

CLINICOPATHOLOGICAL AND MOLECULAR ANALYSIS OF SERRATED LESIONS OF THE APPENDIX

MARK ANDREW ROBSON KEYTER

A research report submitted to the Faculty of Health Science, University of the Witwatersrand, in partial fulfilment of the requirements for the Degree of Master of Medicine (Anatomical Pathology).

Johannesburg 2021

DECLARATION

I, Mark Andrew Robson Keyter, declare that this research report is my own work. It is being submitted for the degree of Master of Medicine in the branch of Anatomical Pathology at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

A handwritten signature in blue ink, appearing to read 'Mark Keyter', is written over a light blue rectangular background.

7th day of April 2021

DEDICATION

This is dedicated to my family and my lovely wife, Andrea, thank you all for your endless support and guidance.

ABSTRACT

A clinicopathological and molecular analysis of serrated lesions of the appendix and the prevalence of incidentally detected serrated lesions of the appendix seen in the Division of Anatomical Pathology, National Health Laboratory Services, Johannesburg, over a five-year period, was performed. To date, there is limited data on the molecular events in serrated proliferations of the appendix, with most data being extrapolated from studies on colorectal serrated proliferations.

The demographics of the patients and the histopathologic characteristics of the serrated lesions were evaluated. Molecular characterisation included immunohistochemistry, polymerase chain reaction and sequence analysis.

A total of ten cases were identified over a period of five years. Sessile serrated lesions/sessile serrated adenomas were seen in 0.14% of all the appendixes received over the five-year study period. All ten cases had characteristic morphological features consistent with a sessile serrated lesion/sessile serrated polyp. The molecular findings were inconclusive, with no definitive association with either BRAF V600E or KRAS codon 12 and 13 mutations. A single case contained a well-described KRAS codon 12 mutation. MLH-1 expression by immunohistochemistry was retained in all ten cases. Two of the ten patients showed microsatellite instability low (MSI-L), with both cases showing a dual peak at the BAT-25 locus. One of the sessile serrated lesions with the MSI-L also had associated BRAF mutations at nucleotide position 1732 (C>Y) and 1798 (A >W).

An overall higher occurrence of sessile serrated lesions compared to other serrated proliferations of the appendix was found. This study highlights the importance of alterations in the MAP Kinase pathway and subsequent microsatellite instability in the pathogenesis of serrated proliferations in the appendix.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr Ewen McAlpine, thank you for all your assistance and constructive criticism throughout this process.

To the National Health Laboratory Service Research Trust, thank you for the financial assistance, enabling the completion of this research.

TABLE OF CONTENTS

DECLARATION	ii
DEDICATION	iii
ABSTRACT	iv
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
LIST OF ABBREVIATIONS	vii
LIST OF FIGURES	viii
LIST OF TABLES	xv
CHAPTER 1: INTRODUCTION	1
1.1 THE VERMIFORM APPENDIX	1
1.2 COLORECTAL SERRATED LESIONS AND POLYPS	1
1.2.1 HYPERPLASTIC POLYPS	2
1.2.2 SESSILE SERRATED LESION	3
1.2.3 SESSILE SERRATED LESION WITH DYSPLASIA	4
1.2.4 TRADITIONAL SERRATED ADENOMAS	5
1.2.5 UNCLASSIFIED SERRATED ADENOMAS	5
1.3 THE SERRATED NEOPLASIA PATHWAY	6
1.3.1 EPIGENETIC DNA PROMOTER HYPERMETHYLATION AND CPG ISLAND METHYLATOR PHENOTYPE (CIMP)	7
1.3.2 BRAF AND KRAS: THE MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) SIGNALLING PATHWAY	8
1.3.3 MICROSATELLITE INSTABILITY – THE ROLE OF THE DNA MISMATCH REPAIR SYSTEM	9
1.3.4 THE WNT SIGNALLING PATHWAY	11
1.4 SERRATED LESIONS OF THE APPENDIX	11
CHAPTER 2: AIMS	17
CHAPTER 3: MATERIALS AND METHODS	18
3.1 CASE SELECTION	18
3.2 PATHOLOGICAL FEATURES	19
3.3 BRAF V600E AND MLH1 IMMUNOHISTOCHEMISTRY	19
3.4 BRAF EXON 15 (V600E) AND KRAS CODON 12 AND 13 SEQUENCE ANALYSIS	21
3.4.1 DNA EXTRACTION	21

3.4.2	<i>BRAF</i> EXON 15 (V600E) SEQUENCE ANALYSIS:	21
3.4.3	<i>KRAS</i> CODON 12 AND 13 SEQUENCE ANALYSIS:	22
3.5	MICROSATELLITE INSTABILITY (MSI) ANALYSIS	23
3.6	STATISTICAL ANALYSIS	24
CHAPTER 4: RESULTS		25
4.1	CLINICAL FEATURES	25
4.2	PATHOLOGICAL FINDINGS	26
4.3	IMMUNOHISTOCHEMICAL AND MOLECULAR CHARACTERIZATION OF SERRATED APPENDICEAL LESIONS	29
4.3.1	<i>BRAF</i> MUTATIONS	29
4.3.2	<i>KRAS</i> MUTATIONS	31
4.4	MLH-1 EXPRESSION AND MICROSATELLITE INSTABILITY ANALYSIS	32
CHAPTER 5: DISCUSSION		35
CHAPTER 6: CONCLUSION		46
REFERENCES		47
APPENDICES		51
APPENDIX A:	HISTOLOGICAL FEATURES USED TO CLASSIFY THE SERRATED APPENDICEAL LESIONS	51
APPENDIX B:	<i>BRAF</i> (196NT FRAGMENT) SEQUENCE ANALYSIS	52
APPENDIX C:	<i>KRAS</i> (173NT FRAGMENT) SEQUENCE ANALYSIS	53
APPENDIX D:	ETHICS CLEARANCE CERTIFICATE	54
APPENDIX E:	TURN IT IN REPORT	55

LIST OF ABBREVIATIONS

APC:	Adenomatous polyposis coli
BRAF:	V-raf murine sarcoma viral oncogene homolog B1
CIMP:	CpG island methylator phenotype
CIMP-H:	CpG island methylator phenotype-High
CIMP-L:	CpG island methylator phenotype-Low
CIN:	Chromosomal instability
CMJAH:	Charlotte Maxeke Johannesburg Academic Hospital
dMMR:	DNA mismatch repair
GCHP:	Goblet cell-rich hyperplastic polyp
HNPCC:	Hereditary non-polyposis colorectal cancer
KRAS:	Kirsten rat sarcoma viral oncogene
LAMN:	Low grade appendiceal mucinous neoplasm
MAP:	Mitogen-activated protein
MAPK:	Mitogen-activated protein kinase
MGMT:	O ⁶ -methylguanine methyltransferase
MLH-1:	MutL homolog 1
MLH-3:	MutL homolog 3
MMR:	Mismatch repair
MSH-2:	MutS homolog 2
MSH-3:	MutS homolog 3
MSH-6:	MutS homolog 6
MSI:	Microsatellite instability
MSS:	Microsatellite stable
MVHP:	Microvesicular hyperplastic polyp
NHLS:	National Health Laboratory Service
PCR:	Polymerase chain reaction
PMS-2:	PMS1 Homolog 2
RAS:	Rat sarcoma viral oncogene
SSA/P:	Sessile serrated adenoma/polyp
SSL:	Sessile serrated lesion
TSA:	Traditional serrated adenoma
WHO:	World Health Organization

LIST OF FIGURES

Chapter 1:

- Figure 1.1.** Two morphological pathways in colorectal carcinoma, the conventional adenoma-carcinoma pathway, and the serrated neoplasia pathway. 7

Chapter 4:

- Figure 4.1.** Age distribution of the cases. The age distribution shows a right shift, with all cases above 20 years of age. 25
- Figure 4.2** Key histological findings in the sessile serrated lesions. 28
- Figure 4.3** *BRAF* V600E immunohistochemistry showing positive, 2+ cytoplasmic staining of the cytoplasm. 30
- Figure 4.4** Chromatogram sequence showing wild type sequence GTG (nt 1798-1800), in the single case which had moderate intensity cytoplasmic BRAFV600E staining 30
- Figure 4.5** Chromatogram sequence of the *KRAS* gene showing a mixed G and T population at nucleotide position 34 (codon 12). 32
- Figure 4.6** Microsatellite stable (MSS) pattern, with all four amplicons (NR-27, NR-21, NR-24 and BAT-25) showing single peaks. 33
- Figure 4.7** Microsatellite instability low (MSI-L) pattern showing a dual peak (allelic variation) at the BAT-25 amplicon. 34

LIST OF TABLES

Chapter 3:

Table 3.1	Primer sequences used for the MSI assay.	24
------------------	--	----

Chapter 4:

Table 4.1	Clinical and histological features of the study group with incidentally detected serrated lesions of the appendix.	26
Table 4.2	Summary of the microscopic findings of the serrated lesions of the appendix.	27
Table 4.3	<i>BRAF</i> V600E mutation and additional incidental <i>BRAF</i> mutations in the serrated appendiceal lesions.	31
Table 4.4	<i>KRAS</i> codon 12 and 13 mutations and additional <i>KRAS</i> mutations in the serrated appendiceal lesions.	32
Table 4.5	Summary of the microsatellite instability findings using the pentaplex polymerase chain reaction.	33

CHAPTER 1: INTRODUCTION

1.1 THE VERMIFORM APPENDIX

The vermiform appendix arises from the medial wall of the cecum (Mills, 2012). Within the right iliac fossa, it can be found in a retro-caecal, pelvic, sub-caecal, pre-ileal, retro-ileal or para-caecal position (Ali Malas *et al.*, 2004). The vermiform appendix has no known function and is currently regarded as a vestigial organ. The appendix reaches its maximum length by four years of age, with subsequent narrowing, secondary to fibrosis, in adulthood (Ali Malas *et al.*, 2004). The layers of the appendix are similar to that of the large bowel, except for the abundance of lymphoid tissue in the mucosa and irregularity of the crypts (Mills, 2012).

The overlying epithelium is columnar, with absorptive cells, goblet cells, neuroendocrine cells and occasional Paneth cells (Katzin and Petras, 2012). The submucosal, muscular and serosal layers are similar to their counterparts in the rest of the lower gastrointestinal tract. Within the muscularis propria, ganglion cells are usually easily identifiable. The mesoappendix is attached to the appendix. It is made up of adipose tissue, it is continuous with the mesentery of the bowel and it contains branches of the appendiceal artery (Mills, 2012).

Due to its inaccessibility by routine endoscopy, there is limited information regarding the biological evolution of lesions of the appendix, particularly, mucosal-based lesions. Data from findings in the gastrointestinal tract are often extrapolated to the appendix, however, this may not be a true reflection of the biological nature of lesions of the appendix. As is the case for serrated lesions of the appendix, which may have different biological and molecular alterations compared to their colorectal counterparts.

1.2 COLORECTAL SERRATED LESIONS AND POLYPS

Colorectal serrated lesions and polyps are characterised by a serrated or sawtooth architecture of the epithelium (Bettington *et al.*, 2014). The terminology and classification of these lesions has been fraught with confusion over the years. The recommended classification includes five groups including; hyperplastic polyp, sessile serrated lesion, sessile serrated lesion with

dysplasia, traditional serrated adenoma and unclassified serrated adenoma (Pai, Makinen and Rosty, 2019). Recently, as per the World Health Organization (WHO) Classification of Tumours of the Digestive System, the terms sessile serrated polyp/adenoma have been replaced with sessile serrated lesion. To avoid confusion, the terms sessile serrated lesion and sessile serrated polyp/adenoma will be used interchangeably and will refer to the same lesion.

The serrated lesions of the colon have variable morphology, are molecularly heterogeneous and differ in their risk of progression to malignant transformation and invasive carcinoma.

1.2.1 Hyperplastic polyps

Hyperplastic polyps are divided into two subtypes, microvesicular hyperplastic polyps (MVHP) and goblet cell-rich hyperplastic polyps (GCHP). The majority of MVHP and GCHP are found within the distal colon and rectum. Endoscopically, hyperplastic polyps are small, usually less than 5 mm, with a single stellate pit (Kudo *et al.*, 1996).

MVHP have a serrated epithelial surface with the serrations limited to the upper two-thirds of the crypt (Pai *et al.*, 2019). The crypts are evenly spaced with a funnel-shaped architecture. This leads to a dilated crypt at the surface, with narrowing of the crypt towards the base, which rests on the muscularis mucosa (Pai *et al.*, 2019). In cross-section, the serrated crypts have a uniform stellate appearance (Pai *et al.*, 2019). There is no basal dilatation or submucosal displacement. The proliferation zones are typically confined to the base of the crypts (Bettington *et al.*, 2017). The cells lining the crypts have abundant, fine apical vacuoles with microvesicular mucin and small, basally-located nuclei. There are variable numbers of intervening goblet cells.

GCHPs were originally described by Torlakovi and colleagues (Torlakovic *et al.*, 2003). These polyps are small, with very subtle morphological alterations that are often overlooked as epithelial hyperplasia or a reactive proliferation (Pai *et al.*, 2019). The crypts are taller and wider than normal crypts and may show occasional branching or tortuosity. In cross-section, the crypt lumina are round rather than stellate, an important feature differentiating GCHPs from MVHPs (Bettington *et al.*, 2014). Similar to MVHPs, the serrations are limited to the upper

two-thirds of the crypts. The lining epithelial cells are predominantly goblet cells, with uniform nuclei, and a luminal based globoid intracytoplasmic vacuole.

1.2.2 Sessile serrated lesion

Sessile serrated lesions have a prolonged history with variable terminology. Since 2003, this serrated proliferation has been referred to as; sessile serrated adenoma, sessile serrated polyp and sessile serrated polyp/adenoma (Bettington *et al.*, 2017). Recently, this serrated proliferation has undergone a name change to sessile serrated lesion (Pai, Makinen and Rosty, 2019). Adenoma was included in the name to emphasise the possible progression to colorectal carcinoma. However, due to the lack of “adenomatous epithelium” in these polyps, certain groups have warned against the inclusion of the word adenoma, to prevent confusion (Pai *et al.*, 2019). Also, as these lesions are seldom polypoid, the use of polyp in the name has been criticised. Hence recent consensus has favoured the new terminology of sessile serrated lesion. To avoid confusion, throughout this manuscript, the terms sessile serrated lesion (SSL) and sessile serrated adenoma/polyp (SSA/P), will be used interchangeably.

Sessile serrated lesions occur predominantly within the proximal colon, appearing as an ill-defined, sessile, pale lesion on endoscopic examination (Hazewinkel *et al.*, 2013). Within sessile serrated lesions, proliferation centres are found at the crypt bases and irregular points along the crypt lumen (Carr *et al.*, 2017; Pai *et al.*, 2019). From the proliferation centres, the epithelial cells spread both basally and luminally (Carr *et al.*, 2017). This gives rise to the characteristic histological features seen in sessile serrated lesions (Pai, Makinen and Rosty, 2019). These histological features include aberrant crypt dilatation with horizontal growth along the muscularis mucosae and exaggerated serrations extending along the entire length of the crypt, extending into the base (Pai, Makinen and Rosty, 2019). Although these characteristic features are easily recognised, the majority of crypts within a sessile serrated lesion don't show these features (Pai *et al.*, 2019). For this reason, a single unequivocal crypt, with typical pathological findings, is sufficient for the diagnosis of a sessile serrated lesion (Pai, Makinen and Rosty, 2019). The lining epithelial cells comprise a mixture of goblet cells, cells with microvesicular mucin and enterocytes (Pai, Makinen and Rosty, 2019). There are often associated foci of neuroendocrine hyperplasia (Pai *et al.*, 2019).

1.2.3 Sessile serrated lesion with dysplasia

A transient step, in the form of dysplasia, may develop in the sessile serrated lesions. The lesions with dysplasia are generally found within the proximal colon (Pai *et al.*, 2019). The presence of dysplasia is associated with increasing age and size of the sessile serrated lesion (Pai *et al.*, 2019). However, the overall occurrence of dysplasia within these lesions is rare, representing only 2-5% of all serrated proliferations of the colon (Pai *et al.*, 2014)

Those serrated lesions with dysplasia are often more protuberant with endoscopic features resembling conventional adenomas (Pai *et al.*, 2019). The presence of dysplasia generally appears as an abrupt area within the sessile serrated lesion. The dysplastic area shows increased complexity in the crypt architecture with associated atypia (Pai *et al.*, 2019). These architectural changes include a more villous-like growth pattern, elongation and crowding of the crypts and complex branching of the crypts (Pai, Makinen and Rosty, 2019). There is often increased luminal serration within the areas of dysplasia (Pai, Makinen and Rosty, 2019). Cytologically, the cells may take the form of two types of dysplasia, either the adenomatous/intestinal type or the serrated type. The serrated type of dysplasia has cells with round atypical nuclei, prominent nucleoli and eosinophilic cytoplasm (Pai *et al.*, 2019). Recently, additional patterns of dysplasia have been described, highlighting the morphologic variety of sessile serrated lesions with dysplasia (Liu *et al.*, 2017). Often, multiple morphological patterns of dysplasia may occur within a sessile serrated lesion (Pai *et al.*, 2019).

