"Ki-67 EXPRESSION IN ENDOMETRIAL HYPERPLASIA AND ENDOMETRIAL CARCINOMA – AN OBSERVATIONAL STUDY"

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Dr. Ranjit Kangle MD,
Professor and HOD
Department of Pathology,
J. N. Medical College,
Belagavi, Karnataka

Date:
Place: Belagavi.

Dr.(Mrs) N. S. Mahantashetti MD (Paed).
Principal
J. N. Medical College,
Belagavi, Karnataka.

Date:
Place: Belagavi.

PLAGIARISM ACCEPTANCE LETTER

JAWAHARLAL NEHRU MEDICAL COLLEGE

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Accredited 'A' Grade by NAAC (2nd Cycle) Placed in Category 'A' by MHRD (GoI)

Nehru Nagar, Belagavi- 590 010, Karnataka, INDIA

0831 - 2471350 0831 - 2470759 www.jnmc.edu principal@jnmc.edu

Ref No: MDC/PG/ Date: 16-11-2021

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KAHER BELAGAVI J. N. MEDICAL COLLEGE

Dr. (Mrs.) N.S. Mahantashetti.
Chairperson-Antiplagiarism Committee &
Principal,
J. N. Medical College, Belagavi.

To,
Reg. No. BN0119012.
Postgraduate Student,
2019-20 Batch,
Department of Pathology,
J. N. Medical College, Belagavi

LIST OF ABBREVIATIONS USED

AH	Atypical Endometrial Hyperplasia
AUB	Abnormal Uterine Bleeding
BMI	Body Mass Index
DNA	Deoxyribonucleic Acid
EC	Endometrial Carcinoma
EH	Endometrial Hyperplasia Without Atypia
EHA	Endometrial Hyperplasia with Atypia
EIC	Endometrial Intraepithelial Carcinoma
EIN	Endometrial Intraepithelial Neoplasia
FIGO	International Federation of Gynaecology and Obstetrics
H&E	Haematoxylin and Eosin
HG-ECC	High Grade Endometrioid Endometrial Carcinoma.
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HRT	Hormone Replacement Therapy
IHC	Immunohistochemistry
Ki-67 LI	Ki-67 Labeling Index
LG-ECC	Low Grade Endometrioid Endometrial Carcinoma
LNM	Lymph Node Metastatsis
LOH	Loss of Heterogeneity
LVI	Lymphovascular Invasion
MIB-1	Mindbomb E3 Ubiquitin Protein Ligase-1
MRD	Medical Records Department
NE	Normal Endometrium

NEEC	Nonendometrioid Carcinomas
PI	Proliferative Index
PNI	Perineural Invasion
POD	Postovulatory Day
SEIC	Serous Endometrial Intraepithelial Carcinoma
WHO	World Health Organisation

ABSTRACT

"Ki-67 EXPRESSION IN ENDOMETRIAL HYPERPLASIA AND ENDOMETRIAL CARCINOMA – AN OBSERVATIONAL STUDY"

BACKGROUND: Endometrial carcinoma is the most common malignancy of female genital tract in developed countries. In India, although the incidence of endometrial carcinoma is low when compared to developed countries, there has been a steady increase, making it the fourth leading cancer and seventh leading cause for cancer deaths in women. Endometrial carcinoma is thought to develop from a continuum of premalignant lesions ranging from endometrial hyperplasia without atypia to hyperplasia with atypia to finally full blown well differentiated adenocarcinoma. Ki-67 is a proliferative immunohistochemical marker and shows expression only during active phases of cell cycle. Its expression progressively increases from non-atypical hyperplasia, atypical hyperplasia to carcinoma and positively corresponds to histological type, grade and stage.

OBJECTIVES: To study Ki-67 expression in Endometrial Hyperplasia and Endometrial Carcinoma and to differentiate these based on Ki-67 expression.

METHODOLOGY: Forty paraffin embedded blocks were retrieved from the Department of pathology, J N Medical College, Belagavi in that 15 cases were of Endometrial hyperplasia without atypia, 10 were Endometrial hyperplasia without atypia and 15 were Endometrial carcinoma; and were stained with Hematoxylin and Eosin and immunohisochemical staining with Ki-67. Ki-67 expression was assessed

as percentage of positively stained nuclei within three high-power fields (x40 magnification) counting at least 1000 nuclei. Its Scoring and Grading was done and compared with various clinicopathological parameters.

RESULTS: Mean Ki-67 Labelling Index and Grade was not significant when compared among Endometrial hyperplasia without atypia, Endometrial hyperplasia with atypia and Endometrial carcinoma cases, but Ki-67 expression positively corresponded to higher histological grade, stage and depth of myometrial invasion in Endometrial carcinoma.

CONCLUSION: Ki-67 may not be helpful in distinguishing endometrial hyperplasia without atypia from atypical hyperplasia or endometrial hyperplasia with atypia from carcinoma, but Ki-67 may serve as important marker in prognosis of Endometrial carcinoma.

KEY WORDS: Endometrial Hyperplasia, Endometrial Carcinoma, Ki-67, Immunohistochemistry.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF GRAPHS

LIST OF PICTOMICROGRAPHS

INTRODUCTION

Endometrial hyperplasia is a non-physiological and non-invasive proliferation of endometrium.[1] Endometrial hyperplasia for many years has been a diagnostic problem for pathologists.[2,3] This is because the condition comprises a spectrum of histological changes from simple exaggeration of the normal proliferated state to changes that are difficult to distinguish from carcinoma.[4,5]

The high degree of morphological variability of endometrial proliferations even within the same sample is also responsible for the difficulty in defining consistent and clinically meaningful diagnostic criteria. A further complication is fragmentation and scantiness of many biopsies. Nevertheless, histological interpretation remains the most accessible, subjective method of evaluating endometrial hyperplasia.[6]

Over the past decades, many studies on different organs have made it clear that in such continuous spectral lesions normal qualitative subjective microscopic evaluations are not completely reproducible, even among experts, and that the resulting differences may be associated with important prognostic variations.[7,8]

In the past, the existence of several classification systems and the use of descriptive diagnostic terms for sub classifying endometrial hyperplasia, have shown low inter-observer and intra-observer diagnostic reproducibility.[9,10,11]

Moreover, previous studies have shown that only 10–20% of endometrial hyperplasia progress to carcinoma when left untreated.[3,4] Diagnosis of histological type

and grade of endometrial cancer is based on preoperative endometrial biopsy, determining the surgical management. However, previous studies have underlined discrepancies between preoperative histological grade assessed by biopsy and final histology. Because the treatment of choice for patients with hyperplasia has often been hysterectomy, it is likely that overtreatment of many women with endometrial hyperplasia has occurred. Conversely, other patients with endometrial hyperplasia might have received adjuvant treatment or no treatment at all as a result of under diagnosis, even though they actually should have received more active treatment.[6,13,14,15]

Thus, routine histology needs to be supported by additional tools like immunohistochemistry to improve diagnosis of endometrial hyperplasia and carcinoma.[6,13,14]

Ki-67 antigen is a non-histone nuclear protein and expressed during the active G1, S, G2 and M phases of the cell cycle. Its expression is a marker of cellular proliferation and is readily detected by immunohistochemistry. An increase of Ki-67 expression shows an increased mitotic activity and cell proliferation. Ki-67 expression is normally increased at endometrium level during the proliferative phase of the menstrual cycle.[16] Ki-67 score positively correlate with grade, stage and depth of myometrial invasion.[17]

The aim of the present study was to analyze the expression of the proliferative marker, Ki-67 in endometrial hyperplasia and endometrial carcinoma.

OBJECTIVES

1. **Primary objective:** To study Ki-67 expression in Endometrial Hyperplasia and Endometrial Carcinoma.
2. **Secondary objective:** To differentiate Endometrial Hyperplasia and Endometrial Carcinoma based on Ki-67 expression.

REVIEW OF LITERATURE

EMBRYOLOGY

The uterus, the fallopian tube and the ovarian surface epithelium are part of the extended müllerian system. A pair of müllerian (paramesonephric) ducts begin their development around the sixth week of fetal life. These structures are formed by an invagination of the celomic epithelium. As the müllerian ducts grow caudally, they approach the midline where the distal portions fuse. Shortly after this fusion, the apposed medial duct walls disappear, bringing the two lumina into continuity to form a single cavity.[18]

The endometrium and myometrium are of mesodermal origin and both structures are formed secondary to fusion of the müllerian ducts. Until the 20th week of gestation, the endometrium is composed of a single layer of columnar epithelium supported by a thick layer of fibroblastic stroma. By the 20th gestational week, the surface epithelium invaginated into the underlying stroma, forming glandular structures that extends towards the underlying myometrium.[19]

ANATOMY

The adult nulliparous uterus is a hollow, pear shaped muscular organ weighing 40 to 80 g and measuring approximately 7 to 8 cm along its long axis, 5.0 cm at its broadest extent (cornu to cornu), and 2.5 cm in anteroposterior dimension. These measurements vary considerably as a function of age, phase of the menstrual cycle,

and parity.[18] Its largest part, the body into which fallopian tubes from both the sides enters and the curved, superior area between the tubes is called the fundus. The uterus narrows in the isthmus and ends in a lower cylindrical structure, the cervix.[20]

The uterine wall has three major layers: An outer connective tissue layer, the perimetrium; A thick tunic of highly vascularized smooth muscle, the myometrium and A mucosal lining, the endometrium, lined by simple columnar epithelium.[20]

The major arterial supply of the uterus derives from the right and left uterine arteries, which arise from the corresponding hypogastric (internal iliac) arteries. The uterine artery divides into ascending and descending branches laterally at the level of the uterine isthmus. Both the ascending and descending uterine arteries give rise to a complex network of circumferentially arranged subserosal arteries: The arcuate arteries. These in turn give rise to a series of radial arteries that penetrate the myometrium. Each of these radial vessels branches, in the inner third of the myometrium, into straight arteries (supplying the basalis) and spiral arteries that become the spiral arteries of the endometrium.[18]

The uterine lymph vessels drain to a rich network of lymph nodes, the main groups being parametrial and paracervical; internal (hypogastric), external, and common iliac; periaortic; and inguinal.[10]

HISTOLOGY

The endometrium is made up of glands and stroma (lamina propria). The stroma of the endometrium contains primarily nonbundled type III collagen fibers

with abundant fibroblasts and ground substance. The surface epithelial lining is simple columnar, and it has both ciliated and secretory cells. The numerous tubular uterine glands that penetrate the full thickness of the endometrium are also lined by the same.[20]

The endometrium has two concentric zones: a deeply seated basal layer and a superficial functional layer. The basal layer is adjacent to the myometrium and has a more highly cellular lamina propria and contains the deep basal ends of the uterine glands. The superficial layer is subdivided into stratum compactum (towards the cavity) and stratum spongiosum (towards the stratum basalis). The functional layer has a spongier lamina propria, richer in ground substance, and includes most of the length of the glands, as well as the surface epithelium. The functional layer undergoes profound changes during the menstrual cycles, but the basal layer remains relatively unchanged.[20]

PHYSIOLOGY

The first day of the menstrual cycle when the bleeding starts is taken as day 1 of the cycle. Proliferative phase is about 10- 12 days and is stimulated by estradiol. Then comes ovulation followed by Secretory phase which last for 14+/- 2days stimulated by both estradiol and progesterone. Thereafter is the period of menstruation that lasts for 4+/- 2days. Then a new cycle starts.[21]

PATTERN OF ENDOMETRIUM[22,23,24]

a) PROLIFERATIVE PHASE:

The stratum functionalis of the normally cycling proliferative endometrium is characterized by nonbranching, nonbudding, similarly shaped glands evenly distributed throughout a stroma composed of monomorphous, undifferentiated stromal cells with scant cytoplasm and indistinct cell margins supplied by a uniformly developed, arborizing vasculature with thin walls. The glands-to-stroma ratio is typically 1:1.

Early in proliferation, the glands are tubular and of narrow caliber; as proliferation continues, they become increasingly coiled, and their caliber increases. Normal proliferative endometria are further marked by pseudo-stratified, mitotically active, elongated epithelial cells with dense chromatin and mitotically active stromal cells. Most of the vessels are inconspicuous and resemble capillaries, particularly near the endometrial surface.

b) INTERVAL PHASE:

An interval endometrium is a late proliferative endometrium in which the glands are coiled and in which fewer than half of the epithelial cells feature spotty, nonuniform subnuclear vacuolation.

c) EARLY SECRETORY (Postovulatory Day(POD) 2-5):

The endometrium during this time features coiled glands composed of cells resembling those found in the proliferative phase, but more than half of the glandular cells contain relatively large cytoplasmic vacuoles. These

vacuoles serve as the marker of early secretory endometria. The glands are set within a predeciduated stroma. The precise date assigned to such patterns depends on the location of the cytoplasmic vacuole (subnuclear or supranuclear) and the number of mitotic figures present.

d) MID SECRETORY PERIOD (Postovulatory Day 6-8):

The midsecretory endometrium is characterized by fully coiled secretory glands lined by cells with round, often vesicular nuclei. The cytoplasm of such cells does not contain large cytoplasmic vacuoles, but luminal secretions may well be present. The stroma has not begun to undergo predeciduation. The absence of extensive vacuolation and predecidua is the most useful marker of midsecretory endometria.

e) LATE SECRETORY PERIOD (Postovulatory Day 9-14):

At the beginning of this phase in the cycle, the spiral arteries are prominent, in part as a result of the thickness of their walls but also largely because of the cuffs of predeciduated stromal cells around them. Predeciduation begins initially around spiral arteries (POD10) and then extends to form islands in the superficial reaches of the endometrial stroma. It marks the beginning of the last third of the secretory phase. At the end of the late secretory phase, these decidual islands become confluent and then, as menstruation becomes imminent, are dissected by interstitial hemorrhage.

Associated with the progressive predeciduation of the endometrial stroma is the increase in the number of stromal granulocytes. These cells have

bean-shaped, dense nuclei; inconspicuous cytoplasm; and cytoplasmic granules visible with special stains and occasionally on Haematoxylin and eosin preparations. These enigmatic cells, once thought to be derived from the endometrial stroma, have been shown by immunoperoxidase techniques to be endometrial granular lymphocytes (natural killer cells).

f) MENSTRUATION:

Menstrual endometrium features disintegrating fragments of fully developed secretory endometrium. The glands are dilated and lined by flattened cells, often with frayed borders (secretory exhaustion), and the stroma is fully predeciduated. Karyorrhectic fragments are present in the subnuclear area of some glands (apoptosis), the cell margins are frayed, the epithelial nuclei are pyknotic and fibrin thrombi are present in vessels and sometimes within the stroma. As menstruation proceeds, the glands breakup into strips, the stroma crumbles, and the epithelial cells lose cohesion.

Neutrophils are seen in large numbers in areas of tissue degradation and are very rare during the other days of the cycle. They should be distinguished from stromal granulocytes, which do not stain for chloroacetate esterase.[10]

Regeneration:

Regeneration of the denuded epithelium is already in progress before the menstrual bleeding has stopped and is completed 48 hours after the end of menstruation. Immediately after menstrual shedding ceases and before proliferation

begins, regenerative phase sets in, lasting 1 to 2 days during which the endometrium appears to be limited to stratum basalis and exhibits re-epithelialization by extension of residual glandular epithelium over the denuded surface. Endometrial stromal cells, similar to myofibroblasts, participate in wound healing.[22,23]

PRECURSOR LESIONS OF ENDOMETRIAL CARCINOMA

Endometrial neoplasms show a spectrum of morphological alterations ranging from endometrial hyperplasia to different types of endometrial carcinomas. Hyperplasia and carcinoma are two extreme points of this spectrum.

ENDOMETRIAL HYPERPLASIA

Endometrial hyperplasia, an important cause of abnormal bleeding, is defined as an increased proliferation of the endometrial glands relative to the stroma, resulting in an increased gland-to-stroma ratio when compared with normal proliferative endometrium. Endometrial hyperplasia requires special attention because of its relationship with endometrial carcinoma. Clinicopathologic and epidemiologic studies have supported the malignant potential of endometrial hyperplasia and the concept of a continuum of proliferative glandular lesions, culminating in some cases in carcinoma. Molecular studies have confirmed this relationship, since endometrial hyperplasia and carcinoma share specific molecular genetic alterations.[9]

There have been many classifications of endometrial hyperplasia over the years. The classification proposed by Kurman and Norris, divides hyperplasia into

simple and complex on the basis of architecture and subdivides each into typical and atypical on the basis of cytology. [25,26]

CLASSIFICATION OF ENDOMETRIAL HYPERPLASIA BASED ON ARCHITECTURE AND CYTOLOGICAL FEATURES[10,23,27,28]

TYPE OF HYPERPLASIA	**PROGRESSION TO CANCER (%)**
1. Hyperplasia without cytological atypia (non-atypical hyperplasia)	
a) Simple	1
b) Complex	3
2. Hyperplasia with cytological atypia (atypical hyperplasia)	
a) Simple	8
b) Complex	29

Simple hyperplasia without atypia, also known as cystic or mild hyperplasia, is characterized by glands of various sizes and irregular shapes with cystic dilatation. There is a mild increase in the gland-to-stroma ratio. The epithelial growth pattern and cytology are similar to those of proliferative endometrium, although mitoses are not as prominent.[28]

Complex hyperplasia without atypia shows an increase in the number and size of endometrial glands, marked gland crowding and branching of glands. As a result, the

glands may be crowded back-to-back with little intervening stroma and abundant mitotic figures. However, the glands remain distinct and non-confluent and the epithelial cells remain cytologically normal.[28]

Simple hyperplasia with atypia is uncommon. Architecturally, it has the appearance of simple hyperplasia, but there is cytological atypia within the glandular epithelial cells as defined by loss of polarity, vesicular nuclei and prominent nucleoli. Morphologically, the cells become rounded and lose the normal perpendicular orientation to the basement membrane. In addition, the nuclei have an open chromatin pattern and conspicuous nucleoli.[28]

Complex hyperplasia with atypia has considerable morphologic overlap with well-differentiated endometrioid adenocarcinoma and an accurate distinction between complex hyperplasia with atypia and cancer may not be possible without hysterectomy.[28]

The past subdivision of endometrial hyperplasia into simple and complex hyperplasia, which was based on the degree of glandular architectural complexity, is no longer done as it is not a clinically relevant.

