Phenotype and Biology of Early Colorectal Cancers

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Background

The increased detection of pT1 colorectal cancers (CRC) in the National Health Service Bowel Cancer Screening Programme (NHSBCSP) raises new concerns for clinicians. The aim of this study is to investigate the phenotypic features and biology of screened and symptomatic pT1 CRC and to assess current and new high risk features associated with lymph node metastasis (LNM). The second aim of this study is to investigate the inter-observer variation of reporting screened pT1 CRC between pathologists.

Methods

Symptomatic and screened pT1 CRC were identified from two databases (Northern and Yorkshire Cancer Registry and Information Services [NYCRIS] and NHSBCSP database). Phenotypic features of the pT1 CRC were evaluated and compared from both cohorts.

The second part of the study investigated the inter-observer variability in the qualitative and quantitative assessments of screened pT1 CRC. Participating pathologists were asked to perform quantitative and qualitative assessments on 41 screened pT1 CRC. The level of agreement was determined using Fleiss Kappa statistics and intraclass correlation coefficient testing.

Results

Symptomatic CRC with LNM had a significantly wider area of invasion (p=0.001), a greater area of submucosal invasion ($p < 0.001$) and a higher proportion of tumour stroma ($p = 0.005$) compared to CRC without LNM. Symptomatic pT1 CRC were also significantly bigger in size than screened pT1 CRC.

The inter-observer variation study showed that quantitative factors had better levels of agreements than qualitative factors.

Conclusion

This study has shown that screened pT1 CRC are quantitatively smaller to their symptomatic counterparts suggesting that the NHS BCSP detects earlier pT1 CRC. This study also showed that novel quantitative factors such as width of invasion, area of submucosal of invasion and PoTS could be used as valid parameters in determining the rate of LNM. Finally, this study highlights the need for better guidelines/definitions in the evaluation of screened pT1 CRC.

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Thesis List of abbreviations

AJCC	American Joint Committee of Cancer
bFGF	basic Fibroblast Growth Factor
CAF	Cancer-associated fibroblasts
CMS	Consensus molecular subtype
CI	Confidence interval
CIMP	CpG island methylator phenotype
CRC	Colorectal cancer
CRT	Chemo-radiotherapy
CT	Computer tomography
CXCL12	Chemokine Ligand 12
DFS	Disease free survival
DNA	Deoxyribonucleic acid
EGF	Epidermal Growth Factor
EMR	Endomucosal resection
EMT	Epithelial – mesenchymal transition
EQA	External Quality Assessment
FAP	Fibroblast associated protein
FIT	Faecal immunochemical testing
FOBt	Faecal occult blood testing
GIST	Gastro-intestinal Stromal Tumour
H&E	Haematoxylin and eosin

HGF	Hepatocyte Growth Factor
HNPCC	Hereditary non-polyposis colorectal cancer
HR	Hazard ratio
ICC	Intraclass correlation coefficient
IF- γ	Interferon – gamma
IL	Interleukin
IQR	Inter-quartile range
JSCCR	Japanese Society for Cancer of the Colon and Rectum
K	Kappa value
LNM	Lymph node metastasis
MMC-1	Monocyte Chemotactic Protein - 1
MMR	Mismatch repair
MMP	Matrix metalloproteases
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
NHS	National Health Service
NHSBCSP	National Health Service Bowel Cancer Screening Programme
NK	Natural Killer
NKT	Natural Killer T
NYCRIS	Northern and Yorkshire Cancer Registry and Information Service
OR	Odds ratio
OS	Overall survival

PDGF	Platelet Derived Growth Factor
PoTS	Proportion of tumour stroma
ROC	Receiver operating characteristic
SD	Standard deviation
SDF – 1	Stromal-Derived Factor - 1
sm	Submucosa
SSL	Sessile serrated lesions
TAMs	Tumour-associated macrophages
TANs	Tumour-associated neutrophils
TEMS	Transanal endoscopic microsurgery
TGF-β	Tumour Growth Factor – β
TH 1	T helper 1
TNM	International Union Against Cancer (UICC) Tumour Nodes Metastasis Classification of Malignant Tumours
Tregs	T regulatory cells
TSA	Traditional serrated adenomas
UK	United Kingdom
WHO	World Health Organisation
VEGF	Vascular Endothelial Growth Factor

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Figures 2.11 Modified ROC curve showing the optimal for the area of submucosal invasion of carcinoma. The optimal value is determined from the peak of the curve (indicated by the arrow) [Area under curve = 0.747].

Figure 2.12 Diagram illustrating pT1 cancers (indicated by the boxes with the red diagonal lines) with different width (x and y) but similar depths of invasion. The wider cancer has a bigger area of invasion compared to the cancer with the smaller width. Hence, with the greater area, the wider cancer has more chances of coming into contact and invading lymphatic vessels.

Figure 2.13 Diagram illustrating pT1 cancers with similar widths (x) but different depths of invasion. The pT1 cancer with SM3 invasion has progressed further in depth and therefore may have a greater area of invasion within the submucosa when compared to the pT1 cancer with SM1 depth invasion. The length of invasive front is also increased in wider and deeper cancers but this study has shown no significant difference in cases with and without LNM.

Figure 5.43 Overall results in round 2 for each case in deciding whether the lesion should receive a radical resection excluding the non-assessable cases.

Chapter 1: Colorectal cancer

Colorectal cancer (CRC) is the third most common cancer worldwide after lung and breast cancer with 60% of all CRC presenting in more developed countries. CRC is an important public health problem as nearly 1.36 million new cases are diagnosed worldwide each year with around 694,000 deaths. In the UK, CRC is the fourth most common cancer in the UK where around 41,600 people are diagnosed with CRC each year (CRUK 2015a).

In England and Wales, the overall five-year survival rate for CRC has improved over the last 30 years. The 5-year survival rate for males and females are 59.2% and 58.2% respectively (CRUK 2015b). Much of the improved survival rate is attributed to earlier cancer detection, improved surgical techniques, the use of therapy post-operatively and for pre-operative down staging by radiotherapy and chemotherapy. Endoscopy (colonoscopy and/or flexible sigmoidoscopy) and imaging modalities such as computer tomography (CT) and magnetic resonance imaging (MRI) scans have helped detect and stage CRC and also dictate the best treatment options (Salerno, Daniels and Brown 2006, Levin et al 2008, Beets-Tan and Beets 2011). Advancing surgical techniques (Heald 1988, Ptok et al 2007, Guerrieri et al 2008, Hohenberger et al 2009) sometimes with CRT (IMPACT 1995) have reduced the recurrence rate of CRC and have helped increase the survival rates in patients with favourable staging. However, there are associated morbidity and mortality risks with surgery (Canivet et al 1989, Brown et al 1991, Bokey et al 1995, Wolpin et al 2007) and CRT (Ooi et al 1999, Andre et al 2004, De Dosso et al 2009). In everyday clinical practice, it is difficult to balance the risks of morbidity and mortality associated with surgery and CRT versus the potential risk of under treating the

malignancy. This is highlighted most in the area of early cancer, stage pT1, where there is a difficult decision making process in determining local endosopic or surgical excision versus a major surgical excision.

1.1 Anatomy of the large bowel

1.1.1 Gross anatomy of the large bowel

The large bowel is the most distal segment of the gastrointestinal tract. It is around 1.5 meters in length and originates in the lower right quadrant of the abdominal cavity, starting at the ileocaecal valve. The large bowel can further be divided into different sections namely the caecum, ascending, transverse, descending and sigmoid colon. The large bowel eventually terminates at the rectum. The rectum is the terminal part of the large bowel and is located within the pelvic cavity. The contents of the large bowel are then evacuated via the anal canal.

The large bowel can be differentiated from the small bowel by its wider transverse diameter, sacculations of the wall (haustra), thickened longitudinal muscle bands (taeniae coli) and the presence of omental appendages (appendices epiplociae). The transverse diameter diminishes continually towards the distal end of the gastrointestinal tract except for the dilatation known as the rectal ampulla.

The arterial supply to the large bowel is from the superior and inferior mesenteric arteries arising from the aorta. The arterial supply from the rectum is via the inferior mesenteric artery and also branches of the internal iliac arteries (middle and inferior rectal arteries). The main venous drainage of the large bowel is by the way of the portal venous system into the liver where absorbed nutrients are metabolised.

There is also a secondary drainage system for the large bowel in the form of the lymphatic system. The lymphatic system is composed of network lymphatic vessels and channels that subsequently drain lymph via lymph nodes into the cisterna chyli in the upper abdomen. The lymph circulates from the cisterna chyli via the thoracic duct into the left subclavian vein.

1.1.2 Microscopic anatomy of the large bowel

The architecture of the large bowel wall is similar to the organisation of the entire gastrointestinal tract as observed from proximal oesophagus to the anus. It contains five distinctive layers; the mucosa, sub-mucosa, muscularis propria, subserosa and serosa.

1.1.2.1 Mucosa

The colonic mucosa lines the inner surface of the large bowel and appears smooth at the gross level due to the absence of villi (figure 1.1). The mucosa is lined by a single layer of columnar epithelium composed of a variety of cell types such as goblet cells, absorptive colonocytes and neuroendocrine cells. The composition of the cells depends on the location within the large bowel and position in the crypt. The crypt has a basement membrane and on the lamina propria side a thin layer of myofibroblasts. The colonic glands are maintained within a connective tissue matrix (lamina propria), which in turn is bounded by a thin smooth muscle layer, the muscularis mucosae, a layer that defines the lower limit of the mucosa.

1.1.2.2 Muscularis Mucosae

The muscularis mucosae represents the lower border of the mucosa and consists of a thin layer of smooth muscle fibres with mixed transverse, oblique and longitudinal orientation. Vascular and lymphatic structures as well as neural branches traverse this muscle layer. The muscularis mucosae is an important pathological structure as the breach of this structure by neoplastic colorectal epithelium indicates invasive carcinoma (figure 1.1) (Walsh and Carey 2013).

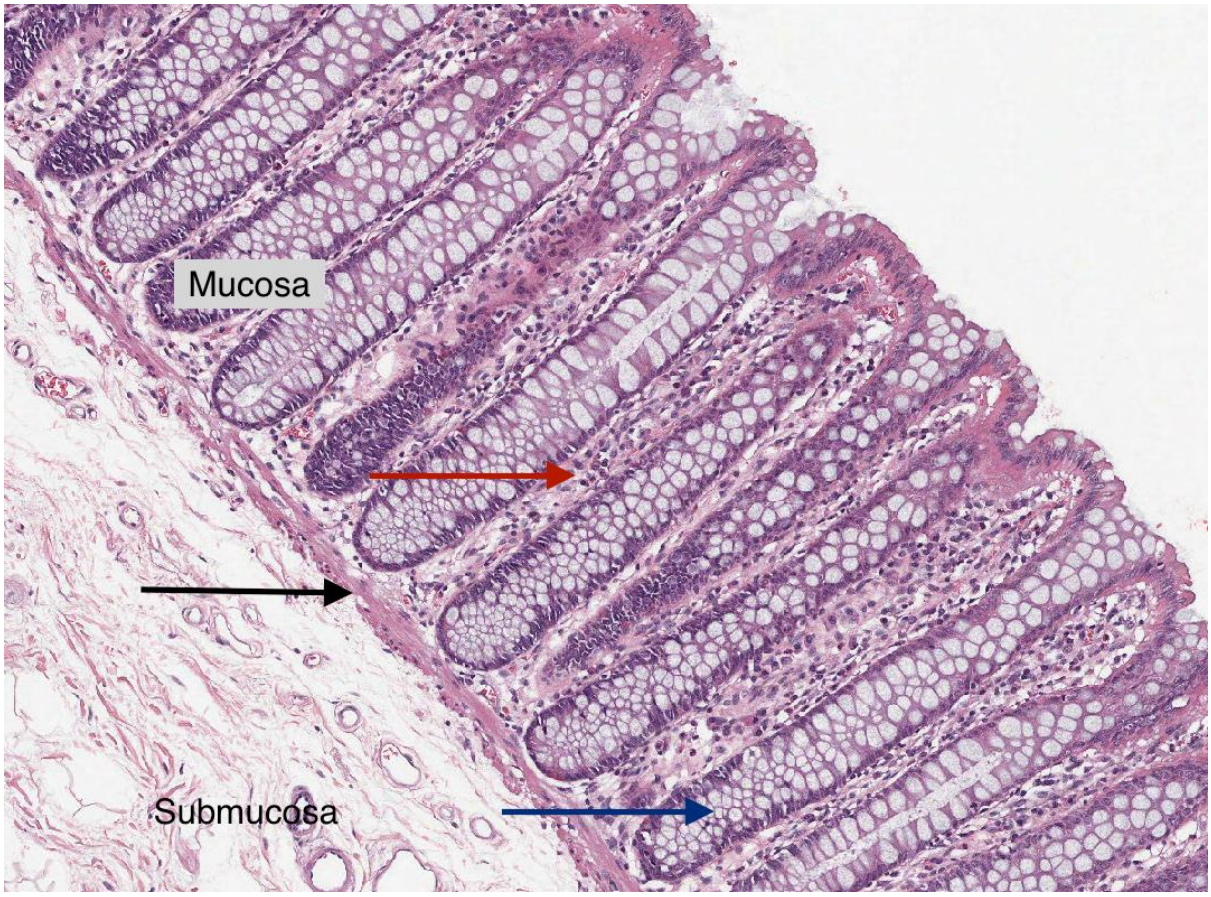


Figure 1.1: The mucosa composed of crypts and lamina propria. The blue arrow indicates the crypts of the colonic mucosa and the red arrow indicates the lamina propria. The black arrow indicates the muscularis mucosae at the lower border of the mucosa. Magnification is at 100x.

1.1.2.3 Submucosa

The submucosa underlies the muscularis mucosae and consists of loose connective tissue such as blood vessels, lymphatics, smooth muscle fibres, collagen and adipose tissue (figures 1.1 and 1.2). It also contains two enteric neural plexi; the inner submucosal plexus (Meissner's plexus) and the outer submucosal plexus (Schabadasch's or Henle's plexus). The enteric nervous system serves to transmit

central, sympathetic and parasympathetic nerve signals to the large bowel in order to regulate its function.

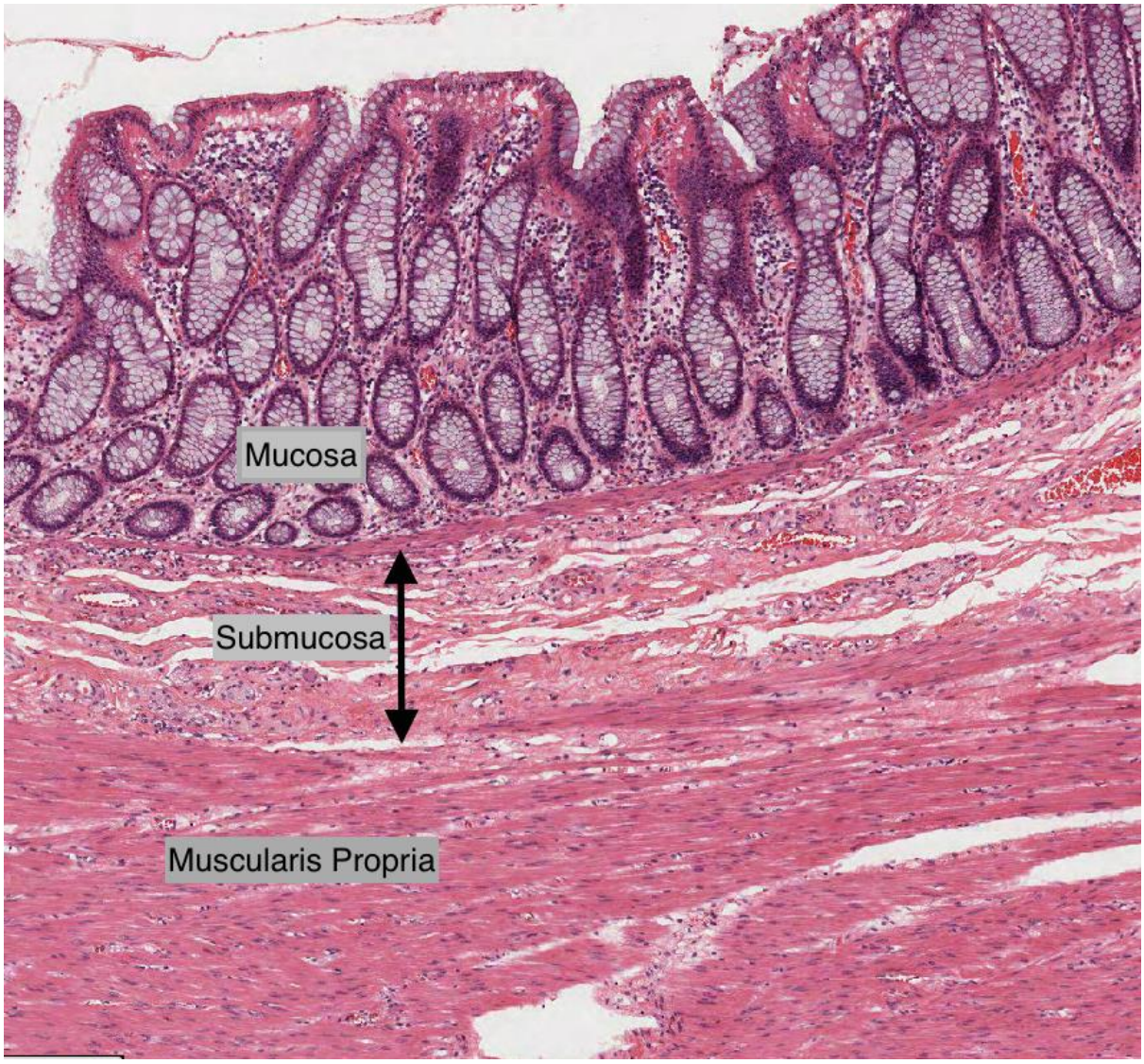


Figure 1.2: The submucosa occupies the area between the muscularis mucosae and the muscularis propria as indicated by the arrow. Magnification is at 40x.

1.1.2.4 Muscularis propria

The muscularis propria comprises of an inner circular smooth muscle layer and an outer longitudinal layer (figures 1.3). In between the muscle layers is the third plexus of the enteric nervous system, the myenteric plexus (Auerbach's plexus) that provides motor innervations to both muscles and secretomotor innervations to the mucosa. The muscularis propria also contains blood vessels and lymphatics.

1.1.2.5 Sub-serosa, serosa and peritoneum

The sub-serosa is the outermost layer of the large bowel and its outermost surface is covered by the serosa consisting of connective tissue with a surface of mesothelium (figure 1.3). The sub-serosa has the same composition as the mesentery. Within the peritoneal cavity, the serosa is also known as the visceral peritoneum and continues over the abdominal wall as the parietal peritoneum.

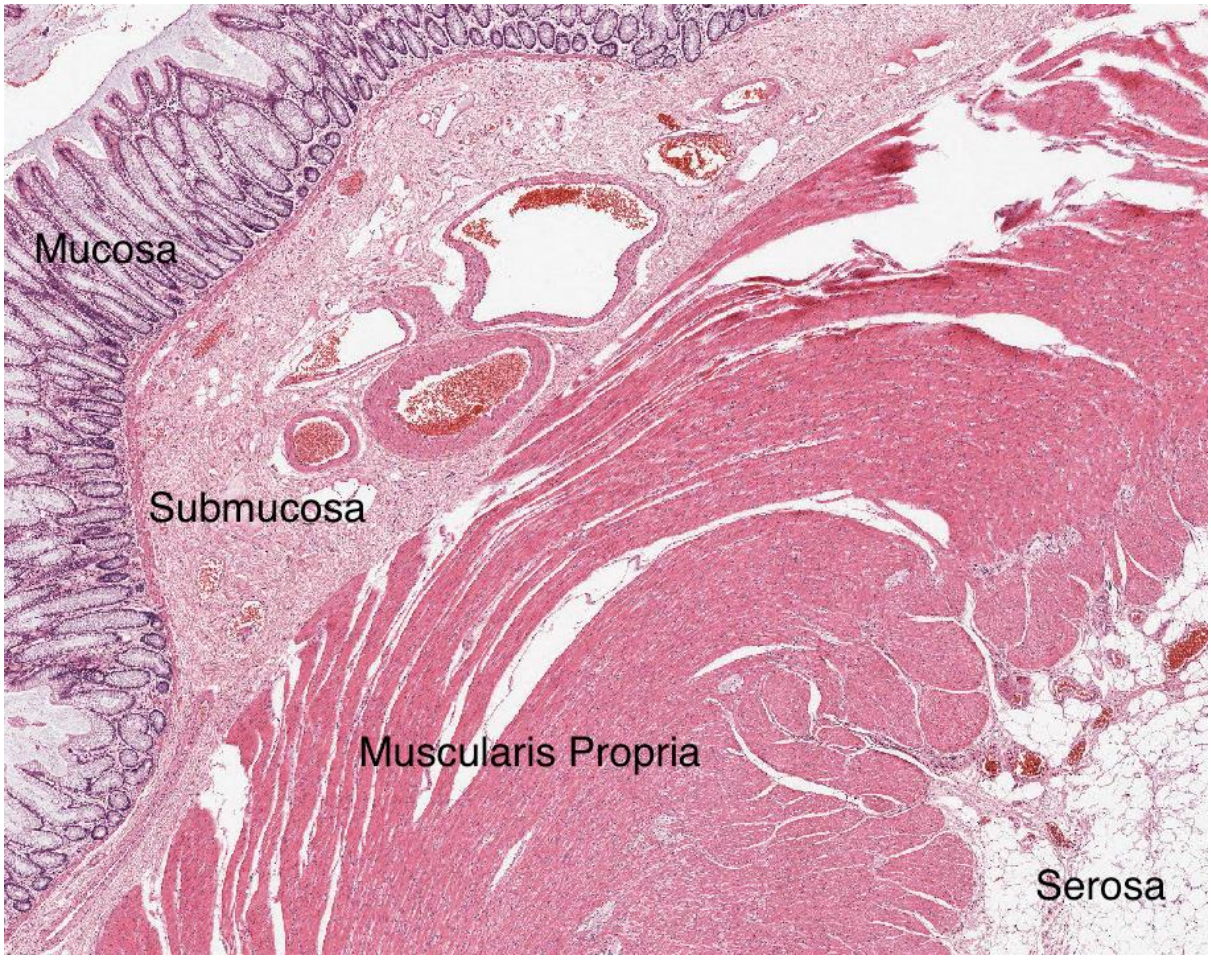


Figure 1.3: Microscopic view of the layers of the colon. Magnification is at 10x.

1.2 Epidemiology of colorectal cancer (CRC)

Around 110 new cases of CRC are diagnosed each day in the UK and it is the fourth most common cancer. In 2011, there were 41,600 new cases of CRC registered in the UK with 95% of CRC occurring in people aged 50 years and over. Most of the CRC are diagnosed in the left side of the colon with around 60% occurring in the sigmoid colon, recto-sigmoid junction and rectum (CRUK 2015a) (figure 1.4).

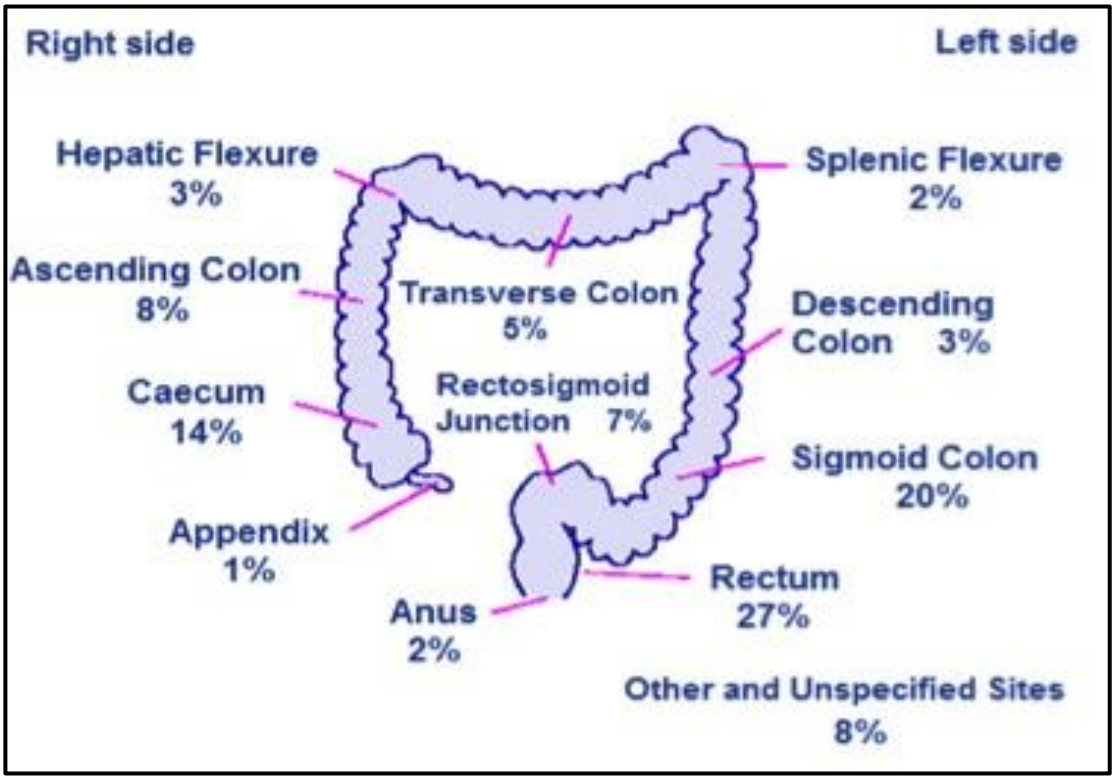


Figure 1.4: Distribution of cancers within the colon, Great Britain, 2007 – 2009. Diagram obtained from Cancer Research UK website [http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/bowel-cancer/incidence#heading-Zero]

CRC is the third most common cancer in both males and females separately. Up to the age of 50, men and women have similar proportions for CRC, but later in life, the male proportion increases (figure 1.5). The lifetime risk for men and women being diagnosed with CRC in the UK is approximately to 1 in 14 and 1 in 19 respectively (CRUK 2015b).

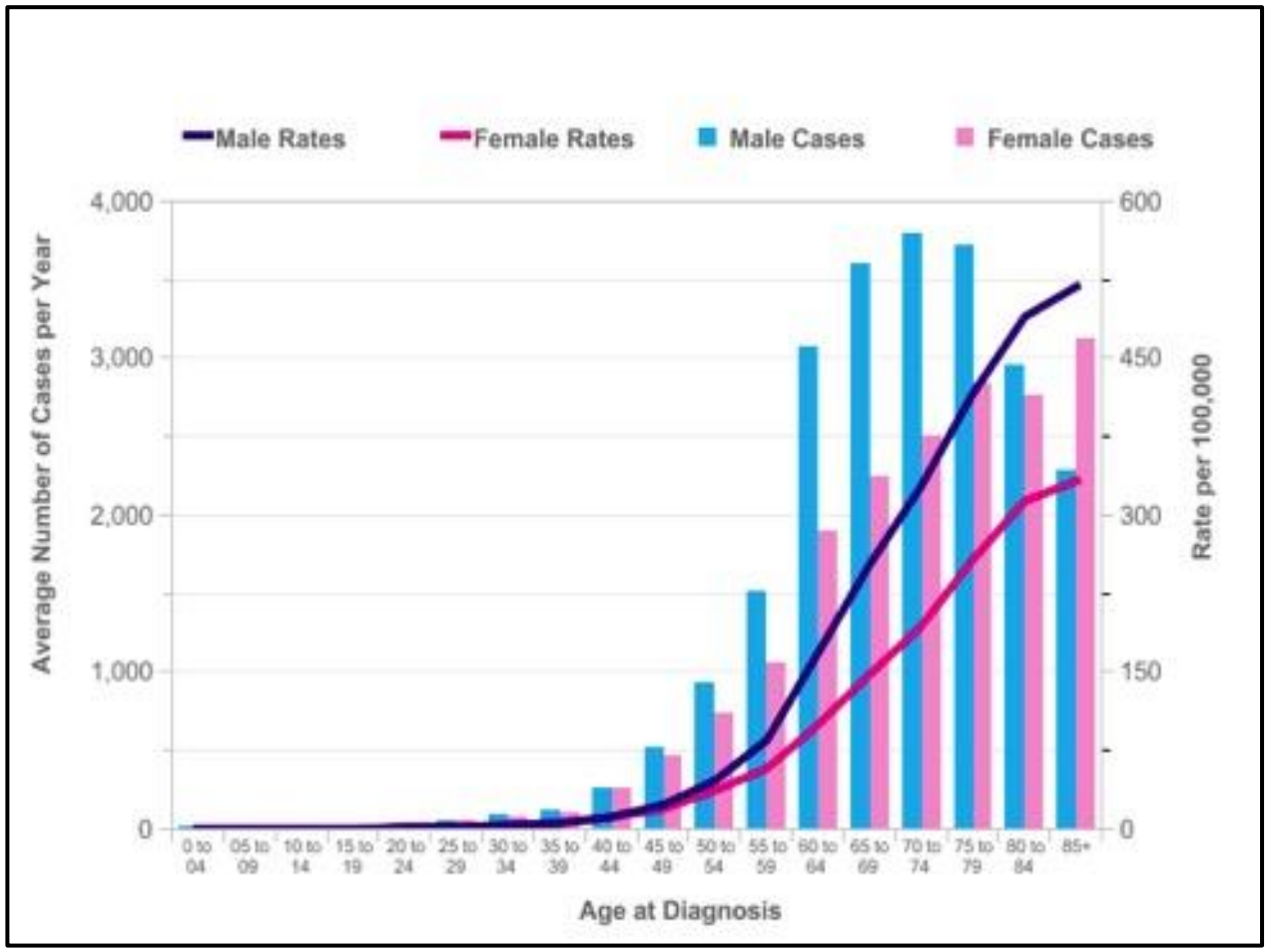


Figure 1.5: Average new number of CRC cases per year and age-specific incidence rates, UK 2009 – 2011. Diagram obtained from Cancer Research UK website [http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/bowel-cancer/incidence#heading-Zero]

1.3 Development of CRC

CRC is a complex disease that arises from a sequence of genetic and molecular events. It has been recognized that normal colonic mucosa produces adenomas that progress onto carcinomas if left untreated. This pathway is most commonly known as the adenoma – carcinoma sequence (Morson 1974). CRC are sporadic, familial (inherited) or associated with inflammatory bowel disease. Sporadic CRC accounts for 70 – 80% of colorectal malignancies. Progression from normal colonic epithelium through to adenoma and finally to carcinoma requires several genetic alterations to normal tumour suppressor genes and proto-oncogenes (Hardy, Meltzer and Jankowski 2000).

Hanahan and Weinberg proposed six biological hallmarks that contribute to the development of cancer in general which can be applied to CRC (Hanahan and Weinberg 2011). The six hallmarks that were proposed are:

- Self-sufficiency in growth signals
- Insensitivity to antigrowth signals
- Ability to evade apoptosis
- Limitless replicative potential
- Sustained angiogenesis
- Tissue invasion and metastasis

The first four hallmarks are acquired through genomic instability by alterations to proto-oncogenes, tumour suppressor genes and DNA mismatch repair genes.

1.3.1 The adenoma – carcinoma pathway

CRC is a heterogeneous disease with multiple molecular pathways leading to different phenotypes As a result of germ line defects or damage and changes in the somatic DNA, normal colonic mucosa epithelium can transform into a benign neoplasm (adenoma) and subsequently into invasive carcinomas (Markowitz and Bertagnolli 2009, Pritchard and Grady 2011). The progression of tubular, tubullo-villous and villous adenomas into CRC has long been recognized, but there is evidence that another pathway to malignancy exists; the serrated neoplasia pathway. This serrated pathway involves a small percentage of hyperplastic polyps progressing to serrated lesions and eventually to CRC (Pritchard and Grady 2011). The serrated adenoma pathway will be discussed further later in this chapter.

Currently, there are three distinct molecular pathways that lead to the transformation of normal colonic mucosa to an adenomatous lesion and subsequently CRC. The three molecular pathways are the chromosomal instability pathway, microsatellite instability pathway and the CpG Island Methylator Phenotype (CIMP) pathway.

1.3.1.1 Chromosomal instability pathway

Chromosomal instability is the most common cause of genomic instability in CRC and is found in up to 85% of sporadic CRCs (Pritchard and Grady 2011). Chromosomal instability results from defective chromosomal segregation leading to aneuploidy, telomere dysfunction or defects in DNA damage response mechanism (Pino and Chung 2010). This most frequently results in loss of heterozygosity at the tumour suppressor gene loci and chromosomal rearrangements. Tumours with

chromosomal instabilities are distinguished with the accumulation of mutations in specific oncogenes (KRAS, BRAF, PIK3CA) and tumour suppressor genes (APC, TP53), thereby triggering the development of CRC.

The most common gene implicated in chromosome instability is the APC gene. The function of the APC is to regulate the spindle microtubule formation and is required to detect misaligned chromosomes during mitosis (Caldwell and Kaplan 2009). The autosomal condition familial adenomatous polyposis, in which numerous adenomatous colonic polyps develop leading to a 100% lifetime risk of developing CRC (Woods et al 2010, Kastrinos and Syngal 2011) is the consequence of the alteration to the APC gene.

The other important example of tumour suppressor gene loss is the TP53 gene. TP53 gene is a key tumour suppressor gene that is mutated/altered in about half of all CRCs (Pritchard and Grady 2011). The TP53 gene has multiple functions. It has been shown to be involved in cell cycle regulation, apoptosis, DNA excision repair and chromosomal segregation (Wsierska-Gadek and Horky 2003, Ewing et al 2014). Loss of function of the TP53 gene often results in the malignant transformation of colonic adenomas (Markowitz and Bertagnolli 2009, Pritchard and Grady 2011).

All or most oncogenes are alterations of normal genes called proto-oncogenes. Proto-oncogenes are normal genes that encode proteins that are responsible for the control of key functions of cell growth, division and development. The proto-oncogenes normally facilitate signal transmission of extracellular growth signals to the nucleus of the cell and also regulate cellular signal transduction. The mutated proto-oncogene is called an oncogene. Oncogenes typically exhibit increased function or production of proteins that are vital in cellular development thus leading to

unregulated cell growth and division. An example of an oncogene often implicated in CRC is the KRAS oncogene which occurs relatively early in the adenoma-carcinoma sequence (Pritchard and Grady 2011). There are two types of KRAS oncogenes that are found in CRC; the normal (wild-type) KRAS oncogene and the abnormal mutated KRAS oncogene. In CRC, up to 60 – 70% of patients have the wild type KRAS oncogene and the remaining 30 – 40% have the mutant version of the oncogene (Schuch, Kobold and Bokemeyer 2009).

The BRAF gene is another proto-oncogene often implicated in CRC and other cancers such as malignant melanoma, papillary thyroid and lung cancers. The BRAF gene encodes for the protein B-raf which is a member of the Raf kinases family of phosphorylating enzymes that control cell differentiation and division. Mutations or alterations to the BRAF gene results in BRAF acting as an oncogene.

1.3.1.2 Microsatellite instability (MSI) pathway

Microsatellites are repeated sequences of DNA found throughout the entire genome. The repetitive nature of microsatellites makes them a vulnerable target to transcription errors during replication. The development of CRC with microsatellite instability (MSI) involves the inactivation of genes responsible for DNA mismatch repair (MMR) through either through germline, somatic mutation or aberrant methylation (Pritchard and Grady 2011). The somatic inactivation of the MMR genes is found in approximately 15% of cases of sporadic CRC and associated with older age, the female sex and the proximal distribution of the CRC (Markowitz and Bertagnolli 2009, Bogaert and Prenen 2014).

The germline mutation/ alteration of MMR genes is responsible for Lynch syndrome or hereditary non-polyposis colorectal cancer (HNPCC). Loss of function in the MMR gene has been identified in four genes: MLH1, MSHS2 (accounting for majority of cases), MSH6 and PMS2 (Woods et al 2010, Kastrinos and Syngal 2011). Lynch syndrome is inherited in an autosomal dominant fashion and is the most common hereditary CRC syndrome amounting to 2 – 3% of all cases (Kastrinos and Syngal 2011). Affected individuals have an 80% lifetime risk of developing CRC (Woods et al 2010) and are also at risk of developing extra-colonic malignancy that includes endometrial and ovarian cancers (Al-Sohaily et al 2012).

1.3.1.3 CpG Island Methylator Phenotype (CIMP) Pathway

Changes to gene expression or function without changing the DNA sequence of that particular gene can happen with epigenetic alteration. Epigenetic alteration is usually caused by aberrant DNA methylation. In particular, the aberrant methylation occurs within the promoter-associated CpG islands leading to the loss of function of the MMR gene hMLH1 (Issa 2004, Markowitz and Bertagnolli 2009). The hypermethylation of promoters containing CpG islands is known as the CpG island methylator phenotype (CIMP) (Pritchard and Grady 2011) and is observed in 15% of CRCs where there is loss of MLH1 expression resulting in MMR deficiency and associated MSI (Donehower, Creighton and Schultz 2013).

Another gene involved in DNA repair is the MGMT gene. The MGMT gene encodes for the MGMT protein (methylated DNA protein cysteine methyltransferase) that is

involved in repair of DNA. This MGMT gene can be inactivated by hypermethylation of its promoter sequence and thus impair its DNA repairing properties.

1.3.2 Serrated colorectal lesions

The significance of colonic serrated lesions and their importance as an alternate pathway to the development of CRC have long been recognised. First used in 1990, Longacre and Fernoglio-Preiser had described the term ‘serrated adenoma’ for colonic polyps that had features of a conventional adenoma and a hyperplastic polyp. This lesion had subsequently been termed as the ‘traditional serrated adenoma (TSA) (Longacre and Fernoglio-Preiser 1990). A few years later, Torlakovic and Snover had identified polyps that showed a constellation of features that were specific for hyperplastic poylps and TSAs and this led to the recognition of the entity of sessile serrated lesions (SSL) (Torlakovic and Snover 1996).

The terminology of serrated lesions is complex as there are differences in opinion between UK, European and US pathologists in regards to the constitution of a serrated lesion. In the UK, it has been proposed that serrated lesions are given one of the following names based on their morphological features: hyperplastic polyp, sessile serrated lesion, sessile serrated lesion with dysplasia and traditional serrated adenoma (Bateman and Shepherd 2015). These lesions will be described in detail in the next headings.

1.3.2.1 The spectrum of sessile serrated lesions

A spectrum of colonic polyps with partial or wholly serrated architecture is now recognised. The spectrum of these polyps ranges from polyps with no dysplasia (hyperplastic polyps, sessile serrated lesions without dysplasia) to polyps with definite dysplasia (sessile serrated lesions with dysplasia, traditional serrated adenomas) (Bateman 2014).

1.3.2.2 Hyperplastic polyp

Hyperplastic polyps are very commonly encountered by both the endoscopist and pathologist. They constitute approximately 25 – 30% of all resected colonic polyps and have an estimated prevalence of 10 – 20% in the Western adult population (Bettington et al 2013). Hyperplastic polyps occur at all site of the large bowel but are more common distally. Three morphological variants exist – microvesicular, goblet cell and mucin poor types (Bateman 2014).

Despite possessing both the BRAF and KRAS mutations, the malignant potential of hyperplastic polyps are considered to be low and under current UK guidelines, no additional surveillance is required (Bettington 2013, Bateman and Shepherd 2015).

1.3.2.3 Sessile serrated lesions (SSL)

SSLs share similar histological features with the microvesicular variant of hyperplastic polyps (Torlakovic and Snover 1996). However, SSLs are bigger than hyperplastic polyps and contain several other key histological features such as an

irregular distribution of crypts, dilatation of crypt bases, presence of serration at crypt bases, branched crypts, horizontal extension of crypt bases, dysmaturation of crypts and herniation of crypts though the muscularis mucosae (Bateman and Shepherd 2015).

Similarly to hyperplastic polyps, SSLs are associated with BRAF gene mutations and therefore, it is possible that hyperplastic polyps and SSLs are part of the same spectrum of serrated lesions with small hyperplastic polyps at one end and large SSLs at the other (Bateman 2014).

1.3.2.4 SSL with dysplasia

Dysplasia can arise in a SSL and the dysplasia can be low or high grade in nature. It is also believed that SSLs with dysplasia are associated with a quicker progression to adenocarcinoma compared to the 'classical' adenoma (Bettington et al 2013). In SSL with dysplasia, there is loss of function of the DNA mismatch repair gene, hMLH-1, increased DNA methylation (the CpG island methylator phenotype) as well as mutation to the BRAF gene (Bettington et al 2013).

1.3.2.5 Traditional serrated adenomas (TSA)

TSAs are uncommon lesions and are distinct from SSL. TSA frequently occur more within the left side of the large bowel and their histological features include tubulovillous architecture, eosinophilic cytoplasm, elongated ('pencillate') nuclei and the presence of multiple tiny crypts extending from the primary crypts (ectopic crypts)

that give a serrated appearance to the lesion (Longacre and Fenoglio-Preiser 1990). These serrated areas are usually mixed with areas displaying a more 'classical' adenoma growth pattern and transition between these areas is abrupt. Dysplasia may occur within a TSA and progression to adenocarcinoma can take place (Makinen 2007).

1.3.3 The serrated carcinoma pathway

The serrated carcinoma pathway currently has two main mechanisms that involve BRAF and KRAS mutations. The BRAF pathway is further subdivided into two further pathways depending on its MSI status.

