COMPARISON OF HAEMATOXYLIN AND EOSIN AND CALRETININ DETECTION OF GANGLION CELLS IN COLORECTAL BIOPSIES SUSPECTED OF HIRSCHSPRUNG'S DISEASE

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Department of Pathology



COMPARISON OF HAEMATOXYLIN AND EOSIN AND CALRETININ DETECTION OF GANGLION CELLS IN COLORECTAL BIOPSIES SUSPECTED OF HIRSCHSPRUNG'S DISEASE.

By

Investigator: Mbazi Shemwetta

"A Dissertation research report submitted in partial fulfilment of the requirement for one degree of MMed (Anatomical Pathology) of the Muhimbili University of Health and Allied Sciences".

October, 2020

CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by Muhimbili University of Health and Allied Sciences a dissertation entitled "Comparison of Haematoxylin and Eosin and Calretinin Detection of Ganglion Cells in Colorectal Biopsies Suspected of Hirschsprung's Disease*"* in partial fulfilment of the requirements for the degree of Master of Medicine in Anatomical Pathology of Muhimbili University of Health and Allied Sciences.

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DECLARATION AND COPYRIGHT

I, **Mbazi Dunstan Shemwetta**, declare that this dissertation is my own original work and that it has not been presented and will not be presented to any other University for a similar or any other degree award.

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DEDICATION

I dedicate this hard work to my wife.

I dedicate my work to my son Benedict and daughter Berenice.

I dedicate my work to my parents.

I dedicate my work to my siblings.

ABSTRACT

Background: Hirschsprung's disease is a functional obstruction of the gastrointestinal tract due to the congenital absence of ganglion cells. The diagnosis and extent of resection for the management of Hirschsprung's disease depend on specific identification of ganglion cells. Recently, calretinin immunohistochemistry has been introduced as a new, simple and reliable tool for the diagnosis of Hirschsprung's disease compared to haematoxylin & eosin. This study compared detection of ganglion cells by the haematoxylin & eosin and calretinin immunohistochemistry, at Muhimbili National Hospital.

Methodology: The study was a retrospective hospital based, of all suspected Hirschsprung's disease cases diagnosed either by clinical, histopathological or both methods in a 1 year period from 1st January 2018 to 31st December 2018. All 99 cases were stained by haematoxylin & eosin, and all cases which were negative for ganglion cells by haematoxylin & eosin (36 cases) and those positive with adequate submucosa or muscular layers (23 cases) were stained by calretinin immunohistochemistry. Data was recorded and analysed by Statistical Product and Service Solutions version 23. Statistical methods used were descriptive and inferential (p-value and Cohen's Kappa value). Concordance of haematoxylin & eosin and calretinin immunohistochemistry in ganglion cells' detection was calculated.

Results: A total of 99 cases were studied, 69 males and 30 females. The median age was 3 years with range of 1 month to 19 years. Using the routine haematoxylin & eosin stain, ganglion cells were seen in 63 cases and no ganglion cells were seen in 36 cases. Calretinin immunohistochemical stain found ganglion cells in all 23 cases that showed ganglion cells by haematoxylin & eosin and 6 cases that did not show ganglion cells by haematoxylin & eosin, making 30 confirmed cases by calretinin immunohistochemistry. There was a substantial agreement between haematoxylin & eosin and calretinin immunohistochemistry with concordance of 90% (Cohen's kappa=0.790). There was a Positive predictive value of 100%, negative predictive value of 83%, sensitivity of 79% and specificity of 100% in detection of ganglion cells. Most cases (50.5%) presented with constipation. The sites of biopsies submitted were mostly from the rectum (64.6%) and most (69.7%) of the submitted biopsies were full thickness. Location of the ganglion cells as seen by the haematoxylin & eosin for majority of the biopsies 31/63(49.2%) were on both submucosa and muscularis propria nerve plexuses. The proportion of Hirschsprung's disease for the year 2018 among the submitted biopsies was 36/99 (36.4%). Male to female ratio of positive cases was 2:1 (p=0.62) and 39% were between 1 to 3 years of age, which was the age group with the highest frequency (p=0.37).

Conclusion: Comparison of detection of ganglion cells by the routine haematoxylin & eosin and calretinin immunohistochemistry has shown a very high concordance rate with a slightly higher detection rate by calretinin for the cases that were missed by the routine haematoxylin & eosin. The overall capability of ganglion cells' detection by calretinin immunohistochemistry has been accompanied by a significant shorter time and simplicity in sighting out the ganglion cells.

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ABBREVIATIONS

AChE - Acetyl cholinesterase

CAL - Calretinin

CPL - Central Pathology Laboratory

DAB - 3, 3'-Diaminobenzidine

DPX - Distyrene, Plasticer and Xylene

FFPE - Formalin Fixed Paraffin Embedded

H&E - Hematoxylin and eosin

HD - Hirschsprung's Disease

HP - Histopathology

IHC - Immunohistochemistry

MNH - Muhimbili National Hospital

MUHAS Muhimbili University of Health and Allied Sciences

PAP - Peroxidase Anti Peroxidase

RSB - Resection Suction Biopsy

SOP - Standard Operating Procedure

SPSS - Statistical Package for the Social Sciences

OPERATIONAL DEFINITIONS

Hirschsprung's disease is a developmental disorder characterized by the absence of ganglia in the distal colon, resulting in a functional obstruction.

Immunohistochemistry (IHC) is a method for detecting antigens or haptens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. The antibody-antigen binding can be visualized in different manners.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Hirschsprung's disease (HD) is a functional obstruction of the gastrointestinal tract, interfering peristalsis due to the congenital absence of ganglion cells in the myenteric (Auerbach's) and sub mucosal (Meissner's) plexuses of the distal bowel. Gastrointestinal motility requires neural network connections to function properly as well as intact muscular layers (1).

In healthy term infants, the passage of meconium occurs in 95% within 24 hours and in 99% within 48 hours of birth. Delayed meconium passage may result from the absence of the enteric nervous system, as typically described in HD (2). Clearly, early differentiation between HD and functional constipation is necessary to prevent severe complications of HD (3).

The incidence of HD is estimated to be approximately one in 5000 live births, with a male-to-female ratio of 4 to 1.15 (2). In a study done in Zaria, Nigeria HD accounted for 11(7.3%) of the neonatal intestinal obstruction cases, representing 20% of all patients presenting with HD to Ahmadu Bello University Teaching Hospital (4).

In Kenya, there were 117 patients with a clinical diagnosis of HD from 2002 to 2006 at Kenyatta National Hospital, 15% females and 85% males (gender ratio 5.5: 1). At presentation, 27.7% of the patients were younger than two weeks of age while the rest (82.3%) were older. The mean age of the group was 709 ± 400 days (range, one day to 17 years). The age at diagnosis was <30 days in 44 children (39%), one month to one year in 26 (24%), and > one year in 42 (37%) (5).

At Bugando Hospital, Tanzania, out of 286 patients who had colonic and rectal biopsies performed for suspected HD from July 2008 to June 2013, 116 patients were histologically confirmed as cases of HD. The age of patients at presentation ranged from 7 days to 10 years with a median age of 24 months (+IQR of 22 to 26 months). Only six (5.5%) patients were in the neonatal period, with a male to female ratio of 3.6: 1 (6).

There are four types of HD; In 80% of individuals, aganglionosis is restricted to the rectosigmoid colon (short-segment disease), In approximately 15%-20%**,** the aganglionosis extends proximal

to the sigmoid colon (long-segment disease**),** In approximately 5% of individuals, aganglionosis affects the entire large intestine (total colonic aganglionosis) and Rarely, the aganglionosis extends into the small bowel or even more proximally to encompass the entire bowel (total intestinal aganglionosis) (7,8).

There is a reduction in nerve fibres storing vasoactive intestinal peptide (VIP), substance P (SP), and a total depletion of nerves storing enkephalin and gastrin-releasing peptide in aganglionic intestine. In the mucosa, on the other hand, the innervation was virtually unchanged. Thus, it appears that the mucosa secretory and sensory innervation is not reduced and that the defects in peptidergic innervation in HD mainly involve motor neurons (9,10). When the presence of c-kit positive cells was investigated from patients with HD, a few c-kit positive cells were found in the muscle layers of aganglionic bowels from HD, in contrast to many c-kit positive cells in ganglionic bowel segments from controls. A lack of c-kit might be of significance for autonomic gut dysmotility in aganglionic bowel segments of patients with HD and allied disorders such as chronic idiopathic intestinal pseudo-obstruction (11).

Contrast enema, anorectal manometry, and rectal suction biopsy are the most common tests used in the diagnostic work-up of HD. Classically; the diagnosis of HD is based on histochemical evaluation of the rectum. This examination typically shows absence of ganglion cells in the submucous and myenteric plexus and an increase in acetyl cholinesterase activity in the parasympathetic nerve fibres of the lamina propria mucosa, muscularis mucosa, and circular muscles. Two methods are used to obtain tissue for histochemical evaluation, rectal suction (partial thickness) and full thickness biopsies.

In rectal suction biopsy, because the specimens are small compared with full-thickness specimens, the examination remains difficult even for experienced pathologists. The introduction of staining for acetyl cholinesterase activity on a suction biopsy has made the morphologic diagnosis of HD easier and more reliable. Absence of ganglion cells in a full-thickness biopsy confirms the diagnosis of HD (2). Without early diagnosis and appropriate treatment, many children will go on to develop serious complications such as acute enterocolitis or toxic megacolon. Delayed presentation may also influence overall management protocol and preclude one-stage operative treatment. The net result may be increased morbidity and mortality. Most reports from developing countries indicate that majority of children with HD present late to a

tertiary health facility for management. This late presentation, among other factors, may be responsible for the less than optimal outcome for this disease in some developing countries (12).

Initial management varies depending on the need for decompression (washouts vs. defunctioning stoma) before proceeding to a pull-through operation to remove the aganglionic section of the bowel (13). In addition to H&E stained tissue sections, Acetyl cholinesterase histochemistry (AChE) and calretinin immunohistochemistry may be useful as confirmatory stains to assess nerves in cases clinically suspicious for HD (14). The diagnosis and extent of resection for the management of HD depend on the sensitive and specific identification of ganglion cells. However, documenting aganglionosis is often difficult and tedious on routine H&E stained sections; thus, either H&E evaluation of serial sections from full-thickness biopsies or AChE staining from fresh superficial biopsies is preferred for histopathologic diagnosis (5). Since its introduction, AChE has been used intraoperatively to guide the surgeons on the extent of resection. Later on, H & E and calretinin staining can be done after processing. AChE has evolved as the gold standard in diagnosing HD; but to date, it has not gained worldwide utilization and applicability.

Recently, calretinin, a calcium-binding protein that plays an important role in the organization and functioning of the central nervous system, has been introduced as a new simple and reliable tool for the diagnosis of HD (6). Moreover, studies in which the results were compared with those of AChE staining protocols showed that calretinin was potentially superior to AChE as a confirmatory diagnostic method for evaluating suction rectal biopsies for HD (7-9). Absence of calretinin expression was first reported in patients with HD in 2006 resection specimens. Two follow-up studies confirmed these findings in Resection Suction Biopsies (RSBs). Holland et al in 2011 described a sensitivity of 100% and a specificity of 83% when classifying HD status based on calretinin stain results (15).

Owing to the limited number of relevant studies on this and the need for a reliable and simple diagnostic method in our routine practice with HD, material for calretinin immunohistochemistry were reviewed, and results compared with standard method used for diagnosing HD (16).

