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सेवा में,

The Dean (Academics)
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Jodhpur

Subject: Submission of M.D thesis.

आदरणीय महोदय,

This is to submit that the M.D thesis by the Academic Junior Resident (July 2020 Batch) of our Department has been duly completed and signed and is ready for submission. Please accept the same.

Details of her thesis are attached herein:

Name of candidate	Thesis topic
Dr. Sangeeta Pradhan	Expression of immunohistochemical marker INSM1 and its comparison with traditional markers in Neuroendocrine tumors along with comparison of PHH3 and Ki67 labelling index

धन्यवाद,

भवदीया

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Expression of immunohistochemical marker INSM1 and its comparison with traditional markers in Neuroendocrine tumors along with comparison of PHH3 and Ki67 labelling index

THESIS

Submitted to

All India Institute of Medical Sciences, Jodhpur

In partial fulfillment of the requirement for the degree of

DOCTOR OF MEDICINE (MD)

PATHOLOGY

JULY, 2020

AIIMS, JODHPUR **Dr. SANGEETA PRADHAN**

All India Institute of Medical Sciences, Jodhpur

DECLARATION

I hereby declare that the thesis titled "**Expression of immunohistochemical marker INSM1 and its comparison with traditional markers in Neuroendocrine tumors along with comparison of PHH3 and Ki67 labelling index**" embodies the original work carried out by the undersigned in All India Institute of Medical Sciences, Jodhpur

Sangeeta Pradhan

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CERTIFICATE

This is to certify that the thesis entitled **"Expression of immunohistochemical marker INSM1 and its comparison with traditional markers in Neuroendocrine tumors along with comparison of PHH3 and Ki67 labelling index"** is the bonafide work of **Dr. SANGEETA PRADHAN** carried out under our guidance & supervision, in the Department of Pathology & Lab Medicine, All India Institute of Medical Sciences, Jodhpur.

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Dr. Sangeeta Pradhan

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SYNOPSIS

In the present study, we assessed the expression of Insulinoma-associated protein 1(INSM1) in Neuroendocrine tumors by immunohistochemistry and compared its expression with traditional neuroendocrine markers like synaptophysin, chromogranin A and CD56. Phosphohistone protein 3(PHH3) labelling index was also compared with Ki67 labelling index. INSM1 and PHH3 LI were applied in all 152 cases of neuroendocrine tumors. INSM1 intensity was graded as weak, moderate and strong. The percentage of tumor cells staining for INSM1 was also evaluated. PHH3 LI was compared with Ki67 labelling index. Congo red stain was applied to highlight stromal amyloid deposits.

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
ADK	Adenosine kinase
ATRX	Alpha-thalassemia/mental retardation, X-linked
CGA	Chromogranin A
CNS	Central nervous system
CoGNET	Composite Gangliocytoma/neuroma-Neuroendocrine Tumor
DAXX	Death domain associated protein-6
DCG	Dense core granules
DFS	Disease free survival
ENETS	European Neuroendocrine Tumor Society
G1	Grade 1
G2	Grade 2
G3	Grade 3
GEP-NEN	Gastro-entero-pancreatic neuroendocrine neoplasms
GU-GHNEC	Genitourinary high grade neuroendocrine carcinoma
HE	Hematoxylin and eosin
HGNEC	High grade neuroendocrine carcinoma
IBC	Invasive breast carcinoma
IHC	Immunohistochemistry
INSM1	Insulinoma associated protein 1
IQR	Interquartile range
LCNEC	Large cell neuroendocrine carcinoma
LI	Labelling index
MAI	Mitotic activity index
MANEC	Mixed adenoneuroendocrine carcinoma
MEN-1	Multiple endocrine neoplasia-1

MF	Mitotic figure
MiNEN	Mixed neuroendocrine non-neuroendocrine neoplasm
MRM	Modified radical mastectomy
NE	Neuroendocrine
NEC	Neuroendocrine carcinoma
NEN	Neuroendocrine neoplasm
NET	Neuroendocrine tumor
NPV	Negative predictive value
OSCC	Oral squamous cell carcinoma
PHH3	Phosphohistone 3
PI3K	Phosphoinositide-3-kinase
PPV	Positive predictive value
RB	Retinoblastoma
RBC	Red blood cell
SEER	Surveillance, Epidemiology, and End Results
SCLC	Small cell lung carcinoma
SCNEC	Small cell neuroendocrine carcinoma
SD	Standard deviation
SSTR	Somatostatin receptor
SYP	Synaptophysin
TC	Typical carcinoid
TP53	Tumor protein 53
TURBT	Transurethral resection of bladder tumor
WDHA	Watery diarrhea, hypokalemia, and achlorhydria
WHO	World Health Organization

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INTRODUCTION

The neuroendocrine system implies the cells have features of both nerve cells and endocrine cells. The nerve cell feature is based on the identification of dense core granules (DCGs) which store monoamines. The endocrine property refers to the synthesis and secretion of these monoamines, peptides and hormones. (1)

The neuroendocrine cell system is divided mainly into two cell types group. The first group includes cell types that comprises of pituitary (adenohypophysis), the parathyroids, the paraganglia and the adrenal medulla. The second group includes the disseminated neuroendocrine cell types found scattered in the exocrine parenchyma, such as endocrine cells of the digestive tract, skin, thyroid, lung, thymus, pancreas, gastrointestinal tract, biliary tract and urogenital tract.(2)

The early history of neuroendocrine (NE) cells dates 40 years back, when Feyrter described a system of clear cells (Helle-Zellen) scattered in the epithelia of various organs. Further studies by Frohlich and Feyrter described these clear cells as argyrophilic. In the mid-1960s, Bensch et al., using electron microscopy, described these cells with cytoplasmic neurosecretory granules as Kultschitzky cells and suggested that bronchial carcinoids and oat-cell carcinomas are derived from these cells.(3)

Neuroendocrine tumors account for 0.5% of all malignancies. The incidence is approximately 2/100,000 with a female preponderance under the age of 50 years. The primary sites are the gastrointestinal tract (62-67%) and the lung (22-27%). 12–22% of neuroendocrine tumors present with metastatic disease.(1)

The nuclear marker for neuroendocrine differentiation of tumor cells is insulinoma-associated protein 1 **(INSM1)** which is a transcriptional regulator with a zinc-finger DNA-binding domain.(4) It is abundantly displayed in fetal pancreas and neuroendocrine tumors.(5) INSM1 is not expressed in non -neuroendocrine tumors. This transcription factor is generated from an intronless gene located on chromosome 20p11.2. The amino acid region between positions 167 and 262 at the N-terminus is responsible for its transcriptional activity.(4)

Various studies have proven that INSM1 has higher sensitivity compared to that of synaptophysin and CD56, and specificity compared to that of chromogranin A.(6)

Since INSM1 is highly expressed in tumors of neuroendocrine origin, it has been suggested that its promoter may be used for targeted therapy in NETs. Lilo et al. described use of INSM1 immunohistochemistry to improve the detection of sentinel lymph node metastases.(7)

Various literature mentions that INSM1 is a better marker for identification of high grade pulmonary neuroendocrine tumors as its sensitivity and specificity is higher than traditional markers. However, studies showing expression of INSM1 in extra-pulmonary neuroendocrine tumors are limited.

Ki 67 is a marker for cell proliferation which is used for grading of the tumor. Ki-67 is categorized to grades G1 (≤2 %), G2 (3– 20 %), or G3 (>20 %) according to the European Neuroendocrine Tumor Society (ENETS) guidelines and the 2010 World Health Organization (WHO) classification.(8) The major limitation of Ki67 immunohistochemistry is possible nonspecific staining in apoptotic cells.

Phosphohistone H3 (PHH3), a recent mitosis specific marker is a core histone protein in the chromatin of eukaryotic cells and in mammalian cells. PHH3 is negligible during interphase but reaches the maximum level during chromatin condensation in mitosis.

Anti-PHH3 is highly specific for phosphorylated histone H3, thus serving as a specific marker for mitosis.(9)

PHH3 may have advantage over Ki67 as it does not highlight cells undergoing cell death or apoptosis. This gives PHH3 an advantage over Ki67 index and routine H&E mitotic count as it decreases the possibility of misidentified apoptotic figures, thus making it a better tool to assess grading in NETs.(10)

PHH3 has been studied in other tumors where mitotic count is important for classification, such as meningioma, melanoma, and astrocytoma. However, the investigation of PHH3 in the setting of NETs has been limited.(11) PHH3 can be used to predict prognosis in patients with neuroendocrine tumors.(12)

REVIEW OF LITERATURE

Incidence of Neuroendocrine tumors:

The incidence of NETs was 0.244 per 100,000 in 1996 and increased to 3.162 per 100,000 in 2015. (13)Worldwide, nine thousand one hundred twenty patients were diagnosed with neuroendocrine tumor between 2010 and 2015, of which 42.25% of the patients were females, while 57.75% were males, and mean age at diagnosis was 62.58 years.(14)

The rising incidence of NETs is due to the increased awareness of NETs by the physicians and radiological investigations.(13)

Etiological factors

Most NETs occur sporadically, regardless of disease site. However, a positive family history of cancer is associated with the risk of developing NETs which did not arise in the context of other hereditary syndromes. The hereditary conditions that cause NETs are multiple endocrine neoplasia type 1(MEN-1), von Hippel-Lindau syndrome, neurofibromatosis type 1, tuberous sclerosis, and nonpolyposis colon cancer. NETs are seen most frequently in patients with MEN1 syndrome, which is an autosomal dominant disorder characterized by parathyroid hyperplasia, pituitary adenomas, and pancreatic tumors. The occurrence of familial NETs not associated with hereditary syndromes is rare as most NETs occur as non-familial (sporadic) tumors.(15)

Smoking and alcohol consumption were not associated with NETs in either men or women. However, neuroendocrine tumors of lung particularly small cell carcinoma are associated with heavy smokers. Gastric NETs has been seen in women with diabetes. (16)

Clinical signs and symptoms

Neuroendocrine tumors (NETs) are a unique group of malignant growth, known for their ability to secrete bioactive peptides. The tumor may be found as an incidental finding or may be suspected from clinical symptoms. When NETs cause clinical symptoms due to hormone secretion, they are termed “functioning”.

Some symptoms may indicate the diagnosis and location of a NET. Small intestinal NETs may cause extensive fibrosis, resulting in recurrent abdominal pain secondary to small bowel obstruction or mesenteric ischemia.

Bronchopulmonary NETs tend to present with centrally located lesions that may result in bronchial obstruction, recurrent obstructive pneumonitis, cough and hemoptysis. Bronchopulmonary NETs may be a source of ectopic adrenocorticotrophic hormone (ACTH) production, leading to cushing syndrome.(17)

Table 1: Functional neuroendocrine tumor syndromes:

Tumor	Tumor location	Hormone	Symptoms and signs	Syndrome
Atypical carcinoid	Foregut	5-HTP, histamine	Pruritus, cutaneous wheals, bronchospasm	Atypical carcinoid
Carcinoid	Small intestine, lung (<5%), pancreas (< 1%)	Serotonin, tachykinin, prostaglandins	Flushing, diarrhea, valvular disease, bronchospasm	Carcinoid
Insulinoma	Pancreatic β cells	Insulin, proinsulin	Hypoglycemic symptoms	Whipple triad
Gastrinoma	Gastrinoma triangle†	Gastrin	Diarrhea, peptic ulcer disease	Zollinger–Ellison
Glucagonoma	Pancreatic α cells	Glucagon	Diabetes, deep vein thrombosis, depression, dermatitis (necrolytic migratory erythema)	4D syndrome
Somatostatinoma	Pancreatic δ cells	Somatostatin	Diabetes, cholelithiasis, steatorrhea, weight loss, achlorhydria	Somatostatinoma
VIPoma	Non-β islet cells	Vasoactive intestinal peptide	Watery diarrhoea (profuse), hypokalemia, achlorhydria	Verner–Morrison (WDHA syndrome)

Table 2: Presenting symptoms for neuroendocrine tumor:

Symptom	Percentage of cases
Gastro-entero-pancreatic:	
Abdominal pain	28–79
Bowel obstruction	18–24
Diarrhea	10–32
Carcinoid heart disease	8–19
Flushing	4–25
Gastrointestinal bleed	4–10
Bronchopulmonary:	
Cough	5–27
Hemoptysis	23–32
Recurrent infection	41–49

General characteristics of Neuroendocrine Neoplasms in digestive system:

Neuroendocrine neoplasms can arise in most epithelial organs of the body and can have varied etiology, clinical features, morphological and genomic findings and outcomes. Historically, NETs of various anatomical sites have been classified separately and thus have caused considerable confusion. In 2018, WHO published a uniform classification framework for all neuroendocrine neoplasms. This novel system published two new categories: neuroendocrine tumors that are well-differentiated (NETs) which were initially described as carcinoid tumors of the gastrointestinal tract and a second category for neuroendocrine carcinomas (NECs) that are poorly differentiated which have a poor prognosis. The classification of NENs into NETs and NEC is supported by genetic evidence as well as clinical, epidemiological, histological and prognostic differences. (18)

Table 3: Classification and grading criteria for Neuroendocrine Neoplasms:

Terminology	Differentiation	Grade	Mitotic rate (mitosis/2 mm^2)	Ki-67 index
NET,G1	Well differentiated	Low	<2	<3%
NET,G2	Well differentiated	Intermediate	2-20	3-20%
NET,G3	Well differentiated	High	<20	>20%
NEC, small cell type (SCNEC)	Poorly differentiated	High	<20	>20%
NEC, large cell type (LCNEC)	Poorly differentiated	High	<20	>20%
MiNEN	Well or poorly differentiated	Variable	Variable	Variable

Well differentiated NENs: NETs

Neuroendocrine tumors (NETs) are well differentiated epithelial neoplasms with morphological and immunohistochemical features of neuroendocrine differentiation. The cells resemble the non-neoplastic neuroendocrine cells. They show characteristic histological pattern including nests, cords, ribbons and organoid architecture with uniform nuclei and coarsely granular chromatin. The cytoplasm show intensely granularity, reflecting abundant neurosecretory granules. NETs can be low grade (G1), intermediate grade (G2, or high grade (G3).

Poorly differentiated NENs: NECs

NECs can be small cell NEC, which shows fusiform nuclei with finely granular chromatin, scant cytoplasm and nuclear moulding, or large cell NEC which has round nuclei, prominent nucleoli and moderate amount of cytoplasm. All NECs are high grade neoplasms. For gastrointestinal tract, the differentiation between NET and NEC has improved due to new molecular genetic insights. In NEC, which is most aggressive

forms of NEN, p53 and retinoblastoma protein 1 (RB1) have turned out to be important biomarkers. Aberrant p53 expression (TP53 inactivation) and the loss of RB are features of pancreatic and gastrointestinal NEC.(18)

Mixed neoplasms: MiNENs

In 2010, mixed neoplasms from the gastrointestinal tract containing a neuroendocrine and an exocrine component, where each of them are present in at least 30% of the tumor mass and being malignant, were classified by the World Health Organisation (WHO) as separate entities and named "mixed adeno-neuroendocrine carcinomas" (MANECs). In 2017, the WHO renamed MANECs, as "mixed neuroendocrine non-neuroendocrine neoplasms" (MiNENs), where "exocrine" was substituted by the more general term "non-neuroendocrine". It was done to include histological variants that cannot be referred to as exocrine (e.g., squamous or sarcomatoid phenotypes), and the term "carcinoma" was substituted by the term "neoplasm" to recognise the fact that occasionally, one or both components are low-grade malignant. (18)

Neuroendocrine tumor of lung:

This classification of NETs is based on macroscopic, microscopic and immunohistochemical features. Therefore, the mitotic activity (mitosis/2 mm^2) as well as the rosette-like structure, palisading, trabecular pattern, organoid nesting and necrosis are common characteristics of pulmonary neuroendocrine tumors. Neurosecretory granules can be demonstrated by electron microscopy.(19)

The neuroendocrine tumor include typical carcinoid, atypical carcinoid, small cell lung carcinoma (SCLC), and large cell NE carcinoma (LCNEC).(20)

Table 4: Criteria for diagnosis of neuroendocrine tumor in lung:

Typical carcinoid	• A tumor with carcinoid morphology and <2mitosis/2mm^2, lacking necrosis and more than equal to 0.5cm.
Atypical carcinoid	• A tumor with carcinoid morphology and 2-10 mitosis/2mm^2 and/or necrosis.
Large cell neuroendocrine carcinoma	• A tumor with a neuroendocrine morphology (organoid nesting, palisading, rosettes, trabeculae) • High mitotic rate: >10 mitosis/2mm^2. • Necrosis (often in large zones) • Cytological features of a non-small cell carcinoma: large cell size, low nuclear-to-cytoplasmic ratio, vesicular, coarse to fine chromatin. • Positive immunohistochemical staining for one or more neuroendocrine markers (other than neurone specific enolase) and/or neuroendocrine granules by electron microscopy.
Small cell carcinoma	• Small size (less than diameter of 3 resting lymphocytes) • Scant cytoplasm • Nuclei: finely granular nuclear chromatin, absent or faint nucleoli • High mitotic rate: >10 mitosis/ 2mm^2. • Frequent necrosis (often in large zones)

HIGHLIGHTS OF WHO 2022 UPDATE:

The 2022 WHO Classification of Endocrine Tumors has, for the first time, included NENs of non-endocrine organs. In the pancreas, there are only a few changes like the change of terminology used for small lesions<0.5 cm which were previously called “microadenoma” but the current classification recommends “neuroendocrine

microtumor". The new section on NENs in non-endocrine organs includes a broad discussion of well-differentiated neuroendocrine tumors (NETs) that occur in the gastrointestinal tract, lung, upper airways, urogenital system, breast, and skin. While these are not always the source of clinically relevant hormone excess, they can be associated with hereditary syndromes and thus may be multifocal disorders, both within a given organ/system or in other sites.

Two unusual tumors that were initially thought to be paragangliomas are now reclassified as "paraganglioma-like" NENs. The tumor that occurs mainly in the duodenum and was formerly known as "gangliocytic paraganglioma" is now been recognized as a composite gangliocytoma or ganglioneuroma with an epithelial duodenal NET; this lesion has been renamed "composite gangliocytoma/neuroma and neuroendocrine tumor," abbreviated as "CoGNET". Similarly, the tumor previously known as "cauda equina paraganglioma" is now recognized to be an epithelial NET and has been reclassified as "cauda equina neuroendocrine tumor. The tools available to pathologists for accurate classification include the conventional biomarkers of neuroendocrine lineage and differentiation like INSM1, synaptophysin, chromogranin, and somatostatin receptors (SSTRs), and also include transcription factors that can identify the site of origin of a metastatic lesion of unknown primary site. The recognition of highly proliferative, well-differentiated NETs has resulted in the need for biomarkers that can distinguish these G3 NETs from NECs, including stains to determine expression of SSTRs. Global loss of RB and aberrant p53 in pancreatic NECs compared with loss of ATRX, DAXX, and menin has been seen in pancreatic NETs. The concepts of mixed neuroendocrine and non-neuroendocrine (MiNEN) and amphicrine tumors are clarified with information about how to approach these lesions in routine practice.

Immunohistochemistry:

In diagnosis of neuroendocrine tumors, IHC may be required for confirmation of epithelial and neuroendocrine nature of NETs. Gastrointestinal NETs express synaptophysin (usually diffuse and strong) and chromogranin A (usually more focal and apical). They also express neuron-specific enolase and CD56. Functional NETs may express hormones like gastrin, insulin, glucagon, PP and somatostatin.

Chromogranin A (CgA) and synaptophysin are currently considered the most specific immunohistochemical markers for NENs.

CgA is an acidic glycoprotein of the granin family, being expressed in NENs and tends to be only focally positive in PD-NECs/SC-NEC. PD-NECs do not react strongly with CgA antibodies. CgA may have limited sensitivity with some tumors, such as hindgut carcinoids (originating from left transverse colon to distal colon, rectum, and anus) and is found to stain only few cases.

Synaptophysin is a membrane glycoprotein, representing a good marker of neuroendocrine cells with a diffuse cytoplasmic immunostaining. (21).

INSM1 is another marker expressed in NETs. In this study, evaluation of expression of IHC marker INSM1 and comparing it with traditional markers (Chromogranin A, Synaptophysin) in neuroendocrine tumors was done.

STRUCTURE AND FUNCTION OF INSULINOMA-ASSOCIATED PROTEIN 1(INSM1):

Insulinoma-associated protein 1(INSM1) is a zinc-finger transcription factor and the protein structure of INSM1 is highly conserved among homologues of different species. INSM1 (formerly IA-1) contains five zinc-finger motifs. Based on the deduced protein sequence, INSM1 can be divided into two major domains. The major amino terminal domain (aa 1-250) that contains a high percentage of proline, glycine, and alanine residues. Proline rich (20-30%) sequences occur in mammalian transcription factors and serve as protein-protein interacting domains that mediates both transcriptional activation and repression.(22) The dibasic amino acids are cleavage recognition sites for processing peptide hormone precursors such as insulin, glucagon, somatostatin and pancreatic polypeptide. An α-amide group is common to many bioactive neuroendocrine peptides. The carboxyl-terminal sequence (aa251-510) contains five putative Cys2-His2-type zinc-finger motifs. These five zinc-finger motifs are symmetrically spaced at the carboxy terminus. Two tandem repeated zinc-finger motifs from either end are spaced by 45/46 aa from the middle zinc finger.(22). INSM1 functions as a transcriptional repressor that simultaneously regulates entry into the cell cycle and controls expression of a neuroendocrine phenotype.(23) INSM1 is also directly responsible for the transcription of synaptophysin and chromogranin A.(23)

INSM1 regulates downstream target genes and exhibits various extranuclear activities associated with multiple signalling pathways, including Sonic Hedgehog, PI3K/AKT, MEK/ERK, ADK, p53, Wnt, histone acetylation, LSD1, cyclin D1, Asc1, and N-myc.(24).

INSM1 is encoded by the insulinoma associated-1 (IA-1) gene of cDNA which was first identified by Goto et al. in 1992 in human pancreatic insulinoma tissues and murine insulinoma cell lines(25). The localization of the INSM1 gene at the start arm of chromosome 20 was revealed by Lan et al. in 1994.

Johan Staaf et al studied the diagnostic value of insulinoma associated protein 1(INSM1) in pulmonary neuroendocrine tumors. The study included 54 pulmonary NE tumors and 632 non-small cell lung carcinomas (NSCLCs) and they were stained with INSM1, CD56, chromogranin A and synaptophysin. 419 cases of metastasis to lungs were also stained with INSM1.A positive staining with INSM1 was seen in 72% of NE tumors and 1% of NSCLCs. The study confirmed that combination of INSM1 and synaptophysin along with CD56 should be the choice for pumonary high grade NE tumors.(26)

Kelsey E. McHugh et al conducted a retrospective review in 110 gastrointestinal neuroendocrine neoplasms from year 2008 to 2018. They were stained with INSM1, synaptophysin, chromogranin A, CD56 and Ki 67. INSM1 showed a specificity of 95.7% which was higher than that of synaptophysin(86%), chromogranin A(87.3%), CD56(86%). (5)

Isaac E. Kim Jr et al studied 32 cases of small cell neuroendocrine carcinoma of the bladder in 2018 where the immunohistochemical expression of INSM1was compared with established neuroendocrine markers. INSM1 was positive in 87% cases which was higher compared to positivity of other markers like CD56 (75%), synaptophysin (60%), chromogranin A (44%). This shows that INSM1 is a sensitive marker for small cell neuroendocrine differentiation of urinary tract.(27)

Rooper et al conducted a study in 2017 on 86 blocks of neuroendocrine tumors of the thoracic cavity where they found that INSM1 demonstrated a sensitivity of 96.4% across all grades of thoracic neuroendocrine tumors which is significantly more than 87.4%, using the panel of traditional markers. This suggested that INSM1 is sufficiently

sensitive and specific to serve as a first-line marker of neuroendocrine differentiation.(28)

Mukhopadhyay et al, in 2022, conducted a study to determine the utility of INSM1 in 345 whole-tissue sections of primary lung neoplasms. INSM1 stained 100% of carcinoid tumors, except one atypical carcinoid tumor, which was negative for INSM1. The sensitivity of INSM1 for neuroendocrine lung neoplasms as a group (95%) which was similar to synaptophysin (98%) and CD56 (97%), but higher than chromogranin A (84%). The specificity of INSM1 for neuroendocrine lung neoplasms (97%) was similar to chromogranin A (98%) but higher than synaptophysin (90%) and CD56 (87%). The study concluded that, INSM1 is a reliable marker of neuroendocrine differentiation in primary lung neoplasms, with sensitivity similar to synaptophysin and CD56, and specificity similar to chromogranin A.(29)

Zou et al investigated immunohistochemical expression of INSM1 in 75 gynecologic high grade neuroendocrine carcinomas (HGNECs) using full tissue sections. The study proved that INSM1 is a highly specific marker (95% specificity) for gynecologic HGNECs with high sensitivity (92%), but it is less sensitive than synaptophysin (96% sensitivity). The literature review reveals that INSM1 has consistently (the same antibody clone A8 used for all reported studies) shown higher or similar sensitivity to chromogranin A (for all 3 chromogranin A antibody clones LK2H10, DAK-A3, DAKO polyclonal). (30)

Maleki et al examined INSM1 expression in various NETs. Pulmonary NETs, including small cell lung carcinoma, large cell NE carcinoma, atypical carcinoid tumor and typical carcinoid tumor, expressed INSM1 with high specificity (97%) that was similar to CGA (98%) but greater than CD56 (87%) and SYP (90%). The study also included pancreatic NETs where INSM1 displayed 100% sensitivity, specificity, PPV, and NPV. This also helped in differentiating pancreatic NETs from non-NE pancreatic tumors. Thus concluded that INSM1 is a reliable immunostain for the characterization of NETs with high sensitivity and specificity.(32)

Fujino et al studied 102 NET cases with evaluation of INSM expression. The staining intensity and extent was calculated via H-score. INSM1 expression was seen in 100 out of 102 NETs (98%), compared to other conventional markers, like chromogranin A,

synaptophysin and CD56. The nuclear immunoreactivity for INSM1 was greater than conventional cytoplasmic NE markers.

