NEUROENDOCRINE NEOPLASMS OF THE DIGESTIVE TRACT: RETROSPECTIVE CLASSIFICATION ACCORDING TO THE 2019 WHO GRADING SYSTEM, AND EVALUATION OF THE IMMUNOHISTOCHEMICAL MARKER INSM1.

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DECLARATION

I, Dr Alessandro Pietro Aldera, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ABSTRACT

Neuroendocrine Neoplasms (NENs) of the Gastrointestinal Tract (GIT) are a heterogeneous group of tumours with varied biologic potential and clinical outcomes. They are classified as well differentiated neuroendocrine tumours (WD NET) or poorly differentiated neuroendocrine carcinomas (PD NECs) based on morphology. WD NETs are further subtyped (grade 1, 2 or 3) by evaluating the mitotic rate and Ki-67 proliferative index. The most recent grading system was published in 2019 by the World Health Organisation (WHO). Insulinoma-associated protein 1 (INSM1) is a transcription factor that is expressed in neuroendocrine cells, and recent studies have shown that it is a sensitive and specific marker for neuroendocrine differentiation.

The aims of this study were to grade NENs of the GIT according to the 2019 WHO grading system, and to evaluate the expression of INSM1 in order to assess its sensitivity and specificity as a marker of neuroendocrine differentiation compared to chromogranin A (CgA) and synaptophysin (SYN).

Sixty-nine GIT NENs diagnosed between 2003 and 2017 at Groote Schuur Hospital were included in this study. The mitotic rate and Ki-67 proliferation index were evaluated for each case. We identified 38 grade 1 NETs, 16 grade 2 NETs, 1 grade 3 NET, 13 small cell type NECs and 1 large cell type NEC. INSM1 immunohistochemical staining was performed on all cases. To assess specificity, we evaluated the expression of INSM1 in other GIT primary tumours (adenocarcinoma, gastrointestinal stromal tumour, lymphoma, leiomyoma and Kaposi sarcoma). Eighty percent of our NEN cases stained with INSM1. We found the sensitivity of INSM1 to be higher than CgA (68%), but lower than SYN (87%) and the combined use of CgA-SYN (94%) when considering all NENs. When evaluating only the PD NEC cases, INSM1 had a higher sensitivity than CgA (50%) and SYN (64%), and an equal sensitivity to the combined use of CgA-SYN (79%).

We conclude that the high sensitivity and specificity of INSM1 make it a valuable standalone marker for assessing neuroendocrine differentiation. The nuclear reactivity and high sensitivity of INSM1 make it the preferred neuroendocrine marker for PD NEC.

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LIST OF ABBREVIATIONS

AJCC	-	American Joint Committee on Cancer
APUD	-	Amine precursor uptake and decarboxylation
AUC	-	Area under curve
CgA	-	Chromogranin A
EC	-	Enterochromaffin
ECL	-	Enterochromaffin-like cell
GI	-	Gastrointestinal
GIT	-	Gastrointestinal tract
GIST	-	Gastrointestinal stromal tumour
H&E	-	Haematoxylin and Eosin
HIAA	-	Hydroxyindoleacetic acid
IHC	-	Immunohistochemistry
INSM1	-	Insulinoma-associated protein 1
LIS	-	Laboratory information system
MEN	-	Multiple endocrine neoplasia
MiNEN	-	Mixed neuroendocrine-non-neuroendocrine neoplasms
NEC	-	Neuroendocrine carcinoma
NEN	-	Neuroendocrine neoplasm
NET	-	Neuroendocrine tumour
NF1	-	Neurofibromatosis type 1
PanNET	-	Pancreatic neuroendocrine tumour
PBS	-	Phosphate buffered saline
PD	-	Poorly differentiated
ROC	-	Receiver operating characteristic
SEER	-	Surveillance epidemiology and end results
SYN	-	Synaptophysin
WD	-	Well differentiated

WHO	-	World Health Organisation

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Neuroendocrine neoplasms (NENs) occur in epithelial organs throughout the body where they demonstrate a wide variety of clinical presentations, morphological features, genetic findings, and outcomes.

In 2018, the WHO published a uniform classification schema for NENs which separates them into two distinct morphologic groups: well differentiated neuroendocrine tumours (WD NETs) and poorly differentiated neuroendocrine carcinomas (PD NECs).(1) Morphologic features and proliferation rates are used to distinguish these. This distinction is supported by clinical, epidemiological, genetic and prognostic differences. The recently published 2019 WHO classification of tumours of the digestive system has adopted this framework for the classification of gastrointestinal (GI) NENs.(2) Neuroendocrine lineage is proven by immunohistochemistry. The traditional neuroendocrine immunohistochemical markers have varying sensitivities and specificities depending on the grade and anatomic site of the tumour. There is interest in a novel neuroendocrine transcription factor Insulinoma-associated protein 1 (INSM1) which has shown promising results in the lung, head and neck and skin. The utility of this marker in the digestive tract has only recently been reported, long after the current study protocol was finalised.(3)

The prevalence of NENs in South Africa is not well documented, and the frequency at which the different tumour grades occurs is also not known. The role of INSM1 in demonstrating neuroendocrine differentiation in GI NENs has similarly not been investigated in the South African context.

1.2 Historical perspective

Siegfried Oberndorfer was the first to recognize the difference between malignant epithelial tumours of the terminal ileum (carcinomas) and a group of more benign-behaving lesions which he termed carcinoid tumours.(4) Since his original description of *"karzinoides"* in 1907, NEN terminology and classification has undergone several revisions.

Oberndorfer first described carcinoids in the terminal ileum as multiple superficial tumourlets with an indolent behaviour. Years later, he recognised that some carcinoids behaved in a malignant manner and were able to metastasize. In 1963, Williams and Sandler proposed classifying WD NETs based on their embryological origin.(5) Tumours were thus grouped as foregut (lung, thymus, stomach, duodenum, pancreas, proximal jejunum), midgut (distal jejunum, ileum, appendix, caecum) and hindgut (colon and rectum) tumours. In 1966, Pearse recognised the biochemical characteristics of neuroendocrine cells and proposed the term APUD (amine precursor uptake and decarboxylation).(6) For a time the term "apudoma" prevailed in the literature.
In the 1980 WHO classification the term carcinoid applied to all tumours derived from the diffuse neuroendocrine system. These were then subclassified based on silver and other staining characteristics into enterochromaffin (EC) cell carcinoids, gastrin cell carcinoids and other carcinoids.

Carcinoid is a historic term that is widely prevalent in the literature. However, it does not sufficiently convey the heterogeneity in behaviour and outcomes of WD NETs. This was recognised in the early 1990s, and in 2000 the WHO eliminated the term carcinoid from their classification system. This classification grouped tumours by anatomic site and recognised the importance of prognostic groups.(7, 8) However, terminology was still confusing and the distinction between NET and NEC was based on the presence of metastases in the latter.

The distinction between WD NETs and PD NECs was recognised in the 2010 WHO classification system. This distinction was based on morphology, mitotic rate and the Ki-67 proliferative index. It is now appreciated that WD NETs are distinct from conventional adenocarcinomas with neuroendocrine differentiation and PD NECs. NECs develop from a surface epithelial precursor and are closely related to conventional adenocarcinomas. WD NETs develop from cells of the diffuse neuroendocrine cell system. This distinction is important as there are marked differences in clinical presentation and prognosis between the two groups.

The 2019 WHO classification of digestive system tumours further emphasizes the importance of morphology in separating the NETs from NECs. The concept of a grade 3 WD NET has been introduced, which has the same cut off values for mitotic count and Ki-67 index as PD NEC, but resembles a WD NET morphologically and behaves in a less aggressive manner than PD NEC.(2)

1.3 Epidemiology

1.3.1 Incidence

The Surveillance Epidemiology and End Results (SEER) database provides the most comprehensive data on the incidence of GI NENs in the United States.

Small intestinal NENs are reported in the literature with an annual incidence of 1.2 cases per 100000 and account for approximately 27% of GI NENs.(9-11) Appendiceal NENs occur in the general population at a rate of 0.15 to 0.6 per 100 000 annually.(11) Colonic NENs are the least common GI NEN and occur with an annual incidence of 0.2 per 100 000.(11) Many of these tumours occur in the caecum and it has been suggested that there may be overlap with appendiceal and ileal NENs, and that true colonic NENs are even rarer than this data suggests. The annual incidence of rectal NENs is the highest in the GIT at 1.2 per 100 000.(11) The incidence of gastric NENs has increased over the past

decade due to increasing use of upper endoscopy. The estimated annual incidence of gastric NENs in the USA and Europe is approximately 0.4 cases per 100 000.(11, 12) Gastric NECs account for 21% of all gastric NENs and approximately 20.5% of all GIT NECs.(13)

To our knowledge, there is no data on the incidence of GI NENs in South Africa.

1.3.2 Geographic distribution

Rectal NETs occur most frequently among the Asian-Pacific Island, American-Indian and African-American populations, compared to Caucasians.(2) Colonic NETs are more common in Caucasians. NENs of the stomach, small bowel and appendix show no geographic predilection.

1.3.3 Age

GI NENs are predominantly tumours of adulthood and occur only rarely in children. The mean age at diagnosis for small intestinal NENs is 65 years.(9) NENs of the stomach present at a mean age of 64 years. Rectal NENs occur in a younger age group and are diagnosed at a mean age of 57 years. For colonic NENs the mean age is 64 years. The highest incidence of appendiceal NENs is before the age of 40 years with a mean age at presentation of 33.2 years.(14, 15)

1.3.4 Gender

The prevalence for gastric, small intestinal, colonic and rectal NENs is similar amongst men and women.(2) Appendiceal NENs have a reported female predominance of 60.5%.(15)

1.4 Aetiopathogenesis

GI NENs occur sporadically for the most part, although they may rarely occur in association with familial endocrine syndromes with germline mutations. These tumours are generally localized to the duodenum and may be seen in association with multiple endocrine neoplasia type 1 (MEN1) and neurofibromatosis type 1 (NF1). The aetiology and pathogenesis of the sporadic small intestinal NENs is unknown.

Gastric type 1 ECL-cell WD NETs are the most common (80-90%) gastric NET and are associated with autoimmune gastritis. Type 2 ECL-cell WD NETs are seen in association with gastrinomas in the context of MEN1. The pathogenesis of both type 1 and type 2 ECL-cell WD NETs is related to unregulated gastrin stimulation of ECL cells.(16) There are no specific aetiologic factors for type 3 gastric WD NETs and PD NECs, and the pathogenesis of both is unknown.

The aetiology and pathogenesis of appendiceal and colorectal NENs is unknown.(2) A recent meta-analysis of colorectal NENs showed an increased risk with alcohol consumption.(17)

1.5 Clinical features

Jejunoileal NENs may present with bowel obstruction, ischaemia or intussusception.(18) Up to 75% of patients with small intestinal NENs present with liver metastases, and at the time of diagnosis the primary intestinal NEN has usually not been identified. Intestinal NENs may be functional and produce a variety of hormones. The most common and well-known are serotonin and other vasoactive substances which, when released from liver metastases, can cause the carcinoid syndrome. Carcinoid syndrome manifests as episodic flushing of the face and neck, sweating, diarrhoea, wheezing and heart disease.(19)

Duodenal NENs are more likely to be functional. Patients with incidentally detected NENs are usually asymptomatic. They may have elevated serum chromogranin A and urine 5-hydroxyindoleacetic acid (HIAA) or even liver metastases.(18)

The majority of appendiceal NENs occur in the tip of the organ, are clinically silent, and are discovered incidentally.(18) Up to 10% of cases can occur more proximally and may present as acute appendicitis.

Rectal NENs are mostly small (<1cm) and detected incidentally on routine colonoscopy. Larger rectal NENs may cause mucosal ulceration and rectal bleeding.
Most patients with gastric NEN are asymptomatic. The commonest clinical sign at presentation is anaemia; symptoms such as dyspepsia, vomiting and abdominal pain may also occur.

1.6 Pathology

1.6.1 Macroscopic pathology

Small intestinal, colonic and rectal NETs generally appear as small firm nodules or polypoid submucosal lesions without mucosal involvement. Jejunoileal NETs may be multifocal in up to a third of cases.(20) On cut surface they are white to yellow in colour. Mesenteric involvement is common.