1.2 PROBLEM STATEMENT

Worldwide, the estimated incidence of HD is approximately 1 in 5000 live births (30,31). HD is the commonest cause of functional intestinal obstruction in children and poses diagnostic and therapeutic challenges in resource-limited countries including Tanzania.

Precise and quick diagnosis of HD is the key to accurate treatment. Patients undergo multiple surgeries in the management of HD especially in our settings which may even be more if pathologists do not detect ganglion cells in the submitted specimens.

Detection of ganglion cells poses a challenge due to various reasons including in neonates who develop a small number of irregularly placed ganglion cells of very small size which are often immature (dysplastic), which leads to problems in differentiation from endothelial cells (newly shaped vessels) and fibroblasts. These difficulties contributed to the development of methods that could potentially simplify the HD diagnostic protocol

The diagnostic effectiveness of the various methods applied for confirmation of clinical suspicion of HD can fluctuate, sometimes leading to failure of proper diagnosis. Recently, anal biopsy has been considered as the important diagnostic tool, by using conventional H & E stain (2,32).

However, these conventional methods have their inherent deficiencies as H&E requires multiple full thickness biopsies and the interpretation of ganglion cells is often very difficult. Similarly, AChE requires fresh frozen section and this is not practised in Tanzania. The number of misdiagnosed results with potential overtreatment stands in need for reliable staining to prevent harm from unnecessary surgery and mortality.

1.3 RATIONALE

The study was to provide information on the accuracy of detection of ganglion cells and hence diagnosis of HD by comparing the detection by H & E and by calretinin IHC marker as Detection of ganglion cells poses a challenge due to various reasons including in neonates who develop a small number of irregularly placed ganglion cells of very small size which are often immature (dysplastic), which leads to problems in differentiation from endothelial cells and fibroblasts.

Immunohistochemical markers including calretinin are becoming more readily available in pathology laboratories, so if the superiority over H&E was confirmed, would provide an alternative, more accurate method for detection of HD.

The project will assist clinicians in proper early treatment to avoid severe complications later in life; reducing a number or surgeries that patients have to go through before H&E detects ganglion cells which could have been missed even if present in the first surgery. It would also bring easiness in detecting ganglion cells as compared to H&E.

1.4 RESEARCH QUESTIONS

1 What is the proportion of HD among the suspected colorectal biopsies submitted?
2 Is calretinin immunohistochemical marker better than H & E in detection of ganglion cells?

1.5 OBJECTIVES

1.5.1 Broad Objective

To compare H&E and calretinin immunohistochemistry in detecting ganglion cells in colorectal biopsies suspected of Hirschsprung's disease at MNH.

1.5.2 Specific Objectives

1. To determine the proportion of HD among the suspected colorectal biopsies submitted at MNH by the routine H&E.
2. To compare the method sensitivity and easiness in detection of ganglion cells by H & E and calretinin at MNH.

1.6 LITERATURE REVIEW

1.6.1 Epidemiology

The worldwide incidence of HD approximates 1 in every 5,000 live births. This has been consistently reported in many countries, including South Africa, where a reported large series found a prevalence of 1 per 5,726 live births which included all ethnic groups. An approximate 4:1 male-to-female ratio was substantiated, but appeared to vary between the various South African ethnic groups, being 3.6:1 in Caucasians and 3.9:1 in black African patients, but only 2.5:1 in those of mixed descent (17). HD is accepted as being a sex-linked heterogeneous disorder with differing phenotypic expressions and degrees of severity. It has a clear inheritance risk and recurs in certain families in autosomal dominant, recessive, and multigenic transmission patterns (17).

In Nigeria, a total of 120 colorectal operations were performed in 90 patients with age ranging from 1 to 13 years. The major diagnoses were anorectal malformations (64.4%) and HD (31.1%)(22)A total of 110 patients (M: F ratio= 3.6:1) with a median age of 24 months were studied. Six (5.5%) patients were in the neonatal period. Sixty-four (58.2%) patients had complete intestinal obstruction whereas 42 (38.2%) and 4(3.6%) patients had chronic intestinal obstruction and intestinal perforation respectively. No patient had enterocolitis(6).

In Kenya, 117 patients had clinical diagnosis of HD, 15% females, 85% males). 27.7% of the patients were younger than two weeks of age while the rest (82.3%) were older. The mean age of the group was 709 ± 400 days (range, one day to 17 years). The age at diagnosis was <30 days in 44 children (39%), one month to one year in 26 (24%), and > one year in 42 (37%) (5).

In Tanzania, Bugando Hospital, from July 2008 to June 2013, out of 286 patients who had colonic and rectal biopsies performed for HD, 116 patients had histologically confirmed cases of HD. The age of patients at presentation ranged from 7 days to 10 years with a median age of 24 months (Interquartile range of 22 to 26 months). Only six (5.5%) patients were in the neonatal period, with a male to female ratio of 3.6: 1 (6).

1.6.2 Calretinin immunohistochemistry

In addition to H&E stained tissue sections, acetyl cholinesterase histochemistry (AChE) and calretinin immunohistochemistry on RSBs may be useful as adjuncts to assess nerves in cases clinically suspicious for HD.(17)

A study done in USA, the diagnosis of HD was usually established by histopathological examination of suction biopsies from the distal rectum. Features in H&E stained sections include sub mucosal aganglionosis and sub mucosal nerve hyperplasia and hypertrophy, but a confident diagnosis based on these findings often requires tedious examination of 50 or more sections and depends greatly on the amount of sampled submucosa.

The challenge is exacerbated by the normal sparse nature of sub mucosal ganglion cells in the distal 1–2 cm of the distal rectum and inability to accurately establish the position of the biopsy device relative to the dentate line.

As a consequence, ancillary enzymatic or immunohistochemical studies have been advocated to simplify identification of ganglion cells or to function as sensitive and specific surrogate markers of aganglionic bowel.

In Turkey, immunohistochemistry for calretinin provided comparable results with H&E findings in HD and non-HD patients, except in one HD patient with very rare nerve staining at the distal surgical margin (16). The rate of non-conclusive results was significantly higher in infants younger than 1.5 months. Calretinin staining tends to reduce the inconclusive results of RSB. Further studies are necessary to determine if this technique is useful to improve RSB results in infants younger than 1.5 months (23).

In Iran, there was great concordance between the final diagnosis of both pathologists and gold standard. Calretinin immunostaining showed 100% specificity and positive predictive value and more than 90% sensitivity and negative predictive value. High agreement was present between two pathologists ($k > 0.9$).It was concluded that calretinin IHC is a very convenient, useful and valuable method to demonstrate aganglionosis in HD patients. Loss of calretinin immunostaining in lamina propria and submucosa is characteristic of HD (24).

In USA, the presence of ganglion cells consistently correlated with calretinin-positive thin nerve fibrils in the lamina propria, muscularis mucosa and superficial submucosa. These nerve fibrils were absent in the aganglionic segments of bowel and in the areas without ganglion cells from the junction of normal with diseased rectum. Calretinin was strongly expressed in the sub mucosal and sub-serosal nerve trunks in the ganglionic segment. It had faint expression in the thick nerve trunks from the areas without ganglion cells 1.6-2.5 cm proximal to the normal rectum. No calretinin expression was seen in the nerve trunks in the rest of the aganglionic segment (15).

In South Korea, of 95 patients, only 21 showed some ganglion cells. All 61 non-HD cases revealed no abnormal AChE-positive fibres. Of 34 HD patients, 32 exhibited abnormal AChE fibres, but 2 showed no stained fibres. None of the tissues from the HD patients exhibited calretinin immunoreactivity. Test sensitivity and specificity of AChE histochemistry alone were 93.5% and 100.0%, respectively, while calretinin immunohistochemistry were 100.0% and 85.2%, respectively. It was concluded that AChE histochemistry is a good diagnostic method for HD, if feasible, and a combination of AChE histochemistry and calretinin immunohistochemistry will help increase the accuracy of the diagnosis of HD (25).

In India, of the 36 biopsy specimens, 19 cases of HD were diagnosed by H&E staining earlier. In 2 patients, ganglion cells were seen so HD was ruled out. In 15 cases, there was a diagnostic dilemma and calretinin was used during which ganglion cells were found in 3 specimens and nerve fibres in 5. In all 24 resected specimens, calretinin correlated with the findings on H and E staining. It was Concluded that calretinin was extremely useful in resolving the suspicious and indeterminate cases of HD, where it can serve as a valuable cost effective diagnostic aid in the centres where AChE enzyme histochemistry was not available (26).

In Brazil, of the 50 cases evaluated by the HE technique, only 5 contradicted the diagnosis based on AChE, with a Kappa value of 0.800 and an accuracy of 90%. In the comparison between calretinin and AChE, 8 cases were discordant, with a Kappa value of 0.676 and an accuracy of 84%.It was concluded that the concordance of results from AChE and H&E methods was satisfactory, allowing for the potential use of the H&E method for fragments of mucosa and submucosa as a valid alternative in the diagnosis of HD.

The immunohistochemical technique of calretinin did not show good agreement with the AChE activity in this study (27).

Contrast enema has the lowest sensitivity and specificity of all the 3-index investigations which include contrast enema, anorectal manometry, and biopsy with histology. The latter procedure seems to have the highest sensitivity and specificity. AChE staining on fresh-frozen material has been found to have slightly higher rates of sensitivity and specificity when compared with H&E only (28). Calretinin is a reliable marker of the presence of ganglion cells, and, together with nerve hypertrophy, it helps to identify the transition zone. Length and nerve size of proximal segment in resected specimen did not affect the postsurgical intestinal function. Reduced b-tubulin expression along the entire colonic tract, included proximal ganglionic segments, may represent a potential impairing factor for the enteric neural transmission (29).

To the best of my knowledge, there is no study that has been done in Africa on the use of calretinin immunohistochemistry.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Study design

This was a retrospective study design conducted at MNH from 1st January to 31st December 2018.

2.2 Study area/ setting

The study was conducted at Muhimbili National Hospital, the largest teaching and referral hospital in Tanzania located in Ilala District in Dar es Salaam region.MNH is the national referral hospital with 1500 beds, admitting 1000-1200 inpatients per day and providing care to more than 1000 outpatients per week. Central Pathology Labaratory receives about 12,000 biopsies per year, including colonic biopsies suspected of HD. The biopsies from different Health facilities in Tanzania are also referred there.

2.3 Eligibility criteria

2.3.1 Inclusion Criteria

All biopsies or resection cases with suspicion of HD as per the request form, during the study period, received from 1st January 2018 to 31st December 2018.

2.3.2 Exclusion Criteria

Cases missing the tissue blocks

2.4 Study period

Cases received during one year, from 1st January 2018- 31st December 2018. This period was conveniently prefered because the blocks would be easily retriavable as they were recent and all available in the archive after the test results were disseminated.

2.5 Sampling Procedure

Convenience sampling techniques was used by which all HD cases diagnosed by clinical, histopathological or both from 1st January 2018 to 31st December 2018 were sampled.