An important event in the pathogenesis of dysplasia is the loss of MLH-1 expression, which leads to the impaired mismatch repair function and subsequent microsatellite instability. In subtle cases, loss of MLH-1 expression within areas worrisome for dysplasia may be helpful (Liu *et al.*, 2017). Loss of MLH-1 expression generally involves the full length of multiple adjacent crypts (Liu *et al.*, 2017). This should not be confused with the incidental loss of MLH-1 expression restricted to the base of occasional crypts (Pai *et al.*, 2019). Another pitfall with the use of MHL-1 is that certain dysplastic patterns may not be MLH1-deficient (Liu *et al.*, 2017). This emphasises the morphological evaluation in the assessment of dysplasia.

1.2.4 Traditional serrated adenomas

Traditional serrated adenomas (TSA) are the least common type of serrated polyps, representing 2% of all colorectal polyps (Bettington and Chetty, 2015). The distal colon and rectum are the most common sites. During endoscopic evaluation, these serrated adenomas appear as large protuberant polyps (Bettington *et al.*, 2014).

Traditional serrated adenomas were originally described by Longacre and Fenoglio-Preiser in 1990 (Longacre and Fenoglio-Preiser, 1990). The prototypical morphology includes a villiform architecture, uniform cytology, slit-like serrations and numerous ectopic crypt foci (Pai *et al.*, 2019). Ectopic crypt foci resemble the base of normal crypts (Pai *et al.*, 2019). They appear as small buds of epithelial cells along the luminal aspect of the crypt. Although a common finding in traditional serrated adenomas, ectopic crypt foci are not required for the diagnosis. Ectopic crypt foci may be seen in tubulo-villous adenomas (Bettington *et al.*, 2016). The lining epithelial cells have abundant eosinophilic cytoplasm, with bland oval palisaded nuclei (Pai *et al.*, 2019).

Proximal traditional serrated adenomas often harbour *BRAF* mutations, whereas distal ones are often *KRAS*-mutated (Pai *et al.*, 2019). Dysplasia may develop in advanced lesions, and when present, can either take the form of intestinal type, resembling dysplasia in conventional adenomas, or serrated type dysplasia (Pai *et al.*, 2019).

1.2.5 Unclassified serrated adenomas

Unclassified serrated adenomas have been increasingly recognised over the past few years (Pai, Makinen and Rosty, 2019). These serrated polyps may have overlapping features of sessile serrated lesions, traditional serrated adenoma and conventional tubulo-villous adenomas. Some authors have termed these polyps with a mixed appearance as serrated tubulo-villous adenomas (Bettington *et al.*, 2016). While other groups believe these polyps are simply traditional serrated adenomas overgrown by conventional adenomatous type dysplasia (Pai, Makinen and Rosty, 2019). Together with the variable histomorphology seen among serrated colorectal lesions, there is also increasingly variable molecular pathways driving these serrated proliferations.

1.3 THE SERRATED NEOPLASIA PATHWAY

Currently, three molecular pathways of colorectal carcinogenesis are recognised, the chromosomal instability pathway, the microsatellite instability pathway and the serrated neoplasia pathway. Although separately defined, there is overlap between these pathways, both at the molecular level and with overlapping histological findings.

The chromosomal instability pathway derives from the classic adenoma-carcinoma sequence, which involves a transition from normal mucosal epithelial cells to adenomas and subsequent carcinomas (Müller, Ibrahim and Arends, 2016).

The microsatellite instability pathway arises due to defects in the mismatch repair (MMR) genes, resulting in the accumulation of microsatellites, leading to dysplasia and ultimately invasive carcinoma (Vilar and Gruber, 2010). This accounts for approximately 15% of colorectal carcinomas (Eshleman and Markowitz, 1996). Of this 15%, 12% arise sporadically, most commonly due to epigenetic silencing of the MLH1 gene, and 3% occur due to germline mutations in one of the four mismatch repair genes, MLH1, MSH2, MSH6, PMS2 (Liu *et al.*, 2017).

The third pathway, the serrated neoplasia pathway is complex, with a large amount of overlap with the microsatellite instability pathway (Müller, Ibrahim and Arends, 2016). The serrated neoplasia pathway has three key characteristics. These include aberrant hypermethylation of promoter regions, leading to downstream microsatellite instability, alterations in MAP kinase signalling pathway and activation of the Wnt signalling pathway (Müller, Ibrahim and Arends, 2016). The key events in these three molecular pathways are highlighted in Figure 1.1. The initiating events and molecular characteristics of the serrated neoplasia pathway are discussed below.

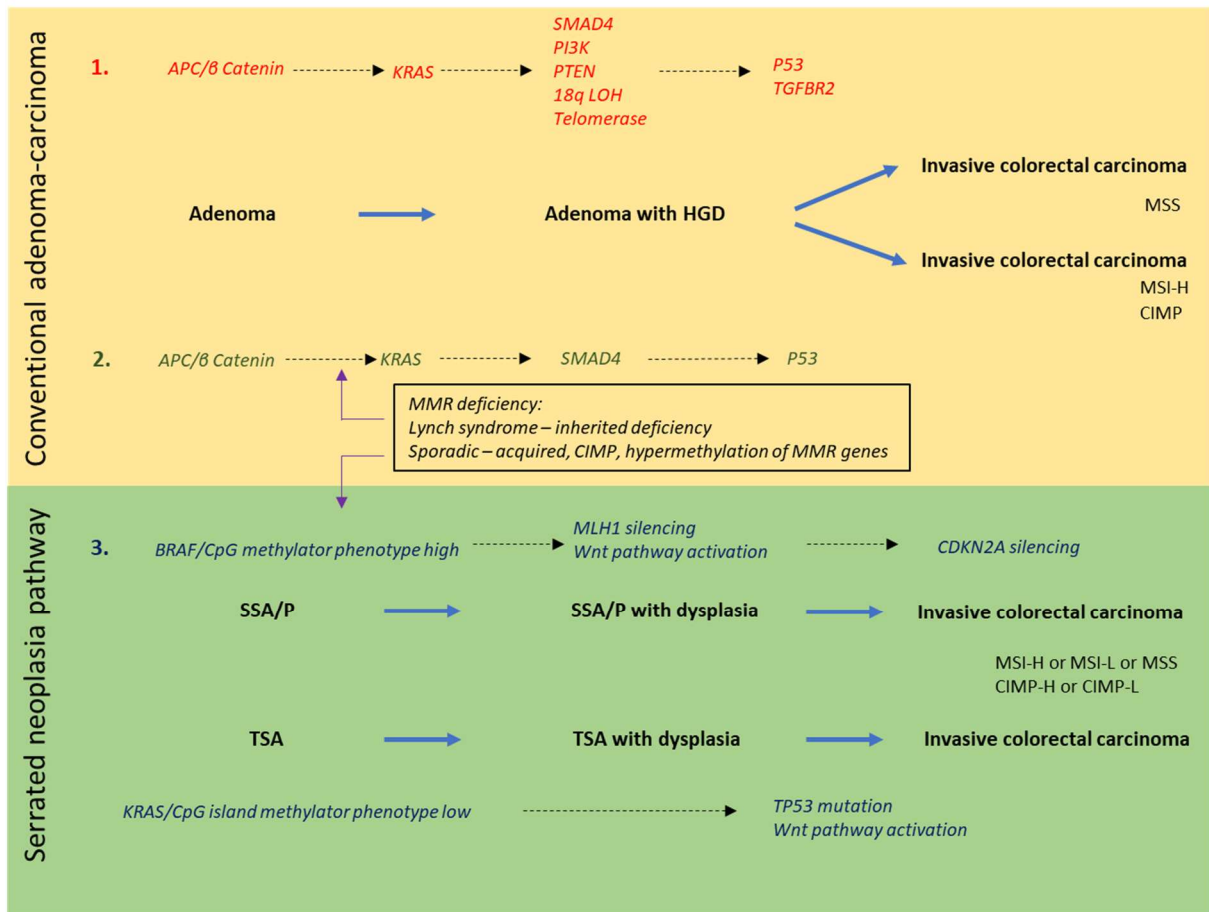


Figure 1.1. Two morphological pathways in colorectal carcinoma, the conventional adenoma-carcinoma pathway and the serrated neoplasia pathway. The three molecular carcinogenesis pathways include (1) chromosomal instability (CIN) pathway, (2) defective DNA mismatch repair leading to microsatellite instability (MSI), and (3) epigenetic DNA promoter hypermethylation leading to the CpG island methylator phenotype (CIMP). Defects in the MMR pathway can be inherited or acquired sporadically, give rise to MSI, which can progress via either to conventional adenoma-carcinoma pathway or via the serrated neoplasia pathway. Information compiled from (Kudo *et al.*, 1996; Yamane *et al.*, 2014; Bettington *et al.*, 2016; Bettington, Brown and Rosty, 2016; Müller, Ibrahim and Arends, 2016).

1.3.1 Epigenetic DNA promoter hypermethylation and CpG island methylator phenotype (CIMP)

Gene expression can be modified via histone modification and/or modification of promoter sequence methylation status (Yamane *et al.*, 2014). DNA methylation occurs via the addition of a methyl group to a cytosine base (Eshleman and Markowitz, 1996). This is carried out via

DNA methyltransferases, which include de novo DNA methyltransferases and a maintenance DNA methyltransferase (Yamane *et al.*, 2014).

Within the human genome, the combination of cytosine followed by guanine (so-called CpG dinucleotides) is uncommon within the coding region of genes (Yamane *et al.*, 2014). CpG islands, which are clusters of CpG dinucleotides, are found in the promoter region of numerous regulatory genes (Kane *et al.*, 1997). CpG island methylation, particularly within promoter regions, is a normal cell regulatory mechanism of gene expression (Kane *et al.*, 1997). Hypermethylation of the *MLH1* gene leads to impaired DNA-mismatch repair and microsatellite instability. This occurs within the traditional adenoma-carcinoma sequence and the serrated neoplasia pathway (Kane *et al.*, 1997).

There are variable degrees of DNA methylation, leading to the terms, CpG island methylator phenotype – High (CIMP-H), and CpG island methylator phenotype – Low (CIMP-L). These differ from one another based on the degrees of methylation (Müller, Ibrahim and Arends, 2016). The mechanisms leading to CIMP-H and CIMP-L phenotypes, specifically those in the serrated neoplasia pathway, have not been fully elucidated. Recently, however, the role of BRAF and KRAS mutations affecting the methylation of certain promoter regions has been described (Müller, Ibrahim and Arends, 2016).

1.3.2 BRAF and KRAS: The mitogen-activated protein kinase (MAPK) signalling pathway

BRAF and *KRAS* are key regulatory proteins in the mitogen-activated protein kinase (MAPK) signalling pathway (Yamane *et al.*, 2014). The role of *BRAF* in promoting DNA hypermethylation has recently become apparent, whereas the role of *KRAS* is less fully understood and seems to be more related to the development of colorectal cancer via traditional serrated adenoma (TSA) precursor lesions displaying a CIMP-L phenotype (O'Brien, Zhao and Yang, 2015).

The identification of *BRAF* mutations, together with CpG-island methylator phenotype in the serrated neoplasia pathway has led to increased research to determine the link between the two. Although high levels of CpG island methylation have been observed, the cause of this has remained unanswered, until recently. The initiating role of *BRAF* has been demonstrated in *BRAF* V600E knock-in mice, in which it was shown that *BRAF* V600E initiates the serrated neoplasia pathway and progression to colon adenocarcinomas in the mice with the *BRAF* V600E knock-in mutation (Rad *et al.*, 2013). Although this showed the importance of *BRAF* in initiating the serrated neoplasia pathway, how *BRAF* mutations cause promoter hypermethylation and CIMP remained unclear. A new study demonstrated the role of *BRAF* mutant proteins in promoter CpG island methylation (Fang *et al.*, 2014). These mutant *BRAF* proteins guide the assembly of the MAFG co-repressor to specific promoter regions, leading to increased cytosine methylation (Fang *et al.*, 2014). Although this is only a single study, it does provide a putative link between *BRAF* and CpG island methylation.

Following the development of *BRAF* mutations and CpG island methylation, there may be a decreased expression of DNA-mismatch repair genes. This leads to downstream microsatellite instability. Thereby providing a possible link with certain DNA-mismatch repair genes and the development of microsatellite instability in the serrated neoplasia pathway.

1.3.3 Microsatellite instability – the role of the DNA mismatch repair system

Dysfunction of the DNA mismatch repair (dMMR) system and subsequent microsatellite instability (MSI) has been well documented in the traditional adenoma-carcinoma pathway of colorectal cancer (Chen, Swanson and Frankel, 2017). This abnormal functioning of the dMMR pathway occurs in sporadic cases of colorectal carcinoma and colorectal carcinoma occurring due to hereditary associated germline mutations (HNPCC/Lynch syndrome) (Chen, Swanson and Frankel, 2017). Recently an overlap with the serrated neoplasia pathway has been observed, in which certain serrated lesions and colorectal carcinomas arising from the serrated neoplasia pathway, have also shown features of microsatellite instability (O'Brien, Zhao and Yang, 2015).

The dMMR pathway is one of the pathways used by cells to repair incorrectly inserted nucleotides during DNA replication (Chen, Swanson and Frankel, 2017). The proteins

functioning within the dMMR system include MLH1, PMS2, MSH2, MSH6, MLH3, MSH3, PMS1 and EXO1 (Chen, Swanson and Frankel, 2017). The dMMR proteins detect DNA replication errors that occur during the S-phase of the cell cycle (Chen, Swanson and Frankel, 2017). These replication errors are mainly in the form of nucleotide mismatches occurring between the two strands of DNA. The dMMR proteins form heterodimers around the DNA strand, excise the incorrectly inserted nucleotide and replace it with the correct one (Chen, Swanson and Frankel, 2017). In colorectal carcinomas, the MLH1/PMS2 and MSH2/MSH6 heterodimers are the most commonly implicated in the carcinogenesis of microsatellite unstable colorectal carcinomas (Chen, Swanson and Frankel, 2017).

Microsatellites consist of mono- to hexa-nucleotides that constitute a repeated motif (Chen, Swanson and Frankel, 2017). Microsatellites are highly susceptible to incorrect base pairing of nucleotides. If the dMMR is dysfunctional, there is an increased risk for incorrect base pairing and mutational changes within these microsatellites (Chen, Swanson and Frankel, 2017). When this happens, microsatellite instability occurs. This is characterised by alterations in the number of mono- or di-nucleotide repeats in coding or regulatory sequences (Chen, Swanson and Frankel, 2017).

Although microsatellite instability is well known in the traditional adenoma-carcinoma pathway of colorectal cancer, its involvement in the serrated neoplasia pathway has only recently been described. As mentioned previously, serrated precursor lesions progress along a sequence of events involving mutations in BRAF or KRAS, with the additional CIMP in some cases. An important step involved in the transition to dysplasia and subsequent carcinoma, is CpG island methylation, particularly methylation induced silencing of the dMMR gene *MLH1* (Liu *et al.*, 2017). This leads to impaired functioning of the dMMR system, causing an increase in mutations in these microsatellite regions. This leads to the phenotype of microsatellite instability (Borowsky *et al.*, 2018) Microsatellite instability is heterogeneous within tumours, with some tumours showing higher degrees of microsatellite instability when compared to others (Liu *et al.*, 2017).

Serrated precursor lesions and colorectal carcinomas, that develop from the serrated neoplasia pathway, have variable degrees of microsatellite instability. Tumours showing a high level of

microsatellite instability are referred to as microsatellite instability-high (MSI-H) tumours, and those that show less, or absent microsatellite instability are referred to as microsatellite instability – low (MSI-L), and microsatellite stable (MSS) tumours, respectively (Yamane *et al.*, 2014).

Tumours arising predominantly in the background of BRAF mutations and high levels of CpG island methylation (*i.e.* the CIMP phenotype), tend to be MSI-H, whereas those arising in the background of KRAS mutations, generally tend to be MSI-L or MSS (Chen, Swanson and Frankel, 2017).

1.3.4 The Wnt signalling pathway

Abnormal Wnt signalling in the sessile serrated neoplasia pathway has also recently been proposed (Borowsky *et al.*, 2018). In the conventional adenoma-carcinoma sequence, where it is well described, activation most commonly occurs through mutations of two key proteins, namely, adenomatous polyposis coli (APC) and β -catenin (Fu *et al.*, 2012). In the serrated neoplasia pathway, activation occurs via a different mechanism. Here, activation of Wnt signalling seems to occur secondary to epigenetic changes, more specifically, methylation induced silencing of key regulators proteins, SFRP4, MCC, and AXIN (Murakami *et al.*, 2015). Activation of the Wnt signalling pathway occurs as a late event in the serrated neoplasia pathway, often associated with serrated proliferations with dysplasia (Borowsky *et al.*, 2018).

