LBC process type used in your laboratory). The fixative, vial and processing equipment are **not** interchangeable between ThinPrep and SurePath systems.

It is important that the cells removed from the cervix give a good representation of as much of the cervix as possible. It is particularly important that the sample includes the area of the SCJ and transformation zone as this is the most important area for the development of cervical neoplasia.

Taking the Sample

The importance of sampling the SCJ junction and as much of the transformation zone as possible has been discussed. It is imperative that the sample taker positions the woman to allow clear visualisation of the cervix before taking the sample. The sample taker should discuss the procedure fully with the woman, ensure that she is relaxed and consider any particular needs she may have. The reason for taking the sample should be made clear i.e. that the detection of early cellular changes can allow treatment to prevent pre-malignant and malignant change. The need for regular screening should also be discussed. The NCSP provides pamphlets for women outlining the important issues and processes involved in having regular samples taken for cytology screening.

Sample takers should also discuss high-risk HPV testing (hrHPV testing). This is particularly pertinent to patients who are 30 years of age or older and who have not had an abnormality in the previous 5 years because the laboratory will automatically perform a hrHPV test if the sample shows possible or definite low-grade changes under these circumstances. If the high-risk HPV test is positive after a low-grade cytology result, the woman will be referred for colposcopy so this conversation needs to occur at the time the sample is taken.

Before carrying out the procedure, talc-free gloves should be worn. Talc/starch crystals can mask cells if they are mixed in with the sample and can also be an irritant to human tissues, resulting in inflammation. Once the speculum is inserted, the complete cervix should occupy the space between the open blades of the speculum. If possible, lubricants should not be used to help insert the speculum, as these can compromise the sample if the lubricant gets into the LBC vial. This is particularly important with ThinPrep samples because lubricant in the sample can clog up the ThinPrep filter during sample processing. Instead, the speculum may be

moistened with warm water, which will also warm the speculum. Where lubricant is needed to locate the cervix, it is recommended that lubricant is used sparingly and only on the sides (not the tip) of the speculum. Some lubricants are more problematic than others. Aqueous-based lubricants only should be used. Lubricants containing carbomer or carbopol polymer should be avoided. Any other gynaecologic examinations should be done after taking the cervical cytology sample, so that the cells on the cervix are not disrupted before the sample is taken.

The woman is then ready to have her sample taken. Any clinical features such as vaginal discharge, bleeding or atypical appearances of the cervix should be noted on the request form that accompanies the sample to the laboratory.

Plastic sampling devices only are used for LBC samples. Conventional wooden spatulas are specifically not used as wooden fragments can compromise LBC processing.

Sampling devices.
1. Broom
2. Endocervical brush

The Broom device is recommended by LBC manufacturers. One commonly used brand is the Cervex Brush®

The cervibroom is the recommended sampling device for cervical screening as it collects sufficient cells for both cytology and HPV testing, and effectively samples the endocervical/transformation zone. It is normally the only device needed to collect a satisfactory sample and is suitable for use with both ThinPrep and SurePath systems.

- The cervibroom is appropriate for vaginal vault samples
- A cytobrush can be used in addition to a cervibroom in situations where sampling the endocervical canal is difficult or of particular interest.

If the Rovers Cervex Brush® (broom) is used, the manufacturers recommend up to **5 full 360^0 clockwise rotations** (stop if contact bleeding is induced) because the bristles of the device have a specially bevelled edge to maximise cell sampling. Due to the texture of the broom, the endocervical canal is best sampled during the 3rd-5th rotation.
If it is doubtful that the SCJ has been sampled or if an endocervical lesion is suspected, then a cytobrush sample may be taken to complement the broom sample. The brush is inserted into the os and rotated between one quarter and one half rotation. This is to minimise distortion artefact of fragile glandular cells. The cytobrush should not be inserted too high otherwise the

sample may contain an abundance of lower uterine segment cells. As the cytobrush can cause bleeding, it is best taken after the broom. A cytobrush should not be used in pregnant women. Using a cytobrush sample alone is not recommended as the cervix is not adequately sampled.

If two sampling devices are used to sample the cervix, both samples can be placed in one LBC vial. However if two samples are specifically taken from two different sites such as the cervix and the vagina (usually specialist testing) then the different samples must be placed in separate LBC vials with each site clearly identified on each vial.

LBC samples

There are two commercial LBC products used in NZ: Hologic ThinPrep® and BD SurePath®. The processing methods of the two techniques are different but both aim to produce a high quality representative sample with the following features: exceptional and consistent fixation, removal of unwanted background material, prevention of cells being obscured i.e. a clear sample that is easy to screen. The samples are also designed to allow for automated screening and other ancillary tests such as HPV testing. All cervical cytology reporting in New Zealand has been done using LBC samples (not conventional sample) since 1st July 2010.

For **ThinPrep samples**, the broom is rinsed and swirled in the ThinPrep vial containing fixative and then the entire sampling device is removed, including the head of the device. With **SurePath samples** the broom head is separated from the broom handle and dropped into the SurePath vial containing fixative. The vials are then sent to the laboratory for processing.

It is essential that the appropriate manufacturer's vial is used for the corresponding technique. The vials used by ThinPrep and SurePath are different in size. The SurePath vial lid has a central area that is pierced during the processing phase (or is uncapped if a laboratory is using the Totalys automated system). Both products use a low strength alcohol fixative. The ThinPrep fixative is methanol-based, and the SurePath fixative is ethanol-based. Failure to collect the sample into the vial that corresponds with the type of LBC system used to process the sample invalidates the technique and the results.

Collection of LBC (Liquid Based Cytology) samples

SUREPATH

Once the cervix is sampled the broom should be quickly introduced into the SurePath vial – use the larger opening to aid in detaching the broom head in the vial. Do not use the small opening. This is only for use by the automated Totalys machine in the lab to obtain an aliquot of the sample for ancillary tests such as HPV testing. If more than one sampling device is used, it is recommended that these be placed in the same vial.

THINPREP

For ThinPrep samples, the recommendation is to rinse the broom as quickly as possible into the PreservCyt® Solution vial by pushing the broom into the bottom of the vial 10 times, forcing the bristles apart. As a final step, swirl the broom vigorously to further release material. Discard the collection device. Tighten the cap so that the torque line on the cap passes the torque line on the vial.

Labelling LBC vials and completing the requisition/electronic laboratory order form

All **vials must be labelled** with at least two primary identifying demographics to clearly identify the woman e.g. Full Name (must include first name and surname) and National Health Index (NHI), or date of birth if the NHI is not available. It is important that the vial is pre-labelled just prior to the sample being taken, to prevent a potential mix-up of samples from different women.

A **fully completed requisition form/electronic laboratory order** must accompany the sample as the cytoscientist/cytotechnician's interpretation can be influenced by clinical conditions. It is very important to ensure that all of the details on the LBC vial match those on the requisition /electronic laboratory order. Laboratories apply strict rules about compromised specimens and may reject samples if there is a mismatch of the details on the LBC vial compared with the requisition/electronic laboratory order form. Every attempt should be made to obtain and record a correct NHI number as this is a key patient identifier for clinicans, laboratory records and the NCSP register.

The following demographic information must be given:

- Full name and any other names known by
- NHI number
- Date of Birth
- Gender
- Contact details/location
- Ethnicity

The following clinical information must be given:

- Date of test
- Type of sample collected (ThinPrep or SurePath)
- Site of sample (e.g. cervix or vagina)
- Test/s collected
- Gynaecological history: LMP (include if pregnant, post-partum or post-menopausal), parity, contraception (including IUCD), is she has had a hysterectomy(total/subtotal),
- Previous treatment to the cervix e.g. cautery, radiation
- Abnormal clinical findings: postcoital or intermenstrual bleeding, pelcic pain, vaginal discharge, abnormal-appearing cervix
- Relevant diseases e.g. immunodeficient
- Relevant drug therapy e.g. immunosuppressive drugs, tamoxifen
- Previous screening history and biopsy/histology results

Sample taker information is also required:

- The health facility identifier number
- The name and address of the clinic
- The sample taker's registration number
- The sample taker's name

1.1.7 Questions

1. What is the importance of the anterior pituitary with regards to hormones of the female genital tract?

2. Describe the menstrual cycle during the three main phases of menstruation, follicular phase and luteal phase.

3. Describe the hormonal effects during the following:
   - Pregnancy
   - Climacteric
   - Old age

4. What is the importance of the squamo-columnar junction (SCJ)?

5. Discuss the precautions that should be observed when taking a cervical cytology sample.

6. What are the main differences between using ThinPrep and SurePath vials when obtaining cervical cytology samples?

References

- Chang AR. 1988. How to obtain an optimal cervical smear. *Professional Bulletin.* Report No.2. Cancer Society of New Zealand Inc.

1.2 CORE TOPIC: CYTOPREPARATION

1.2.1 Introduction

All samples received by the laboratory should be treated as potentially hazardous and processed according to laboratory protocols as documented in method, safety and quality manuals. Cytoscientists and cytotechnicians must be conversant with these documents in order to provide a safe environment for themselves as well as their colleagues.

1.2.2 Fixation

General comments

Fixation can be regarded as the first step of the staining process and is of critical importance in obtaining precise morphology and correct staining. Poor fixation results in poor and variable staining and can result in false negative or even false positive results.

The introduction of LBC has removed many of the difficulties that used to occur with fixation of conventional smears, but sample takers still need to be advised that the time between taking the sample and placing it into the LBC vial should still be as short as possible.

The ThinPrep fixative uses 58% methanol (methyl alcohol) whereas SurePath uses 24% ethanol (ethyl alcohol). The relatively low alcohol concentrations used for LBC (compared with 95% alcohols previously used to fix conventional smears) allow a range of ancillary tests to be performed from the LBC vial.

Principles of fixation

Fixation needs to consistently produce a sample suitable for Papanicolaou staining and this requires that all cellular constituents are preserved and presented as close as possible to their "real" state. LBC allows multiple slides to be made from one sample either at the time of first processing or at a later date, after the initial slide has been screened. Storage of cells in fixative in the vial enables the laboratory to hold the remaining sample for repeat slides or ancillary tests if and when required.

Brief history of fixation

Fixation is not a recent discovery. As far back as 400BC Hippocrates and the Persians knew something about the biological effects of mercury and its salts. Alcohol use as a preservative has also been known for a long time but little was done systematically to investigate fixatives until the second half of the 19th century when workers such as Virchow made detailed cell and tissue examinations.

One major realisation was that no one fixative was optimal for all procedures and investigations. A chaotic list of mixtures of various fixatives resulted until 1960 when Baker began to define them. He classified fixatives as:

- Coagulants
- Non-coagulants

He also gave each fixative the name of the person who originally introduced it. Hopwood further classified fixatives as follows:

Aldehydes	***Oxidising agents***	***Protein-denaturing agents***	***Unknown mechanism***
Formaldehyde	Osmium tetroxide	Ethyl alcohol	Mercuric chloride
Glutaraldehyde	Potassium permanganate	Methyl alcohol	Picric acid
Acrolein	Potassium dichromate		

The protein denaturing agents are the most commonly used fixatives for cytology.

Main objectives of fixation

Fixation is a complex series of chemical events that differs for the different groups of substances found in tissues and cells. Fixation should render tissues and cells as close to their living state as possible and ideally no small molecules should be lost. In cytology this requires the preservation of morphological detail of the cell in as perfect condition as possible. Sometimes there still needs to be a compromise by determining which features matter the most. For example, lipids are lost with alcoholic fixatives unless special precautions are taken.

Preserving morphological detail for cytological examination can be achieved by rapid inactivation of cellular enzymes and coagulation of cell protein, which is the mode of action of most fixatives. All fixatives must be capable of penetrating the cell membrane. A good fixative should also inhibit the growth of the bacteria that are inevitably present in cell samples, as these otherwise proliferate and cause cell lysis.

Theoretical aspects of fixation

The most important reaction of a fixative particularly for morphological assessment is stabilising protein. The mechanism of this process is not fully understood but it appears that fixatives have the ability to produce cross-links between proteins to form a gel, ideally preserving the in vivo relationships. Soluble proteins are fixed to structural proteins and thus rendered insoluble, which gives the overall structure some mechanical strength. Fixation also causes a change in the chemical and physical state of RNA and DNA, but less is known about the fixation of nucleic acids compared with proteins.

Formalin (aldehyde fixative) is not a good fixative of nucleic acids. DNA and RNA in their native states do not react significantly with formalin (or glutaraldehyde) at room temperature. Some reaction begins to take place if heated: to above 45^0C with RNA and at 65^0C with DNA. This is related to uncoiling of DNA/RNA at these temperatures.

Up to 30% of nucleic acids may be lost during fixation so for cytology, alcoholic fixatives are used as they cause little chemical change. DNA collapses in ethanol and methanol but when the denatured DNA is rehydrated, it reverts substantially to the original form. The presence of salts is known to be essential for maximal precipitation of nucleic acids in alcohol. Alcoholic fixatives also penetrate cells and tissues rapidly, whereas glutaraldehyde for example is extremely slow.

Fixation causes changes in cell and tissue volume, an artefact that is critical to consider if quantitative work is to be carried out. Formalin followed by wax embedding causes 33% shrinkage. Changes in osmolality will also cause shrinkage or swelling.

Fortunately, fixation of individual cells for cytology is much less problematic than fixation of whole pieces of tissue for histology. For example, the rate of penetration of the fixative is not a problem for individual cells in a fixative fluid, whereas it can be a problem when fixing large tissue specimens.

Finally, one must also consider safety. Aldehyde fixatives (e.g. formalin, glutaraldehyde) are particularly toxic if used without safety precautions. Contact with skin must be avoided and the air threshold values must be minimised (there are maximum values set for New Zealand). Fixatives such as alcohols are particularly flammable, so caution must be taken with storage, naked flames and electrical sparks. The correct safety procedures must be followed and safety equipment which is tested regularly must be available.

SurePath Preservative fluid and ThinPrep Preservcyt

Alcohol fixation is rapid and is accomplished within 10-15 minutes but its major disadvantage is that it is flammable. Alcohols act by coagulating cell protein and dehydrating the cell. About 80% of the water is removed, mostly from the protein ultrastructure.

	SUREPATH	THINPREP
Appearance	Clear colourless liquid	Clear colourless liquid
Constituent	24% ethanol, 1.2% methanol	58% methanol
pH	7.2	5.5
Boiling point	86°C	64.4°C
Materials/Conditions to Avoid	Oxidising agents, high temp, sources of ignition, direct sunlight	Oxidising and reducing agents, heat, flames, sparks

1.2.3 Sample processing prior to staining

The processing of LBC samples prior to staining is a specialised procedure with significant differences between ThinPrep and SurePath. For the VRPCC, you should become familiar and competent with the theoretical basis and processing procedures of the LBC method used in your laboratory, including the manufacturer's instructions and references. This includes use of appropriate equipment, troubleshooting, daily maintenance, and safety considerations. A basic understanding of the other LBC method (SurePath or ThinPrep) is also required. Further details about SurePath and ThinPrep processing methods are provided in the *VRPCC Workbook: Core Topic 1.3 LBC Platforms Page 32*.

Primary differences between ThinPrep and SurePath processing

Process	ThinPrep	SurePath
Sample	Aggressive agitation of broom device in fixative vial Broom device then discarded, **HEAD NOT LEFT IN** the vial	Place broom head in fixative vial. No agitation needed Broom **HEAD REMAINS IN** the vial
Cells sampled	Cells that part from the broom at the time of collection	Cells that part from the broom after agitation in the laboratory
Fixative	58% methanol	24% ethanol
Fixative vial	Standard lid	Lid with pierceable centre
Basis of process	Vial placed directly into machine where fluid is drawn through a filter capturing cells Separates epithelial cells from debris, blood and inflammatory cells Filter inverts to place cell sample directly onto a pre-treated slide	Agitation and mixing removes cells from broom head Centrifugation concentrates cells Density gradient separates epithelial cells from blood and inflammatory cells Gravity sedimentation deposits cells onto a pre-treated slide
Staining	Separate step after slide preparation	Automated as part of gravity sedimentation process
Cell presentation	Thin cell layer	Combination of single layer and overlapping cell groups
Sample dimensions	Diameter = 20mm Area = 314mm	Diameter = 13mm Area = 133mm

Although there are significant differences in processing and in the final area for cells on the slide, both methods result in similar cell content as the cells on SurePath slides are more concentrated than on ThinPrep slides.

The minimum cell content for adequacy (a satisfactory sample) on LBC slides is **5000 well-preserved and well-visualised squamous cells**. **Less than this is regarded as unsatisfactory (unless abnormal cells are seen).** A repeat sample can be made and this may produce a satisfactory slide, particularly if further treatment of the sample is used to reduce excessive blood or inflammatory material. Adequacy is still based on having one individual slide with at least 5000 squamous cells, not this number across a combination of slides from the same vial.

For borderline cellularity cases, a squamous cell count is required. An estimate based on viewing fields with 40X objective across the diameter of the preparation, including the centre of the cell circle is used. The count for minimum cells that renders the sample satisfactory is **9 cells for SurePath and 4 cells for ThinPrep present in at least 10 of the fields viewed**. A lower count means the sample is unsatisfactory. Sample adequacy is discussed further under *VRPCC Workbook: Subsection 4.1.3 Structure of The Bethesda System Page 254.*

In the current environment of 100% LBC utilisation in New Zealand, the unsatisfactory sample rate is very low, averaging at around 1.2% for all New Zealand laboratories. There is a difference between ThinPrep laboratories and SurePath laboratories resulting from the different processing methods. Unsatisfactory rates at ThinPrep laboratories are in the 1.5-2.5% range

and at SurePath laboratories the range is about 0.5 – 1.0%. The main reason for unsatisfactory samples is insufficient cellularity. Other reasons for unsatisfactory samples are usually excessive blood, inflammation, lubricant or cytolysis. On rare occasions, preparations may be unsatisfactory due to an excessive endocervical component with very few squamous cells – this may result from poor sampling technique or can be caused by the presence of a large ectropion.

Inflammatory debris, blood or mucus that has accumulated on the cervix should be carefully removed by the sample taker before taking the sample, or this may appear on the final slide preparation. If lubricant is used it can interfere with the transfer of cells to the glass slide – this is more of an issue with ThinPrep than SurePath because lubricant clogs up the ThinPrep filter in the T2000 or T5000 processor.

Both LBC types are suitable for high risk HPV testing and HPV genotyping (subtyping) as well as cytology. Other tests, such as testing for chlamydia, may be performed on the residual sample as long as HPV testing and cytology are prioritised and are not compromised. Commercial companies that market LBC systems provide mimumum volumes of fluid that may be removed from LBC vials for such tests (called ancilliary testing).

1.2.4 Papanicolaou staining

The principles of the Papanicolaou stain are described below. The trainee should be familiar with the method employed in their laboratory and become competent in the operation, daily maintenance, troubleshooting procedures, and safety aspects of any equipment used for staining.

The theory of staining is a detailed subject covering a wide range of chemical reactions and dyes. The Papanicolaou technique is a modified (or extended) haematoxylin and eosin stain. It uses a polychromatic differential staining reaction developed to demonstrate variations of cell morphology, including features which demonstrate cell maturity and metabolic activity.

There are four main steps to the procedure:

- Fixation – wet fixation using an alcoholic fixative
- Nuclear staining - generally using an alum haematoxylin
- Cytoplasmic staining - use of two dye mixtures, Orange G and EA (combined EA/OG used with SurePath PrepStain machine)
- Clearing and mounting - complete dehydration and clearing of the slide and mounting in synthetic mounting media

Fixation: discussed above

Nuclear Staining

The nucleus is stained using an alum haematoxylin, which stains the chromatin (DNA) a blue colour. *Haematoxylon campechianum* is a tree originating in the Mexican state of Campeche, but nowadays is mostly cultivated in the West Indies. Haematoxylin is extracted from the heartwood of the tree with hot water and is then precipitated out from the aqueous solution using urea.

Haematoxylin by itself is not a stain. It has to be oxidized to haematin, which is the natural dye. In the ripening process, haematoxylin ($C_{16}H_{14}O_6$) loses two hydrogen atoms to become haematin ($C_{16}H_{12}O_6$).There are two methods of oxidation:

1. Natural oxidation termed “ripening” is caused by exposure to light and air. This method is slow and can take up to 3-4 months, but the final product seems to maintain its staining properties for longer than with chemical oxidation.

2. Chemical oxidation utilising chemicals such as sodium iodate (e.g. Mayer's haematoxylin) or mercuric oxide (e.g. Harris's haematoxylin). These chemicals convert haematoxylin to haematin almost instantaneously. The useful shelf life seems shorter and eventually the continuous exposure to light and air destroys much of the haematin.

- Haematin is a basic dye and stains acid components such as the chromatin of nuclei. Chromatin consists of DNA plus associated structural proteins and it is these proteins that bind with the haematoxylin solution. As the DNA: structural protein relationship is constant this gives a controlled staining artefact.
- Haematin alone is an inadequate nuclear stain and requires the presence of a mordant. The most common mordants for haematoxylins are salts of the metals aluminium, iron and tungsten. The type of mordant selected will strongly influence the type of tissue components stained and their final colour. Most mordants are included in the stain components. The type of mordant used classifies the haematoxylin eg Alum haematoxylin, Iron haematoxylin.
- Aluminium mordants are usually in the form of "potash alum" (aluminium potassium sulphate) or ammonium alum (aluminium ammonium sulphate).

The rationale of alum haematoxylin solutions is that the nuclei are stained by oxidized haematoxylin (haematin) through mordant bonds of metals such as aluminium. The constituents of the haematoxylins are:

- An alum - the mordant of which the aluminium cation is the main reactant.
- An acid - this aims at making staining more precise (accelerator).
- An oxidizing agent - to give speedy conversion of haematoxylin to haematin.
- Glycerol - this slows the oxidation of naturally oxidizing haematoxylin and thus improves the keeping properties.

Mordants are salts of certain metals that radically alter the behaviour of particular dyes. A mordant is capable of forming a chemical combination with a dye and the resulting substance is called a lake (dye lake). One of the big advantages of the use of dye lakes is that when the tissue-mordant-dye complex is formed it becomes insoluble in all neutral fluids used. This makes the subsequent colouring with a large range of other dyes easier and there is no need for rapid dehydration.

The haematoxylin staining process:
After the haematin (haematoxylin stain) has been applied the nucleus is stained a red colour. This is then converted to the familiar blue (to black) when the slide is washed in a very weak alkali which is referred to as "blueing". This is usually done using tap water with a pH slightly above 7.0. The procedure should be carried out for about 5-10 minutes and if the tap water is not alkaline enough, the colour change will not occur. A substitute to speed up the blueing process is the use of Scott’s tap water, which is alkaline and particularly good for use on an automated staining machine. The formula is:

Potassium bicarbonate	2g
Magnesium sulphate	20g
Distilled water	1000mL

This procedure takes around 30 seconds - 1 minute. An even more rapid blueing agent is saturated lithium carbonate where the nuclei are blued in a matter of seconds. The disadvantage with lithium carbonate is that it may lift some cells from the slide. If a blueing agent is used, the slide should be washed in water prior to and after this step.

As there are a variety of haematoxylins available, the laboratory selects the haematoxylin of choice, based mainly on preferred staining characteristics, the speed of the staining procedure and adaptation to automated staining. The use of automated screening devices for reporting cervical cytology is now a major determinant of the stain procedure used for gynaecologic cytology. Laboratories that use The ThinPrep System Imager are required to use the ThinPrep proprietary stain supplied by the manufacturers. With the SurePath system, the proprietary stain is not compulsory but most SurePath laboratories use it if they are using the FocalPoint imaging device. Proprietary stains are used because they standardise the staining step, as this affects performance of the imaging devices used with each LBC system. Many laboratories now run a different stain protocol for gynaecological and non-gynaecological cytology samples.

Understanding the different ways that different haematoxylins are used is still important. Traditionally, most cytology laboratories used either Harris's or Gill's Haematoxylin. Both give consistent results and are easily adapted to automated staining. Gill's is a relatively new formula (1974) and has become particularly popular with cytoscientists/cytotechnicians because it does not need differentiating and does not readily overstain, although the working solution may be diluted to meet staining preferences (e.g. can be used at half strength). Harris's haematoxylin requires differentiation.

Gill's and Harris's haematoxylins are good examples of the two different haematoxylin procedures.

- Gill’s haematoxylin is used **progressively**
- Harris's haematoxylin is used **regressively**

Progressive staining is adding sufficient dye until only the required components e.g. nuclei are stained, and requires no differentiation.

Regressive staining is over-staining all the cellular components and then removing the excess dye until just the wanted components e.g. nuclei are stained. The removal is called differentiation and for alum haematoxylins the differentiator used is acid/alcohol (hydrochloric acid/ethanol) solution (usually less than 1% HCL).

The disadvantage of differentiation is that a constant check is required, especially because haematoxylin becomes weaker with age so that the same degree of differentiation causes paler results over time. Some regard regressive haematoxylins (especially Harris's) as giving superior crisp results although for routine automated staining, Gill’s haematoxylin is the simpler of the methods.

The precise action of differentiation is not clear. Some workers consider that using an alcoholic solution of hydrochloric acid achieves differentiation by the acid attacking and breaking the tissue-to-mordant bond, rather than the mordant-to-dye bond.

Differentiation is carried out prior to blueing. Following a wash, the blueing step is done. If differentiation is not complete (by microscopic check) the process can be repeated.
Some years ago a large shipment of haematoxylin was lost due to the "ship going down". This immediately caused a world-wide haematoxylin shortage that prompted investigation of haematoxylin substitutes. These will not be dealt with here but the reader is referred to: "Some haematoxylin substitutes in biological microtechnique": Gurr E. *Lab Practice* March 1975.

Cytoplasmic Staining

A combination of dyes is used to counterstain the cytoplasm and display the many variations of cellular morphology and degrees of differentiation and metabolic activity. The cytoplasmic dyes used are acid dyes (i.e. have an affinity for basic structures). Solutions of high alcoholic content provide clear visualization through areas of overlapping cells, mucous and debris. All the stains used are synthetic dyes with chemical formulae that have been standardised.

The two staining combinations usually employed for the Papanicolaou technique are:

- Yellow dye - orange G (0G6)
- EA stain (EA 36, 50 or 65) - EA 50 is the most commonly used.

Orange G is a monochromatic stain and stains keratin orange. Combined OG/EA stains are also commercially available, reducing staining steps and time.

EA 50 (eosin azure) is polychromatic and contains eosin yellowish, light green and Bismark brown. The principle of how the differential staining occurs is not clear. Eosin stains the cytoplasm of mature cells, nucleoli and cilia as well as *Candida*. Light green stains the cytoplasm of other metabolically active cells as well as *Trichomonas vaginalis*. The effect of Bismark brown is not clear. It is important to note that conditions such as infection, inflammation, and other conditions can markedly affect the final cytoplasmic staining properties. Imperfect fixation will also lead to poor and altered staining characteristics.

Clearing/Mounting

These are the final steps following complete dehydration after staining. “Clearing” produces the cellular transparency needed for microscopic examination. It is also the step that allows the use of water-free synthetic mounting media to be used e.g. DPX or Clearium. The choice of mounting media is made by the laboratory. Xylene is probably the most commonly used clearing agent. It is imperative that the slides are completely dehydrated before immersion in xylene as xylene will not tolerate the presence of any water. If water is present the final preparation will become "cloudy", seen with the naked eye as a white haze in the preparation.

Xylene has a refractive index of approximately 1.5 which is compatible with mounting media and glass. Clearing agents such as xylene are also colourless and do not interfere with the final colour product. However, xylene does impose major safety hazards.
1. It is highly toxic (benzene derivative). New Zealand has maximum recommended threshold limits for usage.
2. It is a potential carcinogen and any contact must be avoided. It also attacks rubber, so gloves selection needs to be appropriate (e.g. nitrile gloves) and it must be used in accordance with the manufacturer’s instructions. Appropriate and effective fume extraction is essential.

3. It is highly flammable and like alcohol, should be stored in special explosafe containers with appropriate pouring devices. Only the required daily usage should be held immediately in the working area of the laboratory, with bulk storage in a suitable flammable liquid/chemical store. Xylene must be disposed of correctly and must not be tipped down sinks or drains.

Substitutes for xylene are commercially available but are generally more expensive than xylene and have not been widely accepted in NZ. Proper usage and good practice allows xylene to be used in a safe way.

Automated staining

Automated staining has become the normal approach for laboratories that are processing large numbers of slides on a daily basis. Benefits of automated staining machines are:

- Controlled and consistent final product
- Allows large volume bulk and batch processing
- Improves turnaround time
- Allows staff to undertake other activities
- Reduces repetitive manual tasks
- Cost effective
- Time saving
- Protection from chemicals and reagents
- May reduce quantity of chemicals and reagents

Coverslipping

The stained slide is covered to protect the sample and allow easy microscopic examination. Marks can be placed on the coverslip to locate abnormal cells that are identified by microscopy. Coverslips are traditionally glass, maintaining a constant refractive index (see section 1.3.2). There are alternatives e.g. plastic coverslips or special tape coating but these have not proved to be practical so most laboratories use glass coverslips.

Slide labelling

Most automated staining machines have an in-built system for labelling the slides. The final step in cytopreparation is matching these to requisition forms and NCSP-Register histories. There should be sufficient information on the glass slide to allow exact matching of the slide label. Some automated LBC processors use pre-labelled slides which are scanned and matched with the LBC vial as an integral part of slide preparation. Quality control procedures are needed to ensure that the primary screening cytoscientist/cytotechnician has correctly labelled slides that correlate with corresponding request forms and online NCSP histories. Bar-coding and request form imaging significantly reduce matching errors. Computer-generated slide labels are also an advantage as they reduce transcription errors and reading errors.

It is recommended that a slide label contain at least two and preferably three identifiers (such as Full name, NHI number, Date of Birth) well as the unique laboratory number.

Your laboratory will have documented procedures including quality control processes for slide labelling and case matching. You must be fully conversant with these and be competent in both

practical aspects and troubleshooting procedures when errors do occur. Mixing up two patients' samples can result in serious errors in patient management.

Any failure in process should be documented and investigated. Once an error has been rectified, procedures should be put in place to prevent further similar incidents. Communication and educational follow-up is critically important before closure of the error.

1.2.5 Contaminants and artefacts

There are a number of unwanted contaminants and artefacts that can cause difficulties in screening and interpretation of the stained sample. A serious problem is the carry-over of cells from one sample to another during staining. Carry-over of abnormal cells to a sample that is actually negative but interpreted and reported as abnormal, can result in a patient being treated unnecessarily.

Your laboratory will have quality control procedures in place to:

1. Minimise unwanted artefacts and contaminants
2. Identify a problem should it occur
3. Eliminate these should any occur
4. Preventative actions to stop reoccurrence

You should be fully conversant with such procedures. Adherence to daily and weekly cleaning and maintenance protocols is the key to eliminating contaminants and artefacts arising in the laboratory. These will be clearly documented in method and quality manuals. Clear and concise instructions to sample takers and sample couriers will help eliminate externally caused contaminants and artefacts prior to processing and staining.

Contaminants

Contaminants result from the introduction of foreign material to the sample at some stage during collection or processing. This can happen at:

- Sampling and transport
- Processing of the specimen
- Staining and mounting the specimen

The more common contaminants encountered are:

- Fibres from sampling materials such as cotton wool and cellulose. Cellulose fibres are birefringent.
- Human hair may contaminate the sample.
- Talc/starch crystals from gloves appear as translucent, refractile bodies and demonstrate a Maltese cross when polarized.
- Lubricating fluid or locally applied medical creams can adhere to cells preventing proper staining and/or masking cellular detail.
- Airborne contaminants such as pollen and fungi.
- Mineral oils due to faulty batches of mountant. The batch should be discarded and is due to the solvent of the mountant evaporating.

- Poor dehydration may lead to water droplets in the final preparation. The slides become opaque and resolution is poor. The droplets can be seen in the xylene baths and the xylene must be changed, the sample re-hydrated and then re-dehydrated in fresh reagents. If the mountant is contaminated with water it must be discarded.
- Cells from the male urogenital tract may be seen in cervical samples (spermatozoa and seminal vesicle cells).
- Parasites from the intestinal tract may be observed in cervical samples. These include *Enterobius vermicularis* and *Ascaris*. Pubic lice may be seen from the external genitalia. *Schistosoma* may also be seen from the urinary tract as a contaminant of cervical samples.

Floaters deserve special mention as they have the potential risk of causing a false positive diagnosis. Floaters from one sample to another are usually caused by excess or poorly adhered cells. The floaters generally appear on a different focal plane to the rest of the sample and if the coverslip is lightly pressed they may move. Cross-contamination of cells may occur with automated staining machines and also from vigorous agitation during hand staining. Certain types of sample e.g. urines and body cavity fluids are a common cause of this type of contaminant indicating the need for separate processing of these samples. Reagents must be regularly changed and/or filtered and staining baths regularly cleaned. The final xylene bath is a common trap for floaters.

Artefacts

Almost every stage of processing introduces artefacts but these are strictly controlled and form the basis of reactions and the final product. Improper technique can lead to unwanted artefacts. Common unwanted artefacts are:

- Delayed fixation will cause air drying which will cause cells to swell and produce washed-out staining. Staining characteristics may also change. Poor fixation can lead to serious diagnostic errors if not rejected.
- Excessive bleeding due to harsh sampling or clinical heavy bleeding.
- If the clearing agent following staining evaporates, a black or brown deposit may be seen on the cells from the xylene. It is thought that these may be minute bubbles.
- Larger air bubbles may be the result of improper mounting or retraction of the mountant.
- Detergents used for cleaning staining baths and equipment will cause pale staining. All baths must be thoroughly rinsed and dried before use.
- Crystals from old mounting media may be seen.
- Stained slides will be bleached if left in direct sunlight or bright light. Even after a few years of storage in the dark, stains tend to fade.

1.2.6 Preventing cross-contamination of vials with HPV

Introduction

As the molecular biology test to detect hrHPV types is a very sensitive test, all precautions must be taken to ensure that no cross-contamination occurs when LBC vials are received in the laboratory. There are some basic measures that can be taken to ensure that ThinPrep or SurePath samples are not inadvertently contaminated with HPV in the laboratory. The following broad guidelines have been formulated by the National Cervical Pathology Training Service (NCPTS) to promote the safe handling of LBC samples by specimen entry and cytopreparation staff. It is not an exhaustive protocol and laboratories are expected to expand on these to develop more specific protocols for their laboratory.

If pre-aliquots are taken from LBC vials, this must be done in a biohazard cabinet using sterile pipettes. The NCSP endorses the following manufacturers' guidelines:

With both LBC systems, taking an aliquot from the vial for additional tests such as HPV testing is done with the understanding that this may impact on the quality of the cervical cytology sample in certain circumstances.
ThinPrep: A maximum of 4mL may be aliquoted and must be taken as a "one-only" aliquot.
SurePath: A maximum of 0.5mL may be aliquoted and must be taken as a "one-only" aliquot.

Disinfection

The disinfectants below have been recommended by molecular scientists in New Zealand. Other equally suitable disinfectants may be available for use.

1% bleach is made up by mixing commercially available Chlorodux bleach in a ratio of 200mLs bleach to 800mLs water. Good "turnover" of bleach is recommended so for example, this could be made up on a Monday morning and then all containers emptied out on Friday afternoons with a fresh batch made up in readiness for the following week. An expiry date of not more than 5 months from the date the solution is made up should be written on the container. Trigene is also recognised as a suitable disinfectant. Usually a 1% solution is recommended as a bench wipe disinfectant and a 10% solution to decontaminate spills, breakages in machines, etc.

Change gloves often, especially following procedures where aerosol formation is likely – (gloves are worth less than 10 cents each whereas a contamination issue can be HUGE to resolve).

Other than when dealing with spillage, always work with bleach on a paper towel – this will ensure that there is no "pooling". Bleach is a strong oxidising agent which causes corrosion when left in contact with metals, even stainless steel. Following bleach use always ensure adequate 70% ethanol / water–wipe-away. Take care that bleach aerosols are not generated and that bleach is not sprayed near "open" specimens.

SurePath Note: these guidelines do not consider the use of the Totalys machine.

• The shelf above and around the PrepMate must be clutter-free to minimise collection of dust. The shelf must be cleaned regularly with 70% alcohol.

• Fresh gloves must be worn to place syringes in the white PrepMate racks.
• Fresh gloves must be worn to place stickers over the hole in the lid once the PrepMate has removed the required aliquot of sample from the vial for the cytology slide or a new vial cap must be used.
• Rubbish bins must have a lid on them at all times and must not be close to the PrepMate.
• The PrepMate racks must be sprayed with 1% bleach, left for a few minutes, rinsed and sprayed with 1% Trigene, then rinsed and dried again. Note that the wells at the bottom of the PrepMate rack where the syringe tip usually rests must be flooded with Trigene to eliminate any possible cross contamination.
It is advisable that no absorbent pads are used around the PrepMate; a bench that can easily be disinfected daily is a better surface to work with.
• Wipe the bench around the PrepMate with 1% Trigene once per week; wipe the metal draining part of the sink itself with 1% bleach
• Wipe the PrepMate itself with 1% bleach – use paper towels.
• All SurePath vials must be refrigerated whenever possible; this ensures that any hrHPV test that may need to be done is not compromised. Testing using the Roche platform requires that vials can remain at room temp for no more than 2 weeks; thereafter they can remain in the fridge for up to 6 months. With the Abbott platform, SurePath vials can be stored at 15-30C for 2 months and up to 6 months at 2-8C.
• The SurePath syringes must be stored in a box with the lid closed when not in use.

A sentinel sample needs to be run on a regular basis, weekly at a minimum. The sentinel sample result tests for cross/environmental contamination during routine SurePath samples preparation and needs to be placed in a different position on the PrepMate rack each week.
Place an unused SurePath vial in the PrepMate rack and process with other vials for sample extraction using a syringe. Once a sample has been removed by the machine for the cytology slide (which does not actually need to be done), the vial is sent for HPV testing.
The sentinel should always test as "Invalid – HPV/Beta-globin DNA not detected". If DNA is detected then potential sources of contamination need to be investigated.

ThinPrep

If a glacial acetic acid wash is required to remove excess blood, gloves and sterile pipettes must be used. These specimens should have 4mls pre-aliquoted prior to the addition of acetic acid, in case HPV testing is required.

Before aliquoting a sample for HPV testing from ThinPrep vials, Hologic recommends vortexing the vial to ensure homogeneity of the sample. The procedure is:
1. Vortex the vial at high speed for 8 to 12 seconds.
2. Carefully remove the vial cap.
3. Using a pipetting device, withdraw an aliquot of up to 4 ml from the vial. Take care to avoid contaminating gloves with solution. If gloves should become contaminated, replace with a clean pair before proceeding to the next specimen.

The following should be carried out on the ThinPrep T5000, as a minimum:

- Empty filter wastes into the bin after every batch and change the fixing baths daily or after 100 slides, whichever comes first.
- On a weekly basis, remove the carousel and clean around the bottom of the processing area, using distilled water and lint-free towels. Do not dislodge the carousel sensors, but do keep the area around them clean and make sure nothing blocks them
- Clean carousel dispersion area and clean pneumatic suction holders weekly. A lint-free cloth soaked with 70% alcohol may be used to wipe down the surfaces of the slide holder cups. Be sure to let the alcohol completely evaporate (5–10 minutes) before attempting to process slides on the instrument.
    - Clean the carousel and dust cover, change absorbent pads, remove and clean drip trays on a monthly basis. The plastic drip trays are located on the underside of the T5000 Processor. They slide all the way out for inspection and cleaning.
    - Wash down with soap and water.
    - Allow them to dry thoroughly before returning them to the processor

Hologic has no formal recommendation on the frequency of sentinel testing, and suggests that weekly testing is appropriate. Run an un-used ThinPrep vial and an un-used filter for sentinel testing.

1.2.7 Specimen reception and registration

Specimen reception and registration accuracy are critical to accurate sample identification. As each sample is allocated a unique laboratory number, errors at the stage of processing have the potential to result in a mix-up of one patient's sample with that of another patient. The process of specimen reception and registration will be clearly defined in your IANZ approved laboratory manuals and you must be conversant with the practical aspects and policies for this key section. This should include quality control procedures and audit.

1.2.8 Slide/report filing and retrieval

It is a requirement for all NCSP contracted laboratories to have an effective filing and retrieval system in place for both reports and slides. This includes paper and electronic storage of reports and documents. The process must be systematic and allow quick retrieval of material. Some laboratories utilise commercial off-site storage facilities and the same principles apply. The NCSP, IANZ, and the RCPA have guidelines for the minimum retention of reports, documents, and slides. The *NCSP National Policy and Quality Standards (NPQS)* require cytology slides to be retained by the laboratory for a minimum of 10 years. Copies of request forms must be retained for a minimum of 15 years and laboratory results for a minimum of 20 years from the time of the test.

The process of filing and retrieval will be clearly defined in your IANZ approved laboratory manuals, and you must be conversant with the practical aspects and policies for this key section. This should include quality control procedures and audit.

1.2.9 Health and safety

Every employee staff member has a responsibility to ensure that the working environment is safe for themselves, their co-workers and any other person who may enter the laboratory or come in contact with the sample.

There are national regulations for health and safety in the workplace that are monitored and audited by the employing organization in conjunction with local council bylaws, Occupational Safety and Health, and IANZ. For some activities there are also special regulations and international guidelines.
All aspects of health and safety will be clearly defined in your IANZ approved laboratory manuals, and you must be conversant with the practical aspects and policies for this key section. This should include quality control procedures and audit.

1.2.10 Quality control and quality assurance

Every aspect of the laboratory reporting process has quality control procedures defined in the laboratory quality assurance policy. In addition there is ISO15189 and the *NCSP National Policy and Quality Standards* for cervical cytology (Section 5 relates to laboratory practice).

Quality assurance policy and quality control procedures will be clearly defined in your IANZ approved laboratory quality manuals, and you must be conversant with the practical aspects and policies for this key section.

Quality assurance programmes (QAP) are an integral part of any high quality cytology laboratory. They are implemented to maintain the highest standards of technical and diagnostic practice at all times, with the aim of requiring laboratories to provide a quality service, ensuring a high level of public safety. Important aspects of a quality system are transparency, regular review and audit, and excellent documentation. All staff should follow quality control procedures and participate in QAP. It is essential that as part of the audit trail individuals undertaking a task can be identified, so that remedial action in the form of further training and education can be undertaken if there is a system failure, however minor that may be.

There is much international debate regarding QAP in relation to interpretation, diagnosis and reporting in cervical cytology. Such programmes continually evolve and in NZ are intended to have an educational approach to improving quality as well as identifying under performance.

Sampling, processing and techniques

We can apply internal quality control to various stages of sampling, processing and techniques and these take many forms such as:

- Microscope analysis
- Automated procedures
- Certified and validated tests and equipment
- Clear documented procedures
- Known positive and negative control samples
- Quality grade reagents and certified stains
- Data analysis and statistical information
- Data plots giving trends in results
- Regular checks and maintenance procedures

- Continuing education and up-skilling

External checks can be made such as:

- Evaluation of random samples for processing etc.
- Sending specific samples to the laboratory for processing and staining

External assessors examine the final product (e.g. slides produced, reports issued) to ensure that a minimum level of quality was achieved.

Interpretation, diagnosis, and reporting

Consideration of the quality of the final report is paramount. The report must be clear, precise and accurate, predicting the histology as closely as possible. The laboratory must minimise false positive/false negative rates. A high rate of either false positives or negatives is unacceptable and can lead to over or under treatment.

Given a well-sampled and prepared specimen, how do we ensure accurate screening and interpretation with concise reporting? First and foremost is quality staff who are well trained and educated. Cytology is a demanding and very subjective science and requires highly motivated, experienced and appropriately qualified staff. Continuing education such as update courses, attendance at conferences and in-house seminars are essential to maintain the required skills. Environmental and working conditions are important and workload must be balanced. Primary and secondary screening, case reviews and histo/cyto correlation are also key internal quality control procedures. The NCSP requires that all abnormal cases are reported by a cytopathologist.

You must become fully conversant with and practice within the standards and policy statements of the *NCSP National Policy and Quality Standards*.

Laboratories are required to participate in external QAP programmes. There are two relevant RCPA programmes available for cytology screening performance, one assessing laboratory performance and the other assessing individual performance. There are separate modules for ThinPrep and SurePath. Another RCPA QAP programme provides samples to laboratories for HPV Testing. Other options include the ASCP Check Sample programme which provides digital images on a monthly basis along with a questionnaire, educational text and references. There are also numerous web-based programmes, including the NSW Challenges in Cytology educational package that is highly recommended.

1.2.11 Questions

1. What are the main functions that a good fixative tries to achieve?

2. Describe the main steps in the Papanicolaou staining method.

3. With regards to haematoxylin, explain what you understand by oxidation and a mordant.

4. Describe the difference between regressive and progressive Papanicolaou staining methods.

5. What are some of the precautions to be observed when using xylene?

6. What processes and procedures should a laboratory have in place to avoid cross-contamination of LBC vials with HPV?

References

- Bancroft JD, Stevens A. (Editors) 1996. *Theory and Practice of Histological Techniques* (4th Ed). Edinburgh: Churchill Livingstone.

- Coleman DV, Chapman PA. 1989. *Clinical Cytotechnology*. London: Butterworths.

1.3 CORE TOPIC: LBC PLATFORMS

1.3.1 Introduction

This topic provides an overview of equipment and techniques pertaining to the two Liquid-Based Cytology (LBC) systems used in New Zealand to process gynaecological cytology samples. The processing of ThinPrep samples and SurePath samples are not interchangeable systems i.e. samples collected in one type of vial must be processed and reported entirely using the technology for that LBC type. At the time of writing (September 2016), there are six laboratories in NZ using SurePath or ThinPrep for cervical cytology, as listed below:

LabPlus, Auckland City Hospital – ThinPrep with ThinPrep imaging
Anatomic Pathology Services – ThinPrep with ThinPrep imaging
Pathlab Bay of Plenty – SurePath with FocalPoint imaging
Medlab Central – ThinPrep with ThinPrep imaging
Canterbury Health Laboratories - SurePath with FocalPoint imaging
SCL Dunedin - SurePath with FocalPoint imaging

VRPCC candidates are expected to have some knowledge of both LBC systems and more detailed knowledge of the LBC system used at their training laboratory. All staff using LBC system technology are required to undertake additional training stipulated by the manufacturer of the system they are using, and must achieve competency sign-off before being able to process and report samples. If a practitioner changes the type of LBC they are reporting with, additional training in the new technique is required (ref: *NCSP National Policy and Quality Standards*)

1.3.2 SurePath equipment and processing

The BD SurePath™ Cell Enrichment process separates and reduces obscuring debris (e.g. blood, mucous) and inflammatory cells using a density reagent.

The PrepMate machine prepares the sample for the enrichment process by mixing and dispensing the specimen onto the BD density reagent in a 12mL tube. It can process between 1 and 12 samples **per** cycle. To reduce the possibility of contamination, the SurePath vial caps are not removed but are pierced using a syringe, and vials, syringes and tubes are not re-useable. The prepared tubes are then placed into a centrifuge.

PrepMate

The first centrifugation traps debris which is then decanted, enriching the cellular material remaining in the sample. After the second centrifugation, an enriched pellet of cellular material is produced, from which up to five slides can be made.

PrepStain Processor

Most laboratories processing SurePath samples use the SurePath PrepStain processor which prepares and stains the slides, producing slides with a 13mm diameter circle of cells. The cell enrichment step removes non-diagnostic material such as mucus, blood and inflammatory cells. This increases the concentration of epithelial cells (including any abnormal cells) on the slide.

For cervical cytology samples, the sedimentation time of the PrepStain programme is fixed by the instrument at 10 minutes and can't be changed. There is a separate "drying time" towards the end of the programme (i.e. how long cells are left to "dry" onto the slide and flatten) which can be set from a minimum of 55 seconds up to 300 seconds. The longer the drying time is, the less multi-layered the slide preparation will be, making screening the slide easier.

The SurePath method of processing using a separate PrepMate and PrepStain (i.e. not Totalys) requires careful concentration. When the PrepMate rack is set up with the labelled SP vial, syringe and labelled 12mL tube, these must be carefully and thoroughly checked to ensure they match, before loading onto the PrepMate. It is a good idea for laboratories to consider using a second person to double-check these and record that these checks have been done. The same applies to the PrepStain; a careful check is needed to ensure that labelled tubes match labelled slides. Again a second person verifying this and signing a record sheet is good practice. Alternatively labs may choose to use the optional PrepStain barcode scanner to ensure robust checking. Failure to apply robust checking methods during these steps could result in sample mix-ups. "Chain of custody" is a term used to describe the systems that ensure that accurate identification of each sample is preserved throughout the processing sequence.

Another recently available alternative is the Totalys system for SurePath samples. This is an automated processing machine that eliminates the need to separately centrifuge, decant and concentrate the sample.

The Totalys™ MultiProcessor provides full automation and ensures chain of custody for cervical cytology testing and molecular aliquots for ancillary tests such as HPV testing. Up to 480 cytology samples can be processed with preparation of molecular aliquots in an eight-hour shift. The system allows continual loading and unloading of samples.

Totalys MultiProcessor

1.3.3 ThinPrep equipment and processing

ThinPrep processing (T2000 and T5000)

All ThinPrep machines process samples in the same way. Laboratories use the ThinPrep 5000 (T5000) machine to process batches of samples. The ThinPrep 2000 (T2000) processes one sample at a time and is usually reserved for repeat preparations and as a backup.
The final slide preparation yields a 20mm diameter circle of cells that is stained in a separate staining machine. The processors:

1. Disperse the cells evenly in the fixative by agitating the fluid in the vial.
   - The dispersion step breaks up blood, mucus and non-diagnostic debris, then thoroughly mixes the sample.
   - This ensures that the cells on the slide are representative of the sample in the vial.
   - Any further slides made from the same vial should contain the same range of cellular material.

2. Collect cells by sucking the vial fluid up through a filter to trap a sample of cells.
   - With a series of negative pressure pulses, fluid is drawn though the Transcyt filter which has 5 micron pores, collecting a thin layer of cells on the filter membrane. The vials and membrane filters are not re-usable.
   - The processor constantly monitors the rate of flow through the filter, to collect an appropriate amount of material for testing.

3. Transfer the cells from the filter to a slide.
   - The cellular material is transferred to a glass slide using computer-controlled mechanical positioning and positive air pressure. The filter component inverts to deposit the cells on the glass slide, which is located above it.

The slide is then ejected into a cell fixative bath, ready for staining and evaluation.

The T2000 processes one vial at a time, which is loaded and unloaded manually. The T2000 is reserved for back-up use or to process single repeat slides.

The ThinPrep T2000

The T5000 ThinPrep Processor

The ThinPrep 5000 processes batches of vials with up to 20 samples per batch, each batch taking approximately 45 minutes. A carousel with a capacity to hold up to 20 vials, slides and filters is used. The processor uncaps and recaps each vial and

matches the barcode on the vial with the slide label prior to processing each sample. Slide racks with processed slides can easily be transferred to automated staining machines for slide staining.

The T5000 with Autoloader

The T5000 with Autoloader allows loading of 1 to 160 samples with up to 8 hours walkaway capability. It also features automatic slide etching.

1.3.4 Automated screening devices

Laboratories using automation with LBC systems are required to follow the *NCSP National Policy and Quality Standards* which outlines initial training requirements and sign off for cytology staff before they can commence reporting using automation. On-going competency requirements are also defined.

More details can be found at the following URL:
https://www.nsu.govt.nz/system/files/page/ncsp_policies_and_standards_section_5_providing_a_laboratory_service_oct_2013.pdf

The SurePath FocalPoint Profiler

The FocalPoint machine is essentially a high speed video microscope. Prepared coverslipped slides are placed in black metal racks and loaded into the input hopper in the machine. The machine feeds each tray through its optical path, does a calibration before and after each tray and systematically analyses each slide using 4X and 20X objectives. An initial scan of 1000 fields of view (FoV) is done using the 4X objective. Predetermined areas are then closely examined using the 20X objective.

The machine captures 11 FoV for cytoscientists/cytotechnicians to examine under a Guided Screening (GS) microscope. The first FoV is a location confirmation field i.e. to allow the screener to calibrate and set up the stage correctly for the

The FocalPoint Slide Profiler

subsequent fields of view. The subsequent 10 FoV constitute the diagnostic FoV's. It takes about 40 minutes for the FocalPoint Profiler to scan a tray of 8 slides. Slides in batches of between 80 and 120 slides are ranked and scored on the basis of the images captured, according to how likely they are to contain abnormal cells. The first quintile ranking has the highest probability of containing abnormal cells and the last (fifth) quintile has the highest probability of being normal/benign. The machine also reports on the presence or absence of an endocervical component.

Imaging a SurePath slide in the FocalPoint Profiler

The ThinPrep Imaging System

The ThinPrep imager automatically scans every cell and cell cluster, highlighting areas of interest for analysis by a cytoscreener. Abnormal cells are selected on the basis of the staining characteristics of the nuclei. Cell clusters are also analysed, with a minimum of two cell clusters selected for microscopic review for every slide.

Slides are stacked vertically into cartridges carrying up to 25 slides each which are then loaded into the Imager. Cartridges can be loaded/unloaded as ready/complete with the Imager running. One Imager can hold up to ten cartridges at any one time and process 300 slides per day (80,000 per year).

The ThinPrep Imager

Hologic's proprietary DNA stain is used to stain cervical cell nuclei. This is a stoichiometric stain where the degree of darkness of the nucleus reflects the DNA content. Nuclei in abnormal cells have increased amounts of DNA and therefore take up more stain than the nuclei in normal cells. Abnormal cell nuclei are often larger with an irregular nuclear membrane.

The ThinPrep imaging system scans each slide and identifies 22 fields of view (FoV) that contain cells of interest. The cytoscreener then reviews those 22 fields using an automated Review Scope microscope which locates each of the FoV based on stored coordinates from the Imager using an automated stage control system. If all fields are judged to be normal, "no intraepithelial lesion" is reported. If the cytoscreener judges cells in any field to be definitely or potentially abnormal, the slide is reviewed manually and abnormal cell groups marked for further review by a pathologist.

1.3.4 Questions

1. Describe the cell enrichment process used in SurePath processing and explain what this achieves.

2. Explain the differences between the vials used for SurePath and ThinPrep. This should include the vial configuration and the contents.

3. What are the unique properties of the ThinPrep Transcyt filter? Describe how it is used to produce a ThinPrep slide.

4. What are the precautions to be aware of when using the SurePath Prepstain machine to ensure there is no sample mix-up?

5. Is there any potential for sample contamination when using the ThinPrep T2000 or T5000 machines? What measures could be taken to mitigate these?

6. Discuss the NCSP requirements for laboratories using automated screening devices for cervical cytology with LBC systems.

References

Further information is available at:

SurePath: http://www.bd.com/tripath/products/outside_us/index.asp

ThinPrep: http://www.hologic.com/products

1.4 CORE TOPIC: MICROSCOPY

1.4.1 Introduction

The microscope is the most important tool for the cytoscientist/cytotechnician. This precision instrument will give years of high quality imagery as long as it is well maintained, regularly serviced and correctly set up for optimal resolution. The cytoscientist/cytotechnician should understand the basics of bright field microscopy to enable best use for screening and interpretation.

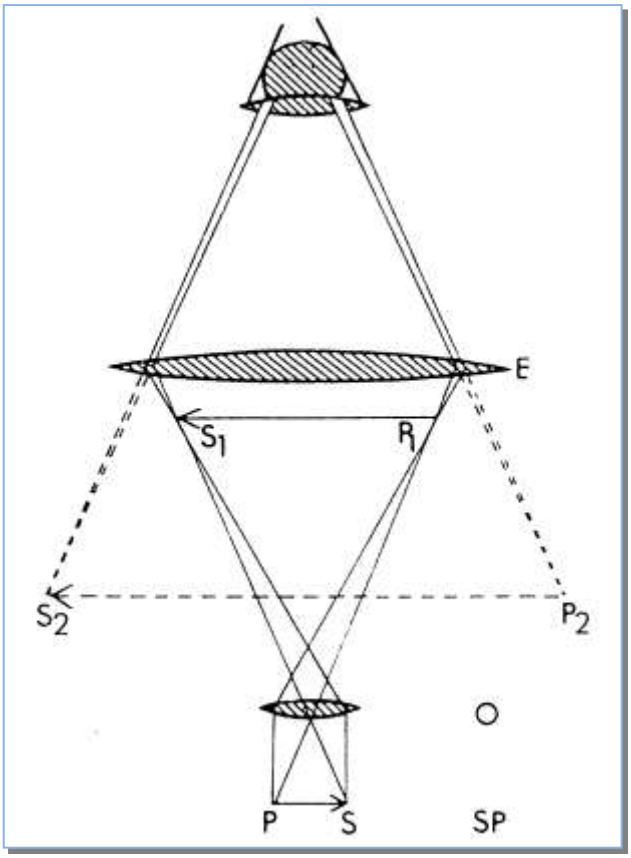
The art and practice of microscopy dates back at least 6 centuries when lenses were used to examine objects. Early inventions still underpin current light microscopy.

15th Century (or earlier)	Earliest use of lenses to examine objects
1609 Galileo	Use of two lenses as a microscope by reversing a telescope to examine spores
1640 Hooke	Single lens microscope and published works The Royal Society of Microscopy established
1702	Examination of blood cells
1830	Compound lens
1850	Photomicrographs
1880 Lindsey	Oil immersion lens

1.4.2 The compound microscope

Modern compound microscopes are extremely complex with an array of lenses and prisms. The principles however, are the same as a basic two-lens compound microscope.

- At least two lenses are used in a compound microscope which are set at a significant distance apart to:
    - Produce greater magnification
    - Help overcome lens aberration and faults
    - Magnify the specimen twice but only invert once, resulting in an upright (virtual) image

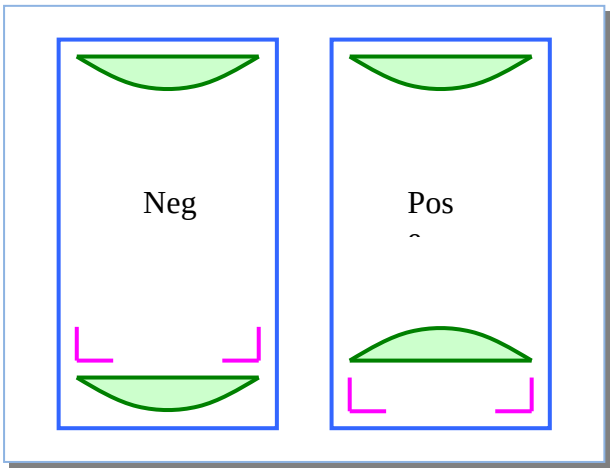


The object (P-S) is magnified twice with this basic two-lens configuration, with a first real inverted image (S1-P1) and a second upright virtual image of the first image (S2-P2).
In practical microscopy terms the object is the specimen (SP), the first lens the objective (O), and the second lens the eyepiece (E).

1.4.3 Components of the compound microscope

Eyepieces

- These have 2 planoconvex (flat and curved sides) lenses either with
  - both with the flat side (plano) facing upwards (negative)
  - or the concave facing each other (positive)
- Eyepieces give a virtual image to the eye which appears in the plane of the object
- Eyepieces are usually marked with the eyepiece viewable diameter e.g. 22mm and the numerical aperture
- Huygens eyepieces are the simplest type but are not corrected for chromatic aberration
- A graticule can be placed at the field stop for microtomy (measuring objects)



Planoconvex lenses.
Left: Negative with the plano (flat) sides facing upwards.
Right: Positive with the convex sides facing in towards each other.
A field stop may be included to hold a measuring graticule.

Objectives

Details of the ways objectives are defined have been discussed above.

- Objectives are generally marked with Type, Magnification/Numerical Aperture, Tube length/cover-glass thickness mm e.g. Plan Achro x 10/0.25;160/0.17

Body tube

- The optical tube length for most microscopes is set at 160 mm
- With newer technology tube length can vary due to infinity corrected lenses.

Stage

- These are mostly mechanical but automatic stages can also be purchased
- The stage generally has a vernier gauge for relocation of cells of interest

Condenser

- The purpose of the condenser is to concentrate the light into the plane of the object.
- The substage condenser should have the same numerical aperture as the objective in use (check the condenser NA for the range of objectives) and should form a true image of the light source
- Abbe condensers are the simplest but are not really suited for critical microscopy, where an achromatic condenser should be used
- A flip top lens can be used to focus light into a field for use with a high magnification objective lens
- The condenser will have an iris diaphragm, which alters the cone of light passing into the condenser, ridding it of any stray light that may interfere with the rays used to form the image. The iris is restricted more with high magnification objectives.

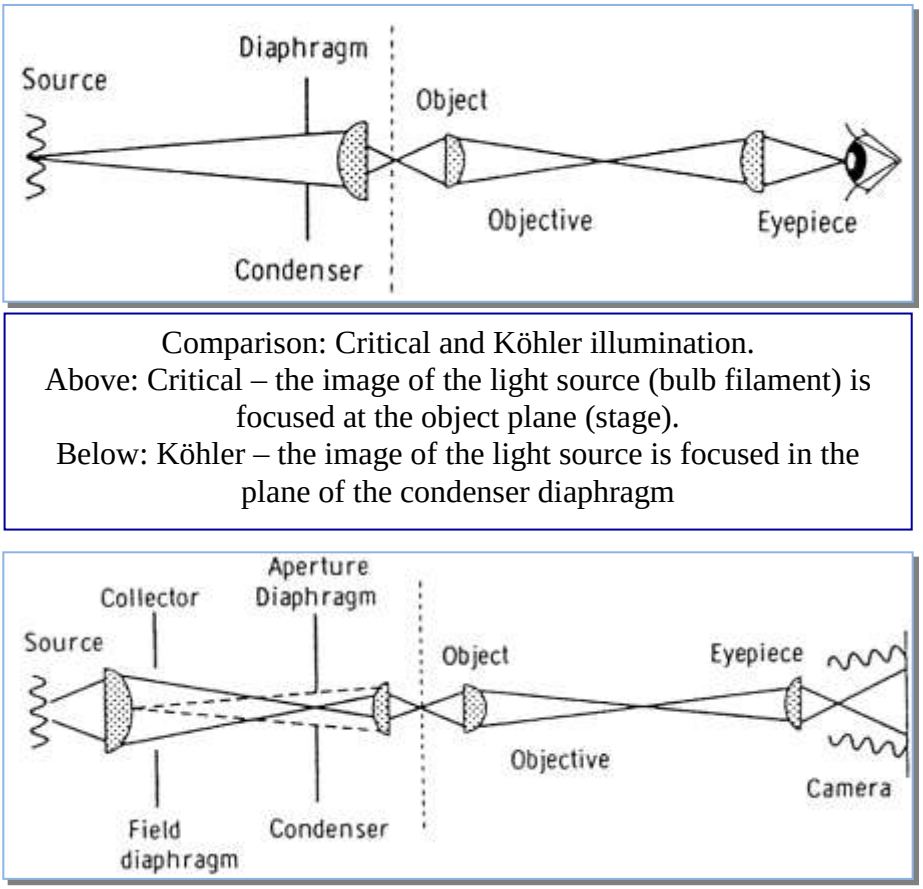
Illumination

It should be noted that a bar filament bulb gives a more even light than a coil filament bulb.

For bright field microscopy two different illumination systems are considered:

- Critical illumination (Nelson)
    - The image of the light source is focused on the object plane. The illuminating rays are symmetrically disposed along the long axis of the microscope. This system has "hot spots" and the filament can be seen at low power. It is not suitable for photography.

- Köhler illumination
    - Includes an adjustable collector lens in front of the lamp, which focuses the light source in the plane of the iris diaphragm of the condenser. This eliminates the problems associated with critical illumination.



Comparison: Critical and Köhler illumination.
Above: Critical – the image of the light source (bulb filament) is focused at the object plane (stage).
Below: Köhler – the image of the light source is focused in the plane of the condenser diaphragm

1.4.4 Adjusting the microscope – getting started

The following is a guide to setting up the microscope. For detailed instructions consult the manufacturer's instructions.

1. Centre the Lamp

Take a ground glass filter (or tissue paper) and place it on the dust protection glass on the base of the microscope. Then move the bulb, either with centring screws or by moving the holder, until the filament is in focus and centred. On many modern standard microscopes the bulb setting is prefixed.

2. Adjust condenser

Select a low power objective (x 10) and place a suitable stained and cover-slipped specimen on the stage. Focus on the specimen to establish the plane of the object. Open the aperture diaphragm (in the condenser) and close the field diaphragm (at the base of the microscope). You will see the outline of the field diaphragm. By adjusting the length of the condenser, focus the field diaphragm. Then centre the image by adjusting the condenser screws.

3. Focus the eyepieces

Most binocular microscopes have one fixed non-focus eyepiece and one focus adjustable eyepiece. When adjusting eyepiece focus, both eyes should be open. Use a piece of card to cover one eye when adjusting the opposite eyepiece. Focus with the non-adjustable eyepiece first by using the coarse and fine focus dials. Then change eyes and focus the adjustable eyepiece.

4. Inserting the Slide

It is always good practice to lower the stage and use a low power objective before inserting the slide. Focus down carefully. Once the specimen is in focus, remove the eyepiece and you will be looking at the back lens of the objective. Close the condenser diaphragm until it is the same diameter as the objective lens.

The microscope is now ready for use. Minor adjustments will be required for critical work for each objective and specimen. It is good practice to check the above items regularly and to clean and maintain your microscope. Special lens tissue and blow brushes may be used but avoid solvents e.g. xylene as this may affect the glue that holds the lenses in place.

1.4.5 Questions

1. Explain how you would set up a modern microscope for optimal visualisation of a stained cover-slipped cervical cytology slide (Kohler illumination).

2. What precautions should you take when changing a microscope light bulb?

3. Describe the regular cleaning maintenance schedule you would undertake for your microscope.

References

- Culling CFA. 1974. *Modern Microscopy Elementary Theory and Practice*. London: Butterworths.

1.5 CORE TOPIC: THE SCREENING PROCESS

1.5.1 The primary screening process

Manual Screening method

Primary screening is the most significant step of cytology screening for the detection of pre-malignant lesions of the cervix. Abnormal changes missed at primary screening are less likely to be detected with secondary/QC review re-screening.

The primary screening method requires intense concentration for long periods. A conducive environment, effective ergonomics, a well-maintained quality microscope and self-maintenance of personal well-being are critically important. Cytoscientists/cytotechnicians are dedicated staff and need to follow a programme of continuing education to maintain and develop their skills and expertise. These activities are governed by ISO15189 (Medical Laboratory Standards) in conjunction with the *NCSP National Policy and Quality Standards.*

The fundamental requirement of primary screening is to examine **all** the cells within the sample to identify any abnormal cell changes or patterns i.e. any change that is not within normal limits. Cytoscientists/cytotechnicians need to be fully conversant with the normal cytology of the cervix as well as other cellular and non-cellular components that may be found in the sample. Until recently, all cells in the sample were screened manually. With the introduction of automation, screening performed by automated screening devices means that the cytoscientist/cytotechnician only needs to look at fields selected as those most likely to contain abnormal cells.

After identifying that abnormal changes are present, the cytoscientist/cytotechnician interprets the abnormality identified. The process of learning to become competent at both identification and interpretation of abnormality requires patience and commitment but is extremely rewarding as these skills develop.

Coverslip size

All of the stained cells on an LBC slide should be covered by the coverslip. As a ThinPrep slide has a 20mm diameter circle of cells and a SurePath sample has a cell preparation diameter of 13mm, the minimum coverslip width for a ThinPrep slide should be 24mm whereas a narrower width coverslip may be used for a SurePath slide. The coverslip length for either preparation type can be between 45mm and 50mm.

Manual Screening: full cell content/field overlap

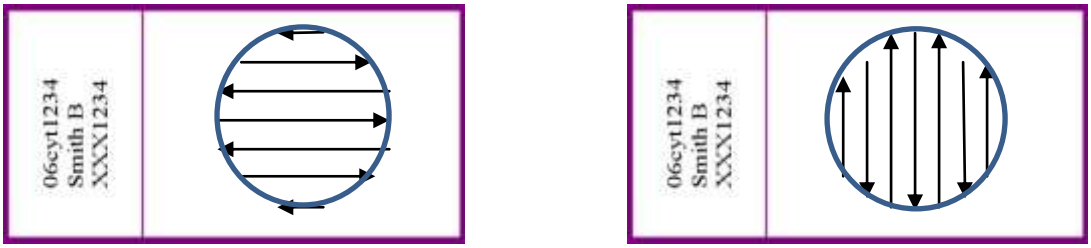
All the cellular material within the cover-slipped area must be examined. When commencing screening as a new practitioner, expect to take more time examining cells than your qualified colleagues. It can be a daunting task to examine the 5,000 to 20,000 cells that may be present on an LBC slide. With experience, the task will become faster until you achieve accurate assessment combined with time efficiency. The brain becomes programmed to let normal changes filter by while recognising abnormality. It is essential that you do not allow speed of screening to compromise quality as this can lead to significant false negative results.

It can take between 3-8 minutes for an experienced practitioner to screen an LBC slide. With SurePath preparations the cell density is greater and the cells are more multilayered, compared

with ThinPrep preparations where the cells are more spread out in a thin cell layer. However, the time taken to screen each type of LBC slide is comparable. SurePath slides with only a 13mm diameter cell preparation area (compared to 20mm area for TP) take as long to screen because the cell population is denser and more "up and down" focusing is required through each field of view.

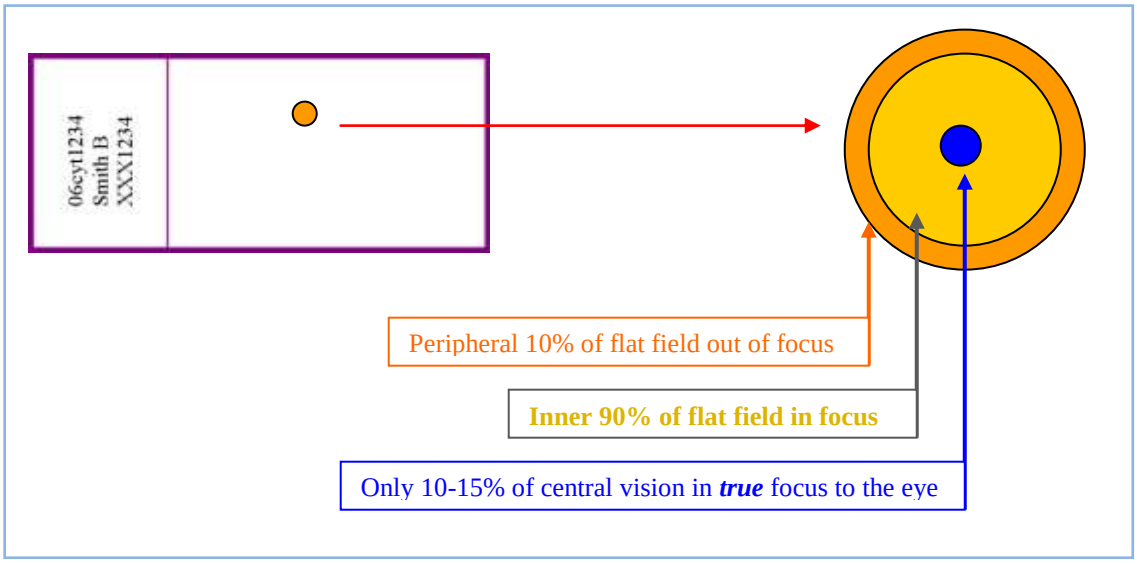
So how does the cytoscientist/cytotechnician ensure that all the cells have been examined?

Firstly, the cytoscientist/cytotechnician develops a consistent pattern of screening. Slides are either screened in a top-to-bottom, up-and-down pattern (i.e. vertically across the narrow aspect of the slide), or in a side-to-side, backward/forward pattern (i.e. horizontally across the wide aspect of the slide) as demonstrated in the diagrams below.



Two directional methods for primary screening:
Left: Backward/forward method screening horizontally across the slide
Right: Up/down method screening vertically across the slide
Note: Always put the label end of the slide the same way – either to the left or to the right

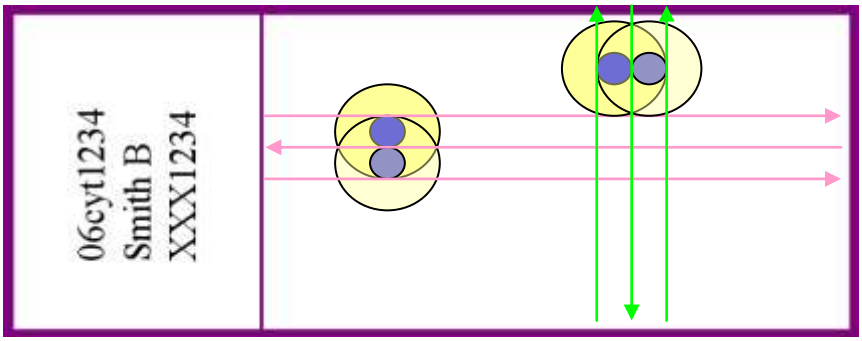
Modern microscopes have flat field objectives where up to 80-90% of the field of view is in focus. However, this does not mean that all the cells within that focus band are in focus to the human eye. In fact if you look at a field of cells on 10X objective magnification for example, you will note that at most, only 10-15% of the cells are in focus. Try looking at the centre of the field and without moving your eye, note that the cells in peripheral vision appear progressively out of focus from your central fixed view. The number of fields you need to examine to be confident that you have seen all the cells in focus is significantly more than the slide area divided by the area of the field of view.



Actual "true" focus to the eye in a standard field of view, with flat field objectives emphasising the need to overlap fields when screening.
Not to scale.

How do we compensate for the limited area of focus within the field of view? There are two common methods that cytoscientists/cytotechnicians use:

1. The first is to maintain a fairly fixed eye (little to no eye movement) to the centre of the field of view. Commence screening from one corner of the slide. Then continually move the stage at a generally steady speed from field to field. When you reach the end of each screening line move the stage up one width of *your* in-focus focal view – **NOT** the full field of view. Then screen back in the same manner, and repeat the process until the complete slide has been examined.
2. The second is to have a degree of eye movement within each field of view. It is essential that you are consistent in the area you scan within each field that you examine from field to field. Commence screening from one corner of the slide. When you reach the end of each screening line move the stage up one width of *your* in-focus field of view – **NOT** the full field of view. Then screen back in the same manner, and repeat the process until the complete slide has been examined.



Overlapping objective fields of view to ensure full visualisation of all cells.
The large circles (yellow) represent full flat objective fields of view and the small circles (blue) are the true in-focus vision to the eye: 10-15% of the objective field. The arrows represent screening paths demonstrating why screening requires overlap of fields of view. *Not to scale*.

It should be noted that screening also requires each cell to be in focus for examination and you will develop the ability to continually make fine focus adjustments to compensate for unevenness of the sample and examination of any multi-layer cell groups and fragments.

Marking cells

Any cell or other component you observe outside normal limits should be identified by a permanent mark or dot adjacent to the abnormal cells. This marking should be consistently placed for easy reference at any time by any other person reviewing the sample.

To achieve this consistency, **always**:

1. Place the slide the same way on the microscope stage i.e. always have the label to the left or to the right
2. Mark the cells or changes in the same manner i.e. if your laboratory marks to the right hand side of the cells do this consistently. Keep your marks at a consistent size so that they are big enough to see with the naked eye, but only obscure a minimal number of cells under the mark. Before you place the mark, be sure that you are not covering significant cell changes that may be crucial to interpretation and grading of the disease process.

When first commencing screening, most practitioners mark slides prolifically - the surface of the screened slide may be akin to a snowstorm. This is normal and eventually you will become skilled in what and when to mark. It is essential that you identify the worst cellular changes that may be present in order to predict the worst level of abnormality.

Environment, ergonomics and exercises

These aspects can be easily overlooked but with current requirements of occupational health and safety and greater employer awareness, the environment and ergonomics that suit your stature should be a serious consideration from the onset of your employment. Should you experience any aspect that may compromise your performance you should bring this to the attention of your employer or their representative. Regular short exercise activity through the day will help prevent occupational overuse syndrome. It is beyond the scope of this workbook to detail environment, ergonomic and exercise requirements but these will be readily available from your employer and are a consideration at every annual laboratory accreditation by IANZ.

Be proactive and do not let minor aches and pains develop into major limitations!

1.5.2 The secondary screening process

Secondary screening (often referred to as QC review) is an essential and compulsory activity applied to all primary screened slides. There are two methods of secondary screening:

- Rapid review
- Full review

Rapid review involves rescreening the slide with a minimum screen time of 60 seconds. For LBC samples all of the slide will be covered, within the shorter rapid review time frame. Under the *NCSP National Policy and Quality Standards*, laboratories that manually screen slides or have not fully converted to automated screening must conduct rapid rescreening of all manual

primary-screened slides categorised as 'Negative for intraepithelial lesion or malignancy' (excluding negative slides requiring a full rescreen) prior to reporting. The outcomes of all rapid re-screens must be recorded.

Full secondary screening review occurs in specific situations. This includes all samples where an abnormality is detected on primary screening, where there are abnormal clinical indicators, and cases with an abnormal screening history. The sample is rescreened in the same manner as the primary screen but by a different practitioner.

For full details of the requirements for full rescreening refer to Section 5 of the *NCSP National Policy and Quality Standards.*

While you are completing the VRPCC you will only be asked to perform primary screening. Once you have successfully completed the VRPCC and have obtained an annual practicing certificate from the Medical Sciences Council of NZ, you will be eligible for further training in your laboratory to perform rapid review secondary screening (if used in your laboratory) to achieve competency sign-off as a rapid reviewer.

To undertake full rescreening you will be required to have completed additional years of experience as detailed in the *NCSP National Policy and Quality Standards*.

1.5.3 Automated screening

All laboratories in NZ processing gynaecological cytology use automation. Conversion to automation mostly occurred between 2008 and 2010. Automation eliminates the need for full examination of all the cellular material on slides as it allows cytoscientists/cytotechnicians to go directly to selected fields of view only. Automation results in greater efficiency i.e. less staff are required to achieve the same work output. The *NCSP National Policy and Quality Standards* sets a maximum of 140 slides screened (FOV review after automated screening) per cytoscientist/cytotechnician per day.

Automated screening devices do not report abnormality, but rather select those fields where abnormality is most likely to be located. Interpretation is the responsibility of the cytoscientist/cytotechnician.

The VRPCC candidate is expected to understand and learn about the automated cytology screening processes in more detail than is outlined in this workbook. This will occur in the laboratory of employment and will be specific to either ThinPrep with Imaging or SurePath with the FocalPoint Profiler. The systems may be set up in various ways including unique ways of interfacing between the individual manufacturer's software and the laboratory information system (LIS).

SurePath

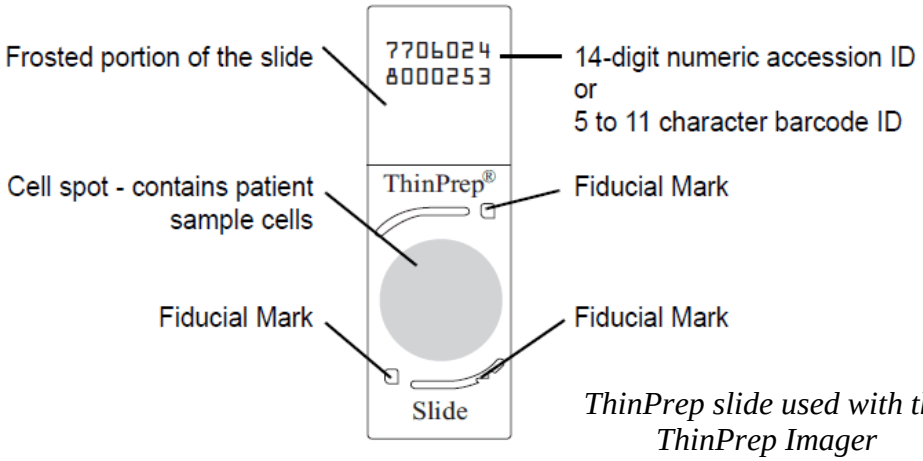
The Focal Point Profiler and SurePath Guided-Screening system provides a computer-assisted primary screening system. Approximately 288 barcoded slides can be processed through the SurePath Focal Point Profiler in 24 hours. The machine utilises a central scanning microscope with 3 high quality digital cameras to screen each slide. Data for each slide is sent to the Guided Screening server. Attached to the server are the Guided Screening Review Microscopes.

SurePath Guided-screening Review Microscope

These microscopes have an automated stage unit. The cytoscientist/cytotechnician scans the slide using the barcode scanner on the Guided Screening Review station, and the data as determined by the FocalPoint Profiler is retrieved for that slide. The Location Confirmation (LC) is always the first FoV presented. LC is a distinct object presented in the slide. This has to be matched with what is being seen down the microscope. LC ensures that the cytoscientist/cytotechnician is evaluating the correct slide as well as the correct areas on the slide by making sure that the slide is correctly aligned. Using the mouse or a foot pedal, the cytoscientist/cytotechnician is then able to advance through the 10 diagnostic fields of view (FoV) on the automated stage unit. The diagnostic FoV are presented for evaluation in rank order according to the likelihood of abnormality. It is up to the cytoscientist/cytotechnician to decide how to interpret the changes being presented and to report through either the GS software or through the Laboratory Information System.

ThinPrep

ThinPrep automation is a computer-assisted primary screening system. The ThinPrep microscope slide used by the ThinPrep Processor in preparing the patient slide is a specially prepared slide with fiducial marks (fixed reference points) permanently printed on the slide. These are used to register the slide position on the stage. A coordinate system based on the fiducial marks is used to locate objects of interest in the cell sample area (cell spot).



ThinPrep slide used with the ThinPrep Imager

The ThinPrep Imaging System uses computer-assisted screening with an automated microscope to locate the cells of interest. The ThinPrep Imaging System pre-screens each slide prioritizing individual cells and cell clusters of interest based on the DNA content, determined by assessing nuclear optical density. A slide identification camera reads the slide label identification (ID) and compares it with the slide ID already in the computer database. The ThinPrep Imaging System scans each slide and identifies 22 fields that contain cells of interest. The entire cell spot is imaged in approximately 90 seconds. The coordinates of the 22 Fields of View (FoV) are recorded and are stored in the system database with the slide ID. The cytoscientist/cytotechnician then reviews the 22 fields using an automated microscope where the slide ID is read and an automated stage locates the fields of interest based on the Imager coordinates. The cytoscientist/cytotechnician reports "no intraepithelial lesion" if all fields are judged to be normal. If cells in any field are deemed to be suspicious, the entire slide is reviewed by the cytoscientist/cytotechnician who marks the abnormal cell groups for further review by a second cytoscientist/cytotechnician and a pathologist if appropriate.

The ThinPrep Imaging System uses either of two automated Imager-integrated microscopes, the Review Scope or the Review Scope Manual Plus. Laboratories can use either or both microscopes.

With the Review Scope, the cytoscientist/cytotechnician uses a “pod” with one hand to control FoV selection (next, back), autoscan (for full screen), objective selection (10X or 40X), and electronic and actual marking. Automated full screens can be done on this microscope but the slide may be taken to a manual microscope for practical reasons of case workflow.

The Review Scope Manual Plus utilises a touch screen, manual objective selection (40X, 10X, 4X) and the option of full control manual screening on the same microscope.

ThinPrep Imager-integrated Automated Microscope

To use either microscope, a prepared ThinPrep slide is first loaded onto the stage. The automated stage presents the 22 FoV to the cytoscientist/cytotechnician who interacts with the review

controls both via sub-stage hand controls and by pod/touch screen. Each FoV is presented to the cytoscientist/cytotechnician at 10X magnification. The cytoscientist/cytotechnician can switch objectives via the pod or manually. During the slide review, the cytoscientist/cytotechnician has the option to electronically mark an area for subsequent review. One or more electronic marks trigger a review of the entire cell spot. After detecting the abnormal cells, the cytoscientist/cytotechnician decides on an interpretation of the changes seen and this can be entered into the laboratory information system.

1.5.4 Reporting

In New Zealand we report using The Bethesda 2001 New Zealand Modified Reporting System (TBS). TBS was initially developed in the United States of America and is now an internationally accepted reporting system used in many countries. It is management focused and ensures standardised report descriptors aligned to a series of codes. This is essential so that there is no ambiguity regarding the interpretation of the cytoscientist/cytotechnician i.e. the report is understood by all sample takers, colposcopists, cytoscientists/cytotechnicians, histopathologists and NCSP staff nationwide.

TBS also allows women's screening histories to be stored on the NCSP register (NCSP-R) and to be readily accessible for determining follow-up and recall. Every sample you screen or rescreen will have the NCSP-R history available for review. You will be required to provide a recommendation in your report based on your assessment of the cytology sample, the previous NCSP-R history (cytology and/or histology) and any clinical information provided by the sample taker.

The method for determining the next event i.e. follow-up or referral is based on the *NCSP Clinical Management Guidelines* which may be accessed on the website www.nsu.govt.nz under NCSP/For Health Professionals.

1.5.5 Questions

1. What are the two fundamental processes involved in primary screening?

2. Describe the main *NCSP National Policy and Quality Standards* relating to screening cervical cytology samples.

3. Describe the main factors that must be considered when primary screening a cervical cytology sample.

4. What are the important factors to remember when marking slides?

5. Under the *NCSP National Policy and Quality Standards*, in what circumstances must full rescreening be carried out?

6. Outline the use of the Bethesda 2001 (TBS 2001 NZ Modified) reporting system in NZ.

7. Discuss any precautions that need to be taken when using automated screening as this applies to the LBC system used in your laboratory.

SECTION 2.0
MORPHOLOGY: CYTOPATHOLOGY and HISTOPATHOLOGY

2.1 CORE TOPIC: THE CELL IN HEALTH AND DISEASE

2.1.1 Introduction

Studying fundamental biological processes in normal, benign and malignant epithelial cells is essential in order to develop an understanding of the many changes and diagnostic features encountered in diagnostic cytopathology. An excellent reference for this topic is:

Monographs in Clinical Cytology Vol 2
The Cell in Health and Disease
Author: Frost J K.
Published: Karger 1969, revised 1986

The first edition provides a good understanding of the topic and is recommended as more accessible than the second more complex expanded revision.

The study of cells in health and disease is fascinating and constantly provides a challenge to the cytologist. The variations on a theme are endless, but observing and following basic principles in cell morphology will help achieve accurate cytologic interpretation and diagnosis and minimise false negative and false positive cytology reports.

The Cell in Health and Disease will be addressed under two major headings:

Part A **General activity**: state of the nucleus in relation to cellular activity
Part B **Functional differentiation**: state and activity of the cytoplasm

The details of cell behaviour described below are applicable to changes seen in **epithelial cells** from many body sites and are not unique to cervical epithelium. Consistent application of basic criteria will assist with interpreting cell appearances when morphological presentations are challenging and do not match classic textbook images.

2.1.2 Part A: GENERAL ACTIVITY

The general activity of an epithelial cell is determined almost exclusively by changes in the nucleus and these changes are often reflected in nuclear morphology. The state of the nucleus seen in the following situations will be discussed.

1. The normal healthy cell
2. Decreased cell activity: cell degeneration and cell death
3. Increased cell activity in benign (non-neoplastic) states
4. Increased cell activity in invasive malignancy (carcinoma)

Descriptors such as “retroplasia” and “euplasia” are scientific biological terms and are cross-referenced with terminology in common usage in laboratories.

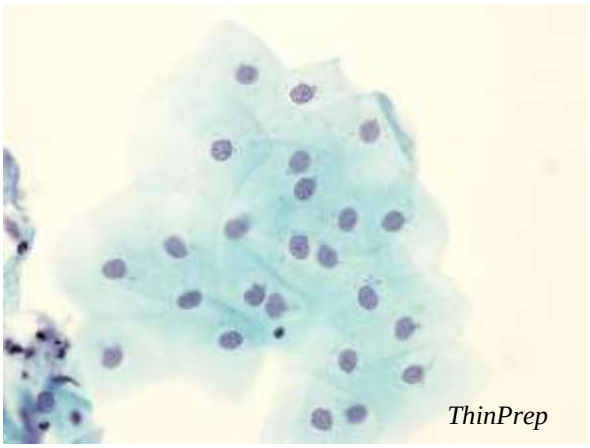
In discussing nuclear changes of epithelial cells that reveal the general activity state of the cell, it is emphasised that no one criterion is used for a final interpretation. A single criterion may be a feature of either benign or malignant cells, but its significance is apparent when

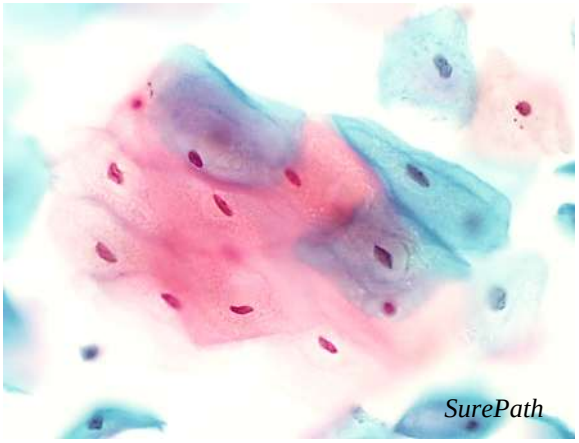
reviewed in association with other features i.e. interpretations need to be based on an assessment of all nuclear and cytoplasmic criteria. The sample needs to be optimally preserved and representative of the cell population of interest.

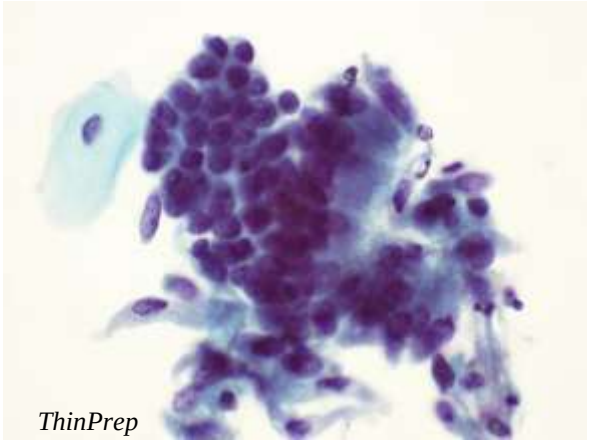
2.1.3 The normal healthy cell: Euplasia

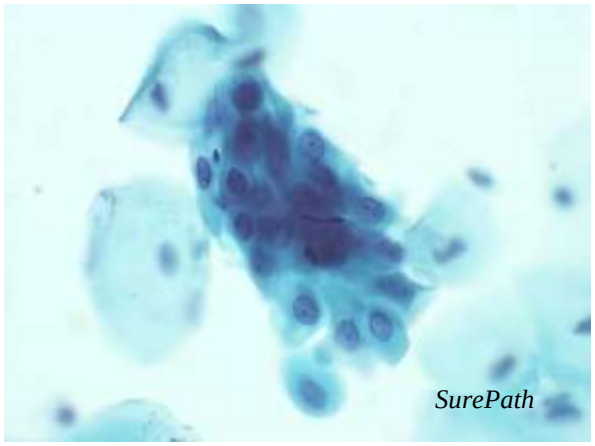
Euplasia refers to the cell in a normal healthy state. The cells are in a synchronous state with neighbouring cells which all show similar uniform cell features. Within the nucleus the chromatin, parachromatin, and nuclear membranes stain basophilic and the nucleolus is acidophilic. The chromatin is made up of DNA and associated structural proteins which have a constant relationship to each other. The nucleolus is the centre of ribosome production. When ribosomal RNA travels from the nucleolus through the nuclear membrane, the proteins associated with the ribosomes change from being acidophilic to basophilic.

The nucleus is round and the chromatin has a uniform pattern. The nuclear membrane (also referred to as the chromatinic rim) is regular. Cells of the same cell type at the same stage of maturation or activity show a monotonous uniformity which is demonstrated well in tissue fragments. Nuclear constituents such as chromatin granules, nucleoli and chromocentres all have round and uniform features and are distributed regularly within the nucleus. If present, the number of nucleoli per cell is uniform across cells within a tissue fragment. Within cell groups, there is strong cell-to-cell cohesion. **Uniformity and predictability from cell to cell is the key feature of euplasia.** Any distortion of these features requires an explanation.









Cells showing predictability and uniformity from one cell to another within cell clusters and groups.
Top left: Intermediate squamous cells. Top right: Superficial squamous cells.
Bottom left: Endocervical cells. Bottom right: Metaplastic squamous cells.

2.1.4 Decreased cell activity, degeneration and cell death: Retroplasia.

Retroplasia refers to decreased cell activity and includes cell degeneration, death or injury. As a cell dies it follows a programmed pattern of degeneration (apoptosis). Retroplasia occurs with all cell processes including neoplasia. Often, the more rapidly proliferative a malignant cell population is the more evidence there is of retroplasia as well.

The common changes observed with retroplasia in the order that they occur are:

1. Initial shrinkage—the nucleus shows creasing across the diameter and wrinkling of the nuclear membrane.
2. Karyopyknosis—the nuclear chromatin becomes condensed as the nucleus continues to shrink, becoming an amorphous mass.
3. Karyorrhexis—the nucleus breaks up into fragments within the cell.
4. Karyolysis—the nuclear fragments disintegrate and are removed from the cell.
5. Anucleation—the cytoplasm remains intact but with no visible nucleus. There may be an outline where the nucleus was, creating a "ghost cell" appearance.

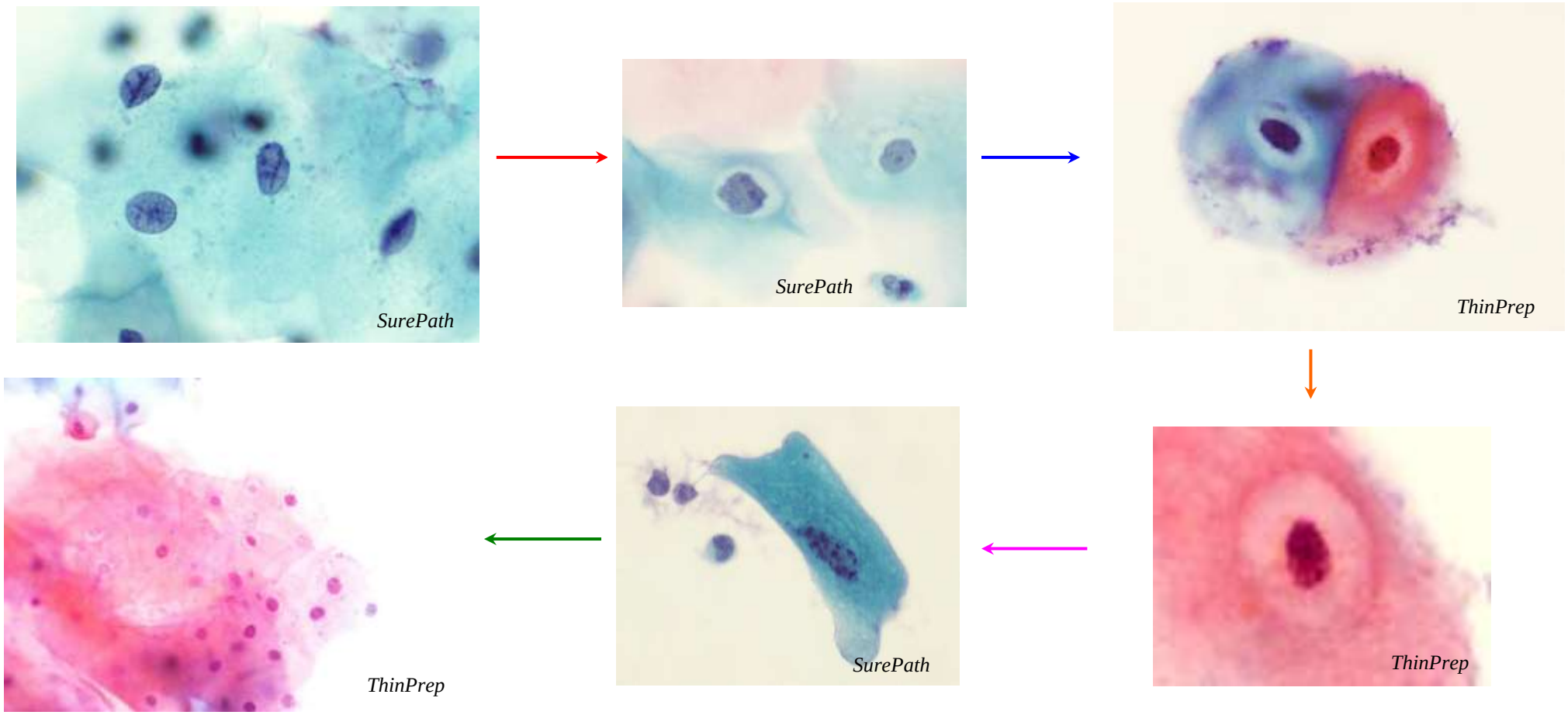
During these phases of cell death, the cytoplasm often becomes less distinct. Degenerative vacuoles may appear. A perinuclear halo (clearing around the periphery of the nucleus) appears as the nucleus begins to shrink and condense (karyopyknosis) and pseudo keratinisation may develop. The cytoplasm eventually disrupts (cytolysis) as the nucleus progressively fragments (karyorrhexis).

Within a cell group, cytoplasmic borders may be lost producing pseudosyncytia of nuclei. Cilia degenerate and are lost very early in cell death. Cell death associated with some viral infections may cause ciliocytophthoria, where ciliary tufts attached to a terminal plate break free from the rest of the cell and are seen as dissociated fragments.

The nucleus may become large, pale and washed out with retroplasia, or may become pyknotic as the chromatin and nuclear membranes shrink and condense leaving a small, amorphous, dark shrunken nucleus. The inner nuclear membrane becomes blurred, while the outer membrane remains crisp (one of the earliest signs of degeneration). Chromatin clumps and thickenings on the nuclear membrane become more rounded. Later in cell degeneration the chromatin clumps marginate to the nuclear membrane producing a beading effect. The nuclear membrane finally becomes wrinkled and nucleoli may either shrink or become larger and swollen. Parachromatin clearing may become marked leaving large clear spaces within the nucleus.

If the nucleus does enlarge becoming large and dark, the changes must be carefully examined for a squamous intraepithelial lesion. The staining usually appears washed out when degeneration is present. Retroplasia results in small dark nuclei, not large dark nuclei.

Some features of retroplasia may mimic criteria of malignancy so careful observation and interpretation is critical to accurate interpretation. If fixation or visualisation is poor or there is limited material present, caution is needed as the potential for misinterpretation is increased. It is unwise to make a definitive interpretation on degenerating cells alone. The context in which the sample has been taken and the other cells observed in the sample must always be considered.



The images above show typical steps in retroplasia/degeneration. The nucleus begins to shrink and shows a crease across its diameter. As shrinkage continues, the nucleus shows a wrinkled nuclear membrane with karyopyknosis and a cytoplasmic perinuclear halo develops. The nucleus then continues a process of apoptosis with increasing beading of the chromatin as the nuclear structure breaks up (karyorrhexis). The fragmented chromatin gradually disappears (karyolysis) resulting in an anucleate "ghost" cell.

2.1.5 Increased cell activity in benign states: Proplasia

Proplasia refers to increased cell activity in benign states such as regeneration, reaction, repair and inflammation and may be associated with non-neoplastic "atypia". When marked, the cytologist may have difficulty differentiating benign proplasia from malignant change.

Epithelial cells undergo a number of changes that reflect increased activity. These changes mostly occur in the nucleus which becomes round and large, up to 4-6 times its normal size (karyomegaly). The nuclear membrane usually maintains a uniform thickness, although this may vary slightly and become wavy (undulating) in outline, a feature that increases with increasing cell activity. There should be no abrupt angular changes or bites in the nuclear membrane. Multinucleation is commonly observed with proplasia.

The chromatin often becomes more granular, but not coarsely granular as in pre-neoplastic (SIL) or neoplastic conditions. Both the size and chromasia of the granules increase with a rise in nuclear activity, but the chromatin remains evenly distributed within the nucleus in benign proplasia.

Chromatin is composed of various fractions. Euchromatin is the genetically active portion, made up mainly of genetic material. Heterochromatin is the genetically inactive part of chromatin. Parachromatin is a looser term reflecting the pale or non-staining areas of the nucleus and is comprised of various elements including the spindle in replicating cells. Parachromatin becomes clearer with increased cell activity. Transcription and duplication of DNA occurs in areas of parachromatin. In proplasia, chromatin granules remain uniformly distributed in the nucleus, without the irregularly distributed areas of chromatin ("clumping and clearing") seen in malignancy.

Nucleoli are not found in normal maturing squamous epithelial cells but can be prominent in glandular cells, as well as in normal squamous metaplastic cells. Nucleoli are the energy centre of the cell so increased activity is generally mirrored by an increase in nucleolar size. During repair there is an increase in ribosomal synthesis by nucleoli that contributes to the increase in nucleolar size. Nucleoli can break the "rule of roundness in benign states" because in some cases of marked inflammation and repair, nucleoli with irregular outlines can be seen. Multiple nucleoli also occur. Enlargement of nucleoli and more variability in size from one cell to another may also increase. Variation in nucleolar size can range up to 2 to 3 times between different cells in proplasia. If nucleolar size variation equals or exceeds a factor of 3, neoplasia should be considered. These nucleolar changes must however always be considered in the context of other nuclear changes present in the cell population.

With increased activity, the ability of the cytoplasm to mature declines. This results in an increase in the N:C ratio and a reduction in the specific features of functional differentiation normally seen in maturing cells. Columnar cells may lose their shape and become rounded. Squamous cells may also be more rounded with thicker less abundant cytoplasm in proplasia.

One important diagnostic feature that helps to distinguish marked non-neoplastic atypia (as seen in marked inflammation and repair) from malignancy is the fact that benign cells still maintain strong cell-to-cell cohesion. Tissue fragments will show strong cell-to-cell relationships and large numbers of cells in a fragment.

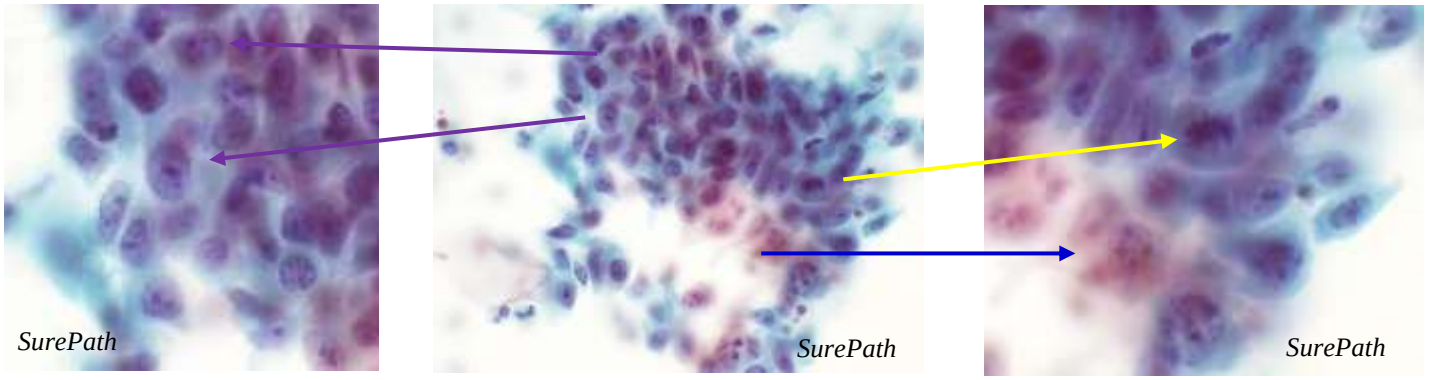
In summary, the following features are found in varying degrees with increased non-neoplastic cell activity (proplasia):

- Karyomegaly and variation in nuclear size
- Hyperchromasia
- Thickened but smooth nuclear membranes
- Wavy nuclear membranes i.e. no angular changes in direction
- Prominent nucleoli that vary in size (up to 2-3 times), number and shape
- Biphasic and eosinophilic cytoplasmic staining
- Increased N:C ratio
- Mitotic figures
- Good cell-to-cell cohesion
- Relatively flat sheets without piling up of cells

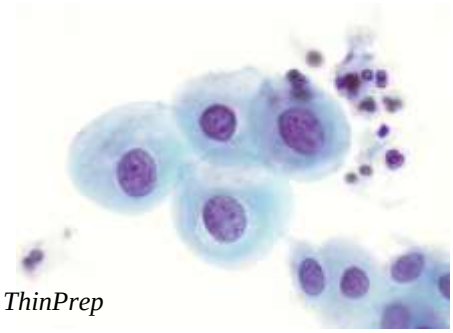
The following are particularly helpful if the changes are marked but non-neoplastic:

- Nuclear membranes do not show any evidence of membrane angularity (may be thickened and wavy, but remain smooth)
- Strong cell-to-cell cohesion is maintained

Immature squamous metaplasia shows a mild degree of increased activity indicated by slight karyomegaly, mild hyperchromasia, little or no change in nucleoli, marginal thickening of nuclear membranes and a slightly undulated/wavy membrane (red arrows). Note the excellent cell-to-cell cohesion.



Three images from one cohesive relatively flat sheet showing increasing cell activity. Biphasic staining is present (blue arrow) and neutrophils are present. Nucleoli vary somewhat in size and shape (purple arrows). However, hyperchromasia is only mild. Note the normal mitotic figure (yellow arrow), a sign of cell proliferation. The nuclear membranes are generally unremarkable. N:C ratios are increased.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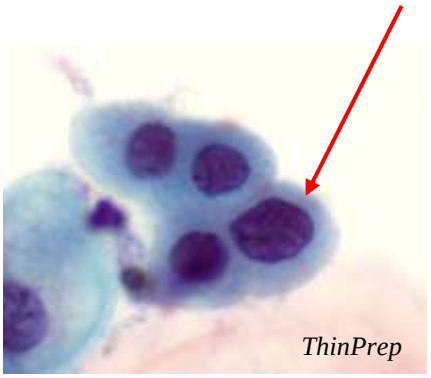
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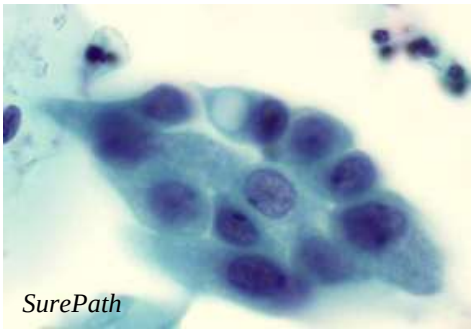
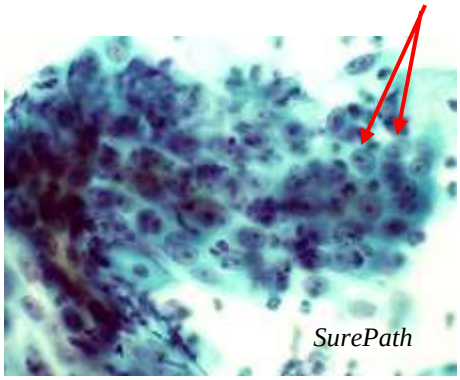

SurePath

Karyomegaly and hyperchromasia are the significant features in these images of proplasia. The central image shows slight undulation/wavy appearance of the nuclear membrane (red arrow) that can become pronounced as activity increases.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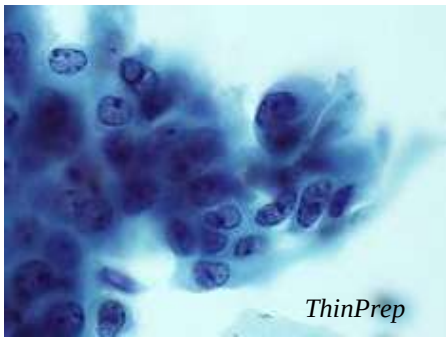
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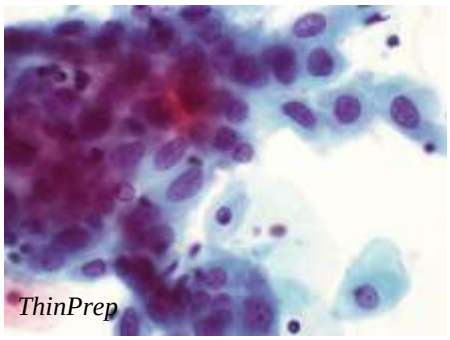
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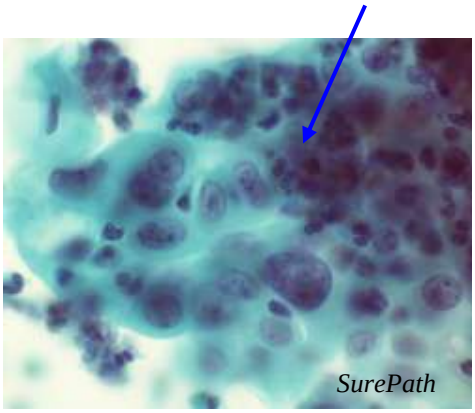
More marked increased activity. Nuclei show karyomegaly, variation in size and hyperchromasia with a corresponding increase in N:C ratio. Nucleoli are variable in size (red arrows). Nuclear membranes are smooth with no irregularities and are not thickened. The cells are still cohesive.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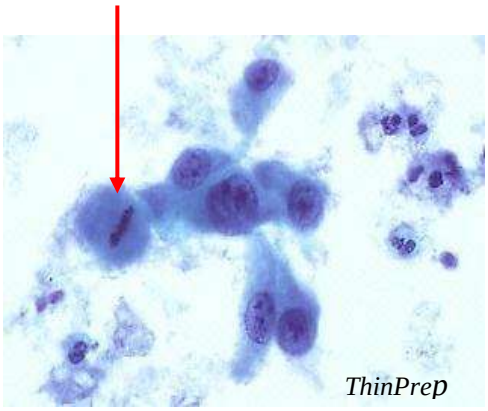
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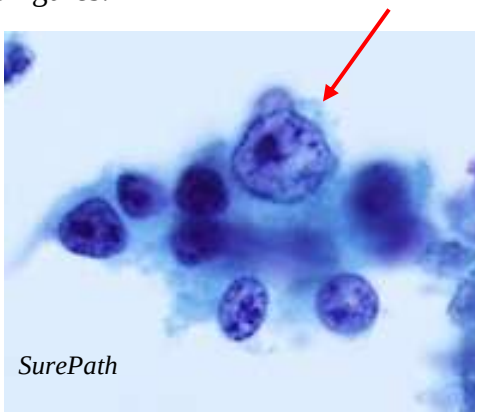
Marked increase in activity. Leucocytosis is pronounced (blue arrow). Nucleoli are prominent and vary in size, shape and number (purple arrow). A normal mitosis is present (red arrow). These more extreme features seen with marked increased activity may overlap with features seen with neoplasia. Careful examination of all features is essential. But as is typical of non-neoplastic proplasia, there is still good cell cohesion, and nuclear membranes are thickened but smooth without sharp angular contours.

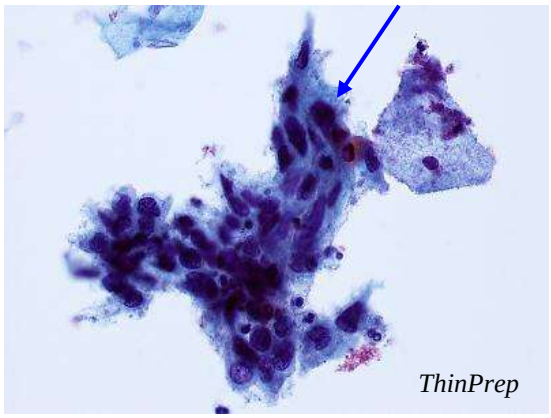
2.1.6 Increased cell activity in malignancy: Neoplasia

Malignant neoplasia refers to invasive cancer and for epithelial cell lesions this is carcinoma. No single cellular or nuclear morphologic feature is diagnostic of malignancy so all features must be assessed and considered. The nuclear features seen in carcinomas vary from case to case and may include:

General nuclear features

- Large and variable size. Can be extreme karyomegaly.
- Irregular, variable, bizarre, and unpredictable shapes.
- Bi and multinucleation are not specific for malignancy, but are commonly seen.
- Abnormal mitotic figures.