1.6.2 Microscopic pathology

WD NETs show a variety of growth patterns which are related to the cell of origin and the peptide secretory product. The classic jejunoileal WD NETs which are thought to derive from serotonin expressing EC-cells are also the commonest type found in the colon and appendix. They show well-defined nests with solid, insular, and acinar growth. Appendiceal WD NETs with this morphology are unique in that the nests are often surrounded by S100 positive spindled cells.(21) WD NETs derived from L-cells producing

glucagon-like peptides and peptide YY are typically found in the rectum and are the second most common architectural pattern in the appendix. Chromogranin A immunohistochemical staining is known to be variable in tumours derived from L-cells.(18) WD NETs derived from L-cells show trabecular and ribbon-like growth within abundant collagen-rich stroma. Appendiceal WD NETs can show a tubular growth pattern (least common) which needs to be distinguished from goblet cell carcinoids and adenocarcinomas.

The cells comprising WD NETs contain eosinophilic cytoplasmic secretory granules which are accentuated at the periphery of the cell nests. The cells are evenly spaced and there is minimal cytonuclear pleomorphism. Nuclei are round to ovoid in shape with stippled chromatin. Both perineural and lymphovascular invasion are commonly observed.

PD NECs show a solid growth pattern composed of sheets or nests of cells. Trabecular growth is less common.(22) The neoplastic cells in small cell PD NEC vary from round to polyhedral to spindled and have scanty cytoplasm with high nuclear-to-cytoplasmic ratios. Nuclear molding is commonly observed. Mitoses and apoptosis are conspicuous. Necrosis is frequently seen.

The clinical significance of the histologic subclassification into small cell and large cell groups has not been established.(18) Large cell PD NECs are defined by the presence of large polyhedral cells with moderate basophilic cytoplasm, and enlarged vesicular nuclei with prominent nucleoli. They are prone to form organoid structures with scattered rosettes and pseudorosettes.(22)

1.6.3 World Health Organization (WHO) Grading system

The 2019 WHO classification of tumours of the digestive system has separated WD NETs from PD NECs based on tumour morphology, mitotic count and Ki-67 proliferative index. We have chosen to use this grading schema for the purposes of our study (Table 1).

Table 1: The 2019 WHO classification of GI NENs

	Mitotic count (per $2mm^2$)	Ki-67 proliferative index
Well differentiated NET		
Grade 1	< 2	< 3%
Grade 2	2 – 20	3 – 20%
Grade 3	> 20	> 20%
Poorly differentiated NEC		
Small cell type	> 20	> 20%
Large cell type	> 20	> 20%

1.6.3.1 Mitotic count

Mitotic rates are to be expressed as the number of mitoses per $2mm^2$. This area equates to 10 high-power fields at 400x magnification when using a microscope with an ocular field diameter of 0.5mm. Most modern microscopes have a wider field diameter and this needs to be considered when determining how many high-power fields should be counted. It is currently recommended to count mitoses in an area of $10mm^2$ and then report the rate as per $2mm^2$. These recommendations are endorsed by the WHO, American Joint Committee on Cancer (AJCC) and College of American Pathologists.(2, 23)

1.6.3.2 Ki-67 proliferative index

The Ki-67 proliferative index is determined by identifying the region of highest immunolabelling (hotspot) and counting at least 500 cells in this region. There is no universally accepted technique for performing this count. Most experts advocate a manual count which can be done digitally or on a printed photomicrograph.(24) Another acceptable technique is using digital software packages which perform automated cell counting. This has limitations which include the software's inability to detect overlapping cells, staining of non-tumour cells and background brown pigment like haemosiderin.

Estimating the Ki-67 index by the "eyeball" technique is controversial as studies have shown different interobserver variabilities.(25-27) Eyeballing is now discouraged due to challenges with interobserver variability.(26)

1.7 Immunohistochemistry

1.7.1 INSM1

Insulinoma-associated protein 1 (INSM1) is a zinc-finger transcription factor which was initially described in pancreatic insulinomas.(28) Subsequently, it has been found to be central to early neuroendocrine differentiation in developing embryonal tissues, and is directly responsible for the transcription of synaptophysin and chromogranin A.(29) The *INSM1* gene is located on chromosome 20p11.23.

INSM1 is the only commercially available neuroendocrine marker for diagnostic immunohistochemistry that shows nuclear localisation. This is advantageous particularly in the evaluation of small cell PD NECs where minimal cytoplasm is present to stain for the traditional neuroendocrine markers, chromogranin A and synaptophysin. In the past three years INSM1 has emerged as a reliable marker in the identification of NENs.(30) Studies have established its role as a sensitive and specific immunohistochemical marker in thoracic NENs, head and neck NENs and Merkel cell carcinoma.(31-35)

Recently, a study by Gonzalez *et al*. evaluated the sensitivity and specificity of INSM1 in a series of 30 gastroenteropancreatic-NENs.(3) They found a sensitivity of 100% and a specificity of 96%. There was only one NEC (1/30) in this study.

INSM1 has not been evaluated in the South African context.

1.7.2 Chromogranin A

Chromogranin A is an acidic protein which belongs to a family of granins present in the secretory granules of neuroendocrine cells and tumours.(36) Chromogranin A has been studied extensively in human tumours and it is present in most NENs. Granins are located primarily in secretory granules within the cytoplasm, and thus immunolabelling may be reduced in tumours which have minimal cytoplasm such as small cell NECs.(37, 38) Chromogranin A immunohistochemistry is typically strongly positive in well to moderately differentiated NETs, whereas it is only focally positive or lost in PD NECs.(39, 40) The sensitivity of chromogranin A in detecting NENs in the left colon and rectum is 20-50%.(41)

1.7.3 Synaptophysin

Synaptophysin is a 38-kDa transmembrane glycoprotein found in the membranes of presynaptic vesicles in neurons and neuroendocrine cells.(38) These cytoplasmic vesicles are different from the granules in which chromogranin is found and therefore the staining pattern is different.(38) Synaptophysin is not specific for GI NENs and also stains other tumours showing neuroendocrine differentiation such as paragangliomas, phaeochromocytomas and medullary thyroid carcinomas. Adrenocortical adenomas and carcinomas typically also label with synaptophysin.

Synaptophysin is considered the most sensitive marker for neuroendocrine differentiation in the GIT.(42) However, it is not entirely specific and its combined use with chromogranin A is recommended.(43) Synaptophysin is cited as being positive in only approximately 50% of gastric NETs.(18, 22)

1.7.4 Ki-67

The Ki-67 antibody recognizes a nuclear protein expressed by actively dividing cells in the G1, G2, S and M phases of the cell cycle. This protein is not found in cells in the resting (G0) phase. The Ki-67 proliferative index is a frequently reported parameter in a variety of

neoplasms and is central (together with the mitotic rate) to the classification and grading of NENs. Increased ischaemic time, from vascular clamping and resection to formalin fixation, significantly decreases the number of mitotic figures.(44) Therefore the Ki-67 is often higher than the mitotic rate and determines the NEN grade.(42) Evaluation of the Ki-67 index may be influenced by the antibody clone, different staining protocols, tissue section thickness and density of tumour cells.(42)

The protein recognised by the Ki-67 antibody can be detected by immunohistochemical staining with the MIB-1 monoclonal antibody. Strong dark brown granular nuclear staining is interpreted as positive.(45)

1.8 Study design

1.8.1 Study aims

The aims of this study were as follows:

1. To describe the epidemiological features of GI NENs seen in the Division of Anatomical Pathology at Groote Schuur Hospital from 2003 - 2017.
2. To reclassify the NENs according to the latest (2019) WHO criteria.
3. To determine the sensitivity and specificity of the immunohistochemical stain (INSM1) in the South African setting and compare it to chromogranin A and synaptophysin.

1.8.2 Study objectives

Reclassification of the NENs seen in the Division of Anatomical Pathology at Groote Schuur Hospital from 2003 - 2017 according to the latest (2019) WHO grading system. This will be achieved by performing a formal mitotic count and evaluation of the Ki-67 proliferative index.

Comparison of the sensitivity and specificity of the new neuroendocrine immunohistochemical marker INSM1 to that of the traditional markers, synaptophysin and chromogranin A. This will be done in order to determine whether INSM1 can be used to replace the combination of synaptophysin and chromogranin A as a panel for neuroendocrine differentiation.

2. MATERIAL AND METHODS

2.1 Ethics approval

Ethics approval for this study was obtained from the University of Cape Town Faculty of Health Sciences Human Research Ethics Committee (Reference number: 176/2018). Scientific approval for this MMed study was obtained from the Department of Pathology Research Committee.

Funding for this study was obtained from the National Health Laboratory Service (NHLS) Research Trust Development Grant (Reference number: 2018-1DEV21-MLO01).

This was a retrospective study performed on archival material, and patient management was not directly affected in any way. The patient's data was anonymised and each specimen was allocated a study number. The data was stored in an electronic password protected document. The physical materials for the study (glass slides, wax blocks) were stored in a locked cupboard in a locked office with electronic access control to the department.

2.2 Acquisition of cases

This was a retrospective study. Cases of GI NENs (both biopsy and resection specimens) seen in the Division of Anatomical Pathology, National Health Laboratory Service - Groote Schuur Hospital were examined. The study period included cases from 2003 to 2017. We searched the Disa and Trakcare laboratory information systems (LISs) using the terms "carcinoid tumour", "neuroendocrine tumour", "neuroendocrine carcinoma", "small cell carcinoma", and "large cell neuroendocrine carcinoma". The study was limited to the GI tract from the stomach to the rectum and excluded pancreatic NENs. Five primary anatomic sites of interest were identified: stomach, small intestine, appendix, colon and rectum. Liver metastases with no identifiable primary tumour were excluded. Goblet cell

carcinoids and mixed neuroendocrine-non-neuroendocrine neoplasms (MiNEN) were also excluded.

The database search returned 236 cases (134 excision specimens and 102 biopsies). We prioritised the excision specimens during the case selection, as they had more tissue available for further stains. Fifty-nine of the 134 excision specimens were appendicectomy cases (44%). We included 25 appendicectomy cases, and all the excision specimens that were from sites other than the appendix. The remaining cases were made up of biopsies to bring the total from each anatomic site to 25 cases. When selecting the appendicectomy cases and the biopsies, we prioritised the more recent cases as these were felt to be more suitable for antigen retrieval. We excluded biopsies that had a follow up excision specimen from the selection.

We anticipated that not all cases would be suitable for inclusion (due to inaccessible or lost tissue blocks, poorly preserved tissue blocks and cases with small tumour volumes not amenable to further sectioning) and were aiming for 100 study cases.

The slides of the selected cases were retrieved from the archives of the Division of Anatomical Pathology, National Health Laboratory Service – Groote Schuur hospital. The slides were reviewed and a block was selected for immunohistochemistry. The formalin fixed paraffin wax embedded tissue blocks were then retrieved from the archive.
The age, gender, anatomic site and whether an excision or biopsy was performed was recorded from the Disa and Trakcare LISs. It was noted whether synaptophysin, chromogranin A and Ki-67 immunostains were done on the cases, and these original slides were reviewed for possible inclusion in the study. We performed immunohistochemistry for these markers on the cases for which we could not retrieve the slides, the slides were damaged, faded or uninterpretable, or where the markers were not done in the first instance.

2.3 Acquisition of controls

We searched the Trakcare LIS to identify 4 non-NENs from each of the 5 anatomic sites selected for the study. Resection specimens were identified. The terms "adenocarcinoma", "lymphoma", "gastrointestinal stromal tumour", "leiomyoma" and "Kaposi sarcoma" were searched to identify a variety of tumours frequently encountered at these sites.

2.4 Antibodies

The primary antibodies used were INSM1, synaptophysin, chromogranin A and Ki-67 (Table 2). A negative reagent control in which the primary antibody was replaced with PBS was used together with a positive tissue control for each IHC run.