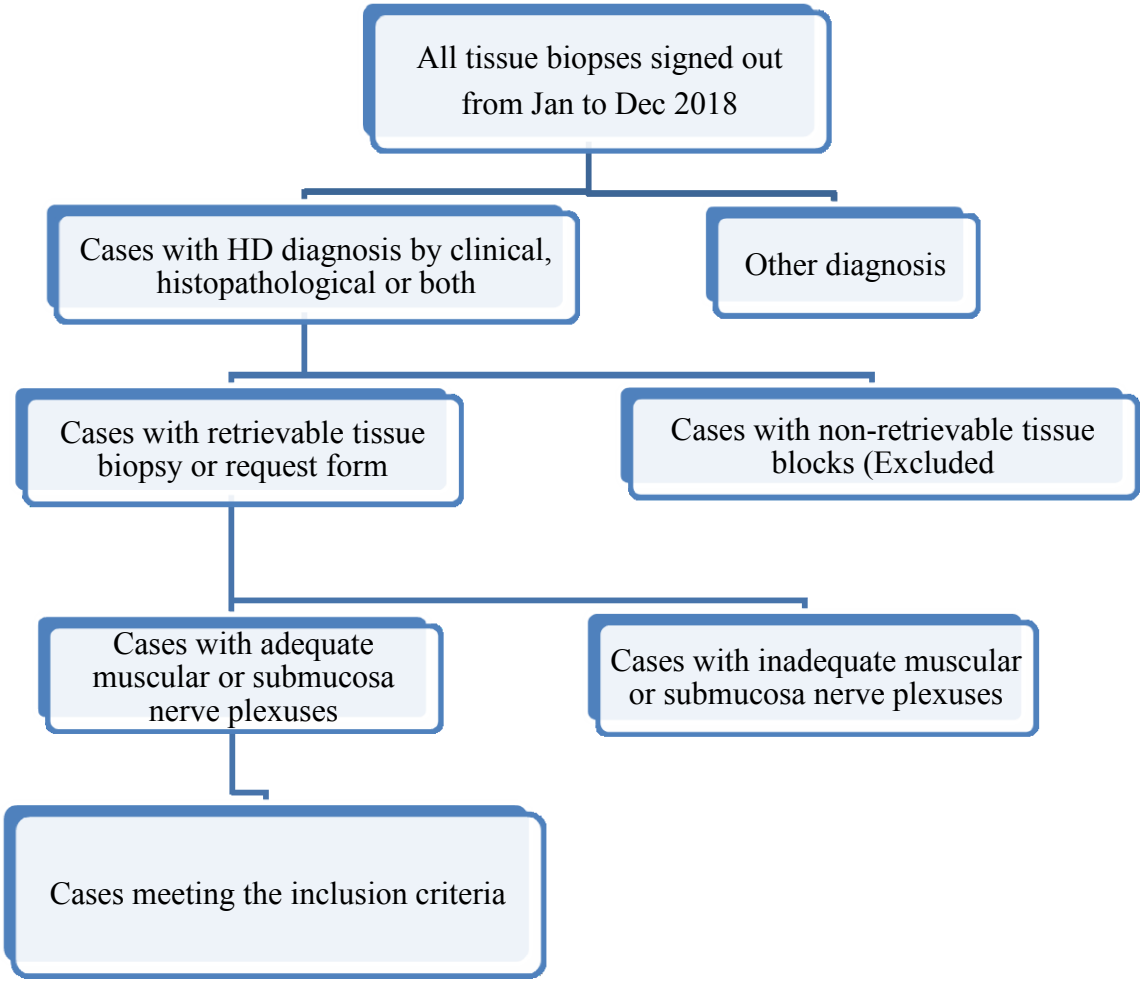


Figure 1: Sampling procedure

2.6: Laboratory procedure

2.6.1: Retrieval of tissue Blocks

FFPE tissue blocks were obtained from the histopathology archive after getting their laboratory identification numbers designated HP numbers in request forms with clinical diagnosis of HD in the study period.

2.6.2: H & E

Formalin fixed paraffin embedded tissue blocks were retrieved, sectioned, dewaxed in Xylene, hydrated through descending alcohol concentrations, stained by H&E, dehydrated through ascending alcohol concentrations, cleared in Xylene and cover-slipped.

All H&E slides were examined by the investigator and confirmed by supervisors. The cases were examined for presence of ganglion cells in the submucosa and/or muscularis nerve plexuses and those with ganglion cells in myenteric and/or sub mucosal plexuses were classified as HD. Previous diagnosis and new diagnosis after review were documented.

2.6.3: Calretinin immunohistochemistry Staining Procedure

Tissue calretinin IHC (Agilent Dako: Monoclonal, Clone DAK-Calret1 M7245 Mouse, diluted 1:10) was performed following this protocol. Tissue blocks were sectioned at 3µm and placed on positively charged slides (Thermo Fisher Scientific) and placed in the hot plate overnight at 61^{o}C. Sections were deparaffinised using xylene for 8 minutes then rehydrated using decreasing grades of alcohol (100%, 90%, and 80%), with 10 dips in each solution. The desired tissue sections were circled with a PAP pen and endogenous peroxidase activity was blocked using 1% peroxidase solution (H_2O_2) for 10 minutes. After the blocking step, slides were washed with distilled water for 1-3 minutes.

Antigen retrieval was performed as follows; first, 1.5L of Cold citrate buffer pH 6.0 (locally made) was added into the pressure cooker and the slide racks placed direct into the pressure cooker and the lid was tightened. The citrate buffer solution was heated to produce steam. After the steam came off, the pressure cooker was removed and put aside to cool for 10 minutes. The retrieval solution was poured and slides were cooled using tap water for another 10minutes. Slides were rinsed using peroxidase washing buffer solution (PBS): 1st dip for 3 minutes and 2nd dip for 10 minutes.

Tissue sections were then incubated with calretinin antibody for 30 minutes. Sections were washed using PBS twice, each for 3 minutes. Thereafter, sections were incubated with labelled polymer horseradish peroxidase anti-rabbit/anti-mouse for 30 minutes and washed in PBS twice for 3 minutes each after which they were incubated with 3, 3'-Diaminobenzine (DAB) for 3 minutes followed by rinsing in running tap water for 1 minute. The Staining with haematoxylin for bluing was done for 3 minutes. Dehydration in the ascending alcohol grades (80%, 90% and

100%) and xylene each 10 dips, followed, before cover slipping using DPX (Distyrene, Plasticiser and xylene) mounting medium on a Tissue-Tek cover slipper.

2.6.3.1 Calretinin immunohistochemistry staining evaluation

Nuclear and cytoplasmic staining of ganglion cells in the nerve plexuses of submucosa and muscularis propria was considered a positive result (34). All IHC slides were initially interpreted by the investigator and confirmed by supervisors (Senior Pathologists).

2.7: Data Collection

2.7.1: Data collection Tools

Demographic data were retrieved from request form.

Demographic data collected were age and sex.

A special form (Appendix I) was used to collect data that helped in determining the concordance of Haematoxylin and Eosin and calretinin in detection of ganglion cells.

2.8: Quality Assurance

The slides were reviewed by the principal investigator and supervisors who are senior pathologists. A known calretinin-positive normal bowel segment was used as positive control.Tissue blocks were handled as per MNH Central Pathology Laboratory routine IHC protocol.

2.9 Variables

2.9.1 Independent variables:

- Calretinin immunohistochemistry marker
- Hematoxylin and Eosin Technique

2.9.2 Dependent Variable

Detection of ganglion cells

Variables for specific objectives

- Qualitative (Demographic data and detection of ganglion cells). Two by two table and Chi-Square test was performed.
- Quantitative (Proportions): P value was used in comparison of proportions

2.10 Statistical analysis

The data were electronically transcribed from the data collection sheet and analysed using SPSS version 23. Data was summarized into proportion and frequency tables.

Objective 1: Summary statistics with number of cases and percentage for categorical variables were used to describe the proportion of cases with HD. The relationship between HD with age and sex was performed using Chi square to assess the statistical significance.

Objectives 2: Positive and negative concordance between H&E and calretinin IHC using Cohen's kappa coefficient (k) was performed. Currently, kappa is interpreted as; <0 No agreement, 0-0.20 Slight, 0.20-0.40 Fair, 0.41-0.60 Moderate, 0.61-0.80 Substantial and 0.81-1.0 Perfect. Statistical significance was determined using McNemar test; p-value of ≤0.05 was considered to demonstrate statistically significant difference. In addition, performance characteristics, including sensitivity and specificity were evaluated

2.11: Ethical Consideration

Ethical review and approval was obtained from MUHAS ethical clearance committee and permission to conduct the study was obtained from the Executive Director of MNH through the Head of Histopathology unit.

The patients' information were strictly kept confidential, no patient names and other clinical information were revealed to any person not involved in this research.

This study did not have adverse effects on subjects' health as no patients were involved. Research identity numbers were used instead of name of patients. The retrieved blocks and slides were returned to their corresponding archives after processing. Calretinin immunohistochemistry was used for research purpose and no change of previous patient's diagnosis was done.

CHAPTER THREE

3.0 RESULTS

3.1 General and demographic characteristics of the study population

In this study, 107 samples were submitted in the histopathology laboratory, from 1st January to 31st December 2018, with clinical diagnosis of HD. Tissue blocks of 8 cases were missing so they were excluded from the study (figure 2), leaving the study sample to be 99. The study included 69 males and 30 females. The age varied from 1 month to 19 years with median age of 3 years. Most cases were between 1 and 3 years old (36.4%) and between 4 and 10 years (36.4%) (Table 1).

Out of 36 cases that were diagnosed as positive for HD, 24(67%) were males and 12(33%) were females, making a male to female ratio of 2:1 (p=0.62). Fourteen (39%) cases out of HD positive cases were between 1 to 3 years of age, which was the age group with the highest frequency. On the other hand, 4 cases were of above 10 years and between 0.6 to 1 year of age (p=0.37). H&E Stain of the cases, showed ganglion cells in 63 cases and 36 cases showed no ganglion cells and were reported positive for HD. Calretinin IHC was done in 59 cases that included all 36 cases with no ganglion cells detected by H&E and 23 cases with ganglion cells detected by H&E that had adequate sub mucosal or muscular nerve plexuses. Thirty cases were true HD after staining with calretinin IHC.