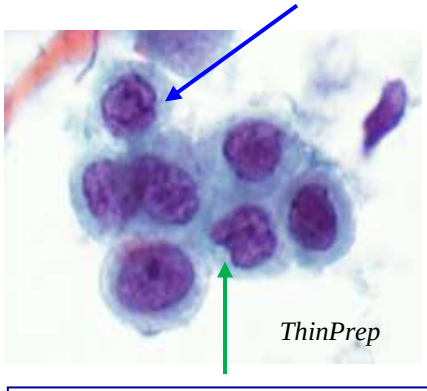


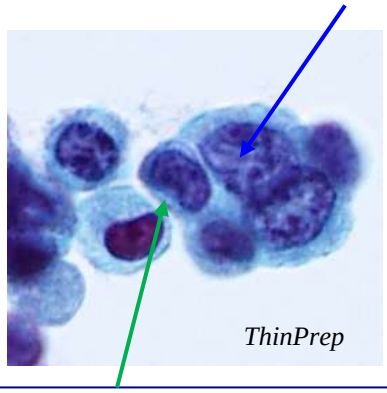


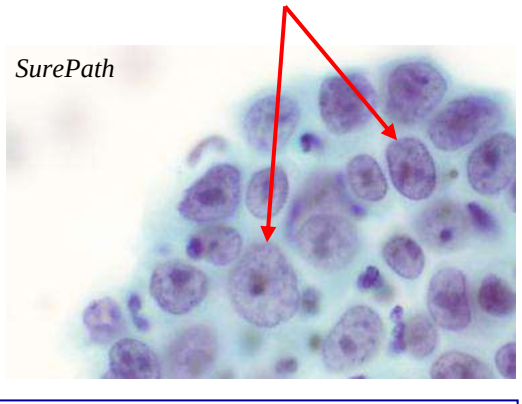
Nuclear pleomorphism: nuclei vary in size and shape within cell groups and the appearance and distribution of chromatin varies. Note malignant cells engulfing other cells (green arrow), extreme karyomegaly (red arrow) and marked hyperchromasia (blue arrow). In addition to pleomorphic nuclei, the cells also vary in size and shape resulting in a range of N:C ratios. Cell-to-cell comparison is easy in these small cell groups. Cells remain cohesive but there is no polarity within the groups.

Nuclear membranes

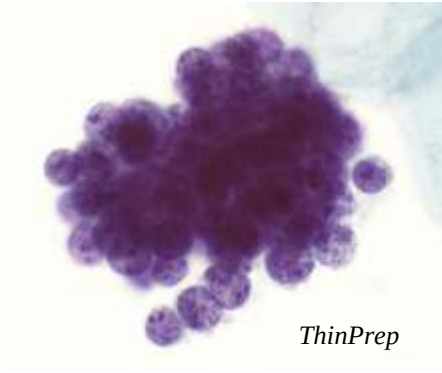
- Abrupt changes and extremes in the thickness and thinness of nuclear membranes
- Irregular outlines with sharp angled projections inwards and outwards
- "Bites" in the membrane







Nuclear membrane irregularity is a critical discriminator for identifying epithelial neoplasia.
Sharp angular nuclear membranes (blue arrows). "Bites" in the nuclear membrane (green arrow). Variation in nuclear membrane thickness (red arrows).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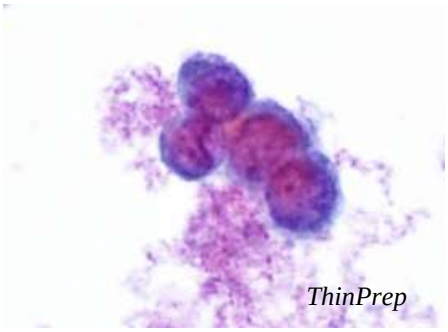
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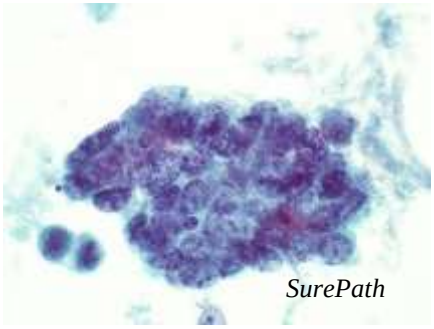
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These images are also carcinoma but the nuclei look less variable on initial impression. On low power screening, small malignant cells can be misinterpreted as lymphocytes or endometrial cells. Careful high-power examination is needed because the cells are small and so the nuclear variations occur on a small scale too. The left image is a small cell neuroendocrine carcinoma, the central image is squamous cell carcinoma with very small cells and the right image is a poorly differentiated endocervical adenocarcinoma. Cell-to-cell cohesion and nuclear moulding can be very helpful in revealing the epithelial nature of the cells. These types of cases are easy to misdiagnose.

Chromatin

- Irregular and altered chromatin pattern and distribution
- Extremely varied granular pattern, from fine to coarse
- Chromatin clumping (clumps are about the size of a polymorph lobe)
- Irregular chromatin granules with pointed projections and indentations
- Commonly hyperchromasia, with variation of the degree of hyperchromasia between different nuclei


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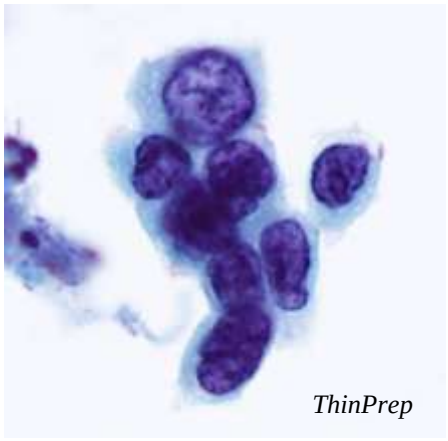
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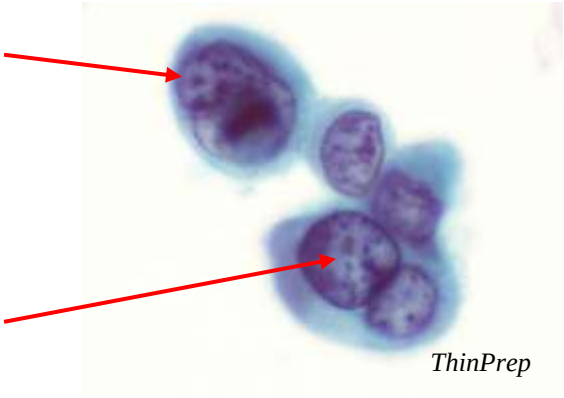
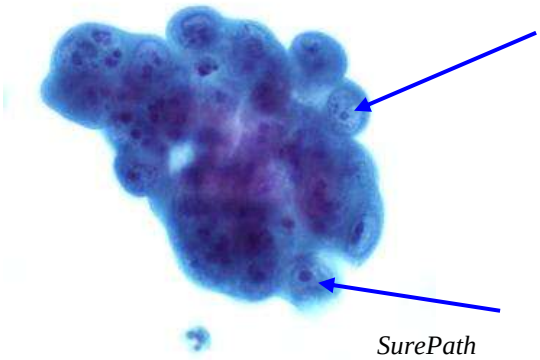
The nuclei above show coarsely granular chromatin with varying degrees of hyperchromasia. Also note the variable distribution of the chromatin within the nucleus. This is seen in invasive malignancy but is not a feature of pre-neoplastic lesions like HSIL.

Parachromatin and paranucleolar chromatin

- Variable clear areas around chromatin (parachromatin clearing)
- Variable clear areas around nucleoli (paranucleolar chromatin clearing)
- Abnormally distributed, large and uneven clear areas with irregular outlines in the nucleus

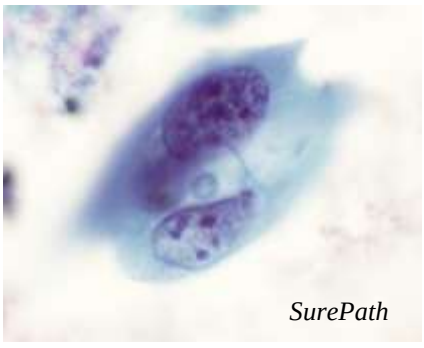


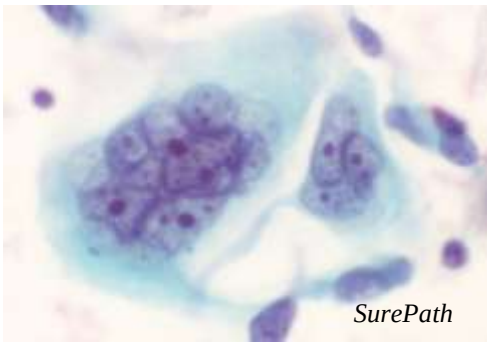


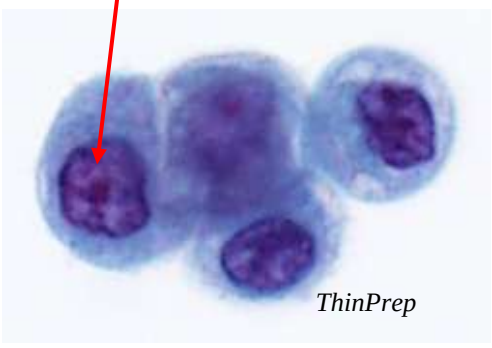
The left image shows variable parachromatin clearing (red arrows), and the right image shows paranucleolar chromatin clearing (blue arrows). Parachromatin and paranucleolar chromatin clearing can be striking on screening magnification but more often it is subtle. It is an important feature because occasionally it is the only indication of invasive malignancy.

Nucleoli

- Large and prominent macronucleoli—comparison with any background erythrocytes can be helpful to assess the nucleolar size
- Sharp angles and pointed projections and indentations
- Numbers increase and vary within a cell and between cells
- Striking eosinophilic staining
- Within a tissue fragment the size and number of nucleoli per nucleus can vary:
  - 1 to 2-fold size variation is seen in normal/reactive cells
  - 3-fold size variation is borderline and should raise suspicion of malignancy
  - 4-fold size variation or greater is characteristic of malignancy







The images above show large eosinophilic nucleoli with the right image containing a macronucleolus (red arrow). The nucleoli are variable in size both within an individual nucleus and also cell-to-cell. The shape of nucleoli also varies.

Cytoplasm

A cell must have intact cytoplasm before being considered malignant, irrespective of how much or little cytoplasm is present. This is important because a bare nucleus indicates degeneration bringing into doubt the validity of other potentially malignant nuclear criteria. In some cases the cytoplasm may be so scant that the nucleus appears bare but when examined on high power, a fine rim of cytoplasm is discernible. Bare atypical nuclei are an atypical feature but only a suspicion of malignancy can be raised in the absence of intact cells.

The cytoplasm has a more minor role in the diagnosis of malignancy than nuclear features. The examination of the cytoplasm is of more value in determining cell differentiation. The presence of necrotic cell debris/tumour diathesis is a good alarm bell for advanced lesions and should alert the cytologist to look carefully for potentially abnormal diagnostic cells.

Nuclear:cytoplasmic ratio (N:C ratio)

- The N:C ratio alone is of limited significance but scanty cytoplasm in a cell with a large atypical nucleus is suspicious of a high-grade malignant lesion. Cells viewed "on end" can appear to have scant cytoplasm so caution is required.
- A very high N:C ratio associated with a malignant nucleus is often seen with undifferentiated or poorly differentiated malignancies. These cells must not be confused with pre-malignant disease such as HSIL where the N:C ratio can also be very high but without the nuclear features of invasive malignancy. The nuclear features are the key primary indicators for establishing a diagnosis of malignancy.

2.1.7 Part B: FUNCTIONAL DIFFERENTIATION

Functional differentiation informs us of the role of the cell (e.g. protective, secretory) and is demonstrated by cytoplasmic features and/or the arrangement of cells in groups. All normal cells exhibit cytoplasmic features of functional differentiation, some of which can be recognised with the light microscope. For example: a columnar shaped cell will suggest a glandular cell. If cilia are also present its main function is likely to be the movement of mucus. Knowing the sample site adds additional information - if a ciliated columnar cell is identified in a sputum sample the cell will be from respiratory epithelium from the lung or nasopharyngeal area whereas in a cervical cytology sample the cell will be from the endocervix.

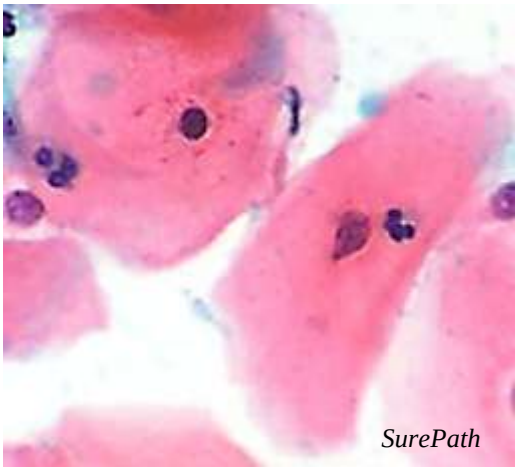
Functional differentiation will not tell us if a cell is malignant - the nuclear features are the key to identifying malignancy. Functional differentiation in a neoplastic cell is an attempt by the cell to continue to develop and mature in order to perform its normal role. In malignant neoplasia, functional differentiation may be expressed in an atypical manner. The degree of expression of functional differentiation in malignant cells is unpredictable and often varies within a lesion. The less differentiated the tumour is, the fewer indicators of functional differentiation are likely to be present. The more differentiated the tumour is, the closer the cellular and cytoplasmic morphology will resemble normality. Very well-differentiated malignant lesions may be misinterpreted as benign because the nuclear features may be only subtly atypical. Such cases are a challenge in cytology.

	Well differentiated	**Moderately differentiated**	**Poorly differentiated**	**Undifferentiated -anaplastic**
Nucleus	Mild pleomorphism, uniformity	Moderate pleomorphism	Marked pleomorphism	Marked pleomorphism, bizarre nuclei
Cytoplasm	Differentiation close to normal	Some differential features, less well formed	Occasional differential features, poorly formed	No differential features, often scanty cytoplasm

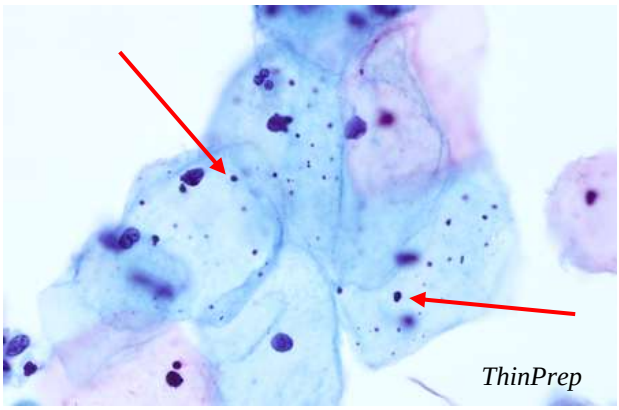
What are the functional differential features that we may observe in normal, reactive or neoplastic cells? There are cytoplasmic features seen in single cells as well as features relating to the arrangement of cells in groups and tissue fragments.

2.1.8 Functional differentiation in squamous cells

Squamous epithelium serves an important protective function, as the vaginal and ectocervical epithelium is exposed to the external environment. The multilayering and basal regeneration of cells with exfoliation from the surface reflect this role and it is also seen at a cellular level. The presence of numerous cytoplasmic tonofilaments gives increasing cell rigidity and strength as the cell matures towards the surface and is the basis for the dense appearance of squamous cell cytoplasm. Developing keratinisation is the most obvious feature of squamous differentiation in cells in cervical cytology samples. Keratohyaline granules are precursors of keratin/hyaline and are part of squamous differentiation. They are blue/black cytoplasmic granules that are normally present in cells from sites that naturally produce keratin as part of their protective function, and may be seen in normal, reactive and neoplastic squamous cells.

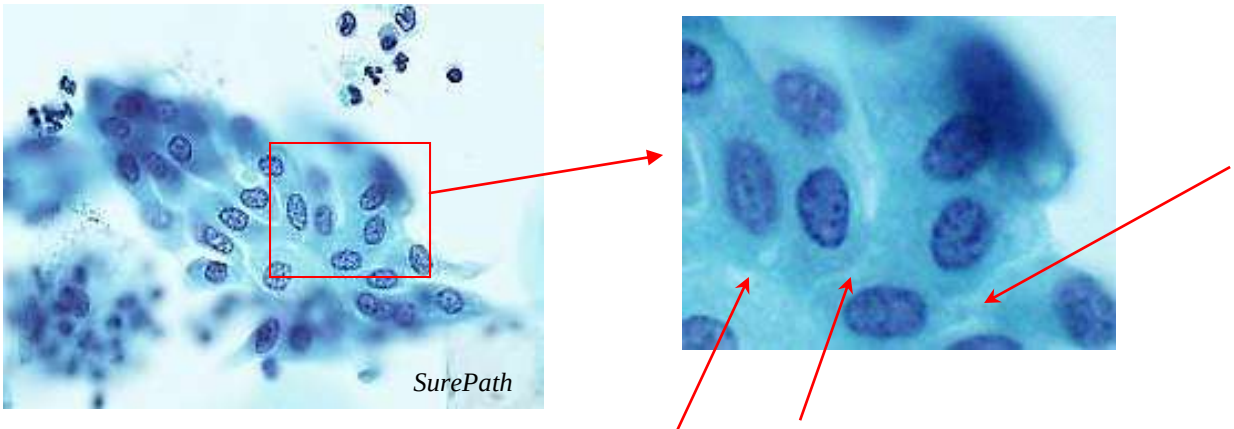


Normal mature superficial squamous cells showing abundant, dense well-keratinised eosinophilic squamous cytoplasm. The nucleus is small and pyknotic in keeping with the degree of maturation, resulting in a low N:C ratio.



Cytoplasmic keratohyaline granules in normal superficial squamous cells

Another feature of squamous cell differentiation is the presence of intercellular bridges. The bridges represent points of strong attachment between neighbouring cells and at an ultrastructural level, are points of desmosomal contact.

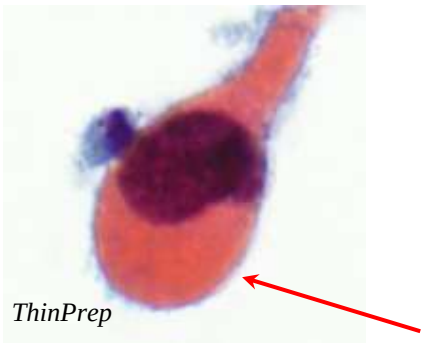


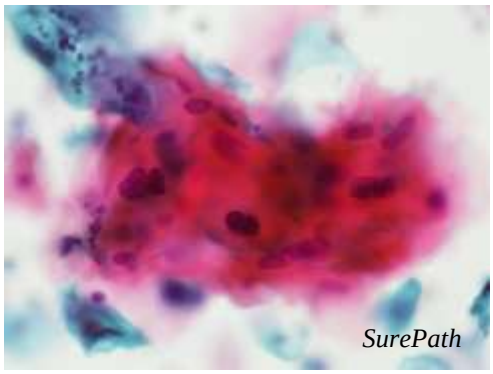
A sheet of normal squamous metaplastic cells. Intercellular bridges (red arrows) give the cells a spidery appearance and identify the functionality of the cells. Intercellular bridges can be seen in normal, reactive and neoplastic epithelial cells and indicate the squamous nature of the cells.

Squamous differentiation is also seen in squamous cell carcinomas. The diagnostic features of invasive squamous cell carcinoma in cervical cytology samples are covered in the *VRPCC Workbook: Core Topic 2.10 Invasive Squamous Cell Carcinoma (SCC) Page 189.* The discussion in this section relates to the cytologic expression of squamous differentiation in squamous cell carcinomas of different degrees of differentiation.

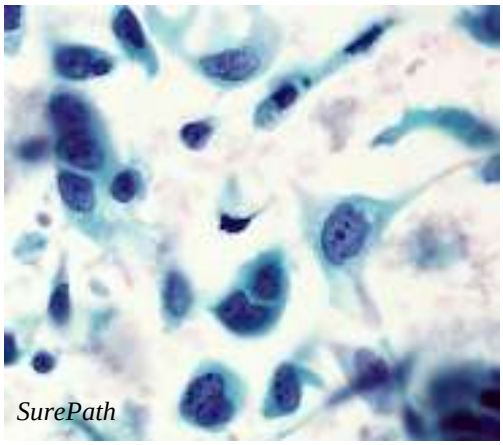
Keratinising SCC: Well-differentiated and moderately-differentiated SCC

Well-differentiated and moderately-differentiated squamous cell carcinomas show many features of squamous differentiation. Cytoplasmic maturation is seen as keratinisation and the nuclei may be pyknotic, also reflecting squamous maturation. The cytoplasm can be keratinised or hyalinised. In LBC preparations, cytoplasmic keratin is usually intensely eosinophilic but can also be basophilic, while hyalinisation is basophilic. Both have a glassy appearance and may appear refractile. With keratinisation, the cytoplasmic border acquires a hard razor-sharp cytoplasmic edge. Close examination may reveal faint lines within the cytoplasm, corresponding to deposition of layers of keratin.



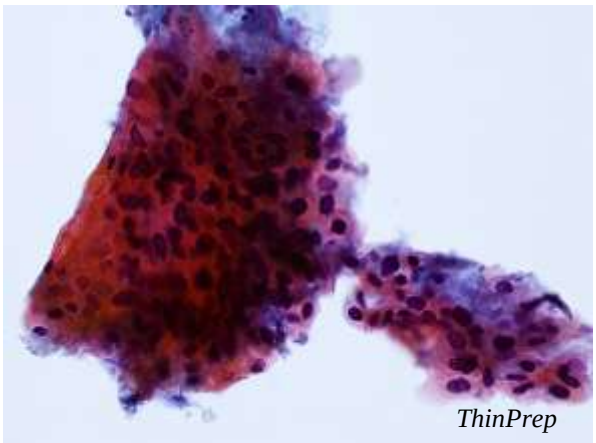


Abundant dense keratinised cytoplasm in malignant squamous cells. Note the razor-sharp edge of the cell border (arrowed).



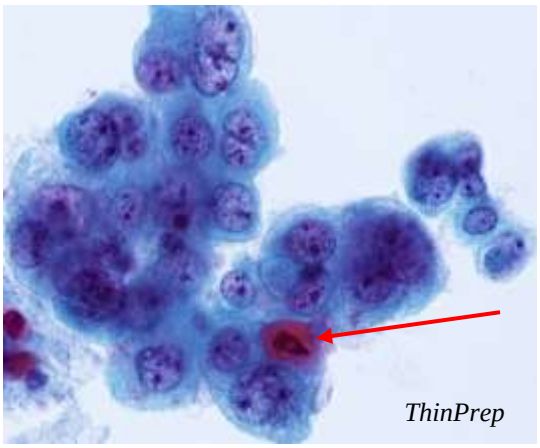
Squamous cell carcinomas showing different and varying cytoplasmic staining characteristics

Malignant epithelial cells usually retain their cohesive nature at least to some extent even though malignant neoplasms are often ulcerated and friable. Large tissue fragments (microbiopsies) may be seen in cervical cytology samples.

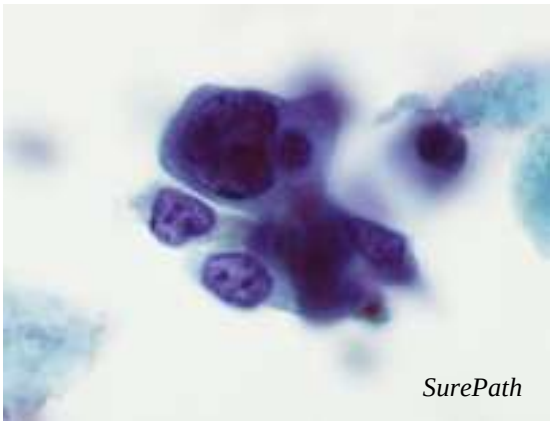


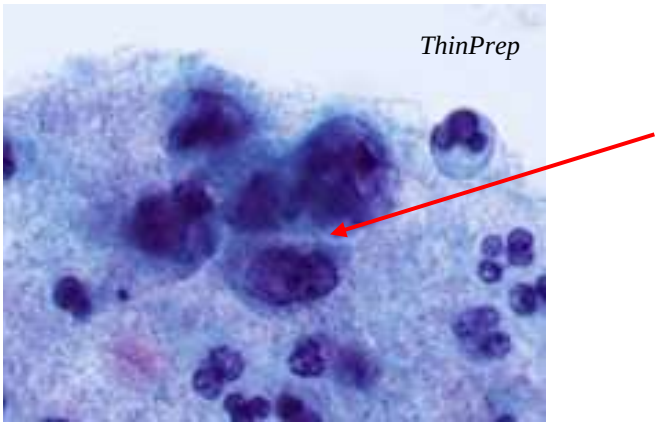
Non-keratinising SCC: Poorly differentiated SCC

The poorly differentiated malignant squamous cell is generally a round, symmetrical cell. The cytoplasm is often scanty (high N:C ratio). The nuclear features are critical in determining whether the cell is from an in-situ pre-invasive lesion (HSIL) or an invasive malignancy. There may only be subtle cytoplasmic features such as focal keratinisation or small cell tails to indicate that the lesion is of squamous type.



Focal cytoplasmic keratinisation (red arrows) may be evident in poorly differentiated SCC. Note the fine faint lines indicating deposition of cytoplasmic keratin (green arrows).





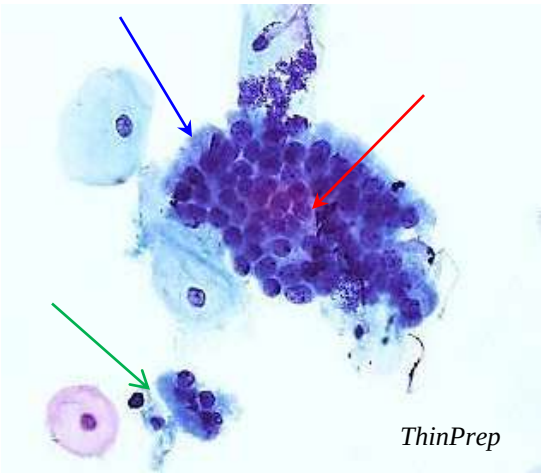
Even when poorly differentiated, SCC retains some cell cohesion reflecting its epithelial origin. Intercellular bridges may be evident in some groups (arrowed).

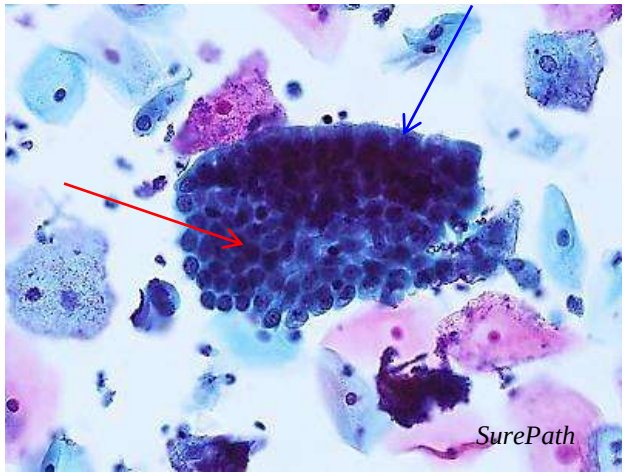
2.1.9 Functional differentiation in glandular cells

Glandular epithelium has a secretory function and this is reflected in the cell structure. The epithelium lining the endocervix is predominantly of endocervical type but various metaplasias may also be present, the most common of which is tubal metaplasia. Many different types of adenocarcinoma arising from within and beyond the genital tract may be seen in cervical cytology samples. Some of the variations in appearance reflect the site of origin of the type of adenocarcinoma present as the functional differentiation seen in glandular cells at the site of origin varies. A wider discussion of the variety of adenocarcinoma cells that may be seen in cervical cytology samples is given in the *VRPCC Workbook: Core Topic 2.11 Glandular lesions Page 210.* The comments below discuss functional differentiation in endocervical glandular cells and endocervical adenocarcinoma in particular.

Normal endocervical cells are columnar in shape with basal nuclei and apical mucin-filled abundant cytoplasm. Mucin is located adjacent to the luminal border of the cell in preparation for secretion into the endocervical canal. Cilia are present at the luminal aspect in some cells such as those that have undergone tubal metaplasia. These cells are concerned with the directional flow of mucus across the epithelial surface. Some of the differential cytoplasmic features by which normal endocervical glandular cells are recognised are:

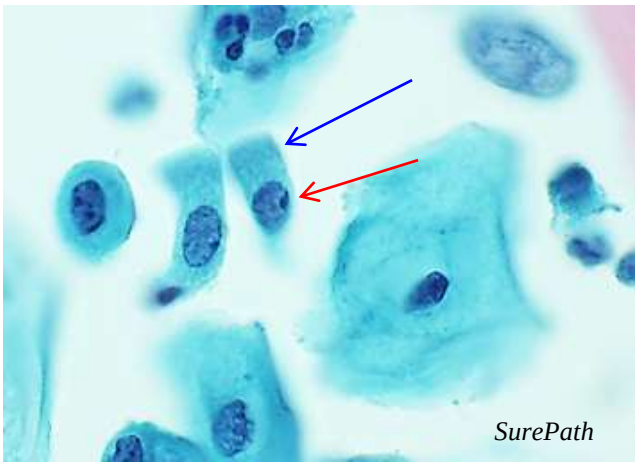
- Palisade or honeycomb pattern. There is good cell cohesion and a "common border" at the luminal aspect, seen as a sharply defined continuous edge at one side of the cell group.



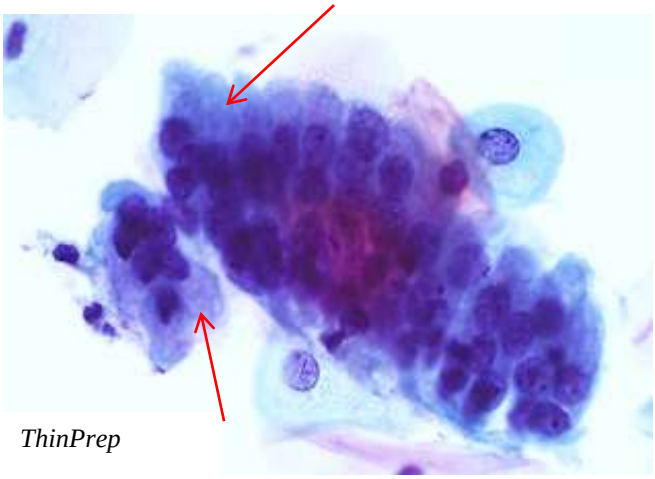


Groups of normal endocervical glandular cells showing both palisading of cytoplasm (blue arrows) in a side-on view of columnar cells, and the honeycomb effect of nuclei when looking down on cells (red arrows). A small strip of normal endocervical glandular epithelial cells is also seen in the left image (green arrow).

- Mucin-filled cytoplasm. Glandular cytoplasm is often described as "delicate" reflecting the paler less dense nature compared with the robust dense cytoplasm seen in squamous epithelial cells. Mucin may be present in small or large cytoplasmic vacuoles.

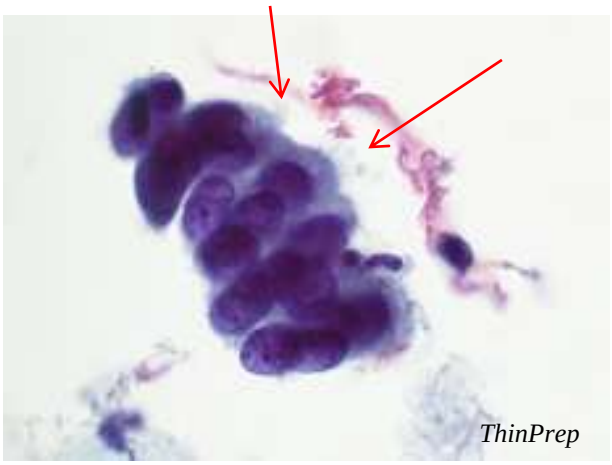


The left image shows the basal orientation of the nucleus (red arrow) and the mucin-filled apical cytoplasm (blue arrow). The basal end of the cell is tapered where it has been pulled off the basement membrane whereas the apical (luminal) edge is flat.

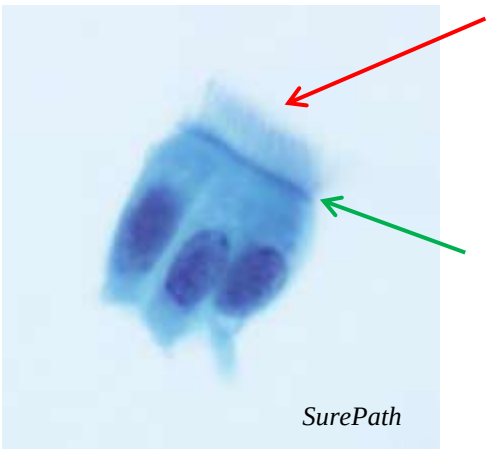


Endocervical glandular cells with abundant cytoplasmic mucin in fine cytoplasmic vacuoles (red arrows).

- Cilia. Normal endocervical glandular cells are occasionally ciliated but ciliated cells are much more likely to have come from an area of tubal metaplasia.



Cilia (red arrows) are seen at the luminal edge of these glandular cells. The configuration of a small somewhat jumbled group of benign ciliated cells is typical of tubal metaplasia.

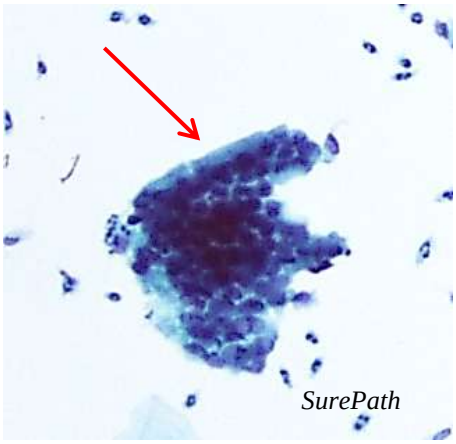


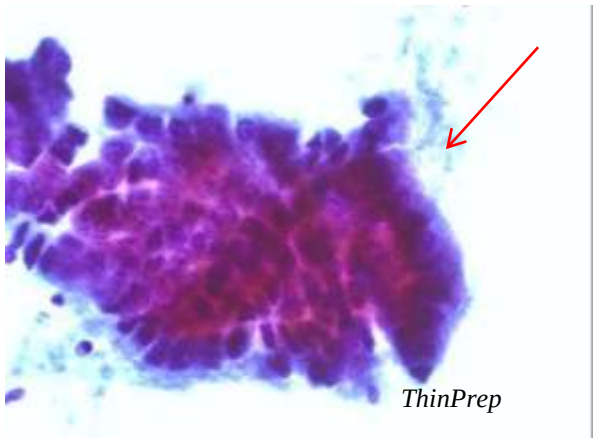
Cilia (red arrow) and the terminal bar (green arrow) where they attach to the cell are clearly seen.

Malignant endocervical glandular cells are recognised by the architecture of groups of malignant cells as much as by the cytology of individual cells. The diagnosis of invasive endocervical adenocarcinoma in cervical cytology samples is covered in the *VRPCC Workbook: Subsection 2.11.7 Morphology of Invasive Endocervical Adenocarcinoma Page 232.* The discussion in this section relates to the cytologic expression of glandular differentiation in endocervical adenocarcinoma. Again it must be emphasised that these functional differential features can also be present in normal and benign glandular cells. A **diagnosis of malignancy is made using nuclear criteria** while the cytoplasmic features assist with classifying the type of malignancy present.

Cells from an endocervical adenocarcinoma tend to exfoliate as tissue fragments/cell clusters. There is a wide range of differentiation from very well differentiated adenocarcinomas which may only have subtle nuclear features indicative of malignancy, to undifferentiated tumours which cannot be classified as endocervical or even glandular on the basis of cytologic morphology alone. Between these extremes, are cases where large undifferentiated cells predominate with some cells showing differential glandular features, cases of adenocarcinoma associated with adenocarcinoma-in-situ (AIS) allowing localisation of the neoplasm to the endocervical canal and better differentiated cases where classic features of endocervical adenocarcinoma are present. Examples of features of functional differentiation seen in better differentiated endocervical adenocarcinomas are given below.

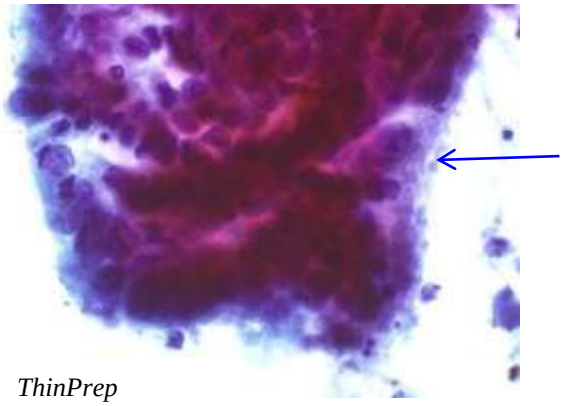
- Cells with a columnar arrangement and a glandular border (common/communal border). Apical mucin lies at the luminal border with the nuclei set back from the border edge.





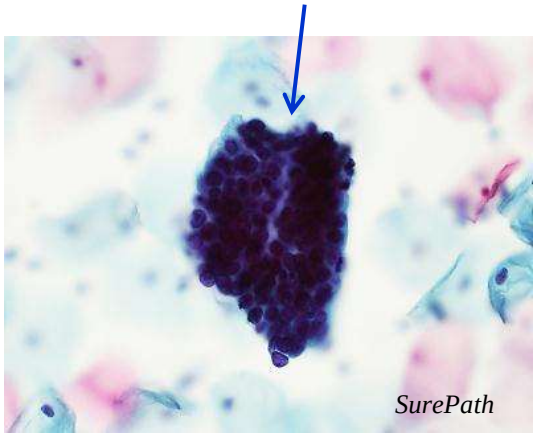
There is a columnar/palisade arrangement of the cells forming a glandular or communal border (red arrows) at one edge of a crowded cell group. Apical mucin-filled cytoplasm lies adjacent to the luminal border with more basally situated nuclei sitting deeper within the group.

- Gland openings and acinar structures within a sheet or cluster of cells. The nuclei of acini are placed at the basal end of the cell (i.e. near the basement membrane) with the secretory cytoplasm on the luminal side allowing secretion into the lumen. Small gland formations (acini) can be difficult to recognise particularly in more poorly differentiated lesions.



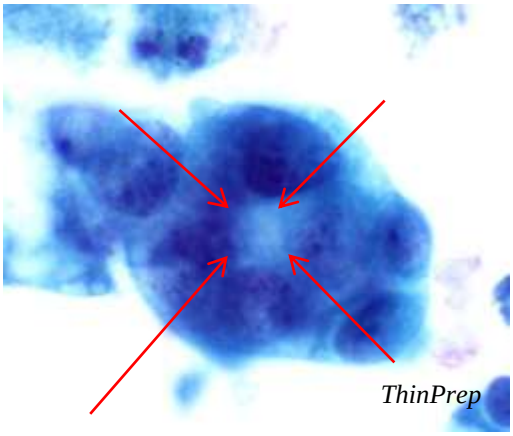
ThinPrep

There is a large gland opening (blue arrow) leading into a gland structure within this thick group of cells from an endocervical adenocarcinoma.



SurePath

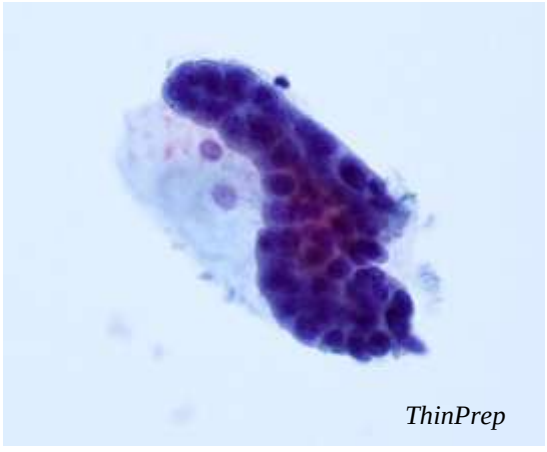
A gland opening (blue arrow) is seen in this crowded group from a case of adenocarcinoma in situ (AIS)



ThinPrep

Close examination of this small cell cluster shows a gland opening at the tips of the red arrows. The orientation of nuclei around the lumen has been lost in this relatively poorly differentiated adenocarcinoma.

- If a papillary (or polypoid) structure is sampled, finger-like papillary formations of a tissue fragment can be seen.



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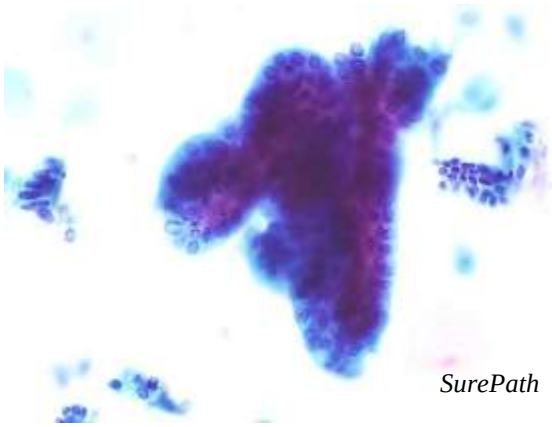
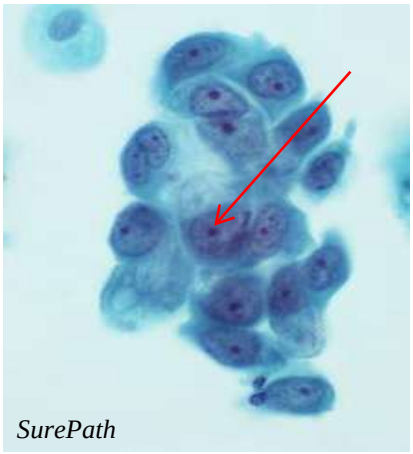
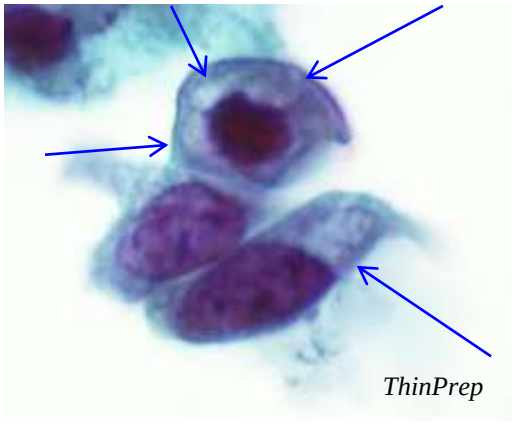


SurePath

Finger-like papillary structures from two endocervical adenocarcinomas

- Secretory vacuoles are generally large and may be multiple. Vacuoles can be so large as to distend the cell and push the nucleus to the periphery of the cell.





Large cytoplasmic mucin vacuoles in endocervical adenocarcinoma. On the left, inspissated material can be seen as a small dot inside the vacuole (red arrow). On the left the vacuoles are multiple and compress the nucleus slightly (blue arrows).

2.1.10 Questions

1. What are the features of cells and groups of cells in the state of euplasia?

2. Describe the features that would help you differentiate retroplasia from malignancy.

3. Repair may present with sheets of cells that may mimic malignancy. What features would help you with this differential diagnosis?

4. Describe the general features observed in nuclei in cases of malignancy.

5. Define the term "functional differentiation" and discuss its significance with reference to cytology in general and malignancy in particular.

6. Explain the caution to be exercised with well-differentiated malignant lesions.

7. Discuss the cytological features of squamous cell carcinoma and adenocarcinoma from a point of functional differentiation.

References

- Frost JK. 1986. *Monographs in Clinical Cytology* (2nd Ed. Vol 2) The Cell in Health and Disease. Basel: Karger.

2.2 CORE TOPIC: NORMAL CYTOLOGY AND HISTOLOGY

2.2.1 Introduction

The features of a normal cervical cytology sample need to be thoroughly understood to provide the basis for evaluating disease processes when screening. Focussed concentration is required to screen cytology samples, as each slide contains thousands of cells and may include only one or two abnormal cells. The cytoscientist/cytotechnician must screen thousands of samples in order to become a competent safe proficient screener, to minimise the chance of missing abnormal cells.

The cytoscientist/cytotechnician also needs enough clinical knowledge to be able to interpret clinical information. Knowledge of the histopathology of normal and abnormal cervical tissues is required in order to understand the significance of the cells encountered in the cytology sample as the aim of cytology is to accurately predict histopathology.

2.2.2 Cytological content of a normal sample.

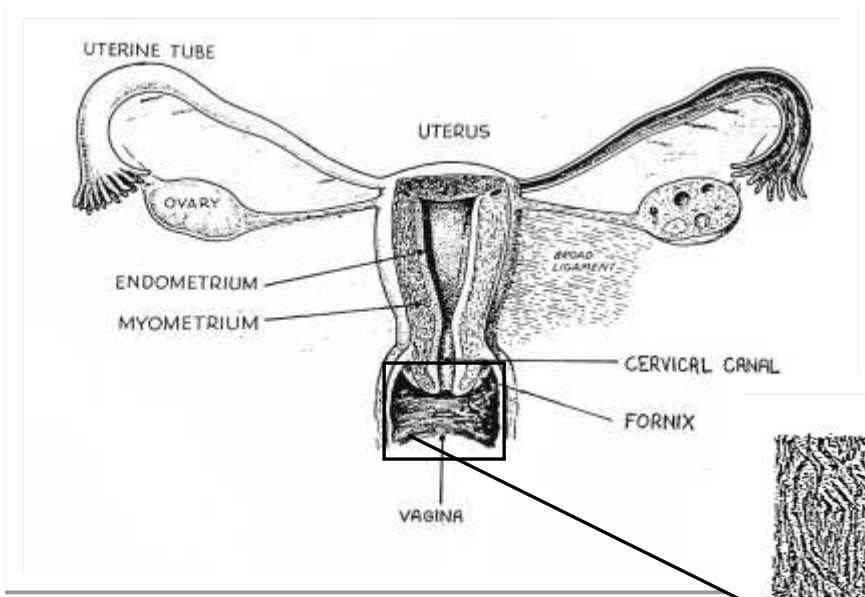
Below is a list of cells and other material that may be seen in a normal cervical cytology sample. The cells present include those sampled from the surface of the epithelium using the sampling device, cells that have exfoliated naturally from the surface of the cervix/vagina and material trapped in cervical mucus. LBC samples usually have only small numbers of leucocytes and erythrocytes and little (if any) mucus on the prepared slides, as much of this material is removed as the sample is processed.

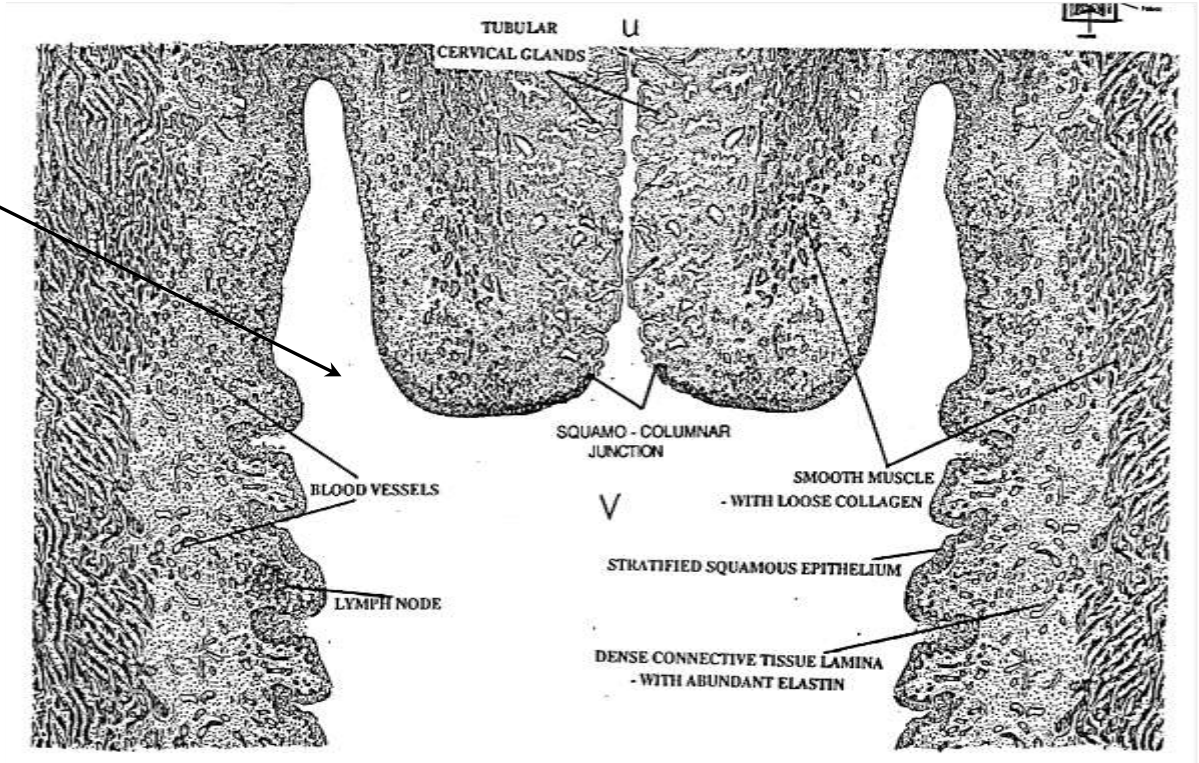
The proportions of the types of cells present will vary depending on clinical factors such as age and hormonal status. Accuracy of sample taking can also affect the proportions of cell types present. Some cell types such as endocervical cells may be absent in some samples.

The normal cervical cytology sample may include:

- Squamous epithelial cells from the ectocervix and vagina
- Glandular epithelial cells from the endocervical canal
- Squamous metaplastic cells from the transformation zone
- Reserve cells
- Stromal cells and glandular cells from the endometrium
- Leucocytes (neutrophils, histiocytes)
- Erythrocytes (red blood cells)
- Mucus
- Normal vaginal flora e.g. Döderlein bacilli (lactobacilli)
- Spermatozoa
- Miscellaneous contaminates e.g. lubricating jelly

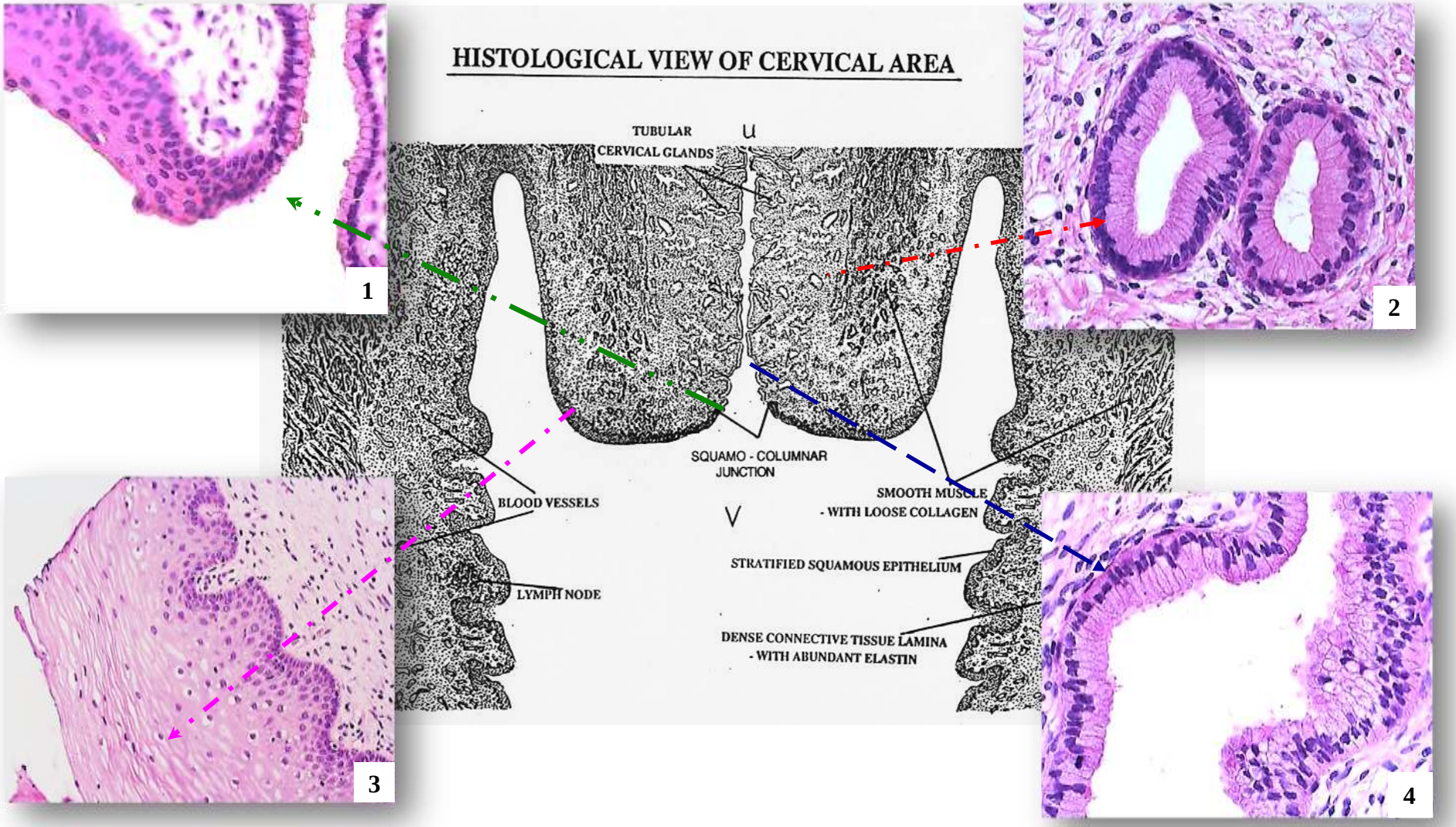
2.2.3 Histology of the cervix





Right: Histology of the cervix and upper vagina (V = vaginal cavity).

The endocervical canal is lined by simple columnar epithelium and extends from the squamo-columnar junction up to the lower uterine segment (U).
The cervix and vagina are lined by non-keratinising stratified squamous epithelium.



Higher power views of selected areas of cervix. (Haematoxylin and eosin stain). 1. Squamo-columnar junction with squamous metaplasia. 2. Endocervical glands. 3. Mature squamous epithelium. 4. Surface endocervical cells lining the endocervical canal.

2.2.4 Squamous epithelium

The squamous epithelium covering the cervix is of non-keratinising stratified type. There are four defined layers within the epithelium. From the surface layer downward to the basement membrane, these are:

- Superficial cells
- Intermediate cells
- Parabasal cells
- Basal cells

A minimum of 5,000 well-visualized/preserved squamous cells are needed for an LBC slide to be considered satisfactory. Not every specimen requires a cell count to assess cellularity as most samples are clearly adequately cellular. If the cytoscientist/cytotechnician feels there may be less than 5000 well-visualized/ preserved squamous cells present, a counting scheme is used. For most microscopes, **minimum numbers of cells needed in each of at least 10 fields** counting across the diameter of the cell circle are:

  - **SurePath: 9 cells per 40X** in each of 10 Fields of View (FoV)
  - **ThinPrep: 4 cells per 40X** in each of 10 Fields of View (FoV)

- Guidelines for estimating cellularity in LBC preparations using different microscopes are available in *The Bethesda System for Reporting Cervical Cytology 2nd Edition. Solomon D and Nayar R.* Pages 4 and 8.
- Strict objective criteria are difficult to apply in some cases. For example, slides with cell clustering, atrophy, or cytolysis are technically difficult to count. Laboratories should apply professional judgment when evaluating these slides.
- Sample adequacy is discussed further under *VRPCC Workbook: Subsection 4.1.3 Structure of The Bethesda System Page 254.*

Any specimen in which abnormal cells are identified is satisfactory for evaluation, by definition.

Superficial squamous cells

Superficial squamous cells are the most mature of the spectrum of squamous epithelial cells and indicate that the squamous epithelium has matured fully under the influence of oestrogen.

The superficial cell is a large, polygonal-shaped flat cell with dense cytoplasm. It measures approximately 35-50μm in diameter but can vary above or below this. The nucleus is small and pyknotic (in comparison with the intermediate cell nucleus) and is rarely more than 5μm. The shape of the cytoplasm reflects the considerable rigidity of the cell which is due to an abundance of tonofibrils. The cytoplasm of most superficial cells stains pink (eosinophilic). Under normal circumstances these cells do not contain keratin, but may possess kerato-hyaline granules (keratin precursors). The granules are sometimes seen as dark (blue-black) granules in the cytoplasm of superficial squamous cells.

These mature cells form the most superficial layer of the epithelium and eventually undergo cell death and exfoliation from the surface. Features of degeneration and cell death can therefore be observed in superficial cells. Perinuclear halos are a common feature, along with karyorrhexis, karyolysis and occasionally, anucleate (ghost) cells.

Intermediate squamous cells

Intermediate cells are about the same size (approximately 35μm in diameter) as superficial squamous cells and are larger than parabasal cells. The cytoplasm is cyanophilic and reasonably dense, although more superficial intermediate cells may stain eosinophilic. The cytoplasm is less angular than superficial cells and less rounded than parabasal cells. Intermediate squamous cells are flat.

The only accurate way to distinguish intermediate cells from superficial cells is by examining the nucleus. Intermediate cells have a round to oval vesicular nucleus which is approximately 8-10μm in diameter. A "vesicular nucleus" is the type of nucleus seen in intermediate cells i.e. round to oval and well-preserved with a defined nuclear membrane surrounding homogeneous nucleoplasm. Nuclear membranes are clearly defined and the chromatin is well preserved. Chromocentres and sex chromatin (Barr bodies) may be observed. Intermediate cells may form squamous pearl formations.

In the latter part of the menstrual cycle (luteal phase), in pregnancy and during menopause, intermediate cells may be elongated and boat-shaped with a yellow-brown cracked substance in the cytoplasm. This is glycogen. These glycogen-rich boat-like intermediate cells are sometimes called navicular cells. Cytoplasmic borders are thickened and the vesicular nuclei may be eccentrically placed.

Parabasal squamous cells

Parabasal cells are not common in normal samples because of their location in the lower layers of the squamous epithelium. Their presence increases in women over 35 years of age, in post-natal women and in menopausal/post-menopausal women because of hormonal effects. In some pathological conditions, the number of parabasal cells may increase sharply.

The cells measure between 15-30μm in diameter. The nucleus is usually about a third of the total cell volume, but this may vary considerably reflecting the transition between basal and intermediate cells. Chromatin granules and chromocentres are present but nucleoli are rarely seen. Nuclei are slightly larger than the nuclei of intermediate squamous cells. N:C ratios are lower than those of basal cells. The cytoplasm stains cyanophilic. Well-fixed forcibly removed parabasal cells can appear very similar in morphology to more mature squamous metaplastic cells, including intracellular bridge formations. They can also appear well rounded when naturally exfoliated. Close examination of subtle nuclear and cytoplasmic details will help differentiate parabasal cells from metaplastic cells.

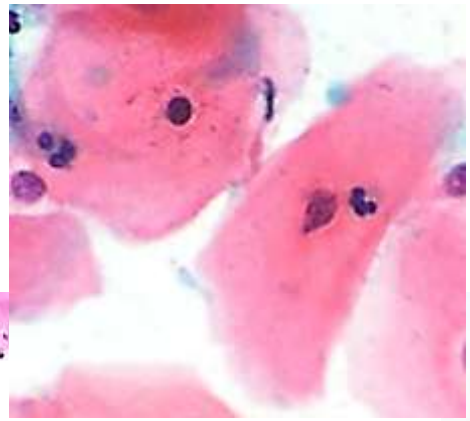
Basal squamous cells

Basal cells form the lowest squamous epithelial cell layer, lying directly above the basement membrane. They are rarely seen in cervical cytology samples and if present, indicate damage to the upper layers of the squamous epithelium. These cells are responsible for regenerating squamous epithelium and are the most immature type of squamous cell.

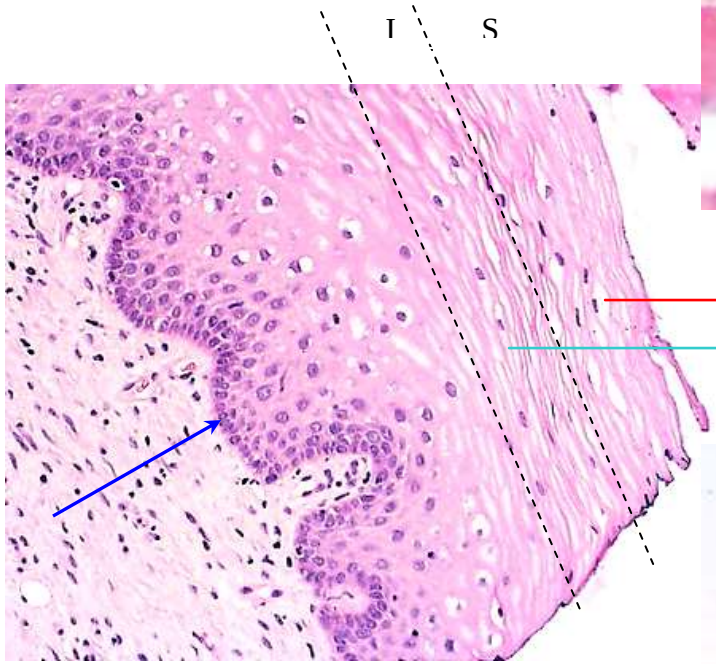
Basal cells look like parabasal cells morphologically but are much smaller and exhibit evenly distributed scanty cytoplasm with a large nucleus, giving a high N:C ratio. The cytoplasm stains cyanophilic. The nucleus shows chromatin granules and nucleoli may be observed.

NORMAL CYTOLOGY

Squamous cells
Superficial (S) and Intermediate (I) type

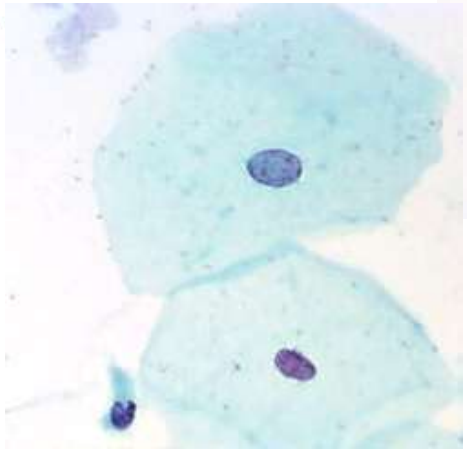


Superficial cell with a pyknotic nucleus and eosinophilic cytoplasm.
Nuclear diameter approx 5µm
Cytoplasmic diameter approx 35-50µm



Superficial cells

Intermediate cells



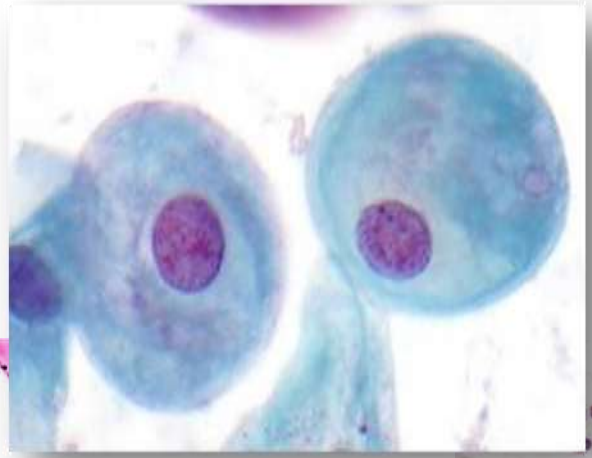
Histology of mature squamous epithelium with uppermost superficial cells (S) and intermediate cells (I) directly beneath. The basement membrane is located between the basal epithelium and the underlying stroma and is shown by the blue arrow.

Intermediate cell with a vesicular nucleus and basophilic cytoplasm. The lower of the two cells is close to being a superficial cell as the nucleus is starting to condense.
Nuclear diameter approx 8-10µm
Cytoplasmic diameter approx 35µm

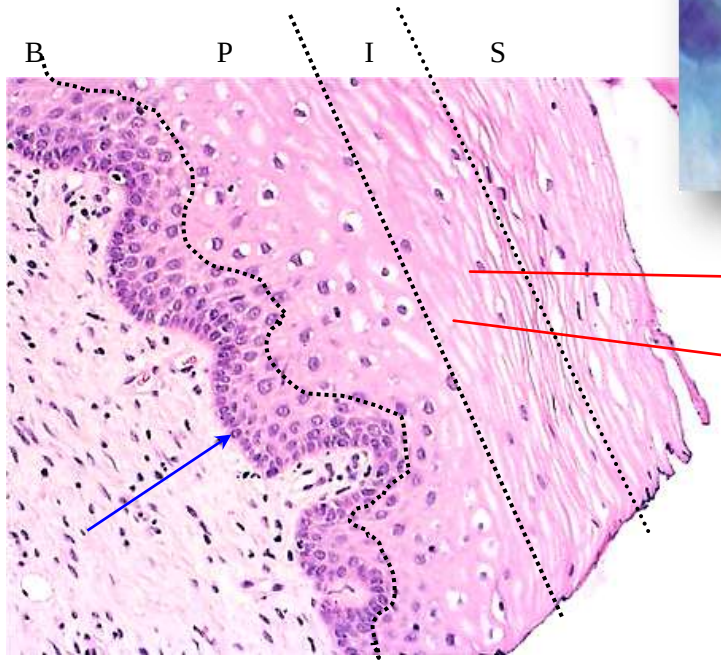
NORMAL CYTOLOGY

Squamous cells

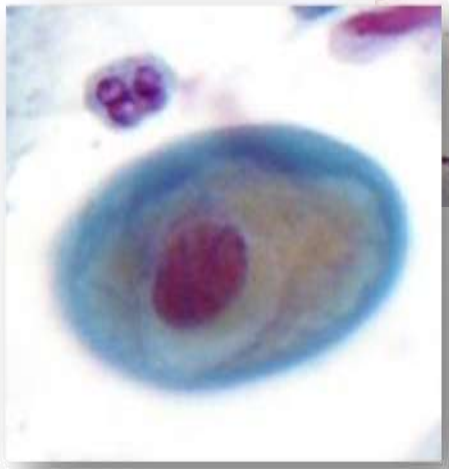
Parabasal (P) and basal (B) type

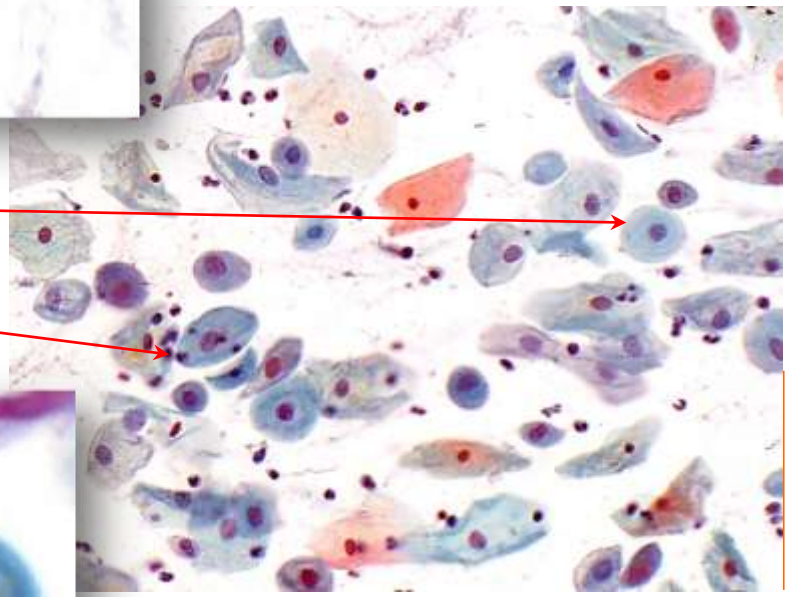


Parabasal cells with open nuclei. The N:C ratio will vary considerably depending on the degree of transition between a basal and an intermediate cell. The cytoplasm is round to oval with a smooth dense cytoplasmic border. It may be difficult to differentiate parabasal cells from maturing squamous metaplastic cells.

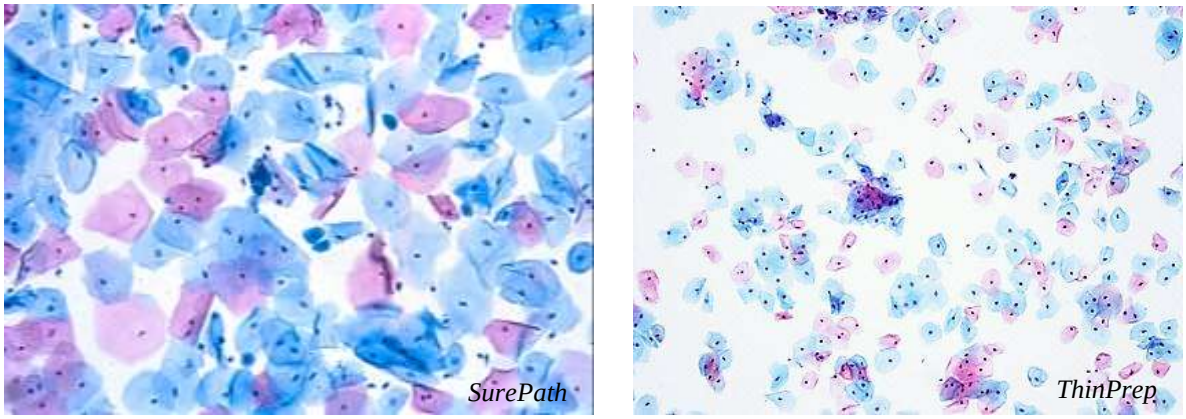


Histology of squamous epithelium with parabasal (P) and basal (B) layers directly beneath the intermediate cell layer (I). The degree of maturation from the basal layer is influenced by oestrogen. The deep basal cells are not seen in normal samples. The blue arrow indicates the location of the basement membrane.





Some low oestrogen states such as post-partum samples may have numerous parabasal cells. These cells may contain glycogen (yellow cracked appearance) within the cytoplasm and also be more angular, almost boat shaped (referred to as "navicular" cells).



Typical low power views of LBC preparations showing squamous cells

2.2.5 Endocervical epithelium

In a well-sampled normal cervical cytology sample, endocervical cells are usually present and well preserved. At least 10 well-preserved endocervical or squamous metaplastic cells should be identified before reporting that a transformation zone component is present. In some clinical situations the squamo-columnar junction is situated up in the endocervical canal (e.g. post-menopausal women) and endocervical/metaplastic squamous cells may not be sampled.

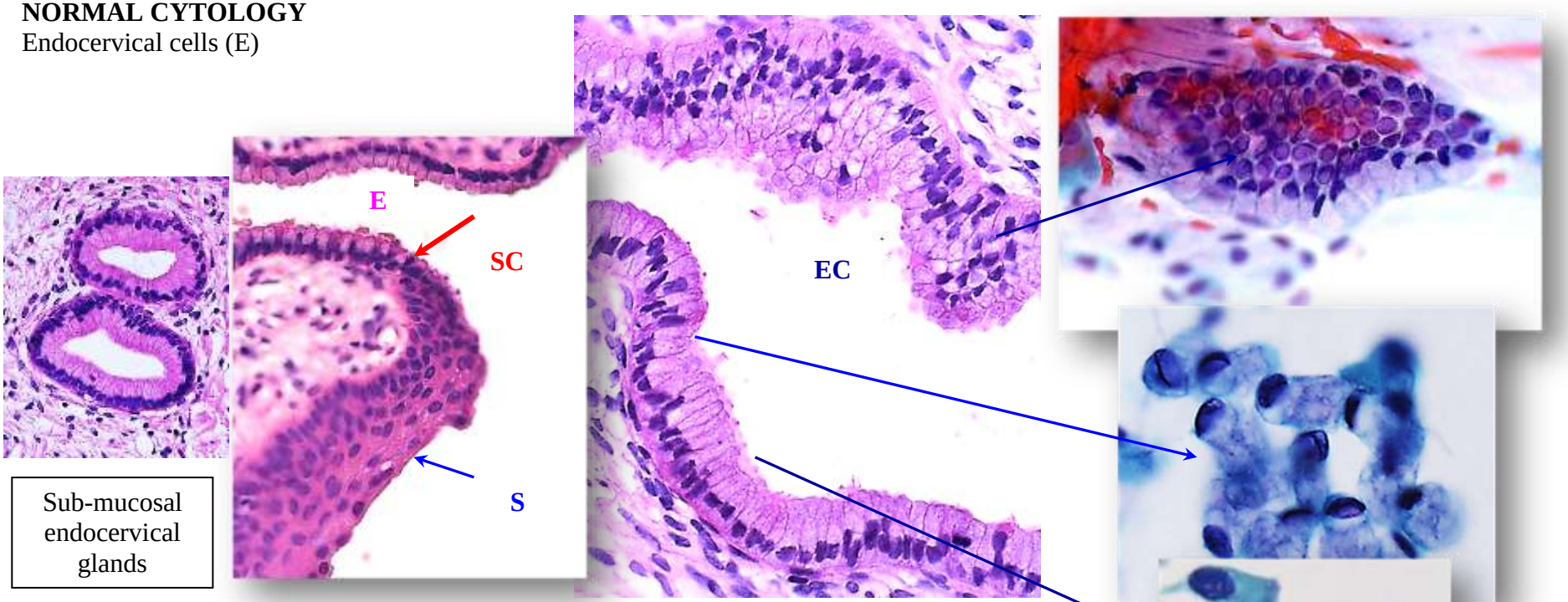
Endocervical cells are tall columnar cells and the cytoplasm is usually finely vacuolated and cyanophilic. The nucleus is basally placed at the end of the cell that was adjacent to the basement membrane. The elongated cytoplasm protrudes out to the apical portion of the cell at the end that was adjacent to the endocervical canal. This allows mucus to be secreted into the endocervical canal. A small number of cells will exhibit cilia supported by a terminal plate. The ciliated cells have a one-directional ciliary beat to move mucus down into the vagina providing both lubrication and an extra protective barrier.

The nuclei of endocervical cells have finely granular grey-blue chromatin, and are approximately the size of intermediate/parabasal cell nuclei (10µm in diameter). One to two small nucleoli are frequently observed reflecting the normal metabolic activity of this type of cell. A small dense dark nipple-like protrusion may be observed on endocervical nuclei, particularly at maximum oestrogen activity during mid-cycle. The cells usually appear in tightly cohesive flat sheets and can be seen either forming a palisade (side on view) or a honeycomb pattern (vertical view from above).

Endocervical cell cytoplasm is fragile and fragments may be seen interspersed with bare free nuclei. Rarely, tufts of cilia supported by the terminal plate with an attached fragment of cytoplasm may be seen. This appearance is called ciliocytophthoria (CCP) and its origin is not clear. Suggestions that CCP is associated with viral infections are not substantiated and CCP may be purely a result of cell degeneration.

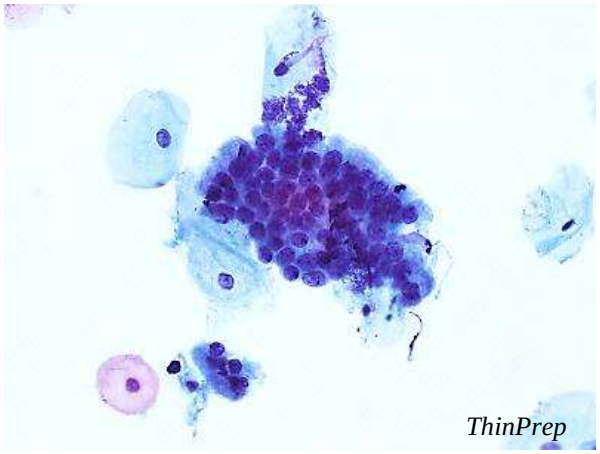
NORMAL CYTOLOGY

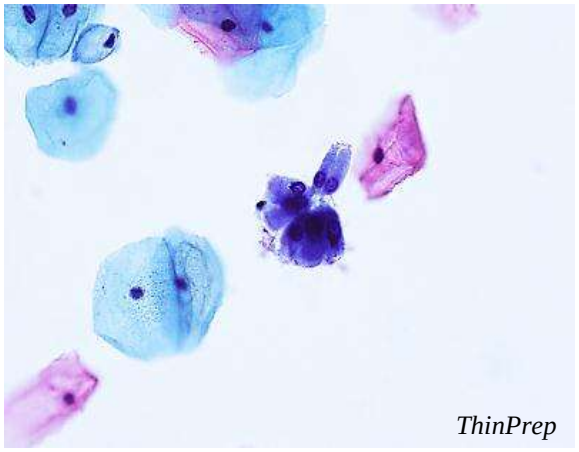
Endocervical cells (E)



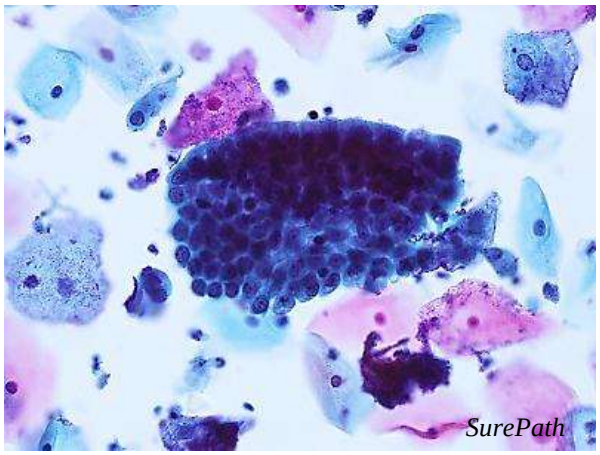
Histology of endocervical epithelium: a single layer of cells with basally placed eccentric nuclei adjacent to the basement membrane. There is an abrupt change between the stratified squamous epithelium (S) and endocervical epithelium (E) at the squamo-columnar junction (SC). The endocervical canal (EC) is seen. Epithelial cells from sub-mucosal endocervical glands (far left) are not be seen in a cervical cytology sample, which only samples surface epithelial cells.

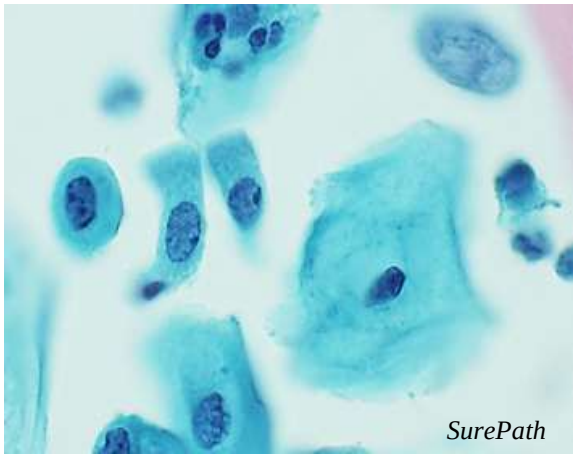
Cytology: The upper cytology image shows a flat sheet of endocervical cells with outer columnar-shaped cells forming a palisade (side-on view) and inner nuclei forming a honeycomb pattern (vertical view). The middle cytology image shows secretory endocervical cells with mucin-filled cytoplasmic vacuoles compressing the basal nuclei. The lowest image demonstrates ciliated cells.





Endocervical cells in ThinPrep





Endocervical cells in SurePath are often dissociated (lying singly). Identification is based on their columnar configuration, eccentric nuclei and vacuolated cytoplasm.

2.2.6 Squamous metaplasia

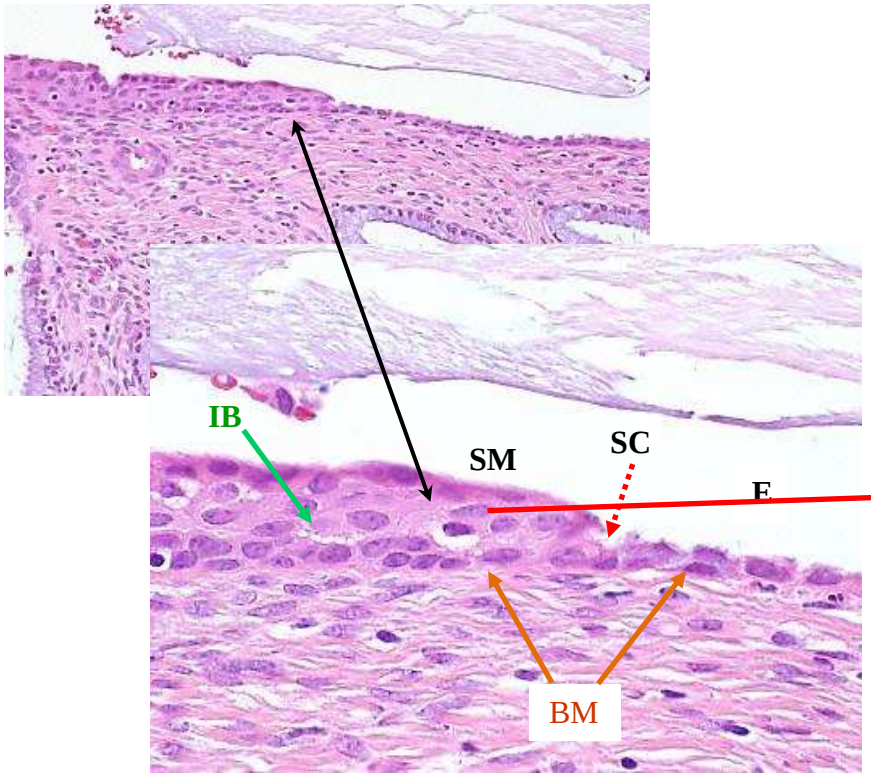
Squamous metaplasia is the process by which some of the original endocervical epithelium is replaced with squamous epithelium. It can be extensive or limited and is a normal physiological process in the cervix. The area between the edge of the original squamous epithelium (i.e. the original squamocolumnar junction) and the current endocervical epithelium (i.e. the new squamocolumnar junction) is called the "transformation zone" (TZ). In the presence of squamous metaplasia, the squamocolumnar junction is the point of change between squamous metaplastic epithelium and endocervical epithelium. At puberty the metaplastic process is triggered by the effect of the lower pH of vaginal secretions on columnar epithelium, which becomes exposed to the vagina as a result of growth. Exposed endocervical epithelium on the outer surface of the cervix (ectocervix) is called an ectropion. The process of squamous metaplasia begins at the tips of the endocervical villi forming metaplastic "islands" which later fuse. During the metaplastic process there is differentiation of endocervical reserve cells into immature metaplastic cells. These cells will eventually mature to a morphological appearance identical to native non-metaplastic squamous cells.

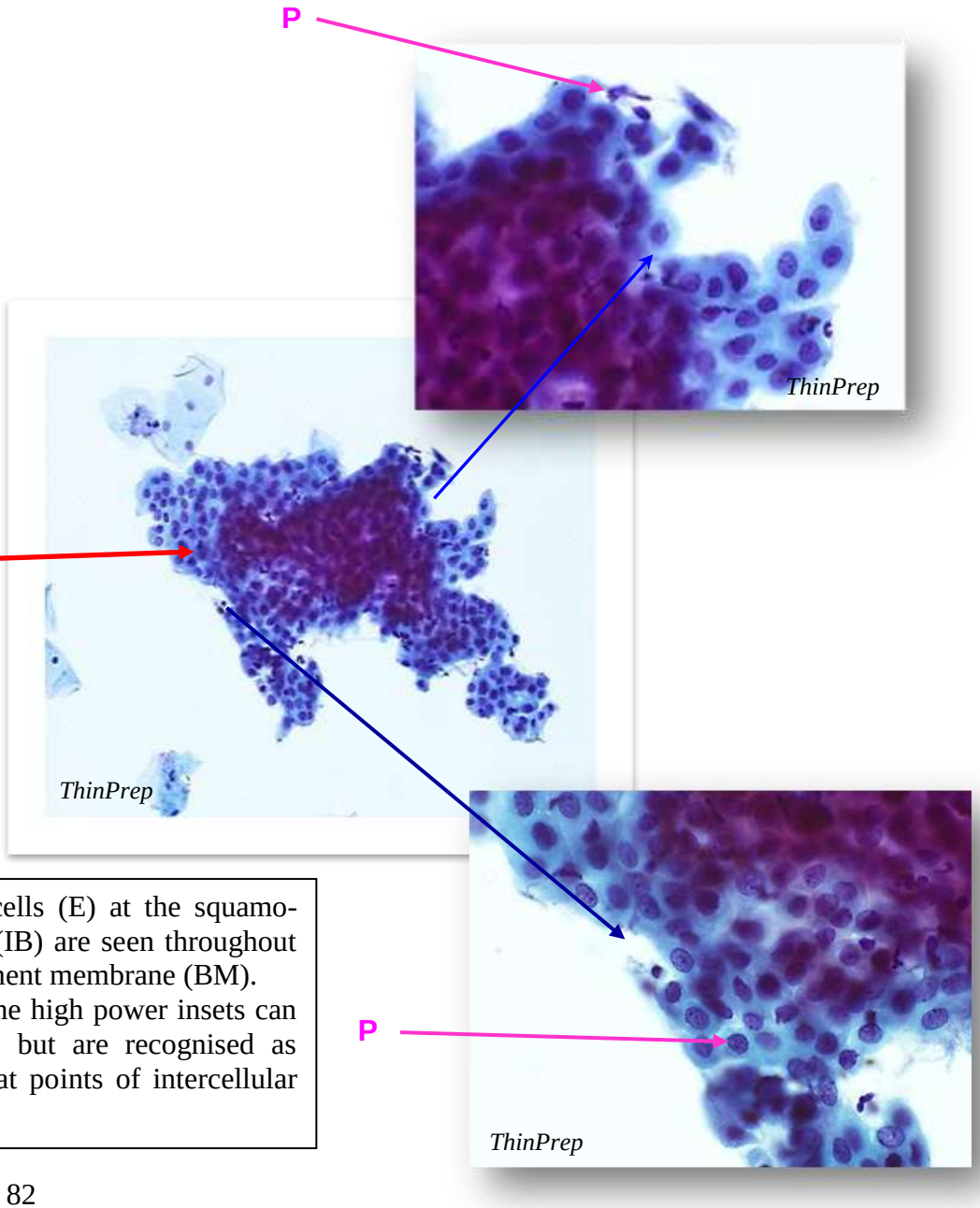
The cytologic appearance of squamous metaplasia varies greatly depending on the maturity of the cells. Mature metaplastic cells tend to appear singly, whilst immature metaplastic cells tend to be in loose groups with cell-to-cell contact by intracellular bridges. The groups have a flat surface appearance. Reserve cells and/or endocervical cells may be associated with immature metaplasia. Fine mucin vacuoles may be observed in less mature metaplastic cells but without confirming the presence of mucin (e.g. using a PAS-Diastase stain) it may be impossible to distinguish these vacuoles from degenerate vacuoles.

Identifying metaplastic squamous cells can be difficult in cytology samples, particularly the distinction from parabasal cells. A useful distinguishing feature of metaplastic squamous cells is the presence of angled projections around the cytoplasmic membrane. In some situations e.g. post-partum, abundant cytoplasmic glycogen may indicate that the cells are parabasal and intermediate cells rather than metaplastic cells. In a correctly sampled cervical cytology specimen it is unusual to see a predominance of all three different squamous cell types of maturation (i.e. superficial, intermediate and parabasal). If numerous superficial and intermediate cells are observed with numerous less mature rounded cells it is more likely that these cells are metaplastic rather than parabasal cells.

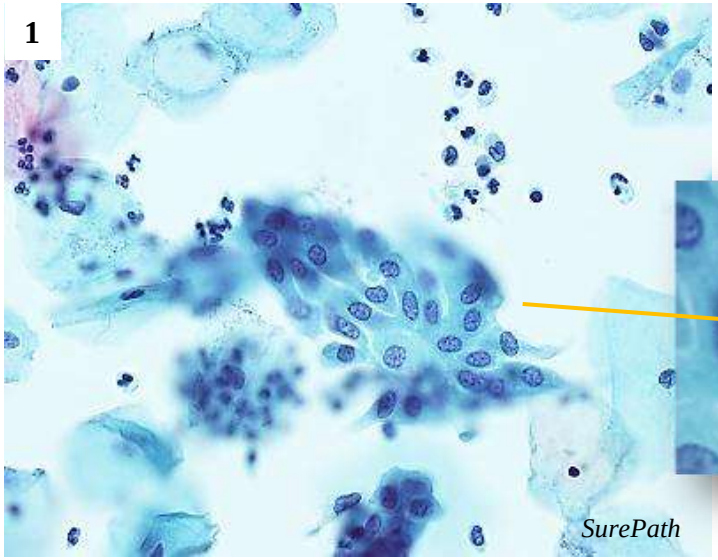
The distinction between parabasal and metaplastic cells is somewhat academic as the important issue is the identification of the cells as normal. The cytoscientist/cytotechnician should focus on the cell nucleus as it is the appearance of the nucleus rather than the cytoplasm that is the most important consideration when deciding if a cell is normal or abnormal.

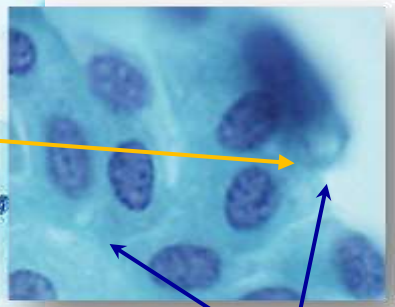
NORMAL CYTOLOGY: Squamous metaplasia

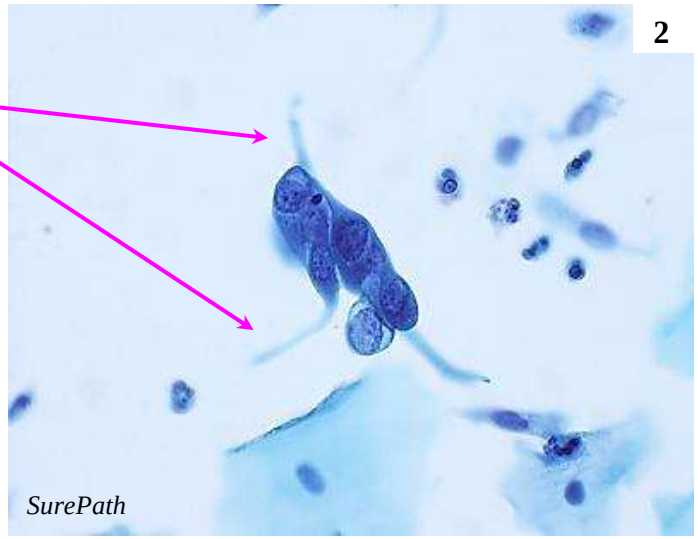


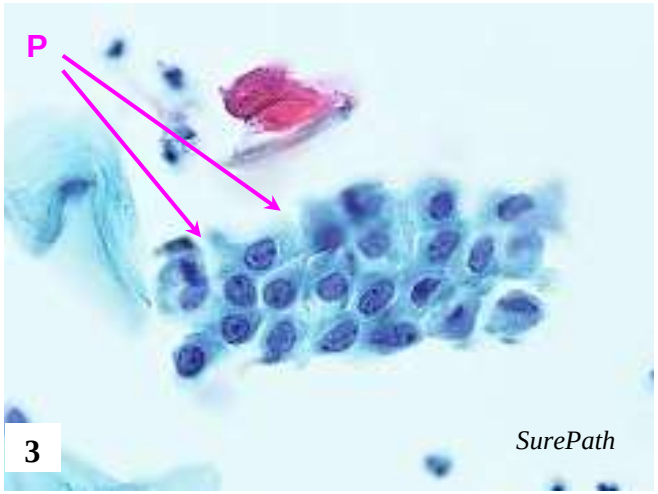


Histology: Maturing squamous metaplasia (SM) alongside endocervical cells (E) at the squamo-columnar junction (SC). Fine cytoplasmic extensions/intracellular bridges (IB) are seen throughout the metaplastic epithelium, particularly in the lower layers close to the basement membrane (BM).
A cluster of metaplastic cells is seen cytologically (blue arrow) which on the high power insets can be seen to be moderately mature. Some cells resemble parabasal cells but are recognised as metaplastic by the fine angular cytoplasmic projections (P) which occur at points of intercellular contact.







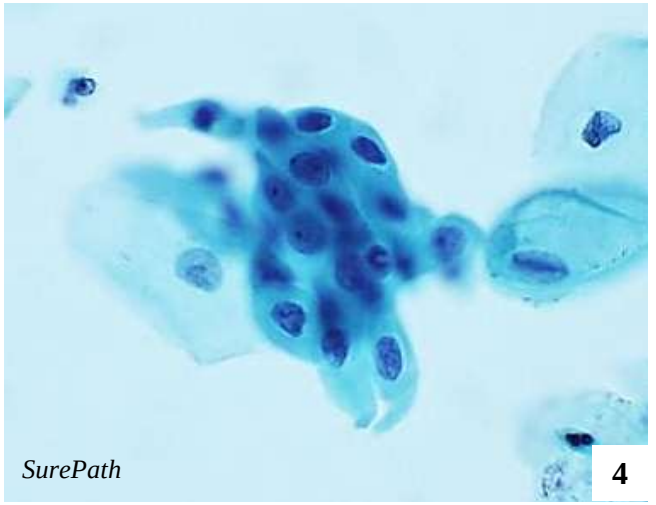


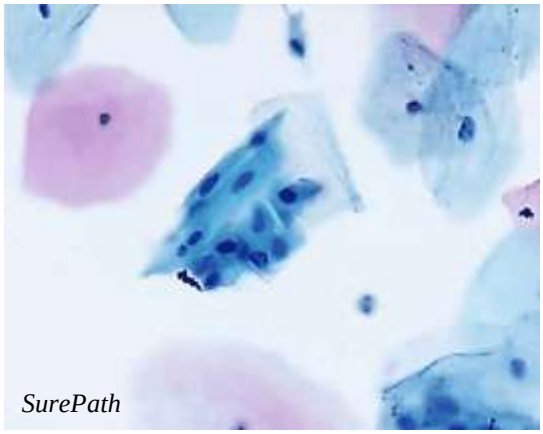
Squamous metaplasia with varying levels of maturation.

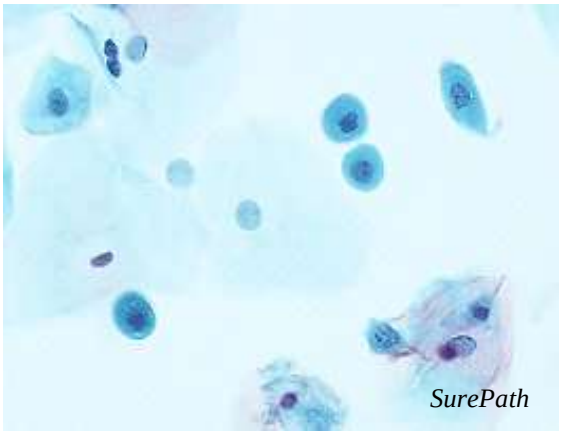
(1) Immature cells show occasional intracytoplasmic vacuoles (**V**) containing mucin.

(2 and 3) Immature cells may also have abundant elongated cytoplasmic processes (**P**).

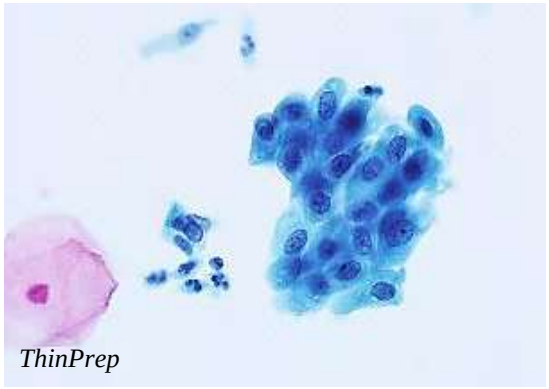
(4) The most mature metaplastic cells have plentiful cytoplasm with low N:C ratios.

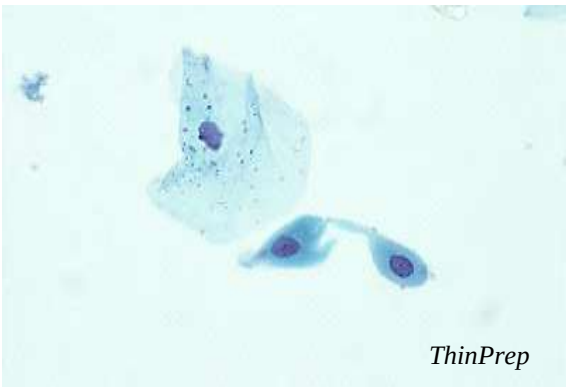






Metaplastic squamous cells in SurePath preparations. Note the dense cytoplasm particularly in the dispersed single metaplastic cells in the right-hand image.





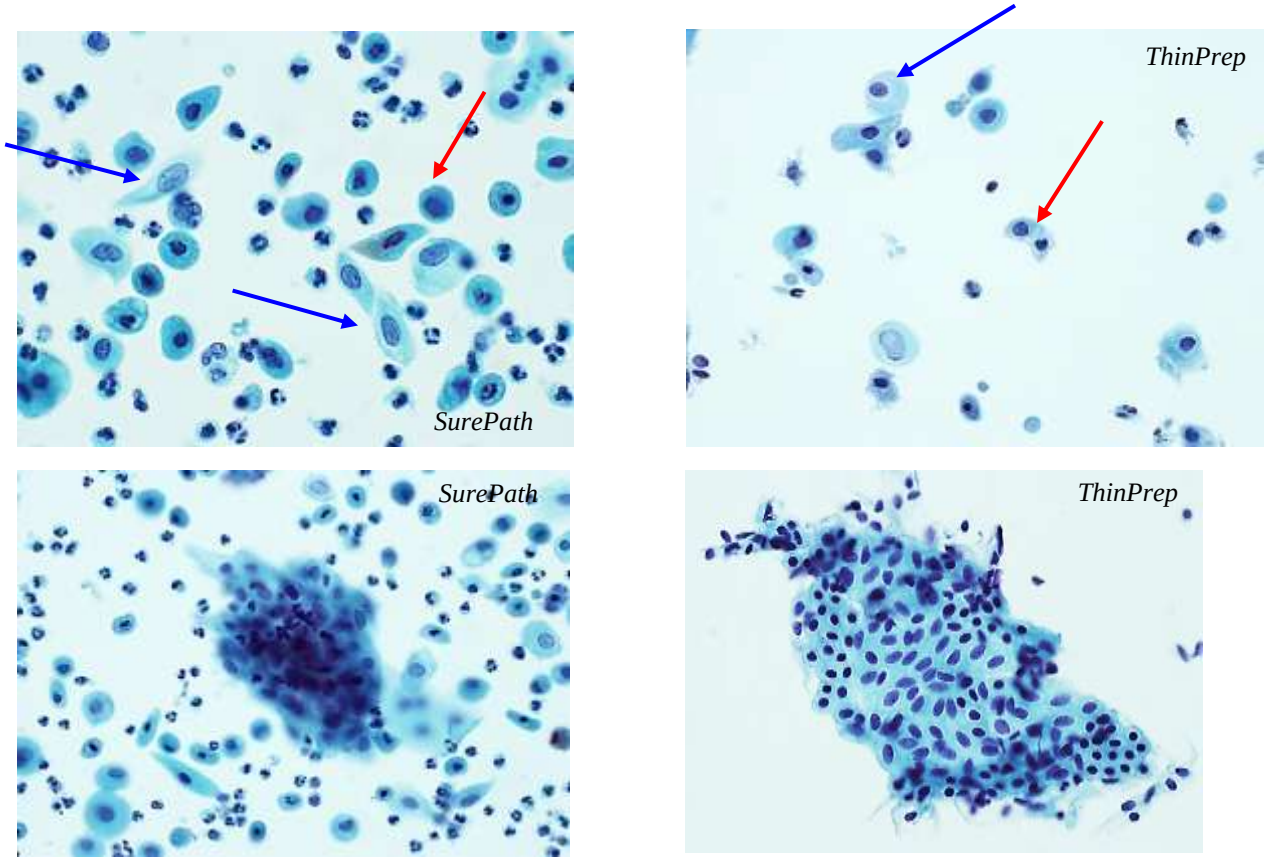
Metaplastic squamous cells in ThinPrep preparations. The cells often lie singly and can show cytoplasmic processes or round up in the vial fluid.

2.2.7 Atrophic squamous cells and Reserve Cells

Atrophy occurs as a result of low levels of oestrogen, or a relative decrease in oestrogen compared with progesterone levels. The three most common settings are the postmenopausal period, postpartum and where women are taking progestagens for contraception (such as Depo Provera). Slide preparations with atrophy may show single dissociated parabasal cells, sheets and loose clusters of atrophic cells or a combination of both. The cells contain increased glycogen and may show cellular degeneration. Stripped nuclei may be present. A granular background of degenerative cellular debris is generally seen but this may have been removed by LBC processing. The degree of atrophy varies considerably from one woman to another. Atrophy can be severe where parabasal cells predominate, or mild to moderate where intermediate squamous cells predominate with a reduction or lack of superficial squamous cells. Atrophy per se is not associated with inflammation or epithelial cell reaction. When these are present, the condition is called atrophic cervicitis/vaginitis which is discussed in the *VRPCC Workbook: Subsection 2.5.1 Atrophic vaginitis/cervicitis Page 123*.

Because atrophic squamous cells have high N:C ratios inherently, assessing the possibility of a significant squamous or glandular lesion can be very difficult when atrophy is present. In particular, a high grade squamous intraepithelial lesion (HSIL) and endometrial cells (either normal or atypical) can be misinterpreted in atrophic samples. The important distinction

between atrophy only and a significant squamous lesion occurring in an atrophic sample, is discussed in the *VRPCC Workbook: Subsection 2.9.5 Differential diagnostic issues with HSIL Page 180*. Experience in examining a large number of normal samples showing atrophy is of considerable assistance in learning how to recognise atrophic samples where a significant lesion is also present.

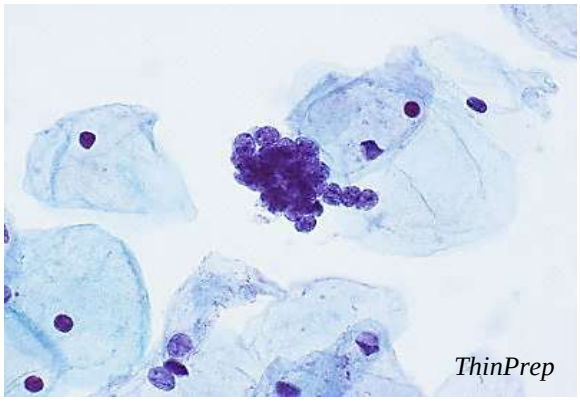


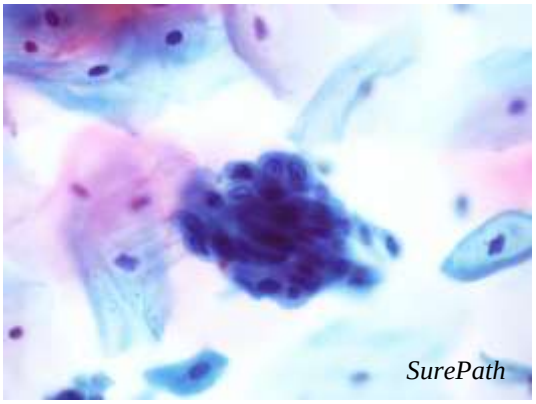
Atrophic squamous cells. The upper images show single atrophic squamous cells, mostly parabasal cells (red arrows) with some larger intermediate squamous cells (blue arrows). The lower images show flat parabasal cell sheets demonstrating cohesion and retention of cell polarity. Neutrophils often accompany atrophy.

Reserve cells are located directly above the basement membrane in the lowest layer of the epithelium. Reserve cells are precursor cells that differentiate into functional mature epithelium. Endocervical reserve cells have the ability to transform into either endocervical cells or metaplastic squamous cells. Basal cells are the precursor cells of mature squamous cells. Genuine reserve cells are seen very infrequently in cervical cytology samples, but may be seen in particular clinical situations such as marked atrophy when the epithelium is thin, immature and fragile.

Reserve cells have small (approximately 10µm diameter), pale-appearing nuclei and the N:C ratio is extremely high. The cells are more oval than round, have a bland chromatin pattern and are often present in grape-like clusters. Distinguishing true reserve cells from bare nuclei which have come from fragile atrophic squamous epithelium can be very difficult. As long as the nuclei are normal, the presence of these cells is not of concern. When reserve cells are seen in clusters, their monotony and predictability easily distinguishes them from the

abnormal bare sticky nuclei that may be seen with high-grade squamous lesions. Lymphocytes have a distinct rim of cytoplasm and a more granular chromatin pattern and should be easily distinguished from reserve cells.





Reserve cells in LBC preparations

2.2.8 Lower uterine segment (LUS) cells

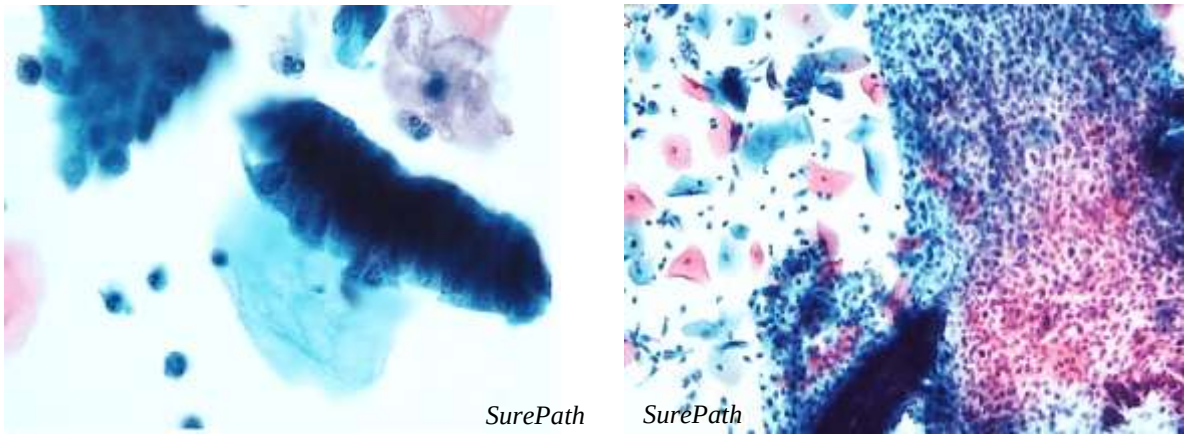
The lower uterine segment, also known as the uterine isthmus, is the narrower lower region of the uterine corpus where the upper endocervical canal ends and the body of the uterus begins. The cells that line the lower uterine segment are called lower uterine segment (LUS or LUSC) cells in cytology. The demarcation point between endocervical cells and endometrial cells in this region is not sharply defined histologically and cells from this region can show elements of both types of epithelia. The cells in the lower uterine segment do not respond to menstrual cycle hormonal fluctuations and are not shed at menstruation, whereas endometrial cells in the body of the uterus do respond and are shed.

Since the introduction of the cytobrush for sampling higher up the endocervical canal, and to a lesser extent with the use of plastic broom sampling devices used for LBC samples, LUS cells have become relative common in cervical cytology samples. Sampling from high in the endocervical canal is sometimes referred to as “high scrape”.

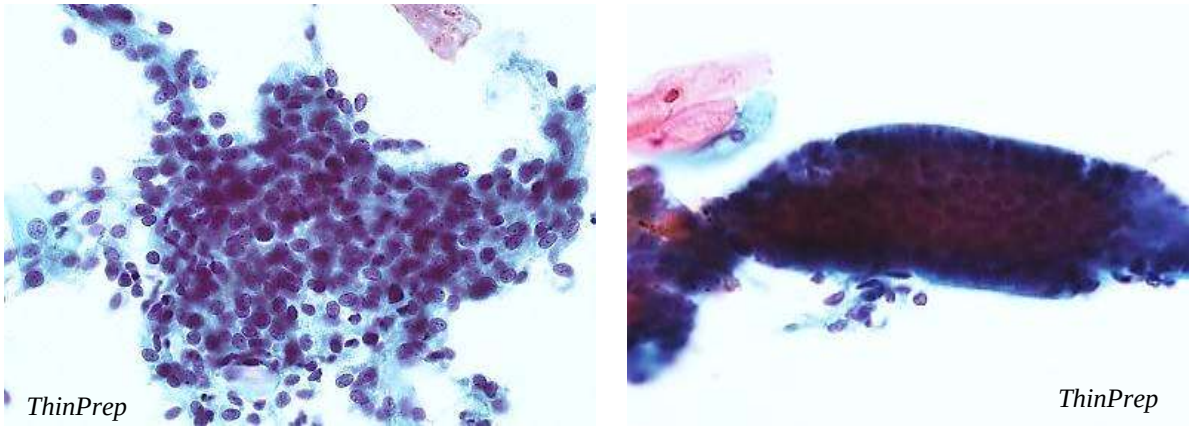
LUS cells usually appear in large multi-layered fragments rather than individual cells or small groups of cells. The fragments may contain epithelial cells forming tubular glands as well as densely packed spindled stromal cells. Finding the typical biphasic pattern of epithelial cells and stromal cells is very helpful in making a correct interpretation of high sampling but stromal cells are not always present. Folding of the fragment over itself may cause a three dimensional appearance. Nuclei retain their polarity within the group and nucleoli may be present. The nuclei overlap and are relatively small and the glandular and stromal endometrial cell nuclei are similar in size. LUS sheets have a similar low power appearance to atrophic squamous cells but are smaller cells which overlap and are more crowded. The chromatin can be granular but is evenly distributed with a uniform granularity. Occasional mitotic figures may be observed if the sampling is high enough to include functioning endometrial cells but these should be normal proliferative mitoses.

When LUS cells form crowded hyperchromatic cell groups they can be of diagnostic concern at low power screening magnification. Like all crowded groups, high magnification examination is required. The very uniform and predictable nature of the nuclei in these large

cohesive sheets identifies the benign nature and origin of the cells. The important distinction between LUS cells and Adenocarcinoma in situ (AIS) is discussed in the *VRPCC workbook: Subsection 2.11.6 Potential diagnostic pitfalls with premalignant endocervical lesions Page 229.*



SurePath preparations showing LUS cells



ThinPrep preparations showing LUS cells

2.2.9 Endometrial cells

Normal endometrial cells may be found from Day 1 up to about Day 10 to 12 following the onset of menstruation. Under The Bethesda System 2001, normal endometrial cells in women under 40 years of age are not reported because endometrial carcinoma in this age group is rare.

The presence of normal endometrial cells in samples from women at or over 40 years of age is reported irrespective of the clinical situation or menstrual history. **Benign-appearing endometrial cells may be associated with endometrial pathology and should be reported to the sample taker.** It is then the responsibility of the sample taker to assess the significance of the cytology report in the clinical context and to decide on appropriate follow-up or referral for specialist care.

Laboratories are able to use their own wording, or use the following suggested comment:

"The presence of endometrial cells in a woman over the age of 40 years can be a normal finding, or seen in association with hormone replacement therapy, or rarely, associated with endometrial pathology including hyperplasia or neoplasia. Please correlate this finding with any symptomatology of uterine pathology, for example abnormal uterine bleeding and refer/investigate accordingly."

Endometrial cells are 5-20μm in size and are smaller than endocervical cells. The cytoplasm is often scanty and can be vacuolated. The vacuoles may displace and compress the nucleus to the periphery of the cell. Polymorphs may be observed within clusters of endometrial cells, often within cytoplasmic vacuoles, and these reflect cell degeneration.

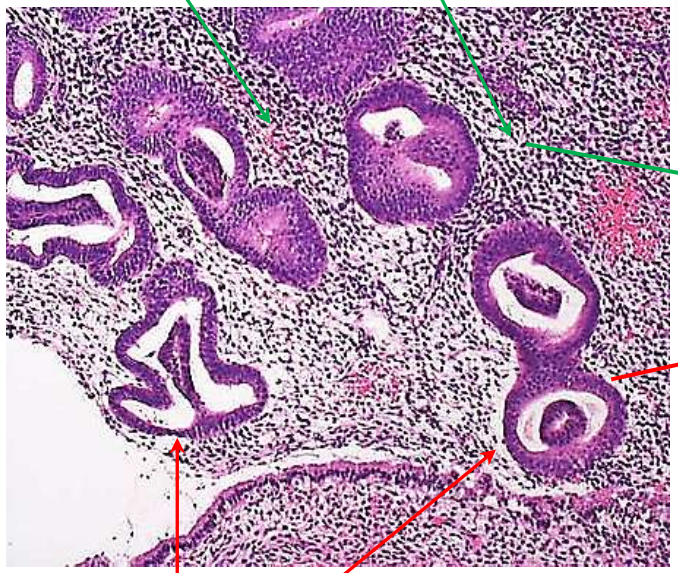
Normal endometrial cells exhibit round to oval nuclei which are relatively monotonous in size and appearance, and are rarely larger than the nuclei of an intermediate or parabasal squamous cell. Nucleoli are small and indistinct. The chromatin is granular and hyperchromatic. It has a characteristic appearance (sometimes called "salt and pepper" chromatin) which helps in the identification of the cells as endometrial in origin. Due to the time between exfoliation and sampling, endometrial cells normally degenerate to some extent, causing shrinkage of nuclei and cytoplasmic vacuolation. Normal endometrial cells are most prominent in cytology samples taken during the 2nd and 3rd days of menstruation.