The classification one in current use is given by the World Health Organization (WHO) in 2014:[10,22]

1. Non-atypical endometrial hyperplasia (benign hyperplasia)
2. Atypical endometrial hyperplasia or Endometrial Intraepithelial Neoplasia (EIN)/well differentiated carcinoma

ENDOMETRIAL HYPERPLASIA WITHOUT ATYPIA(EH) occurs as a result of unopposed estrogenic stimulation of the endometrium and is on a continuum with disordered proliferative endometrium. There is glandular branching, irregularity, and crowding. The diagnostic threshold for diagnosis of endometrial hyperplasia without atypia, rather than disordered proliferative endometrium, is highly subjective, but at the point where glands predominate over stroma, a diagnosis of endometrial hyperplasia without atypia is appropriate. [10]

The risk of progression to carcinoma with endometrial hyperplasia without atypia is low (1%–3%). The chief differential diagnosis is with atypical hyperplasia, and this is based on the assessment of cytologic atypia.[3]

ATYPICAL HYPERPLASIA(EHA) is the precursor lesion of low-grade endometrioid adenocarcinoma. It shows glandular crowding and irregularity in addition to cytologic atypia. The nuclear atypia is characterized by enlargement, rounding, pleomorphism, and presence of nucleoli.[11]

The cytological atypia is more likely to progress into carcinoma than the degree of architectural abnormality in terms of complexity and crowding of glands. For practical use, the hyperplasia with or without atypia is considered as noninvasive proliferative lesion of the endometrium.[11,29]

Patients present with abnormal bleeding. The hallmark of hyperplasia is increase in glandular tissue than stroma with concomitant changes in architecture and cytology. This occurs due to excessive unopposed stimulation by estrogen and it

applies mostly for simple hyperplasia. Administration of unopposed estrogen in postmenopausal HRT (hormone replacement therapy) causes development of hyperplasia.[30]

Anovulatory cycles (menopause and post menopause) leads to hyperplasia. But anovulation in menarchal young women and who are bleeding do not develop hyperplasia of endometrium. Polycystic ovarian disease (Stein-Leventhal) like conditions acquire hyperplasia in reproductive period. Development of hyperplasia is dependent upon estrogenic stimulation. A marked withdrawal effect of estrogen causes breakdown of the tissue.[30,31]

Following menopause estrogen continues to get released from the persistent follicles or by peripheral conversion of andro-steinedione in a fluctuating and in inconstant way. The endometrium continues to increase in size. The inconstant secretion of estrogen is not adequate to support such a volume thereby causing breakdown of the tissue. Besides breakdown, the superficial dilated vessels also contribute in the bleeding process.[30]

ENDOMETRIAL INTRAEPITHELIAL NEOPLASM[30,32,33]

Endometrial intraepithelial neoplasia is a clonal proliferation of architecturally and cytologically altered premalignant endometrial glands. Malignant transformation to endometrioid (Type-I) endometrial adenocarcinoma is at a rate of 26 to 37%. The following factors are involved in the development of EIN such as:

• Genetic mutations such as inactivation of PTEN a tumor suppressor gene is the most common genetic alteration found to occur in cancer preceded by EIN.

• PAX 2 inactivation and microsatellite instability.

• Activating mutations of the KRAS2 cellular oncogene.

• Other factors are unopposed estrogen, obesity, tamoxifen therapy etc.

EIN DIAGNOSTIC CRITERIA

1. **Architecture-** The gland area exceeds the stromal area. EIN lesions consist of aggregates of tubular glands or slightly branching glands, in which the surface area of the glands exceeds that of the stroma. The crowded appearance of the glands is readily visualised under low magnificantion. These EIN glands have an epicentre with maximum glands concentrated in the periphery. The stroma in between the glands is hormone dependant which ranges from stroma of functionalis to more fibrous non cycling stroma of the basalis or polyps. The size of the glands and the distance between them determine the amount of stroma which is expressed as volume percentage stroma. In EIN the volume percentage stroma should be less than 55%. To evaluate this, ocular grids with 50-100 regularly placed points indicated by intersecting lines are used.

2. **Cytology –** The cytology of architectually crowded area is different from that of the background endometrial glands, where the crowded glands show round non-stratified nuclei or elongated pseudostratifed nuclei, with clumped or granular chromatin, with change in nucleoli. The cytoplasm may be of endometrioid differentiation or of non endometrioid differentiation such as tubal, mucinous, secretory or eosinophilic epithelium.

3. **Size-** An EIN lesion should be greater than 1mm in maximum dimension that usually encompasses >5-6 glands.

4. **Exclusion of benign mimics -** Features of EIN overlap with benign conditions which must be carefully discriminated from EIN itself.

   • Endometrial polyps with irregularly placed glands and variable cytology are misinterpreted as EIN. But polyps usually exhibit altered stroma, thick vessels and randomly placed irregular glands.

   • Benign endometrial hyperplasia should not be confused with EIN. Benign hyperplasia changes usually involve the entire endometrial compartment, and have an irregular random pattern of architectural and cytological alteration.

5. **Exclusion of carcinoma**- EIN are composed of clusters of individual glands with simple lining epithelium, whereas adenocarcinoma shows one or more patterns such as solid, cribriform, or complex interlacing maze like growth. These architectural changes of endometrial cancer reflect loss of basement contact between the epithelial cells.

ENDOMETRIAL INTRAEPITHELIAL CARCINOMA(EIC)

It is a precursor lesion of Type 2/ serous adenocarcinoma of endometrium. It mostly affects postmenopausal women on an atrophic endometrium and restricted to small area or in an endometrial polyp. The lesion is characterized by highly pleomorphic cells resembling cells of invasive serous carcinoma. These replace the surface cells and do not invade the stroma. The cells show loss of polarity and have large and atypical nuclei with prominent nucleoli. Abnormal mitotic figures are seen.

The cells show strong positivity for p53 and ki67. A variety of reactive cases can mimic EIC due to cytological atypia and architecture. Data based study reveals that these are not p53 positive.[34,35]

ENDOMETRIAL CARCINOMA

Endometrial carcinoma is the most common malignancy of female genital tract in developed countries.[15] In India, although the incidence of endometrial carcinoma is low when compared to developed countries, there has been a steady increase, making it the fourth leading cancer and seventh leading cause for cancer deaths in women.[36,37]

Endometrial carcinoma mostly occurs in post-menopausal women with mean age at presentation is around is 55 years. There have been many proven studies demonstrating the association between prolonged, unopposed estrogen exposure and development of endometrial carcinoma.[30]

The factors related to prolonged exposure of estrogen leading to endometrial carcinoma are as follows:[38]

a) Use of Tamoxifen in breast cancer. It increases the risk of developing endometrial cancer by 2-folds because of its unopposed estrogen stimulation on the endometrium. Postmenopausal women, on Tamoxifen medication have to undergo screening test for early detection of endometrial cancer.[38]

b) Exogenous unopposed estrogen treatment: This has been the most potential inciting risk factor for endometrial carcinoma. Nowadays combined HRT, which includes both estrogen and progesterone are used to reduce the incidence of this

disease. In one study, noted that postmenopausal women on combined HRT had low risk of developing into endometrial carcinoma than women having only estrogen HRT.[39]

c) Obesity is the most common endogenous cause of excessive estrogen production. Excessive adipose tissue in obese people increases peripheral aromatization of androstenedione into estrogen. Absence of ovulation in post-menopausal women virtually leads to continuous estrogen stimulation. Endometrium proliferate without progesterone effect therefore there is no menstrual withdrawal bleeding.[38]

d) Ovarian lesions like granulosa cell tumor, thecoma, polycystic ovarian disease, all these are associated with excessive prolonged and uopposed estrogenic effect on the endometrium leading to endometrial hyperplasia and endometrial carcinoma.[40]

e) Nulliparity or low parity pose a very high risk for developing endometrial carcinoma. Several studies have shown that infertility and its association with anovulation, no progesterone effect to carcinoma.[41]

f) Other factors like hypertension, diabetes are having indirect source of estrogen production thereby causing endometrial carcinoma. Insulin resistance, hyperinsulinemia, and chronic inflammation are associated with diabetes mellitus and leads to increase in levels of estrogen and androgen and the decrease level of progesterone.[42] Hypothyroidism is associated with metabolic syndrome, polycystic ovarian syndrome and infertility or can directly influence endometrium itself, which leads to the development and progression of endometrial cancer.[43]

Endometrial carcinoma is thought to develop from a continuum of premalignant lesions ranging from endometrial hyperplasia without atypia to hyperplasia with atypia to finally full blown well differentiated adenocarcinoma.[22]

Clinicopathologic studies and molecular analyses support the classification of endometrial carcinoma into two broad categories: [9,10,23,25,44]

1. Type I (estrogen dependent and exhibiting endometrioid morphology)
2. Type II (estrogen independent and have serous/ clear cell morphology).

Endometrial carcinomas have a broad spectrum of phenotypes exhibiting many histological variants. This variation reflects upon the capability of the Mullerian epithelium to differentiate. Endometrial carcinoma follows different pathways of tumorigenesis.[45]

This disease has both sporadic and hereditary population. Norasate et al reported 90% cases of endometrial carcinoma are sporadic and about 10% belong to hereditary. It has been noticed that women with inherited predisposition for this disease, are also seen having an association with disorders like HNPCC (Hereditary Non-Polyposis Colorectal Cancer) and Cowden syndromes.[46]

Majority of the sporadic cases of endometrial carcinoma are **Type I carcinomas.** These are the most common type, accounting for greater than 80% of all cases. The majority are well differentiated and mimic proliferative endometrial glands and as such, are referred to as endometrioid carcinomas. They typically arise in the setting of endometrial hyperplasia and like endometrial hyperplasia; In relation to the

estrogen dependency these occur in younger, pre-menopausal women. These tumors have low propensity for lymphatic spread and myometrial invasion than the non endometrioid adenocarcinoma. They are positive for estrogen and progesterone receptors and generally carry a good prognosis. [47]

These tumors exhibit molecular alterations such as microsatellite instability, inactivated PTEN tumor suppressor gene, K-ras mutations, and abnormalities in b-catenin gene associated with b-catenin nuclear accumulation. Recent molecular studies have provided further evidence that endometrial hyperplasia is a precursor to endometrioid carcinoma.[47]

PROGRESSION OF ENDOMETRIAL HYPERPLASIA AND CARCINOMA[45]

A progression model for Type 1 endometrial carcinoma has been proposed. It is very much similar to the Volgelstein progression model for colorectal carcinoma. This hypothetical model is based on some evident facts such as:

(a) both endometriod carcinoma and endometrial atypical hyperplasia share some similar type of genetic alterations.

(b) There is an increase in genetic alterations of well differentiated endometrial carcinoma compared to atypical hyperplasia

(c) The histological grading increases with increase in genetic alterations.

(d)With the help of comparative genomic hybridization, the detected chromosomal abnormalities were found to be more in endometrial carcinoma than atypical hyperplasia.

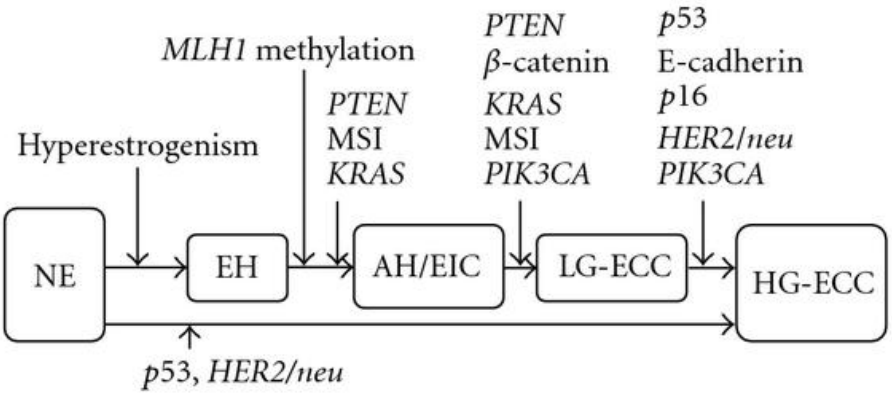


Figure 1 - A progression model for endometrioid carcinoma[45]

NE-normal endometrium; EH-endometrial hyperplasia without hyperplasia, AH-atypical endometrial hyperplasia; EIC-endometrial intraepithelial carcinoma; LG-ECC -low grade endometrioid endometrial carcinoma; HG-ECC -high grade endometrioid endometrial carcinoma.

Tumor initiation and progression are characterized by acquisition of various molecular alterations. The genetic alterations take place in endometrial carcinoma happens to be present at a very early stage. PTEN alterations appear central to the initiation of proliferative lesions that then acquire mutations in other cancer-causing genes (e.g., DNA mismatch repair genes, KRAS, b-catenin) in the carcinogensis. An alternative pathway bypasses atypical hyperplasia and low-grade carcinoma to high-grade carcinoma by p53 mutation and HER2/neu amplification.[45]

Type II carcinomas generally occur in women a decade later than type I carcinoma and in contrast to type I carcinoma they usually arise in the setting of endometrial atrophy. Type II tumors are by definition poorly differentiated tumors and account for approximately 10-20% of cases of endometrial carcinoma. They are not associated with the clinical evidence of estrogen stimulation and typically arise

from atrophic endometrium, frequently in the setting of poorly differentiated phenotype of carcinosarcoma or undifferentiated carcinoma.[48]

They have rapid courses, a high degree of nuclear pleomorphism, frequent aneuploidy DNA content, deeper myometrial invasion, increased lymphatic spread, low sensitivity to progestin and a poor prognosis. In contrast to estrogen dependant tumors these show loss of heterozygosity at different loci, altered p53, and abnormalities in genes regulating mitotic check points.[48]

CARCINOGENESIS OF TYPE 2 CARCINOMA[45]

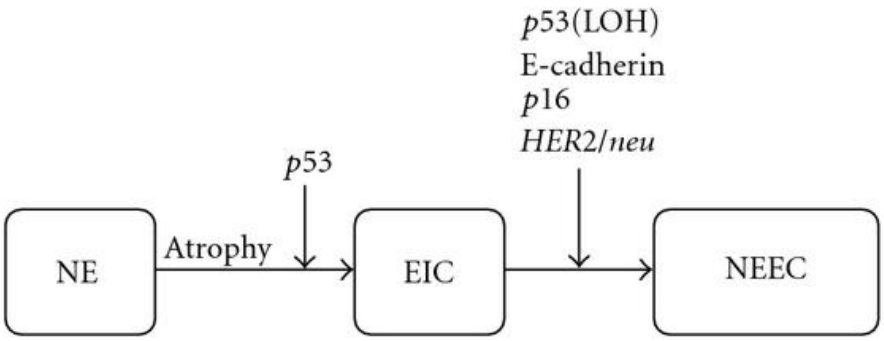


Figure 2 - Progression Model for Nonendometrioid Carcinomas(NEEC)[45]

Mutations of p53 is seen in approximately 80% of EIC, but in most of serous carcinomas, there is no loss of heterogeneity(LOH) at the locus TP53, which means p53 mutation of one allele occurs early, whereas loss of the normal second allele accompanies progression into serous carcinoma. Mutation in E-cadherin, p16, and HER2/neu also affect the progression from EIC to serous carcinoma. Serous carcinoma may also develop from endometrioid carcinoma through p53 mutation.[45]

WHO CLASSIFICATION OF TUMORS OF THE UTERINE CORPUS (2020)(ANNEXURE I)

WHO Classification of tumors also known as WHO blue books are essential tool for standardizing diagnostic practice worldwide. The current fifth volume of classification of Female Genital Tract has described each tumor type based on its location, etiology, epidemiology, clinical features, pathogenesis, histopathology, staging, diagnostic molecular pathology, prognosis and outcome. In recent years, molecular subtyping has become standard of care in Endometrial carcinoma and paradigm of classification has also shifted to molecular pathology.[49]

HISTOLOGICAL TYPES

Endometrioid type or type 1 endometrial carcinoma:

This type is defined as an adenocarcinoma of the endometrium exhibiting glandular pattern. It accounts for 70-80% of endometrial carcinoma compared to serous type. The average age at the time of diagnosis is 63 years. Patients usually present with postmenopausal bleeding. They develop on a hyperplastic endometrium. An atrophic endometrium is frequently associated with high grade lesions and has a great potential to metastasize.[30]

Gross appearance:

All types of endometrial carcinoma have almost similar gross picture. The endometrial surface is shaggy, tan in colour and glistening with focal hemorrhagic areas. They have an exophytic growth pattern even though there is deep invasion. They may also present as a polypoidal mass, or they form one or more discrete nodules. Necrosis is evident only in poorly differentiated forms. The size of the uterus

is enlarged and myometrial invasion is appreciated by well demarcated, firm, gray white tissue with linear extensions into the myometrium. In advanced cases extrauterine involvement is quite evident.[27]

Microscopy findings:

They have a typical glandular or villoglandular pattern. The neoplastic glands are lined by stratified columnar epithelium. These glands are crowded and show complex branchings. The neoplastic cells are columnar with uniform apical border giving a smooth glandular contour at the luminal aspect and have eosinophilic, granular cytoplasm with mild to moderate nuclear atypia and inconspicuous nucleoli. The presence of mitotic figures is quite variable.[11]

The differentiating feature between well differentiated endometrioid carcinoma and atypical hyperplasia is the presence of stromal invasion. It is defined by loss of intervening stroma, confluent glandular pattern and presence of desmoplasia. Grading is primarily made by the architectural pattern and nuclear features.[27]

Architectural grading of endometrial carcinoma:[27]

Grades	Architecture
Grade 1	Well differentiated. Mostly glandular with solid elements less than 5%
Grade 2	Moderately differentiated. 6-50% of the tumor is composed of solid elements.
Grade 3	Poorly differentiated. More than 50% of the tumor is composed of solid elements.