Table 1: Demographic characteristics (stained by H&E)

Characteristic	Frequency (n %) N=99
Age	
0-1 Year	17(17.1)
1-3 years	36(36.4)
4-10 Years	36(36.4)
Above 10	10(10.1)
Sex	
Males	69(69.7)
Females	30(30.3)

Table 2: Clinical and pathological characteristics of the study population

Characteristic	Frequency (n %) N=99
Symptoms	
Abdominal distension	17(17.2)
Constipation	50(50.5)
Abdominal distension and constipation	32(32.3)
Site of Biopsy	
Colon	35(35.4)
Rectum	64(64.6)
Thickness	
Full thickness	69(69.7)
Submucosa	20(20.2)
Muscularis propria	10(10.1)
Ganglion cells' Location(N=63)	
Submucosa and Muscularis propria	31(49.2)
Submucosa	13(20.6)
Muscularis propria	19(30.2)

Table 3: Sex versus HD status

		Diagnosis			
		HD	**No HD**	**TOTAL**	
Sex	**Male**	24(34.8%)	45(65.2%)	69(100.0%)	
	Female	12(40.0%)	18(60.0%)	30(100.0%)	P=0.62
	TOTAL	36(36.4%)	63(63.6%)	99(100.0%)	

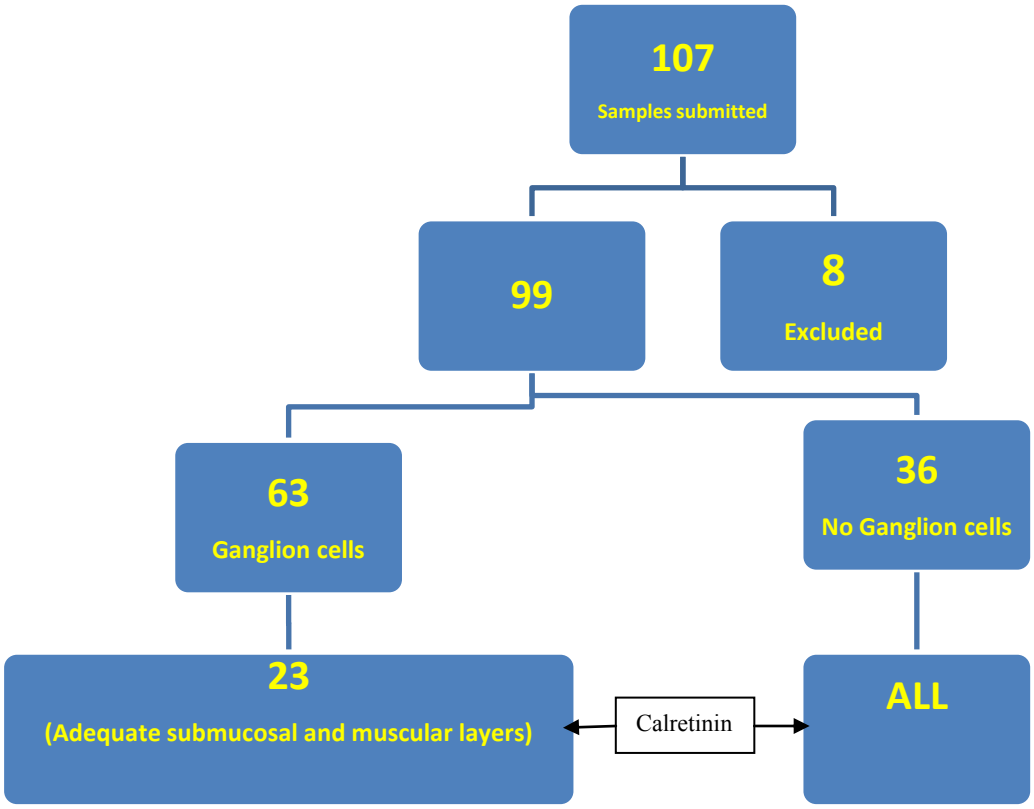


Figure 2: Flow chart showing how cases were selected for calretinin IHC

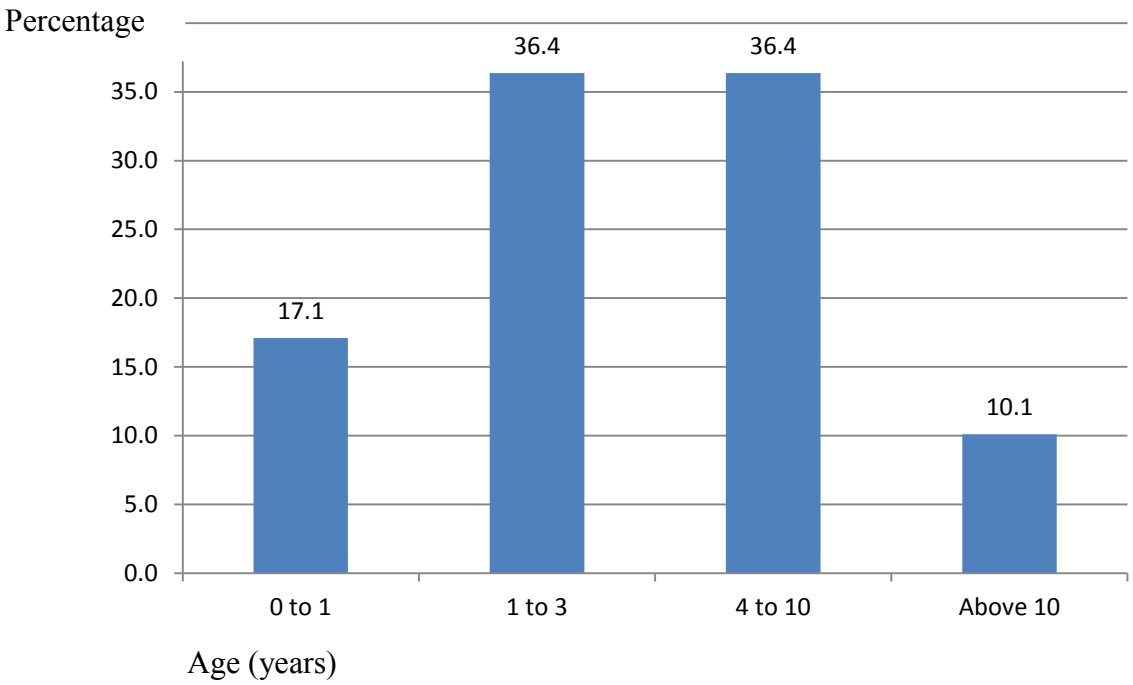


Figure 3: Age distribution in the study population

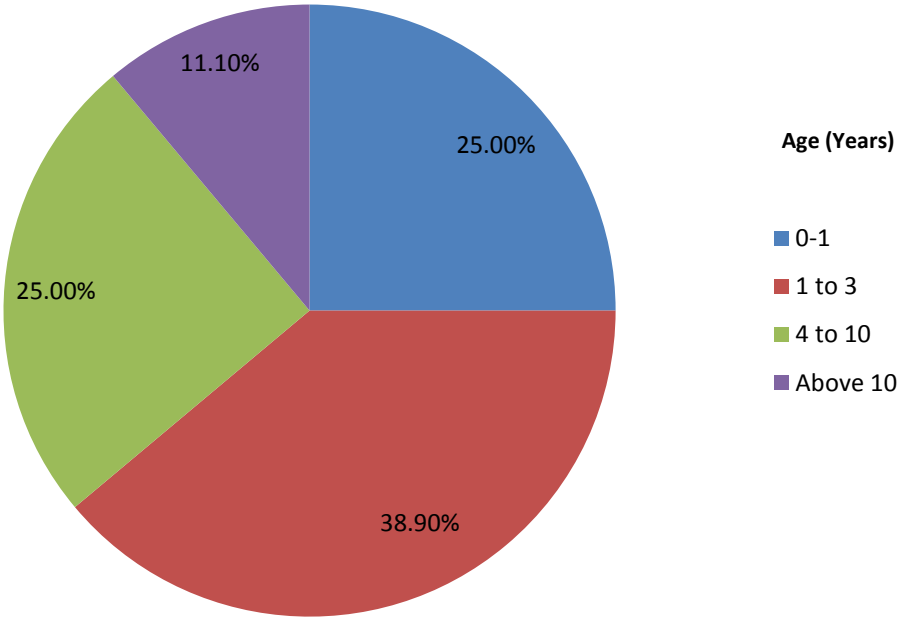


Figure 4: Age versus Hirschsprung's disease

3.2 Clinical and Pathologic characteristics of the study population

Symptoms with which participants presented to the clinicians included abdominal distension, constipation or a combination of both. The most common complaint was constipation (51%), followed by those who presented with both constipation and abdominal distension (32%). The sites of biopsies submitted were rectum and different parts of the Colon. The Rectum was the most frequent site of biopsy (65%), the rest were taken from various parts of the colon. Out of the 63 cases that showed ganglion cells by H&E, 34/63 were in the rectum and 29/63 in the colon.

Most of the submitted biopsies were full thickness 69/99 (70%), followed by biopsies which were up to the level of the submucosa 20/99 (20%). The biopsies submitted as the muscularis propria layer only were also seen 10/99 (10%) (Table 2). Locations of the ganglion cells seen by the H&E for most of the biopsies included 31/63 (49%) which were on both submucosa and muscularis propria layers. Ganglion cells were found on the muscularis propria layer only in 19/63 (30%) and the submucosa layer only in 13/63(21%) biopsies.

3.3 The proportion of HD among the colonic biopsies submitted in suspicion of such.

Out of 99 cases with clinical suspicion of HD, 36 cases showed no Ganglion cells by the routine H&E stain. These were the cases submitted from 1st January to 31st December 2018. The proportion of HD of the year 2018 among the submitted biopsies in suspicion of such was 36/99 (36.4%).

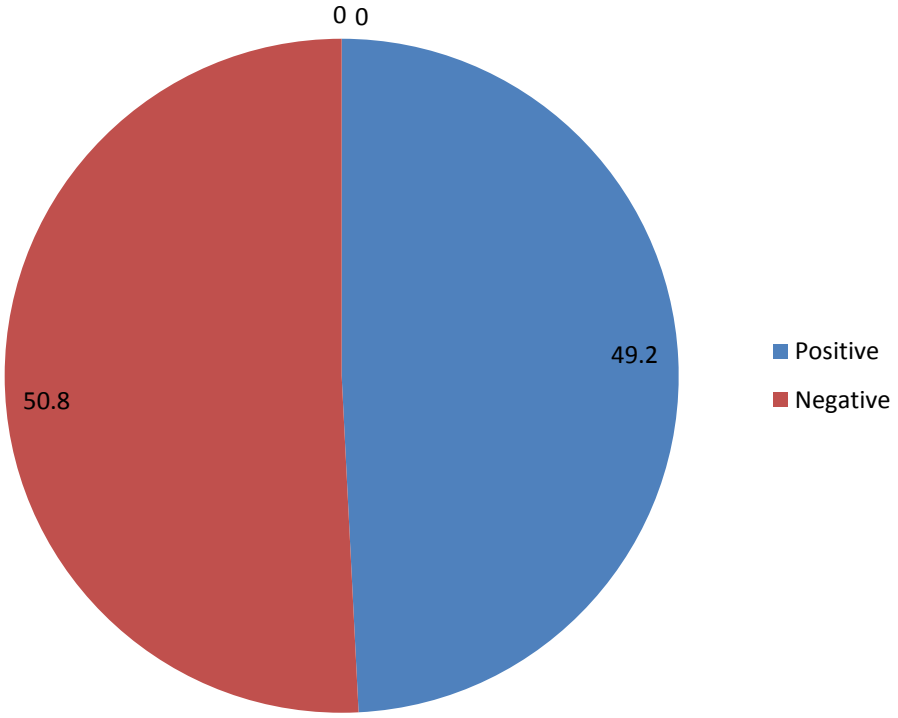


Figure 5: Status of calretinin IHC staining

3.4 Comparison of H & E and calretinin IHC in detection of ganglion cells in colonic biopsies.

The calretinin IHC was performed for 59 cases. The stain was positive for 29 cases and negative in 30 cases (figure 5). Six cases that were negative (showed no ganglion cells) by H&E, were positive (showed ganglion cells by calretinin IHC. Thirty cases that were negative by H&E were also negative by calretinin IHC. All 23 out of 58 cases that were positive by H&E were also positive by calretinin IHC.

Table 4: Comparison of H&E versus calretinin IHC staining of Ganglion cells

		Calretinin IHC		TOTAL	
		Positive	**Negative**		
H&E (Ganglion cells)	**Positive**	23(100%)	0(0.0%)	23(100.0%)	Agreement (Kappa) 0.790 P<0.005
	Negative	6(16.7%)	30(83.3%)	36(100.0%)	
	TOTAL	29(49.2%)	30(50.8%)	59(100.0%)	

The positive concordance (the number of cases that were positive for both) between calretinin and H&E was 23/23 (100%). The Negative concordance (the number of cases that were negative for both) of the two was 30/36 (83%). The overall concordance was found to be 53/59 (90%) and the discordance rate was 10% with Cohen's Kappa agreement of 0.790.