Table 2: Primary antibody information

Primary antibody	Clone	Supplier	Antigen retrieval	Dilution	Incubation time, Temp	Positive control
Chromogranin A	DAK-A3/ Mono	Dako	TEDTA	1:200	1 hour, RT	Normal colon
INSM1	A-8/ Mono	Santa Cruz	TEDTA	1:500	1 hour, RT	Pancreatic WD NET
Ki-67	MIB1/ Mono	Dako	TEDTA	1:100	1 hour, RT	Tonsil
Synaptophysin	27G12/ Mono	Novocastra	Citric acid	1:200	1 hour, RT	Normal thyroid (c-cells)

Abbreviations: RT, room temperature; mono, monoclonal.

Supplier information:

Dako – Denmark

Santa Cruz – Europe

Novocastra – Leica, United Kingdom

2.5 Immunohistochemistry

Immunohistochemistry was performed on sections cut from the formalin fixed paraffin embedded wax blocks according to the following protocol:

1. Three-micron thick paraffin wax embedded tissue sections were cut, picked up onto Histobond slides (Marienfeld-Germany) and heat fixed on a hotplate for 10-15 min.
2. Sections were dewaxed through xylene, cleared in ethanol and rehydrated in water.
3. Endogenous peroxidase activity was blocked by treating the slides with a 3% hydrogen peroxide (H_2O_2) solution for 10 minutes.
4. Slides were washed well in water.
5. Antigen retrieval was performed by pressure-cooking slides in Tris EDTA (TEDTA) or Citric acid (Table 2) for 1 minute 30 seconds at full pressure.
6. This was followed by washing in tap water.
7. Thereafter, slides were rinsed with phosphate buffered saline solution (PBS pH 7.6), (Oxoid-Hampshire, England).
8. Non-specific binding was blocked by treating slides with a 5% Goat Serum Solution (DAKO- Denmark).
9. Serum was then drained off and sections were incubated with primary antibody at room temperature at specified times and dilutions (Table 2).
10. The slides were then washed well with PBS solution.
11. This was followed by incubation with the (Monoclonal) DAKO Envision labelled Polymer, HRP (DAKO- USA) (Table 3) for 30 minutes at room temperature.
12. Sections were washed well with PBS solution.
13. Positivity was developed by applying the chromogenic substrate 3,3 – diaminobenzidine (DAB), (DAKO- USA) for 5-10 minutes.
14. Slides were washed in running tap water and counterstained with Mayers haematoxylin for approximately 3 minutes.
15. After washing in running tap water, sections were blued in ammoniated water.

16. Finally, the slides were dehydrated through alcohols, cleared with xylene and mounted with Entellan, (MERCK- Germany).

Table 3: Kits used in this study

Kit	Supplier
Envision HRP System Labelled Polymer Anti-mouse	Dako - CA, USA
Liquid DAB + Substrate chromogen system	Dako - CA, USA

2.6 Haematoxylin and Eosin method

Cases in which the retrieved H&E stained glass slides were unsuitable for evaluation were recut from the wax blocks and stained according to the following protocol:

MAYERS HAEMATOXYLIN AND EOSIN METHOD:

1. Sections were dewaxed in xylene, brought down through alcohols and washed well in running tap water.
2. The slides were then stained in Mayers haematoxylin for 5 mins
3. Tissue sections were washed well in tap water and the nuclei blued with ammoniated water. The slides were washed again in tap water.
4. The cytoplasm and surrounding tissue were stained with 1% phloxine/eosin for 2 mins.
5. Slides were washed well in water, dehydrated in alcohol and cleared in xylene.
6. Finally, sections were mounted onto coverslips using Entellan.

Results:

Nuclei - blue

Cytoplasm and surrounding tissue - varying shades of pink.

2.7 Interpretation of immunohistochemical staining

Assessment of positivity was determined by granular cytoplasmic staining for synaptophysin and chromogranin A, and nuclear staining for INSM1. The intensity of staining and the proportion of tumour cells which stained positively was recorded. The intensity was scored out of 3 (corresponding to mild, moderate, and strong), and the proportion was recorded as a percentage of the total number of tumour cells present in the section.

The intensity score and proportion of positively staining cells were multiplied to produce a score out of 300 (H-score). For the purposes of this study we regarded positivity to be an H-score of 30 or more out of 300. The minimum proportion of cells required for each intensity score is shown in Table 4.

Table 4: Intensity and proportion values required to meet the H-score cut-off

Intensity	Proportion	H-score
1	30%	30
2	15%	30
3	10%	30

Nuclear staining of tumour cells was assessed to determine the Ki-67 proliferative index, and this was expressed as a percentage of the total number of tumour cell nuclei present in the field of interest.

2.8 Tumour morphology

H&E stained sections were reviewed by light microscopy for all cases to confirm the diagnosis. The distinction between well differentiated and poorly differentiated NENs was

made on light microscopy based on established morphologic criteria. Small cell NEC and large cell NEC were also distinguished based on morphology.

The following cytological features were used, in addition to the characteristic architectural growth patterns, to distinguish WD NET, small cell PD NEC and large cell PD NEC:

WD NET:

1. Medium sized cells with a low nuclear-to-cytoplasmic ratios.
2. Abundant cytoplasm.
3. Small round to ovoid nucleus
4. Dispersed chromatin containing small nucleoli.

PD NEC, small cell type:

1. Small to medium sized cells with high nuclear-to-cytoplasmic ratios.
2. Scant basophilic cytoplasm.
3. Elongated nuclei.
4. Finely dispersed chromatin without nucleoli.

PD NEC, large cell type:

1. Medium to large sized cells.
2. Basophilic cytoplasm.
3. Large ovoid nuclei.
4. Large nucleoli.

2.9 Grading of tumours

We performed a formal mitotic count on H&E stained sections by identifying the area of the tumour with the most mitotic activity on scanning magnification (the so-called hot spot). The number of mitotic figures was then counted in an area of 10mm^2 (42

consecutive high-power fields based on the field diameter of my microscope, 0.55 mm). The mitotic rate was then reported as per $2mm^2$ which is the current recommendation by the WHO. To be as accurate as possible, we did not round off the mitotic rate to achieve a whole number of mitoses. For example: 11 mitoses counted in 42 high power fields was reported as 2.2 mitoses per $2mm^2$.

The Ki-67 proliferative index was determined be identifying the area with the highest number of positively staining tumour cells on scanning magnification (hot spot). This area was photographed at high magnification (400x). Using the Microsoft Paint software program (Washington, USA), a red dot was placed over all the nuclei which showed positive labelling with the Ki-67 immunostain. Black dots were placed over the haematoxylin-stained tumor cell nuclei which did not label with the Ki-67 antibody until a total of 500 cells had been counted. Care was taken to only include tumour cells in this count, and lymphocytes, endothelial cell and fibroblasts were excluded. The Ki-67 proliferative index was expressed as a percentage.

For the purposes of this study we used the 2019 WHO 5^{th} edition classification schema for grading neuroendocrine neoplasms of the digestive system (Table 1). In cases where there was discrepancy between the Ki-67 proliferative index and the mitotic count we assigned the higher tumour grade.

2.10 <u>Site of tumours</u>

The site of the tumours was obtained from the Disa and Trakcare LISs. The anatomic site was grouped as stomach, small intestine, appendix, colon or rectum. The site was confirmed histologically by examination of the H&E stained sections.

2.11 Statistical analysis

Categorical variables were compared in contingency tables using the Fisher's exact test and Chi squared test. Student's t-test was used for hypothesis testing when comparing two sample means. Statistical analysis and interpretation were performed using the IBM SPSS Statistics software package (New York, USA). Results were considered statistically significant if $p < 0.05$.

Sensitivity was defined as the ability of the immunohistochemical stain (INSM1, chromogranin A, synaptophysin) to correctly identify NENs (true positives). This was calculated by dividing the number of cases that were positive for the immunohistochemical stain by the total number of NEN cases.

Specificity was defined as the ability of the immunohistochemical stain (INSM1, chromogranin A, synaptophysin) to correctly identify the non-NEN controls (true negatives). This was calculated by dividing the number of controls that were negative for the immunohistochemical stain by the total number of controls.

3. RESULTS

3.1 Cases and Controls

Sixty-nine cases were retrieved from the archives that had enough remaining tissue in the wax block and were suitable for analysis (Figure 1).

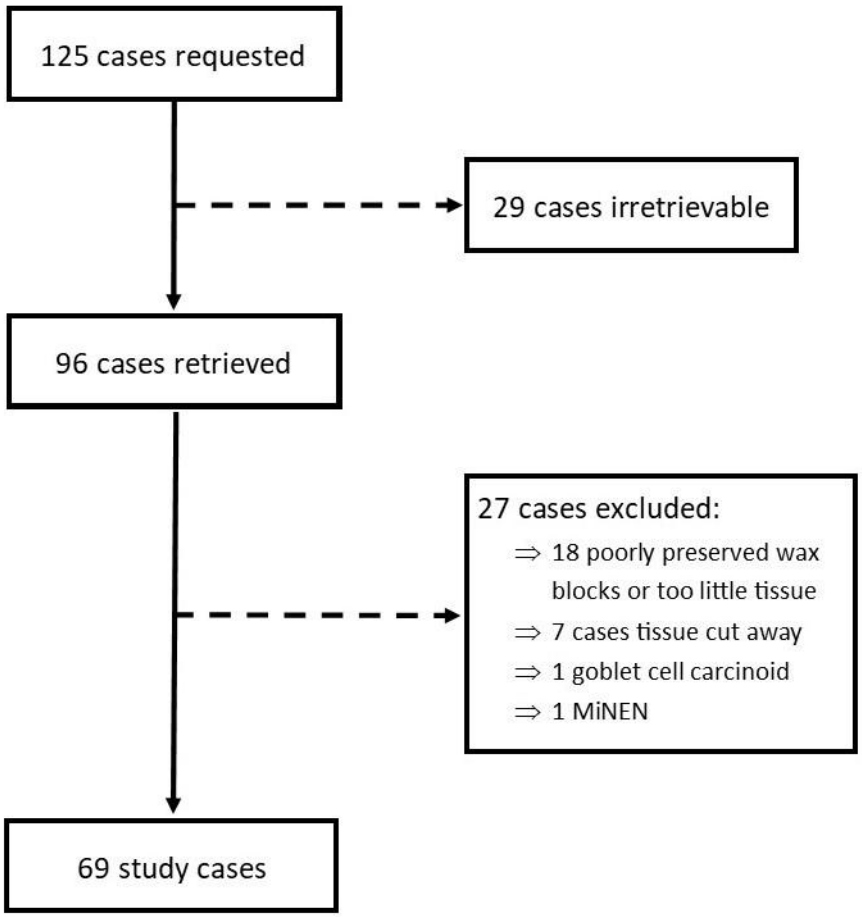


Figure 1: Diagrammatic representation of the case recruitment process

Seventeen tissue control blocks were retrieved from the archives. These specimens originated from a spectrum of anatomic sites and represented a variety of tumour types found in the GIT (Table 5).

Table 5: Controls stratified by anatomic site and diagnosis

	Number
Stomach	
Adenocarcinoma	3
GIST	2
Small intestine	
Lymphoma	3
GIST	2
Leiomyoma	1
Appendix	
Kaposi sarcoma	2
Lymphoma	1
Colorectum	
Adenocarcinoma	3

3.2 Age

Age was available for all cases on the electronic database. The age range for all the cases was 14-85 years with a mean age of 56,7 years (Table 6). WD NETs had a significantly lower mean age of 53,4 (range 14-76 years), compared to the PD NECs (mean 70 years; range 51-85) (t= -3.95, p <0.001). Mean age grouped by anatomic site is shown below in Table 7.

Table 6: Comparison of age (years) between tumour groups

	All cases	WD NET	PD NEC
Number of cases	69	55	14
Age range (years)	14-85	14-76	51-85
Mean age (years)	56.7	53.4	70
Student T-test	$p < 0.001$		

Table 7: Mean age at diagnosis of NENs grouped by anatomic site

	NEN mean age (years)	WD NET mean age (years)	PD NEC mean age (years)
Stomach	64.3	56.1	73.6
Small intestine	54.6	54.6	*
Appendix	28.7	28.7	*
Colon	61.2	58.8	67.5
Rectum	59.8	58.7	65.0

* no cases occurred at these anatomic sites

3.3 Gender

Information on the patients' gender was provided on the hospital sticker as self-identified by the patient. This information was captured in the electronic database and was available for all cases (Table 8).