Chen et al evaluated INSM1 immunohistochemistry in 39 cases of genitourinary high-grade neuroendocrine carcinomas (GU-HGNECs) and compared it to chromogranin A, synaptophysin and CD56. In 33 cases of small cell carcinomas, INSM1 showed similar sensitivity (93.9 %) to chromogranin A (87.8 %), synaptophysin (93.9 %) and CD56 (87.8 %), and stained a similar percentage of tumor cells (52 %) to chromogranin A (49 %) and CD56 (52 %), but lower than synaptophysin (87 %) ($p < 0.0001$). In 8 large cell neuroendocrine carcinomas, INSM1 showed sensitivity similar to chromogranin A, synaptophysin or CD56 (62.5 %, 62.5 %, 75 %, 62.5 %, respectively) and the mean percentage of positively stained tumor cells (21 %, 44 %, 48 %, 37 %, respectively). Thus the study indicated that INSM1 is a sensitive marker for genitourinary HGNECs with high specificity. For genitourinary small cell carcinomas, INSM1 shows similar sensitivity to chromogranin A, synaptophysin and CD56 but stains a lower percentage of tumor cells than synaptophysin. For genitourinary large cell neuroendocrine carcinomas, INSM1 showed similar sensitivity to chromogranin A, synaptophysin and CD56. (24)

Rosenbaum et al evaluated INSM1 in 129 specimen as a semi-quantitative immunohistochemical (IHC) marker for neuroendocrine and neuroepithelial neoplasms and as a quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) marker for gastrointestinal NENs (GI-NENs). As a result, INSM1 expression was highly restricted to nuclei of neuroendocrine cells and tissues. In neoplastic tissue, INSM1 was detected by IHC in 88.3% of 129 NEN specimens. Using qRT-PCR, INSM1 gene expression was evaluated in 113 GI-NEN specimens.(32)

Razvi et al in 2021, evaluated INSM1 as a marker for neuroendocrine differentiation in infiltrating breast cancers (IBC). The expression of INSM1, along with other neuroendocrine markers (synaptophysin, chromogranin A and CD56) was assessed in Invasive breast carcinoma(IBC) cohort using tissue microarray by immunohistochemistry. Overall, 13.1%, 4.6%, 7.0% and 6.5% of the cases were positive for synaptophysin, chromogranin A, INSM1 and CD56. INSM1 expression showed similar clinicopathological profiles as chromogranin A and synaptophysin. Using synaptophysin and/or chromogranin A to define neuroendocrine differentiation,

INSM1 showed a sensitivity of 37.3%, which was more sensitive than chromogranin A (33.5%) and CD56 (16.4%) but less sensitive than synaptophysin (94.6%). (33)

Saijnhaeve et al studied 66 mammary neoplasms in 2021, with known neuroendocrine differentiation as determined by immunohistochemistry for synaptophysin and chromogranin-A. INSM1 immunohistochemistry was validated in these 66 invasive breast cancer biopsies. In the validation cohort, 14 tumors were synaptophysin-positive, of which all except one showed INSM1 immunoreactivity. Eight of the tumors were synaptophysin-negative, of which 3 showed focal nuclear INSM1 expression. Six of the tumors were chromogranin-A-positive, of which one was INSM1-negative. When compared with synaptophysin, INSM1 was more sensitive but less specific than chromogranin-A. In the biopsy cohort, only one invasive carcinoma of no special type showed INSM1 immunoreactivity (i.e, 25% of the tumor cells). Thus the study concluded that neuroendocrine differentiation in invasive breast carcinoma of no special type is a rare finding. Immunohistochemical biomarkers, like INSM1 as well as the first-generation biomarkers chromogranin-A and synaptophysin are useful to distinguish neuroendocrine differentiation in breast neoplasms. The identification of neuroendocrine differentiation can be helpful to establish the diagnosis of special type breast neoplasms such as solid papillary carcinoma.(34)

Kawasaki et al studied on three patients, respectively, 42-, 58-, and 64-year-old Japanese women with breast tumors showing characteristic neuroendocrine morphology. On IHC, these malignancies showed diffuse nuclear expression of INSM1, whereas chromogranin A and synaptophysin did not show any distinct neuroendocrine features in their cytoplasm. Thus the study showed that based on the identification of INSM1, the frequency of detecting neuroendocrine differentiation in systemic neoplasms, including breast neuroendocrine phenotype cancers, is helpful in development of novel treatments including molecular targeted drugs for these tumor entities.(35)

Sándor Turkevi-Nagy et al evaluated the expression of syntaxin-1 and insulinoma-associated protein 1 (INSM1) in 59 cases of breast carcinomas. The sensitivity of syntaxin-1 was found to be 84.7% (50/59) and specificity 98.1%. While, the sensitivity of INSM1 was 89.8% (53/59) and its specificity 88.9% as compared to the sensitivity of chromogranin A, synaptophysin and CD56 which were 98.3, 74.6 and 22.4%,

respectively. The study revealed that Syntaxin-1 and INSM1 are sensitive and specific markers of breast tumors with neuroendocrine features, out-performing chromogranin A and CD56.(36)

Doxtader et al studied 54 cases of primary lung neuroendocrine neoplasms where INSM1 was found positive in 48 of 54 primary lung neuroendocrine neoplasms (92%), including 38 of 41 small cell lung carcinomas (93%), the only large cell neuroendocrine carcinoma (100%), and 9 of 10 carcinoid tumors (90%). For small cell carcinomas, the sensitivity of INSM1 (93%) was lower than the sensitivity of CD56 (100%), equal to the sensitivity of synaptophysin (93%), and higher than the sensitivity of chromogranin A (35%). For carcinoid tumors, the sensitivity of INSM1 (90%) was lower than the sensitivity of all other markers, while specificity was 100%.(37)

Sakakibara et al examined the immunohistochemical expression of INSM1 in 141 neuroendocrine tumors (78 SCLCs, 44 large cell neuroendocrine carcinomas (LCNECs) and 19 carcinoids), and 246 non-NE carcinomas. As a result, INSM1 was expressed in SCLCs (92%, 72/78), LCNECs (68%, 30/44), and carcinoids (95%, 18/19). Also, among SCLCs with no expression of NE phenotype markers like synaptophysin, chromogranin A and CD56 (n=12), 9 (75%) of them were found positive for INSM1. This data suggested, the superiority of INSM1 to other neuroendocrine phenotype markers. Among non-NE carcinomas, only 7% of adenocarcinomas (9/134) and 4% of squamous cell carcinomas (4/112) were positive for INSM1.(38)

Kriegsmann et al studied 493 lung tumors and synaptophysin, chromogranin A, CD56, and INSM1 were done on all cases and evaluated manually as well as with an analysis software. INSM1 was positive in 305 of 493 tumors with expected neuroendocrine differentiation (typical and atypical carcinoids, large cell neuroendocrine carcinomas, small cell lung cancers, and paraganglioma) with sensitivity of 76%. INSM1 was negative in all but 1 of 91 analysed non-neuroendocrine tumors (adenocarcinomas, squamous cell carcinomas with specificity of 99%). All conventional markers, as well as their combination, had a higher sensitivity (97%) and a lower specificity (78%) for neuroendocrine differentiation as compared to INSM1.(40)

El-Kareem et al in 2021 carried out a study on 102 cases of different neuroendocrine tumors and expression of INSM1 was studied. As a result, INSM showed positive nuclear expression in 90 cases (88%). Negative expression was detected in the remaining 12 cases. Thus the study confirmed INSM1 expression in a large data set including pituitary adenomas, head and neck NET, lung NET, Mediastinal NET, GI NET, and pancreatic NET, and found high sensitivity of INSM1 for detecting neuroendocrine differentiation as compared with the currently established markers.(40)

The cell cycle:

The cell cycle is divided into distinct phases: G1 is the interval between mitosis (M phase) and DNA synthesis (S phase).

During G1, the cell is prone to stimulation by extracellular mitogens and growth factors, following which, the cell passes through G1 and proceeds with DNA synthesis in S phase.

G2 phase is the phase between the completion of DNA synthesis of S phase and M phase, which is marked by the generation of bipolar mitotic spindles, separation of sister chromatids and cell division.(42)

The progression of the cell through each phase of the cycle and the transition from one phase to the next are closely regulated by checkpoints.(42)

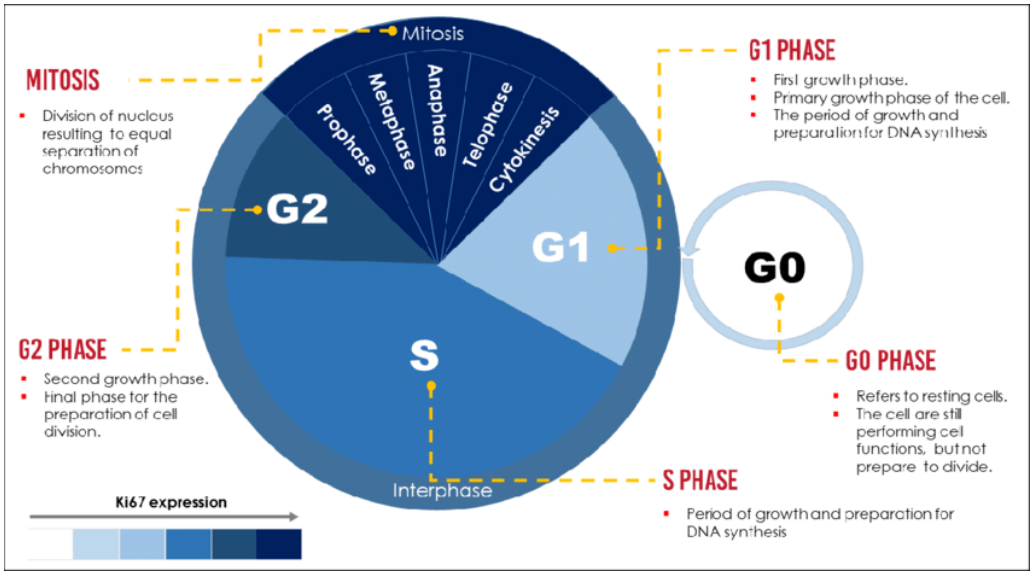


Figure 1: The cell cycle.

TUMOR CELL CYCLE PHASE ANALYSIS:

The WHO grading of neuroendocrine tumors of gastrointestinal tract, lung tumors and pancreatic tumors is based on the mitotic index and/or Ki-67 index. Proliferation index such as mitotic activity and the percentage of S-phase cells have been shown to be of prognostic value in many tumors. The WHO 2017 classification of hepato-pancreatico-biliary neuroendocrine neoplasms has added a new tumor category NET G3. These neoplasms while retaining a well differentiated histologic pattern, show a proliferation index of >20%. No upper limit has been defined for the mitotic index or Ki-67 proliferation index for G3 NET. The value is usually less than equal to 20 mitotic figures/ 10HPF and <55% Ki-67 proliferation index.(43)

Ki67 labelling index:

Ki-67 protein is associated with cell proliferation and is detected within the nucleus, during interphase (G1, S, G2 phases) whereas in mitosis (M phase) most of the protein is relocated to the surface of the chromosomes. Ki-67 is expressed in cells in all active phases of the cell cycle, except for the resting (G0) phase. (44) Tissue anoxia occurring any time from clamping of vessels during surgical resection of the tumor to tissue fixation causes the mitotic counts in surgically resected specimens to decrease abruptly. Thus, grading by the Ki-67 labelling index is always higher than grading by mitosis. Evaluation of the Ki-67 labelling index is also influenced by many factors, such as the use of different clones of the Ki-67 antibody, different Ki-67 staining protocols among laboratories, varying thickness of the sections used for Ki-67 staining, and the density of the tumor cell.(12)

Mitotic count should ideally be calculated from most active areas (or hot spots), which are recognized by scanning the sample under intermediate magnification. This is affected by the presence of mimickers of mitotic figures. Mitotic rate should be reported as number of mitoses/2 mm^2, evaluated in the most active part (hotspot) of the tumor. Only the identifiable mitotic figures should be counted; the hyperchromatic, karyorrhectic, and apoptotic nuclei are excluded. Mitosis mimics include pyknosis, apoptotic bodies (particularly in anaphase and early telophase), or shrunken nuclei. These issues lead to interobserver variability. Mitotic counts determined by PHH3 staining and hematoxylin-eosin staining showed a high concordance rate. (43)

Phosphohistone protein 3:

The PHH3 antibody is recognized as a biomarker of cell proliferation which is specific for cells in mitosis, by identifying phosphorylated histone H3 by IHC. Histone H3 is one of the five different types of histone proteins that are part of nucleosomes, whose phosphorylation at the serine 10 and 28 level, determines the compaction of chromatin during cell division and from this event, the cell enters the M phase of the cell cycle.(10) The PHH3 antibody is characterized by presenting a clear and well contrasted immunostaining limited to cells which are in the M phase of the cell cycle, while interphase cells do not express it or do so minimally.(45)

Study conducted by Villani et al showed that H&E stained sections, Ki67, and PHH3 are excellent predictors of disease-specific survival (DSS). However, PHH3 was superior to H&E and Ki67 in predicting both the disease-free survival (DFS) ($p = 0.006$) and DSS ($p = 0.001$). Evaluation of the PHH3, mitotic count showed 7 mitoses per 10 high-power fields (HPFs), to be the optimal cutoff for differentiating between low-risk and high-risk PNET patients. Thus it concluded that PHH3 is a better predictor of both DFS and DSS than H&E or Ki67 in PNET. In addition, PHH3 appeared to be both easier to interpret and more accurate when compared to current prognostic markers.(47)

Another study conducted by Tsuta et al analysed mitotic figures by using a mitotic-specific antibody of phosphohistone H3 (PHH3) in 113 lung NECs (66 typical carcinoids [TCs], 12 atypical carcinoids [ACs], 20 large cell NECs [LCNECs], and 15 small cell lung carcinomas [SCLCs]). Subdivided by histologic subtype, the mean PHH3-stained MFs (mPHMFs) were 0.09 per high-power field (hpf) in TCs, 0.39/hpf in ACs, 7.84/hpf in LCNECs, and 9.42/hpf in SCLCs. From the 5-year overall survival rate for mPHMFs, a mPHMF of more than 1.0 was the best threshold in all NECs and a mPHMF of more than 0.4 was the best threshold for differentiating ACs from TCs. These values corresponded to 4/10 hpf and 10/10 hpf. Thus the study showed that the PHH3-based mitosis-counting method is a reliable, easy method for counting mitoses in pulmonary NECs.(47)

Jessica Tracht et al did a comparative study of Ki67 and PHH3 in Neuroendocrine Tumors of the Pancreas. Sixty-three cases were included in the study including 29 males and 34 females (M: F 0.9) with a median age of 59 years (ranging 34–84). There was not a significant discrepancy in the stratification of tumor grades for Ki67 and PHH3

but PHH3 significantly predicted lymph node metastasis (p=0.041) suggesting that that PHH3 is an effective marker for determining mitotic activity and can be used as an alternative to Ki67.(48)

Duregon et al studied the diagnostic and prognostic role of phospho-histone H3 in 52 adrenocortical carcinomas, comparing manual and computerized count to standard manual hematoxylin- and eosin-based method and Ki-67 index. Manual hematoxylin and eosin and phospho-histone H3 mitotic counts were highly correlated (r=0.9077, P<0.0001) and better than computer-assisted phospho-histone H3 evaluations. Three of the 15 cases having <5 mitotic figures per 50 high-power fields by standard count on hematoxylin and eosin gained the mitotic figure point of Weiss Score after a manual count on phospho-histone H3 slides. Traditional mitotic count confirmed to be a strong predictor of overall survival (P=0.0043), better than phospho-histone H3-based evaluation (P=0.051), but its is not as strong as the Ki-67 index (P<0.0001). Ki67 helped to segregate adrenocortical carcinomas into three prognostic groups, stratifying cases by low (<20%), intermediate (20–50%), and high (>50%) Ki-67 values. The study concluded that phospho-histone H3 staining was a useful diagnostic complementary tool with high reproducibility to standard hematoxylin and eosin mitotic count, enabling optimal mitotic figure evaluation (including atypical mitotic figures) in adrenocortical carcinomas with a low mitotic index. Ki-67 proved to be the best prognostic indicator of overall survival, being superior to the mitotic index, irrespective of the method (standard on hematoxylin and eosin or phospho-histone H3-based) used to count mitotic figures.(49)

Natalia Tancredi-Cueto et al did a comparative study of PHH3 with ki67 and mitotic activity index (MAI) in 62 cases of oral squamous cell carcinomas (OSCC). As a result, significant association was obtained between the expression of PHH3 (p 0.016) and MAI (p 0.031) with survival time, but similar relationship was not found with Ki-67 (p 0.295). A statistical association between histological grade and Ki-67 (p 0.004) was confirmed while PHH3 did not show a similar relationship (p 0.564). Thereby, confirming the role of the PHH3 antibody as a biomarker for mitotic figures in OSCC and as a potential marker of cell proliferation.(50)

AIMS AND OBJECTIVES

AIMS:

- To study the expression of immunohistochemical marker INSM1 in neuroendocrine tumors.

OBJECTIVES:

- To compare INSM1 immunohistochemical marker with traditional neuroendocrine markers (CGA, Synaptophysin).
- To compare Ki67 labelling index (LI) with PHH3 labelling index (LI).

MATERIALS AND METHODS

Type of study

- The study was an ambispective type of observational study.

Data collection

- The study included small biopsy and large biopsy specimens of neuroendocrine tumors received in the Department of Pathology & Lab Medicine at AIIMS, Jodhpur.
- The study was started after receiving approval from the institutional review board.
- All the slides diagnosed for neuroendocrine tumors were reviewed, antibodies for INSM1 and PHH3 were applied on representative sections.

Ethical clearance:

- The study was approved by the institutional ethics committee on 12/03/2021.
- Certificate No.: AIIMS/IEC/2021/3385

Inclusion criteria:

- All Specimen of neuroendocrine tumors received in the Department of Pathology & Lab Medicine at AIIMS Jodhpur were included in this study.

Exclusion criteria:

- Inadequate samples.
- Neoplasms excluding neuroendocrine tumors.

Statistical analysis:

- Data was entered in excel sheet and analysed by IBM-SPSS software 23.0version.

Sample processing

- After approval from the Institutional ethics committee, the study was started.

Grossing of small biopsy and resection specimen:

- 10% formalin-fixed specimens were measured and processed.
- Paraffin blocks were prepared using routine histopathological techniques.
- Thin sections (4-5µm) were stained with routine Hematoxylin and Eosin (H&E).
- Light microscopy results and histopathological grading were recorded.
- The appropriate representative blocks were subjected to immunohistochemistry (IHC).

I. Steps of block preparation and section cutting

After the representative sections were taken, tissue was processed as follows:

- Dehydration: carried out by passing the sections through a series of ascending grades of ethyl alcohol, from 50%, 70%, 95% to absolute alcohol.
- Clearing: done by passing the tissue through two changes of xylene.
- Impregnation: done in molten paraffin wax which had a melting point of 54 – 62˚C.
- Embedding: Embedding station (Leica EG 1150 H) was used through which a small amount of liquid paraffin was layered into aluminium molds. Properly oriented tissues were placed inside the molds, which were then filled with liquid paraffin 60 – 62˚C and allowed to cool and harden. The lower portion of the cassette with an identification number was used as the final block.
- Microtomy: Microtome (Leica-RM2255) was used and thin ribbons (4-5µm) were cut and floated in warm water (~56˚C) for expansion of the curled sections. These sections were then collected on frosted glass slides and kept for drying.

II. Staining of sections: (for H and E stain)

- Deparaffinization – The glass slides containing the tissue sections were kept over the hot plate at 60 ˚C for 10 minutes, followed by two changes in xylene (Xylene I & Xylene II), 10 minutes each.
- Hydration – Through graded alcohol (100%, 95%, 70%, 50%) to water, 10 minutes respectively.
- Hematoxylin – The sections were kept in Harris's Hematoxylin for 5 minutes.

- Washing – The sections were washed well in water for 2 minutes.
- Differentiation – Done in 1% acid alcohol (1% HCl in 70% alcohol) for 10 seconds.
- Washing – Done under running tap water (usually for 15 – 20 minutes) until the sections 'blue'.
- Eosin – Stained in 1% Eosin Y for 10 seconds.
- Washing – Done in running tap water for 2 minutes.
- Dehydration – Through graded alcohol (50%, 70%, 95%, 100%), 10 minutes each.
- Clearing –Through xylene (Xylene II & Xylene I), 2 minutes each.
- Mounting – The sections were mounted in DPX with a coverslip.

III. Immunohistochemistry

Antibodies used:

Primary antibody: Ready to use.

- INSM1 (Insulinoma associated 1): Prediluted, Clone: BSB-123, Company: BioSB

-PHH3 (Phosphohistone 3): Prediluted, Clone: EP223 , Company: BioSB

Secondary Antibody: Bond Polymer Refine Detection, Leica

- Peroxide block, 3-4%(v/v)
- Post Primary, Rabbit anti-mouse IgG in 10% (v/v) animal serum in tris-buffered saline.
- Polymer, Anti-rabbit Poly-HRP-IgG containing 10% (v/v) animal serum in tris-buffered saline.
- DAB Part 1, in stabilizer solution
- DAB Part B ≤0.1% (V/V) Hydrogen peroxide in stabilizer solution
- DAB Part B ≤0.1% (V/V) Hydrogen peroxide in stabilizer solution
- Hematoxylin, 0.1%

STEPS OF IHC STAINING:

A. Preparation of Buffer–Two types of buffers were used.

- Wash Buffer: Wash buffer preparation: 6 gm powdered TRIS buffer salt was dissolved into 1 liter of distilled water and pH was set at 7.4.

- Antigen Retrieval Buffer (ARB): 6.05 gm TRIS salt and 0.744 gm EDTA salt were dissolved in 1 liter of distilled water, pH was set at 9.0.

B. Preparation of Poly-L-Lysine Solution (PLL Solution):

1 ml of PLL was diluted with 9 ml of distilled water (1 in 10 dilutions).

C. Slide Coating Procedure:

- *Step 1:* Diluted PLL solution was taken in a clean container/Coplin jar
- *Step 2:* Both sides of the glass slides were cleaned with tissue paper
- *Step 3:* The clean slides were immersed in a PLL solution for 5 minutes
- *Step 4:* After 5 minutes, the coated slides were removed and kept overnight for air drying. The coated slides were kept at room temperature. Tissue sections of 4µm thickness were obtained on the PLL coated slides.
- Baking: The slides were kept at 60˚C for 1 hour and then cooled to room temperature.

D. IHC staining procedure

- *Step 1:* Deparaffinization – The slides were kept in Xylene I (10 minutes), followed by Xylene II (10 minutes).
- *Step 2:* Rehydration – The slides were kept in 100%, 70%and 50% alcohol for 5 minutes each followed by running tap water for 5 minutes.
- *Step 3:* Antigen retrieval – by pressure cooker method (38). 200 ml of clean tap water was taken in the empty pressure cooker and heated up to the steam formation. The slides were placed in a rack. 300 ml of ARB was put in the container and the rack with slides was placed inside the container. Then the container containing the rack with slides, was placed inside the pressure cooker and the lid was closed. After two whistles the pressure was released by lifting the air vent and allowed to cool till it reached room temperature.
- *Step 4:* Wash – Slides were washed in Wash Buffer (pH7.4) thrice at a 1-minute interval.
- *Step 5:* Peroxide blocking – Blocking reagent was added to the sections and incubated for 10 minutes in the Humidity chamber at room temperature. This step prevents unwanted, nonspecific background staining.
- *Step 6:* The peroxide was decanted and not washed with buffer.

- *Step 7:* Primary antibody – INSM 1 and PHH3 was added to the sections and incubated in the Humidity chamber for one hour.
- *Step 8:* Wash – After that slides were washed in Wash Buffer (pH 7.4) thrice at a 1-minute interval.
- *Step 9:* Amplifier – Amplifier was added over the sections and incubated for 30 minutes in the Humidity chamber at room temperature.
- *Step 10:* Wash – The slides were washed in Wash Buffer (pH 7.4) thrice at a 1-minute interval.
- *Step 11*: HRP label – The HRP was added and incubated for 30 minutes in the Humidity chamber at room temperature.
- *Step 12*: Wash – The slides were washed in Wash Buffer (pH 7.4) thrice at a 2-minute interval.
- *Step 13*: DAB – The DAB chromogen was applied to the sections and incubated in the Humidity chamber for 10 minutes, avoiding light exposure as much as possible.
- *Step 14:* Wash – The sections were washed in distilled water twice at a 1-minute interval.
- *Step 15*: Counterstain – Slides were counterstained using Harris Hematoxylin for 2-3 minutes.
- *Step 16:* Wash – The slides were washed in running tap water for 5 minutes.
- *Step 17:* Dehydration – was done in graded alcohol (50%, 70%, 95%, 100%), 1 minute each.
- *Step 18:* Mounting – Slides are air-dried, mounted with DPX and examined under the microscope.