1.4 SERRATED LESIONS OF THE APPENDIX

Serrated lesions of the colon and rectum have been extensively evaluated. However, there is a paucity of studies on similar serrated lesions in the appendix. Serrated epithelial lesions of the appendix show similar histological features to the serrated epithelial lesions in the colon. However, this is based on findings in case reports and small series. The main reason for the lack of data on appendiceal serrated lesions is the inaccessibility of the appendix by routine endoscopy (Yantiss *et al.*, 2007). Also, most serrated lesions of the appendix are usually incidentally discovered during a routine appendectomy for acute appendicitis, or at a later stage

with advanced progression to invasive adenocarcinoma. Hence, the natural history of these lesions is poorly defined.

Another factor that has compounded systematic assessment of these is the variable terminology used to describe them over the years, including mucosal metaplasia, mucosal hyperplasia (focal or diffuse), hyperplastic polyp, mixed adenomatous/hyperplastic lesion, serrated adenoma and sessile serrated adenoma (Pai *et al.*, 2019). The use of variable terminology has affected accurate comparative analysis of serrated lesions of the appendix (Pai *et al.*, 2014).

The incidence and the prevalence of serrated proliferations of the appendix are difficult to assess since most of these lesions are incidentally discovered in appendicectomy specimens removed for suspected acute appendicitis. Previous studies have reported an incidence of between 0.002-0.1% (Renshaw, Kish and Gould, 2006; Yantiss *et al.*, 2007; Pai *et al.*, 2014). Serrated appendiceal lesions occur in men and women with an equal frequency and occur more commonly in older adults, usually in the sixth to eighth decades of life (Yantiss *et al.*, 2007; Pai *et al.*, 2014).

Most pathologists have applied colorectal terminology to precursor lesions of the appendix. This includes hyperplastic polyp (both microvesicular and goblet cell variants), sessile serrated polyp/adenoma, sessile serrated polyp with dysplasia, traditional serrated adenoma and adenoma for the conventional adenomatous polyp. This has been maintained to a large extent in the latest WHO Classification of Tumours of the Digestive System (Pai, Makinen and Rosty, 2019). The new terminology of sessile serrated lesion rather than sessile serrated polyp/adenoma has also been applied to lesions occurring in the appendix (Pai, Makinen and Rosty, 2019). Implementation of this terminology has improved uniformity and reproducibility among investigative groups (Pai, Makinen and Rosty, 2019). Although this has improved uniformity among study groups, there remains a paucity of clinical data and assessment of histomorphological features of serrated lesions of the appendix, with most publications being case reports, with a few additional series (Longacre and Fenoglio-Preiser, 1990; Yantiss *et al.*, 2007; Bellizzi *et al.*, 2010).

Of the few publications on serrated appendiceal lesions, most occur in older adults and show an equal sex distribution (Yantiss *et al.*, 2007). Another study also showed hyperplastic polyps occurring in a slightly younger age group compared to sessile serrated polyps (Karabulut *et al.*, 2014). The occurrence of hyperplastic and sessile serrated polyps varies among reports, with a slight predominance of hyperplastic polyps compared to sessile serrated lesions reported by one group of investigators (Yantiss *et al.*, 2007). Another study from Turkey also showed a slight predominance of hyperplastic polyps (50.7%), compared to sessile serrated polyps (46.4%) and traditional serrated adenomas (2.1%) (Karabulut *et al.*, 2014). This differs from a recent study by Pai and colleagues, who showed a predominance of sessile serrated polyps compared to hyperplastic polyps and traditional serrated adenomas (Pai *et al.*, 2014).

The presence of dysplasia varies in the different types of serrated polyps. Yantiss and colleagues reported 38% of their cases of serrated appendiceal lesions had dysplasia (Yantiss *et al.*, 2007). Of these, most cases with dysplasia occurred in serrated adenomas, which was a generic term used in their study for serrated lesions not fulfilling the criteria for a sessile serrated polyp (Yantiss *et al.*, 2007). They found only five of the 20 sessile serrated polyps showing areas of dysplasia (Yantiss *et al.*, 2007). In those cases with dysplasia, the serrated adenomas and sessile serrated polyps were on average larger than those without dysplasia (Yantiss *et al.*, 2007). All cases showed conventional adenomatous type dysplasia (Yantiss *et al.*, 2007). Another study from Turkey also reported dysplasia in 31.4% of sessile serrated polyps, with no dysplasia in the hyperplastic polyps (Karabulut *et al.*, 2014). Again, the foci of dysplasia were typical of more conventional adenomatous type dysplasia, with pseudostratification and elongated pencillate nuclei (Karabulut *et al.*, 2014).

Most often, the appendiceal serrated proliferations are seen in isolation, without an associated invasive adenocarcinoma. In one study, of the 54 serrated appendiceal lesions, only four had an associated invasive adenocarcinoma (Yantiss *et al.*, 2007). All four cases contained invasive intestinal-type glands, with one case showing focal luminal serration (Yantiss *et al.*, 2007). Pai and colleagues also showed a similar occurrence of invasive adenocarcinoma, with four of the 46 serrated polyps having an associated focus of invasive adenocarcinoma (Pai *et al.*, 2014). Of the four cases showing invasive adenocarcinoma, three of them arose from what the authors described as traditional serrated adenoma (TSA)-like appendiceal polyp (3/7, 43%) (Pai *et al.*,

2014). These TSA-like lesions may be a common precursor lesion for invasive non-mucinous adenocarcinoma of the appendix (Pai *et al.*, 2014).

Due to the lack of clinical and histomorphologic characterisation of serrated appendiceal lesions, there is a paucity of data regarding the molecular events in these serrated lesions. To date, only limited series have been published evaluating the molecular events in appendiceal serrated lesions (Bellizzi *et al.*, 2010, Yantiss *et al.*, 2007, Pai *et al.*, 2014). The largest of which was by Yantiss and colleagues, who compared 56 appendiceal serrated polyps to 17 appendiceal mucinous adenomas (Yantiss *et al.*, 2007). This study provided the most extensive molecular analysis of serrated epithelial lesions of the appendix. They found *BRAF* mutations in 29% and *KRAS* mutations in 34% of their cases classified as “serrated appendiceal lesions” (Yantiss *et al.*, 2007).

In another study by Pai and colleagues, a higher occurrence of *KRAS* mutations compared to *BRAF* mutations was found (Pai *et al.*, 2014). Of the 123 cases analysed, *KRAS* mutations were identified in 64 (52%) of them (Pai *et al.*, 2014). Most cases demonstrated codon 12 mutations compared to codon 13 mutations (Pai *et al.*, 2014). There was no significant difference in the presence of *KRAS* codon 12 and 13 mutations in serrated lesions without dysplasia and those with dysplasia (Pai *et al.*, 2014). In addition, there was no difference in the frequency of *KRAS* mutations between lesions classified as hyperplastic polyps compared to those classified as sessile serrated adenomas/polyps (Pai *et al.*, 2014).

Another study evaluated the molecular features of serrated appendiceal lesions in patients with serrated polyposis (Bettington, Brown and Rosty, 2016). In their study of 34 serrated polyposis patients, *BRAF*(V600E) mutations were found in four of the sessile serrated adenomas/polyps and *KRAS* mutations were found in 11 of the polyps (48%). (Bettington, Brown and Rosty, 2016). *KRAS* and *BRAF* mutations are mutually exclusive and highlight the role of the RAS/RAF/MAPK pathway in both serrated lesions of the colon and the appendix.

As mentioned previously, serrated precursor lesions of the colon progress along a sequence of events involving mutations in *BRAF* or *KRAS*, with the additional CIMP in some cases. An

important step involved in the transition to dysplasia and subsequent carcinoma, is CpG island methylation, particularly methylation induced silencing of the dMMR gene *MLH1* (Liu *et al.*, 2017). This leads to impaired functioning of the dMMR system, causing an increase in mutations in these microsatellite regions, creating the phenotype of microsatellite instability. The role of impaired dMMR system and subsequent MSI has not been extensively evaluated in serrated lesions of the appendix.

Yantiss and colleagues evaluated MLH-1 and MSH-2 expression and subsequent MSI analysis in serrated appendiceal lesions and appendiceal mucinous adenomas (Yantiss *et al.*, 2007). In their study, loss of MLH-1 was found with an equal frequency in both non-dysplastic and dysplastic serrated appendiceal lesions (Yantiss *et al.*, 2007). They found that MLH-1 was found with an equal frequency in both hyperplastic polyps and sessile serrated adenomas (Yantiss *et al.*, 2007). Loss of MSH-2 protein expression was not identified in any of the serrated appendiceal lesions or the appendiceal mucinous adenomas (Yantiss *et al.*, 2007). None of the hyperplastic polyps or sessile serrated adenomas showed MSI-H (Yantiss *et al.*, 2007). One of the hyperplastic polyps showed features consistent with MSI-L, with a dual peak at the NR-21 locus (Yantiss *et al.*, 2007). There was no statistical difference between hyperplastic polyps and sessile serrated adenomas regarding MLH-1 expression and the occurrence of microsatellite instability (Yantiss *et al.*, 2007).

The equal distribution of MLH-1 expression among non-dysplastic and dysplastic serrated appendiceal lesions, together with the absence of MSI in the dysplastic polyp should be noted. As this may indicate possible alternative molecular events in the development of dysplasia and invasive adenocarcinoma in serrated appendiceal lesions. The role of abnormal Wnt signalling in the sessile serrated neoplasia pathway has also recently been proposed for colonic serrated proliferations (Borowsky *et al.*, 2018). Evaluation of the Wnt-signalling pathway in the appendix has not yet been evaluated.

As is readily apparent, the clinical, histomorphologic and molecular features of serrated appendiceal lesions are limited, and extrapolation with their colonic counterparts should be cautioned. In light of this, the possible relationship between serrated precursor lesions of the appendix and primary non-mucinous carcinoma of the appendix has been evaluated (Misraji

and Young 2004, Tang *et al.*, 2008). However, both were small series with inconclusive findings. This highlights the need for further analysis of serrated lesions of the appendix and their possible role in the development of appendiceal adenocarcinoma. This will shed light on the possible relationship with their colorectal counterparts, as well as the possible molecular events in serrated appendiceal lesions, and possibly their progression to dysplasia and invasive adenocarcinoma.

To date, evaluation of prevalence and histomorphology of serrated appendiceal lesions has not been investigated in South Africa. Importantly, there is a paucity of data on the molecular events in both colorectal and appendiceal serrated lesions in South Africa.

CHAPTER 2: AIMS

This study aimed to:

1. Determine the prevalence of incidentally detected serrated lesions of the appendix seen in the Division of Anatomical Pathology, National Health Laboratory Services, Johannesburg, over a five-year period, from January 2014-December 2018.
2. Describe the demographics of patients diagnosed with serrated lesions of the appendix, including; gender, age, clinical diagnosis of acute appendicitis and the presence of histologically diagnosed acute appendicitis.
3. Characterise the histopathologic characteristics of serrated lesions of the appendix using the current classification scheme for colorectal and appendiceal serrated lesions as per the 2019 WHO Classification of Tumours of the digestive system. (Pai, Makinen and Rosty, 2019).
4. Determine the molecular alterations of this set of serrated appendiceal lesions including, *BRAF* V600E mutation analysis, *KRAS* codon 12 and 13 mutation analysis, loss of MLH-1 expression, and detection of microsatellite instability using a pentaplex PCR assay of five mononucleotide markers.

CHAPTER 3: MATERIALS AND METHODS

3.1 CASE SELECTION

Following ethics approval (M190635), cases were identified via a retrospective database search using a Systematized Nomenclature of Medicine (SNOMED) based search and a free-text search, over a five-year period from January 2014 to December 2018. This included all cases reported by the Charlotte Maxeke Johannesburg Academic Hospital (CHJAH) branch of the National Health Laboratory Service (NHLS)/Anatomical Pathology Division, Faculty of Health Sciences, University of the Witwatersrand. The following search phrases were included; “Appendix”, “Polyp”, “Serrated”, “Adenoma”. A total of ten cases were identified using the above-listed criteria

To determine the prevalence of incidentally detected serrated lesions of the appendix seen in the Division of Anatomical Pathology, National Health Laboratory Services, Johannesburg, over a five-year period, a free-text search for the word “appendix” was used. The total number of appendixes received over the five-year period was calculated. This figure was then used to determine the prevalence of incidentally detected serrated appendiceal proliferations.

The patient age, gender, clinical features of acute appendicitis and the surgical procedure performed were obtained from the surgical pathology report. If a mass was seen macroscopically, and the size of the mass documented at the time of macroscopic processing, this was included in the analysis.

The haematoxylin and eosin-stained sections and the formalin-fixed paraffin-embedded tissue blocks were retrieved for all the cases. All cases that had an invasive adenocarcinoma, or had no residual serrated proliferation, were excluded. If there was insufficient tissue remaining in the blocks for additional ancillary testing, these cases were excluded.

A total of ten cases were identified using the above inclusion and exclusion criteria. Project specific ethics approval was granted by the Human Research Ethics Committee (Medical), Faculty of Health Sciences of the University of the Witwatersrand, (M190635).

3.2 PATHOLOGICAL FEATURES

All cases were reviewed by the principal investigator and the supervisor (Dr Ewen McAlpine), at a multi-header microscope and classified blindly based on the recently updated terminology for serrated lesions of the appendix, as set out in the new 2019 WHO Classification of Tumours of the Gastrointestinal Tract. This included; hyperplastic polyp, sessile serrated lesion, sessile serrated lesion with dysplasia, traditional serrated adenoma and unclassified serrated adenoma. The serrated appendiceal lesions were classified into one of these groups regardless of the initial diagnosis. The histological features used to classify the serrated lesion into one of these histological groups are outlined in Appendix A.

The presence or absence of dysplasia was recorded. Dysplasia was defined as any area resembling a conventional adenoma with pseudostratified, hyperchromatic nuclei or an area with large vesicular nuclei and prominent nucleoli. Although two types of dysplasia, namely, adenomatous/intestinal type and serrated type are defined, separation into one of these two subtypes was not performed.

Lastly, the presence of histologically confirmed acute appendicitis was recorded. Diagnostic criteria for appendicitis was defined as the presence of neutrophils within the muscularis propria (Kanishka *et al.*, 2014).

3.3 BRAF V600E AND MLH1 IMMUNOHISTOCHEMISTRY

BRAF V600E immunohistochemistry was performed on all cases using the VENTANA platform (Ventana, Roche). The MLH1 immunohistochemistry was performed using the Dako Envision™ FLEX detection system kit and Autostainer Link 48 Equipment (Dako, Glostrup, Denmark) according to the manufacturer's instructions. Two mouse monoclonal antibodies

were used. The ready-to-use (RTU) anti-BRAF V600E (VE1) mouse monoclonal antibody, (clone VE1, ref 790-4855, ready to use, Ventana, Roche) and the FLEX monoclonal mouse antibody against MLH1, at a dilution of 1:100 (clone ES05, ref NCL-L-MLH1).

The antibodies were applied to 4 µm thick formalin-fixed, paraffin-embedded tissue sections. Blockage of endogenous peroxidase activity was carried out with the EnVision FLEX Peroxidase-Blocking agent (Dako, Glostrup, Denmark) and antigen retrieval performed at 97°C for 20 minutes (pH 9.0). The antigen-antibody reaction was visualised with the DAB (Diaminobenzidine) solution and subsequently counterstained with haematoxylin.

For the BRAF V600E antibody, an invasive colorectal carcinoma with positive Anti-BRAF V600E (VE1) staining, was used as the positive control. MLH1 protein expression within the normal epithelium, lymphocytes and stromal cells was used as the positive control.

The staining intensity of anti-BRAF V600E (VE1) antibody in the serrated epithelial cells was recorded on a 0-3 scale. This was recorded as follows; 3 for strong intensity cytoplasmic staining, 2 for medium intensity cytoplasmic staining and 1 for weak intensity cytoplasmic staining. A score of 0 was given for a complete absence of staining. A positive result was defined as unequivocal, diffuse, uniform, cytoplasmic staining at an intensity of 2 or 3 in the majority of neoplastic cells. An equivocal score was given to all cases displaying ambiguous, heterogeneous, non-uniform cytoplasmic staining in the epithelial cells within the serrated area.

Negative protein expression of the MLH1 protein was defined as complete loss of nuclear staining within the epithelial cells in the serrated areas.

3.4 BRAF EXON 15 (V600E) AND KRAS CODON 12 AND 13 SEQUENCE ANALYSIS

3.4.1 DNA extraction

DNA was extracted from the tissue from formalin-fixed, paraffin-embedded (FFPE) blocks. The serrated lesion was marked on the tissue block and this area was micro-dissected from the FFPE block. Following micro-dissection, the tissue was added to 800 µl of distilled water and heated at 55°C for 1 hour to dissolve the wax. DNA was then extracted using the Zymo Quick-DNA Miniprep Plus kit (Zymogen, Irvine, CA, USA), as per the manufacturer's instructions.