SSLs are associated with an early BRAF mutation followed by in some cases, loss of MLH1 expression. The resulting CRC contains BRAF mutations,are CIMP-high and exhibit MSI. This type of CRC tends to occur in elderly women and is most common in the proximal large bowel. Another characteristic of this CRC is that it frequently presents with a high tumour stage but without nodal or distant metastasis (Bettington et al 2013).

The second alternative BRAF pathway occurs in SSL in which MLH1 is not lost but may otherwise show silencing of p16 function or MGMT loss. The ensuing CRC again contains the BRAF mutation, is CIMP-high but may only display low levels of MSI or none at all. These tumours occur more proximally in the large bowel but are more aggressive as they are often poorly differentiated, mucin producing and have higher rates of tumour budding, lympho-vascular invasion and perineural invasion (Bettington et al 2013).

The other serrated carcinoma pathway involves mutation of the KRAS gene. TSA are commonly associated with KRAS gene mutations and wnt abnormalities. This results in CRCs with KRAS mutations, CIMP-low and is microsatellite stable (Jass et al 2006, Bettington et al 2013). A summary of the serrated carcinoma pathway is shown in figure 1.6.

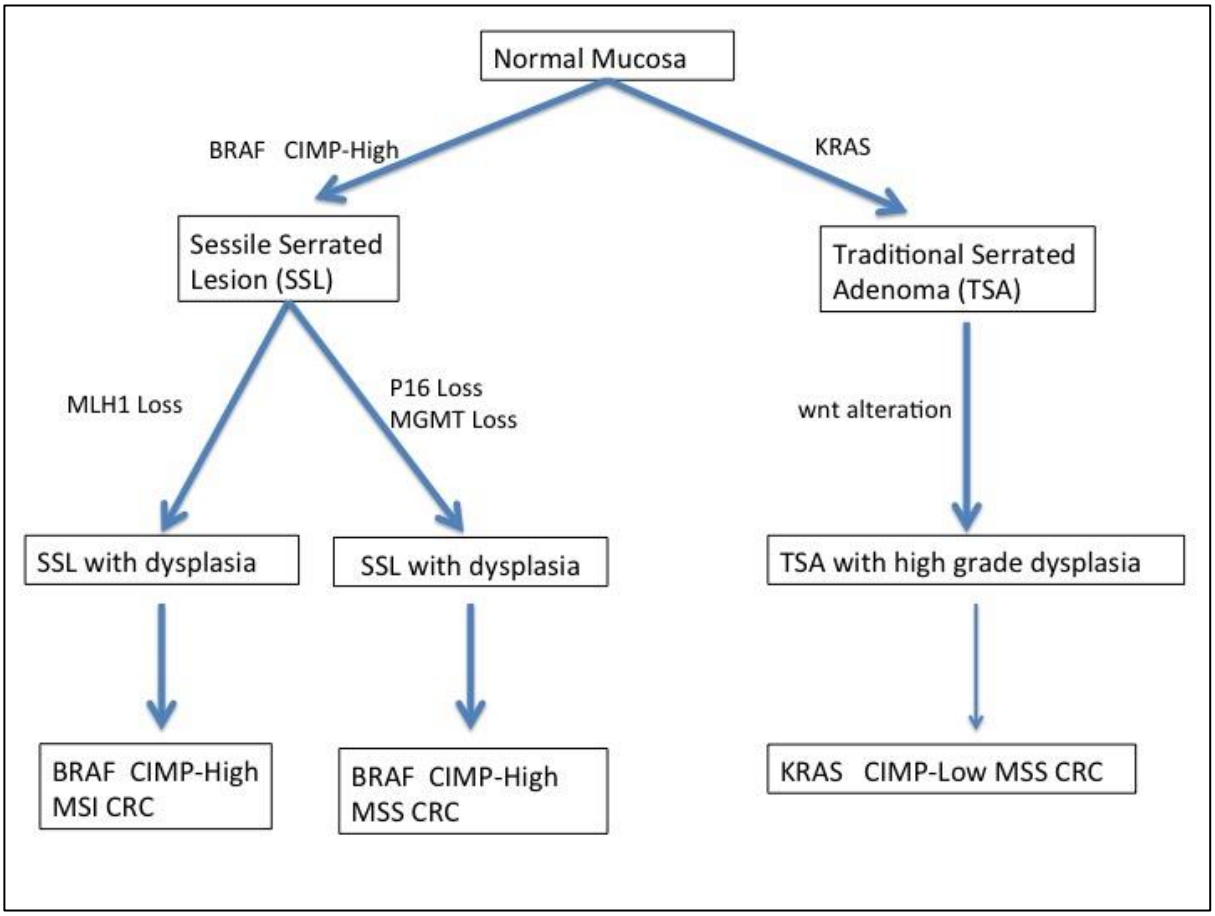


Figure 1.6: Flow diagram of the serrated carcinoma pathways (Figure adapted from Bettington et al 2013).

1.4 Staging and grading of CRC

Staging of CRC describes how far the cancer has progressed and the extent of its spread within the body. Cancer staging aids clinicians in determining the most appropriate treatment for each patient. Staging also gives a reasonable indication of prognosis with an increasing stage denoting a poorer prognosis. In the UK, CRC is staged using the two main systems: the Dukes' and TNM staging systems.

1.4.1 Dukes' and TNM staging system

The Dukes' staging system for CRC was first proposed in 1932 by the British pathologist Cuthbert Dukes (Dukes 1932). At first, the Dukes' staging system consisted of 3 stages; A, B and C but it was later modified to include a subdivision of stage C (stages C1 and C2) (Dukes and Bussey 1958) and further revised by Turnbull to include a stage D (Turnbull et al 1967) (Table 1.1).

Stage	Definition	Expected 5 yr cancer specific survival rate
A	Tumour invades through the muscularis mucosae into the submucosa but not through the muscularis propria. No lymph node involvement.	>90%
B	Tumour invading through the muscularis propria. No lymph node involvement.	60 – 75%
C1	Spread of tumour to local lymph node(s).	40 – 60%
C2	Spread of tumour to apical lymph node.	20 – 40%
D	Spread of tumour to other organs	5 – 40%

Table 1.1: Modified Dukes' staging of CRC and expected 5 year cancer specific survival rate

The TNM staging system was developed by Denoix between 1943 and 1952 (Denoix and Schwartz 1959) and is the most commonly used staging system. This system incorporates depth of invasion along with nodal and metastatic disease status (Table 1.2). Currently, in the UK, the Royal College of Pathologists recommends that CRC staging should be based on the fifth edition of the TNM manual (TNM 5) (Greene et al 2002).

"T" describes the depth of invasion of the tumour through the bowel wall

"N" indicates whether or not there is spread to lymph nodes and if so, how many lymph nodes are involved.

"M" describes the presence of distant metastasis (commonly to the liver or lungs)

Tumour Stage (T)	**Definition**
T0	No evidence of carcinoma
Tis	Carcinoma in-situ
T1	Invasion of the submucosa but no further
T2	Invasion into the muscularis propria
T3	Invasion through the muscularis propria and into the subserosal layers but not through them
T4	Tumour has invaded through the serosal layer and there is direct invasion into other organs or there is perforation of the visceral peritoneum
Nodal Stage (N)	
N0	No regional lymph node metastasis
N1	Metastasis into 1 to 3 regional lymph node(s)
N2	Metastasis into 4 or more regional lymph nodes
Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis present

Table 1.2: Description of each component to the TNM staging system (version 5) (Sobin and Fleming 1997)

1.5 Treatment of CRC

Curative treatment for CRCs consists of 3 options: major surgery, oncological treatment (chemotherapy and/or radiotherapy) and increasingly, local excision for early CRC. All these options can be used in isolation or in combination with each other depending on the CRC stage. For example, in advanced rectal cancers, a patient may initially receive radiotherapy and/or chemotherapy before surgical resection. After surgical resection, the patient then may receive further chemotherapy if there is any evidence of lymph node spread.

1.5.1 Surgery

Surgery is the mainstay of curative treatment and in the UK approximately 80% of patients with CRC undergo surgery (CRUK 2015a). The type of surgery that is offered depends on tumour location. Surgery involves resection of the bowel containing the tumour and its mesentery that contains the vascular supply and draining lymph nodes. If possible, the remaining bowel is re-anastomosed to restore intestinal continuity. However, in certain cases where rectal cancer is low-lying, a permanent stoma is needed due to involvement of the anal sphincter complex.

After resection, the bowel containing the tumour and its respective mesentery are examined by a pathologist. This will accurately determine the completeness of resection, the stage of the cancer and finally the grade of differentiation of the cancer. This information will then further assist clinicians in terms of further treatment if needed.

Risks of surgery include those relating to age and co-existing morbidity and operative and anaesthetic risk. Most common operative risk includes bleeding, infection (wound, chest or intra-abdominal infections), wound dehiscence, thrombo-embolic event, post-operative cardiac event and anastomosis leaks. Overall, morbidity and mortality of colorectal cancer surgery varies from 28% - 37.2% and 3.4% - 5.9% respectively (Bokey et al 1995, Longo et al 2000, Alves et al 2005, Morris et al 2011).

1.5.2 Local procedures

With the increasing number of pT1 CRCs detected within the screening programme, avoidance of major invasive surgery by use of local excision procedures like polypectomy, endomucosal resection (EMR) and transanal endoscopic microsurgery (TEMS) would be ideal.

Polypectomy and EMR are possible for pT1 cancers throughout the bowel and can be done by a trained endoscopist or surgeon. Polypectomy is a technically easier procedure compared to EMR but EMR has the advantage of removing flat or sessile lesions. EMR also identifies lesions that are invading or tethered to the deep submucosa or muscle layer (non-lifting sign) which are unlikely suitable for endoscopic removal and confirms the need for a more radical procedure.

TEMS was first introduced by Buess et al in 1983 (Buess et al 1984, Buess et al 1985; Buess et al 1987, Buess et al 1988a; Buess et al 1988b). TEMS is most commonly done on pT1 cancers affecting the rectum. TEMS is possible due to the accessibility and locality of the cancer within the rectum. Many studies have been

done on the effectiveness of TEM in treating rectal cancers. In 1996, Winde published a prospective randomized trial where TEM (n=24) was compared to anterior resections (n=26) in the treatment of T1N0 rectal cancers (Winde et al 1996). The study showed no statistically significant difference for local recurrence or 5-year survival rates. Langer et al showed that TEM (n=79) had lower rate of recurrence when compared to conventional local excision techniques (n=76) and a lower incidence of complications and less impairment of life quality when compared to radical resection (n=27) (Langer et al 2003).

Ptok et al also showed that local resection (conventional transanal approach and TEMS) were associated with fewer general and specific post-operative complications. However, they showed that the patients with local resections had a significantly higher 5-year local tumour recurrence rate compared to the patients who underwent radical resection. Interestingly, 5-year tumour-free survival and overall survival rate did not differ for both cohorts (TEMS vs radical resection) (Ptok et al 2007). In a prospective study done by Guerrieri et al, they showed that 51 patients who had undergone TEMS did not have any local recurrence or metastasis. The survival rate was 100% at a median follow-up period of 81 months (25^{th} – 75^{th} percentile = 44 – 121 months) (Guerrieri et al 2008).

Local excision procedures like these are optimal as they have lower rates of morbidity (4% - 17%) and mortality (0% - 1.3%) compared to major resections (de Graaf et al 2002, Maslekar et al 2006, Bretagnol et al 2007). However, local excisions do not accurately stage the cancer as lymph node status is unknown. This will have an impact as treatment can be inadequate and the risk of local/distant recurrence will be high.

1.5.3 Chemotherapy and radiotherapy (CRT)

CRT is used as an adjunct to major surgery or local procedures. CRT is also used in isolation as palliation for inoperable cancers. As mentioned already, CRT may be commenced prior to surgery to help reduce tumour size and also downstage the cancer. Post-operative or adjuvant chemotherapy is often administered to patients with node positive disease (stage III) based on the results of the IMPACT trial (IMPACT 1995). The IMPACT trial studied the efficacy of adjuvant 5-flurouracil (5-FU) and folinic acid in treating patients with Dukes' B and C CRC. The results showed a significant reduction in mortality by 22%, increasing 3-year event free survival by 11% and overall survival from 78% to 83%. The successor to the IMPACT trial, the MOSAIC trial showed improvement in the 6-year overall-survival rate with the addition of oxaliplatin to 5-FU (Andre et al 2009). The benefits of adjuvant chemotherapy in stage II (node negative) CRC was investigated by the QUASAR trial (Gray et al 2007) and showed that there is an absolute overall improvement in survival by 3.6%.

Currently in the UK, there are different types of chemotherapy regimens for stage III and high risk stage II colorectal cancers. The more common regimens include:

- Intravenous 5-FU with or without folinic acid
- Combination of folinic acid with intravenous 5-FU and oxaliplatin
- Oral Capecitabine (Capecitabine is an orally administered precursor of 5-FU)
- Combination of Capecitabine and oxaliplatin

The decision of commencing chemotherapy requires a full discussion with the patient with respect to the inconvenience and side effects. Sometimes,

chemotherapy is contraindicated as the risk/side effects outweigh the possible benefits.

1.6 Conclusion

We are slowly beginning to understand the biology and molecular genetics of CRC. This knowledge is being used to help better stratify an individual's risk of developing CRC and also helps clinicians provide a more personalized treatment regimen to specific types of CRC. This knowledge has also help improve the ability to predict the benefits from new biological agents.

Therefore, the role of pathologist remains integral in the management of patients with CRC. The role of pathologist have expanded vastly from issuing an accurate tissue diagnosis to assessing prognostic parameters such as surgical margins, lymphatic and vascular invasion and assessing therapeutic effects in patient who have received neoadjuvant therapy. Pathologist also play a central role in identifying histological features that are suggestive of MSI and selecting the appropriate tissue sections for molecular testing.

The role of pathologist in CRC screening is also important and vital in the treatment of early CRC. CRC screening and the role of pathologist in screening will be discussed further in the chapters below.

Chapter 2 Lymph node metastasis in pT1 colorectal cancers

2.1 Standard predictors of lymph node metastasis (LNM) in symptomatic presentation pT1 CRC

LNM occurs in approximately 8.4 to 24.0% of patients with symptomatic pT1 CRC (Tanaka et al 1995, Nascimbeni et al 2002, Suzuki et al 2003, Shimomura et al 2004, Yasuda et al 2007, Kobayashi et al 2012, Caputo et al 2014, Bosch and Nagtegaal 2014) (see table 2.2 and 2.3). LNM is significant, as persistent malignant disease within the lymph nodes would require the individual to undergo radical resection. LNM is also of prognostic importance as it indicates that the carcinoma possesses the propensity to metastasize and upstages the patient from stage I to stage III.

With the potential of LNM apparent in pT1 cancers, it is essential that the most appropriate treatment is provided be it a local excision or a radical resection. However, such treatment options have their pros and cons in balancing the benefits of a potential cure to the risks of recurrence, morbidity, mortality and disease spread. Therefore, the key to successful treatment is to identify and balance the high risk factors of LNM and systemic metastasis versus the possible mortality and morbidity associated with the treatment.

There are several described risk factors of LNM in pT1 CRC. These include site of carcinoma, grade of differentiation of carcinoma, lymphatic and vascular invasion, depth, width and area of invasion and tumour budding.

2.1.1 Site of carcinoma

Studies have shown that the site of the carcinoma within the bowel plays a factor in the risk of LNM. The rectum is the location that is often associated with LNM and adverse prognostic outcomes (Haggitt et al 1985, Nascimbeni et al 2002). Okabe et al compared the risk of LNM between the locations within the bowel and showed that LNM were more common within the rectum when compared to the left colon ($p=0.04$) or right colon on univariate analysis ($p=0.003$) (Okabe et al 2004). Nascimbeni et al showed that the lower third of the rectum had higher risk of LNM compared to the rest of the colon ($p=0.007$) (Nascimbeni et al 2002).

However, when Nascimbeni compared the risk of LNM between the rectum, left and right colon, there was no difference. Several other studies have also confirmed that the site of carcinoma is not prognostic factor in LNM (table 2.1).

Author	Number of cases				p-value
	Left colon	Right colon	Rectum	Total	
Nascimbeni et al 2002	234		119	353	0.243
Sakuragi et al 2003	119	63	96	278	0.9
Chok and Law 2007	28		44	72	0.89
Choi et al 2008	54	18	96	168	0.529

Table 2.1: Studies showing that the site of carcinoma is not a risk factor for LNM

2.1.2 Grade of differentiation of carcinoma

Tumours are normally graded on how differentiated they appear. Differentiation is primarily based on the architecture and specifically gland or tubule formation. The criteria for poor differentiated tumours are either irregularly folded, distorted and often small tubules or the absence of any tubular formation (Loughrey, Quirke and Shepherd 2014).

Normally, the grading of differentiation of the tumour can be divided into 4 groups; well-differentiated, moderately-differentiated, poorly-differentiated and undifferentiated (Bosman et al 2010). Most commonly, the grades are split into 2 groups; low-grade (well-differentiated/moderately-differentiated) and high-grade (poorly-differentiated/ undifferentiated) (Loughrey, Quirke and Shepherd 2014).

In general, the higher the tumour grade, the poorer the prognosis. In a pooled-data analysis, Hassan et al showed that the presence of poorly differentiated carcinoma is associated with a higher cancer-related mortality (odds ratio 9.2, $p<0.05$) (Hassan et al 2005). In terms of LNM, several studies have shown that poor differentiation/ high tumour grade is a predictor of LNM in early stage CRC (Wang et al 2005, Ricciardi et al 2006, Rasheed et al 2008, Tateishi et al 2010).

2.1.3 Lymphatic and vascular invasion

Even though pT1 CRC are considered 'early' carcinomas, they do have potential to spread because the submucosa of the large bowel contains a rich lymphatic and vascular supply. Invasion of the lymphatic and/or vascular system is generally regarded as a significant risk factor for regional involvement and spread of malignancy to the surrounding lymph nodes and distant organs is a very important prognostic factor in CRC. Table 2.2 and 2.3 summarises the studies that have investigated lympho-vascular invasion as a predictive factor for LNM.

Author	Total patients	Lymph Node Metastasis (LNM)						
		Total LNM cases, n (%)	Vascular invasion absent, n (%)	Vascular invasion present, n (%)	p-value	Lymphatic invasion absent, n (%)	Lymphatic invasion present, n (%)	p-value
Tanaka et al 1995	177	21 (12)	Not assessed			6 (3.3)	15 (8.5)	<0.01*
Sakuragi et al 2003	271	21 (7.7)	16 (5.9)	5 (1.8)	0.025	2 (0.7)	19 (7)	<0.001*
Wang et al 2005	159	16 (10.1)	15 (9.4)	1 (0.6)	0.192	9 (5.7)	7 (4.4)	0.023*
Yasuda et al 2007	86	21 (24)	3 (3.5)	18 (20.9)	0.001*	Not assessed		
Sugimoto et al 2014	102	14 (13.7)	6 (5.9)	8 (7.8)	0.03	3 (2.9)	11 (10.8)	0.25

Table 2.2: Studies investigating lymphatic and vascular invasion as a predictive factor for LNM. * indicates multivariate analysis.

Author	Total patients	Lymph Node Metastasis (LNM)			
		Total LNM cases, n (%)	Lymphovascular invasion absent, n (%)	Lymphovascular invasion present, n (%)	p-value
Nascimbeni et al 2002	353	46 (13)	37 (10.5)	9 (2.5)	0.001*
Okabe et al 2004	428	43 (10)	15 (3.5)	28 (6.5)	0.003*
Choi et al 2008	168	24 (14.3)	18 (10.7)	6 (3.6)	0.019
Kye et al 2012	55	8 (14.5)	4 (7.3)	2 (3.6)	0.232*
Kobayashi et al 2012	68	6 (8.8)	1 (1.5)	5 (7.4)	0.074*
Caputo et al 2014	48	6 (12.5)	5 (10.4)	1 (2.0)	1.000*

Table 2.3: Studies investigating lympho-vascular invasion as single qualitative predictive factor for LNM. * indicates multivariate analysis.

2.1.4 Depth of invasion

Many studies have investigated the risk that is associated with the depth of invasion of pT1 CRC. Depth of invasion of pT1 CRC has been assessed by three methods. The first two methods were investigated by Haggitt and Kikuchi and were based on the shape of the lesion. The third method developed by Japanese researchers involved measuring the vertical depth of invasion from the muscularis mucosae of the lesion (or from the luminal surface if muscularis mucosae is not present) (Watanabe et al 2012).

Haggitt et al first described the levels of invasion within a pedunculated polyp that contained carcinoma. Haggitt described 4 levels of invasion; level 1 invasion involves limited invasion into the head of the polyp, level 2 involves invasion extending to the neck of the polyp, level 3 involves invasion into the polyp stalk and level 4 denotes invasion beyond the stalk into the muscularis propria. Haggitt showed that level 4 invasion is an adverse prognostic factor in terms of local disease spread and mortality (Haggitt et al 1985).

For sessile lesions, Kudo had initially classified the relative level of submucosal invasion into 3 distinctive levels; the superficial, middle and deep thirds of the submucosa (sm1, sm2 and sm3) (Kudo 1993). Based on this classification, Kikuchi et al had showed that the deepest level of invasion was a significant risk factor for the development of LNM and local recurrence (Kikuchi et al 1995).

Nascimbeni et al studied the carcinoma-related variables that were deemed as risk factors of LNM and one of these variables was depth of invasion. In this study, the depth of invasion was based on Kikuchi's system and they showed that in both

univariate and multivariate analysis, sm3 invasion was a significant predictor of LNM. showed that the risk of LNM did increase with deeper sm level invasion with sm1, sm2 and sm3 having 2%, 8% and 23% risk respectively (Nascimbeni et al 2002).

As mentioned above, the risk of LNM is thought to rise with increasing depth of the invading tumour. Interestingly, the rate of LNM is almost comparable for the different depths of invasion in pT1 CRCs. Table 2.4 summarises the studies that have investigated the risk of LNM in relation to depth of submucosal invasion.

Depth	Author	LNM Rate	Univariate analysis	Multivariate analysis	
			p – value	p - value	Odds ratio
≥ 1000 µm	Kitajima et al 2004	87/865 (10.1%)		0.006	5.4
	Tateishi et al 2010	46/322 (14.3%)	0.05	NS	
	Nakadoi et al 2014	38/322 (11.8%)	<0.005	NS	
≥ 1800 µm	Nakadoi et al 2012	41/499 (8.2%)	<0.0001	0.0077	3.27
≥ 2000 µm	Sakuragi et al 2003	21/278 (7.6%)	<0.001	0.022	13.1
	Ueno et al 2004	33/254 (13%)	0.0045		
	Egashira et al 2004	13/140 (9%)	0.01	<0.05	1.45
	Tominaga et al 2005	19/155 (12.3%)	0.035	NS	
	Yasuda et al 2007	11/86 (13%)	0.003	NS	
	Yamauchi et al 2008	16/64 (9.8%)	0.007	NS	
≥ 2700 µm	Sugimoto et al 2014	14/102 (13.7%)	0.02		
≥ 3000 µm	Okabe et al 2004	43/428 (10%)	0.018	<0.05	2.7
≥ sm 2	Hase et al 1995	11/79 (13.9%)	0.01	NS	
sm 3	Kikuchi et al 1995	13/182 (7.1%)	0.0001	NS	
	Nascimbeni et al 2002	46/297 (13%)	0.001	<0.001	5
	Choi et al 2008	24/168 (14.3%)	<0.001	0.018	7.1

Table 2.4: Summary of investigations looking at depth of invasion and risk of LNM. (NS – not significant)

2.1.5 Width/Area of invasion of carcinoma

As pT1 CRC are classed as early invasive carcinomas, the width of the invading carcinoma has often been studied to determine the risk of LNM and to verify whether a local excision would be sufficient in providing cure. Ueno et al showed that the incidence of nodal involvement in a tumour with a width less than 4000μm was much lower than when compared to tumours with width of equal or more than 4000μm ($p=0.0005$) (Ueno et al 2004).

This had also been shown earlier by a study by Suzuki et al where the width of submucosal invasion was significantly greater in lymph node positive tumours compared to lymph node negative ones ($p=0.001$) (Suzuki et al 2003). Suzuki had also measured the area of submucosal invasion in 65 cases using an image analyzer but demonstrated that there were no significant differences in between node positive and node negative patients ($p=0.09$).

2.1.6 Tumour budding

Tumour budding is defined as the presence of isolated carcinoma cells or small carcinoma cell clusters (less than 5 cells per cluster) scattered in the stroma at the invasive margin of the tumour. Morodomi and colleagues believed that budding would be a valuable factor in predicting LNM when used alongside the degree of differentiation of the carcinoma and evidence of lymphatic invasion (Morodomi et al 1989).

Hase et al studied budding in 663 patients who had curative resections of their CRCs that ranged from Dukes' A to C. The degree of budding was divided into 2 groups:

non/mild budding and moderate/severe budding. Hase's study showed that moderate/severe budding was associated with a poorer 5 and 10-year survival outcome compared to the non/mild budding group where 71% of patients in the moderate/severe budding group had developed recurrences. Hase concluded that budding provides valuable prognostic information independent of Dukes' classification (Hase et al 1995).

Ueno et al also studied budding in rectal cancer and showed that rectal carcinomas with high-grade intensity budding (defined as ≥ 10 foci within a microscopic field) had lower 5-year survival rate as compared to patients with low-grade intensity budding ($p<0.0001$). On multivariate analysis, tumour budding was shown to be a significant independent variable in assessing the aggressiveness of rectal carcinomas. Ueno also showed that grading tumour budding using his system is reproducible (Ueno et al 2002).

Yasuda et al specifically studied the relationship between tumour budding and pT1 CRC and showed that on multivariate analysis, tumour budding is an independent risk factor for LNM ($p=0.003$) (Yasuda et al 2007). Tateishi et al also studied tumour budding as a risk factor and showed that it was also an independent risk factor in LNM ($p < 0.01$) when compared to other well established risk factors such as lymphatic invasion and poor differentiation (Tateishi et al 2010).

More recently, studies by Kye, Nakadoi and Nishida have also demonstrated the significance of tumour budding in predicting LNM in pT1 CRCs (Kye et al 2012, Nakadoi et al 2014, Nishida et al 2014). Table 2.5 summarises studies looking at tumour budding in pT1 CRC with LNM.

Author	Total patients	Total LNM cases (%)	Lymph node metastasis		p - value
			Tumour budding absent/ Low grade (%)	Tumour budding present/ High grade (%)	
Hase et al 1993	79	11 (13.9)	0 (0)	11 (100)	$p < 0.005$
Yasuda et al 2007	86	21 (24.4)	4 (4.7)	17 (19.8)	$p < 0.01$
Tateishi et al 2010	322	46 (14.3)	18 (5.6)	28 (8.7)	$p < 0.01$
Kye et al 2012	55	8 (14.5)	1 (1.8) #	5 (9.1)	$p < 0.05$
Nakadoi et al 2014	322	38 (11.8)	21 (6.5)	17 (5.3)	$p < 0.001$
Nishida et al 2014	265	31 (11.7)	8 (3.0)	23 (8.7)	$p < 0.001$

Table 2.5: Summary of studies investigating tumour budding. # Information regarding 2 patients could not be found.

2.2 Research objectives

The primary aim of this research is to increase the understanding of the prediction of the presence of lymph node metastasis in pT1 CRC.

The objectives of the research include:

1. Investigating the utility of subjective and quantitative features in predicting LNM.
2. Increasing our understanding of the relationship between invasion of the submucosa and the presence of LNM.

2.3: Materials and methods

2.3.1 Analysis of phenotypic features of symptomatic pT1 CRC

2.3.1.1 Database searches

Staged pT1 CRC were selected from the Northern and Yorkshire Cancer Registry and Information Service (NYCRIS). All radical resections of primary CRC within Yorkshire region between 1st January 2000 and the 31st December 2009 were identified by the NYCRIS. Radical resections were included in this study as lymph node status could be confirmed from the surgical procedure. Local exicision and piecemeal excisions of pT1 CRC were excluded in this study.

2.3.1.2 Clinicopathological data

Histopathological staging data was obtained from NYCRIS or official hospital pathology reports. The data included the age, gender of patient, site of cancer, stage of cancer, lymph node involvement, distant metatstasis and lymphatic and vascular invasion status.

2.3.1.3 Quantitative analysis

All microscopy slides were retrieved with the help of the local laboratory technicians. The slide that showed the widest and deepest tumour invasion was selected for digital scanning. These slides were scanned at x200 magnification with an automated digital scanner (Aperio XT Scanner, Aperio technologies, San Diego, Ca USA). Using a digital slide viewer (Imagescope v10.0, Aperio Technologies),

computer based morphometry of the tumours could be performed and analysed. Quantitative factors that were analysed as below:

1) Maximum width of lesion. Lesion is defined as the summation of the adenoma and carcinoma component (Figure 2.1)

2) Maximum width of invasive carcinoma (Figure 2.1)

3) Maximum vertical depth of carcinoma from luminal surface (Figure 2.2)

4) Maximum vertical depth of carcinoma from muscularis mucosae (Figure 2.2)

5) Maximum depth of invasion within neck of polypoid/semi-pedunculated lesions (Figure 2.3)

6) Minimum distance from resection margin (Figure 2.4)

7) Minimum distance from muscularis propria if present (Figure 2.4)

8) Total area of lesion (Figure 2.5)

9) Total area of carcinoma (intramucosal and submucosal) (Figure 2.6)

10) Total area of submucosal invasion by carcinoma (Figure 2.7)

11) Length of invasive front (Figure 2.8)

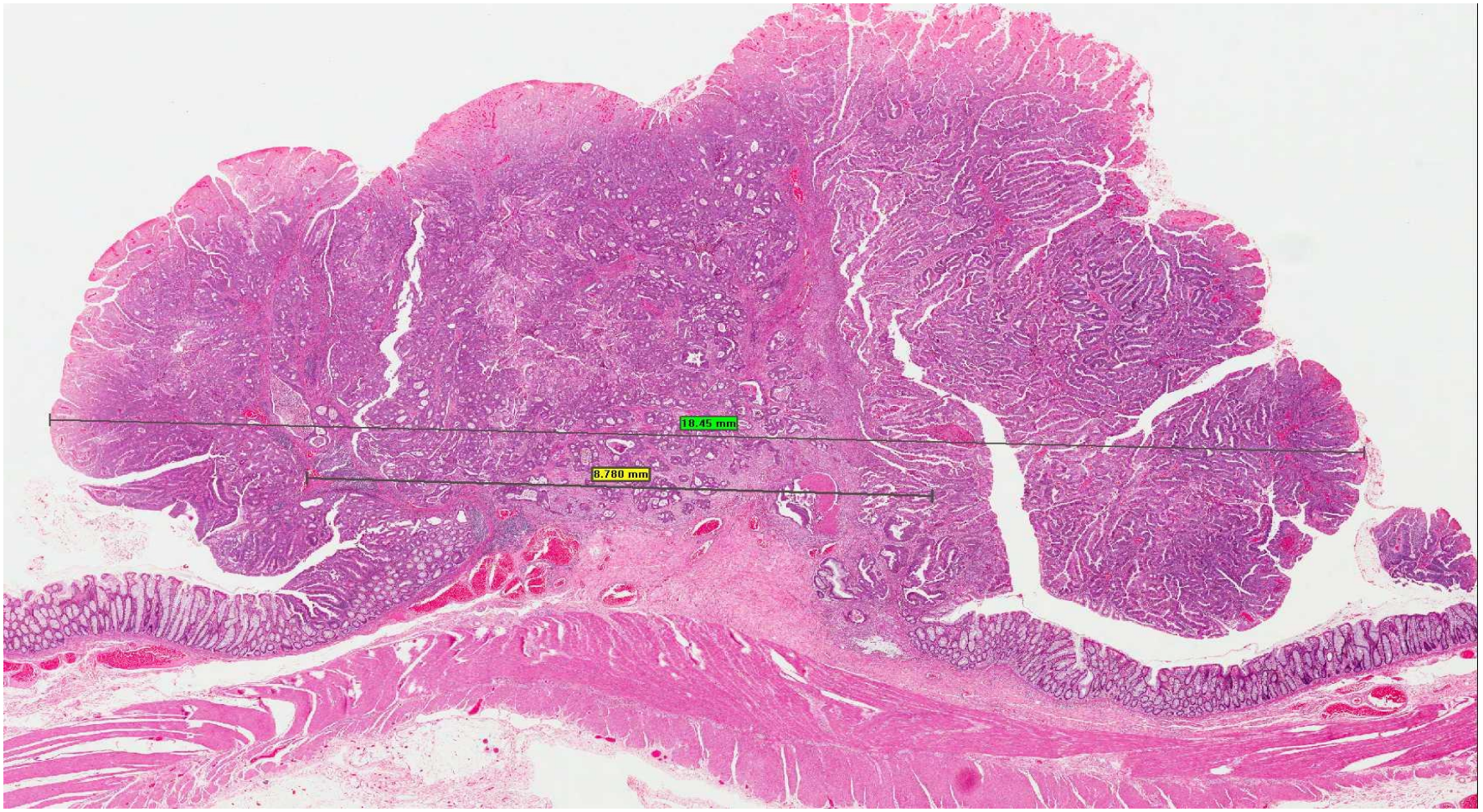


Figure 2.1: Image of a digital slide showing the Imagescope measurements of the width of lesion (long bar) at 18.45mm (green label) and width of carcinoma (short bar) at 8.78mm (yellow label).

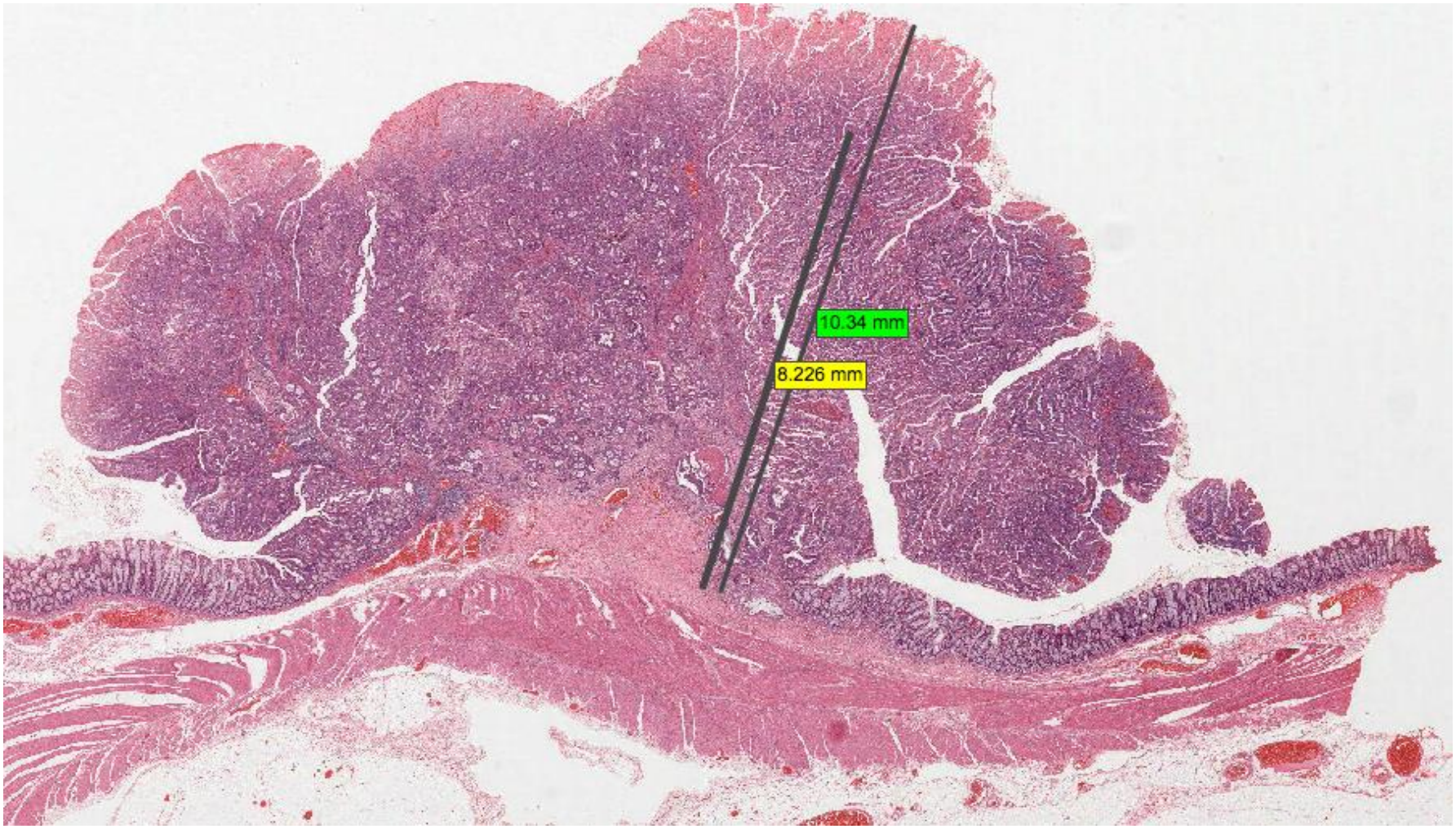


Figure 2.2: Image of a digital slide showing the Imagescope measurements of depth from the surface layer of the lesion (long bar) at 10.34mm (green label) and from the muscularis mucosae (short bar) at 8.226mm (yellow label).

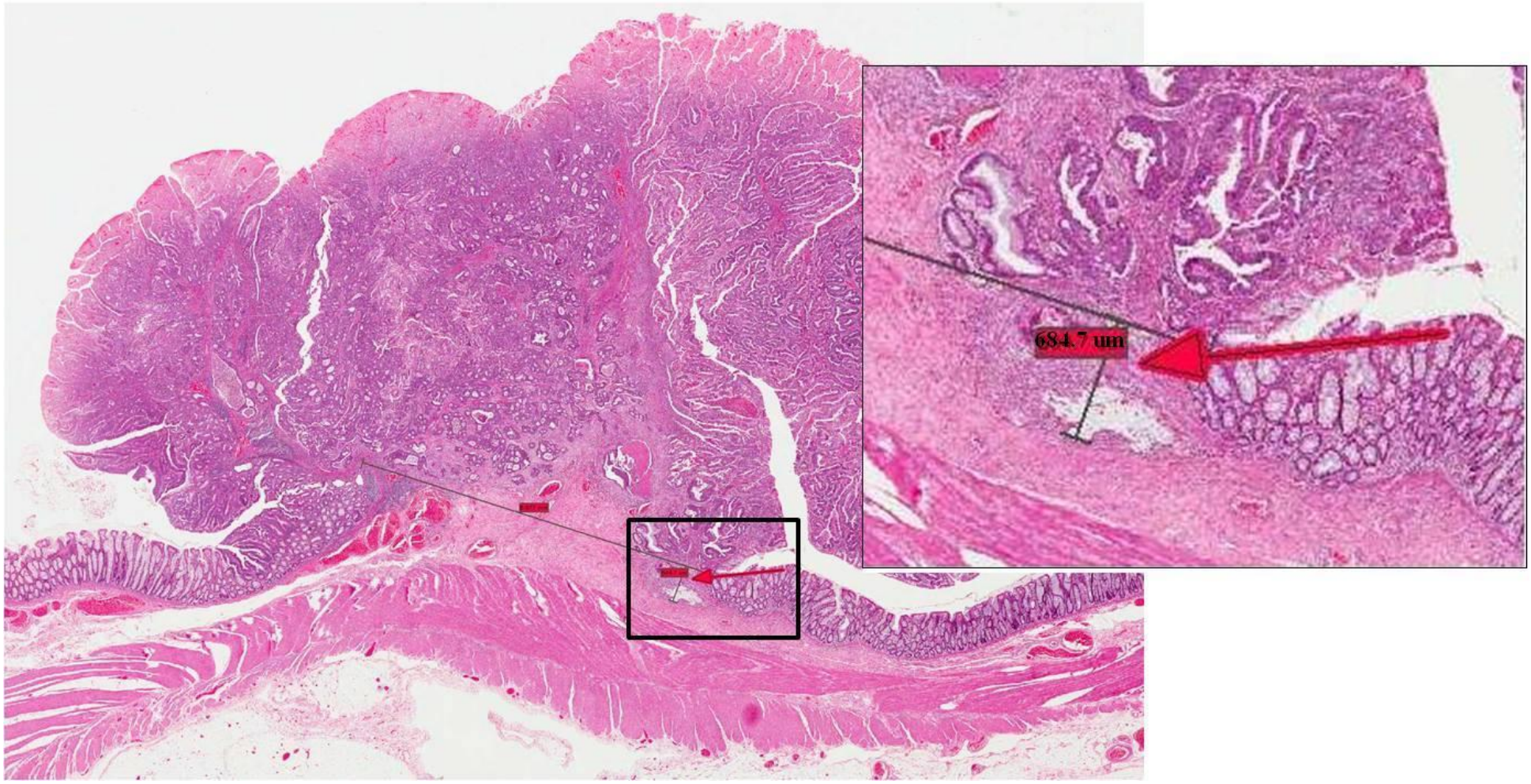


Figure 2.3: For lesions with a stalk (pedunculated/semi-pedunculated), the depth of invasion within the stalk was measured from the reference line which is the boundary between the tumour head and stalk. Red arrow indicates the depth of invasion within the neck which was 684.7 µm.