E. Interpretation of immunohistochemical stains:

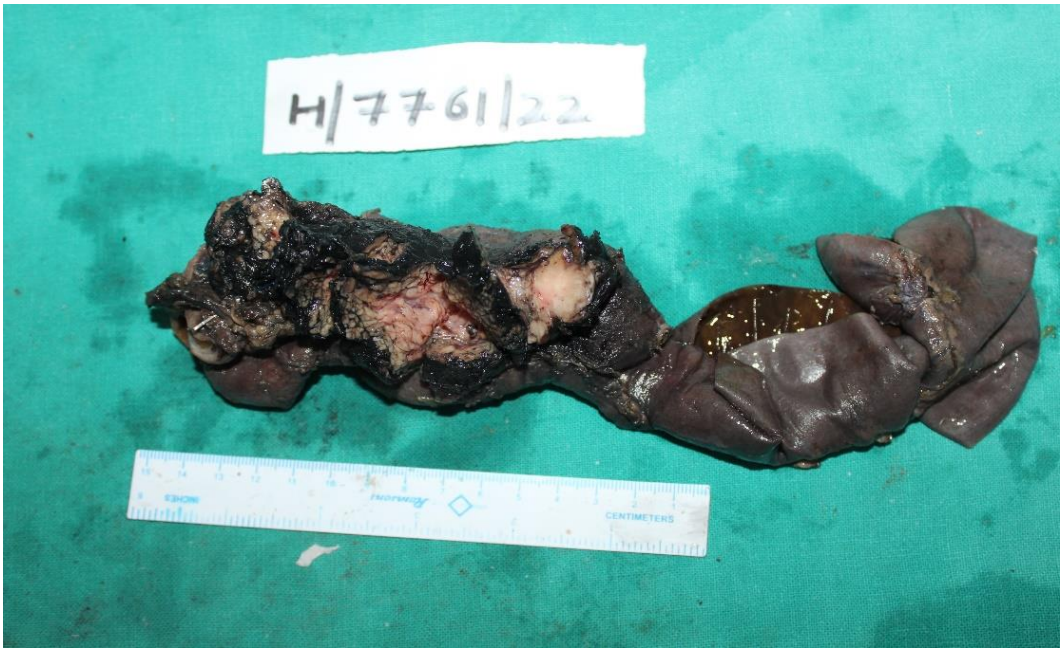
Scoring:

INSM1:

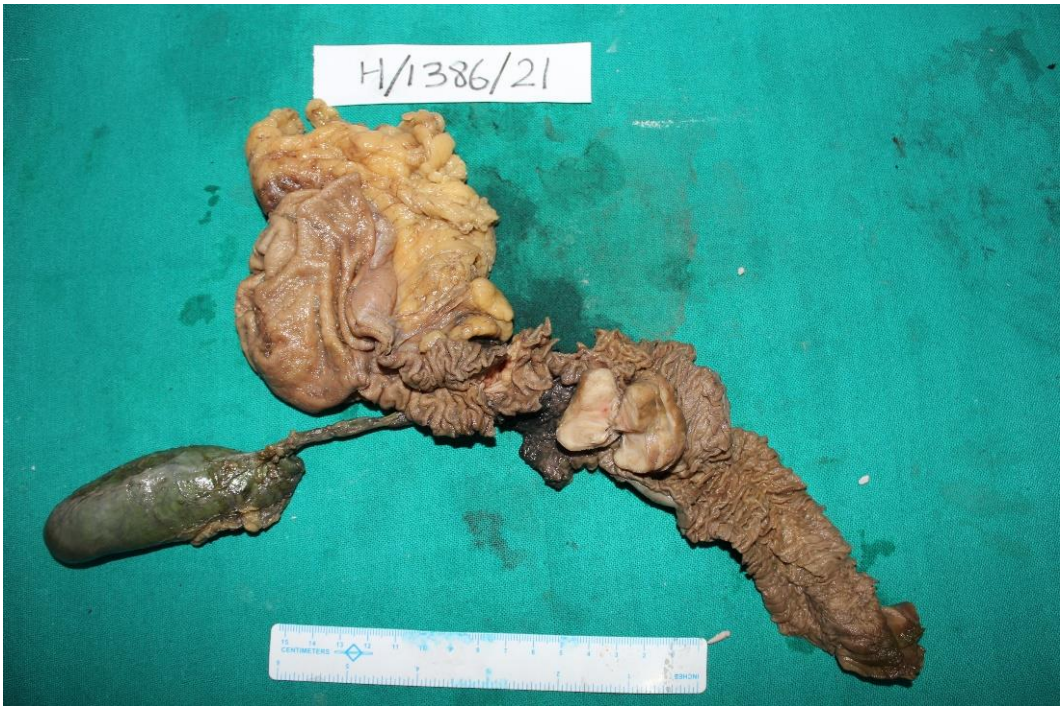
Percentage Score		**Intensity score**	
0	No tumor cells showing positivity	0	Negative
1+	<25% tumor cells showing positivity	1+	Weak intensity
2+	25-50% tumor cells showing positivity	2+	Moderate intensity
3+	50-75% tumor cells showing positivity	3+	Strong intensity
4+	>75% tumor cells showing positivity		

PHH3 LI:

<3%	G1
3-20%	G2
>20%	G3

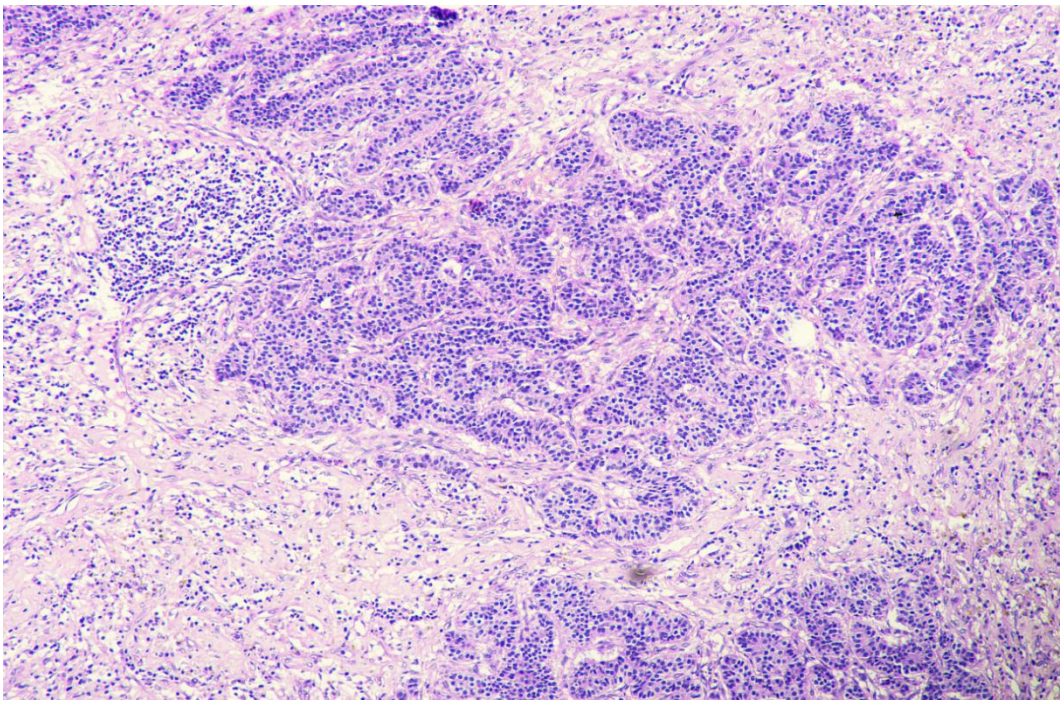


GROSS 1: WHIPPLE'S SPECIMEN- PANCREATIC MASS

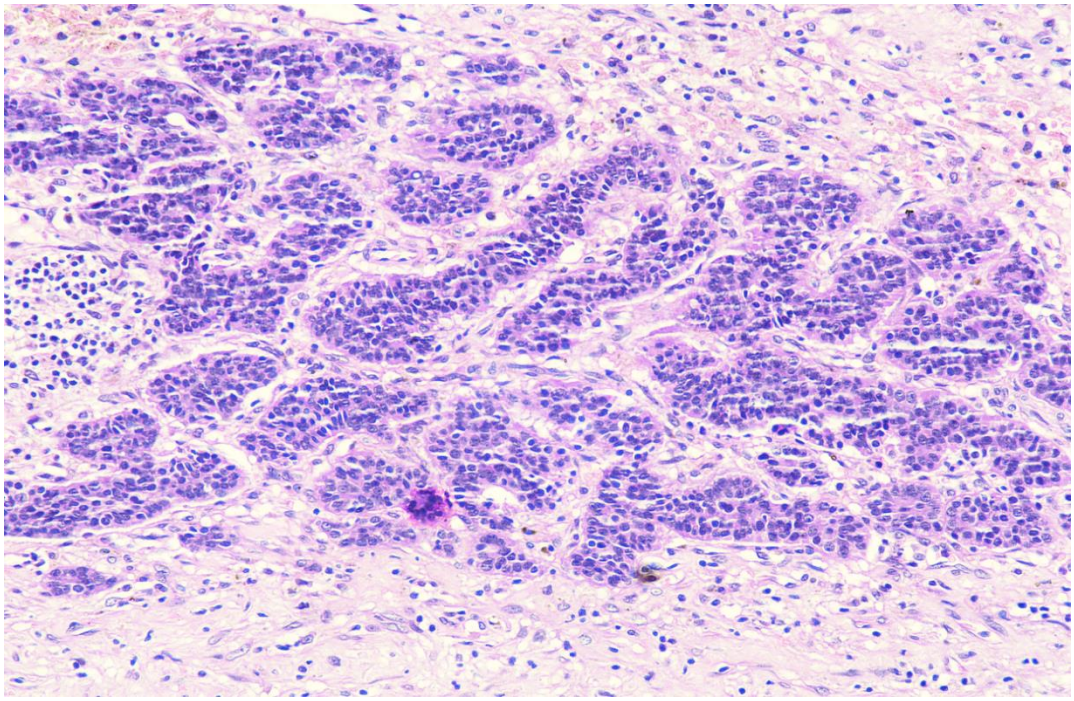


GROSS 2: WHIPPLE'S SPECIMEN-DUODENAL MASS

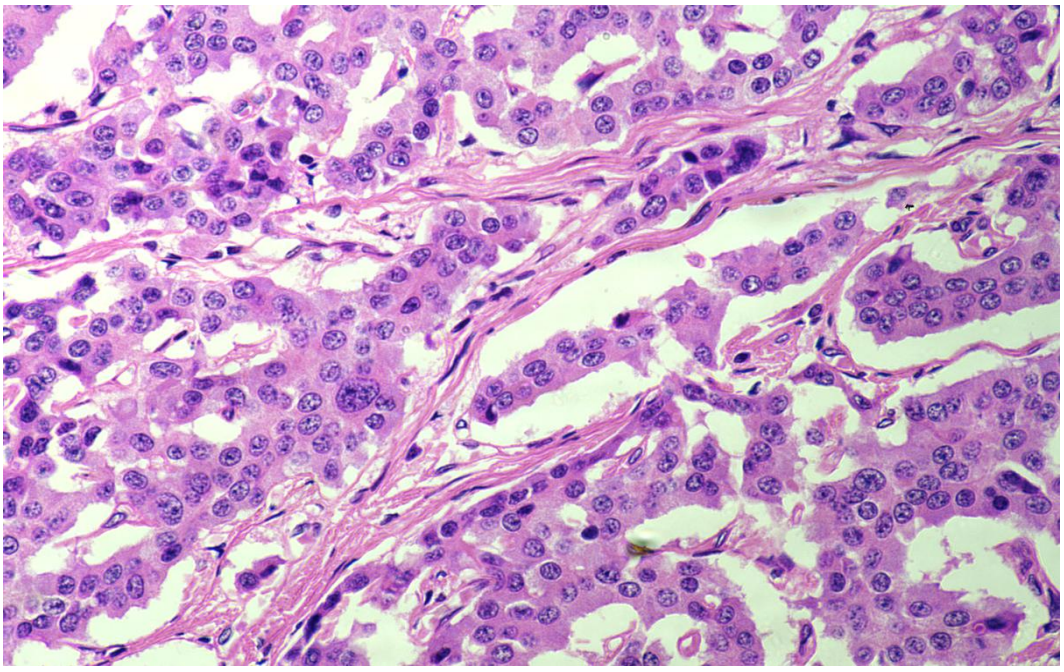
GROSS 3: PARAGANGLIOMA



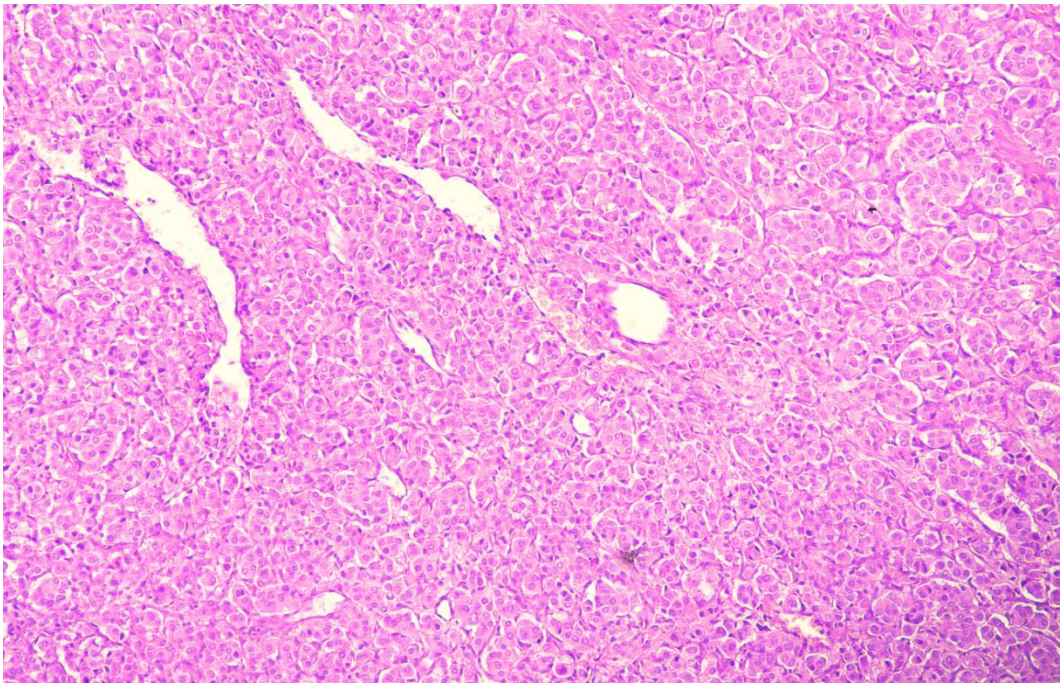
COLOR PLATE 1: GYRIFORM PATTERN IN DUODENAL NET, 100X, H & E stain



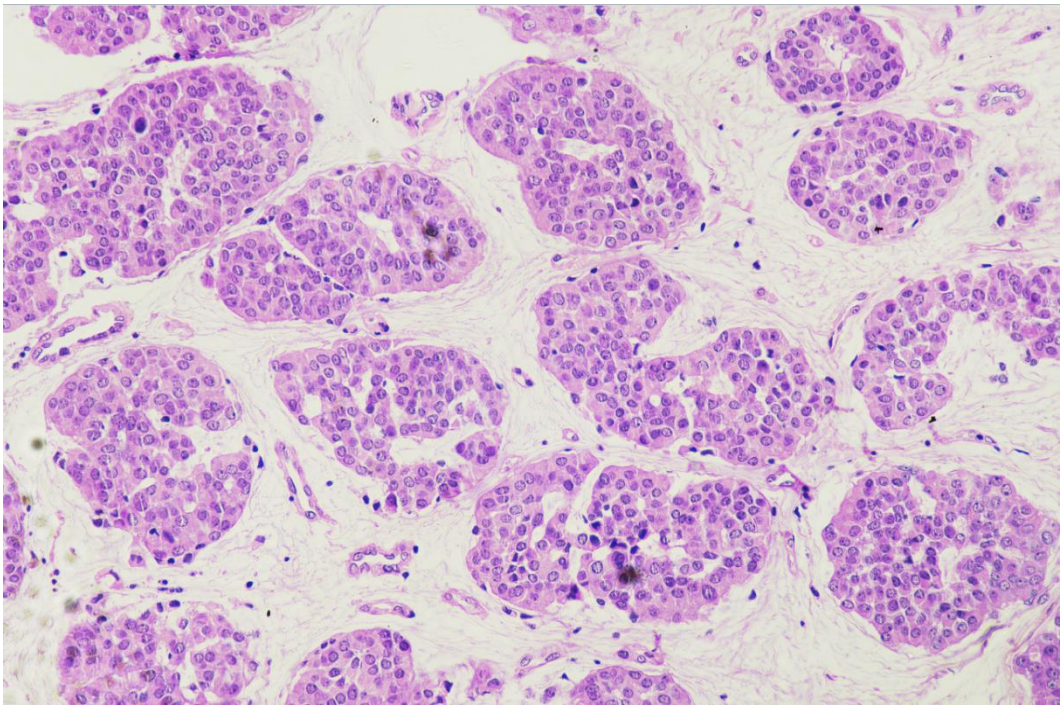
COLOR PLATE 2: GYRIFORM PATTERN IN DUODENAL NET, 200X, H & E stain



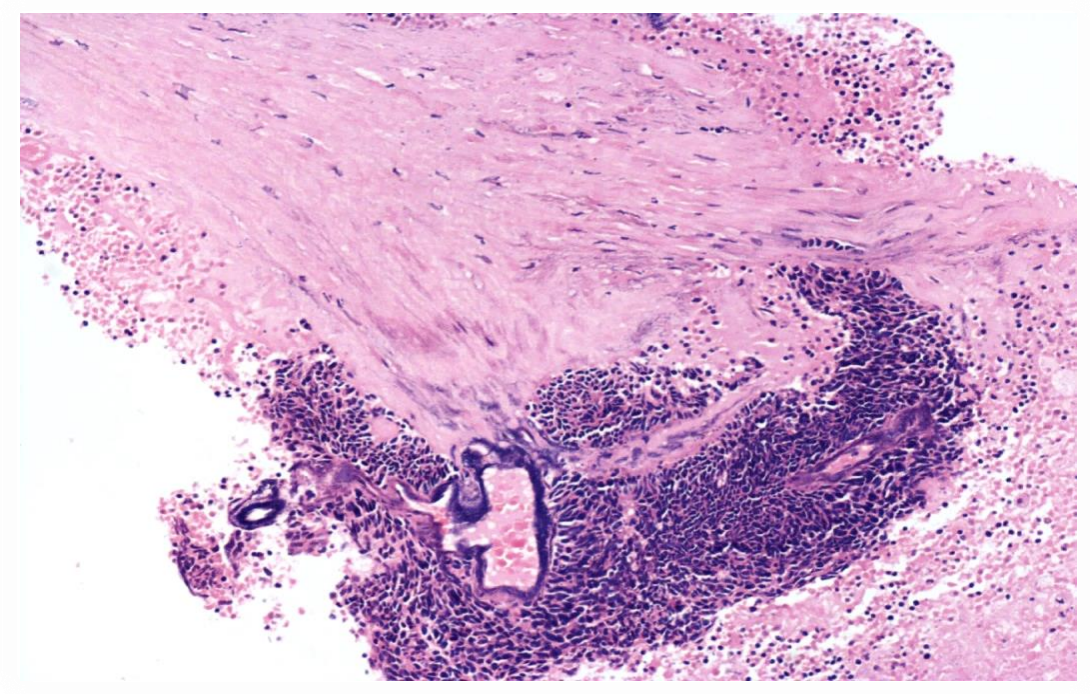
COLOR PLATE 3: NUCLEAR FEATURE- stippled chromatin with focal nuclear atypia, 200x, H & E stain



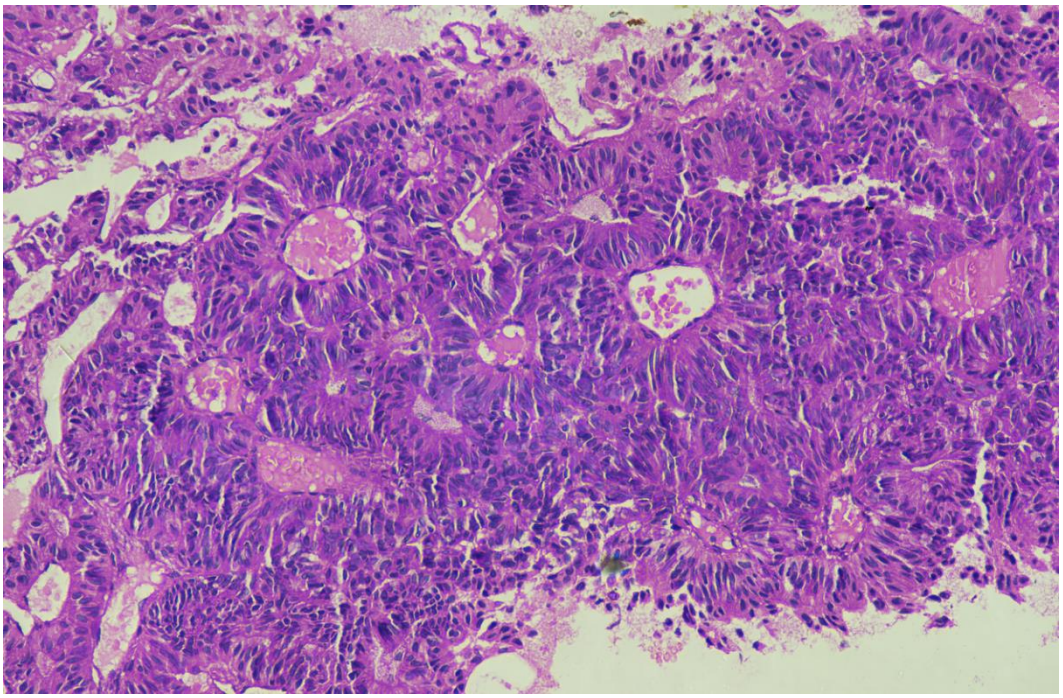
COLOR PLATE 4: ORGANOID PATTERN OF NET IN PARAGANGLIOMA, 100X, H & E stain



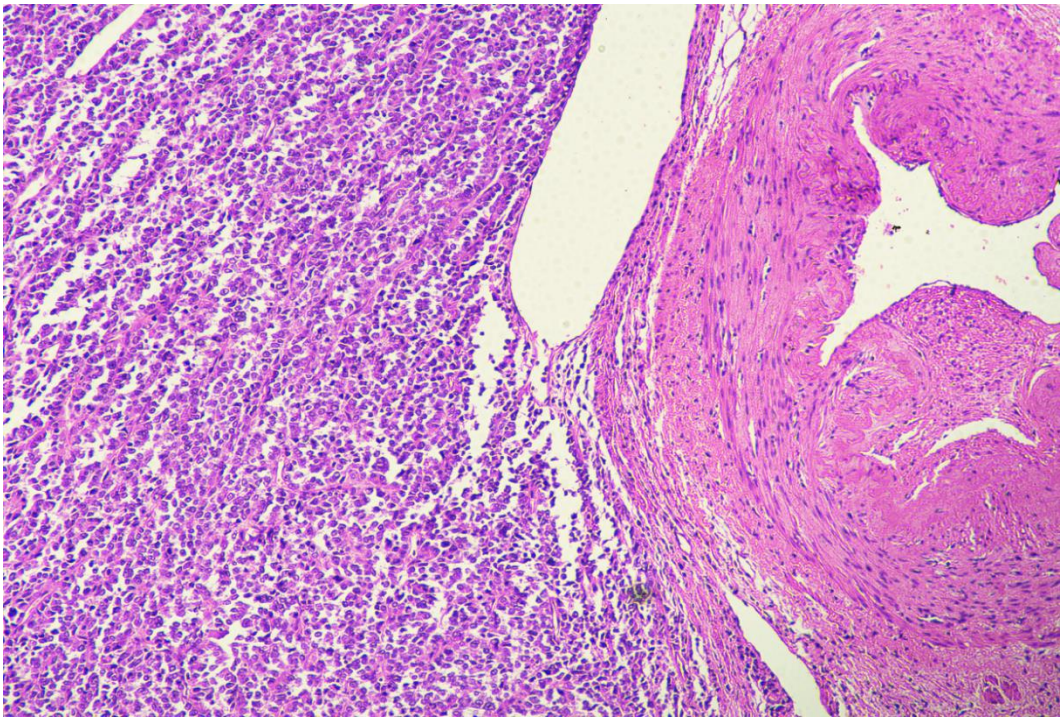
COLOR PLATE 5: MUCINOUS CARCINOMA OF BREAST WITH NE DIFFERENTIATION, 100x, H & E stain



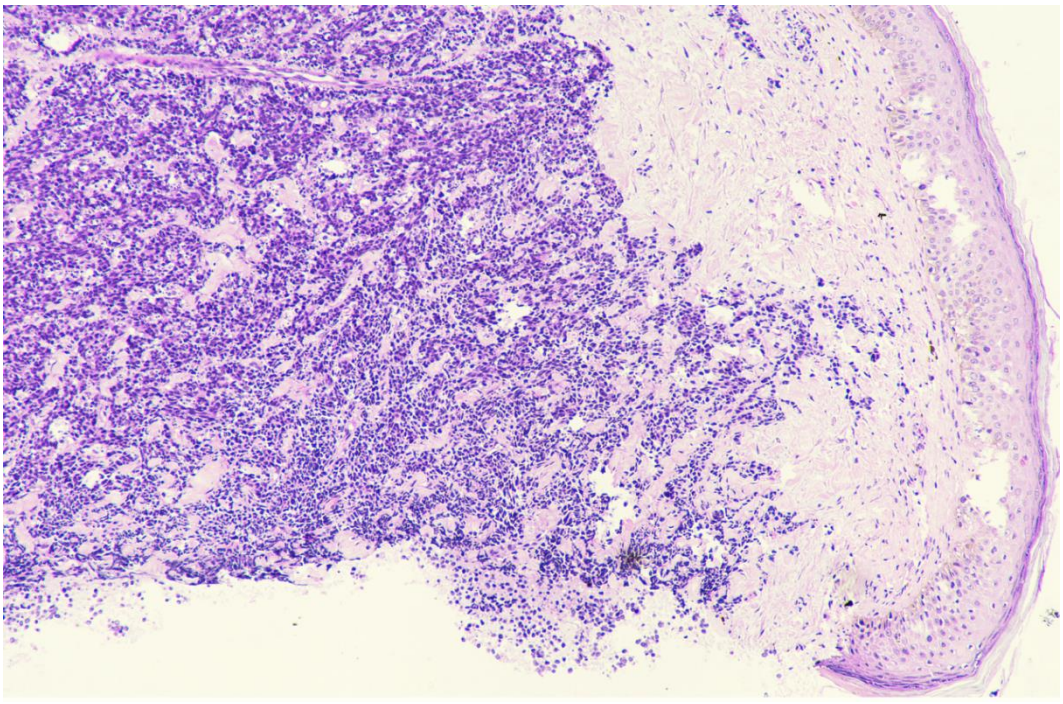
COLOR PLATE 6: AZZOPARDI EFFECT IN LUNG SMALL CELL CARCINOMA, 100X, H & E stain