3.4.2 *BRAF* exon 15 (V600E) sequence analysis

The extracted DNA was then used for the polymerase chain reaction (PCR) amplification of exon 15 of the *BRAF* gene. The primers were as follows, BRAF-F (sense: TCTTCATAATGCTTGCTCTGATAGGA) and BRAF-R (antisense: AACTCAGCAGCATCTCAGGG). The reaction mixture consisted of 10 µl NEB OneTaq 2X MasterMix with Standard Buffer (Catalogue No. M0482S), 1 µl of the BRAF-F primer (10 µM), 1 µl of the BRAF-R primer (10 µM) and 7 µl Nuclease free water (Catalogue No. E476). To this reaction mix, 1 µl of extracted genomic DNA was added, for a total volume of 20 µl. Amplification was performed using the Biorad MJ Mini Thermocycler (BioRad, USA) under the following cycling conditions; initial denaturation at 94°C for 5 min, followed by 35 cycles of, 94°C for 30 seconds, 50°C for 30 seconds and 68°C for 1 minute. A final extension step at 68°C for 10 minutes was also included.

Confirmation of correctly amplified PCR product was performed using a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye. Successful amplification yielded a single 199 bp fragment, which was subsequently sequenced. Before sequencing, the correctly amplified PCR products were cleaned using the ExoSAP Protocol as follows. The Exo/SAP master mix was prepared by adding 50 µl Exonuclease I (20 U/µl) (Catalogue No. NEB M0293L) and 200 µl Shrimp Alkaline Phosphatase (1 U/ul) (Catalogue No. NEB M0371) to a 0.6 ml micro-centrifuge tube. 2.5 µl of this Exo/SAP master mixed was then added to 10 µl of the amplified PCR product in a separate micro-centrifuge tube. This was

then incubated at 37°C for 15 minutes, followed by 80°C for 15 minutes to terminate the reaction.

The cleaned PCR fragments were sequenced using the Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000 according to manufacturer's instructions. The labelled products were then cleaned with the ZR-96 DNA Sequencing Clean-up Kit (Catalogue No. D4053) and the cleaned products injected on the Applied Biosystems ABI 3500XL Genetic Analyser with a 50 cm array, using POP7. Sequence chromatogram analysis was then performed using GeneStudio™ Professional sequence analysis software.

3.4.3 KRAS codon 12 and 13 sequence analysis

The extracted DNA was also used for the PCR amplification of the *KRAS* gene. The primers were as follows, KRAS-F (sense: AAGGCCTGCTGAAAATGACTG) and KRAS-R (antisense: CAAAGAATGGTCCTGCACCAG). The reaction mixture consisted of 10 µl NEB OneTaq 2X MasterMix with Standard Buffer (Catalogue No. M0482S), 1 µl of the BRAF-F primer (10µM), 1 µl of the BRAF-R primer (10µM) and 7 µl Nuclease free water (Catalogue No. E476). To this reaction mix, 1 µl of extracted genomic DNA was added, for a total volume of 20µl. Amplification was performed using the Biorad MJ Mini Thermocycler (BioRad, USA) under the following cycling conditions; initial denaturation at 94°C for 5 min, followed by 35 cycles of, 94°C for 30 seconds, 50°C for 30 seconds and 68°C for 1 minute. A final extension step at 68°C for 10 minutes was also included.

A 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye, was used to confirm the correct PCR amplification. Successful amplification yielded a single 173 bp fragment. The 173 bp fragment was then used for the sequence analysis.

Before sequencing, the correctly amplified PCR products were cleaned using the ExoSAP Protocol as follows. The Exo/SAP master mix was prepared by adding 50 µl Exonuclease I (20U/µl) (Catalogue No. NEB M0293L) and 200 µl Shrimp Alkaline Phosphatase (1 U/ul) (Catalogue No. NEB M0371) to a 0.6ml micro-centrifuge tube. 2.5 µl of this Exo/SAP master

mixed was then added to 10 µl of the amplified PCR product in a separate micro-centrifuge tube. This was then incubated at 37°C for 15 minutes, followed by 80°C for 15 minutes to terminate the reaction.

The cleaned PCR fragments were sequenced using the Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000 according to manufacturer's instructions. The labelled products were then cleaned with the ZR-96 DNA Sequencing Clean-up Kit (Catalogue No. D4053) and the cleaned products injected on the Applied Biosystems ABI 3500XL Genetic Analyser with a 50 cm array, using POP7. Sequence chromatogram analysis was then performed using GeneStudio™ Profesional Sequence analysis software.

3.5 MICROSATELLITE INSTABILITY (MSI) ANALYSIS

The extracted DNA was subjected to a pentaplex PCR panel of 5 markers comprising mononucleotide repeats amplified by 5 sets of primers, i.e. (BAT-25, BAT-26, NR-21, NR-24, and NR-27) as described by Haghighi and colleagues (Haghighi *et al.*, 2010). The primers sequences and the size of the amplicons are shown in table 3.1 Each antisense primer was labelled with 1 of the fluorescent markers FAM, HEX, or NED. The Applied Biosystems® True Allele® PCR master mix was used according to the manufacturer's instructions. The fluorescent PCR products were subjected to capillary electrophoresis on the Applied Biosystems PRISM 3500 automated genetic analyser. The allelic size was then determined using Genemapper 4.0 software (Applied Biosystems).

Samples with no allelic size variations in any of the 5 microsatellite markers were classified as microsatellite stable (MSS). If allelic size variation was found in 1 of the 5 microsatellites markers, these were then classified as microsatellite instability-low (MSI-L). If allelic size variation was found in 2 or more microsatellite markers, these were then classified as microsatellite instability-high (MSI-H) (Haghighi *et al.*, 2010).

Table 3.1. Primer sequences used for the MSI assay. Adapted from (Haghighi *et al.*, 2010)

Primer name	Gene	Genbank number	Repeat	Primer sequences	Amplicon size (bp)
NR-27	Inhibitor of apoptosis protein-1	AF07067427	27 A 5'UTR	F-AACCATGCTTGCAAACCACT	87
				R-CGATAATACTAGCAATGACC	
NR-21	SLC7A8	XM 033393	21 T 5'UTR	F-GAGTCGCTGGCACAGTTCTA	109
				R-CTGGTCACTCGCGTTTACAA	
NR-24	Zinc finger 2	X60152	24 T 3'UTR	F-GCTGAATTTTACCTCCTGAC	131
				R-ATTGTGCCATTGCATTCCAA	
BAT-25	c-kit	X06182	25 T intron	F-TACCAGGTGGCAAAGGGCA	153
				R-TCTGCATTTTAACTATGGCTC	
BAT-26	hMSH2	U04045	26 A intron	F-CTGCGGTAATCAAGTTTTTAG	183
				R-AACCATTCAACATTTTAAACC	

3.6 STATISTICAL ANALYSIS

All data obtained was entered into a Microsoft ExcelTM programme and tabulated. The relationship between the clinicopathological features of the serrated lesions and the *BRAF* V600E mutation, *KRAS* codon 12 and 13 mutations, loss of MLH-1 expression and the occurrence of MSI, was evaluated. Categorical data was described as frequencies and percentages. Numerical data was described by medians and interquartile range.

CHAPTER 4: RESULTS

4.1 CLINICAL FEATURES

A total of ten cases with incidentally detected appendiceal lesions showing serrated morphology were identified over the five-year period from January 2014- December 2018. All cases were reported by a pathologist/pathologists at the Charlotte Maxeke Johannesburg Academic Hospital (CHJAH) branch of the National Health Laboratory Service (NHLS)/Anatomical Pathology Division, Faculty of Health Sciences, University of the Witwatersrand.

The age distribution is shown in Figure 4.1. The median age was 38 years, with an age range of 22 years to 69 years, and an interquartile range of 28.25 – 60.25 (Table 4.1).

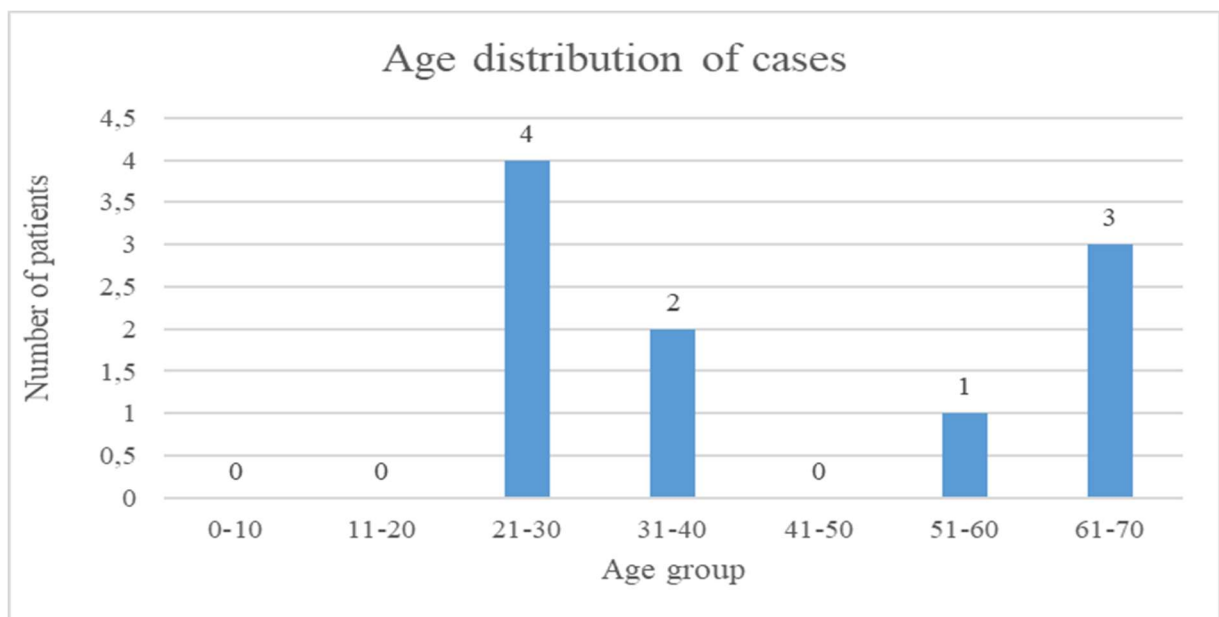


Figure 4.1. Age distribution of the cases. The age distribution shows a right shift, with all cases above 20 years of age.

In three of the patients, clinical features of acute appendicitis were not provided. All patients underwent an appendicectomy, barring one, who underwent a right hemicolectomy for an appendiceal stump abscess, following prior appendicectomy. Of note, the prior appendicectomy specimen for this case showed histological features of acute appendicitis, with no associated

serrated epithelial proliferation. Within the right hemicolectomy specimen, an incidental sessile serrated lesion was identified at the orifice of the appendix, as it entered the caecum.

Sessile serrated lesions/sessile serrated adenomas were seen in 0.14% of all the appendixes received. Over the five-year study period, a total of 6931 appendixes were received. This included 1264, 1318, 1462, 1327, 1560 cases, over the years, 2014, 2015, 2016, 2017 and 2018 respectively. Of the 6931 cases, 10 were diagnosed as sessile serrated lesions/sessile serrated adenomas.

Table 4.1: Clinical and histological features of the study group with incidentally detected serrated lesions of the appendix.

	Total cases (n = 10)
Gender	
Male	4 (40%)
Female	6 (60%)
Age	
Median	38
Range	22-69
Interquartile range	28.25-60.25
Acute appendicitis	
Clinical diagnosis	7 (70%)
Histological diagnosis	8 (80%)
Serrated lesion	
Sessile serrated lesion/polyp	10 (100%)
Hyperplastic polyp	0
Traditional serrated adenoma	0
Dysplasia	
Present	0
Absent	10 (100%)

4.2 PATHOLOGICAL FINDINGS

All ten cases were classified as sessile serrated lesions/polyps, based on the histological criteria provided in Appendix A. A macroscopic mass or polyp was not identified in any of the cases

at the time of macroscopic processing. There were no features of abundant extracellular mucin extravasation described at the time of macroscopic processing. A single case was noted to have an area of rupture seen macroscopically.

Eight cases had histologic features consistent with acute appendicitis. All eight cases had a dense infiltrate of neutrophils within the muscularis propria. Three of the eight cases with histologically confirmed acute appendicitis had an associated overlying serositis. Dense fibrous obliteration of the appendiceal lumen was identified in a single case. There was no associated neutrophilic inflammatory infiltrate in this case. The single case with an absence of neutrophilic inflammation, had a single sessile serrated lesion, with no features of dysplasia.

All ten cases had histological features consistent with the diagnosis of a sessile serrated lesion as per the revised terminology in the recent WHO Classification of Tumours of the Digestive System. A summary of the key microscopic findings is highlighted in Table 4.2. Classic, basally located horizontal growth and expansion of the crypt base was present in eight of the ten cases. This imparted the classic inverted T-shape or L-shape appearance at the base of the crypt (Figure 4.1 A and B). Five of the ten cases had a circumferential growth pattern.

Table 4.2 Summary of the microscopic findings of the serrated lesions of the appendix

	Basal crypt dilatation	Funnel shaped evenly spaced crypts	Circumferential growth pattern	Serrations at the base	Ectopic crypts	Aberrant proliferation centres	Dysplasia
Present	10 (100%)	0	5 (50%)	10 (100%)	3 (30%)	8 (80%)	0
Absent	0	10 (100%)	5 (50%)	0	70 (70%)	2 (20%)	10 (100%)

Although differentiation from reactive epithelial hyperplasia overlying areas of marked inflammation can be difficult to differentiate from a serrated lesion, the serrated crypts are often found overlying areas devoid of a dense inflammatory infiltrate (Figure 4.2 B and C). Although a serrated appearance can often be seen overlying areas of inflammation, in our series, all of

the ten cases showed the classic serrated morphology in areas devoid of a dense acute inflammatory infiltrate.

In cross-section, the typical stellate or star-shaped pattern was seen in the crypts. This was a common feature in all of the sessile serrated lesions, in which a cross-section focus of the crypts was present.

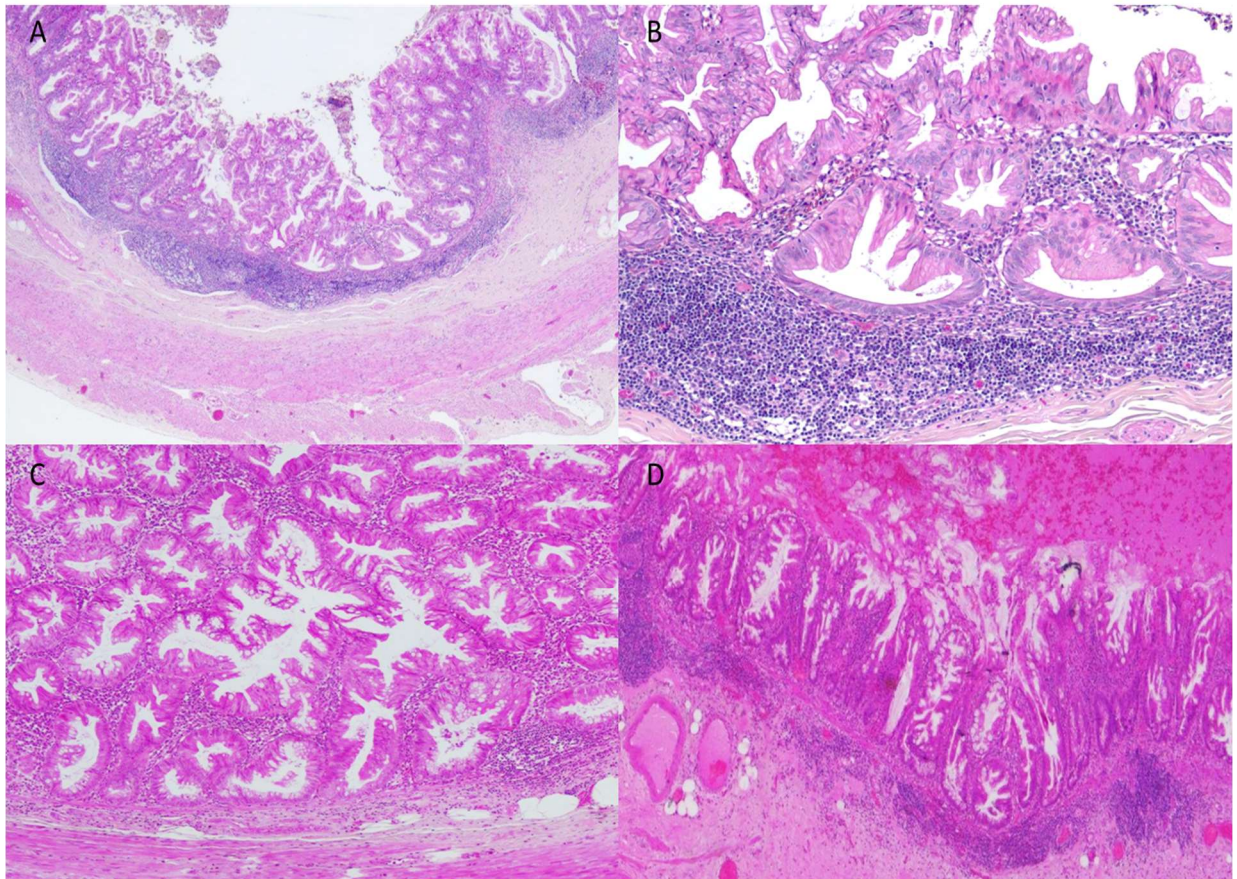


Figure 4.2 Key histological findings in the sessile serrated lesions. The sessile serrated lesions (A-D), showed circumferential involvement of the appendiceal mucosa, with luminal serration of the crypt epithelium and dilated, horizontal expansion at the crypt base. Although serration may be seen in hyperplastic epithelium overlying dense inflammation, the serrated crypts can be seen overlying areas lacking this inflammatory infiltrate (C-D).