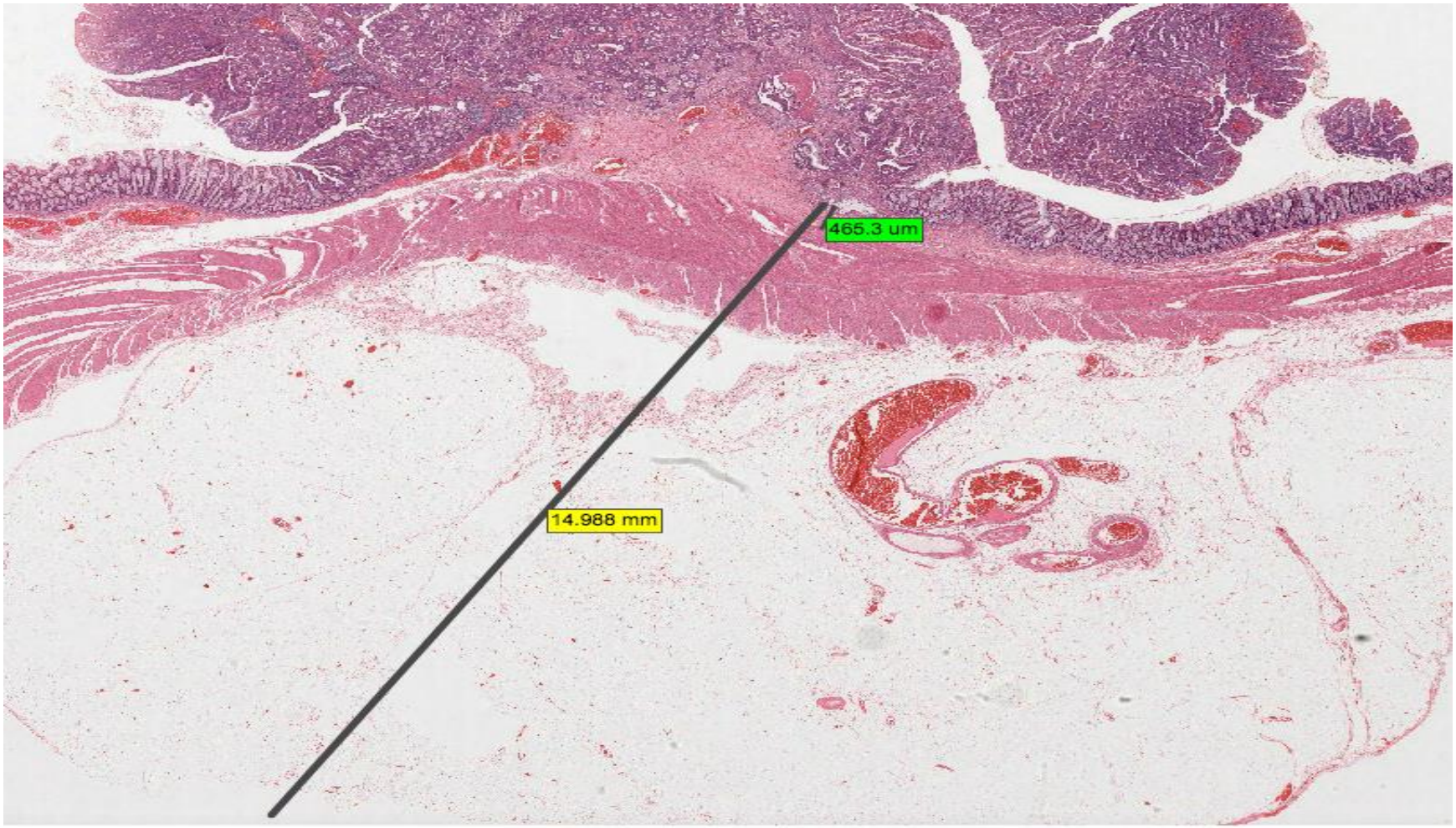


Figure 2.4: Image of a digital slide showing the Imagescope measurements of minimum distance of invading lesion from the nearest muscularis propria (short bar) at 465.3 µm (green label) and resection margin (long bar) at 14.988 mm (yellow label).

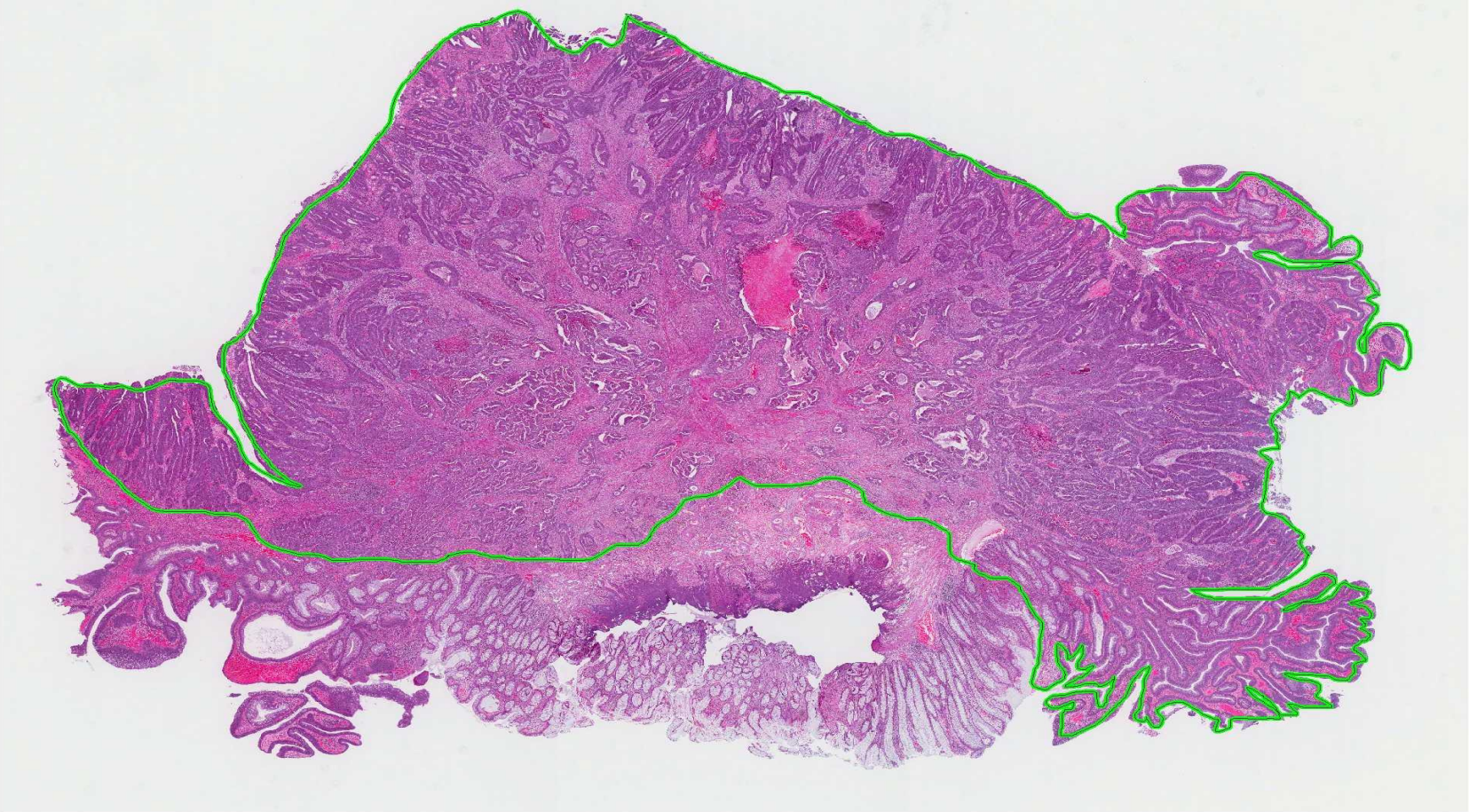


Figure 2.5: Image of a digital slide showing the Imagescope measurements for total area of lesion (contained with the green area) of 75.28mm^2.

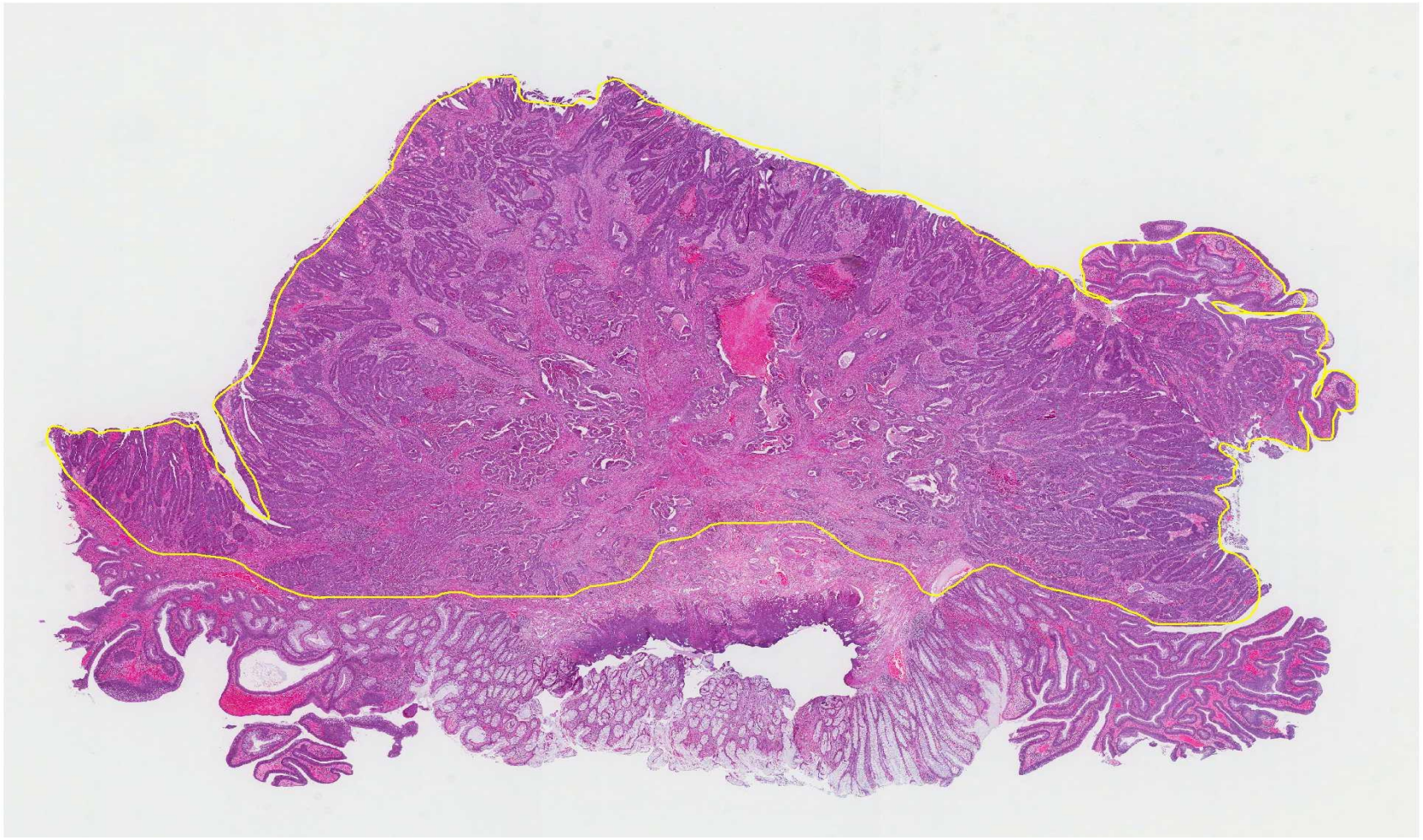


Figure 2.6: Image of a digital slide showing the Imagescope measurements for thetotal area of carcinoma (intramucosal and submucosal) (contained within the yellow area) of 65.27mm^2.

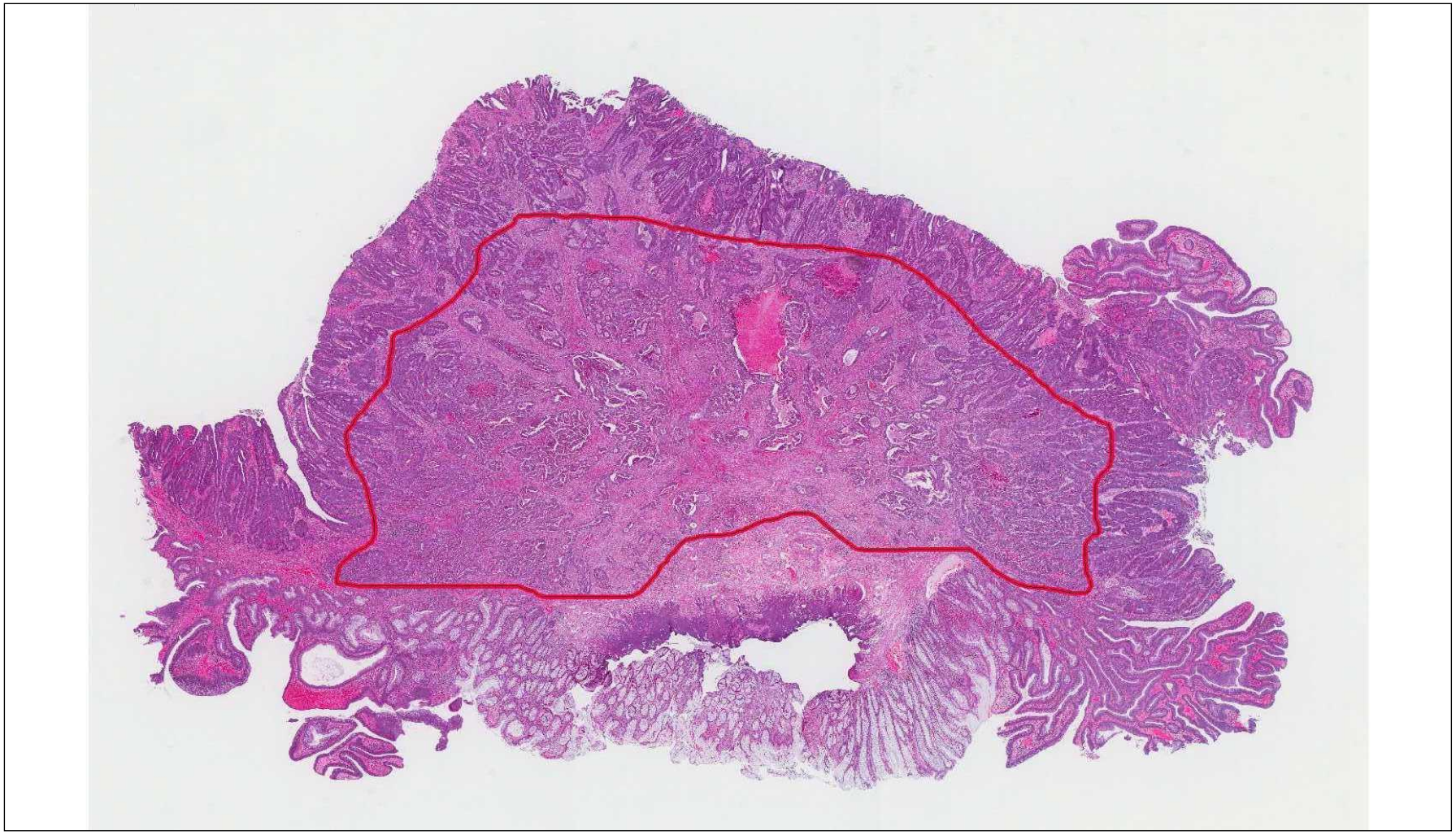


Figure 2.7: Image of a digital slide showing the Imagescope measurements for the estimated total area of submucosal invasion (contained within the red area) of 41.42 mm^2.

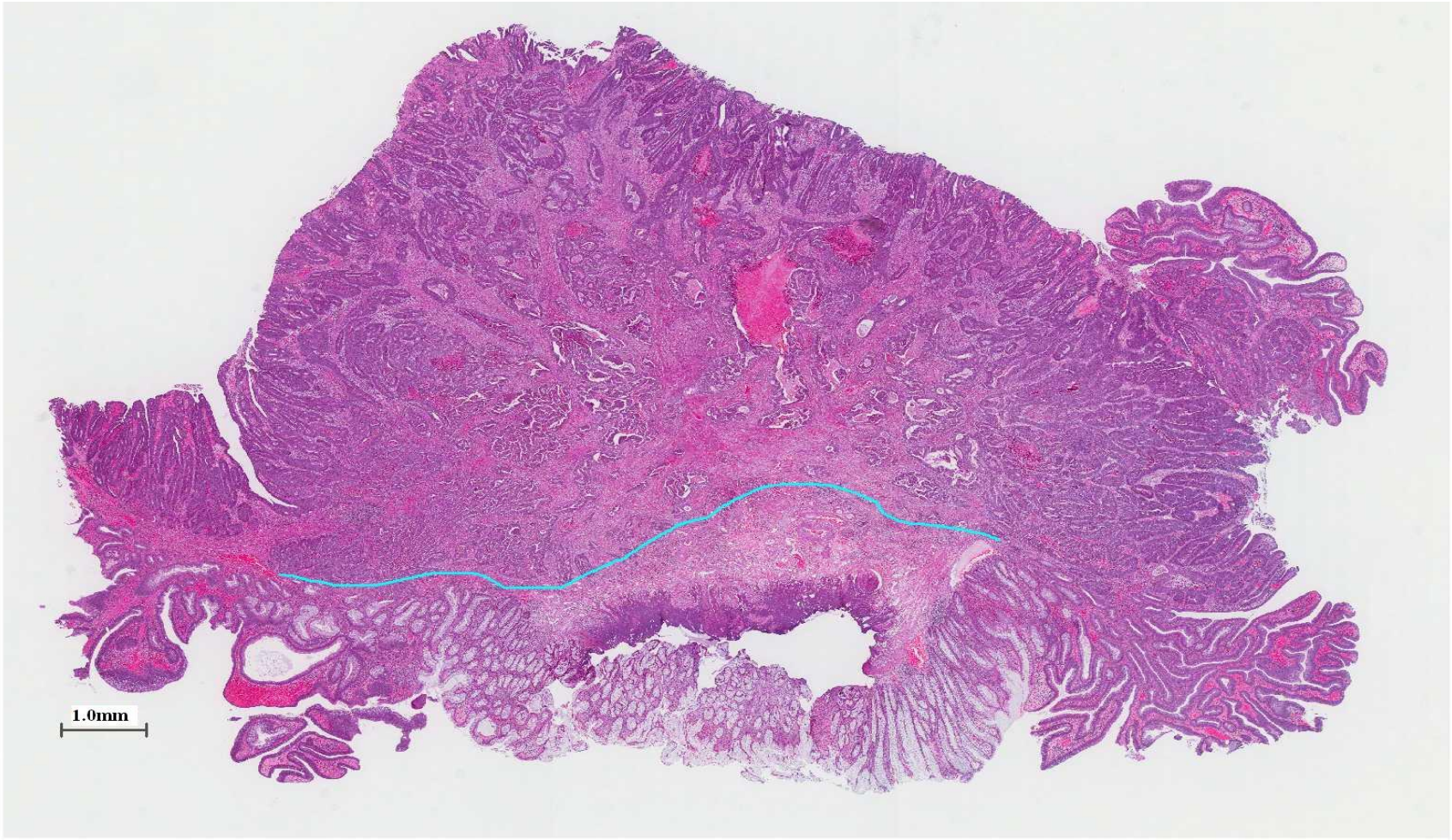
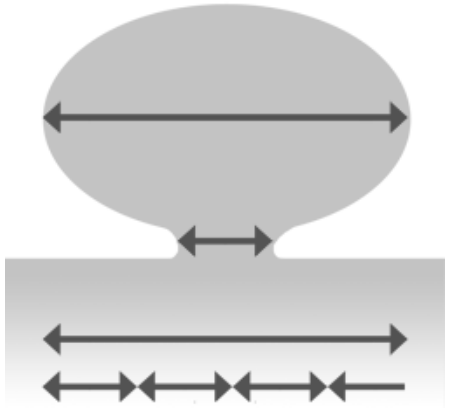
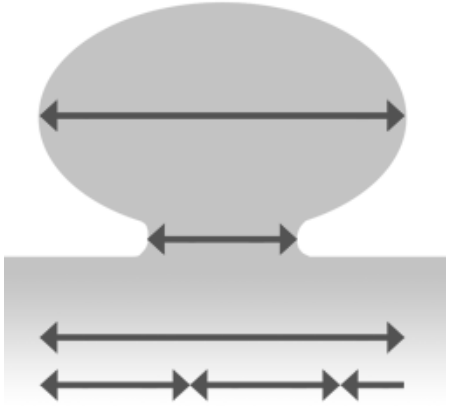


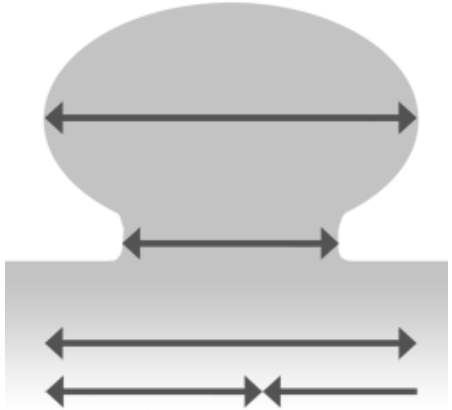
Figure 2.8: Image of a digital slide showing the Imagescope measurements for the length of invasive front of 9.5 mm.

We have qualitatively divided the lesions into three types of shapes; pedunculated, semi-pedunculated and sessile. We have used the relative measurements of the maximum width of the lesion and its base in determining the classification of the shapes of the lesions. In pedunculated lesions, the width of the base is less than one third of the widest part of the body or the head of the lesion. In semi-pedunculated lesions, the base is greater than 33% but less than 67% of the widest part of the body/head. For sessile lesions, the base is equal or greater than 67% the widest part of the lesion. Figure 2.9 shows a summary of the shape classification of the lesions.

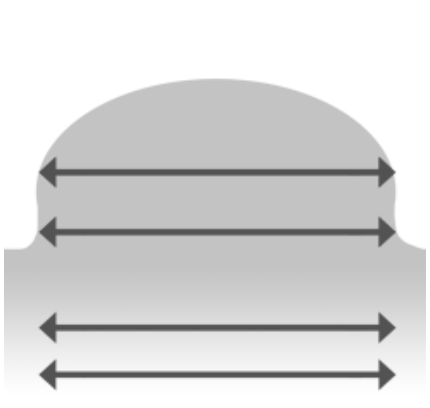


Pedunculated: base is less than 33% of the widest part of body of the head.





Semi-pedunculated: base is greater than33% but less than 80% of the widest part of the body of the head.



Sessile: base is 80% or equal to the widest part of the body.

Figure 2.9: Demonstrations of the qualitative shape classification of the lesions.

2.3.1.4 Qualitative analysis

Information regarding qualitative factors such as vascular and lymphatic invasion and grade of differentiation were obtained from the original issued pathology reports. Vascular invasion was defined as either the presence of intra- or extra-mural vascular invasion or both. Lymphatic invasion was defined as the presence of carcinoma within a lymphatic vessel. Grade of differentiation was divided into two groups: poorly differentiated vs non-poorly (well/ moderate) differentiated as per Royal College of Pathologist reporting guidelines (Loughrey, Quirke and Shepherd 2014).

2.3.2 Inter/Intra-observer variation study

To assess the reproducibility of the study, an inter-observer variation study was performed between the main and senior author (PQ). Both authors had measured the quantitative factors in a random sample of 10 lesions. Intraclass correlation coefficient (ICC) values were calculated between the main and senior author.

An intra-observer study was also performed by the main author (ET). The same quantitative measurements were performed on 10 random lesions with a period gap of 4 weeks. The ICC values were then calculated.

ICC values can be interpreted as follow: ≤ 0 indicates no agreement, 0.01 – 0.20 indicates poor agreement, 0.21- 0.40 indicates fair agreement, 0.41-0.60 indicate moderate agreement, 0.61 – 0.80 indicates strong agreement and > 0.81 indicates almost perfect agreement.

2.3.3 Statistical analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS v21.0, Chicago, IL, USA). Statistical analyses of continuous variables between the symptomatic group with and without LNM were performed using the Mann-Whitney U test. Associations between categorical data and LNM were performed using a Chi-Square test and Fishers exact tests. A modified receiver operating characteristic (ROC) curve was generated with the recommendation of a biostatistician (P McShane 2013, personal communication) to determine the cut-off values for significant quantitative factors that were analysed in the Mann-Whitney U test. Logistic regression analysis was also used to investigate the significant quantitative factors that influenced LNM.

Statistical analyses of continuous variables between symptomatic cases were performed using the non-parametric Mann-Whitney U test. A p value of < 0.05 for all the tests was considered significant.

2.4: Results

2.4.1 Relationship of quantitative factors in symptomatic pT1 CRC and its association with LNM

The NYCRIS database search identified 382 patients. Out of the 382 patients, 207 were used in this study as 105 patients' microscopy slides were missing, 35 slides were damaged and were unsuitable for digital scanning and 26 cases were rectal carcinomas which were down-staged to a pT1 stage after receiving pre-operative treatment. Nine cases had synchronous tumours that were staged pT2 - 4 with lymph node metastasis. All 207 patients had radical resections of their cancer and none had received pre-operative chemotherapy or radiotherapy.

LNM was noted in 19 (9.2%) of the 207 cases. In the patients with LNM, 17 (89.5%) were staged pN1 and 2 (10.5%) patients were staged pN2. This did not correlate with gender, age or tumour site. There were no significant differences in the occurrences of LNM between the shape of the lesions. The summary of the clinico-pathological features is in Table 2.6.

CRC with LNM had a significantly wider area of invasion (p=0.004) and a greater area of submucosal invasion (p=0.002) compared to CRC with no LNM. Table 2.7 and 2.8 summarizes the other quantitative and qualitative factors that were analysed respectively. The cut-off value for the width of invasion and area of submucosal invasion that was determined by the modified ROC curve were 11.5 mm and 35 mm^2 respectively (Figures 2.10 and 2.11).

		Lymph Node Metastasis		p - value
		Negative (n = 188) [%]	Positive (n = 19) [%]	
Sex	Male	113[60.1]	14 [73.7]	0.247
	Female	75 [39.9]	5 [26.3]	
Age	(Median, IQR)	71, 63 - 77	69, 57 - 75	0.536
Site	Caecum	12 [6.4]	0	0.439
	Ascending Colon	12 [6.4]	0	
	Transverse Colon	5 [2.7]	0	
	Descending Colon	9 [4.8]	0	
	Sigmoid Colon	66 [35.3]	9 [47.4]	
	Rectum	83 [44.4]	10 [52.6]	
	Unknown	1 [0.5]	0	
Shape of Lesion	Pedunculated	48 [25.5]	3 [15.8]	0.537
	Semi-pedunculated	86 [45.7]	11 [57.9]	
	Sessile	54 [28.7]	5[26.3]	

Table 2.6: Characteristics of pT1 carcinomas. p-values obtained using Chi-Square test.

Quantitative Factors	Lymph Node Metastasis Median (IQR: SD)		p – value
	Negative	Positive	
Maximum width of lesion (mm)	15.14 (13.08 – 19.26: 4.67)	17.56 (14.85 – 19.74: 3.77)	0.209
Maximum width of carcinoma (mm)	11.46 (9.12 – 14.50: 3.91)	15.58 (12.18 – 17.04: 2.56)	0.001
Maximum depth of invasion of carcinoma from surface (mm)	5.80 (3.81 – 8.96: 3.99)	6.27 (.09 – 8.94: 2.30)	0.215
Maximum depth of invasion of carcinoma from muscularis mucosae (mm)	4.40 (3.21 – 7.08: 3.05)	5.62 (3.89 – 7.42: 2.24)	0.105
Depth of invasion within neck (mm)	1.16 (0.45 – 2.12: 1.07)	2.53 (1.94-4.77: 1.64)	0.002
Distance of carcinoma to margin (mm)	4.89 (3.44 – 7.35: 3.22)	4.93 (2.72 – 7.59: 3.88)	0.894
Distance of carcinoma to muscularis propria (mm)	1.69 (0.98 – 3.27: 2.53)	0.60 (0.25 – 4.77: 2.59)	0.14
Total area of lesion (mm^2)	74.16 (48.53 – 108.27: 50.41)	96.26 (66.88 – 116.27: 42.87)	0.211
Total area of carcinoma (mm^2)	64.38 (41.08 – 105.78: 43.01)	81.32 (63.99 – 111.60: 45.74)	0.064
Total area of carcinoma within submucosal layer (mm^2)	36.16 (19.60 – 56.21: 29.31)	54.10 (39.97 – 77.86: 28.32)	< 0.001
Length of invasive front (mm)	8.92 (6.99– 10.97: 4.27)	12.20 (6.57 – 12.51: 3.52)	0.49

Table 2.7: Quantitative factors of symptomatic pT1 CRC that have been analysed. P-values obtained using Mann-Whitney U test. Values in red identify significant factors.

Qualitative factor		Lymph node metastasis		p-value
		Negative, n = 188 (%)	Positive, n = 19 (%)	
Vascular invasion	No	182 (96.8)	16 (84.2)	0.039*
	Yes	6 (3.2)	3 (15.8)	
Lymphatic invasion	No	184 (97.9)	16 (84.2)	0.018*
	Yes	4 (2.1)	3 (15.8)	
Grade of differentiation	Non-poor	184 (97.9)	14 (73.7)	< 0.0001*
	Poor	4 (2.1)	5 (26.3)	

Table 2.8: Qualitative factors of symptomatic pT1 CRC. p-values obtained using Fisher's Exact Test.

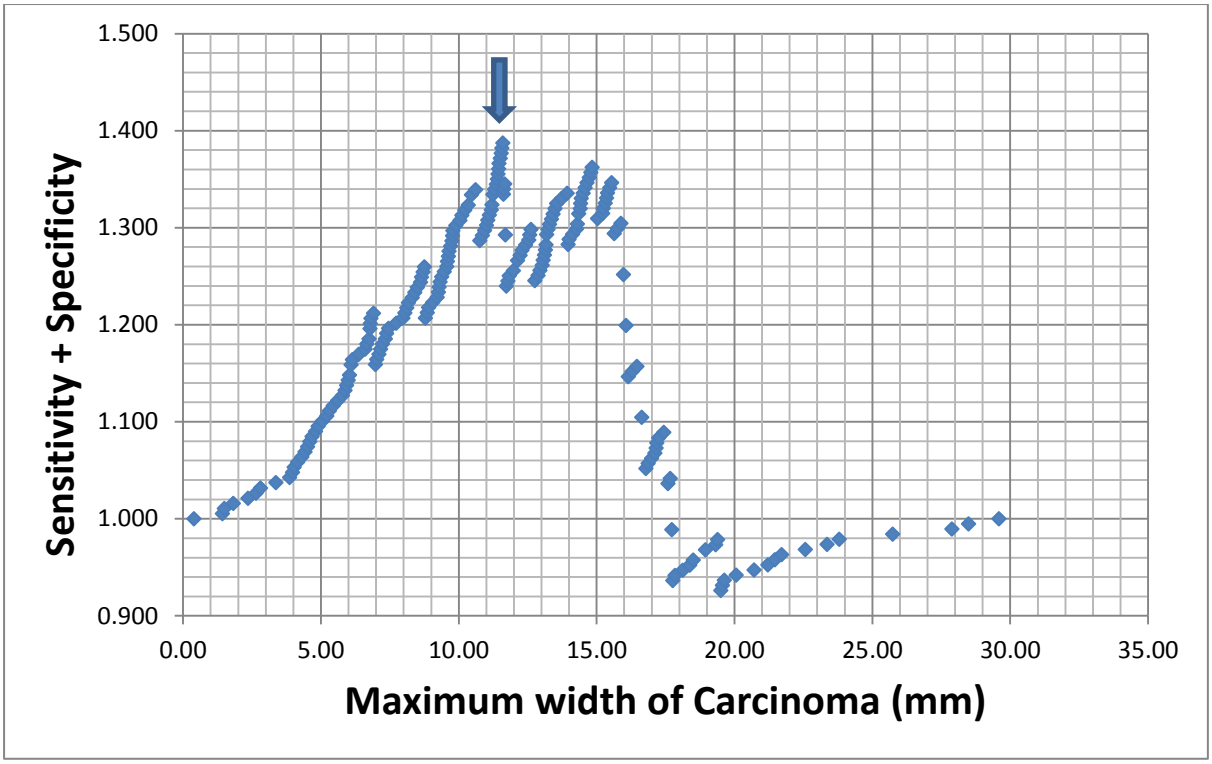
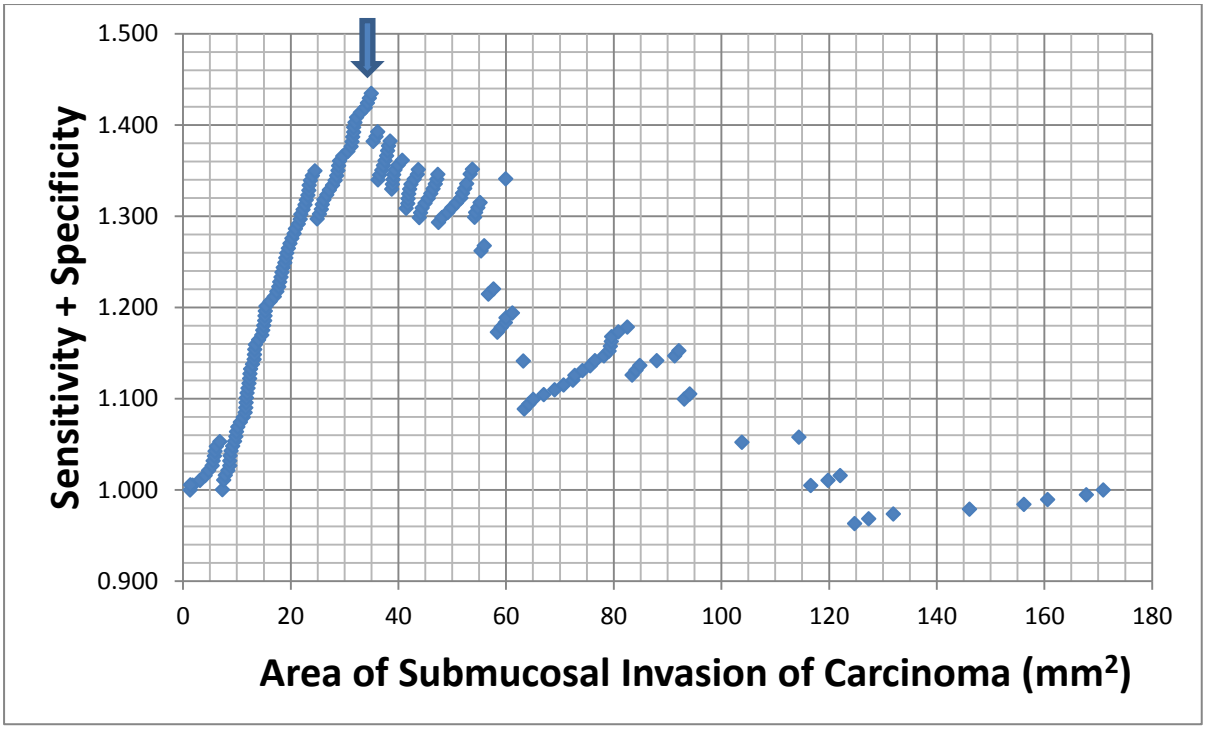


Figure 2.10: Modified ROC curve showing the optimal value for maximum width of carcinoma. The optimal value is determined from the peak of the curve (indicated by the arrow) [Area under curve = 0.723].



Figures 2.11 : Modified ROC curve showing the optimal value for the area of submucosal invasion of carcinoma. The optimal value is determined from the peak of the curve (indicated by the arrow) [Area under curve = 0.747].

Width of invasion as chosen from the ROC curve with the maximum sensitivity and specificity greater than 11.5mm and this was predictive of LNM on univariate analysis (Odds ratio [OR] =9.46, Confidence interval [CI]=2.13 – 42.07, p=0.003) but not on multivariate analysis (OR=5.59, CI=0.92 – 34.12, p=0.062). Area of submucosal invasion as chosen from the ROC curve with the maximum sensitivity and specificity was greater than 35mm^2 and this was predictive of LNM on both univariate (OR=20.9, CI=2.73 – 159.74, p=0.003) and multivariate analysis (OR=12.48, CI=1.44 – 108.07, p=0.022) (Table 2.9).

Factors		Lymph node metastais (LNM) positive (n=19) [%]	Multivariate analysis	
			p value	Odds Ratio (CI)
Width of Carcinoma	<11.5mm (n=101)	2[10.5]	0.062	5.59 (0.92 – 34.12)
	≥11.5mm (n=106)	17[89.5]		
Area of Submucosal Invasion of Carcinoma	<35 mm^2 (n=102)	1 [5.3]	0.022	12.48 (1.44 – 108.07)
	≥ 35mm^2 (n=105)	18 [94.7]		
Vascular invasion	Yes (n=9)	3 [15.8]	0.08	5.51 (0.82 – 37.18)
	No (n=198)	16 [84.2]		
Lymphatic invasion	Yes (n = 7)	3 [15.8]	0.02	12.86 (1.50 – 110.02)
	No (n = 200)	16 [84.2]		
Grade of differentiation	Non-poor (n = 198)	14 [73.7]	< 0.0001	28.36 (3.05 – 263.50)
	Poor (n = 9)	5 [26.3]		

Table 2.9: Multivariate analysis of quantitative and qualitative factors affecting LNM. p-values obtained using logistic regression analysis.

2.4.2 Results for the inter/intra-observer variation study

For the intra-observer variation study, the ICC values for the quantitative parameters showed almost perfect agreement (table 2.10). Focusing on the significant quantitative parameters found in this study, the ICC for width of carcinoma is 0.95 (95% CI 0.81 – 0.99, $p < 0.0001$) and area of submucosal invasion is 0.89 (95% CI 0.63 – 0.97, $p < 0.0001$).

For the inter-observer variation study, the ICC values for the quantitative measurements varied from 0.18 to 0.99 (table 2.11). The significant quantitative parameters did show strong agreement; the ICC for width of carcinoma is 0.76 (95% Confidence Interval 0.31 – 0.93, $p= 0.004$) and area of submucosal invasion is 0.95 (95% CI 0.81 – 0.99, $p < 0.0001$).

Quantitative Factors	Intra-class Correlation Coefficient score (95% confidence interval, p – value)
Maximum width of lesion	0.99 (0.97 – 0.99, p < 0.0001)
Maximum width of carcinoma	0.95 (0.81 – 0.99, p < 0.0001)
Maximum depth of invasion of carcinoma from surface	0.99 (0.97 – 0.99, p < 0.0001)
Maximum depth of invasion of carcinoma from muscularis mucosae	0.97 (0.89 – 0.99, p < 0.0001)
Depth of invasion within neck	0.97 (0.87 – 0.99, p < 0.0001)
Distance of carcinoma to margin	0.98 (0.93 – 0.99, p < 0.0001)
Distance of carcinoma to muscularis propria	0.99 (0.95 – 0.99, p < 0.0001)
Total area of lesion	0.99 (0.99 – 1, p < 0.0001)
Total area of carcinoma	0.86 (0.57 – 0.96, p < 0.0001)
Total area of carcinoma within submucosal layer	0.89 (0.63 – 0.97, p < 0.0001)
Length of invasive front	0.89 (0.64 – 0.97, p < 0.0001)

Table 2.10: Results of intra-observer variation study involving the main author.

Quantitative Factors	Intra-class Correlation Coefficient score (95% confidence interval, p – value)
Maximum width of lesion	0.89 (0.63 – 0.97, p < 0.0001)
Maximum width of carcinoma	0.76 (0.31 – 0.93, p < 0.0001)
Maximum depth of invasion of carcinoma from surface	0.85 (0.54 – 0.96, p < 0.0001)
Maximum depth of invasion of carcinoma from muscularis mucosae	0.83 (0.16 – 0.96, p < 0.0001)
Depth of invasion within neck	0.18 (-0.45 – 0.70, p < 0.0001)
Distance of carcinoma to margin	0.99 (0.97 – 0.99, p < 0.0001)
Distance of carcinoma to muscularis propria	Not assessed
Total area of lesion	0.58 (0.14 – 0.87, p = 0.014)
Total area of carcinoma	0.92 (0.69 – 0.95, p < 0.0001)
Total area of carcinoma within submucosal layer	0.95 (0.81 – 0.99, p < 0.0001)
Length of invasive front	0.88 (0.70 – 0.94, p < 0.0001)

Table 2.11: Results of inter-observer variation study between main author and senior author.

2.5 Discussion

With the potential of LNM in pT1 cancers, it is essential that the most appropriate treatment is provided, be it a local excision or a radical resection of the cancer. However, each treatment option has their pros and cons in balancing the benefit of a potential cure to the risks of spread of the disease and the mortality and morbidity that accompanies such treatments. Therefore the key to success in providing successful treatment is to identify those patients at high risk of LNM.

2.5.1 Suitability of Haggitt/ Kikuchi systems

Both Haggitt and Kikuchi formulated treatment guidelines for early CRC. However, neither Haggitt (for pedunculated tumours) nor Kikuchi (for sessile tumours) systems are always simple to use in pathological practice and neither can be applied to all lesions. For both Haggitt and Kikuchi systems to be applied, the lesions need to be removed whole and not in fragments. The Haggitt system is of no value in lesions that are sessile in nature. For the Kikuchi system, the lesion should be sessile and a definite muscularis propria needs to be identified so that the submucosal levels can be identified. This is frequently not the case for patients who have undergone biopsy removal or submucosal excision of their lesion during colonoscopy. Sub-pedunculated lesions are common and sometimes both systems are applied in the assessment of these lesions.