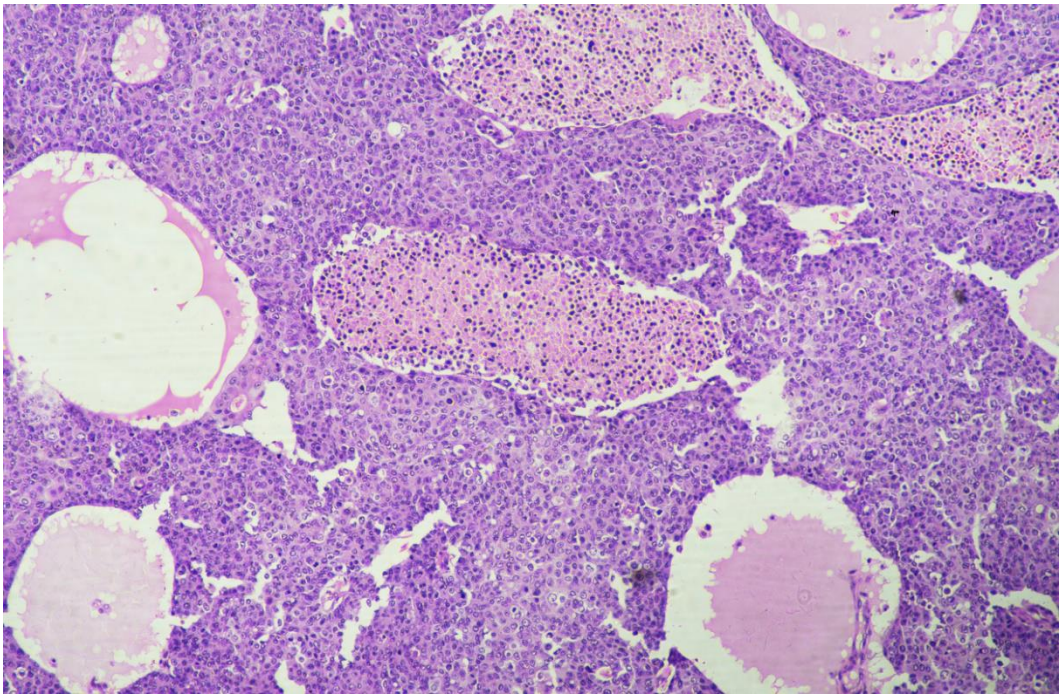
COLOR PLATE 7: ROSETTE FORMATION IN LIVER NET, 200X, H & E stain



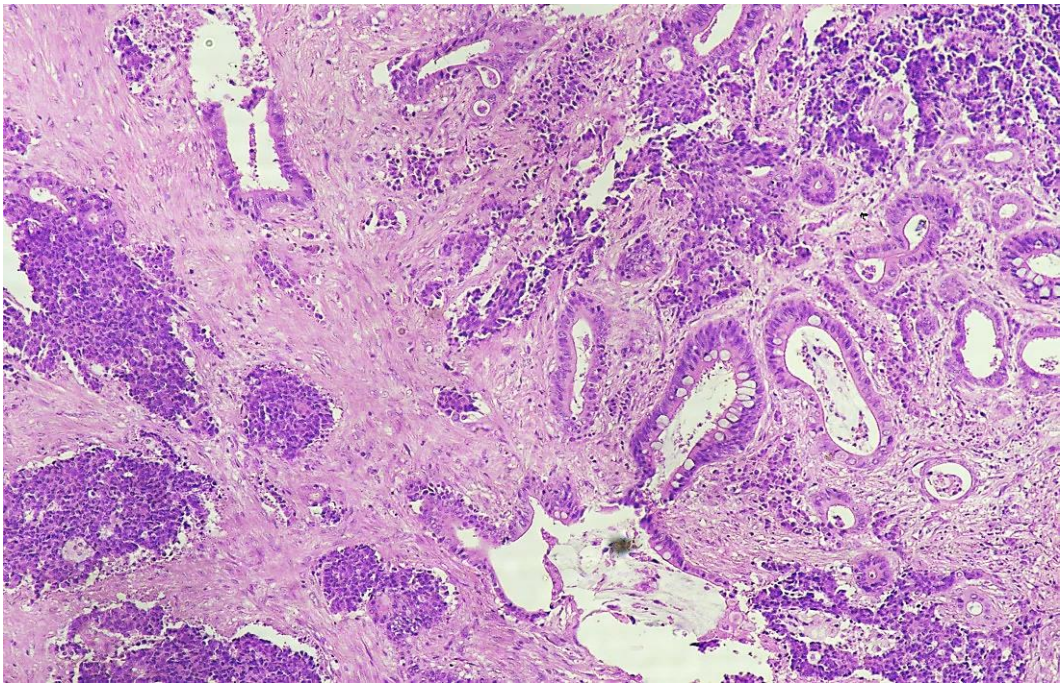
COLOR PLATE 8: NEC OF ENDOMETRIUM WITH MYOMETRIAL THICK WALLED BLOOD VESSEL,100X, H& E stain



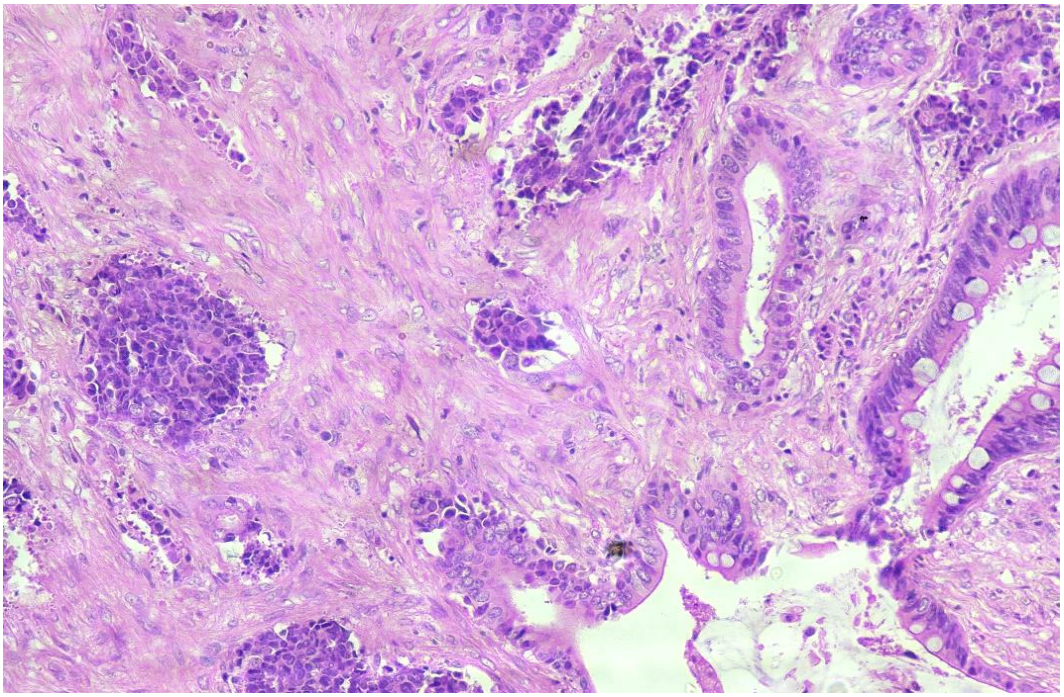
COLOR PLATE 9: MERKEL CELL CARCINOMA OF SKIN, 100X, H& E stain



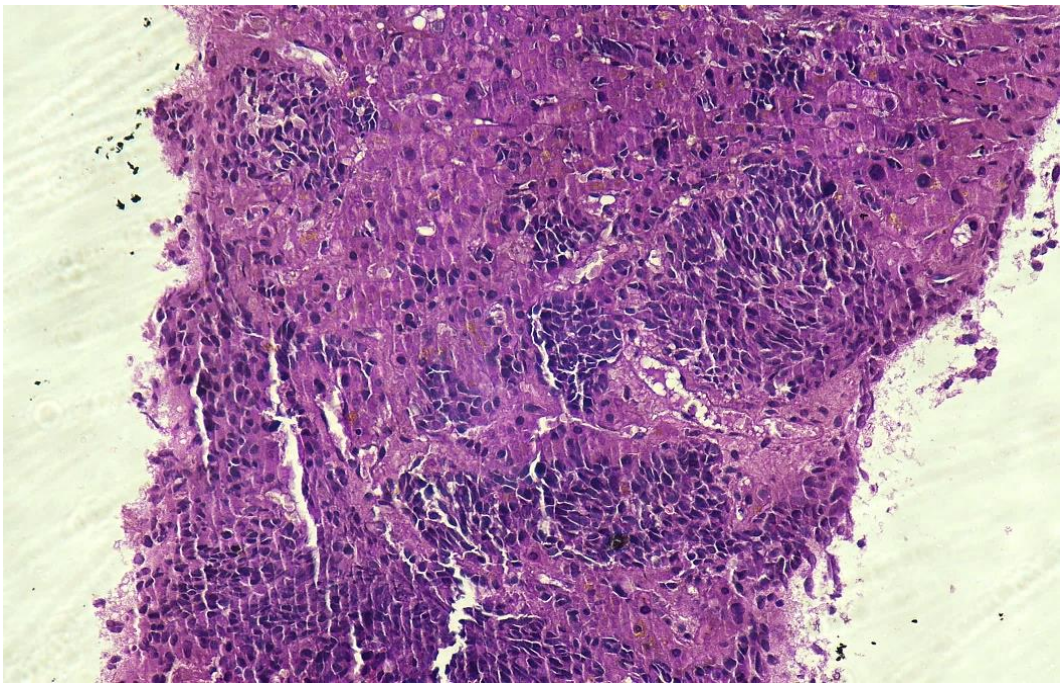
COLOR PLATE 10: NEUROENDOCRINE CARCINOMA OF GIT WITH COMEDO NECROSIS, 100X, H& E stain



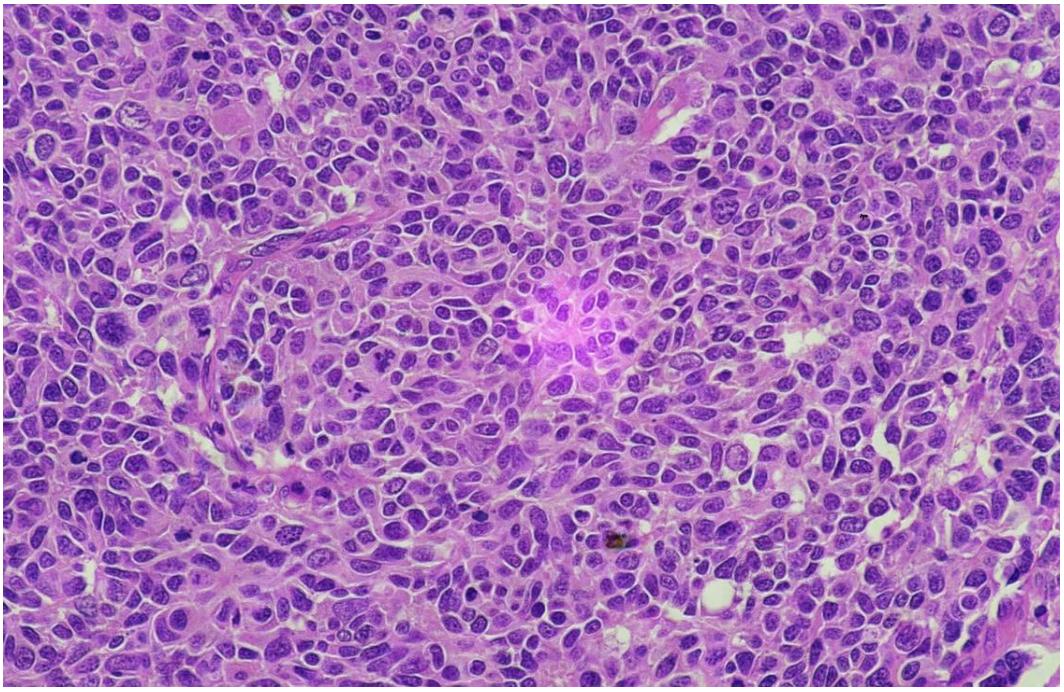
COLOR PLATE 11: MiNEN, 100X, H & E stain



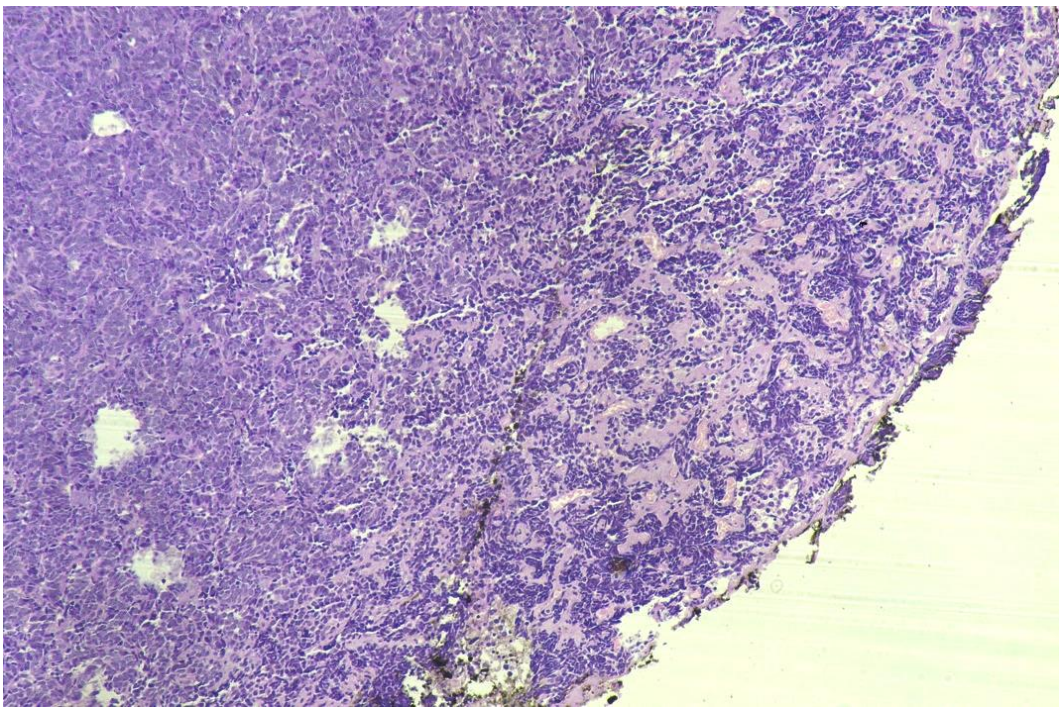
COLOR PLATE 12: MiNEN, 200X, H & E stain



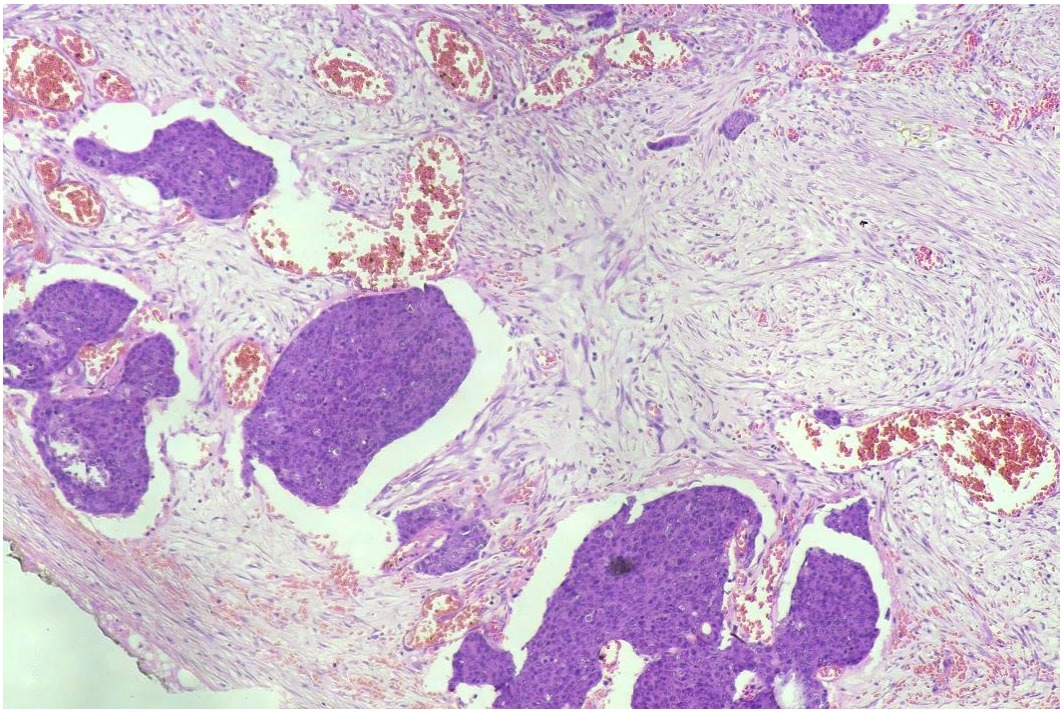
COLOR PLATE 13: LIVER METASTASIS, 200X, H & E stain



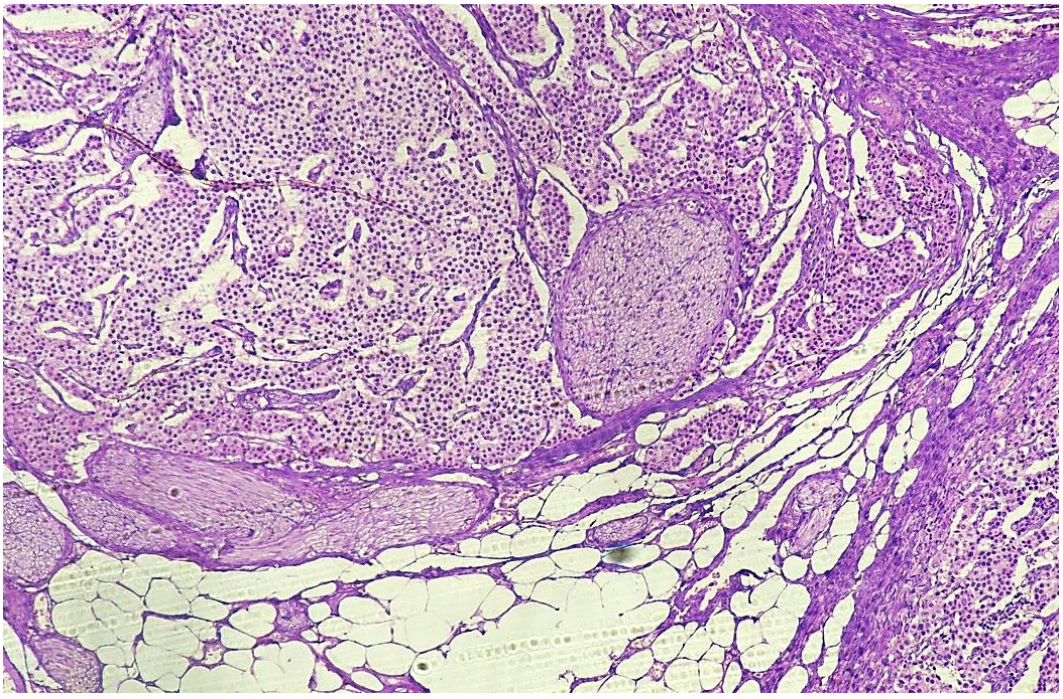
COLOR PLATE 14: LARGE CELL NEC WITH PRESENCE OF ATYPICAL MITOSIS,200X, H& E stain



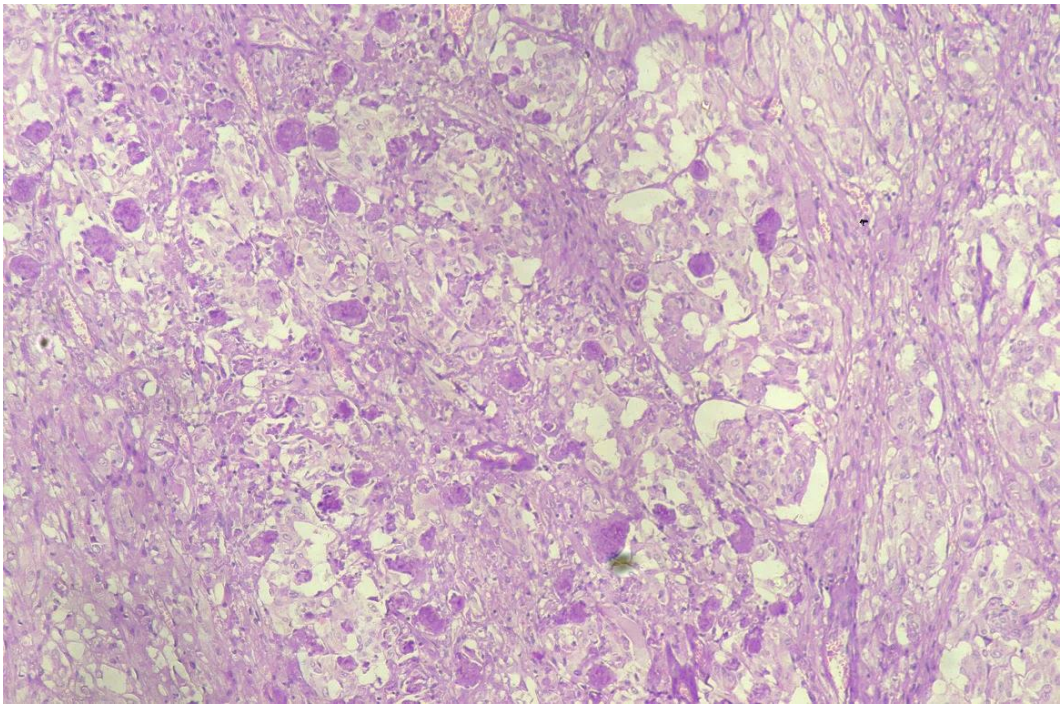
COLOR PLATE 15: LYMPH NODE METASTASIS-100X, H& E stain



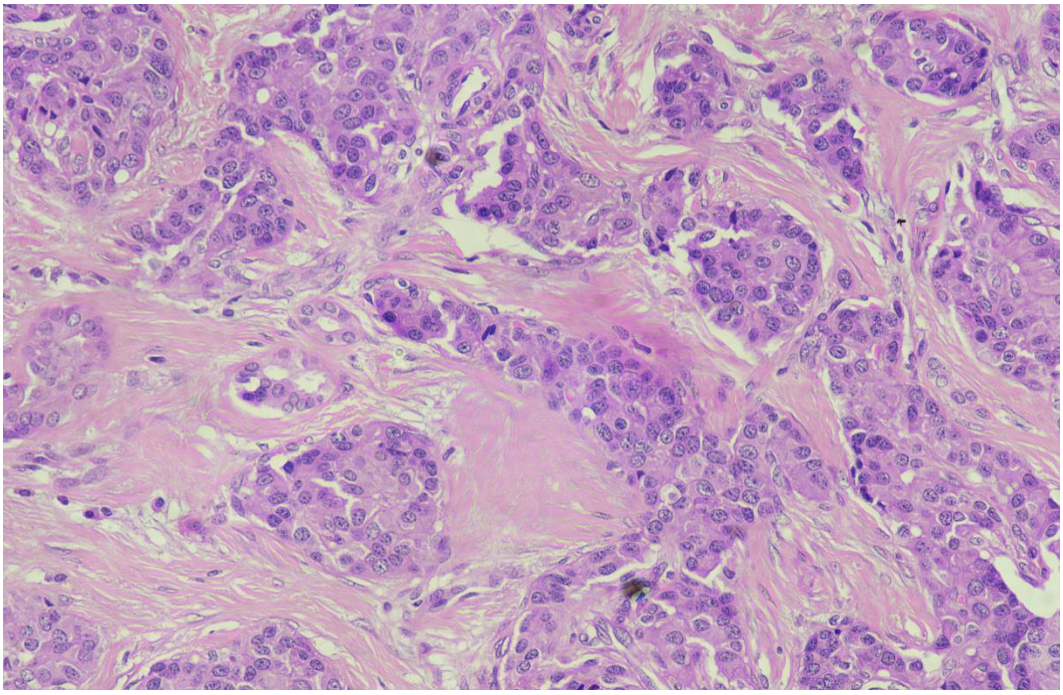
COLOR PLATE 16: LYMPHOVASCULAR INVASION-100X, H & E stain