Table 8: Comparison of gender between tumour groups

	All cases	WD NET	PD NEC
Male (%)	34 (49%)	27 (49%)	7 (50%)
Female (%)	35 (51%)	28 (51%)	7 (50%)
Total cases	69	55	14
Fisher's exact test	$p = 1.00$		

There was no gender predilection demonstrated in either the WD NET or the PD NET groups. Table 9 shows the number of male and female cases grouped by anatomic site.

Table 9: Gender composition of NENs grouped by anatomic site

	Male	Female
Stomach	8 (53%)	7 (47%)
Small intestine	6 (38%)	10 (62%)
Appendix	4 (57%)	3 (43%)
Colon	7 (50%)	7 (50%)
Rectum	9 (53%)	8 (47%)

3.4 Type of sample

The type of specimen (excision or biopsy) was available from the original histology report and confirmed by examination of the glass slides (Table 10). PD NECs were more likely to be found in biopsy cases than WD NETs, which were the more common tumour type in the excision cases (p <0.002).

Table 10: Comparison of specimen type (biopsy/excision) and NEN group

	All cases	WD NET	PD NEC
Excision (%)	41 (59%)	38 (69%)	3 (21%)
Biopsy (%)	28 (41%)	17 (31%)	11 (79%)
Total cases	69	55	14
Fisher's exact test	$p < 0.002$		

3.5 Site of tumour

The sites of the tumours were available from the original histology reports and this data is summarised in the pie chart (Figure 2). Of note, we were only able to include 7 cases from the appendix. This is due to the irretrievability of some wax blocks and the small volume of tumour present in appendiceal WD NETs.

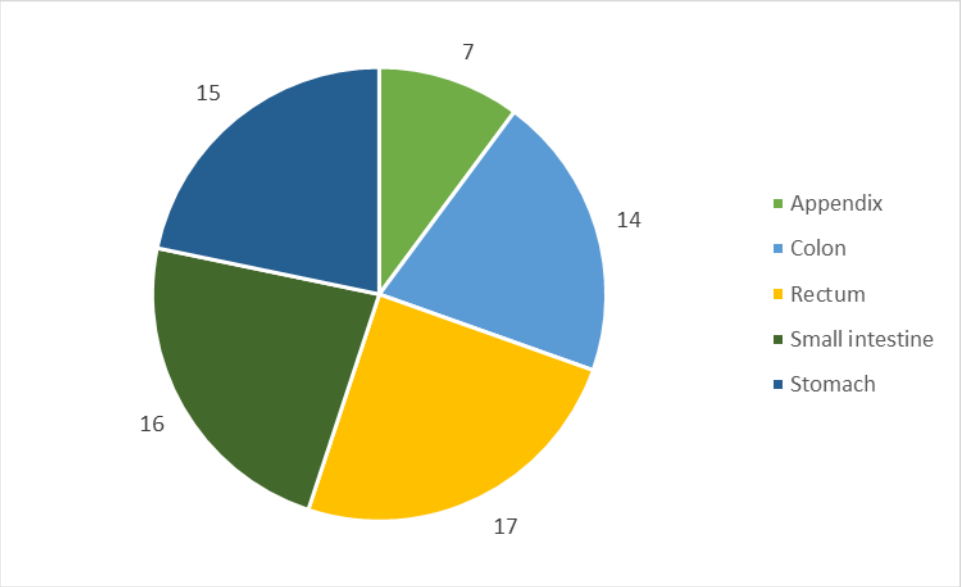


Figure 2: Proportion of study cases illustrated according to anatomical site

3.6 Grading

Ki-67 immunohistochemistry was evaluated on all 69 cases. The technique used is demonstrated in Figure 3. Two of the 69 cases were unsuitable for evaluation of mitotic count due to smearing artefact. Both of these cases were poorly differentiated neuroendocrine carcinomas, one from the rectum and one from the stomach. Based on the tumour morphology, mitotic count, and Ki-67 proliferative index there were 14 cases (20%) classified as PD NEC and 55 (80%) classified as WD NET (Table 11). Examples of cases that were classified as WD NET and PD NEC are shown in Figures 4 to 8.

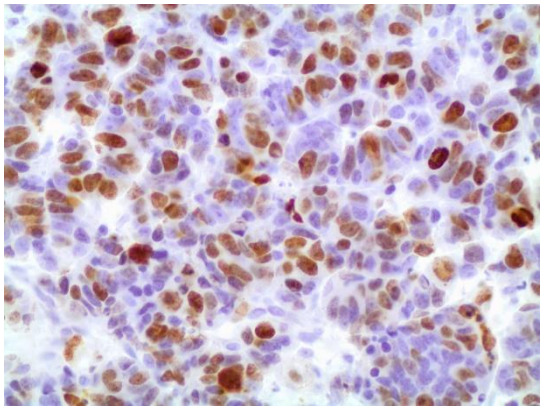
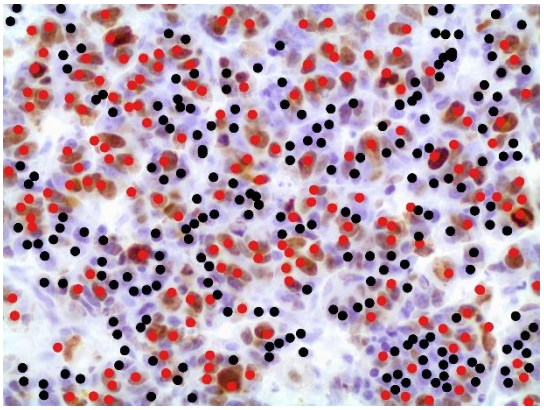
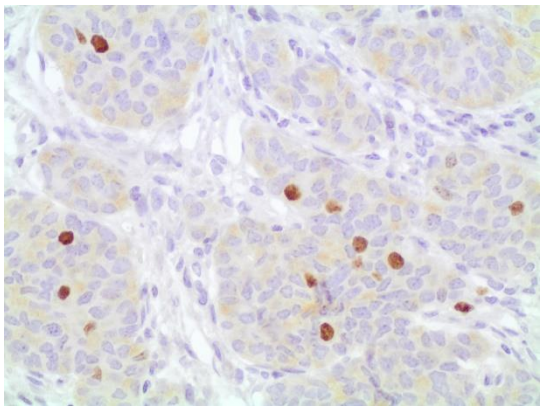
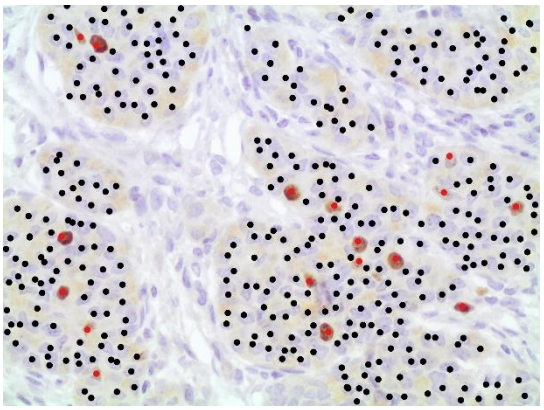
	
NEN_07	176/376 = 46.8%
	
NEN_19	17/385 = 4.4%

Figure 3: Examples of Ki-67 manual counting technique (400x magnification)

Table 11: Number of tumours graded according to 2019 WHO classification system

Well differentiated NET	
Grade 1	38 (55%)
Grade 2	16 (23%)
Grade 3	1 (1.5%)
Poorly differentiated NEC	
Small cell-type	13 (19%)
Large cell-type	1 (1.5%)

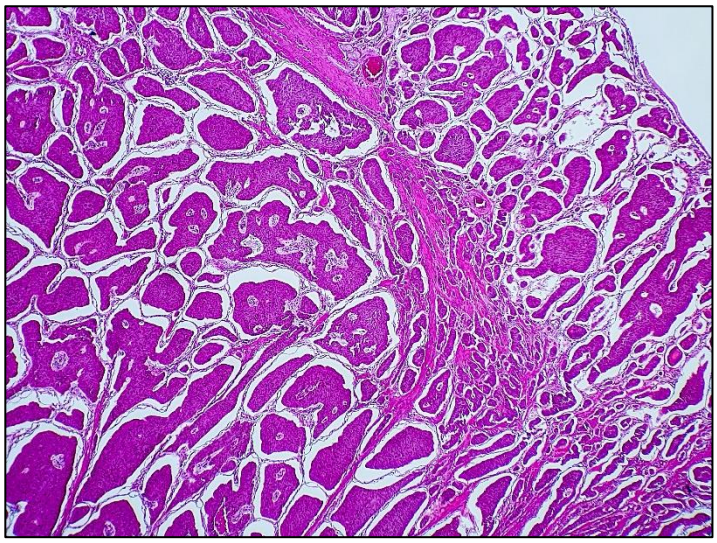


Figure 4: WD NET (NEN_03) low magnification showing insular growth (H&E, 40x)

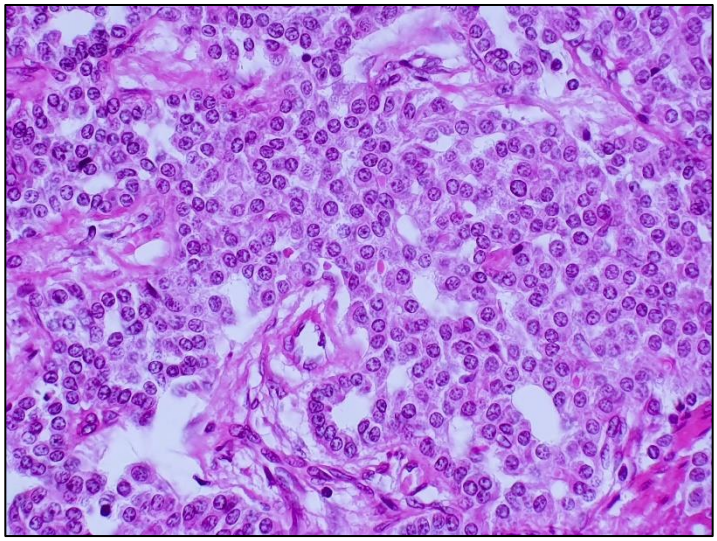


Figure 5: WD NET (NEN_03) high magnification showing round nuclei with stippled chromatin (H&E, 400x)

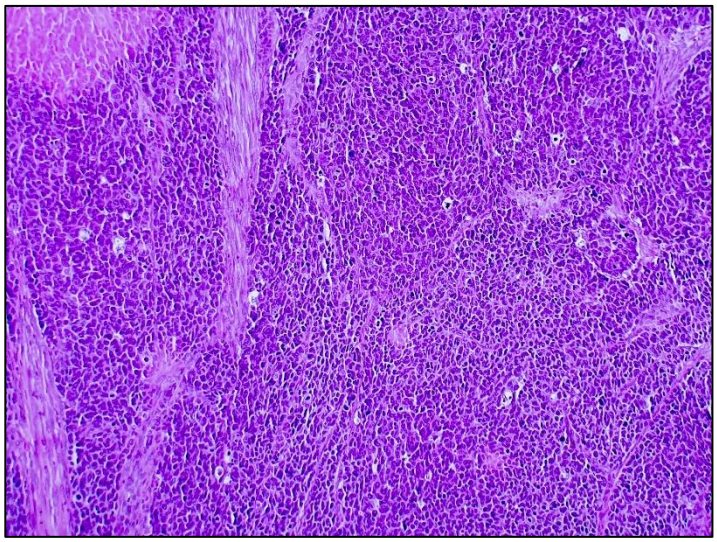


Figure 6: PD NEC small cell type (NEN_02) showing sheets of tumour cells, intermediate magnification (H&E, 100x)