The FIGO (International Federation of Gynaecology and obstetrics) system divides into 3 grades depending upon glands to solid elements. The presence of solid elements signifies poorer differentiation[27,34]

Grade	Grade 1	Grade 2	Grade 3
type	Well differentiated	Moderately differentiated	Poorly differentiated
Glands %	>/=95%	>50%	</=50%
Solid elements%	<5%	</=50%	>50%

Nuclear grading is determined by variation in nuclear size and shape, distribution of chromatin and the nucleolar size.

Grade 1- Nuclei are mildly enlarged, oval in shape with evenly distributed chromatin.

Grade 2- Nuclei are having feature in between grade 1 and grade 3.

Grade 3- Nuclei are markedly enlarged, pleomorphic with irregular borders, has coarse chromatin and prominent eosinophilic nucleoli.

Note: Cytological features used in formulating the final grade

1. High degree of nuclear atypia not correlating with the architectural grade raises the grade1 or grade2 tumors by one level.
2. In carcinomas such as serous adenocarcinoma, clear cell adenocarcinomas and areas of squamous differentiation nuclear grade takes primacy over the architecture.[30]

Low grade endometrioid carcinoma

The low grade endometrioid carcinoma consists of less than 5% of solid growth. These tumors are characterized by malignant glands lined by columnar cells similar to that of the normal proliferative endometrium, where the nucleus are arranged uniformly maintaining the polarity. But the glands are more irregular and larger than benign endometrium. Many of the variants comes under this category which includes, villoglandular variant, endometrioid adenocarcinoma with squamous differentiation, secretory variant, those showing mucinous differentiation and tubal differentiation.[27]

Intermediate grade endometrioid carcinoma

These tumors are categorized as endometrial adenocarcinoma which has solid growth that constitute 5-50% of the tumor. The cytological grade is characterized by high degree of nuclear pleomorphism than grade-1 tumors, which includes, three to four times variation in the nuclear size and even more coarsely clumped chromatin.[27]

High grade endometrioid adenocarcinoma

High grade endometrioid adenocarcinoma is characterized by the presence of solid growth greater than 50%. Some degree of solid growth pattern with higher nuclear grade pushes the grade to grade-3. Nonendometrioid tumors such as serous carcinomas, neuroendocrine carcinoma and undifferentiated carcinomas come under grade-3.[27]

Variants of endometriod carcinoma are:[11]

a) **With squamous differentiation**- About 10-25% of the endometriod carcinoma have a foci of squamous differentiation which are identified with the presence of keratin pearl formation, intercellular bridging or solid nest of large polygonal cells having abundant eosinophilic cytoplasm with distinct cell border. These may be present as morules or seen at the stromal interface. It is important to recognize this feature, as it is not included in the estimation of solid elements for grading. Grading is done only based on glandular component. The presence of squamous component can either be metaplastic or neoplastic. In tumors where the squamous component is well differentiated, is called as Adenoacanthoma. But when both glandular as well as the squamous components are poorly differentiated then the neoplasm is called as Adenosquamous carcinoma.

b) **With secretory differentiation** -Here the endometriod carcinoma are lined by columnar cells which have single, large, sub/supra nuclear vacuolation of glycogen instead of eosinophilic cytoplasm. They constitute less than 2% of the typical endometriod carcinoma. This variant resembles the secretory endometrium of reproductive age group. Most of them are found in non-treated postmenopausal women and are almost always well differentiated.

c) **Villoglandular variant** is a rare subtype and has a papillary architecture. These papillary fronds are composed of delicate fibrovascular core covered by columnar cells having bland nuclei. Median age of the patients for this variant

is 61 years. Microscopic findings show thin, delicate fronds covered by stratified columnar epithelial cells. The nuclei are oval in shape, exhibiting mild to moderate pleomorphism with nuclear grading of 1 or 2. Abnormal mitotic figures are rare in this variant. Myometrial invasion is mostly superficial. This variant has to be differentiated from serous carcinoma of the endometrium where the papillae are mostly arranged in tufts and nuclei exhibiting severe pleomorphism.

d) **Ciliated carcinoma**- This is a rare entity and is hardly present in malignant tumors. If 75% of the tumor has ciliated cells, then only it can be termed as ciliated cell carcinoma. The diagnosis of ciliated cell carcinoma has to be made with extra caution as the hyperplastic endometrium with complex proliferation may also exhibit cilia, which is a benign lesion. The only features supporting the diagnosis has to be presence of myometrial or lymphatic invasion.

e) **Sertoliform differentiation**- This variant of endometriod carcinoma showing areas composed of glands which resemble sex cord-stromal tumors. These glands are arranged in closely packed tubules /trabeculae, and the cells have basally located nuclei and clear cytoplasm. The adjacent area (non sertoliform) is representing typical endometriod carcinoma.

Genetic profile of endometriod carcinoma shows most frequent alterations in PTEN (inactivation/mutation) comprising > 50% of carcinomas, mutations in

PIK3CA (30%), ARID1A (40% of low grade tumors), KRas (20-26%), and TP53(30% of grade 3 endometriod carcinoma).

Microsatellite instability is accounted in 35% of the tumors and mostly seen in the sporadic type of endometriod carcinoma due to hypermethylation of the MLH1 gene promoter.

Serous Adenocarcinoma / Type 2 Endometrial Adenocarcinoma

This is an aggressive form of endometrial carcinoma characterized by complex papillary and/or glandular architecture with diffuse and marked nuclear pleomorphism. Patients mostly belong to post-menopausal age group, present with postmenopausal bleeding. Most often they are multiparous and obese.[30] Tumor has a tendency to develop on an atrophic endometrium. Serous carcinoma causes an increase in CA125 like ovarian carcinoma.[11] The uterus is generally small as the tumor most often arises from atrophic endometrium of old women. Otherwise rest of the features in gross, is almost similar to other endometrial carcinomas. Sometimes this tumor arises in an endometrial polyp.

Serous endometrial intraepithelial carcinoma (SEIC) either develop in atrophic or directly on a polyp. The lesion in SEIC is confined to the surface epithelium. Even though myometrial invasion is absent, there is always a chance for extra uterine metastasis due to shedding of the tumor cells. Hence intraperitoneal spread such as omental cake is often seen even though myometrial invasion is absent. They exhibit complex papillary architecture. The papillae are of various size and width ranging from short and stout to long and thin, with few of the papillae exhibiting branching and features of hyalinization. The papillae have a fibro vascular core and are lined by

large atypical nuclei with prominent eosinophilic macronucleoli and scanty cytoplasm. The luminal surface appears scalloped or frayed. Numerous mitotic figures are seen. Myometrial invasion is evident by the presence of gaping glands and shows higher incidence of lower uterine segment involvement. 25% of the cases show psammoma bodies. Most of the patients diagnosed with serous carcinoma show striking vascular invasion.[11]

80 to 90 % cases show TP53 mutation. There may be a germ line BRCA1/2 mutations present in association with this serous carcinoma. The patients have unfavourable prognosis, therefore comprehensive staging has to be done to determine the risk of recurrence of the tumor.

Mucinous carcinoma

It is a type of endometrial carcinoma composed of cells containing prominent intracytoplasmic mucin. More than 50% of the neoplasm must contain mucinous cells to call it as a mucinous carcinoma. It accounts for 1-9% of all endometrial carcinoma. Women diagnosed with this carcinoma present with vaginal bleeding similar to the clinical presentation of typical endometrioid carcinoma. These are mostly well differentiated and present as stage 1, therefore the prognosis is favourable. The most frequently occurring mutation in this tumor is the KRAS mutations.[30] Treatment for this type of endometrial carcinoma is surgery.[11]

Clear cell carcinoma

It is characterised by papillary and tubulocystic architecture. The cells are polygonal or exhibiting hobnail appearance, with clear or eosinophilic cytoplasm and

at least focal high grade nuclear atypia. 2/3rd of this tumor contain densely eosinophilic extracellular globules / hyaline bodies. These tumors accounts for 3-6% of all endometrial carcinoma. The mean age of the women diagnosed with this carcinoma is 65years. Somatic mutations in PTEN, P53 have been reported in 30 to 40 % of this carcinoma. Prognosis is poor with 5 years and 10 years disease free survival reported in this type of carcinoma are 43-68% and 39% respectively.[11]

Neuroendocrine tumors

They are a diverse group of tumors which share a morphological neuroendocrine phenotype. The incidence is rare and accounts for less than 1% of all endometrial carcinoma. Patients with this neoplasm present with postmenopausal bleeding. On gross, the tumor is bulky, exophytic, polpoidal masses with variable myometrial invasion. Prognosis of small cell neuroendocrine carcinoma and large cell neuroendocrine carcinoma is poor but a study reported that these tumors may have favourable prognosis if the lesion is confined to the endometrial polyp.[30]

Mixed carcinomas

Endometrial carcinomas composed of two or more histological types with one component should be serous carcinoma. The prognosis is poor if 25% or more of the aggressive component of the tumor present in mixed carcinoma.[50]

Undifferentiated carcinoma

It is an endometrial carcinoma that lacks proper architecture. The neoplasm is composed of solid sheets of medium sized, monotonous epithelial cells which has no pattern. Overall prognosis is poor. Stroma is mostly absent / scant.[11]

PROGNOSTIC FACTORS IN ENDOMETRIAL CARCINOMA:[27]

1) Histological type

Type 1/ Endometrioid type endometrial carcinoma and its variations have good prognosis whereas, non endometrioid type, clear cell, carcinosarcoma and undifferentiated have unfavorable prognosis. Pure squamous cell carcinoma of endometrium has poor prognosis

2) Histological Grade

FIGO (International Federation of Gynaecology and obstetrics) is the most common grading system which is used to grade the tumor according to the degree of architectural and nuclear pleomorphism.

Nuclear grading of endometrial carcinoma

1) Round nuclei

2) Variable size and shape of the nuclei

3) Hyperchromatic nuclei

4) Coarse and clumped chromatin

5) Prominent nucleoli

The grade of the tumor increases with significant nuclear atypia.

3) Stage and depth of invasion

The tumor staging system was recommended by FIGO, which determines the outcome of the women with endometrial carcinoma.

TNM and FIGO classification for endometrial carcinoma (Annexure II)

4) Myometrial invasion

Previously, myometrial invasion was reported as proportion of the uterine wall invaded by the tumor and is expressed in thirds. Recent classification includes the feature wherein the maximum depth of invasion is measured in mm and further expressed in % of invasion. Myometrial invasion independent of tumor grade is a crucial parameter for prognostic depiction. The frequency of nodal metastasis is related to the depth of invasion.

When grading was analyzed against myometrial invasion, it was seen that Grade 1 tumors invading inner one third do not show metastasis, whereas when invasion up to outer third causes pelvic node metastasis in 25% of the cases.[51]

5)Cervical involvement:

Cervical involvement can determine the risk of recurrence as well as the possibility of extra uterine metastasis of the tumor. Hence presence of cervical involvement is associated with increasing grade, depth of invasion, and recurrences, thereby determining the prognosis of the patient.[52]

6)Peritoneal cytology:

Only 5-15% of patients have positive peritoneal cytology and therefore signifies the manifestation of extrauterine spread of the tumor and generally these patients are staged as 3A. Larger studies with multivariate analysis support the presence of malignant cells in peritoneal washing as indicative of poor prognosis.[53]

7)Vascular invasion and lymph node invasion:

Vascular invasion is most uncommon in type1 but there is increase in frequency when there is evident deep myometrial invasion, aggressive cell type, and

decrease in histological differentiation. Therefore, presence of positive aortic lymph nodes is the most important factor in predicting the prognosis of the patient.[54]

Atypical endometrial hyperplasia shows favourable prognosis as it is correlated with low grade tumors. Atrophic endometrium is always associated with high grade tumors therefore shows unfavourable prognosis.

8)Steroid receptors:

The presence of steroid receptors, its quantity and their distribution have been correlated with histological differentiation, FIGO stage and survival outcome. Early stage tumors and well differentiated tumors are having association with positive hormone receptor status (i.e. ER and PR positive).[55]

Bcl2 is the marker of apoptosis. It inhibits programmed cell death. The expression varies with menstrual cycle. In endometrial carcinoma it has an antiapoptotic effect. Therefore, overexpression of BCL2 will have poor prognosis.[56] Her 2 neu receptors were seen in high grade and stage tumors.

Angiogenesis and vascular endothelial growth factor permits tumor growth. This neovascularization could result from many factors that are released from the tumor itself or from the surrounding matrix. The tumor vascularity is associated with the increasing stage, decreased differentiation, lymphovascular invasion and increase in micro vessel count. Hence micro vessel density has been proved to be one of the prognostic markers of high potential utility.[27]

Ki-67 IMMUNOHISTOCHEMISTRY

Immunohistochemistry(IHC) is an important application in monoclonal as well as polyclonal antibodies analyses to detect specific antigens in tissue sections. IHC stains are widely used to determine the stage and grade of tumor and identifying cell type and origin of a metastasis to find the site of the primary tumor. This method is also used by the pathologists as an aid in the differential diagnosis and classification of cancer and for certain diseases. The result of the staining enables the pathologist to count the cancer cells and decides the grade of tumors.[57,58]

FIGO grade, stage, histologic type, depth of myometrial invasion, peritoneal cytology, steroid receptor status, and lymphovascular space invasion are all recognized prognostic indicators in patients with cancer of the endometrium.[59]

Cell proliferation has also an important role in deciding the clinical behavior and aggressiveness of tumors. Determination of proliferative activity of a tumor has been reported to be of diagnostic and prognostic significance. Many methods are used to estimate the number of proliferating cells. Ki-67 antigen immunostaining by MIB-1 antibody is one such reliable marker which is expressed in proliferating tissues and absent in quiescent cells.[60]

Ki-67 gene is present on the long arm of chromosome no 10. Ki-67 antigen nuclear protein can present in two isoforms of 345 and 395 kDa. These proteins are expressed in proliferating cells and reacts with Mindbomb E3 ubiquitin protein ligase-1 antibody (MIB-1 antibody). Ki-67 immunostaining are present during all active phases of cell cycle that is, G1, S, G2 and M-phase but absent in resting phase, that is

G0 phase. Hence, Ki67 is a good proliferation marker for cancer cells since they aggressively grow and divide.[61,62]

Ki-67 immunostaining estimates the Proliferative Index (PI) of a neoplastic lesion which is quantitatively expressed as Ki-67 Labeling index (Ki-67 LI)/ MIB-1 labeling index.[63]

Ki67 positivity increase as the severity of endometrial lesions increased from EH to EHA to endometrial carcinoma. In all cases of endometrial carcinoma, mean Ki67 was increasing as the grade increase.[64] Also, Ki67 immunohistochemistry show higher expression in type-II endometrioid adenocarcinoma than Type-I carcinoma.[65]

In endometrial carcinoma, there is association between Ki-67 score and other pathological variables such as depth of myometrial invasion, stage, grade, and outcome.[66,67]

Advanced stages and higher grades show high Ki-67 LI whereas early stages and lower grades shows relatively lower Ki-67 LI.[68,69] In addition, EC with lymphatic metastasis show significant higher Ki-67 LI than the EC without lymphatic metastasis.[70]

Therefore, higher expression of Ki-67 antigen is associated with tumor aggression(grade) and reserved prognosis (stage).[63] Lower expression is associated with prolonged survival[71] and hence Ki-67 expression can be used to guide the clinical management of the tumors.

MATERIALS AND METHODS

The present study was carried out at the Department of Pathology of KLE University's JN Medical College, and Dr. Prabhakar Kore Hospital, and Research Centre, Belagavi.

Study design: This was an observational study

Study period: January 2020 to December 2020

Study Population: All the new cases of Endometrial carcinoma and Endometrial hyperplasia with atypia admitted to KAHER'S DR. PRABHAKAR KORE HOSPITAL from January 2020 to December 2020 were included. From four year retrospective cases, data as well as tissue blocks were retrieved from archives of Department of Pathology, J N Medical College, Belagavi. For Endometrial hyperplasia without atypia, randomly selected one year prospective cases of KAHER'S DR. PRABHAKAR KORE HOSPITAL were taken.

Inclusion criteria:

1. Endometrial hyperplasia without atypia (simple, complex)
2. Endometrial hyperplasia with atypia (simple, complex)
3. Endometrial carcinoma

Exclusion criteria:

1. Infective conditions e.g. Endometritis
2. Metastatic lesions of endometrium

Sample size: Total 40(15 cases of Endometrial Carcinoma, 10 cases of Endometrial Hyperplasia with atypia and 15 cases of Endometrial Hyperplasia without Atypia)

Sampling procedure: Purposive sampling for Endometrial hyperplasia without atypia, Universal Sampling for Endometrial carcinoma and Endometrial hyperplasia with atypia.