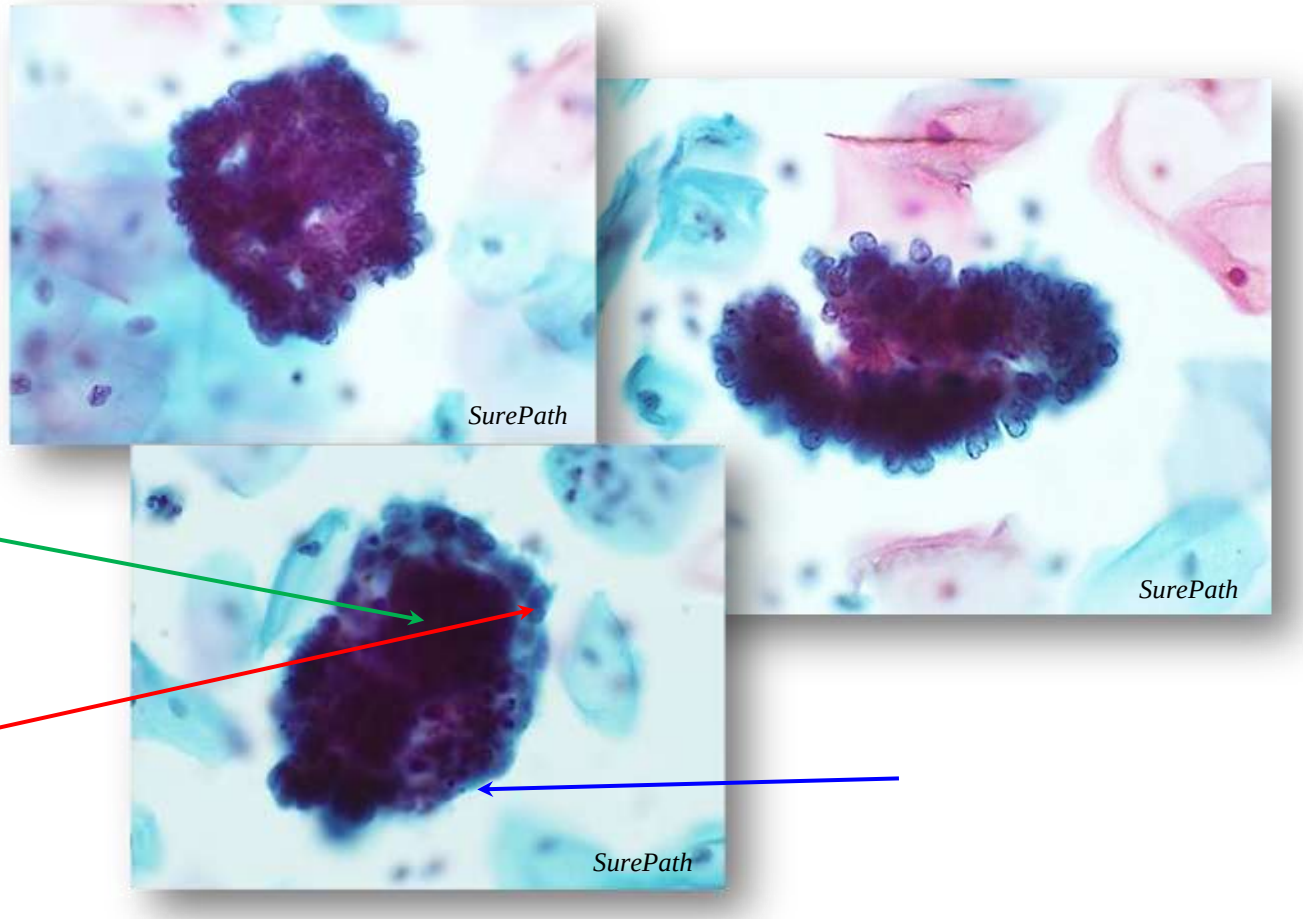
Endometrial cells are smaller than endocervical cells and are shed in clusters whereas endocervical cells usually form flatter sheets. Focusing through the cluster reveals the 3-D nature of the cell group. Well-preserved endometrial cells are sometimes seen as single glandular/short columnar cells. These cells can be difficult to distinguish from small histiocytes and if the chromatin is granular, may be confused with single cells shed from high-grade squamous lesions. The important distinction of normal endometrial cells from HSIL cells is further discussed in the *VRPCC Workbook: Subsection 2.9.5 Differential diagnostic issues with HSIL Page179.*

The presence of an intra-uterine contraceptive device (IUCD) can have a marked effect on endometrial cells, with exfoliation throughout the menstrual cycle. A chronic reaction during the first few months following insertion of an IUCD causes endometrial cells to show enlarged hyperchromatic nuclei, multinucleation, and prominent nucleoli. Marked vacuolation may be observed in the cytoplasm and can be confused with neoplasia. A mild local reaction may continue indefinitely. This highlights the need for accurate clinical information.

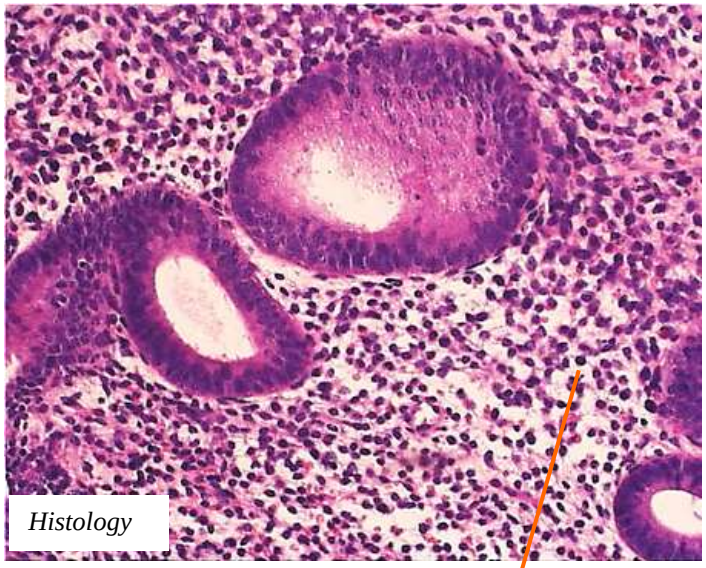
During menstruation, exfoliated endometrial glandular cells may be accompanied by endometrial stromal cells as a result of endometrial shedding. It can be difficult to distinguish between the stromal cells and glandular cells. Stromal cells can appear very similar to small histiocytes or can be spindled in shape. Clusters of shed endometrium may contain a dense central core of small, elongated, densely packed stromal cells surrounded by large glandular cells. These glandular cells are arranged in a fairly orderly manner around the stromal cells (morula appearance). Another appearance often seen after the 5th to 6th day of the cycle is endometrial cells surrounded by many small histiocytes.

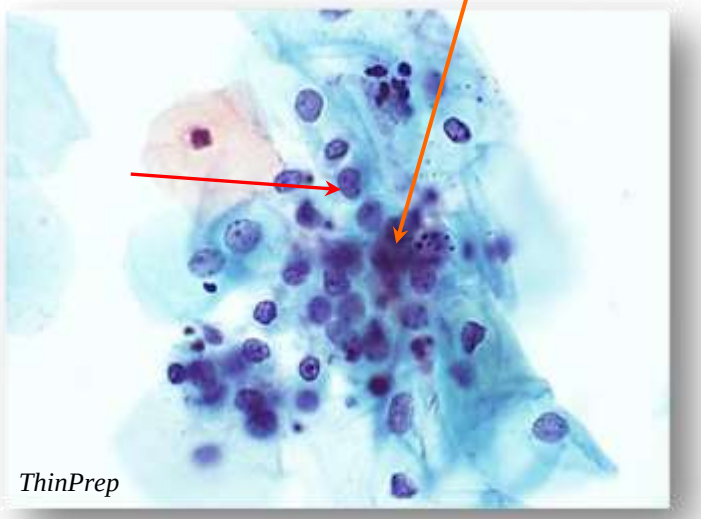
NORMAL CYTOLOGY
Endometrial cells



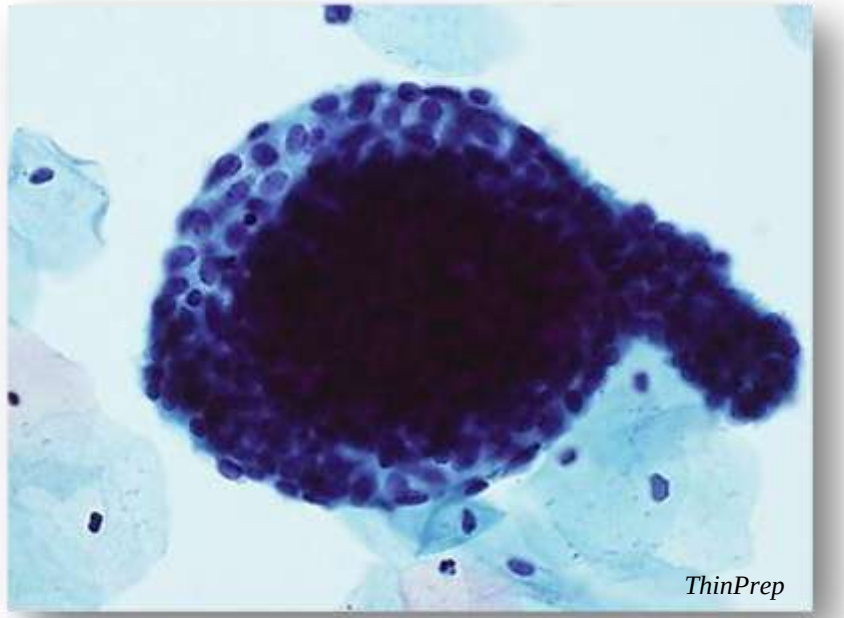


Tightly packed clusters of overlapping glandular endometrial cells. The cells are hyperchromatic but this is partly due to cell degeneration and shrinkage. Note the different group presentations from almost ball-like clusters to papillary-like forms. Uterine debris is also present including granular material and leucocytes (blue arrow). While these cells are morphologically normal, they are reported in a woman at or over 40 years of age as they could be associated with endometrial pathology.

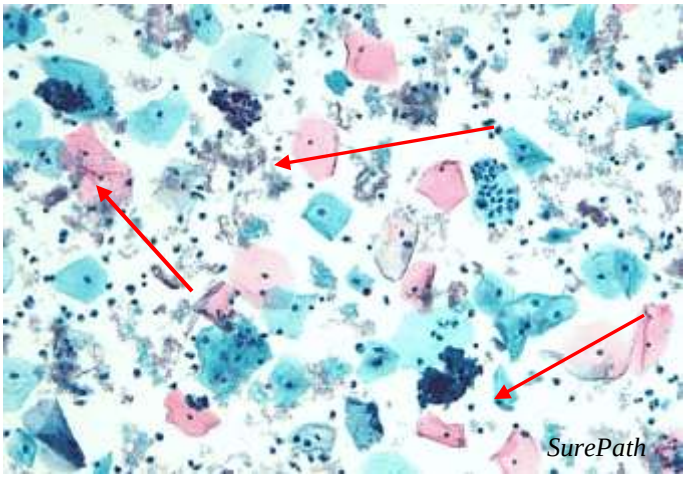




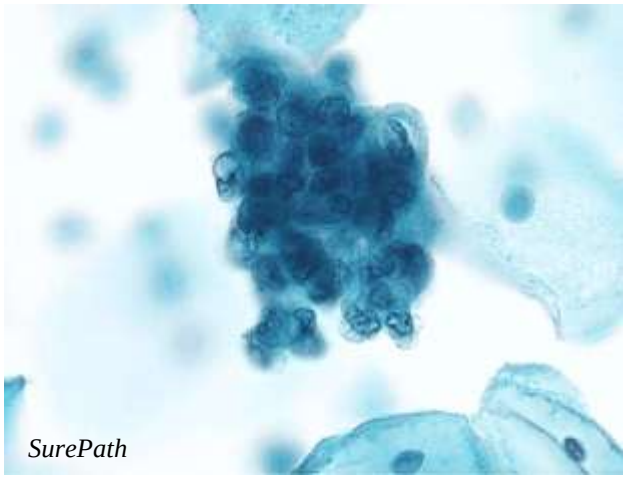
Comparative histology/cytology illustrating endometrial stromal cells. Cytologically the cells appear similar to small histiocytes. There is likely to be a mix of stromal endometrial cells and histiocytes in the cytology sample shown. The cells with bean-shaped nuclei are more likely to be histiocytic (red arrow).



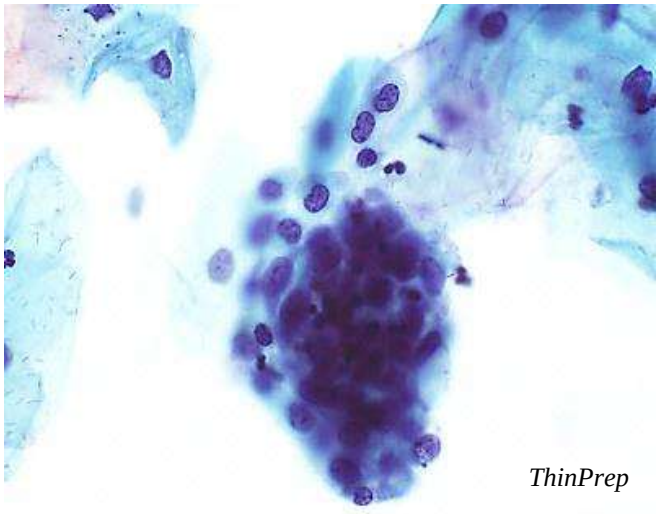
An exfoliated fragment of outer glandular and central stromal endometrial cells.



Low power view showing endometrial clusters. These appear hyperchromatic and should always warrant closer examination to confirm that they are endometrial cells.



Higher power view of an endometrial cell cluster. Note the finely granular chromatin with some chromocenters and delicate cyanophilic cytoplasm.



Normal endometrial cells

2.2.10 Other cellular and non-cellular constituents

Leucocytes and Erythrocytes

Leucocytes and erythrocytes may be found in LBC preparations to varying degrees but generally do not obscure epithelial cells. An increase in leucocytes will be found during menstruation, as will an increase in the number of erythrocytes. Erythrocytes may also be a result of bleeding due to the trauma if the sample is taken vigorously or if the epithelium is fragile.

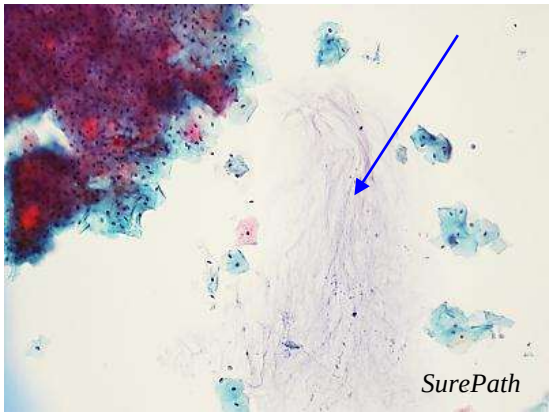
ThinPrep: Haemolytic agents in the ThinPrep fixative fluid usually remove red blood cells prior to slide preparation. For samples containing excessive blood, an acetic acid wash can be used to further lyse red blood cells before ThinPrep slide processing.

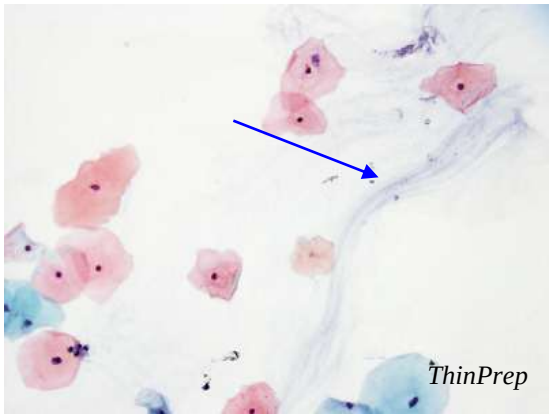
Inflammatory material is evenly dispersed on ThinPrep slides and can cling to epithelial cells. Occasionally the background appears dirty and tattered with few well-preserved squamous epithelial cells. When this type of background is observed, it is important to consider the presence of an infectious agent, cytolysis, and/or malignant disease (tumour diathesis).

SurePath: The density reagent used during SurePath slide preparation removes small particles such as aggregated proteins, disrupted membranes, microbial and red cell fragments, based on particle size. Non-diagnostic debris and excess inflammatory cells are partially removed from the sample. Air-drying artefact and debris obscuring and overlapping cellular material are largely eliminated. The number of leucocytes is significantly reduced, allowing easier visualization of epithelial cells, diagnostically relevant cells and infectious organisms.

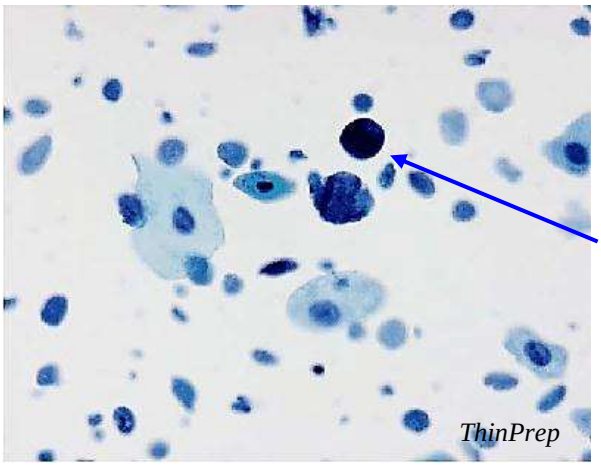
Mucus

Mucus produced by endocervical cells is present in small quantities in LBC preparations as a natural entity. It appears as amorphous and sometimes "stringy" material. In atrophic women mucus may also appear as amorphous blobs, sometimes called “blue blobs”. These are an amorphous grey colour, are approximately the size of a small parabasal cell and may have a darker central core. They can be caused by inspissated mucus or degenerating parabasal cells.





Mucus in SurePath and ThinPrep samples



“Blue blob” in an atrophic LBC preparation. These are globular collections of basophilic amorphous material reflecting either degenerated parabasal cells or inspissated mucus.

Histiocytes

Histiocytes are single cells that do not truly adhere together. They are commonly seen in cervical cytology samples and because of their morphology, are sometimes difficult to distinguish from epithelial cells. Histiocytes are single cells but may clump together giving an epithelial-like appearance. The clumps tend to have a rounded appearance and close examination will reveal "windows" between the cytoplasmic borders of individual cells.

Histiocyte cytoplasm is either foamy or vacuolated which can be confused with secretory activity. The vacuoles are usually multiple and of various sizes, and have indistinct inner vacuole borders. Debris may or may not be observed.

Histiocytes increase in number in various benign clinical settings and occasionally are associated with epithelial pathology. For example, small histiocytes with a mature squamous cell pattern and no inflammatory changes in a cervical cytology sample from a post-menopausal woman, is a pattern that may be associated with endometrial adenocarcinoma.

The nucleus is usually eccentric and round to oval when in the resting (non-active) state, and may be touching the cytoplasmic membrane at one point. When the histiocyte becomes active certain changes can be observed which can cause diagnostic problems. These features are:

- The nucleus can become irregular in shape showing flattening of part of the membrane and even indentation giving a "kidney-bean" appearance. Lobulation may be observed.
- Multinucleation is a common feature with some disease processes eg. granulomatous inflammation. The nuclei in different histiocytes are usually identical to each other.
- Chromatin patterns change and can become uneven with clumping and chromatin clearing (all signs of activity). The chromatin does however remain rounded or smudged but does not have irregular jagged angles as seen with cancer.
- The nuclear membranes can become thickened and uneven, but not sharply angled.
- Nucleoli can become extremely prominent and multiple.
- Mitotic figures may become frequent with increased histiocytic activity.
- Multinucleated cells can become giant in size and the nuclei may form a mirror image of each other. In some conditions the nuclei form a syncytium at one side of the cell and a "horseshoe" type of arrangement may be seen.
- Syncytia can also be formed following the early breakdown of the cytoplasm of degenerating histiocytes. The cells tend to stick together and the nuclei come together to form a pseudo-syncytium.
- Engulfed cytoplasmic debris can help with the differential diagnosis but must be used with caution. The debris in histiocytes is usually of varying size which helps distinguish the cell from a tumour cell.

Commensal Organisms

A variety of organisms are present in the cervix/vagina in the absence of disease as natural flora. Some organisms are cause an epithelial reaction and even damage under conditions which allow their numbers to increase.

Common commensal organisms are:

- Döderlein bacilli (lactobacilli)
- *Gardnerella vaginalis*
- Streptococci
- Staphylococci
- Micrococci
- Non-pathogenic *Neisseria*
- *Pseudomonas*
- *Mycoplasma*
- *Leptothrix*
- *Candida* species

Döderlein bacilli (*Bacillus vaginalis*) are the most common organisms found in the normal vagina and belong to the lactobacilli group. In the normal cycle they proliferate mid-cycle and if particularly abundant, can cause cytolysis in the luteal phase of the menstrual cycle.

Döderlein bacilli

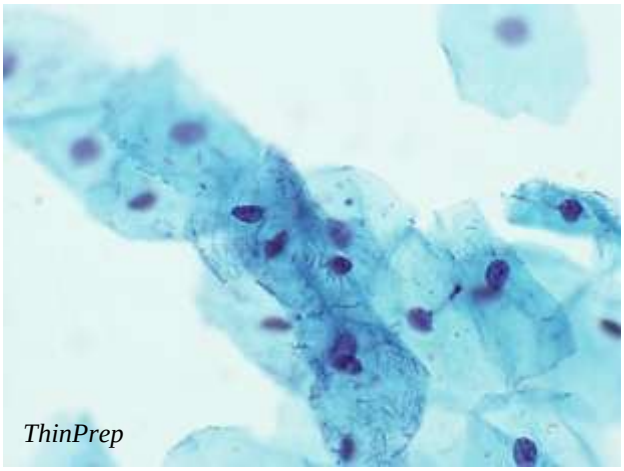
Döderlein bacilli are a heterogenous group of commensal bacilli normally present in the vagina. They are slender, gram-positive rods (2-6 microns in length) which stain blue. They are a member of the group lactobacilli and survive at a pH of 5.0 (acid). Doderlein bacilli help maintain the acid pH of the vagina by hydrolysing glycogen from the cytoplasm of intermediate squamous cells into lactic acid (called glycolysis). This is a natural regulatory process and aids in defence against infections by maintaining a constant pH. When lactobacilli multiply rapidly, as often occurs in the luteal phase of the menstrual cycle, they can have a cytolytic effect on epithelial cells (without causing inflammation).

The bacilli are present throughout the cycle but become more conspicuous in the last few days of the secretory phase (luteal phase day 21 plus). When cytolysis occurs they can be seen among cell debris and bare nuclei. Cytolysis may be so marked that the sample may be unsatisfactory due to insufficient viable intact cells.

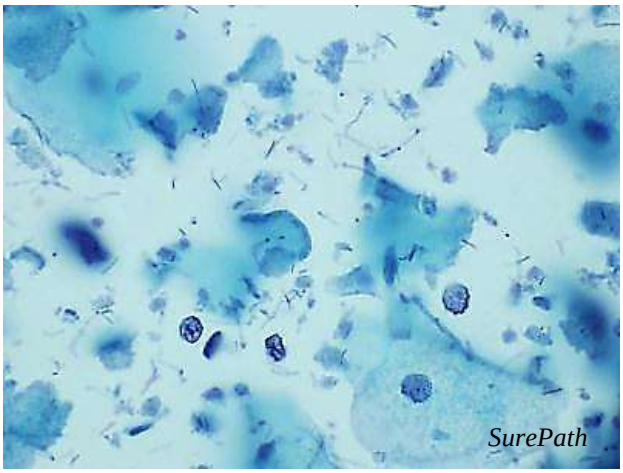
In pregnancy, the glycogen-rich environment produced by intermediate cells may cause an increase of Döderlein bacilli in the second and third trimester. Cytolysis is also common in the early postmenopausal period prior to the later atrophic phase.

The bacilli are commonly found in approximately 60% of normal samples and in samples with cervical intraepithelial neoplasia (CIN), but only in 10% of cases of early invasive cancer and in 1% of cases with advanced cancer. Dysplastic cells (i.e. CIN cells) are not cytolysed as they do not produce glycogen.

Döderlein bacilli (lactobacilli) convert glycogen from intermediate squamous cells into lactic acid to regulate the vaginal pH. Where the bacilli are numerous, cells may be observed in different states of cytolysis ranging from cells with ragged cytoplasm to bare nuclei in a background of cytolytic debris.



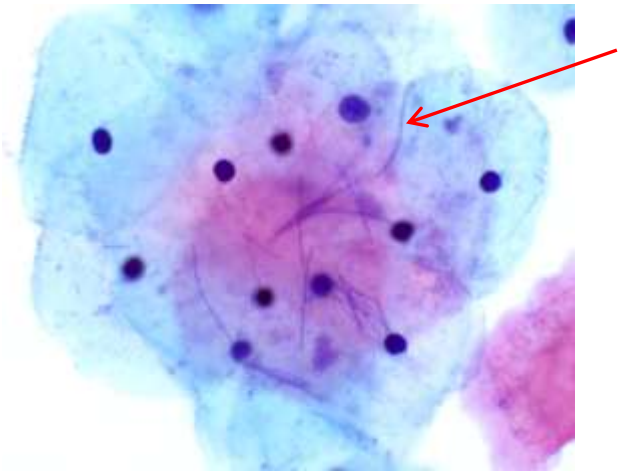
Normal squamous cells and lactobacilli



Cytolytic squamous cells associated with numerous lactobacilli

Leptothrix

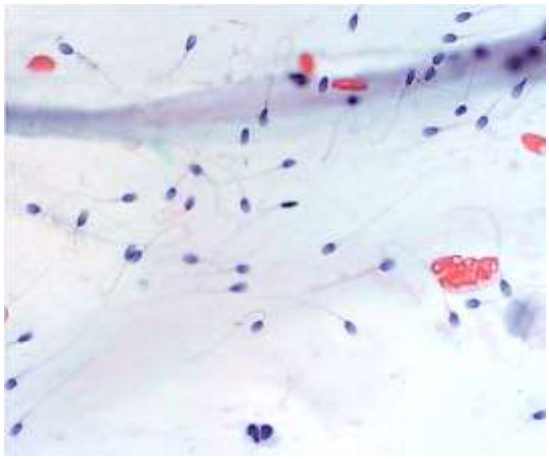
Leptothrix filaments are less commonly present than lactobacilli, are non-pathogenic and do not cause inflammation, although are often associated with Trichomonas vaginalis. The filaments form long or short, grey, thin thread-like structures which are segmented and may branch, but do not form spores. These can be as short as Doderlein bacilli when they are indistinguishable from them cytologically. Leptothrix may assume a characteristic "S" shape.



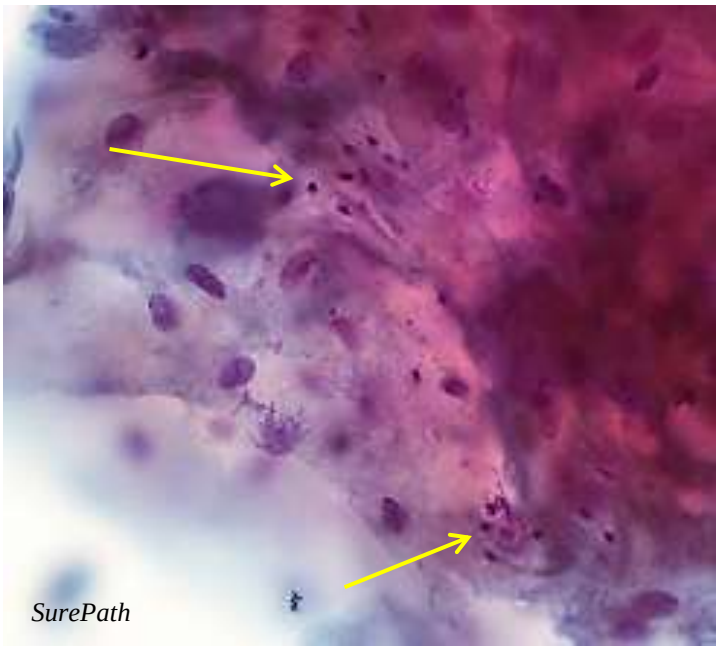
Leptothrix: There is a mix of short and long rods. The characteristic curved and "S-shaped" forms of leptothrix are evident in the long rods (arrowed). The shorter forms are indistinguishable from long Doderlein bacilli.

Spermatozoa

Spermatozoa are easily identified in post-coital samples. They have small round to oval bodies about the size of a polymorph lobe. This is the head of the sperm and stains with haematoxylin producing a two-tone effect. High power examination may or may not reveal the tail. Epithelial cells from seminal fluid may also be present and can show nuclei that can be mistaken for dysplastic nuclei.



Spermatozoa showing the pale and dark zone of the sperm head when stained with haematoxylin.



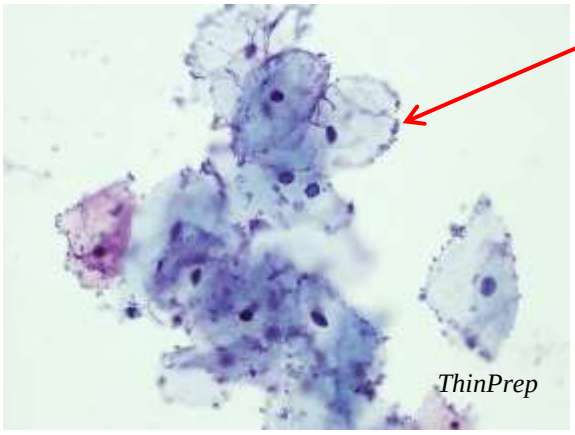
Spermatozoa amongst squamous cells

Contaminants and artefacts

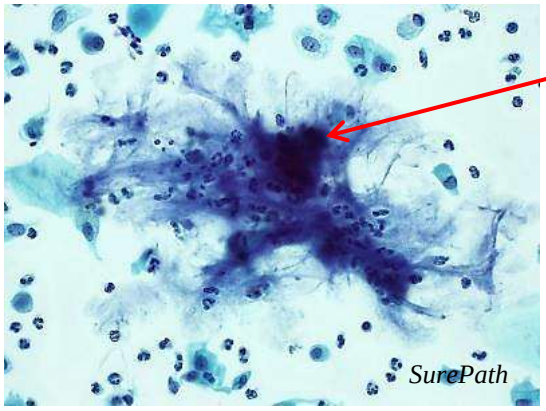
Creams, lubricating jelly, talc/glove powder, and other introduced contaminants may be observed. These tend not to cause too many problems with LBC preparations except for lubricants. As an aid for patient comfort, lubricants can be used during the pelvic examination to make insertion of the vaginal speculum prior to taking a cervical cytology sample, easier. Problems that this can cause are:

- Abundant lubricant on the cervix requires removal by swabbing the cervix prior to the sample being taken for cytology and this could remove exfoliated diagnostic cells.
- Residual lubricant can interfere with the endocervical brush or cervical broom in the acquisition of cervical cells.
- Lubricant can clog up the filter in the ThinPrep processor if present in the sample, resulting in a less cellular slide preparation. Sample takers using ThinPrep vials need to be instructed not to use lubricant when taking cervical samples if possible, or to use it sparingly and only down the slides of the speculum to avoid getting lubricant into the ThinPrep vial.
- Lubricant in the LBC vial could create a potential immiscible interface in alcohol-based solutions leading to potential agglutination and cellular loss.

Collection of samples in LBC vials ensures immediate, good fixation. A background of blood may give an impression of hyperchromasia especially in endocervical cells, as a result of the colour contrast between the red (red blood cells) and blue (nuclear) staining.



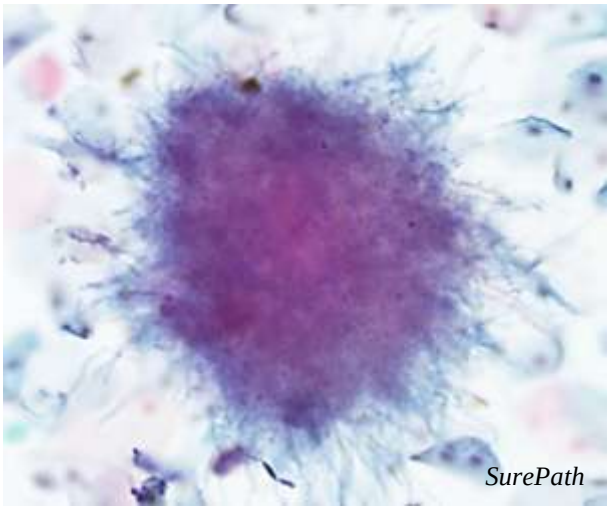
ThinPrep preparation showing excessive lubricant. Superficial and intermediate cells are clumped into a three dimensional group and coated with a granular precipitate.



SurePath preparation containing lubricant

Above: Vegetable cell contaminant in a cervical sample.
Below: fungal contaminant in the same sample.

Xylol precipitate can obscure cell and nuclear detail



2.2.11 Hormonal effects

This section is designed to give an overview of hormonal effects on cervical/vaginal squamous epithelium. Physiological and endocrinological aspects are referred to in VRPCC Section 1.

Menstrual Cycle

Phase	Day range	Cellular observations
Menstruation	1 to 6	• Presence of some blood in LBC preparations • Endometrial cells single and in clusters • Polymorphs • Squamous cells clumped, folded and degenerate during days 1-3 • Squamous cells better preserved, less folded in 4-5 days
Follicular/ Proliferative	6 to 12	• Gradual disappearance of blood (day 6-7) • Well preserved clusters of endometrial cells up to day 10-12 • Endometrial cells accompanied by large numbers of histiocytes • Squamous cells predominantly basophilic intermediate type and gradually replaced by more mature flat eosinophilic superficial squamous
Pre-ovulatory	12 to 14	• Mature flat eosinophilic superficial squamous cells • Clean background
Post-ovulatory/luteal/ secretory	14 to 24	• Cytoplasmic folding in superficial squamous cells reappears • Increase to numerous intermediate squamous cells often in clusters or clumps • Decrease in superficial cells
Late luteal/premenstrual	24 to 28	• Cytoplasmic degeneration and increased folding of squamous cells • Cytolysis with free nuclei, cytoplasmic debris and Döderlein bacilli. This appearance persists to the onset of menstruation

Pregnancy (antenatal) and Post-partum (post-natal)

Phase	Day range	Cellular observations
Pregnancy	After 2nd month	• Predominance of intermediate squamous cells • Glycogenation of intermediate squamous cell cytoplasm (navicular cells) • Clusters of epithelial cells • Extensive cytolysis may be seen in later pregnancy • Increased numbers of endocervical cells in some • Larger than normal endocervical cells
Pregnancy	Reaches term	• Decrease in numbers of cell clusters

		• Increase in single cells • Increase in superficial squamous cells (these changes are variable and may not always occur)
Pregnancy	Rupture of foetal membranes	• Numerous anucleated squamous cells from foetal skin • Small parabasal-like cells
Pregnancy	Abortion	• Trophoblastic cells of syncytial type. These cells are large, irregular, basophilic or eosinophilic, contain a few large often-dark nuclei with fine granular texture. It is uncertain if these represent the Langhans' layer of the placenta • Smooth muscle fibres from uterine wall (rare) • Sudden increase in superficial cells may be associated with threatened abortion
Postpartum	First few weeks and whilst lactating	• Atrophic cell pattern • Predominance of parabasal cells • Navicular cells (glycogenated) in lactating women • Squamous metaplasia • Epithelial inflammatory changes

Menopause

Phase	**Day range**	**Cellular observations**
Menopause	Early (slight oestrogen deficiency	• Reduction in numbers of superficial squamous cells
Menopause	Crowded (moderate oestrogen deficiency	• Crowded clusters of numerous intermediate and parabasal cells • Well preserved epithelial cells • Pale nuclei • Glycogenated cytoplasm of some cells • Cytolysis may be present
Menopause	Advanced (atrophic)	• Diminished or absent mucus production • More cuboidal appearing columnar epithelial cells • Mostly parabasal cells • Parabasal cells with karyopyknosis and eosinophilic cytoplasm • Karyorrhexis • Possible marked cell size/shape variation with senile vaginitis • Marked hyperchromasia • Cytoplasmic fragments • Scarce to absent endocervical cells • Blue-blobs (inspissated mucus) • Mononucleated histiocytes • Multinucleated histiocytes Many of the above features are due to inflammation and may be associated with the irritation caused by reduced mucus on the thin dry epithelium found in marked atrophy

Effects of oral contraceptives

Progesterones, androgens, low levels of oestrogens, and combinations of oestrogens and progesterones result in the maturation of the intermediate cells layers, whereas oestrogens in the absence of progesterones mature the superficial cell layers. Hence women who take the combined oral contraceptive pill (oestrogen and progesterone) or the progesterone-only pill (mini pill) will tend to have an intermediate cell pattern, although with the mini pill this maybe more variable as oestrogen is only suppressed during some cycles. With both types of pill the mucus plug becomes thick and periods occur (due to progesterone).

Other conditions influencing the cervical epithelium

Condition	Process	Cellular observations
Therapeutic oestrogen	Hormone replacement therapy HRT	• Intermediate cell pattern and/or superficial cells with HRT (No direct link between the dose of hormone and the response of the squamous epithelium with HRT)
Therapeutic oestrogen	Direct vaginal oestrogen cream	• Squamous maturation with superficial cells following direct vaginal oestrogen creams
High dose androgens	During child bearing years	• Intermediate cells containing abundant glycogen (androgens compete with oestrogens)
High dose androgens	During late post menopause	• Some maturation giving mixed cell pattern of parabasal and intermediate cells (androgens may be metabolised into oestrogens)
Androgen secreting tumours	Ovarian or adrenal tumours secreting androgens	• mostly parabasal with some intermediate cells. Rare occurrence– effects of low oestrogen and progesterone.
Damaged liver cells	Hepatic insufficiency	• superficial cells. (Liver may not be able to metabolise oestrogen leading to increased circulating oestrogen).
Ovarian tumours	Production of oestrogen	• no cellular effects if during child-bearing years, but in pre-puberty and late menopause will show superficial cells
Testicular feminisation syndrome	Cells with female phenotype but XY chromosome	• mature cell pattern with intermediate and superficial cells but no sex chromatin
Turner's syndrome	Absence of one (or part of) X chromosome	• no evidence of maturation so parabasal cells. (Only 45 chromosomes: reproductive organs are usually poorly developed and menses absent)
Anorexia nervosa with secondary amenorrhea	Decrease in secretion of pituitary gonadotrophins	• complete atrophic pattern, may show atrophic vaginitis (Secondary ovarian failure)

2.1.12 Questions

1. Draw a labelled schematic diagram of the female genital tract. Label and describe the main types of epithelia lining and covering the fallopian tubes, endometrium, cervix and vagina.

2. Describe the cytological features of the different types of normal squamous epithelial cells comprising the epithelium covering the ectocervix.

3. Discuss the importance of the presence of endocervical cells and/or metaplastic cells in cervical cytology samples. What is the reasoning behind a normal (3-yearly) recall in New Zealand for women with samples that do not contain endocervical cells and/or metaplastic cells?

4. Discuss the significance of the presence of reserve cells in cervical cytology samples. What are the distinguishing cytological features of these cells, compared to sticky bare nuclei from a high-grade lesion?

5. What are navicular cells? Describe these cells and list the clinical conditions under which one could expect to see them in cervical cytology samples.

6. Describe with the aid of schematic diagrams, contaminants that may be found in cervical cytology samples.

7. Describe the findings in a "high-scrape" cervical cytology sample. What are the main cytological features that indicate the benign nature of these samples?

8. List the clinical conditions during which one could expect to find benign-appearing endometrial cells in a cervical cytology sample.

9. Discuss the criteria to be applied to assess adequate cellularity of either a SurePath or a ThinPrep slide. Describe what cell counts should be used and how these are performed.

10. What preparation technique and reagents are involved in ensuring that limiting elements do not affect either ThinPrep or SurePath adequacy?

11. You are working in a cytology laboratory and receive a phone call from a sample taker who queries why she repeatedly gets cytolytic samples from a patient. Write an explanation of your response to this query, including the physiology of what this is and what steps the sample taker could take to minimise the possibility of obtaining a further cytolytic sample from the patient.

References

- Farnsworth A, Ford J, et al. 1989. The importance of the cell sample in cervical cytology. *QASEC Handbook No.1*. RCPA Quality Assurance Programmes Pty Ltd

2.3 CORE TOPIC: INFLAMMATION, REACTION and REPAIR

2.3.1 Introduction

The presence of non-specific inflammation and epithelial cell reactive change in cervical cytology samples is common. Inflammatory cells occur as the local response to tissue injury or infection and can also be present for physiological reasons. Minor changes are not of clinical significance and are not reported. Marked inflammation or severe reaction/repair may require reporting so that the sample taker can consider the cytology result in the clinical setting and offer appropriate treatment and follow-up.

It is common to have an associated inflammatory response to infection. If a specific infection is identified clinically, by cytology or by microbiological tests, this will determine treatment. Some infections have a characteristic background pattern in cervical cytology samples that becomes familiar with experience. Such changes are not necessarily unique to the infection but may alert the cytoscientist/cytotechnician to the potential presence of an infectious agent.

Some organisms are commensal. This means they co-exist as normal flora without any adverse effect under normal physiological conditions. These organisms are usually of low virulence but may cause disease under particular circumstances. Some organisms may cause an initial infection with inflammation and then remain inactive (latent) for prolonged periods of time. Reactivation may occur in response to an altered clinical state e.g. HSV (Herpes simplex virus) can be reactivated if a woman becomes immunosuppressed.

In many cases, inflammation is non-specific or associated with non-infectious causes such as localised tissue trauma (e.g. post-partum), therapeutic procedures (e.g. laser, cautery, surgery and irradiation) or the presence of foreign bodies (e.g. IUCDs, pessaries). All clinical information provided needs to be considered when interpreting cervical cytology.

Changes caused by inflammation, reaction and repair can be marked and result in significant cytologic atypia that in extreme cases may be difficult to differentiate from a neoplastic process. Careful examination of cell groups and individual nuclear features with application of consistent criteria will reduce misinterpretation of marked changes where atypia is present. The primary role of cervical screening is to identify pre-malignant changes. Inflammation and infection can co-exist with neoplasia, so although the inflammation/infection may be readily apparent when screening, the cytoscientist/cytotechnician must remain vigilant while screening the rest of the sample. If in doubt, further opinions regarding the interpretation of the appearances should be sought.

2.3.2 Common features of inflammation, reaction and repair.

Common features observed with inflammatory, reactive and reparative changes are:

Architecture of repair

- Flat sheets
- Retention of polarity within sheets
- Inflammatory cells within epithelial sheets

Nuclear changes

- Karyomegaly (increase in nuclear size)
- Marked variation in nuclear size within a group of reactive endocervical cells
- Hyperchromasia
- Increased chromatin granularity
- Uniformly thickened nuclear membranes
- Undulated nuclear membranes but no abrupt changes in contour or angularity
- Bi- and multinucleation
- Typical mitotic figures
- Enlarged and prominent nucleoli
- Multiple nucleoli with variable nucleolar size
- Irregularly shaped nucleoli

Cytoplasmic changes

- Altered staining
- Increased nuclear:cytoplasmic ratio
- Immature metaplastic appearance

*Features that are **not typical***
The presence of any of these features should raise concern and further opinions sought.

- Abnormal mitoses e.g. asymmetrical aggregation of chromatin during division
- Thick sheets (4 or more cell layers)
- Embedded mitoses
- Apoptotic material in sheets – very small, dark staining “granules”
- Large nucleoli (macronucleoli)

Squamous epithelial cells and endocervical cells have somewhat different reaction patterns. Squamous cells that are reacting to the same stimulus (e.g. an infection or local trauma) tend to react in a similar way. Reactive squamous cells may show significant nuclear enlargement with nucleoli for example, but all the cells tend to look similar to each other. This feature assists in identifying the cells as benign because one of the fundamental characteristics of dysplastic squamous cells is nuclear variability. Reactive endocervical cells however, often show considerable nuclear variation from one cell to another. The architecture of the cell groups (rather than individual cell morphology) is much more important when distinguishing reactive endocervical cells from endocervical glandular lesions.

Background

The background of the sample can vary immensely ranging from clean to very dirty with mixed bacteria, cell debris and numerous leucocytes. Reaction in epithelial cells results in increased cellular activity and proliferation of cells. As the inflammatory process becomes more marked, the amount of cell degeneration also increases so that both regenerative (proplastic) and degenerative (retroplastic) changes often co-exist in the same sample.

Leucocytes

There are different phases of the inflammatory response i.e. acute and chronic inflammation. Acute inflammation is reflected by the presence of many neutrophils (polymorphonuclear leucocytes). Chronic inflammation will include a mix of macrophages and lymphocytes, with diminishing numbers of neutrophils. Granulomatous inflammation is a specific type of chronic inflammation that includes multinucleated and epithelioid histiocytes, as well as plasma cells. A combination of leucocytes and debris with or without fibrin (appears as a fibrillary pale-staining material) is referred to as inflammatory exudate.

The presence of leucocytes in cervical cytology samples in the absence of organisms or reactive epithelial cells is of no clinical significance. Many women will have increased numbers of leucocytes in their cytology samples, particularly at the time of menstruation. The presence of numerous leucocytes may be problematic if they obscure significant numbers of epithelial cells.

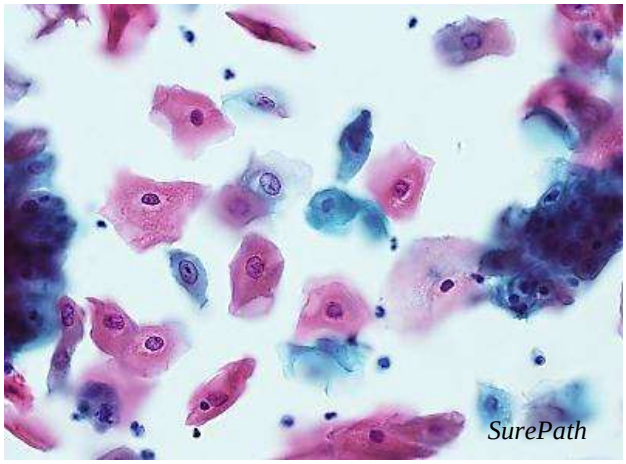
Occasionally it is relevant to comment on the presence of leucocytes. Multinucleated giant histiocytes and epithelioid histiocytes have been described in association with genital tract tuberculosis, for example.

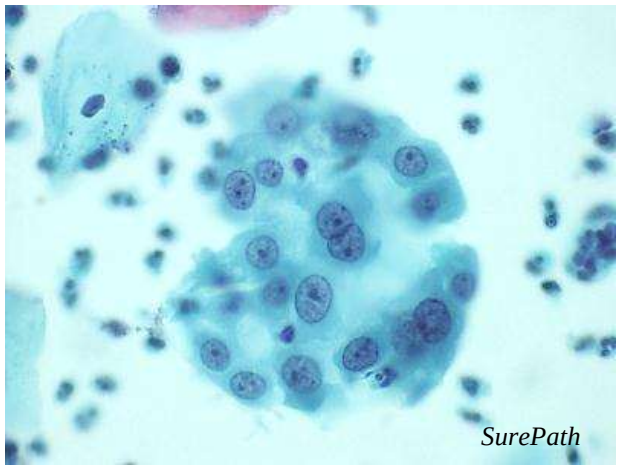
2.3.3 Mild to moderate reactive change

Mild to moderate inflammatory/reactive change:

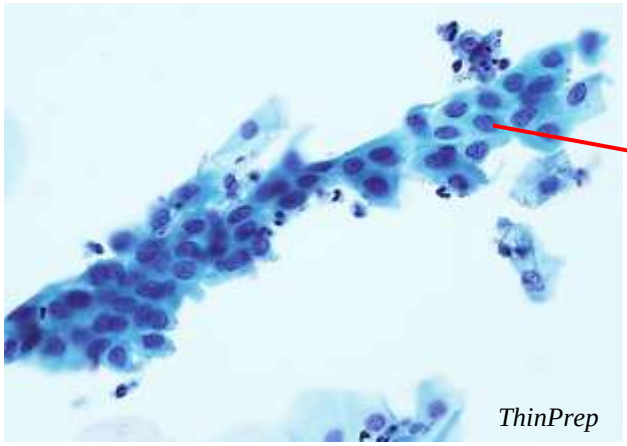
- Cytoplasmic eosinophilia (pink stain)
- Biphasic (two colours) or polychromatic (several colours) cytoplasmic staining
- Karyomegaly (nuclear enlargement) and cytomegaly (cell enlargement) with low N:C ratios
- Undulating nuclear membranes
- Increased nucleolar size
- Small soft-edged perinuclear halos
- Ragged “moth-eaten” appearance of the cytoplasm

In the absence of other cytological features, in most situations these changes will not be reported as their significance is of little consequence. Reporting minor inflammatory or reactive change may lead to unnecessary treatment and cause anxiety for women. Mild to moderate inflammatory change in keeping with an identified infection or a known clinical condition may also not be reported. Individual laboratories will have their own policy on such reporting issues.

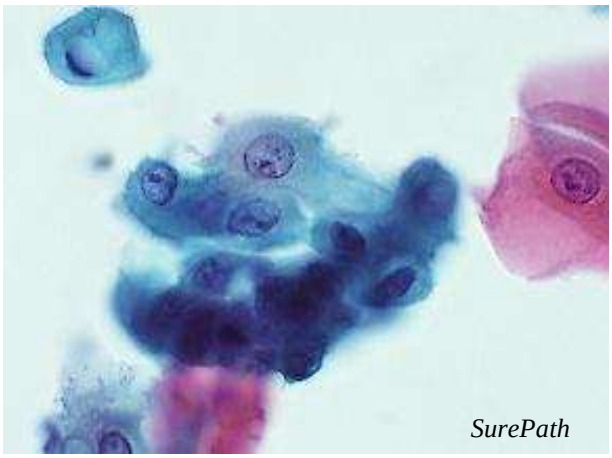


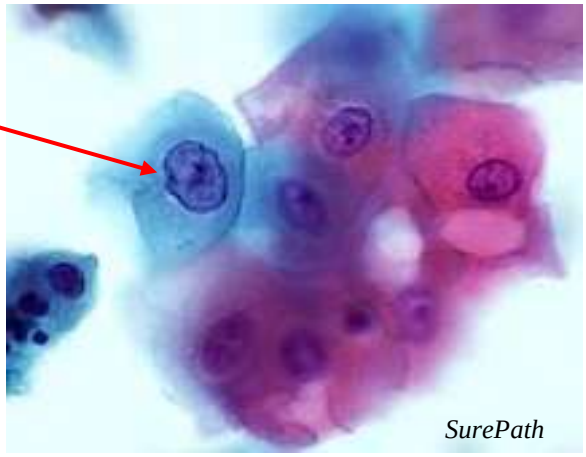


Mild reactive change. The left image shows small soft-edged perinuclear halos and an undulating ragged appearance of cell membranes. The right image shows cells with good cell cohesion, nuclear enlargement and nucleoli.

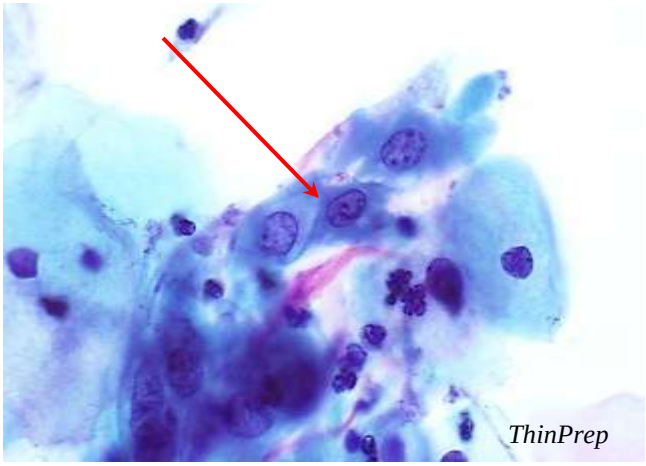


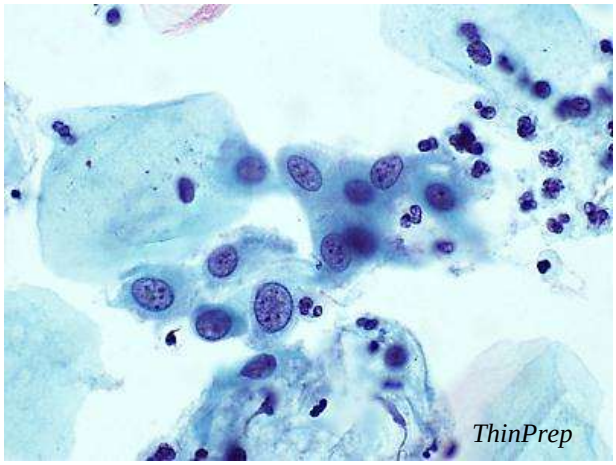
Sheet of reactive squamous cells showing retention of cell cohesion and polarity of cells. There is nuclear enlargement with small nucleoli but little variation between nuclei in the sheet.



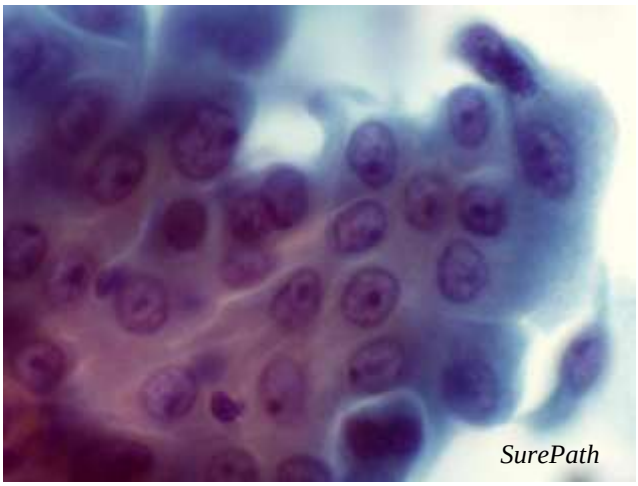


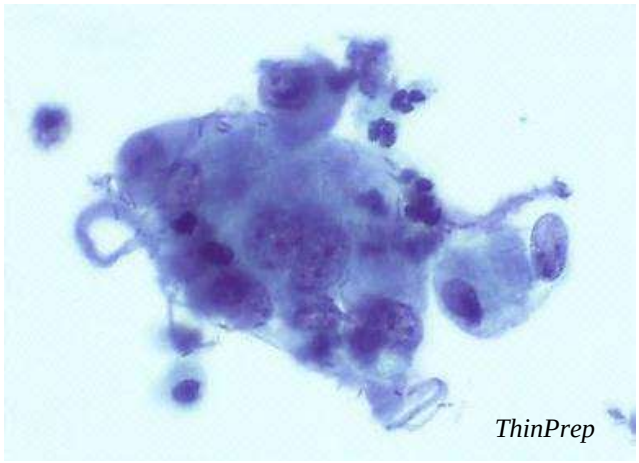
Higher power examples of reactive squamous cells. The left image shows cells which retain good cohesion while there is nuclear enlargement (karyomegaly) and nucleoli. On the right there are enlarged nuclei with nucleoli, undulating nuclear membranes (arrow) and small soft-edged perinuclear halos.

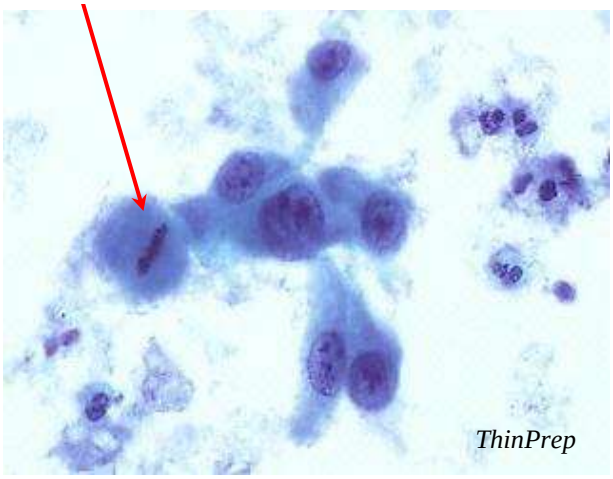




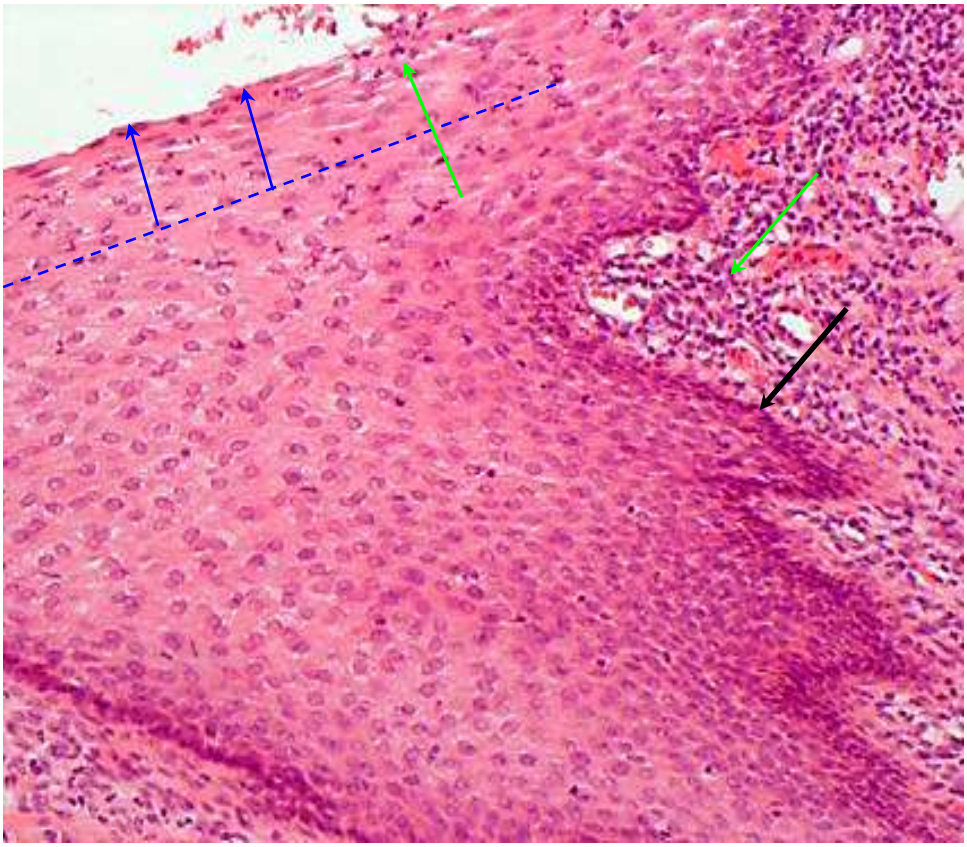
The left image shows cohesive reactive metaplastic squamous cells with nuclear enlargement and slight thickening of nuclear membranes (arrow). In the right image there is moderate variation in nuclear size but the nuclei otherwise closely resemble each other.



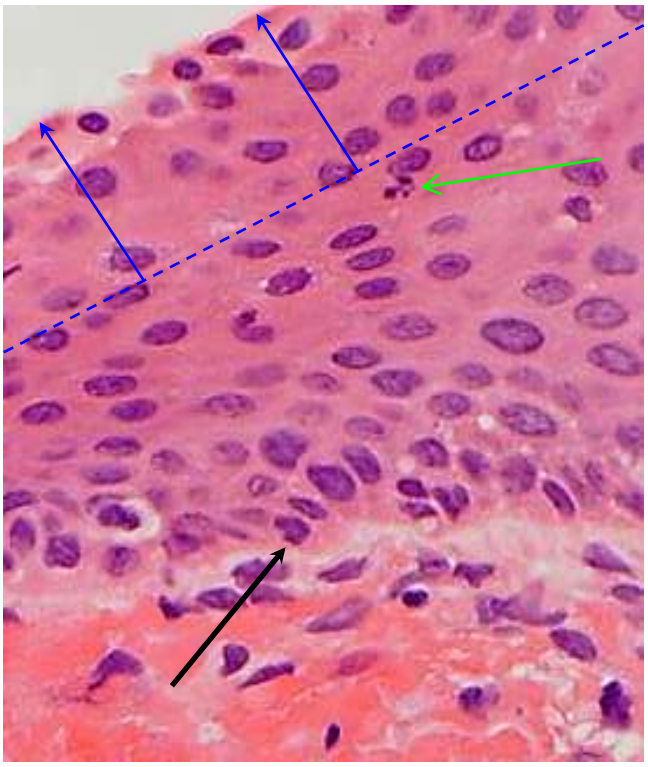




Reactive endocervical cells. There is often a lot more nuclear variation in size, shape and chromasia in reactive endocervical cells, compared with reactive squamous cells. Nucleoli may be very prominent. The lower left image shows a small reactive endocervical strip. A normal mitosis is present on the lower right image at the red arrow.



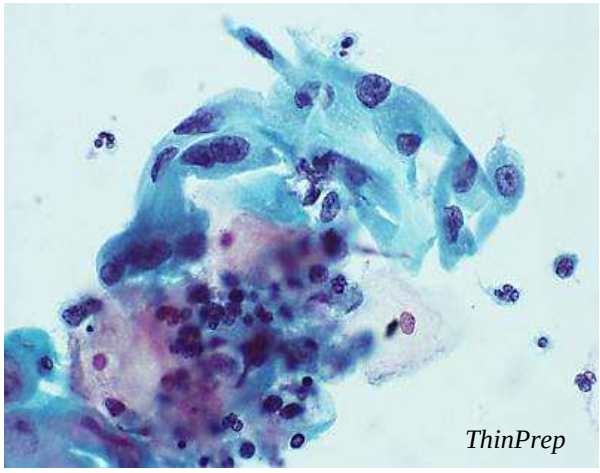
Histology of mild reactive/inflammatory change. Cells for cytology are sampled from the upper layers of the epithelium (blue arrows and line). The basement membrane is indicated by the black arrows. In the upper photo there is an inflammatory infiltrate in the stroma below the basement membrane as well as scattered neutrophils in the epithelium (green arrows).

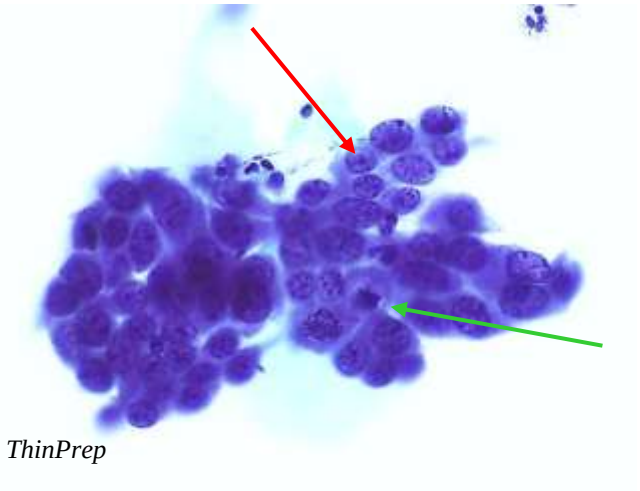


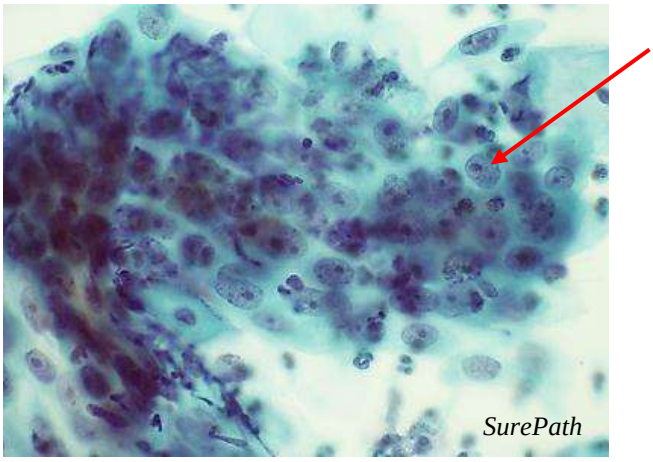
2.3.4 Marked reactive change

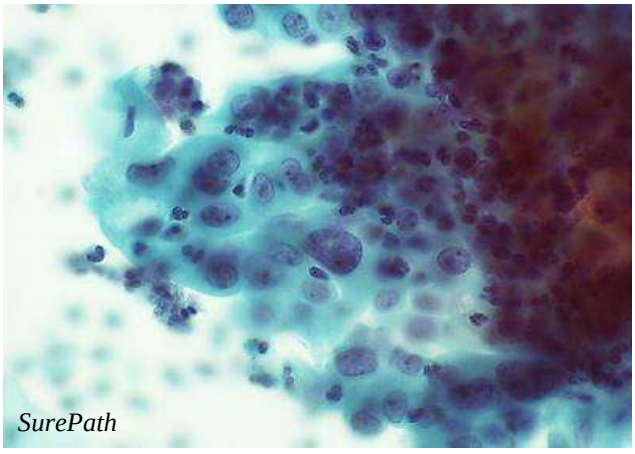
Marked inflammatory/reactive change shows features of inflammatory change as described above as well as:

- Variation in nuclear size (particularly endocervical cells)
- Chromatin clumping
- Chromocenters
- Mitotic figures (but not atypical forms)
- Binucleation and/or multinucleation (not usually in endocervical cells)
- Leucophagocytosis (engulfment of leucocytes in epithelial cells)
- Increased nuclear:cytoplasmic ratios (not in endocervical cells)
- Thickened nuclear membranes (evenly thickened)
- Multiple nucleoli
- Irregular nucleoli (not in endocervical cells)
- Presence of keratin and an increase in keratohyaline granules in squamous cells

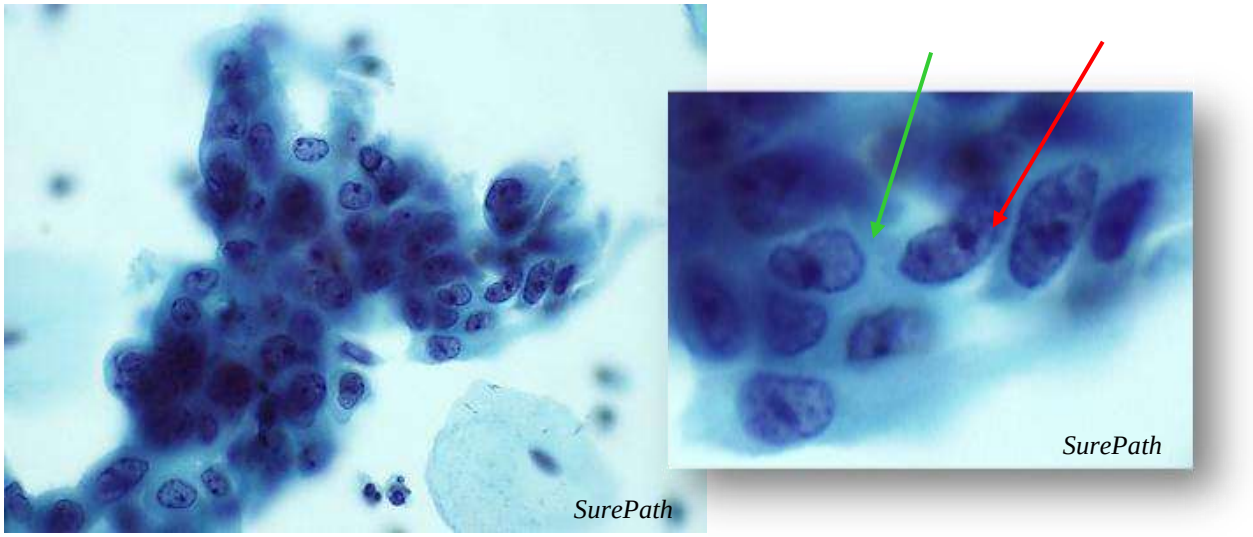




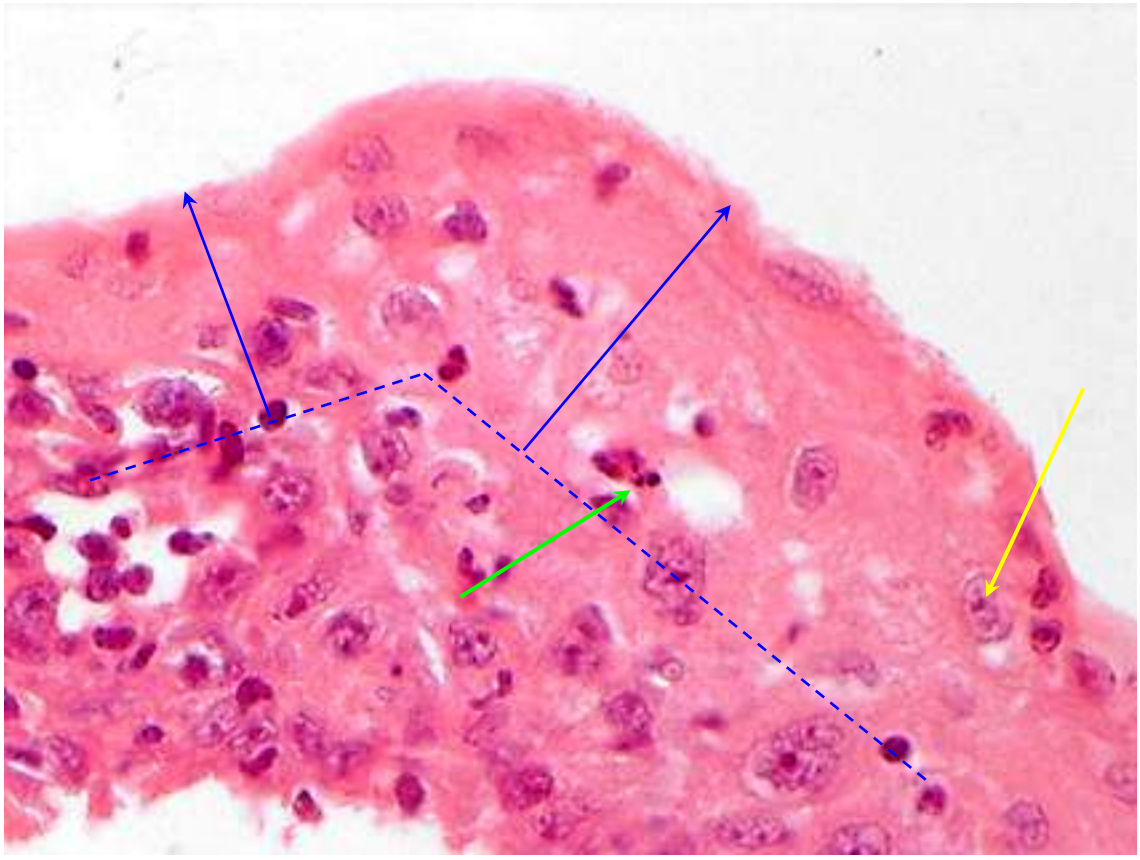




Groups showing marked inflammatory/reactive change in squamous metaplastic cells. There is nuclear variation, hyperchromasia, thickened nuclear membranes, and some nuclear overlapping. There is a normal mitotic figure (green arrow) and prominent nucleoli (red arrow).



Marked reactive change. The cells are cohesive but show some rounding of the cytoplasmic membranes particularly when in sheets. On high power nucleoli are clearly seen (red arrow), and the nuclear membranes are wavy and thickened but uniformly so (green arrow).



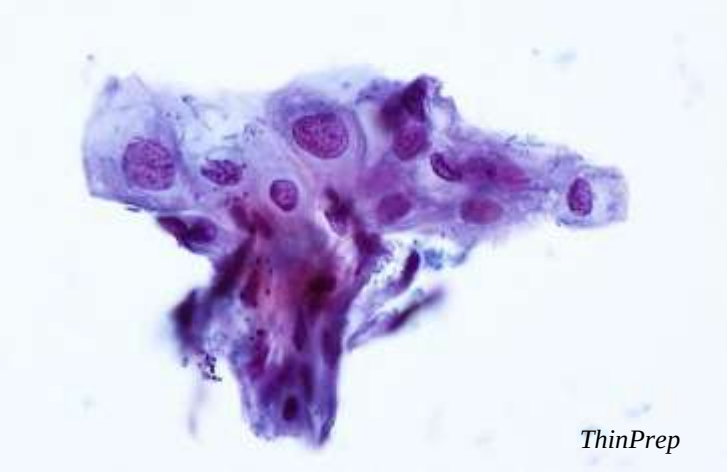
Histology of marked reactive change. The cells seen in a cytology sample would be from the upper layers (blue arrows and line). Neutrophils are present within the epithelium (green arrow). Nucleoli are prominent and frequent (yellow arrow) and reflect increased cellular activity. The surface epithelium still shows a degree of maturation depicted by cells with a lower N:C ratio compared with the immature cells in the basal layers. Sometimes even biopsies are reported as “atypia, favouring reactive atypia”.

2.3.5 Reactive Atypia vs. ASC-US (possible low-grade squamous lesion)

It is important to correctly identify inflammatory/reactive change so that it is not over-reported, for example as a possible low-grade squamous intraepithelial lesion (ASC-US). Cytologic interpretation is a subjective skill and there will be some differences of opinion between practitioners about the distinction of reactive change from squamous atypia. Laboratories develop and monitor their own approach to this issue.

Some useful features that help to distinguish inflammatory/reactive epithelial cell changes from ASC-US (Atypical Squamous Cells of Undetermined Significance) are:

- Strong cell-to-cell cohesion with well-defined cell-to-cell borders, intracellular bridges and relatively ordered group architecture and cell polarity
- Large flat sheets of cohesive cells
- No sharp angles in nuclear membranes
- Leucophagocytosis seen as neutrophils ingested within the cytoplasm

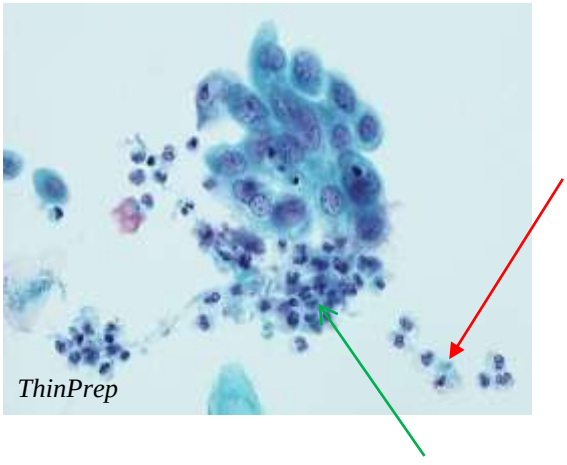


Flat sheet of atypical cells showing some reactive features but with more variation in nuclear size and greater chromatin granularity that is usual for reactive squamous cells. The N:C ratios remain low. The distinction between a reactive sheet and a low grade squamous lesion is difficult and the case was reported as ASC-US.

2.3.6 Marked Reactive Atypia vs. ASC-H (possible high-grade squamous lesion)

The epithelial cell changes may be markedly atypical, causing difficulty in distinguishing marked reactive change from a high-grade lesion, even squamous cell carcinoma. If marked reactive change is favoured but the possibility of a high-grade squamous or glandular lesion cannot be excluded, this should be stated in the cytology report under a heading of ASC-H (Atypical Squamous Cells, possible High-grade) so that the uncertainty about the diagnosis is conveyed to the clinician. If a high-grade lesion cannot be excluded, referral for colposcopy is recommended.

In this case of trichomonas infection (red arrow) and inflammation (green arrow), squamous epithelial cells are atypical. It is difficult to tell if all the changes are due to marked reaction, or whether there is also a high-grade lesion present. The case was reported as ASC-H. Follow up was HSIL.

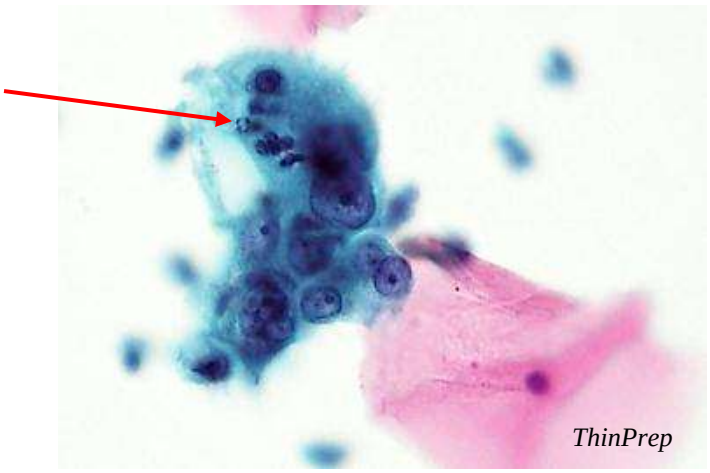


2.3.7 Repair

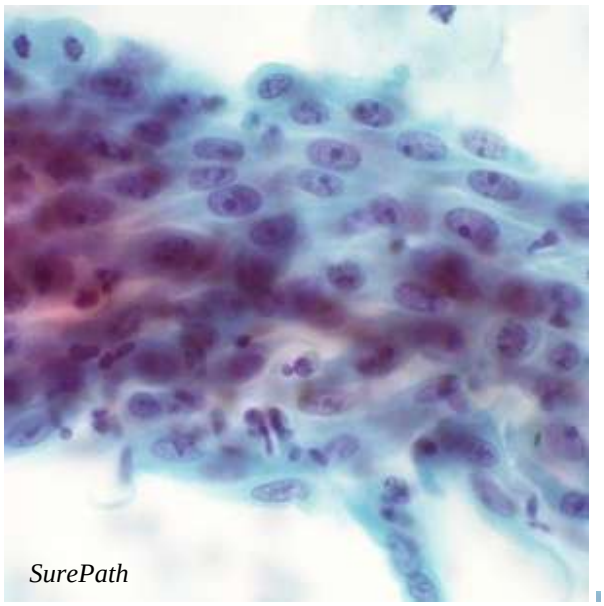
Repair is a term given to cytological change that occurs as part of regeneration of damaged epithelium. As with inflammatory changes, repair can be cytologically atypical to the extent that some features raise the suspicion of neoplasia. In reality there is a fine line between what constitutes reactive/inflammatory change from changes due to repair, and these processes commonly co-exist. However, there are characteristic cytological features of repair.

The main features associated with repair are:

- Immature metaplastic squamous cells
- High nuclear:cytoplasmic ratios
- Prominent and multiple nucleoli
- Irregular nucleoli in severe cases (extreme increased activity)
- Thickened nuclear membranes
- Usually no significant hyperchromasia
- Mitotic figures (not abnormal forms) increase in numbers with increased proliferation
- Cytoplasmic vacuoles (may stain positive with PAS-diastase reflecting immature metaplastic origin and presence of mucin)
- Normal cell group architecture and polarity is preserved
- Large monolayered sheets with a high degree of cell cohesion – single lying **atypical** cells are **not** a feature of repair
- Leucophagocytosis



Sheet of moderately immature squamous metaplastic cells showing karyomegaly, prominent nucleoli, and thickened nuclear membranes. Note strong cohesion between cytoplasmic processes, and also leucophagocytosis (arrow).



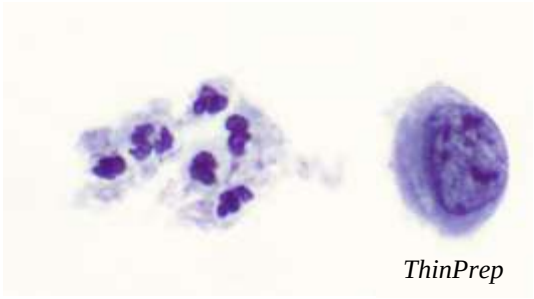
Sheet showing features of repair in a sample from a postmenopausal woman. Nuclei closely resemble each other within the group. The prominent nucleoli reflect increased metabolic activity.

2.3.8 Degenerative features associated with inflammation, reaction and repair

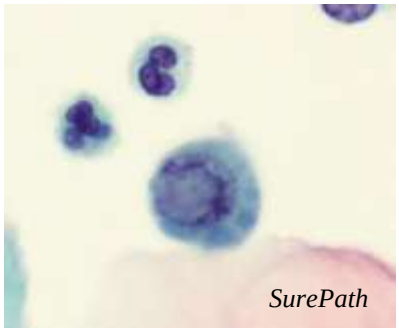
As already mentioned, as cell activity and proliferation increases, there is a corresponding increase in cell apoptosis and degeneration.

Features observed with degeneration are

- Karyopyknosis
- Karyorrhexis
- Karyolysis
- Nuclear ghosting (anucleate squamous cell)
- Nuclear folds and creases
- Pale indistinct chromatin
- Cytolysis
- Cytoplasmic vacuolation
- Fragmented and broken cytoplasmic membranes
- Decreased intensity of staining
- Perinuclear halos
- Background cell debris



Above: Large degenerate squamous cell showing cytoplasmic vacuolation and clumped degenerate nuclear chromatin.



Left: Degenerate squamous cell shows degenerating cytoplasm and clumped degenerate chromatin with a large nuclear "vacuole", possibly an intranuclear cytoplasmic inclusion related to the nuclear degeneration.

2.3.9 Questions

1. What are the causes of inflammation of the cervix?

2. How does the background associated with inflammation vary from that of tumour diathesis?

3. Discuss the significance of the presence of histiocytes in a postmenopausal sample.

4. What features would you use to help differentiate repair (especially marked atypical repair) from changes seen with malignancy?

5. What are the differential features that can be used to aid in deciding that changes being looked at are reactive changes associated with an inflammatory/reactive process rather than a high-grade intraepithelial lesion?

6. Define apoptosis.

7. Describe the cytologic changes associated with degeneration.

2.4 CORE TOPIC: INFECTIONS (excluding HPV)

2.4.1 Bacteria

Bacterial vaginosis (BV)

The vagina is an ecosystem that normally contains numerous species of bacteria, with a predominance of lactobacilli. Bacterial vaginosis is a clinical condition that correlates cytologically with replacement of the normal lactobacilli by a mix of other bacteria, most of which are short bacilli or cocci. These bacteria include *Gardnerella, Mobiluncus, Bacteroides,* and *Mycoplasma*. The bacteria do not produce lactic acid, and so the vaginal pH increases to above 4.5.

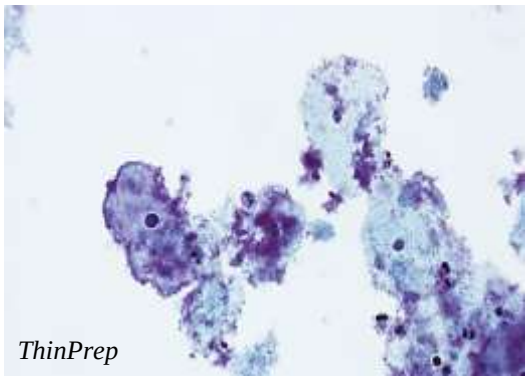
Gardnerella vaginalis (previously called *Haemophilus vaginalis* or *Corynebacterium*) is the most commonly identified of the various bacteria present in bacterial vaginosis. Gardnerella are small, gram-negative bacilli (rods) which adhere to epithelial cells covering the cell surface producing a characteristic "clue cell" appearance. The adherent bacilli are closely and uniformly packed and marginated, giving the cell a grainy appearance. Apart from producing the appearance of clue cells, *Gardnerella vaginalis* is difficult to differentiate from other bacteria by cytology.

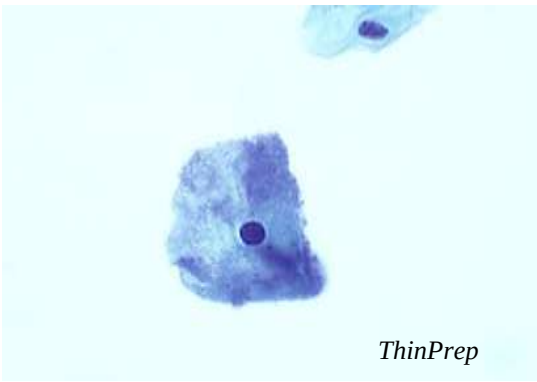
Clinically, bacterial vaginosis can be associated with a fish-smelling vaginal discharge but may also be present without causing symptoms. The diagnosis of bacterial vaginosis is a clinical one, and cytology can only suggest the possibility based on the flora present in the cervical cytology sample. BV is successfully treated with antibiotics. It is particularly important to report the possibility of bacterial vaginosis in pregnancy as bacterial infections can be transmitted to the baby if BV is present at the time of delivery.

As well as the presence of clue cells, features of note in bacterial vaginosis are:

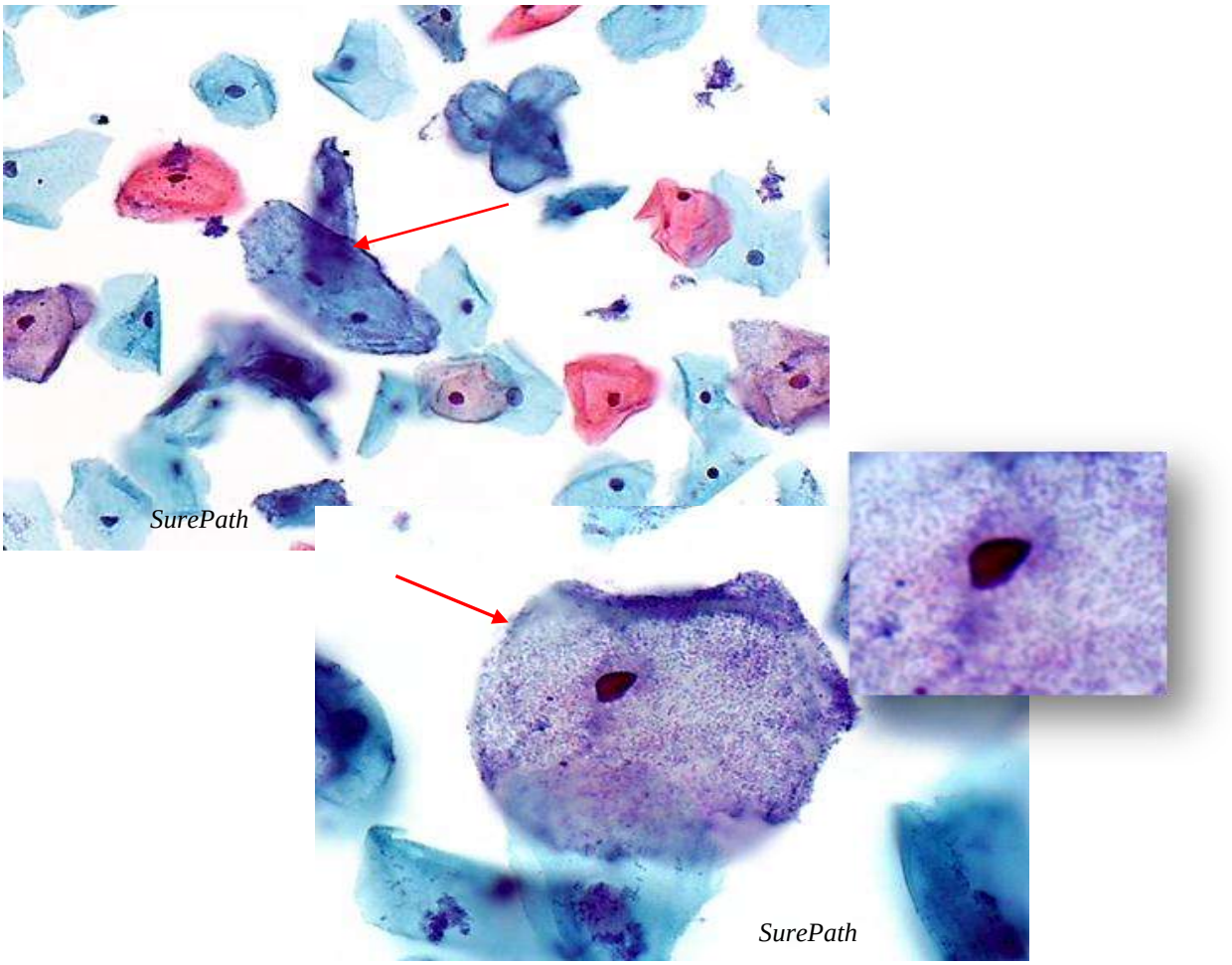
- Lactobacilli are absent
- Leucocytes are scanty
- A mix of small rods or cocci may be seen in the background although often the background is clear in LBC samples because of LBC processing.

Some laboratories only comment on the possibility of bacterial vaginosis if associated with reactive epithelial cells and inflammation because BV is often asymptomatic.


ThinPrep


ThinPrep

Clue cells and background coccoid and bacilliary organisms in a sample suggestive of bacterial vaginosis.



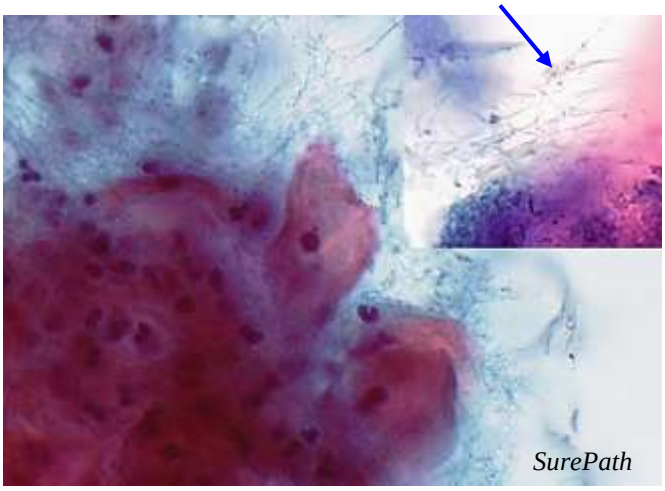
Bacterial Vaginosis. Note the clean background. Clue cells are a prerequisite for reporting BV and are indicated by the presence of bacilli coating squamous cells (red arrows). The rod-shaped bacilli can be seen in the high magnification inset.

Actinomyces

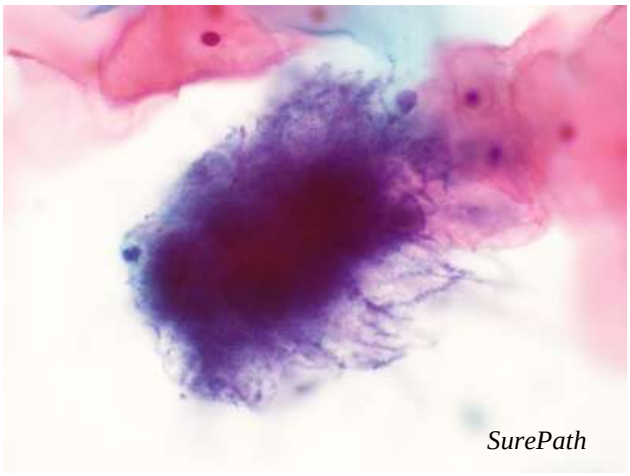
Opportunistic infection with *Actinomyces israelii* is often present in intra-uterine contraceptive device (IUCD) users. Rates reported in studies suggest an incidence of 5% to 12% in the first year of IUCD use, with an increasing incidence related to longer use. Diagnosis of these bacteria depends on the identification of filamentous, branching threads. These often have a fluffy appearance, with acute-angle branching filaments radiating from a dark central dense ball of filaments. The "sulphur granules" commonly seen in sputum are rarely seen in cervical cytology samples. The filaments may be fine or broad in diameter. Coccoid bacteria may accompany the infection. Inflammatory cells and macrophages may surround clusters of organisms. These aggregates of organisms and bacteria have been referred to as Gupta bodies. *Actinomyces* stains blue. Sometimes the filaments possess radially arranged clubs which are host proteins. Filaments must be observed before an interpretation of *Actinomyces* is made.

It is uncertain how the organism arrives in the cervix, but self-contamination from the anus to the vagina, from orogenital or anal sex or spread from another area of infection are possibilities.

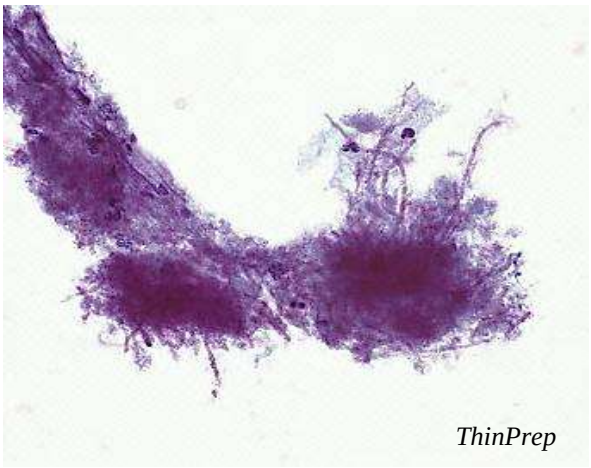
Treatment in *symptomatic* women is essential to prevent potential complications including pelvic inflammatory disease and sterility. The device is removed, the woman treated and a new device may then be inserted. *Asymptomatic* women with infection are not normally treated.

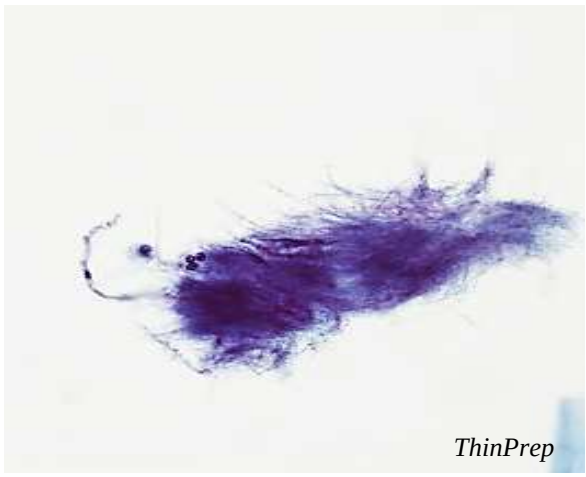


Actinomyces: Well-preserved fine filamentous structures. Small granules can be seen along some filaments (inset).



Dark central woolly ball with radiating fine and intermediate filaments.



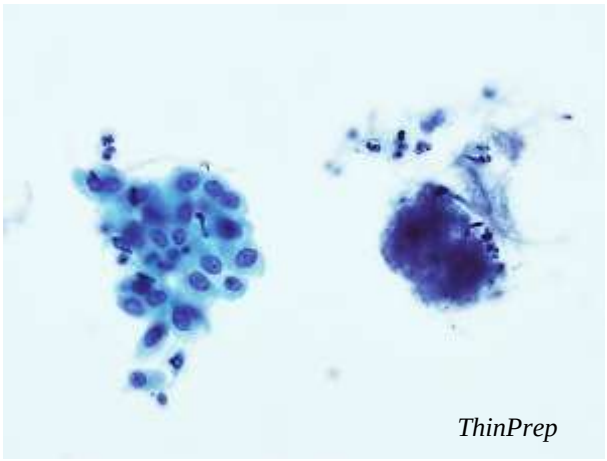


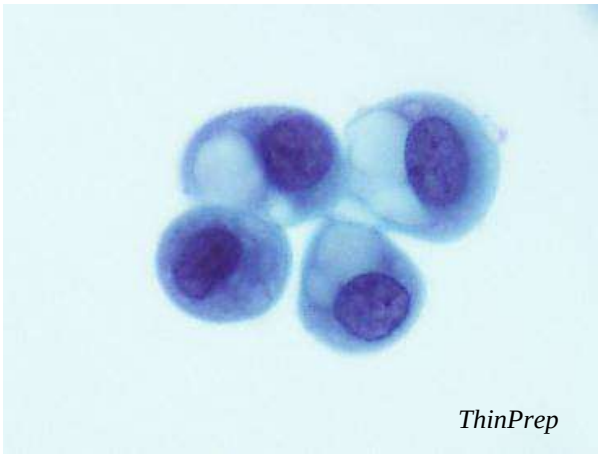
Thick and thin filaments in actinomyces

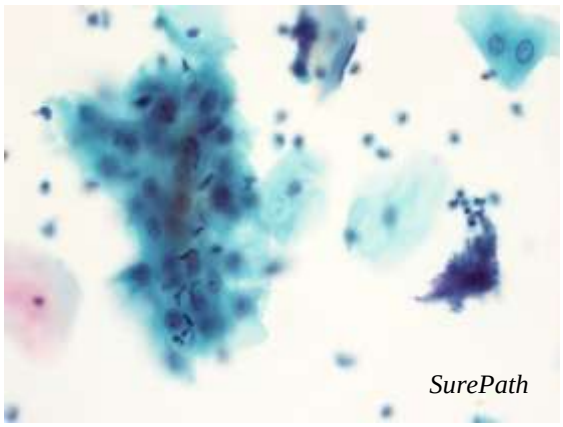
The cytology sample will often show varying degrees of epithelial reaction or atypia, caused by the presence of the IUCD and the irritant effect of the device. Cellular features commonly seen in cytology samples in IUCD wearers include:

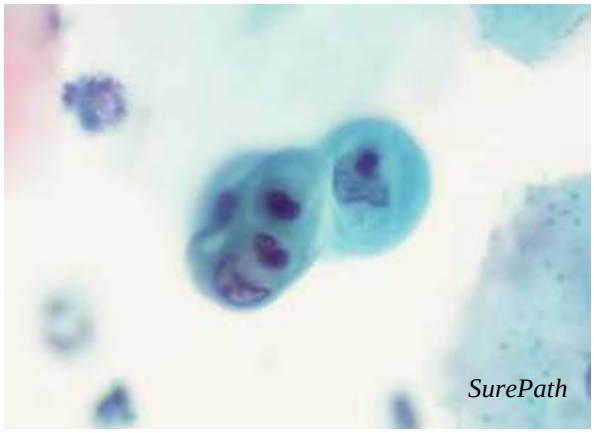
- Endometrial cells throughout the menstrual cycle
- Reactive changes in endocervical cells
- Vacuolated squamous metaplastic cells of varying degrees of maturity
- Mild to marked reactive, inflammatory, and reparative changes

If associated epithelial changes are marked or atypical, a repeat sample may be recommended.









Left images: Non-specific reactive change associated with *actinomyces*.
Right images: Cytoplasmic vacuolation with reactive nuclear change consistent with IUCD effect.

2.4.2 Fungi

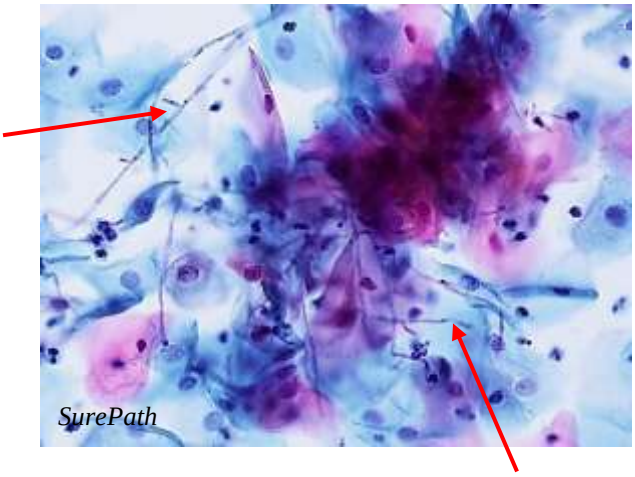
Candida albicans

Candida is so common in the vagina that it is regarded as normal flora. It is usually reported when identified in cervical cytology samples but this does not imply that the woman has an infection or needs treatment. This is a decision that the smear taker makes when correlating the cytology report with the clinical situation. Reporting *Candida* in the absence of inflammation varies between different laboratories, as some regard it as normal flora when the woman is asymptomatic. The presence of *Candida* is associated with a lower vaginal pH (increased acidity), diabetes, pregnancy, use of antibiotics and immunosuppression.

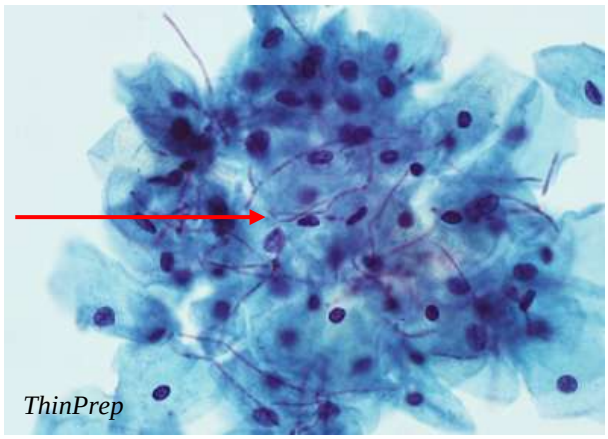
When *Candida* proliferates, it can cause a clinical infection. Candidiasis is a clinical term describing an inflammatory reaction to Candida accompanied by a thick, cheesy vaginal discharge and intense itching with redness of the mucous membrane. The most common causative agent is *Candida albicans.*

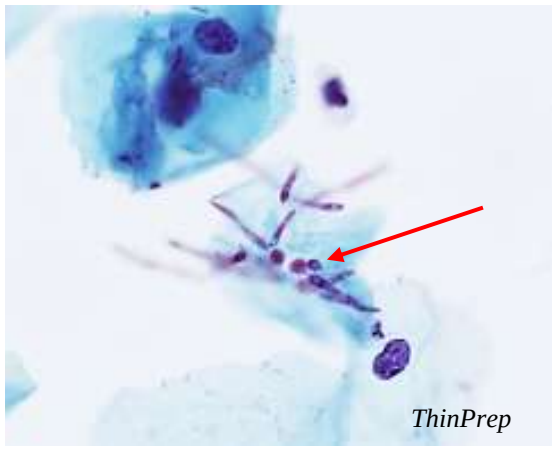
Cellular changes are characterised by clumped squamous cells, vacuolated cytoplasm, perinuclear halos, and marked squamous eosinophilia. The infection can be associated with a normal epithelial cells and a clean or cytolytic cell background, through to marked inflammatory and reactive epithelial cell changes.

Candida is not actually a fungus, but is a yeast that reproduces by budding. The budding spores are small, oval, encapsulated bodies approximately 1.5-8µm which elongate to form pseudohyphae. (In true hyphae or mycelia, protoplasm is continuous between main stem and branches). The hyphae can be short or long, are septate and generally stain pink. Identifying the septations is the way to distinguish *Candida* from other strands of eosinophilic debris in cervical cytology samples.



Typical presentation of *candida* with pseudohyphae associated with a stack of epithelial cells producing a "shish kebab" effect. Branching and septate hyphae are seen at the red arrows





Left: *Candida* showing septations (arrowed) in pseudohyphae
Right: Spores (arrowed) and short budding pseudohyphae

Torulopsis glabrata

Torulopsis glabrata (Cryptococcaceae family) is similar to *Candida* but is an encapsulated 2-8µm diameter yeast which looks like a circle with a central dot. The spores can be in small groups or single, and may surround epithelial cells. It does not form pseudohyphae so should be considered if spores only are present. *Torulopsis* has little clinical effect but may cause slight pruritus or burning.

2.4.3 Parasites

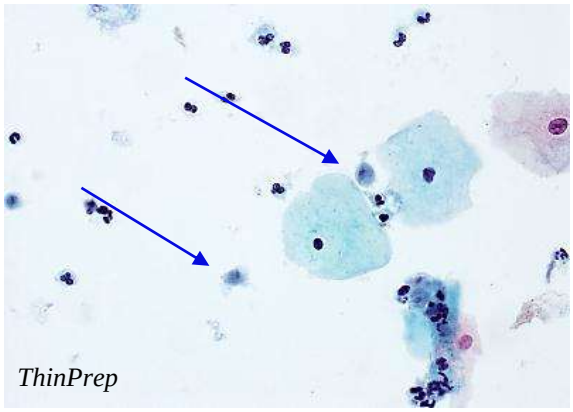
Trichomonas vaginalis

Trichomoniasis is a relatively common, sexually transmitted infection that causes a foamy, yellow-green discharge, irritation, soreness and odour. It is often the cause of a clinical appearance of the cervix referred to as a "strawberry cervix" (haemorrhagic granulation).

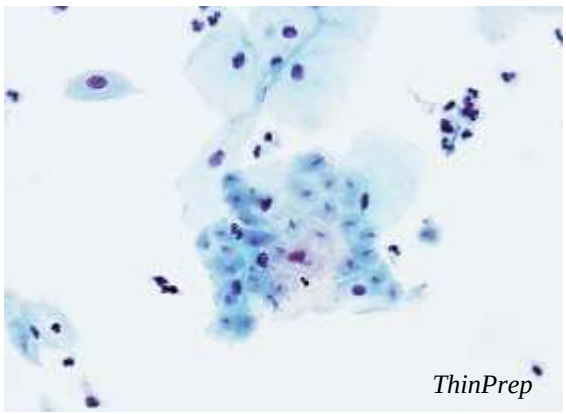
Trichomonas vaginalis (TV) is an oval or pear-shaped flagellated protozoan, 10-30µm in size. In cervical cytology samples, *trichomonas* has greenish-grey cytoplasm with a small, hazy, smudged oval nucleus and fine eosinophilic granules scattered through the cytoplasm. If well preserved, flagella may be identified in LBC samples.

Epithelial cell changes characteristic of *trichomonas* infection may be marked and include:

- Cytoplasm - intense eosinophilia, polychromasia and peri-nuclear halos
- Nucleus - complete range of inflammatory changes that may include nuclear atypia and multinucleation
- Smear background – slightly granular with debris and bacteria
- Leucocytes - mixed with snowballing of neutrophils commonly seen

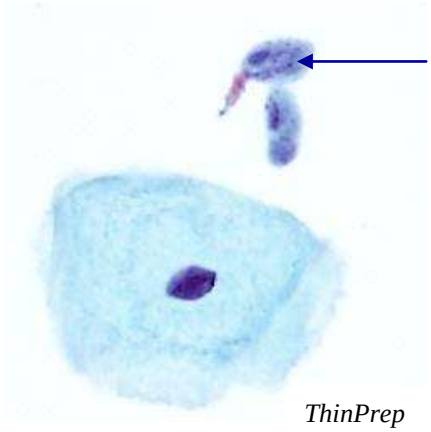


ThinPrep



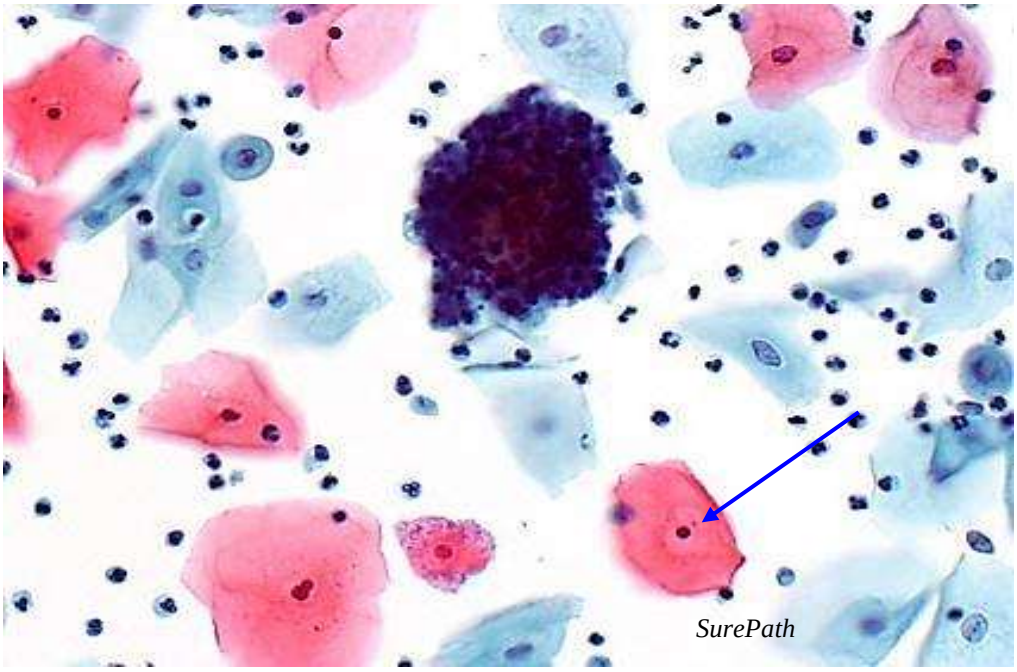
ThinPrep

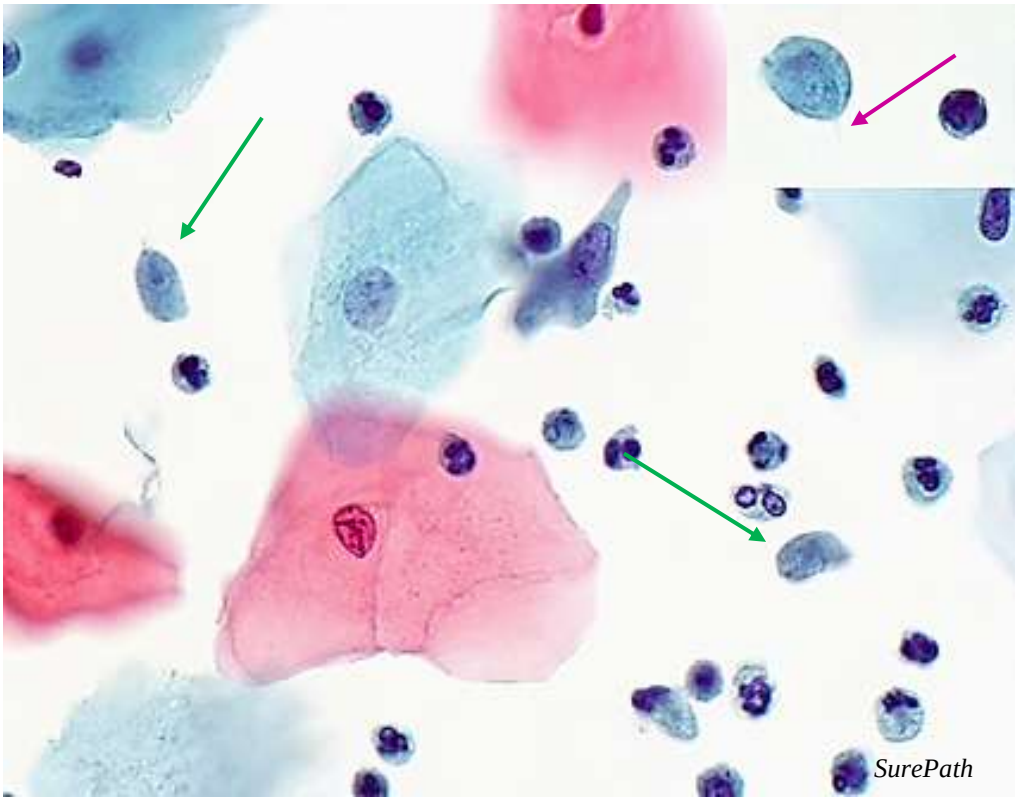
Low power: Organisms (arrowed) are indistinct, round to oval with eccentric nuclei. Large masses (right) are seen less frequently than dispersed single organisms.



ThinPrep

High power: the elongated nucleus of *Trichomonas* and the fine eosinophilic cytoplasmic granules can be seen. As many organisms are degenerate when sampled, the granules are not always visible.

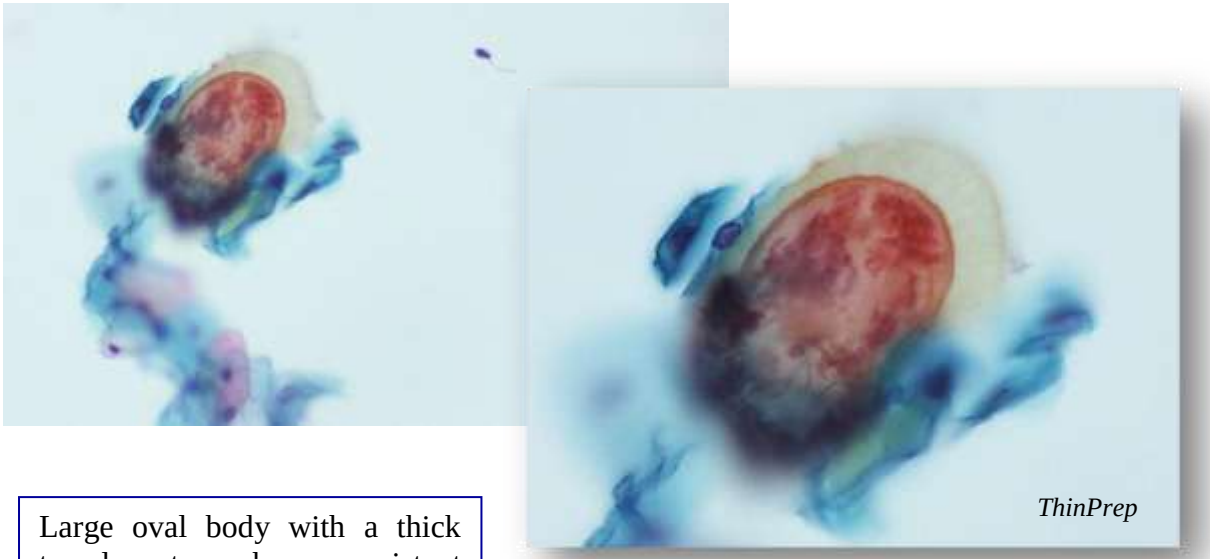




Trichomonas vaginalis. The low power view shows snowballing of neutrophils, perinuclear halos (blue arrow) and neutrophils in the background. At high power, the trichomonads are well preserved (green arrows) with elongated nuclei and granular cytoplasm. Flagella can sometimes be seen in LBC samples (pink arrow).