For calretinin IHC, True positives were 23; true negatives 30, false negatives 6 and no false positives. Positive predictive value was 23/23(100%) and negative predictive value was 30/36(83%). For calretinin IHC, True positive rate (sensitivity) was 23/29(79%) and True negative rate (specificity) 30/30(100%).

For H&E, the True positive rate (sensitivity) was 23/53(43.4%) and True negative rate (specificity) 6/6(100%). This indicated that calretinin staining detected more cases than H&E.

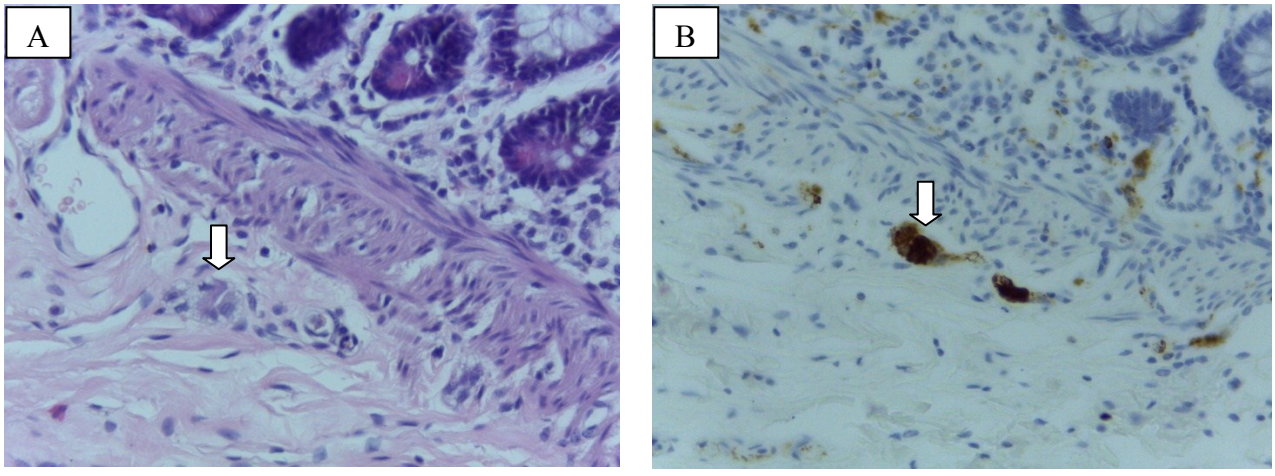


Figure 6: Normal ganglionic segment of bowel demonstrating ganglion cells (arrow) in the submucosa (Meissner's) plexuses by routine H&E morphologic examination (A, x40) and by calretinin immunohistochemical analysis (B, x40). Same case.

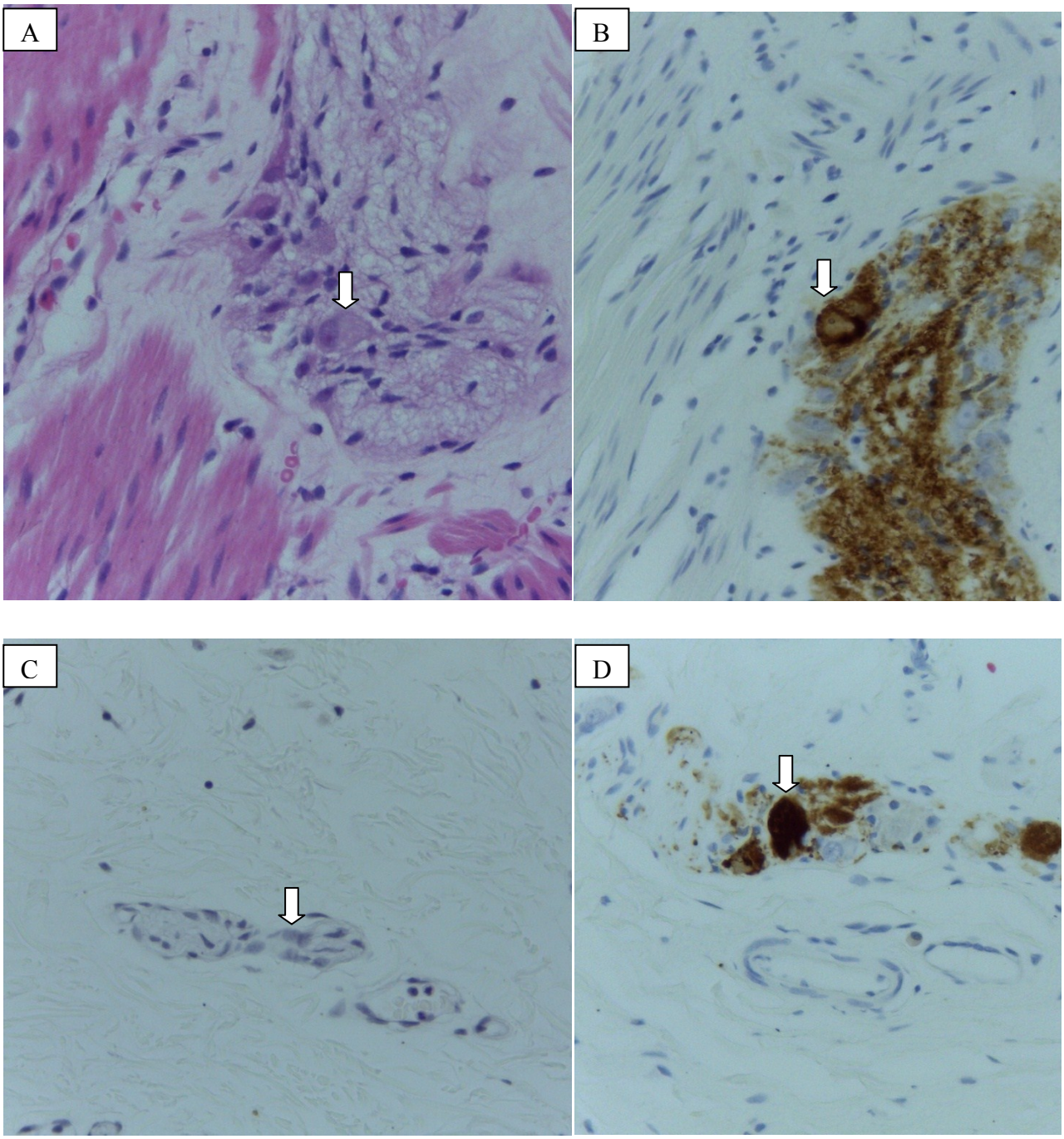


Figure 7: Reported as No HD; Concordant pairs of ganglionic segment of bowel demonstrating ganglion cells (arrow) in the myenteric (Auerbach) plexuses by routine H&E morphologic examination (A&C, x40) and by calretinin immunohistochemical analysis (B&D x40).

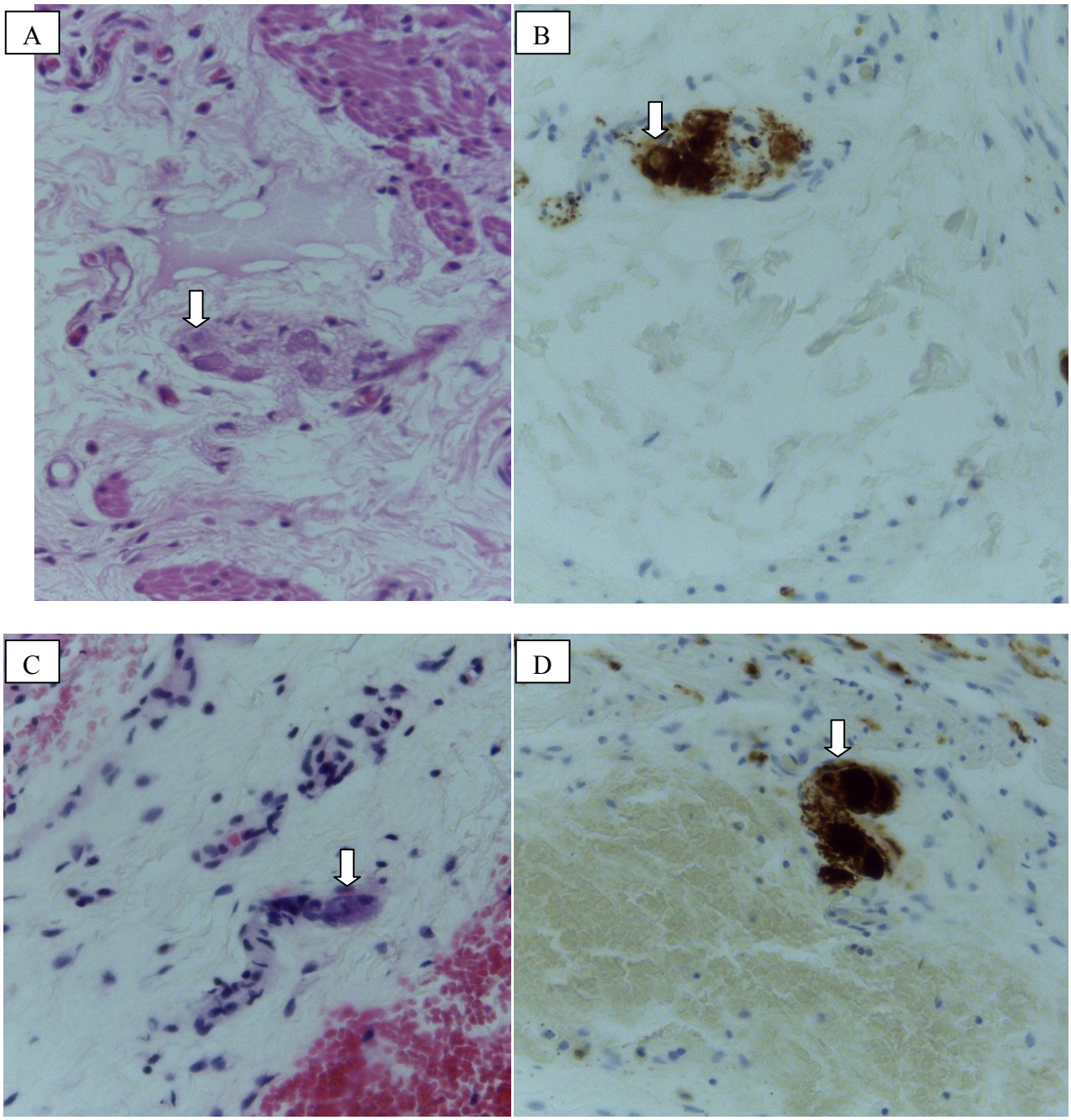


Figure 8: Reported as No HD; Concordant pairs of ganglionic segment of bowel demonstrating ganglion cells (arrow) in the submucosa (Meissner's) plexuses by routine H&E morphologic examination (A&C, x40) and by calretinin immunohistochemical analysis (B&D x40).