2.5.2 Issues in measuring depth and area of submucosal invasion

The muscularis mucosae is an important structure as it signifies the beginning of the submucosal layer and Japanese researchers have used this structure as a reference point when measuring the depth of invasion. In carrying out this study, there was a degree of difficulty in identifying the muscularis mucosae as it is commonly destroyed by the CRC. This leads to increased variability when measuring the depth and area of submucosal invasion of the CRC. In measuring the carcinoma width, we do not need to rely on the muscularis mucosae as a reference point and this would reduce the variability between pathologists when reporting pT1 CRC.

Ueno et al demonstrated a relationship between the width and depth of invasion and the probability of LNM. In this study, there was a significant difference in the rate of LNM between cases with depth of invasion ≥ 2000 μm and width of invasion ≥ 4000 μm compared to cases with depth of invasion < 2000 μm and width of invasion <4000 μm (Ueno et al 2004). Ueno suggested that cases with invasion depth of less than 2000 μm (and width of < 4000 μm) could be appropriately cured with endoscopic resection provided that there is an absence of other high risk features such as unfavourable grade of tumour, tumour budding and vascular invasion (Ueno et al 2004). If a depth of 2000 μm (2mm) or even 1000 μm (1mm) were to be used (Watanabe et al 2012) instead of sm3 or Haggitt 4 as one of the criteria for radical resection, this would lead to much higher rates of resection in symptomatic and screen detected pT1 CRCs than currently predicted. This will further be reviewed in the discussion section of Chapter 3.

2.5.3 The lymphatic system within the submucosa and LNM

In a recent study, Smith et al had showed that mucosal lymphatic vessels were present just above the muscularis mucosae and were significantly smaller than the submucosal vessels but interestingly, the study showed that lymphatic vessels were significantly more numerous within the sm1 level as compared to the sm3 level (Smith et al 2011). How does this relate to the risk of LNM with depth and width of invasion? As mentioned already, several studies have shown that LNM increases with deeper levels of invasion within the submucosal layer but in Smith's study, the deeper levels (sm3) had fewer lymphatic vessels.

This study has shown that lymph node positive pT1 cases have a significantly greater width of invasion ($p=0.004$) and area of submucosal invasion ($p=0.002$) compared to lymph node negative cases. What are the potential implications of these findings in the risk of LNM in pT1 CRC? This could be explained in terms of basic anatomy. Carcinoma cells develop within the mucosa and are termed invasive, because of the risk of metastatic spread, as soon as they breach the muscularis mucosae. The first layer that the carcinoma invades is the superficial level of the submucosa (sm1). With a wider and therefore greater area of invasion (figure 2.12), the chances of a carcinoma coming into contact and invading a lymphatic vessel would be higher due to the fact that lymphatic vessels are more numerous within the sm1 level as shown by Smith *et al*.

How does this explain the increased rates of LNM in sm3 invasion as shown by Kikuchi and Ueno? When comparing two pT1 CRC of equal width but

different depths of invasion, the area of submucosal invasion will typically be larger for the tumour with a deeper extent of invasion than that of a more superficial carcinoma (figure 2.13). This could also explain why tumour budding is a risk factor for LNM as tumour budding increases the surface area of submucosal invasion but the relative significance of this is currently unknown and needs further investigation.

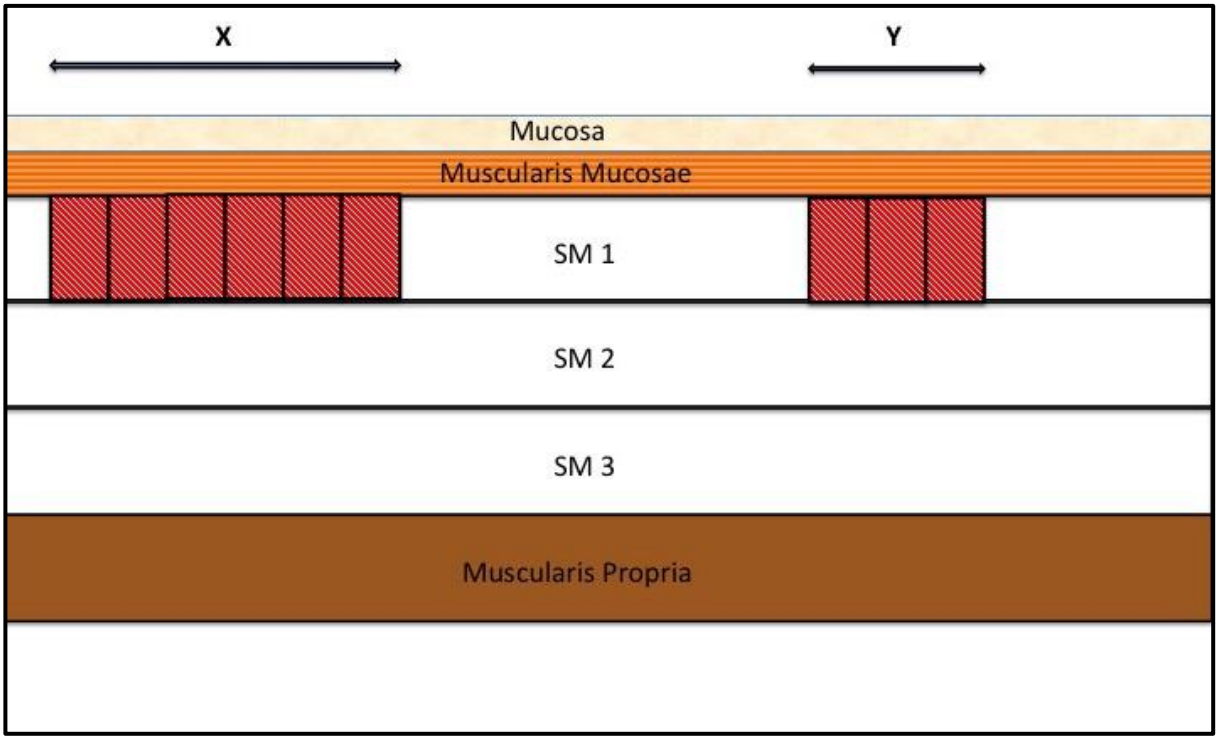


Figure 2.12: Diagram illustrating pT1 cancers (indicated by the red boxes with different widths (x and y) but identical depths of invasion. The wider cancer has a bigger area of invasion compared to the cancer with the smaller width. Hence, with the greater area, the wider cancer has more chance of coming into contact and invading lymphatic vessels especially within the lymphatic rich superficial submucosa.

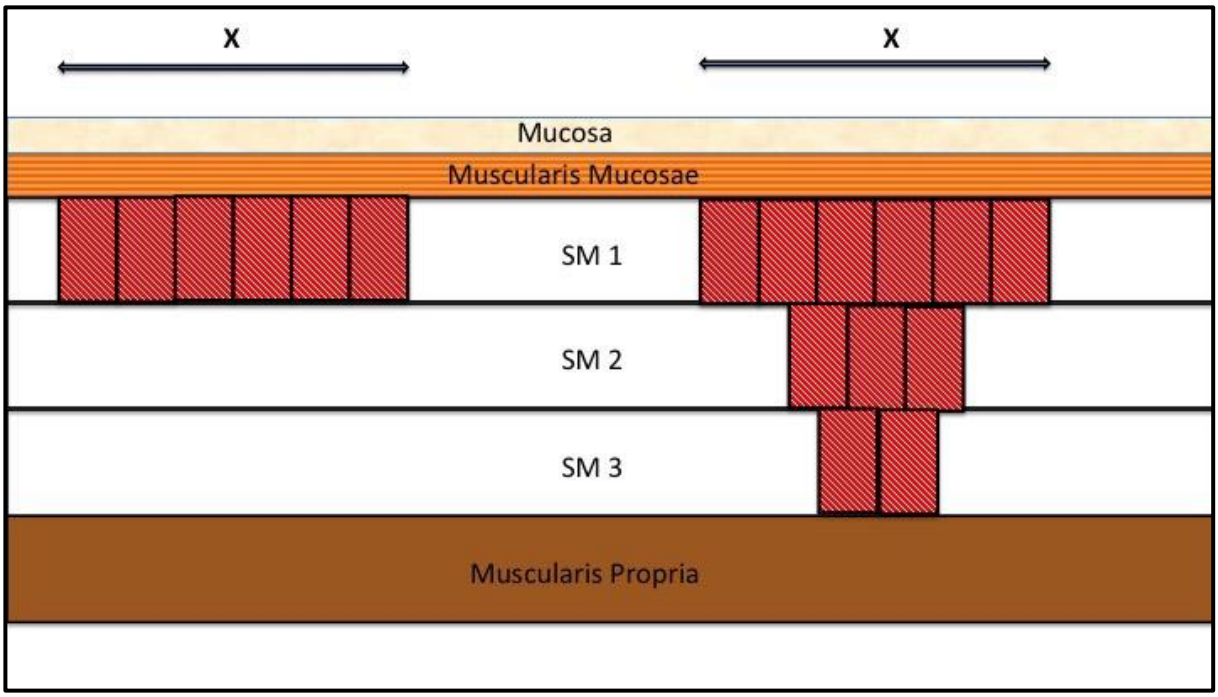


Figure 2.13: Diagram illustrating pT1 cancers with identical widths (x) but different depths of invasion. The pT1 cancer with SM3 invasion has progressed further in depth and has a greater area of invasion within the submucosa when compared to the pT1 cancer with the same extent of SM1 invasion. The length of invasive front is also increased in wider and deeper cancers but this study has shown no significant difference in cases with and without LNM for this parameter.

2.6 Conclusion

In this present study, we have found that the width and area of submucosal invasion can be considered as quantitative risk factors for LNM in pT1 CRC but the area of submucosal invasion in more predictive than width. The area of submucosal invasion may replace the depth of invasion as a better predictor in routine practice. With the advent of digital microscopy, this factor is more easily measurable and being quantitative, with a low inter- and intra-observer variation, less variation would arise between reporting pathologists. In tumours where the muscularis mucosae has been destroyed, there is a need for a subjective assessment as to where it would have been. This finding needs to be tested in a large prospective series and will hopefully help in determining the right type of treatment for patients with pT1 CRCs.

Chapter 3 Differences between screened and symptomatic pT1 colorectal cancers

3.1 Screened pT1 CRC within the National Health Service Bowel Cancer Screening Programme (NHSBCSP)

Screening is the process of detecting or identifying a disease in its early state, with the intention of halting its progression. Screening is performed by means of tests, examinations and other procedures that can be applied rapidly. The formal definition of screening is the process, by which unrecognized disease or defects are identified, using tests that can be applied rapidly and on to large numbers of people.

A screening test should be able to distinguish a healthy individual from those who have the disease. Normally, the screening process involves an initial test or examination that is not diagnostic and requires the appropriate follow up investigations and treatment. Screening is warranted as early intervention or treatment of the disease is more successful and cost-effective to treat and thus lowering the mortality and morbidity from the disease.

There are several types of screening, each with specific aims:

- Mass screening involving large populations (for example chest x-rays for tuberculosis).
- Multiple or multiphasic screening involving the use of several screening test on the same occasion (for example, annual health check ups).

- Targeted screening of groups with specific exposure to environmental and occupational hazards.
- Proactive or systematic screening involving using population registers to invite members of the population at risk for screening at appropriate time intervals.
- Case finding (or opportunistic screening) is a form of screening restricted to patients who consult a health practitioner for another purpose.

In screening for a disease, there are principles that should be met before the screening programme is initialized. These principles are based on the World Health Organization guidelines, which were adopted from the paper published by Wilson and Jungner (Wilson and Jungner 1968). The principles are as below:

1. The condition should be an important health problem.
2. There should be a treatment for the condition.
3. Facilities for diagnosis and treatment should be available.
4. There should be a latent stage of the disease being screened.
5. There should be a test or examination for the condition.
6. The test should be acceptable to the population.
7. The natural history of the disease should be adequately understood.
8. There should be an agreed policy on whom to treat.
9. The total cost of finding a case should be economically balanced in relation to the medical expenditure as a whole.

10. Case finding should be a continuous process, not just a “once and for all” project.

Based on the principles outlined above, CRC is an ideal disease for which to provide screening. CRC is ideal because it is a disease in which the natural history is sufficiently understood. CRC also has an identifiable and treatable “pre-cancerous” stage (adenoma) with treatment in the form of removal of the adenoma preventing the development of CRC. This has led to the introduction of the National Health Service Bowel Cancer Screening Programme, which will be discussed as below.

3.1.1 National Health Service Bowel Cancer Screening Programme (NHSBCSP)

In 2000, a pilot study was established in the UK to assess the feasibility of population based screening for CRC using faecal occult blood testing (FOBt) (UK Colorectal Cancer Screening Pilot Team 2003). This was the UK pilot of Bowel Cancer Screening and consisted of two rounds. It was initially conducted at two sites; the West Midlands in England and Tayside, Grampian and Fife in Scotland. Screening began at the Scottish and English sites in March and September 2000 respectively. The first round of the UK pilot of the Bowel Cancer Screening demonstrated that screening for CRC using FOBt as shown by previous randomised trials (Towler et al 1998) could be replicated in a population-based pilot programme.

A second round of the pilot was then initiated with the aim of getting a clearer picture on how a screening programme would work where the population would be invited on a periodic basis. Furthermore, the aims of the second round (or incidence round) could help determine the effects of the same process on a population who had already been invited to screening. The second round of this pilot also had the advantage of being able to identify interval cancers from the first pilot round and to calculate the sensitivity of the FOBt.

The second round of the pilot had begun in February 2003 and took place in Nottingham with invitations being sent to previous participants in the West Midlands. The results from both rounds of the UK pilot of the Bowel Cancer Screening confirmed that CRC screening in the UK is feasible and worthwhile.

Consequently, in England, the National Health Service (NHS) has devised and introduced the NHS Bowel Cancer Screening Programme (NHSBCSP). This screening programme was first piloted in July 2006 and was based in Coventry and North Warwickshire. The pilot ended in March 2007 with 271,646 participants. The overall rate of detecting cancer was 1.62 per 1000 people screened. The positive predictive value was 10.9% for cancer and 35.0% for adenoma. In this pilot study, 552 cancers were detected by screening, 92 were polyp cancers and 48 per cent of all screened cancers were Dukes' stage A. At time of diagnosis, only 1 per cent of the cancers had metastasised (NHSBCSP 2011).

Following this pilot study, the NHSBCSP has 58 local screening hubs and centres that cover all the regions in the England. Currently, the NHSBCSP

offers screening every two years to men and women aged 60 – 69 and currently extending this age limit to 75 years old. This specified age group was set as CRC increases with age with 85% of bowel cancer occurring in people over 60 years old.

3.1.2 UK Flexible Sigmoidoscopy Screening Trial

A multi-centre randomised controlled trial by Atkin *et al* (2010) which aimed to look at bowel cancer incidence and mortality reduction 11 years after a single screening examination. The screening examination that was used was flexible sigmoidoscopy. The trial recruited 170,000 people and the main objective was to examine the effectiveness of a once only flexible sigmoidscopy screen on people aged 55 to 64 years. The trial involved removing any small colonic polyps (< 10mm) found during the screening process. If any high-risk adenomas (≥ 3 polyps, ≥ 10 mm, ≥ 25% villous, high grade dysplasia) were detected, the participant would then go on to have a full colonoscopy.

The trial showed a reduced cumulative incidence in distal cancers (rectum and sigmoid) by 50% and a reduction of 33% for overall CRC incidence. The trial also showed that CRC mortality was further reduced by 43%. Most importantly, the trial proved that a once only flexible sigmoidoscopy for the population aged between 55 and 64 years old is a safe practical examination and offers a longstanding benefit in terms of reducing mortality and morbidity

from CRC (Atkin et al 2010). This trial has now lead to a full roll out to two thirds of England by 2015.

With the NHSBCSP in place and the roll out of the UK flexible sigmoidoscopy screening programme, there is an increase in the number of stage I cancers detected from the screened population as compared to the non-screened population (Steele et al 2011, Rajaskehar et al 2012, Morris et al 2012). This has directly increased the number of pT1 CRC detected within the screened population. This raises the important issue that we do not as yet know the optimal management for screened pT1 CRCs. Does a screened pT1 have the same risk as a symptomatic pT1? Should we be treating screened pT1 CRC the same way as those that arise in the general population? Over treatment of screened pT1 CRC may lead to a higher morbidity and mortality than is absolutely necessary. This is important, as participating individuals are normally fit and healthy prior to entering the screening programme.

3.2 Research objectives

This research will establish whether screened pT1 CRC are a different population and require different indicators for treatment when compared to symptomatic CRC. Therefore, the objectives of the research include:

1. To characterise pT1 CRC presenting in the NHS BCSP and comparing these cancers to symptomatic CRC to establish phenotypic differences.
2. To identify clinically useful phenotypic features of pT1 CRC within the NHSBCSP that will help optimise decision making for patient management.

3.3: Materials and methods

3.3.1 Database searches

pT1 CRC were selected from the NHSBCSP and Northern and Yorkshire Cancer Registry and Information Service (NYCRIS).

3.3.2 Symptomatic pT1 CRC

All radical resections of primary CRC within Yorkshire between 1st January 2000 and the 31st December 2009 were identified by the NYCRIS were included in this study.

3.3.3 Screened PT1 CRC

Screened pT1 CRC throughout the country were identified by the NHSBCSP database. All but two of the screened pT1 CRC that were included in this study were local excision/ polypectomy specimens. Piecemeal specimens were excluded from the study.

3.3.4 Clinicopathological data

Histopathological staging data was obtained from NYCRIS, NHSBCSP or pathology reports. The data included the age, gender of patient, site of cancer, stage of cancer, lymph node involvement, distant metastasis and lympho-vascular invasion status (in symptomatic cases).

3.3.5 Quantitative analysis

A local pathologist retrieved all microscopy slides. The slide that showed the widest and deepest tumour invasion was selected for digital scanning. These slides were scanned at x20 magnification with an automated digital scanner (Aperio XT Scanner, Aperio technologies, San Diego, Ca USA). Using a digital slide viewer (Imagescope v10.0, Aperio Technologies), computer-based morphometry of the tumours could be performed and analysed. Quantitative factors that were analysed are identical to the ones that were used in the previous chapter and are listed as below:

1) Maximum width of lesion. Lesion is defined as the summation of the adenoma and carcinoma component (Figure 2.1)

2) Maximum width of invasive carcinoma (Figure 2.1)

3) Maximum vertical depth of carcinoma from luminal surface (Figure 2.2)

4) Maximum vertical depth of carcinoma from muscularis mucosae (Figure 2.2)

5) Maximum depth of invasion within neck of polypoid /semi-pedunculated lesions (Figure 2.3)

6) Minimum distance from resection margin (Figure 2.4)

7) Minimum distance from muscularis propria if present (Figure 2.4)

8) Total area of lesion (Figure 2.5)

9) Total area of carcinoma (intramucosal and submucosal) (Figure 2.6)

10) Total area of submucosal invasion by carcinoma (Figure 2.7)

11) Length of invasive front (Figure 2.8)

The screened pT1 lesions were also divided into the 3 types of shapes that were described in the previous chapter (figure 2.9).

3.3.6 Statistical analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS v21.0, Chicago, IL, USA). Statistical analyses of continuous variables between the screened and symptomatic cases were performed using the non-parametric Mann-Whitney U test. A p value of < 0.05 for all the tests were considered significant.

3.4 Results

3.4.1 Relationship of quantitative factors in screened pT1 CRC and its comparison to symptomatic pT1 CRC

164 screened cases have been collected. 2 out of the 164 cases were from radical resections and the remainder of the cases were excised via polypectomies or a local procedure. Table 3.1 compares the shape of the pT1 CRC between the two groups showing an increase in semi – pedunculated lesions and a reduction in sessile lesions.

		pT1 Colorectal Carcinomas		
		Screened (n=164) [%]	Symptomatic (n=207) [%]	p-value
Shape of Lesion	Pedunculated	42 [25.6]	51 [24.6]	0.015
	Semi-Pedunculated	100 [61.0]	97 [46.9]	
	Sessile	22 [13.4]	59 [28.5]	

Table 3.1: Comparison of the shape of pT1 CRC between the screened and symptomatic groups. p-value obtained using Chi-Square test.

The quantitative measurements are shown in Table 3.2. Screened pT1 CRC were smaller in nearly all dimensions except for 1 measurement (maximum depth of invasion of carcinoma within the neck).

Quantitative Factors	pT1 Colorectal Carcinomas		p - value
	Screened (n=164), Median (IQR: SD)	Symptomatic (n=207) Median (IQR: SD)	
Maximum width of lesion (mm)	11.98 (9.69 – 16.25: 5.49)	16.73(13.1 – 21.57: 7.36)	<0.0001
Maximum width of carcinoma (mm)	8.73 (6.15 – 11.29: 3.86)	11.55 (8.11 – 14.84: 5.15)	<0.0001
Maximum depth of invasion of carcinoma from surface (mm)	4.97 (3.59 – 6.88: 2.79)	5.82 (3.73 – 8.71: 3.78)	0.03
Maximum depth of invasion of carcinoma from muscularis mucosae (mm)	3.50 (2.50 – 5.12: 2.01)	4.36 (2.81 – 8.96: 3.08)	0.01
Depth of invasion within neck (mm)	1.02 (0.53 – 1.91: 1.21)	1.49 (0.96-2.36: 1.21)	0.44
Distance of carcinoma to margin (mm)	1.33 (0.58 – 4.18: 2.58)	5.31 (2.94 – 8.21: 4.21)	<0.0001
Total area of lesion (mm^2)	52.65 (34.78 – 89.63: 58.00)	79.63 (47.63 – 124.47: 87.96)	<0.0001
Total area of carcinoma (mm^2)	44.49 (27.76 – 68.90: 43.00)	70.71 (38.05 – 109.30: 85.28)	<0.0001
Total area of carcinoma within submucosal layer (mm^2)	22.31 (14.93 – 37.39: 22.00)	34.98 (17.25 – 54.39, 32.74)	<0.0001
Length of invasive front (mm)	6.48 (4.89 – 9.04: 4.11)	8.15 (5.01 – 11.21: 4.94)	0.009

Table 3.2: Comparison of quantitative factors between screened and symptomatic pT1 CRCs. p-values obtained using Mann-Whitney U test. Values in red identifies significant factors

3.5: Discussion

3.5.1 Implications for screened and symptomatic pT1 CRC based on the Japanese Society for Cancer of the Colon and Rectum (JSCCR) treatment guidelines

The guidelines for the JSCCR have recommended that a pT1 CRC with a depth of submucosal invasion ≥ 1000μm would require a radical resection (Watanabe et al 2012). If this recommendation was applied to the screened pT1 CRC in this study, all the pT1 CRCs would require a bowel resection if depth of invasion was measured from the luminal surface and 99.4% (163/164) would require a resection if the depth of invasion was measured from the muscularis mucosae (Table 3.3).

If this recommendation were to be applied in the West, this would have major implications for all pT1 CRC. Firstly, the rate of radical resections for both screened and symptomatic pT1 CRC will rise with nearly all lesions being resected. This would lead to an unnecessary increase in the morbidity and mortality that are associated with these resections. Secondly, with the increased number of radical resections performed, this will further burden the healthcare system financially. Thirdly, this may have a negative impact on the NHSBCSP as it may deter the general public from entering screening with the fear of operative morbidity and mortality.

Therefore, we again emphasised the importance of selecting the proper treatment for patients with pT1 CRCs. The decision on whether a patient should receive a radical resection or a local procedure should be made by a

multi-disciplinary team and aided by the histopathological features (quantitative and qualitative) of the pT1 CRC and the patient's pre-operative state.

	Depth of invasion ≥ 1000 μm		Depth of invasion ≥ 2000 μm		Depth of invasion ≥ 3000 μm	
	Luminal surface	Muscularis mucosae	Luminal surface	Muscularis mucosae	Luminal surface	Muscularis mucosae
Screened pT1 CRCs	164/164 (100%)	163/164 (99.4%)	162/164 (98.8%)	147/164 (89.6%)	141/164 (86%)	104/164 (63.4%)
Symptomatic pT1 CRCs	207/207 (100%)	198/207 (95.7%)	197/207 (95.2%)	180/207 (87%)	179/207 (86.5%)	151/207 (72.9%)

Table 3.3: Breakdown of depth of invasion from luminal surface and muscularis mucosae of screened and symptomatic pT1 CRCs

3.5.2 Comparison between screened and symptomatic pT1 CRC

This study has shown that screening pT1 CRC had a smaller width (lesion and carcinoma) and area (total area of lesion, total area of carcinoma and total area of submucosal invasion) of invasion and shorter length of invasive front. There was also a significant difference in the depth of invasion from the muscularis mucosae and luminal surface between screened and symptomatic pT1 CRC. Understandably, the distance of the invasive border to clear

margins were greater in symptomatic cases as all these cases had undergone radical resections.

Screening pT1 CRC appear to have a different, possibly earlier morphological pattern. They are quantitatively smaller than their symptomatic counterparts. Not surprisingly, it is clear that screening does not only help identify CRC in their earlier stages but also identifies CRC that are smaller in size within the pT1 staging and therefore potentially curable with local procedures. The greater difference in depth from the neck of the lesion in screening cases is probably a reflection of the frequency of polypoid lesions in the screened population.

However, it is worth noting that this study was performed using symptomatic pT1 CRC that had been resected through a radical resection. Symptomatic cases that underwent a radical resection were chosen, as we wanted to know the lymph node status of each case. This may have consequently introduced some biasness as the majority of screened pT1 CRC were resected by local procedures such as polypectomies, EMR and TEMS. Ideally, it would have been better to compare the screened pT1 CRC to symptomatic pT1 CRC that have been resected through a local excision as this may help to reduce the biasness.

3.5.3 The value of local procedures in the treatment of screened pT1 CRC

Currently, the gold standard for treatment of early CRC is a radical resection but local procedures are becoming a suitable alternative especially for distal early CRC. The main goal with performing a local procedure for a pT1 CRC is to achieve a curative outcome whilst reducing mortality, morbidity and maintaining a good quality of life for the patient. Local procedures such as EMR and TEMS are an attractive alternative to radical resection for several reasons. Local procedures are less invasive and are associated with less postoperative pain with a shorter length of inpatient stay including less post-procedure morbidity. Another advantage of a local procedure is that normal bowel function is preserved without the need for stoma formation. Furthermore, there have been great advances in both equipment technology and the endoscopist/surgeon skill set, leading to even less post-procedure complications and better surgical margins.

However, the single most detrimental disadvantage of a local procedure is that the nodal status is not obtained, as lymph nodes are not excised. This could mean that if lymph node metastasis was present, the local procedure was not considered a curative procedure and the patient will then need to undergo a further treatment. This can then lead to an increase in morbidity and mortality in that patient as well as increased rates of local recurrence and a failure to salvage the patient, leading to death from cancer that may have been avoidable by the performance of a radical resection.

The key to a successful and potentially curative local procedure for an early CRC is to select the appropriate patient and to provide them with the correct local procedure. Key factors that will help decide whether the early CRC would be appropriate or not for a local procedure includes location of the CRC within the rectum (Bhangu et al 2013, Althumairi and Gearhart 2015), poorly differentiated cancers (Nascimbeni et al 2002, Ueno et al 2004, Choi et al 2008), lymphatic, and/or vascular invasion (Sakuragi et al 2003, Yamamoto et al 2004, Kitajima et al 2004, Yasuda et al 2007) and tumour budding (Ueno et al 2004, Kitajima et al 2004, Yasuda et al 2007). Therefore, it is absolutely vital that patients who undergo a local procedure for their early CRC, should be selected based on the key factors mentioned above as this would minimise morbidity and mortality and help improve oncological outcomes.

3.6 Conclusion

This study has shown that the NHS BCSP is identifying earlier pT1 CRCs that are smaller in terms of width and area and also less invasive in regards to the depth of invasion from the muscularis mucosae when compared to their symptomatic counterparts. This should have been expected as one of the main objectives of the NHS BCSP is to detect early CRCs and the shift between stages from late to early should have been expected to generate earlier pT1 CRCs.

Chapter 4 The significance of tumour stroma in pT1 colorectal cancers

Colorectal carcinomas are composed of malignant epithelial cells and non-malignant cells. These non-malignant cells create the tumour stroma that has various complex interactions with the cancer cells (Coussens and Werb 2002, Allen and Jones 2011, Pickup, Mouw and Weaver 2014). The tumour stroma has been investigated extensively but as yet, it is still poorly understood. The non-malignant cells of the stroma have a dynamic relationship with the tumour cells at all stages of carcinogenesis (Hanahan and Coussens 2012). Intracellular communication is driven by the complex network of cytokines, growth factors, receptors and inflammatory and matrix remodeling enzymes (Allen and Jones 2011, Pickup, Mouw and Weaver 2014).

4.1 Cells of the tumour stroma

The tumour stroma is composed of several different types of cells. These cells are described below.

4.1.1 Cancer-associated fibroblasts

Cancer-associated fibroblasts (CAF) are the most prominent cell type within the tumour stroma of many cancers. CAF are different from host fibroblasts

as they express α-smooth muscle actin as cytoplasmic microfilaments and sometimes desmin. Host or local fibroblasts express vimentin as intermediate filament proteins (Sappino, Schurch and Gabbiani 1990). CAF are contractile and secretory cells that produce an extracellular matrix that is rich in type III and V collagen, which may be considered responsible for the hard consistency of many carcinomas (Liotta, Rao and Barsky 1983).

CAF are believed to originate from multiple origins such as from resident/ local fibroblasts, bone marrow derived progenitor cells or trans-differentiating epithelial cells (Anderberg and Pietras 2008, Augusten 2014). Resident/ local fibroblasts are activated to form CAF by cancer-derived growth factors such as Tumour Growth Factor – β (TGF-β), Platelet Derived Growth Factor (PDGF) and basic Fibroblast Growth Factor (bFGF).

The second source of CAF is derived from the bone marrow progenitor cells. These cells are pluripotent and when recruited to tumour sites, are activated to form CAF by cytokines and growth factors such as Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), Hepatocyte Growth Factor (HGF), bFGF, PDGF and Monocyte Chemotactic Protein-1 (MMC-1) (Hall, Andreeff and Marini 2007, Dwyer et al 2007, Spaeth et al 2008, Feng and Chen 2009, Allen and Jones 2011).

The third source of CAF is from epithelial cells and to a certain extent, tumour cells. Tumour epithelial cells undergo transformation to CAF through the epithelial – mesenchymal transition (EMT) process. EMT is a complex process in which the epithelial and tumour cells lose their innate characteristics and gain new mesenchymal properties (Augusten 2014). CAF

play a vital role in tumour progression by providing a supportive environment for tumour growth and proliferation. Once activated by tumour-secreted factors, CAF produces a whole host of growth factors (PDGF, VEGF, and HGF), proteases (matrix metalloproteases [MMP] 2, 9, 13 and 14) and cytokines (Stromal-Derived Factor - 1 [SDF-1], Interleukin [IL] 6, Chemokine Ligand 12 [CXCL12]) that are essential in tumour development, angiogenesis and metastasis (Owusu et al 2015).

4.1.2 Pericytes

Pericytes are contractile cells in close proximity to endothelial cells in capillaries and venules. These cells are an integral component of tumour vasculature as they provide structural support to blood vessels during angiogenesis. (Armulik, Genove and Betsholtz 2011)

The role of pericytes in tumour progression is still poorly understood. Clinical studies in bladder and colorectal cancers (Yomenaga et al 2005, O'Keefe et al 2008) suggest that low pericyte coverage around vessels correlated with poor prognosis and increased metastasis. Using genetic mouse models and pharmacological inhibitors, pericyte depletion suppressed tumour growth at the early stages of tumour angiogenesis but metastasis is enhanced in the advanced stages of tumour angiogenesis (Cooke et al 2012). This may suggest that the role of pericytes changes or evolves with the progression of the tumour.

4.1.3 Vascular endothelial cells

Many angiogenic factors within the tumour stroma such as VEGF and platelet-derived growth factors (PDGF) and chemokines stimulate endothelial cells and their associated pericytes to form new vessels. These new vessels supply the tumour with nutrients that will aid cancer growth (Carmeliet and Jain 2011). The vessels that are formed are usually abnormal with non-uniform distribution, irregularly shaped and are variably 'leaky' (Jain 2005). This characteristic 'leakiness' of the vessel increases the tumour interstitial pressure leading to uneven blood flow and oxygenation and simultaneously creating poor nutrient and drug distribution within the tumour stroma. This in turn leads to further hypoxia and facilitates tumour metastasis (Jain 2005).

4.1.4 Cells of the immune system

Most malignant processes induce an inflammatory response within the host and this response occurs via both the innate and adaptive pathways (Coussens and Werb 2002, Pickup, Mouw and Weaver 2014). The inflammatory response generated by the tumour involves various pro-inflammatory cytokines and cell mediated responses that play a role in modulating an individual's response to the malignancy. Therefore, it is common to see a variety of cells of the immune system within the tumour stroma (Quail Joyce 2013).

4.1.4.1 T-lymphocytes

There are a variety of T-cell populations within the tumour stroma. Among these, the cytotoxic CD8+ memory T cells are capable of killing tumour cells and are strongly associated with a good prognosis. CD8+ T cells are supported by CD4+ T helper 1 (TH 1) cells that produce cytokines IL-2 and interferon – γ (IF- γ). High amounts of these cytokines correlate with a good prognosis (Fridman et al 2012).

The CD4+ T cells associated with tumour growth are the immunosuppressive T regulatory cells (Tregs), which are recognized by their expression of FOXP3 and CD25 (Hsieh, Lee and Lio 2012). Tregs exert an immunosuppressive function through the productions of IL-10, TGF-β and cell-mediated contact through cytotoxic T-lymphocyte antigen 4. This inhibits the recognition and clearance of tumour cells by the immune system (Campbell and Koch 2011). High amounts of Tregs within the tumour stroma have been correlated with worse prognosis in breast, ovarian and pancreatic cancer (Curiel et al 2004, Bates et al 2006, Hiraoka et al 2006). Tregs can also be tumour suppressive in some B cell cancers as their presence in Hodgkin's lymphoma indicates a good prognosis (Tzankov et al 2008, Koreishi et al 2010, Fozza and Longinotti 2011).

4.1.4.2 B-cell lymphocytes

B-cell lymphocytes are normally found within the draining lymph nodes and lymphoid structures in the tumour stroma but can also be found at the

invasive margin of tumours. Infiltration of B-cells in the tumour stroma is associated with a good prognosis in some breast and ovarian cancers (Coronella et al 2001, Milne et al 2009). However, in studies involving mouse models, there is a contrast as B-cells inhibit tumour specific cytotoxic T-cell responses (Qin et al 1998) and more recently, Andreu et al and de Visser et al showed that B cells have a tumour promoting role (de Visser et al 2005, Andreu et al 2010).

4.1.4.3 Natural Killer (NK) and Natural Killer T (NKT) cells

NK and NKT cells also exist within the tumour stroma but are not normally found to be in contact with tumour cells. The presence of NK and NKT cells appear to predict a good prognosis in many cancers such as colorectal, gastric, lung, renal and liver cancers (Tachibana et al 2005). However, it is believed that NK and NKT cells may not be able to exert their full tumour killing ability due the effects of TGF-β that is released by the tumour cells (Fridman et al 2012).

4.1.4.4 Tumour-associated macrophages and neutrophils

Tumour-associated macrophages (TAMs) are abundant within the tumour stroma and are normally derived from monocytes that are recruited largely by monocyte chemotactic protein chemokines (Coussens and Werb 2002). TAMs are thought to have a dual role in tumourigenesis. TAMs have the potential to kill cancer cells following activation by IL-2, interferon and IL-12

(Brigati et al 2002, Tsung et al 2002, Quail and Joyce 2013). However, TAMs are also believed to be pro-tumourigenic as they produce a number of angiogenic and lymphangiogenic growth factors, cytokines and proteases, all of which are potent mediators of tumour progression (Schoppmann et al 2002). TAMs also produce IL–10 which reduce the anti-tumour response by cytotoxic T cells.

The role of tumour-associated neutrophils (TANs) in tumour growth and metastasis is still being researched. There is evidence that TANs promote primary tumour growth by enhancing tumour angiogenesis (Nozawa, Chiu and Hanahan 2006, Shojaei et al 2008), increasing degradation of the extracellular matrix (De Larco, Wuertz and Furucht 2004) and reducing the immune response to the tumour (Youn and Gabrilovich 2010).

Despite convincing evidence in support of TANs promoting tumour development, there is also evidence that TANs demonstrate anti-tumour activity. TANs possess cytotoxic granules that can be used to eliminate surrounding cancer cells (Borregaard, Sorenson and Theilgaard-Monch 2007). TANs are also considered indirect anti-tumour effector cells as they secrete cytokines and chemokines that may direct and activate other anti-tumour effector cells (NK cells).

4.1.5 Role of tumour stroma in malignancies

The role of tumour stroma in cancer progression is still being researched. Many investigators have studied the role of tumour stroma in the progression of different types of cancers and the interactions between tumour stroma and cancer cells are still unclear (Mesker et al 2007, Mesker et al 2009, West et al 2010, Courrech Staal et al 2010, de Kruijf et al 2011, Wang et al 2012, Huijbers et al 2013).

Recently, researchers have acknowledged the importance of tumour stroma in cancer growth, invasion and metastasis. The complex interactions between tumour stroma and cancer cells have been investigated extensively through different types of methods. These methods vary from visual evaluation of the tumour stroma to analysis of the molecules of the tumour stroma itself.

In trying to identify another marker for further adjuvant therapy to colorectal cancer treatment, Mesker studied the carcinoma-stromal components in 122 patients with stage I – III colorectal cancer (Mesker et al 2007). Mesker had visually estimated the carcinoma-stromal percentages on routine H & E stained histological sections. They showed that patients with a high stromal percentage within the most invasive part of the primary tumour had lower overall (OS) and disease free (DFS) survival compared to patients with low stromal percentages (OS – 2.13 years vs 7.36 years, Hazard Ratio [HR] = 3.73, p = <0.0001, DFS – 1.51 years vs 6.89 years, HR = 4.18, p < 0.0001). Mesker also showed that a high stromal percentage remained an independent variable after adjusting for tumour stage (OS: HR – 0.39, 95% CI = 0.22 – 0.71, p <0.001; DFS: HR – 0.34, 95% CI = 0.19 – 0.60, p< 0.0001) and lymph

node status (OS: HR – 0.37, 95% CI = 0.20 – 0.68, p <0.001; DFS: HR – 0.34, 95% CI = 0.19 – 0.61, p<0.0001). They concluded that a high stromal percentage could be used as an extra parameter in identifying high-risk patients (Mesker et al 2007).

In 2009, Mesker again used the similar method of calculating tumour stroma to further identify a sub-group of stage I - II colorectal cancer patients who may benefit from additional treatment. In addition to calculating tumour stroma, Mesker had undertaken extra immunohistochemical staining to identify elements that are involved in the pathways of tumour stroma production (TGF – β – R2, SMAD4 and β – catenin). This study analyzed 135 patients and showed that patients with a high proportion of stroma (< 50% tumour cells) had poorer survival (OS: HR – 2.73, 95% CI = 1.73 – 4.30, p <0.001; DFS: HR – 2.43, 95% CI = 1.55 – 3.82, p < 0.001). A further high-risk group was identified with high tumour stroma and SMAD4 loss. This high-risk group showed a low 5-year survival rates for patients with low tumour stroma with positive SMAD4 staining (OS: HR – 7.98, 95% CI = 4.12 – 15.44, p = 0.008; DFS: HR – 6.57, 95% CI = 3.43 – 12.56, p = 0.005) (Mesker et al 2009).

West et al (2010) further confirmed that a low proportion of tumour cells (high proportion of stroma) were related to a poor cancer-specific survival in 145 patients of various stages (I – IV) of CRC. In contrast to Mesker's studies, tumour stroma was quantified digitally rather than estimated visually via a microscope. Quantification of tumour stroma was performed by point counting using scanned tissue sections (this method will be described later in

the methods section). West et al (2010) showed that patients with high tumour stroma (low proportion of tumour) had significantly lower cancer-specific survival when compared to patients with low tumour stroma (high proportion of tumour) (HR = 2.087, 95% CI = 1.088 – 4.003, p = 0.024). On multivariate analysis, high tumour stroma was an independent poor prognostic marker when the study model was adjusted for age, pT stage, pN stage and extramural vascular invasion (p = 0.017) (West el al 2010).

More recently, Huijbers et al (2013) also confirmed that a high intra-tumour stroma percentage (proportion of tumour stroma > 50%) was a strong prognostic factor for stage II and III CRC patients within the VICTOR trial. Again the proportion of tumour stroma was evaluated and scored by simple microscopic visualization. Huijbers et al had investigated 710 patients of stage II and III CRC and showed that OS and DFS times were significantly lower in stroma high groups (OS: HR = 1.96, 95% CI = 1.40 – 2.74, p < 0.001; DFS: HR = 2.15, 95% CI = 1.60 – 2.90, p < 0.001). The 5-year OS was 69% versus 83.4% and DFS 58.6% versus 77.3% for stroma- high versus stroma-low patients (Huijbers et al 2013).

The studies by Mesker, West and Huijbers have shown that tumour stroma could potentially become a valid prognostic factor in colorectal cancer. Equally useful is that information regarding tumour stroma is easily obtainable from normal H & E slides at no extra cost.