Enterobius vermicularis – pinworm

This is the most common worm found in cervical cytology samples. The eggs (ova) are the most frequent finding. They are oval, 25 -60 μm, and stain bright lavender to orange with a birefringent, translucent thick membrane, one edge of which is folded. These contaminate the vagina from the anus.



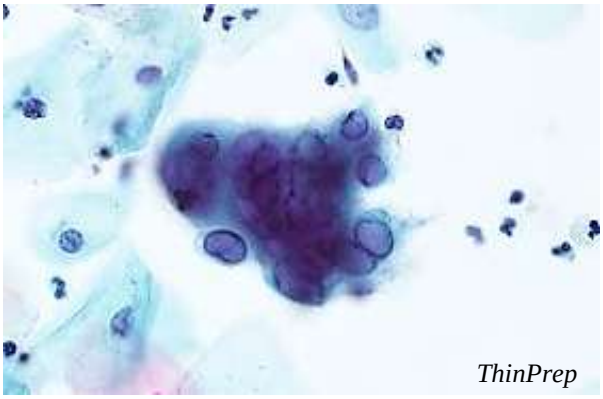
Large oval body with a thick translucent membrane consistent with *Enterobius vermicularis* (pinworm).

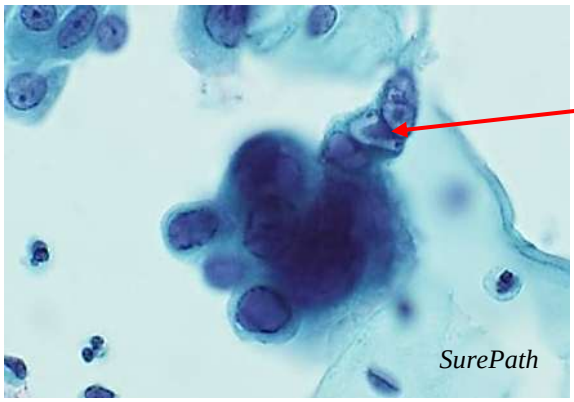
2.4.4 Viral infections (excluding HPV)

Herpes simplex Virus Type II

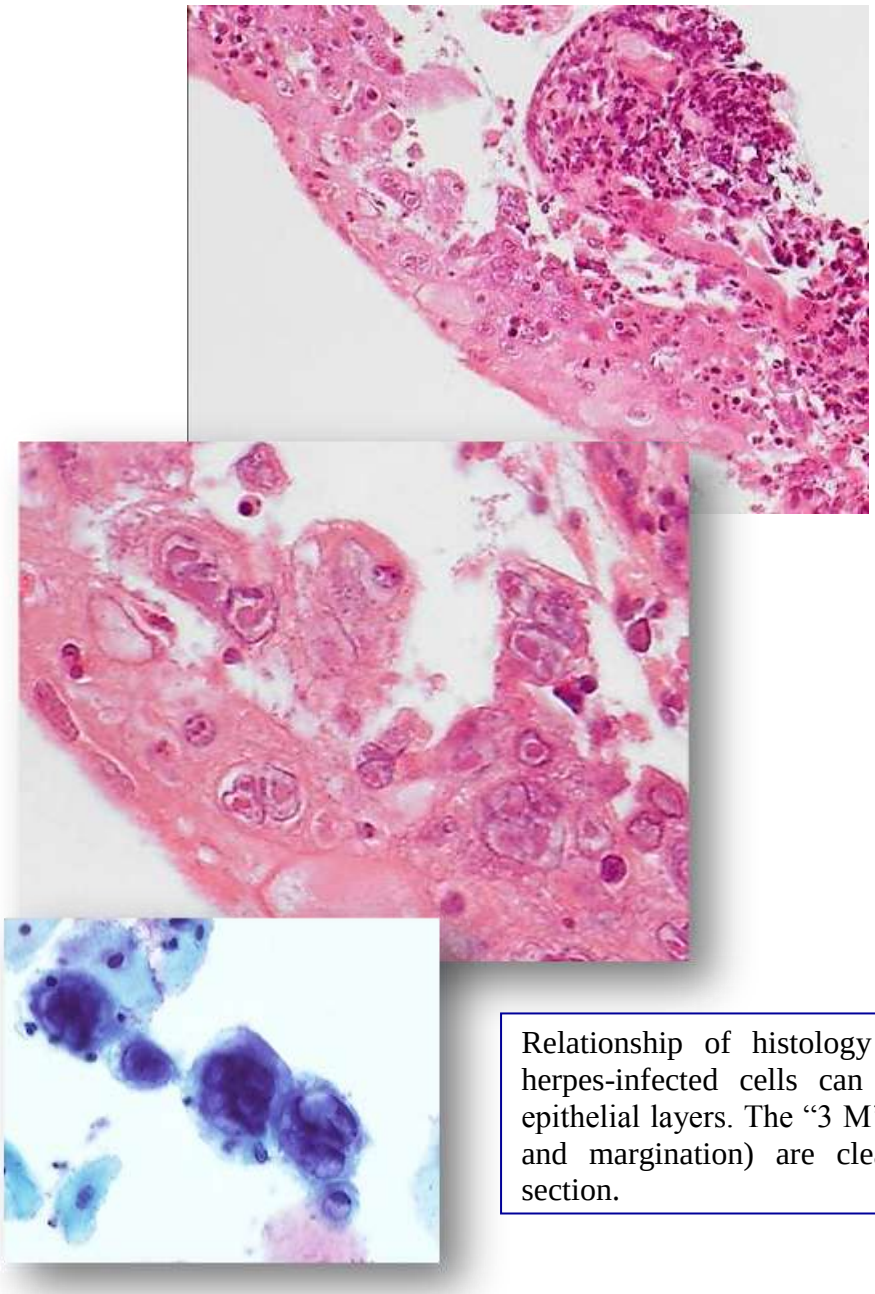
Herpes simplex virus infection (HSV) of the lower female genital tract is sexually transmitted and usually caused by *Herpes genitalis*, referred to as *HSV type II*. *Herpes simplex* symptoms can be severe with fever, lymph node gland enlargement and ulcers or blisters. *HSV* is a DNA virus which can be asymptomatic while still being transmitted sexually to infect or re-infect another person.

Characteristic features are seen in both squamous and endocervical columnar cells. The infected cell may be mono- or multinuclear, but usually contains several nuclei that are tightly packed and mould against each other. The nuclei vary in size and shape and may contain large, single, eosinophilic viral inclusions. The chromatin is altered and may show increased granularity followed by fading. Characteristically the chromatin has a "ground-glass" appearance caused by margination of chromatin (retroplastic effect caused by the virus) to form a distinct thickened nuclear membrane with hypochromasia centrally. Cell size and nuclear size increase, with variable N:C ratios. Large red intranuclear viral inclusion bodies may be present. The surrounding cytoplasm is dense and may be eosinophilic (keratinised) or cyanophilic (hyalinised). *Herpes* cells must be differentiated from foreign-body giant cells, multinucleate malignant cells, Langhans' giant cells and syncytiotrophoblasts (rare).





Cytopathic effect of *Herpes simplex* virus showing multinucleation, nuclear moulding and chromatin margination. The chromatin in the centre of the nucleus is pale and very finely granular ("ground-glass"). A large intranuclear inclusion is arrowed.

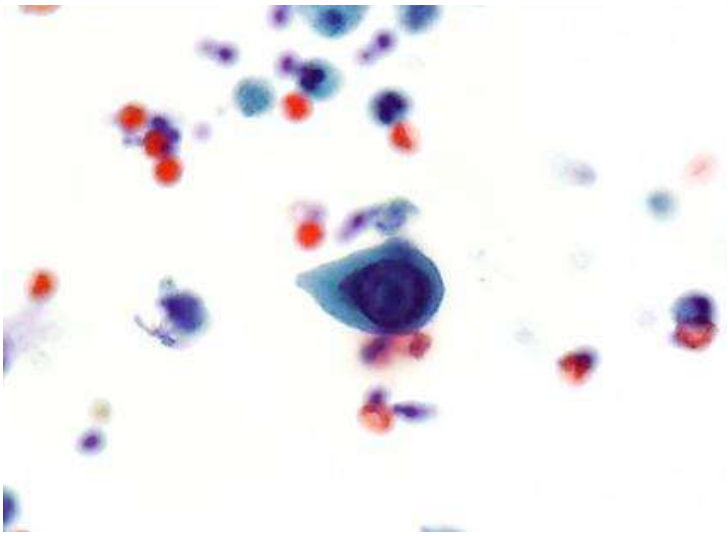


Relationship of histology and cytology showing how herpes-infected cells can be sampled from the upper epithelial layers. The "3 M's" (moulding, multinucleation, and margination) are clearly visible in the histology section.

Cytomegalovirus (CMV)

CMV is a member of the *Herpes* family of viruses. Its presence is characterised by the presence of a large basophilic intra-nuclear inclusion surrounded by a clear halo, hence the term "owls eye" appearance. A punctate deposit of chromatin marks the outline of the affected nucleus. Often there are fine basophilic intra-cytoplasmic inclusions in the cytoplasm.

Endocervical cells are most often affected, and the changes may be indistinguishable from *Herpes* infection. One distinction is that *CMV* presents as a mononuclear cell, without the multinucleation usually seen with *Herpes simplex* infection.



Cell infected by *Cytomegalovirus*. The large single nucleus shows clearing of the chromatin with a developing central inclusion. It can be difficult to distinguish cytologically between *CMV* and *HSV* with mononuclear cells. Virological confirmation is recommended.

2.4.5 Questions

1. Describe the clinical presentation of bacterial vaginosis.

2. Describe the changes that may be seen in cervical cytology samples from IUCD wearers.

3. Describe the cervical cytology cell changes seen with *Candida* infection.

4. Describe the cervical cytology features associated with *Trichomonas*. What are some of the other entities that this organism may be misinterpreted as?

5. Describe the changes seen with *Herpes* viral infection. What differential diagnoses of these changes need to be considered?

2.5 CORE TOPIC: OTHER BENIGN CONDITIONS

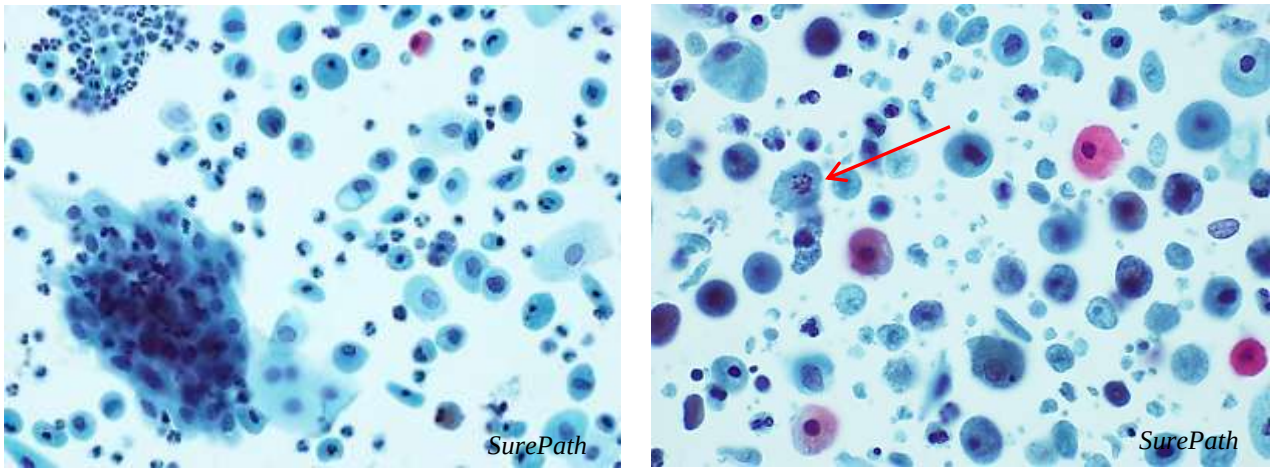
This section outlines a miscellaneous group of benign conditions which may be seen in cervical cytology. They are important to recognise so that they are not confused with dysplastic epithelial lesions. Most are inflammatory in nature but are not related to an infectious agent.

2.5.1 Atrophic (senile) vaginitis/cervicitis

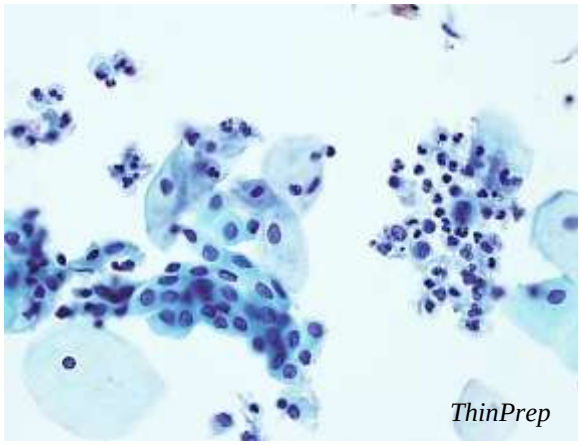
Atrophy of epithelial cells occurs when oestrogen levels are low. This occurs in the postmenopausal period, in the postpartum period where the length of time that atrophy persists is highly variable (usually several months) and in women taking progestational agents such as Depo Provera. Atrophy per se is not associated with inflammation and reactive change in epithelial cells.

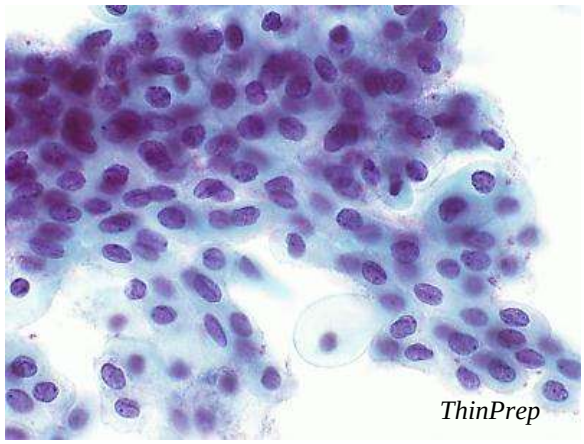
Atrophic vaginitis/cervicitis describes the situation where atrophy is associated with inflammation. Clinically there may be a brown blood-stained vaginal discharge. In situations where low oestrogen levels persist (such as in the postmenopausal period), a thin fragile atrophic squamous epithelium often results. There is reduced mucous secretion from endocervical cells and the resulting lack of lubrication leads to chronic irritation of the cervix and vagina, causing chronic inflammation and reactive changes in epithelial cells. In low oestrogen level settings, the cell sample may show increased glycogen, cell degeneration and stripped bare nuclei. A granular background of degenerate cell debris may be seen although much of this is removed by LBC processing.

The cytology sample is characterised by sheets of atrophic squamous cells including many small round parabasal cells showing a variety of staining reactions including cyanophilia, eosinophilia and biphasic/polychromatic staining. Small keratinised cells may also be seen. The cells show all stages of cell degeneration, a pronounced feature of atrophic vaginitis/cervicitis which ranges from karyopyknosis through to anucleate “ghost” cells. Squamous metaplasia may also be seen. The cytoscientist/cytotechnician must always be aware of the potential for neoplasia to co-exist with atrophy. Small, well-differentiated keratinised abnormal squamous cells may easily be overlooked as atrophy only.



Characteristic pattern seen with atrophic vaginitis: atrophic squamous cells with leucocytes, debris, and bacteria. Some squamous cells show signs of degeneration (arrow).





Sheets of parabasal cells are common in atrophic samples. The sheets are flat and although the cells have moderately high N:C ratios, the nuclei within the sheet closely resemble each other.

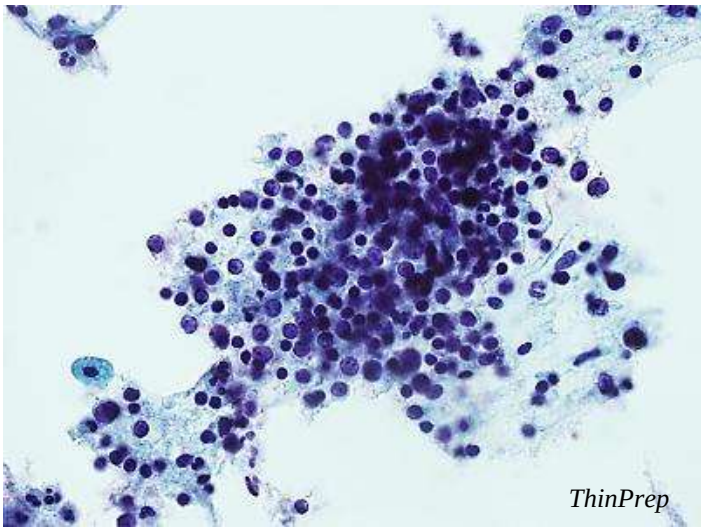
The background characteristically contains bacteria, neutrophils and macrophages (occasionally multinucleated forms), thin strands of mucous and spherical mucous structures termed blue blobs. Free bare epithelial nuclei may also be seen either from atrophic squamous cells and/or reserve cells. These cells are round to oval with bland indistinct chromatin. Care must be taken not too overlook abnormal endometrial cells in this age group.

2.5.2 Follicular (lymphocytic) cervicitis

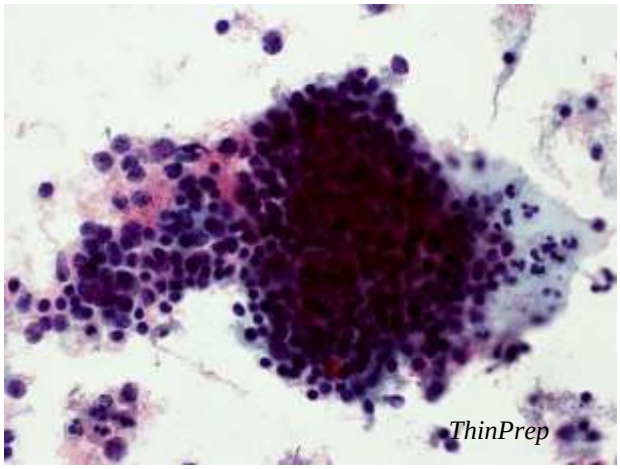
Sub-epithelial lymphoid follicles can form within cervical stroma i.e. beneath the basement membrane of the cervical epithelium. If a follicle is sitting close to the surface epithelium it may rupture when the sample is taken, expelling the contents of the follicle onto the surface where the cells can be collected by the sampling device. Follicular cervicitis has been associated with chlamydial infection in the past, but occurs in a wide range of other settings as well and is too non-specific to indicate the presence of chlamydia.

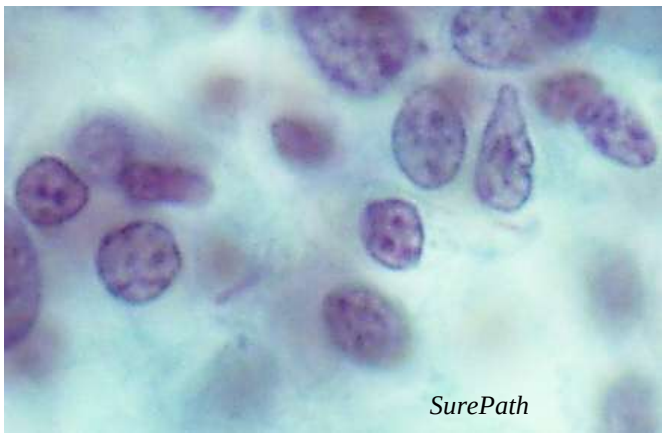
Follicular cervicitis is seen cytologically as loose clusters of lymphoid cells consisting of a range of lymphoid germinal centre cells and small mature lymphocytes. Large immunoblastic cells admixed with plasma cells, neutrophils and macrophages are commonly seen. Tingible body macrophages may also be present. These are macrophages containing phagocytosed nuclear debris from the active follicle. The appearances are very similar to those seen in an FNA of a reactive lymph node. If the cells are closely packed together, they may be mistaken on low power examination for endometrial cells. However, high power will show that the cells are single and non-cohesive. Care must be taken to thoroughly examine the cell aggregates. A monomorphic population of lymphoid cells would raise the suspicion of lymphoma. This is rare in the cervix and when present, usually occurs in the context of widespread systemic lymphoma.

Lymphoid cervicitis is not reported as a specific entity under The Bethesda System 2001. Recognising lymphoid cervicitis is important so that the appearances are not misinterpreted as endometrial cells or a high-grade squamous intraepithelial lesion.

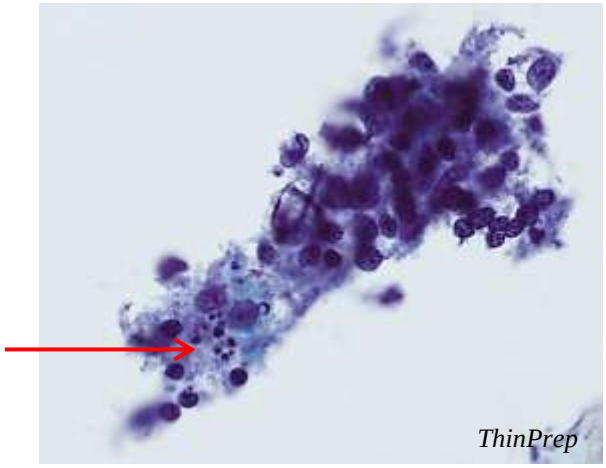


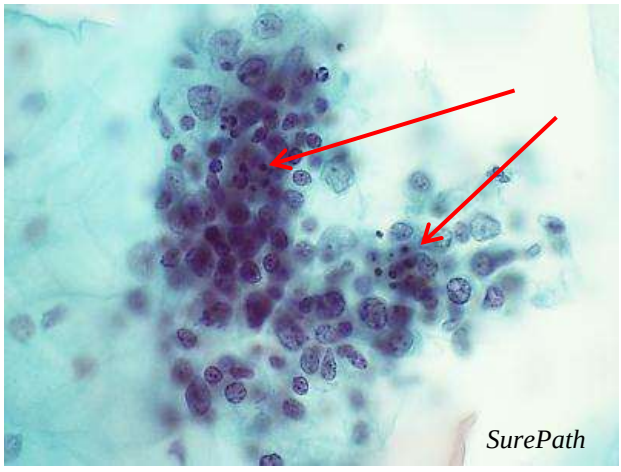
Cluster of cells showing a mixed lymphoid population. Occasional neutrophils and plasma cells may be seen on higher magnification.



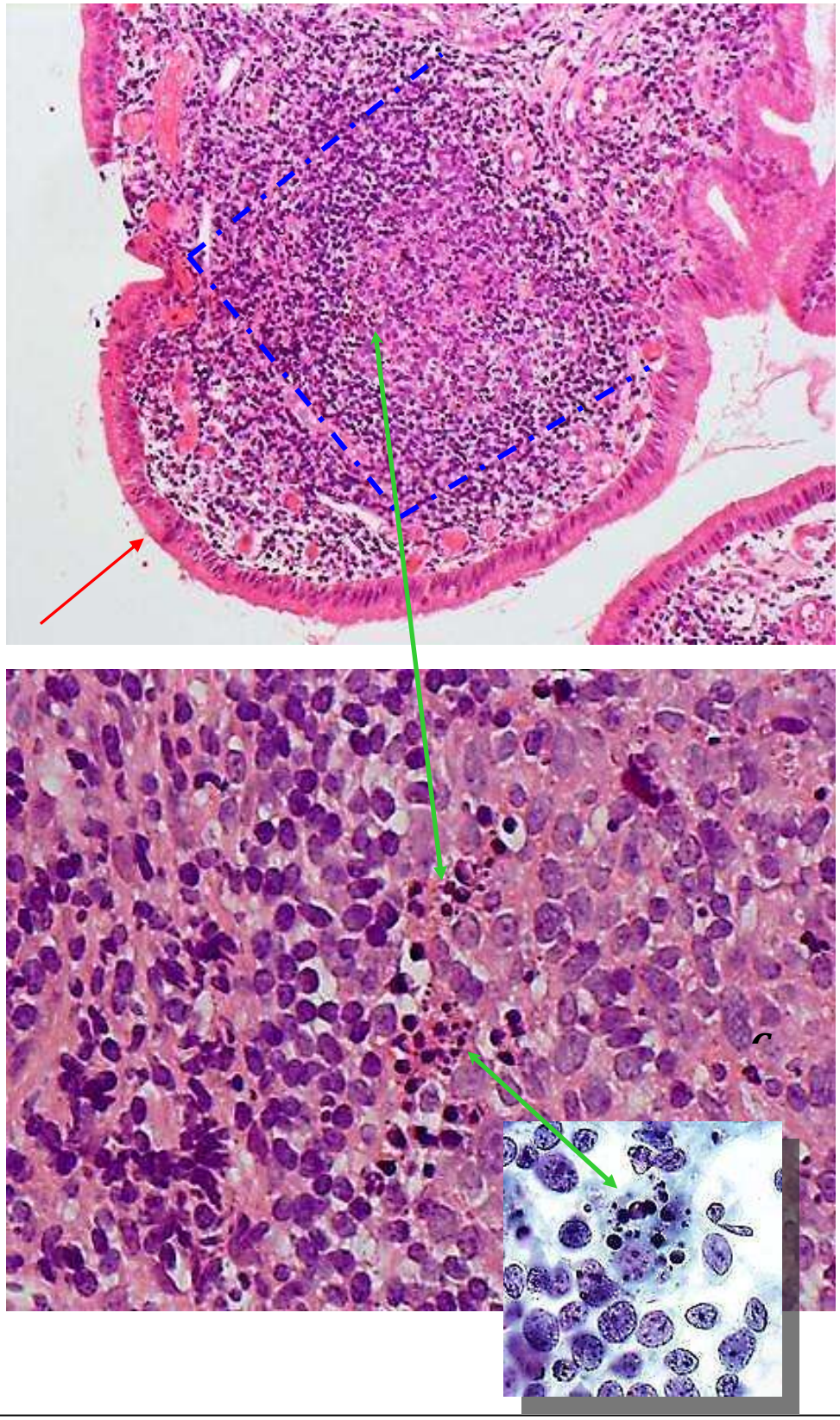


Dense aggregates of lymphoid cells could be mistaken for endometrial cells. At high magnification, the chromatin detail can be seen.





Tingible body macrophages are seen (arrow) containing phagocytosed debris.

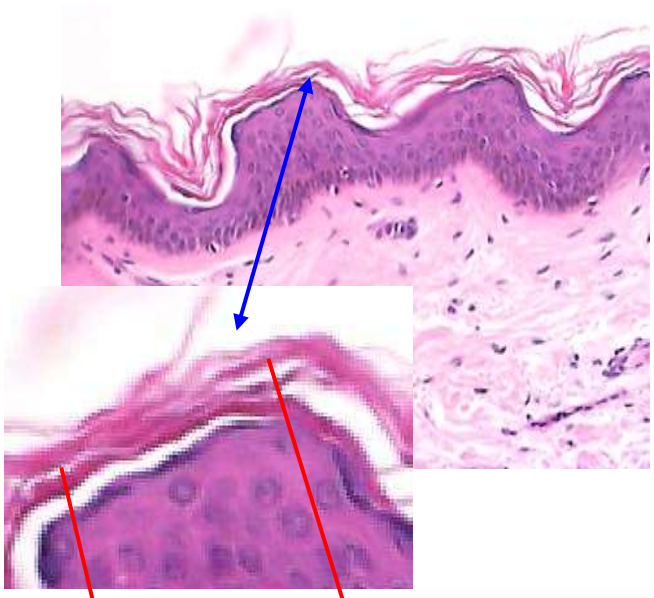


Top: Lymphoid follicle (within blue dashed line) in cervical stroma beneath the surface endocervical epithelium (red arrow).
Bottom: High magnification of the follicle centre showing pale lymphoid cells within the germinal centre (G), and two tingible body macrophages (green arrows), giving rise to the cytological picture seen in the inset.

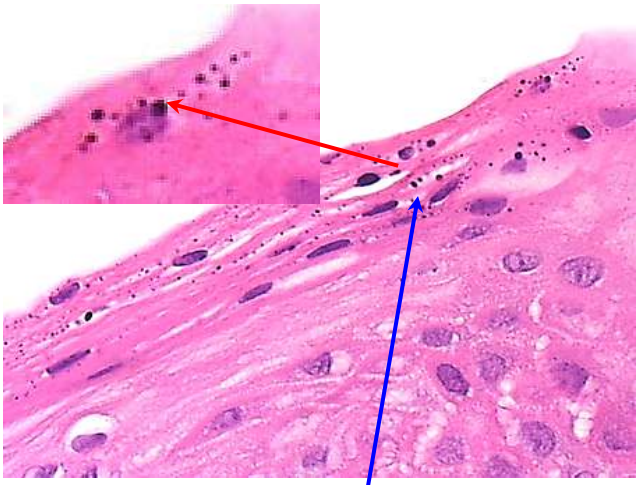
2.5.3 Hyperkeratosis and parakeratosis

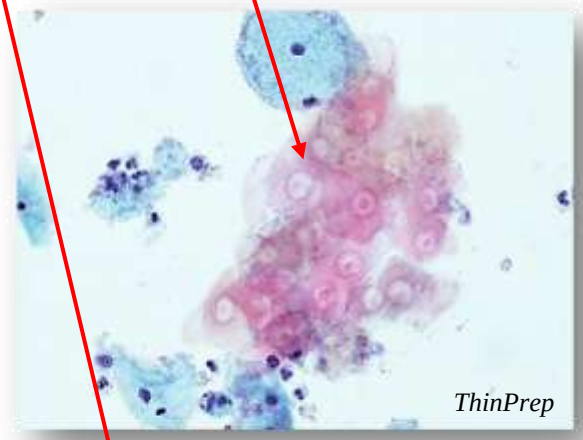
Hyperkeratosis

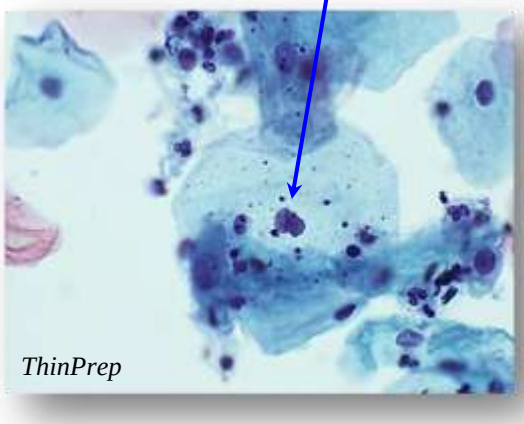
Hyperkeratosis is present when keratinising squamous epithelium is covered with a layer of anucleated squamous cells and keratin. Cytologically, keratinised anucleated squamous debris is seen. Ghost outlines of degenerate squamous cell nuclei may be visible in the keratinised debris. Superficial squamous cells with cytoplasmic keratohyaline granules may also be present. Clinically the appearance is called leukoplakia because it looks like a white plaque on the cervix. Leukoplakia is a problem at colposcopy as the colposcopist cannot see down through the white plaque to examine the underlying epithelium.



Left: Squamous epithelium with hyperkeratosis (histology) results in clumps of keratinous debris (blue arrow) and degenerate squamous cells with ghost nuclei (red arrow) in cytology samples.
Right: Mature squamous cells with an abundance of keratohyaline granules seen in histology and cytology.







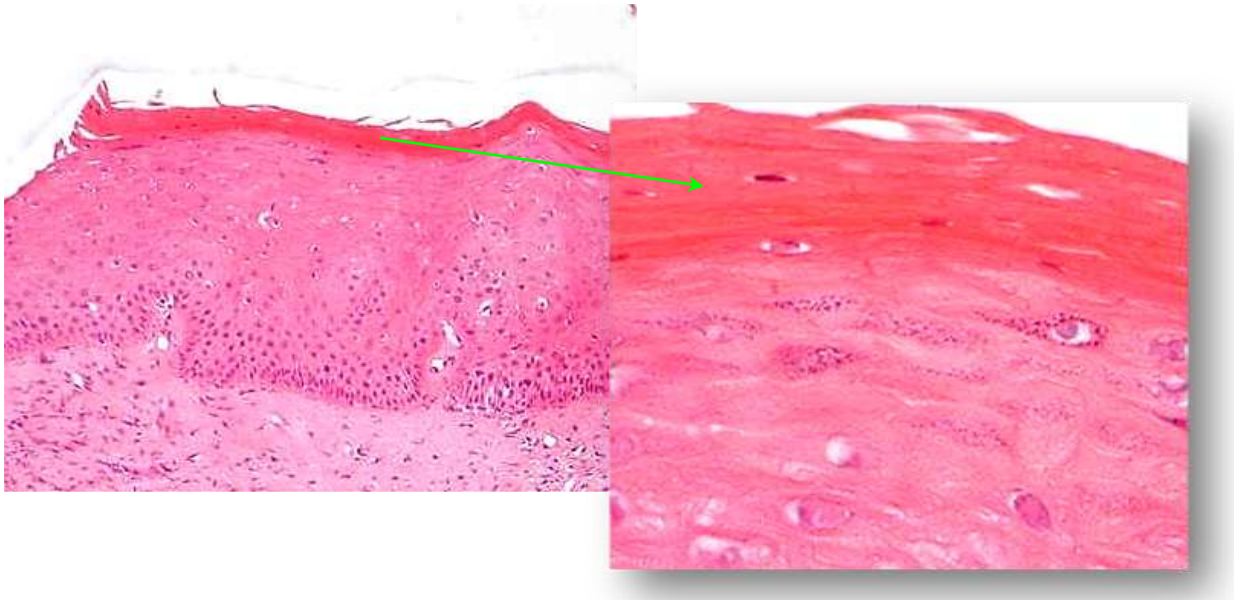
Hyperkeratosis is not reported as a specific entity under The Bethesda System 2001. Recognising hyperkeratosis should alert the cytoscientist/cytotechnician to take extra care in examining the cervical cytology sample, as hyperkeratotic material can overlie squamous epithelial lesions and the colposcopist may not be able to conduct a thorough colposcopic examination.

Parakeratosis

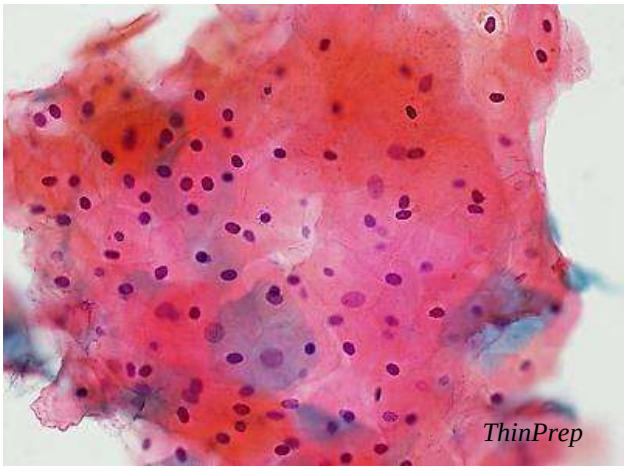
In this condition the features are similar to those of hyperkeratosis except that the keratinised cells are nucleated. The cells are usually fairly small and polygonal with bright red/orange cytoplasm (keratin) and round hyperchromatic or pyknotic nuclei. Parakeratotic cells are often seen in inflammatory atrophic conditions e.g. atrophic cervicitis/vaginitis. In some cases, parakeratosis may overlie squamous neoplasia and as the parakeratotic layer can reduce the chance of the neoplastic cells being dislodged during sampling, the cytoscientist/cytotechnician should take particular care to examine the nuclei in parakeratotic material. Parakeratotic cells can occur at any point along the full squamous spectrum from normal to invasive squamous cell carcinoma. It is the nuclear features of the parakeratotic cells that will distinguish these entities cytologically.

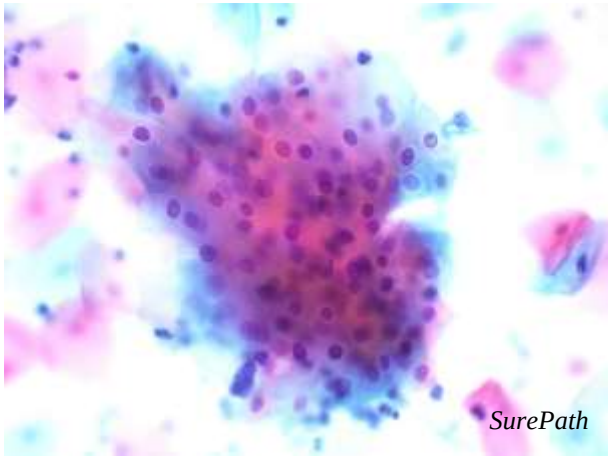
The term “atypical parakeratosis” (previously dyskeratosis) refers to abnormal maturation where there is dys-synchronous maturation of the nucleus and the cytoplasm i.e. the cytoplasm is mature and well-keratinised while the nucleus is less mature than it should be for the degree of cytoplasmic maturation. Atypical parakeratosis is one manifestation of HPV infection of squamous cells and will be discussed in the next section of the workbook.

Normal parakeratosis is not reported as a specific entity under The Bethesda System 2001. Recognising parakeratosis is important so that particular attention can be given to examining the nuclear features of the parakeratotic cells so that the cells can be appropriately classified and reported without abnormal squamous cells being overlooked.



Histology of parakeratosis showing thick surface layer of nucleated keratinised cells. Sampling this surface gives a cytology specimen containing parakeratotic squamous cells.





The parakeratotic cells are small polygonal keratinised cells with pyknotic nuclei. They may be seen in sheets, small clusters or as single cells. Note that the smaller nuclei are also the darker nuclei because the chromatin condenses as the nucleus shrinks.

2.5.4 Radiation and chemotherapy effects

Radiation and chemotherapy can cause short and long term effects on cells. Radiation can result in bizarre cells which can mimic carcinoma, dysplasia, and/or HPV infection. Clinical information is essential and any suspicion of radiation change should be confirmed by contacting the sample taker if insufficient clinical information is provided on the request form. Radiation effect is well documented in the literature. The effects of chemotherapy are less well defined but share some of the features seen following radiation treatment.

There are two stages of radiation effect, short term acute and long term chronic. The effects can persist for many years, even for the remainder of a woman's life time. When the radiation change does subside, atrophy often then dominates the cytology sample.

Cervical cytology samples should not be taken within 8 weeks following radiation treatment as viable tumour cells may still occur along with degenerating tumour cells in this period. At approximately 8 weeks post treatment, the sample should rapidly become clear of tumour cells. There may still be a background of diathesis including blood, fibrin, mixed leucocytes, and necrosis along with features of regeneration and repair but diathesis is often minimal and subtle in LBC preparations. Severe ulceration can occur resulting in the presence of granulation tissue which may include many macrophages (including multinucleated giant forms), epithelioid histiocytes, capillaries, and active fibroblasts, which are elongated with pleomorphic nuclei. Once the condition becomes chronic, the features of repair and granulation will generally disappear, although the sample background may still be proteinaceous.

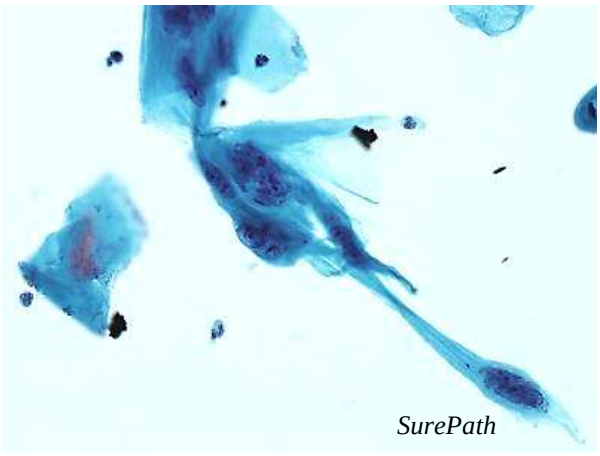
The epithelial changes are similar in both the acute and chronic stages. These present a striking picture of large to giant squamous cells (macrocytes). The cells have abundant cytoplasm with a corresponding nuclear enlargement so the nuclear:cytoplasmic ratio remains low (normal ratio for the cell type). The cells often have indistinct washed-out features. The cytoplasm may be polychromatic and vacuolated, and may contain ingested cells and debris. Bizarre forms and multinucleation are common. The chromatin may appear granular or smudged. If the ovaries have been removed or are inactive the features will be seen within the context of an atrophic cell

pattern. Hormone replacement therapy may be given to some patients who have had radiation for treatment of cervical carcinoma. A full range of degenerative features (retroplasia) will be seen. Similar effects are seen in glandular cells.

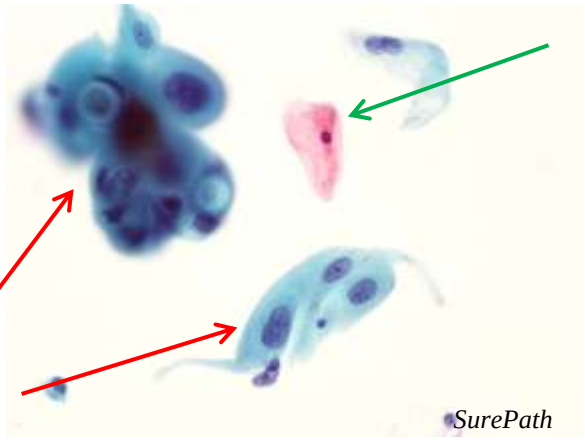
Cytology is not sensitive for detecting tumour recurrence but is specific. The most important feature when considering **recurrence of cervical carcinoma** is

- The presence of abnormal cells with a **high** nuclear:cytoplasmic ratio.

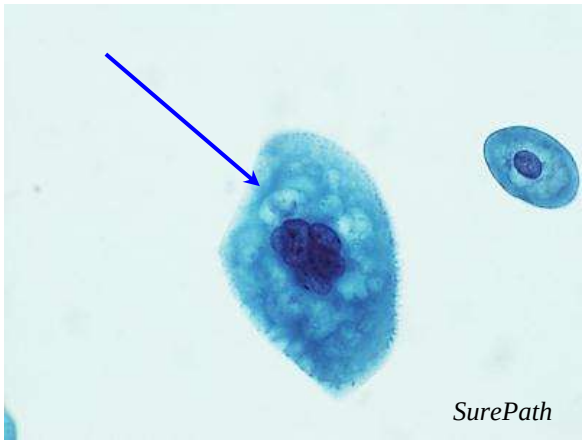
In contrast the cytologic features of mild radiation dysplasia are the presence of abnormal samples with a low nuclear:cytoplasmic ratio, with similar appearances to conventional dysplasia. Keratinisation is common.

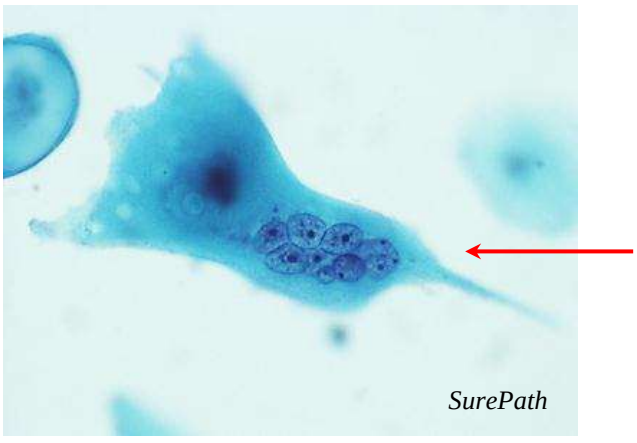


Radiation change - sheet of reactive epithelial cells showing bizarre nuclear and cell morphology

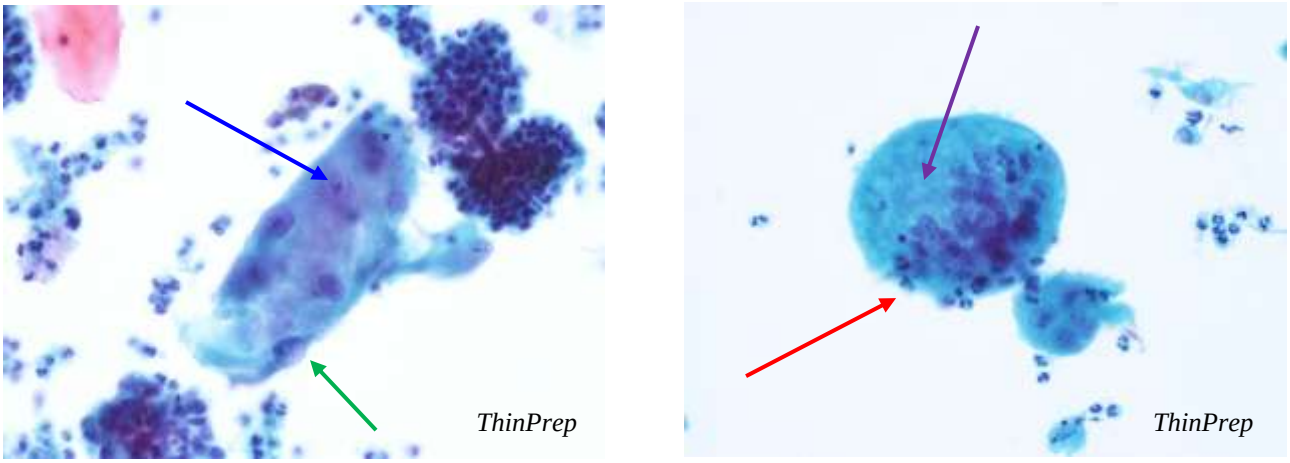


Radiation-affected cells (red arrows) show large atypical nuclei but the cytoplasm is also increased, preserving normal N:C ratios. Compare with the normal superficial squamous cell (green arrow).





Cell morphology can be bizarre as a result of radiation effect and include cytoplasmic vacuoles containing debris and ingested cellular material (blue arrow). Multinucleation is also common (red arrow).



Typical pale washed-out appearance seen with radiation effect. Note the bland chromatin (green arrow), prominent nucleoli (blue arrow), and the normal N:C ratios. The cell on the right shows multinucleation (red arrow) and cytoplasmic vaculoation (purple arrow).

Assessing cervical cytology samples which have abnormal cells after radiation treatment is one of the most challenging areas of cervical cytology. Some clinicians prefer not to take any cervical cytology samples after radiation treatment for cervical carcinoma and to follow patients colposcopically instead. Any grade of radiation dysplasia occurring within the first three years post treatment has a higher risk of tumour recurrence. Mild radiation dysplasia is a relatively common finding (up to 25% of cases) with both acute and chronic radiation change. Any suspicion of high grade dysplasia or recurrence would be referred back for further clinical assessment and colposcopy.

Cervical cytology is also used to detect recurrent endometrial carcinoma in vaginal vault samples. Depending on the stage of the disease when treated for endometrial cancer, some women will have had a hysterectomy only whereas others will have had radiation treatment as well. Recurrent endometrial adenocarcinoma may be cytologically bland so any glandular-appearing cells in samples from these women need careful evaluation.

2.5.5 Endocervical polyps

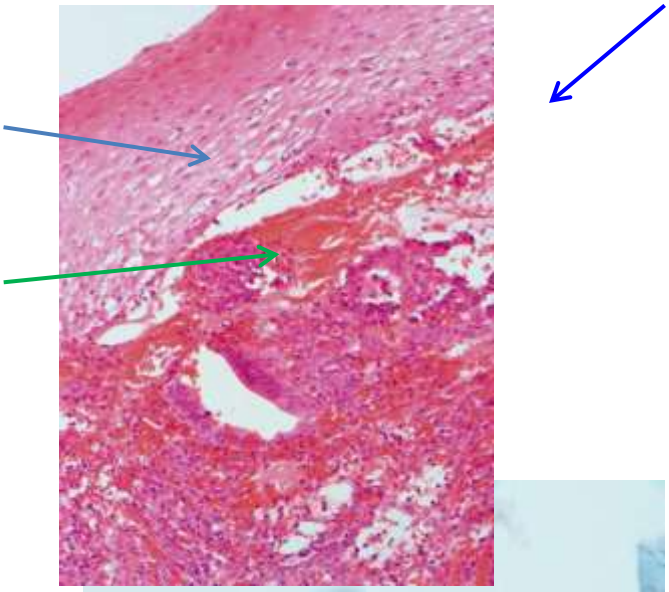
Structurally endocervical polyps are composed of a central connective tissue stalk covered by gland-forming endocervical mucosa. They originate in any area of the endocervical canal. They cannot be accurately diagnosed cytologically, but low cuboidal to columnar epithelial cells may be seen along with plasma cells and lymphocytes. Squamous metaplastic cells can replace portions of the covering epithelium of the polyp and may be seen in cytology samples. Small highly keratinising cells with fairly large pyknotic nuclei may also be seen. These are shed from areas of atypical squamous metaplasia, with surface keratinisation due to pressure from the polyp. Reactive squamous epithelial cells are common in the presence of a polyp. The surface epithelium can have the full spectrum of squamous abnormality from ASC-US to invasive squamous cell carcinoma. The cells in the cytology sample must be assessed by the usual criteria for these lesions even in the presence of a known polyp. Conversely, endocervical polyps can be a source of appreciable cytologic atypia that is due only to reactive change caused by the polyp, with no underlying histologic squamous lesion.

2.5.6 Endometriosis

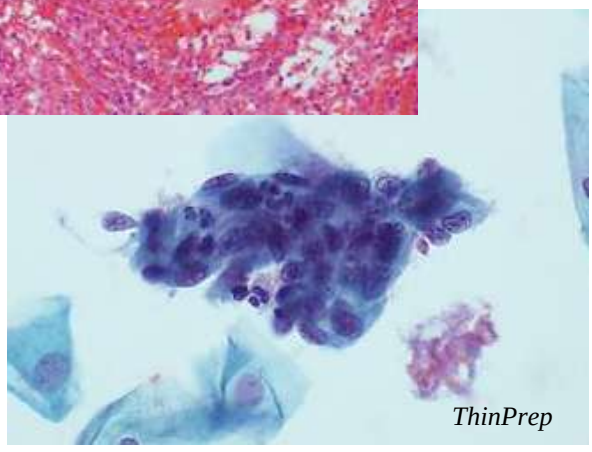
Endometriosis in the cervix can be idiopathic or can occur after surgical treatment of the cervix. After a lletz or cone biopsy, endometrial cells shed during menstruation can seed into the raw area of the cervix before it re-epithelialises, resulting in cervical endometriosis.

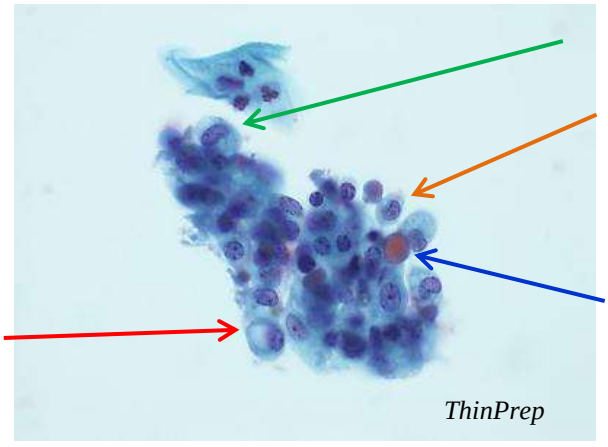
Endometriosis is seen cytologically as endometrial glandular cells and irregular clusters of endometrial stromal cells. Stromal cells have scant cytoplasm, small oval or vesicular nuclei and often show bare nuclei which appear to be falling off the edges of the groups. Small nuclear size, fine regularly distributed chromatin and tiny nucleoli are helpful diagnostically. Useful indicators that can assist in raising the suspicion of endometriosis are a previous lletz or cone biopsy, endometrial cells that are unexpected for the phase of the woman's cycle, and excellent preservation of endometrial glandular cells. When endometrial cells are sampled from an area of cervical endometriosis they are well preserved in the cytology sample, compared with partly degenerate exfoliated endometrial cells originating from the endometrial cavity.

Endometriosis is not reported under The Bethesda System 2001 but laboratories may add a free comment to report it as this can be helpful to the colposcopist when correlating the cytology findings with the colposcopic appearances.



Histology shows surface squamous epithelium (blue arrow) with sub-epithelial endometriosis comprised of both glandular (red arrow) and stromal (green arrow) endometrial cells. There is often associated haemorrhage because the endometriosis is functional and responds to hormonal influences.





Endometriosis: Cluster of endometrial cells (left) and a mix of cells (right) including epithelial cells (vacuolated, red arrow), histiocytes with bean-shaped nuclei (green arrow) and stromal cells (orange arrow). The very small nature of the well-preserved cells along with their uniformity is consistent with benign endometrial type cells. Degenerate blood is also present (blue arrow).

2.5.7 Reserve Cell Hyperplasia

A proliferation of reserve cells is called reserve cell hyperplasia. Foci of reserve cell hyperplasia are common in postmenopausal women. Cytologically, numerous free oval nuclei may be seen which may lie close to groups of endocervical cells. The nuclei resemble those of endocervical cells but are smaller and some have a pointed tip at one end. The chromatin is finely granular and a small nucleolus may be observed. The cells usually lie in dense clumps or side by side in pairs. The tip of one nucleus tends to lie over the edge of the next nucleus giving an overlapping appearance. Nuclear moulding may be observed in dense clumps.

Reserve cell hyperplasia is not reported under The Bethesda System 2001 as it is of no clinical consequence.

2.5.8 Folic acid/Vitamin B12 deficiency

"Folate" is the food form of the B vitamin folic acid, before being ingested. It is found mainly in fresh raw dark green leafy vegetables and some fruits (kiwifruit and citric fruits such as oranges). Our bodies turn folate into folic acid when ingested. It is further transformed into important co-enzymes that enhance and protect metabolic processes, particularly those involved in DNA synthesis and gene expression. All cells require folic acid to divide into new cells and to grow and function to their full potential. Without folic acid, DNA synthesis is suspended and cell division halts. A deficiency can manifest as unique cellular changes in cervical samples due to the hormone sensitive nature of these rapidly proliferating cells and may render the cells more susceptible to viral (HPV) infection.

The cytologic features are of generalised enlargement of intermediate squamous cells (macrocytes) to a diameter of 70μm or larger, along with nuclear enlargement to 14μm or more with a very low nuclear:cytoplasmic ratio. This appearance is referred to as cytomegaly. Cytoplasmic vacuolisation and multinucleation may be seen in a small number of cells. Other features such as phagocytosis and clumping of nuclear chromatin may be seen. Little emphasis is placed on cytological features associated with folic acid/vitamin B12 deficiency because the nuclear changes are very minor and the nuclear:cytoplasmic ratio is extremely low. Folic acid associated changes are occasionally reported as atypical (ASC-US).

Macrocytosis is not reported under The Bethesda System 2001 and is not specific for Folate/Vitamin B12 deficiency. However, a free comment may be helpful to the clinician to promote further investigation for possible folate/vitamin B12 deficiency, particularly if the woman concerned is pregnant.

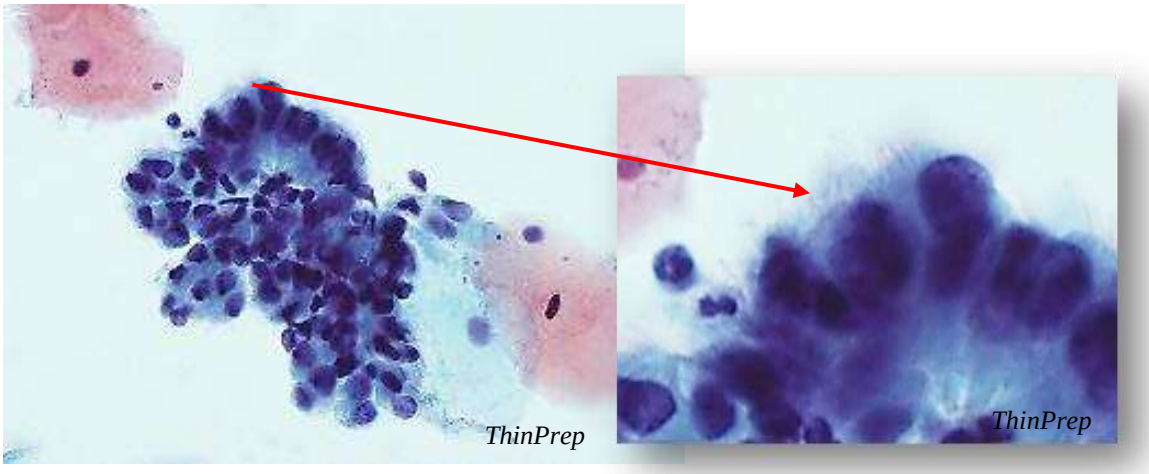
2.5.9 Post-treatment effects

Marked changes in cervical cytology samples after surgery/treatment are not commonly seen because clinicians do not take samples in the first six months after treatment. However minor changes may persist after treatment (such as a lletz or cone biopsy) and it is important not to over interpret these as abnormal epithelial cells. Trauma due to surgery/treatment results in an initial acute phase of degeneration of the affected tissue and responding regeneration. Laser treatment is followed by rapid regeneration with little or no evidence of necrosis in the cytology sample, whereas cautery can result in necrosis for some weeks after the treatment. Active squamous metaplasia and repair may be seen. Tubal metaplasia (see below) is reasonably common.

Cone biopsy and Iletz (large loop excision of the transformation zone) biopsies shorten the length of the endocervical canal. This can lead to the presence of sheets of lower uterine segment (LUS) cells being present in subsequent cervical cytology samples. There may be diagnostic difficulties in distinguishing these from a high grade squamous lesion (HSIL), particularly if this was the original lesion being treated. Careful examination will show regular and uniform nuclei (described in *VRPCC Workbook: Subsection 2.1.8 Functional Differentiation in Squamous Cells Page 62*) compared with the variable nuclei seen in HSIL.

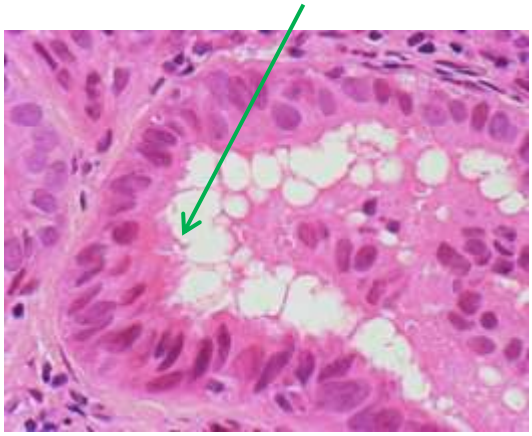
2.5.10 Tubal, tubo-endometrioid and transitional metaplasias

Tubal metaplasia is a common phenomenon in endocervical canal epithelium. The epithelium resembles that found normally in the fallopian tube and includes ciliated columnar cells, peg cells and goblet cells. Cytologically, small clusters of oval to elongated cells with enlarged cigar-shaped hyperchromatic nuclei with nuclear crowding and stratification may be seen in strips or tightly crowded aggregates. These can be misinterpreted as atypical endocervical cells or a high-grade squamous lesion. Discrete cytoplasmic vacuoles, terminal bars and cilia will identify tubal metaplasia but these features will not be present in all cell groups. If cilia and terminal bars are identified in some cell groups, compare the nuclei in these groups with other groups without cilia to see if they all fit within the same spectrum of change. Because tubal metaplasia is common, it will co-exist with other lesions in some samples.



Ciliated cells from the endocervical canal seen in tubal metaplasia.

Tubo-endometrioid metaplasia (TEM) refers to endocervical glands lined by endometrial type epithelium, but without supporting endometrial stroma. It is probably a metaplastic process occurring after injury to the cervix. The cells are often ciliated but differ from tubal metaplasia by showing more pseudostratification and secretory apical snouting (green arrow) in tubo-endometrioid metaplasia and can be misinterpreted as glandular neoplasia. Both tubal metaplasia and TEM occasionally show marked nuclear atypia and if no cilia are identified, these cases can be very difficult diagnostically.



Transitional cell metaplasia is a metaplastic change in endocervical epithelium seen most commonly in postmenopausal women, where the epithelium comes to resemble that found in the urinary tract. The cells have high N:C ratios and are a source of overdiagnosis of high grade squamous intraepithelial lesions (HSIL). Cells from an area of transitional cell metaplasia closely resemble each other (little cell-to-cell variability) and have a characteristic longitudinal groove along the long axis of the elongated nuclei. The absence of the nuclear variability that is seen in HSIL is the feature that distinguishes transitional cell metaplasia from a high grade squamous lesion.

Transitional cell metaplasia with characteristic longitudinal grooves in nuclei.

Tubal and transitional cell metaplasias are not reported under The Bethesda System 2001 as they are of no clinical consequence. Their recognition is important so that they are not confused with cervical lesions as discussed.

2.5.11 Questions

1. Describe the cervical cytology appearances observed in atrophic vaginitis/cervicitis. What particular potential lesions need to be considered when examining samples showing atrophic vaginitis?

2. Discuss what you would see in lymphocytic cervicitis in a cervical cytology sample. What are the differential diagnostic features of cells from lymphocytic cervicitis compared to endometrial cells?

3. Describe the cellular changes that may be seen after pelvic radiation treatment. Differentiate between acute and chronic changes.

4. Describe the cellular findings in reserve cell hyperplasia.

5. Describe some of the findings that may be observed in a cervical cytology sample taken one year after a cone biopsy.

6. A cervical cytology sample taken after a cone biopsy may contain endometrial cells. Explain the reasons why this might occur and what you see cytologically in the situations you describe.

7. Describe the cytological findings of tubal and transitional metaplasia.

References

- Hirschowitz L, Eckford SD, Phillpotts B, et al. 1994. Cytological Changes Associated with Tuboendometrioid Metaplasia of the Uterine Cervix. *Cytopathology* 5: 1-8.

2.6 CORE TOPIC: HUMAN PAPILLOMAVIRUS (HPV)

Human Papillomavirus (HPV) plays an essential role in the development of low and high-grade pre-invasive and invasive lesions of the cervix. Differences in the pathogenesis of low-grade and high-grade lesions resulting from the cellular effects of HPV reinforce the importance of accurate interpretation of cervical cytology samples, particularly the distinction between low and high-grade lesions. This is particularly important because of the different ways in which low-grade and high-grade lesions are managed clinically.

2.6.1 Introduction and history

The identification of sexually transmitted viral infections and their relationship to cervical cancer has been the subject of intense investigation. Human Papilloma Virus (HPV) infection occurs in epidemic proportions in most countries in the world and is now known to cause cervical cancer and its precursor lesions. There are many subtypes of HPV. Some cause benign lesions such as genital warts while others have strong associations with cervical cancer.

The history of genital warts dates back many centuries, being recognised as a venereal disease in the ancient world. Genital warts were known to be associated with homosexual practices and were recorded by erotic poets and satirists as well as in medical writing. The term Condyloma Acuminatum derives from the Greek "kondulos" (a knuckle) and the Latin "acuminare" (to make pointed). The word "ficus" (a fig) was commonly used through to the 19th century. Only during the 19th century were genital warts accepted as a distinct entity unrelated to syphilis or gonorrhoea. Irritants such as dirt and genital discharges were considered to be factors in the development of genital warts, but this didn't explain why both sexual partners were affected. Similarities with skin warts were noted in the 1st century but the viral aetiology of human skin warts was not suspected until early in the 20th century. Viral particles were demonstrated in aqueous extracts of skin wart tissue in 1949. In 1954 transmission of genital warts by sexual contact was reported in service men returning from the Far East and the incubation period for the development of genital warts in their wives was shown to be four to six weeks.

In 1975, Professor Harald zur Hausen hypothesized that a common sexually transmitted virus called Human Papillomavirus was a necessary cause of cervical cancer.

Professor zur Hausen received the Nobel Prize for Medicine in 2008 in recognition of his work identifying the causal relationship of HPV with cervical cancer.

2.6.2 Human Papillomaviruses

Papilloma viruses are classified under Subgroup A of the Papova virus group. Polyomavirus, SV40 and other viruses comprise Subgroup B. The papilloma virus genome exists as a circular double-stranded DNA molecule enclosed within a protein capsid. As a DNA virus, it replicates in the nuclei of susceptible host cells. The viral particles are 55nm in diameter and have icosahedral symmetry with 72 capsomeres. The many different papilloma viruses are associated with a range of different diseases.

Many subtypes of HPV specific to a range of disease processes in humans have been identified. The most common subtypes that involve the genital tract are listed below. Those given in **bold** text are the **high-risk (oncogenic) subtypes** which are strongly associated with cervical cancer and precursor lesions.

The most common HPV subtypes found in the genital tract in humans are:

- Subtypes 6,11,**16,18**,**31,33,35,39**,41,**42**,43,44,**45, 51, 52, 56, 58, 59, 68**

Subtypes 6 and 11 are the two HPV subtypes associated with 90% of cases of genital warts (papillary condylomata). Subtypes 16, 18, 31, 33, and 45 are the most common high-risk HPV (hrHPV) subtypes found in pre-invasive (intraepithelial) and malignant lesions. Together, type 16 or type 18 is present in approximately 70% of all invasive cervical cancers. Identification of high-risk HPV subtypes assists in identifying patients at higher risk of developing invasive cancer. Low-grade lesions caused by HPV subtypes 6 and 11 have an extremely low malignant potential.

HPV Testing is used to detect the most common high-risk subtypes of HPV. **A positive high-risk HPV test means that at least one of 14 high-risk subtypes included in the test is present.** Because of our understanding of low and high-risk HPV subtypes and their associations with low and high-grade lesions, cases can be managed in a more efficient way when HPV testing is used. For example, women in New Zealand who are 30 years of age or over with a ASC-US/LSIL cytology result (with no other abnormal result in the previous 5 years) are referred directly to colposcopy if they are hrHPV positive but have repeat cytology after 12 months if the first sample is hrHPV negative. This is possible because women with a negative high-risk HPV test are at very low risk of developing cervical lesions in the next few years, even if a low-risk HPV subtype is present. Women with low-risk HPV subtypes can be safely followed cytologically without having unnecessary colposcopies and biopsies.

Specific genotyping to identify the individual HPV subtype/s present is more costly and is used for research purposes but not for regular HPV testing. Both HPV testing systems currently in use in New Zealand can provide partial genotyping for HPV 16 and HPV 18, with the remaining 12 types being reported in one category called “Other”. Most laboratories now include this information in reports where hrHPV is detected. The specific high-risk subtype present is not part of the current patient management guidelines in New Zealand but this is likely to change in the near future.

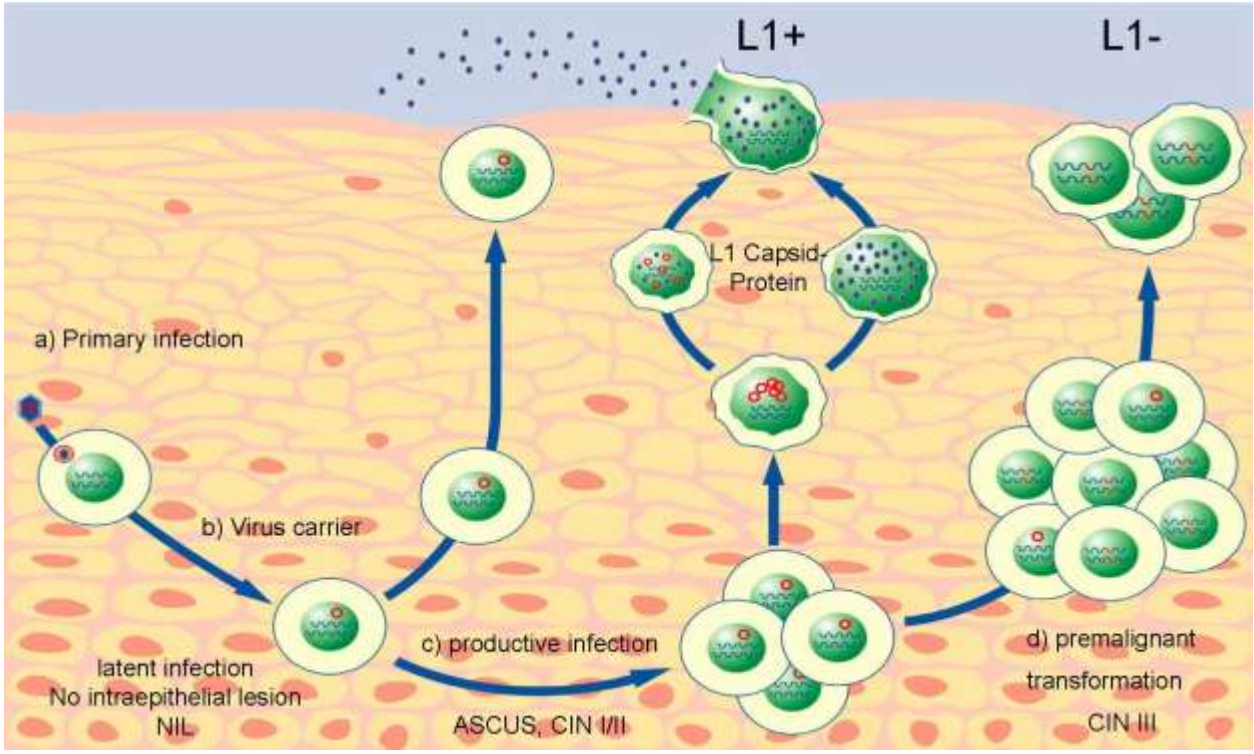
HPV infection begins in basal epithelial cells, with the virus entering through small microabrasions in the surface epithelium. The transformation zone of the cervix is the area most susceptible to infection because of a combination of a thin less robust metabolically active metaplastic epithelium (compared with mature squamous epithelium) and exposure to the virus

on the ectocervix. It was already known to be the cellular region where squamous intraepithelial lesions arise most commonly, before the significance and carcinogenic potential of HPV infection was appreciated.

HPV infection may be latent or productive.

- In latent infections, no clinical or morphological (colposcopic, histologic, cytologic) changes are seen and HPV can only be identified by molecular techniques. In latent infections the HPV DNA is present in the nucleus as a circular episome i.e. it is not integrated into the host cell DNA.

- In productive infections viral replication occurs, predominantly in intermediate and superficial epithelial cells. The assembly of virions in superficial squamous cells often results in koilocytic morphology, as seen in cytology samples. At this stage, the HPV DNA is still episomal.



Davy Vanden Broeck, ISBN 978-953-51-1072-9, Published: April 30, 2013 © The Author(s).
Chapter 4: HPV L1 Detection as a Prognostic Marker for Management of HPV High Risk Positive Abnormal Pap Smears. Ralf Hilfrich

Further details of these processes:

- The presence of viral particles in immature basal cells is not detectable by cytology. The late gene expression manifested by the production of viral capsid proteins depends on the state of differentiation of epithelial cells and probably requires the expression of certain host cellular structural proteins. Viral particle assembly in the differentiated cells of the intermediate and superficial squamous epithelial cell layers is reflected by the altered cell morphology that we are able to identify cytologically.

- As the squamous epithelium matures, the cytoplasmic membrane becomes more permeable and the cell develops a protein envelope that consists in part of a component called involucrin. Development of this envelope is known as terminal differentiation and represents the final stage of squamous differentiation. Normally, involucrin is absent in the basal layer of the squamous epithelium in the cervix but occurs in increasing amounts from the parabasal layers upwards i.e. there is a close relationship between cell maturation and involucrin expression.

- There is also a close relationship between the expression of involucrin and the presence of HPV capsid protein. HPV-infected squamous epithelium shows an abnormal distribution of involucrin in that it is present through the full thickness of the epithelium, including the basal layer. This suggests that HPV infection of squamous cells uncouples normal cellular DNA synthesis and cell maturation, resulting in the expression of involucrin in all epithelial layers.

Malignant transformation of squamous epithelial cells requires persistent infection with a high-risk HPV subtype and almost always involves integration of viral DNA into the host cell DNA. Integration first requires linearisation of the viral DNA (from its round episomal form) and typically the split occurs in the region of the E2 gene in the viral DNA. The resulting integration inactivates the E1 and E2 viral genes. As E2 normally represses E6 and E7, integration into the host DNA results in over-expression of viral E6 and E7 with a resulting increase in cell proliferation and genetic instability.

- The protein product of E6 binds to and inactivates host p53. Sometimes called the genomic "policeman", p53 normally prevents accumulation of potentially oncogenic mutations by cell cycle arrest or by induction of apoptosis.
- The protein product of E7 binds to the protein product of another host tumour-suppressor gene retinoblastoma (RB). Retinoblastoma protein (pRB) controls entry of the cell into the cell cycle and its inactivation by binding of the E7 protein inactivates this control, allowing cell proliferation to proceed.
- Over-expression of p16 (a cyclin-dependent kinase inhibitor) is commonly associated with high-risk HPV infection. p16 normally acts to keep cell proliferative activity in check. High p16 levels are seen in cells where inappropriate replication is occurring because of the consequences of HPV E7 protein binding with pRB.

In approximately 20% of women, HPV viral infection is persistent and HPV DNA becomes integrated into the host DNA resulting in high E6 and E7 protein levels and a blockage of p53 expression. The cellular telomerase is responsible for switching on cell death, but this is also blocked by E6 and E7 allowing the infected cell to continue living and dividing, resulting in the development of high-grade intraepithelial lesions or invasive carcinoma.

2.6.3 Association of HPV and neoplasia

Although HPV is the dominant carcinogen in cervical cancer, other factors are also involved in the development of invasive cancer. Some of these factors have been identified but others are the subject of on-going research.

1. A robust host immune response is known to clear the virus in many women, or limit the progression of disease. Immunosuppression in patients with HIV infection, renal transplantation

and other situations requiring immunosuppressive drug therapy, are associated with an increased risk of disease progression.

2. Cigarette smoking has been shown to independently increase the relative risk for cervical neoplasia in a dose-dependent manner (correlated with the duration of smoking and the number of cigarettes smoked). Products of tobacco smoke and their metabolites have been found in cervical mucus.

In previous years, there has been intense investigation into a possible role for other infectious agents such as Herpes simplex Type II, trichomonas and chlamydia, perhaps acting in concert with HPV. There is currently no evidence that other infectious agents play a causative role or potentiate the effect of HPV infection.

Numerous research studies have been conducted concerning HPV infections and their association with cervical lesions. The results from different studies vary to some extent, because of differences in study design, the population studied, diagnostic criteria used and differences in sensitivity and specificity for HPV detection by different techniques. Collectively however these studies have provided a wealth of information about the importance of HPV viruses and their aetiologic role in the development of cervical lesions including invasive cervical cancer.

- Numerous studies have confirmed the transmission of HPV by sexual contact. One study showed that 76% of females who had sexual contact with men with genital HPV had clinical evidence of lower genital tract HPV infection and that 33% of women who were the sole sexual partner of men with penile condylomata for 12 months or more, developed CIN lesions.

- HPV is common in sexually active women with a peak age of 20-24, but the prevalence falls to less than 7% in women over the age of 30 years. The median duration of infection is approximately 8 months. Women with a positive high-risk HPV test have a 116 times higher chance of developing CIN 3.

- A large study showed that the mean age of women with condylomata was 26.2 years with a peak incidence in the 21-25 year age group. 76.1% of the women with HPV infection were under 30 years and 29.7% were teenagers. HPV infection was thus diagnosed somewhat earlier than CIN (mean age 28 years).

- Quantitative DNA analysis, DNA hybridisation and clinical studies show two distinctive types of cervical condylomata which genetically are:
    - Euploid or polyploid. These regress spontaneously, are of low malignant potential and are most likely to be associated with HPV subtypes 6 or 11.
    - Aneuploid. These tend to persist, have a high malignant potential and contain HPV subtypes 16 or 18.

- HPV 6 and 11 tend to be present in lower grade lesions such as CIN I and are uncommonly found in high-grade lesions. HPV is also found in normal cervical tissue, reflecting the fact that other factors such as host immunity are important in determining when lesions develop in the presence of HPV infection.

- A high-risk HPV subtype is detected in 85-90% of invasive cervical carcinomas in many studies. Detection rates of hrHPV in invasive cervical carcinomas is:

  - Approximately 87% of squamous cell carcinomas
  - Approximately 76% of endocervical adenocarcinomas.

- Rare invasive cervical cancer cases are associated with low-risk HPV subtypes and some rare types of cervical cancer are known to be HPV-negative, particularly some types of endocervical adenocarcinoma and non-epithelial malignancies.

World Health Organisation (WHO) data for 2012 show that cervical cancer is currently the fourth most common cancer and the seventh most common cause of cancer death in women worldwide. In 2012 it is estimated that approximately 528,000 women developed invasive cervical cancer (incidence) and 266,000 women died from it (mortality) worldwide. A high proportion of these women live in less developed regions of the world, where 9 out of every 10 deaths from cervical cancer occurred in 2012. The importance of understanding HPV and its effects on the cervix is critical in the quest to keep reducing this burden of disease. New Zealand and Australia are countries with some of the lowest incidence and mortality rates for cervical cancer in the world. In New Zealand currently (2016), about 160 women develop cervical cancer each year and about 50 women die from it.

The tests and systems used to detect cervical lesions are currently undergoing a period of rapid change. This is occurring partly because of the introduction of new technologies in cervical screening such as liquid-based cytology (LBC) with automation, but also because of new technologies and knowledge relating to the detection and understanding of the significance of HPV subtypes. As knowledge about HPV and the way it influences disease progression in the cervix expands and as new molecular techniques for detecting HPV and identifying HPV subtypes become available, new approaches to the detection of cervical lesions are being introduced worldwide.

2.6.4 Vaccination against HPV

In addition to new ways to detect and mange women with cervical lesions, the potential to prevent the majority of lesions developing in the first place (primary prevention) is also rapidly becoming possible through the use of vaccines against the most common subtypes of HPV that infect the human genital tract.

Two vaccines (Gardasil® and Cervarix®) effective against hrHPV types 16 and 18, are now registered for use in New Zealand. A publicly funded HPV immunisation programme to vaccinate 13 year-old girls commenced in September 2008 in New Zealand and with time this will have a marked effect on the incidence of and mortality from cervical cancer. As the disease prevalence drops following widespread vaccination, there will be fewer women with abnormal cervical cytology and fewer women requiring colposcopy and treatment for cervical disease. Vaccination against HPV subtypes 16 and 18 will prevent about 70% of cases of cervical cancer.

- The HPV vaccine *Gardasil* (manufactured by Merck) is a quadrivalent vaccine that induces antibodies to four HPV subtypes. These are the two high-risk types 16 and 18 and the two low-risk subtypes 6 and 11. Vaccination with Gardasil therefore vaccinates against genital warts as well as providing protection against cervical cancer.
- The HPV vaccine *Cervarix* (manufactured by GlaxoSmithKline) is a bivalent vaccine that induces antibodies to the high risk HPV subtypes 16 and 18.

The HPV vaccine used in the New Zealand immunisation programme is Gardasil®. The programme started on 1 September 2008 for women born in 1990 and 1991. In 2009 the programme was extended to girls born from 1992 onwards and is currently funded for women up to the age of 20 years. The Gardasil® vaccine contains HPV virus-like particles (VLPs) of HPV types 16, 18, 6 and 11. These particles are proteins from the outer shell of the virus. The vaccines contain no viral DNA, are not live and cannot cause HPV infection. The particles mimic the HPV virus so that the immune system makes antibodies against it. Three doses of the vaccine protects against HPV infection for at least ten years after immunisation, and ongoing antibody studies suggest protection will be long lasting, as is the case with the Hepatitis B vaccine.

Each 0.5ml dose of the vaccine contains a small amount of aluminium, which stimulates the immune response. The vaccine also contains tiny amounts of sodium chloride (salt), L-histidine (an amino acid), Polysorbate 80, sodium borate and sterile water. The vaccine does not contain preservatives, antibiotics or any human or animal materials.

Gardasil® vaccine targets the types of HPV responsible for 70 percent of cervical cancer and 90 percent of genital warts. Clinical trials show that it is highly effective in preventing infection with these types of HPV in young women who have not previously been exposed to them.

Research developing further HPV vaccines and studying their effects in different populations is actively continuing. This is an exciting and rapidly evolving field. Widespread vaccination against HPV is already a cost-effective option for countries that do not have the resources to implement an effective cytology screening programme.

2.6.5 New Zealand studies of HPV subtype prevalence.

1. Prevalence of high-risk HPV subtypes in high-grade cervical lesions in NZ women[1]

Because widespread vaccination against HPV 16 and 18 will change the profile of HPV subtypes present in New Zealand, a study published in 2013 was conducted to establish the pre-vaccination prevalence of high-risk HPV subtypes in women with high-grade squamous and glandular lesions. Women aged 20–69 years participating in the New Zealand National Cervical Screening Programme (NCSP) were analysed in two groups:
(1) Women with high-grade cytology with a subsequent histologically-confirmed high-grade cervical intraepithelial neoplasia (CIN2/3) or adenocarcinoma *in situ* (AIS)
(2) A wider group of women who had a cytological prediction of high-grade squamous disease or a glandular abnormality (ASC-H/ HSIL and/or AGC/AIS).

Methods: Women aged 20–69 years with a cytology record of ASC-H/HSIL and/or AGC/AIS on the NCSP Register between August 2009 and February 2011 were invited to participate and 594 women were recruited into the study. Liquid-based cytology specimens were tested for 37 HPV subtypes using Linear Array genotyping. The prevalence of type-specific HPV infection was reported for women with histologically-confirmed CIN 2/3 or AIS and within the wider group with a cytology report of ASC-H/HSIL and/or AGC/AIS. Age-specific trends for the relative proportions of HPV 16/18 and other oncogenic types were assessed.

Results:
1. Oncogenic HPV was detected in 86.7% (515) of the 594 women with a high-grade cytology report and a valid HPV test. This proportion increased to 94.9% when the study population was restricted to women with a histologically-confirmed diagnosis of CIN2+/-AIS.
2. Among women with a high-grade cytology report and a valid HPV test, the most common HPV types detected were HPV 16 (44.1%), followed by HPV 52 (16.8%) and HPV 31 (15.2%). In the sub-group with confirmed disease (CIN2+/-AIS) the prevalence of each of these subtypes was 51.2%m 18.9% and 17.1% respectively.
3. The overall prevalence of HPV 16/18 in CIN2/3 was similar in Maori and non-Maori women, implying that the effects of vaccination will be similar in the two groups.

In this study, the prevalence of HPV 16 in confirmed high-grade disease in New Zealand was comparable to that observed in Australia and European countries. The prevalence of HPV 18 was comparable with Australia and North America but higher than that reported in Europe. Test positivity rates for HPV 52 was higher than in comparable studies in other developed countries. A higher proportion of high-grade lesions in younger women was associated with HPV 16/18 infection, than in older age groups.

2. Prevalence of HPV subtypes women with invasive cervical cancer in NZ [2]

The distribution of HPV subtypes in pre-invasive lesions is not the same as the prevalence in invasive lesions because not all pre-invasive lesions become invasive and those that do, progress to invasion at different rates. This study looked at the pre-vaccination prevalence of HPV subtypes in tissue samples from invasive cervical cancers in New Zealand women.

Methods: Woman 18 years of age and older diagnosed with invasive cervical cancer from five New Zealand hospitals were invited to participate. Tissue samples from 227 cancers were

analysed, including 159 (70%) squamous cell carcinomas (SCC), 61 (27%) adenocarcinomas (ADC) and 7 (3%) adenosquamous carcinomas (ASC).

Results: HPV of any type was identified in 88.5% overall, 93.1% of squamous cell carcinomas and 77.9% of adenocarcinomas. HPV 16 (51%) and 18 (21%) were the most frequent subtypes. HPV 16 was present in 56% of SCC and in 40% of ADC/ASC. HPV 18 was present in 15% of SCC and 35% of ASC/ADC.

For the 201 HPV positive cases, HPV 16 and 18 accounted for 81.1%. Frequent non-16/18 subtypes were 31,45,52,59 and 33. Two low-risk subtypes 11 and 70 were identified, both in squamous cell carcinomas. The presence of low-risk HPV subtypes in invasive cancers emphasises that these are low-risk HPV subtypes, not no-risk subtypes. 5.5% of cases had multiple HPV subtypes present.

The HPV infection rates and subtype distributions for these invasive cancers were comparable with results for other international studies. Subtype 52 was present but was not over-represented. There is less variation internationally in the HPV subtypes present in invasive cancers, than the variation seen with pre-invasive lesions between different countries.

2.6.6 Technologies used for high-risk HPV testing.

There are numerous HPV assays that can be used for HPV testing. New Zealand laboratories are required to use NCSP approved systems and are not allowed to use in-house detection assays for HPV testing. This standardizes results across the country and makes inter-laboratory comparison for monitoring purposes possible. At the time of writing (2016) the six laboratories in New Zealand performing high-risk HPV testing use either the Roche Cobas 4800 or Abbott RealTime HPV platforms.

Both systems can be used with either ThinPrep or SurePath samples. Both systems utilize a mixture of primers and probes for amplifying and detecting different types of HPV DNA. The L-gene is targeted for amplification in both assays. Each system includes a human beta-globin gene from cervical cells to provide a target for internal quality control (QC). The beta-globin gene should be detected in all samples containing any cellular material, and prevents a negative HPV result being issued with a sample that doesn't contain any cells at all.

1. The Cobas HPV Test is an automated test, on the Cobas 4800 processor which identifies if human papillomavirus (HPV) DNA from any of 14 high-risk genital HPV subtypes that are commonly associated with cervical cancer is present. The test specifically identifies types HPV 16 and HPV 18 while concurrently detecting a group of 12 "Other" high risk types: 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. If test results are positive, a high-risk HPV subtype has been detected in the sample.

The Cobas 4800 Processor

2. The Abbott RealTime High Risk (HR) HPV assay uses an m2000 rt automated analyzer for PCR amplification for the detection of high risk human papillomavirus (HPV) DNA in cervical cells collected in liquid cytology media.

The Abbott RealTime HR HPV assay detects 14 high risk HPV genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and partially genotypes 16 and 18 from the other 12 high risk genotypes.

The Abbott RealTime Processor

Many other HPV assays are in use in other countries. Some of these are:

3. Onclarity™ HPV assay (BD)

The Onclarity HPV assay targets E6/E7 DNA oncogenes to identify the presence of HPV DNA. As with the Cobas and Abbott RealTime systems, it provides a pooled result as well as discrete genotyping for Type 16 and 18. The Onclarity HPV assay also can provide additional discrete genotyping for six other high-risk human papillomavirus (HPV) genotypes (16, 18, 45, 31, 51 and 52) and will stratify the remaining eight high-risk types into three groups (33, 58), (56, 59, 66) and (35, 39 and 68) in line with their relative risk. High-risk and genotype assay results are obtained from the same sample with no additional processing steps required.

4. Hybrid Capture® 2 test (Digene, Qiagen),

This test begins by releasing DNA from the cervical cells: normal DNA and if present, HPV DNA. Probe molecules are then added that combine with the DNA to form a DNA-Probe-Hybrid. This Hybrid recognizes many types of high-risk HPVs. Light-producing antibodies are added next and these combine with the DNA-Probe Hybrid. The amount of light produced above a certain level indicates the presence of HPV. Anything below that level indicates either the absence of HPV, or HPV levels that are too low to detect.

5. Aptima® HPV assay (Gen-Probe)

The Aptima HPV Assay reagents are used with the Tigris DTS system to identify human papillomavirus (HPV) RNA from 14 high-risk genital HPV types that are commonly associated with cervical cancer. This assay detects messenger RNA of two HPV viral oncogenes, E6 and E7 and so differs from all other currently approved HPV assays, which detect HPV DNA. The test claims to be more specific at identifying cervical lesions rather than the presence of HPV per se.

6. Cervista (Hologic)

This method uses DNA isolated from a liquid-based cytology sample. Subsequently isolated DNA is mixed in reaction wells with probes that specifically recognise HPV DNA. This reaction is detected by another substance that produces light, which is then measured to determine the presence of HPV in the cervical sample. Using Invader Call Reporter TM software, human papillomavirus HPV DNA from 14 high-risk HPV types can be identified.

HPV Genotyping techniques

Genotyping is performed by amplifying each genotype using primers and probes specific for each HPV type. Differences in nucleotide sequence between HPV genotypes make it possible to amplify and detect each HPV type individually by PCR.

Sequencing is another approach for genotyping. With this method, a woman's HPV DNA is amplified and sequenced. The sequence of the amplified product can be compared with reference sequences with known genotypes using various data bases.

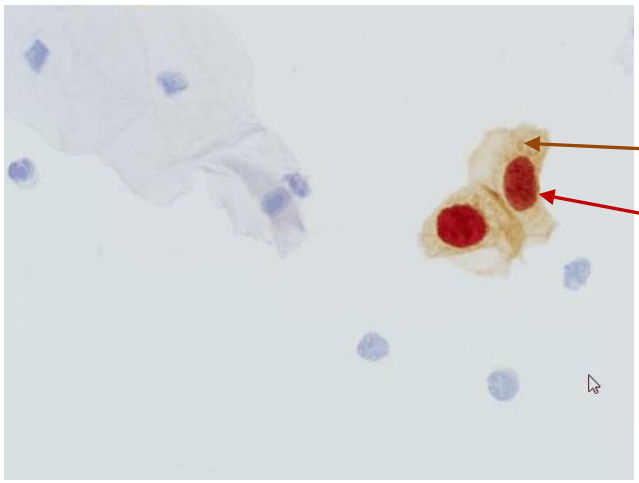
2.6.7 Biomarkers p16 and Ki67

p16 (also known as cyclin-dependent kinase inhibitor 2A and multiple tumor suppressor 1) is a tumour suppressor protein which plays a key role in preventing a wide range of cancers. In humans, p16 is encoded by the *CDKN2A* tumour suppressor gene which is frequently mutated or deleted in a wide variety of cancers, notably melanoma, oropharyngeal squamous cell carcinoma, cervical cancer and esophageal cancer.

p16 plays an important role in cell cycle regulation by decelerating cell progression from G1 phase to S phase thereby acting as a tumour suppressor. When the cell cycle is inappropriately turned on as a result of integration of HPV DNA into the host genome, p16 levels in the cell rise dramatically, as the cell attempts to turn off the inappropriate replicative activity. Overexpression of p16 can be identified in histologic cervical biopsies by immunohistochemistry and is used regularly to improve the accuracy of a histological diagnosis of CIN 2/3 by morphology alone.

Antigen KI-67 also known as Ki-67 or MKI67 is a protein that in humans is encoded by the *MKI67* gene and can be identified by the monoclonal antibody Ki-67. It is a nuclear protein that is associated with and may be necessary for cell proliferation and is involved with ribosomal RNA transcription. Inactivation of antigen KI-67 leads to inhibition of ribosomal RNA synthesis.

P16 and Ki67 biomarkers are currently being explored as adjunctive tools in the diagnosis of high-risk human papillomavirus-associated high-grade squamous lesions to supplement morphological assessment by cytology. A repeat LBC slide preparation is made and stained for the biomarkers. The most useful results are obtained when p16 and Ki67 are used together in a combined dual stain. Over-expression of p16 implies deregulation of the normal controls on cell proliferation and ki67 expression means that the cell is actively replicating. If staining for both p16 and ki67 are present in the same cell, this is good evidence that the cell is abnormal and contains integrated HPV DNA. This is a developing area in cytology but has potential to improve the specificity of diagnosis by cytology alone.



A cytology slide showing dual positive staining for p16 and Ki67.
1. The cytoplasm stains brown indicating **over-expression of p16**
2. The nucleus stains red indicating **Ki67 expression** associated with active cell replication

The other squamous cells present are negative (light blue background counterstain).

2.6.8 New strategies for cervical screening using HPV Technologies

Rapid developments are occurring worldwide in the approach to the prevention of cervical cancer. With the raft of new technologies and strategies now available, many studies are investigating novel potentially more effective and/or less expensive ways of preventing cervical lesions by vaccination (primary prevention) or improving the way we detect and manage pre-invasive cervical lesions (secondary prevention) before invasive cancer develops.

The rationale for using HPV testing for primary screening is based around the fact that the initial screening test should be a highly sensitive test so that all those at risk can be identified. This needs to be followed by a more specific test in order to establish which of the women who tested positive on the initial screening test, need additional investigation or treatment. Not all women who are high-risk HPV positive will have a cervical lesion. HPV testing is a sensitive way of identifying women who are ***at risk*** for cervical disease but it is not as specific as cytology i.e. cytology is better at identifying women who do have a cervical lesion. Performing a sensitive HPV test first followed by the more specific cytology test should optimize the detection of women with cervical lesions.

A review of all recent developments is beyond the scope of this workbook. Two examples of recent and current studies are outlined below. Additional information is widely available on the internet and on the www.nsu.govt.nz website.

1. The ATHENA trial [3-5] is a large multicentre prospective clinical study that investigated the use of HPV Testing (using ThinPrep samples and the Cobas HPV test) for primary cervical cancer screening. The study also set out to evaluate the value of testing for 12 pooled hrHPV subtypes (non16/18 types) as well as specific genotypes 16 and 18 individually. There were 47,000 women 21 years of age or older enroled, all undergoing regular cervical screening. Cytology and HPV testing were performed on all women and all those with abnormal cytology (ASC-US or greater) and /or a positive high-risk HPV test, were sent for colposcopy and biopsy. The study was performed in the USA. Some aspects of the study were designed to address issues in cervical screening in the USA.

Three different populations were investigated:

i. Women > 21 years of age with ASC-US cervical cytology. The study aimed to investigate the performance of the HPV test in identifying high-grade cervical lesions and compared the risks for women with a positive HPV test with those with a negative test in this population.

ii. Women > 30 years of age with normal cervical cytology. The aim in this group was to compare the risk of having CIN2+ for those with a positive HPV test vs. a negative HPV test, for those with HPV16/18 vs. a negative HPV test and for those with a positive HPV16/18 test vs. those with any other high-risk HPV subtype.

iii. An overall screening population (> 25 years) to explore HPV as a first-line screening test (on-going longitudinal 3 year study).

Results: ATHENA demonstrated that primary HPV testing performed better than cytology alone at identifying high-grade cervical lesions in women >25 years of age. The trial also quantified the risk of precancer and cervical cancer in HPV 16+ and/or HPV 18+ women.

- For women with ASC-US cytology, the risk of CIN2+ in women was 18.6 fold higher for those who were HPV positive (all subtypes) compared with those whose who were HPV negative.
- Women who were HPV 16 positive were at the highest risk of cancer. Women who were HPV 16 positive with normal cytology had a 13.6% absolute risk of ≥CIN2. Nearly 1 in 7 women who tested positive for HPV 16 had high-grade cervical disease that was missed by cytology.

2. The **COMPASS** trial is currently underway in Victoria, Australia. The study is being conducted by the VCS (Victorian Cytology Service) in Melbourne in collaboration with the University of New South Wales. This is a randomised controlled clinical trial comparing 3-yearly cytology-based cervical screening with 6-yearly primary HPV screening in Australian women aged 25-64. It is the first large-scale trial to be conducted in an HPV-vaccinated cohort of women. A New Zealand arm of the COMPASS trial took place in Auckland in which 500 women were enrolled to assess the feasibility of HPV primary screening in New Zealand.

Numerous countries around the world are adopting or seriously considering new strategies for cervical screening using HPV testing rather than cytology as the primary screening test. Cytology is usually being used as a second follow-on test for women who test positive for high-risk HPV. England, Denmark, The Netherlands, Italy, Latin American countries, South Africa, Canada, USA, Australia and China are either considering or have adopted new screening regimes using HPV testing for primary screening. New Zealand is planning to move to HPV testing for primary screening in 2018.

2.6.9 Questions

1. Describe briefly the process by which HPV invades and affects epithelial cells.

2. Name at least five high-risk HPV subtypes. Which subtype is the most common cause of cervical cancer?

3. Will all HPV infections lead to cancer? Provide some evidence to justify your answer.

4. Which molecular method is used by the two commercial assays in use in New Zealand for high-risk HPV testing?

5. Which subtypes of HPV are included in the Gardasil vaccine used in NZ? Why have these subtypes been selected for the vaccine?

References

- Type-specific oncogenic human papillomavirus infection in high grade cervical disease in New Zealand. Leonardo S, Lewis H, Smith M, Neal H, Bromhead C, Canfell K. *BMC Infectious Diseases* 2013, 13:114

- Type distribution of human papillomavirus among adult women diagnosed with invasive cervical cancer (stage 1b or higher) in New Zealand. Sykes P, Gopala K, Tan AL, Kenwright K, Petrich S, Molijn A. Chen J. *BMC Infectious Diseases* 2014, 14:374

- Primary cervical cancer screening with human papillomavirus: End of study results from the ATHENA study using HPV as the first-line screening test. Wright TC, Stohler MH, Behrens CM, Sharma A, Zhang G, Wright TC. *Gynecol Oncol (2014), http://dx.doi.org/10.1016/j.gyno.*2014.11.076

- High-risk Human Papillomavirus Testing in Women With ASC-US Cytology, Results from the ATHENA HPV Study. Stoler MH, Wright TC, Sharma A, Apple R, Gutekunst K, Wright TL, and the ATHENA Study Group. *Am J Clin Pathol*. 2011;135(3):468-475

- Performance of carcinogenic human papillomavirus testing and HPV 16 or HPV 18 genotyping for cervical cancer screening of women aged 25 years and older: a sub-analysis of the Athena Study. Castle P, Stohler MH, Wright TC, Sharma A, Wright TL, Behrens CM. *Lancet Oncol*. 2011:12(9):880-890

2.7 CORE TOPIC: TERMINOLOGY: SQUAMOUS INTRAEPITHELIAL LESIONS (SIL) & CERVICAL INTRAEPITHELIAL NEOPLASIA (CIN)

2.7.1 Terminology of SIL and CIN

Squamous intraepithelial lesions (SIL) are of paramount importance in cervical cytology. The ability to detect and treat women with squamous intraepithelial lesions before invasive cancer develops is the main reason why cervical screening has made it possible for well-organised cervical screening programmes to reduce the incidence and mortality of squamous cell carcinoma so effectively.

The terminology used to describe these lesions has evolved over time as our understanding of underlying disease processes has developed. The use of different terms can initially be confusing. The term "Squamous Intraepithelial Lesion (SIL)" is used in cytology under The Bethesda System 2001, whereas an older term "cervical intraepithelial lesion (CIN)" is used in histopathology. Colposcopists may refer to "low-grade" and "high-grade" lesions or use either the CIN or SIL terminology. "Dysplasia" is a generic term used to cover all grades of CIN and can be divided into grades: mild dysplasia (CIN 1), moderate dysplasia (CIN 2) and severe dysplasia (CIN 3). The term "carcinoma in situ" is no longer used but is synonymous with severe dysplasia/CIN 3. All these lesions are confined to the epithelium i.e. the abnormal cells remain above the basement membrane without invading through into the underlying cervical stroma.

CIN terminology is defined by the histological appearance of lesions. The use of words is self-explanatory:

- *Cervical* - indicates the location of the lesion
- *Intraepithelial* - indicates that the lesion is located within the epithelium i.e. it has not breached the basement membrane (not invasive)
- *Neoplasia* - neo meaning "new" and "plasia" derived from the word plasma which means "formation, creation" i.e.: abnormal growth of tissue

The word *intraepithelial* is the most significant as it defines the fact that the abnormal cells have not penetrated the basement membrane and are therefore not invasive. The major implication is that the lesion can be successfully managed and treated. For women, words such as *pre-cancer* or *pre-neoplasia* are realistic and appropriate and are less anxiety-provoking than previous terminology such as carcinoma-in-situ.

SIL terminology was introduced when The Bethesda System was developed. The Bethesda System (TBS) is a system for reporting cervical and vaginal cytology results. It was introduced in 1988, and revised in 1991 and again in 2001. The name comes from the location of the conference that established the system (Bethesda, Maryland USA). The terminology was developed to address the need for a standardised clear way of communicating cervical cytology sample results to primary care physicians and gynaecologists, using terminology reflecting the current understanding of cervical disease. A group of experts representing a broad spectrum of interested professional organisations met in Bethesda under the auspices of the National Cancer Institute on December 12 and 13, 1988 to formulate this system of reporting that has since been widely adopted throughout the world.

SIL terminology includes low-grade intraepithelial lesions (LSIL) encompassing HPV effect and CIN 1 (a low-grade lesion resulting from HPV infection), and high-grade intraepithelial lesions (HSIL) encompassing CIN 2 and CIN 3.

Two important shifts in concept and reporting occurred when The Bethesda System was introduced.

1. The previous three-tier way of thinking about intraepithelial lesions in three grades (mild=CIN 1, moderate=CIN 2 and severe=CIN 3) based on the morphologic appearances in histology and cytology, was superseded. We now understand that CIN 1 lesions caused by infection with HPV (where the virus is still episomal) differ from CIN 2 and CIN 3 lesions, where the HPV virus is integrated into the host genome and the risk of progression to invasion is appreciably higher. This new two-tier approach is reflected in The Bethesda System which defines LSIL (low-grade) lesions and HSIL (high-grade) lesions.

2. The first version of The Bethesda System in 1990 recognised that although the distinction between HPV infection only and CIN 1 is made on histology, the use of cytology to predict this difference was not reliably reproducible. For this reason, definite low-grade lesions are all reported as LSIL without an attempt to differentiate between HPV infection alone and CIN 1, as reported in histologic biopsies.

For clarity the relationship of each is as follows:

- CIN 1 = **LSIL** = mild dysplasia → Low-grade lesions
- CIN 2 = **HSIL** = moderate dysplasia → High-grade lesions
- CIN 3 = **HSIL** = severe dysplasia/carcinoma in situ (CIS) → High-grade lesions

New Zealand cervical cytology laboratories use The Bethesda System 2001 with SIL terminology but to assist clinicians, CIN terminology is included. e.g. "There are abnormal squamous cells consistent with a low-grade squamous intraepithelial lesion (LSIL; CIN 1/HPV)". For HSIL, laboratories are not required to sub-classify cases as CIN 2 or CIN 3 (both HSIL) but laboratories may choose to do so in their reports.

2.7.2 CIN as a disease process

The best way to understand CIN is to study it in histology. To recap, the squamous epithelium lies above the basement membrane and matures from the basal cell layer adjacent to the basement membrane up to the superficial (mature) layer, which naturally desquamates (exfoliates). This could be described as cervical intraepithelial "euplasia".

In neoplasia, abnormal squamous cells arise in the basal layer where HPV infection first occurs. The severity of the intraepithelial abnormality is reflected in the degree of involvement of the epithelium as the cells mature towards the epithelial surface. In general:

CIN 1 Abnormal cells in the lower 1/3 with squamous maturation in the upper 2/3rds of the epithelium.
CIN 2 Abnormal cells in the lower 2/3 with squamous maturation in the upper 1/3.
CIN 3 Abnormal cells through the full thickness of the epithelium with no squamous maturation in the superficial layers.

The significance of grading intraepithelial lesions of the cervix is not as clear-cut as previously believed, with respect to progression of disease to invasive carcinoma. Evidence suggests that

any precancerous lesion regardless of the degree of CIN can progress to invasive cancer and that earlier beliefs that invasive cancer only arose following progression of CIN 3 are false. The grading of CIN does however carry a graduated level of risk of progression, with the majority of invasive lesions arising from CIN 3 while most low-grade HPV lesions regress. Risk of progression is also related to the extent (size) of the lesion and differences in risk with different HPV subtypes.

In an extensive review of the literature [(1)] Oster calculated that:

- Mild dysplasia (CIN 1) - 60% regress and 1% progress to invasive carcinoma
- Severe dysplasia (CIN 3) – 33% regress and 12% progress to invasive carcinoma

2.7.3 Diagnosis of CIN by histopathology

Of all neoplastic lesions in the cervix, 90% are squamous and begin in the transformation zone close to the squamo-columnar junction. The remaining 10% originate in endocervical epithelium giving rise to glandular lesions. Squamous lesions occasionally occur further out on the ectocervix or in the vagina. Although vaginal lesions are much less common than cervical lesions, this site needs to be considered if abnormal cells are present in a cytology sample without a lesion identified on the cervix at colposcopy.

CIN is characterised by loss of polarity of cells and disruption of the ordered stratification of squamous cells normally seen in cervical epithelium. The grading of CIN is based on:

- The proportion of the thickness of the epithelium occupied by undifferentiated cells
- The level in the epithelium at which mitotic figures are found
- The presence of abnormal mitoses
- The severity of nuclear abnormalities such as hyperchromasia, pleomorphism, chromatin changes

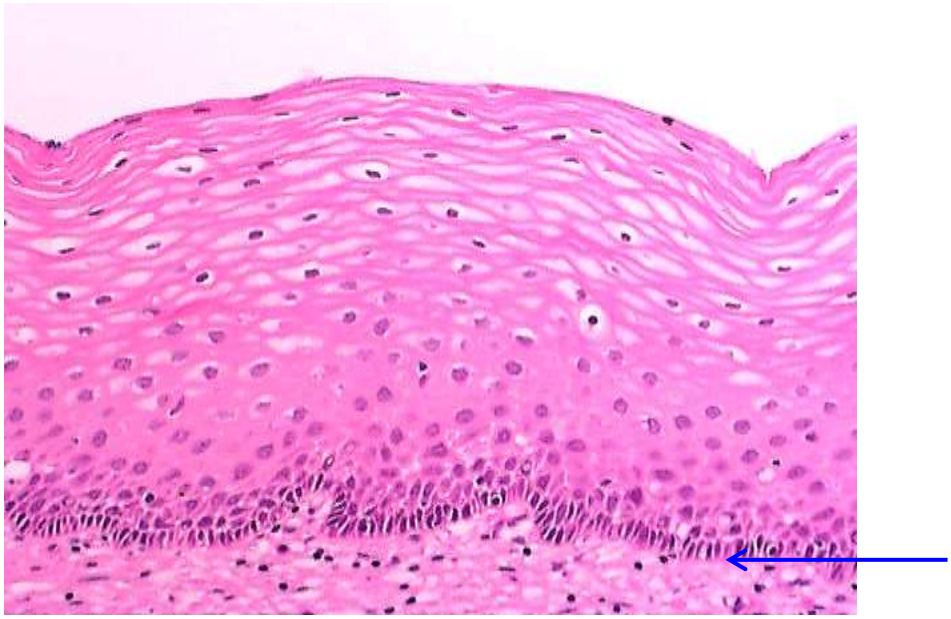
FEATURES IN THE HISTOLOGICAL GRADING OF CIN

	Maturation	**Nuclei**	**Mitoses**	**Comments**
CIN I	Upper 2/3	Pleomorphic	Infrequent	
CIN 2/3	Upper 1/3	Pleomorphic	Increased frequency	
Types of CIN2/3:				
small cell undiff	Nil	Marked pleomorphism Crowded Appear monotonous	Frequent Abnormal	Small undifferentiated basaloid cells
large cell undiff	Nil (see comments)	Marked pleomorphism	Abnormal in upper 1/3	Large parabasaloid cells, more cytoplasm
large cell keratinised	Nil	Marked pleomorphism	Frequent Abnormal	Large cells, lower N:C ratio keratinised surface

Some pathologists sub-classify CIN in histology into keratinising, non-keratinising and metaplastic cell types but this information is not included in most histopathology reports. In practice, the differentiation between low-grade (CIN 1) and high-grade (CIN 2 and 3) lesions is the most important distinction as this has major implications for patient management.

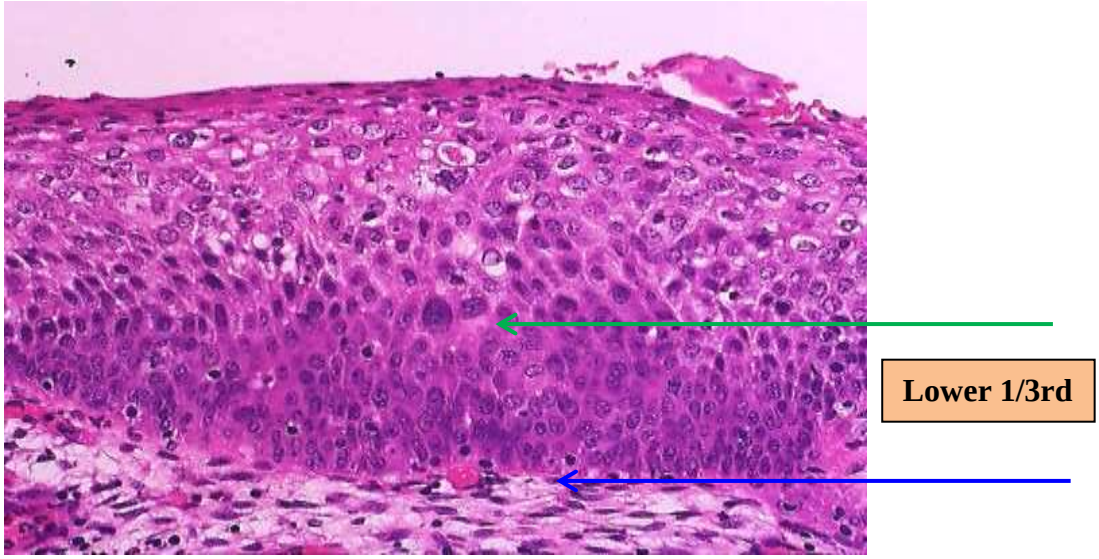
When looking at the following illustrations of histology, remember that cervical cytology only samples the top few layers of cells –look at these layers in the histology photographs below to predict the types of changes seen cytologically as LSIL and HSIL.

CIN 1/LSIL – Low-grade disease

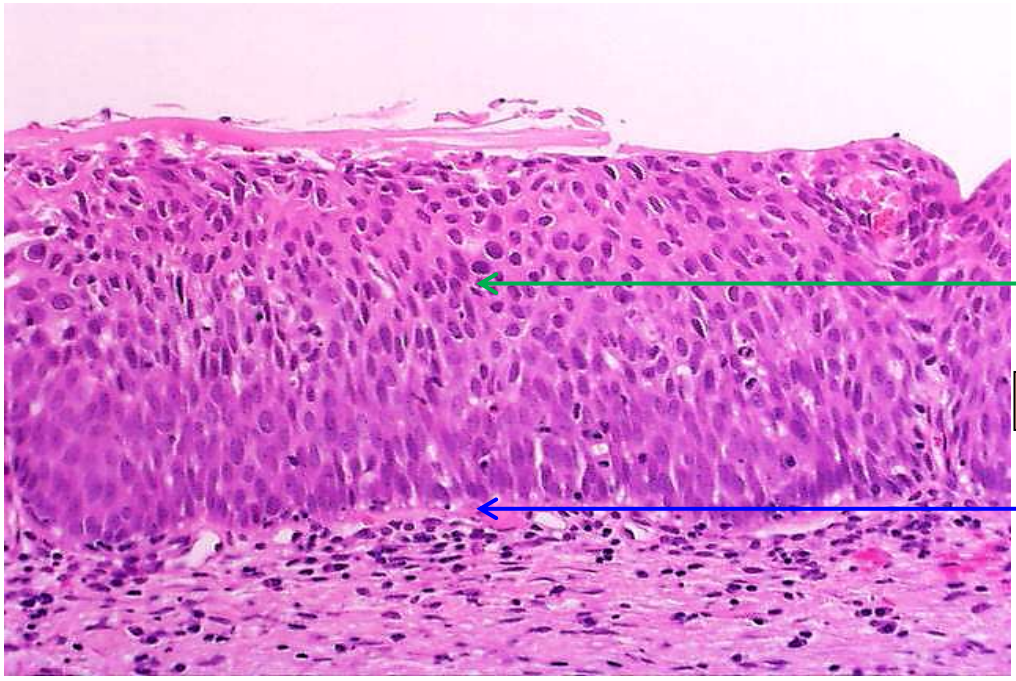


Above: Normal squamous epithelium showing full maturation. The basement membrane as indicated by the blue line.

Below: CIN 1 with evidence of HPV. The immature neoplastic cells occupy the lower third of the epithelium from the basement membrane upwards (between blue and green lines). The upper two thirds show degrees of maturation.