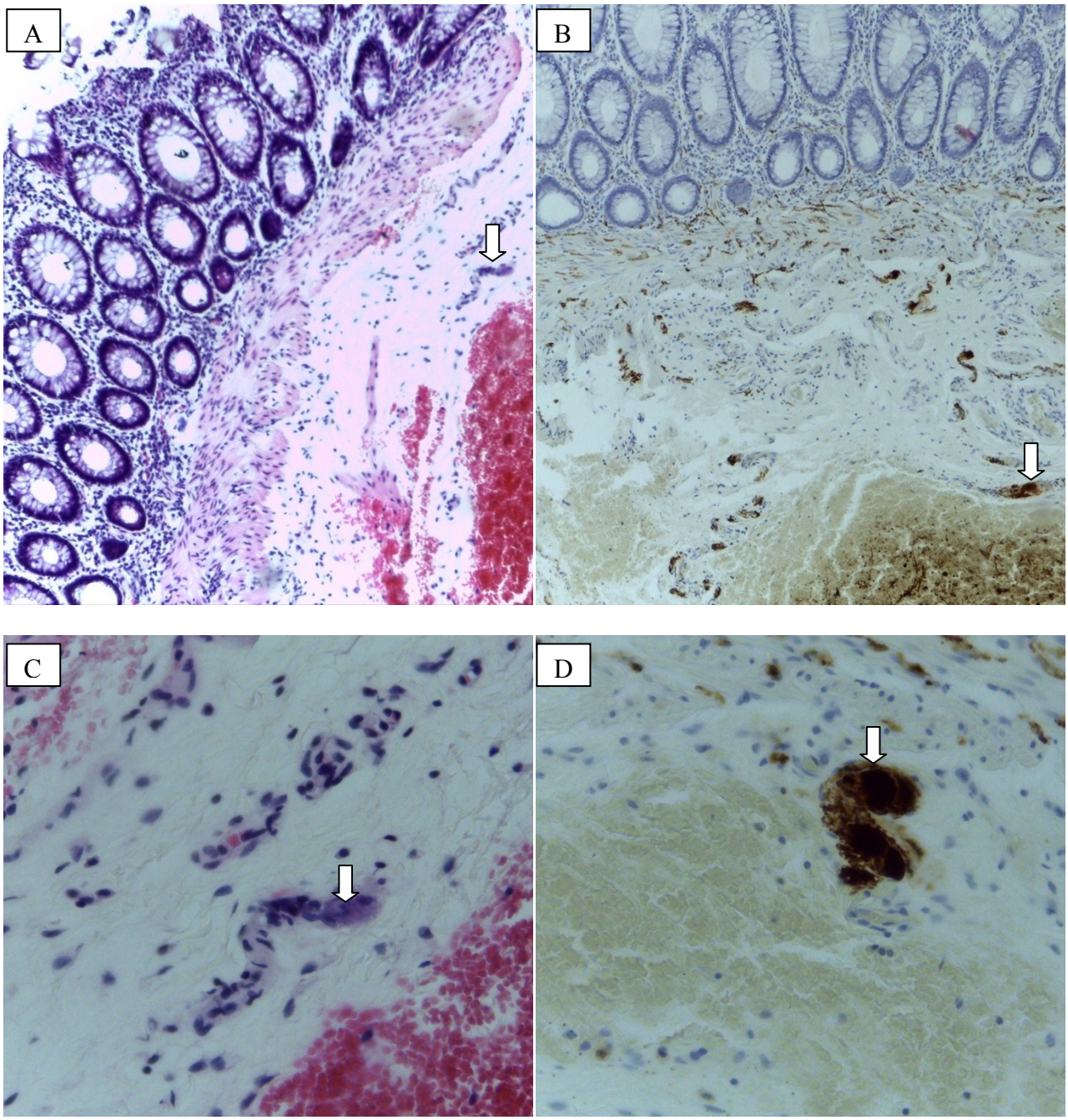


Figure 9: Simplicity in lower power detection; concordant pairs of ganglionic segment of bowel demonstrating ganglion cells (arrow) in the submucosa (Meissner's) plexuses by routine H&E morphologic examination (A&C) and by calretinin immunohistochemical analysis (B&D). A&B x10, C&Dx40.

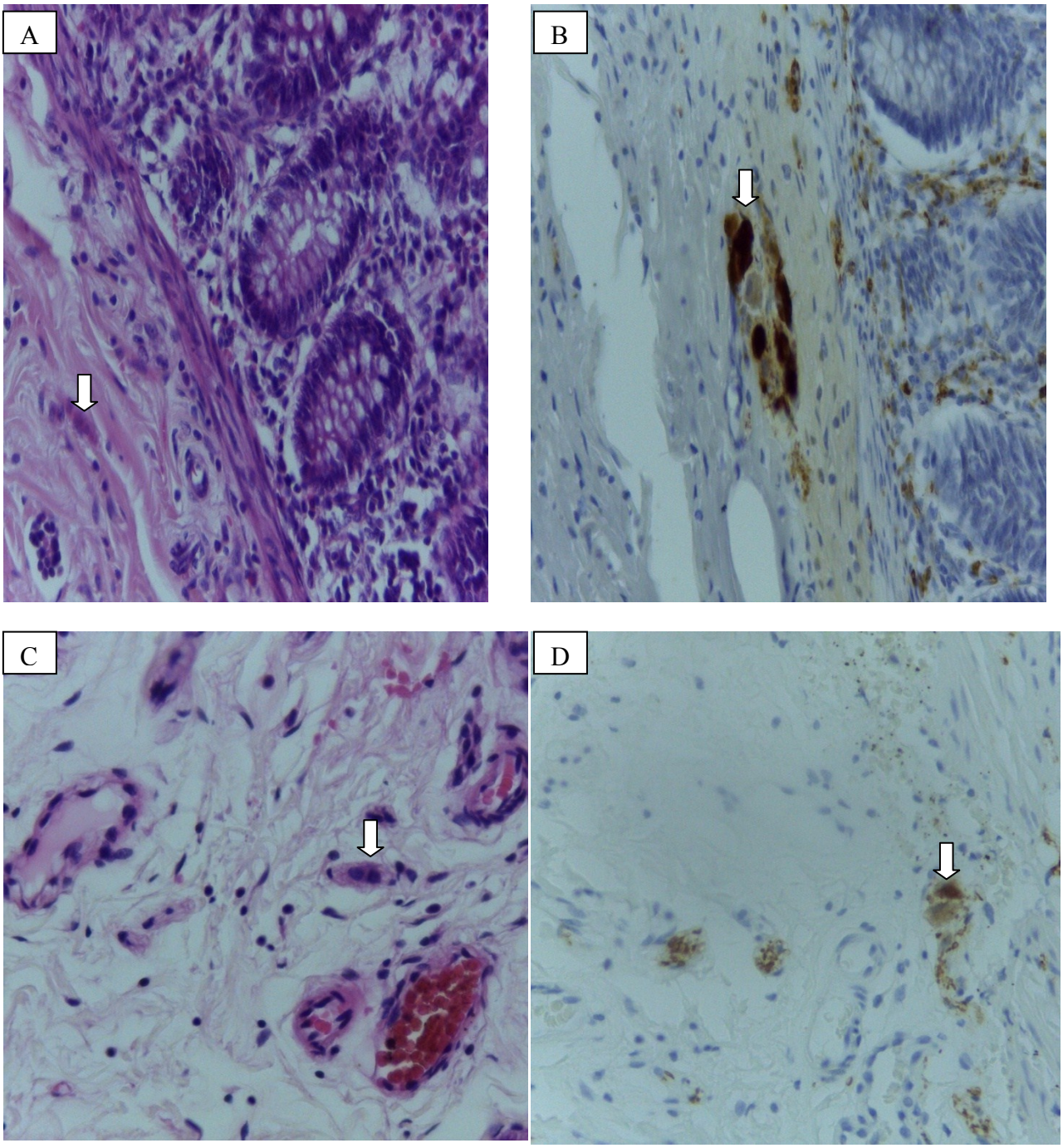


Figure 10: Challenging detection by H&E; concordant pairs of ganglionic segment of bowel demonstrating ganglion cells (arrow) in the submucosa (Meissner's) plexuses by routine H&E morphologic examination (A&C, x40) and by calretinin immunohistochemical analysis (B&D x40).

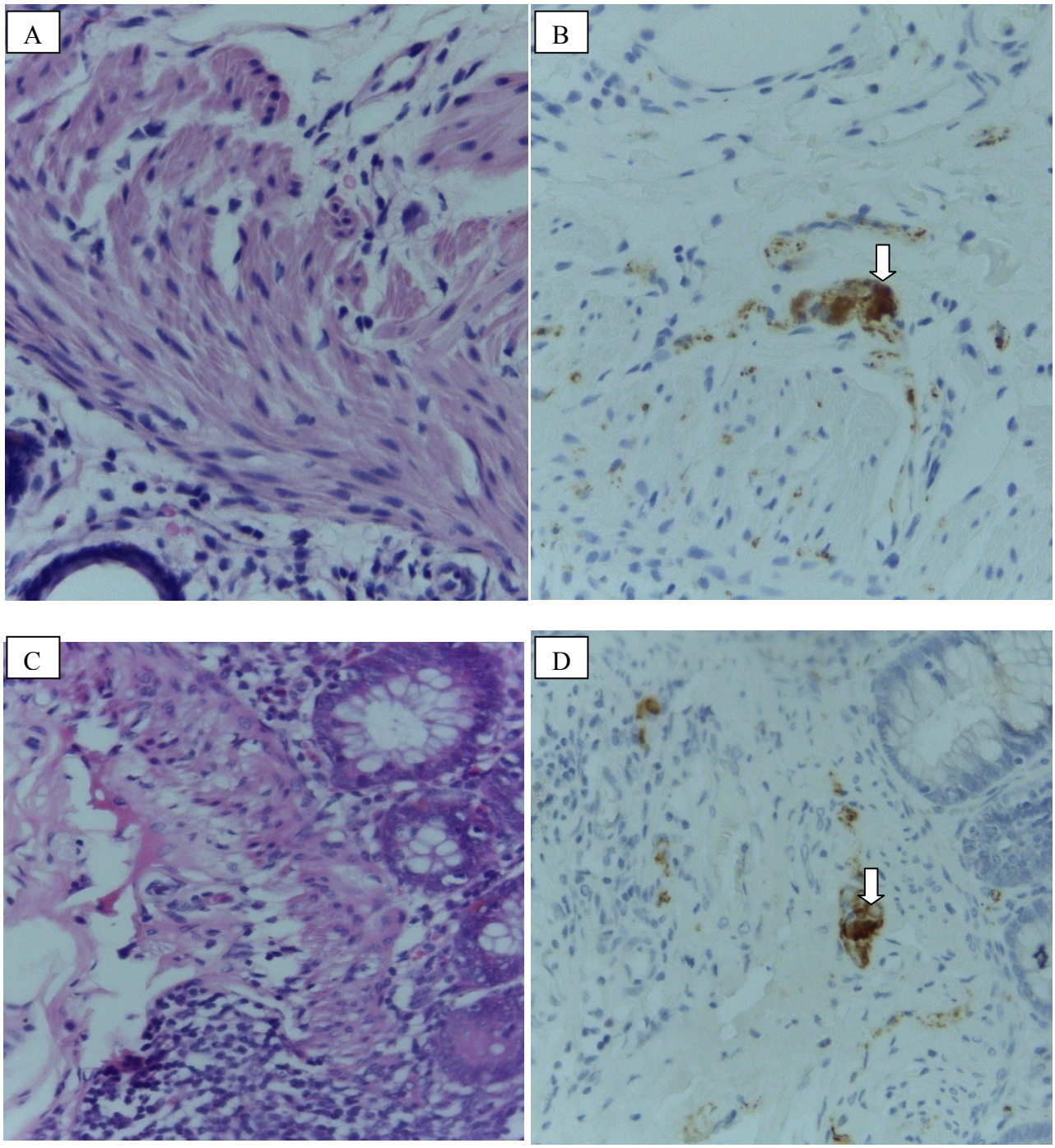


Figure 11: Reported as HD; Segment of bowel demonstrating no ganglion cells in the submucosa (Meissner's) plexuses by routine H&E morphologic examination (A&C, x40) but ganglion cells (arrow) seen by calretinin immunohistochemical analysis (B&D x40).