To further reinforce the role of tumour stroma in tumourigenesis, Roepman et al had investigated the molecular and genetic differences in CRC and revealed a sub-type of CRC with a high EMT index. This sub-type of CRC

showed increased mesenchymal gene expression and were termed ‘mesenchymal type’ CRC (Roepman et al 2014). Guinney et al further validated this by identifying four consensus molecular subtypes (CMS) of CRC. The four CMS contain distinguishing features: CMS1 (microsatellite instability immune – hypermutated, microsatellite unstable and strong immune activation); CMS2 (canonical – epithelial, marked WNT and MYC signalling activation); CMS3 (metabolic – epithelial and evident metabolic dysregulation) and CMS4 (mesenchymal – prominent TGF-β activation, stromal invasion and angiogenesis) (Guinney et al 2015).

Clinically, patients with CMS4 CRC have poorer relapse-free and overall survival amongst the other CMS CRC (Guinney et al 2015). Previous studies by Yang, Roepman, Singh and Settleman have also shown poor chemotherapy responsed in ‘mesenchymal type’ CRC (Yang et al 2006, Singh and Settleman 2010, Roepman et al 2014).

4.1.5.1 Tumour stroma in other malignancies

The role of tumour stroma in other malignancies has also been investigated. Researches have shown that tumour stroma does play a prognostic role in breast and oesophageal cancers. de Kruijf et al (2011) had investigated the tumour-stroma ratio in breast cancer and proved that it was an independent prognostic factor for the relapse free period in breast cancer patients especially in the triple negative subpopulation. The research had shown that stroma-rich tumours had a shorter relapse free period ($p=0.001$) and overall

survival (p=0.025). Within the triple negative subpopulation, patients with stroma rich tumours had a 2.92 times higher chance of relapsing (p=0.006) compared to their stroma poor counterparts. Dekker et al (2013) further validated the prognostic importance of the tumour stroma ratio showing that stroma rich tumours had a higher disease relapse rate independent of other clinical parameters (P <0.001). They also showed that tumour stroma ratio was independently associated with locoregional recurrence in younger patients (age ≤ 40 years old) (hazard ratio = 2.201, 95% CI 1.038 – 4.669, p = 0.04) (Dekker et al 2013).

Courrech Staal et al (2010) had evaluated the prognostic value of the tumour stroma ratio in patients with oesophageal adenocarcinoma. They showed that patients with stromal rich oesophageal carcinomas had significantly poorer disease-free survival compared to stroma poor cancers. In the multivariate analysis, the stroma rich characteristic was a highly significant prognostic factor for overall survival (hazard ratio = 2.0, 95% CI 1.181 – 3.407, p = 0.01) independent of other clinicopathological factors such as depth of tumour invasion, nodal status, TNM stage, histological grade and type of resection.

Wang et al (2012) had investigated the prognostic value of tumour-stroma ratio in oesophageal squamous cell carcinoma. They showed that oesophageal squamous cell carcinomas that were stroma rich (tumours with more than 50% tumour-stroma ratio) were associated with poorer prognosis and an increased risk of relapse. Specifically, stroma rich tumours had lower 3 year overall and disease-free survival rates.

4.2 Research objectives

To assess whether the relative proportion of tumour stroma (PoTS) could be used as a prognostic factor for the prediction of the presence of lymph node metastasis (LNM) in patients with stage pT1 CRC.

4.3 Materials and Methods

4.3.1 Patients and clinico-pathological data

Patients with pT1 CRCs from the NYCRIS registry were used in this study. This cohort of patients was identical to the cohort used in the previous studies as described in chapters 2 and 3. Clinicopathological data were extracted from official histopathology reports. These data included histological information such as grade of differentiation, evidence of vascular and lymphatic invasion and lymph node involvement. Other demographical data like gender and age of the patient were also obtained from the official histopathology reports.

4.3.2 Measurement of proportion of tumour stroma (PoTS)

The measurement of PoTS was based on the method employed by West et al (2010). The H & E – stained slide that best represented each pT1 CRC were selected and scanned at X 400 magnification with an automated scanning system (Aperio XT, Aperio Technologies, Vista, CA, USA). Using a digital slide software program (ImageScope v10.0, Aperio Technologies), slides were inspected after scanning. An area of 9 mm^2 was selected from the area that was deemed most invasive on digital microscopy. A grid with systemic random sample of 300 points was superimposed on this 9 mm^2 area using a virtual graticule software (RandomSpot, University of Leeds, Leeds, UK). This grid/ virtual graticule was placed at the tumour invasive front and did not include any of the normal colonic submucosa (figure 4.1). Each point was

scored using the following categories; tumour (the points fall onto a cancer cell), stroma, tumour lumen, necrosis, vessel, inflammation and non-informative (unclassifiable) (figure 4.1 and table 4.1). The main author (ET) was trained by an experienced pathologist (PQ) in recognizing the different categories and subsequently navigated through each point and categorized the material underneath the point while blinded to the lymph node status. After scoring, the informative points were then categorized into two groups: a tumour group and a stroma group (Table 4.1).

The percentage of tumour stroma was calculated and expressed as a percentage fraction of all the informative points per case.

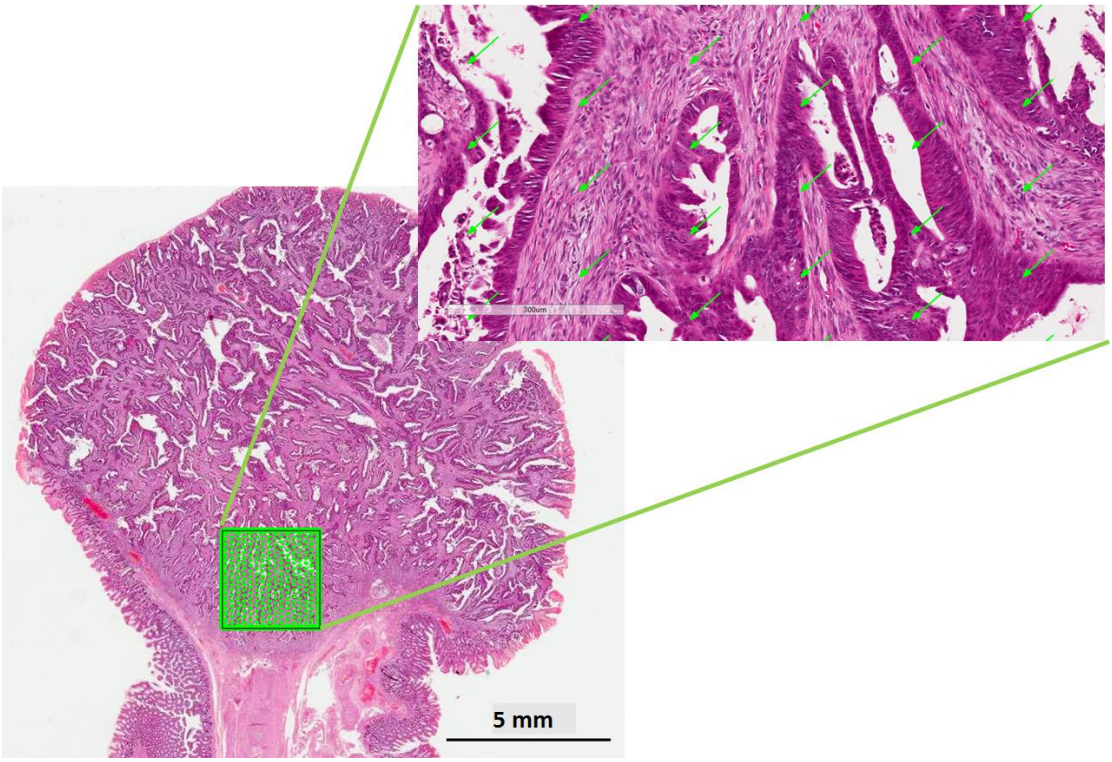


Figure 4.1: A 9 mm^2 boxed area is inserted at the invasive front of the cancer. This boxed area contained approximately 300 points.

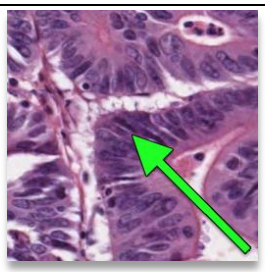
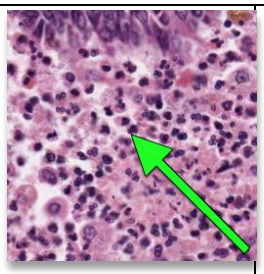
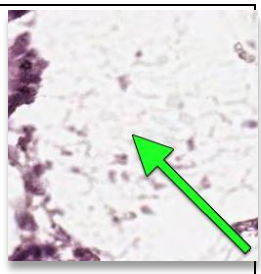
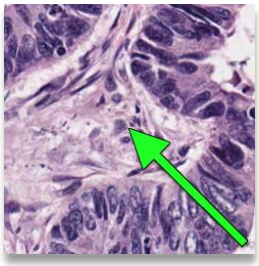
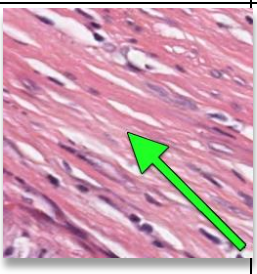
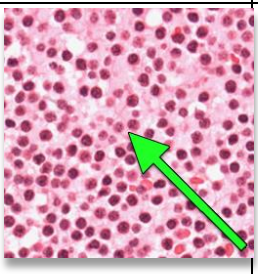
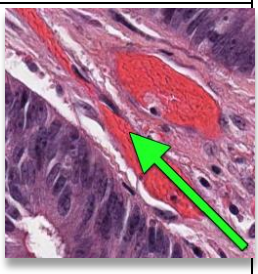
Tumour				
	Tumour	Lumen	Necrosis	Mucin
Stroma				
	Stroma	Muscle	Inflammation	Vessel

Table 4.1: A summary of how each point was classified based on where the spot had landed. There is a 9th class for unclassifiable data if a spot landed on an area that is non-informative.

4.3.3 Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS v15.0, Chicago, IL, USA). Using a modified receiver operating characteristic (ROC) curve recommended by a biostatician (P McShane – personal communication, 2013), the cut off for dichotomization of PoTS with the highest sensitivity and specificity regarding LNM was calculated. With this approach, PoTS was classified as either PoTS – high or PoTS – low.

Statistical analyses of continuous variables between these two groups were performed using the Mann – Whitney U test. Associations between categorical data (for example qualitative data) and LNM were performed using a Chi – Square test and Fishers exact tests where appropriate. Multi-variable analyses were performed using binomial logistic regression analysis.

A p value of < 0.05 for all tests was considered statistically significant.

4.4 Results

4.4.1 Clinicopathological data

Similar to the previous chapter, the NYCRIS database search identified 382 patients and from this total, 207 patients were used in this study as 106 patient's microscopy slides were missing, 35 patients microscopy slides were damaged and were unsuitable for digital scanning and 25 cases were rectal carcinomas which were down-staged to a pT1 stage after receiving pre-operative radiotherapy. Nine cases had synchronous tumours that were staged pT2 – 4 with lymph node metastasis. All 207 patients had primary resections of their cancers and none had received pre-operative chemo-radiotherapy. Table 2.6 shows the demographical data of the population of pT1 cases evaluated.

4.4.2 Lymph node metastasis and relative proportion of tumour stroma

The PoTS for the pT1 population followed a normal distribution with values of PoTS ranging from 9.38 to 84.46% (figure 4.2). The median PoTS value was 40.07% (interquartile range from 29.73 to 51.01%).

LNM was noted in 19 (9.2%) of the 207 cases. In the patients with LNM, 17 were staged pN1 and 2 patients were staged pN2. Figure 4.3 displays the distribution of the pT1 CRCs with and without LNM.

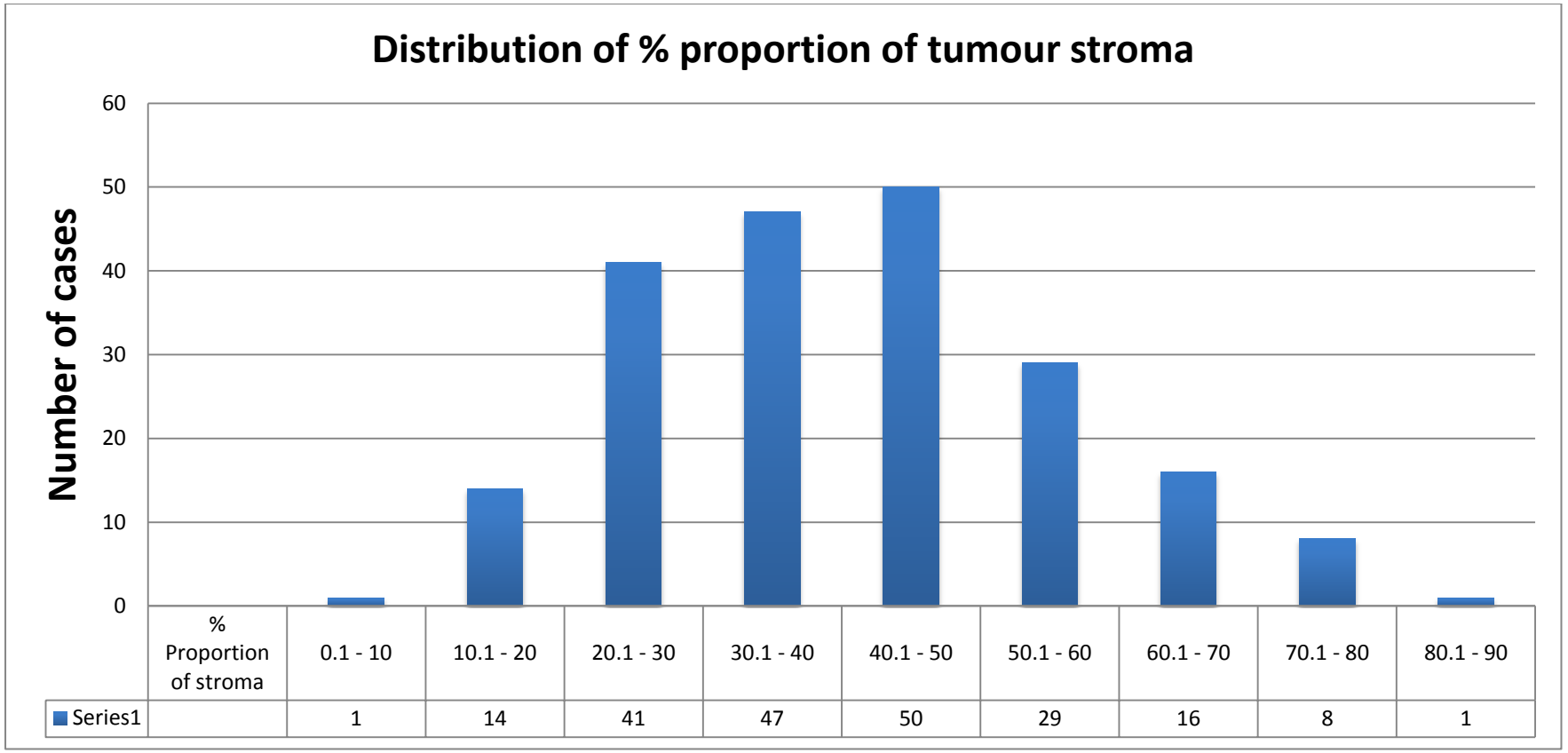
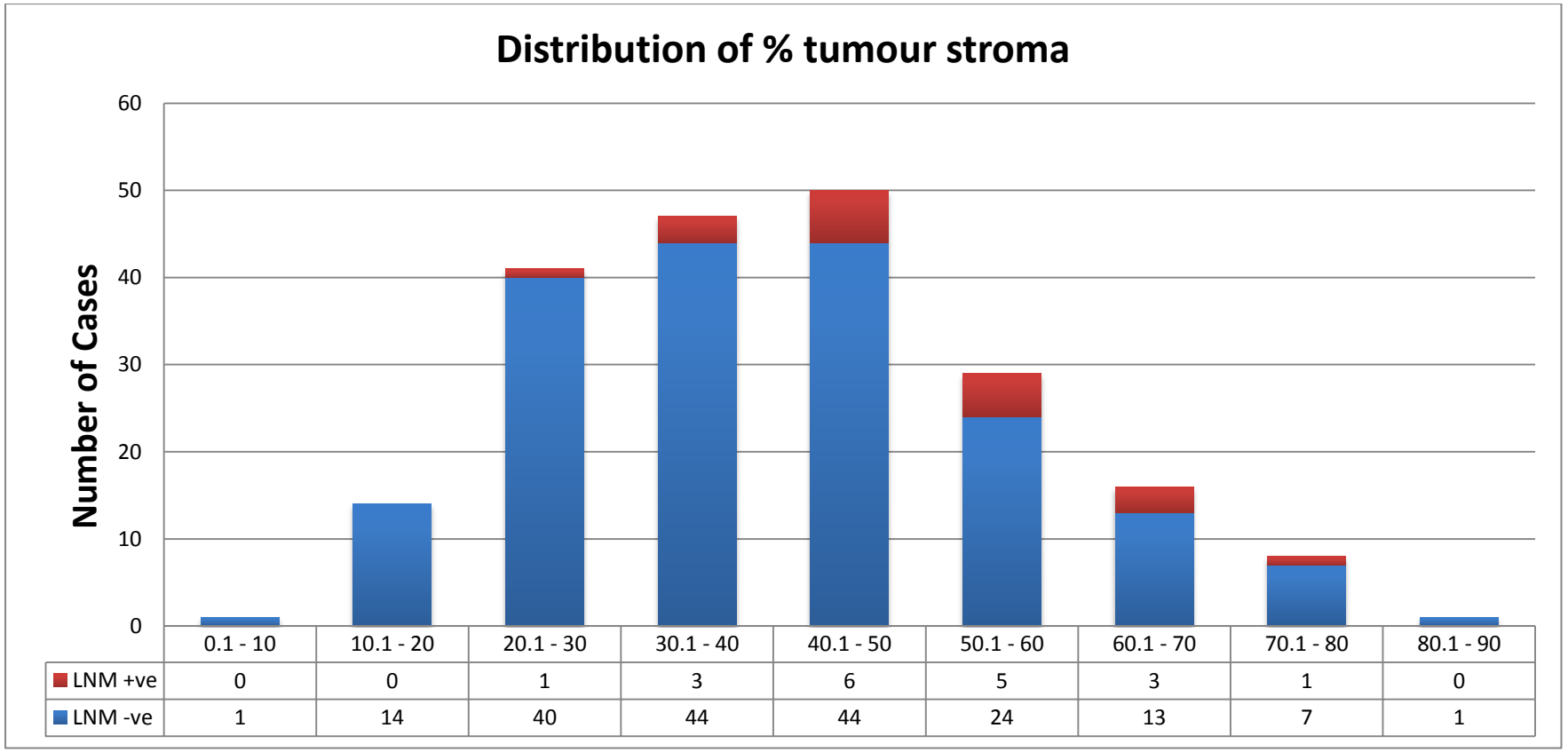


Figure 4.2: The distribution of the percentage PoTS across the patient population.



	0.1 - 10	10.1 - 20	20.1 - 30	30.1 - 40	40.1 - 50	50.1 - 60	60.1 - 70	70.1 - 80	80.1 - 90
LNM +ve	0	0	1	3	6	5	3	1	0
LNM -ve	1	14	40	44	44	24	13	7	1

Figure 4.3: The distribution of the percentage of PoTS for the pT1 CRCs with and without LNM

There were no statistical differences between the groups with and without LNM for gender, age and the site of the tumour (table 4.2). However, pT1 cancers with LNM had a higher PoTS compared to pT1 cancers without LNM and this was statistically significant. This data is displayed in table 4.2.

		Lymph Node Metastasis		p-value
		Negative (n=188) [%]	Positive (n=19) [%]	
Sex	Male	113 [60.1%]	14 [73.7]	0.247
	Female	75 [39.9]	5 [26.3]	
Age	Median, IQR	71, 63 - 77	69, 57 – 75	0.536
Site of tumour	Colon	104 [55.3]	9 [47.4]	0.439
	Rectum	83 [44.4]	10 [52.6]	
	Unknown	1 [0.5]	0 [0.0]	
Proportion of tumour stroma (%)	Median, IQR	39.50, (28.73 – 49.83)	48.31, (43.92 – 56.61)	0.005

Table 4.2: Correlation between clinicopathological data, PoTS and lymph node metastasis status. p- values obtained using Fischers exact test.

The modified ROC curves had generated a cut off value of PoTS of 43.5% (area under curve = 0.702, sensitivity = 0.789 and specificity = 0.612) (figure 4.4).

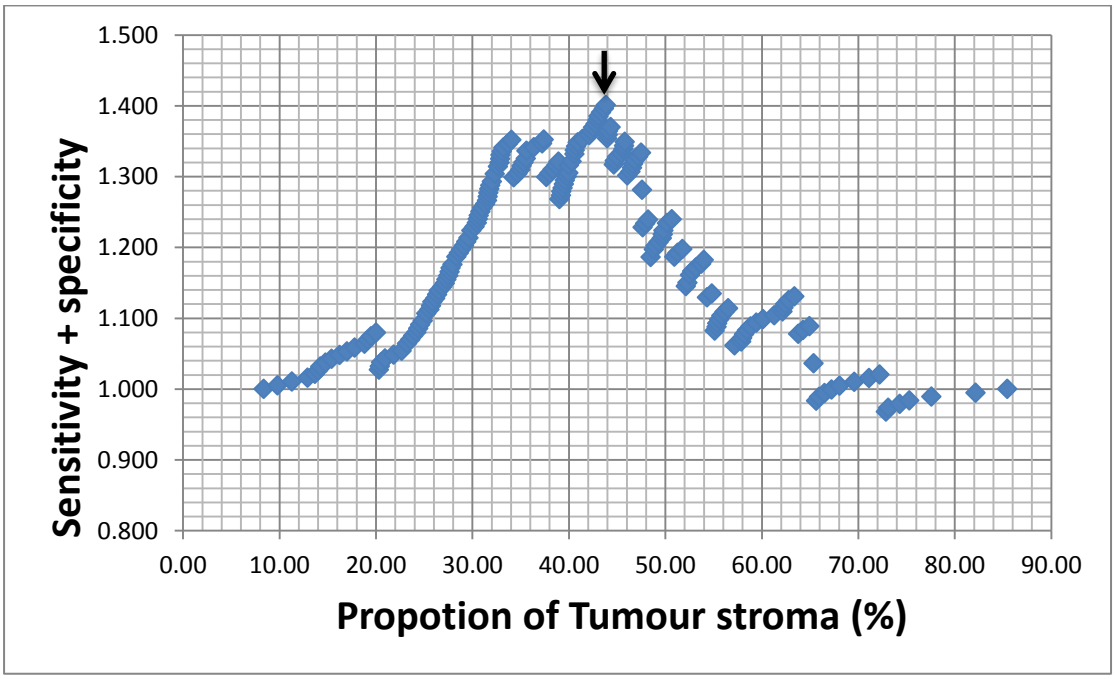


Figure 4.4: Modified receiver operating characteristic curve used to determine the optimal cut-off value for PoTS to dichotomize the patient cohort. The area under the curve was 0.702.

Using the cut off value that was generated by the modified ROC curve, the pT1 cancer population was dichotomized into a tumour stroma high group (PoTS ≥ 43.5%) and a tumour stroma low group (PoTS < 43.5%). There was a significant difference between the two groups with regard to LNM where the majority of pT1 cancers with LNM (15/19 cases [78.9%]) had PoTS ≥ 43.5% ($p = 0.001$, OR = 5.78, 95% CI = 1.85 – 18.08) (table 4.3).

	Lymph node metastasis		p-value	Odds ratio (95% confidence interval)
	Negative (n=188) [%]	Positive (n=19) [%]		
Tumour stroma < 43.5%	114 [60.6)	4 [21.1]	0.001	5.78 (1.85 – 18.08)
Tumour Stroma ≥ 43.5%	74 [39.4]	15 [78.9]		

Table 4.3: Univariate analysis of PoTS (low vs high) and lymph node metastasis. p- values obtained using Chi-Square test.

When analysed on multivariate analysis with other established histopathological factors such as poor differentiation, vascular and lymphatic invasion, a PoTS ≥43.5% was predictive of LNM (p = 0.017, HR = 4.34, 95% CI = 1.30 – 14.47). Both lymphatic invasion and poor differentiation were also significant in predicting LNM (p values 0.017 and 0.003 respectively). Details of these data are in table 4.4.

Qualitative factor	p-value, hazard ratio (95% confidence interval)
Vascular invasion	0.054, 5.23 (0.97 – 28.16)
Lymphatic invasion	0.017, 9.49 (1.51 – 59.74)
Grade of differentiation	0.003, 11.6 (2.36 – 57.06)
Proportion of tumour stroma ≥43.5%	0.017, 4.34 (1.3 – 14.47)

Table 4.4: Multivariable analysis of well-established histopathological factors and high proportion of tumour stroma. p-values obtained using binomial logistic regression analysis.

When the area of submucosal invasion ≥ 35 mm^2 was included in the multivariate analysis with the PoTS ≥43.5% and the other established histopathological factors, the PoTS was still predictive of LNM (p = 0.002, HR = 9.30, 95% CI = 2.27 –38.12). Details of these data are in table 4.5.

Qualitative factor	p-value, hazard ratio (95% confidence interval)
Vascular invasion	0.149, 4.84 (0.57 – 41.08)
Lymphatic invasion	0.017, 14.70 (0.61 – 134.05)
Grade of differentiation	0.004, 22.68 (2.69 – 191.31)
Proportion of tumour stroma ≥43.5%	0.002, 9.30 (2.27 – 38.12)
Area of submucosal invasion ≥ 35 mm^2	0.001, 42.23 (4.44 – 460.63)

Table 4.5: Multivariate analysis of well-established histopathological factors, high proportion of tumour stroma and area of submucosal invasion greater than 35 mm^2. p-values obtained using binomial logistic regression analysis.

4.5 Discussion

There is increased appreciation of the importance of tumour stroma and its relationship with colorectal cancer progression. The tumour stroma promotes the proliferation and survival of cancer cells and facilitates epithelial-mesenchymal transition (EMT) (Liu et al 2013). Furthermore, the tumour stroma may play a role in local invasion and metastatic dissemination (De Weaver and Mareel 2003) and may provide resistance to chemotherapy agents (Petty et al 2009) however others differ (Hutchins et al 2015). Not surprisingly, studies have already shown that a higher proportion of tumour stroma has been associated with a poorer survival outcome in patients with colorectal cancer (Mesker et al 2007, Mesker et al 2009, West et al 2010, Huijbers et al 2013, Hutchins et al 2015).

There are several theories as to why a high PoTS within a tumour may increase the risk of LNM. Tumour stroma contains a rich network of non-malignant cells that produce key growth factors and cytokines such as VEGF, EGF, PDGF, IL-10, and TGF-β. These appear vital in the development, maintenance and progression of the malignancy.

Secondly, the tumour stroma may play a role in helping tumour cells evade the host immune response directed towards them. It is accepted that lymphocytes play a role in providing anti-tumour immunity (Titu, Monson and Greenman 2002). Researchers have shown that increased numbers of lymphocytes within the tumour stroma offer better survival in patients with CRC (Jass 1986, Ropponen et al 1997, Ogino et al 2009). Lieubeau et al (1999) showed that cancer associated fibroblasts may prevent penetration of

immune cells such as lymphocytes within the tumour environment and create a physical barrier between the tumour cells and the immune cells. Therefore, tumour growth and progression is not halted by actions of the immune cells (Lieubeau et al 1999). A high density of cancer-associated fibroblasts has also been associated with tumour budding (Ueno et al 2004, Tsujino T et al 2007), which is an adverse prognostic factor.

Thirdly, a high tumour stroma may be indicative of how aggressive a cancer is. In their cohort study, Mesker et al (2007) showed that there was a greater amount of patients with higher tumour stroma percentage in stage III patients compared to stage I patients (68.7% vs 7.7%). Mesker et al (2009) further validated this by showing that in a tumour stroma high group, there was a greater amount of patients within stage II of the disease compared to stage I (94.1% vs 5.9%) (Mesker et al 2009). Park et al (2014) also showed in their study of patients with primary operable CRC, there were a greater amount of pT3 and pT4 disease compared to pT1 and pT2 disease within the high tumour stroma percentage group (91% vs 9%). In this study, we focused on pT1 CRC and showed that 78.9% of the pT1 CRC in the PoTS high group had LNM compared to 21.1% in the PoTS low group ($p < 0.001$). This further substantiates the hypothesis that a high tumour stroma content within a cancer may be reflective of how aggressive it is in invading and spreading locally and distantly. However, this needs to be validated with a study comprising of a bigger sample size.

This study has focused on early CRC and showed that pT1 CRC with LNM had significantly higher PoTS compared to pT1 CRC without LNM. This

would suggest that evaluation of the tumour stroma using routine pathological specimens might help identify high risk pT1 CRC. Moreover, evaluating the tumour stroma is simple and adds no extra cost to the preparation of the pathological specimen. It is hoped that when tumour stroma is assessed together with other well-known high-risk histopathological features, this will lead to better risk stratification for patients undergoing local or radical treatment for their pT1 CRC.

4.5.1 Tumour stroma as a potential future treatment target

Tumour cells are genetically unstable. This makes targeted chemotherapy towards tumour cells difficult and often gives unpredictable results. In contrast to tumour cells, cells of the tumour stroma are genetically more stable thus making these cells an ideal target for chemotherapy agents and immune therapy.

A successful example of therapy targeting the tumour stroma is bevacizumab, which is an anti-VEGF antibody (Presta et al 1997). VEGF is a unique product generated by tumour stroma in conditions of hypoxia where VEGF promotes tumour angiogenesis. Bevacizumab was first believed to exert its direct antiangiogenic effect on tumour vasculature (Willet et al 2004). However, it is now believed that bevacizumab alters tumour physiology (reducing tumour interstitial fluid pressure, potentially increasing tumour oxygenation and lowering extracellular pH (Wildiers et al 2003, Kramer and Lipp 2007, Los, Roodhart and Voest 2007). These factors help to increase

the sensitivity of a tumour towards chemoradiotherapy (Willet et al 2005, Pavlidis and Pavlidis 2013).

Another potential target within the tumour stroma are the CAF and its associated protein, fibroblast associated protein (FAP). Therapeutic strategies that target CAF have shown promising results in animal models. However, these treatment strategies have not been widely used in the clinical setting due to lack of evidence within the human models (Brennen, Issacs and Denmeade 20112).

The over expression of FAP is believed to lead an increase in tumour growth and metastasis (Brennen, Issacs and Denmeade 2012). Loeffler et al (2006) reported a 70% greater uptake of chemotherapeutic agents in carcinoma-induced mice vaccinated specifically against fibroblast activation protein (FAP). In Loeffler's study, the vaccinated mice showed a 3-fold prolongation of lifespan when vaccinated for FAP. Wikberg et al (2013) have also shown high intra-tumoural FAP was associated with a poorer prognosis in CRC patients (HR = 1.72, 95% CI 1.07 – 2.77, p – 0.025). However, more research is still needed in human studies. Several phase I and II studies targeting FAP with a humanized monoclonal antibody (sibrotiozumab) failed to produce clinical benefits in CRC (Scott el at 2003, Hofheinz et al 2013).

We are slowly beginning to understand the complex relationship between tumour cells and their stroma. There is no doubt that tumour stroma does play an integral role in tumour development and metastasis. Therefore, the tumour stroma may prove to be a crucial target for future treatments of CRCs, especially higher staged CRCs.

4.6 Conclusion

The understanding of the detailed roles of tumour stroma in the development and progression of CRC is important as it may help identify new prognostic and predictive factors. The tumour stroma may also prove to be a target for new treatments for CRC and thus providing a more precise and personalized therapy.

This study has shown that the calculation of PoTS by point counting on digital microscopy could potentially be a useful quantitative prognostic factor for LNM in pT1 CRC. This low cost and reproducible method of calculating tumour stroma could be valuable in identifying high risk pT1 CRC and should be easily transferable to routine histological diagnostic practice. Furthermore it could be automated increasing its value and likelihood of adoption.

Chapter 5 Inter-observer variations in the reporting of pT1 colorectal cancers

With the NHS BCSP being fully rolled out, the pathology service plays a vital role in colorectal screening as the programme depends on high quality, accurate evaluation of the lesions found within the large bowel. Pathology also plays an important role in determining the type of treatment that a patient requires, be it a local excision or a radical resection and in determining the period of surveillance that a patient requires after screening.

5.1 Reporting of lesions within the NHS BCSP

Histopathological diagnosis of lesions discovered within the screening programme is mostly straightforward. However, there are times when the diagnosis of these lesions are difficult and may cause some discrepancy between pathologists. To aid in reducing these discrepancies, national guidelines have been produced for reporting lesions discovered through screening (NHSBCSP 2011, 2016). The European recommendations appeared in 2011 (Quirke et al 2011).

5.1.1 Pathological reporting of lesions within the NHS BCSP

Histopathological reporting of lesions within the screening programme should contain key diagnostic data that should help both pathologist and clinicians in making treatment decisions. For colorectal adenomas, the key diagnostic features that require reporting are as below:

5.1.2 Site of lesion

The origin of each lesion should be identified by the endoscopist and provided to the pathologist on the request form.

5.1.3 Size of lesion

The size of a lesion is an important objective measurement and is best performed during histopathological analysis. Microscopic measurements of lesions are more accurate compared to endoscopic measurements. Accurate measurements of these lesions are clinically important as these measurements would help dictate the period of surveillance colonoscopies and help decrease the burden on endoscopists (Taylor et al 2016). Normally, the largest diameter of the lesion on the glass slide is taken as the measurement of the size and a hierarchy of measurements is recommended.

5.1.4 Type of lesion

Three broad groups of lesion are commonly reported; classical adenomas, serrated lesions and other polyps.

5.1.4.1 Classical adenomas

Classical adenomas must show a degree of dysplasia and can further be sub-divided into tubular, tubulovillous or villous types. Previously, the definitions of these sub-types are based on the '20% rule' as described in the WHO classification (Hamilton 2000). For a polyp to be classified as a tubulovillous adenoma, at least 20% of the estimated volume of the adenoma should be villous. In a villous adenoma, 80% of the volume must comprise of villous. All other lesions are classified as tubular. However, the current guidelines (NHS BCSP 2016) now state that for a polyp to be classified as a tubulovillous adenoma, at least 25% of the estimated volume of the adenoma should be villous and in a villous adenoma, more than 75% of the volume of the polyp must comprise of villi.

5.1.4.2 Serrated lesions

These lesions have a common serrated pathology. The spectrum of lesions with a serrated growth pattern is subdivided into hyperplastic polyps, sessile serrated lesions, traditional serrated adenomas and mixed

hyperplastic/adenomatous polyps. The serrated lesion spectrum is still being researched and remains controversial in terms of their malignant potential.

Hyperplastic polyps are common and have a very low malignant potential. These polyps are frequently found within the distal large bowel and are composed of simple elongated crypts with a serrated structure in the upper half. Hyperplastic polyps are only significant when they are numerous in numbers, proximally located within the large bowel and/or are of a large size (≥ 10mm), features raising the possibility of serrated (formerly hyperplastic) polyposis syndrome.

Sessile serrated lesions are lesions that show a serrated morphology with structural changes that are consistent with mucosal neoplasia. If dysplasia is present then they are classified as sessile serrated lesions with dysplsia.

Traditional serrated adenomas are histologically distinct from sessile serrated lesions. These poylps contain histological features that include eosinophilic cytoplasm, pencillate nuclei, ectopic crypts and variable degrees of dysplasia (Bateman and Shepherd 2015).

Mixed hyperplastic/adenomatous polyps are lesions that have both non-dysplastic hyperplastic type epithelium showing serrated glandular architecture and areas of adenomatous dysplastic epithelium (Jass et al 2006). In context to the screening programme, these lesions should be completely removed and if there is an adenomatous or dysplastic component, the lesion should be under surveillance in the same manner as for adenomas.

A summary of the different type of polyps is provided in table 5.1.

Type	Criteria
Classical Adenomas	Classification is based on the relative proportions of tubular and villous components
Tubular	Adenomas that contain a villous component less than 25%
Tubulo-villous	Adenomas that contain more than 25% but less than 75% villous component
Villous	Adenomas with a villous component more than 75%
Serrated Lesions	
Hyperplastic polyps	Characterized by serrated glandular profiles caused by variable degrees of epithelial hyperplasia, without dysplasia.
Sessile serrated lesions (SSL)	Morphologically similar to the microvesicular variant of the hyperplastic polyp, but with one or more key histological features that include irregular distribution of crypts, dilatation of crypt bases, serration present at crypt bases, branched crypts, horizontal extension of crypt bases, dysmaturation of crypts and herniation of crypts through the muscularis mucosa.
SSL with dysplasia	Similar histological features to SSL but containing cytological features of dysplasia (low or high grade).

Traditional serrated adenoma	Histologically distinct from SSLs. Histological features include the presence of dysplasia together with variable proportion of the lesion showing eosinophilic cytoplasm, pencillate nuclei and ectopic crypts (Bateman and Shepherd 2015).
Mixed hyperplastic/adenomatous polyps	Contain areas of both dysplastic adenomatous epithelium and hyperplastic polyp-type hyperplasia

Table 5.1: Summary of the classical adenomas and serrated lesions (NHS BCSP 2016, Bateman and Shepherd 2015).

5.1.4.3 Other types of polyps

There are other polyps that can be identified within the bowel cancer screening programme. These polyps include inflammatory polyps, juvenile polyps and Peutz-Jeghers polyps. There are also other different types of lesions found in the screening programme such as lipomas and leiomyomas. Rarely, unusual forms of stromal lesions, which include ganglioneuroma, neurofibroma, gastro-intestinal stromal tumour (GIST), various forms of vascular tumour, perineurioma, fibroblastic polyp and epithelioid nerve sheath tumour can present as polypoid lesions in the bowel.

5.1.5 Grading of dysplasia

In reporting the grade of dysplasia, it is recommended that high grade dysplasia and low grade dysplasia are used instead of mild, moderate and severe dysplasia. By definition, adenomas have at least low grade dysplasia. Thus, an adenoma is classed as low grade dysplasia unless it has any features of high grade dysplasia.

Adenomas with high grade dysplasia usually involve more than just one or two glands and should exhibit architectural abnormalities and appropriate cytological features. Architectural abnormalities include:

- Complex glandular crowding and irregularity
- Prominent budding
- Cribiform appearance and ‘back to back’ glands
- Prominent intraluminal papillary tufting

Under low power microscopy, the epithelium appears thick, disorganised and ‘dirty’.

Cytological features that accompany the architectural abnormalities include:

- Atypical mitotic figures
- Loss of cell polarity or nuclear stratification to the extent nuclei are approximately equally
- Significantly enlarged nuclei often with dispersed chromatin pattern and a prominent nucleolus

- Prominent apoptosis, giving the epithelium of the lesion a ‘dirty’ appearance

5.2 Reporting of pT1 CRC within the NHS BCSP

Meticulous and accurate assessment of pT1 colorectal cancer is an integral part in determining the appropriate treatment. Reporting of the screened pT1 colorectal carcinomas include similar key diagnostic features as described above, but there are extra features that need to be reported as well. These features are as described below.

5.2.1 Tumour grade

As described earlier, tumours are graded on how differentiated they appear. Based on the NHS BCSP guidelines for reporting lesions, poorly differentiated carcinomas can be identified by either:

- The presence of irregularly folded, distorted and small tubules or
- The lack of any tubular formation

When there is lack of the evidence as above, it is recommended that the grade of differentiation should be applied to the polyp carcinoma when any area of the lesion displays poor differentiation. Testing of such lesions for deficient mismatch repair is also now recommended to exclude this as a cause of poor differentiation.

5.2.2 Lymphovascular invasion

Identifying lymphatics and vascular structures on microscopy can be challenging to pathologists. Blood vessels are usually more easily identified as they have a thicker muscular wall and contain red blood cells within their lumen. However, lymphatic vessels are thinned-walled, of irregular size (often smaller than blood vessels) and frequently have no visible contents at all. This makes detecting lymphatic vessels difficult and additional immunohistochemistry stains may be required. These stains include D2-40 and CD 34 stains.

Any cases with uncertain lymphovascular invasion should be recorded and discussed by the multidisciplinary team.

5.2.3 Margin involvement

HIstopathological assessment should include the recording of deep (intramural) resection margin involvement by the invading carcinoma. It is also vital to record the mucosal resection margin that is involved by the carcinoma. There have been recent discussions and a change in recommendations (NHSBCSP 2016) regarding the degree of clearance that is acceptable. Previously a clearance of less than or equal to 1mm would be defined as margin involvement and would require further therapy. Recently this has been revised to any degree of histological tumour clearance, even less than 1mm, can be regarded as non-involvement of the margins unless there are infiltration of malignant glands into the diathermy zone associated with

morphological distortion to the extent that it is not possible to identify tumour clearance (NHSBCSP 2016).

5.2.4 Sub-staging of pT1 CRC

As already described, pT1 carcinomas can be further sub-staged based on their shapes; the Haggitt system for polypoid lesions and the Kikuchi system for sessile lesions respectively. However many case are semi-pedunculated and do not fall into these categories. Each of these systems has their advantages and disadvantages and will be described in the chapter ahead.

5.3 Inter-observer variations in reporting pT1 CRC

Inter-observer variation in the reporting of colorectal polyp cancers has been studied in various ways and has been largely unsatisfactory. There is no formal training or selection process for histopathologists working within the NHS BCSP. However, quality assurance is maintained by a combination of annual training sessions, inspections and an online External Quality Assessment (EQA) scheme. Currently, there are 150 pathologists within this scheme of whom all are consultant histopathologists (details of the GI and BCSP pathology EQA schemes available at http://www.gieqa.org.uk/overview).