COLOR PLATE 17: PERINEURAL INVASION, 100x, H & E stain

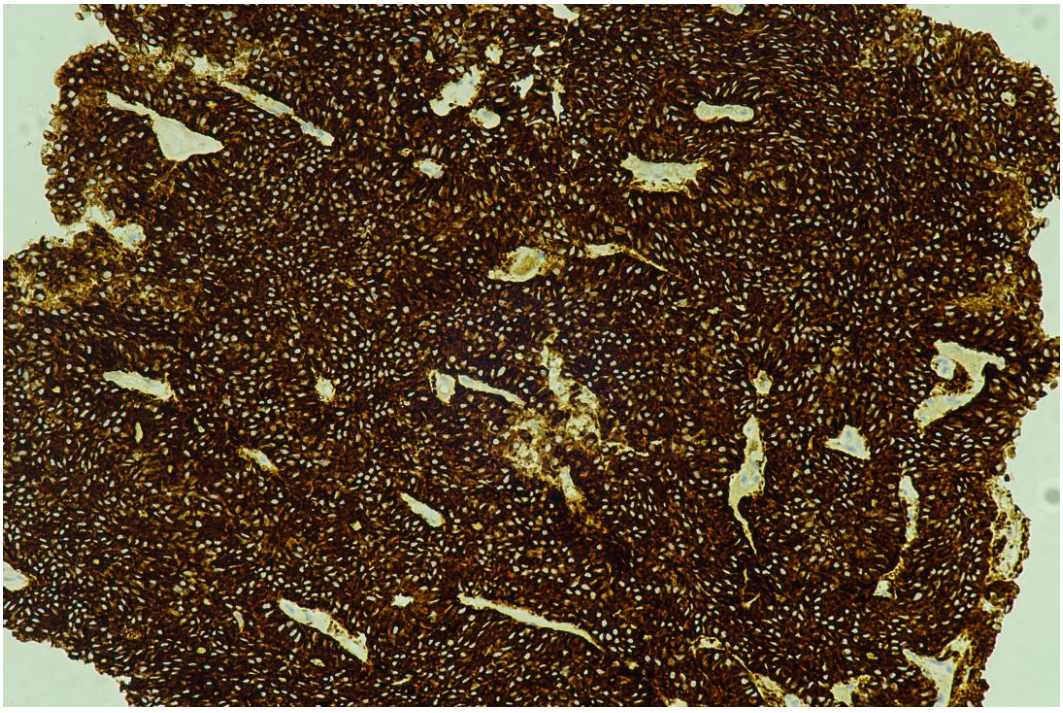


COLOR PLATE 18: PSAMMOMATOUS CALCIFICATION IN NET, 100x, H & E stain

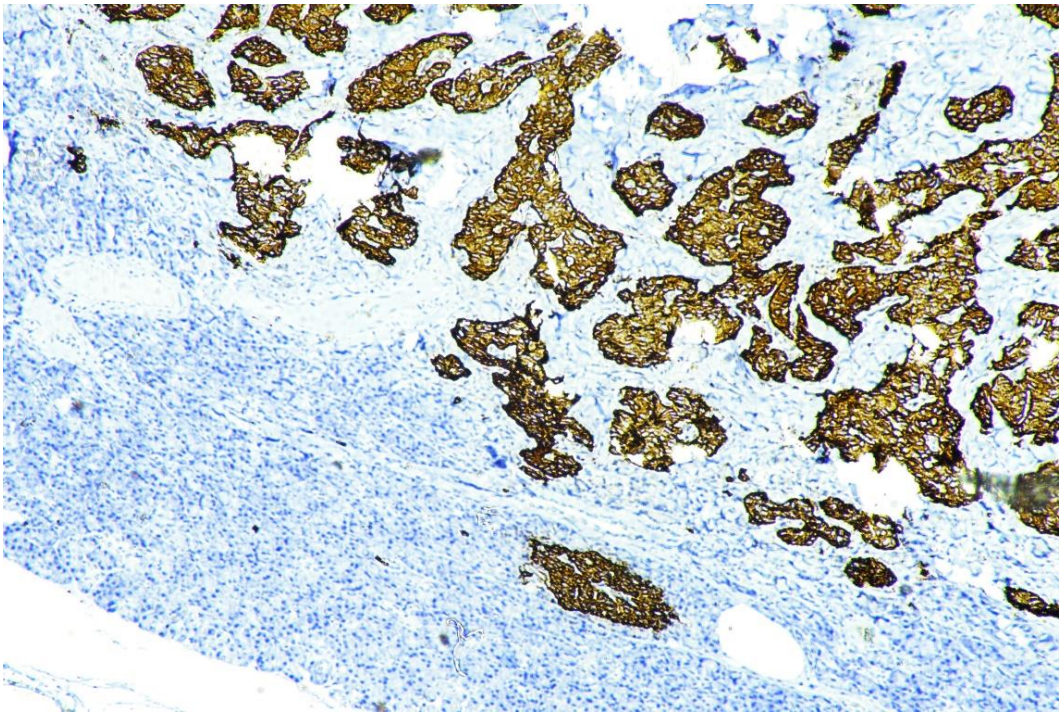


COLOR PLATE 19: AMYLOID DEPOSITION – 200X, H & E stain

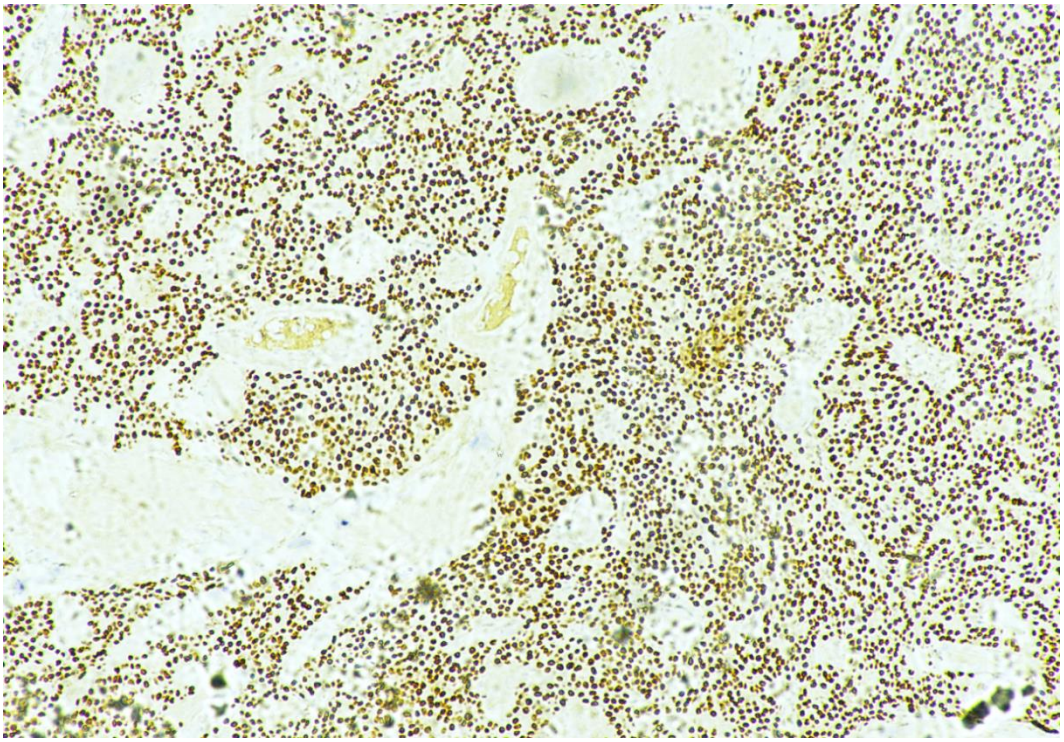
COLOR PLATE 20: CONGO RED STAIN UNDER POLARIZING MICROSCOPE HIGHLIGHTING AMYLOID DEPOSIT, 100X



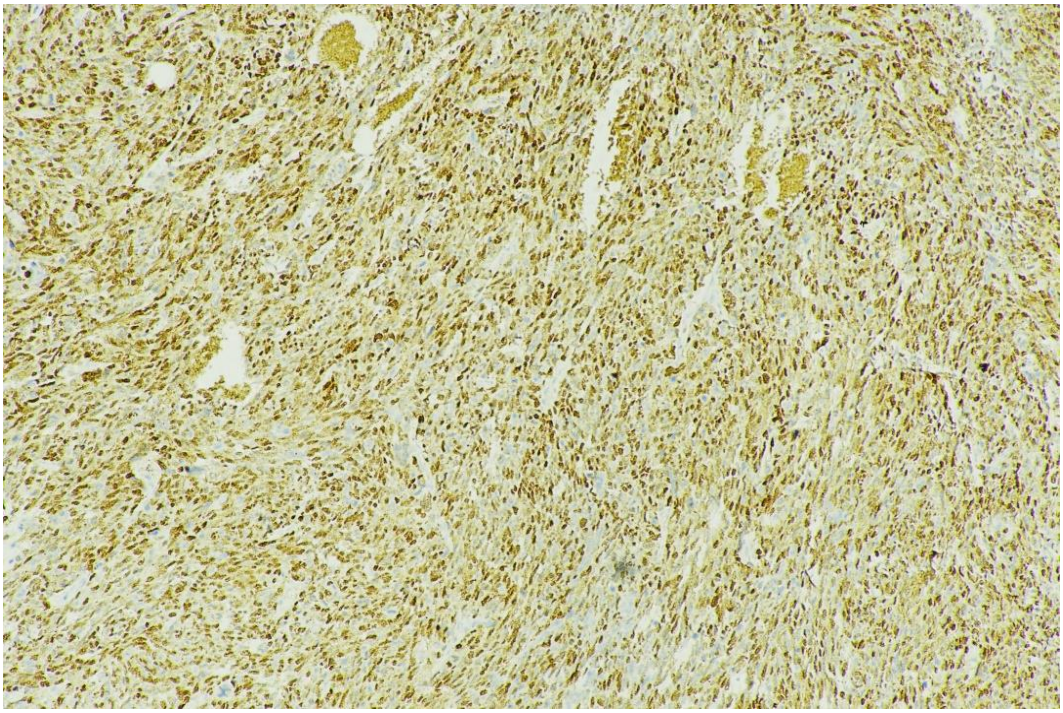
COLOR PLATE 21: SYNAPTOPHYSIN, 100X



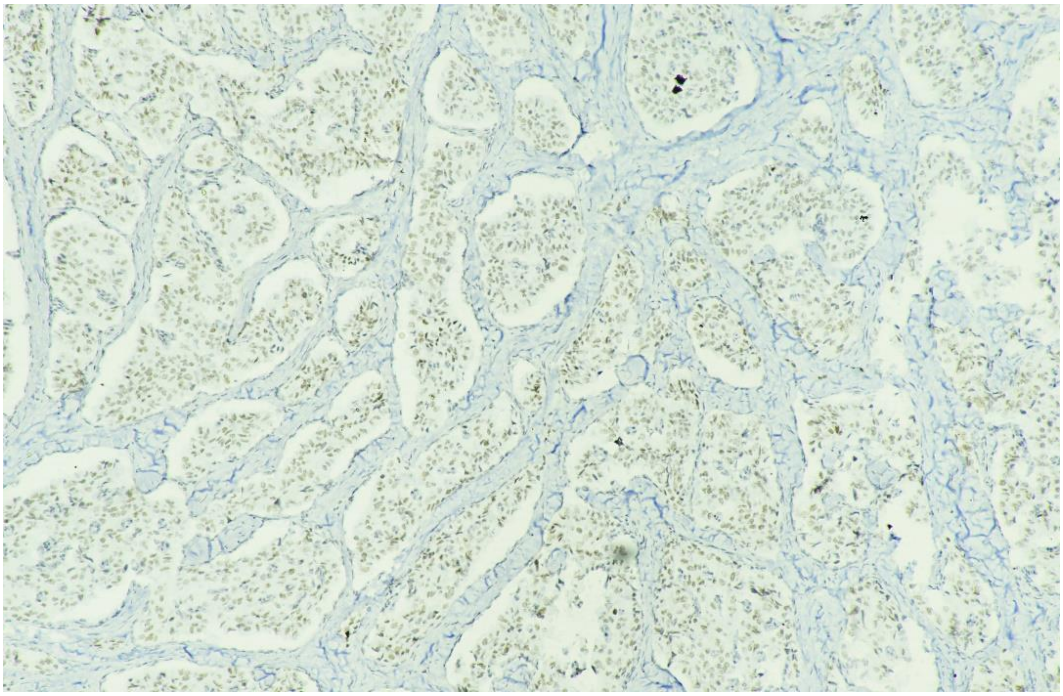
COLOR PLATE 22: CHROMOGRANIN A IN PANCREATIC NET, 100X



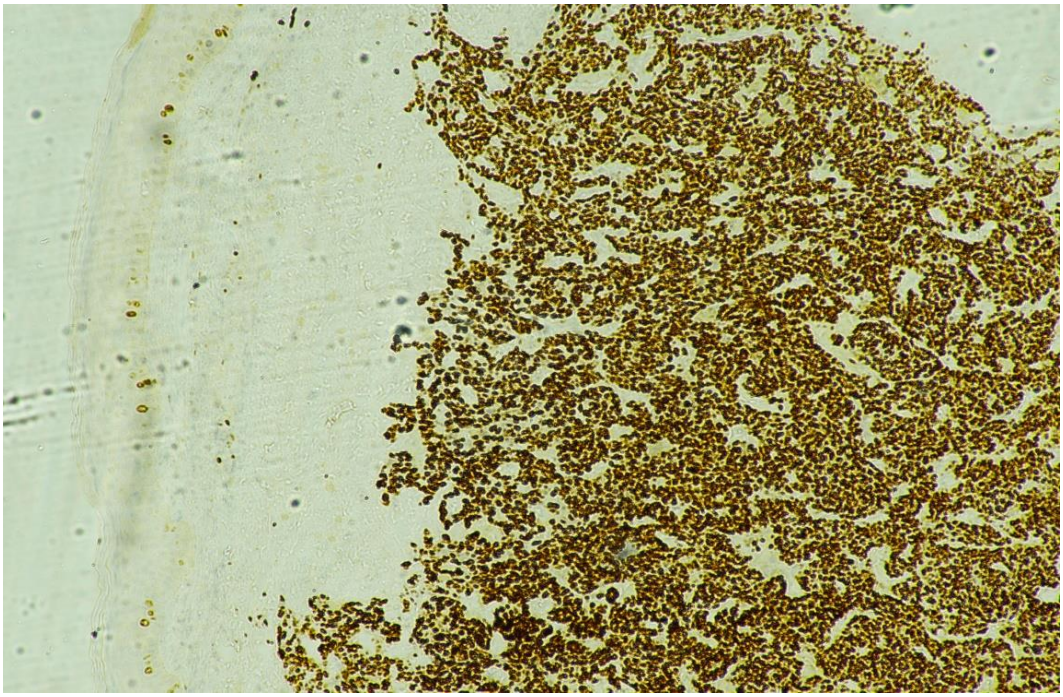
COLOR PLATE 23: INSM1 3+ INTENSITY, 100X



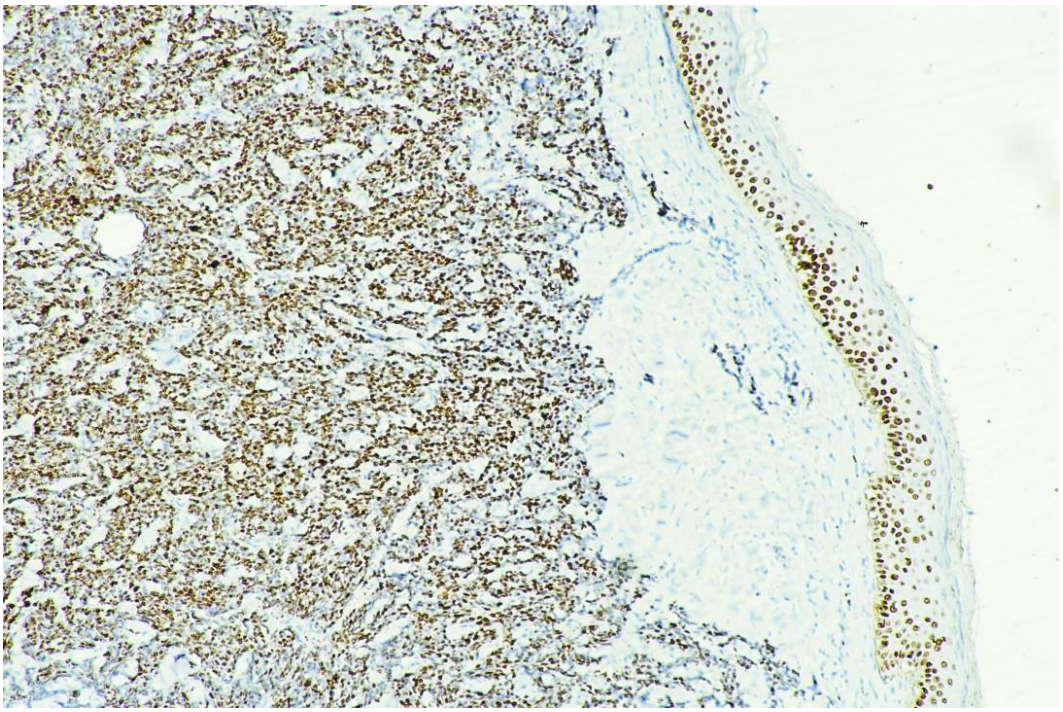
COLOR PLATE 24: INSM1 2+ INTENSITY, 100X



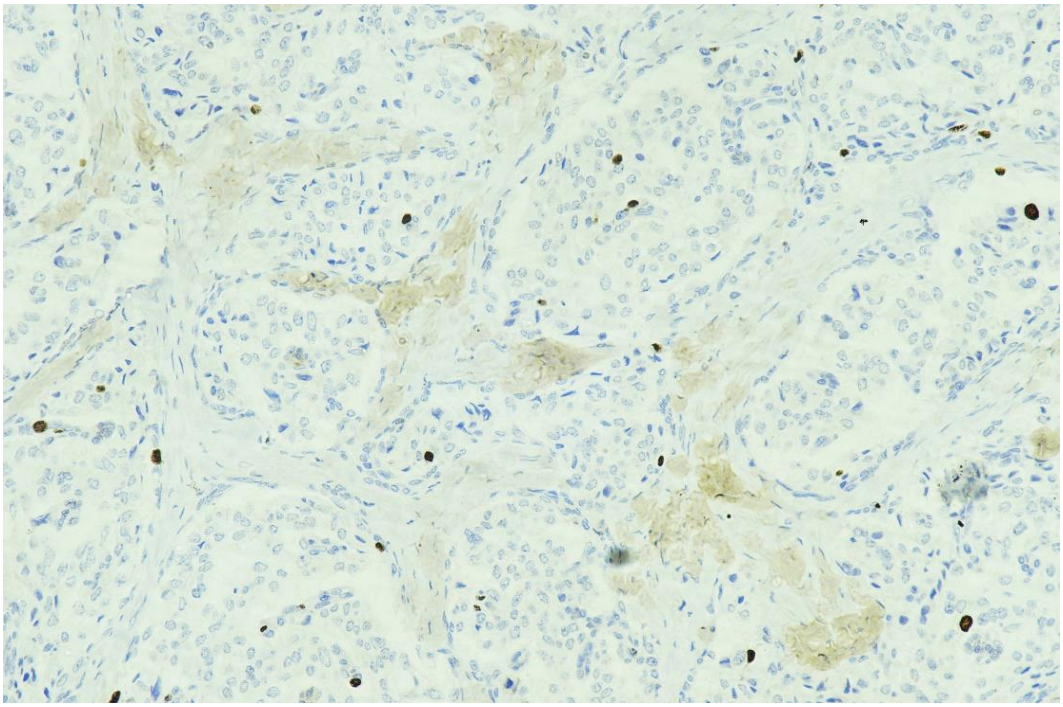
COLOR PLATE 25: INSM1 1+ INTENSITY, 100X



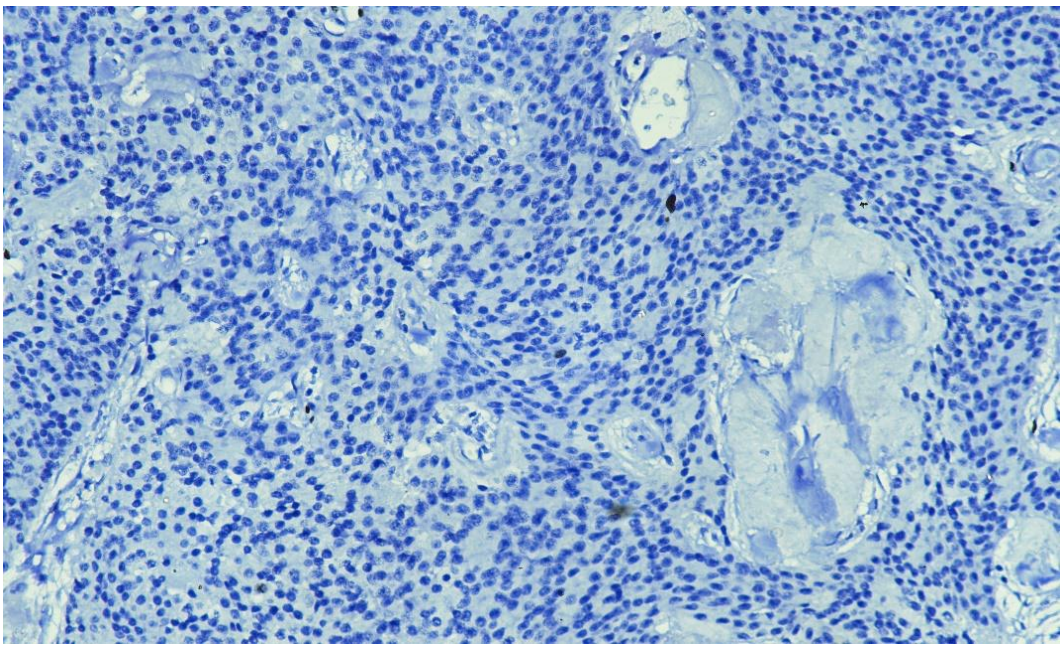
COLOR PLATE 26: 100% Ki67 LI in MERKEL CELL CARCINOMA, 100X



COLOR PLATE 27: 90% PHH3 LI in MERKEL CELL CARCINOMA 100X



COLOR PLATE 28: <3% Ki67 LI, 100X



COLOR PLATE 29: PHH3 LI <3%, 100X

OBSERVATIONS AND RESULTS

A total of 152 cases of neuroendocrine neoplasms, including primary and secondary neuroendocrine tumors and neuroendocrine carcinomas of liver, pancreas, gallbladder, upper and lower gastrointestinal tract, female genital tract, urinary bladder, lymph node metastasis and metastasis to CNS were selected based on the availability of paraffin blocks and the adequacy of tissue. The slides and paraffin blocks were retrieved from the archives of the Department of Pathology & Lab Medicine.

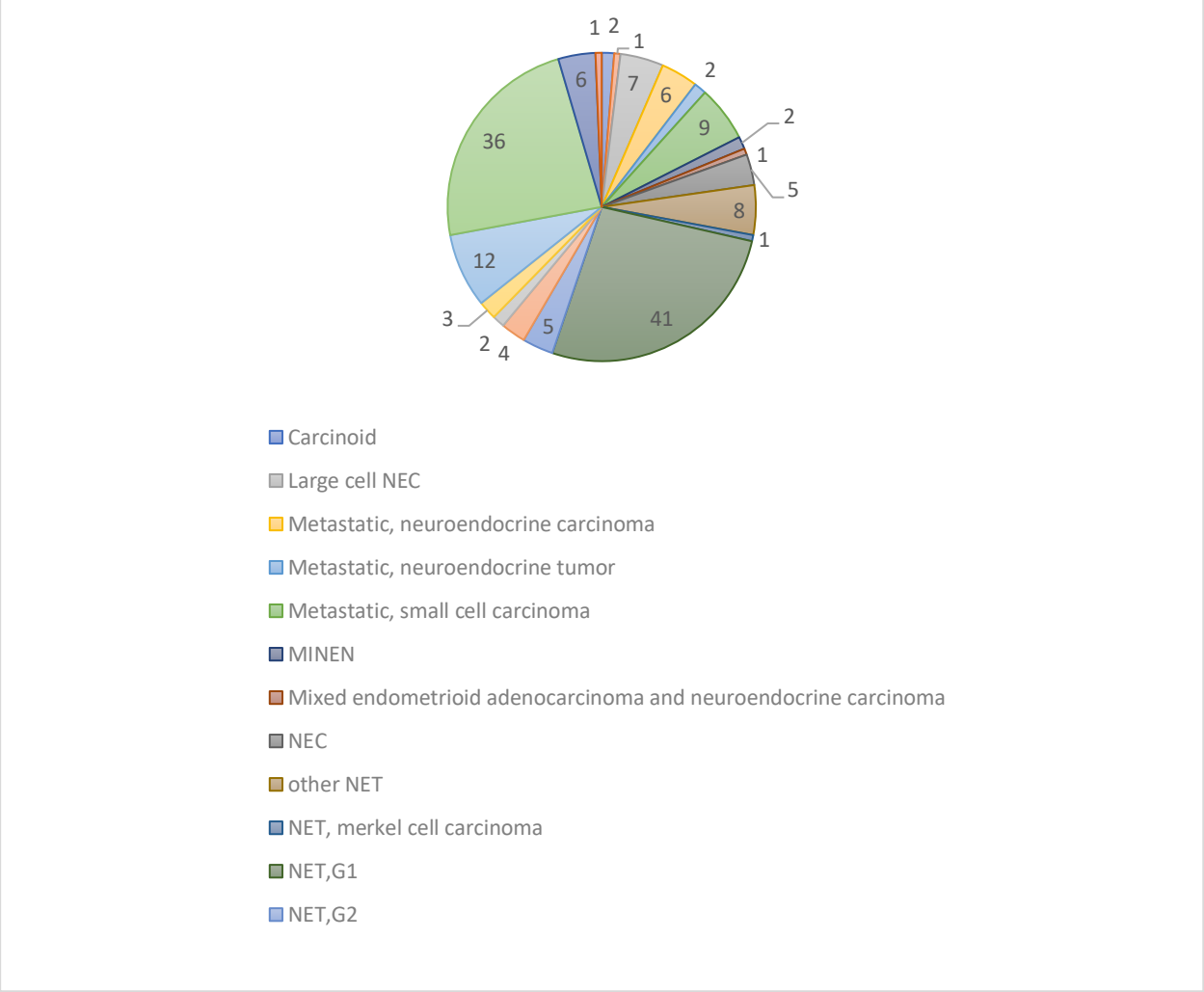


Figure 2: Relative frequency of different types of neuroendocrine tumors.