Ethical clearance: The present study was approved by Jawaharlal Nehru Medical College's Institutional Ethics Committee on Human Subjects Research. (Ref.: MDC/DOME/265) (Annexure III)

Data Collection:

The present study was conducted on endometrial hyperplasia and all endometrial carcinoma cases admitted in KAHER'S Dr Prabhakar Kore Hospital & Medical Research Centre, Belagavi.

Information regarding old cases were obtained from Medical Records Department (MRD) records. The information regarding clinical parameters including age, menopause status, surgery type was obtained from patient's records from MRD.

For selected retrospective cases, representative tissue blocks were retrieved from the storage of the Department of Pathology, KAHER. 4-5 micron thick sections were cut from paraffin embedded blocks. One section from each block was taken for staining with Haematoxylin & Eosin(H&E) and one for the immunohistochemistry with Ki-67(Annexure VI).

The Hematoxylin and Eosin stained slides were evaluated for following points: phase of menstrual cycle; hyperplastic changes; presence or absence of atypia; histopathological diagnosis, sub-typing, grading, status of Lymphovascular Invasion (LVI) and pathological staging of endometrial carcinoma.

Ki-67 staining was performed manually using a pre-diluted antiKi-67 antibody (Vitro Master diagnostica, Clone SP6) using peroxidase-antiperoxidase method and H2O2-diaminobenzidine as substrate-chromogen. The processing of the sample, prior to incubation with the primary antibody, included antigenic unmasking by heating in Tris-EDTA buffer at pH 8 in a pressure cooker at 95^{o}C.

The percentage of cells positive for Ki-67 was scored manually. Each slide was evaluated at 4X and 10X magnification in order to find areas with maximum positive cells ("hot-spot area"). Then randomly selected 3 areas across tumor were examined at 400X magnification and the percentage of positive cells to total cells was calculated. At least 1000 cells were counted and only the cells that were definitely positive for the desired marker were considered. Criteria for Ki67 positivity were strong and complete brown yellowish nuclear coloration. Nuclei of stromal cells and lymphocytes which took up the stain were not counted.

Grading criteria used for assessing Ki-67 labelling index (Ki-67 LI)[52]

Percentage of cells (Ki-67 LI)	**Grading**
<10%	1
10-39%	2
40-69%	3
>70%	4

Statistical Analysis: Descriptive and inferential statistical analysis was carried out in the present study. The data was analyzed using The SPSS Software version 25 and Microsoft word and Excel were used to generate Graphs, tables etc.

Descriptive statistics such as Mean, & Percentages were calculated. The relationship between expression of markers and other clinicopathological variables was analyzed using chi-square test, Spearman's rank correlation coefficient method and one-way ANOVA. Probability (P) value: < 0.05 was considered significant.

RESULTS AND OBSERVATIONS

Out of the total number of 40 patients, 15 patients of endometrial hyperplasia without atypia(EH), 10 cases having endometrial hyperplasia with atypia(EHA), and 15 of Endometrial carcinoma(EC) cases were included in this study.

Table1: Comparison of three groups (EH, EHA, and EC) with age

Age groups	EH group	%	EHA group	%	EC group	%	Total
<=40yrs	4	26.67	3	30.00	1	6.67	8
41-50yrs	8	53.33	3	30.00	3	20.00	14
51-60yrs	3	20.00	1	10.00	6	40.00	10
>=61yrs	0	0.00	3	30.00	5	33.33	8
Total	15	100.00	10	100.00	15	100.00	40
Mean age	43.33		51.70		55.87		50.13
SD age	8.49		15.37		10.32		12.24
Chi-square=11.8760m p=0.0650, NS							

The mean age of the patients for EHs 43.33 ± 8.49 years, for EHAs 51.70±15.37 years, and for ECs 55.87±10.32 years was noted. The age distribution of patients is shown in Table 1 and Figure 1. It was noted that there were more number of cases between 41- 50 years of age for EHs and between 51-60 years of age for ECs. For EHAs no peak incidence was seen for age. There was no statistical significance found(p=0.0650) as determined by the chi-square test.

It is still noteworthy that EHs were found in the earlier age group while ECs found to be more frequent in the older age group.

Figure1: Comparison of three groups (EH, EHA, and EC) with age

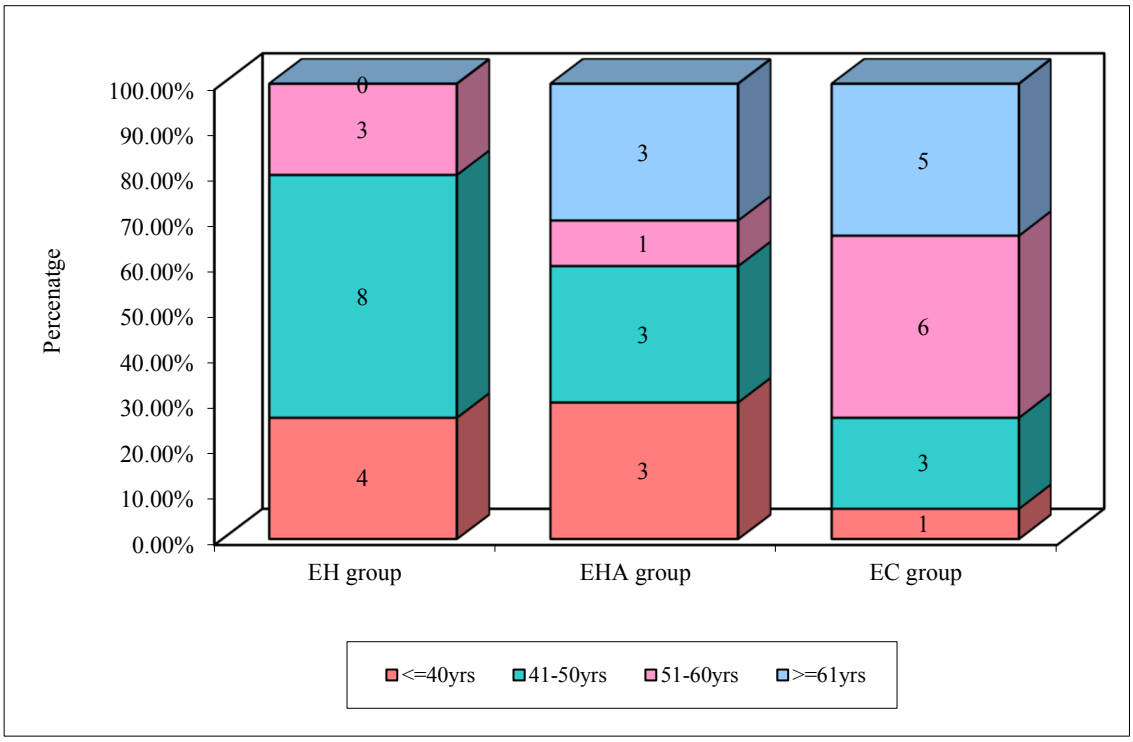
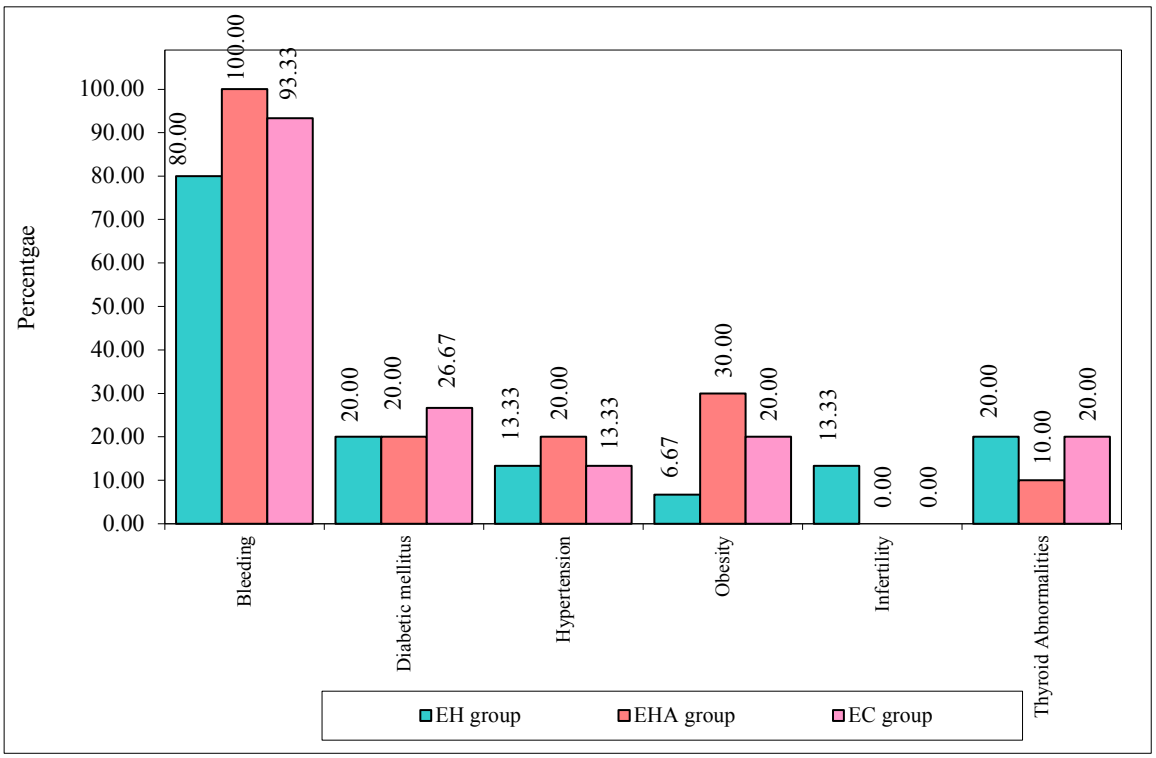


Table 2 and Figure 2 shows the distribution of clinical features among cases of EH, EHA, and EC and shows bleeding manifestations are the most common feature to be present and seen in 36 patients out of 40, followed by diabetes(N=9), obesity(N=7), thyroid dysfunction(N=7), Hypertension(N=6) and infertility(N=2) in decreasing order of presentation. The associations between these components were found to be not statistically significant as determined by the chi-square test.

Table2: Comparison of three groups (EH, EHA, and EC) with the presence of clinical features

Clinical features	EH	%	EHA	%	EC	%	Total	%	Chi-square	p-value
Bleeding	12	80.00	10	100.0	14	93.33	36	90.00	2.9630	0.2270
Diabetic Mellitus	3	20.00	2	20.00	4	26.67	9	22.50	0.2390	0.8870
Hypertension	2	13.33	2	20.00	2	13.33	6	15.00	0.2610	0.8770
Obesity	1	6.67	3	30.00	3	20.00	7	17.50	2.3670	0.3060
Infertility	2	13.33	0	0.00	0	0.00	2	5.00	3.5090	0.1730
Thyroid Dysfunction	3	20.00	1	10.00	3	20.00	7	17.50	0.5190	0.7710

Figure2: Comparison of three groups (EH, EHA, and EC) with the presence of clinical features



Ki-67 LI expression is depicted in table 3a and figure 3. In EH cases, Ki-67 minimum expression was 1.2% and the highest expression was 75.1% with the mean being 34±21% with SE of 5%. 95% Confidence interval for mean in EHs was between 22% to 45%.

EHA expression varied from 11.5% to 42.1% with a mean of 32±10% with SE of 3% and giving 95% confidence interval for the mean between 25% to 39%.

The minimum expression of 1.3% was recorded for EC cases and the maximum expression was 61.5% with a mean of 31±17% and SE 4%. 95% Confidence interval for mean in ECs was between 19% to 37%.

Table3a: Summary of Percentage of Ki-67 scores in three groups (EH, EHA and EC)

Groups	Min	Max	Mean	SD	SE	95% CI for Mean	
						Lower Bound	Upper Bound
EH group	0.01	0.75	0.34	0.21	0.05	0.22	0.45
EHA group	0.12	0.42	0.32	0.10	0.03	0.25	0.39
EC group	0.01	0.62	0.28	0.17	0.04	0.19	0.37
Total	0.01	0.75	0.31	0.17	0.03	0.26	0.37

Figure3: Comparison of three groups (EH, EHA, and EC) with mean Ki-67 score

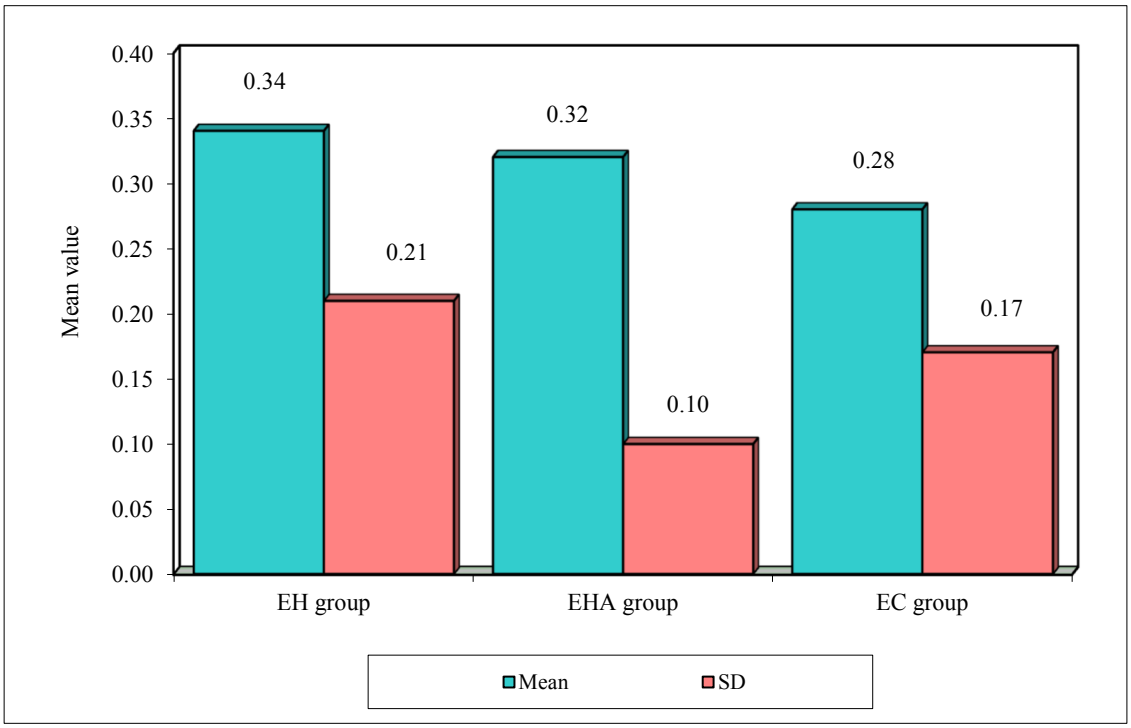


Table 3b depicts Between group analysis and Within group analysis among EHs, EHAs, and ECs with mean Ki-67 LI by one-way ANOVA. The Ki-67 expression pattern was compared between all the study groups EC, EH, EHA, and no statistically significant difference (P = 0.6150) was observed between the groups. Further, comparing the expression profile of Ki-67 among the various (paired) groups, no statistically significant difference was observed among the results of EC and EH, EC and EHA, EH and EHA.

Table 3b: Comparison of three groups (EH, EHA, and EC) with mean Ki-67 scores by one-way ANOVA

Sources of variation	Sum of Squares	df	Mean Square	F-value	p-value
Between groups	0.0290	2	0.0140	0.4930	0.6150
Within groups	1.0800	37	0.0290		
Total	1.1080	39			

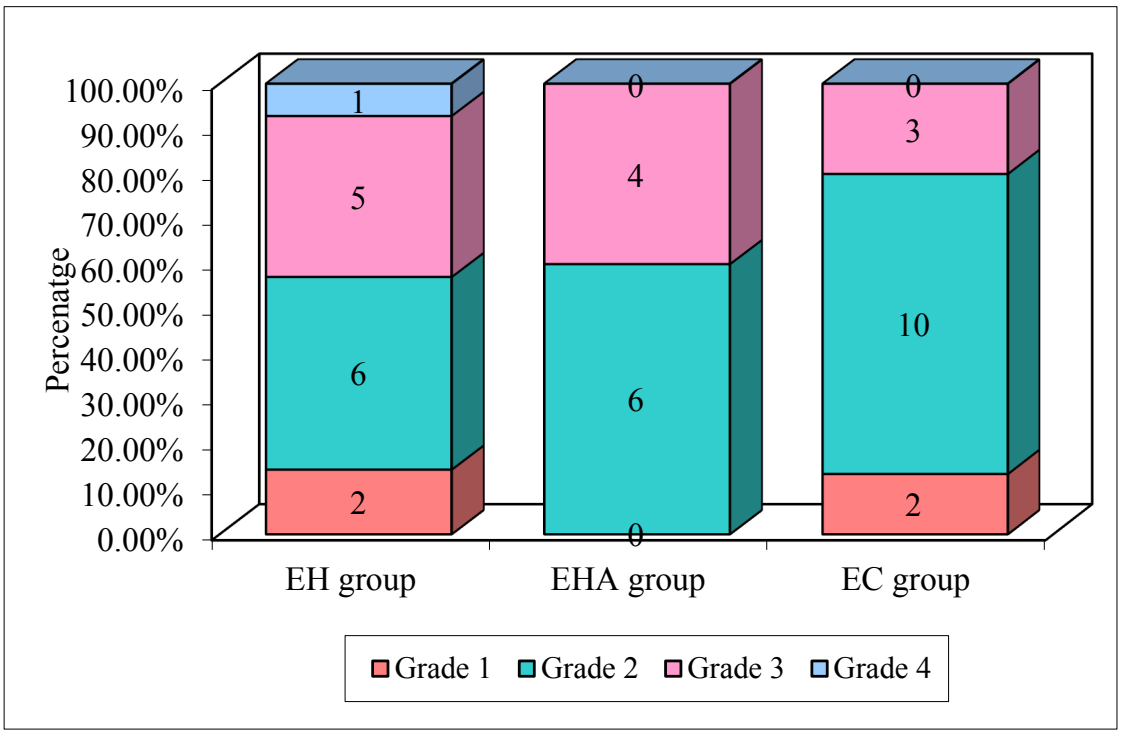
Table 4: Comparison of three groups (EH, EHA, and EC) with Ki-67 grades

Ki-67 grades	EH group	%	EHA group	%	EC group	%	Total	Total
Grade 1	2	13.33	0	0.00	2	13.33	4	10.00
Grade 2	6	40	6	60.00	10	66.6	22	55.00
Grade 3	5	33.3	4	40.00	3	20.00	12	24.00
Grade 4	1	6.66	0	0.00	0	0.00	1	2.50
Total	15	100.00	10	100.00	15	100.00	40	100.00
Chi-square4.8834, p=0.5588								

Table 4 and Figure 4 depict Ki-67 grade comparison of EH, EHA, and EC cases. It was noted that grade-2 was most common for all the groups in the present study.