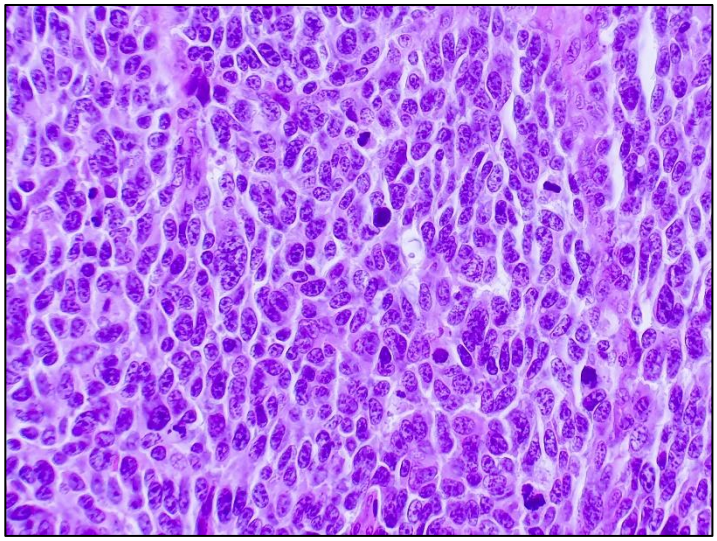


Figure 7: PD NEC small cell type (NEN_02) showing round to polyhedral cells with scanty cytoplasm and high nuclear-to-cytoplasmic ratios, high magnification (H&E, 400x)

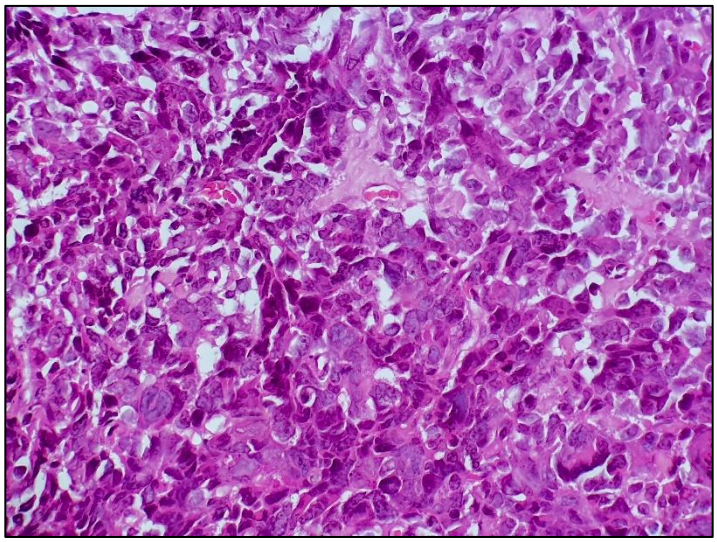


Figure 8: PD NEC large cell type (NEN_45) showing medium sized cells with basophilic cytoplasm and large ovoid nuclei, high magnification (H&E, 400x)

The PD NECs had a mean mitotic count of 16.1 per 2mm^2 (range 0.5-43). The mean Ki-67 index for these tumours was 66.5% (range 32-100%). The WD NETs had a mean mitotic count of 0.4 per 2mm^2 (range 0- 2.2) and a median Ki-67 index of 2.0% (mean 3.7%; range 0-64%). The skewed Ki-67 index mean value is due to the single grade 3 WD NET which demonstrated a Ki-67 proliferative index of 64.1%.

The PD NECs occurred in the stomach and colorectum, and none were found in the small intestine and appendix. All of the tumours in the small intestine and appendix were WD NETs (grade 1 or 2). There was a single grade 3 WD NET identified in the colon (Figures 9 and 10). This tumour demonstrated a mitotic rate of 1 per 2mm^2 and had a Ki-67 proliferation index of 64%. The breakdown of tumour grades at the different anatomic sites is shown in Table 12.

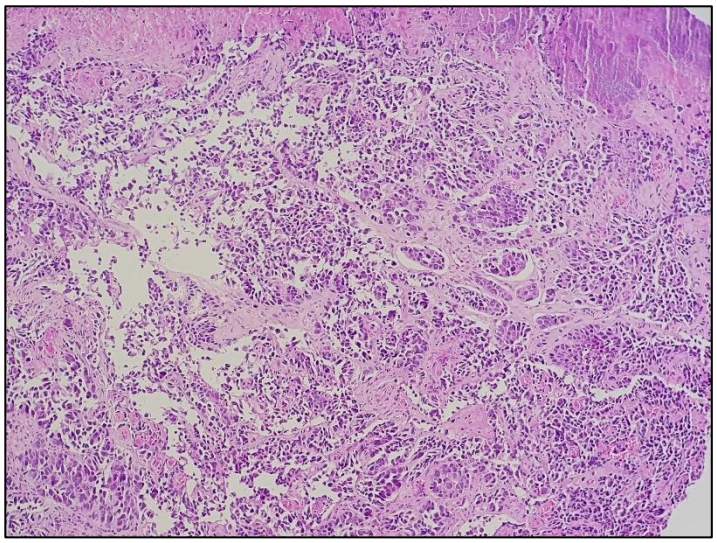


Figure 9: WD NET grade 3 (NEN_32) showing nests of tumour cells underlying a mucosal ulcer, intermediate magnification (H&E, 100x)

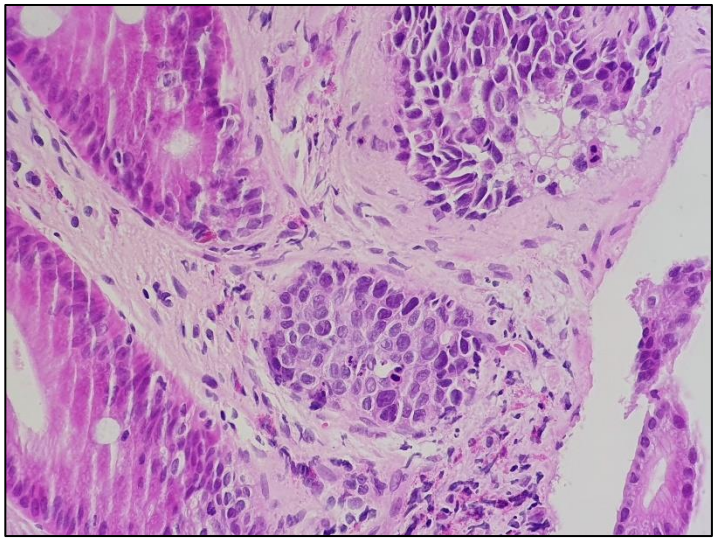


Figure 10: WD NET grade 3 (NEN_32) showing tumour cells with moderate eosinophilic cytoplasm and nuclei with stippled chromatin, high magnification (H&E, 400x)

Table 12: Comparison of the NEN grades found at different anatomical sites

	WD NET			PD NEC		
	Grade 1	Grade 2	Grade 3	Small cell	Large cell	Total
Stomach	5	3	0	7	0	15 (22%)
Small intestine	10	6	0	0	0	16 (23%)
Appendix	6	1	0	0	0	7 (10%)
Colon	8	1	1	3	1	14 (20%)
Rectum	9	5	0	3	0	17 (25%)
Total	38	16	1	13	1	

3.7 Immunohistochemistry

3.7.1 INSM1

INSM1 immunohistochemistry was performed on all 69 cases and the 17 control cases. Figure 11 shows the various staining intensities. Fifty-five of the 69 cases (80%) showed positive staining with INSM1 (Table 13). The single grade 3 WD NET was negative for INSM1 (not shown in Table 13). None of the 17 control cases met our H-score criteria for positivity with INSM1. There were however isolated positive cells which showed neuroendocrine differentiation in all of the adenocarcinoma cases. The colonic adenocarcinoma case which was positive for synaptophysin according to our H-score criteria, had an INSM1 H-score of 25.

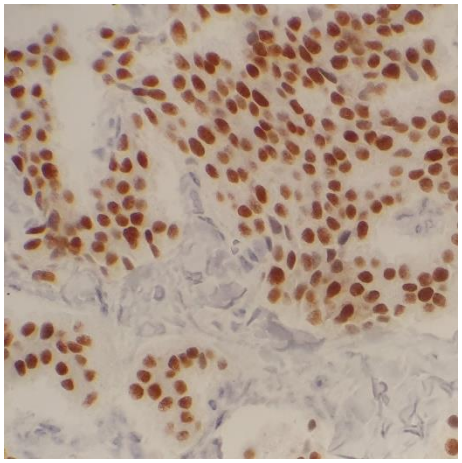
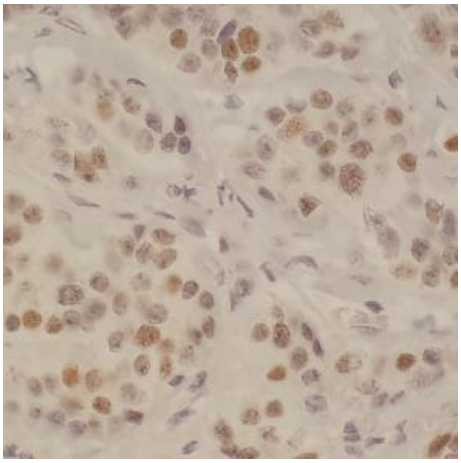
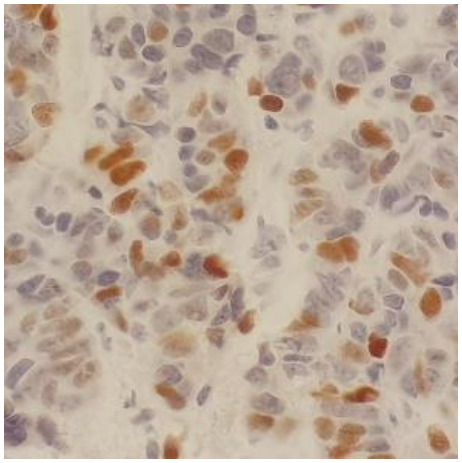
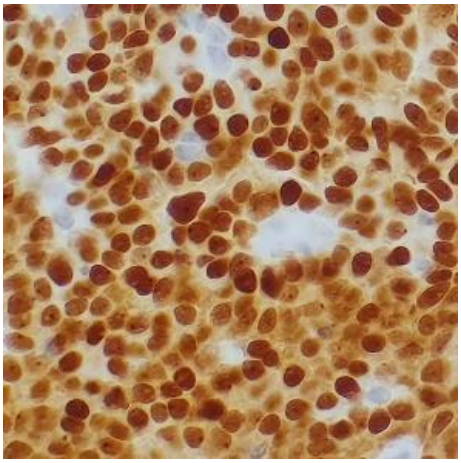
	
Positive control (PanNET)	1+
	
2+	3+

Figure 11: Staining intensities of INSM1 (400x magnification)

Table 13: INSM1 staining stratified by tumour grade and anatomic site

	INSM1		
	Positive	Negative	*p**
Tumor Grade			.919
WD G1	31 (81.6%)	7 (18.4%)	
WD G2	12 (75%)	4 (25%)	
NEC	12 (85.7%)	2 (14.3%)	
Site			.058
Appendix	7 (100%)	0 (0%)	
Colon/Rectum	27 (87.1%)	4 (12.9%)	
Small Intestine	9 (56.3%)	7 (43.8%)	
Stomach	12 (80%)	3 (20%)	

*Fisher's exact test

INSM1 staining was not significantly different between WD and PD NENs ($p = 0.919$). There was no significant difference between the WD NET grades. The single grade 3 NET was negative for INSM1. A high proportion of tumours in the stomach, colorectum and appendix showed positive staining (80-100%). Only 56% of small intestine tumours were positive, but this finding was not statistically significant ($p = 0.058$).

3.7.2 Synaptophysin

Synaptophysin immunohistochemistry was performed on all 69 cases and 17 controls. Sixty of the 69 cases (87%) showed positive staining with synaptophysin. Figure 12 shows examples of the synaptophysin staining intensities. The grade 3 WD NET case was positive for synaptophysin (not shown in Table 14). One of the 17 control cases (6%) showed positive staining. This positive control case was a colonic adenocarcinoma with an H-score of 40 (intensity 1, proportion 40%).