Only two cases had abundant luminal mucin. Luminal mucin was not seen “tracking” into the submucosa or the muscularis propria. All sessile serrated lesions had a maintained underlying lamina propria and muscularis mucosa. A dense band of fibrosis and absence of the lamina

propria was not identified, thus precluding the diagnosis of a low grade appendiceal mucinous neoplasm (LAMN) in those cases with increased extracellular mucin. Both cases with increased luminal mucin had a prominent serrated architecture, rather than the typical long slender filiform villi seen in LAMN.

Occasional sessile serrated lesions had luminal based goblet cells, however, additional features of a hyperplastic polyp were not present in these cases. Although occasional cases had increased mitotic figures, this was not a prominent finding and was limited to the aberrant proliferation centre located throughout the length of the crypts.

Dysplasia, in the form of conventional adenoma-like dysplasia or serrated type dysplasia, was not identified. However, in two of the cases, patchy cytologic atypia with scattered dystrophic goblet cells was present.

4.3 IMMUNOHISTOCHEMICAL AND MOLECULAR CHARACTERISATION OF SERRATED APPENDICEAL LESIONS

4.3.1 *BRAF* mutations

All cases underwent *BRAF* mutations analysis in the form of BRAF V600E immunohistochemistry and *BRAF* exon 15 sequencing. A single sessile serrated lesion showed positive BRAF V600E staining, with moderate intensity (2+) staining of the cytoplasm (Figure 4.3).

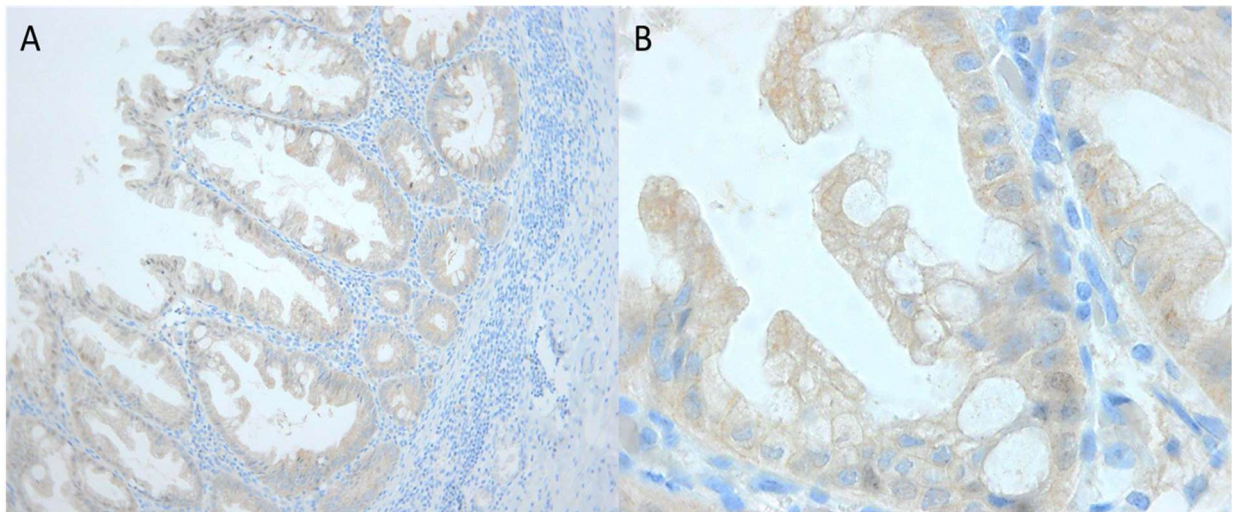


Figure 4.3 BRAF V600E immunohistochemistry showing positive, 2+ cytoplasmic staining of the cytoplasm. (A, 200X; B, 1000X).

This case, however, did not show the corresponding GTG→GAG (t1799a) mutation following sequence analysis (Figure 4.4). All other nine cases did not show BRAFV600E positivity and a wild type GTG sequence at nucleotide positions 1798-1800nt (Appendix B).

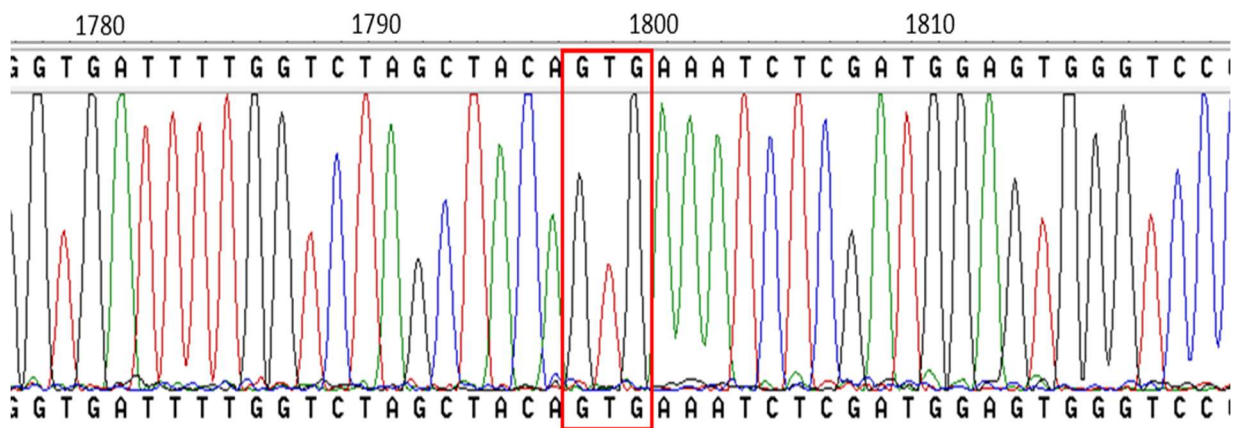


Figure 4.4 Chromatogram sequence showing wild type sequence GTG (nt 1798-1800), in the single case which had moderate intensity cytoplasmic BRAFV600E staining.

Three cases had incidental nucleotide alterations in the 199bp fragment of the *BRAF* gene (Table 4.3). All three cases in which *BRAF* nucleotide changes were identified, had classic histological features of a sessile serrated lesion/polyp. There was no dysplasia in any of the cases showing nucleotide changes in the *BRAF* gene. There was no associated or concomitant

KRAS mutation in any of the three cases in which *BRAF* mutations were identified. One of the cases, with the dual 1731C→Y and 1797A→W mutations, had a low frequency of microsatellite instability, in which one of the markers (BAT25), showed a dual peak with allelic variability, indicative of instability at this mononucleotide repeat, (amplicon size 153bp) (Figure 4.6).

Table 4.3 *BRAF* V600E mutation and additional incidental *BRAF* mutations in the serrated appendiceal lesions.

	Total cases
BRAFV600E Immunohistochemistry:	
Positive	1 (10%)
Negative	9 (90%)
GTG→GAG (t1799a) mutation:	
Present	0
Absent	10 (100%)
Additional <i>BRAF</i> mutations:	
1814G>R	2 (20%)
1779T>Y/1781A>W/1814G>R	1 (10%)
1731C>Y/1797A>W	1 (10%)

4.3.2 *KRAS* mutations

A single 173 bp fragment of the *KRAS* gene, which included codons 12 and 13, was sequenced following successful PCR amplification. Of the ten serrated lesions analysed, *KRAS* mutations were identified in 2 (20%) of the cases. A single case had a codon 12 mutation, with a mixed G and T population at nucleotide position 34. (Figure 4.5). The other *KRAS* mutations did not occur within either codon 12 or 13 and were found at nucleotide positions 23 (A→D), and 86 (T→K) respectively (Table 4.4). Codon 13 mutations were not present in any of the cases. Of note, the case with the *KRAS* nucleotide 23 (A→D), and 86 (T→K) mutations, showed positive BRAFV600E immunohistochemical staining, with moderate intensity, (2+) staining of the cytoplasm in the sessile serrated lesion. Both these nucleotide changes would be best described a synonymous substitution, or silent substitution, as the amino acid sequence of the protein is not altered, and hence are unlikely to have any effect on the functioning of the protein. This case therefore likely only has a *BRAF* V600E, with no significant alteration in the *KRAS* proteins, hence, in keeping with mutual exclusivity of *BRAF* and *KRAS* mutations.

KRAS mutations were seen in younger patients, compared to those with *BRAF* mutations, in which the mean age was 45 years, compared to 26.5 years.

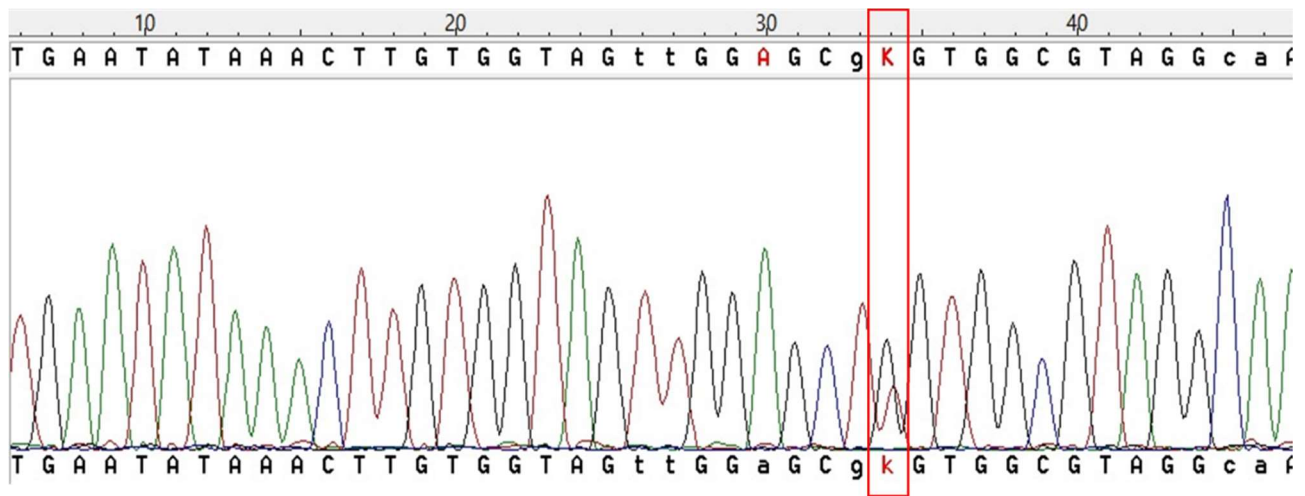


Figure 4.5. Chromatogram sequence of the *KRAS* gene showing a mixed G and T population at nucleotide position 34 (codon 12).

Table 4.4 *KRAS* codon 12 and 13 mutations and additional *KRAS* mutations in the serrated appendiceal lesions.

	Total Cases
KRAS Codon 12 mutation	
Present	1 (10%)
Absent	0
KRAS Codon 13 mutation	
Present	0
Absent	0
Additional KRAS mutations	
23A>D/86T>K	1 (10%)

4.4 MLH-1 EXPRESSION AND MICROSATELLITE INSTABILITY ANALYSIS

MLH-1 expression by immunohistochemistry was retained in all ten cases. Of the five loci analysed, only four were successfully amplified using the set of 5 primers. The BAT-26 primer, which amplifies a 183bp fragment of the hMSH2 gene was unsuccessful in all ten cases analysed (Table 4.5).

Table 4.5 Summary of the microsatellite instability findings using the pentaplex polymerase chain reaction.

	Pentaplex PCR panel				
	NR-27	NR-21	NR-24	BAT-25	BAT-26
Successful amplification	10 (100%)	10 (100%)	10 (100%)	10 (100%)	0
Allelic variability (instability)					
Absent	10 (100%)	10 (100%)	10 (100%)	8 (80%)	NA
Present	0	0	0	2 (20%)	NA

Eight of the cases were microsatellite stable (MSS), with dual peaks, indicative of allelic variability, not detected at any of the four loci that were successfully amplified using the pentaplex PCR (Figure 4.6).

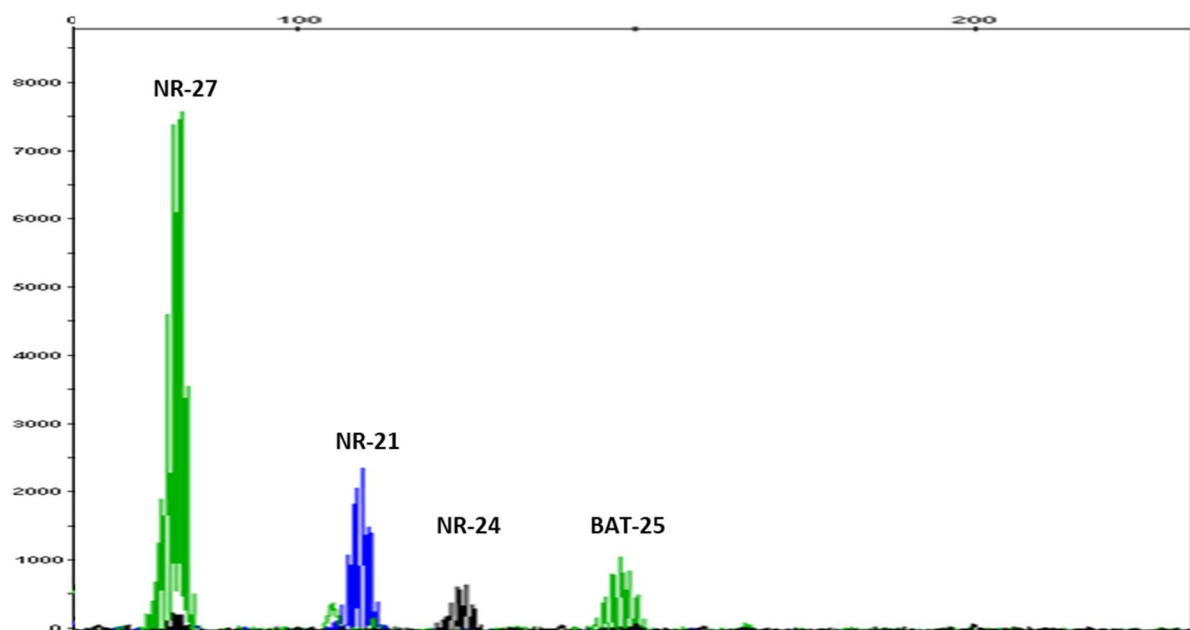


Figure 4.6 Microsatellite stable (MSS) pattern, with all four amplicons (NR-27, NR-21, NR-24 and BAT-25) showing single peaks.

Two of the ten patients showed microsatellite instability low (MSI-L), with both cases showing instability with a dual peak at the BAT-25 locus (Figure 4.7). One of the sessile serrated lesions with the MSI-L also had associated BRAF mutations at nucleotide position 1732 (C>Y) and 1798 (A >W). Both serrated lesions with MSI-L did not show any areas of dysplasia or an invasive component.

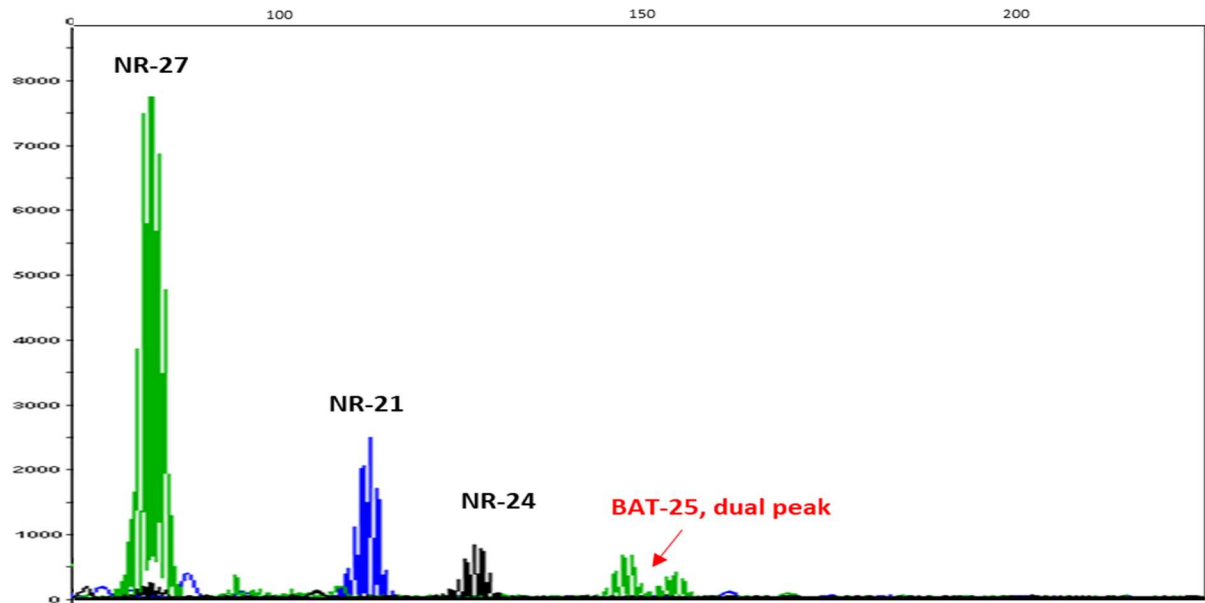


Figure 4.7 Microsatellite instability low (MSI-L) pattern showing a dual peak (allelic variation) at the BAT-25 amplicon.