Most inter-observer variation studies have focussed on colorectal adenomas rather than colorectal carcinomas. Most of these inter-observer variation

studies have looked at the agreements in diagnosing the polyp histologic type, grade of dysplasia and completeness of the excision margin. Below, we will discuss these factors that have been studied and also studies that evaluated inter-observer variation in colorectal cancers.

5.3.1 Inter-observer variations in polyp histologic type

The inter-observer agreement in discriminating adenomatous and non-adenomatous polyps is generally good. As part of a Dutch population-based randomized screening trial, van Putten (van Putten et al, 2011) had showed that there was a very good kappa value (κ) of 0.88 (95% confidence interval [CI] 0.83 – 0.94) between 23 general pathologists who had reviewed a total of 440 polyps that were detected in the screening programme. Equally impressive is a similar kappa value that was obtained when two expert gastrointestinal pathologist were asked to review the same 440 polyps (κ = 0.85, 95% CI 0.73 – 0.98).

More recently, Foss (Foss et al, 2012) studied the inter-observer agreement between two gastrointestinal pathologists who had reviewed a total of 239 polyps that were detected within the NHS BCSP. The discrimination between adenomatous and non-adenomatous polyps also showed very high levels of inter-observer agreement (κ = 0.83).

The inter-observer agreement with respect to determining the histologic type of adenoma has ranged from fair to moderate. Within the screening population, Foss showed that there was moderate agreement in identifying

tubular adenomas (κ = 0.61, 95% CI = 0.51 – 0.71) but only poor and fair on the classification of villous sub types (tubulo-villous adenoma - κ =0.38. 95% CI 0.26 – 0.49, villous adenoma - κ =0.18, 95% CI 0.02 – 0.34). Other studies within a screening population (Denis et al 2009, van Putten et al 2011) have shown a moderate amount of inter-observer agreement (κ = 0.44 - 0.55) when distinguishing the histologic type of adenoma when there were 3 categories (tubular adenoma, tubulo-villous adenoma and villous adenoma).

Turner (Turner et al 2013) had expanded the histologic types into 5 categories (hyperplastic polyp, serrated adenoma, tubular adenoma, tubullo-villous adenoma and villous adenoma). This study showed that there was still a moderate level of agreement (κ = 0.45, 95% CI 0.34-0.59) between BCSP pathologists in Wales.

5.3.2 Inter-observer variation in grading of dysplasia

Grading of dysplasia has produced satisfactory Kappa values. Studies have often categorised grading of dysplasia into two categories (low grade/ high grade) or three categories (no dysplasia/ low grade/ high grade or low grade/ high grade/ infiltrating carcinoma). Kappa values for studies with two categories ranged from 0.59 to 0.69 (van Putten et al 2011, Foss et al 2012) and studies with three categories had kappa values of 0.54 to 0.67 (Costantini et al 2003, Denis et al 2003, Turner et al 2013).

5.3.3 Inter-observer variation in assessing excision margin status

There has been great variability in the reporting of the completeness of a polyp excision margin. Turner (Turner et al 2013) had looked at the histological reporting of 28 pathologists based in Wales. The 28 pathologist had to report on 12 screened cases based on the BCSP recommended guidelines. The kappa value for the histological reporting of the degree of excision (complete/incomplete/ uncertain) was only fair (κ= 0.24, 95% CI 0.07 – 0.43).

In contrast, an earlier study by Foss and colleagues (Foss et al 2012) showed that there was a higher level of concordance in determining the degree of excision (κ= 0.75, 95% CI 0.64 – 0.84) in screened polyps. However, Foss's study only had two pathologists participating. Another study by Komuta (Komuta et al 2004) also showed that there was good agreement in assessing excision margin status within malignant colorectal polyps κ= 0.668.

5.3.4 Inter-observer variation in the grading of CRC

Chandler and Houlston (Chandler and Houlston 2008) had carried out a nationwide study looking at the inter-observer agreement in grading of colorectal cancers. This study had enlisted the participation of 104 consultant histopathologists. They were instructed to grade 20 carcinomas within three grades (well differentiated, moderately differentiated and poorly differentiated). The overall κ value for the grading results using the three grades was 0.351 indicating fair agreement. The study had also evaluated the

inter-observer agreement when the grading was reduced into two categories (low and high grade). The inter-observer agreement was still fair, κ value = 0.358.

Komuta et al evaluated the inter-observer variability in the histopathological assessment of malignant colorectal polyps. Three experienced gastrointestinal pathologists had reviewed 88 polyps. The inter-observer agreement was poor with respect to histopathological grading of these polyps (κ = 0.163) (Komuta et al 2004).

The results from the studies above have shown that using both two and three grade system pathologists had only achieved fair levels of agreement. This implies that grading of cancers can be subjective and better definitions and criteria should be developed.

5.3.5 Inter-observer variation in assessing lymphovascular invasion in CRC

As mentioned already, assessment of lymphovascular invasion is often difficult and there is a high level of inter-observer variability. Komuta had looked into the inter-observer variability in assessing lymphovascular invasion in malignant colorectal polyps. The polyps were assessed on normal H & E slides and the inter-observer agreement was poor (κ = -0.017) (Komuta et al 2004).

The use of immunohistochemical stains was expected to lower inter-observer variability. Harris et al evaluated the use of immunohistochemical stains in

identifying lymphovascular invasion. Six gastrointestinal pathologists assessed small and large vessel invasion based on 50 cases of colorectal cancers that were stained with H & E, CD31 and D2-40 stains. Inter-observer agreement was only fair for H & E small vessel invasion (κ = 0.28, 95% CI 0.22 – 0.34) and poor for H & E large vessel invasion (κ = 0.18, 95% CI 0.11 – 0.26). When the immunohistochemical stains were applied to the same cancers that were being assessed, agreement was not improved (CD31 stains; large vessels κ value = 0.42 95% CI 0.2 – 0.63, small vessels κ value = 0.26, 95% CI 0.1 – 0.42; D2-40 stains, κ value = 0.32, 95% CI 0.21 – 0.42) (Harris et al 2008).

Similar results were obtained in a study conducted by the Pathology Working Group of the Japanese Society for Cancer of the Colon and Rectum (Kojima et al 2013). This study had involved eight pathologists assessing 20 tumours for lymphatic and vascular invasion on H & E slides and on slides with immunohistochemical stains (D2-40 and elastic stains). Inter-observer agreement was only moderate in identifying vascular invasion (κ=0.574, 95% CI 0.441 – 0.606) and poor in lymphatic invasion (κ = 0.216, 95% CI 0.133 – 0.209). This was no better when the cancers were prepared with immunohistochemical stains (κ value for vascular invasion = 0.502, 95% CI 0.419 – 0.584, κ value for lymphatic invasion = 0.153, 95% CI 0.071 – 0.236).

5.4 Research objectives

The main aim of this study is to investigate the reproducibility of qualitative and quantitative factors that may contribute to LNM in pT1 CRC and thus the decision as to whether a major resection is required.

The secondary aim of this study is to assess the reproducibility of the novel quantitative factors that were studied (width of invasion and area of submucosal invasion).

5.5 Materials and methods

5.5.1 Inter- and intra-observer variation studies

H & E slides from the NHS BCSP were selected for the inter-observer and intra-observer study. NHS regional leads confirmed all the cases as stage pT1 CRC. The screened pT1 cases were scanned in an identical fashion as described in section 4.3. In selecting the cases for the inter-observer study, a single digital image of the cancer was selected based on its best representation of the cancer as a whole. Digital images of these screened pT1 cancers were uploaded to a web page. The web page is available for viewing at the address listed within the appendices.

Regional lead pathologists who regularly reported within the bowel cancer screening programme were invited to participate in this study. An invitation was emailed to all pathologists together with instructions for the tasks to be performed and a proforma to complete (see appendix I). The proforma was a Microsoft Excel form that consisted of the selected cases (and the respective URL web page links) and tasks that the pathologists had to perform. The participating pathologist could access the cases through the web page.

The participating pathologists were requested to perform the 10 key tasks listed as below:

1. To define the shape of the lesion (pedunculated, semi-pedunculated, sessile or not assessable)
2. To define the grade of differentiation (non-poor/poor)
3. To identify the distance of the lesion to the nearest margin

4. To identify the presence of lymphatic invasion
5. To identify the presence of vascular invasion
6. To state whether the lesion can be assessed by Haggitt's levels and identify the level of invasion (Level 1/ 2/ 3/ 4)
7. To state whether the lesion can be assessed by Kikuchi's levels and identify the level of invasion (SM1/ SM2/ SM3)
8. To measure the width of the carcinoma
9. To measure the depth of invasion of the carcinoma
10. To state whether the lesion/ carcinoma is fully excised and/or should be resected

The pathologist had to analyse 41 pT1 cases and were given a six week time limit to complete the analysis of the cases.

For the intra-observer study, the pathologists were requested to repeat the study on the same cases 3 months after the first round. After the first round of the inter-observer study, new recommendations were formulated for the method of measuring the distance from the carcinoma to the nearest margin and the depth of invasion of the carcinoma.

In this second round, the same cases as above were given to the pathologist but not in the same order as the first round. However, in the second round, the pathologists were asked to perform 11 instead of 10 tasks (see appendix II). The 11 tasks are as below:

1. To define the shape of the lesion (non-sessile/ sessile)
2. To measure the width of the lesion and carcinoma

3. To identify the distance of the lesion to the nearest margin based on the recommendations from the first round of the inter-observer study
4. To measure the depth of invasion of the carcinoma based on 4 methods (epithelial surface to deepest tumour cell, virtual muscularis mucosae curved line method to deepest tumour cell [Figure5.1], virtual muscularis mucosae straight line method to deepest tumour cell [Figure 5.2] and from the muscularis mucosae that is visible)
5. To measure the area of invasion by the carcinoma below the muscularis mucosae (submucosal invasion)
6. To define the grade of differentiation (non-poor/ poor)
7. To identify the presence of lymphatic invasion
8. To identify the presence of vascular invasion
9. To state the Haggitt's level (level 1/ 2 /3/ 4)
10. To state the Kikuchi's level (SM1/ SM2/ SM3)
11. To state whether the carcinoma should be resected based on the qualitative and quantitative features identified

The pathologists were again given 6 weeks to complete the analysis of the cases.

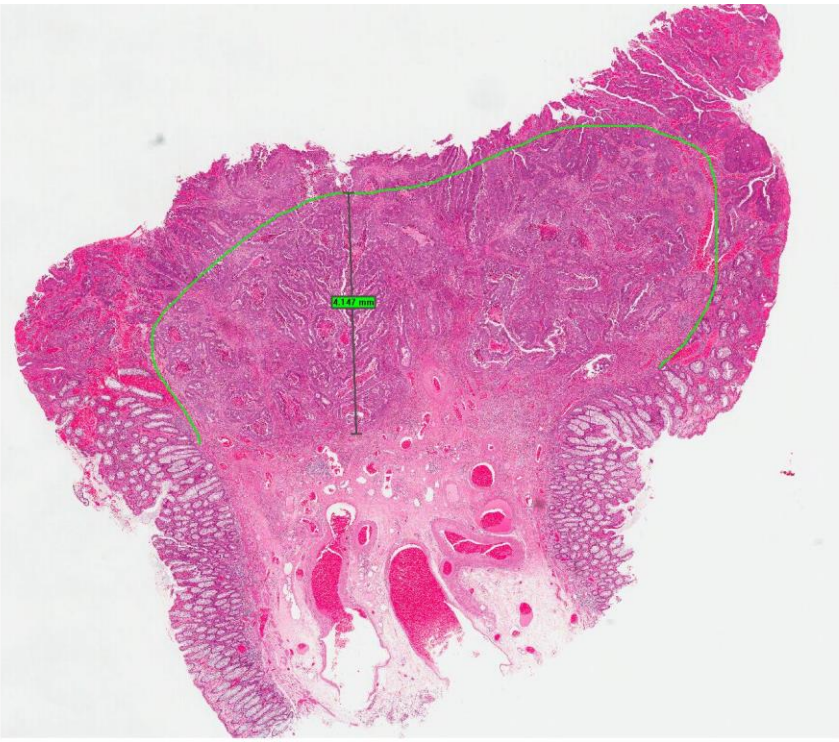


Figure 5.1: Virtual muscularis mucosae curved line method. A virtual muscularis mucosae is created by estimating where the muscularis mucosae would have lain if not destroyed by tumour or eroded and the depth of invasion is measured from this virtual muscularis mucosae.

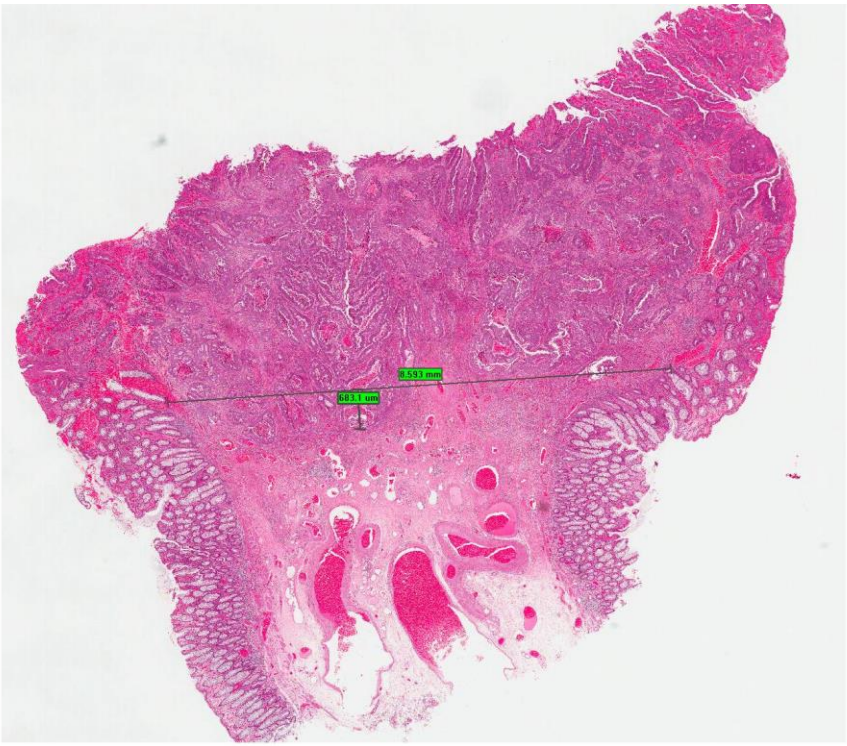


Figure 5.2: Virtual muscularis mucosae straight line method. A straight line is drawn from the highest point in the lesion where residual normal epithelium is seen containing normal muscularis mucosae to the same site on the opposite side. The depth of invasion is measured perpendicular to this straight line up to the deepest part of the invading cancer.

5.5.2 Statistical analysis

The Kappa (κ) statistic was used to assess agreement between pathologists (inter-observer) for qualitative data. Specifically, the Fleiss' κ was used as this method allowed for multiple assessors and categorical ratings. The Cohen's κ was used to calculate the intra-observer agreement. Both the Fleiss' and Cohen's κ were recommended by a senior biostatician (H Thygesen – personal communication, 2013). A value of 0 indicates agreement no better than what would be expected by chance alone. Values of <0.20, 0.21 – 0.40, 0.41 – 0.60, 0.61 – 0.80 and > 0.80 correspond to poor, fair, moderate, substantial and almost perfect agreement.

For quantitative data, the inter-observer agreement was obtained using the intra-class correlation coefficient (ICC). The ICC is a popular method to assess agreement between quantitative measurements taken from different observers. The ICC was calculated using the Statistical Package for the Social Sciences (SPSS v21.0, Chicago, IL, USA). Similar to the κ value, the ICC value can take on any value from 0 to 1 (values of <0.20, 0.21 – 0.40, 0.41 – 0.60, 0.61 – 0.80 and > 0.80 correspond to poor, fair, moderate, substantial and almost perfect agreement).

5.6 Results

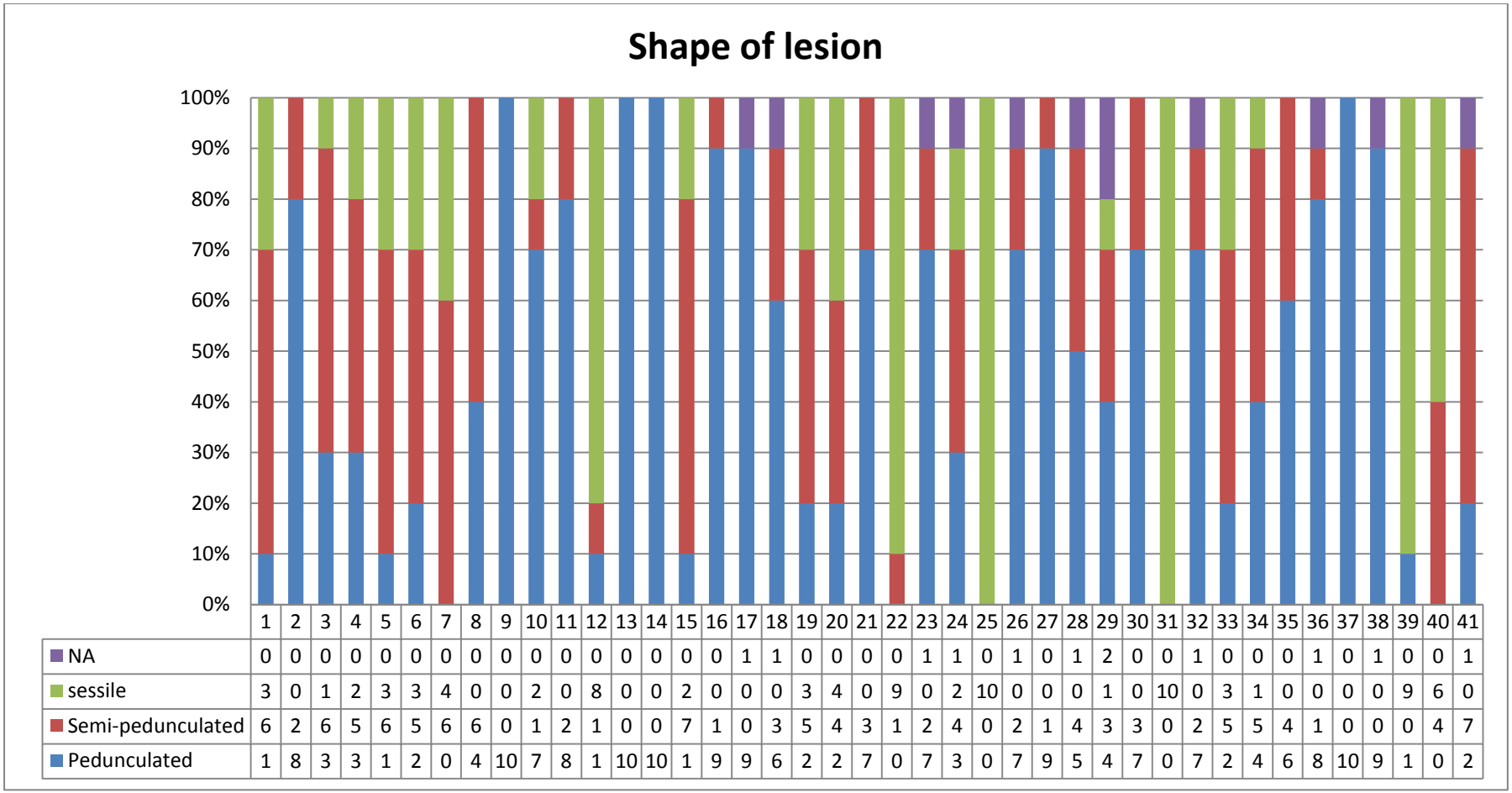
A total of 19 histopathologists were invited to participate in the inter- and intra-observer studies, however, only 10 histopathologists participated in the study. They were all lead regional histopathologists who regularly participated in the reporting of colonic polyps and cancers from the NHS BCSP.

5.6.1 Inter-observer variation round 1 results

5.6.1.1 Qualitative factors

5.6.1.1.1 Shape of lesion

The inter-observer agreement in deciding the shape of the lesion generated a κ value of 0.33 (95% CI 0.30 – 0.36, $p < 0.001$). Figure 5.3 demonstrates the breakdown of the shape of the lesion that the pathologist had decided on each case.

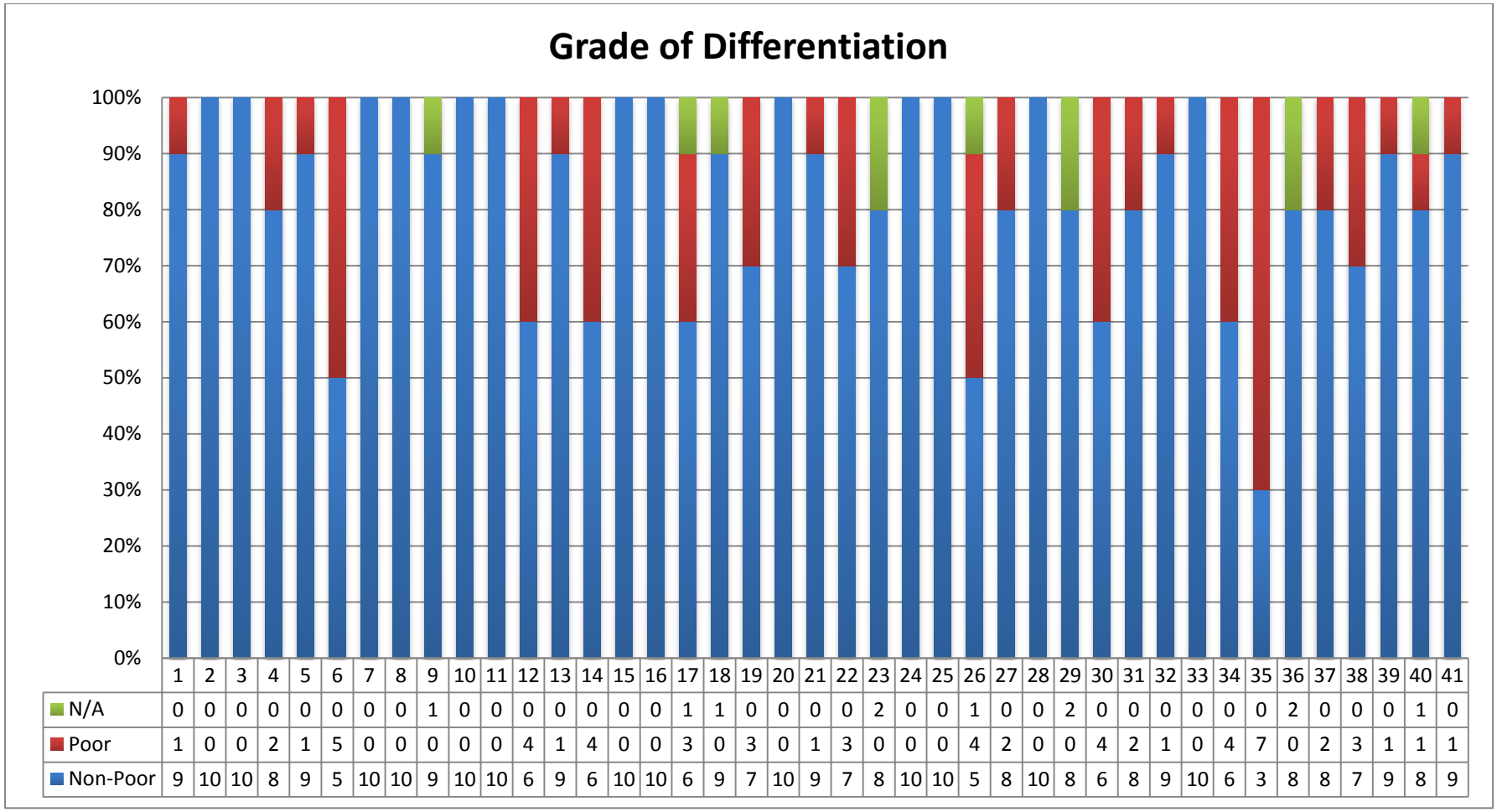


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
NA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	0	1	0	1	2	0	0	1	0	0	0	1	0	1	0	0	1
sessile	3	0	1	2	3	3	4	0	0	2	0	8	0	0	2	0	0	0	3	4	0	9	0	2	10	0	0	0	1	0	10	0	3	1	0	0	0	0	9	6	0
Semi-pedunculated	6	2	6	5	6	5	6	6	0	1	2	1	0	0	7	1	0	3	5	4	3	1	2	4	0	2	1	4	3	3	0	2	5	5	4	1	0	0	0	4	7
Pedunculated	1	8	3	3	1	2	0	4	10	7	8	1	10	10	1	9	9	6	2	2	7	0	7	3	0	7	9	5	4	7	0	7	2	4	6	8	10	9	1	0	2

Figure 5.3: Overall results for each case in deciding the shape of the lesion

5.6.1.1.2 Grade of differentiation

The overall κ score for the reporting of the grade of differentiation (where the three possible categories were non-poorly, poorly differentiated and not assessable) was 0.13 (95% CI 0.09 – 0.18, $p < 0.001$). Figure 5.4 demonstrates the breakdown of the grade of differentiation for each case.

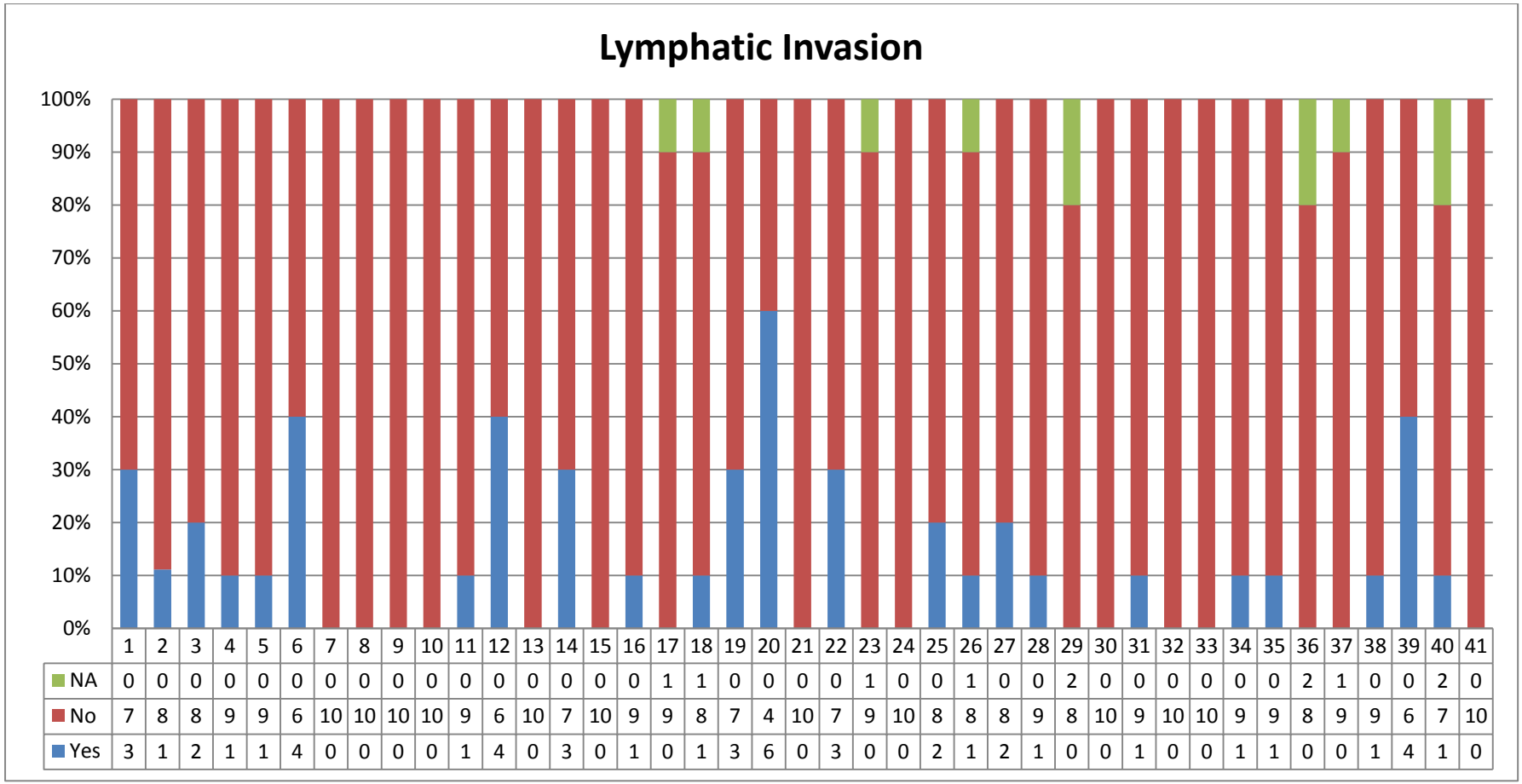


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
N/A	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	2	0	0	1	0	0	2	0	0	0	0	0	0	2	0	0	0	1	0
Poor	1	0	0	2	1	5	0	0	0	0	0	4	1	4	0	0	3	0	3	0	1	3	0	0	0	4	2	0	0	4	2	1	0	4	7	0	2	3	1	1	1
Non-Poor	9	10	10	8	9	5	10	10	9	10	10	6	9	6	10	10	6	9	7	10	9	7	8	10	10	5	8	10	8	6	8	9	10	6	3	8	8	7	9	8	9

Figure 5.4: Overall results for each case in deciding the grade of differentiation.

5.6.1.1.3 Lymphatic invasion

The overall κ score for reporting the presence of lymphatic invasion was 0.08 (95% CI 0.03 – 0.12, $p<0.001$). Figure 5.5 displays the breakdown of each case.

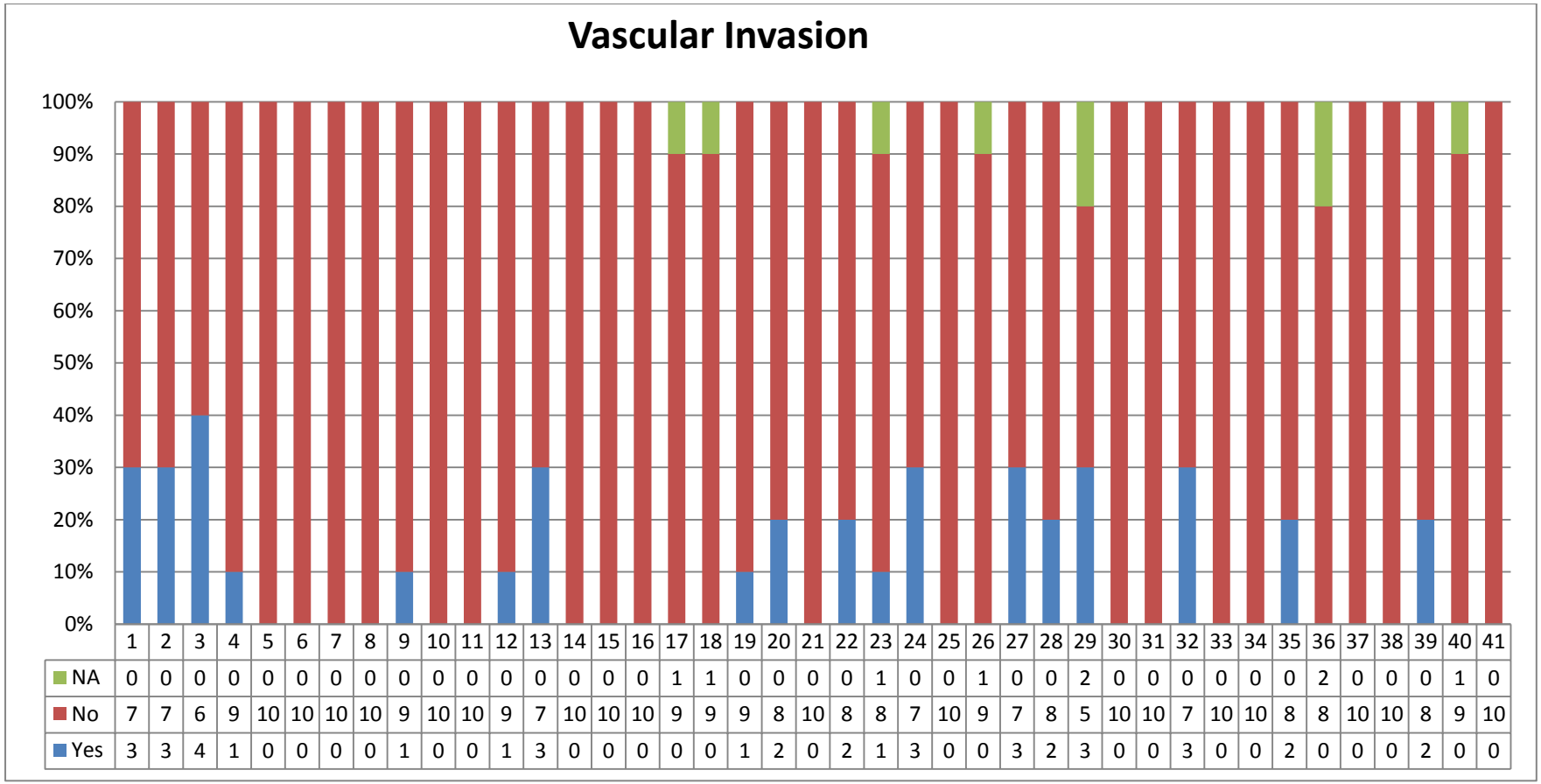


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
NA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	0	2	0	0	0	0	0	0	2	1	0	0	2	0
No	7	8	8	9	9	6	10	10	10	10	9	6	10	7	10	9	9	8	7	4	10	7	9	10	8	8	8	9	8	10	9	10	10	9	9	8	9	9	6	7	10
Yes	3	1	2	1	1	4	0	0	0	0	1	4	0	3	0	1	0	1	3	6	0	3	0	0	2	1	2	1	0	0	1	0	0	1	1	0	0	1	4	1	0

Figure 5.5: Overall results for each case in deciding the presence of lymphatic invasion

5.6.1.1.4 Vascular invasion

The overall κ score for reporting the presence of vascular invasion was 0.08 (95% CI 0.03 – 0.19, p<0.001). Figure 5.6 displays the breakdown of each case.

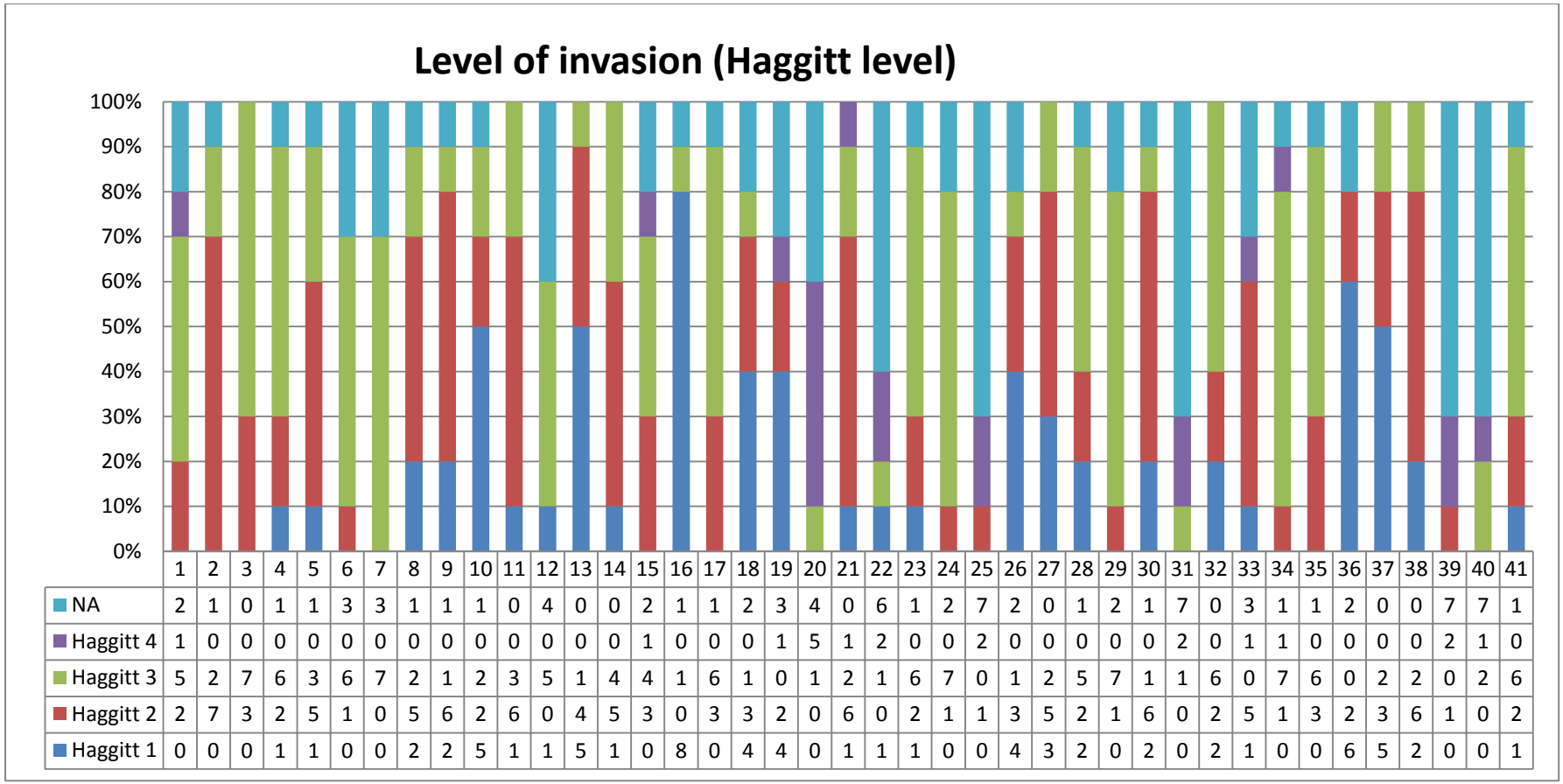


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
NA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	0	2	0	0	0	0	0	0	2	0	0	0	1	0
No	7	7	6	9	10	10	10	10	9	10	10	9	7	10	10	10	9	9	9	8	10	8	8	7	10	9	7	8	5	10	10	7	10	10	8	8	10	10	8	9	10
Yes	3	3	4	1	0	0	0	0	1	0	0	1	3	0	0	0	0	0	1	2	0	2	1	3	0	0	3	2	3	0	0	3	0	0	2	0	0	0	2	0	0

Figure 5.6: Overall results for each case in deciding the presence of vascular invasion.

5.6.1.1.5 Haggitt level

The overall κ score for the reporting of Haggitt level (where the five possible categories were Haggit level 1 to 4 and not assessable) was 0.17 (95% CI 0.15 – 0.19, $p < 0.001$). Figure 5.7 demonstrates the breakdown of the Haggitt level for each case.

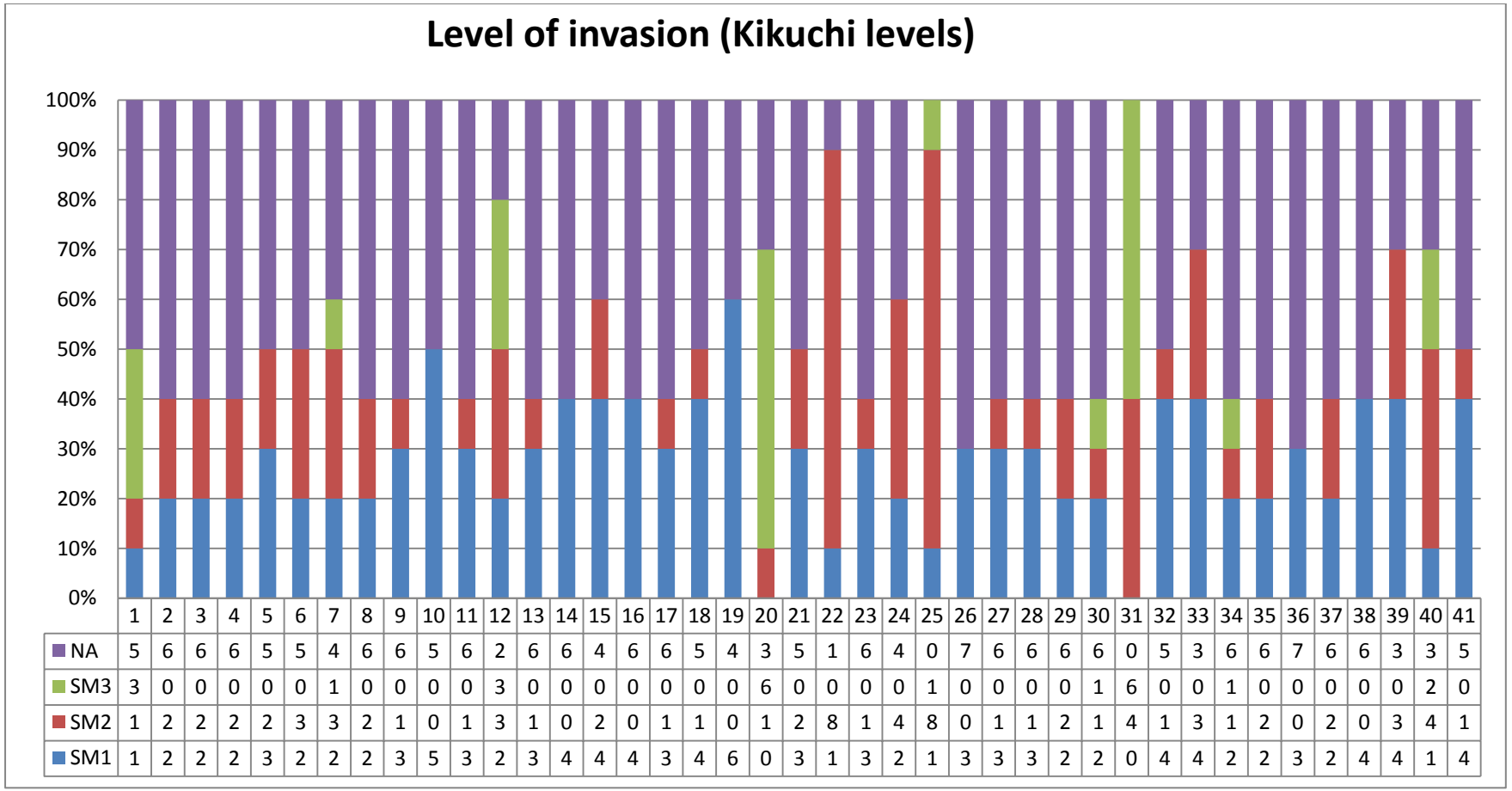


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
NA	2	1	0	1	1	3	3	1	1	1	0	4	0	0	2	1	1	2	3	4	0	6	1	2	7	2	0	1	2	1	7	0	3	1	1	2	0	0	7	7	1
Haggitt 4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	5	1	2	0	0	2	0	0	0	0	0	2	0	1	1	0	0	0	0	2	1	0
Haggitt 3	5	2	7	6	3	6	7	2	1	2	3	5	1	4	4	1	6	1	0	1	2	1	6	7	0	1	2	5	7	1	1	6	0	7	6	0	2	2	0	2	6
Haggitt 2	2	7	3	2	5	1	0	5	6	2	6	0	4	5	3	0	3	3	2	0	6	0	2	1	1	3	5	2	1	6	0	2	5	1	3	2	3	6	1	0	2
Haggitt 1	0	0	0	1	1	0	0	2	2	5	1	1	5	1	0	8	0	4	4	0	1	1	1	0	0	4	3	2	0	2	0	2	1	0	0	6	5	2	0	0	1

Figure 5.7: Overall results for each case in deciding the Haggitt level of invasion.