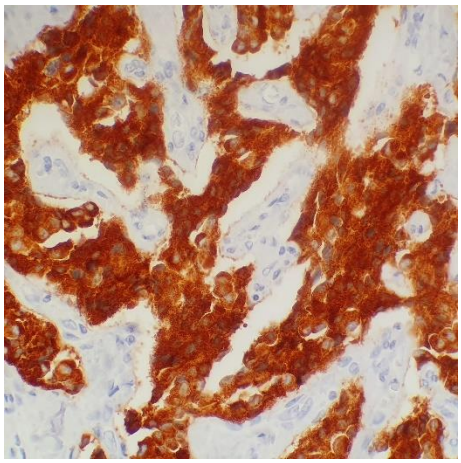
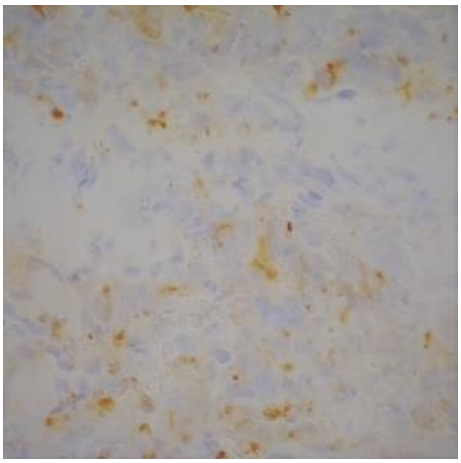
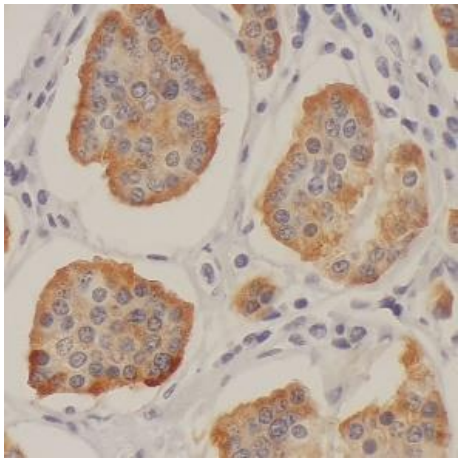
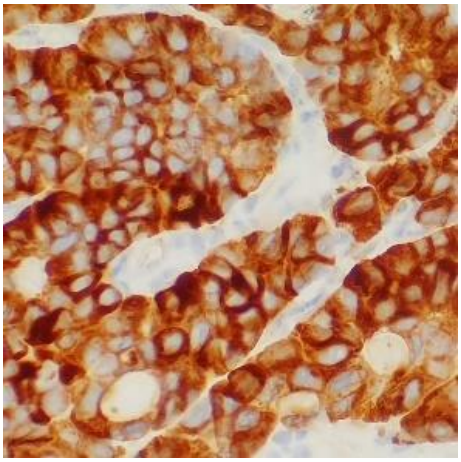
	
Positive control (PanNET)	1+
	
2+	3+

Figure 12: Staining intensities of synaptophysin (400x magnification)

Synaptophysin was less likely to be positive in the PD NECs as compared with the WD NETs (Table 14). This difference was statistically significant (p = 0.012). There was no significant difference in the proportion of positively staining cases when stratified by the anatomic site (p = 0.161).

Table 14: Synaptophysin staining stratified by tumour grade and anatomic site

	Synaptophysin		
	Positive	Negative	*p**
Tumor Grade			.012
WD G1	34 (89.5%)	4 (10.5%)	
WD G2	16 (100%)	0 (0%)	
NEC	9 (64.3%)	5 (35.7%)	
Site			.161
Appendix	5 (71.4%)	2 (28.6%)	
Colon/Rectum	27 (87.1%)	4 (12.9%)	
Small Intestine	16 (100%)	0 (0%)	
Stomach	12 (80%)	3 (20%)	

*Fisher's exact test

3.7.3 Chromogranin A

Chromogranin A immunohistochemistry was performed on all 69 cases and 17 controls. Forty-seven of the 69 cases (68%) were positive with chromogranin A. The grade 3 WD NET was positive for chromogranin A (not shown in Table 15). Examples of cases positive for chromogranin A are shown in Figure 13. Two of the 17 control cases (12%) were positive with chromogranin A. These represented one small bowel diffuse large B cell lymphoma (H-score = 30), and one gastric adenocarcinoma (H-score = 30).

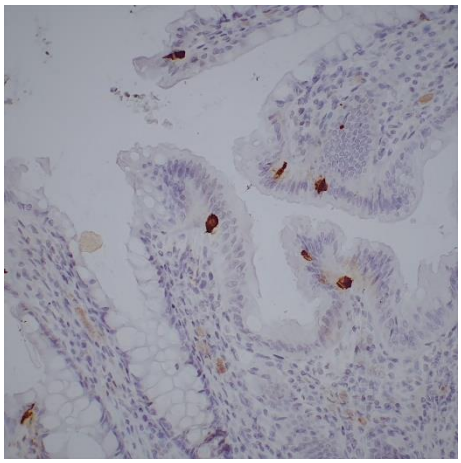
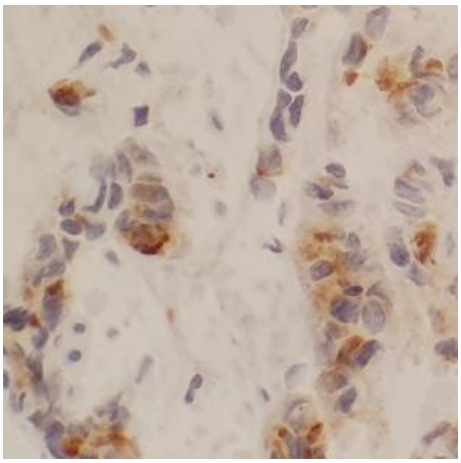
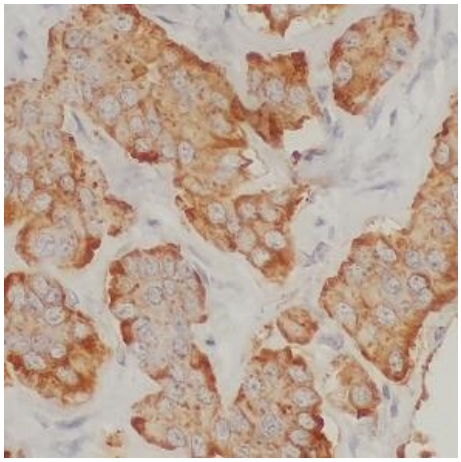
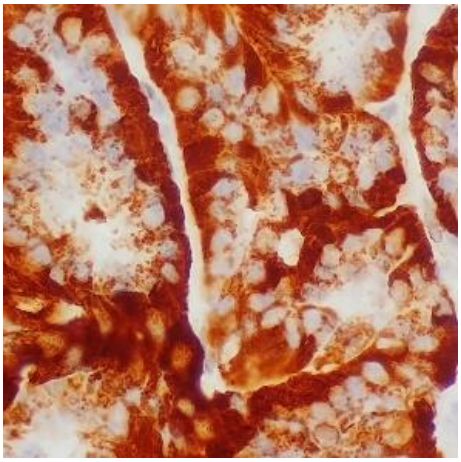
	
Positive control (colon)	1+
	
2+	3+

Figure 13: Staining intensities of chromogranin A (400x magnification)

There was no statistically significant difference in chromogranin A positivity between the well differentiated NETs and poorly differentiated NECs ($p = 0.318$). The proportion of colorectal cases which showed positive labelling with chromogranin A was significantly lower than at the other anatomic sites ($p < 0.001$) (Table 15).

Table 15: Chromogranin A staining stratified by tumour grade and anatomic site

	Chromogranin A		
	Positive	Negative	*p**
Tumor Grade			.318
WD G1	27 (71.1%)	11 (28.9%)	
WD G2	12 (75%)	4 (25%)	
NEC	7 (50%)	7 (50%)	
Site			<.001
Appendix	7 (100%)	0 (0%)	
Colon/Rectum	13 (41.9%)	18 (58.1%)	
Small Intestine	16 (100%)	0 (0%)	
Stomach	11 (73.3%)	4 (26.7%)	

*Fisher's exact test

3.8 Sensitivity and Specificity of Immunohistochemical Stains

Twenty-eight cases showed positivity for all three neuroendocrine markers examined in this study (Table 16). The majority of cases (54%) were positive for only 2 markers. In the 4 cases that were only identified by a single neuroendocrine immunohistochemical marker, INSM1 was the positive stain. These cases were 3 small cell type PD NECs (2 cases from the rectum, 1 case from the stomach) and 1 grade 1 WD NET (rectum).

Table 16: Cases positive for different numbers of immunohistochemical markers

Immunohistochemical positivity	Number of cases
All three NE markers	28 (41%)
Any two markers	37 (54%)
Any one marker	4 (5%)

The area under the curve (AUC) calculation demonstrates that the expression for all the stains (with the exception of chromogranin A in the PD NEC group) were better than chance (Table 17).

Table 17: Sensitivities and specificities of IHC markers based on tumour type and grade

	AUC	Std Error	p	95% CI	Sensitivity	Specificity
All cases						
INSM1	0.899	0.033	<.001	0.84 - 0.96	79.7%	100%
CGA	0.782	0.058	<.001	0.67 - 0.90	68.1%	88.2%
SYNAP	0.905	0.041	<.001	0.83 - 0.99	87%	94.1%
CGA-SYNAP	0.883	0.057	<.001	0.77 - 0.99	94.2%	82.4%
WD G1 NET						
INSM1	0.908	0.040	<.001	0.83 – 0.99	81.6%	100%
CGA	0.796	0.064	.001	0.67 – 0.92	71.1%	88.2%
SYNAP	0.918	0.044	<.001	0.83 – 1.00	89.5%	94.1%
CGA-SYNAP	0.899	0.057	<.001	0.79 – 1.00	97.4%	82.4%
WD G2 NET						
INSM1	0.875	0.068	<.001	0.74 – 1.00	75%	100%
CGA	0.816	0.079	.002	0.61 – 0.97	75%	88.2%
SYNAP	0.971	0.034	<.001	0.90 – 1.00	100%	94.1%
CGA-SYNAP	0.912	0.057	<.001	0.80 – 1.00	100%	82.4%
PD NEC						
INSM1	0.893	0.069	<.001	0.76 – 1.00	78.6%	100%
CGA	0.691	0.099	.071	0.50 – 0.89	50%	88.2%
SYNAP	0.792	0.088	.006	0.62 – 0.97	64.3%	94.1%
CGA-SYNAP	0.810	0.084	.004	0.64 – 0.97	78.6%	82.4%

AUC= area under curve

Overall INSM1 had a sensitivity of 79.8% and specificity of 100%. The sensitivity of INSM1 was higher than chromogranin A, but lower than synaptophysin and the combined use of chromogranin A and synaptophysin (Figure 14).

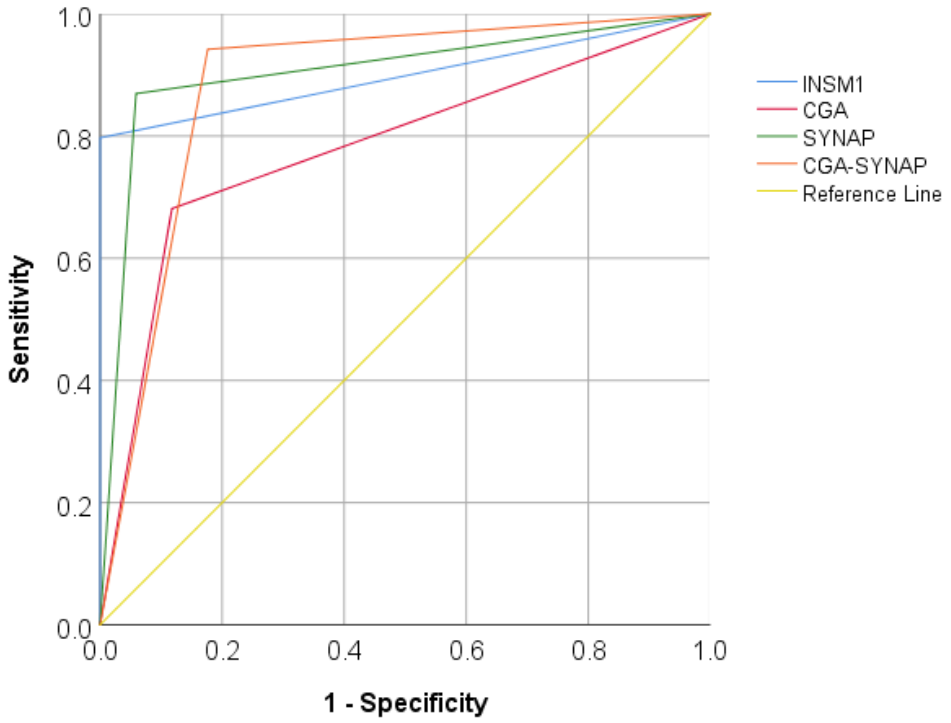


Figure 14: ROC curve considering all cases of NEN

When only considering cases of PD NEC, INSM1 had a higher sensitivity than chromogranin A and synaptophysin, and an equal sensitivity to the combined use of chromogranin A and synaptophysin (Figure 15). The use of chromogranin A in isolation for PD NEC was statistically no better than chance (p=.071).