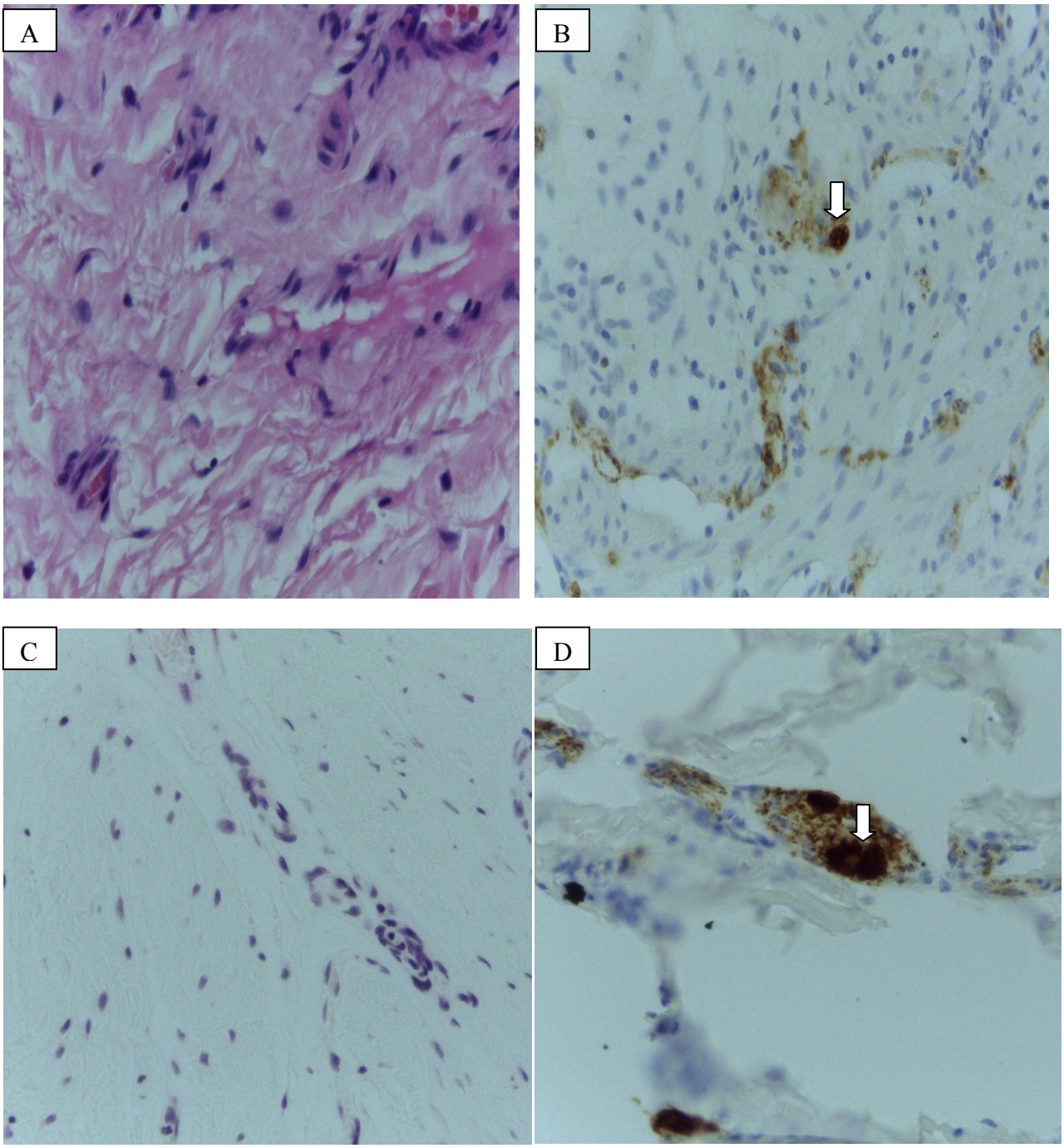


Figure 12: Reported as HD; Segment of bowel demonstrating no ganglion cells in the myenteric (Auerbach's) plexuses by routine H&E morphologic examination (A&C, x40) but ganglion cells seen by calretinin immunohistochemical analysis (B&D x40).

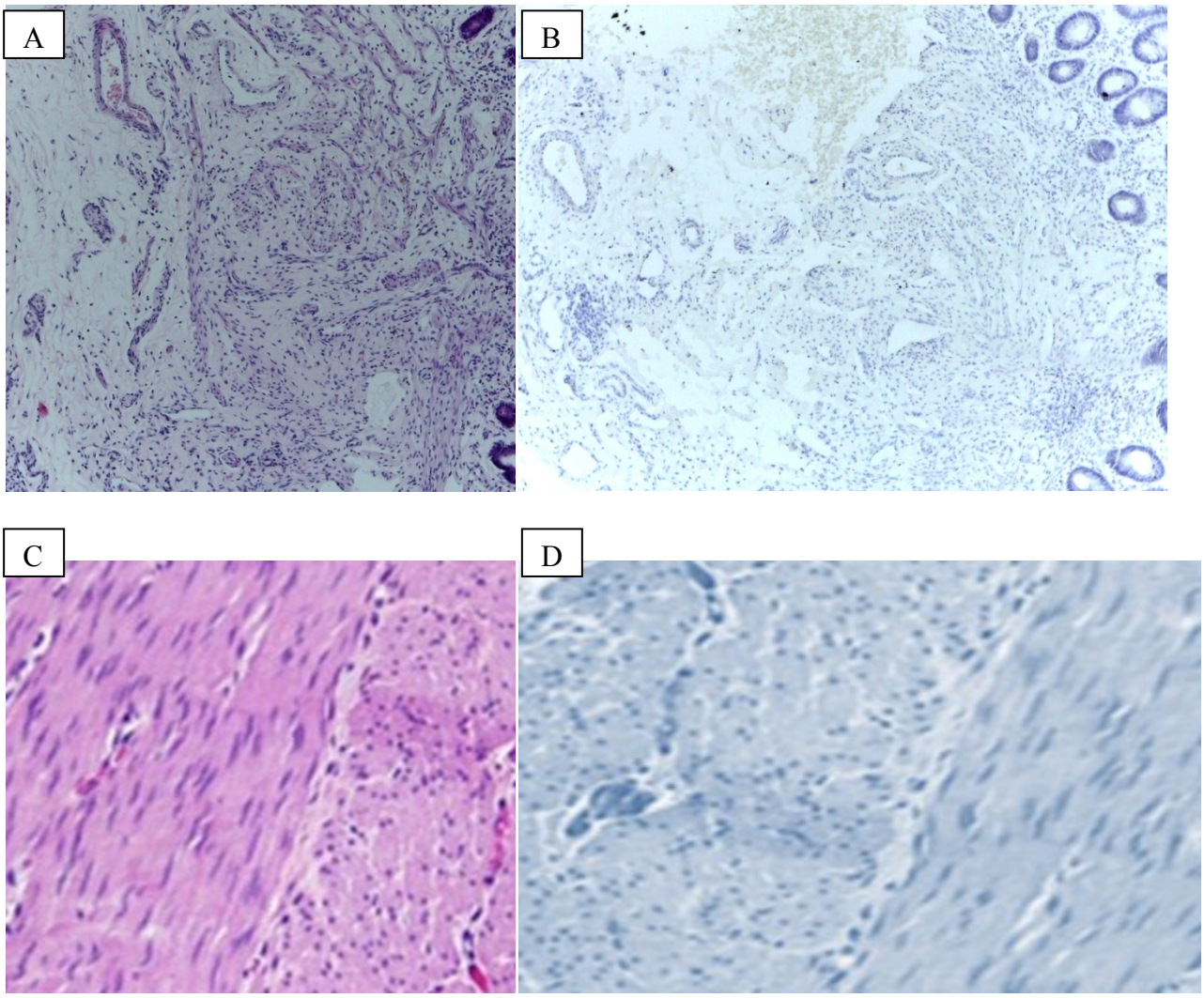


Figure 13: Concordant pairs of Segment of bowel demonstrating no ganglion cells in the submucosa (Meissner's) plexuses (A&B) and myenteric (Auerbach's) plexuses (C&D) by routine H&E morphologic examination (A&C, x10) and calretinin immunohistochemical analysis (B&D x10).

CHAPTER FOUR

4.0 DISCUSSION

H&E AChE and calretinin immunohistochemistry are different diagnostic modalities of HD after clinical suspicion. However, documenting aganglionosis is often difficult and tedious on H&E evaluation of serial sections or AChE staining from fresh superficial biopsies. calretinin IHC has been introduced as a new simple and reliable tool for the diagnosis of HD. Calretinin has shown superiority to AChE as a confirmatory diagnostic method for evaluating suction rectal biopsies for HD, with sensitivity and specificity of above 70% (16,23,24). It has been expected that it would add a substantial value if in routine use. In this study, the Sensitivity of calretinin IHC in ganglion cells' detection is 79% and specificity is 100%. This finding is in agreement with several studies that have been done. Anbardar MH et al, showed sensitivity and specificity of above 90.3% and agreed that calretinin IHC is very useful in detecting aganglionosis in patients suspicious to HD (24); Specificity of 93.3% was reported by Kacar et al (16); Sensitivity and specificity of 97.5 % and 97.1 % respectively, was reported by de Haro et al (23).

The study has clearly shown that calretinin has a very high overall concordance with H&E in detecting ganglion cells. The positive concordance was 23/23(100%) and Negative concordance of the two was 30/36 (83%), making the overall concordance of 53/59 (90%) and the discordant rate was 10%. This means that calretinin is comparable with H&E in accuracy of proving the absence of ganglion cells. Similar finding was also reported in a study by Kannaiyan, et al whereby of the 19 cases reported negative through H and E staining, 17 (89.5%) were reported negative, and 2 (10.5%) positive for calretinin immunohistochemistry for the ganglion cells and nerve fibres, therefore calretinin correlated with H and E examination in both rectal biopsies and the resected bowel specimens; They, however, recommend that it should be used as an aid along with H and E examination(26).A kappa value of 0.790 was revealed in this study, which is considered a good concordance index. Serafini et al in Brazil, found similar concordance index where calretinin immunohistochemistry revealed a kappa value of 0.676 (27), although, the specificity was70% which is lower than of this study.

This study has also shown that IHC for calretinin provides highly comparable results with H&E findings in diagnosis of HD.

A similar finding was reported by Rakhshani N et al where 80 cases were detected negative through H&E, 77 (96.2%) cases were confirmed in terms of absence of ganglion cells by calretinin and values of specificity (96.2%) and specificity (71.4%) presented significantly higher sensitivity and specificity values than H&E staining (P <0.0001), (33).