5.6.1.1.6 Kikuchi Level

The overall κ score for the reporting of Kikuchi level (where the four possible categories were Kikuchi level 1 to 3 and not assessable) was 0.05 (95% CI 0.03 – 0.08, $p < 0.001$). Figure 5.8 demonstrates the breakdown of the Kikuchi level for each case.

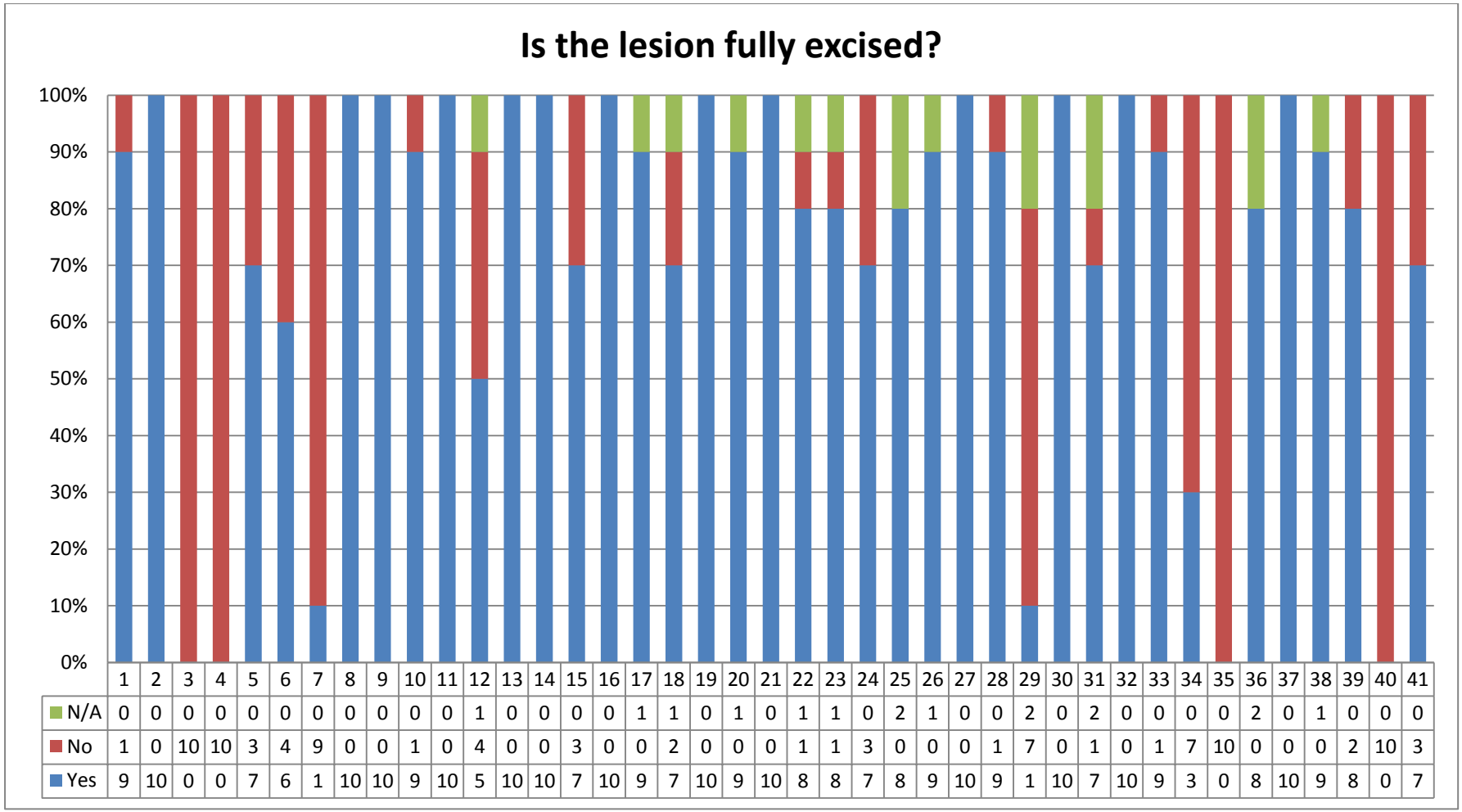


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
NA	5	6	6	6	5	5	4	6	6	5	6	2	6	6	4	6	6	5	4	3	5	1	6	4	0	7	6	6	6	6	0	5	3	6	6	7	6	6	3	3	5
SM3	3	0	0	0	0	0	1	0	0	0	0	3	0	0	0	0	0	0	0	6	0	0	0	0	1	0	0	0	0	1	6	0	0	1	0	0	0	0	0	2	0
SM2	1	2	2	2	2	3	3	2	1	0	1	3	1	0	2	0	1	1	0	1	2	8	1	4	8	0	1	1	2	1	4	1	3	1	2	0	2	0	3	4	1
SM1	1	2	2	2	3	2	2	2	3	5	3	2	3	4	4	4	3	4	6	0	3	1	3	2	1	3	3	3	2	2	0	4	4	2	2	3	2	4	4	1	4

Figure 5.8: Overall results for each case in deciding the Kikuchi level of invasion.

5.6.1.1.7 Is the lesion fully excised?

The overall κ score that was generated by the question posed (is the lesion fully excised?) was 0.49 (95% CI 0.44 – 0.53, $p < 0.001$). Figure 5.9 demonstrates the breakdown of each case in relation to the question posed.

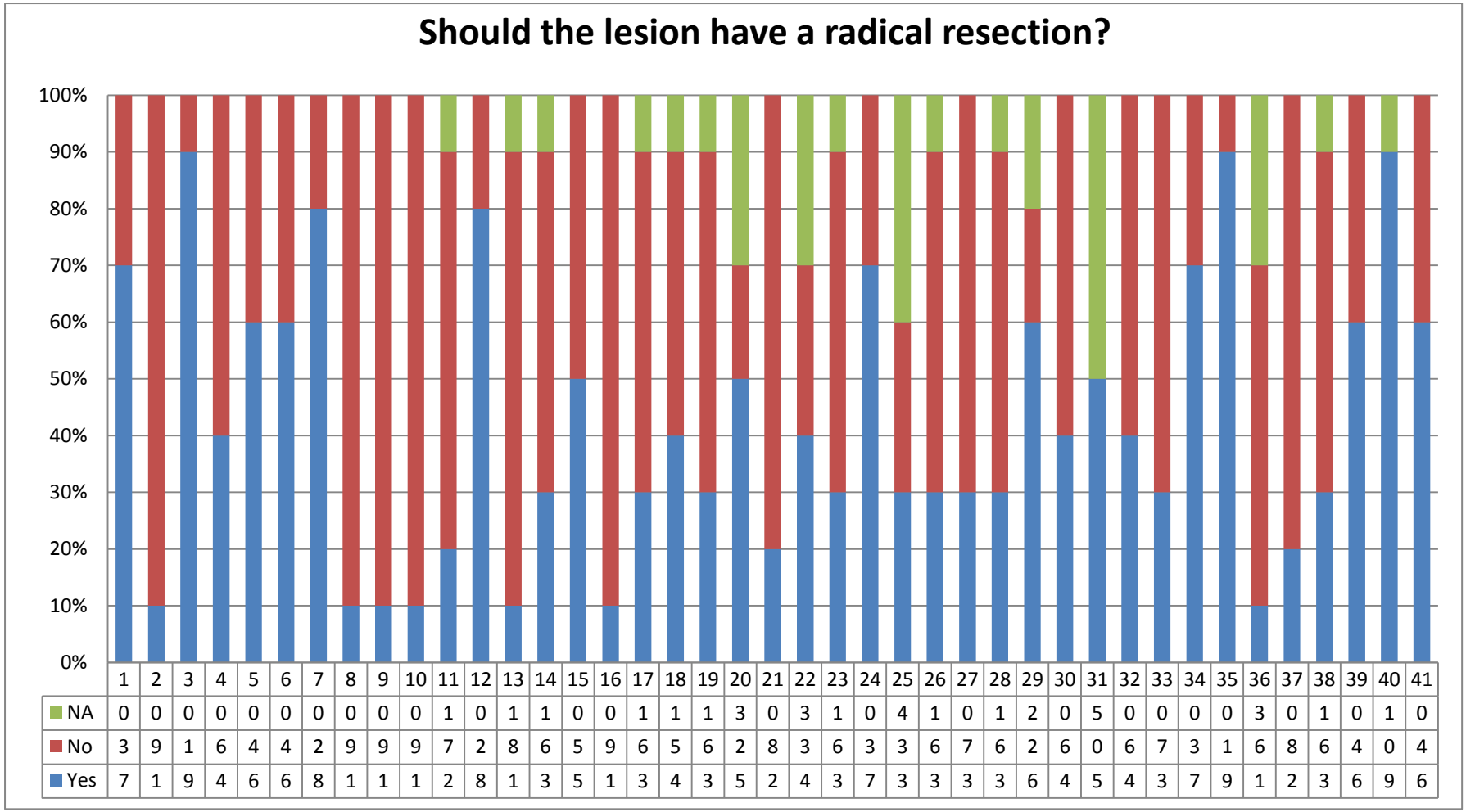


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
N/A	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	1	0	1	1	0	2	1	0	0	2	0	2	0	0	0	0	2	0	1	0	0	0
No	1	0	10	10	3	4	9	0	0	1	0	4	0	0	3	0	0	2	0	0	0	1	1	3	0	0	0	1	7	0	1	0	1	7	10	0	0	0	2	10	3
Yes	9	10	0	0	7	6	1	10	10	9	10	5	10	10	7	10	9	7	10	9	10	8	8	7	8	9	10	9	1	10	7	10	9	3	0	8	10	9	8	0	7

Figure 5.9: Overall results for each case in deciding whether the lesion is fully excised.

5.6.1.1.8 Should the lesion have a radical resection?

The overall κ score that was generated by the question posed (should the lesion have a radical resection?) was 0.18 (0.14 – 0.22, $p < 0.001$). Figure 5.10 demonstrates the breakdown of each case in relation to the question posed.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
NA	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	1	1	1	3	0	3	1	0	4	1	0	1	2	0	5	0	0	0	0	3	0	1	0	1	0
No	3	9	1	6	4	4	2	9	9	9	7	2	8	6	5	9	6	5	6	2	8	3	6	3	3	6	7	6	2	6	0	6	7	3	1	6	8	6	4	0	4
Yes	7	1	9	4	6	6	8	1	1	1	2	8	1	3	5	1	3	4	3	5	2	4	3	7	3	3	3	3	6	4	5	4	3	7	9	1	2	3	6	9	6

Figure 5.10: Overall results for each case in deciding whether the lesion should receive a radical resection.

5.6.1.2 Quantitative factors

The quantitative factors that were studied in round 1 of the inter-observer study were the width of lesion, distance of the lesion to the nearest margin and the depth of invasion that gave ICC values of 0.73, 0.58 and 0.67 respectively. Figures 5.11, 5.12 and 5.13 display the distribution of values for each case assessed by the pathologist.

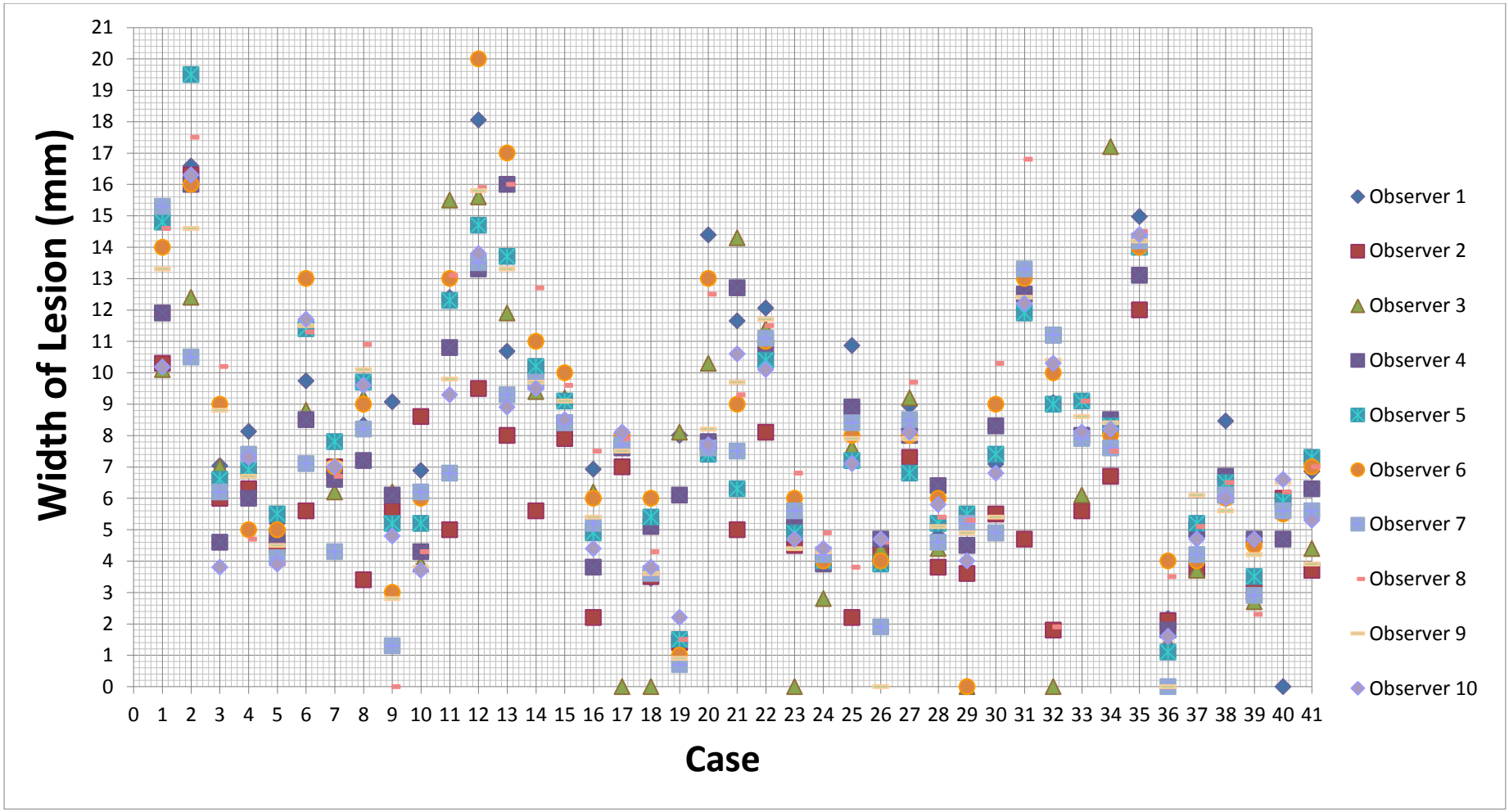


Figure 5.11: Scatter plot showing the overall results for the measurements of the width of the lesion by each pathologist.

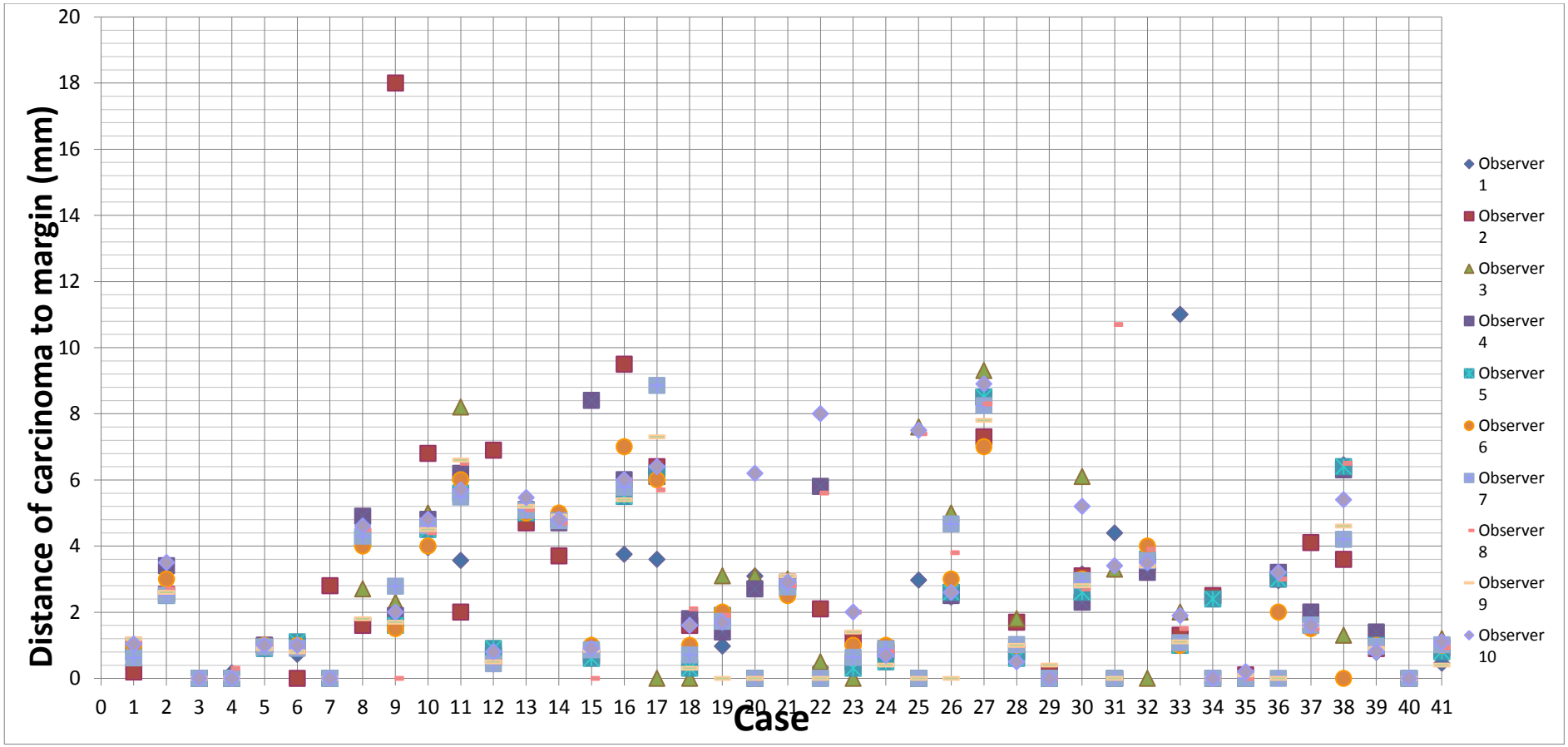


Figure 5.12: Scatter plot showing the overall results for the measurements of the distance of carcinoma to margin by each pathologist.

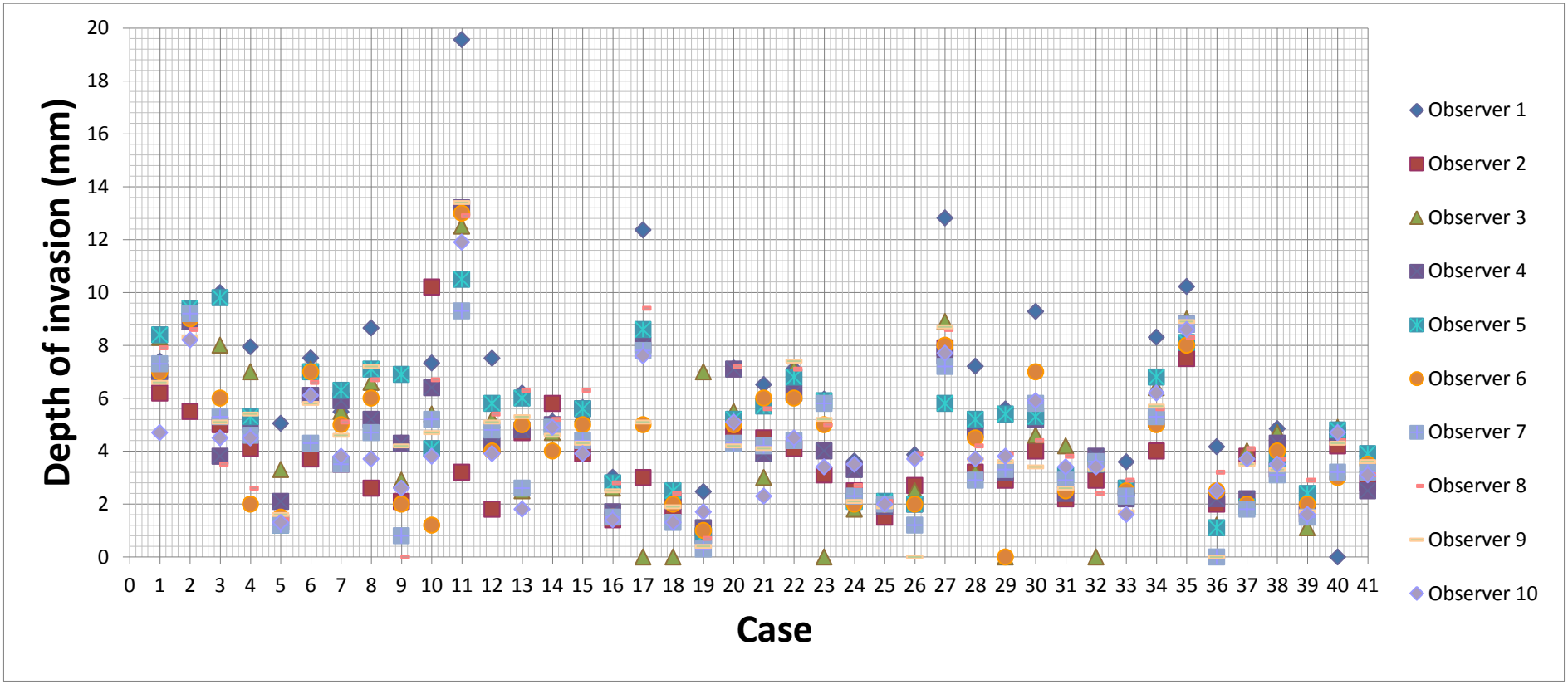


Figure 5.13: Scatter plot showing the overall results for the measurements of the depth of invasion by each pathologist.

Table 5.2 shows a summary of the agreement between the pathologist in assessing the qualitative and quantitative factors of the 41 pT1 CRCs for round 1

Qualitative / Quantitative Factors	Kappa (κ) value / Intra-class Correlation Coefficient (ICC) value (95% confidence interval, p value)	Interpretation of Kappa/Intra-class correlation value
Shape of lesion	κ = 0.33 (0.30 – 0.36, p < 0.01)	Fair agreement
Grade of differentiation	κ = 0.13 (0.09 – 0.18, p < 0.001)	Slight agreement
Lymphatic invasion	κ = 0.08 (0.03 – 0.12, p < 0.001)	Slight agreement
Vascular invasion	κ = 0.08 (0.03 – 0.12, p < 0.001)	Slight agreement
Haggitt level	κ = 0.17 (0.15 – 0.19, p< 0.001)	Fair agreement
Kikuchi level	κ = 0.05 (0.03 – 0.08, p < 0.001)	Slight agreement
Is the lesion fully excised?	κ = 0.49 (0.44 – 0.53, P < 0.001)	Moderate agreement
Should the lesion be fully resected?	κ = 0.18 (0.14 – 0.22, p < 0.001)	Slight agreement
Width of lesion	ICC = 0.73 (0.63 – 0.82, p < 0.001)	Substantial agreement
Distance to margin	ICC = 0.58 (0.45 – 0.69, p < 0.001)	Moderate agreement
Depth of invasion	ICC = 0.67 (0.55 – 0.78, p < 0.001)	Substantial agreement

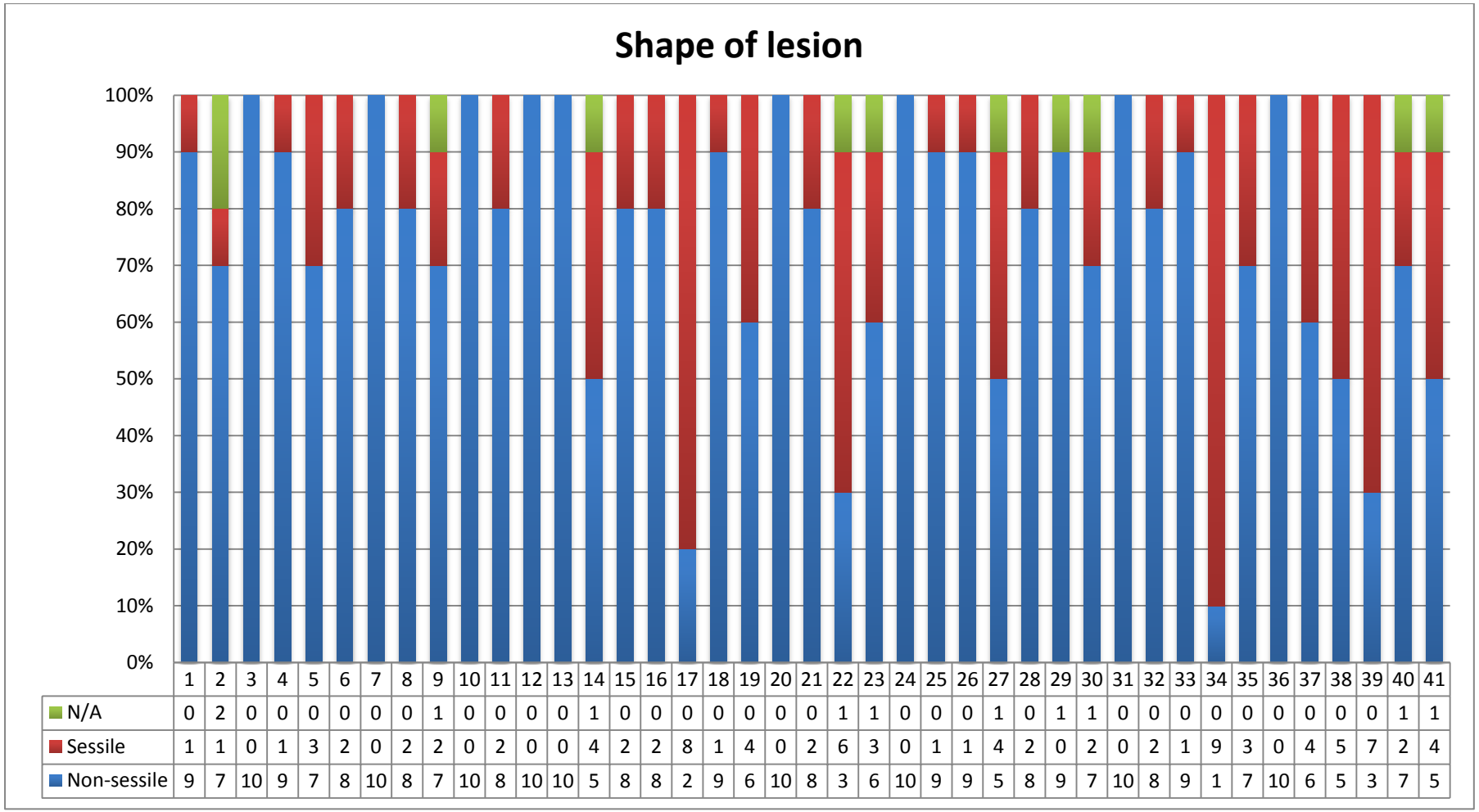
Table 5.2: Summary of the qualitative and quantitative factors that were analysed in round 1 of the inter-observer study.

5.6.2 Inter-observer variation results round 2

5.6.2.1 Qualitative factors

5.6.2.1.1 Shape of lesion

The overall κ score for the reporting of the shape of the lesion in round 2 (where there were three possible categories [sessile, non-sessile and not assessable] compared to four possible categories [sessile, semi-pedunculated, pedunculated and not assessable] in round 1) was 0.21 (95% CI 0.17 – 0.25, $p < 0.001$). Figure 5.14 demonstrates the breakdown of the shape of the lesion for each case in round 2.

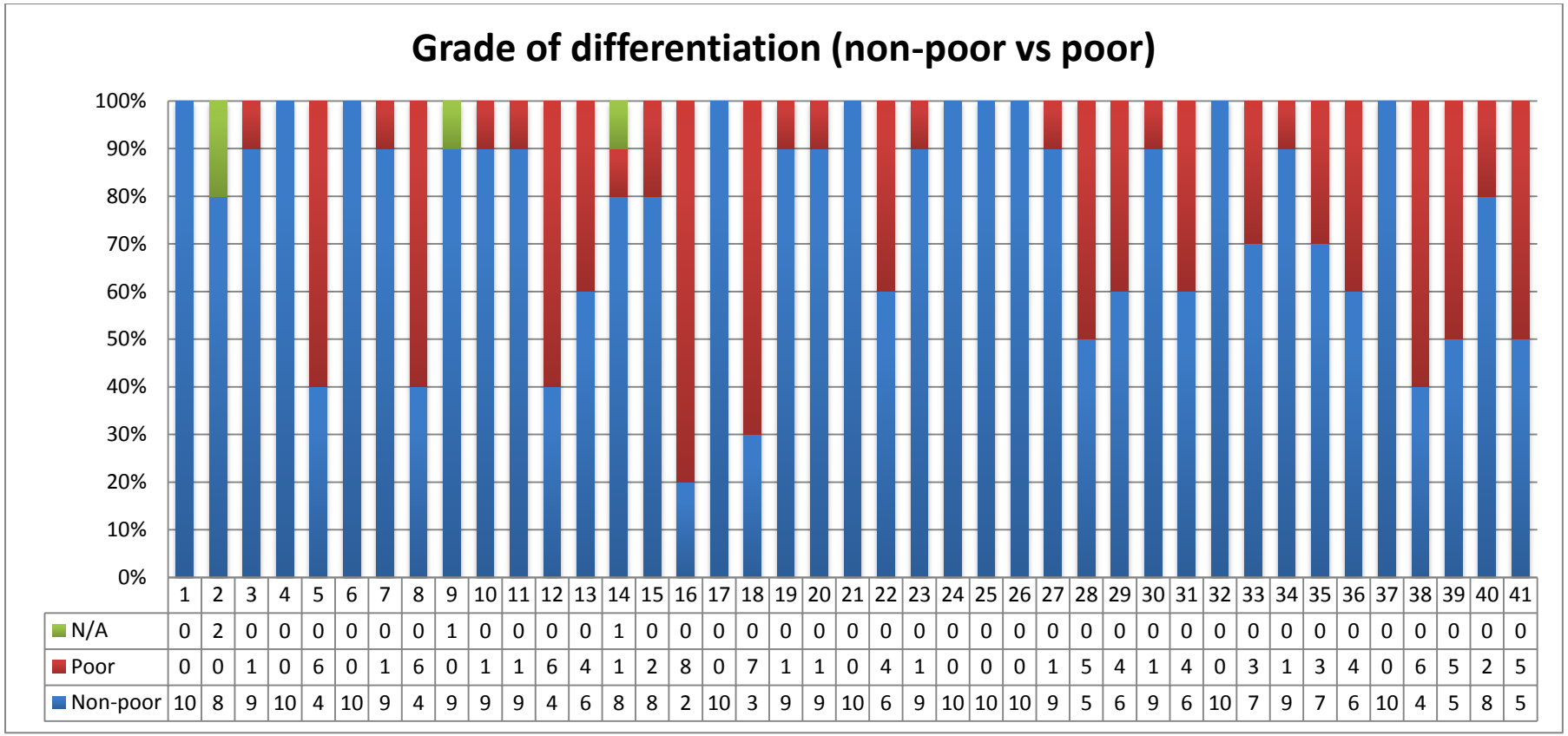


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
N/A	0	2	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	1	1
Sessile	1	1	0	1	3	2	0	2	2	0	2	0	0	4	2	2	8	1	4	0	2	6	3	0	1	1	4	2	0	2	0	2	1	9	3	0	4	5	7	2	4
Non-sessile	9	7	10	9	7	8	10	8	7	10	8	10	10	5	8	8	2	9	6	10	8	3	6	10	9	9	5	8	9	7	10	8	9	1	7	10	6	5	3	7	5

Figure 5.14: Overall results for each case in deciding the shape of the lesion in round 2.

5.6.2.1.2 Grade of differentiation

In round 2, the pathologist had to grade the pT1 CRC into 3 categories (non-poor, poor and not assessable). The overall κ score for the reporting of the grade of differentiation was 0.19 (95% CI 0.15 – 0.24, $p < 0.001$). Figure 5.15 demonstrates the breakdown of the grade of differentiation for each case.

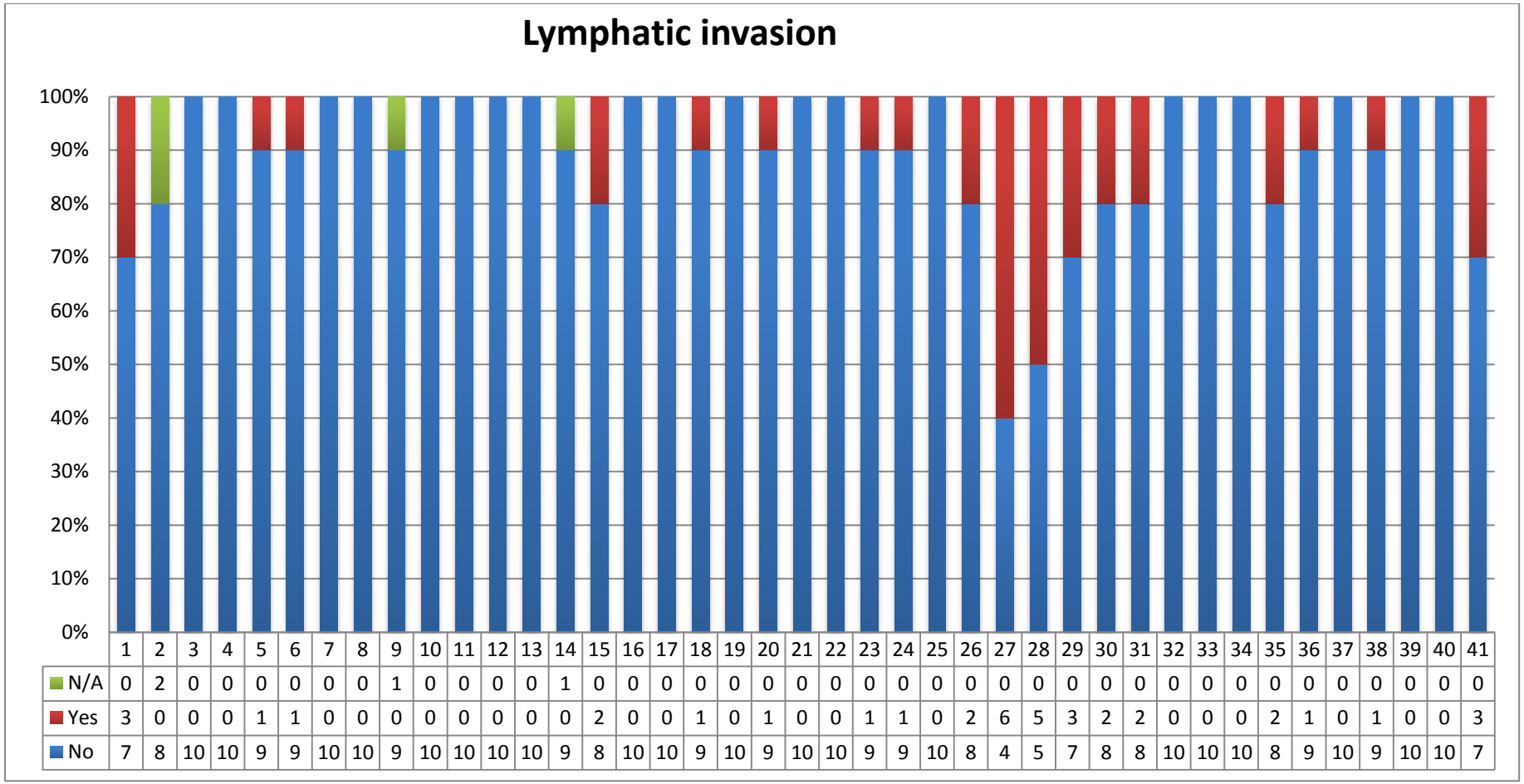


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
N/A	0	2	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Poor	0	0	1	0	6	0	1	6	0	1	1	6	4	1	2	8	0	7	1	1	0	4	1	0	0	0	1	5	4	1	4	0	3	1	3	4	0	6	5	2	5
Non-poor	10	8	9	10	4	10	9	4	9	9	9	4	6	8	8	2	10	3	9	9	10	6	9	10	10	10	9	5	6	9	6	10	7	9	7	6	10	4	5	8	5

Figure 5.15: Overall results for each case in deciding whether the grade of differentiation of the pT1 CRC in round 2.

5.6.2.1.3 Lymphatic invasion

The overall κ score for reporting the presence of lymphatic invasion in round 2 was 0.12 (95% CI 0.08 – 0.17, $p<0.001$). Figure 5.16 displays the breakdown of each case.

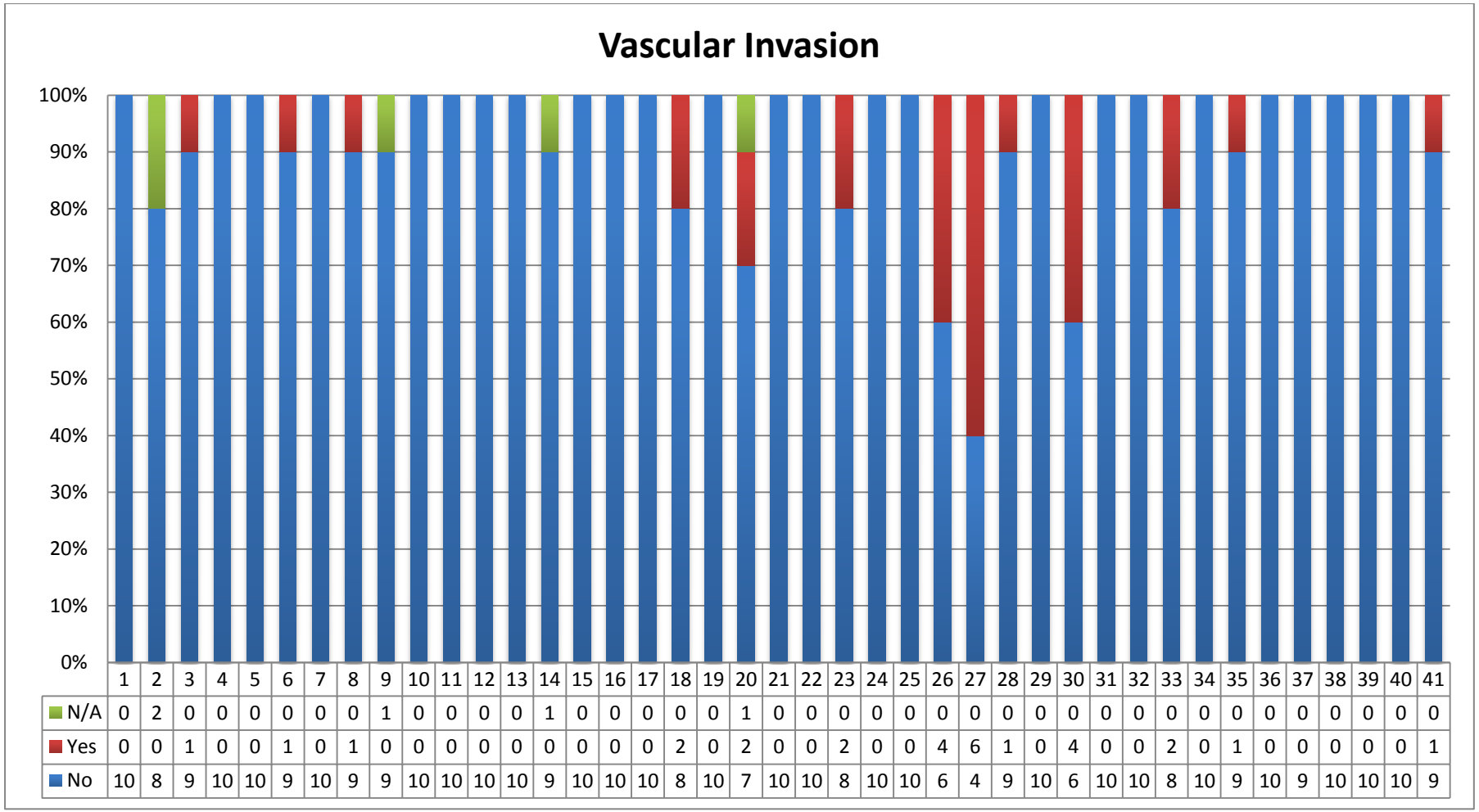


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
N/A	0	2	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Yes	3	0	0	0	1	1	0	0	0	0	0	0	0	0	2	0	0	1	0	1	0	0	1	1	0	2	6	5	3	2	2	0	0	0	2	1	0	1	0	0	3
No	7	8	10	10	9	9	10	10	9	10	10	10	10	9	8	10	10	9	10	9	10	10	9	9	10	8	4	5	7	8	8	10	10	10	8	9	10	9	10	10	7

Figure 5.16: Overall results for each case in deciding the presence of lymphatic invasion in round 2.