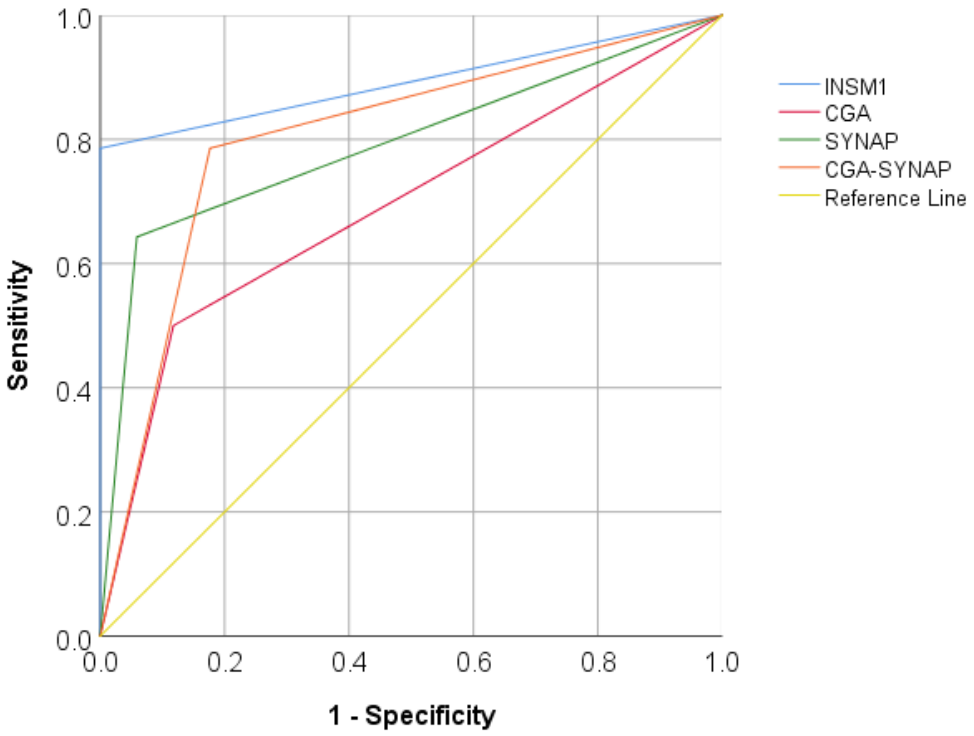


Figure 15: ROC curve considering only PD NEC cases

Synaptophysin alone and the combined use of synaptophysin and chromogranin A demonstrated a higher sensitivity than INSM1 when considering only WD NETs (Figures 16 and 17). INSM1 had a higher sensitivity than chromogranin A when considering only WD NET cases.

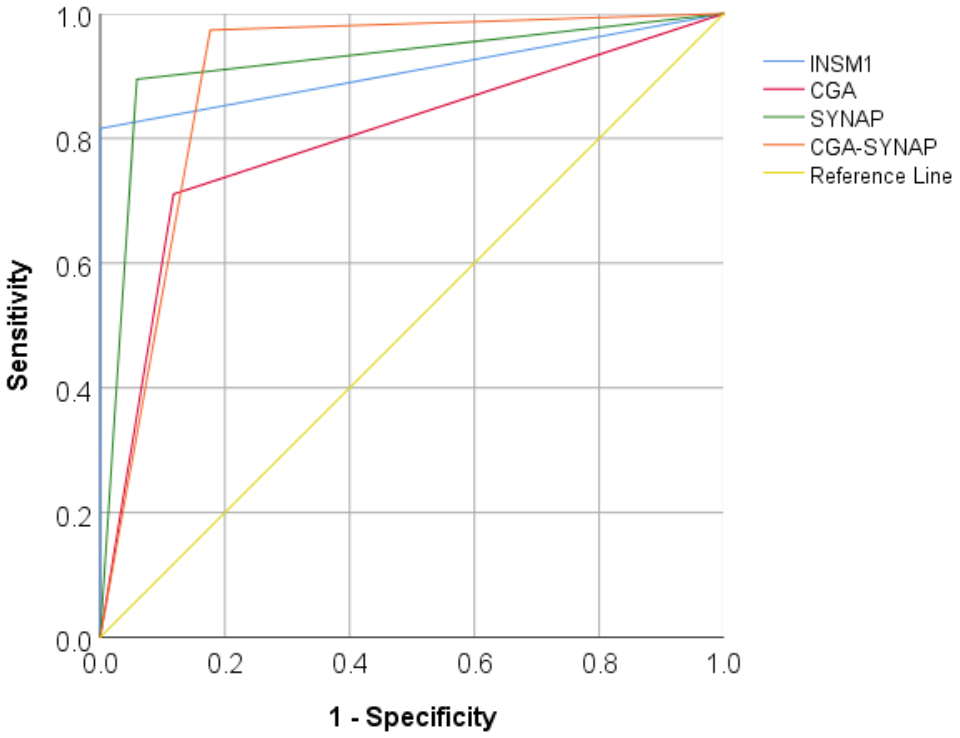


Figure 16: ROC curve evaluating only the grade 1 WD NET cases

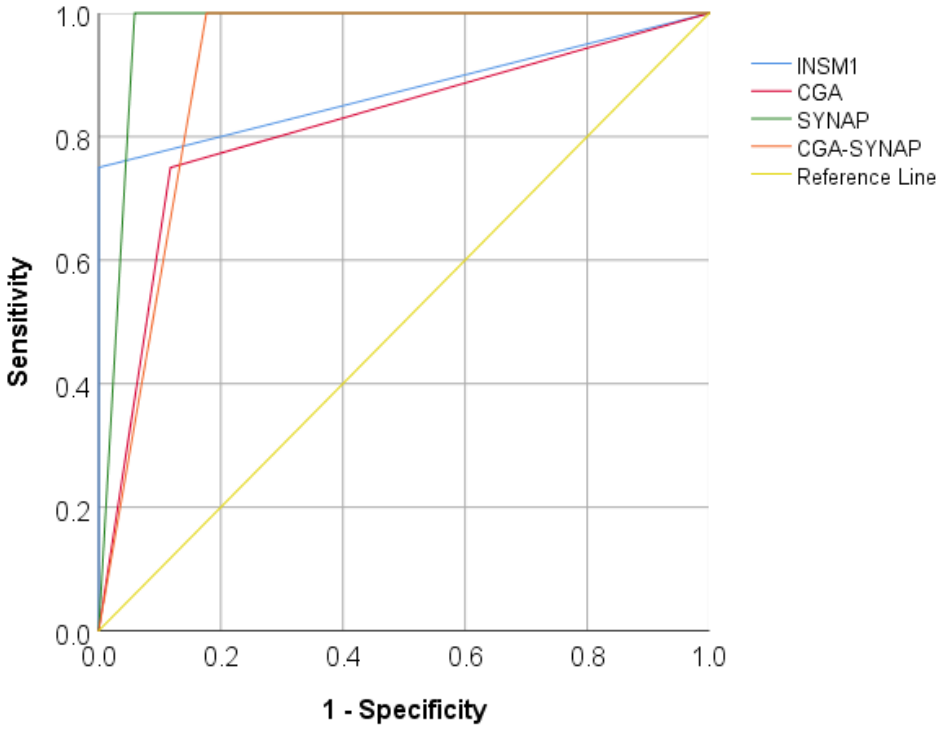


Figure 17: ROC curve evaluating only the grade 2 WD NET cases

4. DISCUSSION

4.1 Age

The mean patient age in this study was 56.7 years (range 14-85). Cases of PD NECs were on average older (mean 70 years) than patients with WD NETs (mean 53.4 years) and this result was found to be statistically significant. Comparing the mean age of NEN cases by anatomic site showed appendix 28.7 years, small intestine 54.6 years, rectum 59.8 years, colon 61.2 years and stomach 64.3 years. This is very similar to what is quoted in the international literature.(2, 9, 14, 15)

4.2 Gender

In our study we had an almost even proportion of males (n=34; 49%) and females (n=35; 51%). This is in keeping with the international literature which reports no gender predilection.(2) When examining cases stratified by anatomic site, a higher proportion of small intestine cases were female (n=10; 62%) compared with male cases (n=6; 38%). This is different to what is reported in the literature. A large study examining 14850 appendicectomy cases in the United Kingdom found appendiceal NETs to have a female predominance of 60.5%.(15) We found the opposite trend with only 43% female cases of appendiceal NENs. This discordance may be due to selection bias and our small sample size, as we only had 7 appendixes in our study.

4.3 Type of sample

Most of our cases (59%) were excision specimens. Of the 41 excision specimens 38 (93%) were WD NETs. This result is expected as PD NECs are more likely to have a higher clinical stage at presentation and may not be amenable to surgical intervention. PD NECs also generally occur in older individuals who may have medical comorbidities contraindicating surgery. Of the 28 biopsy specimens 11 were PD NECs (40%).

4.4 Site of tumour

Our aim was to retrieve 25 cases from each of the 5 anatomic sites identified. We were able to retrieve and include 17 cases (25%) from the rectum, 16 (23%) from the small intestine, 15 (22%) from the stomach, 14 (20%) from the colon, and 7 (10%) from the appendix. The proportion of cases from the appendix was lower than intended. This occurred because most appendiceal NENs are microscopic and so there was insufficient tissue remaining for further immunohistochemical stains.

4.5 Grading

The majority of WD NETs in our study were grade 1 (69%), followed in proportion by grade 2 (29%), with only one grade 3 tumour (1.5%) identified. The proportion of grade 3 WD NETs in our series is lower than that found in other studies. Gonzalez *et al* reported 22% of the NEN cases in their study as grade 3 NETs.(3) Their study did however include pancreatic NENs but they do not specify how many of these grade 3 NETs occurred in the pancreas. Fourteen out of our 69 cases (20%) were classified as PD NEC. Only one case of PD NEC was classified as large cell NEC. This finding confirms the rarity of large cell NEC which is described in the literature.(2)

There were no PD NECs identified in the small intestine or appendix. This supports the international literature in which only isolated case reports have been published of appendiceal PD NECs.(46) Jejunoileal PD NECs are similarly extremely rare.(47) In the small intestine, PD NECs occur almost exclusively at the duodenal ampulla. We did not have any cases of duodenal NENs which explains why no small intestinal PD NECs were identified.

The PD NECs in our study were evenly split between the stomach (50%) and colorectum (50%). Stomach PD NECs are reported to comprise 20.5% of all GI PD NECs.(13) There was a higher proportion in our study. This may be due to selection bias and the small

sample size. The colorectum is the most common site for GI PD NEC reported in the literature.(11) This was supported by our data.

4.6 Immunohistochemistry

4.6.1 INSM1

We found a sensitivity of 79.7% for INSM1 immunohistochemistry with 55 out of 69 cases showing positive staining. This result is lower than reported in the literature. González *et al* found a sensitivity of 100% in their study examining 30 GI NEN cases.(3) This study had a small sample size, only one case of PD NEC and it is uncertain what cut off they used for intensity and proportion in assigning positivity. Our sensitivity result is also lower than those reported for NENs at other anatomic sites in the literature.(32, 33) Thoracic NENs, head and neck NENs and Merkel cell carcinomas are high-grade with small cell morphology. The high proportion of low-grade NETs in our study may account for this discrepancy. In addition, we noted that the immunoreactivity on some of our older cases (pre 2010) was not strong and perhaps poor preservation of the wax blocks led to decreased antigenicity in these cases.