Less than 10% nuclear staining was found in 13.3% of EHs, 0% of EHAs, and 13.33% of ECs. 10-39% nuclear staining for Ki-67 was observed in 40% of EHs, 60% of EHA, and 66.2% of EC. More than 70% score was seen in 6.6% of EHs and 0% of EHAs and ECs whereas less than 70% nuclear staining for Ki-67 was observed in 33.3% of EHs, 40% of EHAs, and 20% of ECs. The associations between these components were found to be not statistically significant (p=0.5588) as determined by the chi-square test.

Figure 4: Comparison of three groups (EH, EHA, and EC) with Ki-67 grades



In ECs, all the 15 cases were of Endometrioid type (Type I) of Endometrial carcinoma. No Type II Endometrial carcinoma were observed. Also, 2 out of 15 ECs, showed squamous differentiation.

Table 5 and Figure 5 depict histological grade in EC cases where grade II was most frequent with 66.67%.

Table 5: Distribution of EC case by Histological grades

Histological grades	Number	Frequency(%)
Grade I	4	26.67
Grade II	10	66.67
Grade III	1	6.67
Total	15	100.00

Figure 5: Distribution of EC case by histological grades

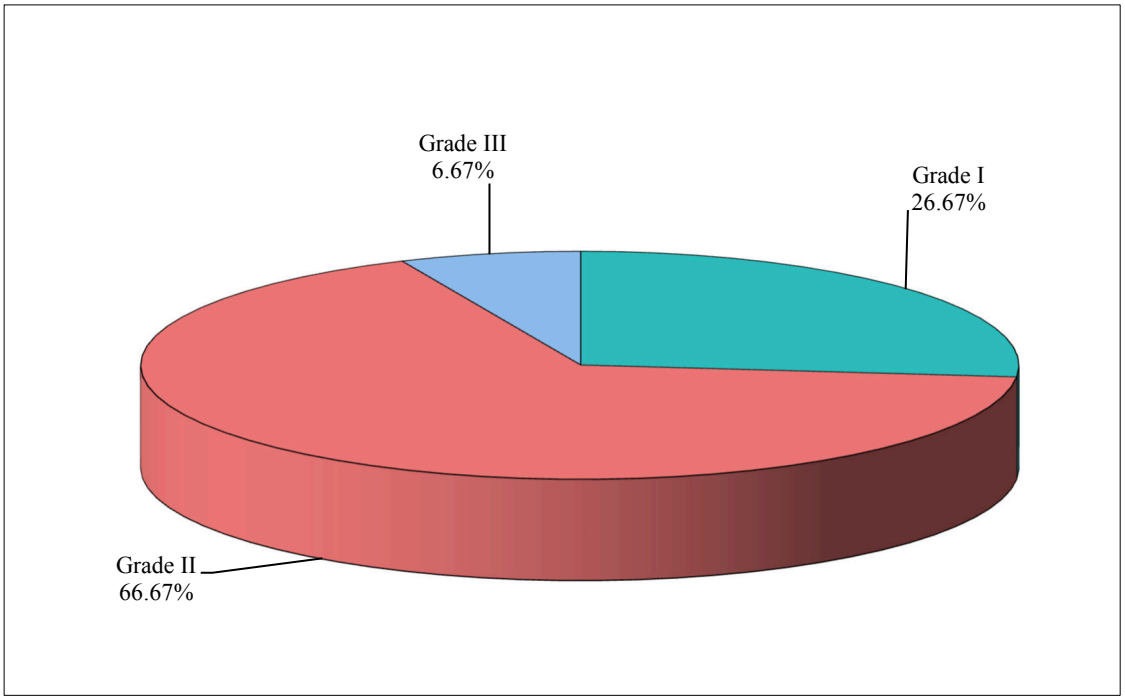


Table 6 and Figure 6 depict the FIGO stage in EC cases where grade IA was most common in this study (53.33%).

Table 6: Distribution of EC case by FIGO stages

FIGO stages	Number	Frequency(%)
Stage IA	8	53.33
Stage IB	4	26.67
Stage II	3	20.00
Total	15	100.00

Figure 6: Distribution of EC case by FIGO stages

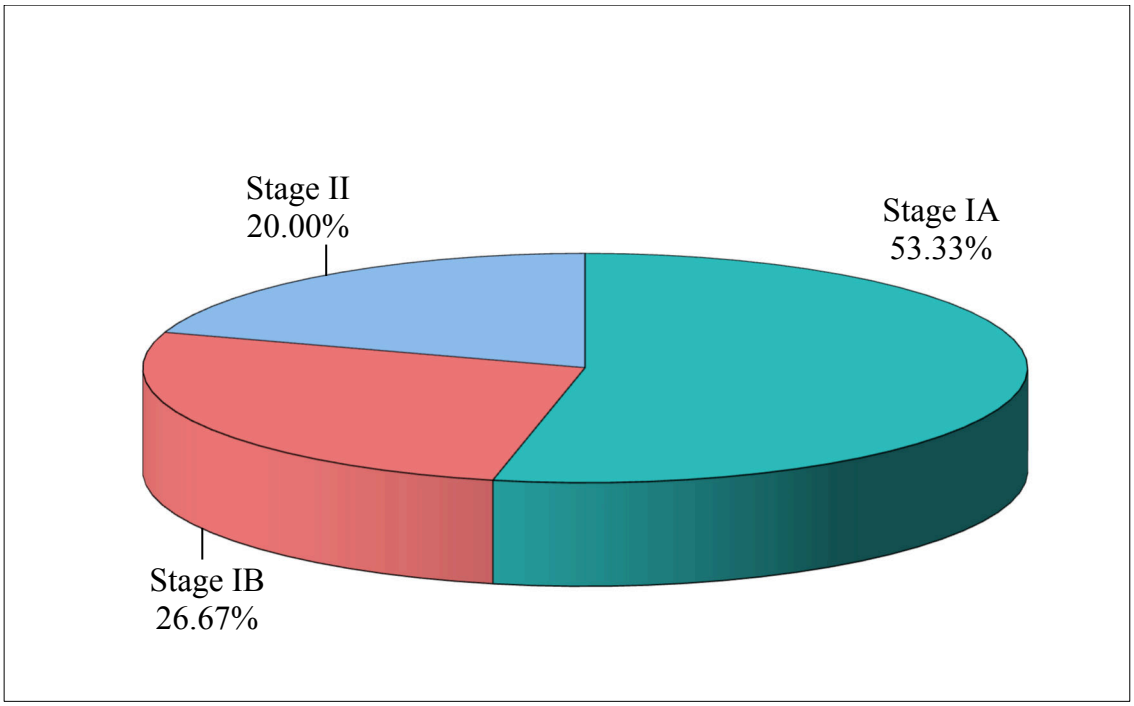


Table 7 and Figure 7 depict Depth of Infiltration in EC cases showing 8 out of 15 cases having infiltration less than half of the myometrial thickness, 4 cases having more than half of myometrial thickness and 3 cases were having infiltration in more than half of the thickness of myometrium and to the cervix.

Table 7: Distribution of EC case by Depth of Infiltration

Depth of Infiltration	Number	Frequency
Less than half thickness of myometrium	8	53.33
More than half thickness of myometrium	4	26.67
More than half thickness of myometrium, cervix	3	20.00
Total	15	100.00

Figure 7: Distribution of EC case by Depth of Infiltration

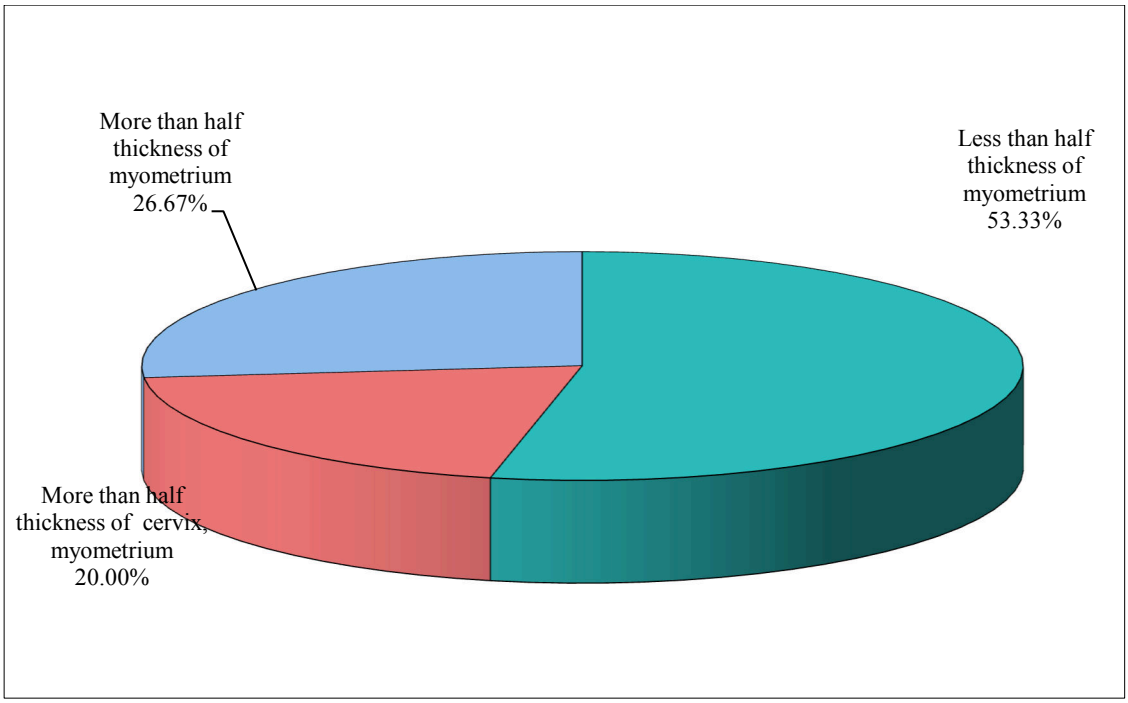


Table 8 and Figure 8 depict a total of 7 out of 15 cases showing PNI, 4out of 15 cases showing LVI and none of the cases showed Lymph node invasion.

Table 8: Distribution of EC case by the status of PNI, LVI, and LNM

Status of	Number	Frequency
PNI	7	46.67
LVI	4	26.67
LNM	0	0.00

Figure 8: Distribution of EC case by the status of PNI, LVI, and LNM

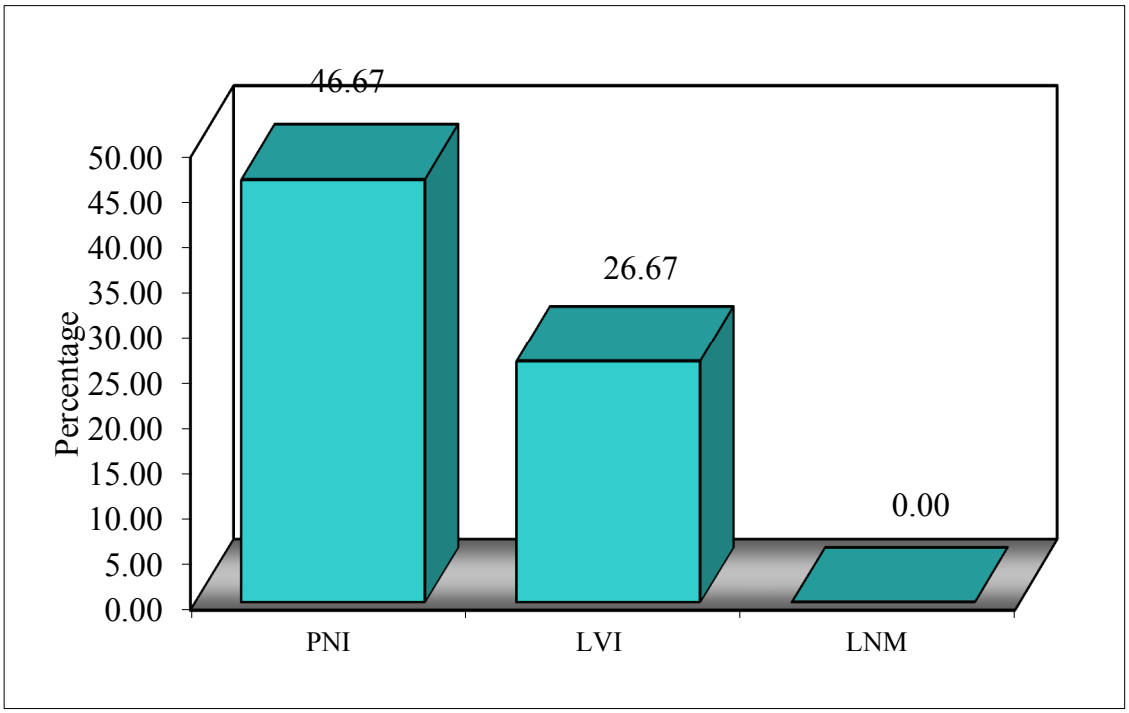


Table 9 and Figure 9 depict Ki-67 grade in EC cases. Grade 2 and 3 both show equal distribution of 40%(N=6).

Table 9: Distribution of EC case by Ki-67 Grade

Ki-67 Grade	Number	Frequency
Grade 1	2	13.33
Grade 2	6	40.00
Grade 3	6	40.00
Grade 4	1	6.67
Total	15	100.00

Figure 9: Distribution of EC case by Ki-67 Grade

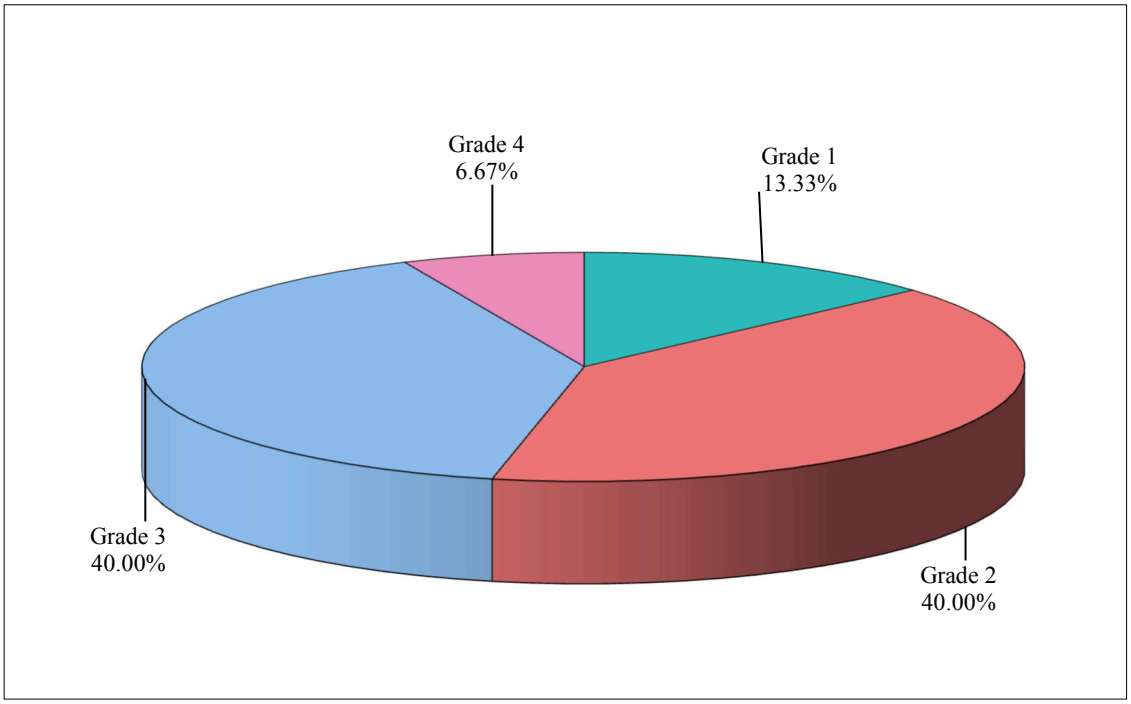


Table 10 depicts the correlation between histopathological grade, FIGO stage, and Mean Ki-67 LI, Ki-67 grade in ECs. With the increase in histopathological grade and FIGO stage, it was observed that there is an increase in Ki-67 score and grade.

Spearman's coefficient was found to be positive and in between 0.40 to 0.69 for all these parameters. That signifies the strong relationship between these parameters. The associations between these components were found to be statistically significant.