The two serrated lesions in which KRAS mutations were present, did not show any microsatellite instability or loss of MLH-1 expression by immunohistochemical analysis.

CHAPTER 5: DISCUSSION

The histomorphology and molecular characteristics of serrated proliferations of the colon and rectum have been extensively evaluated. Serrated proliferations of the appendix have a resemblance to their colorectal counterparts, however, recently, molecular differences between the two have been described (Yantiss *et al.*, 2007; Pai *et al.*, 2014; Tsai *et al.*, 2019).

This is the first study on serrated proliferations of the appendix from a single Anatomical Pathology Centre, in South Africa. Although only a small series of cases is presented, the results show an overall predominance of sessile serrated lesions (100%), with no associated dysplasia and an absence of the characteristic *BRAF* V600E mutation in the majority of our series. A single *BRAF* V600E mutation was identified in a sessile serrated lesion. Only a single *KRAS* codon 12 mutation was found, with a complete absence of *KRAS* codon 13 mutations. All ten cases had retained MLH-1 expression, together with 80% of the sessile serrated lesions being classified as microsatellite stable (MSS).

All ten cases were found incidentally, most after an appendicectomy for suspected acute appendicitis. No cases were identified during endoscopic examination and no cases presented with clinical or imaging findings of a right iliac fossa/appendiceal mass. This is consistent with previous studies, in which all serrated lesions of the appendix were found incidentally following an appendicectomy or a right hemicolectomy (Yantiss *et al.*, 2007; Pai *et al.*, 2014). Sessile serrated lesions/sessile serrated adenomas were seen in 0.14% of all the appendixes received. This is comparable to previous studies, which showed a prevalence rate varying from 0.02-0.1% (Renshaw, Kish and Gould, 2006; Yantiss *et al.*, 2007; Pai *et al.*, 2014).

Although this is a small series, this is the first study to evaluate the clinical and histomorphologic characteristics of serrated lesions of the appendix from South Africa. A potential shortfall of this study relies on an initial diagnosis of a serrated proliferation of the appendix to be made and hence may be a slight underrepresentation of the true prevalence. Serrated proliferations are often subtle, and lack a polypoid configuration macroscopically, and may not always be adequately sampled. Due to this, they may be often overlooked at the time

of histological assessment. Future studies could involve a retrospective histological review of all appendixes received, to assess for subtle, potentially missed serrated proliferations.

All ten cases had morphologic features consistent with a sessile serrated lesion/sessile serrated polyp. The complete absence of hyperplastic polyps in this series differs from those previously reported, in which two previous studies showed a slight predominance of hyperplastic polyps compared to sessile serrated lesions (Yantiss *et al.*, 2007; Karabulut *et al.*, 2014). This may be due to the effect of sampling on the identification of serrated proliferations of the appendix and hence a possible underestimation of hyperplastic polyps. However, this may also represent a predominance of sessile serrated lesions of the appendix in our study population. Also, colonic hyperplastic polyps are found predominantly in the distal colon and rectum. One study has suggested that the high rate of hyperplastic polyps in the appendix may be related to overcalling subtle epithelial proliferations around areas of active inflammation as hyperplastic polyps (Karabulut *et al.*, 2014).

The importance of looking for serrated proliferations overlying areas devoid of inflammation was highlighted in a previous study by Renshaw (Renshaw, Kish and Gould, 2006). They found that reactive epithelial hyperplasia with luminal serrations is often overcalled as a serrated polyp or hyperplastic polyp (Renshaw, Kish and Gould, 2006). Similar to these findings, they also noted that even in sessile serrated adenomas occurring in the background of acute appendicitis, often the serrated changes were found distinctly separated from the inflamed mucosa (Renshaw, Kish and Gould, 2006). This highlights the importance of the low power appearance of the serrated proliferation, which may be subtle, but is often well delineated from the surrounding uninvolved mucosa.

A previous study concluded that sampling the entire appendix was critical for adequate assessment of serrated proliferations of the appendix (Bellizzi *et al.*, 2010). This is because often characteristic features of serrated lesions are only focally present. Hence extensive sampling and deeper levels are often required in suspected small serrated lesions of the appendix (Yantiss *et al.*, 2007). In light of this, a prospective study should be considered, in which all appendixes received are processed in their entirety, to accurately assess the true incidence of serrated proliferations of the appendix.

Comparison with earlier studies is hampered by the variable terminology used to describe serrated proliferations of the appendix. Sessile serrated proliferations of the appendix have been reported under a variety of names, including mucosal metaplasia, mucosal hyperplasia and diffuse hyperplasia (MacGillivray, 1972). The first report on serrated adenomas of the appendix was in 2004, by Rubio and colleagues. In their study, they described adenomas with sawtooth-like dysplastic epithelium, coining the term, serrated adenoma of the appendix (Rubio, 2004). Today, most of these lesions can be extrapolated into either a hyperplastic polyp or sessile serrated lesion (previously termed sessile serrated adenoma/polyp).

Traditional serrated adenomas of the appendix are seldom identified, with only two studies reporting on traditional serrated adenomas of the appendix. The first study reported two traditional serrated adenomas in their series (Karabulut *et al.*, 2014). The other, reporting a total of seven traditional serrated adenomas, all with dysplasia (Pai *et al.*, 2014). All other studies evaluating serrated proliferations in the appendix have not described traditional serrated adenomas in their study population. Most of these traditional serrated adenomas may be better characterised as either sessile serrated lesions or hyperplastic polyps, using the strict morphologic criteria recently proposed (Pai *et al.*, 2019). Hence, traditional serrated adenomas have not been included in the recent classification of appendiceal serrated lesions in the new WHO Classification of Tumours of the Digestive System (Pai, Makinen and Rosty, 2019).

Histologically, all ten cases in the current series showed classic histomorphologic features of a sessile serrated lesion. All ten cases showed classic basal crypt dilatation, with horizontal expansion at the crypt base imparting an inverted T or L-shaped appearance. All ten cases showed serrations extending to involve the base of the crypts. These histological features are well described in colonic sessile serrated lesions, however, only recently have these findings been defined in their appendiceal counterparts (Yantiss *et al.*, 2007; Karabulut *et al.*, 2014). From this study, and others, it appears, that on morphologic appearance, colorectal and appendiceal sessile serrated lesions are identical or near-identical. In light of this, it can be argued, that larger sessile serrated lesions within the cecum, nearby the orifice of the appendix may have arisen in the proximal region of the appendix with extension into the caecum, or vice versa.

Although there are some studies in which the histomorphology and molecular characteristics of colorectal carcinoma in South Africa have been described, to date, serrated polyps of the colon have not been evaluated in South Africa (McCabe *et al.*, 2019). Hence accurate comparison with sessile serrated lesions of the colon, with regard to the South African context, is not possible at this stage. In this regard, more studies evaluating both serrated proliferations of the appendix and colon, in a local context, are required.

Nevertheless, this is the first study to assess serrated proliferations of the appendix in South Africa, with findings differing from previous studies in Turkey, Australia and America (Yantiss *et al.*, 2007; Karabulut *et al.*, 2014; Pai *et al.*, 2014). In our small series, hyperplastic polyps were not identified. This could be as a result of the few cases in the current study. Or, it is postulated that subtle changes in hyperplastic polyps are often overlooked at the time of histological assessment. In addition, in the public sector, patients may present late with signs and symptoms of acute appendicitis, often with rupture and abscess formation. This may lead to distortion of the appendix with extensive ulceration, acute inflammation and secondary changes which may obliterate a small underlying hyperplastic polyp. Although these causes have been considered, larger case series are required to more accurately assess the distribution of serrated appendiceal proliferations in South Africa.

There was no dysplasia in any of the ten sessile serrated lesions/polyps in this case series. Serrated proliferations of the appendix with cytologic dysplasia often circumferentially involve the appendiceal mucosa. On gross examination, there is often cystic dilatation of the appendiceal lumen (Pai *et al.*, 2014). This is similar to the gross appearance of low grade appendiceal mucinous neoplasm (LAMN), for which they may often get confused.

In sessile serrated lesions, dysplasia can take the form of conventional adenoma-like dysplasia, serrated dysplasia, traditional adenoma-like dysplasia and lesions with multiple morphological patterns of dysplasia (Yantiss *et al.*, 2007). As per the updated WHO Classification of Tumours of Digestive system, hyperplastic polyps of the appendix may contain mild reactive cytological atypia in the deep crypts, however, true cytological dysplasia is not usually present (Pai *et al.*, 2019).

In the study by Yantiss and colleagues, areas of dysplastic epithelium were present in 23 of the 56 (41%) appendiceal serrated polyps. Of these 23, the majority of lesions with dysplasia occurred in “serrated adenomas” (Yantiss *et al.*, 2007). “Serrated adenoma” was used by this group to distinguish serrated proliferations that did not adequately meet the criteria defined for a sessile serrated polyp or hyperplastic polyp (Yantiss *et al.*, 2007). Five cases were classified as sessile serrated polyps with dysplasia (Yantiss *et al.*, 2007). Another study found a similar rate of dysplasia within serrated proliferations of the appendix, with 18 of 46 (39%) of the serrated polyps having dysplasia (Pai *et al.*, 2014). Of the 18 cases with dysplasia, nine were classified as sessile serrated adenomas, seven as traditional serrated adenomas, and two were classified as appendiceal adenomas with serrations (Pai *et al.*, 2014). The sessile serrated adenomas/polyps (now classified as sessile serrated lesions), typically contained focal areas of conventional adenoma-like dysplasia adjacent to recognisable non-dysplastic areas (Pai *et al.*, 2014).

The absence of dysplasia within the present study differs from those previously published. As not all cases with serrated proliferations of the appendix are processed in their entirety, this may be an under-representation of the presence of dysplasia within these lesions. Further prospective studies are required in this regard.

The absence of an invasive component within the present study also differs from previous studies. Occasionally, invasive adenocarcinomas are seen adjacent to serrated proliferations of the appendix. In their study, Yantiss and colleagues had four (7.1%) cases with an adjacent invasive adenocarcinoma (Yantiss *et al.*, 2007). Similarly, three invasive non-mucinous adenocarcinomas were identified adjacent to serrated dysplastic appendiceal lesions, in a recent study from 2014 (Pai *et al.*, 2014). It has been postulated that non-mucinous adenocarcinomas of the appendix may arise from precursor serrated lesions with dysplasia. However, to date, studies have shown conflicting findings (Yantiss *et al.*, 2007; Pai *et al.*, 2014).

In the present study, a definitive association with sessile serrated lesions and either *KRAS* or *BRAF* mutations was not found. The predominant molecular findings from previous studies evaluating serrated proliferations of the appendix have shown alterations of the MAPK

pathway. *BRAF* mutations are often reported as a key molecular event, in keeping with their colorectal counterparts, that often contain activating mutations in either *BRAF* or *KRAS*.

The *BRAF* V600E (GTG→GAG) mutation was analysed using both BRAF V600E immunohistochemistry and conventional PCR based Sanger sequencing. A single sessile serrated lesion showed positive BRAFV600E staining, with moderate intensity (2+) staining of the cytoplasm (Figure 4.2). This case, however, did not show the corresponding GTG→GAG (t1799a) mutation following sequence analysis. This may represent a false positive using BRAF V600E immunohistochemistry or may be related to a predominant wild type sequence due to contamination by adjacent non-serrated mucosa at the time of microdissection and subsequent DNA extraction. This leads to a higher concentration of the wild type sequence following DNA extraction. A predominant wild type sequence may account for this, as Sanger sequencing methods are only able to detect a mutant allele in a background of 5–20 fold excess of wild-type alleles (Dvorak *et al.*, 2019).

False-positive and false-negative results using the BRAF V600E antibody may occur due to technical considerations. These include variations in the staining protocols, the use of inappropriate antigen retrieval methods, or due to the under-recognition of staining pitfalls. Also, incorrectly discounting of weak, but positive cytoplasmic immunostaining can occur (Bledsoe, Kamionek and Mino-Kenudson, 2014). Most studies have shown a 100% concordance with mutation-specific immunohistochemistry for BRAF V600E and molecular methods, particularly Sanger sequencing (Capper *et al.*, 2011; Ghossein, Katabi and Fagin, 2013). However, a recent study comparing two BRAF immunohistochemical protocols with *BRAF* sequence analysis, showed 71% and 83% concordance with sequence analysis using the two different protocols (Szymonek *et al.*, 2017). These studies highlight potential reasons for discordant results using BRAF V600E immunohistochemistry and sequence analysis.

The reason for the discordant finding within the present study is not known. However, it is postulated, that a predominant wild type sequence may have been detected on sequence analysis due to contamination by adjacent non-serrated mucosa at the time of microdissection and subsequent DNA extraction and PCR analysis.

All other nine cases had concordant *BRAF* V600E negativity and a wild type GTG sequence at nucleotide positions 1798-1800nt. Apart from the GTG→GAG (t1799a) mutation, three cases had incidental nucleotide changes at positions, 1731, 1779, 1781, and 1814. These four changes are all silent nucleotide alterations with no influence on the coding amino acid sequence. These therefore have no bearing on the functional activity of the BRAF protein.

Previous studies looking at the frequency of *KRAS* and *BRAF* mutations in serrated proliferations of the appendix have shown mixed results. Recently published studies have shown a higher frequency of *KRAS* mutations in serrated proliferations of the appendix, compared to *BRAF* mutations. In one of the largest studies on serrated lesions of the appendix, *KRAS* mutations were found in 64 (52%) of the serrated polyps, compared to BRAF V600E mutations, which were identified in only five cases (4%) (Pai *et al.*, 2014). In the present study, *KRAS* mutations were identified in 2 (20%) of the cases. However, another study showed a similar frequency of *BRAF* and *KRAS* mutations, 33% and 27 % respectively, in their series (Yantiss *et al.*, 2007). Also, in their series, they found *BRAF* and *KRAS* mutations at a comparable frequency to those that occur in the colon (Yantiss *et al.*, 2007). In this study, a single case demonstrated a codon 12 mutation, with a mixed G and T population at nucleotide position 34 of the *KRAS* gene. *KRAS* codons 12 and 13 occur within the G domain of the *KRAS*, which comprises residues 1–166 and forms the basis of biological functionality of the GTPase protein (Pantsar, 2020).

The most common codon 12 mutation is the G12R (A or G or T), which has been reported in numerous studies (Yantiss *et al.*, 2007; Pai *et al.*, 2014; Tsai *et al.*, 2019). The single codon 12 mutation in this study, is the same as those previously found, with a G12K (G or T). Other common *KRAS* codon 12 mutations include G12V, G12R and G12C (Pai *et al.*, 2014). *KRAS* mutations have been found in hyperplastic polyps, sessile serrated lesions, as well as sessile serrated lesions with dysplasia. Pai and colleagues found no significant difference in patient age, lesion size and histological findings between serrated appendiceal lesions without a *KRAS* mutation and those with a *KRAS* mutation (Pai *et al.*, 2014). However, this study did show *KRAS* codon 12 and 13 mutations were more frequently identified in serrated proliferations of the appendix compared to BRAF V600E mutations (Pai *et al.*, 2014). A recent molecular analysis of epithelial proliferations of the appendix, which included serrated proliferations and

mucinous lesions, also showed a predominance of *KRAS* codon 12 mutations across the board, with only a few cases showing *BRAF* mutations (Tsai *et al.*, 2019).

The other *KRAS* mutations in this study were in a single case and occurred at nucleotide positions 23 (A→D), and 86 (T→K) respectively. Although these mutations are found within the G domain of the *KRAS* protein, which comprises residues 1–166 (nt: 1-498), these mutations have not been previously reported. Both these nucleotide changes would be best described as synonymous substitutions, or silent substitutions, as the amino acid sequence of the protein is not altered, and hence are unlikely to have any effect on the functioning of the protein.

KRAS codon 13 mutations were not identified in the present study, in keeping with previous studies which showed a predominance of codon 12 mutations compared to codon 13 mutations in serrated proliferations of the appendix (Tsai *et al.*, 2019). None of the cases with *KRAS* mutations had dysplasia or an adjacent invasive component.

The role of microsatellite instability in the serrated neoplasia pathway has been described in serrated colorectal polyps and invasive colorectal adenocarcinomas. An important step involved in the transition to dysplasia and subsequent colorectal adenocarcinoma, is CpG island methylation, particularly methylation induced silencing of the dMMR gene *MLH1* (Liu *et al.*, 2017). Although documented in serrated proliferations of the colon, the extent of microsatellite instability in appendiceal lesions remains largely unknown.