Table 5: Age distribution of neuroendocrine tumors.

Age Group	No. of Cases	Percentage (%)
0-10	1	0.65
11-20	3	1.97
21-30	8	5.26
31-40	10	6.57
41-50	28	18.42
51-60	38	25.00
61-70	50	32.89
71-80	13	8.55
81-90	1	0.65
Total	**152**	**100**

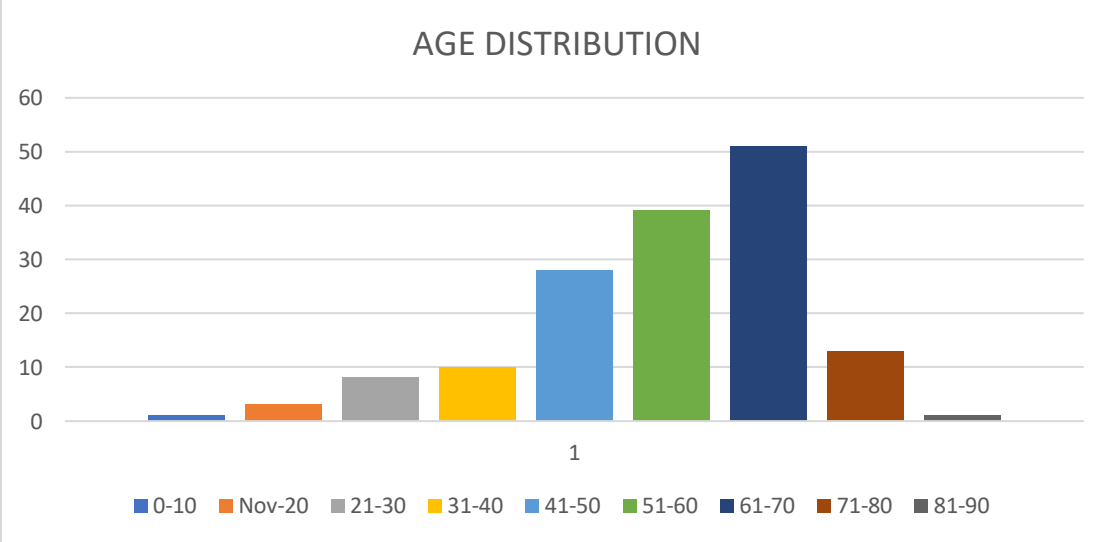
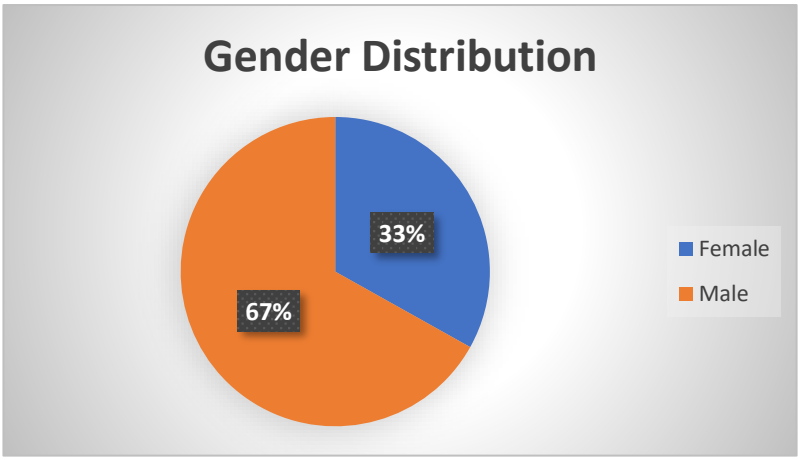


Figure 3: Age distribution of neuroendocrine tumors.

The median age in the present study was 42 years and the mean age was 54.97 years with a range of 2-83 years. The majority (50, 32.89%) of patients were in the 6th to 7th decade. 1 patient (0.65%) was below 10 years of age and 1 patient was above 80 years of age (0.65%).

Figure 4: Gender distribution.



Out of the 152 cases, 102 (67%) patients were males and 50 (32.8%) patients were females.

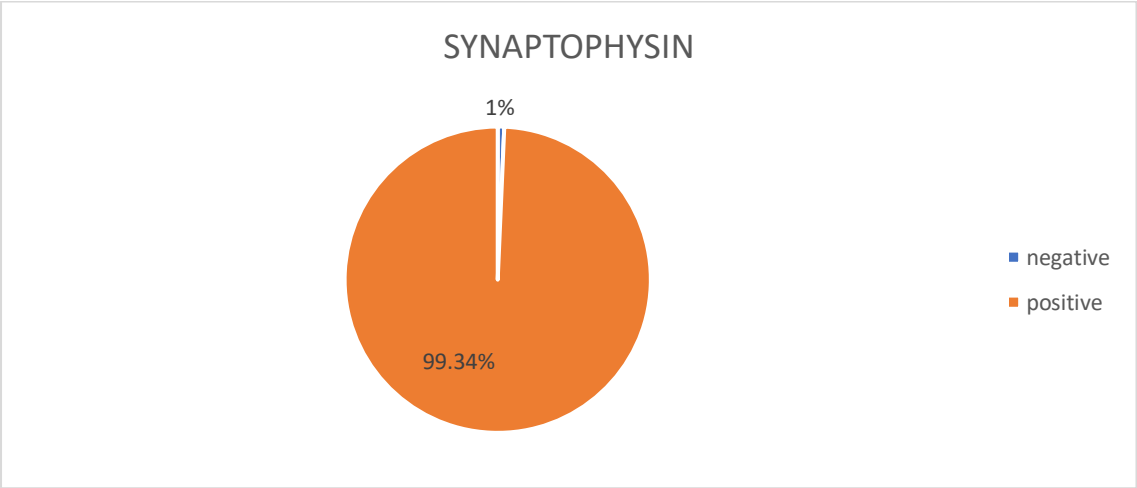
Of the 152 cases, majority were seen in gastrointestinal tract (39 cases) followed by lung (37cases), liver (17 cases, including primary and metastasis), metastasis to lymph node (11 cases), pancreas (8 cases), breast (6 cases), urinary bladder (6 cases), female genital tract (4cases), metastasis to CNS (4 cases), gall bladder (1case) and others (19 cases).

Table 6: Site distribution.

ORGANS	No. of cases	Percentage (%)
Lung	37	24.34
Upper GIT	27	18.18
Others	19	12.5
Liver	17	11.04
Lower GIT	12	7.79
Lymph node	11	7.14
Pancreas	8	5.19
Breast	6	3.90
Urinary bladder	6	3.90
CNS	4	2.60
FGT	4	2.60
Gall bladder	1	0.65
Grand Total	**152**	**100**

Out of 152 cases included in the study, 96 cases were of small biopsies (63%) and 56 cases were resection specimens (37%). The resection specimens included Whipple's pancreaticoduodenectomy, hemicolectomy, gastrectomy, TURBT, MRM, radical cystoprostatectomy, hysterectomy and excised lymph nodes.

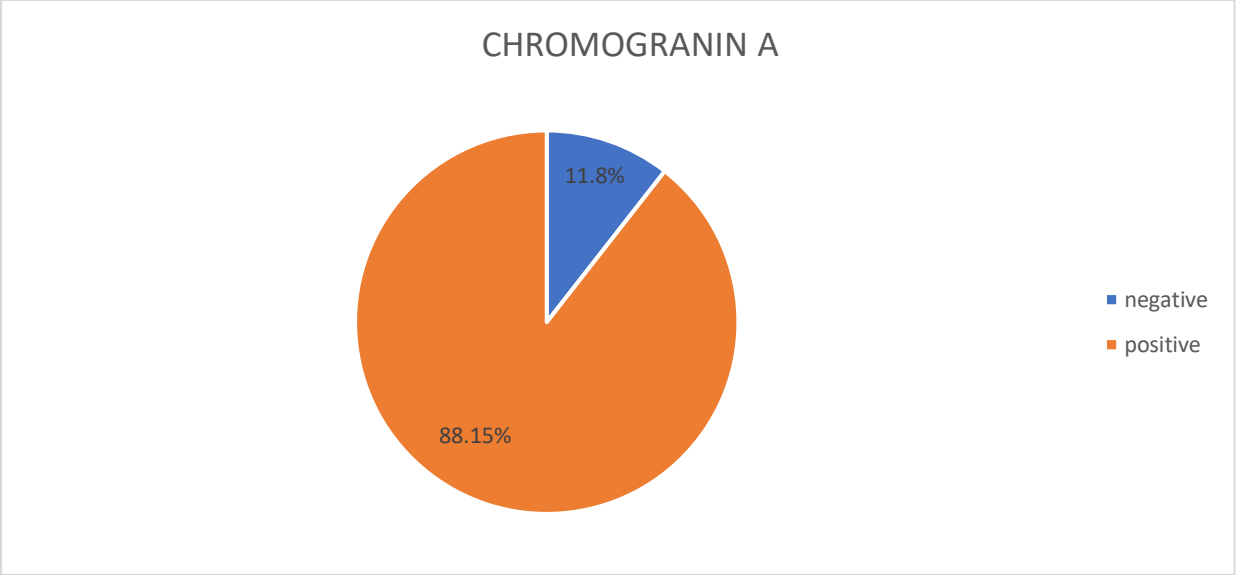
Figure 5: SYNAPTOPHYSIN.



Synaptophysin was applied in all 152 cases, of which 151 cases (99.34%) were immunopositive, while only one case(0.6%) was immunonegative. This case was

immunonegative for chromogranin A while immunopositive for CD56 and INSM1 antibodies.

Figure 6: CHROMOGRANIN A.



Chromogranin A was applied in all 152 cases, of which 134 cases (88.15%) were immunopositive and 18 cases (11.8%) were immunonegative.

CD56 IHC was applied in 30 cases, of which all the cases showed immunopositivity.

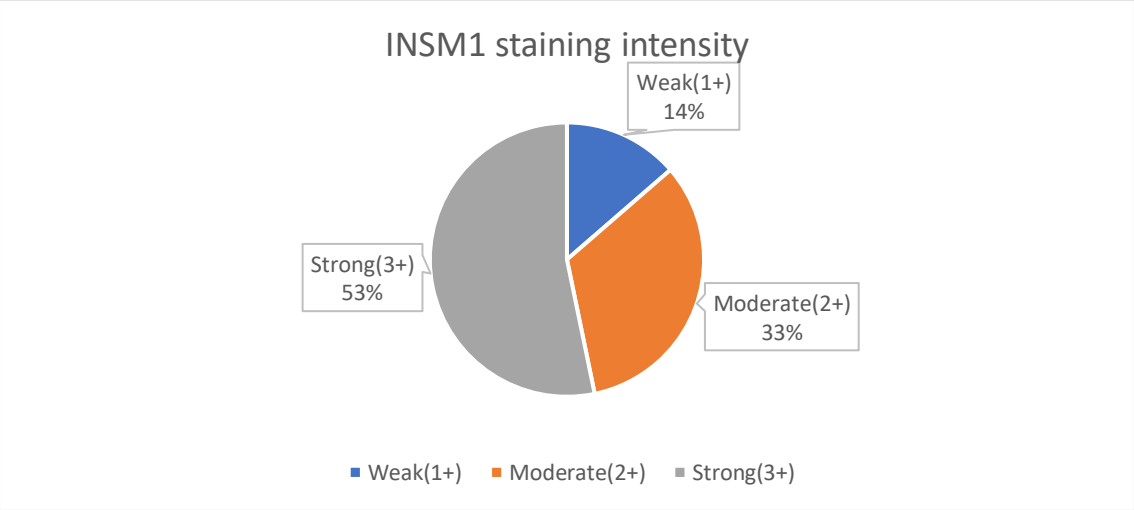
INSM1:

INSM1 was applied in all 152 cases of neuroendocrine tumor and all the cases (100%) showed nuclear positivity for INSM1. INSM1 expression was detected in all samples of primary and metastatic neuroendocrine neoplasms. The percent of tumor cell staining was scored in quartiles (0, <25%, 25% to 50%, 50% to 75%, > 75%).

Staining intensity was interpreted as weak, moderate, or strong. Weak staining is given score 1, moderate staining score 2 and strong staining is given as score 3.

Table 7 and Figure 7: Distribution of cases as per intensity of INSM1 staining.

INSM1 INTENSITY	Frequency	Percentage (%)
Weak(1+)	21	13.8
Moderate(2+)	50	32.8
Strong(3+)	81	53.2
Total	**152**	**100**

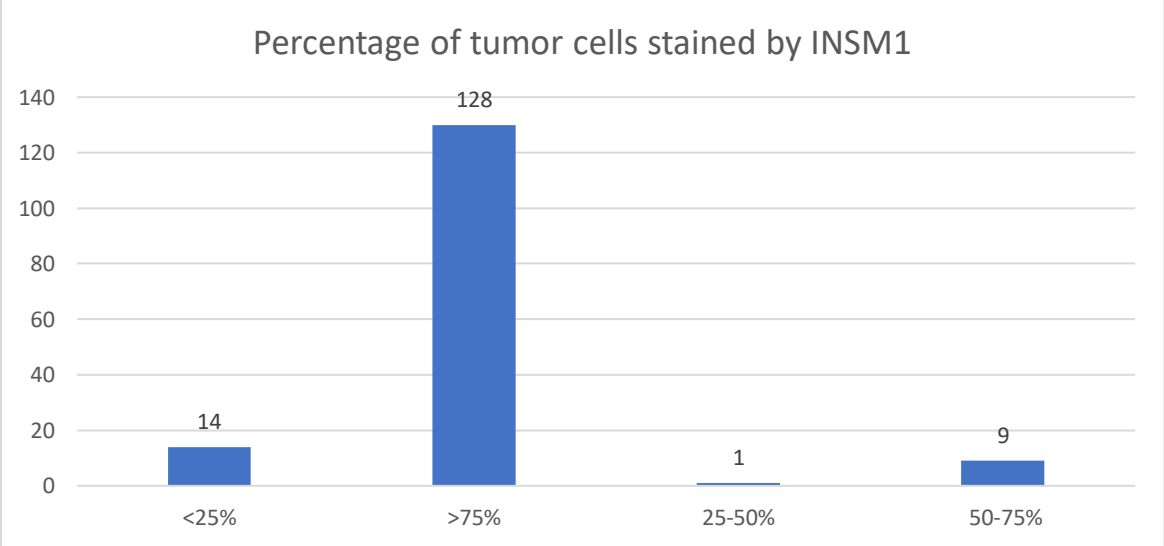


Out of 152 cases, majority, 81 cases (53.2%) had strong intensity of INSM1 staining, while 50 cases (32.8%) had moderate intensity of staining and 21 cases (13.8%) had weak intensity.

Table 8: Distribution of cases as percentage of tumor cells stained by INSM1.

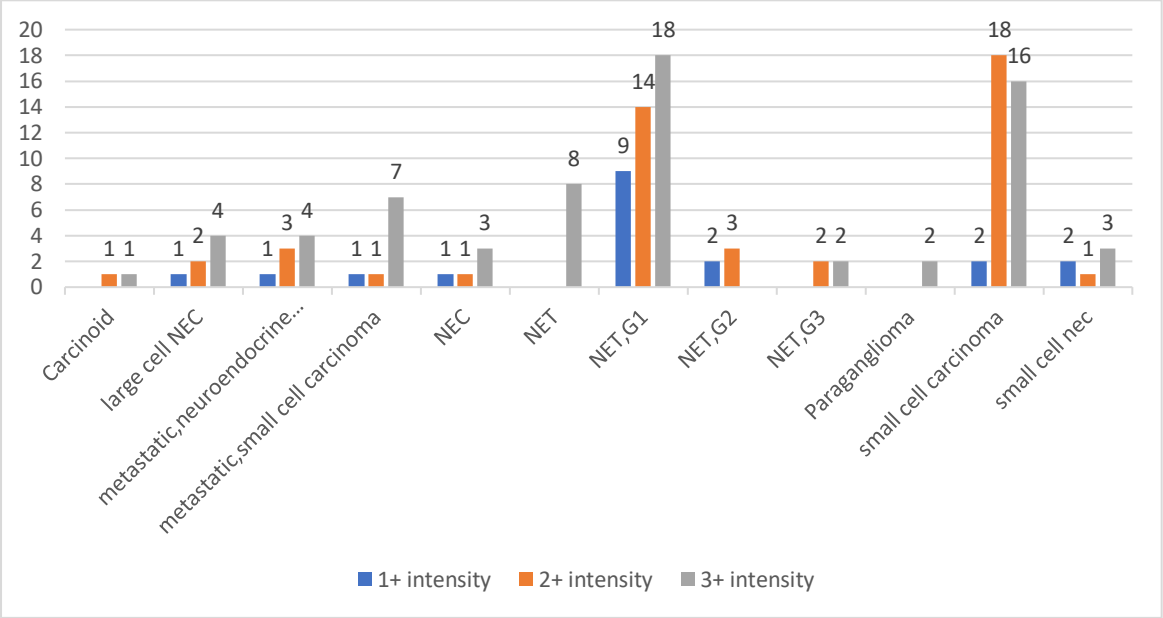
Percentage of tumor cells with INSM1 positivity	Cases	Percent
<25%	14	9.2
25-50%	1	0.6
50-75%	9	5.9
>75%	128	84.2
Total	**152**	**100**

Figure 8: Distribution of cases according to percentage of tumor cells stained positively by INSM1.



Out of 152 cases, 128 cases (84.2%) had >75% cells stained by INSM1. 14 cases (9.2%) had <25% stained cells, 9 cases (5.9%) had 50-75% of stained cells and 1 case (0.6%) had staining of 25-50% cells.

Figure 9: INSM1 intensity scoring among neuroendocrine tumors.



INSM1 positivity was seen in all the cases of neuroendocrine tumors. However, the intensity and percentage of staining showed variable results.

NECROSIS:

Out of 152 cases, 43 cases (28.28%) showed necrosis.

LYMPHOVASCULAR INVASION:

Out of 56 resection specimen, 13 cases showed lymph-vascular invasion.

PERINEURAL INVASION:

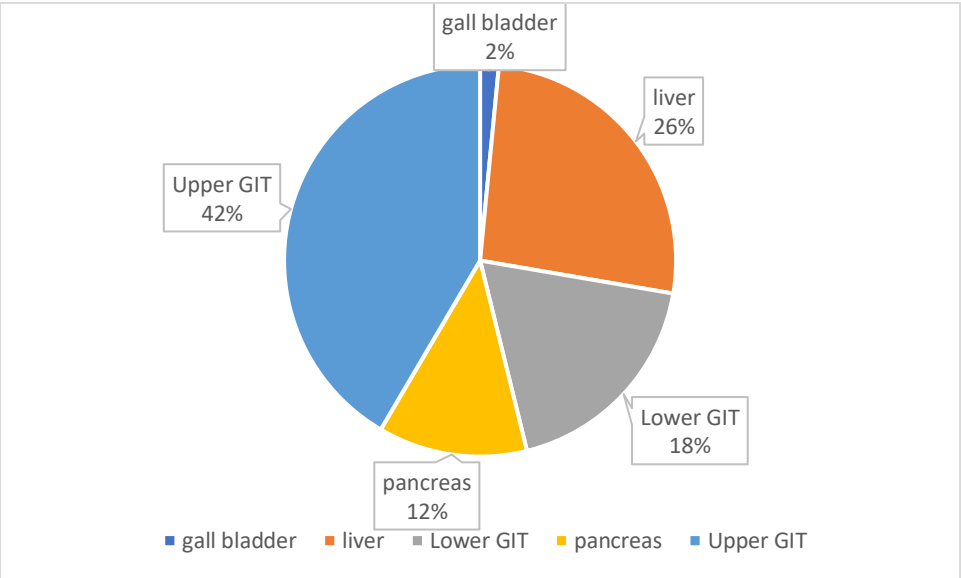
Out of 56 resection specimens, 12 cases showed perineural invasion.

NEN in digestive system:

The category of gastroenteric NETs, including gastric, intestinal, rectal is heterogeneous and may be quite clinically aggressive due to the risk associated with their location, higher grade, and high mitotic index. Out of 152 cases, 65 cases included in the present study are NET of stomach, ileum, duodenum, colon, pancreas, gall bladder and liver. 27 cases (41.53%) belonged to upper gastrointestinal tract, 8 cases (12.3%)were of pancreas, 12 cases(18.46%) of lower gastrointestinal tract, 17 cases

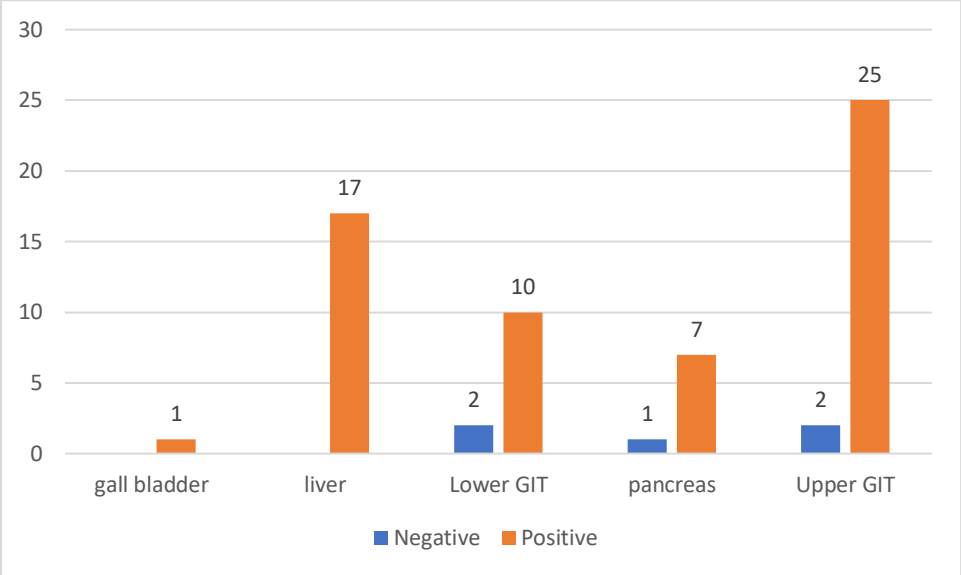
(26.1%) of liver and one case(1.5%) of gall bladder. 64 cases(98.4%) were immunopositive for synaptophysin and one was immunonegative.

Figure 10: Distribution of cases in NEN of gastrointestinal tract.



Conventional neuroendocrine marker, Chromogranin A was applied in 65 cases of digestive tract including pancreatico-biliary tract, out of which 60 cases (94.44%) were immunopositive for chromogranin A. 25cases (41.6%) were of upper gastrointestinal tract (stomach, duodenum and ileum), 17cases (28.33%) were of liver, 10 cases (16.66%) of lower gastrointestinal tract(rectum, caecum and colon), 7 cases of pancreas (11.6%) and 1 case (1.6%) of gall bladder. 5 cases (7.6%) were immunonegative for chromogranin A, one from pancreas, and two each from upper and lower gastrointestinal tract.

Figure 11: Chromogranin A positive and negative cases in NEN of digestive tract.



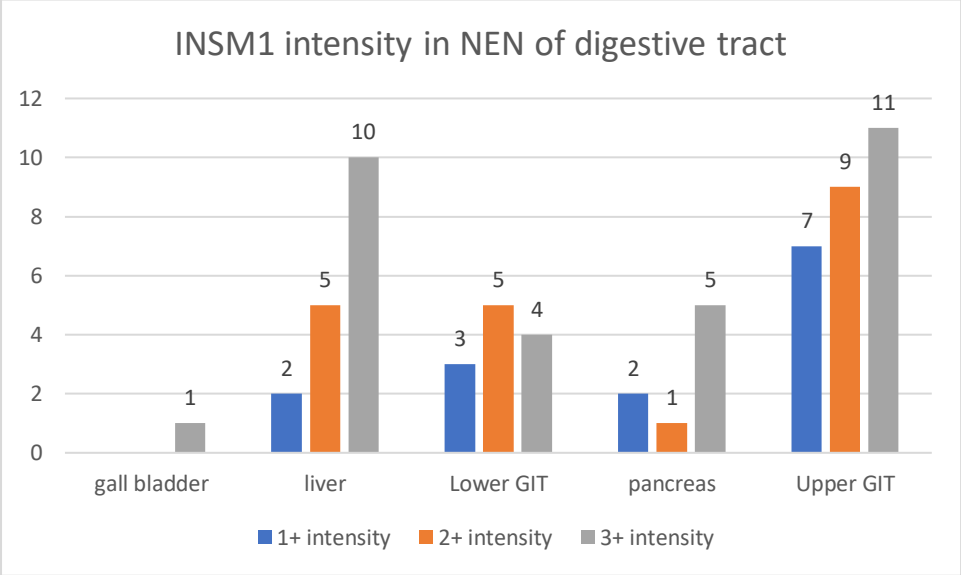
INSM1 in NEN of digestive tract:

All the 65 cases of gastrointestinal tract were immunopositive for INSM1 (100%). Strong INSM1 intensity was seen in 31 cases (46.96%), of which 11 cases (16.66%) were of upper gastrointestinal tract, 10 cases (15.15%) of liver, 5 cases (7.5%) of pancreas, 4 cases (6.0%) of lower gastrointestinal tract and 1 case (1.5%) of gall bladder.

Moderate intensity of INSM1 was seen in 20 cases (31.81%), of which 9 cases (15.15%) were of upper gastrointestinal tract, 5 cases (7.5%) of liver, 1 case (1.5%) of pancreas and 5 cases (7.5%) of lower gastrointestinal tract.