Table 10: Correlation between Ki-67 expression and its grades with Histological grades and FIGO stages in EC group by Spearman's rank correlation coefficient method

Variables	N	Spearman R	t(N-2)	p-value
Mean Ki-67 LI with Histological grades	15	0.5363	2.2907	0.0393*
Ki-67 grades with Histological grades	15	0.6695	3.2498	0.0063*
Mean Ki-67 LI with FIGO stages	15	0.6468	3.0579	0.0092*
Ki-67 grades with FIGO stages	15	0.6732	3.2829	0.0059*

*$p<0.05$

DISCUSSION

Endometrial carcinoma is the most common invasive malignant tumor of the female genital tract. There is a rise in incidence and a corresponding increase in deaths from endometrial cancer.[9]

According to the 2014 World Health Organization (WHO) classification, hyperplasias are classified as hyperplasia without atypia and Atypical Endometrial Hyperplasia (EHA)/Endometrial Intraepithelial Neoplasia (EIN).[10,22]

Endometrial (endometrioid) carcinoma is often preceded by endometrial hyperplasia and currently, it is accepted that there is a continuum of changes that evolve to endometrioid carcinoma.[73] Hyperplasia is usually associated with exogenous estrogen stimulation therefore estrogen is considered as an endometrial carcinogen.[74] Hyperplasia without atypia does not show relevant genetic alterations and less than 2% progress to carcinoma in case endocrine abnormality persists over a long time. However, EHA progresses to Endometrioid Adenocarcinoma in 23% of cases.[3,75]

FIGO stage, histologic type, depth of myometrial invasion, peritoneal cytology, steroid receptor status, and lymphovascular space invasion are all recognized prognostic indicators in patients with cancer of the endometrium.[27,76]

Ki-67 is a proliferation marker for cancer cells since they aggressively grow and divide. Ki-67 immunostaining is present during all active phases of the cell cycle that is, G1, S, G2, and M-phase but absent in the resting phase, that is G0 phase.[61,62] Ki-67 immunostaining estimates the Proliferative Index (PI) of a neoplastic lesion

which is quantitatively expressed as Ki-67 Labeling index (Ki-67 LI)/ MIB-1 labeling index.[63]

The purpose of the study was to study Ki-67 expression in Endometrial Hyperplasia and Endometrial Carcinoma and differentiate these based on its expression.

In the present study, 15 patients of endometrial hyperplasia without atypia(EH), 10 cases having endometrial hyperplasia with atypia(EHA), and 15 Endometrial carcinoma(EC) cases with a total of 40 cases were included.

In the present study, the mean age of the patients for EH 43.33 ± 8.49 years, for EHA 51.70±15.37 years, and for EC 55.87±10.32 years were noted. In the study of Reed et al., peak incidence for EH was 50-54 years while for EHA and EC incidence peaked in women ages 60-64 years.[77]

The pathogenesis of endometrial carcinoma is closely linked with hormones like estrogen and progesterone. The results from previous studies suggested that most women with endometrial hyperplasia and carcinoma present with abnormal uterine bleeding(AUB).[78,79,80] In the present study, 90% of women presented with bleeding manifestation (36/40).

Obesity has been recognized as an important risk factor for endometrial proliferative lesions; accounting for almost 17-46% of uterine cancers. The incidence and mortality rate of endometrial cancer has grown and continues to grow as a result of the worldwide obesity epidemic. The molecular mechanisms related to adipose tissue metabolism, highly contribute to the pathogenesis of proliferative changes of hyperplasia and carcinoma.[38] Giannella L et al postulated an association between

elevated BMI and EC in women with EH.[78] In the present study, a Total of 7 patients presented with obesity. However, no statistically significant correlation was found between obesity and endometrial lesions in this study.

Diabetes, hypertension, thyroid dysfunction, and infertility are also important risk factors, which indirectly increase levels of estrogen and further leads to develop endometrial hyperplasia and carcinoma.[42,43,78] but no statistically significant correlation between these parameters was found in the present study.

Bockman described two pathogenetic types of endometrial carcinomas. Type 1 tumors are usually low-grade endometrioid adenocarcinomas that develop in pre or post-menopausal women and frequently arise in a background of complex or atypical endometrioid hyperplasia. In contrast, Type 2 tumors are nonendometrioid, more aggressive, and less common.[44] Norsate et al postulated that the two types of endometrial carcinoma are pathogenetically and clinically distinct. The molecular pathways of carcinogenesis may follow a similar sequence as that of the Vogelstein model that includes stepwise genetic changes of oncogenes and tumor suppressor genes.[45]

Pollock et al reported that in their study involving 187 cases of endometrial tumors, 118 were type 1 endometrioid and 18 were nonendometrioid or type 2. The rest of the cases included clear cell, carcinosarcoma, and stromal sarcoma.[81] In the study of Risinger et al, 115 out of 136 cases were type 1.[82] In the present study, all 15 cases were type 1 and only 2 showed squamous differentiation.

Pathological staging was done based on the FIGO staging system for carcinoma of the endometrium and in this study, 8 cases were presented in stage 1A, 4 in stage 1B, and 3 in stage 2. Rasty et al reported that in their study,146 cases of

endometrioid carcinomas, 40 cases were Grade 1, 74 cases in Grade 2, and 32 in Grade 3(46).[83] Risinger et al published a similar trend in their study involving 115 cases. In their study, there were 28, 52, and 35 cases of Grade 1,2, and 3 carcinomas respectively (44).[82] In the present study, similar results were found; 4 were Grade 1, 10 cases in Grade 2, and 1 in Grade 3. In this study, any direct relationship between grade and stage of tumors was not observed.

Substantial lymphovascular invasion(LVI) is a significant risk factor for recurrence in endometrial carcinomas. Lymphovascular embolus poses an inverse prognostic factor and also is an independent factor for pelvic regional recurrence and overall survival rate.[54] In a study conducted by Hachisuga T et al on 303 endometrial carcinoma specimens concluded that severe degree of LVI is associated with lymph node metastasis and in turn with a higher grade.[84] In the present study, we have not compared lymphovascular invasion with disease free and overall survival rates, but we observed that lymphovascular invasion was seen in 3 cases in FIGO stage 2 and 1 case in grade 1b. Therefore, we conclude that lymphovascular tumor emboli were more common in higher stages and grades of tumors.

A study conducted by Shevra CR et al states there is a gradual and progressive increase in expression of Ki-67 levels from normal Proliferative Endometrium, hyperplastic endometrium, atypical hyperplastic proliferations to Endometrial Carcinoma. A simultaneous and corresponding increase in the levels of Ki-67 was also observed in these groups (median 22.5%, 20%, 27%, 30%, and 57.5%, respectively).[74] Another study by Truskinovsky AM et al also found a similar finding where the Ki67 (proliferative) index of atypical hyperplasia and carcinoma to be increased and significantly higher than that of non-neoplastic endometrial lesions, and complex hyperplasia without atypia.[85]

In a study conducted by Masjeed NM et al, Ki67 positivity increased as the severity of endometrial lesions increased from EH to endometrial carcinoma. Mean Ki67LI increased from 8.4% in EH without atypia to 9.8% in EHA. Ki67 positivity was seen in all cases of EC with mean Ki67LI increasing from a value of 22.52 % to 40% as the grade increased.[75] Zidan AA et al., in their study on 40 cases of EH and endometrial carcinoma found Ki67 expression increased from EH (10%) and EHA (16.7%) to endometrial carcinoma (38.9%).[17] In the study by Yu C et al, the positive expression of Ki-67 was 64.2%, higher than in normal endometrial tissue (5.7%) and atypical hyperplasia endometrial tissue (34.0%), indicating that Ki-67 expression goes up successively in normal endometrial tissue, atypical hyperplasia endometrial tissue and EC tissue, which may be related to the enhanced cell proliferation.[86]

However, the present study revealed Mean Ki-67 expression for EHs 34±21%, for EHAs 32±10%, and for ECs 31±17% which is not a statistically significant increase (P = 0.6150) as lesions progress from hyperplastic to carcinoma. For EH, the minimum expression was 1.2% and the highest expression was 75.1%. EHA expression varied from 11.5% to 42.1%. The minimum expression of 1.3% was recorded for EC cases and the maximum expression was 61.5%. Also, Ki-67 grades when compared by chi-square test for these categories was non-significant statistically(P=0.5588). Ki-67 grade 2 was most frequent for EHs(40%/N=6), EHAs(60%/N=6) and for ECs(66.6%/N=10).

The results of the present study are similar to the study conducted by Hamid AA et al, which included 12 Endometrial hyperplasia and 37 endometrial carcinoma cases. They observed a wide range of Ki-67 positivity from SH (53.3±2.1%), CH (55.5±2.1%), EHA (56±5.7%) to EC (56.7±26.9%).[87] Gurda GT et al also concluded

that the Ki-67 index does not assist in distinguishing nonatypical hyperplasia from atypical hyperplasia or atypical hyperplasia from well-differentiated carcinoma.[88]

A study by Kato K et al, conducted on 23 normal endometrial tissue, 9 endometrial hyperplasia specimens, and 60 endometrial carcinomas, found the Ki-67 LI did not differ significantly among the proliferative phase endometrium (28.1±15.6), hyperplasias (27.5±8.8), and carcinomas(28.3±2.3).[89] Another study by Tuo X et al also suggested that Ki-67 alone may not be a promising marker for differentiating malignant endometrial lesions from proliferative endometrium.[72] Present study results are comparable with these studies.

In endometrial carcinoma, there is an association between Ki-67 score and other pathological variables such as depth of myometrial invasion, stage, and grade.[66,67] Kosmas K et al also found the expression of Ki-67 in EC tissue was related to the increased staging, grade, depth of myometrial invasion, and presence or absence of lymph node metastasis, illustrating that Ki-67 expression increases with the elevation of tumor malignant degrees.[90] This study also showed an association of Ki-67 expression and histological grade, stage, and depth of myometrial invasion. Thus, Ki-67 expression may play important role in the prognosis of endometrial carcinoma.

LIMITATIONS OF PRESENT STUDY

1. The sample size was limited. If the sample size is increased better results can be obtained.
2. There were no cases of serous or type II carcinomas during the study period. So the clinicopathological and immunohistochemical profiles of these cases could not be compared.

SUMMARY

- A total of 40 cases were studied, including 15 cases of endometrial hyperplasia without atypia, 10 of endometrial hyperplasia with atypia, and 15 of Endometrial carcinoma cases.

  The mean age of the patient included in the study was 43.3 years for EH, 51.7years for EHA, and 55.8 years for EC. 90% of patients presented with AUB.

- All endometrial carcinoma cases were of endometrioid type or type 1. 66.6% of cases were presented in histological grade II. The majority of cases were found to be in stage 1A (53.3%). Lymphovascular invasion was seen in 4 cases of the higher grade of carcinoma.

- Mean Ki-67 Labelling Index was expressed as 34±21% for endometrial hyperplasia without atypia, 32±10% for endometrial hyperplasia with atypia, and 31±17% for Endometrial carcinoma. These results were not statistically significant($p=0.6150$). The majority of the cases($n=22$) presented with Ki-67 Grade 2.

- Higher Ki-67 expression was associated with higher histological grade, stage, and depth of myometrial invasion in Endometrial carcinoma. Statistically significant expression($p<0.05$) was found for these clinicopathological parameters of endometrial carcinoma.

CONCLUSION

- The findings of this study highlight that Ki-67 may not be helpful in distinguishing endometrial hyperplasia without atypia from atypical hyperplasia or endometrial hyperplasia with atypia from carcinoma.

- But Ki67 immunolabelling index can be used in conjunction with other immunohistochemical markers for proper risk stratification to aid in therapeutic intervention and proper prognostication of endometrial carcinomas.

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ANNEXURE I

WHO CLASSIFICATION OF TUMORS OF THE UTERINE CORPUS (2020)

- EPITHELIAL TUMORS AND PRECURSORS

  a) Precursors

    1) Hyperplasia without atypia

    2) Atypical hyperplasia / Endometriod intraepithelial neoplasia

  b) Endometrial carcinomas

    1) Endometriod adenocarcinoma
       - POLE-ultramutated Endometriod adenocarcinoma
       - Mismatch repair deficient Endometriod adenocarcinoma
       - P53 mutant Endometriod adenocarcinoma
       - No specific molecular profile (NSMP) Endometriod adenocarcinoma
    2) Serous carcinoma NOS
    3) Clear cell adenocarcinoma NOS
    4) Carcinoma, undifferentiated NOS
    5) Mixed cell adenocarcinoma
    6) Mesonephric adenocarcinoma
    7) Squamous cell carcinoma NOS
    8) Mucinous carcinoma, intestinal type
    9) Mesonephric-like adenocarcinoma
    10) Carcinosarcoma NOS
    11) Neuroendocrine tumors

- Low grade neuroendocrine tumor- carcinoid

- High grade neuroendocrine carcinoma- small cell and large cell neuroendocrine carcinoma

c) Tumor like lesions- polyp, metaplasias, Arias stella reaction, lymphoma like

- MESENCHYMAL TUMORS
- MIXED EPITHELIAL AND MESENCHYMAL TUMORS
- MISCELLANEOUS TUMORS
- LYMPHOID AND MYELOID TUMORS
- SECONDARY TUMORS

ANNEXURE II

TNM AND FIGO CLASSIFICATION FOR ENDOMETRIAL CARCINOMA

Primary tumor (T)		
TNM	*FIGO stages*	*Surgical-pathologic findings*
TX		Primary tumor cannot be assessed
T0		No evidence of primary tumor
Tis*		Carcinoma in situ (preinvasive carcinoma)
T1	I	Tumor confined to corpus uteri
T1a	IA	Tumor limited to endometrium or invades less than one half of the myometrium
T1b	IB	Tumor invades one half or more of the myometrium
T2	II	Tumor invades stromal connective tissue of the cervix but does not extend beyond uterus**
T3a	IIIA	Tumor involves serosa and/or adnexa (direct extension or metastasis)
T3b	IIIB	Vaginal involvement (direct extension or metastasis) or parametrial involvement
IIIC		Metastases to pelvic and/or para-aortic lymph nodes
IV		Tumor invades bladder mucosa and/or bowel mucosa, and/or distant metastases
T4	IVA	Tumor invades bladder mucosa and/or bowel mucosa (bullous edema is not sufficient to classify a tumor as T4)
***FIGO no longer includes stage 0 (Tis) **Endocervical glandular involvement should only be considered as stage I and no longer as stage II**		

Regional lymph nodes (N)		
TNM	*FIGO stages*	*Surgical-pathologic findings*
NX		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastasis
N1	IIIC1	Regional lymph node metastasis to pelvic lymph nodes
N2	IIIC2	Regional lymph node metastasis to para-aortic lymph nodes, with or without positive pelvic lymph nodes

Distant metastasis (M)		
TNM	*FIGO stages*	*Surgical-pathologic findings*
M0		No distant metastasis
M1	IVB	Distant metastasis (includes metastasis to inguinal lymph nodes, intraperitoneal disease, or lung, liver, or bone metastases; it excludes metastasis to para-aortic lymph nodes, vagina, pelvic serosa, or adnexa)

ANNEXURE III - ETHICAL CLEARANCE LETTER

K.L.E. ACADEMY OF HIGHER EDUCATION AND RESEARCH
(Deemed – to- be- University)
Accredited 'A' Grade by NAAC (2nd Cycle) Placed in Category 'A' by MHRD (GoI)
JAWAHARLAL NEHRU MEDICAL COLLEGE,
NEHRU NAGAR, BELAGAVI-590010 (KARNATAKA-INDIA)
Website: http//www.jnmc.edu
E-Mail : domc@jnmc.edu
Phone: (+ 91-(0)831 Office : 2472550
Principal: 2471701
Fax No. +91 (0)831 – 2470759

Ref: MDC/DOME/ 265 **Date: 24/12/2019**

To,
BN0119012
PG student in Pathology
J.N.Medical College,
BELAGAVI.

Sub: Institutional Ethical Clearance for the study.

With reference to the above, we wish to inform you that your proposed research project titled **"Ki-67 EXPRESSION IN ENDOMETRIAL HYPERPLASIA AND ENDOMETRIAL CARCINOMA – AN OBSERVATIONAL STUDY",** is ethical and justifiable. The proposed research project has been cleared by the JNMC Institutional Ethics Committee on Human Subjects Research.

(Dr. Anita Dalal)
Member Secretary
JNMC Institutional Ethics Committee
on Human Subjects Research,
J.N.Medical College, Belagavi.

(Dr. Roopa M Bellad)
Chairman,
JNMC Institutional Ethics Committee
on Human Subjects Research,
J.N.Medical College, Belagavi.

96

ANNEXURE IV– CONSENT FORM

INFORMED CONSENT

Ki-67 EXRESSION IN ENDOMETRIAL HYPERPLASIA AND ENDOMETRIAL CARCINOMA – AN OBSERVATIONAL STUDY

Purpose of the study: The purpose of this study is to assess effectiveness of Ki-67 expression in Endometrial Hyperplasia from Endometrial Carcinoma which will be helpful in early detection of endometrial malignant lesions and will serve as a prognostic marker.

You are being asked to enroll in this study as you are eligible for participation in this study. If you undergo endometrial curettage, biopsy or hysterectomy for endometrial hyperplasia or endometrial carcinoma, you will be included in this study.

Procedure: During this study, you will be asked questions regarding history and background and you are supposed to answer to the best of your knowledge.

If you agree to enroll yourself in this study, you will be interviewed regarding your present, past and family history and your clinical manifestations.

Risks and benefits: There are no risks involved in taking part in this study and benefit is we will be able to know a better way to assess risk of endometrial cancers which is essential for providing appropriate treatment.

Alternatives: Taking part in this study is voluntary. You may choose not to take part in this study or if you decide to take part now, you can later change your mind and

withdraw from the study. The study doctor may terminate your participation in this study anytime.