As is to be expected, MLH-1 expression was retained in all ten cases, as no cases showed foci of dysplasia. Loss of MLH-1 expression is often reported in foci of dysplasia within serrated lesions of the colon, particularly sessile serrated lesions. This is often the initiating event in the progression from dysplasia to invasive adenocarcinoma. A recent paper by Liu and colleagues, which is quoted in the recently updated WHO Classification of Tumours of the Digestive System, proposes four types of dysplasia in colorectal sessile serrated lesions (Liu *et al.*, 2017). They showed variable loss of expression of *MHL-1* in the different morphologic types of dysplasia (Liu *et al.*, 2017). In their study, adenomatous and serrated dysplasia were typically

MLH-1 proficient, whereas, the proposed minimal deviation dysplasia and dysplasia not otherwise specified, typically show a loss of *MHL-1* expression (Liu *et al.*, 2017). This highlights possible other pathways that could be implicated in the progression to dysplasia and subsequent invasive adenocarcinoma.

Eight cases were classified as microsatellite stable with no allelic variation in the four loci that were successfully amplified (NR-27, NR-21, NR-24 and BAT-25). Two of the ten cases showed microsatellite instability low (MSI-L), both showing a dual peak (allelic variation) at the BAT-25 locus. Unfortunately, a limitation of this study is unsuccessful amplification using the BAT-26 primers which amplifies a segment of the *hMSH2* gene. In this regard, MSI-H cannot be excluded in the two cases which showed MSI-L. If a dual peak was detected at the BAT-26 locus, these two cases would be classified as MSI-H. Likewise, the other eight cases may have shown variability at the BAT-26, and hence could be classified as MSI-L. This is an important pitfall of the present study and despite variable testing parameters, amplification using the BAT-26 primers was unsuccessful in all ten cases.

Variable concentrations of DNA, annealing temperatures and primers dilutions were optimised, however, despite this, successful amplification using the BAT-26 primers was not possible. The BAT-26 locus contains a 26-repeat adenine tract and is located within the fifth intron of the *MSH2* gene (Pyatt *et al.*, 1999). The primers sequences flank this intron containing the poly adenine repeat and hence primer binding should not be affected. The lack of successful amplification of the BAT-26 loci has been queried, however, a definitive cause within the present study is unknown. Further molecular analysis and sequencing of the *MSH2* gene and the flanking primer binding sites is required to assess for possible sequence variations and hence impaired or non-specific primer binding. Polymorphisms in the primer binding site may occur within the South African population and hence impact primer binding and successful PCR amplification. This is however unlikely, as previous MSI analysis of colorectal adenocarcinoma from South Africa showed successful amplification of the BAT-26 locus (McCabe *et al.*, 2019).

Both cases with MSI-L showed retained MLH-1 expression via immunohistochemical analysis. The majority of sporadic cases of MSI are due to *hMLH1* promoter methylation rather than mutations in one of the mismatch repair genes. Although this is often the case, inactivation of

any of several MMR genes, including *hMLH1*, *hMSH2*, *hMSH6*, and *hPMS2*, can result in MSI (Eshleman and Markowitz, 1996). This can be in the form of promoter methylation or a mutation within one of these genes, leading to decreased or impaired expression and hence, a defect in the mismatch repair system. This may explain the MSI-L detected in the present study, but a lack of associated loss of MLH-1 expression. Confirmation of this requires further methylation and mutational analysis of these four genes.

One of the sessile serrated lesions with the MSI-L also had associated BRAF mutations at nucleotide position 1732 (C>Y) and 1798 (A >W). The other case did not show any mutations within *KRAS* or *BRAF*. Both serrated lesions with MSI-L did not show any areas of dysplasia or an invasive component.

There is very limited data on microsatellite instability in epithelial proliferations of the appendix, particularly serrated lesions. Loss of *MLH-1* expression has been reported with an equal frequency in non-dysplastic and dysplastic serrated proliferations of the appendix (Yantiss *et al.*, 2007). Yantiss and colleagues also analysed O⁶-methylguanine methyltransferase (MGMT) expression in their series on serrated appendiceal proliferations. The degree of MGMT expression reflects the methylation status of CpG islands in the promoter regions of the *hMLH-1* gene (Yantiss *et al.*, 2007). They found an association with loss of MGMT expression and loss of MHL-1, highlighting the likely mechanism of decreased MLH-1 expression secondary to promoter hypermethylation (Yantiss *et al.*, 2007). This is similar to sporadic cases in the colon and rectum, which also show a loss of MLH-1 expression. In their study, the loss of MLH-1 was not associated with MSI-H, with only a single case showing MLH-1 loss together with MSI-L (dual peak at NR-21 locus) (Yantiss *et al.*, 2007). In addition, all 56 cases with serrated morphology did not show MSI-H (Yantiss *et al.*, 2007). The lack of microsatellite instability in serrated appendiceal proliferations significantly differs from those in the colon and rectum. Therefore, the involvement of the MMR pathway and MSI may not be as important in the development of sessile serrated lesions of the appendix.

The low frequency of MSI in the appendix may be explained by the higher frequency of *KRAS* mutations compared to *BRAF* mutations in the appendix. Tumours arising predominantly in the background of *BRAF* mutations and high levels of CpG island methylation (i.e. the CIMP

phenotype), tend to be MSI-H, whereas those arising in the background of *KRAS* mutations, generally tend to be MSI-L or MSS (Chen, Swanson and Frankel, 2017). Studies in the appendix have not shown this association, with no definitive correlation between MSI and *KRAS* or *BRAF* mutations (Yantiss *et al.*, 2007; Pai *et al.*, 2014). More studies evaluating the importance of MSI in serrated proliferations of the appendix are required.

A low frequency of microsatellite instability has also been found recently in non-mucinous adenocarcinomas of the appendix (Taggart *et al.*, 2013). In a study from the Mayo Clinic, high-level MSI were only detected in three appendiceal adenocarcinomas, representing just 2.8% of 108 invasive appendiceal adenocarcinomas (Taggart *et al.*, 2013). This differs considerably from colonic adenocarcinomas, with most series showing approximately 15% of colorectal carcinomas overall, and at least 20% of proximal tumours, are MSI-high (Vilar and Gruber, 2010). These findings indicate subtle differences in the molecular pathways in colorectal and appendiceal adenocarcinomas, particularly, those arising via the “serrated neoplasia pathway”.

Additional studies, involving larger cohorts are required to adequately assess the molecular pathways involved in appendiceal adenocarcinomas, particularly those arising via the so-called “serrated neoplasia pathway” and serrated precursors.

CHAPTER 6: CONCLUSION

This is the first study from South Africa to characterise the clinical, morphologic and molecular aspects of incidentally detected serrated proliferations of the appendix. Sessile serrated lesions/sessile serrated adenomas were seen in 0.14% of all the appendixes received, which is consistent with the few other reports which have assessed the incidence or prevalence of serrated proliferations of the appendix.

Although only a small series of ten cases, all were classified as sessile serrated lesions, with no features of dysplasia and no associated adjacent invasive adenocarcinoma. This highlights the predominance of sessile serrated lesions compared to hyperplastic polyps and traditional serrated adenomas, in our local context. This is the first study from South Africa to analyse the molecular aspects of the so-called “serrated neoplasia pathway” and how it may relate to the serrated proliferation of the appendix.

The molecular findings were inconclusive, with no definitive association with either *BRAF* V600E or *KRAS* codon 12 and 13 mutations. A single case contained a well-described *KRAS* codon 12 mutation, however, the occurrence of *KRAS* mutations was significantly less than those previously reported. MSI-L was present in two cases, both showing dual peaks indicative of allelic variability at the BAT-25 locus. Although, MSI-H could not be excluded due to the unsuccessful amplification of the BAT-26 locus in all cases in this series. This study, together with the handful of other studies evaluating serrated proliferations of the appendix, highlights subtle differences with serrated proliferations of the colon. In this regard, serrated proliferations of the appendix should be viewed as inherently different from those occurring in the colon.

Although this study was hampered by the small number of cases and failure to successfully amplify the BAT-26 locus in all the cases, and hence being unable to exclude MSI-H cases, it provides an important stepping stone for future larger retrospective and prospective studies evaluating serrated appendiceal proliferations in South Africa. Studies investigating the molecular characteristics of serrated colonic lesions in South Africa are required, to enable adequate comparison with those occurring in the appendix.

REFERENCES

- Ali Malas, M. and A. Gokcimen. (2004) 'Development of the vermiform appendix during the fetal period', *Surgical and Radiologic Anatomy*, 26(3), pp. 202–207. doi: 10.1007/s00276-003-0208-9.
- Bellizzi, A. M., Rock, J., Marsh, W.L. *et al.* (2010) 'Serrated lesions of the appendix: A morphologic and immunohistochemical appraisal', *American Journal of Clinical Pathology*. doi: 10.1309/AJCP1UJXPX6UURLCH.
- Bettington, M., Walker, N., Rosty, C. *et al.* (2014) 'Critical appraisal of the diagnosis of the sessile serrated adenoma', *American Journal of Surgical Pathology*. doi: 10.1097/PAS.0000000000000103.
- Bettington, M., Walker, N., Rosty, C. *et al.* (2016) 'Serrated tubulovillous adenoma of the large intestine', *Histopathology*. doi: 10.1111/his.12788.
- Bettington, M., Walker, N., Rosty, C. *et al.* (2017) 'Clinicopathological and molecular features of sessile serrated adenomas with dysplasia or carcinoma', *Gut*. doi: 10.1136/gutjnl-2015-310456.
- Bettington, M., Brown, I. S. and Rosty, C. (2016) 'Serrated lesions of the appendix in serrated polyposis patients', *Pathology*. doi: 10.1016/j.pathol.2015.11.012.
- Bettington, M. L. and Chetty, R. (2015) 'Traditional serrated adenoma: An update', *Human Pathology*. doi: 10.1016/j.humpath.2015.04.002.
- Bledsoe, J. R., Kamionek, M. and Mino-Kenudson, M. (2014) 'BRAF V600E immunohistochemistry is reliable in primary and metastatic colorectal carcinoma regardless of treatment status and shows high intratumoral homogeneity', *American Journal of Surgical Pathology*. doi: 10.1097/PAS.0000000000000263.
- Borowsky, J., Dumenil, T., Bettington, M. *et al.* (2018) 'The role of APC in WNT pathway activation in serrated neoplasia', *Modern Pathology*. doi: 10.1038/modpathol.2017.150.
- Capper, D., Preusser, M., Habel, A. *et al.* (2011) 'Assessment of BRAF V600E mutation status by immunohistochemistry with a mutation-specific monoclonal antibody', *Acta Neuropathologica*. doi: 10.1007/s00401-011-0841-z.
- Carr, N. J., Bibeau, F., Bradley, R.F. *et al.* (2017) 'The histopathological classification, diagnosis and differential diagnosis of mucinous appendiceal neoplasms, appendiceal adenocarcinomas and pseudomyxoma peritonei', *Histopathology*. doi: 10.1111/his.13324.
- Chen, W., Swanson, B. J. and Frankel, W. L. (2017) 'Molecular genetics of microsatellite-unstable colorectal cancer for pathologists', *Diagnostic Pathology*. doi: 10.1186/s13000-017-0613-8.
- Dvorak, K., Higgins, A., Palting, J. *et al.* (2019) 'Immunohistochemistry with Anti-BRAF V600E (VE1) Mouse Monoclonal Antibody is a Sensitive Method for Detection of the BRAF V600E Mutation in Colon Cancer: Evaluation of 120 Cases with and without KRAS Mutation and Literature Review', *Pathology and Oncology Research*. doi: 10.1007/s12253-017-0344-x.
- Eshleman, J. R. and Markowitz, S. D. (1996) 'Mismatch repair defects in human carcinogenesis', *Human Molecular Genetics*. doi: 10.1093/hmg/5.supplement_1.1489.
- Fang, M., Ou, J., Hutchinson, L. *et al.* (2014) 'The BRAF Oncoprotein Functions through the

Transcriptional Repressor MAFK to Mediate the CpG Island Methylator Phenotype', *Molecular Cell*. doi: 10.1016/j.molcel.2014.08.010.

Fu, B., Yachida, S., Morgan, R. *et al.* (2012) 'Clinicopathologic and genetic characterization of traditional serrated adenomas of the colon', *American Journal of Clinical Pathology*. doi: 10.1309/AJCPVT7LC4CRPZSK.

Ghossein, R. A., Katabi, N. and Fagin, J. A. (2013) 'Immunohistochemical detection of mutated BRAF V600E supports the clonal origin of BRAF-induced thyroid cancers along the spectrum of disease progression', *Journal of Clinical Endocrinology and Metabolism*. doi: 10.1210/jc.2013-1408.

Haghighi, M. M., Javadi, G.R., Parivar, K. *et al.* (2010) 'Frequent MSI mononucleotide markers for diagnosis of hereditary nonpolyposis colorectal cancer', *Asian Pacific Journal of Cancer Prevention*.

Hazewinkel, Y., Lopez-Ceran, M., East, J.E. *et al.* (2013) 'Endoscopic features of sessile serrated adenomas: Validation by international experts using high-resolution white-light endoscopy and narrow-band imaging', *Gastrointestinal Endoscopy*. doi: 10.1016/j.gie.2012.12.018.

Kane, M. F., Loda, M., Gaida, G.M. *et al.* (1997) 'Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines', *Cancer Research*.

Karabulut, Y. Y., Saras, B., Kursun, N. *et al.* (2014) 'Serrated lesions of the appendix: Do they differ from their colorectal counterparts?', *Turkish Journal of Gastroenterology*. Turkish Society of Gastroenterology, 25(1), pp. 29–34. doi: 10.5152/tjg.2014.4056.

Katzin, W. E. and Petras, R. E. (2012) 'Vermiform Appendix', in *Histology for Pathologists: Fourth Edition*. doi: 10.5005/jp/books/12847_18.

Kudo, S. E., Tamura, S., Nakajima, T. *et al.* (1996) 'Diagnosis of colorectal tumorous lesions by magnifying endoscopy', *Gastrointestinal Endoscopy*. doi: 10.1016/S0016-5107(96)70222-5.

Liu, C., Walker, N.I., Legget, B.A. *et al.* (2017) 'Sessile serrated adenomas with dysplasia: Morphological patterns and correlations with MLH1 immunohistochemistry', *Modern Pathology*. doi: 10.1038/modpathol.2017.92.

Longacre, T. A. and Fenoglio-Preiser, C. M. (1990) 'Mixed hyperplastic adenomatous polyps/serrated adenomas. A distinct form of colorectal neoplasia', *American Journal of Surgical Pathology*. doi: 10.1097/00000478-199006000-00003.

MacGillivray, J. B. (1972) 'Mucosal metaplasia in the appendix.', *Journal of clinical pathology*. doi: 10.1136/jcp.25.9.809.

McCabe, M., Perner, Y., Magobo, R. *et al.* (2019) 'Microsatellite Instability assessment in Black South African Colorectal Cancer patients reveal an increased incidence of suspected Lynch syndrome', *Scientific Reports*. doi: 10.1038/s41598-019-51316-4.

Mills, S. E. (2012) *Histology for Pathologists: Fourth Edition*, *Histology for Pathologists: Fourth Edition*.

Müller, M. F., Ibrahim, A. E. K. and Arends, M. J. (2016) 'Molecular pathological classification of colorectal cancer', *Virchows Archiv*. doi: 10.1007/s00428-016-1956-3.

Murakami, T., Mitani, H., Saito, T. *et al.* (2015) 'Distinct WNT/ β -catenin signaling

activation in the serrated neoplasia pathway and the adenoma-carcinoma sequence of the colorectum', *Modern Pathology*. doi: 10.1038/modpathol.2014.41.

O'Brien, M. J., Zhao, Q. and Yang, S. (2015) 'Colorectal serrated pathway cancers and precursors', *Histopathology*. doi: 10.1111/his.12564.

Pai, R., Hartman, D.J., Gonzalo, D.H. *et al.* (2014) 'Serrated lesions of the appendix frequently harbor KRAS mutations and not BRAF mutations indicating a distinctly different serrated neoplastic pathway in the appendix', *Human Pathology*, 45(2), pp. 227–235. doi: 10.1016/j.humpath.2013.10.021.

Pai, R. K., Bettington, M., Srivastava, A. *et al.* (2019) 'An update on the morphology and molecular pathology of serrated colorectal polyps and associated carcinomas', *Modern Pathology*. Nature Publishing Group, pp. 1390–1415. doi: 10.1038/s41379-019-0280-2.

Pai, R. K., Makinen, M. and Rosty, C. (2019) 'Colorectal serrated lesions and polyps', in *WHO Classification of Tumours: Digestive System Tumours*, 5th Edition. 5th Editio, pp. 163–169.

Pantsar, T. (2020) 'The current understanding of KRAS protein structure and dynamics', *Computational and Structural Biotechnology Journal*. doi: 10.1016/j.csbj.2019.12.004.