Weak intensity of INSM1 was seen in 14 cases (21.21%), of which 7 cases (10.60%) were of upper gastrointestinal tract, 2 cases (3.03%) of liver, 2 cases (3.03%) of pancreas and 3 cases (4.5%) of lower gastrointestinal tract.

Figure 12: INSM1 staining intensity in NEN of digestive tract.

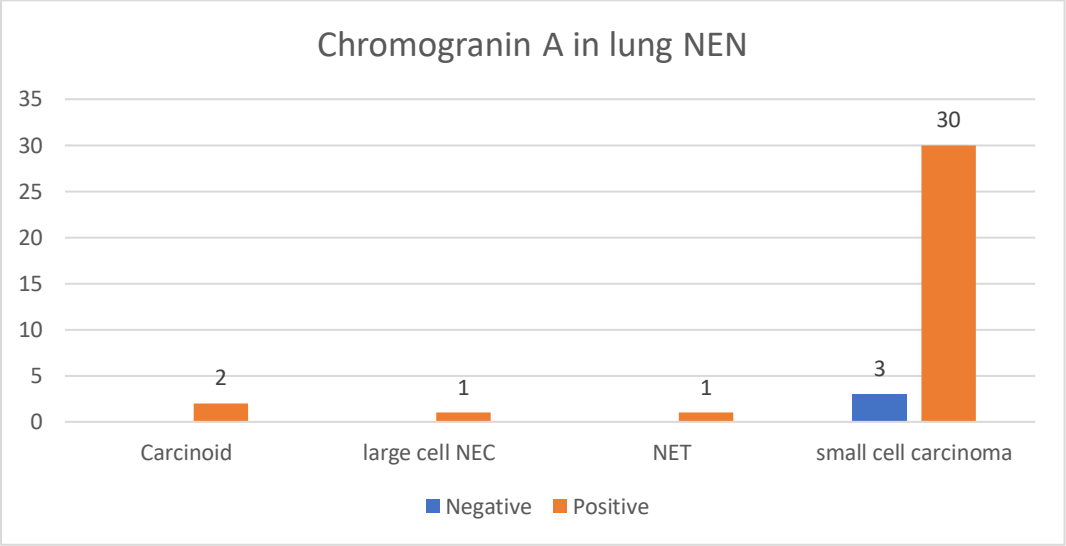


Pulmonary neuroendocrine neoplasm.

A total of 37 cases are included in the present study. 2 cases are of LCNEC, 3 cases of carcinoid and 32 cases of small cell carcinoma. All the cases (100%) were immunopositive for synaptophysin.

Chromogranin A was applied in 37 cases of lung NEN, of which 34 (91.89%) are immunopositive for chromogranin A. 3 cases (8.1%) of small cell carcinoma are immunonegative for chromogranin A.

Figure 13: Chromogranin A in lung NEN.



INSM1 intensity in lung NEN.

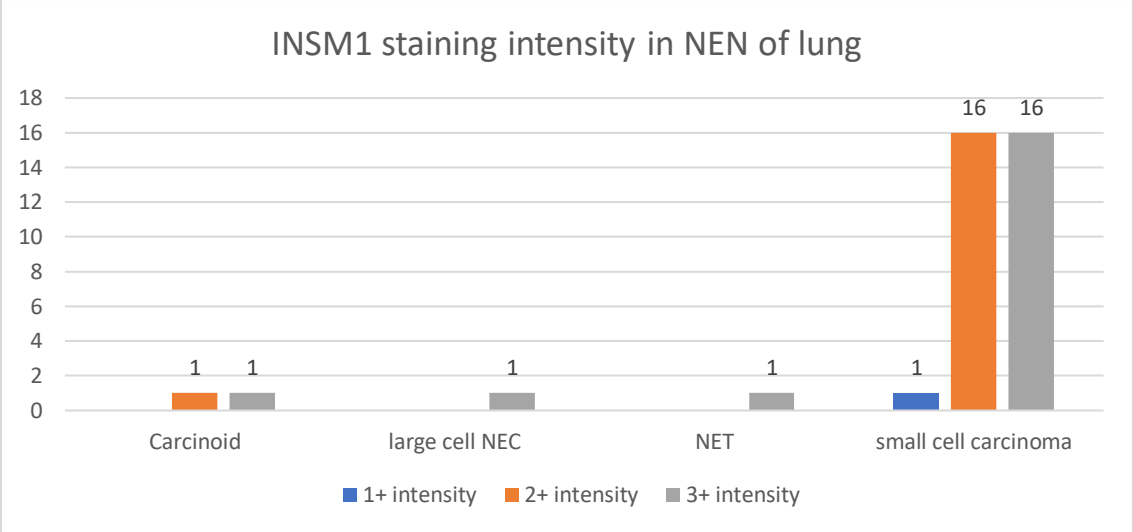
All the 37 cases were immunopositive for INSM1 (100%). Strong INSM1 intensity was seen in 19 cases (51.35%), of which 15 cases (40.54%) were small cell carcinoma, 2 cases (5.40%) of carcinoid, 2 cases (5.40%) of large cell NEC.

Moderate INSM1 intensity was seen in 17 cases (43.58%), of which 16 cases (41.02%) were small cell carcinoma and 1 case (2.5%) of carcinoid.

Weak INSM1 staining intensity was seen in one case of small cell carcinoma.

Table 9 and Figure 14: INSM1 staining intensity in NEN of LUNG.

LUNG NEN	1+	2+	3+	Grand total
Carcinoid		1	1	3
Large cell NEC			1	2
Small cell carcinoma			1	1
Grand Total	1	16	16	32
	1	17	19	37

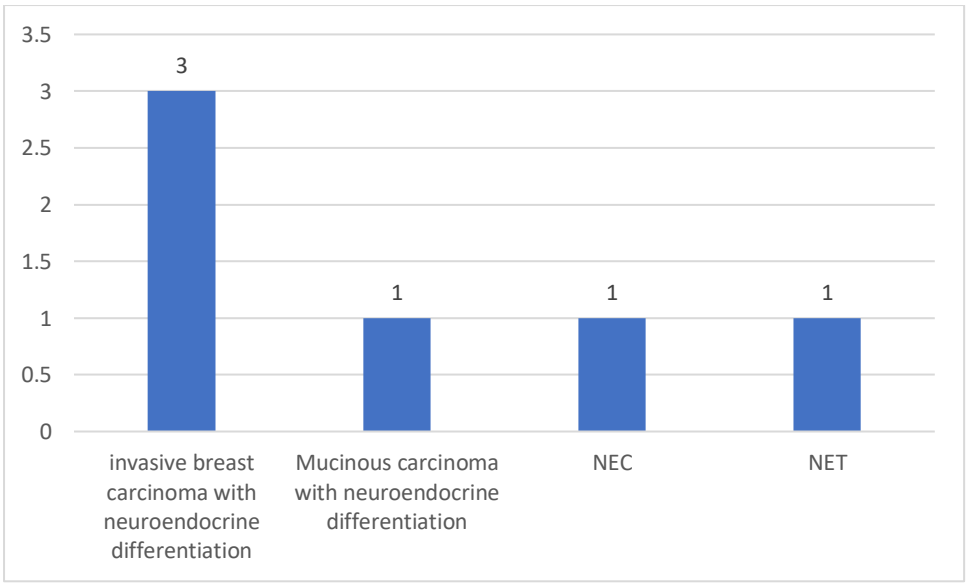


NEN in Breast:

6 cases of neuroendocrine neoplasm were included in the present study. Morphologically, NETs of the breast were categorized into well differentiated NETs, poorly differentiated neuroendocrine carcinomas and invasive breast carcinoma with neuroendocrine differentiation. 1 case (16.6%) was well differentiated NET and 4 cases (66.66%) were IBC with neuroendocrine differentiation and one case (16.6%) was of neuroendocrine carcinoma.

Synaptophysin was applied in all cases, and all the cases were immunopositive (100%).

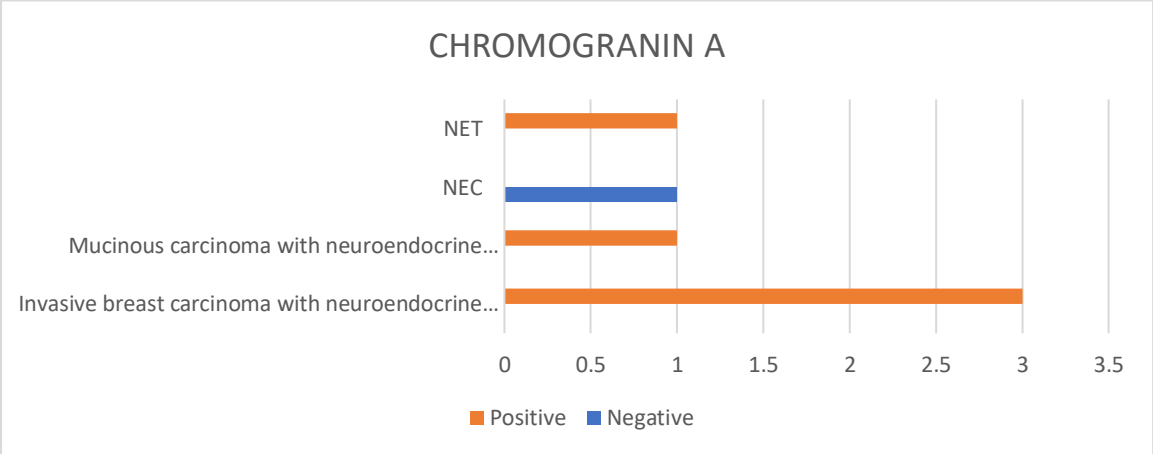
Figure 15: Breast neuroendocrine neoplasm.



Chromogranin A expression was noted in 5 cases. One case of neuroendocrine carcinoma was negative for chromogranin A.

Table 10 and Figure 16: Chromogranin A expression in NEN of breast.

NEN of Breast	negative	positive	Grand Total
Invasive breast carcinoma with neuroendocrine differentiation		3	3
Mucinous carcinoma with neuroendocrine differentiation		1	1
NEC	1		1
NET		1	1
Grand Total	**1**	**5**	**6**



INSM1 expression in breast NEN:

INSM1 was applied in all the breast NEN cases, where all were immunopositive for INSM1. However, Strong INSM1 intensity was seen in 5 cases (83.33%), of which 3 cases (50.00%) were IBC with neuroendocrine differentiation, 1 case (16.66%) of NET and 1 case(16.66%) of NEC.

Moderate INSM1 intensity was seen in 1 case (16.66%) of IBC with neuroendocrine differentiation.

Table 11: INSM1 intensity in NEN of breast.

NEN of breast	1+	2+	3+	Grand Total
Invasive breast carcinoma with neuroendocrine differentiation		1	2	3
Mucinous carcinoma with neuroendocrine differentiation			1	1
NEC			1	1
NET			1	1
Grand Total		**1**	**5**	**6**

The percentage of cells stained by INSM1 was more than 75% in all the cases of NEN of breast.

NEN in lymph nodes:

11 cases of metastatic NEN were included in present study. Synaptophysin and Chromogranin A was applied in all the cases. Synaptophysin was positive in all the 11 cases, while chromogranin A was positive in 8 cases (72.72%) and negative in 3 cases (27.27%). CD56 was applied in 4 cases and all the cases (100%) showed immunopositivity.

INSM1 intensity in metastatic NEN of lymph nodes:

All the 11 cases were immunopositive for INSM1. Strong intensity was seen in 6 cases (54.54%), moderate intensity was seen in 4 cases (36.36%) and weak intensity was seen in 1 case.(9.09%)

Table 12: Distribution of cases as per intensity of staining of INSM1.

Metastatic NEN of lymph node	1+	2+	3+	Grand Total
Large cell NEC		1		1
Metastatic neuroendocrine carcinoma			2	2
Metastatic neuroendocrine tumor	1	1		2
Metastatic small cell carcinoma		1	2	3
NEC			1	1
NET,G1		1	1	2
Grand Total	**1**	**4**	**6**	**11**

INSM1 in Genitourinary tumors:

Out of 152 cases, 10 cases of genitourinary tumors were included in the study of which 6 cases (60%) were from urinary bladder, 3 cases (30%) were from cervix and one case (10%) was of endometrial adenocarcinoma with neuroendocrine differentiation.

Synaptophysin was positive in all the cases (100%). Chromogranin A showed immunopositivity in 7 cases (77.77%) and 3 cases (30%) were negative.

INSM1 showed strong intensity in 5 cases (50%), moderate intensity in 4 cases (40%) and weak intensity in 1 case(10%).

Table 13: Distribution of cases as per intensity of staining of INSM1.

Genitourinary tumor	1+	2+	3+	Grand Total
Large cell NEC		1		1
Metastatic small cell carcinoma			1	1
Mixed endometrioid adenocarcinoma and neuroendocrine carcinoma		1		1
Poorly differentiated carcinoma with neuroendocrine differentiation			2	2
Small cell carcinoma		1		1
Small cell NEC	1	1	2	4
Grand Total	**1**	**4**	**5**	**10**

NEN in skin:

One case of merkel cell carcinoma was seen in the study. The tumor cells showed immunopositivity for chromogranin A, synaptophysin and INSM1. INSM1 showed strong intensity with nuclear staining in >75% of the tumor cells.

Ki-67 Labelling Index:

The mean (SD) of Ki67 (%) was 45.52 (41.51). The median (IQR) of Ki67 (%) was 40.00 (2-90). The Ki67 (%) ranged from 1 - 100.

Ki67 based grading tumors:

Using Ki67 alone as a method of grading, the tumors were classified by WHO classification into G1, G2 and G3 neoplasms. 57(37.5%) of the cases showed Ki67 of <3%. 14 (9.2%) of the cases had Ki67 of 3-20%. 81 (53.3%) of the cases had Ki67 of >20%.

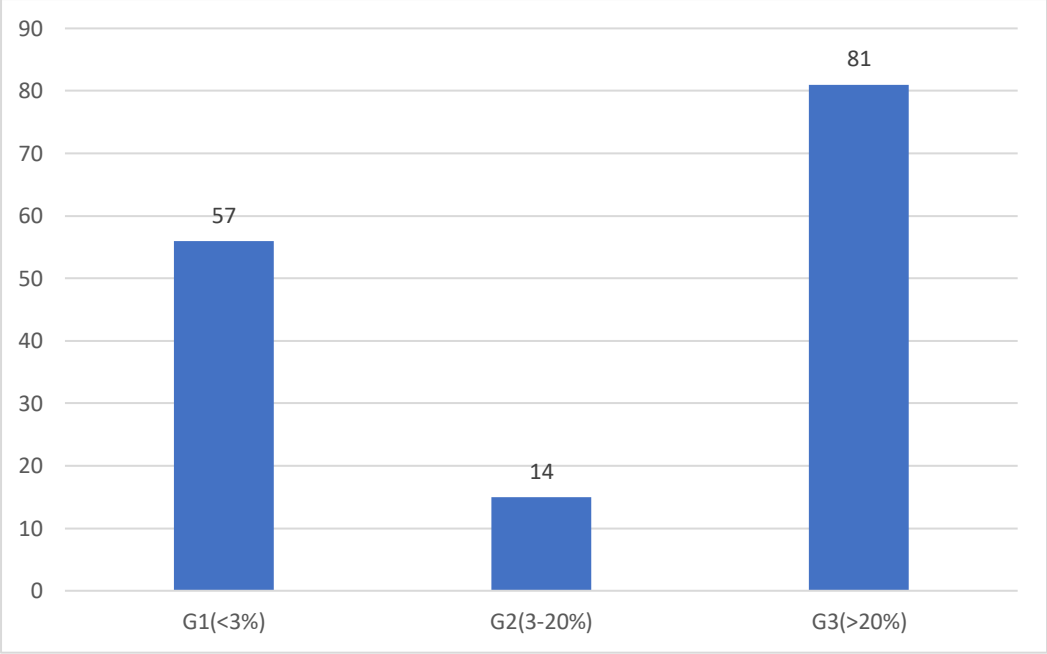


Figure 17: Frequency of cases as per Ki67 labelling index.

PHH3 in grading of NENs:

There is no standard for grading based on PHH3 labelling index. So, tumors in the study were provisionally divided using the same cut-offs as WHO Ki67 labelling index for PHH3 LI.

The mean (SD) of PHH3 (%) was 41.32 (39.03). The median (IQR) of PHH3 (%) was 30.00 (2-80). The PHH3 (%) ranged from 1-90.

60 (39.5%) 1 of the cases had PHH3 of <3%. 13(8.6%) of the cases had PHH3 of 3-20%.

79 (52.0%) of the cases had PHH3 of >20%.

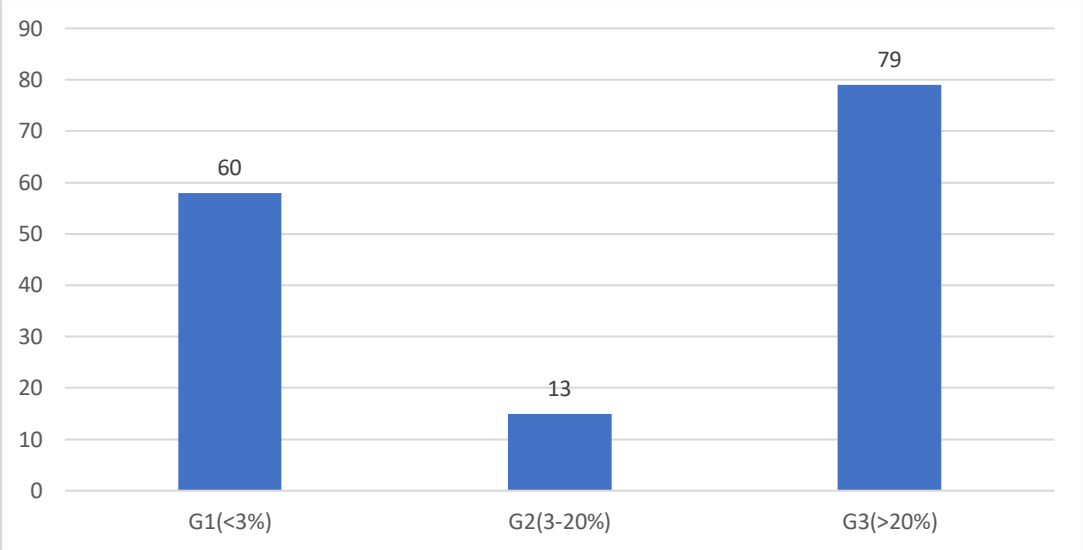


Figure 18: Frequency of cases as per PHH3 labelling index.

Five cases which showed discordance between grade of PHH3 and Ki67 grade.(3.2% discordance).

Using PHH3 MI cut offs for WHO Ki67 criteria, it was found that all the 57 G1 cases were in concordance with WHO G1 cases. Out of 14 WHO G2 cases, 11 were concordant with WHO Grade 2 and 3 cases were downgraded to G1 by PHH3 MI cut off. Similarly, 81 cases of WHO G3 neoplasms, 79 were concordant with G3, 2 were downgraded to G2 using PHH3 MI cut offs.

Table 14: Association between Ki67 and PHH3:

PHH3	Ki67				Fisher's Exact Test	
	<3%	3-20%	>20%	Total	χ2	P Value
<3%	57 (100.0%)	3 (21.4%)	0 (0.0%)	60 (39.5%)	243.908	<0.001
3-20%	0 (0.0%)	11 (78.6%)	2 (2.5%)	13 (8.6%)		
>20%	0 (0.0%)	0 (0.0%)	79 (97.5%)	79 (52.0%)		
Total	57 (100.0%)	14 (100.0%)	81 (100.0%)	152 (100.0%)		

There was a significant difference between the various groups in terms of distribution of PHH3 ($\chi 2 = 243.908$, $p = <0.001$).

Strength of association between the two variables (Cramer's V) = 0.9 (High Association) Strength of association between the two variables (Bias Corrected Cramer's V) = 0.89 (High Association)

Table 15: Association between INSM1 and Synaptophysin:

Synaptophysin	INSM1		Chi-Squared Test	
	Positive	Total	$\chi 2$	P Value
Positive	151 (99.3%)	151 (99.3%)	-	-
Negative	1 (0.7%)	1 (0.7%)		
Total	152 (100.0%)	152 (100.0%)		

Chi-squared test was used to explore the association between 'INSM1' and 'Synaptophysin'.

There was no significant difference between the various groups in terms of distribution of Synaptophysin ($\chi 2$ = -, p = -).

Table 16: Association between INSM1 and Chromogranin A:

Chromogranin A	INSM1		Chi-Squared Test	
	Positive	Total	$\chi 2$	P Value
Positive	134 (88.2%)	134 (88.2%)	-	-
Negative	18 (11.8%)	18 (11.8%)		
Total	152 (100.0%)	152 (100.0%)		

Chi-squared test was used to explore the association between INSM1 and Chromogranin A.

There was no significant difference between the various groups in terms of distribution of Chromogranin A ($\chi 2$ = -, p = -).

DISCUSSION:

Neuroendocrine neoplasms (NENs) seem to be a fairly homogeneous group but are heterogeneous neoplasms with great differences in origin and biology. NENs occur in almost every organ or region of the body and originate from cells having a neuroendocrine phenotype. These tumors share the expression of general neuroendocrine markers such as synaptophysin and chromogranin A but are diverse in terms of special histologic features, proliferation, hormonal production, molecular profile, and clinical aggressiveness. Among these, the classification of the gastro-entero-pancreatic NENs (GEP-NENs) plays a central role since the digestive system NENs constitute 70% of all NENs.

This is an observational ambispective, hospital-based observational study conducted on 152 cases of neuroendocrine tumors at the All India Institute of Medical Sciences (AIIMS), Jodhpur in the Department of Pathology & Lab Medicine.

In this study, the expression of INSM1 immunohistochemical marker in neuroendocrine tumors was studied and compared with the traditional neuroendocrine markers (CGA/ Synaptophysin/ CD56). Expression of Ki67 labelling index was also compared with PHH3.

Age distribution of neuroendocrine tumor cases:

The present study comprised of 50 cases (32.89%) in ≤ 50 years age group, and 102 cases (67.10%) in >50-years age group. The mean age of the cases affected by neuroendocrine tumor was 54.97 years, with a range of 2-83 years.

According to Surveillance, Epidemiology, and End Results (SEER) database from the United States, the patients with neuroendocrine tumor presented between 40-74 years and the mean age at diagnosis was 60 years.(51) Studies on the Indian population by Kulkarni et al found the mean age to be 49 years which was younger than western population.(53) Neuroendocrine neoplasm rarely occurs in the pediatric population. However, in our study one patient was diagnosed at age of 2 years.

Gender distribution of neuroendocrine cases:

In the present study, males (67%) were affected more than females (32.8%). According to the SEER database from the United States, males(47.3%) are less affected than female(52.7%).(53)

In various literature, there was no significant difference in females and males. Study conducted by Mengfei Fu et al showed that incidence was moderately higher in females (52.8%) vs. 47.2% in males. Indian studies showed an increased prevalence amongst males. Study conducted by Manoharan et al and Kulkarni et al showed males are more affected than females with male to female ratio being 1.85:1.(54) (53) .Hence, the prevalence of neuroendocrine tumor is variable in different ethnicity and populations.

Site of carcinoma

In the present study, gastrointestinal tract involvement was seen in 39 cases(25.65%), which included colon in 10 cases(6.5%), duodenum in 14 cases(9.2%),ileum in 7 cases(4.5%), stomach in 6 cases(3.9%), caecum in 1 case(0.6%) and rectum in 1 case(0.6%). The second most common site was lung primary, which constituted 37 cases (24.3%). Pancreas involvement was seen in 8 cases (5.2%), liver involvement was seen in 17 cases (11.0%). The other sites included in the study are breast (3.9%), CNS (2.6%), urinary bladder (3.9%), skin (0.6%), pituitary (0.6%), pleura (0.6%), vaginal wall (0.6%) and vagus nerve (0.6%).

Primary site	**Present study**	**Kulkarni et al**(53)	**Yao JC *et al*. (US Whites)**(55)	**Yao JC *et al*.**(55) **(US Asians/PI)**	**Hauso O *et al*.**(56)	**Tsai HJ *et al*.**(57)	**Abdulfattah MK *et al*.**(58)	**Kapoor R *et al*.**(59)
1	GIT (25.65%)	Pancreas (35%)	Lung (30%-32%)	Rectum (41%)	Small intestine (26%)	Rectum (25%)	Pancreas (26.3%)	Pancreas (35.2%)
2	Lung (24.3%)	Unknown primary (19%)	Small intestine (18%-19%)	Lung (15%)	Lung (21%)	Lung (20%)	Pelvis (15.8%)	Periampullary (21.5%)
3	Liver (11.0%)	Small intestine (9%)	Unknown primary (13%)	Pancreas (8%)	Colon (8%)	Stomach (7%)	Lung (13.2%)	Small intestine (13.7%)
4	Metastasis to lymph node (7.14%)	Lung (6%)	Rectum (12%)	Small intestine (8%)	Rectum (7%)	Pancreas (6%)	Small intestine (13.2%)	Retroperitoneum (9.8%)
5	Pancreas (5.2%)	Rectum (5%)	Colon (7%-8%)	Stomach (6%)	Pancreas (7%)	Colon (5%)	Mediastinum (10.5%)	Unknown primary (9.8%)

Table 17: Common sites of neuroendocrine tumor in various studies and comparison with present study.