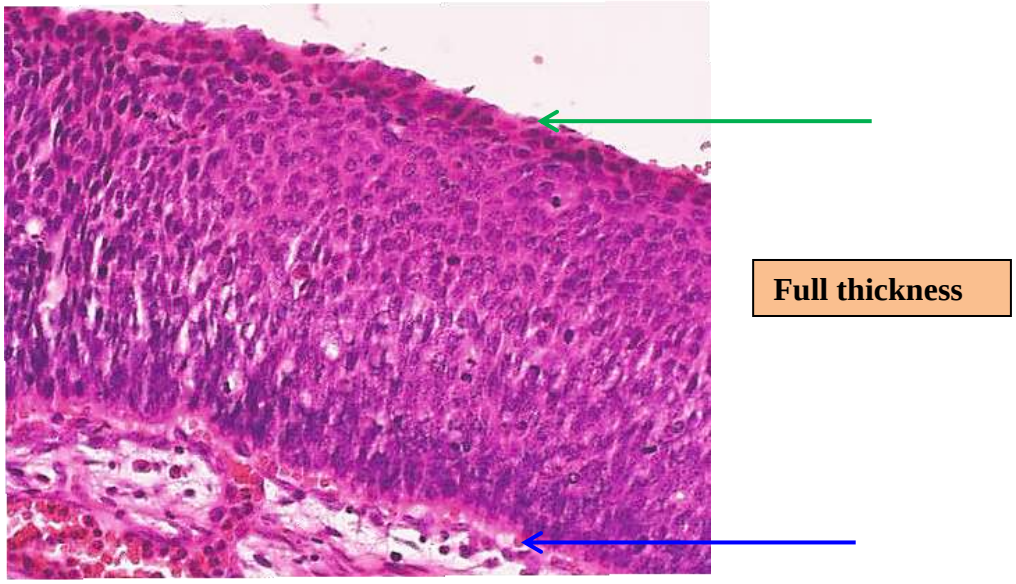


CIN 2/HSIL – High-grade disease



Undifferentiated neoplastic cells are present above the basement membrane in the lower two-thirds of the epithelium (between blue and green lines) with less maturation in the upper third compared to CIN I. Like cytology, the assessment is qualitative and subjective.

CIN 3/HSIL – High-grade disease



There are undifferentiated neoplastic cells extending from the basement membrane to the epithelial surface, with no evidence of any significant maturation.

2.7.4 Diagnosis of SIL by cytopathology: General comments

Cytological grading of SIL into low-grade and high-grade lesions is not always easy because this is essentially a subjective visual task. Reporting cytology under a two-tier system using SIL terminology rather than the three-tiered CIN terminology and removing the requirement to distinguish between HPV infection alone and CIN 1 lesions by cytology, have improved the reproducibility of grading lesions by cytology as there is less variation in reporting between different practitioners. While there is no point in attempting to differentiate between HPV effect alone and CIN 1 by cytology, it can still be helpful in some situations to consider whether a case of HSIL is likely to be CIN 2 or CIN 3 on histology. A New Zealand study in women under 25 years of age with CIN 2 on histology is currently looking at whether this particular group of women could be safely managed more conservatively by following them carefully to see if their disease regresses, rather than proceeding to excisional treatment. The diagnosis of CIN 2 can be more difficult cytologically than CIN 3 so learning to make the distinction in cytology samples can be of value in training practitioners to be alert to subtle differences in the detection of CIN 2. Some New Zealand laboratories distinguish between CIN 2 and CIN 3 as a qualifier in their HSIL reports.

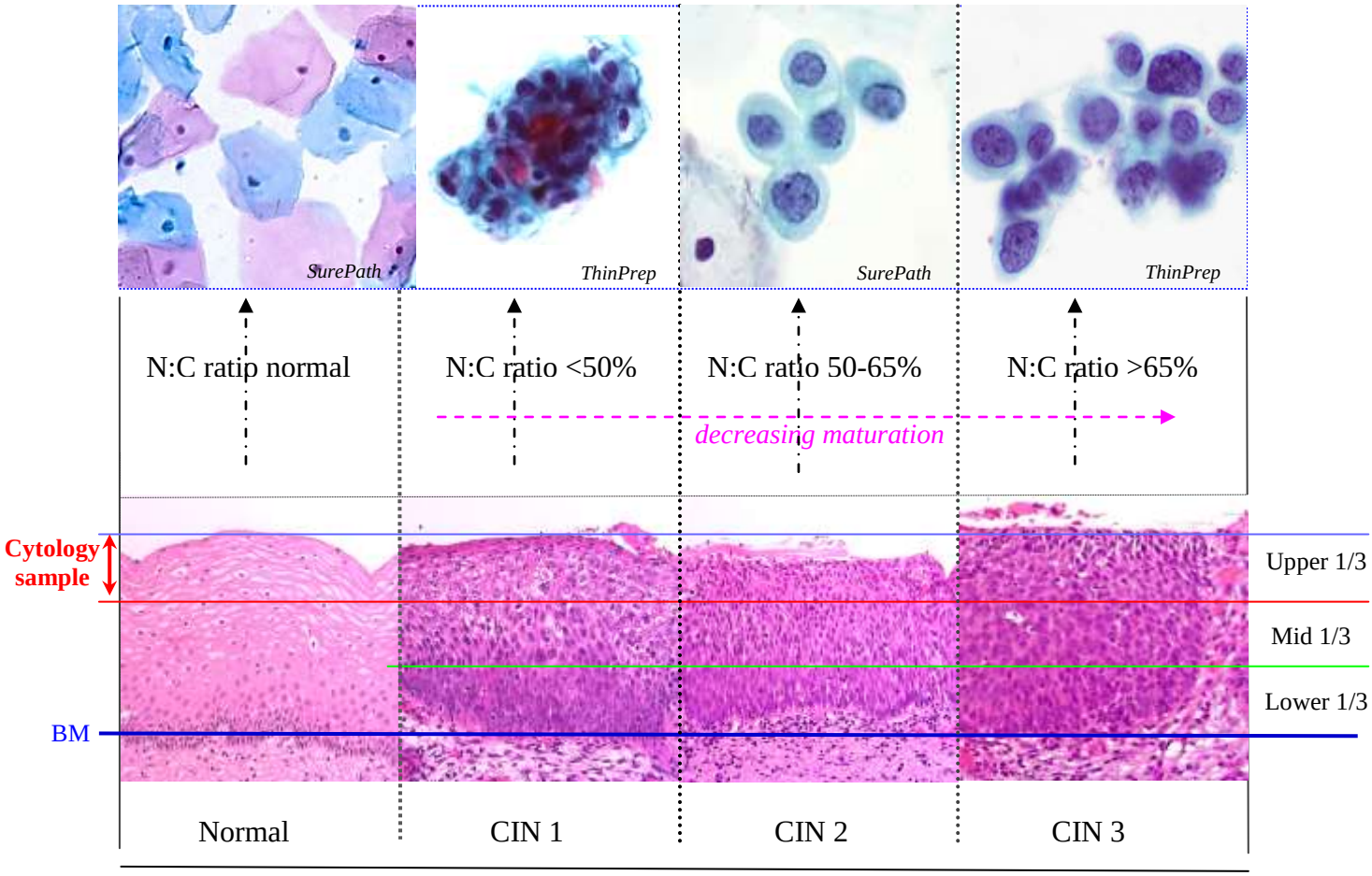
The role of cytology is to predict the histopathology. When reporting abnormal cytology samples it is important to convey to the clinician the most severe histological abnormality anticipated. The cytological assessment is a prediction of histological grade not an absolute indicator, so cytology reports use words such as “consistent with” rather than giving a final definite diagnosis.

The cells in a cervical cytology sample are typically from the surface layers (upper third) of the epithelium. “Microbiopsies” are sometimes present in samples which may include deeper layers. The cells in the cytology sample will show differing degrees of cell maturation and this is a major factor used in the cytological grading of CIN. Cell maturation is reflected by the nuclear:cytoplasmic (N:C) ratio of the cell.

With reference to the histology and cytology of cells from the upper 1/3 of the epithelium:

Lesion	Histology	Cytology	N:C ratio
CIN 1	Maturation in upper 2/3	Maturating abnormal cells Low N:C ratio	Nucleus occupies less than ½ of the area of the cell
CIN 2	Some maturation in upper 1/3	Less mature abnormal cells Increased N:C ratio	Nucleus occupies ½ to 2/3 of the area of the cell
CIN 3	No maturation in any layer	Least mature cells High N:C ratio	Nucleus occupies over 2/3 to almost all of the area of the cell

2.7.5 Summary of comparative histology and cytology in CIN



2.7.6 Discordant results in histology and cytology

There are a number of reasons (apart from misinterpretation) that explain why there may not be correlation between cytology and histology results, even when the samples are taken at the same time at colposcopy. Difficulties that can occur with the histology sample include the lesion not being biopsied adequately by the colposcopist or the lesion not being represented in the histologic sections that the histopathologist sees when reporting.

Situations that can cause difficulties with the cytology include:

1. Small numbers of abnormal cells.
Some cytology samples contain many abnormal cells but some contain very few, and this may make grading very difficult. LBC slide preparations contain a subsample of the cells collected by the sampling device and abnormal cells accordingly will also be a subsample of the abnormal cells collected.

2. Lesion not sampled.
Significant lesions may not have been sampled particularly if the lesion lies in a relatively inaccessible area (such as high in the endocervical canal or in the vagina). Conversely, cytology enables the full circumference of the cervix to be sampled and may detect a lesion that is missed by a small cervical biopsy.

3. Factors compromising the sample.
Even with LBC samples, obscuring elements like blood, inflammatory cells, cytolysis and lubricant may obscure significant abnormal cells.

4. Clinical conditions.
Lesions are often more difficult to diagnose in post-menopausal women with atrophic changes because the cells are less mature due to low levels of oestrogen. The samples are often less cellular and can be more difficult to interpret.

Cases with discordant results are usually discussed at a clinical-cytological-histological review session. This is particularly important where cytology has predicated a high-grade lesion but biopsy and/or colposcopy has demonstrated only a low-grade lesion, or no lesion at all. In situations where the cytology predicted LSIL and HSIL was found on histology, review of the cytology is a quality measure and a learning opportunity.

2.7.7 Questions

1. Discuss the disease process of CIN, including details of histological grading.

2. What is the value of using The Bethesda System for reporting cervical cytology samples?

3. Explain the significance of the terms maturation and differentiation with regards to CIN changes.

4. Elaborate on possible reasons for discordant cytology and histology results. Use the notes about this in the workbook as a guide but add to this by discussing with senior laboratory screening staff.

References

- Ostor AG. Natural History of Cervical Intraepithelial Neoplasia: A Critical Review. *International Journal Gynaecological Pathology* 1993;12: 186-192.

2.8 CORE TOPIC: LOW-GRADE SQUAMOUS LESIONS: LSIL (CIN 1/HPV) and ASC-US.

Using The Bethesda System, cytology samples showing HPV effect alone and those which also show features of CIN 1 as identified by histology, are all reported as LSIL because the cytologic distinction between HPV alone and CIN 1 is not reproducibly reliable. These lesions are also now all managed in the same way so the distinction is not important clinically. The distinction between HPV effect alone and CIN 1 is still made in histology.

In this section, the morphologic appearances in cytology samples of HPV infection alone will be discussed first followed by a discussion of LSIL morphology reflecting CIN 1. Finally, when there are cytologic features that are suspicious of a low-grade lesion (HPV and/or CIN 1) but these are not diagnostic, the case is reported as Atypical Squamous Cells of Undetermined Significance (ASC-US). Although ASC-US is not a definite diagnosis, it is managed in the same way as definite LSIL and therefore included in the low-grade spectrum.

2.8.1 Morphology of HPV infection

The morphological diagnosis of HPV-induced lesions by cytology and histology can be difficult and is by no means 100% accurate. HPV infection often results in characteristic diagnostic appearances but may cause subtle changes that are difficult to recognise as HPV-associated.

There are many published descriptions of the cytological appearances of HPV-infected squamous cells and the histological lesions that result from HPV infection. The positive predictive value (PPV) reflects how accurately cytology correctly predicts the presence of cervical lesions and this is relatively high for HPV effect. Correlation of the cytology result with cervical biopsy results and the appearance at colposcopy can however be variable. This can be due to factors such as sampling, variable interpretation of findings by different practitioners and variations in morphologic/colposcopic appearances. Multidisciplinary meetings (MDM's) are held regularly where cases with discrepant results are discussed to determine patient management.

The criteria for the cytological recognition of HPV infection are discussed below. If HPV change or low-grade CIN is detected cytologically, a high-grade lesion could still be present even if there is no evidence of this in the cytology sample.

Two cell types found in cervical cytology samples are pathognomonic of HPV infection:

- Koilocytes
- Atypical parakeratotic cells (previously called Dyskeratocytes)

Diagnostic koilocytes and atypical parakeratotic cells are not always present with HPV infection. There are also conditions that cause appearances that mimic koilocytes so the cytology always needs to be assessed with care.

Koilocytes

The first detailed description of the morphological appearance of the koilocyte was made by Ayre in 1949. The term "koilocyte" was first used for "balloon" cells by Koss and Durfee in 1956, who described the appearances as a cell change of unknown cause associated with pre-cancer. In 1960 Ayre postulated that lesions showing koilocytic atypia could be related to a viral infection with oncogenic potential.

The term koilocyte is derived from the Greek "Koilos" which means hollow or cavity and is used to refer to the transparent balloon appearance of the cytoplasm around the nucleus. Koilocytes are squamous epithelial cells infected by HPV. Crystalline arrays of viral particles have been demonstrated in these cells using electron microscopy. The perinuclear clear zone is due to an area of perinuclear cytoplasmic degeneration, which is a cytopathic effect of the virus. The sharp demarcation of the perinuclear zone is due to condensation of cytoplasmic fibrils peripheral to the area of degeneration. Transitional forms of koilocytes show that the cytoplasmic necrosis progresses from the perinuclear area out to the periphery of the cell as the infected cell matures. The specificity of koilocytes as a diagnostic marker for HPV infection is demonstrated using DNA hybridisation techniques, which show the presence of HPV DNA in more than 95% of patients expressing koilocytes.

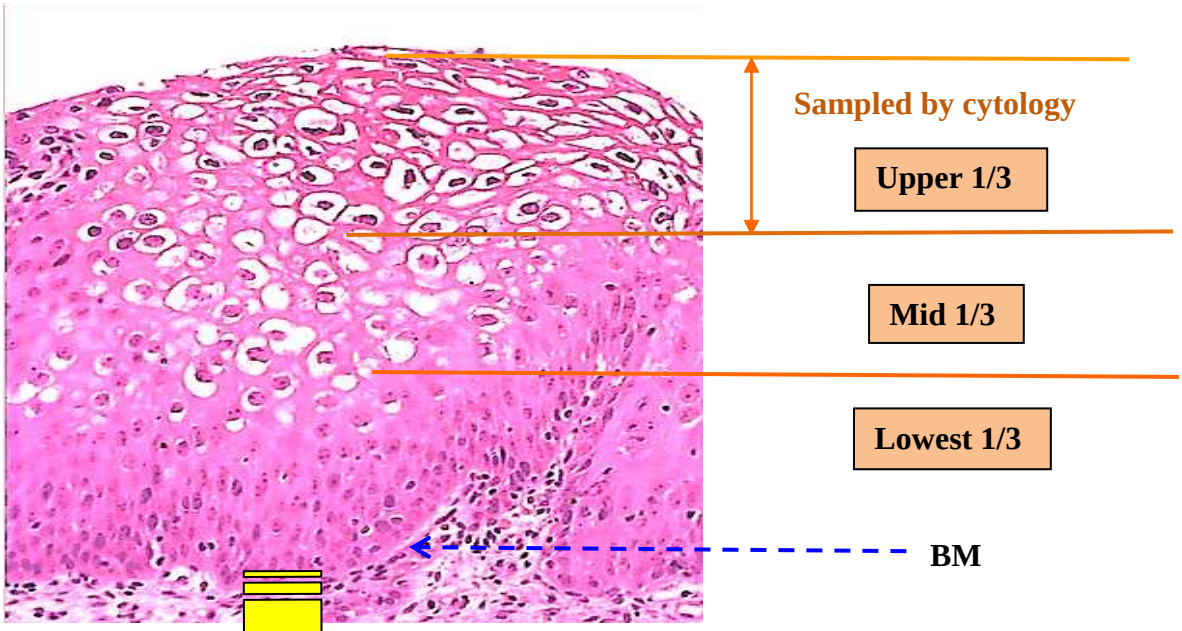
Diagnostic koilocytes are intermediate or superficial squamous cells and must have both characteristic cytoplasmic features and an abnormal nucleus.

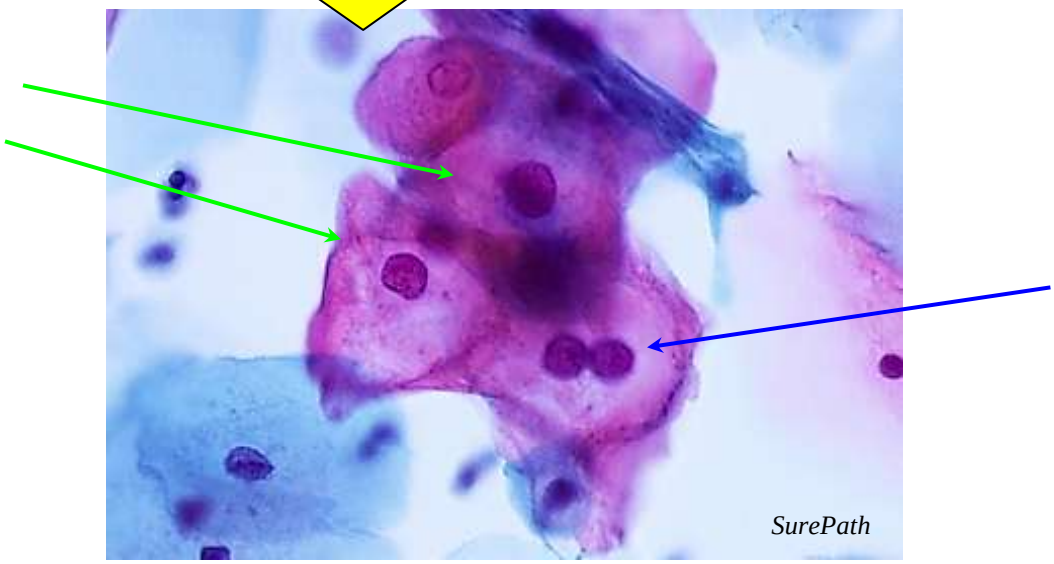
(1) The cytoplasm is abundant and contains a characteristic large perinuclear clearing with a sharply defined edge, and condensation of the more peripheral cytoplasm. The appearance is distinctly different from the small soft-edged perinuclear halos often seen in reactive squamous cells. Within the translucent to transparent clearing there may be remnants of cytoplasm in the form of fragmented debris. The edge of the clearing is sharp and well defined adjacent to the peripheral cytoplasm. The remaining intact cytoplasm is condensed and is usually eosinophilic, sometimes hyaline (glassy) in appearance, but may be cyanophilic.

(2) The nucleus can be abnormal in a variety of ways. The cell may contain one, two or multiple nuclei which may be eccentrically located in the cell. The chromatin is often poorly preserved, smudged and poorly defined or can be granular. The nuclear membrane can be irregular in outline, sometimes markedly so. The degree of nuclear atypia varies. Intranuclear inclusions or nucleoli are not seen.

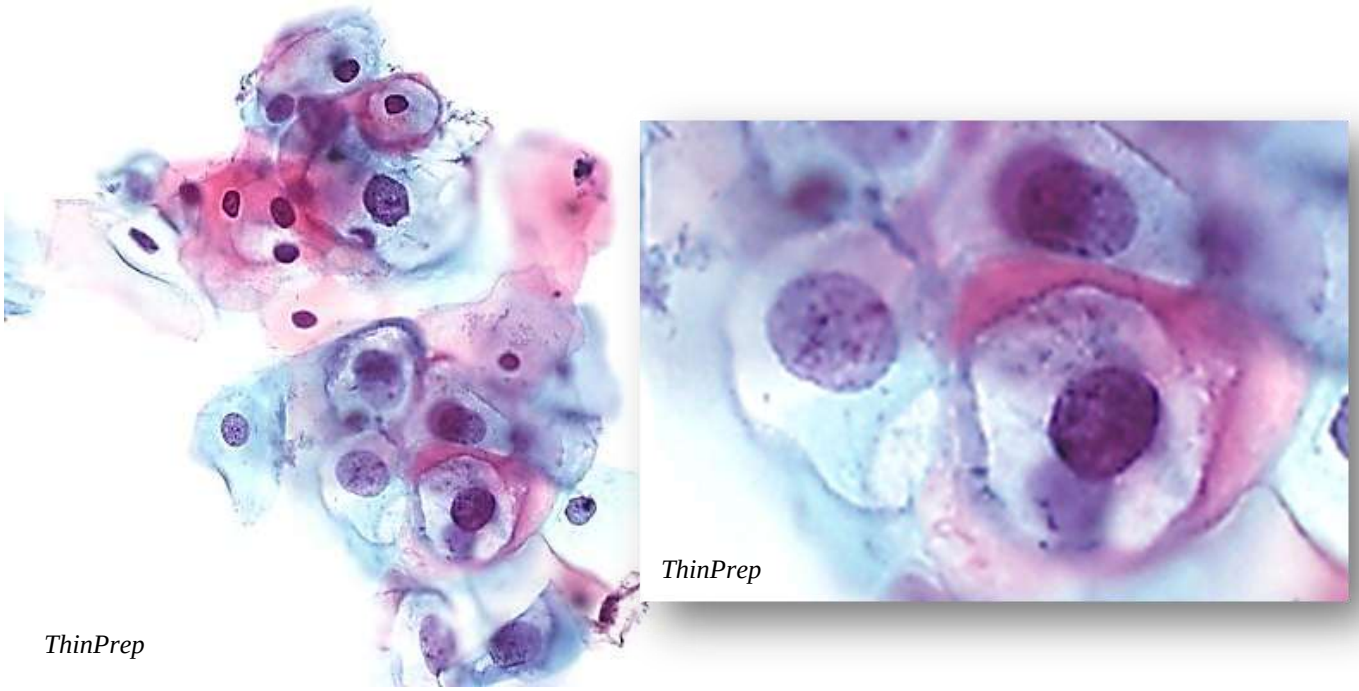
Cells that are suspicious of HPV infection but do not meet all the criteria for diagnostic koilocytes are reported in The Bethesda System terminology as Atypical Squamous Cells of Undetermined Significance (ASC-US).

Histological evidence of HPV infection showing mature koilocytes at the epithelial surface. Cytoplasmic vacuolation is prominent and the nuclei are atypical showing hyperchromasia, size and shape variation, and occasionally bi-nucleation.
Koilocytosis is less evident and patchy in the more immature lower layers of the squamous epithelium and is not evident in the basal layer next to the basement membrane (BM).





Cytological appearance of classic mature koilocytes (low N:C ratio) showing a large cytoplasmic cavity that contains fragments of cytoplasm (bundles of tonofilaments). Note the well-defined outer rim of densely stained eosinophilic cytoplasm. Koilocytes are arrowed, with the blue arrow marking a binucleated koilocyte. The nuclei are atypical showing variable karyomegaly and hyperchromasia, some with a smudged appearance of the nuclear chromatin.



Low power view (left): Koilocytes admixed with normal superficial and intermediate squamous cells (note the similarity in overall size and N:C ratio). The high power image (right) shows clearly defined perinuclear cavities with condensation of peripheral cytoplasm. The cytoplasm can be eosinophilic or cyanophilic.

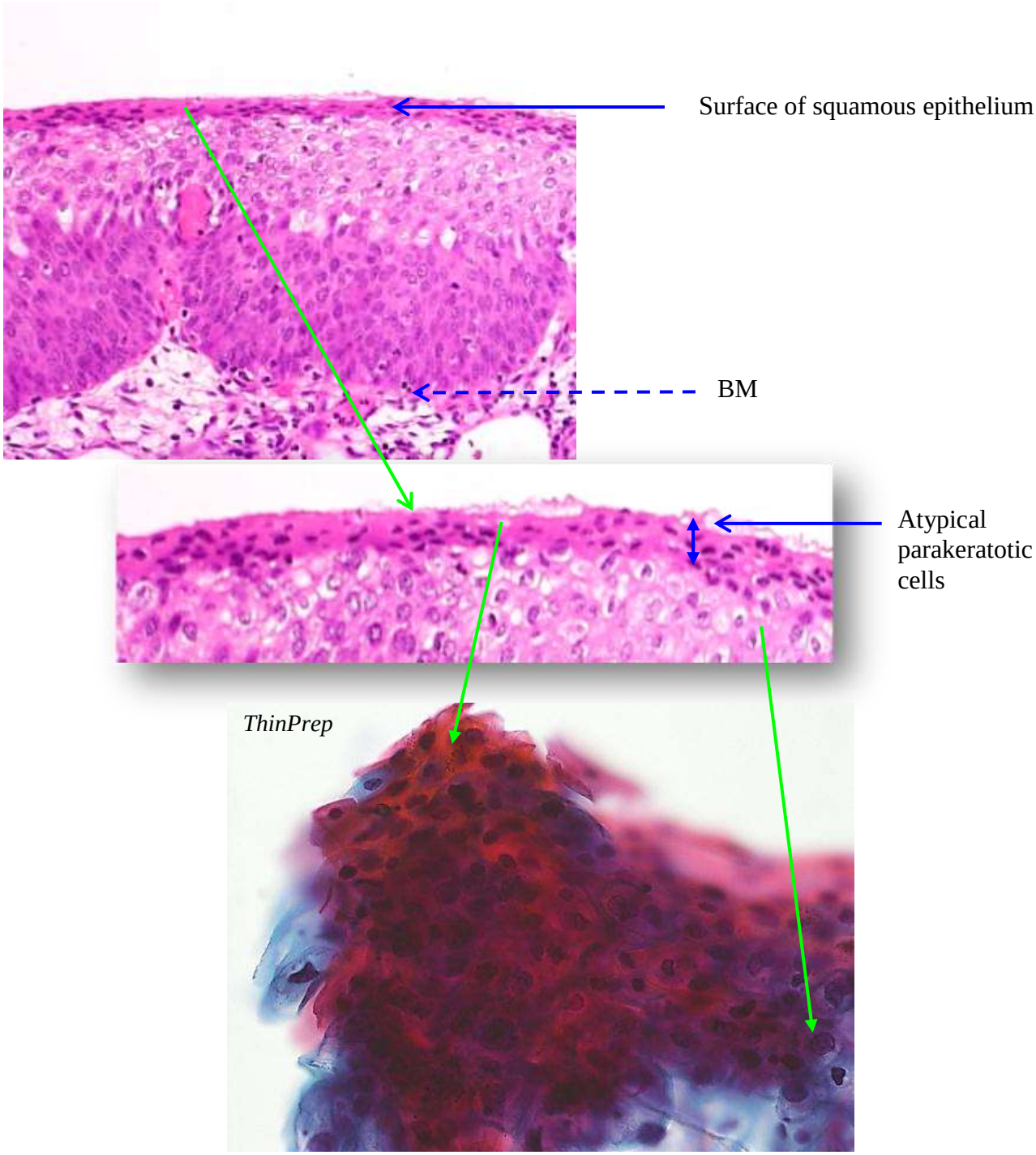
Atypical parakeratotic cells (previously called dyskeratocytes)

Atypical parakeratotic cells occur as small single cells or in groups. Single cells are small keratinised cells with an oval or spindle shape. Groups of atypical parakeratotic cells form dense three-dimensional aggregates made up of several cell layers. The cytoplasm is dense due to the presence of keratin and/or keratin precursors, staining an intense yellow/orange with OG6. The nuclei are usually condensed and pyknotic or hyperchromatic and may be irregular in outline. Although small, the nuclei are larger than the nuclei of superficial cells. These are relatively small cells but the N:C ratio usually remains low with smudged chromatin. The nuclear atypia can vary from mild to marked with obvious changes of dysplasia.

Atypical parakeratotic cells are often present without koilocytes. Atypical parakeratotic cells are distinguished from normal parakeratotic cells by their atypical nuclei. As normal parakeratotic cells mature prior to exfoliation at the epithelial surface, the nucleus condenses and contracts, becoming smaller and darker. This is not true for atypical parakeratotic cells which may already be hyperchromatic. Large dark nuclei within a parakeratotic sheet are therefore a cause for concern, particularly if there are smaller nuclei within the sheet with lighter chromatin staining.

Atypical parakeratotic cells can be seen with any grade of cervical intraepithelial neoplasia (CIN).

Histology: High-grade squamous lesion (HSIL) with associated HPV effect. Koilocytic change is present just below smaller elongated flattened keratinised squamous cells (atypical parakeratotic cells) at the surface. These surface atypical parakeratotic cells show nuclear atypia with karyopyknosis and smudged black chromatin.

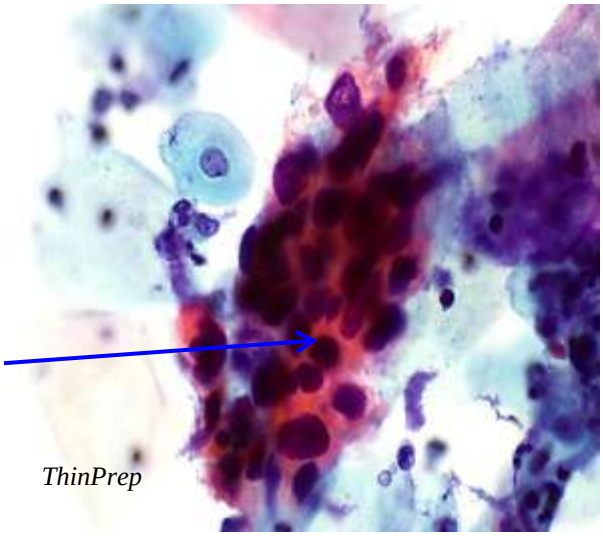


Cytology: Cluster of atypical parakeratotic cells with intense orange cytoplasm associated with koilocytes in adjacent squamous epithelium.

ThinPrep

Single dissociated atypical parakeratotic cells in a case of LSIL. The cytoplasm is intensely orangeophilic because of the keratinisation. In comparison to normal parakeratotic cells, atypical parakeratotic cells have atypical nuclei.

Three-dimensional clusters of atypical parakeratotic cells in a case of HSIL. Note the intense cytoplasmic eosinophilia and the markedly atypical irregular hyperkeratotic nuclei.



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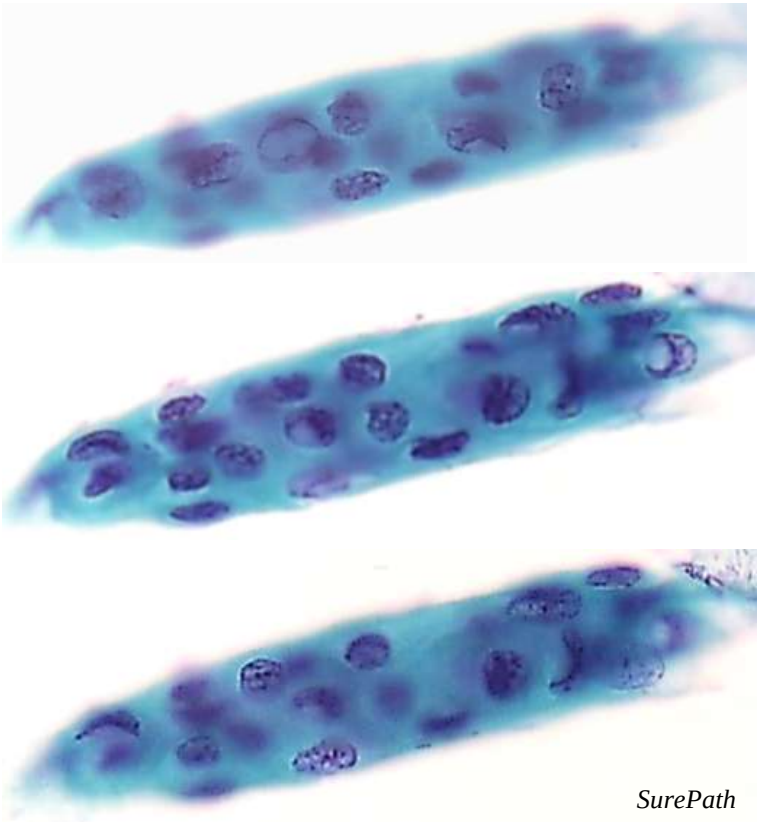


SurePath

A cluster of atypical parakeratotic cells where different focal planes illustrate a three-dimensional aggregate of atypical squamous cells. There is cyanophilic cytoplasm rather than the more common orangeophilia.

2.8.2 HPV: difficulties with cytologic interpretation.

Cytological mimics of HPV effect.

The following are features and situations that may lead to a false positive diagnosis of HPV.

- Cytoplasmic polychromasia and mild perinuclear clearing associated with other infections such as candida or trichomonas.
- Nuclear enlargement and multinucleation after radiation therapy or more rarely, with folic acid or vitamin B12 deficiency.
- Heavily glycogenated or navicular squamous cells with a "koilocytic" appearance (ballooning), particularly when the glycogen has been washed out of the cell during processing.
- Parakeratosis associated with other inflammatory changes causing confusion with atypical parakeratotic cells.
- Cell degeneration resulting in nuclear pyknosis and large cytoplasmic vacuoles and cavities.
- Mature keratinised cells associated with high-grade or invasive keratinising squamous lesions.

Florid HPV effect

Problems with over-diagnosis can occur when HPV effect is pronounced, as may occur with acute recent infection. Florid HPV effect can cause false positive diagnoses, particularly keratinising high-grade squamous lesions. Well-preserved cells with well visualised nuclear detail are essential when evaluating these samples. Any suspicion of high-grade disease should be referred to a pathologist.

The following features may help with the differentiation of atypical parakeratotic cells from high-grade squamous lesions:

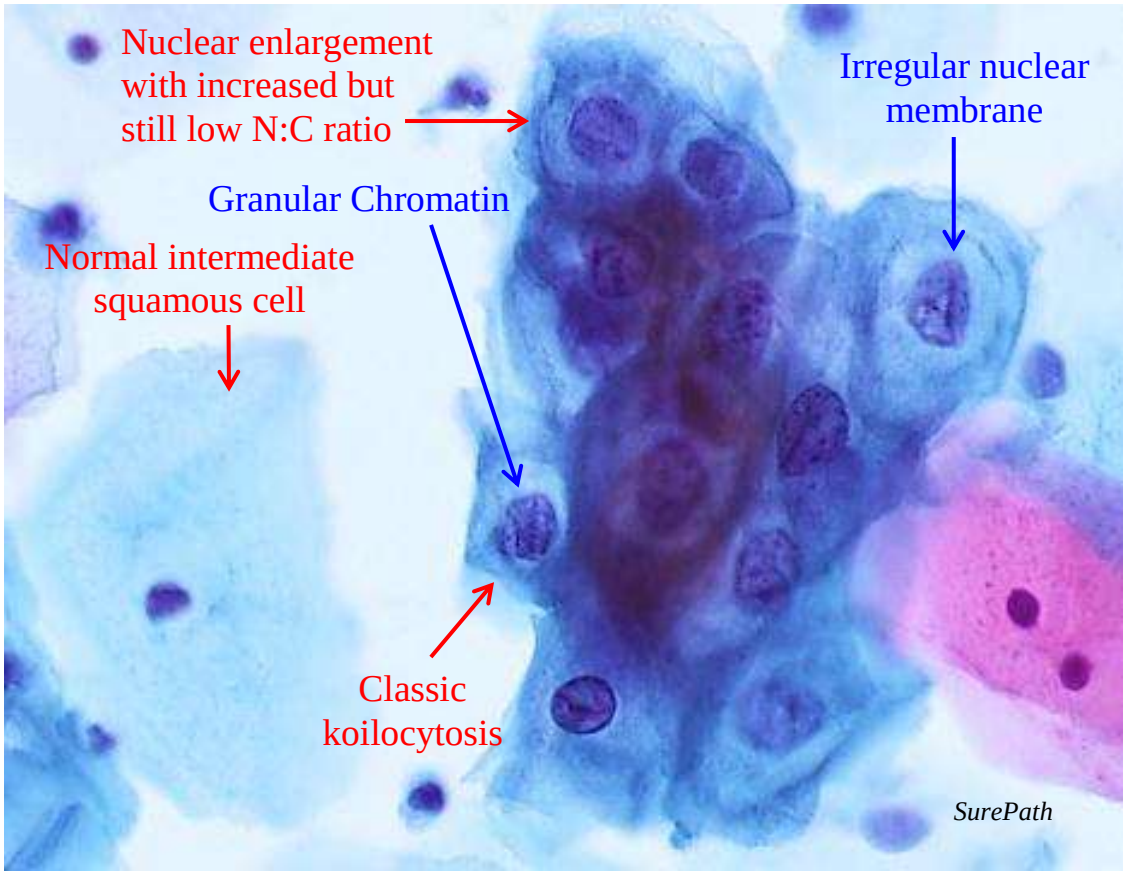
- HPV-affected cells have dark pyknotic nuclei with smudged chromatin whereas the chromatin in high-grade squamous lesions is usually crisp and granular.
- HPV-affected cells show varying degrees of degeneration. Degenerate squamous cells are often seen in invasive squamous cell carcinoma but are uncommon in HSIL.
- Atypical parakeratotic cells are often small in appearance and particularly on low magnification, may give an impression of a high N:C ratio. High magnification examination will show that the nucleus usually occupies no more than half of the cell. This criterion also applies to spindle-shaped cells where the nucleus does not extend to the edges of the cytoplasm.
- The nuclear membrane of atypical parakeratotic cells may look irregular on low magnification, but high magnification will reveal a more wrinkled membrane. Well-preserved, sharp angles in the nuclear membrane contour are suspicious of a high-grade lesion.
- Atypical parakeratotic cells often have polygonal-shaped cell outlines whereas high-grade cells have a more rounded cell outline.
- Tumour diathesis is seen in invasive carcinoma but is not a feature of HPV effect alone.

2.8.3 Cytological appearances of LSIL/CIN 1

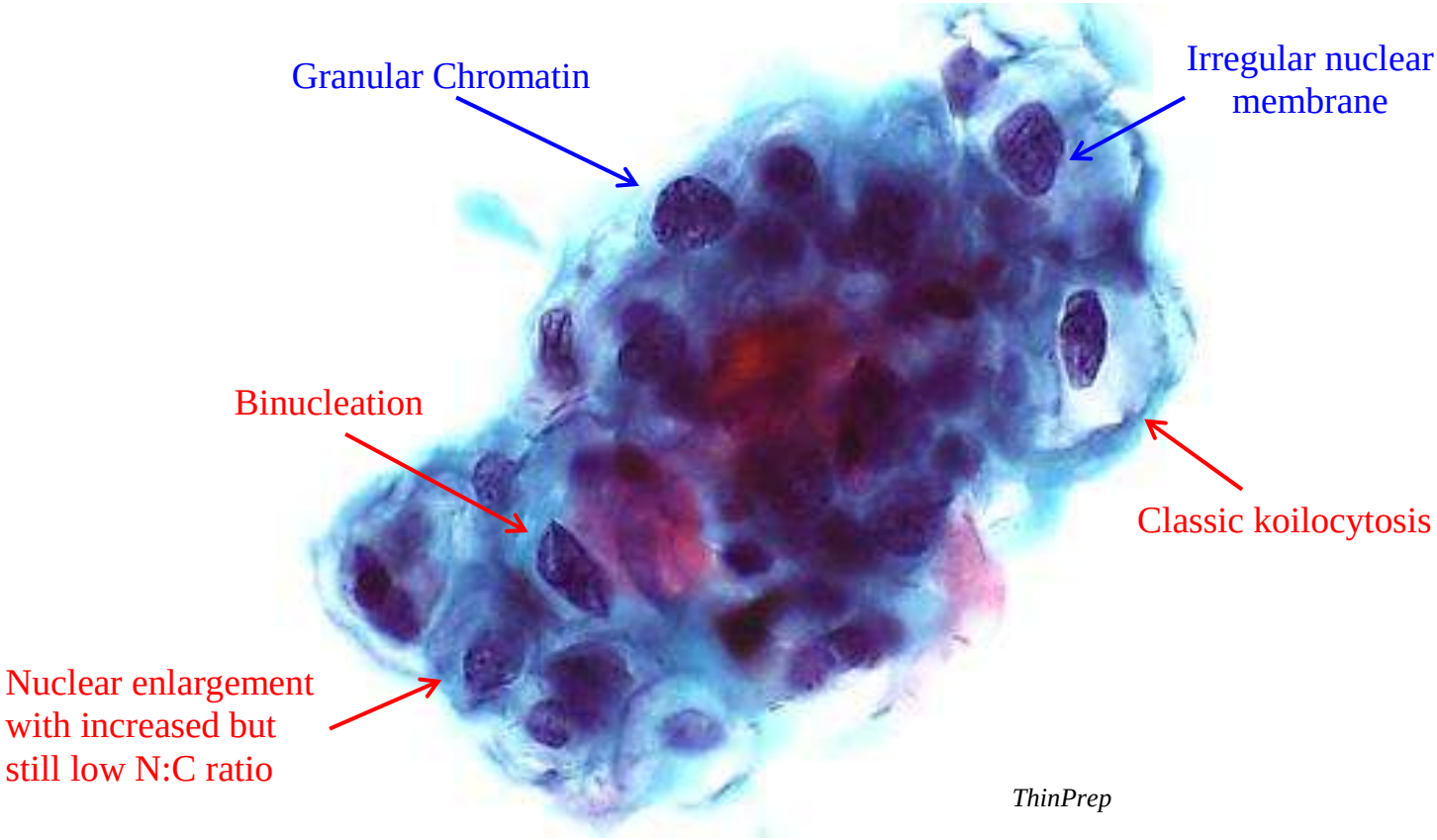
The cytologic features seen in lesions reflecting CIN 1 on histology overlap considerably with those seen in lesions that show HPV effect only on histology. CIN 1 lesions tend to show more nuclear abnormality i.e., greater degrees of enlargement, membrane irregularity and chromatin alteration such as granularity compared to “HPV effect only".

CIN 1/LSIL – Low-grade disease

- Low N:C ratio, typically confined to cells with mature or superficial-type cytoplasm. These mature cells are as large if not larger than normal intermediate/superficial cells. The cytoplasm is abundant with angular borders. Keratinisation may be seen.
- The nucleus is enlarged, typically more than 3x the size of an intermediate cell nucleus.
- Nuclei are hyperchromatic and the chromatin is granular.
- The nuclear membranes are usually irregular and quite marked angularity/cleaving may be observed.
- Binucleation is common.
- Features associated with HPV effect are frequently seen.



LSIL: CIN 1



2.8.4 Atypical Squamous Cells of Undetermined Significance (ASC-US)

There is a spectrum of cytological change for many disease processes and the differences that distinguish different entities are frequently subtle. One common situation where this occurs is when there are atypical cells with low N:C ratios but the changes are not enough to make a definite interpretation of LSIL. Because there is a possibility that there is disease present, we need a way of conveying the non-definitive concern to the clinician in the cytology report so that the woman can be managed in a way that will cover the possibility that she has a lesion. The Bethesda System 2001 provides this by using the term Atypical Squamous Cells of Undetermined Significance (ASC-US).

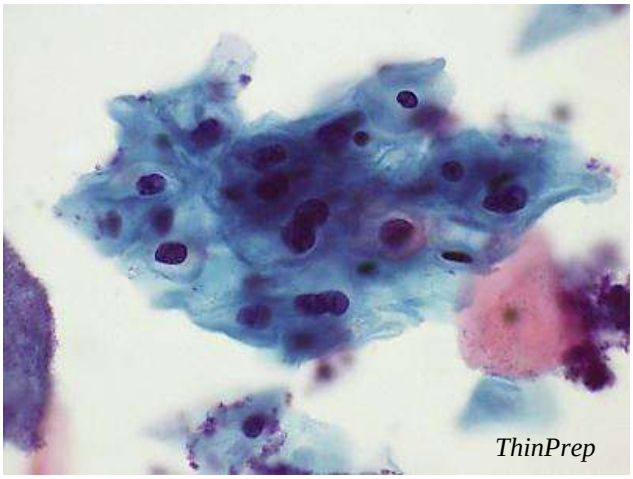
Learning what is "enough atypia" to call a case definite LSIL rather than ASC-US is a skill that cytoscientists acquire with experience. At the other end of the spectrum, experience is also required before practitioners can determine what is enough atypia to justify calling a case ASC-US rather than negative/reactive. This is a more critical call because it results in a much greater difference in patient management. A golden rule is that however minor changes may appear and whether you are primary or secondary screening:

"If in doubt, mark the cells and get a second opinion"

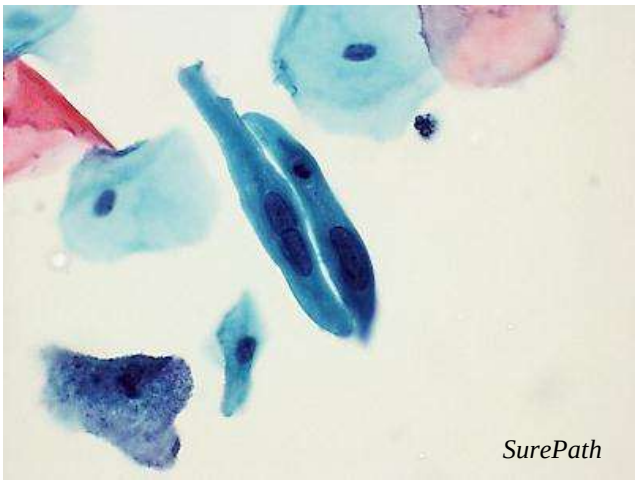
In New Zealand, women with ASC-US cytology are managed in the same way as women with LSIL cytology. An ASC-US or LSIL result is followed by a 12-month repeat cytology sample for a woman under 30 years (with no other abnormal cytology in the previous 5 years) and if the repeat sample is also ASC-US or LSIL then she is referred for colposcopy. HPV testing is not

performed because women under 30 years are frequently hrHPV positive and most HPV infections in this age group resolve without treatment.

For women 30 years of age and older with an ASC-US or LSIL result (and no other abnormal cytology in the previous 5 years), HPV testing is used to determine who is referred directly for colposcopy (HPV detected) and who has repeat cytology at 12 months (HPV not detected). This is a safe way to follow women who have low-grade abnormal cytology but are hrHPV negative, because we know that the risk of a high-grade lesion in these women is very low.



ASC-US: Dark enlarged nuclei without nuclear membrane irregularity and small/moderate perinuclear halos are present. Follow-up cytology was negative.



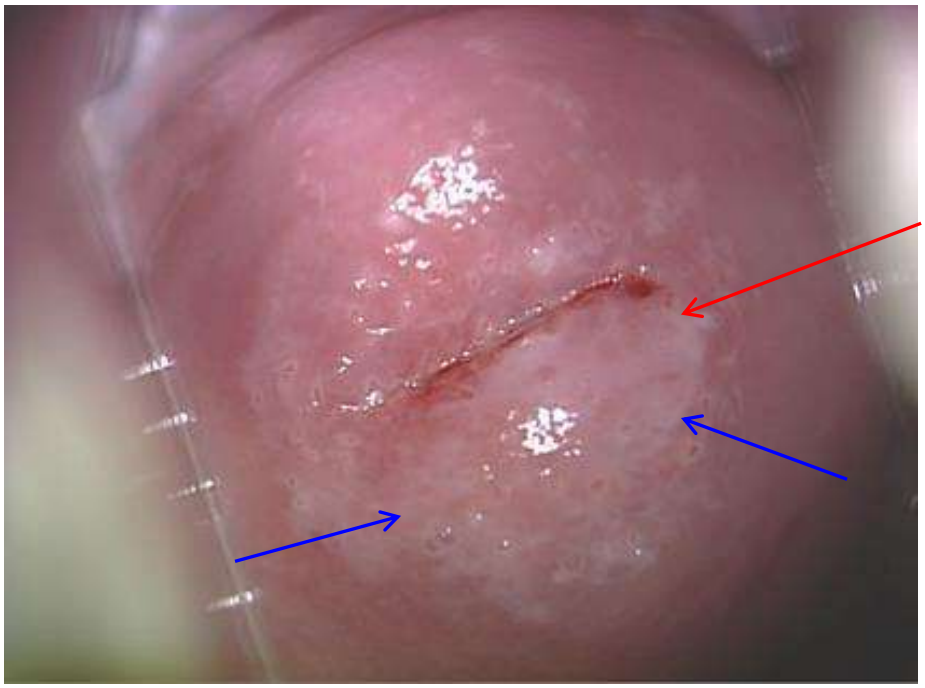
ASC-US: Elongated cells show nuclear enlargement and binucleation with granular chromatin but no nuclear membrane irregularity or perinuclear clearing.

2.8.5 Colposcopic appearances of low-grade lesions

Low-grade condylomata acuminata are papillary lesions and are usually present as multiple discrete warts. As they enlarge, the bases may coalesce to form large papillomatous structures. The colour varies depending on the degree of keratinization and the presence of secondary infection and ulceration. The most common site of infection is the vulva, at the posterior introitus in particular and between the labial folds. The perineum, perianal region, vagina and cervix can also be affected. Visible condylomas are uncommon on the cervix as most cervical lesions are flat (condylomata planum) so are less visible to the naked eye.

Most low-grade lesions involving the cervix are not raised papillary condylomas. The appearances of LSIL (HPV +/- CIN 1) are variable but most are pale flat lesions with indistinct margins that often have an irregular "geographic" border. The lesions may/may not be acetowhite after application of acetic acid. Vascular changes such as punctuation and mosaicism may be present in low-grade lesions as well as high-grade lesions. These colposcopic terms are sometimes used on cytology request forms.

Like cervical cytology, colposcopic assessment of cervical lesions relies on the interpretation of subtle visual changes. Interpretation can be as difficult as it can be in cytology and for this reason, it is very important that colposcopists work closely with cytologists and histologists to achieve the best outcome for women.



Typical LSIL lesion seen at colposcopy. The abnormal area is flat and white with a geographic border (red arrow). Cuffing of abnormal areas around gland openings can be seen (blue arrows). Note: The intensely white areas are light reflecting off the surface of the cervix. *Photograph: British Society of Colposcopy and Cervical Pathology archive*.

2.8.6 Low-grade lesions in histology

Papillary condylomas are sessile papillomas where the epithelium is covered with a superficial layer of atypical parakeratotic cells / koilocytes with perinuclear cavitation. *Spiked condylomas* are characterised by projections of cervical stroma pushing upwards towards the surface producing small spikes covered by a few layers of atypical parakeratotic cells / koilocytes. *Inverted (endophytic) condylomas* show a papillary growth extending into the necks of endocervical glands, replacing and obliterating the columnar epithelium. These types are all uncommon.

Flat condylomas are the most common type found in the cervix. The epithelial thickness may be unchanged, slightly thickened or even decreased. The basal cell layers maintain normal polarity. Squamous cells in the deep epithelial layers have nuclei which are relatively large with finely dispersed chromatin and dense cytoplasm. More superficial squamous cells show the characteristic cytoplasmic clearing of koilocytes. atypical parakeratotic cells are often observed in the most superficial layers. The nuclei of the upper cells show the features seen cytologically in koilocytes, with bi- and multinucleation.

Distinguishing between HPV effect only *vs*. HPV with CIN 1 in histology requires considerable experience at reporting histologic biopsies. Occasionally condylomata are associated with very marked nuclear atypia making distinction from CIN lesions (LSIL or even HSIL) difficult by morphology alone. The use of immunohistochemistry with these histologic biopsies can be of considerable assistance to the histopathologist.

2.8.7 Ancillary techniques with low-grade lesions

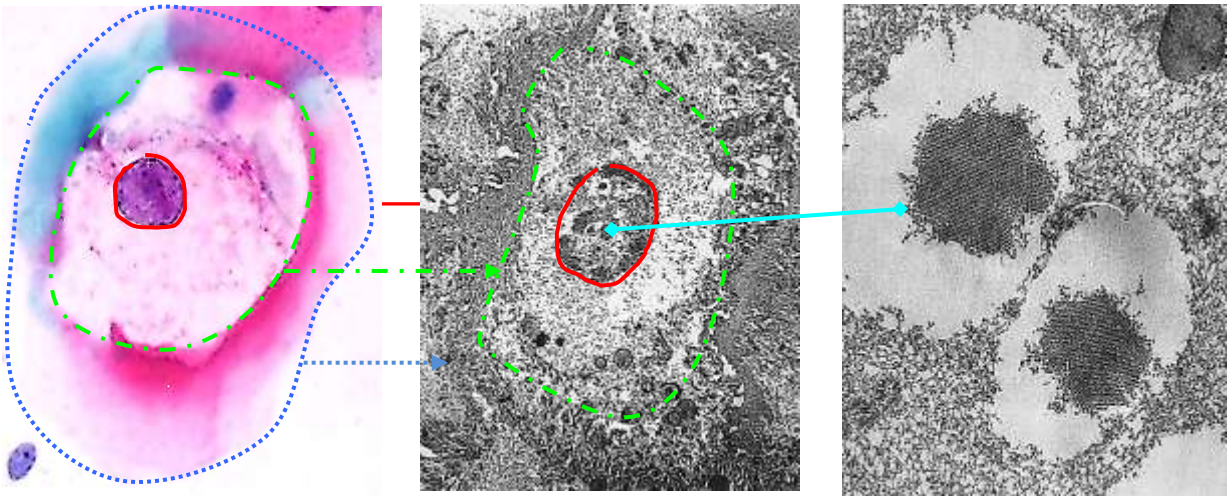
Cervical cytology has successfully played a major role in reducing the incidence and mortality of invasive squamous cell carcinoma of the cervix in countries with cervical screening programmes. More recently, other technologies have become available that can be used in combination with cytology screening or with histologic biopsies, either to increase the effectiveness of screening or as research tools to increase our understanding of the pathogenesis of cervical lesions.

Testing for high-risk HPV was introduced in New Zealand in October 2009 and is funded by the National Cervical Screening Programme for use in three clinical settings. The test is performed on an aliquot taken from the same LBC vial that is used for the cytology sample. The recommendations are discussed in more detail in the *VRPCC workbook: Subsection 4.2.4 Using hrHPV testing in New Zealand Page 275*. The three clinical settings where hrHPV testing is currently used are:

1. Women with a cytology sample showing ASC-US (atypical squamous cells of undetermined significance) or LSIL (low grade squamous intraepithelial lesion), where the patient is 30 years of age or older and where there is no abnormality in the preceding 5 years. The result determines which women are referred to colposcopy (hrHPV detected) and which women have a repeat cytology sample at 12 months (hrHPV not detected).
2. hrHPV testing is used after treatment of a high-grade squamous cervical lesion (HSIL) to ensure that the virus has been cleared by the treatment.
3. hrHPV testing can be requested by specialist colposcopists to assist in the management of women e.g. where there are discrepant cytology, histology and/or colposcopy results.

Immunohistochemistry can be used to demonstrate the presence of viral antigens using monoclonal antibodies but this is not commonly used in routine practice. More usefully, histologic biopsies can be stained using immunohistochemistry to illustrate whether there is over-expression of p16 or Ki67. These stains are widely used in histopathology to confirm and classify cervical intraepithelial neoplasia (CIN). The use of dual staining for p16/ki67 using cervical cytology samples is currently under investigation.

Electron microscopy can identify viral particles but is only used as a research tool.



Cytology of a koilocyte (left) with corresponding transmission electron micrographs (centre and right). Note the cytoskeletal disruption within the halo/cavity, and the peripheral dense cytoplasm. At high magnification, aggregates of actual viral particles can be identified within the nucleus. The peripheral granular material represents nuclear chromatin.

2.8.8 The ASC-US/LSIL Triage Study (ALTS)

The ALTS trial was a major clinical trial published in 2001 that underpins the current use of hrHPV testing in the management of women with low-grade cervical cytology (ASC-US or LSIL). The major hypothesis tested was whether HPV testing could be used reliably to determine the management of women with low-grade cervical cytology. The ALTS trial was undertaken by a collaborative group of investigators in the USA.

The ALTS study looked at three different ways to manage ASC-US and LSIL: immediate colposcopy, conservative management with repeat cytology samples and HPV testing to determine who went to colposcopy.

Methods: The 5060 women enrolled in the study were randomised into one of three arms of the study and were followed closely at 6 monthly intervals for two years to determine how effective each of the strategies was at detecting the presence of cervical lesions. The data for those who had ASC-US cytology at the beginning of the study were analysed separately from those with LSIL at enrolment. The study used ThinPrep LBC cytology and Hybrid Capture-2 for HPV testing. The three management arms investigated were:

- *Immediate Colposcopy*: Women in this arm were referred to colposcopy for biopsy, and treatment if necessary. Repeat cytology was performed every six months.

- *Conservative Management*: Women in this arm were closely followed with repeat cytology every six months. Patients had a colposcopy and a biopsy only if the repeat cytology samples suggested a more severe abnormality than LSIL.

- *HPV Triage*: Women in this arm were managed according to the results of their cytology and an HPV test. If their cytology showed a more severe abnormality than LSIL or if HPV testing was positive for a high-risk HPV subtype, they were referred for colposcopy.

Principal findings of ALTS in ASC-US [(1)]

HPV Testing was confirmed as a viable strategy for determining the management of women with ASC-US cytology.

For women with an ASC-US diagnosis, HPV testing identified 96.3% of women with CIN3+ requiring treatment. About 55% of women with ASC-US cytology were HPV positive so the test was a useful discriminator as about 45 % of women did not need further investigation. For the ASC-US cytology group:

- Approximately 5-10 percent of women with ASC-US already had CIN or (rarely) cancer at colposcopy.

- Of the women who had CIN lesions or cancer at colposcopy, 96.3 percent had a positive HPV test. This means that the HPV test had a 96% *sensitivity* in identifying women with CIN or cancer at colposcopy. (Sensitivity = % of people with disease who have a positive test.)

- Approximately 10-20% of women with a positive HPV test had CIN or (rarely) cancer. The *Positive Predictive Value* of a positive HPV test for high-grade lesions is 10-20%. (Positive

Predictive Value = % of cases with a positive test result who have the disease predicted).

- Approximately 99.5% of the women with a negative HPV test did not have CIN or cancer i.e. the *Negative Predictive Value* of HPV testing in excluding the presence of high-grade disease was 99.5%. (Negative Predictive Value = % of cases with a negative result in whom the disease is absent).

- Approximately 57% of women with ASC-US on their referral cytology sample, had the same or a more serious result on the repeat cytology sample performed when they enrolled in the study. If these "ASC-US-or-worse" results had been used as a basis for assigning women to colposcopy, about 85% with CIN or cancer would have been referred to colposcopy. The sensitivity of repeat cytology using ASC-US+ as the basis for referral to colposcopy was 85%, which is lower than was achieved by HPV testing.

Conclusion: HPV testing is highly sensitive at detecting significant cervical lesions requiring treatment in women with ASC-US cytology.

Principal findings of ALTS in LSIL [(2)]

For women with a LSIL diagnosis, the HPV test is positive in a high proportion of cases.

There were 642 women in ALTS with an initial LSIL diagnosis. In this group:

- 83.6% of women with LSIL were positive for high risk HPV

- Women 30 years of age and older were less likely to be HPV positive.

- Women with three or more lifetime sex partners were more likely to be HPV positive than those with one or two partners.

Conclusion: The high prevalence of HPV infection in women with LSIL limits the usefulness of HPV testing in deciding how to manage LSIL, particularly in women under the age of 30 years.

Additional findings of the ALTS study [(3)] relate to the accuracy of cytologic and histologic sample/biopsy interpretation. A summary of the findings show that experts differ significantly in their diagnoses of cervical abnormalities.

To conduct this analysis, the researchers compared different pathologists' diagnoses of 4,948 LBC and 2,772 biopsy specimens that were collected when women enrolled in ALTS. In each case, the LBC and biopsies were evaluated by an experienced pathologist at the clinical center where the patient had enrolled and by another expert pathologist at an independent quality control center.

- There were substantial differences between the pathologists in interpreting both LBC and biopsies; agreement (reproducibility) was only moderate.

- Agreement on the biopsy specimens was not substantially greater than agreement on LBC.

- Agreement was about the same for biopsy specimens whether they were obtained through punch biopsies or loop electrosurgical excision procedures (LEEP).

Conclusion: Variability among experts in interpreting LBC and biopsies should be taken into consideration when using these interpretations and when developing standards of practice.

2.8.9 Questions

1. Discuss the significance of ASC-US and the precautions to be taken when reporting this entity.

2. What are the differential cytological features of ASC-US compared with diagnostic HPV effect in LBC samples?

3. What specific features in koilocytes, dyskeratocytes or spindle cells would suggest that there might be a lesion that is of higher grade than low grade change only?

4. Describe the use of hrHPV testing for ASC-US and LSIL in New Zealand.

5. Explain the ALTS trial and its fundamental conclusions.

References

- Koss LG, Durfee GR. Unusual Patterns of Squamous Epithelium of Uterine Cervix: Cytologic and Pathologic Study of Koilocytic Atypia. *Annals of New York Academy of Science* 1956;63:1245-61.

- Shroyer KR, Hasey J, Swanson LE, et al. Cytological Diagnosis of HPV Infection. *Diagnostic Cytopathology* 1990;6:178-183.

- Solomon D, Schiffman M, Tarone R. Comparison of Three Management Strategies for Patients With Atypical Squamous Cells of Undetermined Significance: Baseline Results From a Randomized Trial. *Journal of the National Cancer Institute* 2001;93(4):293-9

- Stoler MH, Schiffman M. Interobserver Reproducibility of Cervical Cytologic and Histologic Interpretations: Realistic Estimates from the ASCUS-LSIL Triage Study. *Journal of the American Medical Association* 2001;285(11):1500-05

- The ASCUS/LSIL Study (ALTS) Group. Human Papillomavirus Testing for Triage of Women with Cytologic Evidence of Low-Grade Squamous Intraepithelial Lesions: Baseline Data From a Randomized Trial. *Journal of the National Cancer Institute* 2000; 92:397-402

2.9 CORE TOPIC: HIGH-GRADE SQUAMOUS LESIONS: HIGH-GRADE SQUAMOUS INTRAEPITHELIAL LESIONS (HSIL: CIN 2 and 3) ATYPICAL SQUAMOUS CELLS, POSSIBLE HIGH-GRADE (ASC-H)

2.9.1 Introduction

Reducing the incidence and mortality from invasive cervical carcinoma is the aim of the National Cervical Screening Programme (NCSP). Detecting high-grade intraepithelial squamous lesions by cervical cytology allowing treatment before invasion develops is the most important role that cytology plays in assisting the cervical screening programme to achieve this aim. The variety of different ways that high-grade squamous intraepithelial lesions (HSIL) present in cytology samples needs to be appreciated by cytoscientists and cytotechnologists. In samples where the appearances are suspicious of a high-grade lesion but a definite diagnosis is not possible, the case is reported as "Atypical Squamous Cells, possible high-grade".

2.9.2 High-grade squamous intraepithelial lesions (HSIL): Terminology

In The Bethesda System 2001 the term HSIL incorporates lesions that are reported in histology as CIN 2 and CIN 3. Cytology is not particularly reliable at predicting which cases will show CIN 2 or CIN 3 in subsequent histologic biopsies. Laboratories are at liberty to add a comment to their HSIL cytology reports with a prediction of CIN 2 or CIN 3 and some laboratories chose to do so.

Although laboratories report all high-grade lesions as HSIL, there is merit in discussing the cytological appearances predictive of CIN 2 and CIN 3 separately when learning to appreciate the range of appearances of HSIL in cytology samples.

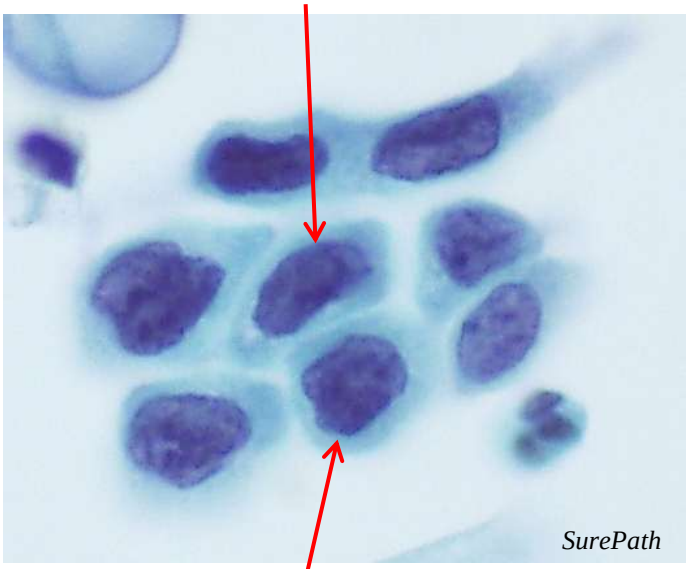
2.9.3 HSIL: Cytomorphology predictive of CIN 3.

Cells can be single, clustered, in crowded groups or in syncytia (sheets). The loss of cell cohesion which occurs in neoplasia usually results in the presence of at least some single HSIL cells, but this is not always the case and only crowded sheets of HSIL may be present.

Nuclear variability is central to the diagnosis of HSIL. Within the abnormal cell population in any one sample, nuclei may vary in size, shape, chromatin pattern and/or nuclear membrane characteristics. Not all features will be present in every case. Comparison of different cells and cell groups is essential to confirm that nuclear variability is present.

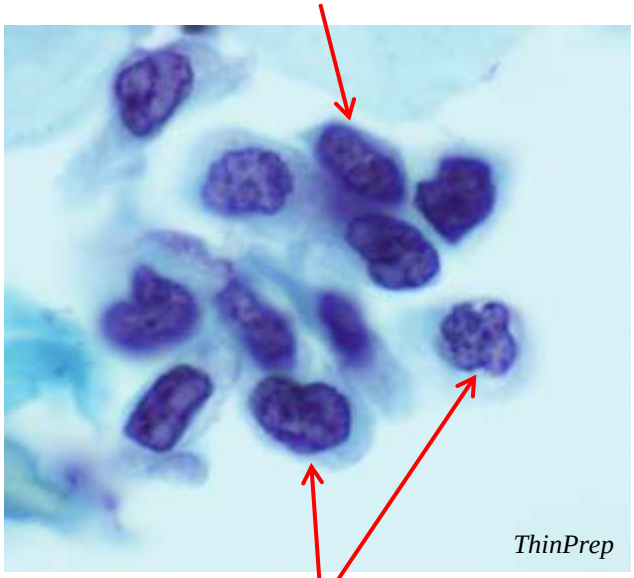
- There is a marked increase in N:C ratio with the nucleus occupying >65% of the diameter of the cell.
- Nuclei are usually hyperchromatic.
- The nuclear membranes are usually thickened. Irregularity or angularity of the nuclear membrane may be marked or subtle, particularly in metaplastic cells.
- The chromatin is finely or coarsely granular and evenly dispersed within the nucleus.
- Macronucleoli are not a feature, but small nucleoli may be observed (2-5% of cases).
- The cytoplasm can be squamoid, delicate, densely metaplastic or keratinised.
- The cytoplasm occupies a small proportion of the cell and only a rim may be seen.

Granular evenly distributed chromatin



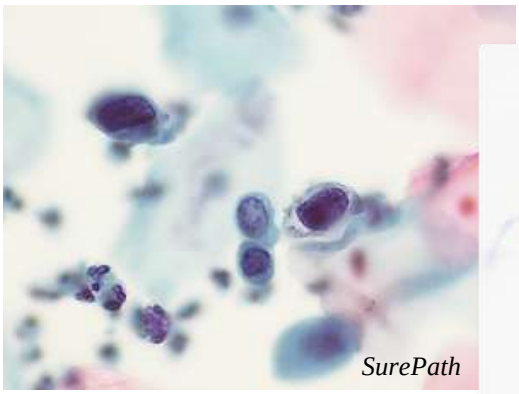
Irregular nuclear membranes

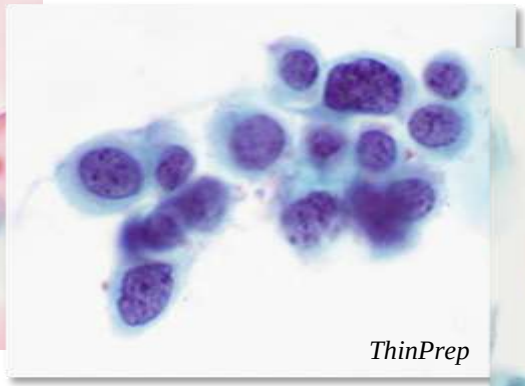
Very high N:C ratios

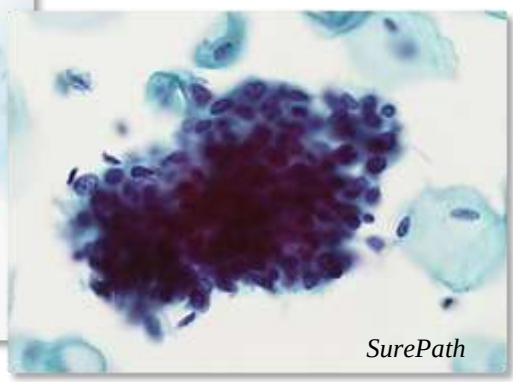


Variation in nuclear size, shape and chromatin pattern

High-grade squamous intraepithelial lesions consistent with CIN 3







HSIL can present as single cells, in small clusters or in large thick groups

Patterns of presentation of CIN 3

Ron Bowditch (an Australian cytoscientist) produced some ground-breaking work during the 1990's identifying the different ways that HSIL presents in cytology samples. His work describes high-risk patterns that should alert the cytoscientist/cytotechnician to consider the possibility of a high-grade diagnosis and to hunt carefully for diagnostic material. He is particularly acknowledged for his work defining the diagnosis of HSIL in crowded sheets. The

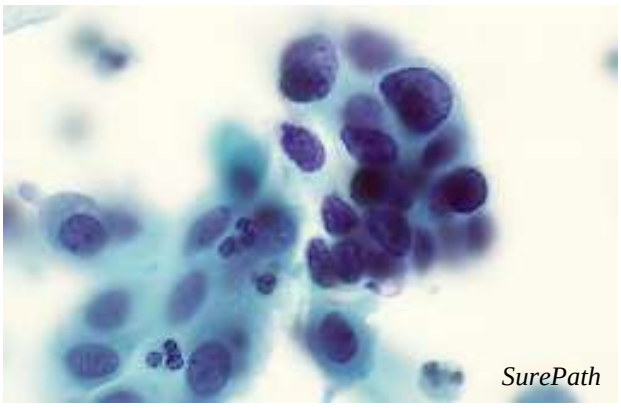
following is based on his approach to the diagnosis of CIN 3, emphasising the three main ways that HSIL presents in cytology samples.

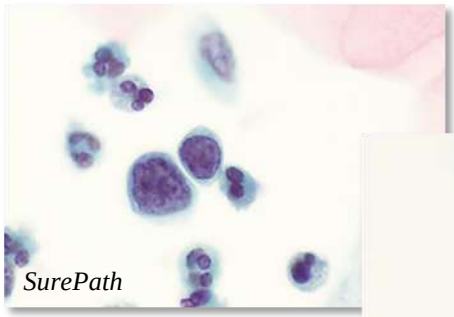
1. Metaplastic CIN 3
2. Crowded sheets of CIN 3
3. Parakeratotic CIN 3

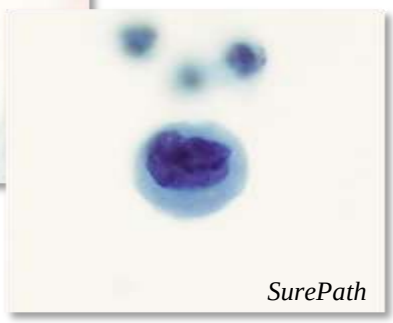
1. Metaplastic CIN 3

Metaplastic CIN 3 refers to the presentation of HSIL as single cells and small groups with cytoplasmic features resembling the cells of immature squamous metaplasia. Features to note:

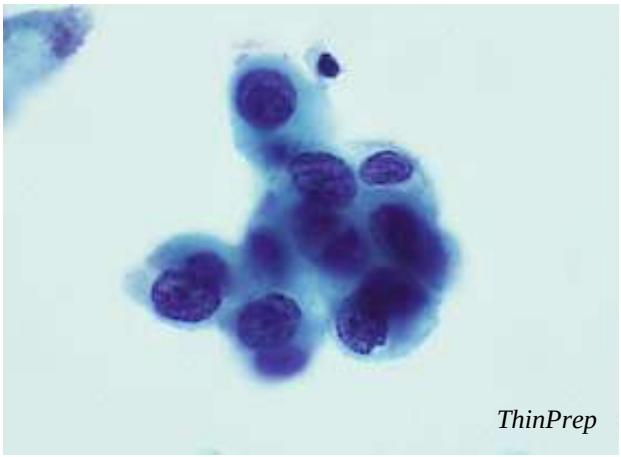
- The cells are often single or in small groups of 2-5 cells, often dispersed across the slide and therefore individually inconspicuous. Cell comparison is difficult particularly when the total number of abnormal cells present is small, because the cells don't lie together.
- Nuclear variation is the key to the diagnosis:
  - Nuclei vary in size, chromatin, structure, chromasia, border thickness and shape
  - Abnormal nuclear shapes are usually present.
  - Cytoplasmic density and texture varies from fine and delicate to thick and squamoid, reflecting the transformation zone origin. May see keratohyaline granules.

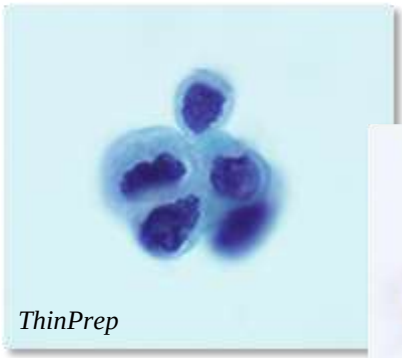


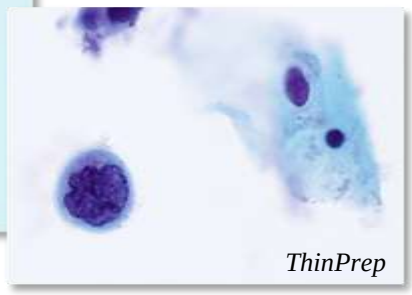




HSIL: Metaplastic CIN 3





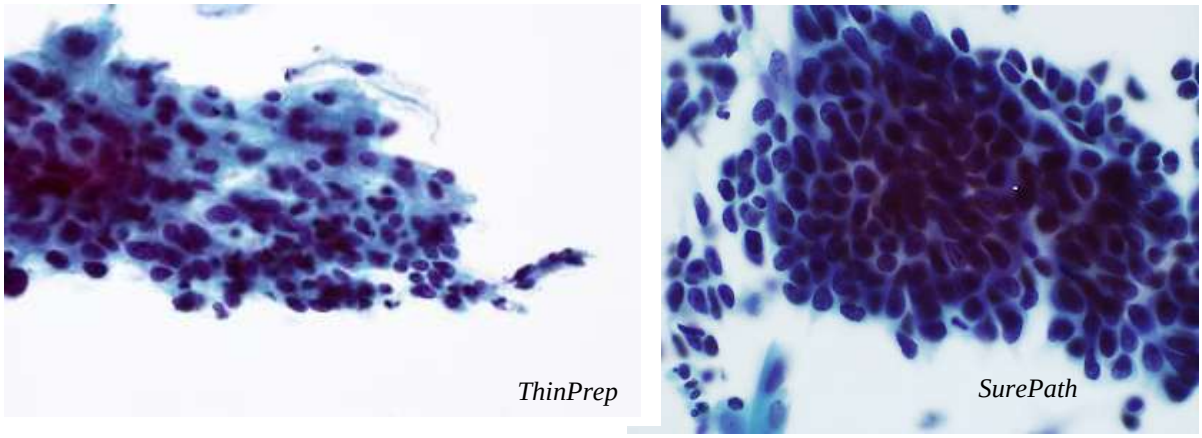


Immature metaplastic cells can be very difficult to interpret. HSIL is easy to miss with this high-risk pattern.

- Variation within the cell population is very important. A single metaplastic CIN 3 cell is not diagnostic. Need to establish variation within a population of abnormal cells.
- Be suspicious of HSIL even if the cells individually look normal as there may only be single inconspicuous HSIL cells scattered through the sample. Compare the nuclei for variability even if the cells are widely dispersed.

2. Crowded Sheets of HSIL

HSIL may present in crowded sheets, often referred to as hyperchromatic crowded sheets/groups (HCG's). There may not be any single HSIL cells in the sample. Sheets or tissue fragments are removed by brushing or scraping the lesion when the cytology sample is taken, so these fragments are in effect, microbiopsies. High power examination is essential for all crowded groups especially those that are hyperchromatic with overlapping nuclei.



CIN 3 presenting in hyperchromatic crowded groups. Focusing through the group is essential to assess the morphology.

This high-risk pattern is well documented as a cause of false negative cytology reports, particularly if the cells are small and only show mild to moderate hyperchromasia. The nuclear changes can be subtle and on screening magnification the chromatin may appear pale and difficult to distinguish from normal chromatin. The cells have a high N:C ratio and may be metaplastic in appearance. Cell dissociation can help if present, and high magnification will show irregular nuclear membranes and variable granular chromatin, albeit lightly stained.

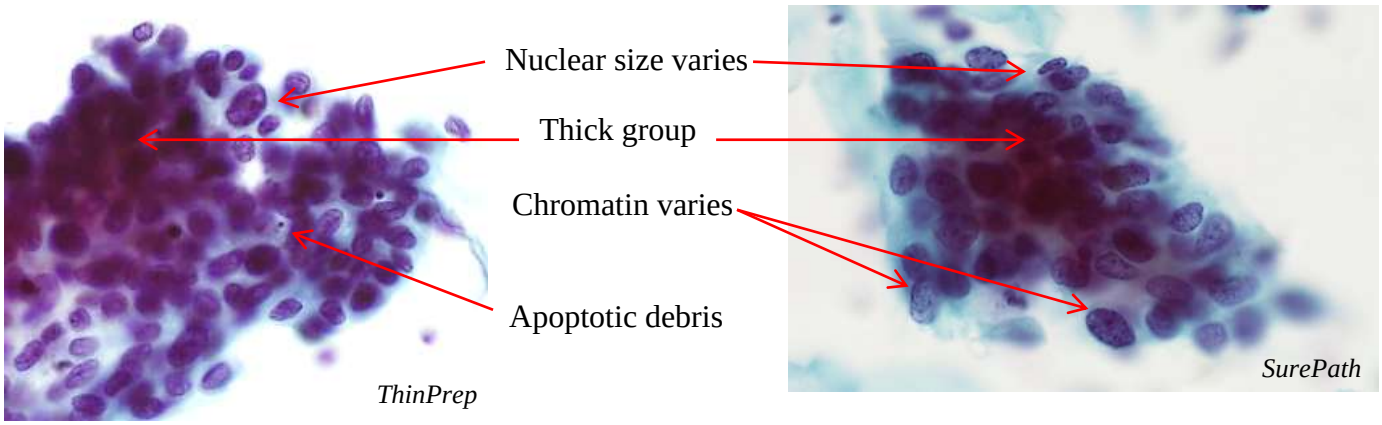
The cytologic assessment of crowded sheets:

- Crowded sheets are easy to detect at screening as they contain 20 to thousands of nuclei. Nuclei are very crowded and overlapped. Sheets are usually at least 4 nuclei thick in HSIL. To assess how thick a group is, carefully focus through the group using high magnification and count through each plane of focus to determine the depth of the sheet.
- Every crowded cell sheet or thick cell group should be assessed as potentially high-grade. Individual cells can often be seen best at the edge of cell sheets, but much valuable information can also be obtained by focusing up and down through sheets.

The key cell parameters within cell groups that should be carefully examined to ascertain if there is a **loss of predictability from cell to cell associated with neoplasia** are:

- Cell-to-cell spacing and polarity
- Nuclear chromasia
- Nuclear structure
- Nuclear membranes
- Karyomegaly
- Nucleolar variation and enlargement
- Embedded mitoses i.e. mitoses in the middle layers of the group as focus through it
- Apoptosis

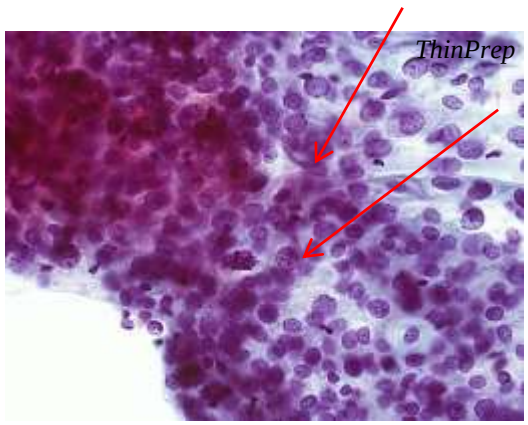
The thickness of the sheet is also a very useful parameter to assess (4 or more cell layers is high-risk) and should be combined with the features above. A consistent approach is essential to minimise false negative samples.



HSIL in hyperchromatic crowded groups

Unpredictable variation is the key to identifying a crowded group as abnormal

- Variation in nuclei: size – more than 2 to 3x variation is suspicious
  chromatin structure – coarse or fine, crowded or open
  nuclear border – thick, or thin, or absent
  chromasia – larger nuclei darker than smaller nuclei
  Note: nuclei in CIN 3 sheets are usually round or oval – the nuclear shape is not often abnormal in a crowded sheet.
- Variation in other cell features: degree of maturation reflected by the degree of keratinisation, cell size and shape and N:C ratio.
- Changes in cell polarity within groups may be helpful but are not always present. May include jumbled cells, flattened cells at one edge.
- Apoptosis may be present, where the granules vary in size and colour. (Need to distinguish from degenerating neutrophils and keratohyaline granules).
- Mitoses are very significant, especially if embedded in a sheet. Assess by focusing up and down through the sheet to determine if the mitosis is in the middle.



Mitoses in a sheet of HSIL

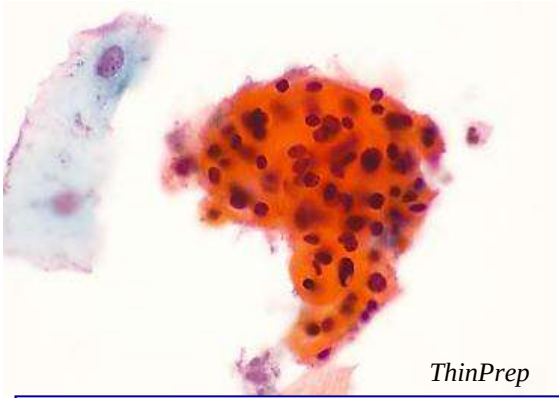
- Sticky big bare nuclei especially if variable in size, may originate from HSIL and should prompt a thorough search for diagnostic cells.
- "Tennis racquet cell" is a cytomorphological description of a cell with a tapering body, a single oval nucleus in the expanded end and a thin rim of cytoplasm surrounding the nucleus. These cells are known to be present in SurePath cases of SIL, hanging from the edges of hyperchromatic crowded abnormal cell groups. It is hypothesized that the presence of "tennis racquet cells" in SurePath preparations may be related to the processing of the sample or the cellular rotation in the fluid medium.

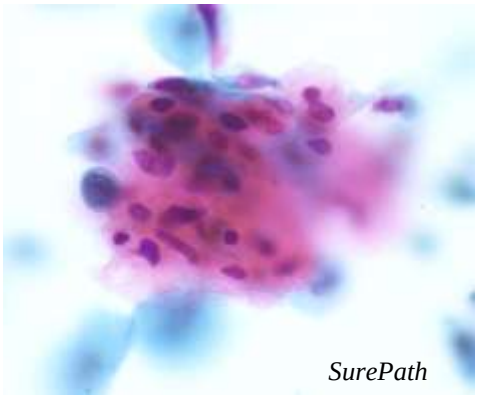
3. Parakeratotic CIN 3

HSIL is often missed when this is the dominant pattern of HSIL

- Maturation at the surface of CIN 2 or CIN 3 can result in shedding of a variety of small mature and hypermature squamous cells with various degrees of keratinisation. Some may present as pale miniature squamous cells, others as dense green or orange keratinised cells.
- These are small cells but have relatively more cytoplasm than is usual with HSIL.
- Nuclei may be small, condensed or pyknotic: parakeratosis or miniature squamous cells are suspicious of HSIL if the nucleus is retained. Look for nuclear variability. May lack the standard abnormal criteria of nuclear enlargement and coarse chromatin. Nuclear size variation may be the only abnormal nuclear feature, especially if pyknotic.
- When comparing pyknotic and non-pyknotic nuclei for variation, allow for the fact that nuclei shrink to 1/3rd their pre-condensation diameter when becoming pyknotic, A smaller nuclei will be darker than a larger nucleus in the process of normal nuclear pyknosis, so larger darker nuclei are a cause for concern.
- May co-exist with small scattered immature metaplastic HSIL cells. These may be inconspicuous and a careful search for them is essential to establish a diagnosis of HSIL, particularly in cases which would otherwise be reported as LSIL or ASC-H.
- Dissociation of cells is usual with HSIL so look for loose groups and many single cells. Pale parakeratotic HSIL cells may only be present as scattered single cells, especially in LBC preparations so be alert to this as a high-risk pattern.

Remember: Parakeratosis and hyperkeratosis may occur in every degree of abnormality in the squamous spectrum, from minimal reactive atypia to HPV through to squamous cell carcinoma. **Any parakeratotic change must be scrutinised with great care.** In benign parakeratosis, you usually see cohesive tissue fragments with normal predictable nuclei and normal N:C ratios.

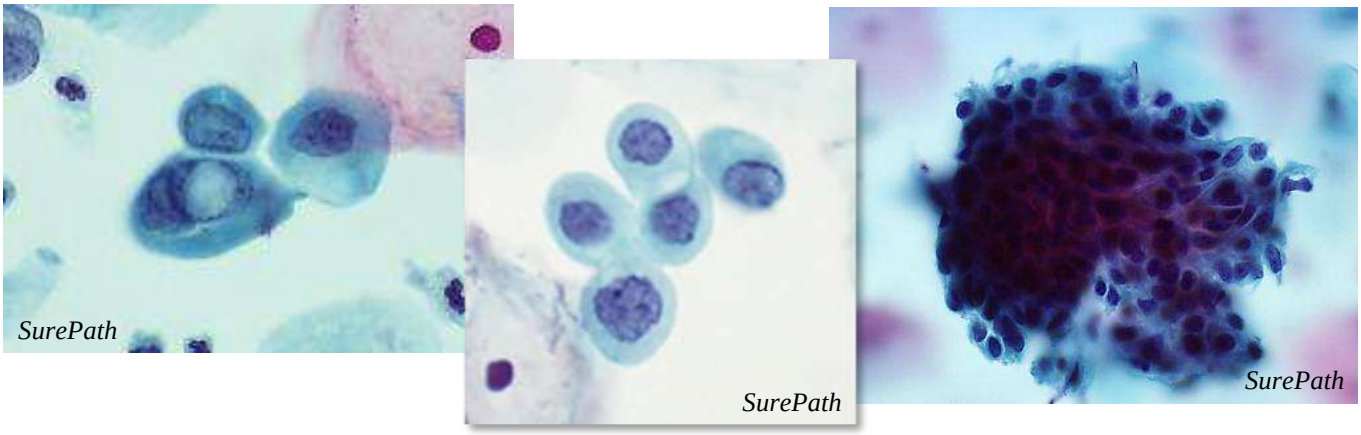




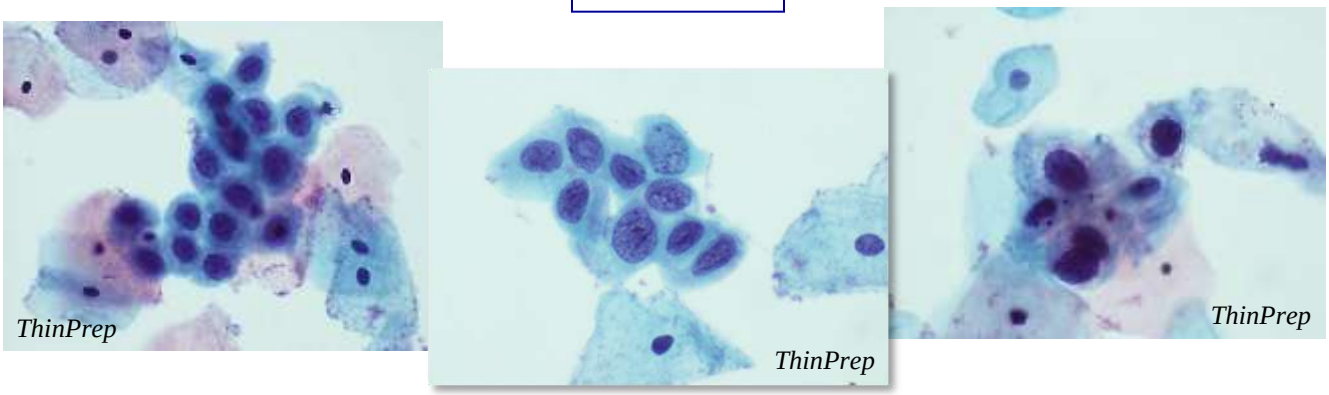
Parakeratotic HSIL: The keratinised cytoplasm may be more abundant than is usually seen in HSIL. Look for nuclei that are larger and darker than other nuclei in the group.

2.9.4 HSIL: Cytomorphology predictive of CIN 2.

- Increased N:C ratio but less so than with CIN 3. The nucleus occupies 50-65% of the diameter of the cell. The nuclear features otherwise show the same features as CIN 3.
- single CIN 2 cells are particularly difficult to detect when the abnormal cells are widely dispersed as the N:C ratios can be similar to normal metaplastic cells.
- CIN 2 often accompanies CIN 1 so a careful search for HSIL cells is always warranted in samples with LSIL.
- When the abnormal cells are keratinised, the distinction between CIN 2 and CIN 3 is not reliable by cytology.



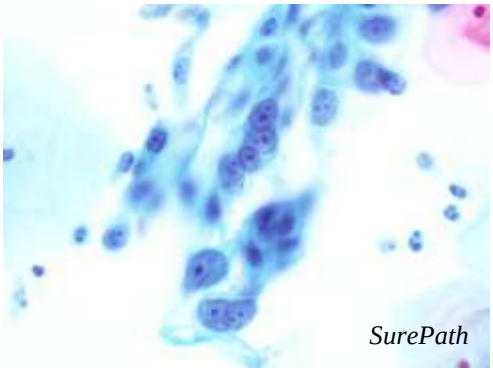
HSIL: CIN 2



2.9.5 Differential diagnostic issues with HSIL

1. Distinguishing **HSIL from reactive atypia.**

- HSIL may resemble reactive metaplasia or repair. HSIL is suggested by fragile foamy disintegrating cytoplasm, bare nuclei, single cells or very high N:C cells. Nuclear variation and densely hyperchromatic nuclei are also important.
- Careful examination of the nuclear membrane in benign conditions should show no irregular cleaved nuclear membranes
- Nucleoli or micronucleoli are common in reactive atypia. They are not a common feature of CIN but may occur with



HSIL with prominent nucleoli

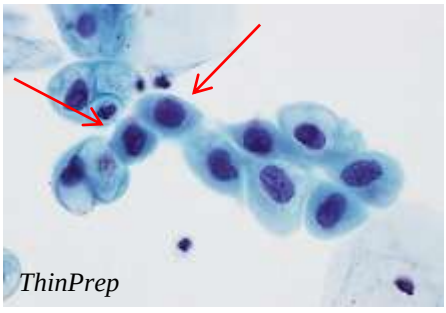
HSIL, usually as micronucleoli and they are frequent in invasive squamous cell carcinoma. The presence of nucleoli must be carefully assessed, considering conditions such as invasive malignancy or reactive/inflammatory changes. Macronucleoli with neoplastic cell changes are generally associated with invasive lesions.

- Mitoses are not common in CIN cells and cannot be relied on before making a diagnosis of HSIL. They can be seen in florid reactive processes. When present in CIN lesions, they are generally associated with high-grade lesions. Their presence in crowded sheets may be helpful to alert one to the possibility of HSIL if they are "embedded mitoses" i.e. they lie within the sheet when focusing up and down, not in either the uppermost or lowermost plane of focus.
- Apoptotic material in crowded sheets is another high risk alert for possible HSIL. Degenerating neutrophil lobes can look similar and are common in cervicitis. On high-power, apoptotic debris often has variation in staining within fragments with some parts being lighter/darker than others. A degenerating neutrophil lobe is uniform in colour.
- Multinucleation is a relatively common feature in HSIL but is nonspecific so not helpful diagnostically. Multinucleation is common in LSIL and can be a feature of reactive conditions.

	Features of HSIL	**Features of reaction/repair (only)**
Arrangement in groups	Appears haphazard, loss of polarity	Flat sheet with retained polarity
Single cells	Presence of 3D asymmetrical abnormal nuclei	Single cells may be present but they lack nuclear abnormalities
Nuclear/cytoplasmic ratio	Increased markedly in favour of nucleus	Presence of abundant cytoplasm
Naked nuclei	Often present	Rare finding
Nuclear membrane	Irregular outline	Smooth outline
Variation between adjacent nuclei	Variation usually includes size, shape, chromasia	Lacks variation, nuclei usually look similar
Apoptosis and mitosis	Abnormal mitotic figures and apoptosis	Normal mitotic figures may be present, no apoptosis
Chromatin	Often clumped and granular	Finely granular, evenly distributed

2. Diagnosing **HSIL in the presence of LSIL**

- Most cases of HSIL will show a range of cells. Cells consistent with CIN 3 are often accompanied by CIN 1 and 2 although a lack of LSIL should not deter one from considering a diagnosis of HSIL.
- When LSIL is predominant in a sample, it is easy to miss a small number of HSIL cells that are also present. Ron Bowditch taught that LSIL is a "high-risk" pattern for HSIL and that all cases of LSIL need to be carefully screened for small numbers of HSIL cells. If there is LSIL as well as cells that are suspicious but not diagnostic for HSIL, the case is


ThinPrep

Range of N:C ratios mainly LSIL but two cells (arrowed) are in the CIN 2 range. Histology showed CIN 3.

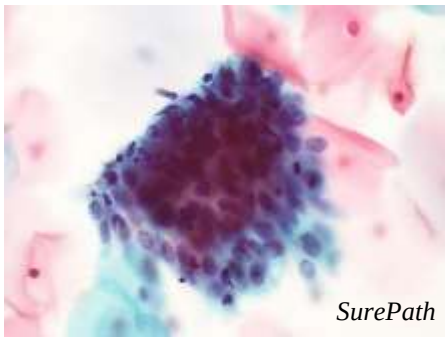
reported as LSIL and ASC-H (refer discussion below under ASC-H).

- If LSIL and only crowded sheets suspicious for HSIL are present, then a report of ASC-H may be more appropriate than a definite diagnosis of HSIL, particularly if this is a first time abnormal diagnosis. The crowded sheets need to be assessed independently from the LSIL cells to ascertain the risk or level of certainty about the possible presence of HSIL.

IF YOU FIND AN ABNORMALITY, ALWAYS CHECK TO SEE IF THERE IS A LESS OBVIOUS BUT MORE SIGNIFICANT ABNORMALITY PRESENT TOO.

3. Distinguishing **HSIL from AIS**

- Need to distinguish HSIL with some glandular features from Adenocarcinoma in situ (AIS). Sheets of HSIL are usually more than 3 nuclei thick and haphazardly arranged whereas AIS is generally 3 or less nuclei thick with a more ordered arrangement.
- Occasionally squamous groups may have an endocervical-like arrangement particularly if the neoplastic cells extend down into endocervical glands (referred to as glandular involvement). Again, the nuclear detail requires high power examination.

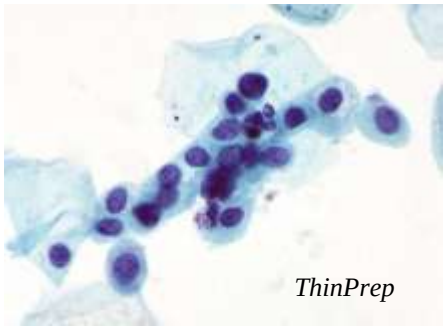


HSIL involving endocervical glands. This is not AIS.

	Features of HSIL	**Features of AIS**
Arrangement of abnormal cell groups	Syncytial sheets, not crowded, more dyshesive	Rosette, strips, crowding, stratification, feathering
Nuclear appearance	Round to oval, lacks moulding	Elongate and moulding with adjacent nuclei
Nuclear membrane	Irregular, uneven, presence of 3D asymmetrical single nuclei	Often smooth nuclear membrane, but irregular membrane can be seen; lack of single nuclei with abnormal 3D features,
Chromatin	Coarse, unevenly distributed	Evenly distributed, can vary from fine to coarse
Cytoplasm	Scant, homogeneous	Scant, vacuolated

4. Distinguishing **HSIL from Atrophy**

- Crowded parabasal cell sheets in atrophic samples make the detection of HSIL difficult. Look for localised nuclear variability, hyperchromasia, coarse chromatin and numerous prominent chromocentres. Changes of HSIL can be very subtle. A repeat sample after oestrogen may help and in indeterminate cases, a report of ASC-H may be used.



These worrying cells were atrophy only.

	Features of HSIL	**Features of Atrophy (only)**
Cell population	Abnormal cells appear as a distinctly different cell population to the rest of the normal cells in the preparation (dual population)	LBC preparation appears to be comprised of one cell population
Arrangement	Single and syncytial sheets with loss of polarity	Single and flat sheets with retained polarity
Nuclei	Marked variation in chromasia, size and chromatinic distribution between adjacent nuclei	Monotony of nuclear features in groups
Chromatin	Granular, uneven distribution	Evenly distributed usually finely granular
Nuclear membrane	Irregular, 3D asymmetrical single nuclei	Even, smooth
Cytoplasm	Scant, high N/C ratio	May be abundant in well preserved cells but often lacking entirely due to fragility
Apoptosis and abnormal mitoses	May be identified in some abnormal groups	Absent

5. Distinguishing **HSIL from endometrial cells**

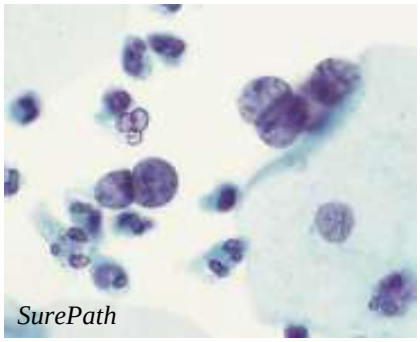
- HSIL can present as tight compact clusters and sheets of very small dark high N:C ratio cells, resembling endometrial cells. Nuclear size variation may be the only abnormality present but can be difficult to appreciate in very small cells.

	HSIL (Small cell type)	Endometrial Cells (Benign)
Cell presentation	Single and loose syncytial groups with loss of polarity	Single and 3D clusters
Cell cohesion	Single-lying HSIL cells present	Loosely cohesive stromal cells present
Stromal histiocytes	Usually lacking	Often in close proximity to glandular groups
Nuclear uniformity	Careful examination shows variation between adjacent nuclei	Uniform-appearing nuclei with predictable features
Nuclear borders	Asymmetrical 3-D nuclear structure abnormality (focus up and down)	Smooth nuclear borders
Nuclear size	Nuclei may also be similar in size to intermediate squamous cell nucleus	Nuclear size comparable to intermediate cell nucleus
Nuclear shape	Nuclei round to oval	Bean-shaped nuclei present in clusters
Nuclear membrane	Unevenly thickened and irregular outlines common	Nuclear membrane may be thickened, have longitudinal grooves, spindle configurations but usually even/only undulating outline
Chromatin	Chromatin coarsely granular and unevenly distributed	Granular and evenly distributed chromatin

Nucleoli Chromocentres	Absent but fine nucleoli may be seen	Chromocentres present
Cytoplasm	Homogeneous, may be scant and ill defined	Scant and vacuolated when present
Cell edges	Lack of spindly configuration	Some cells at edges may appear spindly
N:C ratio	High N:C ratio in favour of nucleus	High N:C ratio in favour of nucleus

6. Other comments:

- **Atypical free nuclei** are sometimes observed in samples and are seen in some cases of HSIL. When clustered, they are referred to as "sticky bare nuclei". These nuclei are not diagnostic of HSIL but are a high-risk alert and should prompt a thorough search for well-preserved intact abnormal cells. Degenerate endocervical nuclei can look similar. Compare the bare nuclei looking for variation as this is more likely to be present in HSIL than in degenerate endocervical nuclei.
- Tubal metaplasia can be confused with HSIL. Look for ciliated cells to identify tubal metaplasia.
- The background of the sample does not usually show tumour diathesis/necrosis or old blood/fibrin in HSIL. If these are present, the possibility of an invasive lesion needs to be considered.



Sticky bare nuclei in HSIL.

2.9.6 Atypical Squamous Cells, possible high-grade (ASC-H)

This is an important category of reporting and accounts for approximately 0.7% of cervical cytology reports in New Zealand. A report of ASC-H is used in situations where there is a cytologic suspicion of a high-grade lesion, but a definite diagnosis is not possible. These patients are all referred for colposcopic evaluation. At colposcopy, about 80% have a discernible colposcopic abnormality that is biopsied. The Positive Predictive Value (PPV) of a cytologic result of ASC-H for predicting a histologically confirmed high-grade lesion is about 45%. i.e. about 45% of cases reported as ASC-H will be confirmed as having a high-grade lesion on biopsies taken at colposcopy.

The need to use the ASC-H category can be related to the number of abnormal cells present or to the morphologic appearance. There may only be a few scattered abnormal cells in the sample, with high N:C ratios, chromatin abnormalities and nuclear membrane irregularities. If the abnormal cells are sparse and a confident diagnosis of high-grade SIL cannot be made, the case is reported as ASC-H. In other cases, the sample may contain definite LSIL but also occasional cells demonstrating features suggestive of a high-grade SIL. In this situation a report of LSIL and ASC-H can be used. Cases with morphology that is difficult to interpret include samples with crowded clusters of hyperchromatic cells that are difficult to visualise but could represent a high-grade lesion. Because using ASC-H reflects a suspicion of disease rather than a definite prediction, its use can be very subjective. These cases can be some of the most difficult to report in cervical cytology.

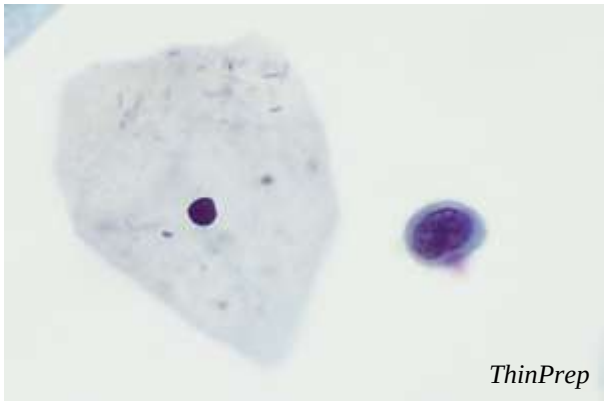
There are differences between ThinPrep and SurePath with regards to how ASC-H cells present in the sample. As discussed in the *VRPCC Core Topic 1.3 LBC Platform, Page 32.*

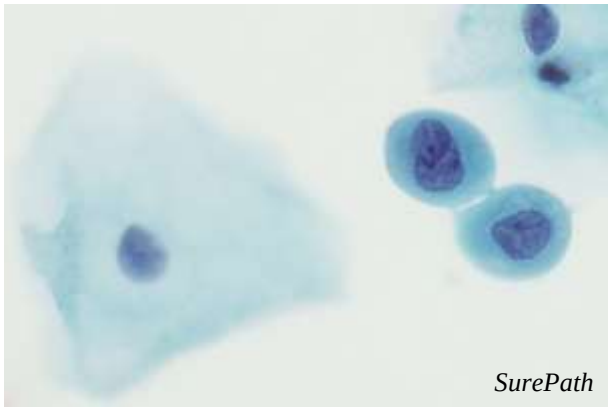
a) ThinPrep utilises a gentle dispersion step that breaks up blood, mucus and non-diagnostic debris, before thoroughly mixing the sample. A series of negative pressure pulses are then generated which draw the vial fluid though a ThinPrep Filter to collect a thin, even layer of diagnostic cellular material. The cellular material is then transferred to a glass slide using computer controlled mechanical positioning and positive air pressure.
b) SurePath concentrates the cellular material in the sample by a cell enrichment process (removing non-diagnostic material such as mucus, blood and inflammation) using centrifugation. During the final slide preparation, the cells in the concentrated sample "settle" onto the slide through a process of gravity sedimentation.
These differences in slide preparation technique result in a higher proportion of cell groups in SurePath samples compared with ThinPrep samples, which comparatively have more single cells.

There are two common appearances for which ASC-H should be considered for both ThinPrep and SurePath. The first appearance is the finding of single atypical cells and the second pattern is the presence of crowded groups/sheets.

1. **Atypical single cells**

ASC-H cells are usually quite small in LBC preparations, often only 2 to 3 times the size of neutrophils and many are metaplastic. Normal small immature metaplastic cells with high N:C ratios can show some nuclear atypia, making them very difficult to distinguish reliably from HSIL cells, particularly in ThinPrep samples. If HSIL cannot be excluded, then ASC-H is an appropriate reporting category.

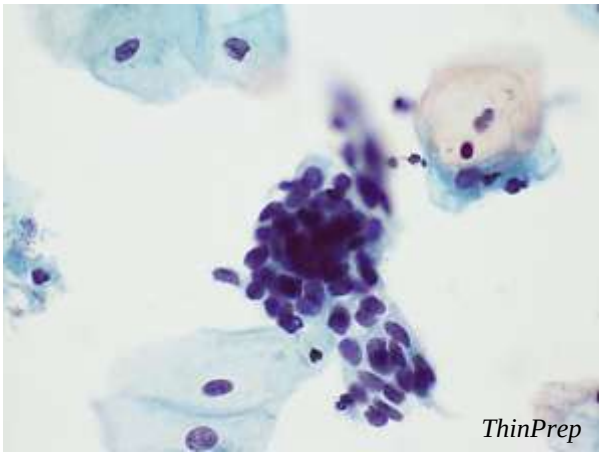

ThinPrep

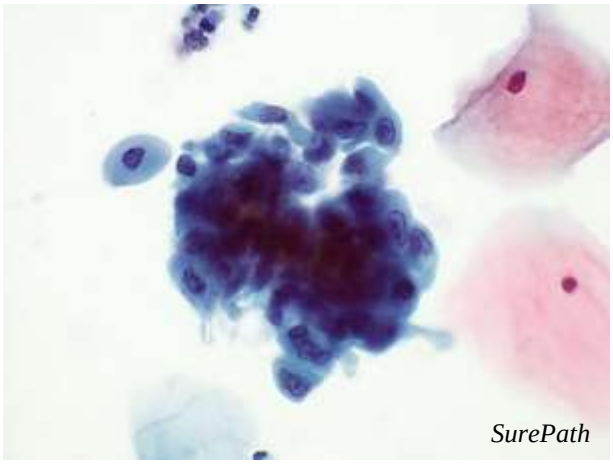

SurePath

Immature squamous metaplasia can closely mimic HSIL in cytology samples. Single metaplastic squamous cells can be difficult to interpret, particularly when the abnormal cells are sparse. Both the above images are from cases reported as ASC-H that were CIN 3 on follow-up.

2. **Hyperchromatic crowded groups**: may sometimes be difficult to examine properly and require careful focussing through the groups. Use the 40X objective to carefully focus particularly on cells around the group edges. Is there variation in the size of nuclei? Is the

chromatin granular in some nuclei and less so in others? Are there any mitotic figures? Remember these are not diagnostic features that are being looked for but enough atypia to suggest that the group is abnormal and suspicious of a high-grade lesion i.e. ASC-H. These crowded clusters occur in ThinPrep samples as well as in SurePath samples but are more common in SurePath preparations.


ThinPrep


SurePath

All hyperchromatic crowded groups need to be examined carefully as this is a high-risk pattern for HSIL. Some HSIL cases will only have crowded groups in the sample, without any single HSIL cells.

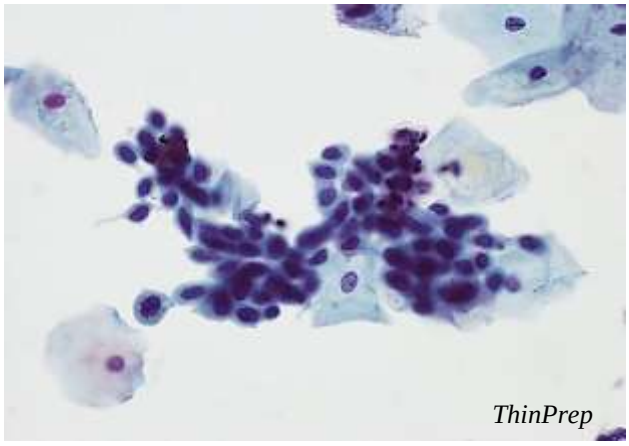
It may sometimes be difficult to distinguish between a glandular and a squamous origin of the atypical cells. Sharp linear cytoplasmic cell edges with nuclei lying parallel to the sheet edge usually favour their being squamous whereas lining up of cells in a group with eccentric, slightly elongated nuclei at right angles to the sheet edge points towards a glandular abnormality.

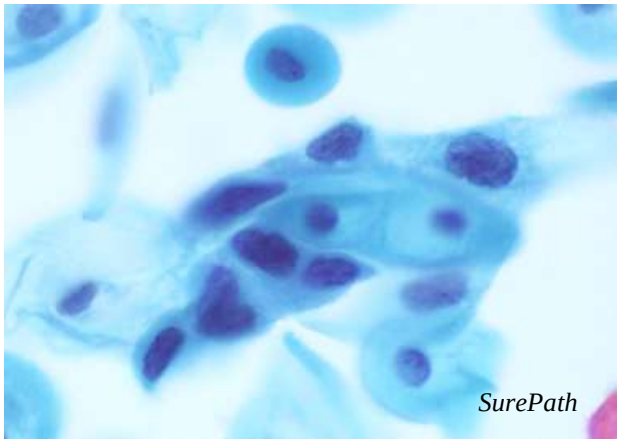
Degenerate endometrial cell groups and macrophages are sometimes reported as ASC-H. Obtaining a menstrual history from the sample taker may alert the cytoscientist/cytotechnician to this possibility. Basic criteria for identifying normal endometrial cells should be applied to the groups. Macrophages usually demonstrate a "streaming" effect with abundant foamy/finely vacuolated cytoplasm and bean-shaped nuclei. The nuclei should not be hyperchromatic but will contain nucleoli. These should not be overcalled as ASC-H. Where it is perceived that abnormal criteria still exist, then report the case as ASC-H.

There are some clinical situations which are benign or reactive, but which are renowned for creating appearances in cervical cytology samples which mimic high-grade lesions. High-grade lesions do occur in these situations so any cytologic suspicion of HSIL does need to be reported as ASC-H and investigated appropriately. Be aware of potential diagnostic difficulties and use ASC-H to report the sample to reduce both false-negative and false-positive reports. The most common of these clinical situations are:

1. Postpartum preparations and ASC-H

The combination of postpartum atrophy and the marked reactive change that can occur post-delivery can make benign cells in cytology samples look highly atypical in postpartum samples. Cytology samples used to be taken regularly at the 6-week clinical check post-partum, but it is better to wait until the postpartum atrophy has resolved, unless there is a particular reason for taken a sample so soon after delivery. Sample takers are now discouraged from taking cytology samples in the postpartum period. The presence of single parabasal cells sometimes with high N:C ratios and granular chromatin may warrant reporting postpartum samples as ASC-H. A postpartum preparation can also present with clusters of hyperchromatic cells that on closer examination, reveal crowding and loss of polarity. The cells will often have dense cytoplasm, polygonal shapes and will demonstrate some variation in size and shape of adjacent nuclei.





These samples taken post-partum show clusters of high N:C cells that could be HSIL or a marked reactive change. The case on the left was CIN 2 on follow-up.