Privacy and confidentiality: All information collected about you during the course of this study will be kept confidential to the extent permitted by law. The code numbers will identify you in this research record. Information from this study will be published but your identity will be confidential in any publication. No information about you or information provided by you during research will be disclosed to other without your written permission except:

1. In emergency to protect your rights and welfare.
2. If required by law.

Financial incentives for participation: You will not be paid / offered any gift /incentives for participating in this study.

Authorization to publish results: The results of this study would be forwarded to the KAHER, Belagavi as a part of requirement towards the completion of MD degree, review and publishing.

Questions: In case you have any questions related to the study in future you can contact:

1. Principal Investigator , Department of Pathology, J.N. Medical College.
2. Guide , Professor, Department of Pathology, J.N. Medical College.
3. If you have any queries about your rights as a study subject, you may call Dr. Roopa Bellad, Professor, Department of Paediatrics, Chairman of J.N. Medical

College Institutional Ethical Committee of Human Subjects Research, Ph No-9448113403, at J.N. Medical College, Belagavi

CONSENT STATEMENT

I voluntarily agree to take part in this study by signing below. I may withdraw at any time. I am not giving up any legal rights by signing this form. My signature below indicates that I have read, or it has been read to me, this entire consent form and have had all my questions answered.

Name of the participant: (signature/thumbprint)

Name of the witness: (signature/thumbprint)

Name of the investigator: (signature)

Date:

ANNEXURE V

PROFORMA

PATIENT HISTORY

Name: Age:

IP No.: BMI:

Brief clinical history:

Obesity: Yes / No Hypertension: Yes / No Diabetes: Yes / No

OCP Intake: Yes / No Irregular Vaginal Bleeding: Yes / No Infertility: Yes / No

Family History and Past History:

PHYSICAL EXAMINATION

OTHER FINDINGS

CLINICAL DIAGNOSIS:

HISTOPATHOLOGICAL DIAGNOSIS:

1. **Hematoxylin and Eosin staining:**

Endometrial Hyperplasia:

Without Atypia / with Atypia

Endometrial Carcinoma:

Grade: Well differentiated / Moderately differentiated / Poorly differentiated

Stage: I – confined to uterus corpus

II – involves uterus and cervix

III – outside uterus but not outside true pelvis

IV – outside true pelvis or involves mucosa of bladder or rectum

Histological type: Endometriod / Non-Endometriod

Lymphovascular invasion: Present / Absent

Depth of Myometrial invasion: <50% / ≥50%

2. **IHC staining:**

Percentage of positively stained nuclei by Ki-67:

ANNEXURE VI

HEMATOXYLIN AND EOSIN STAINING PROTOCOL

Bancroft D, Laylon C. The haematoxylin and eosin, In: Kim SS Ed, Bancroft's Theory and practice of histopathological techniques. 8th Ed., Churchill Livingstone; 2019: p173-87.

1. Deparaffinize in Xylene I and II and III changes. (III change use warmed xylene) (5 minutes in each)
2. Rehydrate using
    a. Absolute ethanol 100% (5 minutes)
    b. Absolute Ethanol 100% (5 minutes)
3. Rinse in distilled water (5 minutes)
4. Rinse in running tap water (5 minutes)
5. Stain in Harris's haematoxylin by progressive method (2 minutes) Fresh and filtered
6. Rinse in running tap water (20 minutes)
7. Decolorize in 1% acid alcohol (1 second)
8. Rinse well in tap water (5 minutes)
9. Immerse in hot water bath, 55˚C for blueing (3 seconds)
10. Rinse in tap water (5 minutes)
11. Counterstain in Eosin (15 seconds)
12. Dehydrate with absolute alcohol 100% (2-4 dips)
13. Clear in xylene I and II (5 minutes)
14. Mount with DPX.

Stock solution – Eosin:

Stock – 1% aqueous Eosin – Y

Stock – 1% aqueous Phloxin B

Working Solution – Eosin:

100ml stock Eosin

10 ml stock Phloxin B

780 ml 95% Ethanol

4 ml glacial acetic acid

Working Solution – Hematoxylin

Harris Hematoxylin, 1 litre

Working solution – 0.25% Acid alcohol

95% Ethanol, 2578 ml

dH2O, 950 ml

HCl, 9ml

Result: Nuclei – blue, cytoplasm – pink, RBCs – red.

PROCEDURE FOR IHC

1. Cut the sections at approximately 4 µm thickness in poly L Lysine coated slides.
2. Float on to the positive charged slides.
3. Slides were air dried overnight at 60 ºC.
4. Two changes of xylene of 10 minutes each for deparaffinization.
5. Hydration.
    - Absolute alcohol – 2dips
    - 80% alcohol -- 2dips
    - 70% alcohol -- 2dips
    - Distilled water -- 2 changes 5 minutes each.
6. Antigen retrieval by heat, using microwave using TRIS EDTA Buffer.
7. Cooling of sections to room temperature.
8. Rinse in distilled water for 3 minutes.
9. Wash in TBS buffer two times for 3 minutes each.
10. Treatment with peroxide block for 10 minutes to block endogenous peroxidase.
11. Wash in TBS buffer two times for 3 minutes each.
12. Treatment with primary antibody (Vitro Master diagnostica, Rabbit Anti-Human Ki-67 Monoclonal Antibody, Clone SP6) for 60 minutes
13. Wash in TBS buffer two times for 3 minutes each
14. Treatment with Target binder for 10 minutes
15. Wash in TBS buffer two times for 3 minutes each
16. Treatment with HRP Polymer for 10 minutes
17. Wash in TBS buffer two times for 3 minutes each

18. Treatment with DAB (secondary antibody) for 3-5 minutes to give brown colour to antigens
19. Wash in distilled water for 3 minutes
20. Counter stain with Harris haematoxylin for 30 seconds to 1 minute
21. Wash in tap water for 3 minutes to remove excess stain
22. Two changes of absolute alcohol for 2 minute each for dehydration
23. Clearing with xylene for two minutes. Dry the slides and mount with DPX

Preparation of reagents

1. Antigen retrieval Buffer

TRIS EDTA Buffer- pH: 8.0 to 8.5

Preparation:

TRIS Base- 1.21 gram

EDTA (atomic number:372)- 0.37 gram

Dissolve in 1000ml of water

2. Wash buffer

TRIS BUFFERED SALINE (TBS)-pH: 7.2 to 7.6

Preparation:

TRIS Base- 8.6 gram

NaCl- 9.6 gram

Dissolve in 1000ml of water.