Discrepancy between suspected clinical cases (99) and histologically confirmed cases (30) has been seen in this study. This could be due to inability to differentiate between immature/non-functioning and mature ganglion cells which can be detected by other methods, and therefore a need for further studies. It could also be due to sampling of unrepresentative area that showed ganglion cells and reported as no HD while the aganglionic part was missed especially in short segment HD.

This study found that the use of calretinin made detection of ganglion cells simpler and quicker even by the use of a lower magnification. The same fact was observed by Alexandrescu et al and de Haro Jorge et al, who concluded that calretinin IHC offered additional diagnostic value in the specimens with inadequate amount of submucosa and rarely seen ganglion cells, and this can be used in clearing doubts in many inconclusive results in ganglion cells' detection (15), (23). Ongeti K et al and Rakhshani N et al reported as being saved from long time spent in searching for the ganglion cells and found calretinin as less tedious and less difficult in detection of ganglion cells (5), (33).

This study included more biopsies from males than females, and among patients diagnosed as positive for Hirschsprung's disease, the majority were males. This male preponderance could have been due to the higher number of biopsies submitted from males because they present more with signs of HD. Other studies done in East Africa have reported similar findings; a study done in Bugando Tanzania by Mabula et al, showed that 78.2% of Hirschsprung's patients were males and 21.8% were females, with a male to female ratio of 3.6: 1 (6); and in Kenya, a study done by Ongeti K. et al, found 85% males and 15% females (gender ratio 5.5: 1)(5).Similar findings were obtained by Ameh E. et al, in Zaria Nigeria that found a male/female ratio of 3:1(4); Bandré E. et al, in Burkina Faso found M: F=3.3:1(35); De Lorijn F. et al, in the Nethelands reportedmale-to-female ratio of 4 to 1.15(2); Moore S. et al, approximated 4:1 male-to-female ratio worldwide (17).

Amiel J. et al, reported sex dependent mutation with higher estimated penetrance in males (72%) than females (51%), explaining higher male to female ratio(36). Many studies have not given out the reason for male predominance.

This study found that, age of the patients varied from 1 month to 19 years with median age of 3 years and the most frequent age groups of between 1 and 3 years (36.4%) and between 4 and 10 years (36.4%). Most of the cases diagnosed with HD were between 1 and 3 years of age (38.9%). A study done by Mabula et al, in Tanzania, reported the age of patients at presentation ranged from 7 days to 10 years with a median age of 24 months and a few were in the neonatal period (6); Ongeti K. et al, in Nigeria, reported that at presentation, majority were older with the mean age of the of 709 ± 400 days (range, one day to 17 years) (5); Bandré E. et al, in Burkina Faso, found age ranged from two days 10 years (median 20 months)(35); Ekenze SO. et al, found median age of 4 years and attributed it to delayed referral, parental ignorance and poverty (12).

In this study, symptoms with which participants presented to the clinicians included abdominal distension, constipation or a combination of the two. The most common complaint was constipation (51%), followed by those who presented with both constipation and abdominal distension (32%). This was in agreement with a study in Mwanza by Mabula et al, who found that constipation and abdominal distension were the most common presenting complaints in 94.5% and 92.7% of cases, respectively (6). In Nigeria, Ongeki K. et al, reported main symptoms as repeated vomiting (48% of children), chronic constipation (90%), delayed passage of meconium (69%), abdominal distension (90%) and failure to thrive (29%) (5). De Lorijin et al, that found more than 40% of cases with functional constipation (2). Nasir A. et al, reported 61.9% of cases presented with chronic constipation (37).This could be due to the fact that constipation is an early complaint and distension develops later. In out setting this could also be due to the fact that the submitted clinical history was very short with possibility of missing important symptoms to be included in the study.

Study limitations

This study could have been more informative if the clinical information submitted with the request form had more details on clinical history and findings, or if such an information could be easily accessible through the hospital information management system. This could be improved by providing the pathologists with full access to clinical details of the patients in the information system.

The immunohistochemical staining was performed on the tissues whose pre analytical stages were not controlled, making a possibility of affecting the IHC staining especially if the fixation protocol was not followed. The current protocol allows tissue processing after 24 hours of fixation. This limitation could be overcome by a prospective study with consideration of fixation time by the investigator.

CHAPTER FIVE

5.0CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Comparison of detection of ganglion cells by the routine H&E and calretinin has shown a very high concordance rate with a slightly higher detection rate (16.7%) by calretinin for the cases that were missed by the routine H&E. The overall capability of ganglion cells' detection by calretinin IHC has been accompanied by a significant shorter time and simplicity in sighting out the ganglion cells.

5.2 Recommendations

5.2.1 To MUHAS and MNH Histopathology unit

a) To adopt the utilization of calretinin in diagnosis of HD in addition to H&E for the cases that H&E fails to detect the ganglion cells.
b) To encourage more studies to be done on calretinin including cost benefits analysis.
c) Clinician should submit specimens with more clinical information.

5.2.2: To the Ministry of Health, Community Development, Gender, Elderly and Children.

Health Insurance funds should cover the costs for calretinin immunohistochemistry for their members, as it would significantly prevent patients from incurring high costs

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6: APPENDICES

6.1: APPENDIX I: DATA COLLECTION SHEET.

1. Date. ……
2. Study code number ……
2. HP number ……
3. Hospital Registration Number ……
4. Age ……
5. Sex ……
6. Patient Signs/Symptoms (It is possible to retrieve this information as the Histopathology Request form has an area for clinical information)
……
……
……
7. Site of the biopsy……
8. Clinical Diagnosis……
9. Previous histopathological diagnosis……...
9. New histopathological diagnosis…….
10. Calretinin immunohistochemistry results……
11. Final Diagnosis……
12. Supervisors' review and comments
……
……

6.2: APPENDIX II: MNH SOP FOR: HARRIS HEMATOXYLIN AND EOSIN

1. Retrieve blocks and make tissue sections using microtome (3-5 microns)
2. Pick the sections on a slide from water floatation and place in a hot plate at 60^0C for 50 minutes.
3. Deparaffinise sections, hydrate through graded alcohol to water
4. Remove fixation pigments if necessary
5. Stain in alum haematoxylin for a suitable time.
6. Wash well in running tap water until section 'blue' for 5 minutes
7. Differentiate in 1% acid alcohol (1% HCL in 70% alcohol) for 5-10 seconds.
8. Wash well in tap water until sections are again 'blue' (10-15 minutes)
9. Stain in 1% eosin Y for 10 minutes.
10. Wash in running water for 1-5 minutes
11. Dehydrate through alcohols (70, 95, 100%), clear and mount.

Appendix III: Modified MNH Tissue blocks immunohistochemistry

1. Section the IHC cell blocks and tissue blocks at 3µm, place on charged slides and place in the on hot plate overnight at 61^0C.
2. Deparaffinise the section into two step xylene to remove the waxy each steps for 8minutes.
3. Rehydrate the section using decreasing grades of alcohol (100%, 90%, and 80%) each steps ten dips.
4. Circle the desired tissue sections using PAP pen and block the endogenous peroxidase activity with 1% peroxidase solution (H_2O_2) for 10minutes and after blocking wash the slide in distilled water.
5. Perform antigen retrieval as follows: First, 1.5L of Cold citrate buffer (pH 6.0) to be added into the pressure cooker and the slide racks containing 20 slides to be placed direct into the pressure cooker and the lid tightened. The citrate buffer solution to be heated to produce steam.
6. After the steam comes off, cool the pressure cooker for 10minutes and remove the metal bucket pour the retrieve solution and cool the slide in running water for 10minutes.
7. Wash the slide with peroxidase washing buffer solution (PBS) 1^{st} dip 3m and 2^{nd} dip 10minutes.
8. Incubate the section with Monoclonal Rabbit, Anti-Human Estrogen receptor α Clone EPI for 30minutes.
9. Wash the slide with PBS 1^{st} step 3minutes and 2^{nd} step 3minutes.
10. Incubate the section with Horseradish Peroxidase (Universal secondary antibody) for 30minutes and wash with PBS 1^{st} step 3minutes and 2^{nd} step 3minutes.
11. Incubate the section with 3, 3'-Diaminobenzine (DAB) for 3minutes and wash the sections in running tap water for 1minutes, dip in haematoxylin (one dip), bluing in tap water for 1minute.
12. Dehydrate the section in ascending alcohol grades (80%, 90% and 100%) and finish with xylene each steps 10dips.
13. Mount the slide with DPX (Distyrene, Plasticiser and xylene) using Tissue-Tek.

Appendix IV: Ethical Clearance Letter

MUHIMBILI UNIVERSITY OF HEALTH AND ALLIED SCIENCES

OFFICE OF THE DIRECTOR OF POSTGRADUATE STUDIES

P.O. Box 65001
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Ref. No. DA.287/298/01A/

20th June, 2019

Dr. Mbazi D. Shemweta
MMed. Anatomical Pathology
MUHAS.

RE: APPROVAL OF ETHICAL CLEARANCE FOR A STUDY TITLED: "COMPARISON OF HAEMATOXYLIN AND EOSIN AND CALRETININ DETERCTION OF GANGLION CELLS IN COLORECTAL BIOPSIES SUSPECTED OF HIRSCHSPRUNG'S DISEASE"

Reference is made to the above heading.

I am pleased to inform you that, the Chairman has, on behalf of the Senate, approved ethical clearance for the above-mentioned study. Hence you may proceed with the planned study.

The ethical clearance is valid for one year only, from **19th June, 2019 to 18th June 2020.** In case you do not complete data analysis and dissertation report writing by **18th June, 2020,** you will have to apply for renewal of ethical clearance prior to the expiry date.

Dr. Emmanuel Balandya
ACTING: DIRECTOR OF POSTGRADUATE STUDIES

cc: Director of Research and Publications
cc: Dean, School of Medicine, MUHAS

Appendix V: MNH Permission letter for data collection

MUHIMBILI NATIONAL HOSPITAL

Cables: "MUHIMBILI"
Telephones: +255-22-2151367-9
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Postal Address:
P.O. Box 65000
DAR ES SALAAM
Tanzania

In reply please quote:

MNH/TRCU/IRB/Permission/ 2019/122 03rd July, 2019

Head of Department
Central Pathology Laboratory
Muhimbili National Hospital

RE: PERMISSION TO COLLECT DATA AT MNH.

Name of Student	**Dr.Mbazi Shemweta**
Title	**"COMPARISON OF HEMATOXYLIN AND EOSIN AND CALRETININ DETERCTION OF GANGLION CELL IN COLORECTAL BIOPSIES SUSPECTED OF HIRSCHSPRUNG'S DISEASES".**
Institution	**Muhimbili University of Health and Allied Sciences**
Supervisor	**Prof. James Kitinya** **Dr. Amos Mwakigonja** **Dr. Angela Mwakimonga**
Period	22th July 2019 to 30th December, 2019

Approval has been granted to the above mentioned student to collect data at MNH.

Named MNH based supervisor must ensure that the student abide to the ethical principles and other conditions of the research approval.

Sincerely,

Dr. Faraja Chiwanga
Head of Teaching, Research and Consultancy Unit

TEACHING, RESEARCH & CONSULTANCY UNIT
P.O. Box 65000
DAR-ES-SALAAM
MUHIMBILI NATIONAL HOSPITAL

c. c DMS
c.c **Dr. Angela Mwakimonga**
c. c **Dr.Mbazi Shemweta**

All Correspondence to Addressed to the Executive Director