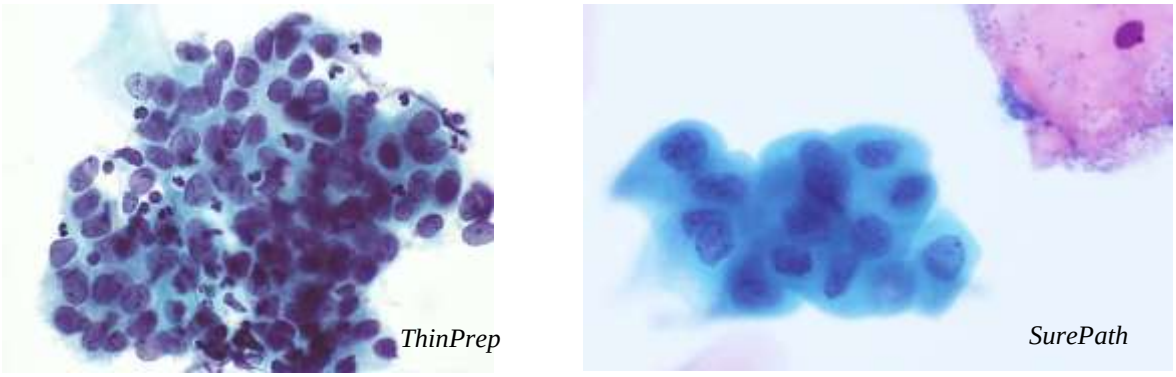
2. Atrophy and ASC-H

Caution needs to be exercised with atrophic samples. In particular, if there are loosely cohesive atrophic cells with high N:C ratios and some slight irregularities of the nuclear membrane contour, then the possibility of reporting as ASC-H should be considered even if the chromatin appears finely granular. At times the presentation may be of clusters of hyperchromatic cohesive cells with a background of atrophic single cells. These require careful examination to try to identify irregularities of the nuclear membrane, granularity of chromatin and N:C ratios which may warrant reporting as ASC-H. If the nuclei appear bland (i.e. no granularity), it is likely that these are just normal atrophic groups but these cases need a second opinion by an experienced cytoscientist/cytotechnician. If there is atypia but the suspicion of a high-grade lesion is low, some laboratories report the case as ASC-US and request a repeat sample after oestrogen rather than using ASC-H with referral for colposcopy. A repeat oestrogenised sample may be much easier to interpret, providing reassurance that there is no SIL (squamous intraepithelial lesion) present. With atrophic vaginitis, small degenerate atrophic orangeophilic keratinised cells may be observed. These should be reported according to the degree of nuclear atypia. Cases with hyperchromatic enlarged nuclei with irregular shapes will require reporting as ASC-H.

Two different cases reported as ASC-H in samples with epithelial atrophy. The case on the left was CIN 3 on follow-up whereas the case on the right was atrophy only.

3. Squamous metaplasia and ASC-H

Immature metaplastic squamous cells occur singly or in small groups and may be difficult to distinguish from HSIL, particularly because the N:C ratio is already high. In HSIL, there will be evidence of nuclear hyperchromasia, some nuclear membrane and chromatin irregularities and abnormal nuclear shapes. There will also be variation in size and shape of nuclei within clusters of cells. But the evidence of a high-grade lesion may not be convincing (because the case would then warrant being reported as HSIL): instead the morphologic features may be subtle.

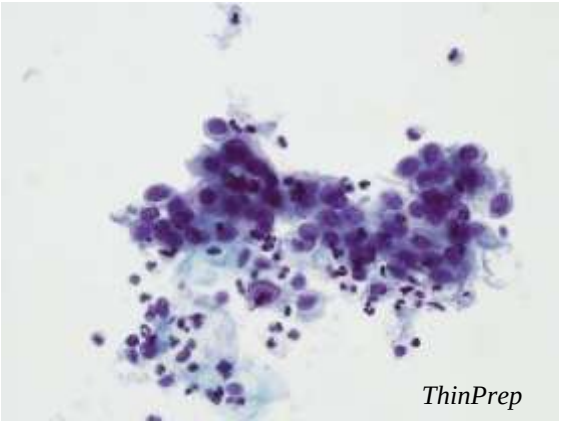


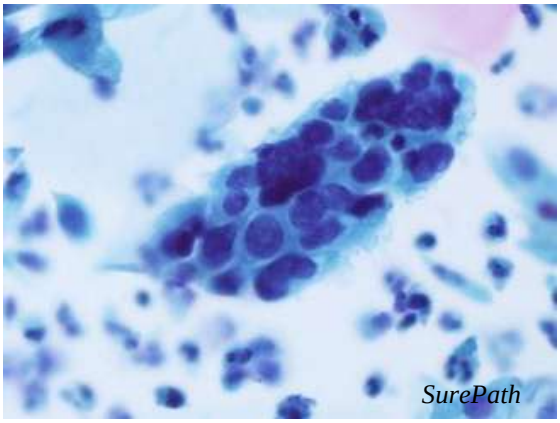
These cases show clusters of metaplastic cells that are suspicious of HSIL although the degree of nuclear variation is subtle. Both were reported as ASC-H. Left = CIN 3

4. Cervicitis, degeneration and ASC-H

When first practising cervical cytology, look at as many cases as possible and consult extensively with an experienced screener to ensure that changes are not over-called when involving cervicitis and degeneration. Experience is critical in this area because the morphologic distinction between a florid reactive process and HSIL can be subtle. IUCD's in particular may result in cytology samples with marked reactive change in metaplastic squamous cells and glandular cells. There may often be numerous single atypical-looking metaplastic cells that are hyperchromatic with a high N:C ratio. Examine the chromatin carefully. Smudged and bland chromatin in these cells should suggest the possibility that the changes are IUCD-related

changes. To complicate this, samples from IUCD wearers may have abundant inflammatory material requiring careful examination. Degeneration often imparts a wrinkled appearance to the entire nuclear membrane; this together with smudged chromatin should caution against an over-call of ASC-H. If there are prominent nucleoli present and flat (i.e. not thick) cells sheets, consider the possibility of repair rather than a report of ASC-H.





Both these cases show crowded groups of high N:C squamous cells infiltrated by inflammatory cells and were reported as ASC-H. The case on the left showed inflammation only on biopsy.

2.9.7 Questions

1. With the aid of schematic diagrams, discuss the various cytologic presentations of CIN 3.

2. You are looking at a cervical cytology sample with low-grade squamous intraepithelial changes. Discuss what precautions you would take to ensure that HSIL cells are not missed.

3. Tabulate differences in nuclear appearance between LSIL and HSIL.

4. Explain how mitoses, apoptosis and crowded sheets can offer clues that you are dealing with HSIL.

5. Discuss the differential features between HSIL and the following:
   - Tubal metaplasia
   - Repair
   - Benign atrophy
   - Benign endometrial cells

6. What precautions could you take to ensure that you do not overcall ASC-H?

References

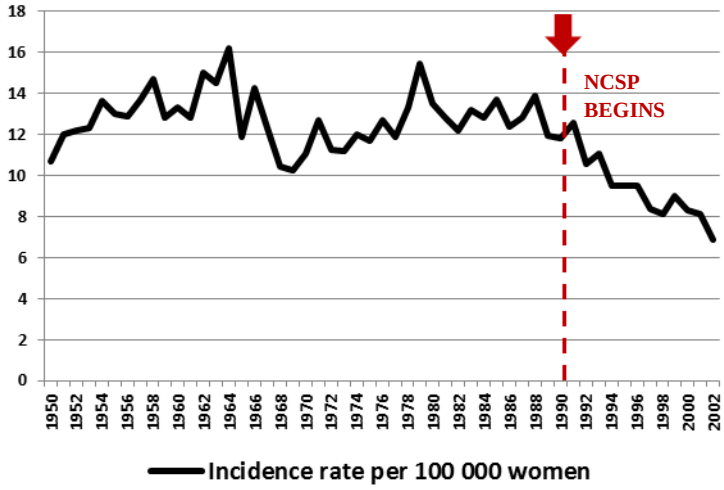
- Bowditch R. 2002. *Challenges in Cytology: Confronting Difficult High Grade Lesions.* Sydney: NSW Cervical Screening Programme.

2.10 CORE TOPIC: INVASIVE SQUAMOUS CELL CARCINOMA (SCC)

2.10.1 Cervical Cancer Rates in New Zealand

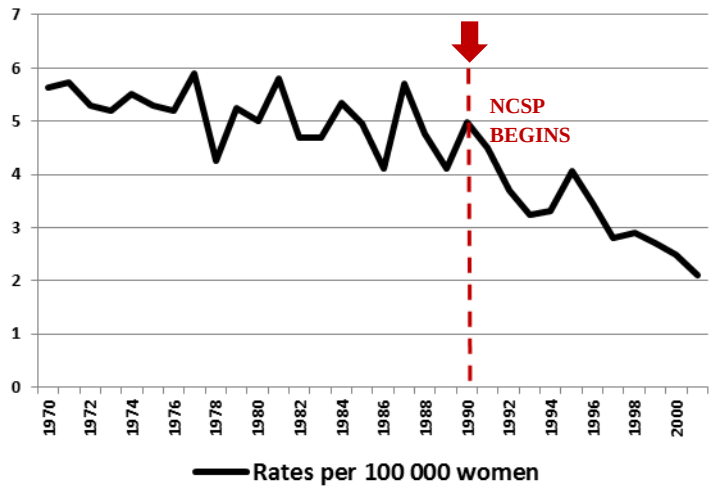
New Zealand statistics show that there were 165 new cases (incidence) of cervical carcinoma and 53 deaths (mortality) as a result of the disease in 2011.(1) These figures include both squamous cell carcinomas and adenocarcinomas arising in the cervix, with the majority (70%) being of squamous type. The incidence of cervical cancer fell by 40% and mortality fell by 60% from 1990 to 2001, i.e. the first decade of operation of the National Cervical Screening Programme (NCSP).(1) Similar rates of decline for both new registrations and deaths have occurred in other countries with well organised cervical screening programmes.

Cervical Cancer Incidence in NZ 1950 - 2002
Age-standardised rate (Segi) per 100,000 women



Cervical cancer 1990-2001:
Incidence fell by 40%

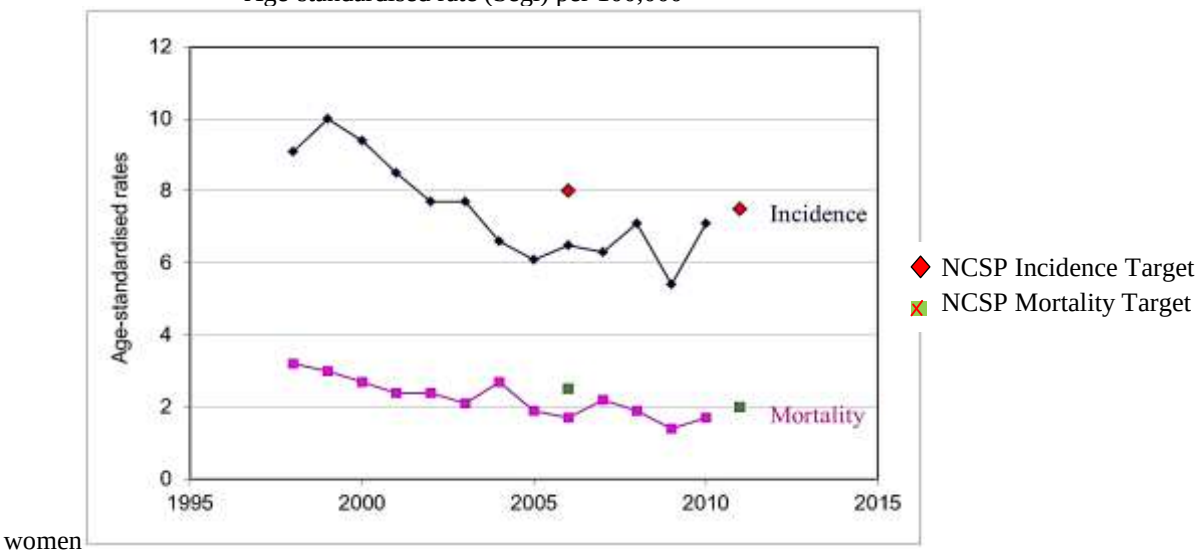
Cervical Cancer Mortality in NZ 1970 - 2001
Age-standardised rate (Segi) per 100,000 women



Cervical cancer 1990-2001
Mortality fell by 60%

The figure below shows the incidence and mortality rates of cervical cancer in New Zealand from 1997 to 2011. Both rates continued to decline until about 2005. New approaches to the detection and prevention of cervical cancer such as HPV screening and vaccination may allow further reductions in disease rates in future years.

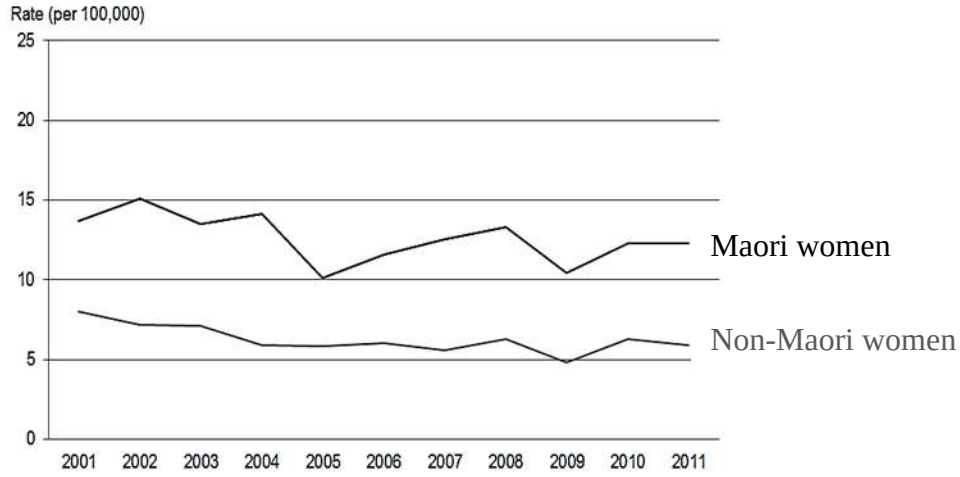
Incidence and Mortality of Cervical Cancer in New Zealand 1997- 2011
Age-standardised rate (Segi) per 100,000



Despite the overall success of the cervical screening programme in New Zealand, there remain challenges. One is the inequality between different ethnic groups within New Zealand. For example, the disparity in the incidence of invasive cervical cancer between Maori and non-Maori fluctuated little between 2001 and 2011. The incidence for Maori women was at least 1.7 times greater than for non-Maori women each year. (1)

Incidence of Cervical Cancer in New Zealand for Maori and non-Maori women 2001 to 2011

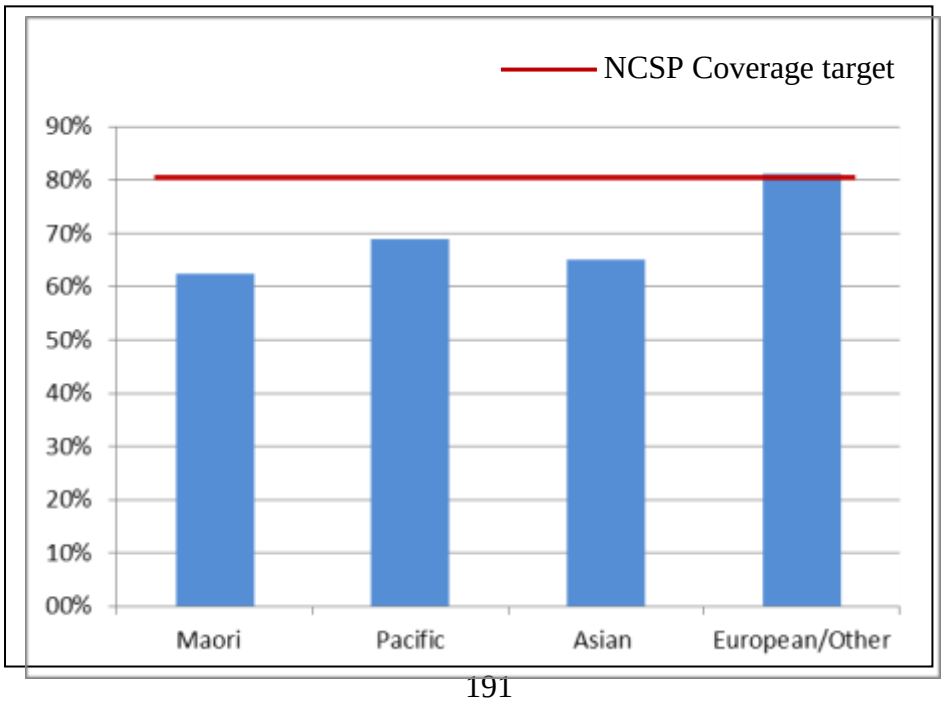
Age-standardised rate per 100,000 women



One of the reasons behind the difference in cervical cancer incidence in different ethnic groups is related to the proportion of women in each population group who have regular cervical cytology samples taken. This is expressed as coverage, i.e. the proportion of women who have had a cervical cytology sample recorded on the NCSP-Register within the previous three years (the screening interval in New Zealand). Coverage data is monitored regularly by the NCSP for Maori, Pacific and Asian women, and for the remainder of the population (called "European/other"). Only the latter group meets the NCSP 80% coverage target.

Coverage for women screened in the three years prior to 30 June 2014, by ethnicity

Hysterectomy-adjusted data



2.10.2 Definition and development of squamous cell carcinoma

Invasive carcinoma is present by definition when there is a breach of the basement membrane with invasion of malignant cells into the underlying stroma. Once the basement membrane is breached there is potential for spread into lymphatics and blood vessels, the risk of which becomes greater with increasing depth of stromal invasion and increasing tumour size. Invasion of lymphatics and blood vessels (lymphovascular invasion) may result in distant metastases. Widespread local invasion of squamous cell carcinoma into adjacent organs may occur. Invasion within the pelvis can cause ureteric obstruction explaining why renal failure was a common cause of death in women with cervical cancer in the pre-screening era, when the disease typically presented at an advanced stage of the disease.

The development of SCC usually evolves over many years, generally by progression of persistent high-grade CIN. HPV DNA was identified in 93% of SCC cases in a recent New Zealand study. [(2)] However the majority of pre-invasive CIN lesions do not progress to SCC even without treatment and when invasion does occur, the time interval from CIN to invasion varies. It is likely to take at least 5 years for SIL to become invasive in most cases and studies suggest a typical time span of 10-15 years from the initial development of CIN through to invasion. The interval between the development of intraepithelial lesions (CIN) and invasive cancer allows time for the detection and treatment of pre-invasive lesions to occur before invasion develops. Detecting persistent high-grade lesions before invasion develops is the aim of cervical screening. Because there is a significant false negative rate using cervical cytology, repeat samples taken at regular intervals are needed to achieve significant reductions in disease incidence. The time frame to the development of invasive cancer should not lead to complacency in the treatment of premalignant disease.

2.10.3 Invasive SCC: Clinical aspects

Invasive carcinomas may be visible on inspection of the cervix with the naked eye, particularly with large advanced tumours. Sample takers should document any abnormal appearance of the cervix on the request form when sending a cervical cytology sample to the laboratory and this clinical history should always be taken seriously. The proportion of SCC cases which are symptomatic at the time of diagnosis has dropped with the use of cervical screening because screen-detected tumours are diagnosed at an earlier stage of the disease. However, about 50% of women diagnosed with invasive SCC in New Zealand have never been screened by cytology and these women present with symptoms.

Typical symptoms, seen particularly when ulceration and tissue destruction are present, are:

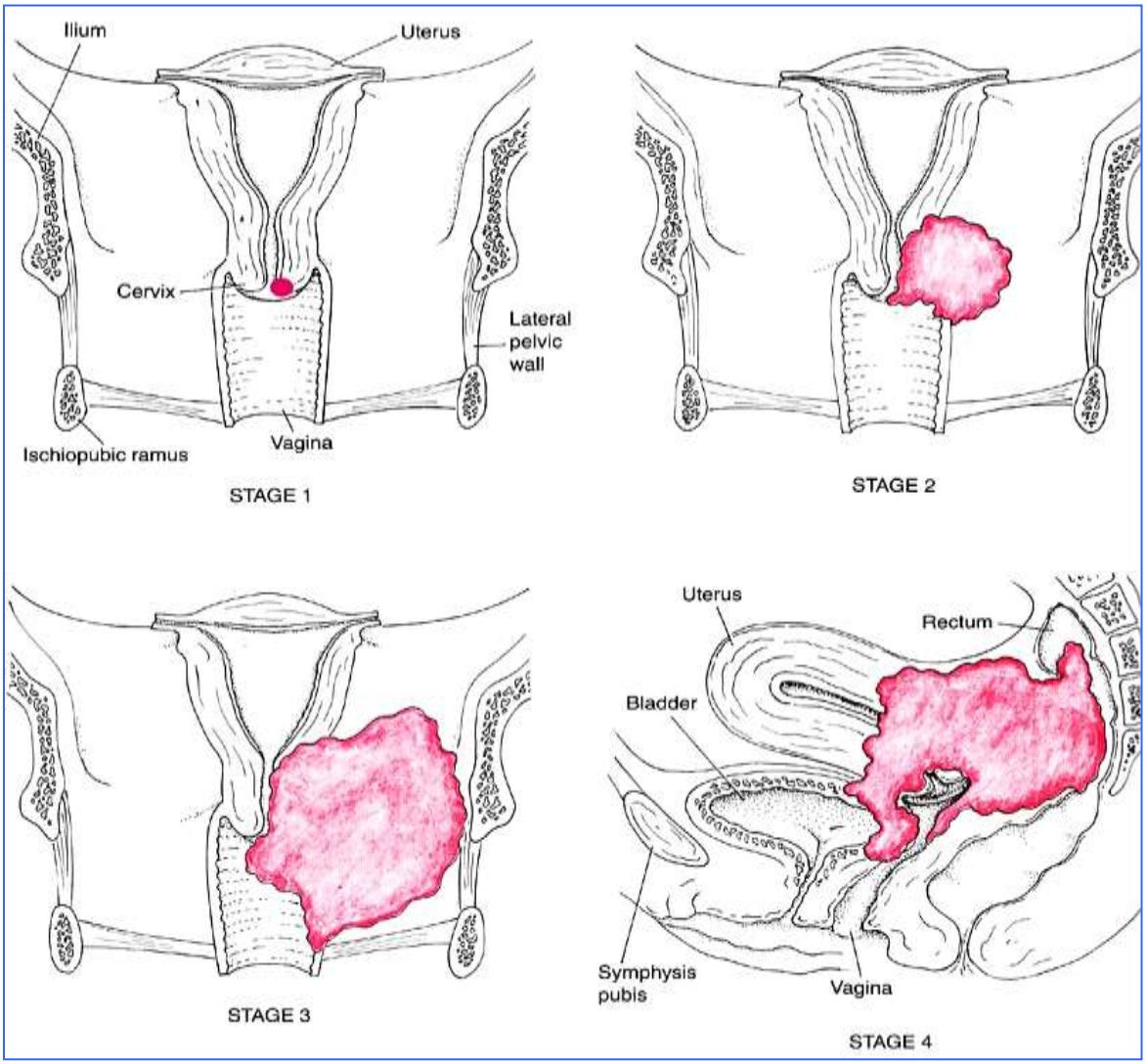
- Yellow or bloody foul-smelling vaginal discharge
- Post-coital/intermenstrual/postmenopausal vaginal bleeding and pain
- Hard feeling of the cervix on palpation.

When visible, invasive carcinomas often appear red because of prominent blood vessels and haemorrhage. Exophytic ectocervical tumours cause a bulging friable mass whilst endophytic tumours appear as an ulcer covered with fibrin and necrotic debris.

The extent of disease is assessed by a clinical staging system based on the location and spread of the carcinoma. The international system (FIGO – International Federation of Gynaecology and Obstetrics) for staging carcinoma of the cervix is as follows:

2009 modification of FIGO staging of carcinoma of the cervix uteri [3]

Stage	Spread
I	Carcinoma is confined to the uterus
IA	Invasive carcinoma diagnosed only by microscopy (all macroscopically visible lesions even with superficial invasion, are Stage 1B)
IA1	Stromal invasion no greater than 3.00mm in depth and 7.00mm or less in horizontal spread
IA2	Stromal invasion more than 3.0mm and not more than 5.00mm with a horizontal spread of 7.00mm or less
IB	Clinically visible lesion confined to the cervix or microscopic lesion greater than IA2
II	Tumour invades beyond the uterus but not to pelvic side walls or lower third of the vagina
III	Tumour extends to the pelvic wall and/or involves the lower third of vagina and/or causes hydronephrosis or non-functioning kidney.
IV	The carcinoma has extended beyond the true pelvis or has involved the mucosa of the bladder or rectum.
IVA	Spread of the growth to adjacent organs
IVB	Spread to distant organs



With invasion to the bladder, recurrent infections of the urinary tract may occur. Invasion to other surrounding organs can lead to complications such as bilateral ureteric obstruction with renal failure or a rectovaginal fistula. Pain is usually a late symptom, developing when metastatic deposits in lymph nodes exert pressure on nerve bundles or when carcinoma invades the sheath of a nerve. The lungs and liver are common sites for advanced spread of cervical SCC. Treatment of invasive carcinoma is either by radiotherapy/chemotherapy, surgery or a combination of both.

2.10.4 Superficially invasive SCC: Stage IA1

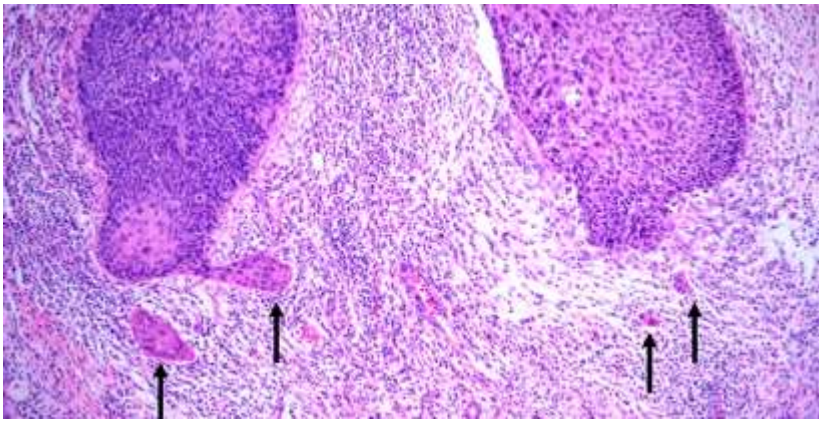
CIN can involve endocervical glands by extending into crypts from the surface epithelium. This is not invasion as the basement membrane has not been penetrated. Early invasion is seen histologically as small buds of neoplastic cells extending through the basement membrane into superficial cervical stroma, often with surrounding stromal oedema and chronic inflammation. These early lesions have traditionally been called "microinvasive squamous cell carcinoma" although other terms such as minimally invasive squamous cell carcinoma or superficially invasive squamous cell carcinoma are now preferred.

Cells in the region of the basement membrane are not seen in cervical cytology samples (unless there is ulceration) as only cells from the surface layers of the epithelium are sampled. Superficially invasive squamous cell carcinoma is not an entity that can be reliably predicted by cytology. When early invasion is present, the cytology sample is likely to be reported as HSIL, HSIL with possible invasion, or invasive SCC. Referral for further investigation will be recommended so that the necessary colposcopic and histologic assessments to establish the diagnosis will occur. A cytology report of "HSIL with possible invasion" is not a prediction of superficially invasive SCC, although will sometimes be associated with it.

Superficially invasive squamous cell carcinoma of the cervix is a lesion that is not visible clinically, has a depth of invasion from the basement membrane into the stroma of $\leq$3mm with a surface extent $\leq$7mm and is completely excised. The risk of vascular involvement and lymph node metastasis increases with increasing depth of stromal invasion. These risk rates are used to determine the parameters that define superficially invasive SCC as a better prognostic category than more advanced lesions.

Most superficially invasive SCC cases are not symptomatic, may not be detected as invasive foci at colposcopy and do not show epithelial ulceration so many cases are first detected histologically. Almost all cases show high-grade CIN 2-3 in the overlying and adjacent squamous epithelium. There are particular histological features which assist the histopathologist to determine that small buds of squamous cells do constitute superficial stromal invasion. These are:

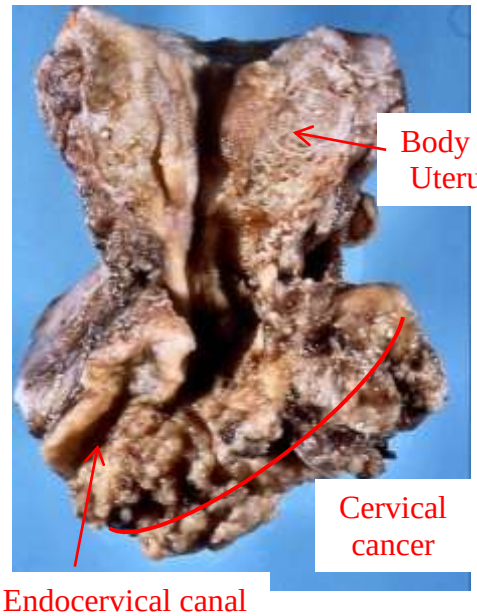
- Increased squamous differentiation i.e. there is more abundant eosinophilic cytoplasm and a lower N:C ratio in the invasive focus compared with the CIN 2-3 in the adjacent surface epithelium.
- Increased cellular and nuclear pleomorphism
- Presence of nucleoli
- The stroma adjacent to the invasive focus shows oedema, chronic inflammation and/or desmoplasia.



Multiple foci of early stromal invasion by SCC in histology. Increased amounts of cytoplasm, an increase in cytoplasmic eosinophilia, larger more pleomorphic nuclei and the presence of nucleoli are seen in invasive lesions in both histology and cytology.

2.10.5 Invasive SCC: Histopathology

For invasive tumours that are more extensive than superficially invasive SCC, the World Health Organisation (WHO) classification divides SCC into two histological subtypes: keratinising and non-keratinising. A third previous subtype called "small cell non-keratinising" is no longer used because the term is too readily confused with another rare type of cervical cancer called small cell carcinoma with neuroendocrine features, which is not a type of SCC.
The SCC subtype has some influence on prognosis with a better 5-year survival rate after radiation therapy being associated with large cell non-keratinising carcinomas [(4)] but the FIGO stage of disease is more important in determining the clinical outcome than the histologic subtype.

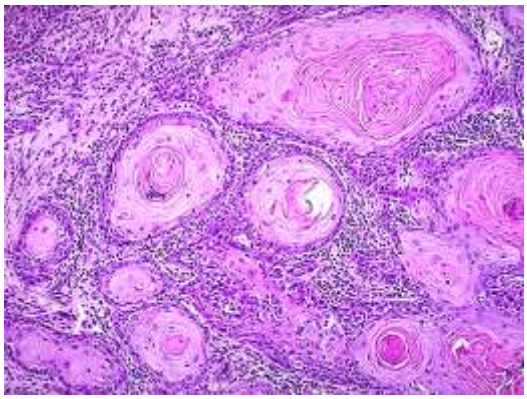


Histology is also used to grade the degree of differentiation although the grade does not predict the clinical outcome.
Well-differentiated (Grade 1) tumours are well keratinised with obvious squamous cell features such as intercellular bridges, keratin pearl formation and mature-appearing squamous cells with abundant dense eosinophilic cytoplasm. Moderately differentiated tumours (Grade 2) are more pleomorphic and still show individual cell keratinisation. Poorly differentiated (Grade 3) tumours are the most pleomorphic, with high N:C tumour cells resembling HSIL. Evidence of keratinisation may be difficult to find, the mitotic rate is high and areas of necrosis are more abundant. Immunohistochemistry helps define these tumours as of squamous origin.

The histological features of the subtypes of SCC are:

Keratinising (Grade 1) SCC

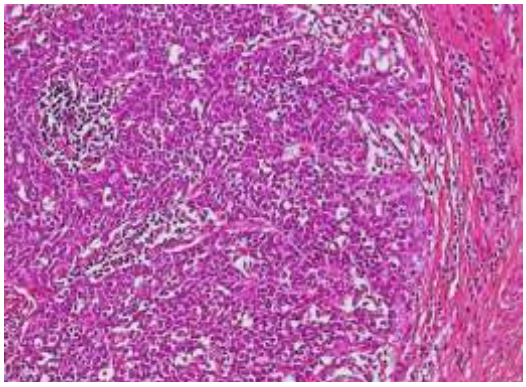
- Good evidence of squamous differentiation
  - Cytoplasmic keratinisation and keratohyaline granules
  - Intercellular bridges
  - Circular whorls of cells forming keratin pearls
- Pushing margin at the tumour edge
- Large hyperchromatic nuclei, irregular coarse chromatin, mitoses in less well differentiated areas.



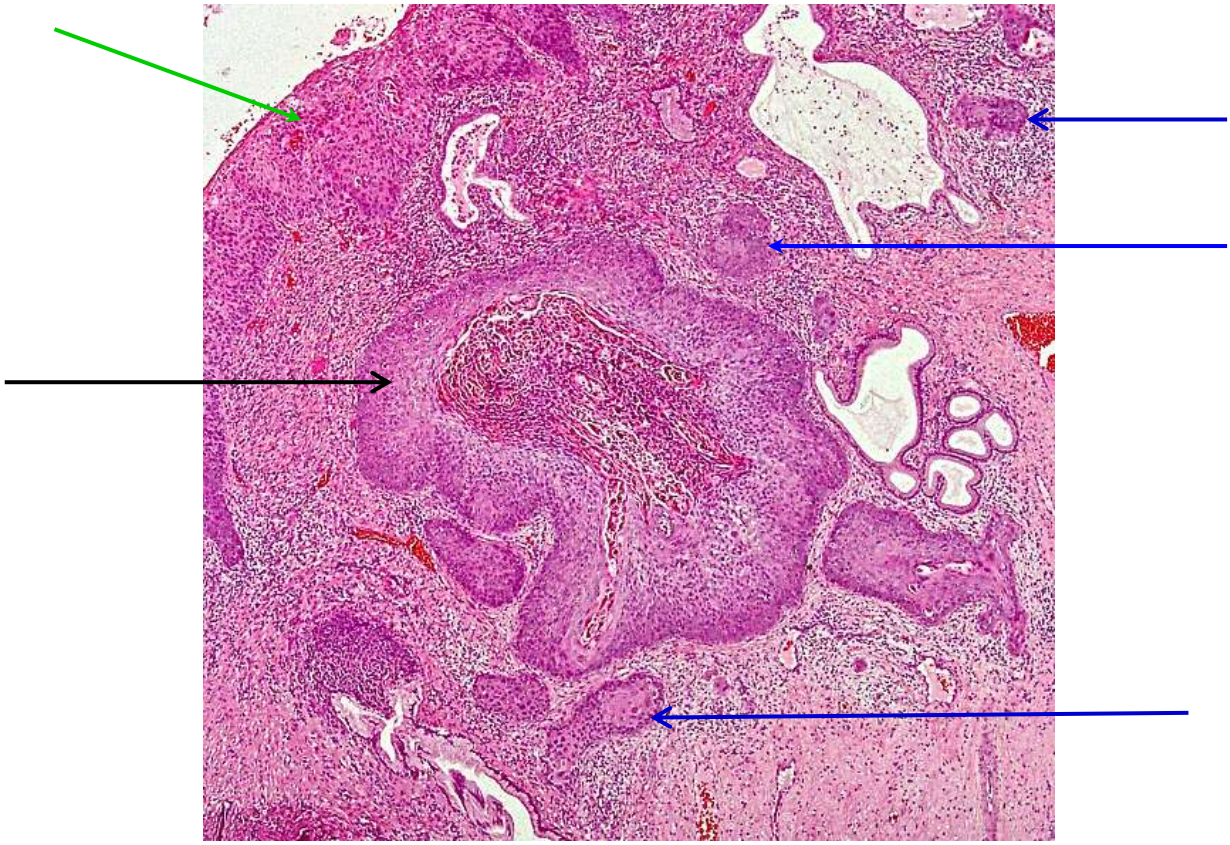
Well differentiated keratinising SCC

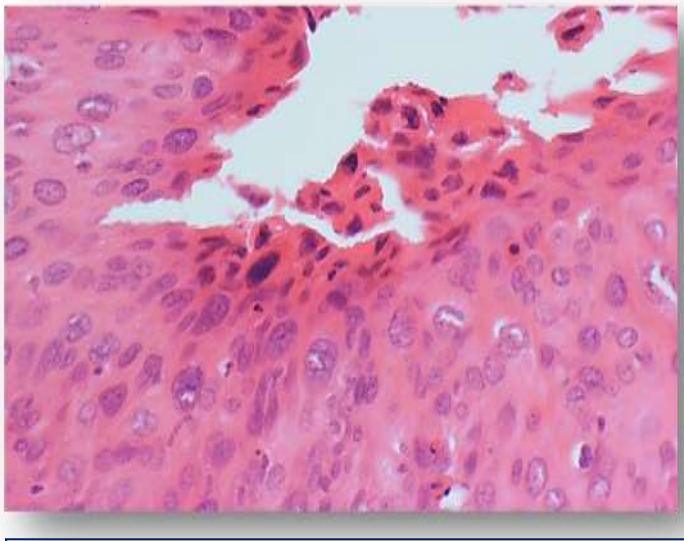
Non -keratinising (Grade 2-3) SCC

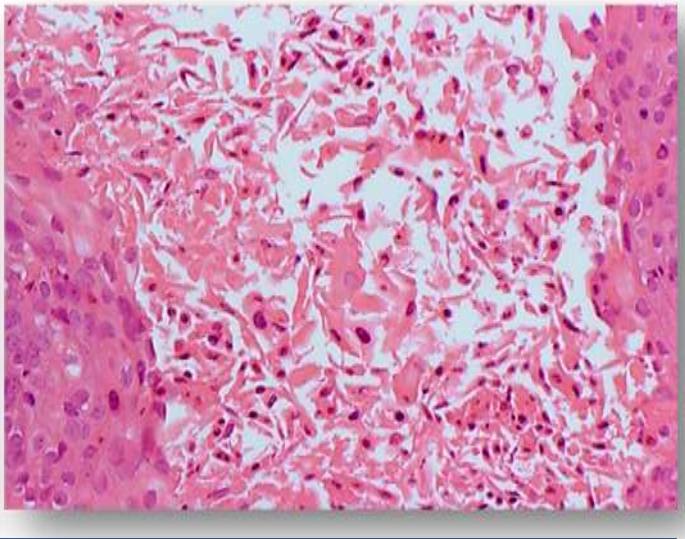
- Less evidence of squamous differentiation
  - Occasional individual cell keratinisation.
- Masses of polygonal cells with buds or cords at the advancing margins
- Moderate cell differentiation
  - Mitoses more frequent throughout
  - Necrosis in the centre of the large cell masses
  - Large, irregular, pleomorphic nuclei
  - Irregular nucleoli



Non-keratinising SCC







Histology of invasive keratinising squamous cell carcinoma.

Top: Multiple nests of neoplastic squamous cells infiltrate the cervical stroma (e.g. blue arrows) showing central keratinisation. There is HSIL in the surface squamous epithelium (green arrow). In the centre (black arrow) there is an endocervical gland lined by epithelium showing HSIL, with cellular debris filling the gland. The contour of the gland is altered and the small adjacent invasive foci of SCC probably arose from this gland.

Bottom left: Surface keratinising malignant squamous cells.

Bottom right: Surface exfoliation of malignant nucleated and anucleated keratinised squamous cells that would be seen as spindle and caudate cells in a cervical cytology sample.

2.10.6 Invasive SCC: Cytopathology

Invasive SCC can be difficult to diagnose by cytology. The sample is often compromised by abundant blood, cell degeneration and debris. Features of invasion can be subtle. A consequence of the decline in the number of cases of invasive SCC in New Zealand since the establishment of the NCSP in New Zealand is that cytologists see fewer cases of invasive SCC in regular practice.

- For accurate cytological interpretation optimal fixation, preparation and staining is essential before an unequivocal diagnosis of malignancy is made.
- If there is any doubt, the sample can be reported as "HSIL with features suspicious of invasion". Thorough colposcopic and histologic investigation will follow.
- Cervical cytology samples consisting predominantly of a mix of blood, leucocytes, and tumour diathesis are sometimes wrongly reported as unsatisfactory or negative. Samples with this low-power appearance need to be screened thoroughly for malignant cells as these may be inconspicuous.
- Because a significant lesion can be missed by cytology, if there is a clinical comment about abnormal bleeding or an abnormal appearance of the cervix on the requisition form, the sample should be referred to a pathologist and considered for referral for specialist assessment **irrespective of the cytology result.**

A range of differentiated and undifferentiated malignant squamous cells may be found in invasive SCC. There is no single feature that determines that a case is invasive so the diagnosis is made by taking all features observed into account. LSIL and HSIL cells can be present but malignant squamous cells (from invasive SCC) usually predominate. Malignant squamous cells show more extreme aberrations both in the nucleus and cytoplasm than HSIL. Close examination of nuclear detail, particularly the chromatin pattern, may be necessary to identify the cells as originating from an invasive lesion. Some features such as nuclear pleomorphism are similar to HSIL but may be more extreme in SCC, whereas other features such as clumping and clearing of nuclear chromatin are not seen in HSIL and when present, are more specific for SCC.

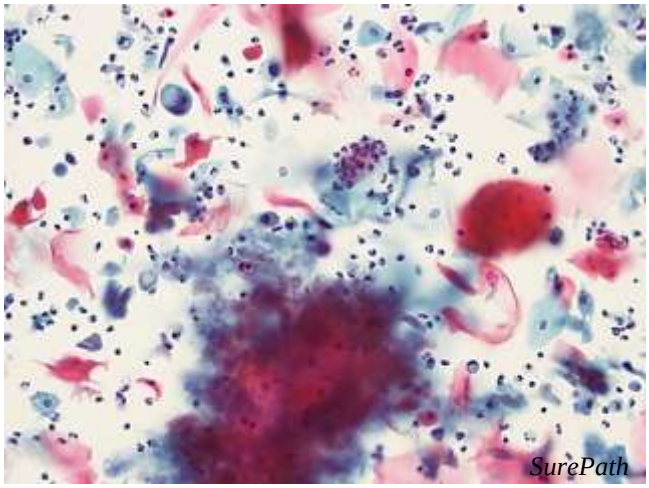
While background and cytoplasmic features can alert the cytoscientist/cytotechnician to the possibility of invasion, **identifying malignant nuclei is essential** in making a diagnosis of invasion. Features suggesting invasion include:

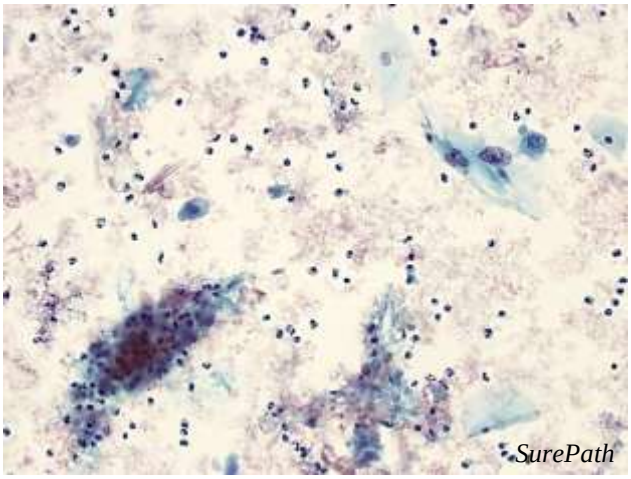
- Marked cellular and nuclear pleomorphism and hyperchromasia. May be extreme.
- Cells often show more cytoplasm than is usually seen in HSIL. Other invasive SCC cells may show very high N:C ratios.
- Coarse irregularly distributed chromatin. The chromatin may show sharp-edged, angular clumps with irregular areas of parachromatin/paranucleolar chromatin clearing producing an appearance sometimes called "clumping and clearing".
- Irregular angular thick nuclear membranes with sharp peaks and indentations.
- Large, prominent, often irregular eosinophilic nucleoli which may be multiple and vary in number between different cells. May be macronucleoli.
- Bare/free and isolated abnormal nuclei, often clustered together.
- Bizarrely shaped keratinised squamous cells including spindle, caudate/tadpole forms
- Whole tissue fragments. Cells within a group can be closely packed and show nuclear moulding.

- Tumour diathesis consisting of degenerate blood, disintegrating leucocytes and necrotic debris. This feature is more frequently observed with non-keratinising SCC than with keratinising SCC.
- Abundant blood in the background. This is as common as tumour diathesis.

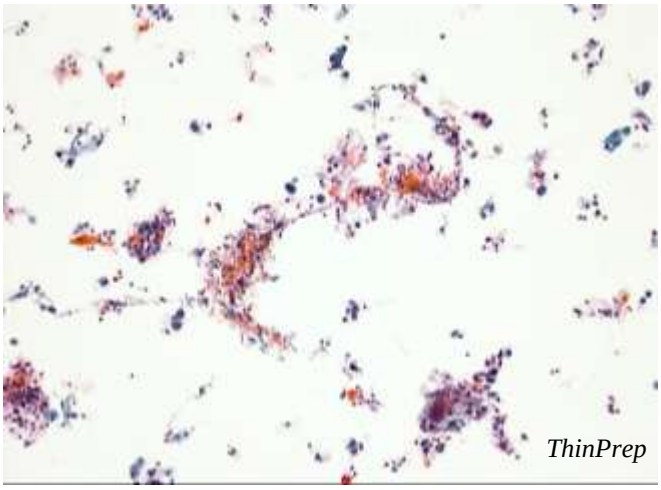
Low-power features

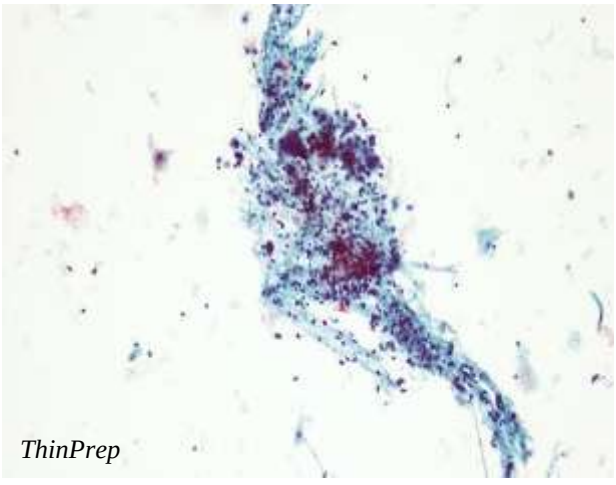
The low power appearance of cases of SCC is not diagnostic, but alerts the cytoscientist/cytotechnician to the possibility of an invasive lesion. The first impression is often of a dirty background and in well-differentiated carcinoma an abundance of anucleated fragments of keratin may be seen. The cells from squamous cell carcinoma are often shed singly or in small groups. There may be clumped and dissociated cells, cell fragments and inflammatory cells, all of which are may be partly degenerate. There may be fresh or degenerate red blood cells or granular debris, either in irregular clusters or around the edges of cell groups ("clinging diathesis"). At low power, ThinPrep samples in SCC often look "scrappy" and difficult to screen. Examination of the cells at high power is essential to confirm or refute a suspicion of invasive malignancy.





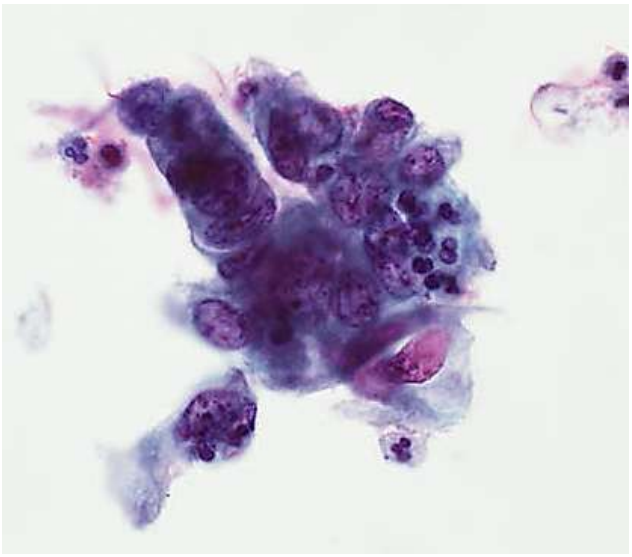
Left: SCC with keratinised spindle cells and a clump of degenerate cells
Right: Abundant blood, debris, neutrophils and suspicious squamous cells





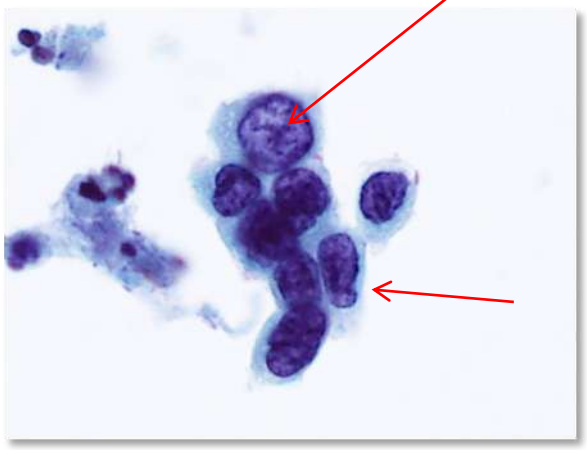
Left: Keratinising SCC with tumour cells, inflammatory cells, debris and blood
Right: Non-keratinising SCC with a ragged clump of debris requiring closer inspection

High power features: ThinPrep

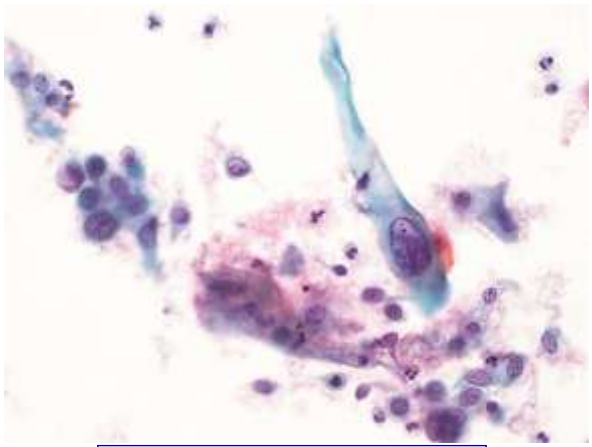


Cluster of malignant squamous cells in SCC

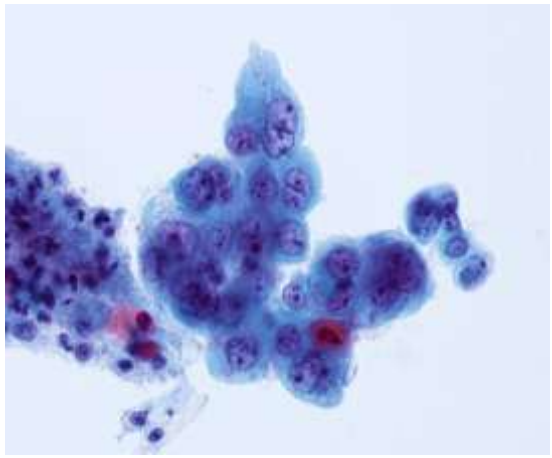
Chromatin clumping and clearing



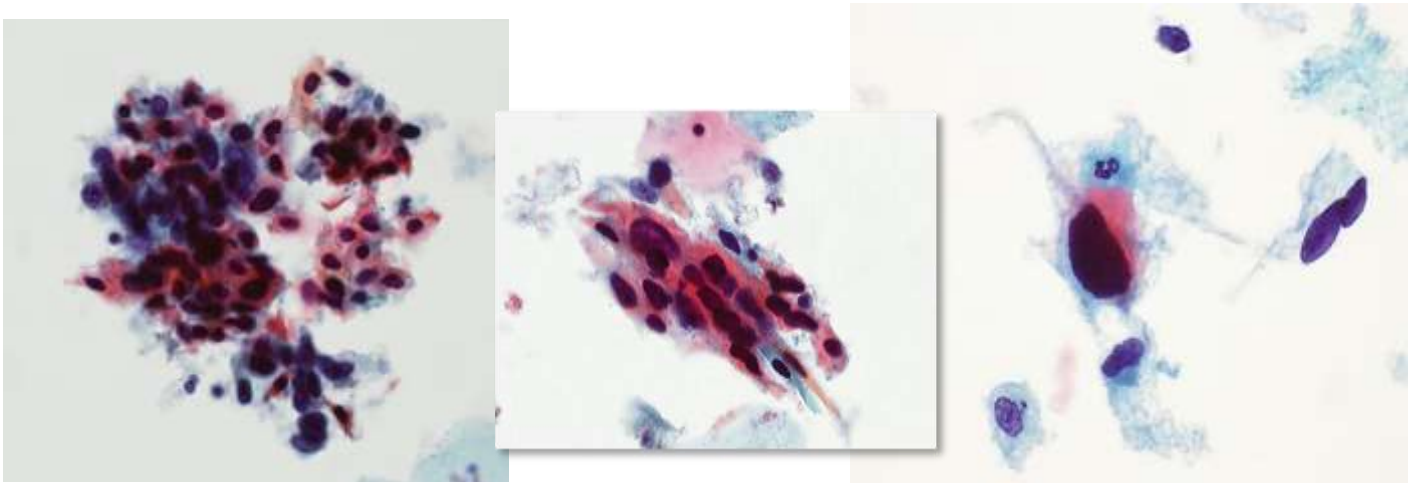
Thickened angular nuclear membranes



Marked pleomorphism

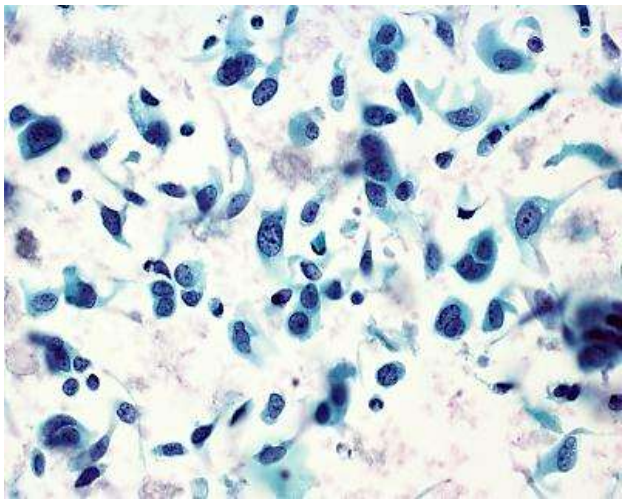


Prominent nucleoli which vary in size and number

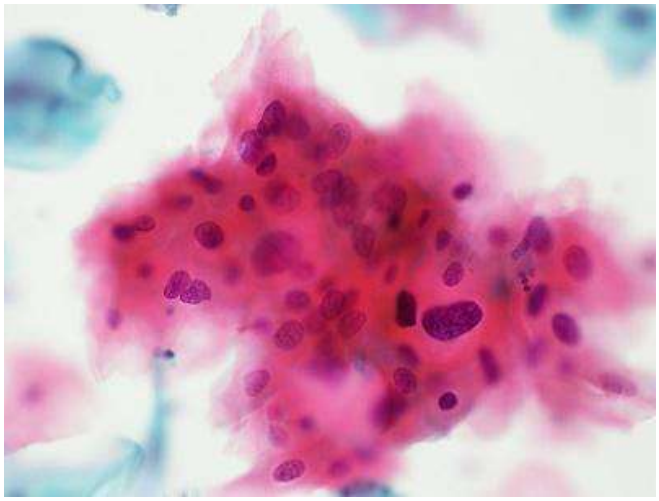


Keratinising SCC showing inky black hyperchromatic nuclei, spindle cells (centre), marked pleomorphism and keratinised cytoplasm.

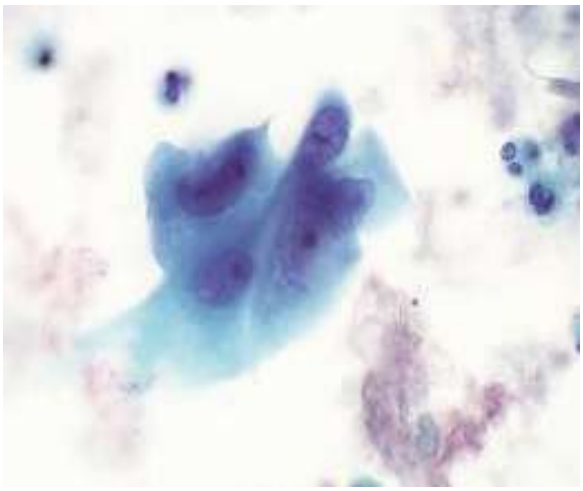
High power features: SurePath

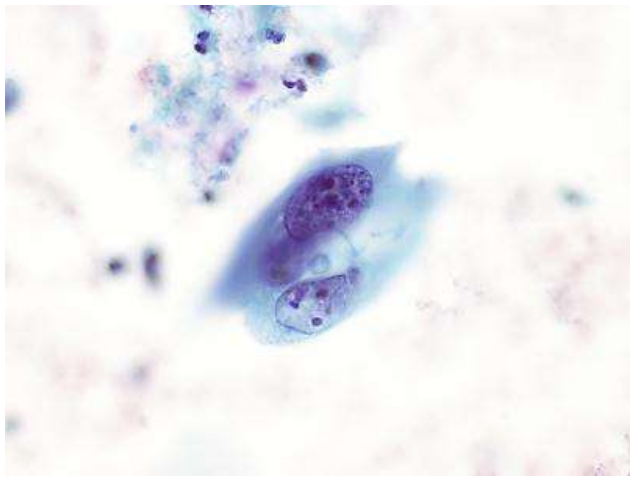


Dissociated malignant squamous cells in SCC

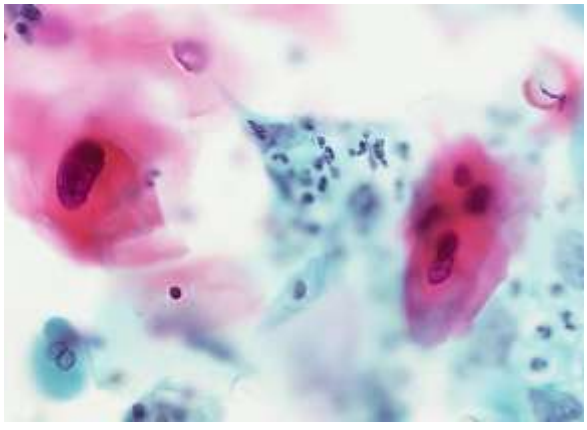


Cluster of keratinising malignant squamous cells

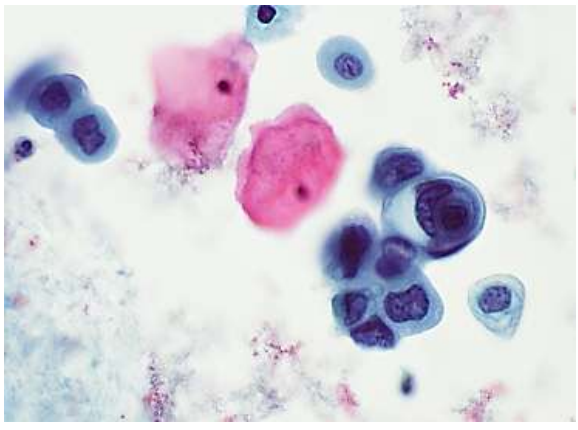




Malignant squamous cells with large nuclei, irregularly distributed chromatin (right image), prominent nucleoli and moderately abundant cytoplasm



Large malignant squamous cells with large hyperchromatic nuclei and abundant well-keratinised cytoplasm



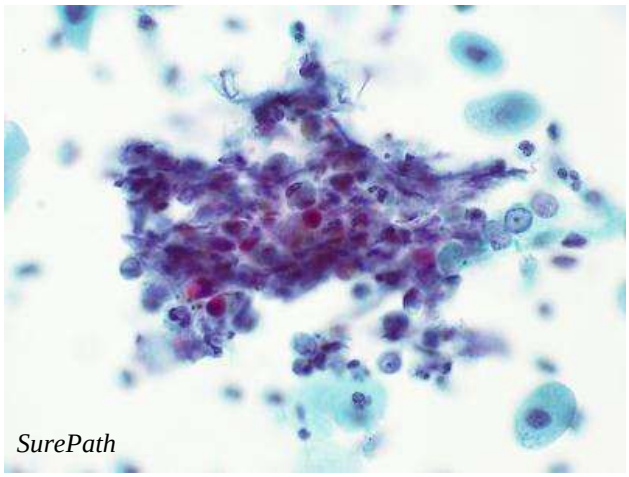
Small malignant squamous cells with high N:C ratios, irregular nuclear membranes, hyperchromasia and cell-in-cell engulfment

Tumour Diathesis

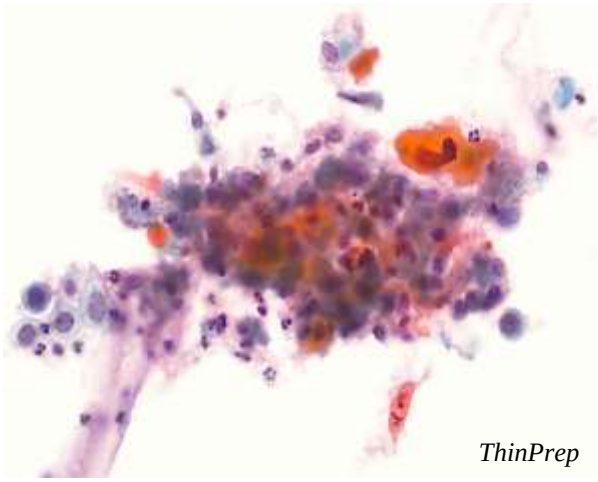
Tumour diathesis consists of blood, leucocytes, and malignant cellular material all in various stages of degeneration, often resulting in granular proteinaceous debris. Viable or degenerate tumour cells may be visible within the material. Tumour diathesis is not always present in invasive SCC and abundant blood in the background is at least as common as tumour diathesis in SCC. When present, tumour diathesis alerts the cytoscientist/cytotechnician to consider the possibility of invasion and to search for diagnostic malignant cells.

- In ThinPrep preparations, the diathesis tends to clump, being comprised of "ratty" debris including blood, fibrin and protein strands, tissue necrosis and cellular debris, with a frayed appearance around the edges. The material can also be observed sticking to epithelial groupings (clinging diathesis).

- In SurePath preparations, tumour diathesis may present as fibrillar or granulated material (remanants of old blood and protein) combined with tumour necrosis. It is composed of small collections of thin proteinaceous material with accompanying fragments of leucocytes and red blood cells, tends to be grey/blue in colour and is often closely associated with malignant cell clusters (clinging diathesis).



SurePath



ThinPrep

Tissue Fragments

Tissue fragments are micro-biopsies and often referred to as “crowded sheets”. High-power magnification allows subtle nuclear changes to be assessed because abnormal cells lie in close proximity to each other. The nuclear features in crowded sheets in invasive carcinomas may closely resemble the crowded sheets seen in HSIL i.e. there is nuclear size and shape variation, chromatin variability (size, shape, and pattern), nucleoli variability (number, size, shape and pattern) and sometimes nuclear moulding. Nuclear features that suggest invasion are clumping and clearing of nuclear chromatin, marked nuclear pleomorphism and a decrease in N:C ratio. The cytoplasm from one cell to another may also be variable.

Very well-differentiated tumours can cause great diagnostic difficulty because many nuclear features do not show the expected marked variability and unpredictability. The nuclei may be quite uniform with only subtle changes within each cell and between cells, and the cytoplasm will have features closely resembling normality. This situation is an interpretative trap and may

lead to false negative reports. Experience at seeing lots of cytology is very helpful when assessing such difficult cases.

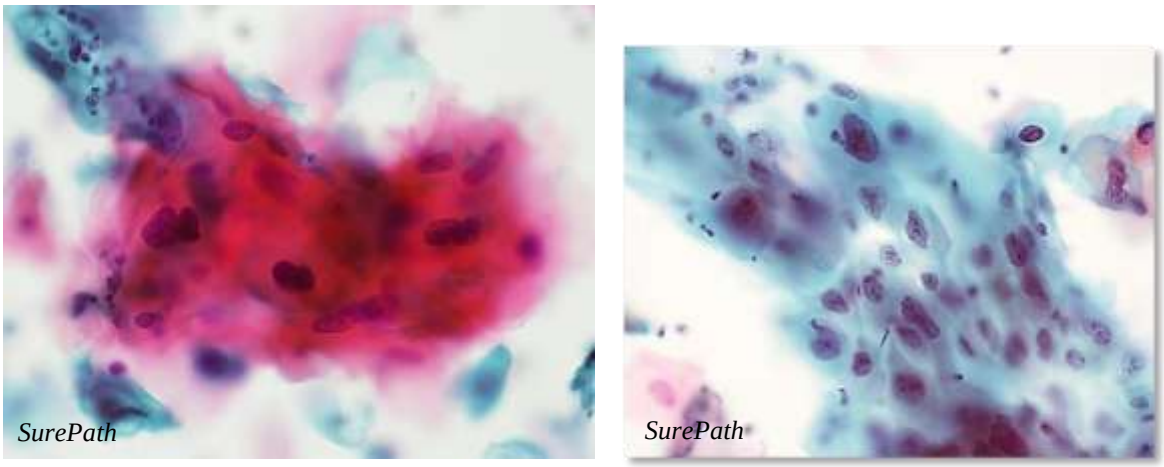
Subtypes of SCC

Subdivision into the subtypes described in histology is not necessary in cytology as SCC subtypes and tumour grade are not reported. In practice, a mix of the cell types and features is often observed in cytology. Discussing the subtypes is still useful to highlight the different ways that SCC can present in cytology samples.

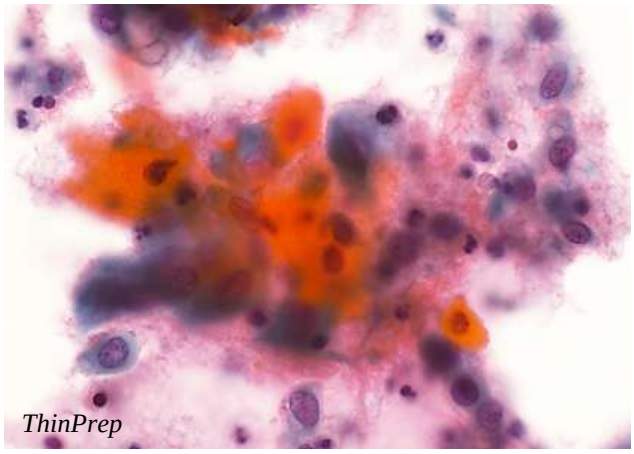
Keratinising SCC

This is classic keratinising SCC as seen at many different body sites in cytology. Cytology samples typically contain many bizarrely shaped orangeophilic cells with large pyknotic/hyperchromatic irregular nuclei.

- Keratinisation usually results in a brilliant glassy eosinophilic/orange appearance. The cytoplasm is dense. In LBC preparations, keratinised cytoplasm can stain a dense hyalinised blue, rather than the classic bright orange.
- Cells may be very large or very small, round or quite irregular or bizarre in shape
- Nuclei are usually large for the cell size but the N:C ratio is often lower than is seen in HSIL because cells acquire more cytoplasm as they become invasive.
- Nuclei may be pyknotic and karyorrhexis may be present.
- Nucleoli are frequent.
- The background may contain keratin and hyaline debris (eosinophilic/basophilic). Background diathesis is less common than with non-keratinising SCC.



Malignant squamous cells. The cytoplasm is abundant and dense and may stain an intense orange (left) or a thick hyalinised blue colour (right).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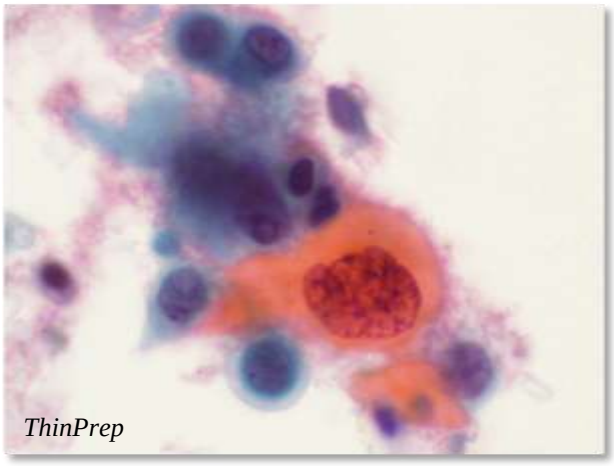
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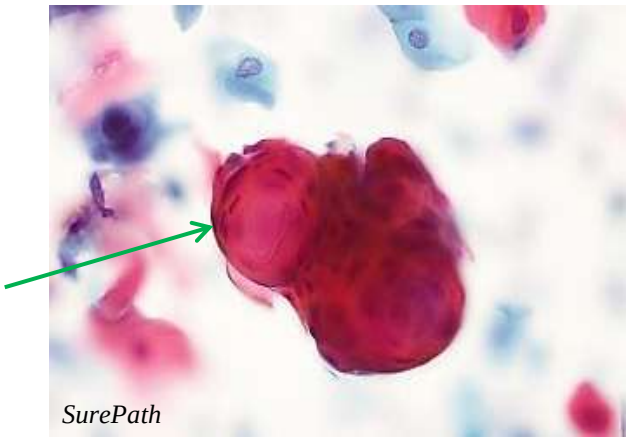
A mix of keratinised and non-keratinised squamous cells is often seen in SCC.

Specific cellular and cytoplasmic features are well described for keratinising SCC. All of the following cytoplasmic forms can also be seen in some benign conditions. It is essential that the cell be classified as malignant based on nuclear criteria.

Keratin Pearls

Pearl formation is a feature of squamous differentiation and may be seen in normal (reactive) or abnormal (neoplastic) cells. Squamous pearls are round structures of stratified squamous epithelial cells that form and radiate out from a central single initial cell. The "newest" or "most recent" cells are on the outer edge of the pearl. The oldest central cell of origin is often keratinised due to maturation.

- Concentrically arranged clusters of malignant squamous cells
- Enlarged, hyperchromatic nuclei
- A deposit of acellular keratin can be observed at the centre of the pearl


SurePath

A fragment of keratinised squamous cells in a case of SCC.

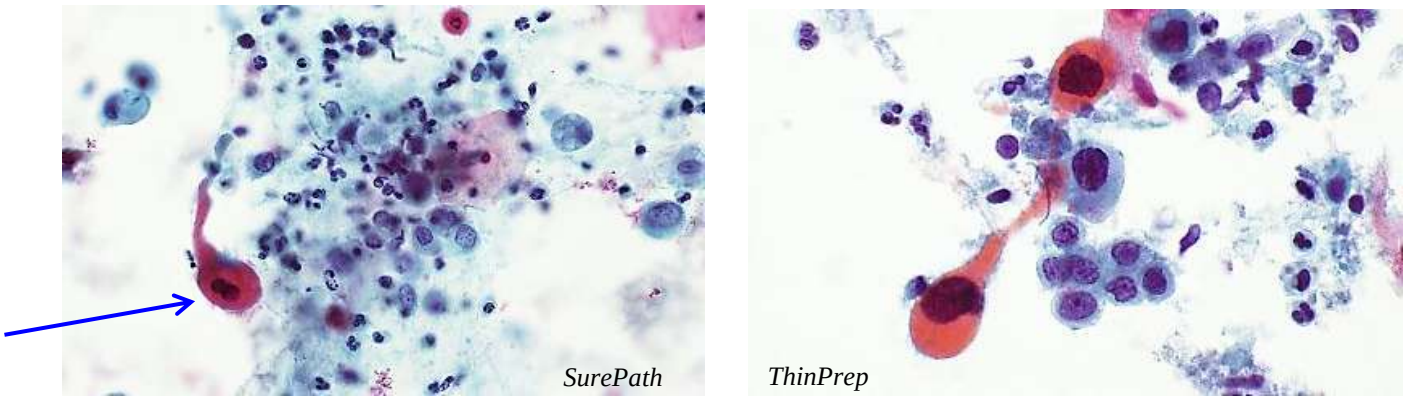
The pearl has a dense keratinised centre with peripheral nucleated cells (green arrow). The centre of the pearl is its origin, with the most recent cells being on the outer periphery of the formation.

Tadpole/caudate cells

The malignant tadpole cell (caudate cell) has its nucleus at the head of the cell with a long elongated, thinned tail of cytoplasm that may have a bulbous end. The nucleus is generally

rounded. Karyopyknosis is common. Tadpole-shaped cells can also be seen in benign processes such as inflammation and HPV infection. These cytoplasmic features are useful to classify the cell type (i.e. squamous) but can not be used to diagnose malignancy.

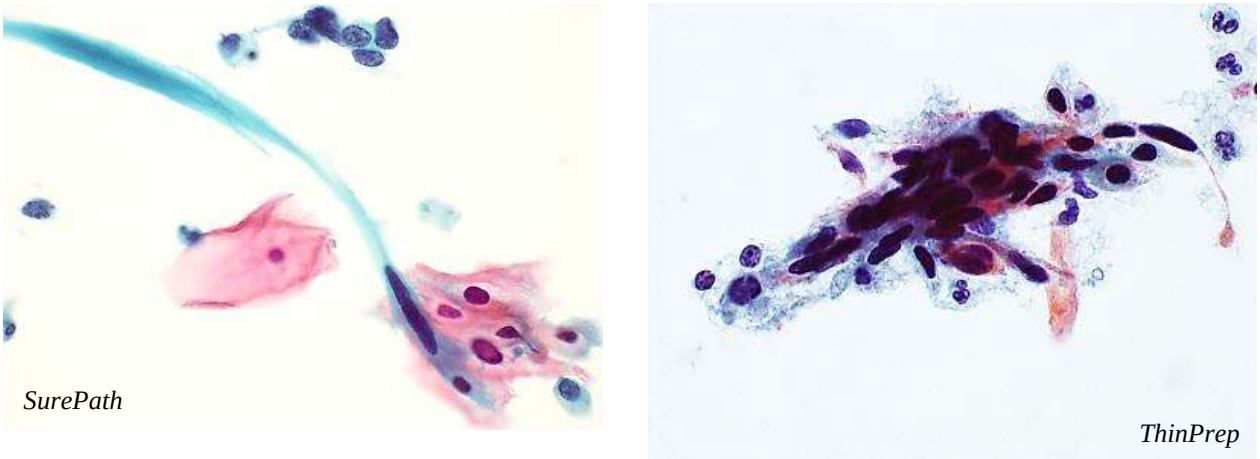
- Elongated, club-shaped cells
- One end (nuclear end) is broad and the other end (tail) is narrow and can be extremely long. The nucleus is usually round and centrally placed but the N:C ratio is high
- Multiple nucleoli may be seen
- Varying degrees of keratinization may be seen. Herxheimer spirals may be seen.



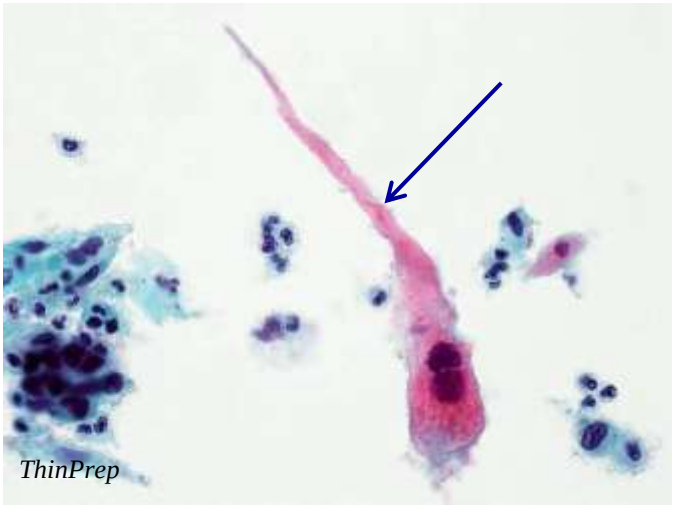
Two images showing keratinised tadpole/caudate forms in SCC. The left image shows a binucleate cell (blue arrow) with a low N:C ratio. Although atypical, this individual cell does not have the criteria of malignancy. The right image has a tadpole cell with a grossly abnormal hyperchromatic nucleus with a high N:C ratio consistent with malignancy.

Spindle or fibre cells

- Narrow, elongated spindle shaped varying in length from10-40µm.
- Keratinization is variable (cyanophilic or eosinophilic). May be Herxheimer spirals.
- Nuclei are elongated (cigar shaped) and hyperchromatic
- The nucleus bulges in the cytoplasm giving a high N/C ratio



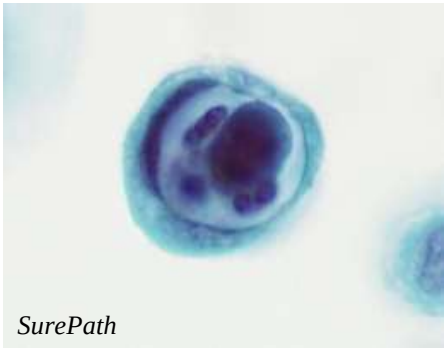
Spindle cells in SCC



Herxheimer spirals running the length of spindle cells in SCC. Note the coiled architecture of these tonofibrils, which are evidence of keratinisation.

Cell-in-cell engulfment (cannibalism)

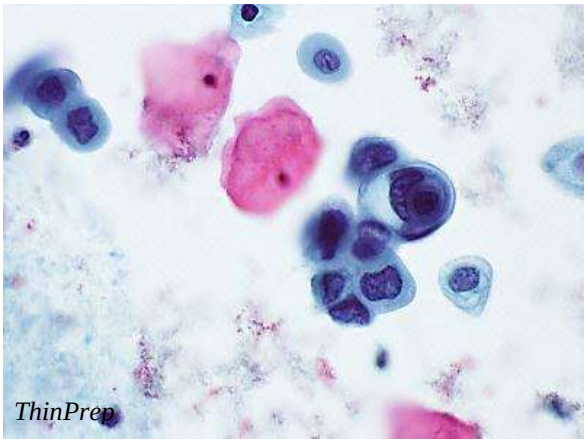
A malignant cell may engulf another cell producing a "cell-in-cell" appearance. Care must be taken to ensure that the appearance is truly cell-in-cell rather than a cell lying over another cell. This appearance may be observed in HSIL as well as in invasive squamous cell carcinoma.

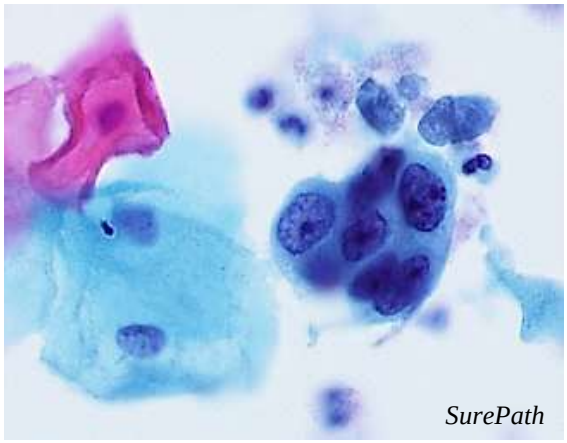


Cell-in-cell engulfment in cases of SCC

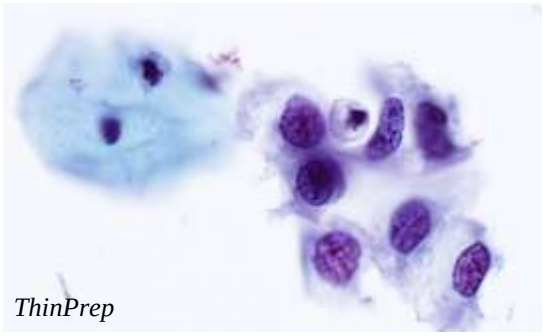
Non-keratinising SCC

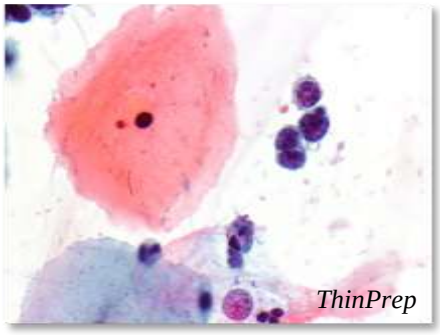
This is the most common subtype of SCC. Classic features of keratinisation are not or only focally present. Individual keratinised cells may be seen. Nuclei are large, pleomorphic and hyperchromatic and contain prominent, irregular nucleoli. The cell size can vary considerably from large non-keratinised cells to small cells that closely resemble HSIL. Tumour diathesis is more common than with keratinised SCC.





Non-keratinising SCC





Malignant squamous cells: Cell size can vary considerably in non-keratinised tumours. Large malignant squamous cells (left) contrast with small malignant cells (right) which closely resemble HSIL. The uneven distribution of nuclear chromatin indicates invasive SCC.

These poorly differentiated squamous cell carcinomas have small tumour cells and can be very difficult to diagnose and to distinguish from a number of other types of malignancy. Often ancillary techniques such as immunohistochemistry and/or lymphoid marker studies are needed to confirm the diagnosis. Any sample with a suspicion of a small cell malignancy should be referred to a pathologist. Diagnostic features include:

- Syncytial groups may mimic glandular clusters but lack the rounded 3-dimensional presentation classic to adenocarcinoma.
- Cells may be single or clustered and as the cytoplasm is scant and fragile, stripped nuclei may be present.
- The nuclei are irregular, small and vary in size with fairly smooth nuclear membranes. The chromatin pattern is often coarsely granular and hyperchromatic. Small conspicuous nucleoli may be evident.

- Squamous carcinomas with small cells consist of a relatively monotonous population of undifferentiated small malignant cells, and must be differentiated from lymphoma. The presence of groups of cells suggests an epithelial origin (carcinoma) as lymphoma cells are not cohesive. Misinterpretation as normal lymphocytes is another pitfall.

- Squamous carcinomas with small cells may have an endometrioid appearance on screening power and can be overlooked as normal endometrial cells. However they will have malignant hyperchromatic nuclei on high-power examination and there are usually other features of malignancy such as a tumour diathesis and/or a marked loss of cell cohesion (single cells). High power examination of crowded hyperchromatic groups is essential to reduce false negative reports in this situation.

- Squamous carcinoma with small cells needs to be distinguished from "Small cell carcinoma with neuroendocrine features", which is not a squamous cell carcinoma. The appearances in cytology can be similar but be distinguished using immunohistochemistry. Small cell carcinoma with neuroendocrine features has a rapid progressive clinical course and needs to be treated aggressively so the distinction is very important.

Other SCC subtypes

Other variants of SCC are described, some of which carry prognostic implications.

- A rare spindle and giant cell variant of squamous carcinoma sheds a combination of squamoid cells and multinucleated giant cells.
- Verrucous carcinoma is also rare. It looks like a large condyloma clinically but is a slow-growing locally invasive malignant lesion. The frond-like papillae on the surface can be seen as papillary groups in cytology samples and as the degree of cytologic atypia is low, it can be under-reported in cytology and histology samples.
- Papillary squamous cell carcinoma is a further rare variant which also has surface papillary projections which may be evident in a cytology sample. There are varying proportions of transitional carcinoma as well as SCC in these lesions. Typical invasive squamous cell carcinoma is present the base of what is often better differentiated squamous epithelium superficially so these lesions can also be under-reported on both cytology and superficial histologic biopsies.
- Mixed squamous and glandular lesions are relatively common. This can be adenosquamous carcinoma showing both squamous and glandular differentiation in the same tumour, or can be "collision tumours" where both squamous and adenocarcinomas are present in the same cervix concurrently. Invasive squamous carcinoma associated with Adenocarcinoma in situ (AIS) or adenocarcinoma associated with HSIL also occurs.

2.10.7 HSIL with features suspicious of invasion

The term "HSIL with features suspicious of invasion" is used in situations where the cytological features extend beyond those observed with HSIL but are inconclusive for invasive carcinoma. It is important that the possibility of invasion is conveyed to the sample taker even if a definite diagnosis cannot be made. Urgent referral for specialist assessment at colposcopy will be recommended. The subtle changes that may be observed in cases of HSIL with features suspicious of invasion include increasing pleomorphism, nucleoli, a tumour diathesis and/or chromatin features suspicious of invasion (such as clumping and clearing). The features may sometimes be present focally (in a few groups only), with typical HSIL in the rest of the sample.

2.10.8 The New Zealand Invasive Cervical Cancer Audit: 2000-2004

A nation-wide audit of women diagnosed with invasive cervical cancer between 1 January 2000 and 30 September 2002 was conducted in New Zealand in 2000-2004. Women were interviewed, medical records were reviewed where consent was given, and cytology slides taken in the four years prior to the histologic diagnosis of invasive malignancy and reported as less than high-grade, were rescreened by a rigorous blinded review process.

Of the 371 women who gave consent to have their medical records reviewed, the types of invasive cervical cancer present were:

Squamous cell carcinoma	287 cases	(78%)
Endocervical adenocarcinoma	57 cases	(15%)
Adenosquamous carcinoma	22 cases	(6%)
Other types	5 cases	(1%)

All the cytology slides reviewed were **conventional smears**, not LBC samples. Some of the key findings of the slide reviews were:

- Cases originally reported as negative and upgraded to SCC by the audit review were more likely to have:
    - Fewer than 50 high-grade cells on the slide in 50% of cases
    - Bland chromatin in 22% of cases
    - Small cell size in 8% of cases
    - Single cells only (no groups) in 33% of cases

  Tumour necrosis was a common background feature in the upgraded cases.

- Cases originally reported as negative and upgraded to adenocarcinoma were more likely to have:
    - Negative samples only reported prior to the histologic diagnosis of invasion.
    - Small cell size (endometrioid subtype) in 10% of cases
    - AIS present
    - Features of intestinal metaplasia present

Other important audit findings were:

- Only 50% of women with invasive cervical cancer had had a cervical cytology sample in the 3-year period prior to their diagnosis of malignancy and only 20% had an adequate screening history. i.e. 70% of women who developed cervical cancer had not had any cervical screening or were under-screened.
- Women with lower stage disease were more likely to have been screened.
- Maori women were less well screened and a greater proportion had later-stage disease compared with non-Maori women.
- The laboratory false negative rate was below the upper threshold limit set by the Audit team prior to the slide review.

More information on the NZ Cervical Cancer Audit can be found at http://www.health.govt.nz/system/files/documents/publications/initialresponse-nzcervicalcanceraudit2004.pdf

2.10.9 Questions

1. Define superficially invasive SCC of the cervix.

2. Describe your understanding of CIN 3, superficially invasive carcinoma and invasive SCC and the histologic and cytologic features that distinguish these entities.

3. List the different ways that invasive cervical carcinoma presents to clinicians.

4. Discuss the histologic features of the following:
- Keratinising SCC
- Non-keratinising SCC

5. Why is it important to undertake a full rescreen by an appropriately experienced cytologist, of a cervical cytology sample that is unsatisfactory because of blood and/or inflammation?

6. Discuss the cytological presentation of the following cancers. Include a discussion about important differential diagnoses for each entity in your answer.
- Keratinising SCC
- Non-keratinising SCC

References

- Lewis H (NCSP), Baker S, Kaminetska Z (NSU). Cervical screening in New Zealand: A brief statistical review of the first decade. 2005. Available at: https://www.nsu.govt.nz/health-professionals/national-cervical-screening-programme/publications-and-reports?page=1

- Ministry of Health. Cancer: New Registrations and Deaths 2011. Published Sept 2014.

- Sadler L, Priest P, Peters J, et al. 2004. *The New Zealand Cervical Cancer Audit; Screening of Women with Cervical Cancer, 2000-2002.* Wellington: Ministry of Health.

- Schreiner A, Hoda S. Herxheimer Spiral on Liquid-Based Papanicolaou Test. *Diagnostic Cytopathology* 2013: 41:60

- Sykes P, Gopala K, Tan AL, Kenwright D, Petrich S, Molijn A, Chen J. Type distribution of human papillomavirus among adult women diagnosed with invasive cervical cancer (stage 1b or higher) in New Zealand. *BMC Infectious Diseases* 2014, 14:374.

- Wentz WB, Reagan JW (1959). Survival in cervical cancer with respect to cell type. *Cancer* 12(2):384-388.

2.11 CORE TOPIC: GLANDULAR LESIONS

2.11.1 Introduction

Cervical screening programmes have reduced the incidence and mortality of cervical cancer predominantly by detecting pre-malignant squamous lesions before invasive squamous cell carcinoma develops. There has been less success with glandular lesions. Endocervical lesions are more difficult to detect by cytology and are also more difficult for the colposcopist to detect and biopsy because they are usually located within the endocervical canal. Endocervical adenocarcinoma is less common than squamous cell carcinoma although the relative frequency of glandular lesions is increasing due in part to the decline in numbers of squamous cancers as a result of organised screening programmes.

This section focuses on the cytological appearances of glandular abnormalities. Glandular lesions offer one of the greatest challenges in cervical cytology. They are much less frequent than squamous lesions so it takes more time to gain experience with these cases. Because glandular lesions are difficult to assess by cytology and colposcopy, all cytologic glandular abnormalities of any grade are referred directly for specialist assessment and colposcopy.

Appreciating that a glandular lesion is or may be present is the critical first step. Second opinions are frequently sought for these cases. The approach for groups or sheets of glandular cells is similar to that used for crowded hyperchromatic squamous sheets: there are high-risk patterns that alert the cytoscreener to a potential abnormality, and suspicious groups then need to be examined closely with high power magnification. The cytologic distinction between high-grade squamous and glandular lesions can be difficult. Interpreting a group of cells as a high-grade endocervical lesion where the biopsy turns out to be a squamous lesion is a relatively common occurrence, particularly where high-grade squamous lesions extend into endocervical crypts giving a gland-like appearance on cytology. It is also relatively common for endocervical and squamous lesions to coexist. In cytology the aim is to predict the histology, but if a high-grade abnormality is detected and the woman is referred, screening has performed its most useful function even if the classification of the high-grade abnormality is different on final histology.

Reviewing incidence data and concepts of aetiology for endocervical lesions helps practitioners to appreciate their importance and understand the context in which they occur.

2.11.2 Incidence of endocervical lesions

Cytology reporting of glandular abnormalities
Glandular abnormalities are reported much less frequently than squamous abnormalities in cervical cytology. In 2014, atypical glandular cells/AIS was reported in 0.10% of all cervical cytology reports compared with 7.2% for ASC-US/LSIL/ASC-H/HSIL reports. Invasive adenocarcinoma (all types) was reported in 0.02% of all cytology reports.

Glandular lesions are over-represented in false-negative cases, reflecting the difficulties in identifying and assessing glandular lesions. In the New Zealand Invasive Cancer Audit 2002-2004, 22% of 65 smears (conventional cytology smears) reported as negative within 4 years prior to invasive adenocarcinoma were upgrade to high-grade on review, compared with 18% of 160 negative smears prior to invasive SCC.

Incidence of endocervical lesions

In New Zealand in 2012, 70% of new cases of invasive cervical cancer were of squamous type and 16% were primary endocervical adenocarcinomas. The remainder were a mix of other types of adenocarcinoma involving the cervix and other types of malignancy.

Numerous studies have reported an increasing incidence of endocervical adenocarcinoma (AC) even in countries with established screening programmes. Key findings are:

- In the United States of America (USA) and Western Europe from the 1950's and 60's approximately 95% of all invasive cervical cancers diagnosed were SCC on histology, whereas adenocarcinoma (AC) accounted for the remaining 5%. From the 1970's this ratio changed to 75%-80% for SCC and 20-25% for AC. Australia, Canada, England and Scotland have annual increases in age-adjusted incidence rates for AC in the range of 6 – 12%, with significant increases occurring in women aged 25-49 years of age compared to women aged 50-74 years.
- An increase in AC appears to have started amongst women born in the 1920's with a cumulative incidence increase of 4.2% per year observed for women born since 1935. Women in the USA and Canada who were born in the mid-1950's are at over three times the risk for cervical AC than women born in the mid-1930's.

Key reasons for the observed increase of *adenocarcinoma in situ* (AIS) and AC in relation to the decrease observed for SCC are:

- There is a true increase in the biological incidence of AC.
- Endocervical sampling devices such as the cytobrush introduced in the early 1980s, improved the sampling of AIS lesions within the endocervical canal.
- HPV was detected in 78% of AIS and AC cases in a 2014 review of HPV genotypes in invasive cancers in New Zealand (Sykes et al 2014), with HPV 18 (40%) and 16 (35%) being the most prevalent subtypes. HPV may act as a direct transforming agent by infecting the reserve cells of the endocervical epithelium which are committed to glandular differentiation, leading to the development of glandular dysplasias, AIS and AC.
- Better recognition and awareness of cytological criteria has allowed cytoscientists and pathologists to make a diagnosis of AIS more confidently.
- Effective screening, detection and treatment of squamous intraepithelial lesions (SILs) has led to a true decrease in the incidence of SCC in western countries.

Endocervical sampling has also led to an increase in false positive diagnoses of endocervical lesions. This is usually the result of higher sampling, as normal upper endocervical canal and lower uterine segment cells in cytology samples can mimic the appearance of AIS and adenocarcinoma.

2.11.3 Aetiology of Adenocarcinoma

AIS as a precursor lesion for AC

Friedell & McKay first reported the concept of a preinvasive lesion for endocervical AC in 1953. They presented histological case reports for two women who had neoplastic endocervical glands interspersed within normal endocervical glands, but showed no evidence of invasion.

It was not until the 1970s that the understanding of this entity developed further. Reasons for renewed interest were firstly a theory that the common sub-columnar reserve cell was the cell of origin for all types of cervical cancer and secondly, there was a desire to categorize glandular lesions in a similar fashion to the precursor lesions of squamous cell carcinoma of the cervix. Ultrastructurally, reserve cells contain small bundles or aggregates of fine filaments with a few secretory granules indicating potential for both squamous and glandular differentiation. These cells have microvilli and adenomatous cellular connections as tight junctions. AIS cells have also been shown to contain tonofibrils and a few secretory granules, supporting the likelihood that AIS arises from reserve cells. It is now accepted that the reserve cell of the endocervical canal is the cell of origin for the development of both squamous and glandular abnormalities.

No plausible explanation exists to explain how or why the neoplastic transformation will develop to involve glandular or squamous epithelium. Bi-directional differentiation of reserve cells may be the reason why a co-existing squamous intraepithelial lesion is seen in approximately 50% of AIS cases.

Evidence that supports the concept that AIS is a precursor lesion to invasive AC includes:

- The mean age for women who develop AIS is approximately 39 years compared with 52 years for women who develop AC. Note that the mean age difference between AIS and AC provides ample time for screening for detection and treatment of AIS before AC develops.
- AIS is frequently found adjacent to invasive AC.
- There are histological and cytological similarities between AIS and AC.
- HPV is found in 40%-90% of AIS cases and is frequently found in AC.
- In reviewing cases of women who have developed AC, AIS was originally missed in some cervical biopsies reported as negative preceding the AC diagnosis. This suggests that if AIS is left untreated it may proceed to AC.

Features of AIS

- AIS usually arises at or close to the transformation zone of the cervix, involving the surface endocervical epithelium with extension to variable depths in endocervical glandular crypts. AIS can occur underneath normal or dysplastic squamous epithelium in the transformation zone. Investigators have reported AIS to occur as a focal or localized lesion, but it may also be extensive. Multi-focal lesions occur in a minority of cases, but the AIS lesion can extend up the canal for a variable distance of up to 30mm or more.
- In approximately 50% of AIS cases there is an accompanying SIL. The detection of the more easily recognised SIL can lead to the incidental finding of an AIS lesion in histologic biopsies.
- The difficulties associated with colposcopic identification of endocervical lesions emphasizes the importance of cervical cytology in detecting endocervical lesions. A careful search for a concomitant glandular lesion should be made every time a case of HSIL is reported.
- Women who are diagnosed with AIS or even invasive AC are often asymptomatic and may have a normal colposcopic examination.
- Women who are diagnosed with Stage 1 AC have a 5 year survival of 80%. This prognosis decreases with advancing stage to 40% for Stage 2 AC, when tumour is

invasive beyond the uterus. Detecting precursor lesions or early stage AC can make a significant difference to prognosis.

2.11.4 Morphology of Adenocarcinoma in situ (AIS)

The normal histologic appearance of endocervical epithelium consists of a single layer of columnar epithelial cells. Cytologically these cells are seen in a "honeycomb" configuration when viewed from above, or like a "picket fence" if viewed from the side.

AIS is seen histologically as hyperchromatic neoplastic cells that vary in size and shape and form a crowded pseudostratified columnar epithelium that remains confined within the basement membrane. Cell borders can be indistinct because the cells are very crowded. The N:C ratio is higher than in normal endocervical cells because nuclei are enlarged and there is usually less cytoplasmic mucin. Nuclei are pleomorphic to a varying degree, often elongated or oval, and lie perpendicular to the luminal surface. Nucleoli may be seen and if present are typically small. Mitotic figures and apoptosis may also be observed.

The cytological presentation of AIS is well described although there is still some debate regarding the significance/emphasis of the different cytological parameters required for an unequivocal report of AIS. Experience with confirmed cases is needed in order to make a confident diagnosis. In 1987/8 Pacey and co-workers in Westmead Sydney published some of the earliest descriptions of AIS in cervical smears that have not really been surpassed. Because of their work, cytologists in both Australia and New Zealand have a heritage of competently and confidently diagnosing AIS by cytology. A specific report category for AIS was used for years in Australasia before being accepted in other parts of the world and before inclusion as a separate category in The Bethesda System.

Morphology of AIS : Two important points to note:

1. **No one single criterion is sufficient for the cytological diagnosis of AIS.** The diagnosis should only be made from collective parameters.
2. **Architecture matters most**, when assessing endocervical glandular lesions. Abnormal glandular architecture must be observed to make a diagnosis of AIS. The individual cell cytology can range from very well differentiated to poorly differentiated.

Architectural Features of AIS

- Often **increased groups/sheets of endocervical cells** which are prominent at low-power screening magnification.
- **Tightly packed/crowded sheets of endocervical-like cells** (hyperchromatic crowded groups) – **potential high-risk pattern: the most consistent and striking feature**
- Isolated **strips** of endocervical cells
- **Pseudostratified nuclei** (nuclei at different distances from the basement membrane/luminal surface)
- Nuclear palisading at periphery of groups
- Communal borders (common linear edge at one side of a group)
- **"Feathered" edges** at periphery of cellular groups that presents as cytoplasmic "nipples" and nuclei splaying out from the edge of tissue fragments
- Rosette or acinar formations
- "Bird-tale" arrangements: wedge-shaped flat groups (particular feature in SurePath)

- Gland-like structures/lumina in crowded endocervical sheets
- Papillary-like projections occasionally
- **"Tennis racquet" or "frying pan" cells** where the nucleus bulges out at the sides of the cell (also called "egg in snake" appearance). Easiest to see in single cells or loose clusters.

Nuclear Features seen in AIS

- Crowded nuclei which may be enlarged
- Oval to elongated nuclei are typical but they may be round. Nuclei may "mould" against one another to accommodate the high rate of cell division
- Increased N:C ratio
- Hyperchromatic nuclei
- Fine to coarse granular **stippled evenly distributed chromatin**
- Small nucleoli may be seen: single or multiple

Other Features

- Mitotic figures
- Apoptotic debris

"Feathering" describes nuclei that appear to be pulling off from the edge of sheets and lie at different levels at the edge of the sheet of cells. The cytoplasm may be lost and nuclei appear attenuated or small cytoplasmic tags may be present.

"Pseudostratification" describes nuclei lying at different levels in neighbouring cells with maintained polarity. In small strips, cells with enlarged nuclei that retain cell-to-cell adherence at the luminal edge can assume a curved shape, after the cells are pulled off the basement membrane during sampling.

A "rosette" is a circular group of cells with nuclei lying at the periphery and the cytoplasm directed to the centre of the circular group (acinar-like formation).

There are differences in the appearance of AIS in ThinPrep compared with SurePath preparations, caused by differences in processing. In ThinPrep preparations, both normal and abnormal glandular groups round up in the fixative fluid. The cells may appear small and crowded. It is essential to closely examine rounded groups of cells with high power magnification, as this is a high-risk pattern and the changes seen in AIS can be subtle. In SurePath, glandular groups can appear more stretched out. It is important to become familiar with the appearances seen with the type of LBC used. Fortunately, most preparations with AIS usually have a number of groups/sheets of abnormal cells – observation of many glandular groups/sheets should be treated as a high-risk pattern, mandating close examination with high power magnification.

The distinction between AIS and endocervical adenocarcinoma can be very difficult. Cytology is not particularly good at distinguishing AIS and invasive endocervical adenocarcinoma because there is an overlap of appearances. The following comments give some guidance to assist with distinguishing AIS from AC.

AIS versus Invasive Endocervical Adenocarcinoma

- The overall presentation of AIS tends to maintain a uniformity of abnormality, and does not usually show bizarre cellular pleomorphism.
- Bizarre architecture/loss of polarity is not a usually a feature of AIS but may be seen with AC. Some AIS is well differentiated cytologically.
- Single malignant cells are more common with invasive lesions although may be seen in AIS in LBC preparations.
- Large variable secretory vacuoles are not usually observed in AIS although abundant cytoplasmic mucin can be present in the intestinal variant.
- Conspicuous prominent nucleoli are not common in AIS but are observed with AC.
- There is usually evidence of diathesis with AC – "clinging" fibrillary cyanophilic and orangeophilic material around groups of malignant cells – but not with AIS.
- AIS frequently coexists with invasive endocervical adenocarcinoma.

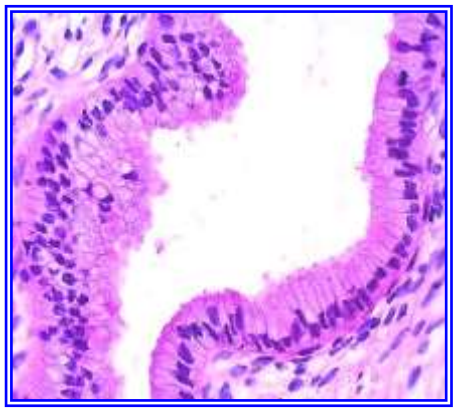
Squamous versus glandular (endocervical) lesions

Groups of cells from high-grade squamous lesions may have glandular-appearing architecture particularly where HSIL extends down into endocervical crypts giving a rounded gland-like appearance of HSIL cell groups in cytology samples. The following points can be useful:

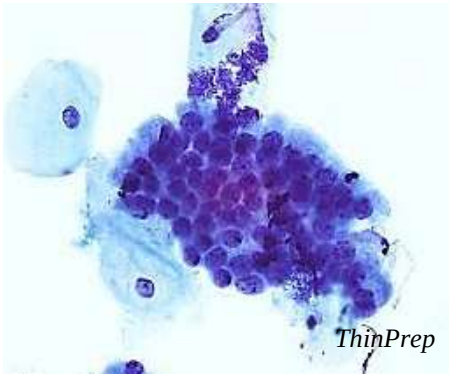
- Examine the edges of cell groups carefully. Look for communal borders with nuclei set back from the outer edge of the cytoplasm i.e. basal nuclei which may have a palisading appearance, suggesting a true glandular group.
- With HSIL extending into glands, the peripheral cells are often flattened and elongated, lying in parallel with the edge of the group.
- Differentiating between a squamous or endocervical lesion can be difficult cytologically although the most important point is detecting the presence of a high-grade neoplastic lesion. A significant number of cytology cases reported as endocervical lesions turn out to be squamous lesions on biopsy.
- Reporting abnormal glandular features is important because their presence will direct the colposcopist to take particular care when examining the endocervical canal, and may result in a longer length of the endocervix being included in a treatment excision specimen. Concurrent HSIL will be detected in the histologic specimen. In contrast, misclassification of a glandular lesion as squamous could result in a positive endocervical margin in an excision specimen, if a glandular lesion is present.
- It is common for squamous and endocervical lesions to co-exist. A report that identifies both components will place the colposcopist in the best position to ensure the best clinical outcome.

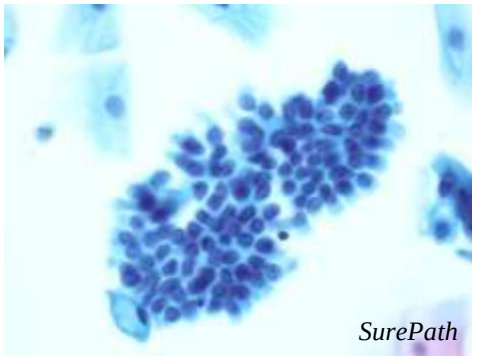
To summarise:

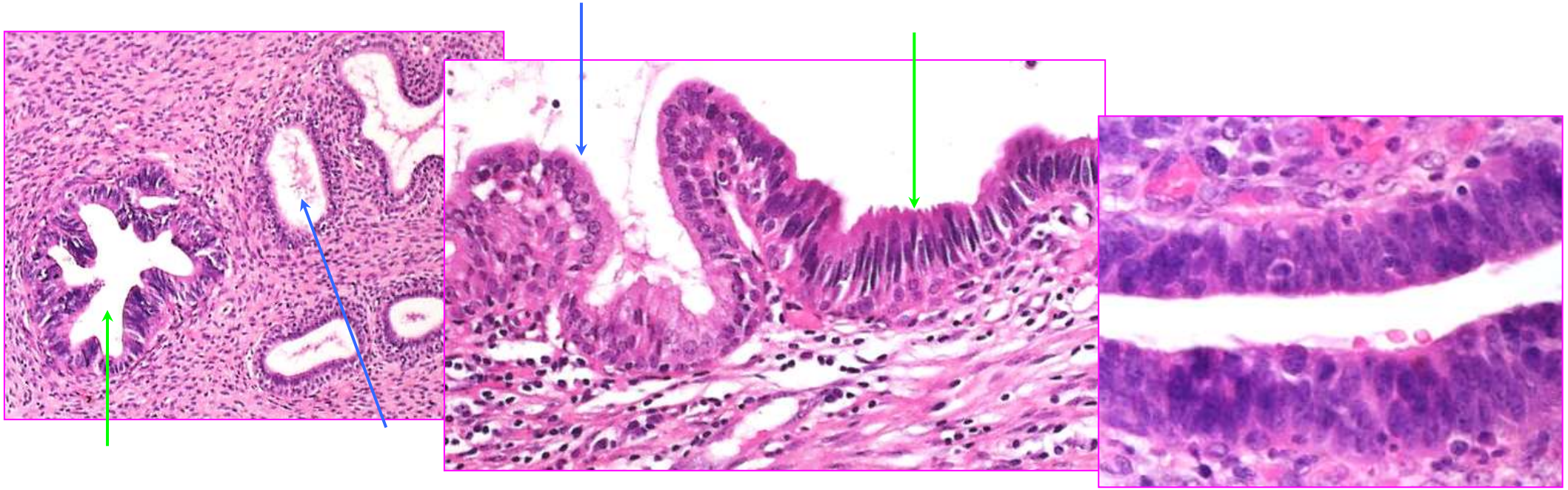
- AIS often presents a striking appearance when observed at a low-power screening magnification with a predominance of crowded cell groups with nuclear crowding but retained basal nuclear polarity and hyperchromasia: **a high-risk pattern**.
- Hyperchromatic crowded groups tend to be flatter (1 to 3 cells thick) than the 3-D thick clusters seen with HSIL (3 to 4+ cells thick).
- On higher magnification, AIS cells can be recognised as tightly packed sheets 1-3 cells thick, cell strips with pseudostratification, feathered/frayed edges, oval to elongated nuclei with increased N/C ratio, and stippled fine to coarse granular chromatin. Apoptotic debris and abnormal mitotic figures may be observed in AIS.



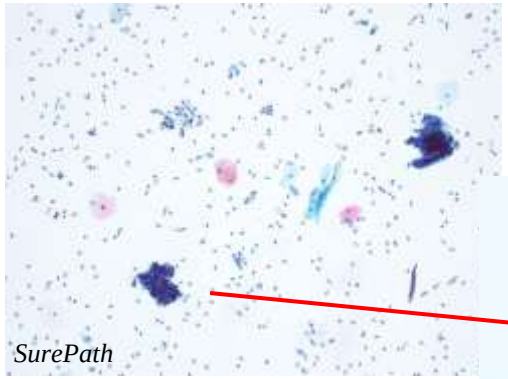
Left: Histology of the **normal endocervical canal** lined with a single layer of endocervical cells.
Right: Cytology of normal honeycomb and columnar endocervical cells. There is no cell overlap, cell polarity is maintained and nuclei are regular and predictable.

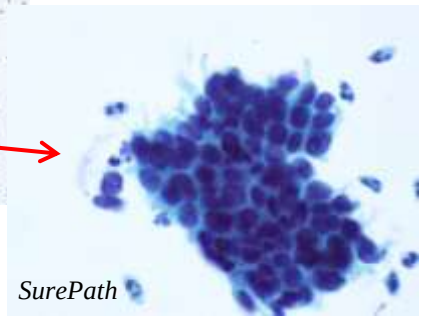


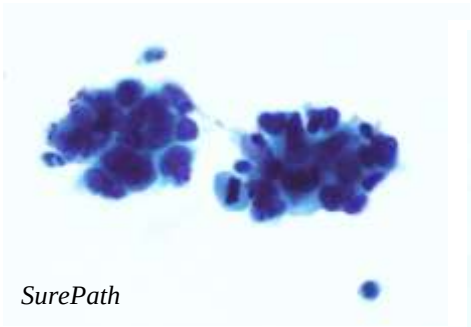


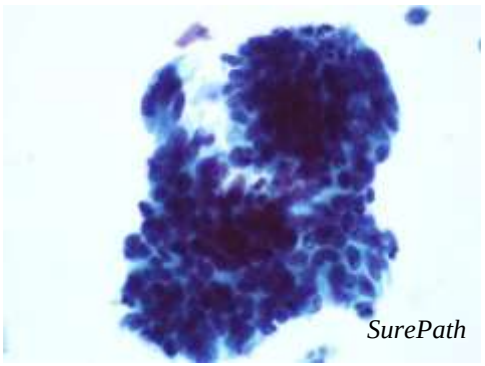


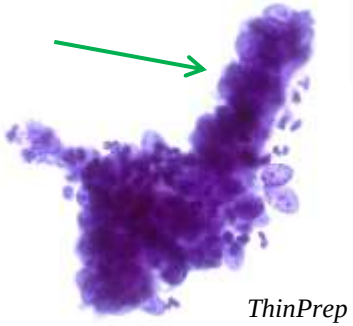
Histology of AIS: *Left:* At low-power endocervical glands with AIS (green arrow) are lined by columnar cells with crowded larger darker (hyperchromatic) nuclei compared with normal glands (blue arrow). *Middle:* The transition from normal endocervical epithelium (blue arrow) to AIS (green arrow) with elongated pseudostratified columnar cells is often abrupt. *Right:* Nuclear crowding, overlapping and pseudostratification in AIS at high-power.











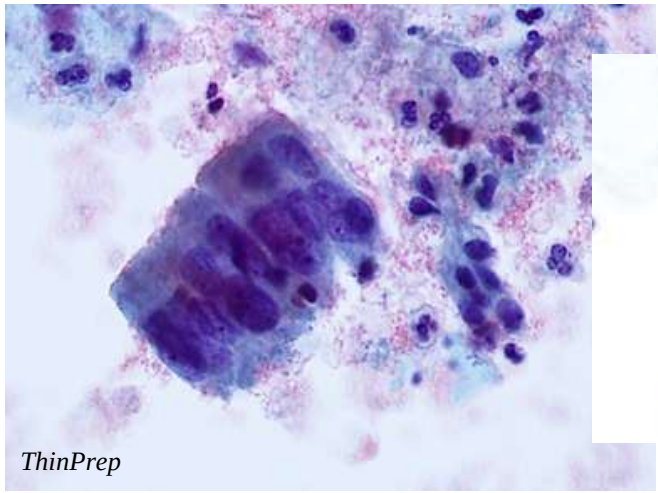
AIS: Irregular crowded groups.

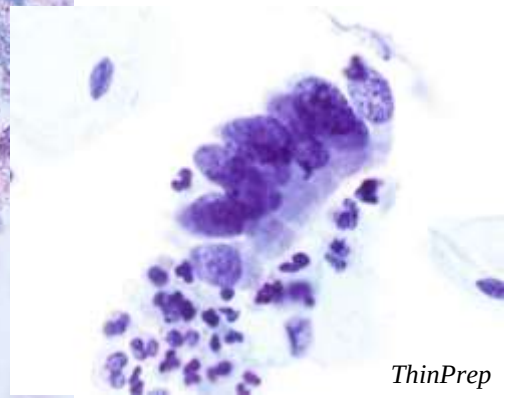
Large and small irregularly-shaped crowded groups of atypical cells are common in AIS. The groups can be small with rounded up edges. The cells are cohesive with nuclei being crowded and overlapped. The nuclear crowding can be intense ("super-crowded" groups). The groups often have well-defined cytoplasmic borders which give a smooth edge and may form a communal border. Specific shapes such as papillary-like formations, abnormal strips, strips coming off the group edge (green arrow), common borders and rosettes are illustrated here and in the following pages. These architectural features are critically important in recognising the glandular nature of the groups. These low power appearances define a high-risk presentation and the groups should not be mistaken for reactive/non-neoplastic endocervical cells or endometrial cells. Close examination and often second opinions are required.