Adjust pH by using concentrated HCl

ANNEXURE VII - PICTOMICROGRAPHS

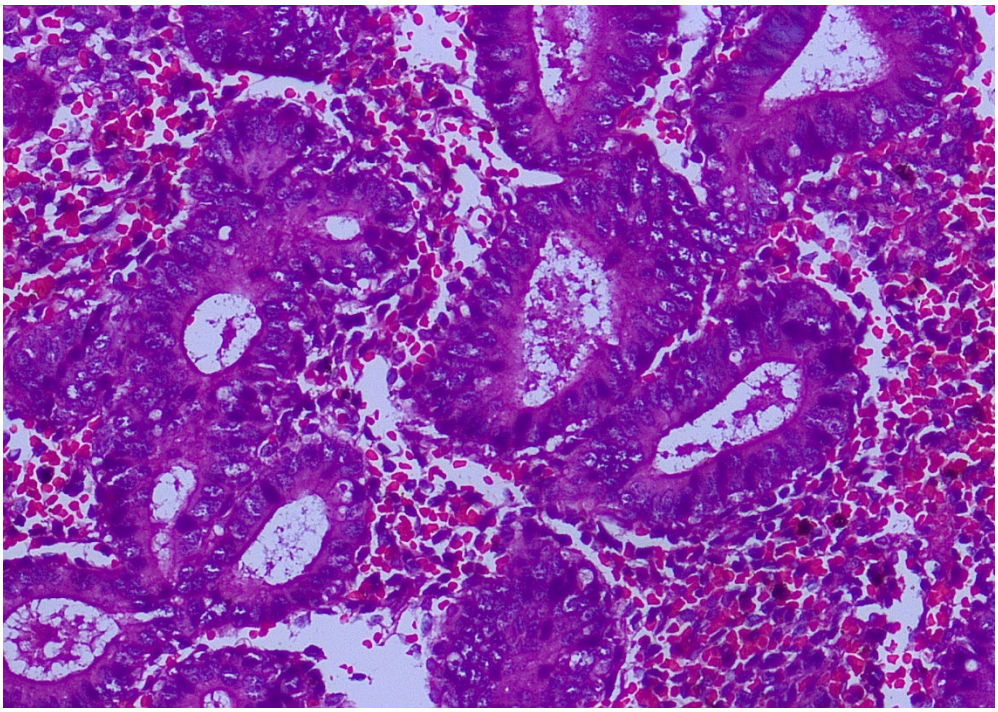

Fig 1 : Endometrial Hyperplasia without atypia. {H & E, 20X}

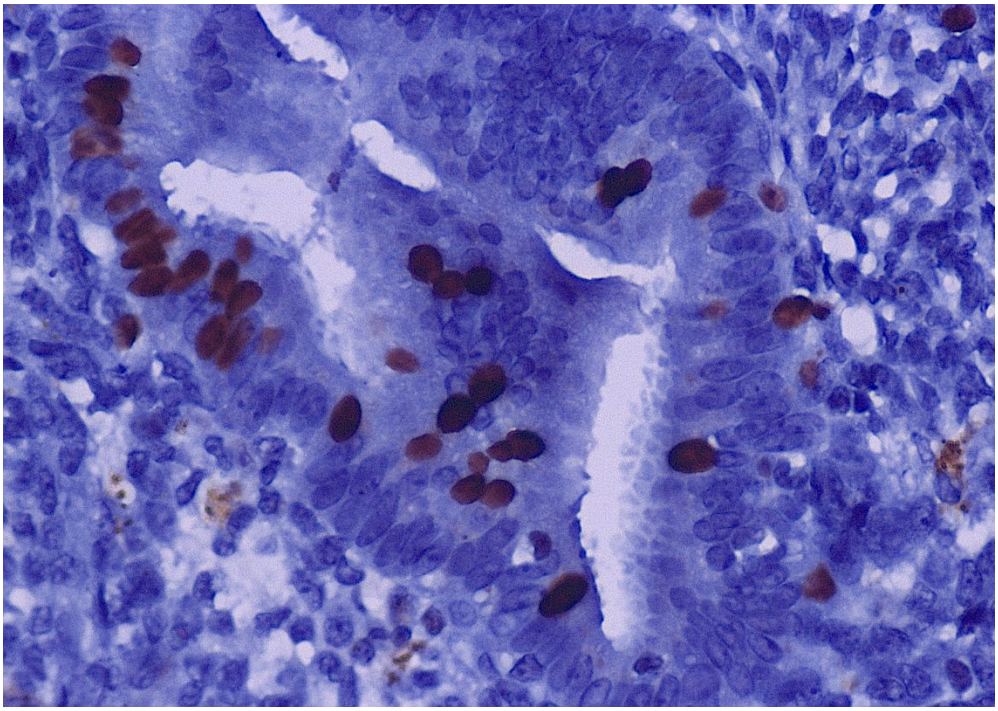

Fig 2 : Endometrial Hyperplasia without atypia staining with Ki-67 {IHC, 40X}

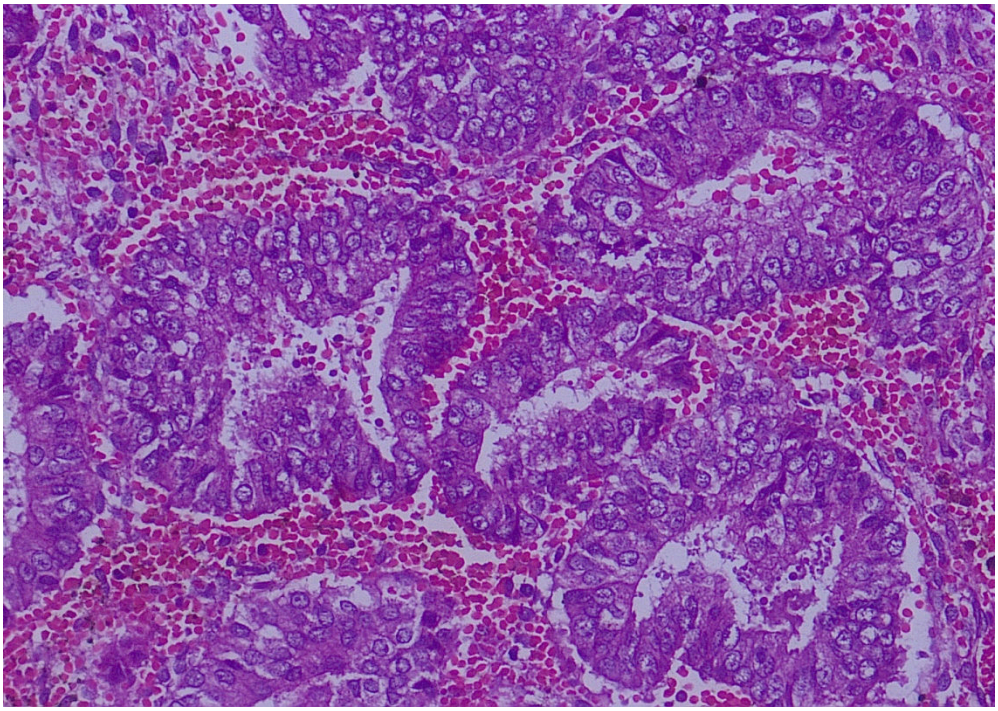

Fig 3 : Endometrial Hyperplasia with atypia. {H & E, 20X}

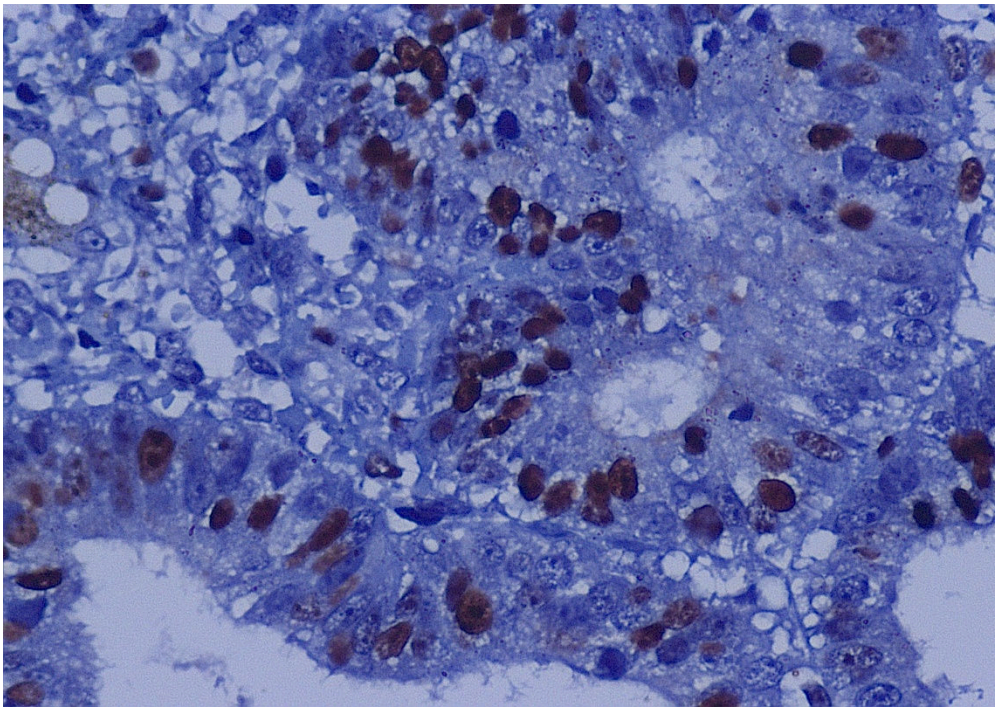

Fig 4 : Endometrial Hyperplasia with atypia staining with Ki-67 {IHC, 40X}

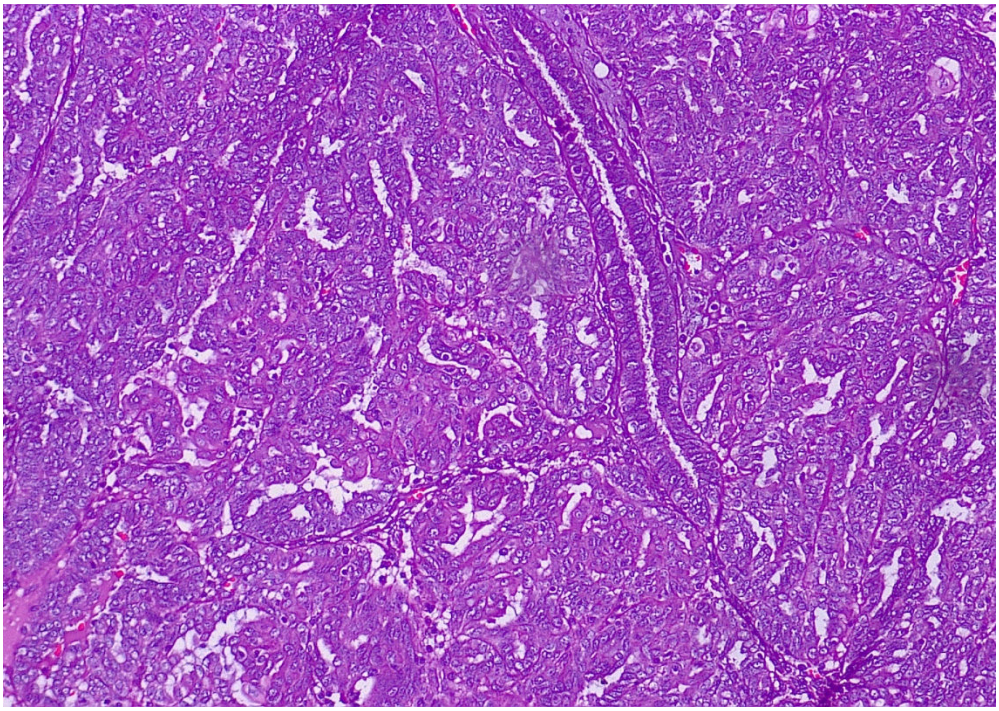

Fig 5 : Well differentiated (Grade I) endometrioid carcinoma. {H & E, 10X}

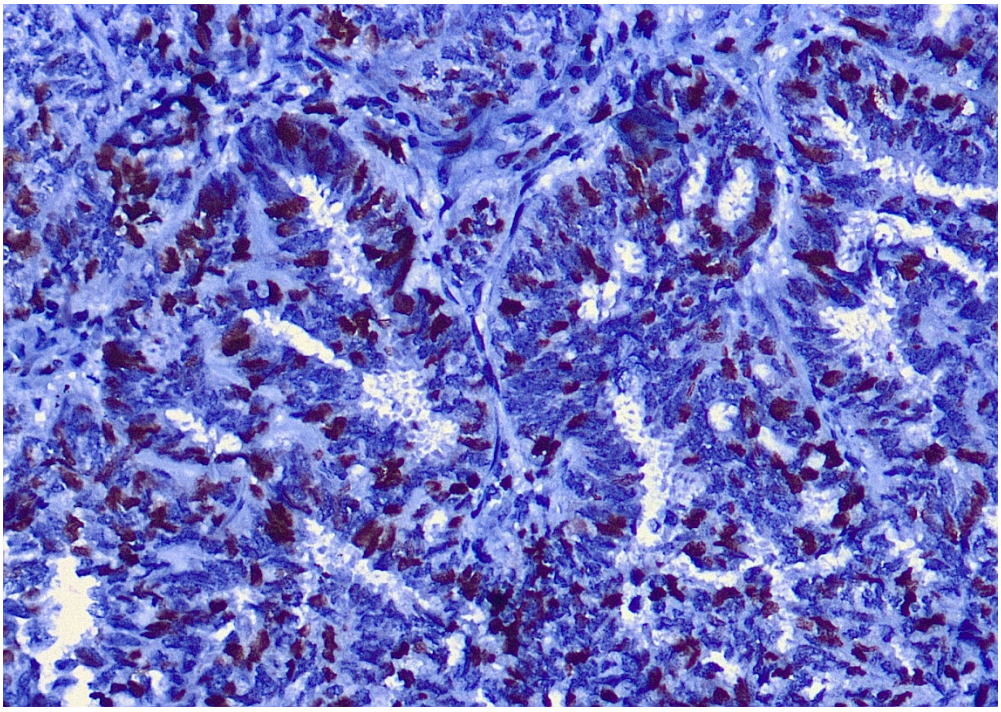

Fig 6: Well differentiated (Grade I) endometrioid carcinoma. {Ki-67, 20X}

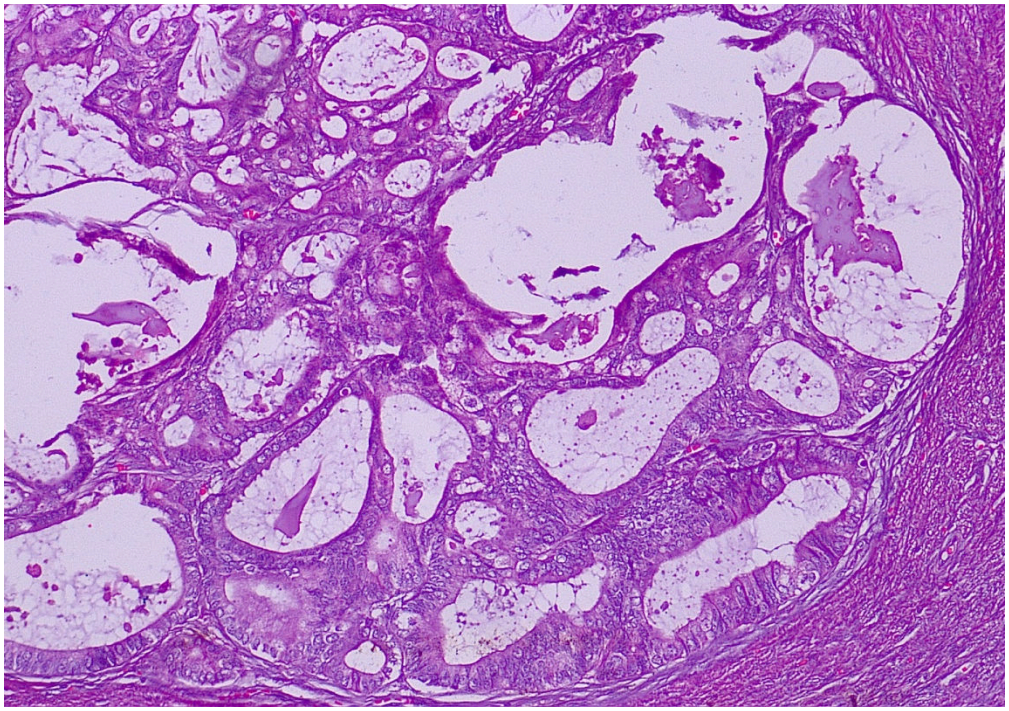

Fig 7 : Moderately differentiated (Grade II) endometrioid carcinoma.{H&E, 10X}

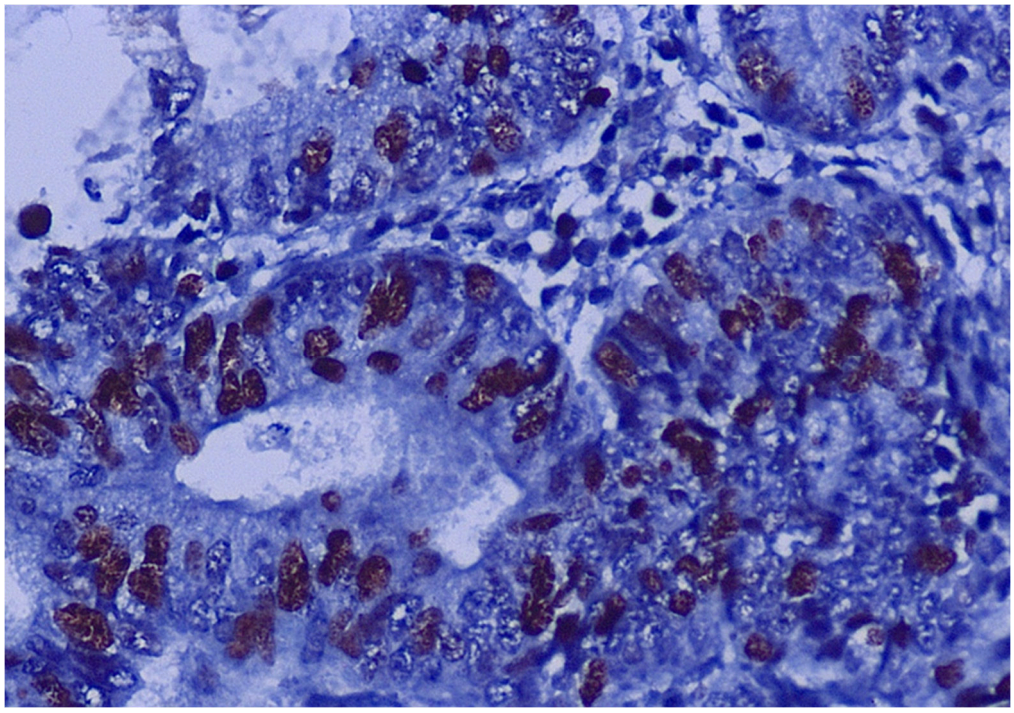

Fig 8 : Moderately differentiated (Grade II) endometrioid carcinoma.{Ki-67, 40X}

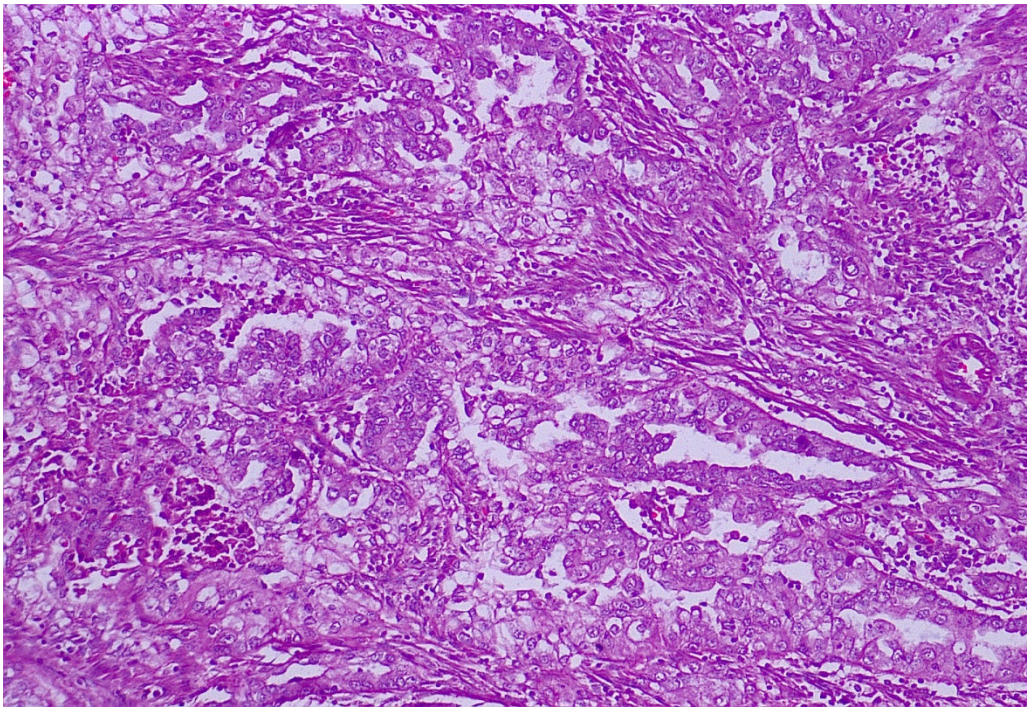

Fig 9 : Poorly differentiated (Grade III) endometrioid carcinoma. {H & E, 10X}

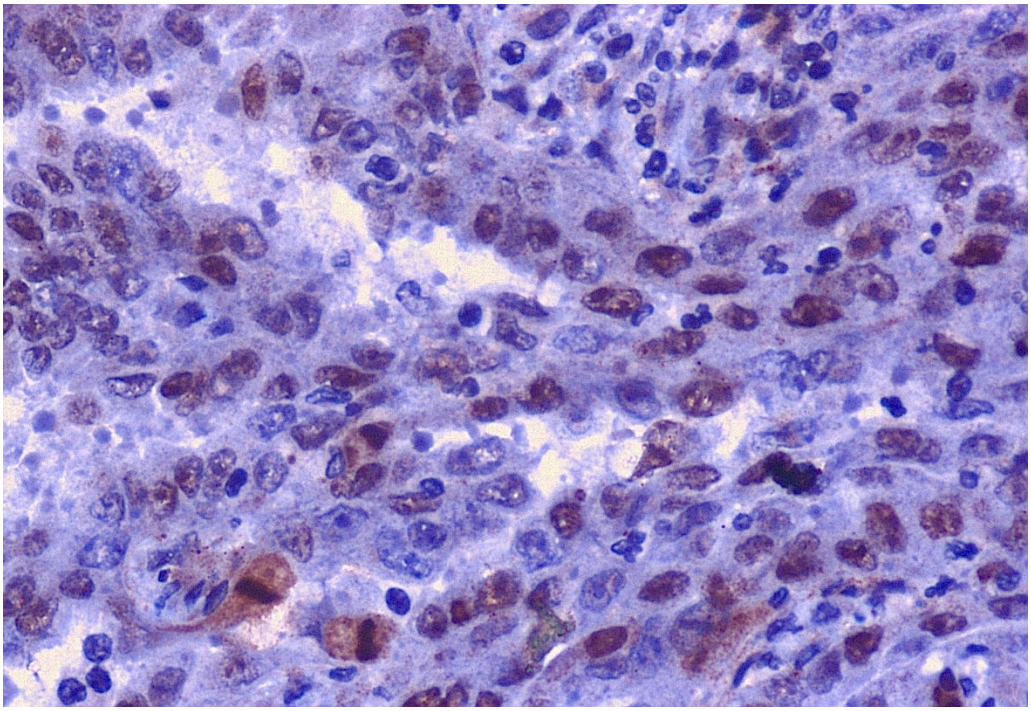

Fig 10 : Poorly differentiated (Grade III) endometrioid carcinoma. {Ki-67, 40X}

ANNEXURES VIII

KEY TO MASTER CHART

D&C	–	Dilatation and Curettage
TAH	–	Total abdominal hysterectomy
TLH	–	Total Laparoscopic Hysterectomy
H&E	–	Histopathological Report using Hematoxylin and Eosin
EH	–	Endometrial Hyperplasia without atypia
EHA	–	Endometrial Hyperplasia with atypia
EC	–	Endometrial carcinoma
EC with SD	–	Endometrial carcinoma with squamous differentiation
Y	–	Present
N	–	Absent

ANNEXURES IX - MASTER CHART

No.	Age	Specimen	History	H&E	Ki-67	Ki-67 Grade
1	46	D&C	AUB	EH	6.90%	1
2	43	D&C	AUB, HTN with IHD	EH	21.60%	2
3	26	D&C	Polymenorrhea	EH	1.20%	1
4	44	D&C	Menorrhagia	EH	50.40%	3
5	44	D&C	Menorrhagia	EH	24.60%	2
6	45	D&C	AUB	EH	54.50%	3
7	49	D&C	Menorrhagia	EH	39.70%	3
8	51	D&C	AUB, DM, Hypothyroidism	EH	35.10%	2
9	52	D&C	Post Menopausal Bleeding	EH	18.80%	2
10	31	TAH	Metrorrhagia	EH	34.80%	2
11	42	D&C	PCOD, Hypothyroidism, Primary Infertility	EH	54.60%	3
12	57	TAH	AUB, DM, Obesity	EH	30.70%	2
13	33	D&C	Primary Infertility on treatment	EH	51.10%	3
14	50	D&C	AUB, DM, HTN, Hyperthyroidism	EH	75.10%	4
15	37	D&C	Menstrual Irregularity	EH	8.80%	1
16	43	D&C	AUB, HTN	EHA	27.90%	2
17	74	D&C	Postmenopausal Bleeding, DM, Obesity	EHA	35.60%	2
18	55	TAH	AUB, Hypothyroidism	EHA	11.50%	2
19	62	D&C	Postmenopausal Bleeding	EHA	24.70%	2
20	34	D&C	Metrorrhagia	EHA	39.80%	3
21	78	D&C	Postmenopausal Bleeding,DM, HTN, Obesity	EHA	24.10%	2
22	45	D&C	AUB, Obesity	EHA	36.50%	2
23	50	D&C	Menometrorrhagia, H/O Progesterone pill	EHA	40.80%	3
24	36	D&C	Menometrorrhagia	EHA	39.80%	3
25	40	TAH	AUB	EHA	42.10%	3

No.	Age	Specimen	History	H&E	Ki-67	Ki-67 Grade	Histological Grade	FIGO	Depth of Infiltration	PNI	LVI	LNM
26	60	TAH	AUB	EC	1.30%	1	I	IA	<50 of myometrium	N	N	N
27	68	TAH	AUB, HTN	EC	33.80%	2	II	IB	>50 of myometrium	Y	Y	N
28	55	TAH	Post Menopausal Bleeding	EC	25.50%	2	I	IA	<50 of myometrium	N	N	N
29	65	TAH	AUB, DM, Obesity	EC	47%	3	II	IA	<50 of myometrium	N	N	N
30	55	TAH	Post Menopausal Bleeding	EC	38.70%	2	II	IB	>50 of myometrium	Y	N	N
31	43	TLH	Metrorrhagia	EC	61.50%	3	II	II	>50 of myometrium, cervix	Y	Y	N
32	54	TLH	Nulligravida, Obesity, Postmenopausal Bleeding, DM, Hypothyroidism	EC	24.40%	2	II	IA	<50 of myometrium	N	N	N
33	74	D&C	Post Menopausal Bleeding, DM	EC with SD	13.60%	2	II	IA	<50 of myometrium	N	N	N
34	40	TLH	AUB, Fibroids, H/O Prosterone pill	EC	14.50%	2	I	IA	<50 of myometrium	N	N	N
35	55	D&C	Post Menopausal Bleeding	EC	31.90%	2	III	IB	>50 of myometrium	Y	N	N
36	65	TLH	AUB, Fibroids	EC	3.00%	1	I	IA	<50 of myometrium	N	N	N
37	52	TAH	AUB, Hypothyroidism, Obesity	EC	33.20%	2	II	IB	>50 of myometrium	Y	N	N
38	65	TAH	AUB, DM, HTN	EC with SD	18.10%	2	II	IA	<50 of myometrium	N	N	N
39	45	TAH	AUB	EC	46.00%	3	II	II	>50 of myometrium, cervix	Y	Y	N
40	42	TAH	AUB, Hypothyroidism	EC	25%	2	II	II	>50 of myometrium, cervix	Y	Y	N