Of 22 cases with metastases, lymph node was the most common metastatic site (7.14%%), followed by liver(3.24%), CNS (1.94%%) and lung (0.6%). According to Chan et al, the most common sites of metastases were regional lymph nodes and liver (53.8 % each).(17)

Neuroendocrine neoplasms in digestive tract and pancreatico-biliary tract:

The category of gastroenteric NETs, including gastric, intestinal, rectal, and appendiceal, is heterogeneous and may be quite clinically aggressive because of the risk associated with their location, higher grade, and high mitotic index. In the present study, conventional NE histochemical markers used in gastroenteric-NET diagnoses are synaptophysin, chromogranin A and CD56. Pancreatic NETs are pancreatic neoplasms with neuroendocrine differentiation. They clinically present as a solid or cystic pancreatic mass.(60) Their various morphology include oncocytic, hepatoid, lipid-rich, plasmacytoid, ductuloinsular, pleomorphic, and paraganglioma-like. In the present study, INSM1 was seen in 100% cases of NET of stomach and intestine while SYP was seen in 98.4% and CGA was seen in 94.44%. INSM1 has proved to be useful in identifying NETs with its strong and diffuse nuclear staining with 3+ intensity and >75% cell staining. McHugh et al found that INSM1 was positive in 94.1% of gastric (16/17), 72.2% of small bowel (13/18), 81.0% of colonic (17/21), and 72.2% of appendiceal tumors (26/36). SYP positivity was 99.1% and chromogranin A positivity was 88.6% in this study.(5)

Another study by Rosenbaum et al found that INSM1 was positive in 90% of the cases while chromogranin A was positive in 70% and synaptophysin was positive in 96.7%.(32) Due to the non-specific background staining along with spotted membranous and cytoplasmic staining seen with SYP and CGA, INSM1 seems to be a superior NE biomarker for gastroenteric NETs. INSM1 can detect primary NETs by their strong and diffuse nuclear expression, particularly in cases with unusual cytomorphologic features. It can be useful as a supplemental stain when conventional staining is difficult to interpret.

A study conducted by Hou et al found that the detection rates of INSM1, CG, and SYN were 100%, 95%, and 100%, respectively.(61)

The amyloid stroma is an uncommon but well-known and characterized feature of syndromic PanNETs, particularly of insulinomas.(62) To the best of our knowledge, no other cases of non-syndromic amyloid-rich PanNETs are reported in the literature. In present study, Congo red staining and polarization microscopy highlighted amyloid stroma in 5 cases of pancreatic NEN.

Pulmonary NET:

Pulmonary NETs are a subset of pulmonary neoplasms that contain secretory granules and express neuroendocrine markers. There are four types of pulmonary NETs: small cell lung carcinoma (SCLC), large cell NE carcinoma (LCNEC), atypical carcinoid tumor (ATC), and typical carcinoid tumor (TC). Since prognosis and management of pulmonary NETs differs significantly, early diagnosis of the pulmonary NETs is important. INSM1 has recently emerged as a reliable prognostic immunostain of neuroendocrine differentiation in human lung neoplasms.

In present study with a cohort of 37 cases, INSM1 demonstrated positivity of 100% which is comparable to positivity of synaptophysin (100%) and higher than chromogranin A (91.89%). The findings indicate that INSM1 alone may be reliable and robust in routine diagnostics of pulmonary NETs. As per literature INSM1 over performs a combined panel of traditional markers. (38) (63) However according to Staaf et al and Kriegsmann et al, all traditional NE markers (SYP, CD56, and CGA) and their combination outperformed in their positivity (85-97%) compared with INSM1.(40) (26). In another cohort of 54 lung NETs, INSM1 performed lower than CD56 (87%), higher than CGA (56%), and lower than SYP (85%). (26) As in present study, smaller number of cases was there, more studies with larger number of cases are needed to propose replacement of traditional neuroendocrine markers with INSM1.

Close attention to cytomorphologic features and clinical presentation of thoracic neoplasms to avoid diagnostic pitfalls and ancillary studies for further characterization of neoplastic cells mimicking NETs is required.

The intensity of INSM1 staining is mostly strong as seen in the present study. However, literature to compare the intensity of INSM1 staining in pulmonary NEN is not available.

In present study, all the carcinoid tumors were positive for INSM1, as seen in a similar study by Mukhopadhyay et al.(64)

	Source	INSM1	SYN	CgA	CD56
		%, n/N	%, n/N	%, n/N	%, n/N
	Present study	**100(33/33)**	**100(33/33)**	**90.9(30/33)**	**100(5/5)**
SCLC	Doxtader et al.	93 (38/41)	93 (37/40)	35 (14/40)	100 (40/40)
	Viswanathan et al.	89(8/9)	78 (7/9)	22 (2/9)	100 (9/9)
	Rodriguez et al.	97 (31/32)	82 (14/17)	62 (10/16)	96 (22/23)
	Narka et al.	97 (36/37)	96 (27/28)	100 (18/18)	100 (6/6)
	Sakakibara et al.	92 (72/78)	55 (43/78)	48 (37/78)	81 (63/78)
	Mukhopadhyay et al.	98 (63/64)	100 (64/64)	83 (53/64)	95 (61/64)
	Present study	**100(1/1)**	**100(1/1)**	**100(1/1)**	**100(1/1)**
LGNEC	Doxtader et al.	100 (1/1)	100 (1/1)	100 (1/1)	100 (1/1)
	Viswanathan et al.	75 (6/8)	63 (5/8)	25 (2/8)	100 (8/8)
	Sakakibara et al.	68 (30/44)	57 (25/44)	44 (18/440	84 (37/44)
	Mukhopadhyay et al.	75 (18/24)	88 (21/24)	46 (11/24)	92 (22/24)
	Present study	**100(3/3)**	**100(3/3)**	**100(3/3)**	**0(0/0)**
Carcinoid, Typical and Atypical	Doxtader et al.	90 (9/10)	100 (10/10)	100 (10/10)	90 (9/10)
	Viswanathan et al.	100 (22/22)	100 (22/22)	100 (22/22)	95(21/22)
	Sakakibara et al.	95 (18/19)	100 (19/19)	100 (19/19)	100 (19/19)
	Mukhopadhyay et al.	98 (63/64)	100 (64/64)	100 (61/61)	100 (58 /58)

Table 18: Expression of various IHC in lung NEN.

NEN in Breast:

Primary NETs of the breast are rare entities and are under diagnosed. NETs of the breast occur most commonly in postmenopausal women. The emergence of INSM1 as a more sensitive "second-generation" biomarker for neuroendocrine differentiation in the breast has improved the diagnosis of breast NETs, which are difficult to distinguish from other types of breast carcinomas based on histologic features alone. To date, only few studies have analysed the usefulness of INSM1 in detecting neuroendocrine differentiation of invasive breast carcinomas. Moreover, only few studies have compared INSM1 to other types of neuroendocrine markers in the context of neuroendocrine breast carcinomas. Neuroendocrine differentiation can also be associated with invasive breast carcinomas in up to 30% of the cases. Neuroendocrine carcinoma of the breast is defined as a subset of invasive breast carcinoma in which neuroendocrine differentiation is expressed in greater than 50% of the tumor cells.

INSM1 has emerged as a sensitive biomarker for the detection of neuroendocrine differentiation in breast neoplasms. In present study, INSM1 was found diffusely positive in all the 6 cases (100%) of NEN, similar to synaptophysin (100%). Chromogranin A was seen in 83.3% cases. This was in concordance to studies found in literature. A study by Roy et al. found that INSM1 was diffusely positive in five out of seven breast carcinoma cases with neuroendocrine differentiation (similar to CgA and CD56 expression) while SYP was expressed in six of the seven cases.(65) In a study on three breast neoplasms from three Japanese female patients, ages 42, 58, and 64, Kawasaki and Kaira found that INSM1 was expressed strongly and diffusely in all three cases (1 NET, 1 mucinous carcinoma, and 1 neuroendocrine carcinoma in situ), while CgA and SYP were negative in all three cases. (35)A study by Seijnhaeve et al. compared SYP and CgA with INSM1 by using a validation cohort of 22 mammary neoplasms. Overall, INSM1 was expressed in 16/22 cases, which was higher than that for SYN (14/22) and CgA (6/22).(66)

INSM1 Expression in Genitourinary Tumors:

NETs can present in variety of locations within the reproductive and urinary tracts. Neuroendocrine carcinomas (NEC) of the female genital tract are rare and aggressive malignant neoplasms. Location plays an important role in INSM1 staining for

neuroendocrine carcinomas (NEC) in the female genital tract. In present study, all the cases were immunopositive for INSM1. However, SYN and CGA were positive in 100% and 77.7% cases respectively. INSM 1 intensity was strong and diffuse in small cell NEC. However, in endometrial NEC the staining of INSM1 was of moderate intensity. In a similar study Ting et al found, INSM1 expression was diffuse and intense compared to SYN, CgA, and CD56 for cervical small cell NECs but not for large cell NECs. However, for endometrial NECs, the other NE markers performed better, as INSM1 was only focally and weakly expressed. (67)

Merkel cell carcinoma (MCC) is neuroendocrine carcinoma of the skin. MCC morphology overlaps with other neoplasms, such as basal cell carcinomas and squamous cell carcinomas, especially when presenting with atypical immunophenotype. Accurately diagnosing MCC can improve the treatment outcomes for this cancer that is known particularly for its aggressive behavior. INSM1 has been found to be a sensitive biomarker for the differential diagnosis of MCC from other non-MCC cases. In present study, MCC showed strong nuclear expression of INSM1 in all the tumor cells. In literature, Lilo et al and Leblebici et al found the INSM1 expression was also strong and diffuse and positively stained more than 75% of the cells for INSM1, which was more diffuse and more intense than the other NE markers. (68) (69)

Proliferation Index:

In this study we explored the diagnostic utility of PHH3 LI as an ancillary mitotic marker in patients with NETs, by comparing WHO grades by Ki67 labelling index and WHO grades modified by PHH3 LI. We found that a PHH3 MI cut off was most similar to WHO grade.

The most accurate evaluation of mitoses in patients with NETs using the WHO grading system remains unclear, because mitoses is mimicked by darkly stained or shrunken irregular nuclei, apoptotic bodies, and karyorrhectic debris, yielding false positives. In addition, diagnosis of mitoses is limited by narrow cut offs in mitotic counts between grades 1 and 2. PHH3 is only expressed during mitosis, not during interphase or apoptosis, thus making PHH3 a specific marker of mitosis.

We found that PHH3 correlated with the Ki-67 LI and the correlation is statistically significant. ($p=<0.001$). PHH3 only stains cells during the late G2 and M phases of

mitosis, whereas Ki-67 is expressed throughout the cell cycle except in the G0 phase. PHH3 would therefore stain fewer tumor cells than Ki-67, resulting in a lower PHH3 LI.

Recently, Voss et al.(9) studied PHH3 in comparison with the current WHO grading scheme of mitotic figure count and Ki67 in 63 well-differentiated pNET resected surgical specimens. Similar to our results, their study showed that PHH3 LI correlated well with Ki67 indices.

Dragonova-Tacheva et al.(70) compared PHH3 LI and Ki67-based grading in 23 pNET cytological specimens. Similar to the present study, they found a correlation between Ki67 and PHH3 grading, with the range of PHH3 positivity being narrower than Ki67. They also suggested the utility of PHH3 as a tool for mitotic count, rather than determining a proliferation index. Fung et al.(71) analysed 19 well-differentiated gastrointestinal cytological specimens not limited to the pancreas and concluded that Ki67 and PHH3 have significantly correlated labelling index.

Dumars et al (72) studied the usefulness of PHH3 LI in assessing discordant grades in 41 primary and metastatic enteropancreatic neoplasms. In concordance with our study, there was a positive correlation with a significant p value.

PHH3 LI is comparable to the current WHO grading system but is superior to H&E and Ki-67, in predicting disease-free survival, with PHH3 appearing to be both easier to interpret and more accurate than current prognostic markers.

	Year of study	Sample size	Type of specimen	PPH3 vs Ki67 LI
Present study	2022	152	NET of all body organs	P=<0.001
Dumar et al	2016	41	Pancreas and intestine	P=<0.001
Voss et al	2015	65	Pancreas	P=<0.05
Dragonova et ale	2013	23	Pancreas	P=0.001
Fung et al	2013	26	Pancreas and GIT	P=0.05

Table 19: Summary of studies comparing Ki67 LI and PHH3 LI

LIMITATIONS OF THE STUDY:

1. INSM1 IHC showed nonspecific cytoplasmic staining in hepatocytes and strong nuclear staining in the RBCs. Thus, interpretation in few cases was challenging.
2. Synaptophysin and Chromogranin A were only interpreted as positive or negative. No further classification was done on basis of intensity of staining, as advocated by few studies.
3. Radiological details and functional status of tumors were not included in the present study.
4. Similar studies with a larger sample size need to be carried out for more accurate quantification methods.
5. Similarly, more studies need to be done to look at the role of PHH3 grading cut-offs for further grading modifications in NETs in comparison to Ki67.

SUMMARY AND CONCLUSION

Expression of INSM1 on immunohistochemistry was studied in 152 cases of neuroendocrine tumors and its expression was compared to those of traditional neuroendocrine markers like synaptophysin, chromogranin A and CD56.

Ki67 LI was compared to PHH3 LI in all 152 neuroendocrine tumors.

- Most of the patients were in their sixth and seventh decades. However, one patient was younger than 10 years of age.
- Males were more commonly affected compared to females.
- Neuroendocrine tumors were seen to occur mostly in gastrointestinal and pancreatico-biliary tract, followed by lung. The other organs involved were liver, breast and genitourinary system.
- Synaptophysin was positive in 151 of 152 cases and one case was immunonegative. The case which was negative for synaptophysin was also negative for chromogranin A. However, this case was immunopositive for INSM1 with 2+ intensity and also positive for CD56.
- Chromogranin A was positive in 134 cases and negative in 18 cases. However, these 18 cases were immunopositive for synaptophysin and INSM1.
- INSM1 is a neuroendocrine (NE) marker that offers ease of interpretation due to its nuclear expression. Its specificity for NE differentiation promises important value in the diagnosis of NETs that either lack or have equivocal expression of "traditional" NE markers.
- INSM1 showed strong staining intensity in 81 cases, moderate staining intensity in 50 cases and weak staining in 21 cases.
- Percentage of cells stained by INSM1 was >75% in 128 cases, 50-75% in 9 cases, 25-50% in 1 case and <25% in 14 cases.
- Ki-67 LI ranged from 1%-100%.

- PHH3 LI ranged from 1% - 90%.
- There was a significant association between the various groups in terms of distribution of PHH3 and Ki67 LI using Fisher exact test. ($\chi 2 = 243.908$, $p = <0.001$).
- PHH3 LI did not show much variation in its value as compared to Ki67 in G3 tumors. PHH3 LI was more useful in G1 and G2 tumors.
- Hence, it is suggested that PHH3 LI could be used in the grading of NETs as a possibly better alternative tool to Ki67 LI as it does not highlight cells undergoing cell death or apoptosis.
- While reviewing the cases, amyloid deposits were suspected in 5 cases. Congo red stain was applied followed by polarization microscopy, which highlighted amyloid deposits in these 5 cases. Hence, it is important to evaluate the NET stroma for amyloid deposits.

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ANNEXURES

1. IEC certificate
2. **Informed Consent form – English – 9A**
3. **Informed Consent form – Hindi – 9B**
4. **Patient Information Sheet – English – 9C**
5. Patient Information Sheet – Hindi – 9D
6. Proforma
7. Master chart

Ethical Justification

- Informed written consent was taken from all study subjects. No pressure or coercion was exerted on subjects for participation in study.
- Confidentiality and privacy were maintained at all stages.
- Enrolment in the study posed no risk to the patient and did not increase the cost of the treatment
- Informed written consent was taken from all the patients as per the attached proforma.

अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर
All India Institute of Medical Sciences, Jodhpur
संस्थागत नैतिकता समिति
Institutional Ethics Committee

No. AIIMS/IEC/2021/3550 Date: 12/03/2021

ETHICAL CLEARANCE CERTIFICATE

Certificate Reference Number: **AIIMS/IEC/2021/3385**

Project title: **"Expression of immunohistochemical marker INSM1 and its comparison with traditional markers in neuroendocrine tumours along with comparison of PHH3 and Ki67 labelling index"**

Nature of Project:	**Research Project Submitted for Expedited Review**
Submitted as:	**M.D. Dissertation**
Student Name:	**Dr. Sangeeta Pradhan**
Guide:	**Dr. Poonam Abhay Elhence**
Co-Guide:	**Dr. Deepak Vedant & Dr. Vikarn Vishwajeet**

Institutional Ethics Committee after thorough consideration accorded its approval on above project.

The investigator may therefore commence the research from the date of this certificate, using the reference number indicated above.

Please note that the AIIMS IEC must be informed immediately of:

- Any material change in the conditions or undertakings mentioned in the document.
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research.

The Principal Investigator must report to the AIIMS IEC in the prescribed format, where applicable, bi-annually, and at the end of the project, in respect of ethical compliance.

AIIMS IEC retains the right to withdraw or amend this if:

- Any unethical principle or practices are revealed or suspected
- Relevant information has been withheld or misrepresented

AIIMS IEC shall have an access to any information or data at any time during the course or after completion of the project.

Please Note that this approval will be rectified whenever it is possible to hold a meeting in person of the Institutional Ethics Committee. It is possible that the PI may be asked to give more clarifications or the Institutional Ethics Committee may withhold the project. The Institutional Ethics Committee is adopting this procedure due to COVID-19 (Corona Virus) situation.

If the Institutional Ethics Committee does not get back to you, this means your project has been cleared by the IEC.

On behalf of Ethics Committee, I wish you success in your research.

Dr. Pravdev Sharma
Member Secretary

Member secretary
Institutional Ethics Committee
AIIMS,Jodhpur

Basni Phase-2, Jodhpur, Rajasthan-342005; **Website:** www.aiimsjodhpur.edu.in; **Phone:** 0291-2740741 Extn. 3109
E-mail : ethicscommittee@aiimsjodhpur.edu.in; ethicscommitteeaiimsjdh@gmail.com

All India Institute of Medical Sciences
Jodhpur, Rajasthan
Informed Consent Form

Title of the project: **Expression of immunohistochemical marker INSM1 and its comparison with traditional markers in Neuroendocrine tumors along with comparison of PHH3 and Ki67 labelling index**

Name of the Principal Investigator : Dr. Sangeeta Pradhan Tel. No. 8249368490

Patient / Volunteer Identification No.:_____

I, _____ S/o or D/o _____ R/o

_____

give my full, free, voluntary consent to be a part of the study "_____", the procedure and nature of which has been explained to me in my own language to my full satisfaction. I confirm that I have had the opportunity to ask questions.

I understand that my participation is voluntary and am aware of my right to opt out of the study at any time without giving any reason.

I understand that the information collected about me and any of my medical records may be looked at by responsible individual from _____ - (Company Name) or from regulatory authorities. I give permission for these individuals to have access to my records.

Date: _____ _____

Place: _____ Signature/Left thumb impression

Date: _____ _____

This to certify that the above consent has been obtained in my presence.

Place: _____ Signature of Principal Investigator

Witness 1	Witness 2
Signature _____	Signature _____
Name: _____	Name: _____
Address: _____	Address: _____

All India Institute of Medical Sciences
Jodhpur, Rajasthan
Informed consent form (Hindi)

थीसिस / निबंधकाशीर्षक: **इम्यूनोहिस्टोकेमिकल मार्कर INSM1 की अभिव्यक्ति और तुलना पारंपरिक मार्करों के साथ न्यूरोएंडोक्राइन ट्यूमर में और PHH3 और Ki67 लेबलिंग इंडेक्स की तुलना**

पीजीछात्रकानाम: डॉ संगीता प्रधान टेलन:8249368490

रोगी / स्वयंसेवकपहचानसंख्या: _____

मैं, _____एस / ओयाडी / ओ _____आर/ओ_____ _____

अध्ययन "" _____

का एक भाग बनने के लिए मेरी पूर्ण, स्वतंत्र ,स्वैच्छिक सहमति दें,जिसकी प्रक्रिया और प्रकृति मुझे अपनी पूरी संतुष्टि के लिए अपनी भाषा में समझाई गई है। मैं पुष्टि करता हूं कि मुझे प्रश्न पूछने का अवसर मिला है।

मैं समझता हूं कि मेरी भागीदारी स्वैच्छिक है और मुझे किसी भी कारण दिए बिना किसी भी समय अध्ययन से बाहर निकलने के मेरे अधिकार की जानकारी है।

मैं समझता हूं कि मेरे और मेरे मेडिकल रिकॉर्ड के बारे में एक त्रित की गई जानकारी को_____(कंपनीनाम) या विनियामक प्राधिकरणों से जिम्मेदार व्यक्ति द्वारा देखा जा सकता है। मैं इन व्यक्तियों को अपने अभिलेखों तक पहुंच के लिए अनुमति देता हूंI

date : _____

जगह: _____ हस्ताक्षर/ बाएं अंगूठे का छाप_____

यह प्रमाणित करने के लिए कि मेरी उपस्थिति में उपरोक्त सहमति प्राप्त की गई हैI

तारीख : _____ जगह: _____

पीजी छात्र के हस्ताक्षर_____

गवाह1: _____ हस्ताक्षर: _____ तारीख : _____	गवाह2: _____ हस्ताक्षर: _____ तारीख : _____

PATIENT INFORMATION SHEET (English)

1. Risks to the patients: No interventions or life-threatening procedure will be done.
2. Confidentiality: Your participation will be kept confidential. Your medical records will be treated with confidentiality and will be revealed only to doctors/ scientists involved in this study. The results of this study may be published in a scientific journal, but you will not be identified by name.
3. Provision of free treatment for research related injury. Not applicable.
4. Compensation of subjects for disability or death resulting from such injury: Not Applicable
5. Freedom of individual to participate and to withdraw from research at any time without penalty or loss of benefits to which the subject would otherwise be entitled.
6. Your participation in the study is optional and voluntary.
7. The copy of the results of the investigations performed will be provided to you or your record.
8. You can withdraw from the project at any time, and this will not affect your subsequent medical treatment or relationship with the treating physician.
9. Any additional expense for the project, other than your regular expenses, will not be charged from you.

रोगीसूचनापत्रक

1. रोगियों के लिए जो खिम: कोई हस्तक्षेप या जीवन-धम की प्रक्रिया नहीं की जाएगी।

2. गोपनीयता: आपकी भागीदारी को गोपनीय रखाजाएगा। आपके मेडिकल रिकॉर्ड को गोपनीयता के साथ इलाज किया जाएगा और केवल इस अध्ययन में शामिल डॉक्टरों / वैज्ञानिकों को पता चलेगा। इस अध्ययन के परिणाम एक वैज्ञानिक पत्रिका में प्रकाशित हो सकते हैं,लेकिन आपको नाम से पहचाना नहीं जाएगा।

3. अनुसंधान संबंधी चोट के लिए नि:शुल्क उपचार कीव्यवस्था: लागू नहीं।

4. ऐसी चोट से उत्पन्न विकलांगता या मृत्यु के लिए विषयों का मुआवजा: लागू नहीं हैI

5.किसी भी समय दंड या लाभों के नुकसान के बिना किसी भी समय भाग लेने के लिए व्यक्ति को स्वतंत्रता लेने और अनुसंधान से वापस लेने के लिए स्वतंत्रता,जिसके तहत विषय अन्यथा हकदार होगाI

6.आपको जुर्माना या लाभ के नुकसान के बिना किसी भी समय भाग लेने और अनुसंधान से वापस लेने की पूरी आजादी है,जिस पर आप अन्यथा हकदार होंगे।

7. अध्ययन में आपकी भागी दारी वैकल्पिक और स्वैच्छिक है।

8. प्रदर्शन की जांच की परिणामों की प्रति आपके रिकॉर्ड के लिए आपको उपलब्ध कराई जाएगी।

9.आप किसी भी समय परियोजना से वापस ले सकते हैं ,और यह आपके बाद के चिकित्सा उपचार या उपचार चिकित्सक के साथ संबंध को प्रभावित नहीं करेगा।

10. परियोजना के लिए कोई भी अतिरिक्तव्यय,आपके नियमित खर्चों के अलावा,आप से शुल्क नहीं लिया जाएगा।

All India Institute of Medical Sciences (AIIMS), Jodhpur
Department of Pathology
PROFORMA

Date:

Name

Age: Sex: I.D:

Address:

Relevant clinical History:

Family history of malignancy:

Histological diagnosis (with histopathological stage if available):

Requested information for optimal patient care:

(1) Known/Previous malignancy: ☐ 2) Clinical tumor staging information: ☐

(3) Immunocompromised: ☐ (4) Chemotherapy: ☐ (5) Radiotherapy: ☐

(6) Immunohistochemistry:

- **INSM1** - Positive ☐ Negative ☐

(a) Intensity of stain

No stain: 0 ☐

Weak intensity: 1+ ☐

Moderate intensity : 2+ ☐

Strong intensity: 3+ ☐

b) Percentage of cell staining:

i. <25%
ii. 25-50%
iii. 50-75%
iv. >75%

- **PHH3 LI**

i. <3%,
ii. 3-20%,
iii. >20%

(7)Other IHC done:

- Synaptophysin
- Chromogranin A
- CD56
- Ki67 LI

(8)Other histochemical stain done: Congo Red.

अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर
ALL INDIA INSTITUTE OF MEDICAL SCIENCES, JODHPUR
विकृति विज्ञान विभाग
DEPARTMENT OF PATHOLOGY & LAB. MEDICINE

संदर्भ :- एम्स/जेडीएच/ वि. वि /6368/2023

दिनांक: 13/01/2023

सेवा में,

The Dean (Academics)
All India Institute of Medical Sciences
Jodhpur

Subject: Submission of M.D thesis.

आदरणीय महोदय,

This is to submit that the M.D thesis by the Academic Junior Resident (July 2020 Batch) of our Department has been duly completed and signed and is ready for submission. Please accept the same.

Details of her thesis are attached herein:

Name of candidate	Thesis topic
Dr. Sangeeta Pradhan	Expression of immunohistochemical marker INSM1 and its comparison with traditional markers in Neuroendocrine tumors along with comparison of PHH3 and Ki67 labelling index

धन्यवाद,

भवदीया

डॉ. पूनम ऐल्हेन्स
आचार्या एवं विभागाध्यक्षा
विकृति विज्ञान विभाग