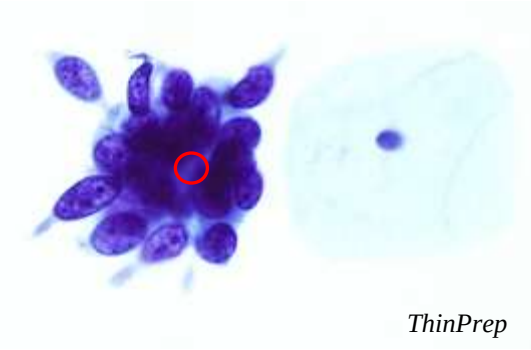
AIS: Retention of cell polarity

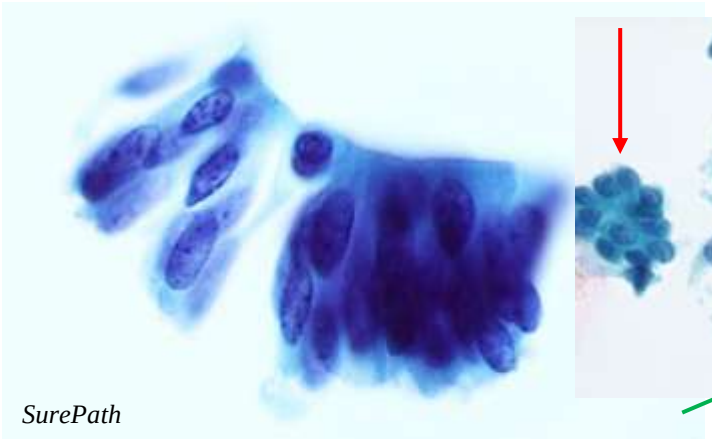
Centre: Histology of endocervical AIS showing elongated overlapping neoplastic nuclei with a distinct polarity from the basal to apical portion of the cells (green arrow) at right angles to the luminal surface (red arrow).

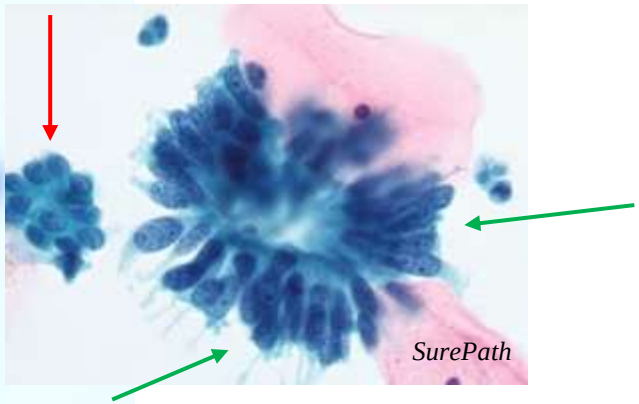
Right and Left: The two cytology images show pseudostratified strips of AIS with the same orientation indicated by the coloured arrows. Other key features are hyperchromatic nuclei with stippled granular chromatin. Nucleoli are present in the SurePath image but are usually inconspicuous. Despite being neoplastic there is often a certain "uniformity" in the morphologic abnormality of the cells.

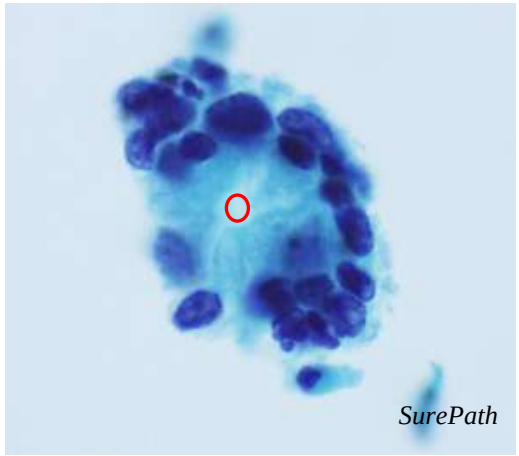








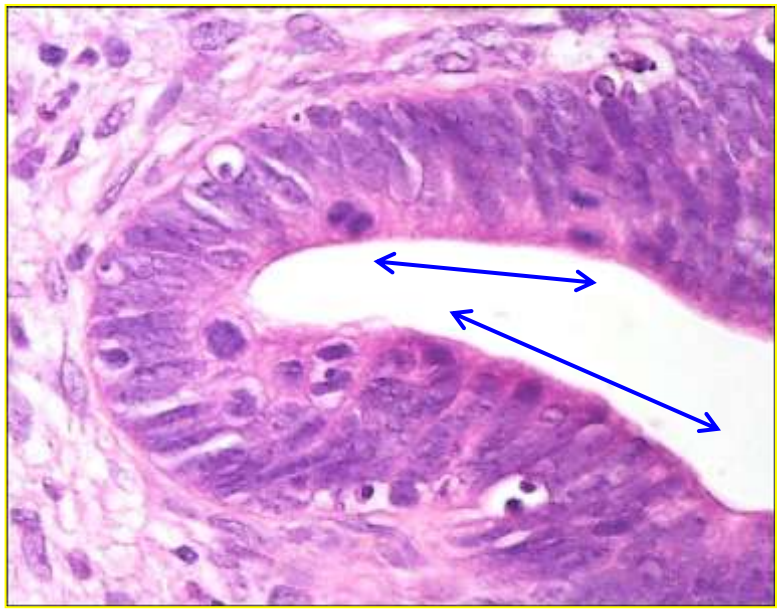


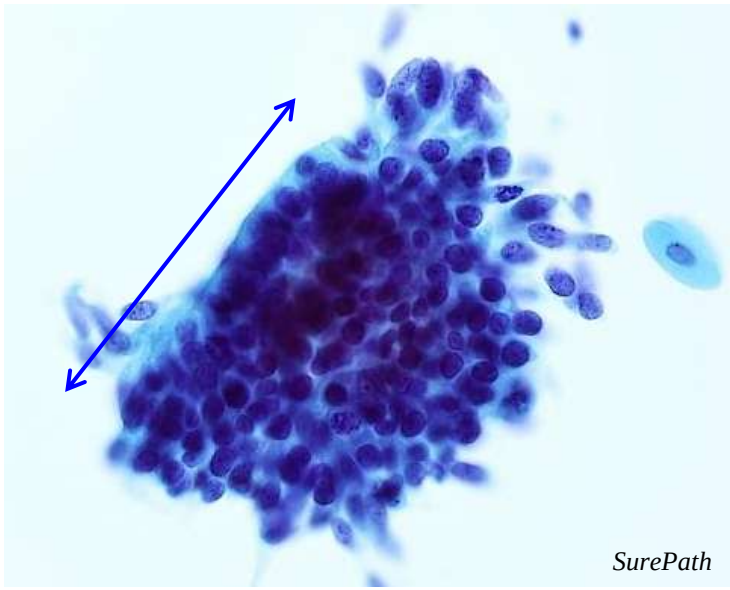


AIS: Strips and Rosettes

Strips where cell polarity is maintained are common in AIS. Strips of normal endocervical cells are also seen in LBC preparations so look for pseudostratification and nuclear abnormality, particularly hyperchromasia in AIS. AIS strips are often curved, with the luminal border at the concave edge. The basal part of the cell expands when the attachment to the basement membrane is lost during sampling, because of the enlarged basal nuclei. Note the feathering (green arrows) and the wedged shaped "bird-tale" group (red arrow).

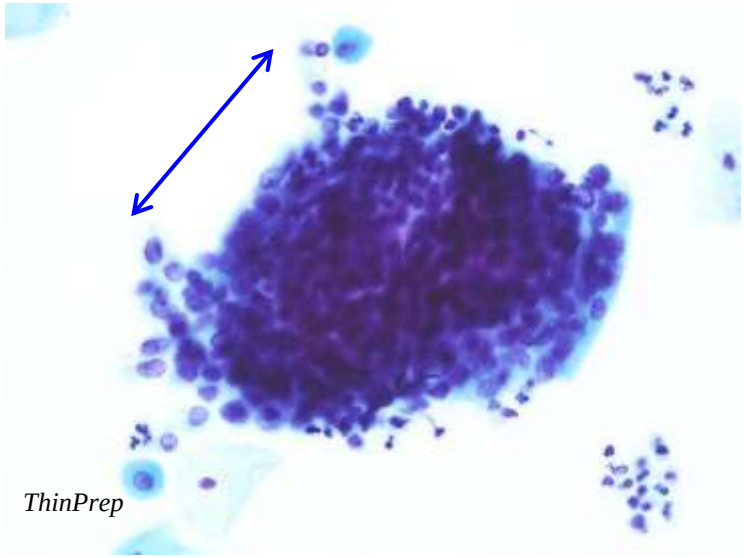
Rosettes are less frequently seen but are a characteristic architectural appearance in AIS. There is mucin in the apical portion of the cells adjacent to a central lumen (red circles) with nuclei radiating out at the periphery of the cluster.





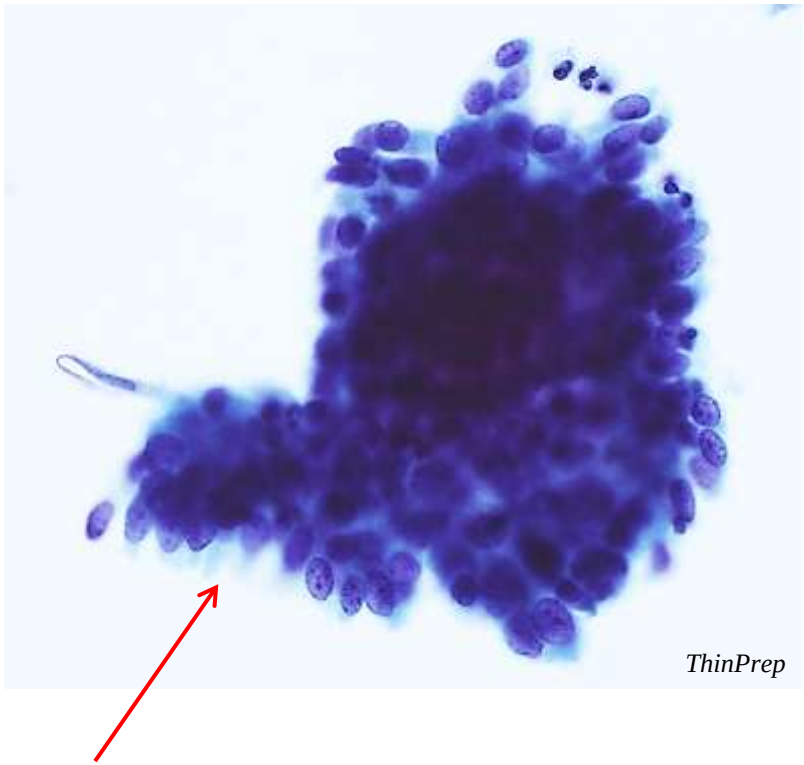
AIS: Common (communal) borders

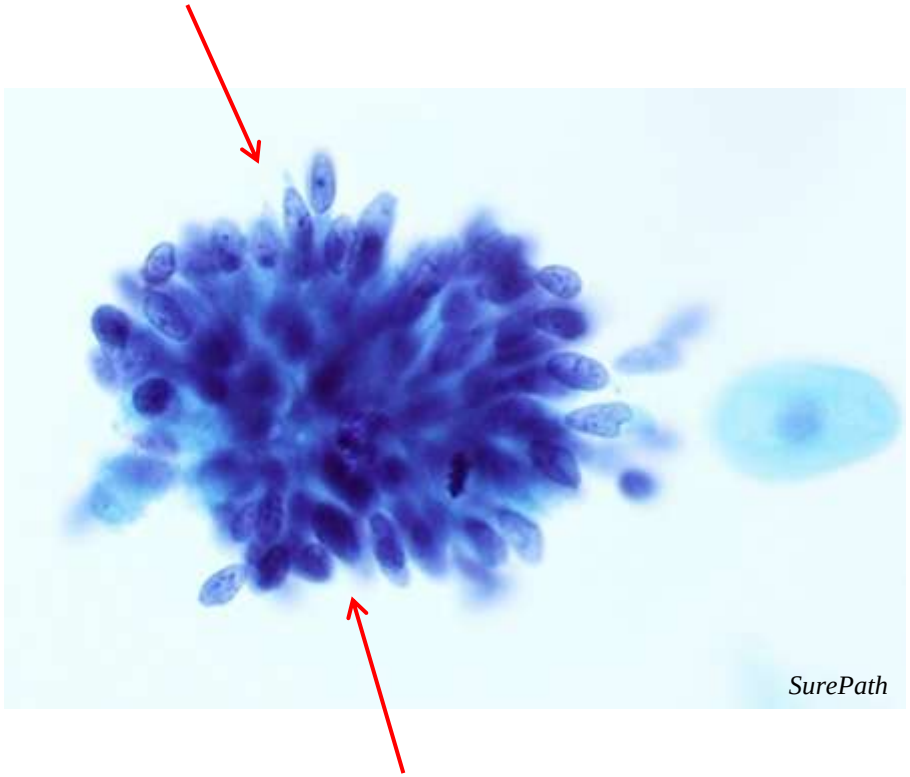
Despite being pseudostratified and crowded, the cells in AIS often maintain their columnar appearance with a glandular or common shared border (blue arrows), seen in histology (above) and in cytology (right). This is a very helpful feature when considering a group of abnormal crowded, overlapped and hyperchromatic cells as it helps to identify the group as glandular.
In these two groups, the nuclei are hyperchromatic but not particularly large. Note the feathering at the edge of the SurePath example.

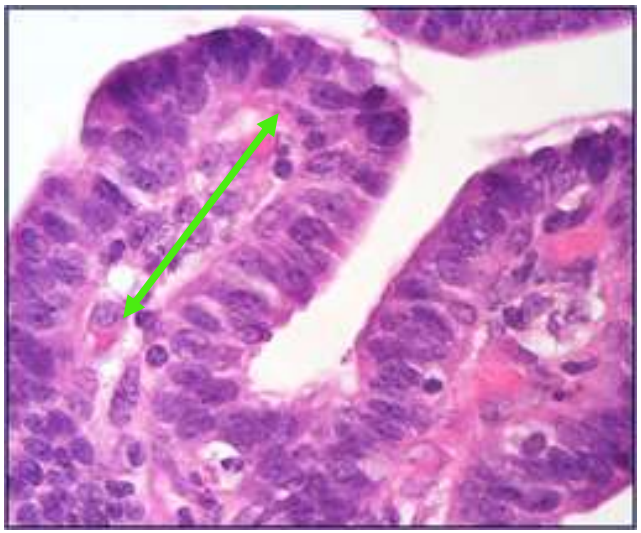


AIS: Feathering.

"Feathering" is the appearance of elongated and splayed out nuclei around the periphery of glandular clusters and is so named because of the resemblance to the tail feathers of a bird. Feathering is quite characteristic on low power screening and is a reliable feature in the differential diagnosis of AIS when present, but only occurs in 10-20% of cases. The nuclei lie at different levels at the edge of the cell cluster. The cytoplasm may appear as small cytoplasmic tags or be lost with nuclei appearing pulled out and attenuated. Feathering is not a feature of reactive endocervical cells.



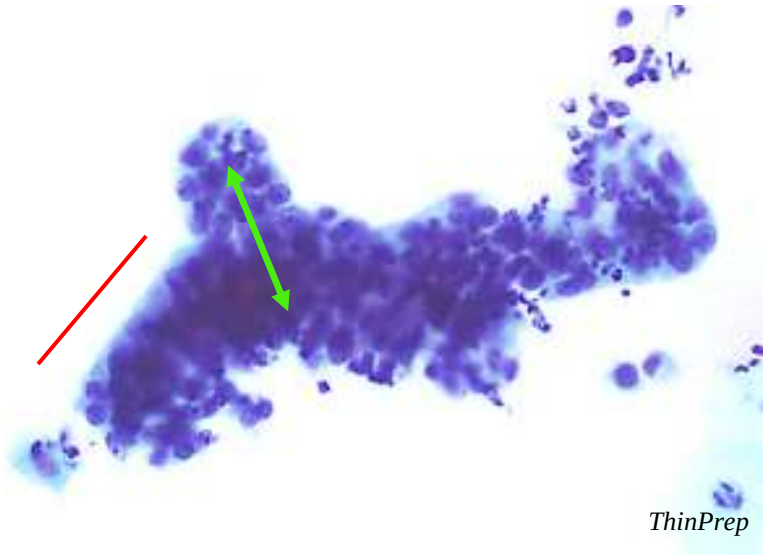




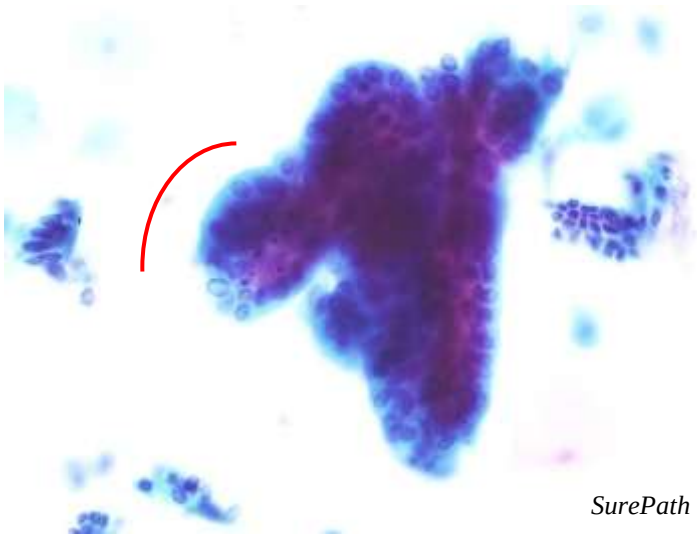
AIS: Papillary-like structures

These indicate a glandular lesion and may be seen in both AIS and in adenocarcinomas from a variety of different sites of origin. The histology of a case of AIS (right) shows surface epithelium covering a finger-like papillary formation with an inner central stromal core (green arrow).

Cytologically, papillary/finger-like arrangements are seen associated with endocervical AIS with many crowded and overlapping nuclei and the nuclear features already described. An inner stromal core is uncommon in cytology and papillary-like configurations are usually identified by a common surface border covering three sides of the cluster. Note the communal edges (red lines).



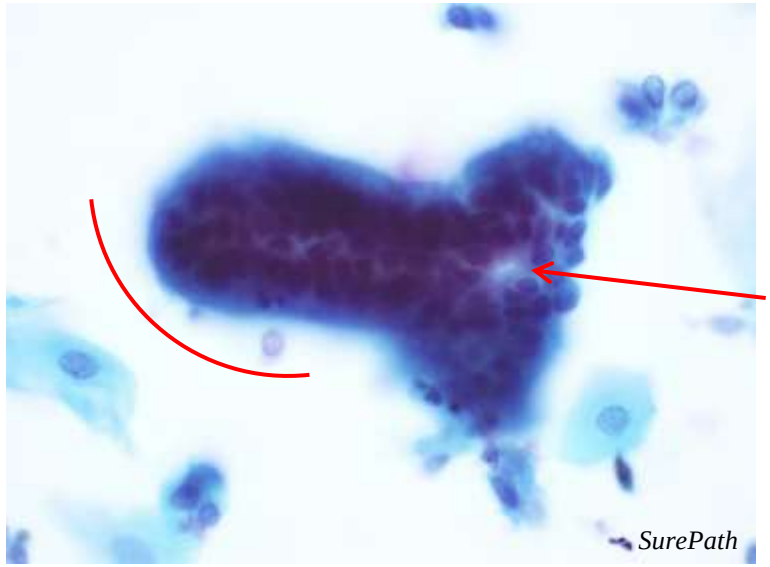
ThinPrep

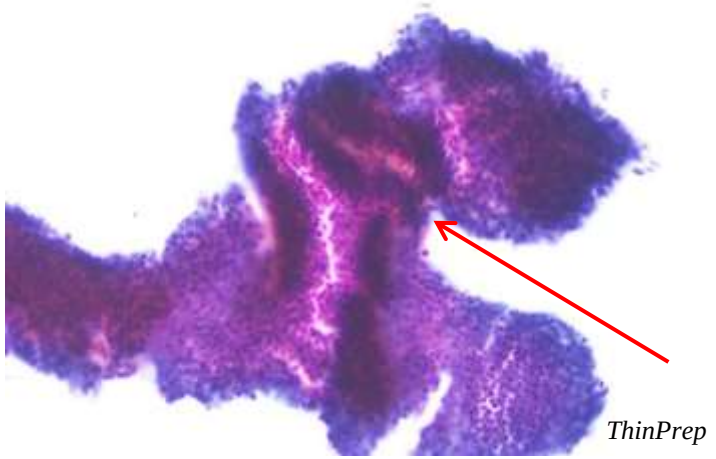


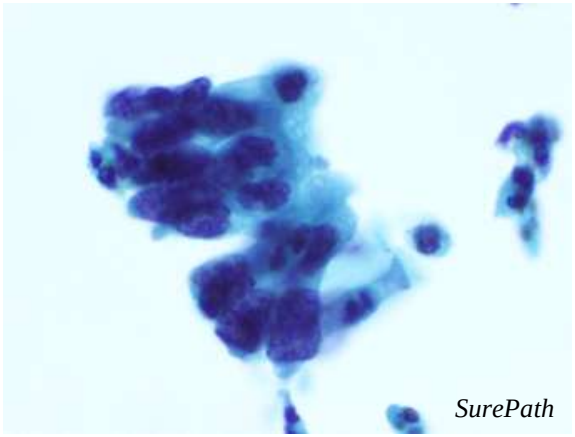
SurePath

AIS: Gland openings

Glandular lumina are occasionally observed within crowded groups shed from endocervical AIS. These lumina will be within the surface epithelium and lead into endocervical crypts (red arrows). Note also the communal edge (red line).







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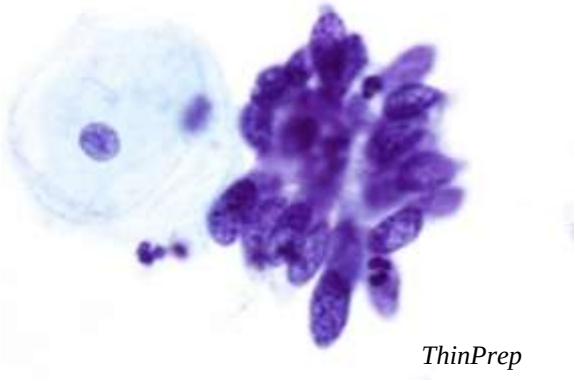


ThinPrep

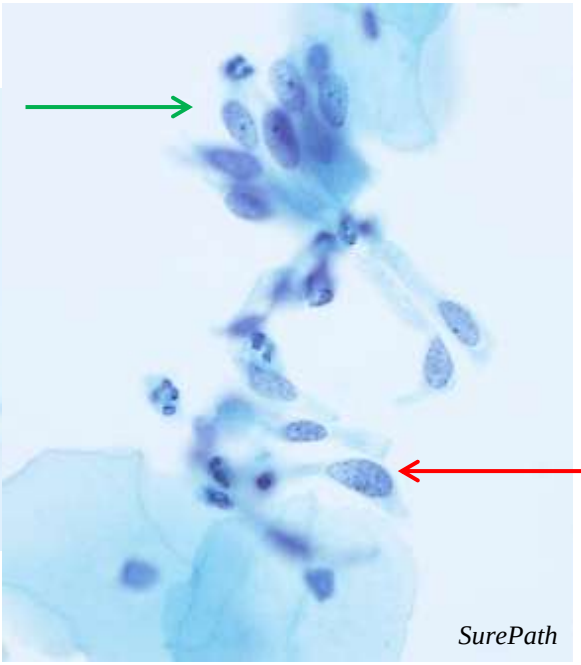
AIS: Nuclear chromatin

AIS nuclei are typically hyperchromatic and usually have a stippled appearance although these nuclear changes can be subtle with very well-differentiated AIS. If present, nucleoli are small and inconspicuous. Prominent nucleoli are not common in endocervical AIS.

Nuclear enlargement

Bulging nuclei in endocervical cells from AIS cause an appearance called "frying pan" cells, or "egg-in-snake". The nucleus (red arrows) is wider in diameter than the cross-section of both ends of the cell where there is cytoplasm only. The nuclear enlargement causes nuclei to splay out at the basal edge of a short strip in the photo on the right (green arrow), giving a wedge-shaped architecture.

ThinPrep



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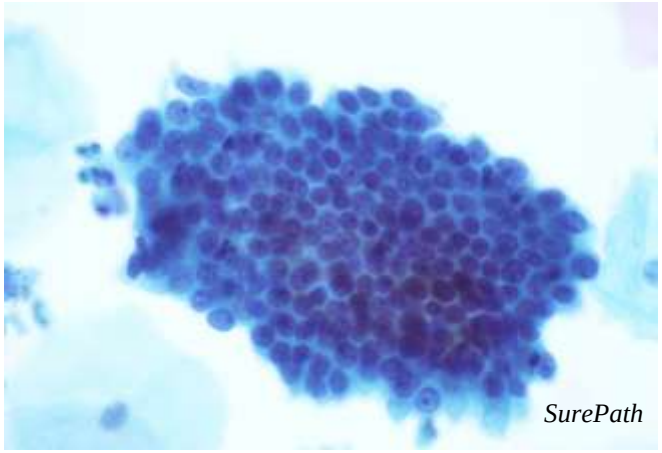
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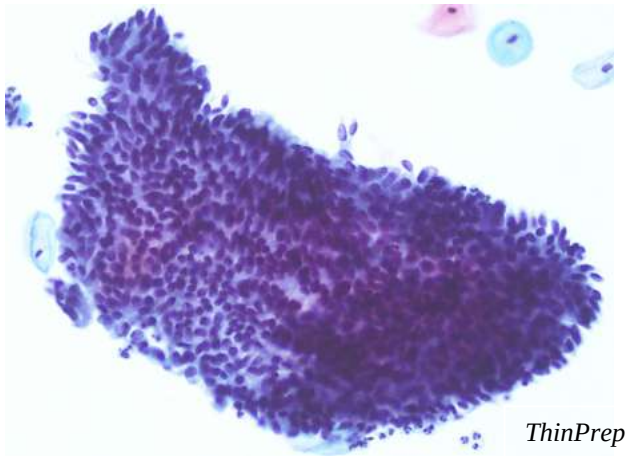
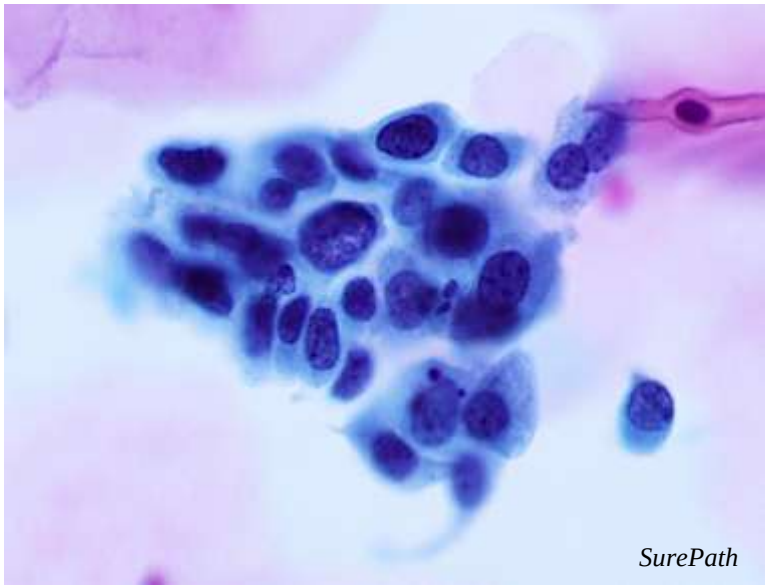

ThinPrep

AIS: Pleomorphism

The degree of nuclear pleomorphism varies in AIS. Most cases are well-differentiated (above) but poorly differentiated AIS also occurs (below). The possibility of invasive endocervical adenocarcinoma needs to be considered in a poorly differentiated case but it but may still be an in-situ lesion.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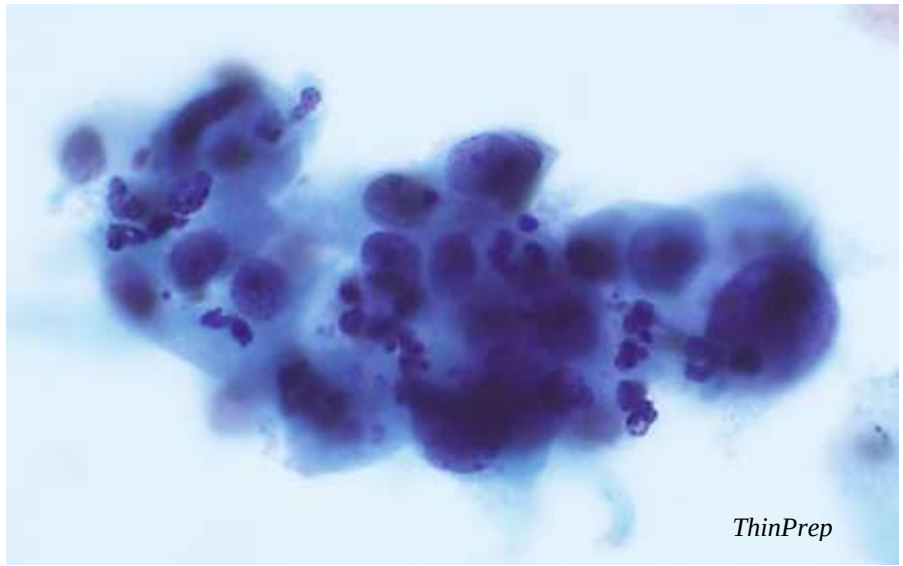
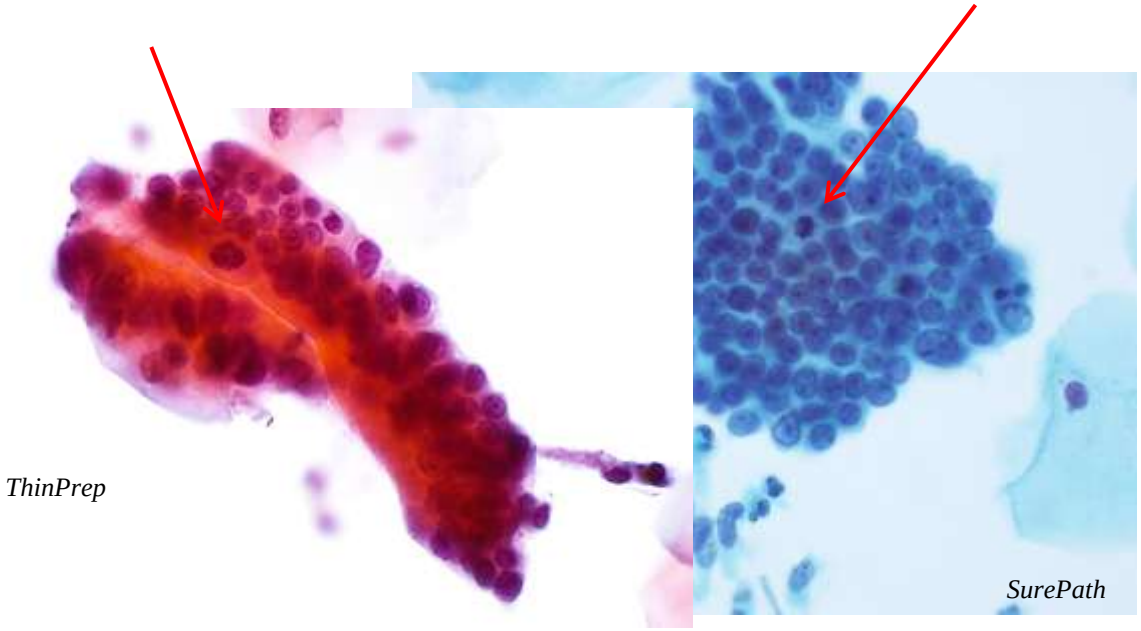

ThinPrep

AIS: Mitoses and Apoptotic debris

Mitoses and apoptotic debris/remnants of nuclei are relatively common findings in cases of endocervical AIS, and along with other features give reassurance when making an unequivocal interpretation. Mitoses are seen in the images on the right (red arrows). Apoptotic debris is identified as variably-sized and shaped blue/black granular material among cell groups (green arrows). On low power, the debris may be difficult to visualise, but methodical examination using high magnification may reveal debris and mitotic figures in some cell clusters. It is important to distinguish apoptotic debris, which may have different shades of colour (see ThinPrep example), from the degenerating lobes of neutrophils which are only black.



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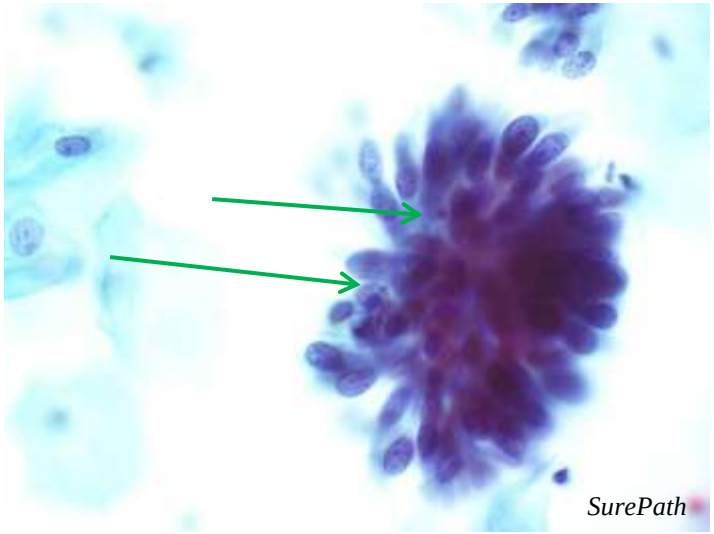

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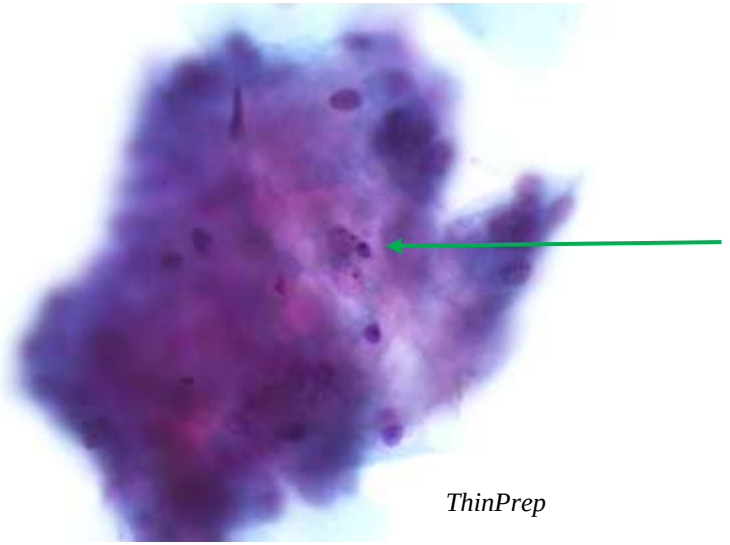
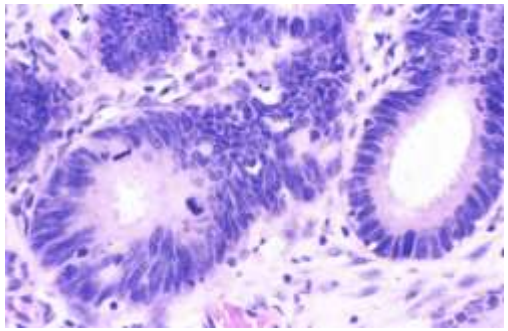


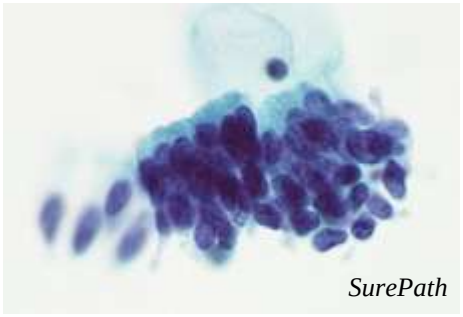
ThinPrep

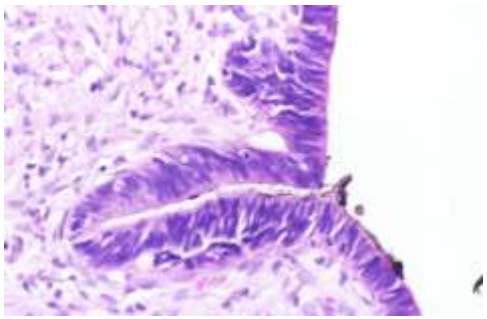
HISTOLOGY



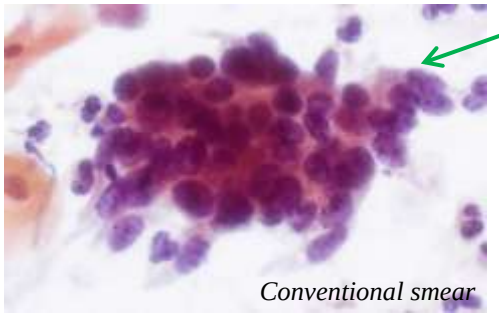
Adenocarcinoma-in-situ (AIS) of usual **endocervical type**.
Cytology: The cells largely retain their columnar configuration with basal nuclei and apical cytoplasmic mucin.

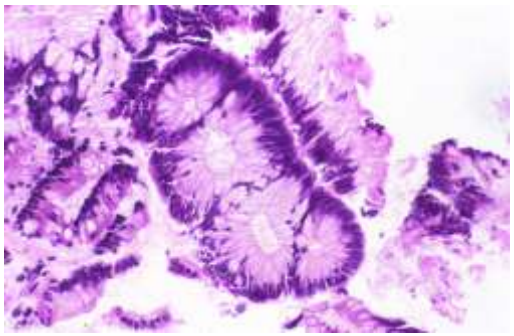
CYTOLOGY



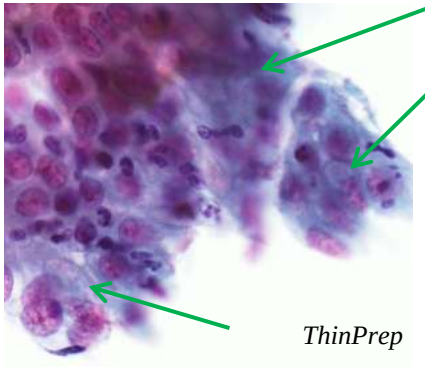


Cervical adenocarcinoma-in-situ (AIS) of **endometrioid type**. This uncommon subtype has columnar or cuboidal cells but little or no cytoplasmic mucin and the nuclei are less often basally situated in the cell. This subtype is the most difficult to recognise in cytology samples.
Cytology: A small strip (green arrow) is seen coming of a tight cluster of cells showing subtle chromatin stippling.





Cervical adenocarcinoma-in-situ (AIS) of **intestinal type** is also less common. The cells are columnar with basal nuclei and abundant cytoplasmic mucin. Goblet cells may be present.
Cytology: When viewed from above, a plane of focus showing only mucin-filled cytoplasm (green arrows) may be evident, with a different plane of focus needed to see the nuclei. This well-differentiated subtype may be missed as AIS if the nuclei are not examined closely.



Adenocarcinoma in situ (AIS) subtypes.

There are three different histological subtypes of adenocarcinoma in situ (AIS). The different histological appearances are reflected in differences in the cytological appearance. The different sub-types do not have prognostic significance but the different appearances need to be appreciated so that the full spectrum of AIS is detected in cervical cytology samples. The different subtypes frequently coexist in one case.

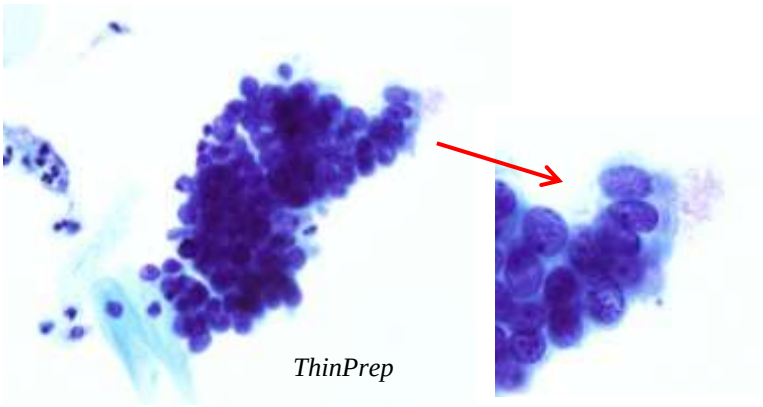
2.11.5 Atypical Endocervical Glandular cells

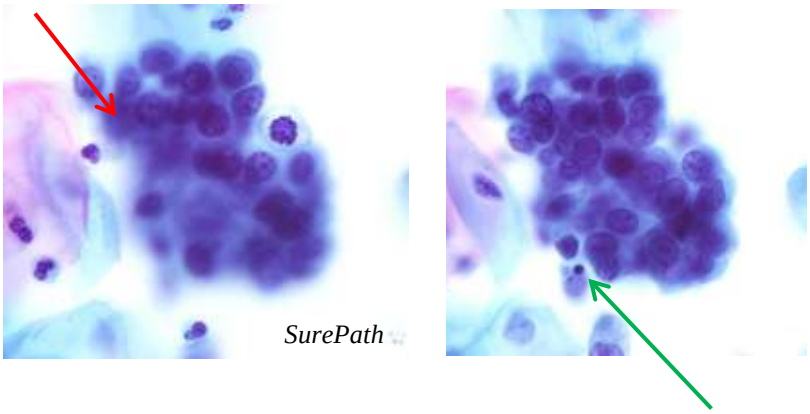
An unequivocal diagnosis of AIS (or AC) **should not be made if there insufficient features present for a confident assessment of AIS**. If there is doubt that there are sufficient criteria present, or if there are only very few abnormal cells, it is acceptable and safer to report such cases as atypical endocervical glandular cells favouring neoplasia, as the woman will still be referred directly to colposcopy.

When reporting atypical glandular cells, the report should reflect the degree of certainty about the origin of the atypical glandular cells and for atypical endocervical cells, the degree of concern about the possibility of AIS. Options for reporting atypical glandular cells in the Bethesda system coding are as follows:

AG1 There are atypical endocervical cells present
AG2 There are atypical endometrial cells present
AG3 There are atypical glandular cells present
AG4 There are atypical endocervical cells favouring a neoplastic process
AG5 There are atypical glandular cells favouring a neoplastic process

Further details about reporting using The Bethesda System codes is provided in the *VRPCC Workbook in Core Topic 4.1: Reporting Cytology using The Bethesda System Page 253.*





Atypical endocervical glandular cells.

The ThinPrep case (left) shows suspicious AIS glandular architecture, with an irregular crowded group of endocervical cells with nuclear overlapping and a strip coming off one edge (arrowed insert). The nuclei are uniform and there is only a suggestion of stippled chromatin. The case was confirmed as AIS.

The SurePath case (right) shows two planes of focus of the same abnormal group. One plane shows a mitosis (red arrow) and the second plane shows some apoptotic debris (green arrow). The nuclear chromatin is slightly stippled. The case was reported as atypical glandular cells favouring a neoplastic process. AIS was confirmed on histologic biopsy.

2.11.6 Potential diagnostic pitfalls with pre-malignant endocervical lesions

Presentations of normal cells from the endocervical canal

The endocervical canal varies in architecture and epithelial structure over its length.

- There is histological variation in the abundance and depth of the endocervical glands, gland neck crowding and pseudostratification.
- Tubal metaplasia is very common particularly in the upper third of the canal and is also noted in cytology specimens from the lower third of the canal in older women.
- In one study, cytology samples from the middle and upper third of the endocervical canal presented a number of endocervical groups that showed concerning morphological features. 25% of cases showed some of the atypical features used in the diagnosis of AIS such as cellular crowding, nuclei overlapping, palisading, tubular glands and feathering, but not all features were present collectively to make a diagnosis of AIS. The presence of these endocervical groups in the cytology specimens correlated well with the corresponding histology sections that showed features of cellular crowding, pseudostratification and tubal metaplasia. This correlation explained the cytological "atypia" observed on the cytology slides.

The use of devices that facilitate endocervical canal sampling and differences in normal endocervical canal topography result in considerable variation in the appearances of normal and reactive endocervical cells in cervical cytology samples. False positive diagnoses of glandular atypia, AIS or even adenocarcinoma can occur.

- Overly aggressive endocervical brushing may result in large cell groups. These groups can appear to have nuclear overlapping, a higher N/C ratio, and hyperchromatic nuclei.
- Endocervical cells are fragile and may lose their cytoplasm. Bare nuclei should not be over-interpreted as indicating HSIL. Bare nuclei in HSIL are "sticky, bare and atypical" compared with single or loosely aggregated bare endocervical nuclei
- Iodine artefact – extreme elongation of endocervical cells affecting both nuclei and cytoplasm producing a picket fence-like appearance can occur if iodine was applied to the cervix before the cytology sample was taken (at colposcopy).

The colposcopist is particularly reliant on the opinion of the cytologist to provide an opinion to guide further management because the endocervical canal is often poorly visualised colposcopically and is less accessible for biopsy, so every effort should be made to correctly classify glandular cells as normal/reactive, atypical or definite AIS/adenocarcinoma. If there is doubt, cases can be reported under "Atypical Glandular Cells" category in The Bethesda Sytem classification.

Lower uterine segment cells

These may be observed in cytology samples as a result of high sampling. The features of these cells are already described in *VRPCC Workbook: Subsection 2.2.8 Lower uterine segment (LUS) cells page 85.* To summarise, LUS cells have the following morphological features:

- Usually appear in large multi-layered biphasic fragments with densely packed spindled stromal cells and tubular glands. The typical biphasic pattern is very helpful in making the correct interpretation of "high sampling"

- The nuclei overlap and are relatively small and similar in size to glandular and stromal endometrial cell nuclei.
- The nuclei in these large crowded cohesive sheets, will reassure the cytologist of the benign nature and origin of the cells.
- The chromatin can be granular, but is evenly distributed with a uniformity of the granularity.
- Occasional mitotic figures may be observed but these should be normal proliferative mitoses

Post-cone/lletz biopsy cytology samples may have fragments of endometrium and high endocervical epithelial cells exhibiting crowded, hyperchromatic, and pseudostratified nuclei. This is essentially "high sampling" because the endocervical canal is shortened as a result of the lletz/cone biopsy.

AIS versus reactive endocervical cells

A common differential to consider is markedly reactive endocervical cells with atypia vs. AIS. Cervicitis is much more common than AIS. Reactive endocervical cells present with a normal N:C ratio, well-defined cell borders, minimal nuclear overlapping, round to oval nuclei with fine chromatin and large, prominent nucleoli. Prominent nucleoli represent increased activity and alone should not be used for a diagnosis of neoplasia. Normal mitotic figures may be seen in reactive cells.

The following features may assist with this important distinction:

- Large prominent nucleoli with few other atypical features in groups with non-overlapping cells are more likely to represent reactive endocervical cells. Conspicuous prominent nucleoli are not commonly seen with AIS.
- Reactive endocervical cell sheets tend to be flat and not overlapped with a visible rim of cytoplasm around the nucleus - the honeycomb pattern is usually preserved.
- The chromatin in reactive endocervical cells may appear hyperchromatic but does not have a stippled appearance. Stippled chromatin appears a consistent criterion for AIS.
- Strips of cells are not pseudostratified if reactive, although thick groups with overlapping nuclei may be seen in a "heavy scrape" sample. High-power magnification will show a non-pseudostratified pattern with reactive cell changes. Pseudostratified strips of cells with abnormal nuclei and abnormal groups showing communal/glandular borders are very good features for AIS.
- Acinar structures or lumina within groups should not be seen with reactive endocervical cell changes.
- Feathering is not seen in reactive endocervical cell groups. Feathering is a useful feature if present but is observed in only 10-20% of AIS cases and is not entirely specific.
- Ciliated cells are almost always benign and unless features of glandular neoplasia are overwhelming, the presence of cilia and/or ciliated cells should lead to a very cautious approach to reporting glandular neoplasia. Conversely, the possibility of glandular neoplasia/AIS should not be dismissed just because of the presence of ciliated cells, particularly because tubal metaplasia can coexist with AIS in the same sample.
- Reactive endocervical cells often have a less uniform appearance from cell to cell within a group compared to cells from AIS.
- Neutrophils are often observed within reactive endocervical cell groups. Apoptotic debris is often observed with AIS.

- Mitotic figures are occasionally seen in reactive endocervical cells but should not be abnormal. Apical mitoses lying close to the luminal border in AIS can be helpful.

It is important – **if you are not 100% confident** that the endocervical cell changes are just reactive then **get a second opinion.** Indeterminate cases can be reported as atypical endocervical glandular cells and referred for colposcopy.

Endometriosis

Endometriosis appears as endometrial glandular fragments and clusters of endometrial stromal cells in cytology specimens. These are seen as irregular clusters of endometrial cells with scant cytoplasm and small oval or vesicular nuclei. Bare nuclei may appear to fall away from the fragments. Nuclear size and presence of fine, regular distributed chromatin and tiny nucleoli are helpful in the differential diagnosis. Useful indicators to raise the suspicion of endometriosis is the presence of the cells out of cycle, and well-preserved endometrial cells that have been scraped from the tissue surface rather than the more degenerate cells that are usually observed as a result of endometrial shedding during menstruation.

Tubal metaplasia (TM) and Tubo-endometrioid Metaplasia (TEM)

TM is seen histologically as the replacement of normal endocervical epithelium by tubal-type epithelium and may be seen in cervical smears as oval to elongated cells in tightly grouped aggregates or strips. The nuclei of these cells may be low columnar to cuboidal and hyperchromatic but the presence of cilia confirms a benign process for the cell group being examined (remember that Tubal metaplasia and AIS can coexist). In much of the literature TM and TEM are referred to as a single entity, but some authors separate them as two conditions. TEM differs from TM in that it shows pseudostratification of nuclei and secretory apical snouting in addition to luminal ciliation. It may be that the loss of cilia sometimes seen with TEM is due to morphological change towards an endometrioid appearance. The conditions are commonly seen in women, particularly post treatment e.g. cone biopsy, loop excision. TM/TEM may exhibit marked nuclear atypia and in the absence of cilia will pose a high-risk pattern.

Tubo-endometrioid metaplasia (TEM) is thought to occur as a result of injury and the resulting cytology may be misinterpreted as either squamous or glandular neoplasia. Distinctive cytological features of TEM are:

- Small cells (smaller than endocervical and metaplastic cells)
- Mixed population – heterogeneous i.e. ciliated, mucous secreting (discrete vacuoles) and sometimes frank goblet cells
- Centrally or basally located round to oval nuclei
- Nuclei show uniform hyperchromasia
- Inconspicuous nucleoli
- Two to three dimensional sheets and glandular structures
- The cell groups are poorly cohesive
- Feathering is not a feature
- Mitotic activity can be seen but is not a prominent feature
- Ciliated cells not always seen – there is debate in the literature regarding this as histologically the cells are ciliated.

2.11.7 Morphology of invasive endocervical adenocarcinoma (AC)

General criteria for diagnosing endocervical Adenocarcinoma are similar to adenocarcinoma from other body sites and are discussed in the *VRPCC Workbook: Core Topic 2.1: The Cell in Health and Disease page 51*. Endocervical adenocarcinoma is often detected at an advanced stage when the cytology sample shows definite invasion. Occasionally the cytology may show predominantly AIS with scattered features suggesting a co-existing invasive lesion.

Cytology is not particularly good at discriminating between AIS and invasive endocervical adenocarcinoma. We do make separate predictions of AIS and adenocarcinoma but accept that the histology may not correlate. Criteria that raise a suspicion of invasion are:

- Tumour diathesis – debris that may be cyanophilic and orangeophilic and either dispersed or appearing to cling to groups of malignant cells. In ThinPrep, this is described as displaying a "woven shroud" appearance. In SurePath, the tumour diathesis may appear granular and fibrillary and often "clings" to cells/cell groups.
- 3-dimensional rounded up balls of cells with multiple focal planes of cell layers
- Super-crowding of cells and syncytia
- Nuclei generally larger with irregular membrane although they tend to be undulating and smooth (compared with the sharp "bites" seen in high-grade squamous lesions).
- Large prominent nucleoli
- Single large central nucleoli or multiple nucleoli with marked variation in nucleolar size
- Paranucleolar and parachromatin clearing
- Bizarre cell and group morphology
- Secretory vacuoles
- Frequent mitoses
- Significant loss of architecture and polarity (more significant with less well-differentiated lesions)

In the absence of bizarre cell features, the three main features suspicious of invasion are:

- Tumour diathesis
- Large prominent nucleoli
- Change from typical AIS features such as rounding and 3D cells

On occasions it may be impossible to differentiate between an endocervical and endometrial adenocarcinoma, or even poorly differentiated squamous cell carcinoma. These cases can be reported as Adenocarcinoma NOS, Carcinoma NOS or Malignant neoplasm NOS. Usually the primary site of origin is apparent clinically or radiologically, or becomes so on further investigation.

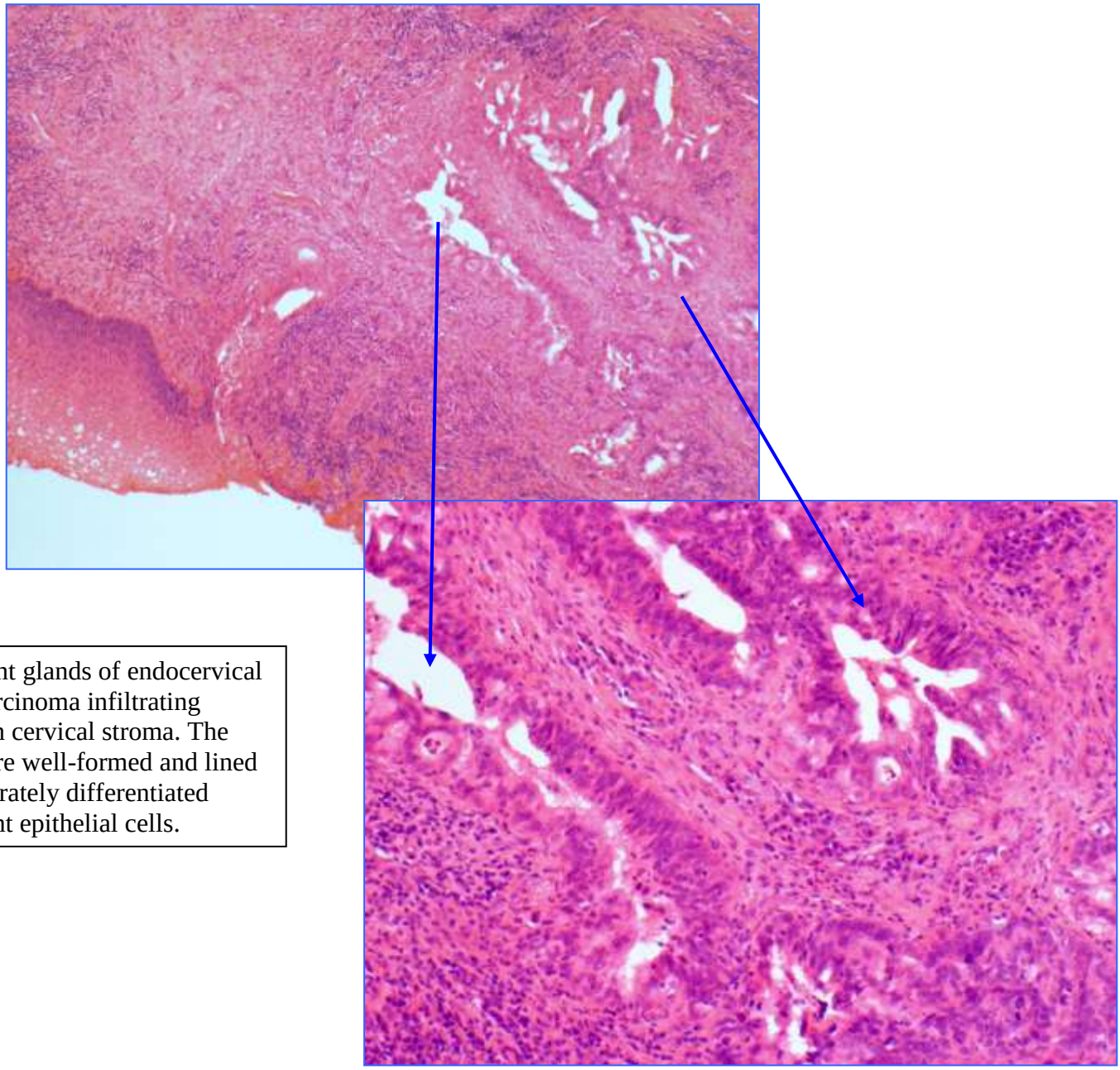
Endocervical adenocarcinoma is the most common type of adenocarcinoma arising in the endocervical canal, but rarely, other histological types of adenocarcinoma may arise as primary tumours in the endocervix too. Some such as endometrioid carcinoma are more common as primary malignancies at other genital tract sites. Types of adenocarcinoma that rarely can arise as primary neoplasms in the endocervical canal are:

- Endometrioid adenocarcinoma
- Papillary serous adenocarcinoma
- Adenosquamous carcinoma

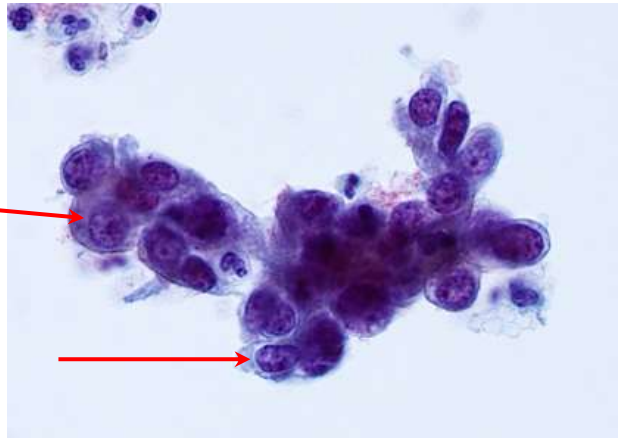
- Clear cell carcinoma
- Intestinal-type adenocarcinoma
- Mesonephric carcinoma
- Minimal deviation carcinoma (adenoma malignum - extremely well-differentiated carcinoma with a well-differentiated appearance but aggressive behaviour)

Most of these lesions will be recognised as adenocarcinoma in a cervical cytology sample, but may be difficult to classify. Referral to a pathologist and correlation with clinical information, radiology and sometimes histology will occur as part of the reporting process.

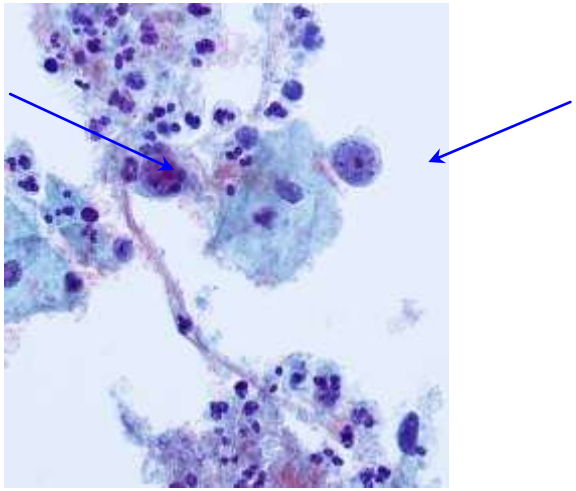
Endometrial adenocarcinoma of uterine body origin (exfoliated cells from the uterine cavity) is the most common type of adenocarcinoma seen in cervical cytology samples.



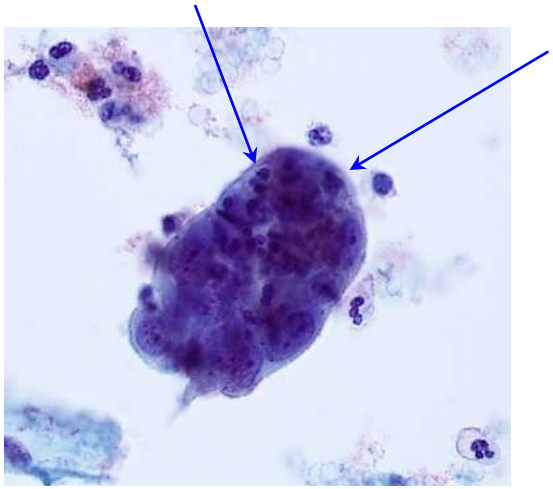
Malignant glands of endocervical adenocarcinoma infiltrating deeply in cervical stroma. The glands are well-formed and lined by moderately differentiated malignant epithelial cells.



Large glandular cells show pleomorphic malignant nuclei with abnormally distributed granular chromatin (red arrows), small nucleoli and delicate vacuolated cytoplasm. This group is irregular in shape without particularly distinctive glandular architecture. All abnormal groups need to be examined to establish the diagnosis.

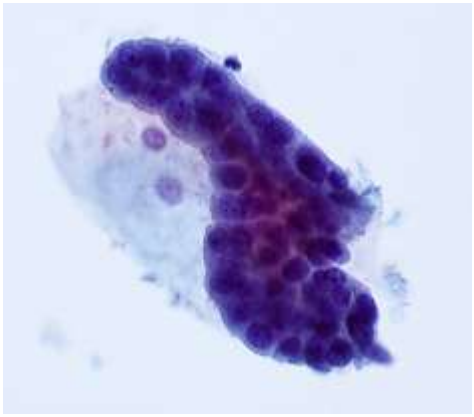


Two single malignant cells (blue arrows) and debris comprised of intact and degenerating neutrophils and degenerate blood.



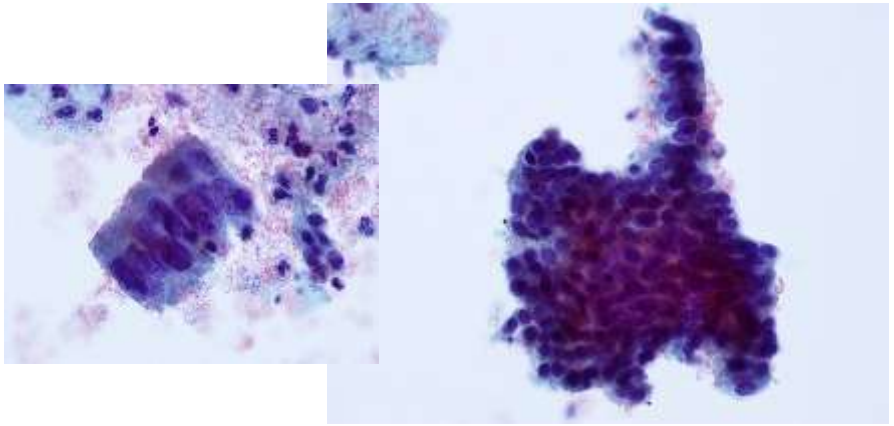
A rounded (probably exfoliated before sampling) group of malignant glandular cells infiltrated by neutrophils. Some neutrophils appear to be ingested (blue arrows). This is a feature of degeneration and so while not specific, is common with malignancies.

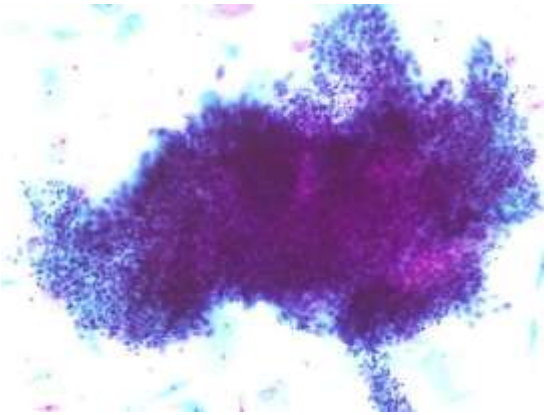
Endocervical adenocarcinoma: a case in ThinPrep



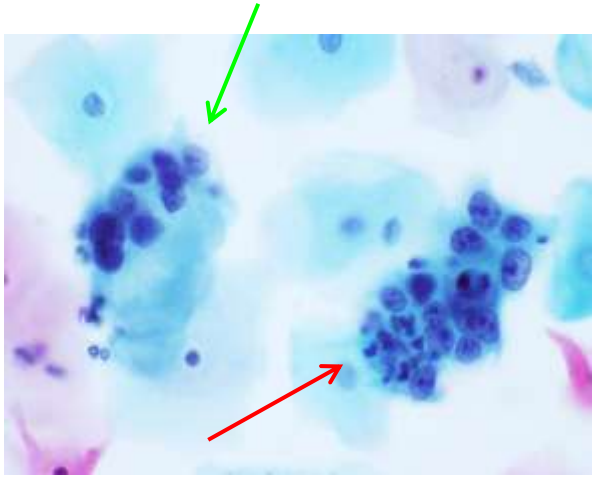
A papillary structure in one group.

Right: A short strip and an irregular crowded group with a strip coming off one edge – features of co-existing AIS that assist with classifying the lesion as endocervical adenocarcinoma

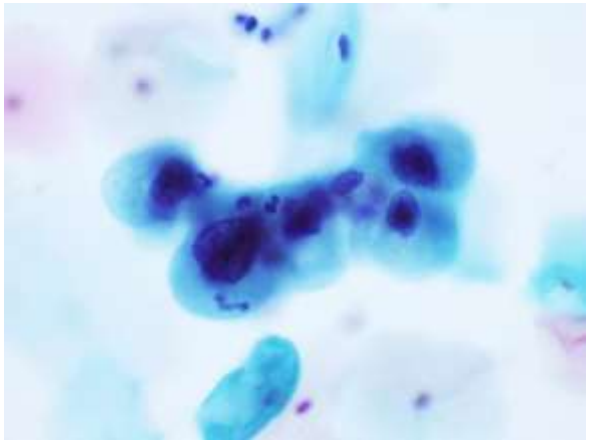




A large irregular densely cellular "microbiopsy" of glandular cells requiring close high-power examination

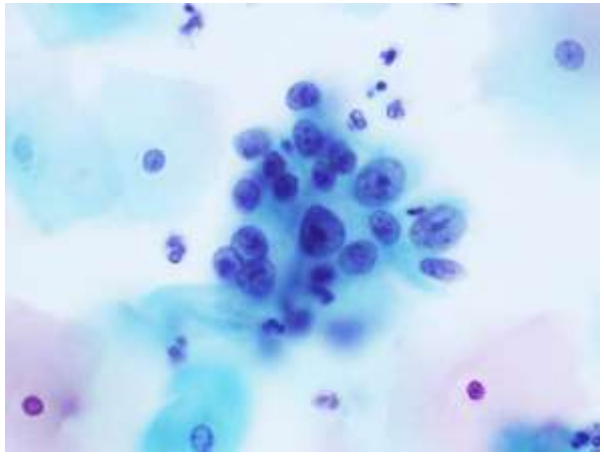


A strip on the left (green arrow) shows basal nuclei, pseudostratification and apical cytoplasm. The group on the right is disorganised which more nuclear pleomorphism, small nucleoli and heavy nuclear outlines, and infiltration by neutrophils (red arrow).



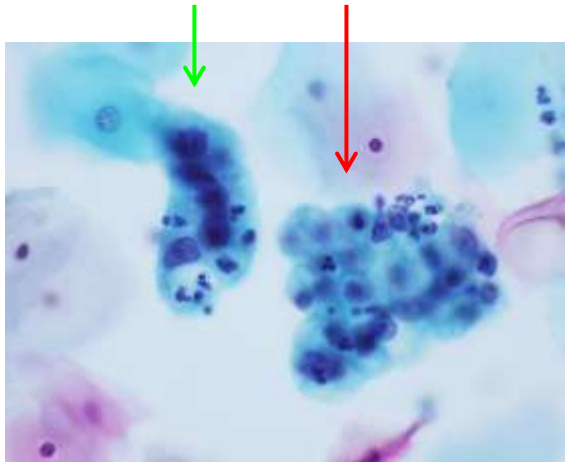
Marked nuclear pleomorphism, stippled chromatin and small nuclei. The cytoplasm is delicate with small vacuoles.

Endocervical adenocarcinoma: a case in SurePath



A cluster of malignant glandular cells showing nuclear pleomorphism, stippled chromatin, nucleoli and heavy nuclear outlines.

Right: An abnormal strip on the left (green arrow) and a group on the right (red arrow) showing abundant cytoplasmic mucin in the plane of focus, with "out of focus" nuclei in behind. Care needs to be taken not to screen over such groups but to focus through the group to examine the nuclei closely.



2.11.8 Endometrial lesions

Normal endometrial cells

The presence and significance of finding normal endometrial cells in cervical cytology samples is discussed in the *VRPCC workbook: Core Topic 2.2 Normal Cytology and Histology Page 71*. **Endometrial cells which are cytologically normal can be detected in cytology samples from women with endometrial adenocarcinoma before symptoms such as abnormal bleeding occur.** For this reason, normal endometrial cells are reported in women who are 40 years of age or older, and in New Zealand are reported under a "Negative for intraepithelial lesion or malignancy" heading, if no other abnormalities are present in the sample.

- Once normal endometrial cells have been reported in a woman 40 years of age or older, a decision needs to be made about whether further investigation is warranted. Clinicians are in a better position to do this than laboratory staff.
- Premenopausal women taking hormonal contraceptive preparations, women with an intrauterine device (IUCD) and postmenopausal women taking hormone replacement therapy (HRT) can shed normal endometrial cells at any time.
- Investigations for possible endometrial pathology include ultrasound imaging and endometrial sampling (by endometrial curetting and/or pipelle sample). These are relatively simple investigations without significant morbidity for the woman concerned.

Early detection of endometrial adenocarcinoma makes a significant difference to survival, as the prognosis is very dependent on the stage of disease at diagnosis. Most women with endometrial adenocarcinoma are diagnosed after investigation of abnormal/postmenopausal bleeding - only a very small number are diagnosed in asymptomatic women because of investigation after the detection of normal endometrial cells in a cervical cytology sample.

The aim of cervical cytology is to detect cervical neoplasia. Cervical cytology is not sensitive or specific for detecting endometrial pathology. Sample takers may take a cervical cytology sample as part of the investigation of a woman with post-menopausal bleeding (PMB) or other clinical reasons to suspect endometrial pathology, but it is not a reliable test in this context – if *abnormal* endometrial cells are found this result may be helpful, but a negative result does not exclude a significant endometrial lesion.

So under The Bethesda System 2001 – the approach is simple:

- **If normal endometrial cells are detected in women 40 years of age or older, then they are reported whatever their morphology and the woman's cyclic/clinical status.**

If the endometrial cells are normal, a clause may be added to the report (at the discretion of the laboratory). The NCSP has provided the following suggestion for wording the clause:

"The presence of endometrial cells in a woman over the age of 40 years can be a normal finding, or seen in association with hormone replacement therapy, or rarely, associated with endometrial pathology including hyperplasia or neoplasia. Please correlate this finding with

any symptomatology of uterine pathology, for example abnormal uterine bleeding and refer/investigate accordingly."

Some key diagnostic points to keep in mind:

- Use strict criteria to determine that the cells are endometrial. The misinterpretation of HSIL or small invasive cancer cells as endometrial is a well-recognised cause of false negative reports. If in doubt, get another opinion.
- When typical endometrial cells are present, be careful to not assume that all other small, high N:C ratio cells with hyperchromatic nuclei will also be endometrial – HSIL may be present as well.
- Assessing whether endometrial cells in cytology samples are normal or abnormal can be very difficult. The nuclear changes in well-differentiated endometrial carcinoma can be subtle and as endometrial cells are usually exfoliated, the cells from both normal endometrium and from malignant lesions are often partly degenerate. Degenerative changes may either mask or mimic neoplastic morphological change.
- Endometrial glandular cells are usually small. This means that the changes of malignancy are also happening on a small scale so small subtle variations can be very significant, particularly with well-differentiated lesions. Any doubt, however minor, as to the normality of glandular cells, should be referred for another opinion.

Atypical endometrial cells

Atypical endometrial cells are always reported, whatever the age of the woman concerned. Assessing endometrial cells as atypical implies the possibility of malignancy, but such cases are sometimes caused by cystic hyperplasia of the endometrium with atypia (a pre-neoplastic condition) or benign lesions such as an endometrial polyp. Because even assessing endometrial cells as atypical is challenging, further sub-classification of atypical endometrial cells is not attempted cytologically and so there is only one reporting category for atypical endometrial cells (compared with atypical endocervical cells which can be reported as *atypical endocervical cells* or as *atypical endocervical cells suspicious of neoplasia*).

- Atypical glandular cells are difficult to assess and it may not be possible to determine whether they are endocervical or endometrial in origin. Such cases are reported as *atypical glandular cells.*

- All atypical glandular cell cases of any type are referred for specialist investigation.

- The shedding of normal and abnormal endometrial cells can be erratic and therefore sequential samples may not contain similar diagnostic material. **Repeat samples to see whether the presence of normal or atypical/abnormal endometrial cells is persistent have no place because of the erratic nature of endometrial shedding.**

Endometrial carcinoma

In the practice of cervical cytology, laboratories see many more cases of endometrial adenocarcinoma than cases of endocervical adenocarcinoma because the former is more common. All glandular lesions are difficult however and because collectively they are a lot less common that squamous lesions, it is recommended that you supplement the cases you

see in routine practice with examples from teaching sets to expand your experience of glandular lesions.

Clinically endometrial adenocarcinomas are divided into two types. The predominant Type I endometrial adenocarcinomas usually occur in premenopausal or perimenopausal women with increased endogenous oestrogen levels or after unopposed exogenous oestrogen administration (i.e. oestrogen supplementation without progestogen). The cytology sample is mature under the influence of oestrogen. Type II tumours represent about a third of endometrial adenocarcinomas, usually affect postmenopausal women and are not associated with hyperoestrogenic states. These types usually present as poorly differentiated neoplasms within an atrophic background.

Morphology of endometrial carcinoma

Carcinomas arising in the endometrium include a spectrum of different tumour types as well as a range in the degree of differentiation. Both these factors influence the appearance seen in cervical cytology samples. With poorly differentiated lesions, the cytologist may only be able to report a case as carcinoma, or even as a malignant neoplasm NOS, without being able to specify the site of origin. This is not usually a problem clinically because the site of origin is usually evident, for example by the location of a mass detectable clinically or radiologically with a histologic biopsy of some sort establishing the site of origin.

Malignant endometrial cells may be single cells or occur in small clusters. Large clusters are not common and are seen mainly in poorly differentiated lesions. Difficulties with endometrial lesions that there may be very few malignant cells present in the sample, the cells are often very small and easily overlooked and because of their small size, they may be masked by leucocytes. The number of malignant cells present varies greatly from only a few malignant cells to numerous malignant cells right through the sample. In general, well-differentiated lesions shed fewer cells than poorly differentiated lesions. Most malignancies of endometrial origin seen in cervical cytology are carcinomas, and even with malignant mixed tumours (carcinosarcoma) it is the epithelial carcinomatous component that is usually seen in cervical cytology samples. Stromal tumours such as endometrial stromal sarcomas can occasionally be seen. Any sample from a woman with postmenopausal bleeding needs very careful screening to ensure that very small numbers of malignant cells are detected, if present.

General features

There are often clues at low-power that alert the cytologist to look very carefully for small numbers of malignant endometrial cells.

- There is usually some evidence of debris in the background – this appears as granular cyanophilic or eosinophilic material and may contain remnants of blood. Fine granular debris often forms membranous sheets of granular degenerate material that may contain neutrophils or histiocytes.
- There may be an increase in histiocytes in the sample generally, although this is a relatively nonspecific feature. The presence of histiocytes in conjunction with a mature smear pattern in a post-menopausal woman should alert the cytologist to the possibility of endometrial pathology.
- Endometrial debris often consists of very small particles of debris which can have a distinctive "bitty" look.
- Prominent emperipolesis (engulfment of cells and debris) should prompt a thorough examination of the nuclei of the cells concerned for malignant characteristics. Engulfment of other cells, neutrophils and debris is a feature of cell degeneration not malignancy per se, but is common in endometrial carcinoma.
- As endometrial malignancies may present exfoliated cells in cervical cytology samples, some degree of cell degeneration is common. Interpreting the effects of degeneration in this setting and distinguishing between degenerative and malignant features can be very challenging.

Nuclear features

- Either hyperchromatic or normochromatic cells.
- Nuclear enlargement may be present. Nuclei are generally smaller than seen with endocervical carcinoma but poorly differentiated endometrial carcinomas may have large nuclei. Conversely, cells and their nuclei can be very small because of partial degeneration. Small nuclei still have malignant features but the variation between malignant nuclei is happening on a small scale too, so can be subtle.
- Nucleoli - can be anything from small to macronucleoli. The "cherry red" macronucleolus is a classically described feature of endometrial adenocarcinoma.
- Eccentrically placed nuclei are classically displaced and indented by a large cytoplasmic vacuole.

Cytoplasmic features

- Variable in amount – can see a short stubby columnar form. Generally malignant endometrial cells have more cytoplasm than normal endometrial cells.
- Vacuolated
- Cyanophilic
- Polymorphs may be engulfed/ingested (reflects cell degeneration)

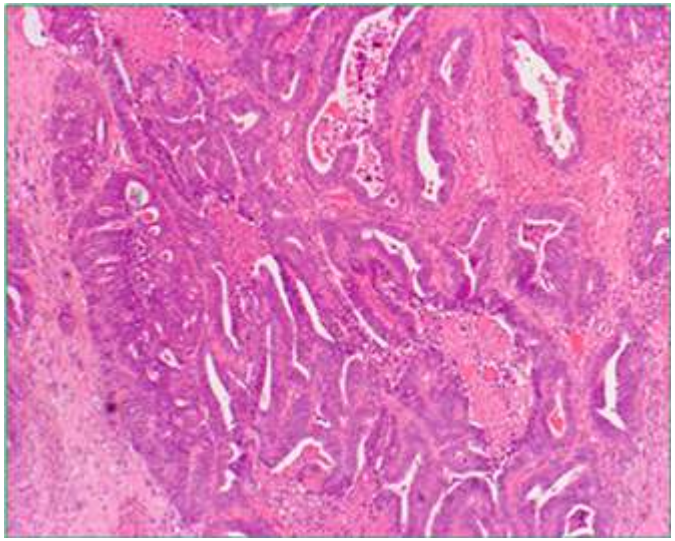
Architecture of cell groups: a wide variety of appearances reflects the wide variety of tumour types seen in the endometrium.

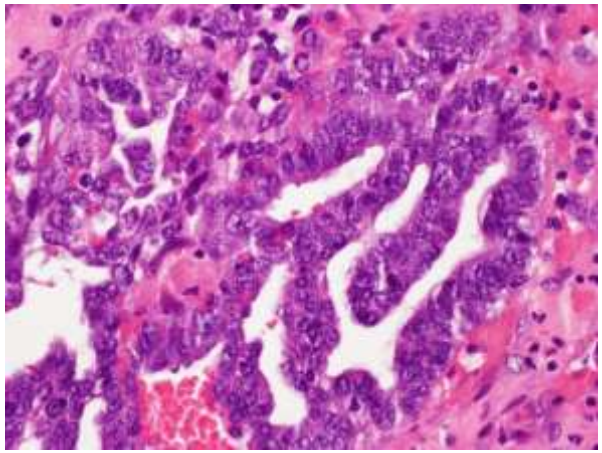
- Loss of polarity
- Glandular appearance
- Acini
- Papillary groups may be present
- Cell balls and clusters are common

- Single cells are common
- Tumour diathesis or granular debris is usually present
- Shed malignant endometrial cells are typically less well-preserved than is seen with directly sampled endocervical lesions

Most endometrial lesions in cervical cytology samples show exfoliated cells and debris that have dropped into the endometrial cavity and passed down through the endocervical canal to be collected by the sample taker when a cervical cytology sample is taken. In contrast, sometimes the endometrial malignancy directly invades the endocervical canal from above, or presents as a polypoid mass that protrudes down into the endocervical canal. In these cases, the tumour may be directly scraped by the sampling device. The appearance of directly scraped material differs from that of exfoliated cells. The amount of abnormal material is generally greater, cell preservation is often better and the cells are not as rounded up into balls. The distinction between directly scraped endometrial carcinoma and endocervical carcinoma can be challenging particularly if the possibility of a directly scraped endometrial malignancy is not considered.

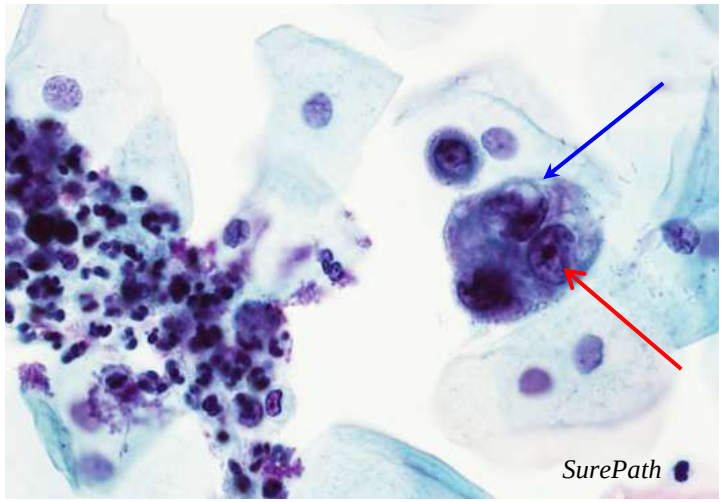
The combination of the right clinical setting, the sample background and the characteristic appearance of the malignant endometrial cells often allows a confident cytologic diagnosis of endometrial carcinoma. Malignant cells must be clearly identified before making a definite diagnosis.

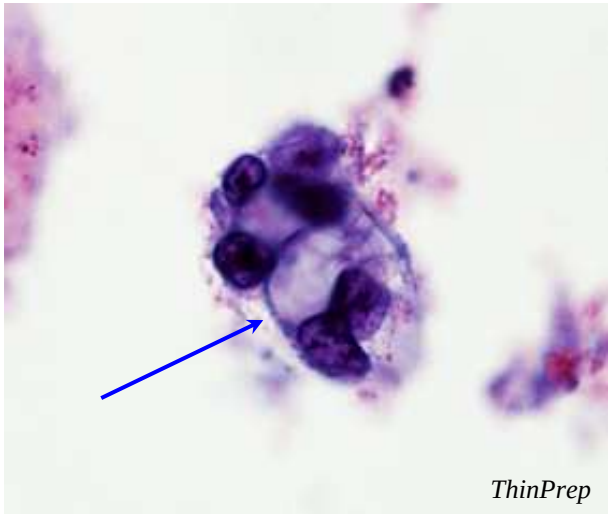




Histology of endometrial adenocarcinoma.

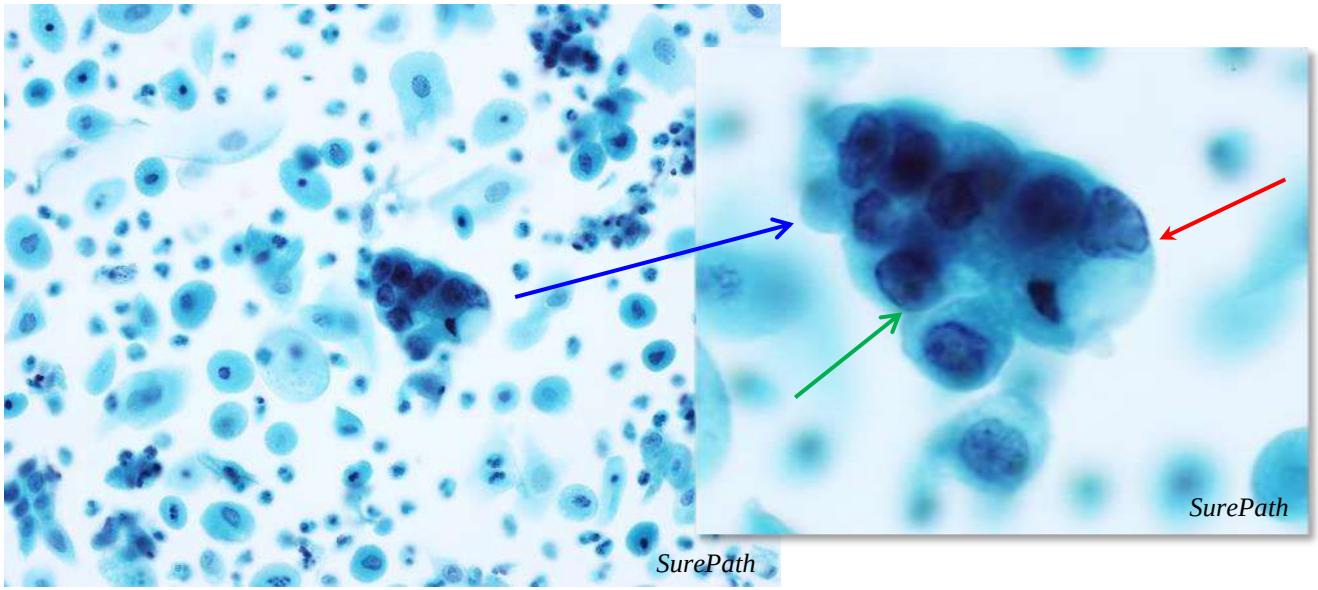
At low power (far right) there is complex glandular architecture with necrotic debris and neutrophils within glands. At high power (right) there are back-to-back **glands without intervening stroma** forming a complex cribriform pattern of glands.



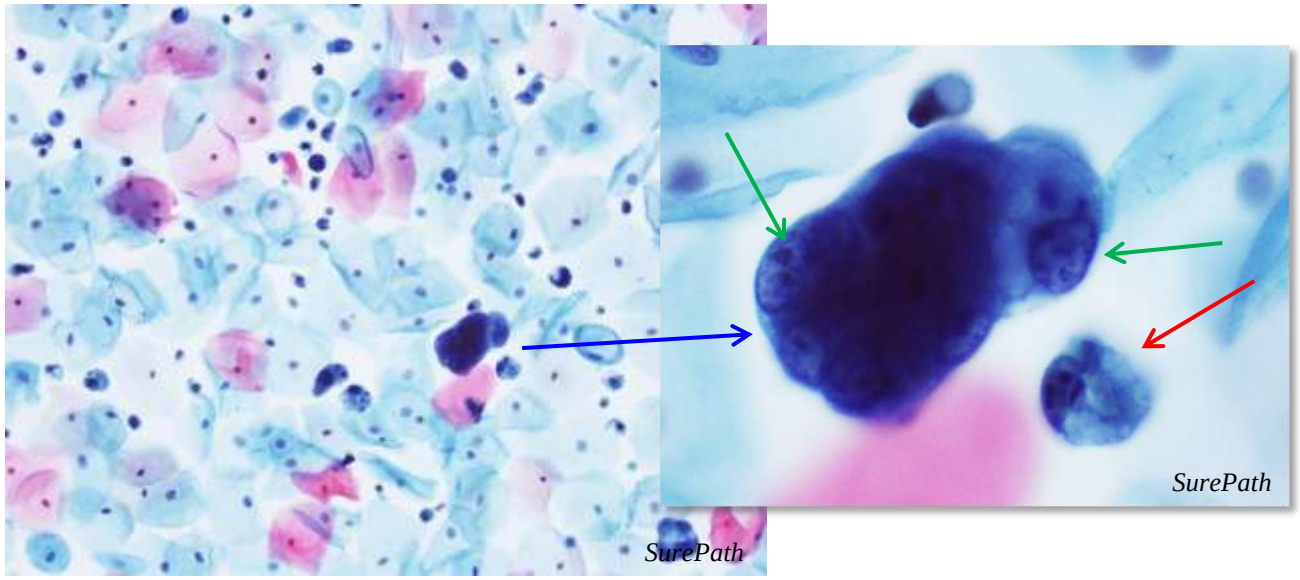


Cytology of endometrial carcinoma.

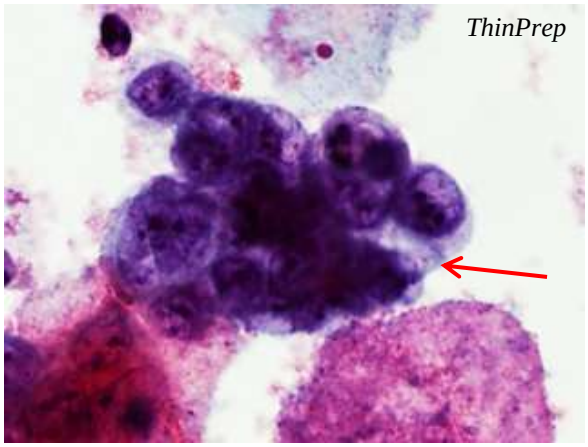
The SurePath case (far right) shows single and clustered malignant glandular cells with prominent nucleoli (red arrow), cytoplasmic vacuoles (blue arrows) that compress the nucleus and associated inflammatory debris. The ThinPrep cluster shows malignant glandular cells forming a rounded-up exfoliated cluster with prominent cytoplasmic vacuoles that indent adjacent hyperchromatic malignant nuclei.



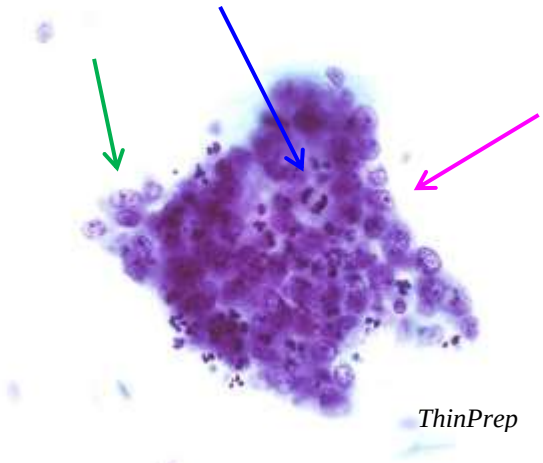
A rounded exfoliated cluster of endometrial adenocarcinoma occurring in an atrophic background. At high power there are eccentrically placed malignant nuclei which are indented by cytoplasmic vacuoles (red arrow). Prominent nucleoli are also present (green arrow).



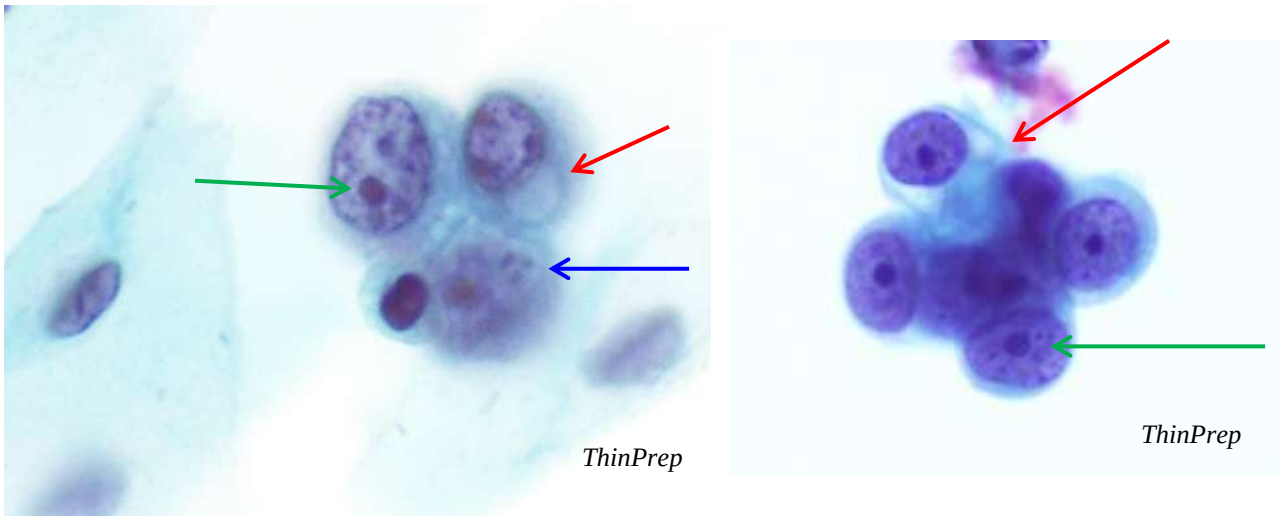
Rounded exfoliated clusters and dissociated single cells of endometrial adenocarcinoma occurring in an 80 year old woman without epithelial atrophy. At high power malignant nuclei have highly abnormal chromatin with margination, thick irregular nuclear membranes and prominent nucleoli (green arrows). Cytoplasmic vacuoles displace and indent the nucleus in some cells (red arrow).



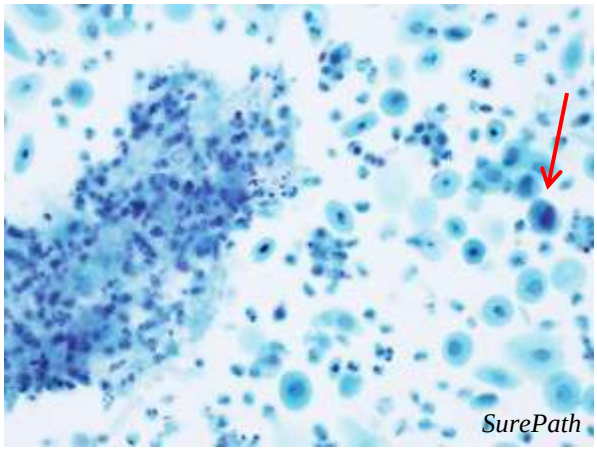
An exfoliated group of malignant epithelial cells from an endometrial adenocarcinoma. Note the abnormal granular chromatin, the disorganised thick nature of the group and the occasional cytoplasmic vacuoles (red arrow).

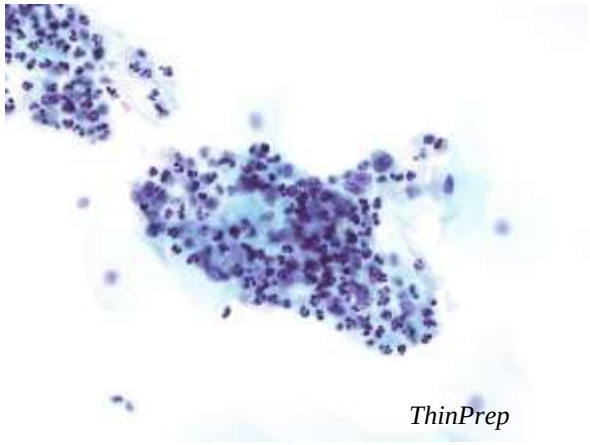


A larger sheet of malignant epithelial cells showing a glandular contour at one edge (pink arrow), mitoses (blue arrow), prominent nucleoli (green arrow) and infiltration by neutrophils.

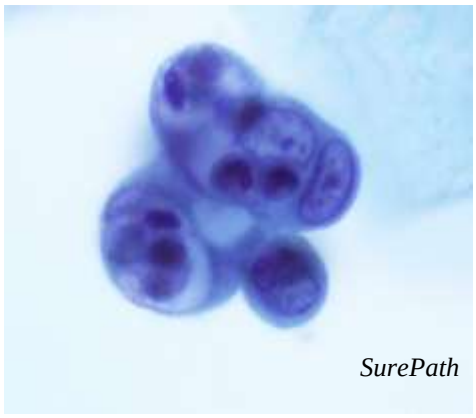


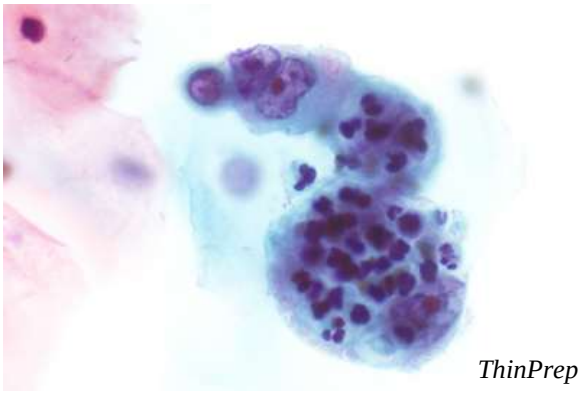
Endometrial adenocarcinoma at high power. The case on the right shows marked nuclear pleomorphism with abnormal granular unevenly distributed chromatin. Both cases show prominent nucleoli (green arrows) and cytoplasmic vacuoles (red arrows) and there is ingestion of a neutrophil (blue arrow) on the right.



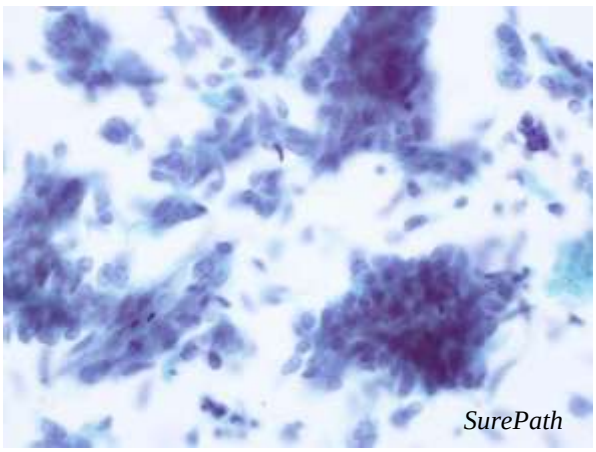


Clusters of granular debris, neutrophils and histiocytes are common and should alert the cytoscreener to the possibility of endometrial adenocarcinoma in a postmenopausal woman. There may or may not be partially degenerate malignant cells associated with the debris. More often, malignant cells are found elsewhere in the sample (red arrow).





Ingested neutrophils are common in the cytoplasm of malignant cells from an endometrial adenocarcinoma and may obscure the malignant nuclei. Ingested neutrophils are common but are not specific for endometrial adenocarcinoma and are a reflection of cell degeneration rather than malignancy per se.



Directly sampled material can be seen where there is direct invasion of the cervix by endometrial adenocarcinoma arising from higher up in the uterus or as in this case, where there is a vault recurrence after hysterectomy. When directly sampled, abundant tumour cells are present and the groups have sharp or frayed edges rather than the rounded up groups seen with exfoliated material.

2.11.9 Extra-uterine, metastatic and non-epithelial malignancies

Malignant cells arising from tumours in sites other than the cervix are very occasionally detected in cervical cytology samples and can present diagnostic difficulties. This can occur in three different ways:

1. Direct invasion of the cervix or vagina by tumours from adjacent sites and organs.
The most common example is endometrial carcinoma that has extended down to directly invade the endocervix. If the malignancy is within reach of the cervical cytology sampler, tumour cells may be directly sampled resulting in abundant malignant material in the sample. Cells are often in flatter sheets with sharp edges rather than the balled-up small groups seen typically with exfoliated endometrial carcinoma arising in the uterine cavity. Another example of direct invasion is colonic carcinoma which occasionally infiltrates directly through to the upper vagina, sometimes resulting in a rectovaginal fistula.

2. Metastastic malignancy in the cervix.
Breast carcinoma and lymphomas for example, can involve the cervix in women with advanced stage disease. The underlying malignancy is almost always already known but rarely, the cervical cytology sample is the first point of identification of the malignancy. Metastatic carcinoma in a cervical cytology is classically described as having a clean background compared with primary cervical carcinoma because surface ulceration and tumour diathesis is usually not a feature associated with metastatic lesions.

3. Exfoliation of tumour cells from the upper female genital tract or the peritoneal cavity.
The cells can pass down through the uterine cavity and the endocervical canal to be picked up in a cervical cytology sample. Examples include malignant cells from ovarian or fallopian tube adenocarcinomas.

If suspicious or malignant glandular cells are present that do not have criteria consistent with endometrial or endocervical lesions, and there is no tumour diathesis (i.e. a clean background), then these more distant lesions should be considered. The presence of psammoma bodies within tumour cell groups (associated with papillary serous carcinoma) and large cytoplasmic vacuoles (associated with mucinous adenocarcinoma) may suggest an ovarian lesion, but without clinical evidence neither is a reliable criterion of malignancy. Cells from advanced lesions of the tubes and ovaries may show degenerative changes in cervical cytology samples. Referral for further opinions will obviously be appropriate with these rare cases.

It is rare to detect other non-epithelial malignancies in cervical cytology but melanomas, sarcomas and primary lymphomas do occur. Experience in non-gynaecological cytology can be helpful in identifying a possible rare malignancy and ensuring that referral of the case to a pathologist who is experienced with different types of malignancies occurs.

2.11.10 Questions

1. Explain how and why the spectrum of invasive cervical cancer is changing with regards to incidence and histological type.

2. Discuss how it is that endocervical adenocarcinoma in situ can remain occult for years before being detected, even in women having regular cervical cytology samples.

3. Define the terms feathering, pseudostratification, rosette and acinar formation, with respect to the cytological features of adenocarcinoma in situ.

4. Tabulate the similarities and differences between adenocarcinoma in situ and invasive endocervical adenocarcinoma.

5. What features would assist in distinguishing between a high-grade squamous lesion and a high-grade glandular lesion in a hyperchromatic crowded group of abnormal epithelial cells?

6. Tabulate the main cytological differences between reactive endocervical cells and endocervical AIS.

7. Discuss the cytological features of endocervical adenocarcinoma.

8. List the clinical conditions in which you could see benign endometrial cells in a cervical cytology sample.

9. Tabulate the cytomorphological differences between benign endometrial cells and benign endocervical cells.

10. Discuss the association of small histiocytes, a mature smear pattern and a post-menopausal patient with regards to potential endometrial pathology.

11. Tabulate the cytological features that help differentiate endocervical adenocarcinoma from endometrial adenocarcinoma.

12. Describe the cytological presentation of tubal metaplasia and important lesions with which it can be confused.

References

- Ayer B, Pacey F, Greenberg M, et al. 1987. The Cytologic Diagnosis of Adenocarcinoma in Situ of the Cervix Uteri and Related Lesions. 1. Adenocarcinoma in Situ. *Acta Cytologica* 31(4): 397-411.

- Friedell GH, McKay DG. 1953. Adenocarcinoma in Situ of the Endocervix. *Cancer* 6(5): 887-897.

- Krumins I, Young Q, Pacey F, et al. 1977. The Cytologic Diagnosis of Adenocarcinoma in Situ of the Cervix. *Acta Cytologica* 21: 320-329.

- Laverty CR, Farnsworth A, Thurloe J, et al. 1988. The Reliability of a Cytological Prediction of Cervical Adenocarcinoma in Situ. *Australia and New Zealand Journal of Obstetrics and Gynaecology* 28: 307-312.

- Pacey F, Ayer B, Greenberg M. 1988. The Cytological Diagnosis of Adenocarcinoma in Situ of the Cervix Uteri and Related Lesions. *Acta Cytologica* 33: 325-330.

- Sykes P, Gopala K, Tan A-L, Kenwright D, Petrich S, Molijn A and Chen J. Type distribution of human papillomavirus among adult women diagnosed with invasive cervical cancer (stage 1b or higher) in New Zealand. *BMC Infectious Diseases* 2014,14:374

SECTION 3.0
PRIMARY SCREENING RECORDS AND SLIDE SETS

3.1 CORE TOPIC: PRIMARY SCREENING RECORDS

The VRPCC logbook contains sheets for you to use to record your screening experience and progress. The number of cervical cytology samples you need to screen to complete the VRPCC programme is a minimum of 3000 LBC samples. This number has been determined to be consistent with other international competency programmes and with *NCSP National Policy and Quality Standards* workloads. However, if you have completed the New Zealand BMLSc degree and successfully completed cytology as a 4th year placement, then you are required to screen a minimum of 2500 LBC samples to complete the VRPCC.

Successful completion of the VRPCC programme will enable you to be signed off as a competent primary cervical cytology screener. After gaining experience and competency as a primary screener, determining when you gain further sign-off authority for rapid review screening or full secondary screening will be the responsibility of the charge cytoscientist at your place of employment. Note also that the sample numbers given to complete the VRPCC programme refer to manual screening, not automated screening methods. For VRPCC candidates to be signed off as competent for automated screening, *NCSP National Policy and Quality Standards: Location-guided screening Policy* requirements must be completed after completing the VRPCC screening requirements.

Candidates are advised to be familiar with the *NCSP National Policy and Quality Standards* which can be accessed on the website www.nsu.govt.nz. Go to the "For Health Professionals/National Cervical Screening Programme" link, then select "Section 5: Providing a Laboratory Service". Minimum volumes to be reported annually by cytoscreeners for both automated and non-automated screening environments are detailed in Section 5.

A record of primary screening outcomes must be recorded in the table in the VRPCC Logbook.

The samples will be either SurePath or ThinPrep, determined by the laboratory where you are completing the VRPCC programme. Please ensure consistency with placing the slide on the microscope stage and the marking of cells with the systems used at your laboratory. Candidates should record discrepancies between their own interpretation and those of other cytoscreeners. In the case of abnormal results, await the final pathologist report before entering the results. The trainer/supervisor should review these cases with you. In some laboratories, candidates may be able to attend multiheader review sessions with the pathologist at sign-out.

In the early months of screening you should not be concerned with a high rate of false negatives. This is to be expected, and will significantly reduce as your experience accumulates. The same applies to cases that are finally reported as ASC-US, ASC-H or AGC's. These cases are difficult to interpret even for experienced cytologists. Screening of cervical samples is a two-step process: detection and interpretation. During the initial stages of screening, the candidate should focus on detection. Interpretive skills will develop gradually.

3.2 CORE TOPIC: NORMAL/BENIGN/REACTIVE TEST SETS

Test sets are a key component of the VRPCC and provide a measure of your developing competency. Your training laboratory will provide the test sets as you are ready to complete them. The final 100-slide test will be supplied on request by the National Cervical Pathology Training Service when you have completed all other components of the VRPCC programme and when both you and your supervisor think you are ready to take the final slide test.

1. It is important that both you and your supervisor discuss the timing of the tests. You should feel confident and ready to undertake each test set. Follow the instructions in the training logbook.

2. Each set will comprise 10-12 LBC slides. The time taken to screen each slide is recorded so that the practitioner/trainer/supervisor can monitor screening progress.

3. A minimum number of 4 normal/benign/reactive test sets should be given to assess competency in identifying individual constituents of samples which are normal, benign, reactive/inflammatory and contain organisms (excluding HPV). All components of these samples (cellular and non-cellular) should be identified and described in detail. Hormonal patterns should also be noted.

4. Mastery of a minimum of four normal/benign/reactive test sets is required before progressing to the advanced test sets. The trainer/supervisor should continue to provide normal/benign/reactive test sets with appropriate combinations of the above conditions until the practitioner shows 80% mastery in identifying and accurately describing changes in each of four sets.

5. After the minimum four sets have been completed with 80% mastery and after the completion of further normal/benign/reactive test sets, the trainer/supervisor should make a recommendation as to whether the candidate should sit more normal/benign/reactive test sets, or can progress to the abnormality test sets.

6. Print off the normal/benign/reactive test set sheets (4 pages) for each set, and retain when completed and signed.

3.3 CORE TOPIC: ABNORMALITY TEST SETS

Upon successful completion of a minimum of 4 normal/benign/reactive test sets, the candidate may proceed to the abnormality test sets. Please follow the instructions in the training logbook. A signed record of test sets results must be retained.

- Each set will comprise 10–12 slides/cases. The time taken to screen each slide set is recorded so that the practitioner/trainer/supervisor can monitor screening progress.

- Abnormal samples should be admixed with normal and benign samples in these sets.

- A minimum number of 4 abnormality test sets should be given to the candidate to assess competency in identifying and interpreting female genital tract lesions including low and high-grade squamous intraepithelial lesions, squamous cell carcinoma, ASC-US and ASC-H, abnormal glandular cells, and adenocarcinoma (endocervical/endometrial). Wherever possible, abnormal samples in the test sets should have histological confirmation.

- Abnormal cell changes should be identified and described in detail. Grading of lesions should be attempted (recognition and description is the major factor).

- Mastery of a minimum of four advanced test sets is required. The supervisor should continue to provide abnormality test sets with appropriate combinations of conditions until the practitioner shows 80% mastery in both identifying and accurately describing changes in each of four sets.

- After the minimum four sets have been completed with 80% mastery and after the completion of further abnormality test sets, the trainer/supervisor should make a recommendation as to whether the candidate should sit more abnormality test sets, or can progress to consideration of sitting the 100-slide test set.

- Please print off the advanced test set sheets (4 pages) for each set, and retain when completed and signed.

- Before the 100 slide test set can be undertaken, the candidate must have achieved:

1. Satisfactory primary screening of 3000 cervical cytology samples (or 2500 samples for BMLSc candidates who have completed a 4th year cytology placement)
2. 80% mastery of a minimum of four normal/benign/reactive test sets and four abnormality test sets.
3. A minimum of 9 months training in the VRPCC programme after the date of enrolment.
4. A level of competency as a primary screener in cervical cytology that both the supervisor and the candidate are satisfied is appropriate prior to sitting the 100-slide test.

3.4 CORE TOPIC: THE FINAL 100-SLIDE TEST SET

The 100-slide test is the last requirement that candidates need to pass to successfully complete the Vocational Registration Programme in Cervical Cytology (VRPCC). The test is a screening test of 100 slides, taken over three consecutive 8-hour days and is administered and marked by the NCPTS.

Candidates must complete all other requirements in the training logbook and be signed off by their laboratory supervisor as competent to sit the test before doing so. The requirements include completion of all other sections of the VRPCC logbook, satisfactory mastery of introductory and advanced slide sets and primary screening of a minimum of 3000 LBC samples, or 2500 samples for BMLSc graduates who have completed a 4th year cytology major. Both the candidate and the laboratory supervisor need to be confident that the candidate is ready to sit the final test set.

The VRPCC is designed to take about 12 months to complete, although this can vary depending on a particular candidate's aptitude and previous experience. The minimum period of time before a candidate can sit the final test set is 9 months from their enrolment in the VRPCC. There is no maximum time set by the NCPTS for training in the VRPCC.

- Laboratory supervisors need to inform the NCPTS with **at least 2 weeks notice** when their candidate is ready to sit the test, so that dates can be arranged, the slides and documentation can be made available and the NCPTS can prepare to mark the test.
- The 100 slides are a mix of normal, benign and abnormal samples.. Test sets are available for both ThinPrep and SurePath and each candidate will only be tested in the type of LBC with which they have been training.
- The test is taken in your laboratory under examination conditions.
- For each case, the woman's age and brief clinical and/or NCSP-Register history details are provided.
- In the examination, candidates are asked to:
    - Mark (dot) infections, benign changes, abnormal cell changes and other significant features, as when screening.
    - Mark an endocervical component if present.
    - Provide an adequacy and an interpretation report using The Bethesda System terminology, either in NCSP standard Bethesda codes or by using written words. An unmarked copy of the NCSP Bethesda codes is permitted in the examination.
    - A report recommendation is not required as the full NCSP history and clinical details will not be available for each case.

- The test is marked by NCPTS staff. The pass criteria are:
    - 95% of high grades are identified as high grade
    - 90% of all abnormal slides are identified as abnormal
    - 85% of all true negatives are identified as negative (for an intraepithelial lesion or malignancy)

- Candidates are informed of the outcome of the test within three weeks of submitting their responses to the NCPTS for marking. Feedback will be provided through laboratory supervisors to all candidates.

    - The NCPTS provides a certificate to successful candidates and informs the Medical Sciences Council of New Zealand (MSCNZ) of the candidate's successful completion of all requirements of the VRPCC.

    - For candidates who fail the 100-slide test, further training will be discussed with the candidate and the laboratory supervisor. A minimum of a further one month of training is required before a candidate is allowed to re-sit the test.

    - Further training requirements for any candidate who fails a second time will be decided by discussion between the NCPTS, laboratory and NCPTS supervisors and the candidate on a case by case basis.

SECTION 4.0
REPORTING SYSTEMS AND MANAGEMENT GUIDELINES

4.1 CORE TOPIC: REPORTING CYTOLOGY USING THE BETHESDA SYSTEM

4.1.1 Introduction

Reports provides the pathway by which the cytological interpretation is conveyed to the sample taker. The report must incorporate a number of key parameters and formats:

- Accurate patient demographics with sufficient identifiers to guarantee that the report issued is for the correct person. Date of birth and name alone carry risks for accurate identification as neither of these identifiers may be unique to one individual. As stipulated in the *NCSP National Policy and Quality Standards* Section 3 (Sample taking), the National Cervical Screening Programme requires the National Health Index (NHI) number to be included in identifying every woman who has a cervical, vaginal or vault cytology sample or histologic biopsy. Each woman has a unique NHI ensuring accurate correlation with her previous results and clinical details on the NCSP-Register with a national link for the individual irrespective of where the testing is done. Laboratories are also required to comply with the IANZ medical testing standard ISO 15189 for labelling forms, LBC vials and slides. This standard requires two matching identifiers, one being the full name (first and surname) and the other being either the date of birth or NHI number.
- The date and time the specimen was taken.
- The date the specimen was received by the laboratory (usually when registered on the laboratory computer system).
- Clear labelling of both the requisition form and the specimen with at least minimum patient demographic information as discussed above.
- The unique laboratory identifier allocated by the laboratory and specific for the individual specimen received. This number is used as the identifier within the laboratory and is cross-referenced to the NCSP-Register history after reporting.
- A clear description of the type of specimen as determined by the Bethesda 2001 NZ modified reporting system (TBS). This will be discussed below in *Section 4.1.2: Bethesda 2001 New Zealand modified reporting system (TBS).*
- Identification details of the laboratory that is interpreting and reporting the sample.
- A clear description of the cytological interpretation using standard national reporting terminology (The Bethesda System 2001) including recommendations for follow-up or referral.
- The identification of the laboratory staff member who issued the final report and who may be contacted to discuss the report if required.
- The date reported and where the report and any copies were sent.