The specificity of INSM1 was 100% with none of our 17 control cases showing positive immunohistochemical labelling. The study reported by González *et al* found positive staining in colorectal adenocarcinoma, adrenal cortical carcinoma, solid pseudopapillary neoplasm of the pancreas and breast carcinoma.(3) They used lower cut off values to define positivity, accepting a proportion of only 5% expression as positive. Our smaller number of controls and higher cut off value for assigning positivity may account for our higher specificity.

Stratification of INSM1 staining by tumour grade revealed the highest sensitivity in the PD NEC group (85.7%), followed by grade 1 WD NET (81.6%) and grade 2 WD NET (75%). The single case of grade 3 WD NET was negative for INSM1. These differences were not

statistically significant (p = 0.919). Our results were all lower than those reported by González *et al* as they found sensitivities of 100% in all groups.(3)

INSM1 showed high sensitivity in the appendix (100%), colorectum (87.1%) and stomach (80%). The sensitivity was only 56.3% in the small intestine. The reason for this is uncertain, particularly since synaptophysin and chromogranin A were very sensitive in the small intestine. There was a high proportion of low-grade WD NETs in the small intestine and this may have contributed to these results.

4.6.2 Synaptophysin

The sensitivity of synaptophysin was 87% (60 out of 69 cases stained positively) and the specificity was 94% (1 out of 17 controls positive). The positive control case was an adenocarcinoma of the colon. Neuroendocrine differentiation in GI adenocarcinomas is well described in the literature. The sensitivity of synaptophysin in our study is lower than that reported in the literature. The study by Gonzalez *et al* found synaptophysin to have a sensitivity of 100% in their series of 32 gastroenteropancreatic NENs.(3)

We found that synaptophysin was less likely to stain PD NEC (sensitivity 64.3%) than WD NET (sensitivity 93%). This difference in sensitivity was not found in the study by Gonzalez *et al*.(3)

Synaptophysin stained 80% of the gastric NENs which is higher than the rate reported in the literature of 50%.(18, 22)

4.6.3 Chromogranin A

The sensitivity of chromogranin A in our study was 68% (47 out of 69 cases showing positive staining). The specificity was 88% with 2 of the 17 controls staining positively. One of these positive controls was a diffuse large B-cell lymphoma of the small intestine. The staining in this case was weak and patchy and best interpreted as aberrant (even

though it did meet the threshold criteria for positivity with an H-score of 30). Chromogranin A labelling of lymphomas has not been reported in the literature.

The sensitivity of chromogranin A in our study is lower than that reported by González *et al* who found an overall sensitivity of 97%. There was no statistically significant difference when positive staining was examined by tumour grade.

Chromogranin A had a low sensitivity in the colorectum, staining only 41.9% of cases (p = .001). This phenomenon is well described in the literature and has to do the cell of origin of the NEN.(41)

4.7 Comparison of INSM1, Synaptophysin and Chromogranin A

Considering all GI NEN cases in this study, INSM1 had a sensitivity (79.7%) that was higher than chromogranin A (68.1%), but lower than synaptophysin (87%) and the combined use of synaptophysin and chromogranin A (94.2%). The combined use of synaptophysin and chromogranin A detected more cases than the use of only INSM1, but this difference was not statistically significant. The study by Gonzales *et al* quoted a better sensitivity for INSM1 but they did not report on statistical significance, possibly due to a small sample size. When considering data on NENs at other anatomical sites, our results are different to the findings of Rooper *et al* who described a sensitivity of INSM1 of 96.4% across all grades of thoracic NENs which was significantly more than the sensitivity of 87.4% achieved by a panel of synaptophysin, chromogranin A and CD56.(31)

Analyzing only the PD NEC cases showed that INSM1 (0.893) had a higher area under the curve (AUC) than chromogranin A (0.691), synaptophysin (0.796), and the combined use of synaptophysin and chromogranin A (0.810). This result was statistically significant.

Comparing the sensitivities and specificities of the three immunohistochemical markers in the WD NET group revealed no statistically significant difference between the use of INSM1 alone and the combined use of synaptophysin and chromogranin A.

It is interesting to note that of the 4 cases which were negative for both synaptophysin and chromogranin A, the INSM1 stain was positive.

4.8 Study design

Our study included both resection and biopsy specimens. We only included cases with sufficient tumour to accurately assess mitotic count and Ki-67 proliferative index according to the international guidelines. This was not possible on many of the biopsy specimens and in many appendicectomy specimens. Excluding these cases may have introduced selection bias.

We were able to identify a sufficient number of cases by searching the laboratory electronic information systems (DISA and Trakcare). Obtaining the wax blocks from our archives was a challenge for various logistical reasons and many cases were irretrievable. If time permitted, we would have reselected more cases and attempted to retrieve their wax blocks. However, we had already requested all the resection specimens, and had we requested additional biopsy specimens it is likely that many of them would also have had insufficient remaining tissue for analysis.

There was only one grade 3 WD NET in this study. It is uncertain why the incidence of grade 3 WD NET was so low in our population.

Several pre-analytical factors could have influenced the outcomes of this study. These include tissue fixation and preservation of tissue in the wax blocks. Underfixation has a

negative impact on antigen retrieval and can result in false negative immunohistochemical stains.(48)

The dilutions of the primary antibodies used in this study were optimized prior to beginning the study. The staining protocol was standardized and carried out in batches. Positive and negative external controls were used for each batch. Positive internal controls were also identified where applicable.

All slides were coded to reduce bias. Intra-observer variability was reduced by scoring the immunohistochemical stains by the same pathologist, using the same microscope, in as few sittings as possible. Scoring of the INSM1 immunohistochemical stains and the synaptophysin and chromogranin A stains was checked by the supervising pathologist.

Future studies should include a larger sample size to achieve statistical significance. Multicentre collaboration would be of value as these tumours are uncommon and it is difficult to find sufficient suitable cases at a single centre.

The low sensitivity of INSM1 in small bowel WD NETs found in our study is of interest and should be investigated further and at different centres.

5. CONCLUSIONS

1. GI NENs are tumours of middle-aged adults and PD NECs occur in older individuals, findings consistent with international data.
2. Within the WD NET group, grade 1 tumours comprise the majority (69%) of cases, grade 2 tumours make up 29% and grade 3 tumours only 2%.
3. There is a very low prevalence of grade 3 WD NETs in our study sample.
4. PD NECs occurred only in the stomach and colorectum in this study.
5. Chromogranin A stained significantly fewer colorectal NENs, a result supported by the literature.
6. Synaptophysin stained the majority of gastric NENs, more than reported in the literature.
7. Although INSM1 stained fewer overall tumours than did the combined use of synaptophysin and chromogranin A, this difference is not statistically significant.
8. INSM1 stained fewer small bowel NENs than synaptophysin in our study, although this finding was not statistically significant.
9. INSM1 has equivalent sensitivity to the combined use of synaptophysin and chromogranin A in PD NECs and a higher specificity, making it a more robust marker in this setting.

6. APPENDICES

Appendix 1: Summary of cases

Case	Age	Gender	Excision/ Biopsy	Site	Reclassification
NEN_01	58	F	E	Stomach	WD NET G1
NEN_02	51	F	E	Colon	NEC, small cell
NEN_03	70	M	E	Small intestine	WD NET G1
NEN_04	18	F	E	Appendix	WD NET G1
NEN_05	33	F	E	Appendix	WD NET G1
NEN_06	72	M	B	Stomach	WD NET G2
NEN_07	62	M	B	Rectum	NEC, small cell
NEN_08	49	M	B	Rectum	WD NET G1
NEN_09	37	M	E	Stomach	WD NET G1
NEN_10	18	F	E	Appendix	WD NET G1
NEN_11	52	M	E	Appendix	WD NET G1
NEN_12	46	F	E	Small intestine	WD NET G1
NEN_13	60	M	E	Small intestine	WD NET G1
NEN_14	55	M	B	Stomach	WD NET G1
NEN_15	51	F	E	Small intestine	WD NET G1
NEN_16	32	F	E	Small intestine	WD NET G1
NEN_17	76	F	B	Rectum	WD NET G2
NEN_18	65	M	E	Small intestine	WD NET G2
NEN_19	14	M	E	Appendix	WD NET G2
NEN_20	82	M	B	Stomach	NEC, small cell
NEN_21	51	M	E	Rectum	WD NET G1
NEN_22	34	F	E	Small intestine	WD NET G2
NEN_23	54	M	E	Small intestine	WD NET G1

NEN_24	62	M	E	Small intestine	WD NET G2
NEN_25	21	M	E	Appendix	WD NET G1
NEN_26	46	F	B	Rectum	WD NET G1
NEN_27	69	F	B	Stomach	NEC, small cell
NEN_28	51	F	E	Small intestine	WD NET G2
NEN_29	68	M	B	Rectum	NEC, small cell
NEN_30	65	F	E	Stomach	NEC, small cell
NEN_31	77	M	B	Stomach	NEC, small cell
NEN_32	42	M	B	Colon	WD NET G3
NEN_33	55	F	B	Colon	WD NET G2
NEN_34	43	M	B	Stomach	WD NET G2
NEN_35	58	F	E	Small intestine	WD NET G1
NEN_36	60	M	B	Rectum	WD NET G2
NEN_37	70	F	B	Rectum	WD NET G2
NEN_38	57	F	B	Rectum	WD NET G2
NEN_39	49	M	B	Rectum	WD NET G1
NEN_40	53	F	E	Small intestine	WD NET G2
NEN_41	57	M	E	Colon	WD NET G1
NEN_42	66	F	B	Stomach	WD NET G1
NEN_43	68	F	B	Stomach	NEC, small cell
NEN_44	61	M	E	Colon	WD NET G1
NEN_45	77	M	B	Colon	NEC, large cell
NEN_46	64	M	E	Rectum	WD NET G1
NEN_47	69	F	E	Rectum	WD NET G1
NEN_48	70	F	B	Stomach	NEC, small cell
NEN_49	45	M	E	Appendix	WD NET G1
NEN_50	46	F	E	Stomach	WD NET G2

NEN_51	55	F	E	Small intestine	WD NET G1
NEN_52	76	F	B	Rectum	WD NET G1
NEN_53	72	M	E	Stomach	WD NET G1
NEN_54	60	F	E	Colon	WD NET G1
NEN_55	57	F	E	Colon	NEC, small cell
NEN_56	70	M	B	Rectum	WD NET G1
NEN_57	63	F	E	Small intestine	WD NET G2
NEN_58	60	M	B	Colon	WD NET G1
NEN_59	70	M	E	Small intestine	WD NET G1
NEN_60	52	F	B	Colon	WD NET G1
NEN_61	48	F	E	Rectum	WD NET G1
NEN_62	70	M	E	Colon	WD NET G1
NEN_63	66	F	E	Colon	WD NET G1
NEN_64	50	F	E	Small intestine	WD NET G1
NEN_65	65	M	B	Rectum	NEC, small cell
NEN_66	65	M	E	Colon	WD NET G1
NEN_67	85	F	B	Colon	NEC, small cell
NEN_68	84	M	B	Stomach	NEC, small cell
NEN_69	37	F	E	Rectum	WD NET G2

Appendix 2: Ethics approval letter

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

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14 May 2018

HREC REF: 176/2018

Dr M Locketz
Division of Pathology
NHLS Anatomical Pathology
D7, NGSH

Dear Dr Locketz

PROJECT TITLE: NEUROENDOCRINE TUMOURS OF THE GASTROINTESTINAL TRACT: A RETROSPECTIVE REVIEW AND RECLASSIFICATION OF CASES ACCORDING TO THE WHO GRADING SYSTEM, AND APPLICATION OF THE NOVEL MARKER OF NEUROENDOCRINE DIFFERENTIATION INSM1 (MMed-candidate-Dr A Aldera)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30 May 2019.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

We acknowledge that the student: Dr A Aldera will also be involved in this study.

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval before the research may occur.
Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

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