Pyatt, R., Chadwick, R.B., Johnson, C.K. *et al.* (1999) 'Polymorphic variation at the BAT-25 and BAT-26 loci in individuals of African origin: Implications for microsatellite instability testing', *American Journal of Pathology*. doi: 10.1016/S0002-9440(10)65131-0.

Rad, R., Cadinanos, J., Rad, L. *et al.* (2013) 'A Genetic Progression Model of BrafV600E-Induced Intestinal Tumorigenesis Reveals Targets for Therapeutic Intervention', *Cancer Cell*. doi: 10.1016/j.ccr.2013.05.014.

Renshaw, A. A., Kish, R. and Gould, E. W. (2006) 'Sessile serrated adenoma is associated with acute appendicitis in patients 30 years or older', *American Journal of Clinical Pathology*. American Society of Clinical Pathologists, 126(6), pp. 875–877. doi: 10.1309/BF5KLH7J547AXAA0.

Rubio, C. A. (2004) 'Serrated adenomas of the appendix', *Journal of Clinical Pathology*. doi: 10.1136/jcp.2004.018317.

Szymonek, M., Kowalik, A., Kopczynski, J. *et al.* (2017) 'Immunohistochemistry cannot replace DNA analysis for evaluation of BRAF V600E mutations in papillary thyroid carcinoma', *Oncotarget*. doi: 10.18632/oncotarget.20451.

Taggart, M. W., Galbincea, J., Mansfield, P.F. *et al.* (2013) 'High-level microsatellite instability in appendiceal carcinomas', *American Journal of Surgical Pathology*. doi: 10.1097/PAS.0b013e318282649b.

Torlakovic, E., Skarlund, E., Suover, D. *et al.* (2003) 'Morphologic reappraisal of serrated colorectal polyps', *American Journal of Surgical Pathology*. doi: 10.1097/00000478-200301000-00008.

Tsai, J. H., Yang, C.Y., Yuan, R.H. *et al.* (2019) 'Correlation of molecular and morphological features of appendiceal epithelial neoplasms', *Histopathology*. doi: 10.1111/his.13924.

Vilar, E. and Gruber, S. B. (2010) 'Microsatellite instability in colorectal cancer the stable evidence', *Nature Reviews Clinical Oncology*. doi: 10.1038/nrclinonc.2009.237.

Williams, G. R., Boulay, C. E. H. D. and Roche, W. R. (1992) 'Benign epithelial neoplasms

of the appendix: classification and clinical associations', *Histopathology*. doi: 10.1111/j.1365-2559.1992.tb00429.x.

Yamane, L., Scapulatempo-Neto, C., Reis, R.M. *et al.* (2014) 'Serrated pathway in colorectal carcinogenesis', *World Journal of Gastroenterology*. doi: 10.3748/wjg.v20.i10.2634.

Yantiss, R. K., Panczykowski, A., Misdraji, Y. *et al.* (2007) 'A comprehensive study of nondysplastic and dysplastic serrated polyps of the vermiform appendix', *American Journal of Surgical Pathology*. doi: 10.1097/PAS.0b013e31806bee6d.

APPENDICES

APPENDIX A: HISTOLOGICAL FEATURES USED TO CLASSIFY THE SERRATED APPENDICEAL LESIONS

Type	Histologic features			
	Crypt architecture	Proliferation zone	Cytologic features	Mucin type
Hyperplastic polyp (microvesicular)	Funnel-shaped crypts, serrations limited to upper 2/3rds of the crypts	Located uniformly in the basal portion of crypts	Small basally located nuclei, no dysplasia	Mixed microvesicular and goblet cells
Hyperplastic polyp (goblet cell)	Elongated crypts that resemble enlarged normal crypts, only a few serrations	Located uniformly in the basal portion of crypts	Small basally located nuclei, no dysplasia	Goblet cells only
Sessile serrated lesion/polyp	Horizontal growth along the muscularis mucosae creating T- and L-shaped crypts, dilatation (often asymmetric) of the crypt base, and/or serrations extending into the crypt base	Proliferation zone may be abnormally located away from the crypt base, and the proliferation zone is variable from crypt to crypt.	Small basally located nuclei with occasional larger nuclei with inconspicuous nucleoli, no dysplasia	Mixed microvesicular and goblet cells
Sessile serrated lesion/polyp with dysplasia	As for sessile serrated polyp	As for sessile serrated polyp with more proliferation in dysplastic component	Varied morphologic appearance to dysplastic component	Varied type
Traditional serrated adenoma	Slit-like serrations, ectopic crypt foci common	Proliferation zone present within ectopic crypt foci and crypt base	Elongated pencillate nuclei with nuclear stratification and cytoplasmic eosinophilia, may develop overt dysplasia (either conventional or serrated)	Occasional scattered goblet cells

Table adapted from (Pai *et al.*, 2019)

APPENDIX B: *BRAF* (196nt FRAGMENT) SEQUENCE ANALYSIS

	1705	1725	1735	1745	1755	1765	1775	1785	1795	1805	1815	1825
BRAFREFSEQ2	GAGATCTACT	GTTTTCCTTT	ACTTACTACA	CCTCAGATAT	ATTTCTTCAT	GAAGACCTCA	CAGTAAAAAT	AGGTGATTTT	GGTCTAGCTA	CAGTGAAATC	TCGATGGAGT	GGGTCCCATC
2736BRAF	GAGATCTACT	GTTTTCCTTT	ACTTACTACA	CCTCAGATAT	ATTTCTTCAT	GAAGACCTCA	CAGTAAAAAT	AGGTGATTTT	GGTCTAGCTA	CAGTGAAATC	TCGATGGAGT	GGGTCCCATC
5811BRAF	GAGATCTACT	GTTTTCCTTT	ACTTACTACA	CCTCAGATAT	ATTTCTTCAT	GAAGACCTCA	CAGTAAAAAT	AGGTGATTTT	GGTCTAGCTA	CAGTGAAATC	TCGATGGAGT	GGGTCCCATC
25711BRAF	GAGATCTACT	GTTTTCCTTT	ACTTACTACA	CCTCAGATAT	ATTTCTTCAT	GAAGACCTCA	CAGTAAAAAT	AGGTGATTTT	GGTCTAGCTA	CAGTGAAATC	TCGATGGAGT	GGGTCCCATC
28314BRAF	GAGATCTACT	GTTTTCCTTT	ACTTACTACA	CCTCAGATAT	ATTTCTTCAT	GAAGACCTCA	CAGTAAAAAT	AGGTGATTTT	GGTCTAGCTA	CAGTGAAATC	TCGATGGAGT	GGGTCCCATC
28939BRAF	GAGATCTACT	GTTTTCCTTT	ACTTACTACA	CCTCAGATAT	ATTTCTTCAT	GAAGACCTCA	CAGTAAAAAT	AGGTGATTTT	GGTCTAGCTA	CAGTGAAATC	TCGATGGAGT	GGGTCCCATC
32295BRAF	GAGATCTACT	GTTTTCCTTT	ACTTAYTACA	CCTCAGATAT	ATTTCTTCAT	GAAGACCTCA	CAGTAAAAAT	AGGTGATTTT	GGTCTAGCTA	CWGTGAAATC	TCGATGGAGT	GGGTCCCATC
44021BRAF	GAGATCTACT	GTTTTCCTTT	ACTTACTACA	CCTCAGATAT	ATTTCTTCAT	GAAGACCTCA	CAGTAAAAAT	AGGYGWT	GGTCTAGCTA	CAGTGAAATC	TCGATGGART	GGGTCCCATC
45779BRAF	GAGATCTACT	GTTTTCCTTT	ACTTACTACA	CCTCAGATAT	ATTTCTTCAT	GAAGACCTCA	CAGTAAAAAT	AGGTGATTTT	GGTCTAGCTA	CAGTGAAATC	TCGATGGART	GGGTCCCATC
5080BRAF	GAGATCTACT	GTTTTCCTTT	ACTTACTACA	CCTCAGATAT	ATTTCTTCAT	GAAGACCTCA	CAGTAAAAAT	AGGTGATTTT	GGTCTAGCTA	CAGTGAAATC	TCGATGGAGT	GGGTCCCATC
48578BRAF	GAGATCTACT	GTTTTCCTTT	ACTTACTACA	CCTCAGATAT	ATTTCTTCAT	GAAGACCTCA	CAGTAAAAAT	AGGTGATTTT	GGTCTAGCTA	CAGTGAAATC	TCGATGGAGT	GGGTCCCATC
	1835	1845	1855	1865	1875	1885	1895	1905	1915	1925	1935	1945
BRAFREFSEQ2	AGTTTGAACA	GTTGTCTGGA	TCCATTTTGT	GGATGGTAAG	AATTGAGGCT	ATTTTCCAC	TGATTAAATT	TTTGGCCCTG	AGATGCTGCT	GAGTT-----	-----	-----
2736BRAF	AGTTTGAACA	GTTGTCTGGA	TCCATTTTGT	GGATGGTAAG	AATTGAGGCT	ATTTTCCAC	TGATTAAATT	TTTGGCCCTG	AGATGCTGCT	GAGT-----	-----	-----
5811BRAF	AGTTTGAACA	GTTGTCTGGA	TCCATTTTGT	GGATGGTAAG	AATTGAGGCT	ATTTTCCAC	TGATTAAATT	TTTGGCCCTG	AGATGCTGCT	GAGTT-----	-----	-----
25711BRAF	AGTTTGAACA	GTTGTCTGGA	TCCATTTTGT	GGATGGTAAG	AATTGAGGCT	ATTTTCCAC	TGATTAAATT	TTTGGCCCTG	AGATGCTGCT	GAGT-----	-----	-----
28314BRAF	AGTTTGAACA	GTTGTCTGGA	TCCATTTTGT	GGATGGTAAG	AATTGAGGCT	ATTTTCCAC	TGATTAAATT	TTTGGCCCTG	AGATGCTGCT	GAGTT-----	-----	-----
28939BRAF	AGTTTGAACA	GTTGTCTGGA	TCCATTTTGT	GGATGGTAAG	AATTGAGGCT	ATTTTCCAC	TGATTAAATT	TTTGGCCCTG	AGATGCTGCT	GAGTT-----	-----	-----
32295BRAF	AGTTTGAACA	GTTGTCTGGA	TCCATTTTGT	GGATGGTAAG	AATTGAGGCT	ATTTTCCAC	TGATTAAATT	TTTGGCCCTG	AGATGCTGCT	GAGTTA----	-----	-----
44021BRAF	AGTTTGAACA	GTTGTCTGGA	TCCATTTTGT	GGATGGTAAG	AATTGAGGCT	ATTTTCCAC	TGATTAAATT	TTTGGCCCTG	AGATGCTGCT	GAGTT-----	-----	-----
45779BRAF	AGTTTGAACA	GTTGTCTGGA	TCCATTTTGT	GGATGGTAAG	AATTGAGGCT	ATTTTCCAC	TGATTAAATT	TTTGGCCCTG	AGATGCTGCT	GAGTT-----	-----	-----
5080BRAF	AGTTTGAACA	GTTGTCTGGA	TCCATTTTGT	GGATGGTAAG	AATTGAGGCT	ATTTTCCAC	TGATTAAATT	TTTGGCCCTG	AGATGCTGCT	GAGTT-----	-----	-----
48578BRAF	AGTTTGAACA	GTTGTCTGGA	TCCATTTTGT	GGATGGTAAG	AATTGAGGCT	ATTTTCCAC	TGATTAAATT	TTTGGCCCTG	AGATGCTGCT	GAGTTACTAG	AAAGTCATTG	AAGGTCTCAA

*** *BRAFREFSeq2*: NG_007873.3

APPENDIX C: *KRAS* (173nt FRAGMENT) SEQUENCE ANALYSIS

	10	20	30	40	50	60	70	80	90	100	110	
KRASREFSeq	ATGACTGAAT	ATAAACTTGT	GGTAGTTGGA	GCTGGTGGCG	TAGGCAAGAG	TGCCTTGACG	ATACAGCTAA	TTCAGAATCA	TTTTGTGGAC	GAATATGATC	CAACAATAGA	GG-
2736KRAS	ATGACTGAAT	ATAAACTTGT	GGTAGTTGGA	GCTGGTGGCG	TAGGCAAGAG	TGCCTTGACG	ATACAGCTAA	TTCAGAATCA	TTTTGTGGAC	GAATATGATC	CAACAATAGA	GG'
5811KRAS	ATGACTGAAT	ATAAACTTGT	GGTAGTTGGA	GCTGGTGGCG	TAGGCAAGAG	TGCCTTGACG	ATACAGCTAA	TTCAGAATCA	TTTTGTGGAC	GAATATGATC	CAACAATAGA	GG'
25711KRAS	ATGACTGAAT	ATAAACTTGT	GGTAGTTGGA	GCTKGTGGCG	TAGGCAAGAG	TGCCTTGACG	ATACAGCTAA	TTCAGAATCA	TTTTGTGGAC	GAATATGATC	CAACAATAGA	GG'
28314KRAS	ATGACTGAAT	ATAAACTTGT	GGTAGTTGGA	GCTGGTGGCG	TAGGCAAGAG	TGCCTTGACG	ATACAGCTAA	TTCAGAATCA	TTTTGTGGAC	GAATATGATC	CAACAATAGA	GG'
28939KRAS	ATGACTGAAT	ATAAACTTGT	GGTAGTTGGA	GCTGGTGGCG	TAGGCAAGAG	TGCCTTGACG	ATACAGCTAA	TTCAGAATCA	TTTTGTGGAC	GAATATGATC	CAACAATAGA	GG'
32295KRAS2	ATGACTGAAT	ATAAACTTGT	GGTAGTTGGA	GCTGGTGGCG	TAGGCAAGAG	TGCCTTGACG	ATACAGCTAA	TTCAGAATCA	TTTTGTGGAC	GAATATGATC	CAACAATAGA	GG'
44021KRAS	ATGACTGAAT	ATAAACTTGT	GGTAGTTGGA	GCTGGTGGCG	TAGGCAAGAG	TGCCTTGACG	ATACAGCTAA	TTCAGAATCA	TTTTGTGGAC	GAATATGATC	CAACAATAGA	GG'
45779KRAS	ATGACTGAAT	ATAAACTTGT	GGTAGTTGGA	GCTGGTGGCG	TAGGCAAGAG	TGCCTTGACG	ATACAGCTAA	TTCAGAATCA	TTTTGTGGAC	GAATATGATC	CAACAATAGA	GG'
5080KRAS	ATGACTGAAT	ATAAACTTGT	GGTAGTTGGA	GCTGGTGGCG	TAGGCAAGAG	TGCCTTGACG	ATACAGCTAA	TTCAGAATCA	TTTTGTGGAC	GAATATGATC	CAACAATAGA	GG'
48578KRAS	ATGACTGAAT	ATAAACTTGT	GGTGTGTTGGA	GCTGGTGGCG	TAGGCAAGAG	TGCCTTGACG	ATACAGCTAA	TTCAGAATCA	TTTTGKGGAC	GAATATGATC	CAACAATAGA	GG'

*** *KRASREFSeq*: NG_007524.2

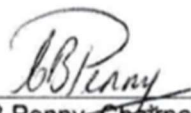
APPENDIX D: ETHICS CLEARANCE CERTIFICATE



R14/49 Dr Mark Andrew Robson Keyter

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

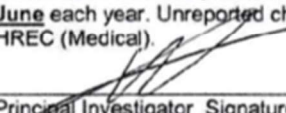
CLEARANCE CERTIFICATE NO. M190635

NAME: Dr Mark Andrew Robson Keyter
(Principal Investigator)
DEPARTMENT: Division of Anatomical Pathology
National Health Laboratory Service
PROJECT TITLE: Clinicopathological and molecular analysis of serrated lesions of the appendix
DATE CONSIDERED: 28/06/2019
DECISION: Approved unconditionally
CONDITIONS:
SUPERVISOR: Ewen McAlpine
APPROVED BY: 
Dr. CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL: 01/07/2019

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. I agree to submit a yearly progress report. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in June and will therefore be due in the month of June each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).


Principal Investigator Signature

Date

04/07/2019

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX E: TURN IT IN REPORT

a0015699:MMED_ResearchReport_MK_09122020-TurnItIn.pdf

ORIGINALITY REPORT

17%

SIMILARITY INDEX

8%

INTERNET SOURCES

15%

PUBLICATIONS

3%

STUDENT PAPERS

PRIMARY SOURCES

1

Submitted to University Der Es Salaam
Student Paper

2%

2

www.nature.com
Internet Source

1%

3

Rish K. Pai, Douglas J. Hartman, David H. Gonzalo, Keith K. Lai et al. "Serrated lesions of the appendix frequently harbor KRAS mutations and not BRAF mutations indicating a distinctly different serrated neoplastic pathway in the appendix", Human Pathology, 2014
Publication

1%

4

Rish K. Pai, Mark Bettington, Amitabh Srivastava, Christophe Rosty. "An update on the morphology and molecular pathology of serrated colorectal polyps and associated carcinomas", Modern Pathology, 2019
Publication

1%

5

www.ncbi.nlm.nih.gov
Internet Source

1%

link.springer.com