4.1.2 Bethesda 2001 New Zealand modified reporting system (TBS)

The original version of The Bethesda System was devised in Bethesda, Maryland, USA in 1989 and introduced to New Zealand in 1991. It was initially developed to provide a standardised method of reporting that a wide number of different health professionals could all use and understand. Two revisions have occurred as cytology practice has evolved to keep pace with

increasing understanding of cervical lesions, particularly HPV and its impact on the development and management of low and high-grade cervical disease.
The TBS 2001 version in current use was introduced in NZ on 1st July 2005 after review and approval by the New Zealand cytology sector in conjunction with the NCSP.

In 2014, the American Society of Cytology sponsored a Bethesda 2014 Update with three aims:

(1) Develop a third edition of the Bethesda Cervical Cytology Atlas
(2) Develop a Bethesda 2014 Website
(3) Conduct a second Bethesda Interobserver Reproducibility Study. In this study, cytology images are sent to cervical cytology experts who are asked to submit their opinion about the diagnosis. This is done to assess the degree of consensus about images used in the Atlas and on the website.

There were minimal changes recommended to the terminology of TBS-2001. These changes are summarised below. The VRPCC candidate is encouraged to look at additional information online. New Zealand has not adopted these recommendations at the time of writing (2016).

- Change to reporting benign endometrial cells in women aged 45 years or older (currently 40+ years).
- Additional guidelines provided for assessing adequacy in specific situations e.g. for hrHPV testing, when interfering substances (like lubricant) are present and in post-radiation samples.
- An additional code for reporting cytomegalovirus, if present.

TBS is **management based** i.e. it is designed to be helpful for patient management and includes a recommendation for follow-up or referral. The recommendation identifies best management practice after considering current clinical factors, the result of the current cytology sample, and any past cytology sample or biopsy results.

Other positive aspects of TBS are:

- Fewer report codes and descriptors compared with previous reporting systems
- Standard terminology used by all laboratories
- Provision for the use of free text as an addition to standard terminology
- Assessment of adequacy of the sample by the provision of a semi-quantitative assessment system
- Single descriptors for Atypical Squamous cells of Undetermined Significance (ASC-US) and ASC- possible high-grade lesion (ASC-H)
- Introduction of a report category of "high-grade squamous, possible invasion"
- All atypical glandular cells (AGC) cases are referred for colposcopy
- Normal-appearing endometrial cells are not reported in women under 40 years of age
- Introduction of a recommendation for referral based on suspicious clinical symptoms or signs irrespective of the cytology result.

4.1.3 Structure of The Bethesda System

There are four sections to TBS preceded by the specimen type and specimen site. These are:

- Specimen adequacy
- General categorisation (heading)
- Interpretation (Result)
- Recommendation

Each of these headings is compulsory in the report, unless otherwise indicated below. TBS codes and coded comments used in New Zealand are given in tables at the end of this section. The older Bethesda system codes and descriptors are also provided as any cytology smear/sample reported prior to 1st July 2005 will be recorded on the NCSP-Register record in the older Bethesda code format. An understanding of all cytology/biopsy history codes is needed in order to determine current report recommendations.

Specimen type/technique
This refers to the method of specimen collection, which must be identified in the report. Only one LBC method may be used by each laboratory – either SurePath or ThinPrep. The LBC method chosen dictates the sampling device/s used as each LBC type is an approved technique only as long as the recommended sample-taking devices for that LBC type are used. Recommendations on how to use the recommended sampling device/s must be provided to sample takers.

Specimen site
The specimen site refers to the site where the sample was taken e.g. cervix or vagina and this must be identified in the report.

1. Adequacy
A statement of adequacy is mandatory. A sample is reported as either satisfactory or unsatisfactory for evaluation based on the number of well-visualised, well-preserved squamous cells in the sample. For satisfactory samples, a second comment is added if there is no endocervical component (endocervical cells or squamous metaplastic cells) identified. A detailed discussion about assessing sample adequacy is provided in the *VRPCC Workbook: Subsection 4.1.6 Assessing sample adequacy Page 263.*

2. General categorisation
The general category is mandatory unless the sample is unsatisfactory. The general categorisation is useful for sample takers who may be handling large numbers of cytology sample reports, most of which are negative for intraepithelial lesion or malignancy (i.e. in a screening population). Abnormal reports can be quickly identified.

The general categorisation provides a summary statement for a satisfactory sample as either

- Negative for intraepithelial lesion or malignancy *or*
- Epithelial abnormality (ASC-US through to carcinoma) *or*
- Other (Non-epithelial abnormality)

3. Interpretation
For a negative sample with no other findings, the general category of *Negative for intraepithelial lesion or malignancy* is in effect, the interpretation. For this reason, an interpretation comment is often not given if the sample is categorised as *Negative for Intraepithelial lesion or Malignancy*. An interpretive comment can be used under a *Negative* category heading for infections, reactive changes, endometrial cells in women 40 years or over and atrophic cellular changes as long as there are no abnormal cells (ASC-US or greater) present in the sample.

An abnormal interpretation must be provided if either of the two abnormality general categories are used.

The interpretation in conjunction with previous cytology/biopsy results and/or clinical details will determine further follow-up or referral. Any high-grade or "possible high-grade" interpretation will result in referral to colposcopy and if the result is suspicious of cancer or unequivocally cancer, this will be urgent. Suspicious clinical observations such as an abnormal-appearing cervix will also result in specialist referral irrespective of the cytology result for the sample.

4. Recommendation

Giving a recommendation for follow-up or referral is mandatory and is determined by whether the sample is negative/abnormal/unsatisfactory, by the clinical history and by previous cytology/biopsy results. The recommendation provides management advice to the sample taker regarding appropriate follow-up or referral. The three most common options are

- Routine cytology follow-up in 3 years
- Earlier repeat cytology e.g. 3 months, 6 months, or 1 year repeat
- Referral for colposcopy or specialist assessment

Recommendation guidelines in use in New Zealand are set out in the *NCSP Guidelines for Cervical Screening* document. This topic is discussed in more detail in the *VRPCC Workbook: Core Topic 4.2.3 Managing women with abnormal cervical cytology results Page 272.*

New Zealand modification of The Bethesda System 2001

One modification to the international version of The Bethesda System 2001 was adopted in New Zealand when it was introduced in 2005. This is the General Categorisation of normal samples that contain normal endometrial cells (women 40 years of age and older). In the international version, these samples are reported under the heading of "Other" but for tracking and classification reasons, in New Zealand normal samples with normal endometrial cells are reported under a "Negative for intra-epithelial lesion/malignancy" heading.

Withdrawing from the NCSP Register

Women have the right to choose not to have their cervical cytology, hrHPV, histology results and colposcopy records recorded on the NCSP-Register. Women need to inform the NCSP Register in writing if they wish their results to be withdrawn from the register record. Legislation was passed in March 2006 requiring laboratories to send all results of cervical cytology and histology to the register. The register then has the responsibility of removing any results received for a woman who has chosen to withdraw her results. The options are "all or none" i.e. the choice is to have all cervical cytology and histology results held by the NCSP Register or to withdraw, in which case all the woman's results are removed. Individual case histories held by the NCSP Register should therefore be complete from March 2005 whereas prior to this date, women could withdraw results on a single-result basis. The number of women who choose to withdraw from the NCSP Register is extremely low, at about 0.004% of women who have samples taken.

The information proved by the NCSP for women who wish to withdraw from the NCSP Register, and the withdrawal consent form are as follows:

Withdraw from the National Cervical Screening Programme (NCSP)
Explanatory Notes

Attached is a 'Withdraw from the Programme' form for you to sign and return. Please make sure you have completed all of your background details. When you have completed this form you will have given your consent to be withdrawn from the Programme.

You may wish to remain in the Programme but have decided to stop having cervical cytology samples and do not want to receive reminder letters from the Programme.

Some of the reasons that may apply are:

- you no longer choose to have any more cervical cytology tests (and you are under 70yrs of age)
- you are going overseas
- you have had a complete Hysterectomy, including removal of your cervix (the need for further cervical cytology tests needs to be discussed with your sample taker or Doctor)
- you are too unwell to have any further cervical cytology samples for other medical reasons.

Under the above circumstances you do not need to complete the "Withdraw from the Programme" form. You will not receive reminder letters, and your records will remain on the NCSP-Register. Please contact us on 0800 50 60 50 and we can update your record.

If you wish to Withdraw from the Programme, once we receive your completed consent form or written request, all of your electronic details, except for your background details, will be deleted from the NCSP Register after 20 working days. Any paper records will also be destroyed unless you have requested these, or a copy of your screening history, be returned to you. You will also receive a letter confirming that your withdrawal from the Programme has been processed.

It is important to note, once you have withdrawn from the Programme, you and your sample taker are responsible for your cervical screening. Please let your sample taker know you have withdrawn so they are aware of this.

If you change your mind at a later date, you are welcome to re-enrol. Please ring us on free phone 0800 50 60 50 to request a 'Re-enrol in the Programme' form, or you can download a form at www.nsu.govt.nz under 'Frequently asked questions'. As the Programme will not have any of your previous information, your screening history would begin with your next cervical cytology result.

Regular cervical cytology tests are your best protection against cervical cancer

Withdraw from the NCSP
Consent Form

The National Cervical Screening Programme supports women and sample takers by:

- making sure a complete record of your cervical screening history exists, regardless of whether you change your sample taker
- sending a reminder if you are a few months overdue for your regular cervical cytology test
- making sure you receive follow-up if you have an abnormal result
- monitoring programme quality and evaluating all stages of screening.

Please see enclosed information about withdrawing from the Programme, or visit our website www.nsu.govt.nz under 'Frequently asked questions'.

I DO NOT wish to take part in the National Cervical Screening Programme.

Background details to be completed

Last Name:

First Name(s):

Other Names known by, including Maiden Name:

Address:

Phone Number: ( )

Date of Birth: / /

National Health Index (NHI) Number (if known):

Ethnicity

Which ethnic group do you belong to?

Mark the space or spaces that apply to you

- ☐ New Zealand European
- ☐ Maori
- ☐ Samoan
- ☐ Cook Island Maori
- ☐ Tongan
- ☐ Niuean
- ☐ Chinese
- ☐ Indian
- ☐ Other (please state):

(e.g. Dutch, Japanese, Tokelauan)

Tick one of the following options:

☐ I wish to have my paper records destroyed OR ☐ I wish to have my paper records returned to me

☐ Please tick this box if you would like a copy of your current cervical screening history from the NCSP-Register

☐ Please tick this box if you agree to your last sample taker being informed of your decision to withdraw from the NCSP-Register

Signed: **Date:**

Please post your completed form in an envelope marked 'Private and Confidential' to:

NCSP-Register Central Team
PO Box 5895, Lambton Quay
Wellington 6145

Alternatively, you may like to fax your request to (04) 460 1100, or email us at info@ncspregister.health.nz

Constructing the cytology report using The Bethesda System

The table below overviews the TBS use of Adequacy, General Categorisation, Interpretation and Recommendation categories. Detailed coding is given in further tables that follow.

Adequacy	General Categorisation	Interpretation	Recommendation
U	**----**	Nil or benign changes*	Usually repeat cytology in 3 months
S1 or S2	**G1**	Nil	Usually repeat in 3 years (if no abnormal clinical or previous cytology/histology results)
S1 or S2	**G2**	ASL,LS ASH, HS1, HS2, SC AG1-AG5 AIS, AC1-AC4	Determined by the cytology result, clinical history and the NCSP-Register history of any previous abnormal cytology/histology results
S1 or S2	**G3**	AC5	Determined by the result and clinical setting

*An unsatisfactory report may be accompanied by an interpretation report identifying organisms, endometrial cells in a woman 40 years of age or over, or atrophy.

KEY: Adequacy codes: U = Unsatisfactory sample
S1 = Satisfactory, endocervical component present
S2 = Satisfactory, no endocervical component present
General Categorisation: G1 = Negative for intraepithelial lesion or malignancy
G2 = Epithelial abnormality
G3 = Non-epithelial abnormality
Epithelial abnormality codes: ASL = ASC-US
LS = LSIL
ASH = ASC-H
HS1 = HSIL
HS2 = HSIL, possible invasion
SC = Invasive squamous cell carcinoma
AG1-AG5 = AGC's
AIS = AIS
AC1- AC4 = Adenocarcinoma
AC5 = Consistent with malignant neoplasm

With experience, cytoscreeners become familiar with clinical terminology used in gynaecology. As part of the screening process, reviewing the NCSP-Register (NCSP-R) history and any clinical information provided by the sample taker is essential. For example, a sample taker may note a cervix. The cytology and the woman's NCSP-R history may both be normal. Without the clinical information, the case would be reported as negative with a recommendation for repeat cytology in 3 years, whereas referral to colposcopy is required because of the suspicious appearance of the cervix. This is an extreme example that highlights the importance of reviewing all information provided. If additional information is held on your laboratory computer records that the NCSP-R record does not have (e.g. a previous high-grade result that is not on the NCSP-

R history) then make the appropriate recommendation and inform the NCSP-R of the additional information.

4.1.4 Tables: Bethesda 2001 New Zealand Modified (TBS) - From 1st July 2005

Detailed tables of TBS codes and coded comments in current use in New Zealand are given below. These tables provide the specific codes used daily in laboratory practice. Cross-references to the previous "old" codes used prior to July 2005 are included in the yellow column.

- **SPECIMEN TYPE AND SITE**

Specimen types - mandatory	
New Code	**Descriptor**
CPS	Conventional pap smear
LBC	Liquid based cytology
COM	Combined (conventional and liquid based)
Specimen site - mandatory	
Code	**Descriptor**
T	Vault
R	Cervical
V	Vaginal

- **ADEQUACY**

Adequacy - mandatory
- Either *one* S code **or** *a maximum of two* U codes are allowed
- Only O1-O5, OT2, OT3 interpretation codes are allowed with U codes

Old code	**TBS2001 code**	**Full New Descriptor**
A1	S1	The specimen is satisfactory for evaluation (optional free text)
A2G	S2	The specimen is satisfactory for evaluation (optional free text). No endocervical/transformation zone component present
A3A	UA	The specimen is unsatisfactory for evaluation because of insufficient squamous cells
A3B	UB	The specimen is unsatisfactory for evaluation because of poor fixation/preservation
A3C	UC	The specimen is unsatisfactory for evaluation because foreign material obscures the cells
A3D	UD	The specimen is unsatisfactory for evaluation because inflammation obscures the cells
A3E	UE	The specimen is unsatisfactory for evaluation because blood obscures the cells
A3F	UF	The specimen is unsatisfactory for evaluation because of cytolysis/autolysis
A3I	UG	The specimen is unsatisfactory for evaluation because … (free text)

- **GENERAL CATEGORISATION**

4.1.5 Deriving General "G" codes

Interpretation	**Derived General "G" codes**
Unsatisfactory	No G code
Negative – no "I" code • Infection • Reactive	G1 G1 + "O" code G1 + "OT" code
Abnormal - except AC5	G2
Abnormal AC5	G3
"O", "OT2", "OT3" codes may be used with "U" codes = no "G" code "O" and "OT" codes may be used with abnormal codes = "G2" or "G3"	
General (previously "category") o General code is mandatory (except when unsatisfactory) o Only one General code is allowed o G codes will be accompanied by either S1 or S2 code	

- Abnormal interpretation codes must be accompanied by either G2 or G3
- G1 doesnt require an interpretation code but may be used with O1-O5 codes and OT1-OT3 codes

Old Code	TBS2001 code	Full New Descriptor	Grade
B1	G1	Negative for intraepithelial lesion or malignancy	N
B2A2	G2	Epithelial cell abnormality: See interpretation/result	ABN
	G3	Other: See interpretation/result	ABN

- **INTERPRETATION**

Interpretation (previously "diagnosis")

- A maximum of *five* interpretation codes are allowed
- G2 code is mandatory with any of the following: ASL,ASH,LS,HS1,HS2,SC,AG1-AG5,AC1-AC4
- G3 code is mandatory with AC5
- Only O1-O5, OT2, OT3 codes are allowed with an unsatisfactory (UA-UG) report
- OT2 may be accompanied with a qualifying clause for sample takers (*see below)
- A combination of LS and ASH may be used to replace the old code C3A2B7

Old code	TBS2001 code	Full New Descriptor	Grade
C1C1	O1	There are organisms consistent with Trichomonas vaginalis	N
C1A1	O2	There are fungal organisms morphologically consistent with Candida species	N
C1B1	O3	There is a shift in microbiological flora suggestive of bacterial vaginosis	N
C1B2	O4	There are bacteria morphologically consistent with Actinomyces species	N
C1D2	O5	There are cellular changes consistent with Herpes simplex virus	N
C2A1 (C2A1A) C2B1A (C2B1B) C2B2 (C2B2A) C2B4	OT1	There are reactive cellular changes present (optional free text)	N
C3B1 C3B1A C3B1B C3B1C	OT2	There are endometrial cells present in a woman over the age of 40 years	N
C2A4 (C2A4A)	OT3	There are atrophic cellular changes present	N
C3A1 C3A1A C3A1B C3A1C C3A1D C3A1F	ASL	There are atypical squamous cells of undetermined significance (ASC-US) present	LG
C3A1E	ASH	There are atypical squamous cells present. A high grade squamous intraepithelial lesion cannot be excluded (ASC-H)	HG
C3A2A C3A2A1 C3A2A2 C3A2A3	LS	There are abnormal squamous cells consistent with a low grade squamous intraepithelial lesion (LSIL; CIN1/HPV)	LG
C3A2B C3A2B1 C3A2B2 C3A2B3 C3A2B4 C3A2B5 C3A2B6	HS1	There are abnormal squamous cells consistent with a high-grade squamous intraepithelial lesion (HSIL). The features are consistent with CINII or CINIII	HG
	HS2	There are abnormal squamous cells consistent with a high grade squamous intraepithelial lesion (HSIL) with features suspicious for invasion	HG
C3A3	SC	There are abnormal squamous cells showing changes consistent with squamous cell	HG

	carcinoma	

OT2: Suggested qualifying clause accompany coded comment for OT2 (endometrial cells in women over 40 years):

"The presence of endometrial cells in a woman over the age of 40 years can be a normal finding, or seen in association with hormone replacement therapy, or rarely, associated with endometrial pathology including hyperplasia or neoplasia. Please correlate this finding with any symptomatology of uterine pathology, for example abnormal uterine bleeding and refer/investigate accordingly."

C3B2B C3B2B1	AG1	There are atypical endocervical cells present	HG
C3B2A C3B2A1	AG2	There are atypical endometrial cells present	HG
C3B2 C3B2C C3B2E	AG3	There are atypical glandular cells present	HG
C3B2B2	AG4	There are atypical endocervical cells favouring a neoplastic process	HG
C3B2D	AG5	There are atypical glandular cells favouring a neoplastic process	HG
C3B3D C3B3E C3B3F	AIS	There are abnormal endocervical cells consistent with adenocarcinoma in-situ (AIS)	HG
C3B3A	AC1	There are abnormal glandular cells consistent with endocervical adenocarcinoma	HG
C3B3B	AC2	There are abnormal glandular cells consistent with endometrial adenocarcinoma	HG
C3B3C	AC3	There are abnormal glandular cells consistent with extrauterine adenocarcinoma	HG
C3B3	AC4	There are abnormal glandular cells consistent with adenocarcinoma	HG
C3C C4	AC5	There are abnormal cells consistent with a malignant neoplasm	HG

- **RECOMMENDATIONS**

Recommendations - repeats, follow up, management

- Recommendation code is mandatory
- There is no change to the policy of determining recommendation codes
- R12 (oestrogen treatment) must be accompanied by interpretation code OT3 (atrophic changes)
- R10 is used with HS2,SC,AC1-AC5
- R14 is a new code and may be used with any report except HS2,SC,AC1-AC5 and when there is a clinical suspicion of invasive cancer indicated on the requisition form

Old code	TBS2001 code	New Full Descriptor
B2B0	R1	The next sample should be taken at the usual screening interval
B2B1	R2	Please repeat the sample within 3 months
B2B4	R3	Please repeat the sample within 3 months of the end of pregnancy
B2B5	R4	Please repeat the sample in 3 months
B2B6	R5	Please repeat the sample in 6 months
B2B7	R6	Please repeat the sample in 12 months
B2B7A	R7	Because a previous sample showed atypical squamous cells or low grade changes, please repeat the sample in 12 months
B2B7H	R8	Annual samples are indicated because of previous high grade abnormality
B2B8 B2B8A	R9	Referral for specialist assessment is indicated
B2B8B	R10	Urgent referral for specialist assessment is indicated
B2B8D	R11	Further assessment is recommended
B2B9	R12	Please repeat the sample shortly after a course of oestrogen treatment
B2B8C B2B13	R13	Under specialist care
	R14	In view of the abnormal clinical history provided, urgent referral for assessment is recommended regardless of cytological findings

4.1.6 Assessing sample adequacy

Squamous cellularity

Bethesda 2001 introduced a semi-quantitative method to determine adequacy of the cellularity for LBC samples based on a minimum number of well-preserved, well-visualised squamous cells. Adequacy of an LBC sample is based on demonstrating sufficient cells to provide reasonable assurance that any abnormal cells in the LBC vial will be represented on the slide. Each slide is judged on its own merit. For each LBC slide, this is the number of cells in **one** slide i.e. if the first slide made from the LBC vial is unsatisfactory, the repeat slide must have the minimum number of required cells **in the repeat slide** for the sample to be reported as satisfactory for evaluation. Adding the cell content from several slides to achieve the minimum number of cells is not permitted.

As previously discussed in the *VRPCC Workbook: Core Topic 1.2 Cytopreparation Page 15* and in *Subsection 2.2.4 Squamous epithelium Page 74*, the cellularity requirements for a satisfactory LBC sample are as follows:

A minimum of 5,000 well-visualized/preserved squamous cells are needed for an LBC slide to be considered satisfactory. Less than this is regarded as unsatisfactory (unless abnormal cells are seen). A repeat sample can be made and this may produce a satisfactory slide, particularly if further treatment of the sample is used to reduce excessive blood or inflammatory material.

Not every specimen requires a cell count to assess cellularity as most samples are clearly adequately cellular. For borderline cellularity cases where there may be less than 5000 well-visualized/preserved squamous cells present, a counting scheme is used. An estimate based on viewing fields with 40X objective across the diameter of the preparation, including the centre of the cell circle is used. For most microscopes, **minimum numbers of cells needed in each of at least 10 fields** counting across the diameter of the cell circle are:

- **SurePath: 9 cells per 40X** in at least 10 of the fields viewed.
- **ThinPrep: 4 cells per 40X** in at least 10 of the fields viewed.

- Guidelines for estimating cellularity in LBC preparations using different microscopes are available in *The Bethesda System for Reporting Cervical Cytology 2nd Edition. Solomon D and Nayar R.* Page 8 and is reproduced as Table 1 on Page 265.

- Strict objective criteria are difficult to apply in some cases. For example, slides with cell clustering, atrophy, or cytolysis are technically difficult to count. Laboratories should apply professional judgment when evaluating these slides.

Any specimen in which abnormal cells are identified is satisfactory for evaluation, by definition.

The endocervical component

A minimum of 10 endocervical or metaplastic squamous cells lying either singly or in a group/s are required for an adequate endocervical component in an LBC sample. The absence of an adequate endocervical component i.e. less than 10 endocervical or metaplastic cells, is still reported as a satisfactory sample, but an additional comment is added to note that an endocervical component has not been identified. A number of factors may result in the absence of these cells including poor sampling technique, the squamo-columnar junction being high in the endocervical canal, and difficulty in visualising the cervix especially in women over 50 years of age. The absence of these cells does not carry an increased risk of cervical disease or affect follow-up recommendations but is included in the report information mainly as a QC indicator for sample takers regarding their sample taking technique.

Tables and diagrams from *The Bethesda Reporting System for Cervical Cytology 2nd Edition. Solomon D and Nayar R* provide a visual representation of adequate cellularity. With experience, cytoscientists/cytotechnicians rapidly form an accurate impression of unequivocally satisfactory and unsatisfactory samples, and only need to apply the semi-quantitative cell counting procedures to samples where the cellularity adequacy is uncertain.

Bethesda 2001 also emphasises that cellularity may be affected by clinical or physiological conditions. For example, postmenopausal women with an atrophic sample may contain clusters of small parabasal cells with areas of the sample containing no cells. On low magnification this may give an impression of insufficient squamous cells, but closer examination of the cell groups may reveal 50-100 cells in a group. Bethesda clearly comments that professional discretion must be applied in addition to the use of cell counts.

After three consecutive unsatisfactory sample reports referral to colposcopy occurs. This situation and even an unsatisfactory sample followed by a 3-month repeat cytology sample, can cause increased anxiety for women. Apply the criteria for measurement with care and diligence.

Bethesda 2001: A guide to practical estimation of sample adequacy for cervical cytology

To assess LBC slide cellularity adequacy based on recommendations from Bethesda 2001 using a rapid semi-quantitative approach:

Cell content: Minimum of 5,000 well-visualized and well-preserved squamous cells are required for a satisfactory sample: The Bethesda 2001 recommendations.

- A minimum of 10 fields should be counted randomly along a diameter that includes the centre of the preparation. “Holes” (spaces) should be included in the field count. The average cell number per microscopic field to achieve 5000 cells is shown in Table 1 on Page 265. Cell clumps should not be included in the cell count. A satisfactory sample including an adequate endocervical/metaplastic component is reported as **S1** Bethesda adequacy code.

- In some instances the cellularity on the prepared slide may not be representative of the collected sample. Cases with fewer than 5,000 cells should be examined to determine if the reason for the scant cellularity is due to a technical problem in preparation of the slide. In those instances, a repeat preparation may yield an adequate cellular preparation. However, the adequacy of each slide should be determined separately and not

cumulatively. The report should clarify whether blood, mucus, or inflammation contributed to an unsatisfactory sample, or if the reason is directly related to low squamous cellularity.

General notes:

- Strict objective criteria may not be applicable in every case. Some slides with cell clustering, atrophy, or cytolysis are technically difficult to count, and laboratories should apply professional judgment and employ hierarchical review when evaluating these slides.
- The minimum count applies only to squamous cells. Endocervical cells and completely obscured cells should be excluded from the estimate.
- When determining cellularity, compare the reference images/charts to specimens in question and determine if they have a sufficient number of fields with approximately equal or greater cellularity than the reference image.
- The sample should be determined as unsatisfactory if 75% or more of the cellular components are obscured by inflammation, blood, bacteria, mucous or artefact (UB – UG codes).
- A sample is not unsatisfactory if there is no endocervical cell/transformation zone cell component present. The absence of less than 10 well-preserved endocervical or squamous metaplastic cells should be reported with an S2 Bethesda adequacy code.

All eyepieces are **x10**		Eyepiece = **FN20** Objective = **x10**		Eyepiece = **FN20** Objective = **x40**		Eyepiece = **FN22** Objective = **x10**		Eyepiece = **FN22** Objective = **x40**	
Prep diam mm	Prep area mm^2	Total no. of fields	No. of cells/field for 5000 cells	Total no. of fields	No. of cells/field for 5000 cells	Total no. of fields	No. of cells/field for 5000 cells	Total no. of fields	No.of cells/field for 5000 cells
13 SurePath	132.7	42.3	118.3	676	7.4	34.9	143.2	559	9.0
20 ThinPrep	314.2	100	50.0	1600	3.1	82.6	60.5	1322	3.8

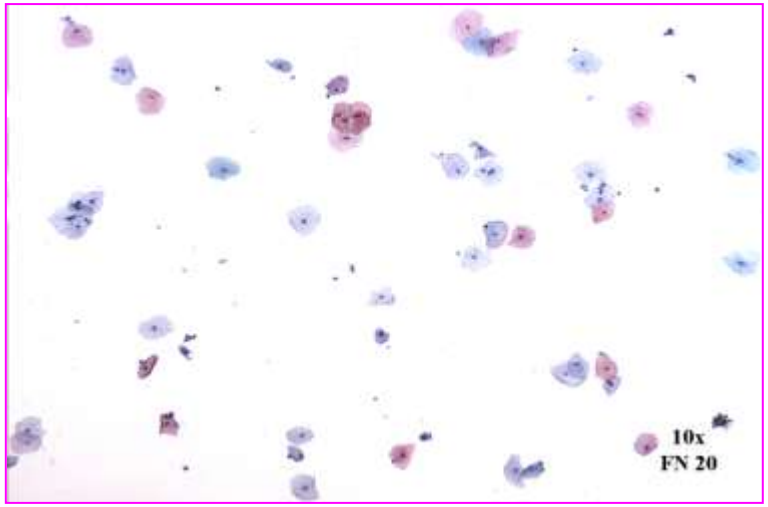
Table 1: Calculated cell counts per field for LBC adequacy based on a minimum of 5000 cells
Ref: The Bethesda System for Reporting Cervical Cytology 2nd Edition. Solomon and Nayar.

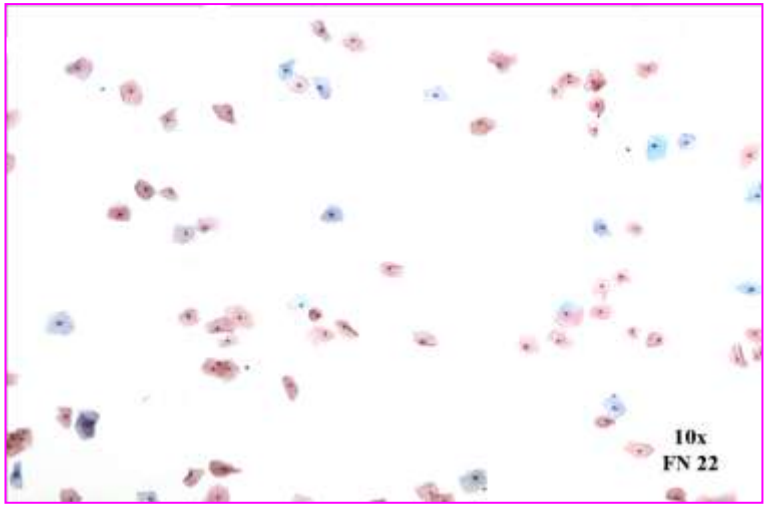
Methodology for calculating field cellularity:
If you have microscope objectives and/or eyepieces with different specifications to those in the tables above, use the following formula to determine the cell content:
[Total number of cells required] ÷ [area of LBC circle mm^2 ÷ ((eyepiece FN ÷ objective magnification) ÷ 2)^2 x π]
For a ThinPrep sample using eyepiece FN20 x10 and objective x40 above as an example, then the formula equates as follows:
[5000] ÷ [314.2 ÷ ((20 ÷ 40) ÷ 2)^2 x 3.142] = 5000 ÷ [314.2 ÷ (0.25^2 x 3.142)] = 5000 ÷ [314.2 ÷ 0.20] = 5000 ÷ 1571 = 3.1

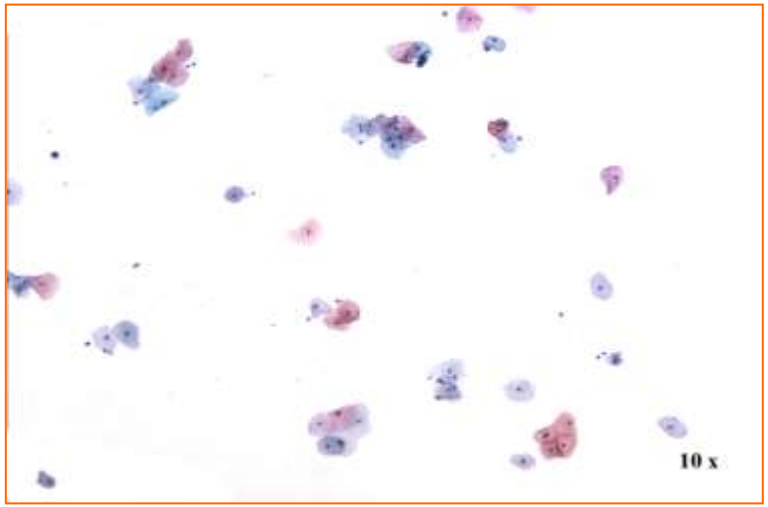
SurePath is a smaller diameter preparation, has more cell overlap, and more cells per field compared to ThinPrep. Therefore assessment of SurePath adequacy is easier using higher objective magnification (e.g. x40).

Microscopic assessment of adequacy

A: ThinPrep







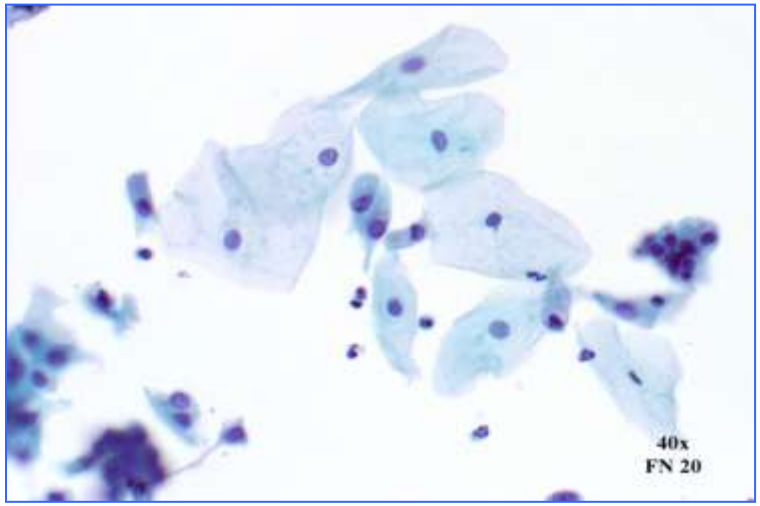
Minimum of 10 fields counted randomly along a diameter that includes the centre of the preparation.

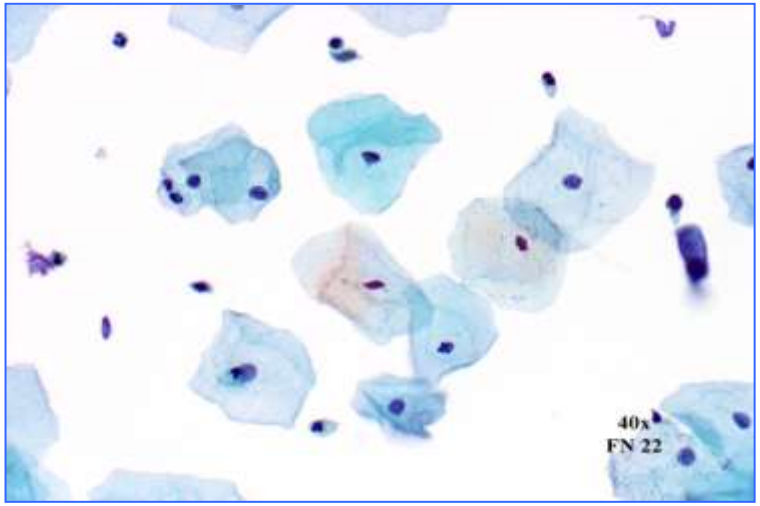
Upper left: ***Satisfactory***
- Eyepiece FN20 x10
- Objective magnification x10
- Number of cells per field = 50

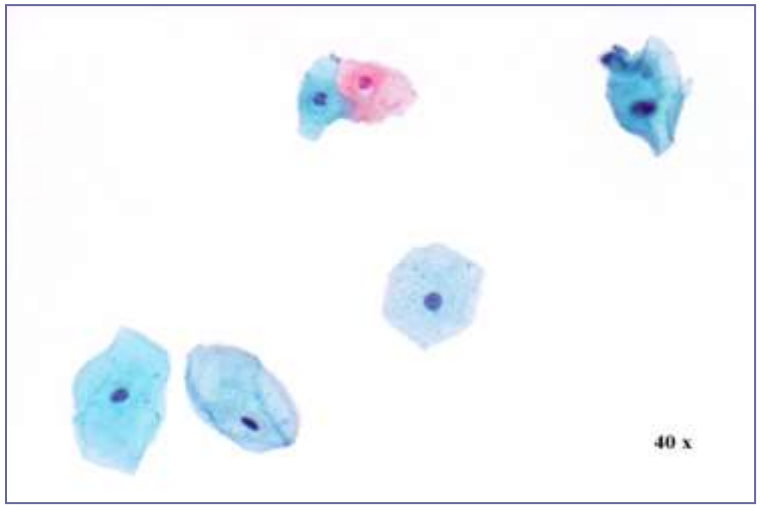
Upper right: ***Satisfactory***
- Eyepiece FN22 x 10
- Objective magnification x 10
- Number of cells per field = 60

Lower left: ***Unsatisfactory***
- Eyepiece FN20 or FN 22 x 10
- Objective magnification x 10
- Number of cells per field = 40

B: SurePath







Minimum of 10 fields counted randomly along a diameter that includes the centre of the preparation.

Upper left: ***Satisfactory***
- Eyepiece FN20 x40
- Objective magnification x10
- Number of cells per field = 7.4

Upper right: ***Satisfactory***
- Eyepiece FN22 x40
- Objective magnification x10
- Number of cells per field = 9.0

Lower left: ***Unsatisfactory***
- Eyepiece FN20 or FN22 x10
- Objective magnification x10
- Number of cells per field = 6.0

4.1.7 SNOMED® Coding for Histology

SNOMED® is an acronym for "Systematized Nomenclature of Medicine". SNOMED® International, a division of the College of American Pathologists (CAP), is focused on advancing excellence in patient care through the delivery of SNOMED terminology. SNOMED® is a complex coding system for histopathology and has been adopted worldwide. There are different versions but all are based on a similar code structure. Further information about SNOMED® can be found at www.snomed.org.

SNOMED codes are used to code histology specimens, not cytology samples. The codes are used for histology specimens recorded on the NCSP-Register so it is important that the cytoscientist/cytotechnician knows how to interpret them.

A SNOMED code subset is used by the NCSP-Register for histology results and histopathologists are asked to restrict the SNOMED codes used when reporting cervical/vaginal histology specimens to the NCSP-approved SNOMED codes. The NCSP-Register uses the 1993 SNOMED version but for historical reasons, two versions are in use in NZ histology laboratories, the 1986 version as well as the 1993 version. The 1986 codes are translated to the 1993 version for the NCSP-Register record.

Tables: Histology codes used by the National Cervical Screening Programme Register January 2013

T = Topography; M = Morphology

Adequacy of specimen		**1986 Code**	**1993 Code**	
Insufficient or unsatisfactory material for diagnosis		M09000	M09010	
There is no code for satisfactory materials.				
Site (topography) of specimen		**1986 Code**	**1993 Code**	
Vagina		T81	T82000	
Cervix (includes endocervix and exocervix)		T83	T83200	
Summary diagnosis	**Code stored on register**	**1986 Code**	**1993 Code**	**Abn**
There will be a maximum of four M codes transmitted to the register.				
Negative result - normal tissue		M00100	M60000	N
Inflammation		M40000	M40000	N
Microglandular hyperplasia		M72480	M72480	N
Squamous Metaplasia		M73000	M73000	N
Polyp		M76800	M76800	N
Other (Morphologic abnormality, not dysplastic or malignant)		M01000	M01000	N
Atypia		M69700	M67000	L
HPV, koilocytosis, condyloma (NOS) Condyloma acuminatum		M76700 ^M76720	M76700 ^M76720	L
Dysplasia / CIN NOS		M74000	M67015	L
CIN I (VAIN I when used with T81/ T82000)		M74006	M67016	L
CIN II (VAIN II when used with T81/ T82000)		M74007	M74007	H
CIN III (VAIN III when used with T81/ T82000) Carcinoma in situ		M74008 M80102 M80702	M67008 M80102 M80702	H H H
CIN II and CIN III when used together		M67017	M67917	H
Microinvasive squamous cell carcinoma		M80765	M80763	C
Invasive squamous cell carcinoma		M80703	M80703	C
Benign glandular atypia		M81400	M67030	N
Glandular dysplasia		M81401	M67031	H
Adenocarcinoma in situ		M81402	M81402	H

Adenocarcinoma, endocervical type		M83843	M83843	C
Adenosquamous carcinoma		M85603	M85603	C
Invasive adenocarcinoma (not endocervical)		M81403	M81403	C
Metastatic tumour		M80006	M80006	F
Undifferentiated carcinoma		M80203	M80203	C
Sarcoma		M88003	M88003	C
Other codes accepted	**Code stored on register**	**1986 Code**	**1993 Code**	**Abn**
Carcinosarcoma	M88003	#M89803	#M89803	E
Choriocarcinoma	M80003	*M91003	*M91003	E
Miscellaneous primary tumour	M80003	*M80003	*M80003	E
Small cell carcinoma	M80003	*M80413	*M80413	E
Malignant tumour, Small cell type	M80003	*M80023	*M80023	E
Melanoma	M80003	*M87203	*M87203	E
Other primary epithelial malignancy	M80003	*M80103	*M80103	E

^This code was stored on the NCSP-Register as M76700-HPV prior to 1 October 2008
#This code was stored on the NCSP-Register as M88003 – Sarcoma prior to 1 October 2008
*These codes were stored on the NCSP-Register as M80003 – Other malignancy prior to 1 Oct 2008

Notes: Abn = Abnormality type used by the register			
C=Cancer of the cervix	F=Secondary / metastatic Cancer to the Cervix	E=Cancer not in the Cervix	
H=High Grade	L=Low Grade	N=Negative	U=Unsatisfactory

In addition, either use the SNOMED ***procedure code*** detailed in the first table below, or a ***specimen type code*** detailed in the second table below.

Procedure Code (entered into the next available diagnosis code field)	**1986**	**1993**	
Hysterectomy	P11001 or P11101	P83350 or P83353 or P83360 or P83380	(stored as H)
Partial hysterectomy with cervical component	P11041	P83352	(stored as S)
Biopsy - Diagnostic	P11481 or P11541	P83425	(stored as B)
Biopsy - Treatment e.g. LLETZ, Laser, Cone	P11011 or P11411 or P11461	P83401 or P83420 or P83423	(stored as T)

Specimen Type (entered into the next available diagnosis code field)		
Either enter a single character or up to 6 characters as specified.		
Hysterectomy	H or HYSTER	(stored as H)
Partial hysterectomy with cervical component	S or PARTIAL	(stored as S)
Biopsy - Diagnostic	B or BIOPSY D or DIAGNO	(stored as B)
Biopsy - Treatment e.g. LLETZ, Laser, Cone	T or TREATM	(stored as T)
Polyp	P or POLYP	(stored as P)

4.1.8 Questions

1. Explain the reasons behind adoption of TBS-2001 in NZ.
2. Read Section 4 of the *NCSP National Policy and Quality Standards* and discuss the importance of supplying mandatory patient details at the time of sample taking.
3. Discuss minimum cellularity for LBC preparations and the criteria to be used for assessing slides of borderline cellularity.
4. Is there any benefit in monitoring unsatisfactory sample reporting rates in a laboratory? Explain reasons for this.
5. Under what circumstances would remakes (repeat slides made from the same LBC vial) be useful or of limited value in cytological assessment?
6. An experienced nurse phones to complain that she has been receiving an increase in the number of her sample results reported as "no endocervical component present". Explain how you would deal with this phone call.
7. Discuss unsatisfactory SurePath and ThinPrep slide preparations and measures that could be taken to mitigate these.

References

- Nayar R, Wlbur DC (eds). 2015. *The Bethesda System for Reporting Cervical Cytology: Definitions, Criteria, and Explanatory Notes* (3rd Ed). New York: Springer.

- Ministry of Health. 2014. *Bethesda 2001 (New Zealand Modified): Codes for Cytology Laboratories*. Available at: https://www.nsu.govt.nz/health-professionals/national-cervical-screening-programme/publications-and-reports

4.2 CORE TOPIC: CERVICAL SCREENING AND MANAGEMENT GUIDELINES

4.2.1 Introduction

The National Cervical Screening Programme (NCSP) determines the policy for cervical screening for New Zealand and provides clinical guidelines for managing women who have abnormal cytology results. The screening policy states the age at which screening is recommended and also the interval (the "screening interval") between successive screening cytology samples. The policy and management guidelines are reviewed and updated at regular intervals. The management guidelines in current use were published in 2008 and updated in 2009 when hrHPV testing was introduced, and are being reviewed again in 2016.

4.2.2 Cervical Screening Guidelines for New Zealand

The *Guidelines for Cervical Screening in New Zealand Incorporating the Management of Women with Abnormal Cervical Smears* provides the following information on the current cervical screening policy in New Zealand. The full document can be accessed at https://www.nsu.govt.nz/system/files/resources/guidelines_for_cervical_screening_in_new_zealand.pdf

When to screen and how often
Regular cervical screening for the prevention of cervical cancer is recommended for all women who are, or have ever been, sexually active.

NCSP policy on screening age and interval

All women who have ever had sexual intercourse should be offered a three-yearly cervical cytology test from age 20 to age 69

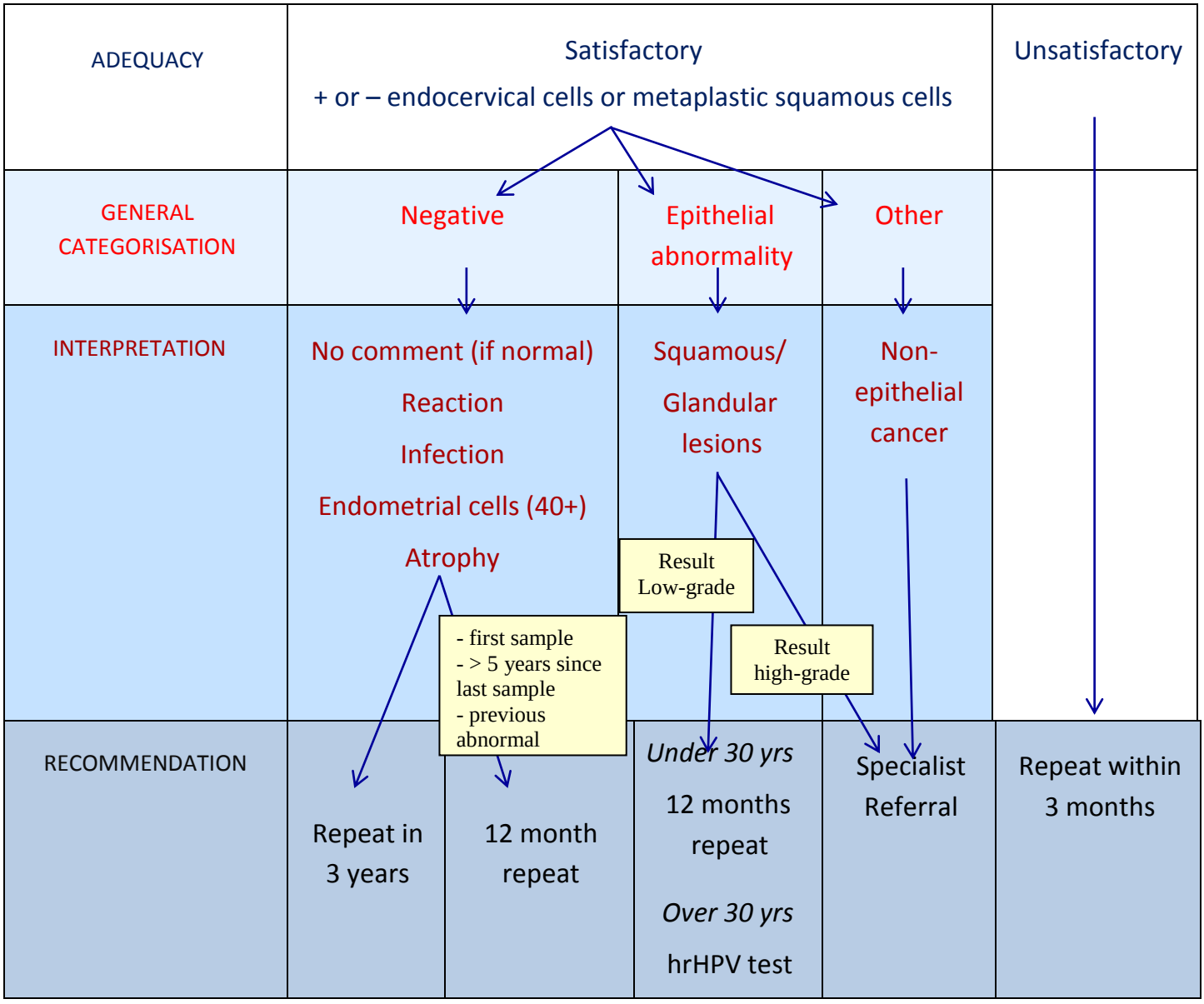
If this is the first ever sample, or more than 5 years have elapsed since the previous sample, a second sample is recommended one year after the first, with three-yearly samples thereafter

Age to start screening
The age-specific incidence of cervical cancer in New Zealand shows that there is no need to screen sexually active women under 20 years of age. Invasive squamous or adenocarcinoma of the cervix has rarely been diagnosed in a woman less than 20 years of age.

Age to stop screening
Good quality cervical cytology samples are difficult to obtain in women many years after menopause, and women aged 65 years and over who have had many samples with a normal result in the previous 10 years, have a very low risk of developing cervical cancer. The current policy in New Zealand is to continue regular screening until aged 69 years.

4.2.3 Managing women with abnormal cervical cytology results

Management guidelines are closely aligned to The Bethesda System reporting codes because The Bethesda System report categories are deliberately selected to be those categories that are important clinically. A brief overview of the relationship of the TBS cytology results to recommendation management guidelines is shown in the table below.



NCSP Management Guidelines for women with abnormal cervical cytology results

The NCSP guidelines for managing women with abnormal cervical cytology results are based on best national and international practice and serve to standardise the way that women are managed in New Zealand. The guidelines are not rigid rules as it is the responsibility of clinicians in discussion with their patients to make treatment decisions that consider the best outcome with the least risk for individual women.

The development and revision of the guidelines is an ongoing process that incorporates new evidence as this becomes available and involves thorough discussion between the NCSP and professionals working in the field. The 2016 guidelines review underway at the time of writing will anticipate a move to the use of hrHPV testing for primary screening.

The NCSP publication *Guidelines for Cervical Screening in New Zealand Incorporating the Management of Women with Abnormal Cervical Smears* documents the current management guidelines in use in New Zealand. A full discussion of the guidelines is beyond the scope of the VRPCC workbook but it is important that cytoscientists/cytotechnicians understand key aspects of the guidelines as they form the basis for formulating recommendations used in laboratories on a daily basis in cervical cytology reporting. In brief:

1. Women who have a **normal cytology** result with a normal clinical history and no previous abnormal cytology or cervical histology results have a repeat cytology sample in three years. If it is the woman's first-ever sample or if it is more than 5 years since her last cytology sample, then the recommendation is for a repeat cytology sample in 12 months before commencing 3-yearly screening (assuming all results are negative).

2. **Unsatisfactory samples** are repeated within 3 months. Cytology samples should not be repeated more frequently than one month as lesions may be missed if samples are repeated before the epithelium has had time to regenerate after a previous sample. If there are three consecutive unsatisfactory samples, the woman is referred to colposcopy because she is inadequately screened.

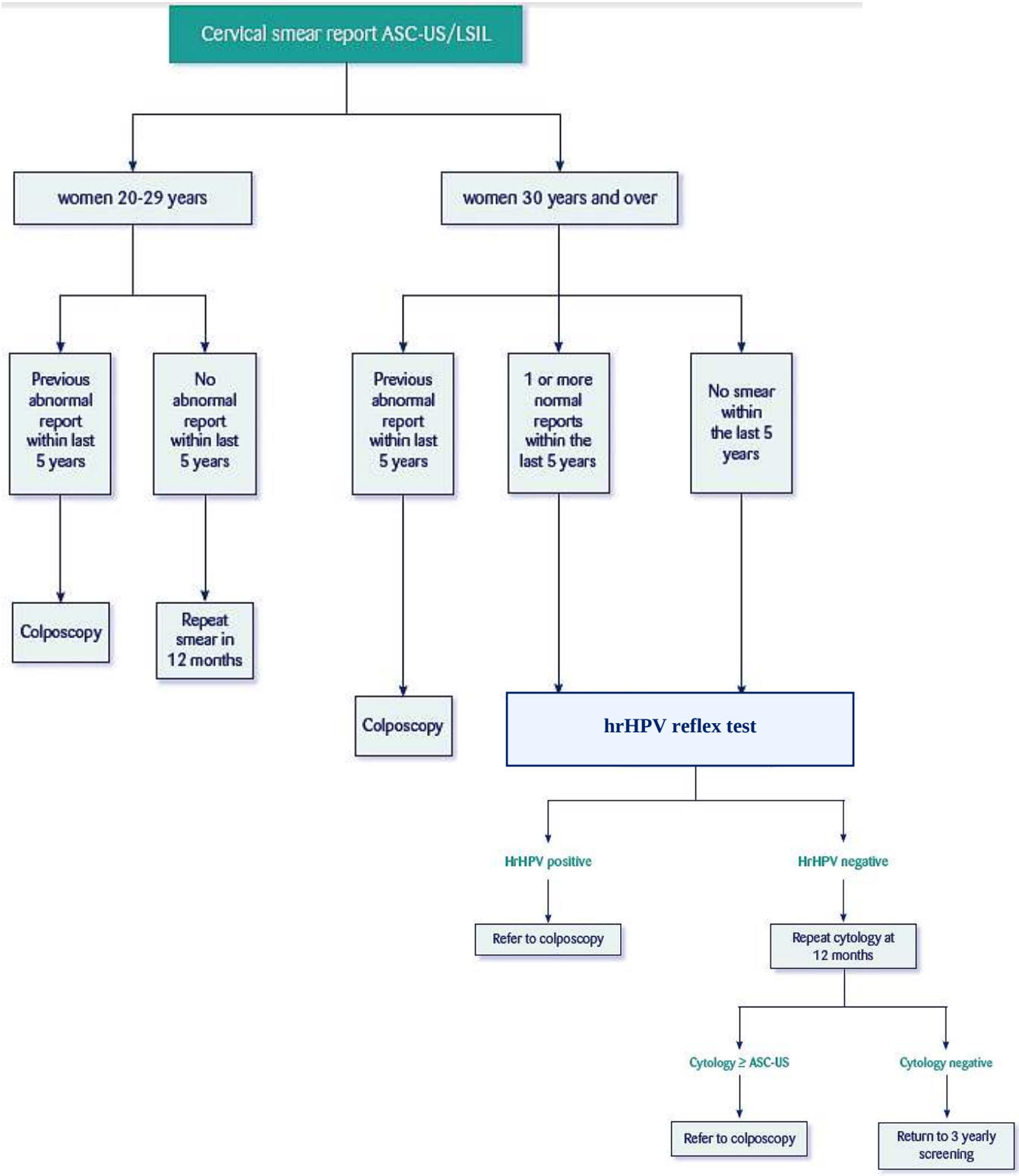
3. Women with **low-grade cytology** are managed in the same way whether the result is ASC-US or LSIL. HrHPV testing is used to determine the management of women who are 30 years of age or older. It is not used in women who are under 30 years of age because the proportion of the population who will test positive for hrHPV in this younger age group is too high for it to be a useful discriminator in identifying women with cervical pathology.

For women who have an ASC-US or LSIL cytology result (with no previous abnormality):

- Women who are ***30 years of age or older*** with no cytology/histology abnormality in the preceding 5 years, have an hrHPV test in conjunction with their cytology (same LBC vial)
    - If the hrHPV test is negative, a 12 month repeat cytology sample is recommended. If the repeat cytology sample is normal she will return to 3 yearly screening.
    - If the hrHPV test is positive, she is referred for colposcopy.
- Women who are ***under 30 years of age*** are recommended to have a 12 month repeat cytology sample. If the repeat is normal, another 12 month repeat cytology sample is recommended and if that repeat is also normal, she returns to 3 yearly screening.

The following flowchart is from the *Guidelines for Cervical Screening in New Zealand* document.

Management of women with low-grade abnormalities: ASC-US or LSIL



4. Women with **high-grade cytology** results.
   - For management purposes high-grade cytology includes ASC-H, HSIL, SCC, all categories of AGC's, AIS, all adenocarcinomas and all other malignant neoplasms.
   - All high-grade cases are referred for specialist assessment/colposcopy.

Subsequent management depends on the colposcopic findings and the results of any histologic biopsies or specimens taken. Learning to make appropriate report recommendations for women who have follow-up cytology after a colposcopy will develop with reporting experience. Further details are available in the *Guidelines for Cervical Screening in New Zealand* document.

4.2.4 Using hrHPV testing in New Zealand

Testing for high-risk Human Papillomavirus (hrHPV) was introduced nationally in New Zealand on 1 October 2009. The test is performed on cellular material from LBC vials (ThinPrep or SurePath). HrHPV testing is **only used in the management of women with squamous lesions**, not glandular lesions.

A positive hrHPV test result means that a woman has acquired at least one subtype of high-risk HPV but it does not mean that a woman has a cervical lesion. Even if no lesion is identified at colposcopy, follow-up is important as women with persistent hrHPV infection are at increased risk of developing cervical intraepithelial lesions.

The hrHPV test is funded by the NCSP for use in three specific clinical situations.

1. Triage of ASC-US and LSIL in women ≥30 years of age (with no abnormal cytology in the previous 5 years).

The test is ordered by the laboratory once the cytology result is known. Use of hrHPV testing in this situation has already been discussed.

2. Test of cure after treatment of HSIL.

The hrHPV test in this case is ordered by sample takers on the same laboratory requisition form as the request for cytology. The test of cure identifies women who have successfully completed treatment for high-grade squamous lesions and who can safely return to three-yearly screening. Their risk of developing further cervical lesions is the same as the rest of the population having screening cytology, if all four results of the Test of Cure are negative.

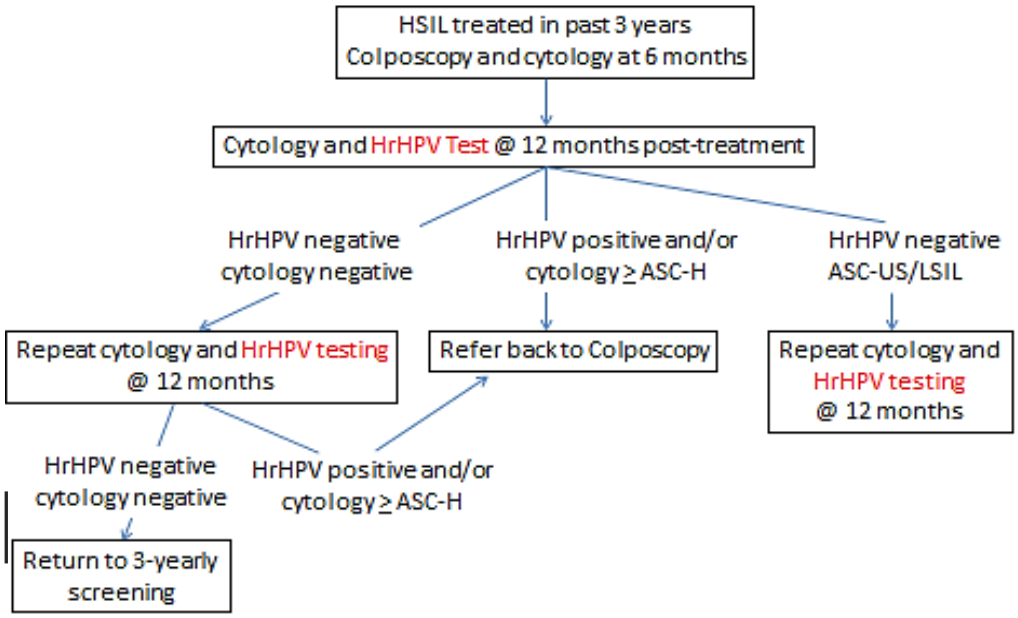
- Cytology with concurrent hrHPV testing is performed on two occasions, 12 months apart
- If all four results are negative, women can return to three-yearly screening. Prior to the use of hrHPV testing as a test of cure, women treated for HSIL remained on annual samples for life as there was no way of predicting those who remained at increased risk of developing further high-grade lesions
- The Test of Cure can not be used for followup after any glandular abnormality so these women remain on annual cytology screening for life

When hrHPV testing was introduced as a test of cure, all women who tested hrHPV positive were sent for colposcopy but it soon became apparent that many colposcopies were negative.

The recommendations were therefore modified to make a distinction between those women who had their high-grade squamous lesion treated within the previous three years, and those who had a high-grade squamous lesion treated more than three years previously (called "historical testing"). Many women having a test of cure for historical reasons will have had several negative annual cytology results since their treatment.

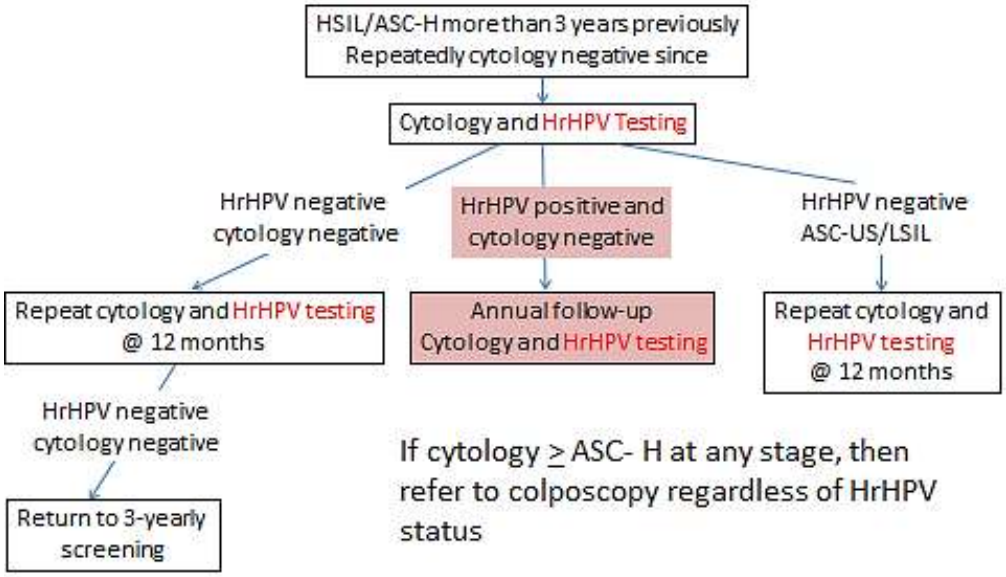
Using Test of Cure hrHPV testing for women treated within the past 3 years

Women who have been treated for a high-grade squamous lesion within the previous 3 years are referred back to colposcopy if they are hrHPV-positive or if they have possible or definite high-grade cytology on any of the tests of cure samples. If they are hrHPV-negative with possible or definite low-grade cytology, they are followed with repeat cytology and hrHPV testing instead.



Using Test of Cure hrHPV testing for women treated more than 3 years previously (historical testing)

Women who were treated for a high-grade squamous lesion more than 3 years previously and have had repeated annual negative cytology results since, are less at risk. Those who are hrHPV-positive but their cytology result remains negative are not referred for colposcopy but are followed annually with repeat cytology and hrHPV testing. Deciding whether to refer a woman for colposcopy if her hrHPV test is persistently positive is a clinical decision made by the woman and her sample taker. A *Test of Cure for historical reasons* can also be used for any woman who has had a previous possible (ASC-H) or definite (HSIL) highgrade squamous cytology result, regardless of whether this was confirmed by histology and regardless of whether she was treated, as a negative Test of Cure provides excellent reassurance about a very low risk of current disease, allowing a return to regular three-yearly screening.



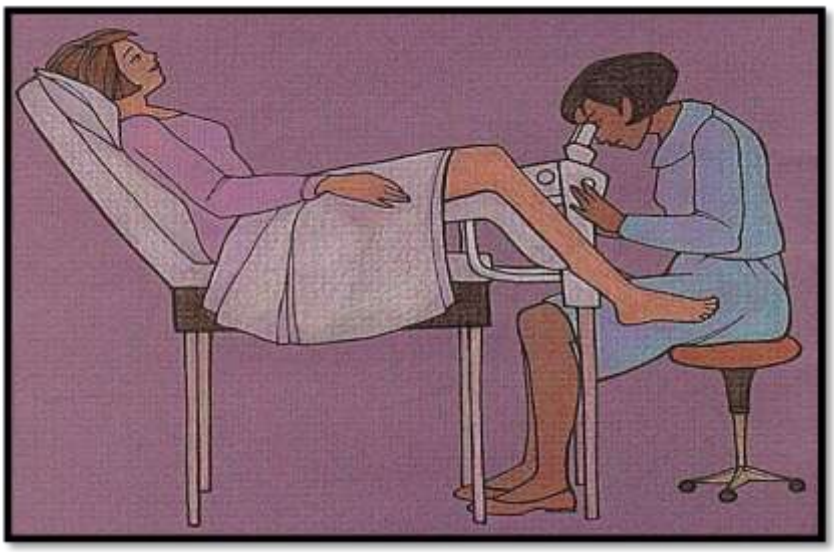
3. Specialist testing after discordant results.

The hrHPV test is ordered by specialist colposcopists on the same laboratory requisition form as the request for cytology.

HrHPV testing may be ordered by colposcopists to assist with case management particularly where there is discordancy between the cytology, histology and the colposcopy findings.

4.2.5 Colposcopy

An understanding of the basics of colposcopy is important for the cytoscientist/cytotechnician as colposcopy is an essential part of the management of women with abnormal cervical cytology. Identifying cervical lesions at colposcopy is a visual skill that requires experience in much the same way as the development of the visual skills needed to appreciate cervical lesions by cytology. There is an element of subjective judgement in both colposcopy and cytology and for this reason, it is particularly important that cytologists and colposcopists work closely together to achieve the best outcome for women.



Women may be referred for colposcopy for the following reasons:

- Cytological abnormalities, either high-grade or persistent low-grade abnormalities including LSIL with a positive hrHPV test in women over 30 years of age
- An abnormal appearance of the cervix, seen by visual inspection (e.g. by a sample taker)
- Abnormal symptoms e.g.: post-coital bleeding, irregular bleeding, pelvic pain
- Follow-up after treatment of a high-grade lesion

Colposcopy is a specialist outpatient procedure and most colposcopies in New Zealand occur in District Health Board (DHB) colposcopy clinics. A smaller number (about 15%) are performed by private colposcopists. There are specific policies and standards that apply to colposcopy that are determined by the NCSP, as there are for all aspects of the cervical screening programme. The *NCSP National Policy and Quality Standards* provide guidance about best practise, care and support for women both before and after colposcopy and the standards also define timeframes within which women should be seen following referral for a colposcopy.

The visual examination of the cervix at colposcopy uses a low-powered microscope called a colposcope. The appearance of the cervix seen through the colposcope allows the colposcopist to predict the presence and grade of cervical lesions and to select the best area of the cervix to biopsy to confirm the diagnosis. Colposcopy is also used to visualise the cervix during treatment procedures.

Women having a colposcopy lie on a reclining chair covered with a sheet. A speculum is used to gently hold the walls of the vagina open so that a light can be shone onto the cervix. The colposcope magnifies the cervix so it can be examined by the colposcopist in detail. The cervix and vagina will then be painted with a weak acetic acid solution. This alters the abnormal areas that will then look white and can be seen more readily. Tissue samples (biopsies) can be obtained using biopsy punches. These small biopsies are taken to confirm the diagnosis prior to treatment (if this is needed). The colposcopy procedure itself usually takes ten minutes, with the entire appointment taking 20 or 30 minutes depending on whether it is a first appointment or not. The biopsies are sent to the histopathology laboratory for processing and microscopic examination by a histopathologist.

Treatment of identified cervical abnormalities depends on the nature and extent of the lesion identified. Low-grade lesions are usually not treated as most resolve naturally so these will be followed by repeat colposcopy to make sure that the lesion does resolve within about 2 years. High-grade lesions require treatment and this involves removing or destroying the abnormal cells. In New Zealand, almost all women are treated by excising the affected area of the cervix. The location and extent of the high-grade abnormality will influence exactly how this is done.

A **Large Loop Excision of the Transformation Zone (LLETZ)** is the most common type of treatment and is performed using colposcopic visual guidance under local anaesthetic. The procedure involves passing an electrified wire loop into the cervix on one side, moving it across the endocervical canal and then drawing it out the other side, removing a wedge of cervical tissue. Bleeding is controlled by the small amount of cautery induced by the wire. The removed cervical tissue is sent to the laboratory for examination and diagnosis.

Loop instruments of varying sizes used during Lletz procedures

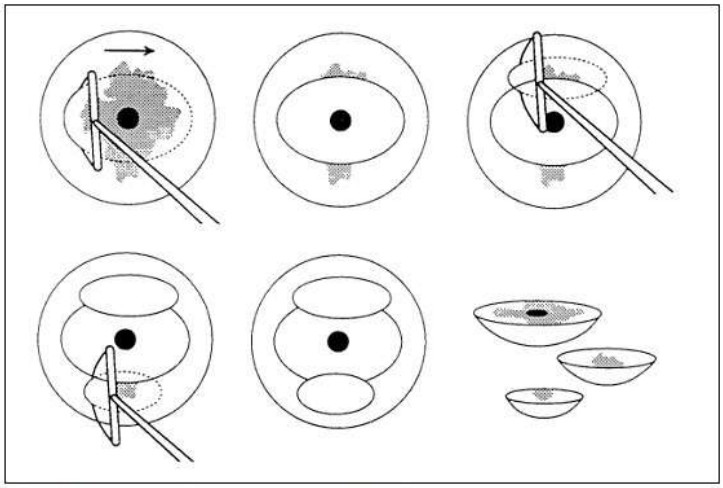
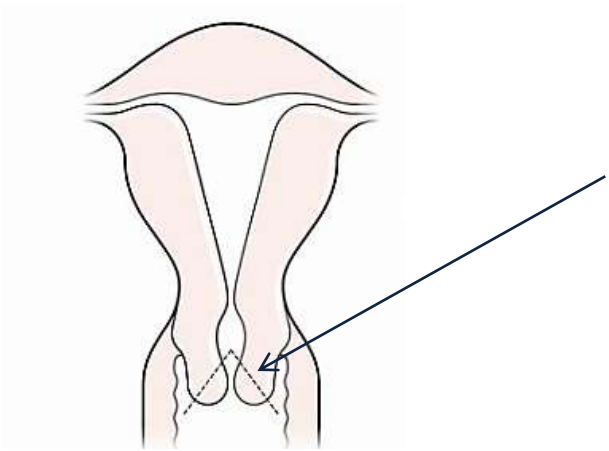


FIGURE 13.6: Excision of an ectocervical lesion with multiple passes

Diagram showing the use of a loop instrument to removed the central portion of the cervix during a Lletz procedure. One pass is recommended removing the entire lesion if possible, whereas three passes are depicted in this illustration.

A **cold knife cone biopsy** is the other main type of excisional treatment method used. In this procedure, a larger cone shaped section of the cervix is removed surgically under a general anaesthetic. This type of procedure is used under particular clinical circumstances, such as an extensive lesion or a glandular lesion where the upper border of the lesion cannot be seen by colposcopic examination. A larger piece of tissue is removed for histopathologic examination.

As well as diagnosing any lesions present in the excised tissue, the histopathologist also reports on whether the lesion has been completely excised or not. Lesions which extend to the excision margins are more likely to recur so this is important information for the colposcopist who will be seeing the woman for follow-up after her colposcopy. This applies to the histologic examination of lletz biopsies as well as cone biopsies.



Schematic diagram showing the wedge of tissue that is removed when a cone biopsy is performed.

After a woman has had a lletz or cone biopsy, the cervix heals. Epithelium regenerates across the area of exposed cervical stroma where the tissue has been removed over a period of several months. Consequences of a previous lletz or cone biopsy are that the endocervical canal is shorter, particularly if the woman has two or even three lletz biopsies performed. This can mean that cells from the lower uterine segment (LUSC) are more readily sampled, which can cause problems with interpretation in post-lletz cervical cytology samples. Cervical endometriosis is also more common in women who have had a lletz or cone biopsy because endometrial cells sometimes seed into the raw stromal bed during menstruation, before the surface epithelial layer has regenerated.

Sometimes women who have had a lletz or cone procedure have difficulty in subsequent pregnancies with miscarriages or early delivery if they have insufficient cervical tissue present to sustain the pregnancy (called an "incompetent cervix"). Treatment of cervical lesions in young women who are likely to want to become pregnant in later years needs to be carefully considered because of this risk.

Occasionally women will have a hysterectomy to remove cervical lesions but this is only done if a hysterectomy is indicated for other medical reasons.

There are other treatment options in use overseas such as laser treatment, cryosurgery and diathermy which destroy abnormal cells rather than excising them. A disadvantage of destructive treatments rather than the excisional methods described above, are that the tissue cannot be examined by a histopathologist as it is destroyed rather than removed.

Post-treatment follow-up

After treatment a woman will see her specialist at about 8-12 months for a follow-up colposcopy. The colposcopist will examine the cervix to see if there is any indication of residual disease. A cervical cytology sample is taken at the same time. The woman will see her sample taker for ongoing follow-up if the colposcopist is happy with the outcome of her treatment. She will need more regular cervical cytology tests as recommended in the NCSP guidelines.

If all the affected tissue has been removed, she will become hrHPV negative after treatment but this takes some time to occur. No hrHPV testing should be done until 12 months after treatment. If she has been treated for a high-grade squamous lesion, she will undergo a test of cure and eventually return to three-yearly screening if she completes the test of cure without any abnormal results.

Although less common, the squamous epithelial lesions seen in cervical epithelium can also occur in the vagina. Women who have a hysterectomy for cervical abnormalities need to continue having cytology samples taken from the top of the vagina. These are called vault samples. Women can still undergo a test of cure (if the cervical lesion was a high-grade squamous lesion) and return to three-yearly screening if all results are negative.

4.2.6 Questions

1. What are the current recommendations for the age and interval of cervical cytology screening in NZ?
2. A doctor phones to ask you why an hrHPV test was not done even though he had requested this on the form. His 31 year old patient has a history of a LSIL abnormality 2 years earlier. What explanation would you provide to the doctor?
3. Read Section 6 of the *NCSP National Policy and Quality Standards* (Providing a Colposcopy Service). Discuss some of the timeliness requirements for colposcopy referrals.
4. Briefly discuss the various types of treatment used for histologically confirmed cervical abnormalities.

References

- Ministry of Health. *Guidelines for Cervical Screening in New Zealand 2010*. Available at:https://www.nsu.govt.nz/health-professionals/national-cervical-screening-programme/cervical-screening-guidelines
- Guidelines for Cervical Screening in New Zealand: Implementation of HPV Testing 2009. Available at: https://www.nsu.govt.nz/publications4/

SECTION 5.0
QUALITY ASSURANCE

5.1 CORE TOPIC: QUALITY ASSURANCE IN CYTOLOGY LABORATORIES

5.1.1 Introduction

Quality Assurance is critically important in cervical cytology because there is a significant reporting error rate. Abnormal cells may not be on the cytology slide examined, may be present but not detected at screening or may be present and detected but misinterpreted. Samples reported falsely as negative are particularly problematic with cervical screening because women may not present for further screening for another three years. During this time, a high-grade lesion such as HSIL may become more extensive requiring a larger area of cervix to be removed for treatment or may progress to an invasive lesion. If an invasive malignancy is missed at screening, the consequences can be very serious. Cervical screening is only effective when there are multiple checks and systems in place to manage this significant risk of error. Finding errors that have occurred through QA processes when the incidence of disease is already low, as it is in a cervical screening population, requires a focused approach.

Laboratories address Quality Assurance on two levels. Internal Quality Assurance refers to the programmes and activities which occur within laboratories, such as monitoring staff reporting rates, slide reviews and correlations of cytology reports with histologic and colposcopic findings. External Quality Assurance programmes refer to those activities where there is monitoring of laboratory procedures and performance by an external body. Examples are International Accreditation New Zealand (IANZ) accreditation which is obligatory for all New Zealand medical laboratories, and participation in the quality assurance programmes run by the Royal College of Pathologists of Australasia Quality Assurance Programme (RCPA QAP).

Because the QA requirements for cervical cytology screening laboratories are stipulated by the NCSP and as all laboratories are externally audited by IANZ on a regular basis, the QA policies and practice in your laboratory will be similar to those of other New Zealand cytology laboratories.

5.1.2 Internal Quality Assurance

Internal QA provides the way for the laboratories to monitor and evaluate their own processes and to monitor the accuracy of all the individuals who process, screen and report cervical cytology samples. General laboratory-wide issues that affect the quality of work performed such as staff qualification, the provision of a conducive work environment and accurate sample collection and identification are as important in cervical cytology as in any other medical laboratory department. This section deals with the particular internal quality assurance procedures that are specific to cervical cytology.

Individual competency to perform a test is part of internal QA. Each practitioner is required to have their competency for the tests they perform signed off annually by their supervisor in order to renew their Annual Practicing Certificate (APC), which is issued by the Medical Sciences Council of NZ (MSCNZ). Annual competency sign-off is also required by IANZ. When practitioners sign their application to the MSCNZ they confirm the following statement:

Every practitioner must practise only within his or her area of competence.

Practitioners must also be enrolled in a re-certification programme approved by the Medical Sciences Council of NZ, such as the CPD (Continuing Professional Development) programme of the NZIMLS (NZ Institute of Medical Laboratory Science).

Under the *NCSP National Policy and Quality Standards*, laboratories are required to:

- ensure consistent registration, processing and staining
- ensure rescreening occurs before reporting
- evaluate individual performance of all staff
- monitor the sensitivity of primary screening: individuals and the laboratory as whole
- correlate cytology with follow-up histology
- review prior negative cytology after high-grade histology has been reported
- ensure that any change in a result is notified to the NCSP-Register and the smear taker

Because of the potential for error in cervical cytology, we need to assure accuracy at every step of the processing, screening and reporting pathway.

Processing and staining LBC slides prior to screening.
Increased use of automation has standardised some of the processing and staining of slides, reducing the potential for day to day variation in the quality of slides produced. Checking the staining of slides on a daily basis is good laboratory practice, to ensure consistency both within a batch of stained slides and from one day to another.

Monitoring the Screening process
Monitoring the maximum numbers of cases an individual primary screener may process on a daily and annual basis is another example of an internal QA requirement. Standards for maximum (daily) and minimum (annual) workloads for cytoscientists/cytotechnicians and for minimum numbers (annually) of samples that pathologists and laboratories must report, are determined nationally through consultation and discussion between the NCSP and sector professionals.

Monitoring the accuracy of reporting
The reporting profile of all individuals screening and reporting cervical cytology is monitored to ensure that there are no "outliers" and that there is consistency of reporting over time. All individuals will have periods of fluctuating focus and accuracy whatever their level of proficiency and it is important that this is monitored to endure that no serious reductions in performance occur. This applies to pathologists as well as cytoscientists/cytotechnicians.

In addition to monitoring reporting rates, regular reviews are conducted to monitor accuracy of reporting. The three key ways this occurs are:

1. Histology-cytology correlation reviews.
After cervical histology reports are issued, cytology laboratories are notified of the histology result if they have reported any cytology samples at their laboratory in the previous 6 months. This is coordinated on a national basis so that laboratories are informed about any histology report issued anywhere in New Zealand. Cytology laboratories then correlate the histology result with their previous cytology result and if there are discrepancies, then the case is reviewed. If there remain discrepancies after review, then clinicians need to be informed. Any revised diagnoses are conveyed to the NCSP-Register so that the register record can be updated.

2. "Prior negative" case reviews.
Laboratories are required to review any cytology slides reported as negative, benign/reactive or unsatisfactory in the 42 months prior to a high-grade or invasive diagnosis on histology. These reviews provide important educational feedback to primary screeners as inevitably some cases that have been under-reported will be identified. Because all the cases have already had a high-grade lesion identified on histology, the women concerned will be under active follow-up and treatment so clinicians don't need to be notified of the outcome of these reviews. The NCSP does monitor the rate at which cases where high-grade or possible high-grade cells are identified in a slide previously reported as negative is occurring for each laboratory, as part of their laboratory monitoring programme.

3. Participation at MDM (Multidisciplinary Meeting) Colposcopy meetings.
Meetings of colposcopists, cytopathologists, cytoscientists/cytotechnicians and histopathologists occur regularly where individual cases are discussed. The cases may be selected because there is a discrepancy between the cytology/histology/colposcopy results, because there is a difficult decision regarding treatment that the colposcopist wishes to discuss with colleagues or because the colposcopist wishes to clarify or review a particular laboratory sample. These meetings are valuable because the cytology result can be considered in the clinical context and provide an opportunity to discuss cases directly with colposcopists.

The laboratory Quality and Procedures manuals detail the internal QA policy and activities that a laboratory undertakes. All procedures have to be documented and approved by designated senior scientists and/or pathologists. VRPCC candidates should review these manuals and make their own notes of each procedure or QA activity. The charge cytoscientist will provide an opportunity for VRPCC candidates to observe these activities in action in the daily work of the cytology laboratory.

5.1.3 External Quality Assurance

External QA refers to the systems and processes that are monitored and evaluated by a source external to and independent of the laboratory. This may be another laboratory or an external organisation such as a professional body or an auditing agency. The key issue is that there is no conflict of interest between the assessor and organisation being evaluated so that an objective unbiased assessment can be made.

The two main aspects of external QA are participation in external QA programmes, and laboratory audits. These are often inter-related. For example, laboratories are required to maintain accurate records of the processes used and the outcomes of participation in external QA programmes including any remedial action required as a result. When the laboratory has its reporting performance evaluated by an external organisation such as IANZ, the external auditor will want to see and review these records as evidence that the laboratory is participating in the external QA programme in the correct manner.

Clear and concise documentation of all QA activities must be kept for laboratory performance and for each individual's educational activities record.

Participation in External QA Programmes

1. Laboratory-based external Quality Assurance Programmes.

All cytology laboratories are required by the NCSP to participate in a laboratory-based external Quality Assurance programme and most choose to participate in programmes run by the Royal College of Pathologists Quality Assurance Programme (RCPA QAP).

The RCPA QAP laboratory programme is a quarterly evaluation on the reporting performance of the laboratory. There are different modules for ThinPrep and SurePath samples and laboratories choose which module/modules they wish to enrol for. The laboratory receives **five slides once every three months**. The samples are screened and reported in the normal manner for that laboratory, without special treatment or attention. The final report for each sample is submitted to the RCPA QAP. The QAP provides a report for each laboratory identifying for each slide, whether the laboratory achieved:

- A Target response: an exact agreement between the laboratory result and the correct result
- An Acceptable response: a response that is close to the target response and does not alter the clinical management of the case.
- An Unacceptable response: a laboratory result which differs significantly from the correct result and which carries implications for clinical management. Unacceptable responses require the laboratory to review the sample for educational purposes. Laboratories are expected to carefully monitor trends in unacceptable responses and to implement any level of remedial action that is required. IANZ would also require a laboratory to demonstrate evidence of remedial action.
- A major error: a laboratory result which is unacceptably different from the correct result and one that would result in a serious error in clinical management. An example would be reporting a high-grade lesion as a negative sample. A major error requires appropriate remedial action to be undertaken by the laboratory. What this is would be determined by the type of error and the circumstances under which it occurred.

The RCPA QAP programme should also be used as an educational tool in the laboratory. Staff who are not involved in reporting a particular set of QAP samples can also review them, offer opinions on the slides and then check their answers against the QAP return. Multiheader review and discussion of the cases can qualify for CME/CPD points.

2. Individual-based quality Assurance Programmes

The introduction of an External Quality Assurance (I-EQA) programme for individual cervical cytology practitioners was a key recommendation of the Ministerial Inquiry into the Under-reporting of Cervical Smears in the Gisborne Region (2001). The programme required considerable development and commenced in 2012. The programme evaluates individual performance for reporting gynae cytology, is comprised of **two surveys per year each containing 5 slides** and is mandatory for all cytoscientists/cytotechnicians and cytopathologists screening or reporting cervical cytology. The RCPA QAP administers the I-EQA under contract to the NCSP. Laboratories enrol in either SurePath or ThinPrep modules. Results are confidential to the individual participant unless there is a significant issue with the level of their performance.

Laboratory Audits

Laboratories that process cervical, vaginal and vault cytology, hrHPV tests and histology for women enrolled on the NCSP Register are contracted to the NCSP. As part of the contract the NCSP audits laboratories against a set of standards stated in the *NCSP National Policy and Quality Standards*. Medical laboratory practice is also accredited against an international standards document called ISO15189 Medical laboratories – Requirements for quality and competence. This standard is based on ISO/IEC 17025 abd ISO 9001 in conjunction with specific criteria for medical testing. Accreditation is undertaken in New Zealand by IANZ http://www.ianz.govt.nz/services/accreditation-2/accreditation/laboratories/medical/.
Laboratories need to comply with these standards in order to be acknowledged as a laboratory with accreditation to practice.

International Accreditation New Zealand (IANZ) in conjunction with the National Cervical Screening Programme (NCSP) accredits and audits cervical cytology, HPV testing and histology laboratories through periodic site visits. Representatives from IANZ and the NCSP visit each cervical cytology laboratory annually, with a more in-depth audit once every three years.

5.1.4 Ethics for cytopathology staff

Ethics is a key issue that will be discussed when you commence employment. It is an essential competency that should be part of your daily practice as a professional.

The Medical Sciences Council of NZ has developed an excellent document entitled *Code of Competencies and Standards for the Practice of Medical Laboratory Science*. The final page of this document is attached. You are strongly encouraged to obtain the full document and commence your career and practice with the Medical Sciences Council of NZ policy and philosophy incorporated in your daily activities.

Your laboratory will also have a defined policy for all aspects of practice.

MEDICAL LABORATORY SCIENCE BOARD

CODE OF COMPETENCIES AND STANDARDS FOR THE PRACTICE OF MEDICAL LABORATORY SCIENCE

MAINTAINING HIGH PROFESSIONAL STANDARDS:
MEDICAL LABORATORY SCIENTISTS AND TECHNICIANS WILL PRACTISE IN A RESPECTFUL, INCLUSIVE, HONEST AND TRUSTWORTHY MANNER

COMPETENCY	Practise as a Professional	Practise as a scientist/technician	Safe Practice	Communication	Culturally competent practice
OUTCOME	Act in accordance with ethical, legal, professional and regulatory requirements	Practise by integrating medical laboratory science knowledge and skills within area of competence and scope of practice	Ensure personal, patient/client, colleague and public safety	Communicate effectively with patients/clients, colleagues, other health professionals and the public	Practise taking into account the socio-cultural values of others
STANDARD	1.1 Comply with the HPCA Act 1.2 Comply with other relevant legislation and codes 1.3 Demonstrate honest and trustworthy practice 1.4 Maintain the privacy and confidentiality of patients/ clients 1.5 Apply the protocols of informed consent 1.6 Apply the principles of Quality Assurance and improvement 1.7 Manage workload and resources 1.8 Demonstrate problem solving skills 1.9 Work collaboratively 1.10 Demonstrate accountability 1.11 Take responsibility for the training, direction and/ or supervision of others 1.12 MLS: Continue to develop knowledge and skills	2.1 Demonstrate the correct safe operation of laboratory equipment 2.2 Demonstrate practical competence in laboratory analytical techniques 2.3 Analyse specimens using the prescribed protocols of the workplace 2.4 Report and interpret laboratory results 2.5 Demonstrate competence in the application and use of relevant information technology 2.6 Demonstrate knowledge of the normal and pathological states of human anatomy and physiology of the major organ systems in health and disease 2.7 Demonstrate an understanding of current knowledge and practical competence in one or more of the MLSB approved health service categories 2.8 MLS: Be able to apply appropriate research methods	3.1 Practise safely in accordance with health and safety legislation and workplace safety policies and procedures 3.2 Identify and manage laboratory hazards 3.3 Handle, store, transport and dispose of hazardous chemical and biological material appropriately	4.1 Demonstrate competence in written and oral English 4.2 Accurately record and report results in a clear, timely and appropriate format for interpretation 4.3 Use a range of communication skills to convey information and instructions	5.1 Recognise own beliefs, values and prejudices and the impact these may have on patients/clients and colleagues 5.2 Recognise cultural diversity as it relates to ethnicity, culture, age, gender, sexual orientation, migrant experience or disability 5.3 Apply the Treaty of Waitangi principle of partnership by making informed decisions through consultation 5.4 Demonstrate culturally competent practice

5.1.5 Questions

1. Explain the value of laboratories in having to meet Standard 522 (reviewing cases with a high grade histology diagnosis) of the *NCSP National Policy and Quality Standards* and how this benefits individual screeners.

2. What parameters should laboratory managers monitor regularly and provide active feedback to individual screeners about to ensure NCSP requirements are met?

3. How is the New Zealand Medical Sciences Council (NZMSC) Annual Practising Certificate (APC) renewal linked to practitioner quality performance?

4. What specific quality measures should be in place in the cytopreparation room to ensure optimal quality of slide preparation? Complete this question for both ThinPrep and SurePath.

5. What are "MDM's" and what is the importance of these for the colposcopist?

CORE TOPIC 5.2 NCSP MONITORING OF LABORATORY PRACTICE

5.2.1 Introduction

Annual laboratory audits that are undertaken by the NCSP and IANZ in all New Zealand laboratories reporting cervical cytology have been discussed in the previous *VRPCC Workbook: Subsection 5.1.3 Page 286.* This topic covers the additional ways that the NCSP monitors laboratory performance. This occurs in two ways: through the *NCSP National Policy and Quality Standards* which sets out the expectations of the NCSP for laboratory practice and provides the standards against which laboratories are audited annually, and through Independent Monitoring Reports which are provided every six months by the Cancer Council of New South Wales for the NCSP Advisory Committee.

5.2.2 NCSP National Policy and Quality Standards

The *NCSP Policy and Standards* document is a working document that is continually being revised and modified. The document covers the whole of the screening pathway from sample taking through to colposcopy. Section 5 deals specifically with laboratory practice (cytology, histology and hrHPV testing). The standards are subject to active ongoing discussion between the NCSP and laboratory staff and are modified as laboratory practice evolves.

A copy of Section 5 of the *NCSP National Policy and Quality Standards* will be provided to all VRPCC candidates. You must be familiar with the document, particularly those standards that pertain to your areas of work and responsibility. These include daily and annual workloads and educational requirements for cytoscientists/cytotechnicians, primary and secondary screening requirements and quality assurance requirements.

There are currently (2016) 22 standards for laboratories included in the *NCSP Policy and Quality Standards* and these are itemised in the table below. This summary should be read in conjunction with the full document.

***NCSP National Policy and Quality Standards*: Section 5 Laboratory Standards (2016)**

Standard Number	Standard	Description
501	Qualifications for pathologists	All pathologists reporting gynaecological cytology and/or histology must be qualified.
502	Senior scientist requirements for laboratories conducting gynaecological cytology screening, histology processing and hrHPV testing	Laboratories conducting gynaecological cytology screening must employ at least one senior registered cytoscientist who has a minimum of five years full-time (or equivalent) cytology experience and who is a named lead senior cytoscientist. Laboratories conducting histology and molecular testing for hrHPV must employ at least one senior histoscientist and senior molecular pathology scientist respectively with a minimum of two years full-time (or equivalent) experience. Each discipline must also be led by a named senior scientist.
503	Continuing professional development requirements for cytoscientists, cytotechnicians, pathologists and hrHPV testing staff	As a minimum, the continuing professional development requirements must be met by all pathologists, cytoscientists, cytotechnicians and hrHPV testing staff and the laboratory must keep a record of the professional development requirements that have been met.

504	Volume of gynaecological cytology cases per laboratory per annum	Each fixed laboratory site will process a minimum of 15,000 gynaecological LBC samples per annum. A single case may include multiple cytology samples per woman at any single patient episode.
505	Number of cases to be reported per pathologist per annum	Each pathologist will report at least 500 gynaecological LBC samples per annum. A single case may include multiple cytology samples per woman at any single patient episode. Each laboratory must have at least two pathologists who are competent in gynaecological cytology to cover for periods of sickness and annual and other leave.
506	Maximum workload for screeners	The maximum workload for any cytoscreener involved in manual primary screening (or equivalent full screens) of LBC samples is 70 cases on any one working day. The maximum workload for any cytoscreener involved in location-guided field of view (FOV) review of LBC samples is 140 cases on any one working day. 3. FOV : LBC = 2 : 1. It is recommended that up to three rapid rescreens = one full primary screen = one full rescreen. Note: A full review of historical conventional cytology slides is counted as being equivalent to one LBC sample full re-screen.
507	Minimum number of cases per annum per cytoscientist/ cytotechnician	Cytoscientists and cytotechnicians must primary screen a minimum of 3000 gynaecological LBC samples per annum. In the case of senior cytoscientists and senior cytotechnicians, this may include a maximum of 1200 full re-screen cases. Cytoscientists and cytotechnicians must complete a minimum of 3000 FOV review cases per annum to maintain competency for location-guided FOV work. In an automated environment for a mixed workload of manual full screens and FOV, cytoscientists and cytotechnicians must complete a minimum of 1000 manual full-screen and 3000 FOV cases per annum. Note: This does not apply to staff who are primary screening in a laboratory that has not fully converted to automation.
508	Qualifications for screening staff reporting gynaecological cytology	All screening staff reporting gynaecological cytology must be qualified cytoscientists or cytotechnicians. For the purpose of screening, a senior cytoscientist or senior cytotechnician is defined as a health professional with three years full-time (or equivalent) (FTE) work experience post-cytology qualification.
509	Rapid rescreening results and recording outcomes	At least 98 percent but less than 100 percent of 'Negative for intraepithelial lesion or malignancy' slides are confirmed as such after rapid rescreening. Outcomes of rapid rescreening must be recorded for all cases.
510	Full rescreening	Full rescreening must be performed for gynaecological cytology in all of the following categories: • abnormal (G2 or G3) gynaecological cytology • gynaecological cytology from women with abnormal screening histories (not high grade) who have not been hrHPV tested before returning to usual (three-yearly) screening after the abnormal diagnosis. Women being followed up with annual samples for a previous high grade require annual samples with a minimum of rapid review (manual screening) or FOV (automated screening) or for squamous only lesions until they have completed appropriate hrHPV testing returning them to usual three-yearly screening. • gynaecological cytology from women with: suspicious clinical conditions, abnormal bleeding, observed cervical abnormalities or immunosuppression (optional at the discretion of the laboratory) • unsatisfactory (U) gynaecological cytology • gynaecological cytology where there has been shown to be a discrepancy between the primary screening result and the rapid re-screening result.

		Samples scanned using automated screening devices where abnormal cell changes are identified by location-guided FOV must have a minimum of one full manual screen performed by a different staff member.
511	Confirmation and reporting for abnormal results	All results confirmed abnormal (G2 or G3) after full rescreening will be sent to the pathologist for confirmation and reporting.
512	Rescreening timing	All rescreening (rapid and full) will take place before the results are confirmed and sent to the sample taker and the NCSP Register
513	Reporting gynaecological cytology and hrHPV test results	Laboratories are required to report 90 percent of final gynaecological cytology results to sample takers within seven working days of receiving a specimen. Laboratories are required to report 98 percent of final gynaecological cytology results and hrHPV test results to sample takers within 15 working days of receiving a specimen. Results for cytology and conjunct hrHPV tests must be reported together to the sample taker.
514	Histopathologist access to cervical cytology results	The histopathologist must have the full and current NCSP Register screening event history available at the time of reporting the gynaecological biopsy.
515	Examining and reporting histology slides	All histology slides must be examined and reported by a histopathologist.
516	Reporting histology results	Laboratories are required to report 90 percent of final histology results to referring colposcopists within 10 working days of receiving a specimen. Laboratories are required to report 98 percent of final histology results to referring colposcopists within 15 working days of receiving a specimen.
517	Cultural sensitivity and appropriateness	Requests regarding culturally appropriate methods of handling and disposal of human tissue will be treated sensitively and in accordance with local protocols.
518	Sending cytology results to the NCSP Register	Ninety-eight percent of all cytology results and hrHPV test results must be forwarded to the NCSP Register, in the approved Bethesda coding and format, within 16 working days of receipt of a specimen.
519	Sending histology results to the NCSP Register	Ninety percent of the histology results must be forwarded electronically to the NCSP Register, in approved format and NCSP SNOMED coding, within 15 working days of receipt of a specimen. Ninety-eight percent of the histology results must be forwarded electronically to the NCSP Register, in approved format and NCSP SNOMED coding, within 20 working days of receipt of a specimen. Histology results must include all appropriate topography, morphology and procedure SNOMED codes.
520	Sending results to the New Zealand Cancer Registry	The laboratory that has analysed the sample must forward all cytology with an interpretation of cancer (or suspicious of) and histology results with a diagnosis of invasive or in situ cancers to the NZCR (at the Ministry of Health).
521	Correlation of histology and cytology slides	All histology results must be correlated with any cytology slides that have management implications and were taken in the previous six months, and the results must be recorded for audit and statistical purposes.
522	Reviewing cases with a high-grade histology diagnosis	All cases with a high-grade/invasive diagnosis on histology must have a review of any cytology slides that have been reported as negative, benign/reactive or unsatisfactory in the previous 42 months.

5.2.3 NCSP Monitoring Reports

Further auditing of laboratory practice by the NCSP occurs through the NCSP Advisory Group Monitoring reports. These six-monthly and annual reports are compiled from data held on the NCSP-Register. Laboratory cytology, histology and hrHPV reporting rates are documented and compared. Six target indicators are monitored for cytology reporting. If a laboratory falls

outside of the targets, an investigation by the NCSP will occur. Laboratories are also expected to use the monitoring reports as part of their own quality assurance processes.

The cytology indicators and targets are:

Cytology reporting rates: The number of cytology sample reports in the following categories as a % of all satisfactory samples: Negative for intraepithelial lesion or malignancy = not more than 96% reported as negative HSIL = not less than 0.5% reported as HSIL Total abnormalities = not more than 10% reported as total abnormalities
Unsatisfactory sample rate: Number of cytology sample reports reported as unsatisfactory: Between 0.1% and 3.0%
Accuracy of cytology reports predicting HSIL/SCC on histology: PPV for HSIL/SCC = between 65% – 85%
Accuracy of negative cytology reports: For women with a histological diagnosis of a high-grade squamous or glandular lesion, the proportion of cytology slides originally reported within the preceding 42 months as negative, benign/reactive or unsatisfactory which are reviewed as: HS1, HS2, SC, AIS, AC1-5 = not more than 10% combined ASC-H, HS1, HS2, SC, AG4-5, AIS, AC1-5 = aim for less than 15%, but not more than 20% combined.
Reporting to sample takers 90% of final reports to sample takers within seven working days of sample receipt 98% of final reports to sample takers within 15 working days of receipt (includes those samples requiring hrHPV testing)

5.2.4 Questions

1. Discuss the value of having NCSP target indicators for laboratory performance. How does this benefit laboratories and individual screeners?

2. Apart from the laboratory section, what other aspects of the screening pathway are covered in other sections of the *NCSP National Policy and Quality Standards*?

3. Investigate and discuss the value of requiring a full rescreen under Standard 510 (Full rescreening policy) for each of the stated scenarios.

4. Read a recent NCSP Monitoring Report and provide a one page summary of the salient points. Remember to record which report this synopsis pertains to.

References

- Ministry of Health. *Operational Policy and Quality Standards 2014. Chapter 5: Providing a Laboratory Service.* Available at: https://www.nsu.govt.nz/health-professionals/national-cervical-screening-programme/policies-and-standards

SECTION 6.0
THE HISTORY OF CYTOPATHOLOGY

6.1 CORE TOPIC: THE HISTORY OF CYTOPATHOLOGY

Author: Dr Harold Neal

"The place, Oxford; the time, around 1845-50. Henry Acland lectured on medicine in a room furnished with `little railroads on which ran microscopes charged with illustrations of the lecture, alternately with trays of coffee'. Dr Ogle, applying his eye to the microscope, screwed a quarter-inch right through the object; and Dr Kidd, after examining some delicate morphological preparation, made answer first that he did not believe in it, and secondly that if it were true he did not think God meant us to know it'".

This apt quote by Tuckwell in 1907 demonstrates the impact of the microscopic examination of cells and disease in early days (Ref: History of Cytodiagnosis. Spriggs A. *Journal of Clinical Pathology* (1977) 30: 1091-1102).

When tracing the history of cytodiagnosis it is impossible to separate cytology, histology and pathology because earlier studies focused on all three.

Anatomical structures along with some causes of disease were described by Kircher (1602-1680), Van Leeuwenhoek (1632-1723), and Marcello Malpighi (1628-1694) as the microscope was developed and used. Malpighi was really the founder of histology and is responsible for the identification of some structures known today, such as:

2 Stratum Malpighi of the skin
3 Malpighian corpuscles of the spleen
4 Malpighian tuft of the kidney

The father of medical microscopy was Johannes Müller who was a physiologist and greatly influenced Virchow. The first volume of *Virchow's Archive* (1847) contained an illustrated article on the nature of cancer. By the 1870s it was realised that the tissues were not fabrics made of woven fibres, but rather colonies of cells living in a fibrous network and that cells originate from pre-existing cells.

Most of the preparations of the early 19th century were unstructured. In 1856 William Perkin discovered aniline dyes, and in 1858 Joseph Gerlach introduced staining as a routine laboratory procedure. The use of stains and dyes were the topic of many articles written by Paul Erlich. Using stained air-dried films, Erlich described the cells of serous effusions and in 1882, the features of adenocarcinoma in three pleural effusions (two ovarian and one breast carcinoma).

The same era led to illustrations of malignant cells from solid tumours. Lambert produced an atlas from unstained preparations showing cancer cells from uterine carcinoma and gastric carcinoma (1857-61). In 1851 he had put forward the idea of tumour puncture although did not develop the method. He also measured nuclei and nucleoli, and noted increased nuclear-cytoplasmic ratios in cancer cells, all features used today in cytodiagnosis.

However with the introduction of paraffin embedding in about 1880, the study of solid tissues led to the general abandonment of cytological methods and in conjunction with the inadequacies in the cytological methods used, cytology came to be regarded with much

scepticism. In fact Roodhouse Goyne in 1919 said in relation to malignant cells: "it is practically impossible to identify these cells in film preparations".

The word cell comes from the Latin “cella” (a small room) and was used by Hooke (1665) to describe the microscopic compartments of cork. In 1831 Brown described the cell nucleus. Without a doubt, the greatest contributor to the practice of cytodiagnosis today was Dr George Nicholaus Papanicolaou (1883-1962). Papanicolaou qualified in medicine in Athens in 1904 and specialised in the experimental study of reproduction. After emigrating to New York in 1913, he continued to study the oestrus cycle in animals and the menstrual cycle in women. In 1917 he introduced the vaginal smear for his studies and a paper: *The sexual cycle in the human female as revealed by vaginal smear* was published in 1933.

From his studies, Papanicolaou presented a paper in 1928 entitled *New Cancer Diagnosis* regarding malignant cells seen in the vaginal smears. This wasn't the first mention of vaginal cytology. Donn (1844) and Pouchet (1847) described the cytology of vaginal secretion, with Pouchet describing the findings at different stages of the human menstrual cycle. Richardson in 1871 used forceps to remove a small amount of secretion from the uterine os in cases of suspected cancer of the womb. Friedlaender (1886) using the same method, warned against this technique for diagnosing carcinoma by cytology alone, and recommended the histological examination of a little piece of excised tissue which became known as a biopsy.

Needless to say Papanicolaou's name was given to the "Pap" smear, which indicates the credit deserved by his work. There was however another worker Aurel Babes, a pathologist from Bucharest, who at about the same time as Papanicolaou's early work (1928), presented a new method for the diagnosis of carcinoma of the cervix. He used a platinum loop to transfer material from the affected area, and stained his air-dried smears with Giemsa. Regrettably, Babes' contribution was virtually forgotten although J. Ayre referred to him in the atlas *Cancer Cytology of the Uterus* (1952).

It still wasn't until the late 1940s that Papanicolaou's work became recognised, accepted and used to promote cytology in the diagnosis of cancer. The influencing publications that were the drivers for a new interest in cytology were *Diagnosis of Uterine Cancer by the Vaginal Smear* (Papanicolaou and Traut 1943) and *The Epithelia of Women’s Reproductive Organs* (Papanicolaou, Traut and Marchette 1948). Papanicolaou's famous *Atlas of Exfoliative Cytology* was published in 1954.

It was now being recognised that precancerous lesions could be diagnosed using Pap smear cytology. In 1947 Papanicolaou ran a course in cytological diagnosis and soon after, many investigations into other applications of cytology were being studied.

Population screening first developed using the vaginal smear and then the cervical scrape with the Ayres Spatula (1947, 1952).

An excellent historical appraisal of cytopathology is a paper by James R Wright (Diagn. Cytopathol. 2015;43:257–263). Wright’s abstract nicely summarises why cytopathology took so long to thrive:

Lionel S. Beale of London made some of the earliest contributions to Cytopathology in the 1850–1860s. Cytopathology then experienced a 60+ year hiatus during which few advances were made. In 1927, Londoner Leonard S. Dudgeon published his wet film method for rapid

intraoperative diagnosis and in 1928 Greek-American George Papanicolaou and Romanian Aurel A. Babeș, independently discovered that cervical cancer can be diagnosed using vaginal smears; these were huge advancements. Yet, there was another hiatus where little progress was made which lasted until the publications of Papanicolaou and Trout in the early 1940s. After that, the field of exfoliative cytopathology immediately flourished. None of the standard histories of Cytopathology explain these two gaps. Primary and secondary historical sources were examined to explain this pattern. The author concludes that the first hiatus is explained by the 19th Century pathology establishment's strong opposition to the doctrine of the uniqueness of cancer cells that was being pushed by only a few maverick pathologists; in fact, for many mainstream pathologists, cancer was rigidly defined by cell behavior (metastases and invasion) and not cell morphology well into the 20th Century. Biopsy-based diagnosis faced similar opposition but advanced more rapidly as it was possible to examine increased numbers of cells in a pattern that partially maintained their normal adjacencies and architecture. The second hiatus is explained by economic pressures supporting intraoperative frozen section diagnoses and, in the instance of vaginal smears, the embryonic state of the public campaign supporting the importance of early cancer diagnosis.

The New Zealand National Cervical Screening Programme (NCSP) was introduced in 1990, following **The Cartwright Inquiry 1988** (lead by Judge Dame Silvia Cartwright) into a clinical study which had started in 1966 at the Auckland National Women's Hospital. The study involved following women with cervical abnormalities without definitively treating them to see if the lesions resolved. Another outcome of the inquiry was legislation in 1994 which established the Office of the Health and Disability Commissioner. The first Commissioner developed a Code of Rights for Health and Disability Consumers and established a national advocacy service which is independent of health care providers.

A further inquiry, **The Gisborne Inquiry 2001** was charged with finding the reasons for a high under-reporting rate of abnormal smears in the Tairawhiti region, and investigating whether there was evidence of wider systemic problems in the NCSP. One of the main areas of focus for the Gisborne Inquiry was the level of quality assurance around laboratory cervical screening services, and in particular during the early years of the NCSP. The inquiry commenced in 1999 with the report issued in 2001. The NCSP Interim Operational Policy and Quality Standards (OPQS) were developed as the Inquiry was proceeding and were introduced in October 2000 along with other initiatives for improving the ongoing development and quality of the NCSP. Improving ongoing education for cytoscreeners and cytopathologists was a further recommendation of the Gisborne Inquiry panel. The publication of the VRPCC in 2006 was a workforce quality improvement initiative for cytoscientists, cytotechnicians and pathology registrars. This revised 2nd edition became necessary because of technological advancements during 2006-2015 in New Zealand cytology laboratories.

The NCSP has matured into a high-quality screening programme delivering reductions in cervical cancer incidence and mortality equal to the best international screening programmes. There are still important challenges associated with lower coverage rates for Maori, Pacific and Asian populations leading to disparities in cancer rates compared to other groups.

The programme is also addressing the benefits of rapidly advancing molecular-based technology by progressing a change to HPV primary screening which is likely to be implemented in 2018. A number of international programmes are also following the same pathway. These changes will address the positive impacts of HPV vaccination as the primary intervention strategy in reducing the burden of cervical cancer, together with screening with a

more sensitive test (hrHPV testing) for both vaccinated and unvaccinated women. Cytology will continue as a second tier interpretive test for women who test positive with hrHPV testing, to maintain high specificity for the diagnosis of HSIL. The NCSP is well placed for a move to HPV primary screening having already converted to 100% LBC and introduced hrHPV testing in selected clinical settings.

The history of cervical cytology continues to evolve and is at a crossroads in its role in cervical screening with an exciting future transitioning from a primary screening test to a targeted interpretive test for the better prevention of cervical cancer.

References

- Ayre JE. 1949. The Vaginal Smear: Pre-cancer Cell Studies using a Modified Technique. *American Journal of Obstetrics and Gynecology* 53: 609-617.
- Spriggs A. 1977. History of Cytodiagnosis. *Journal of Clinical Pathology*. 30: 1091-1102.

SECTION 7.0
CONTINUING EDUCATION

7.1 CORE TOPIC: ADDITIONAL EDUCATIONAL ACTIVITIES

The list below from the VRPCC logbook details many of the different educational activities that are available for continuing education. Under the Health Practitioners Competency Assurance Act, registered practitioners are obliged to maintain competency on an ongoing basis and take part in a continuing professional development programme (CPD). There are three CPD programmes available to medical laboratory scientists that are approved by the Medical Sciences Council of New Zealand (MSCNZ).

1. The New Zealand Institute of Medical Laboratory Science (NZIMLS) Continuing Professional Development (CPD) programme.
2. The New Zealand Hospital Scientific Officers Association (NZHSOA) Continuing Education Programme for Scientific Officers and Recertification Programme for Medical Laboratory Scientists.
3. The Australian Institute of Medical Scientists (AIMS) Australian Professional Acknowledgement Continuing Education (APACE) programme

More information for medical laboratory scientists is available on the MSCNZ website at http://www.mscouncil.org.nz/for-practitioners/recertification/cpd-programmes-and-compliance-audits/medical-laboratory-scientist/

Once enrolled in a CPD programme medical laboratory scientists are required to regularly submit details of ongoing educational activities and gain sufficient points annually before renewal of the Medical Sciences Council of New Zealand (MSCNZ) Annual Practicing Certificate (APC) will be approved.

A medical laboratory technician applying for an APC must have undertaken at least eight hours of approved professional development activity within the previous twelve-month period.

Further information for medical laboratory technicians is available at http://www.mscouncil.org.nz/for-practitioners/recertification/cpd-programmes-and-compliance-audits/medical-laboratory-technician/

CPD programmes are audited regularly and enrolled practitioners are also randomly audited each year. As part of the audit you may be asked to submit *supporting evidence* to confirm the CPD points you have claimed. For this reason, as well as listing the activities on the logbook sheets, you should also keep evidence of your participation, in case you are asked to verify your participation. Examples of evidence are:

4. Attendance at a conference: Notes you have taken and a copy of the conference booklet, abstracts for the lectures/workshops you attended, any handouts given, certificate of attendance.
8. Reading a journal article: Notes from your reading attached to the article.
9. Participation in external QA programmes: Details of the date and the cases with your outcome. The laboratory will have a record of the laboratory results.

As your career develops you should aim to build a portfolio of all important activities. Such documents may be reviewed when IANZ audits your laboratory. Ask your supervisor and other staff to show you their portfolios and the formats they use.

Activities can be wide ranging and may include:

10. Further text book/journal reading
11. In-house teaching sessions including formal microscope review sessions
12. Presentations at in-house or professional meetings
13. Attendance at conferences
14. Journal clubs
15. Attendance at workshops and training courses
16. Clinico-pathology meeting attendances
17. Colposcopy meeting attendances
18. Video reviews
19. Web based self-assessment programmes
20. In-house QA meetings and cytology review sessions
21. External QA programmes eg RCPA QAP
22. Individual external QA programmes (EQA)

Maintaining a current Curriculum Vitae is always a good idea too as this is needed for applications for employment, for scholarships and sometimes for research projects and for professional committees that you may wish to be part of. Some of the activities you record in your continuing education record will be suitable to include in your CV and if you maintain comprehensive training records, these are easy to select for inclusion in your CV.