5.6.2.1.4 Vascular invasion

The overall κ score for reporting the presence of vascular invasion in round 2 was 0.19 (95% CI 0.14 – 0.22, $p<0.001$). Figure 5.17 displays the breakdown of each case.

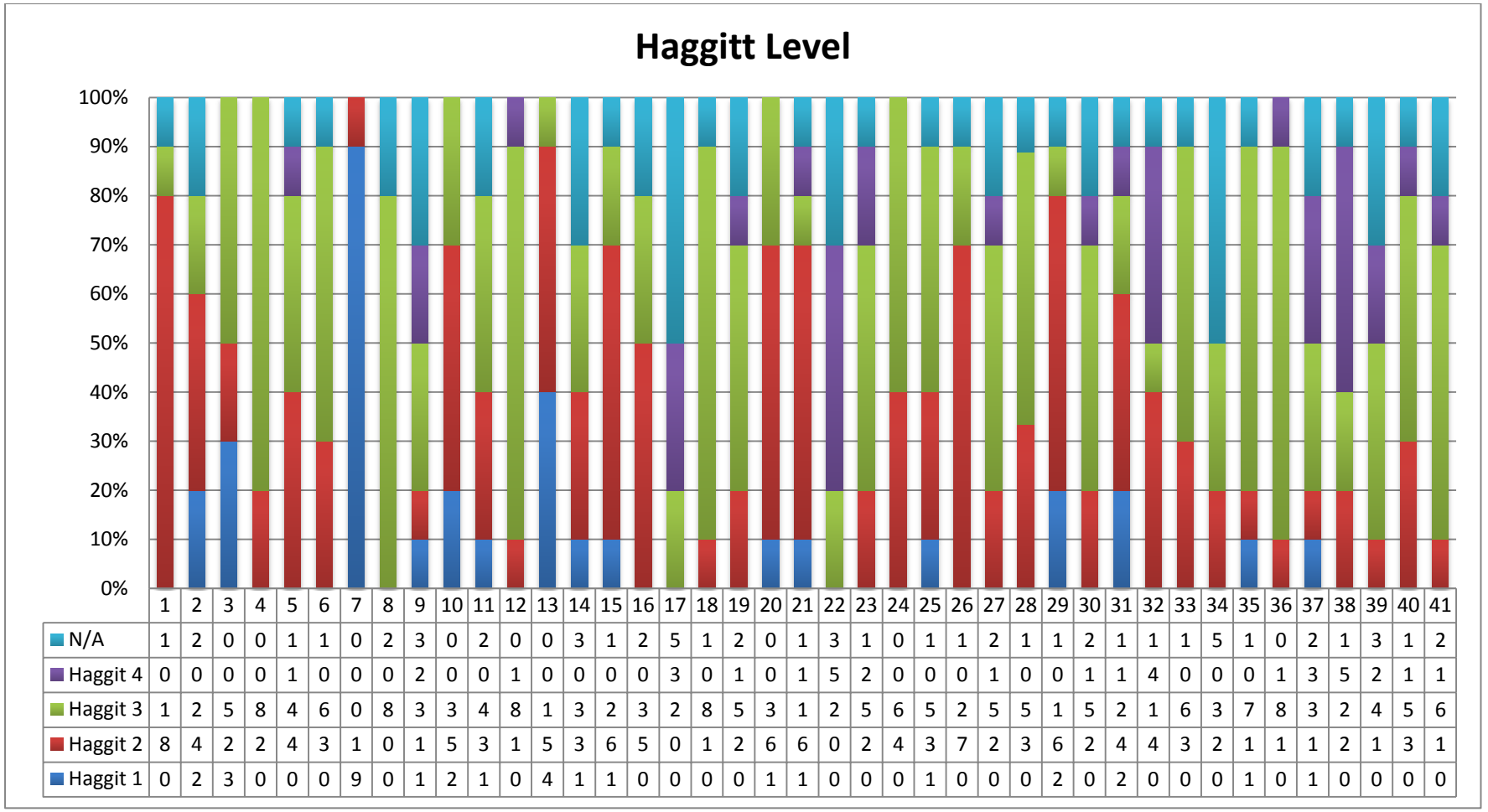


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
N/A	0	2	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Yes	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	2	0	2	0	0	2	0	0	4	6	1	0	4	0	0	2	0	1	0	0	0	0	0	1
No	10	8	9	10	10	9	10	9	9	10	10	10	10	9	10	10	10	8	10	7	10	10	8	10	10	6	4	9	10	6	10	10	8	10	9	10	9	10	10	10	9

Figure 5.17: Overall results for each case in deciding the presence of vascular invasion in round 2.

5.6.2.1.5 Haggitt level

The overall κ score for the reporting of Haggitt level in round 2 (where the five categories were similar in round 1) was 0.15 (95% CI 0.13 – 0.17, $p < 0.001$). Figure 5.18 demonstrates the breakdown of the Haggitt level for each case.

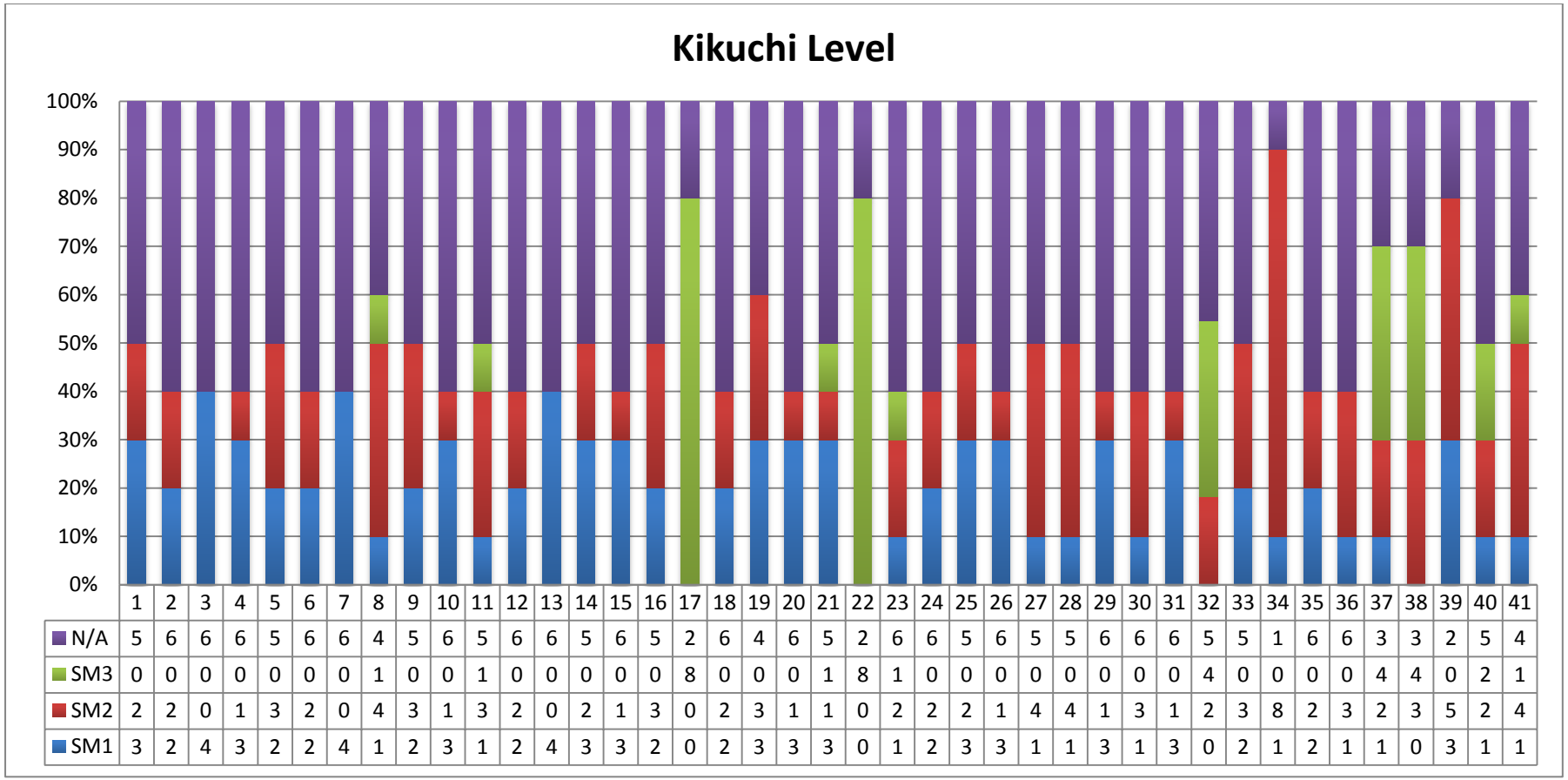


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
N/A	1	2	0	0	1	1	0	2	3	0	2	0	0	3	1	2	5	1	2	0	1	3	1	0	1	1	2	1	1	2	1	1	1	5	1	0	2	1	3	1	2
Haggit 4	0	0	0	0	1	0	0	0	2	0	0	1	0	0	0	0	3	0	1	0	1	5	2	0	0	0	1	0	0	1	1	4	0	0	0	1	3	5	2	1	1
Haggit 3	1	2	5	8	4	6	0	8	3	3	4	8	1	3	2	3	2	8	5	3	1	2	5	6	5	2	5	5	1	5	2	1	6	3	7	8	3	2	4	5	6
Haggit 2	8	4	2	2	4	3	1	0	1	5	3	1	5	3	6	5	0	1	2	6	6	0	2	4	3	7	2	3	6	2	4	4	3	2	1	1	1	2	1	3	1
Haggit 1	0	2	3	0	0	0	9	0	1	2	1	0	4	1	1	0	0	0	0	1	1	0	0	0	1	0	0	0	2	0	2	0	0	0	1	0	1	0	0	0	0

Figure 5.18: Overall results for each case in deciding the Haggitt level of invasion in round 2.

5.6.2.1.6 Kikuchi level

The overall κ score for the reporting of Kikuchi level in round 2 (where the four categories were similar in round 1) was 0.07 (95% CI 0.04 – 0.10, $p < 0.001$). Figure 5.19 demonstrates the breakdown of the Kikuchi level for each case.

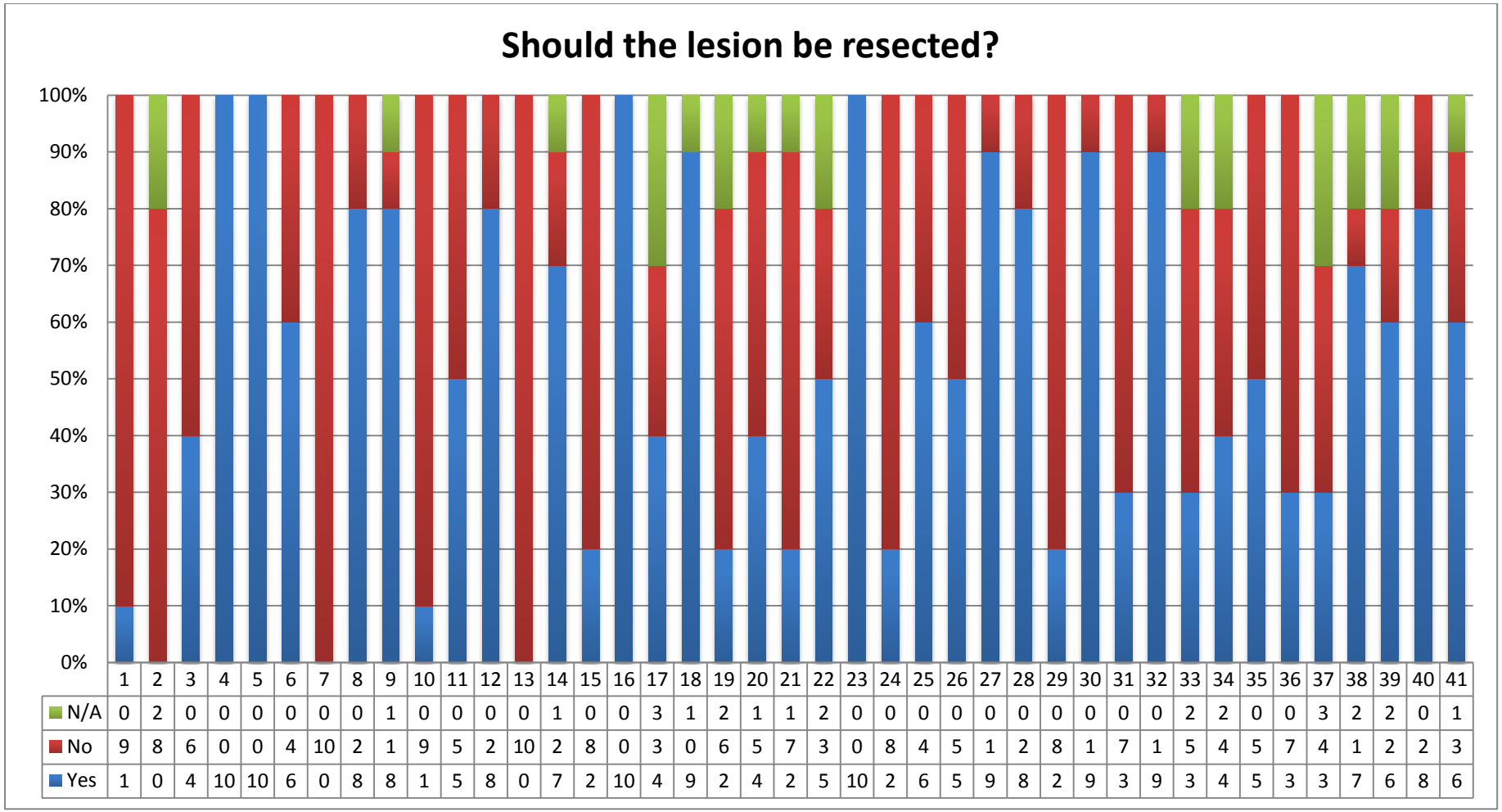


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
N/A	5	6	6	6	5	6	6	4	5	6	5	6	6	5	6	5	2	6	4	6	5	2	6	6	5	6	5	5	6	6	6	5	5	1	6	6	3	3	2	5	4
SM3	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	8	0	0	0	1	8	1	0	0	0	0	0	0	0	0	4	0	0	0	0	4	4	0	2	1
SM2	2	2	0	1	3	2	0	4	3	1	3	2	0	2	1	3	0	2	3	1	1	0	2	2	2	1	4	4	1	3	1	2	3	8	2	3	2	3	5	2	4
SM1	3	2	4	3	2	2	4	1	2	3	1	2	4	3	3	2	0	2	3	3	3	0	1	2	3	3	1	1	3	1	3	0	2	1	2	1	1	0	3	1	1

Figure 5.19: Overall results for each case in deciding the Kikuchi level of invasion in round 2.

5.6.2.1.7 Should the lesion have a radical resection?

In round 2, the overall κ score that was generated by the question posed (should the lesion have a radical resection?) was 0.32 (95% CI 0.28 – 0.36, $p < 0.001$). Figure 5.20 demonstrates the breakdown of each case in relation to the question posed.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
N/A	0	2	0	0	0	0	0	0	1	0	0	0	0	1	0	0	3	1	2	1	1	2	0	0	0	0	0	0	0	0	0	0	2	2	0	0	3	2	2	0	1
No	9	8	6	0	0	4	10	2	1	9	5	2	10	2	8	0	3	0	6	5	7	3	0	8	4	5	1	2	8	1	7	1	5	4	5	7	4	1	2	2	3
Yes	1	0	4	10	10	6	0	8	8	1	5	8	0	7	2	10	4	9	2	4	2	5	10	2	6	5	9	8	2	9	3	9	3	4	5	3	3	7	6	8	6

Figure 5.20: Overall results in round 2 for each case in deciding whether the lesion should receive a radical resection.

5.6.2.2 Quantitative factors

5.6.2.2.1 Width of lesion/ carcinoma

The ICC score for width of lesion in round 2 was 0.95 (95% CI 0.92 – 0.96, p < 0.001) and the ICC score for width of carcinoma was 0.01 (95% CI -0.03 – 0.08, p< 0.001). Figures 5.21 and 5.22 demonstrate the measurements of the individual cases by the pathologists.

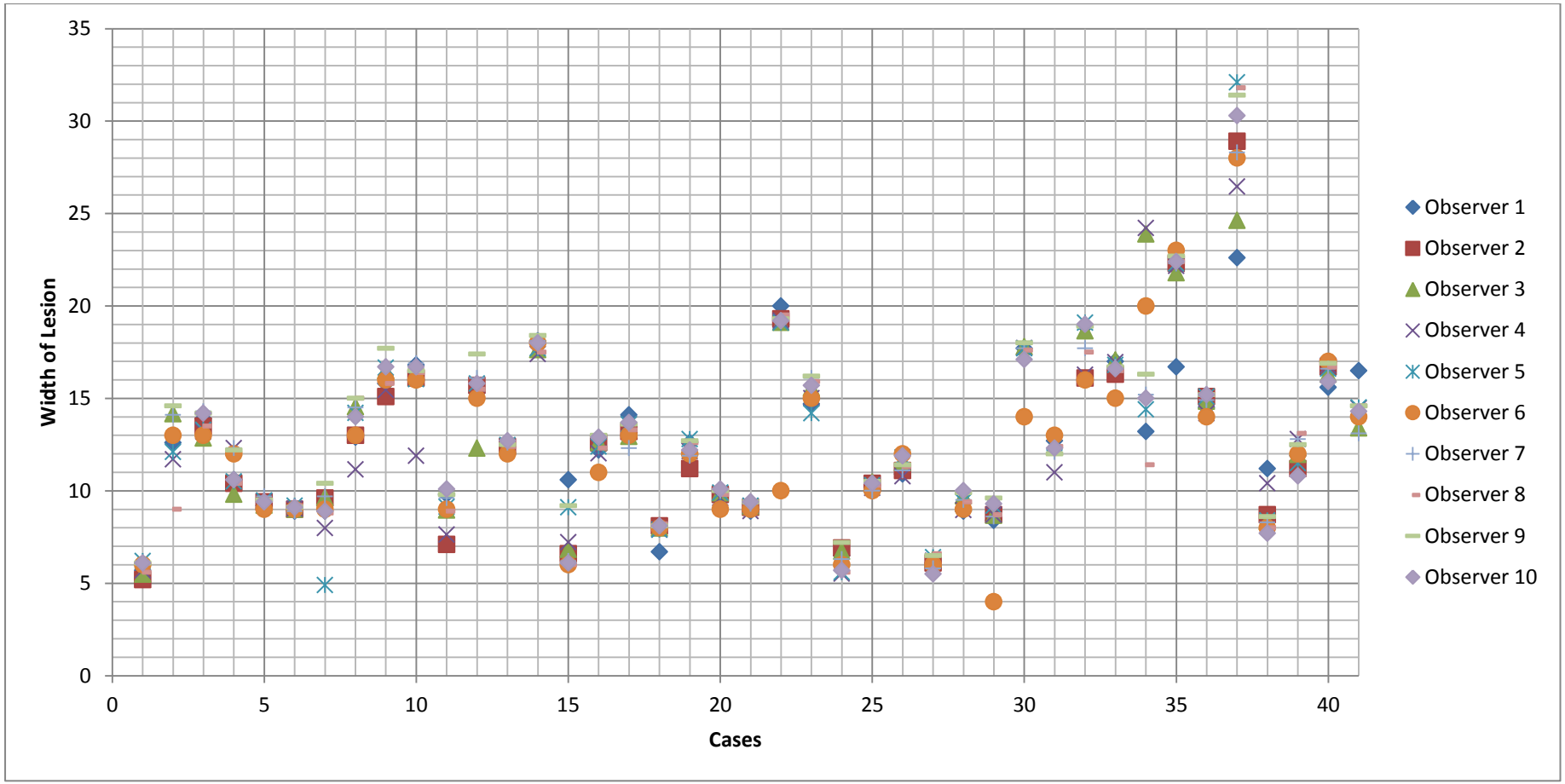


Figure 5.21: Overall results for the measurements of the width of the lesion in round 2.

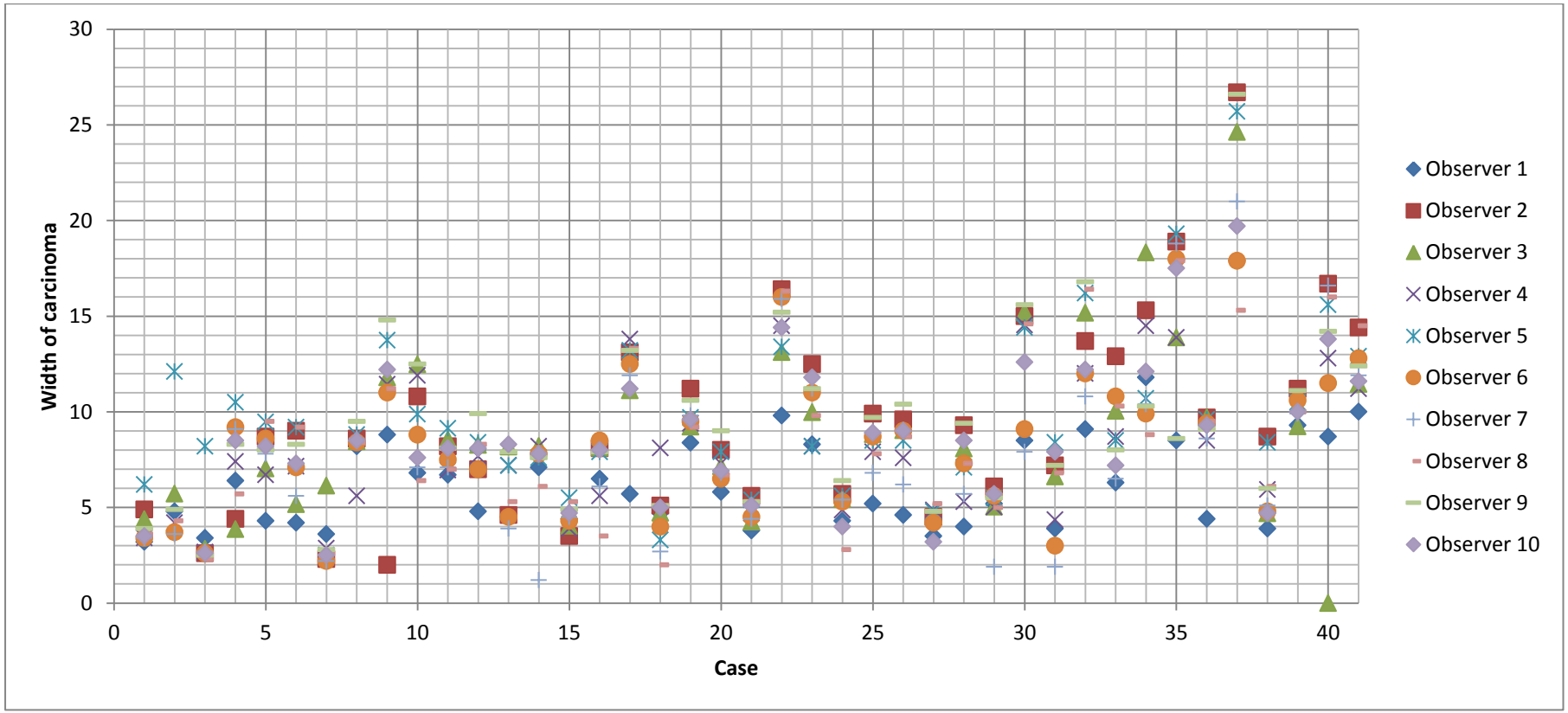


Figure 5.22: Overall results for the measurements of the width of the carcinoma in round 2.

5.6.2.2.2 Distance to margin

In round 2, we had asked the pathologist to measure the distance of the invasive front of the cancer to the nearest margin twice. First, using their own method and secondly, measuring the distance to the margin based on recommendations generated from the first round of the inter-observer study. The ICC score for the distance to margin using the pathologist's own method was 0.79 (95% CI 0.69 – 0.88, P < 0.001) and the ICC score for the distance to margin based on the recommendations was 0.88 (95% CI 0.81 – 0.93, P < 0.001). Figures 5.23 and 5.24 demonstrate the measurements of the individual cases by the pathologists.

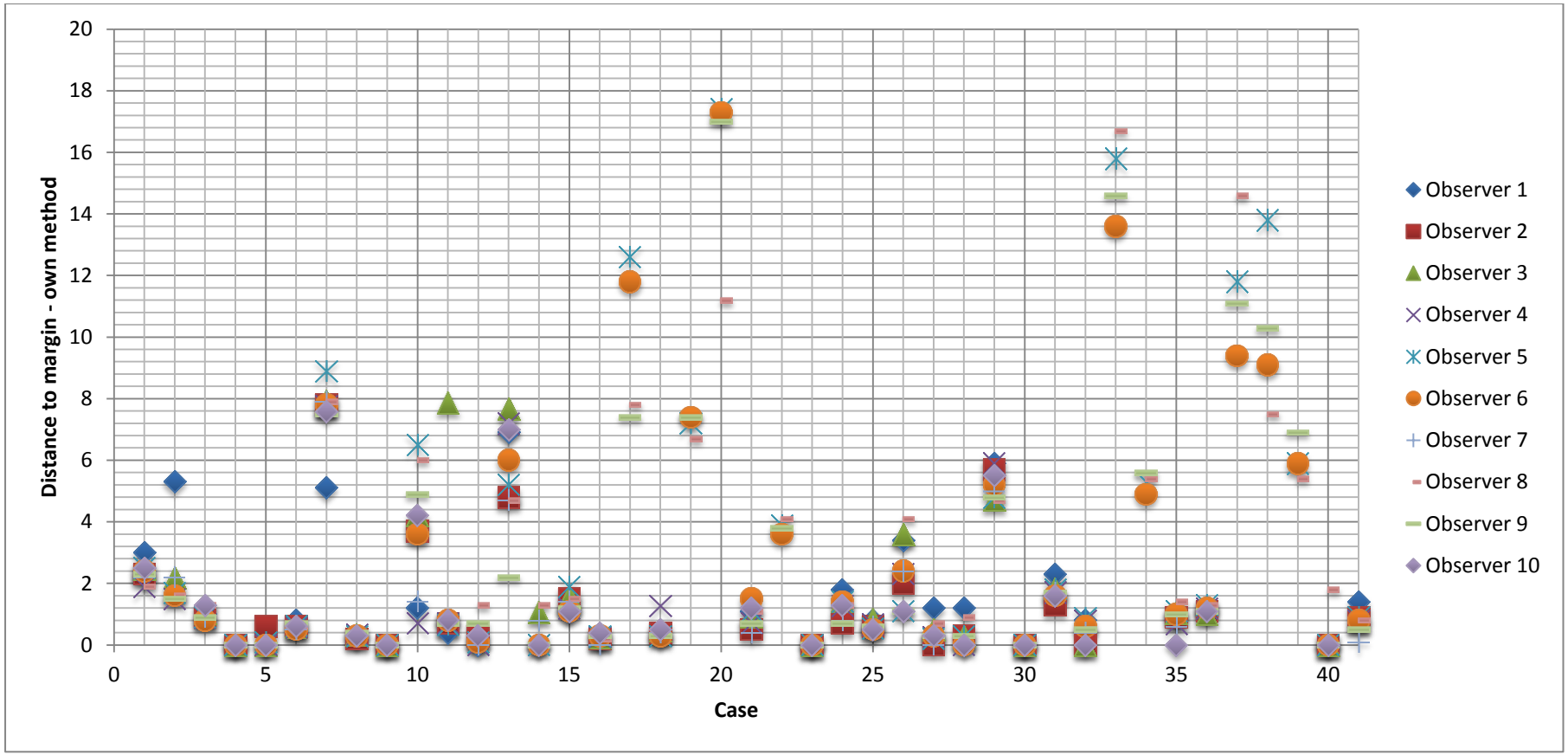


Figure 5.23: Overall results for the measurements of the distance to margin based on the pathologist own method in round 2.

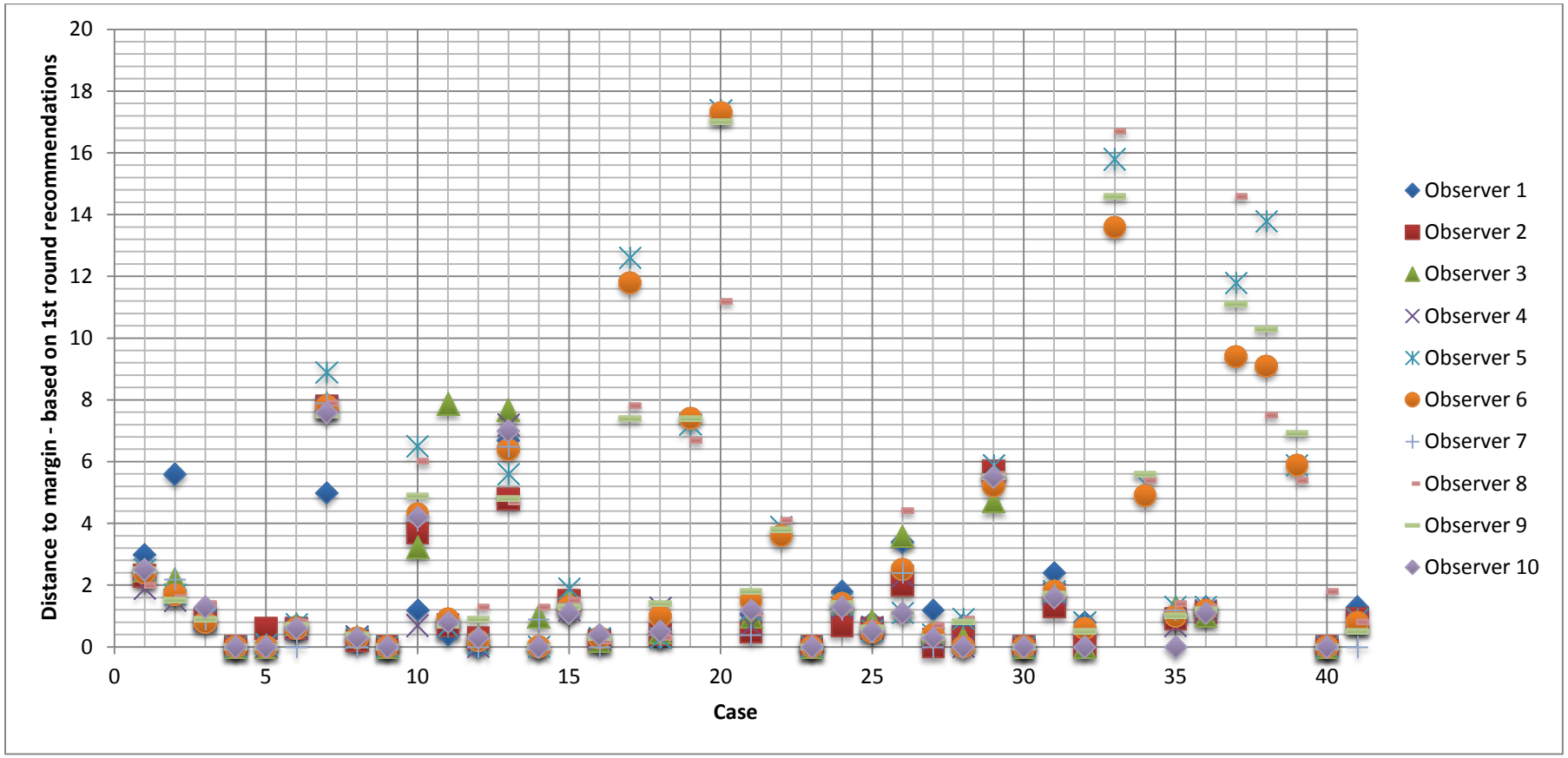


Figure 5.24: Overall results for the measurements of the distance to margin based on round 1 recommendation.

5.6.2.2.3 Depth of invasion

In round 2 of this study, we had asked the pathologist to measure depth of invasion based on 4 methods: epithelial surface to deepest tumour cell, virtual muscularis mucosae curved line method to deepest tumour cell, virtual muscularis mucosae straight line method to deepest tumour cell and from the muscularis mucosae that is visible to the deepest tumour cell.

The ICC scores for depth of invasion from the epithelial surface, virtual muscularis mucosae curved line method and virtual muscularis mucosae straight line method was 0.86 (95% CI 0.79 – 0.91, $p < 0.001$), 0.78 (95% CI 0.67 – 0.87, $p < 0.001$) and 0.40 (95% CI 0.25 – 0.62, $p < 0.01$) respectively. Figures 5.25, 5.26 and 5.27 represents the measurements of the different methods of depth of invasion for each case.

The ICC score for depth of invasion from the muscularis mucosae that is visible to the deepest tumour cell was 0.29 (95% CI 0.04 – 0.95, $p < 0.001$). However, it should be noted in the majority of cases, the pathologists were unable to identify the muscularis mucosae. Figures 5.28 demonstrate the measurements of the depth of invasion from the muscularis mucosae.

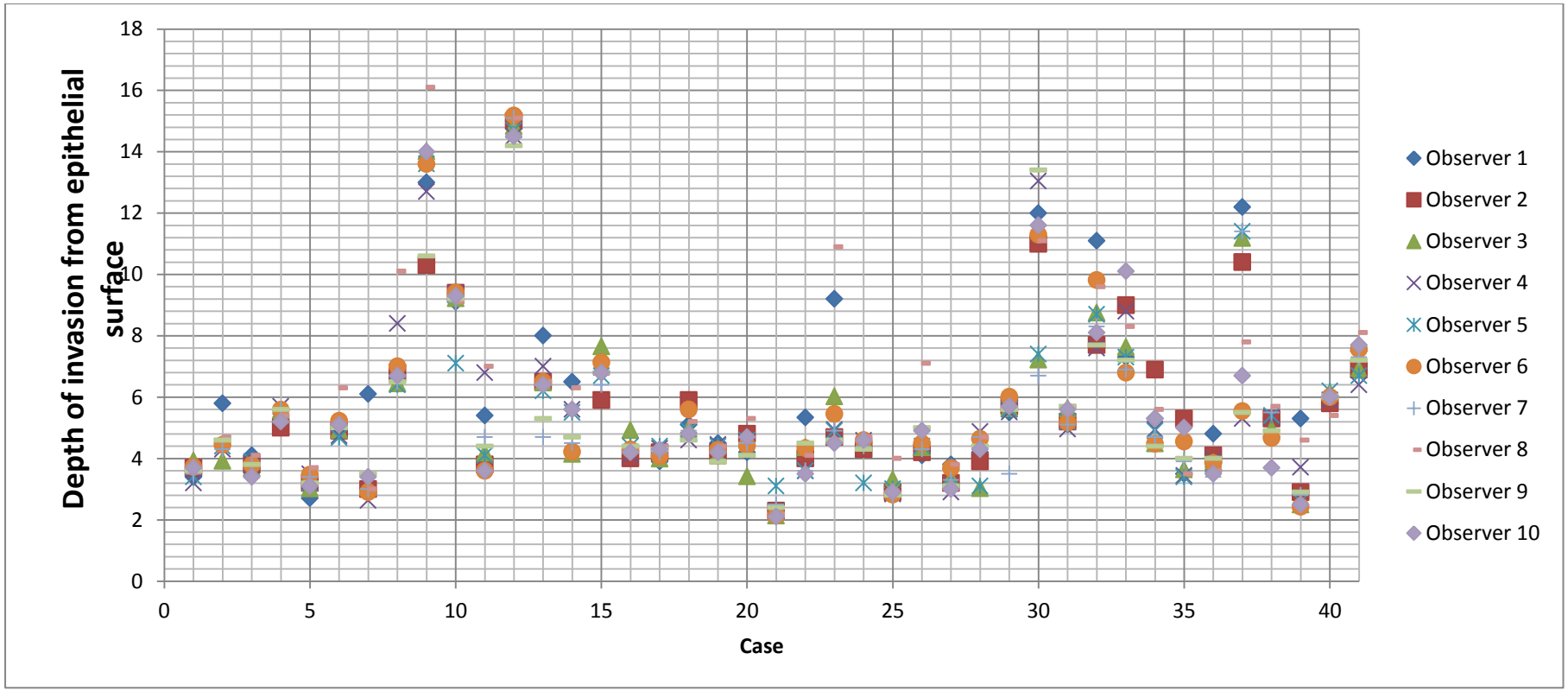


Figure 5.25: Overall results for the measurements of the depth of invasion from the epithelial surface in round 2.

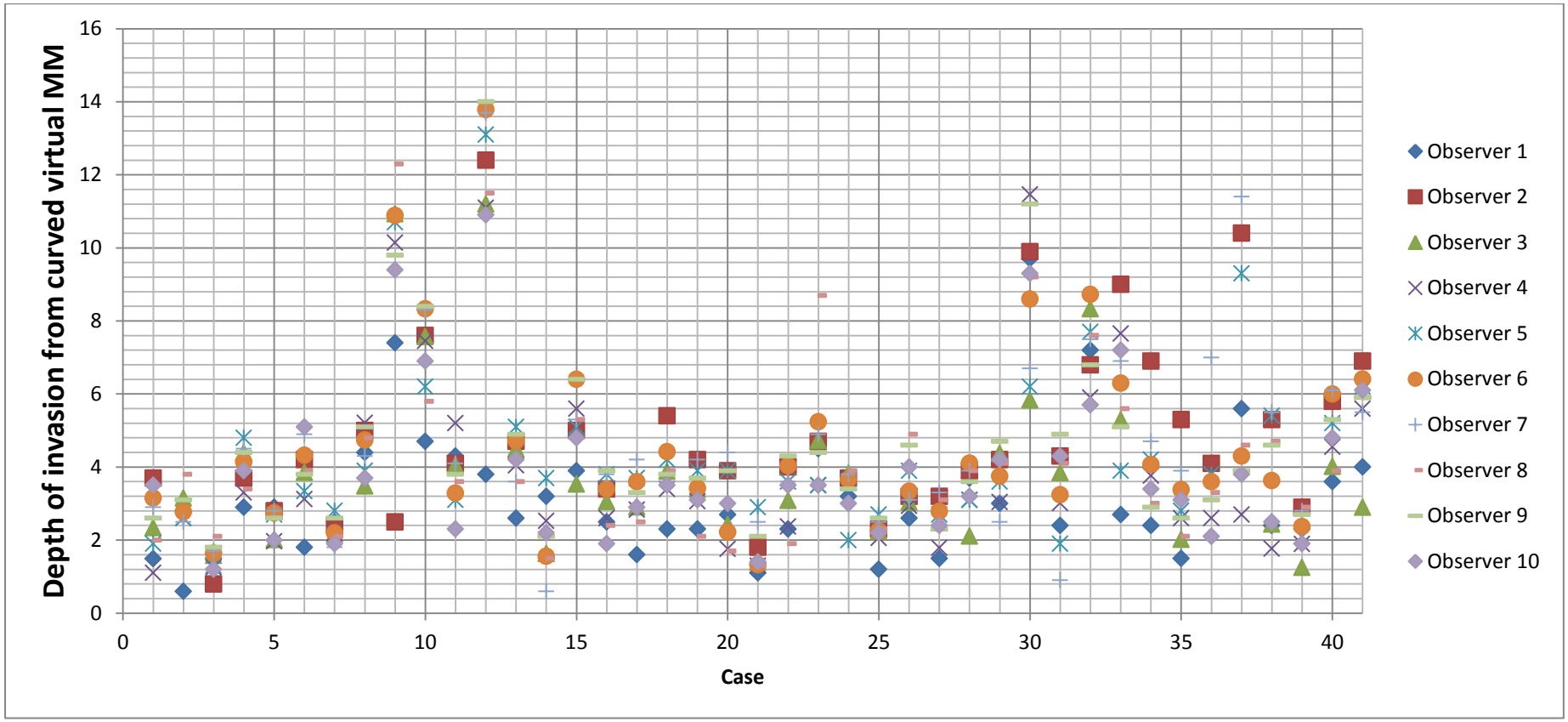


Figure 5.26: Overall results for the measurements of the depth of invasion from curved virtual muscularis mucosae in round 2.

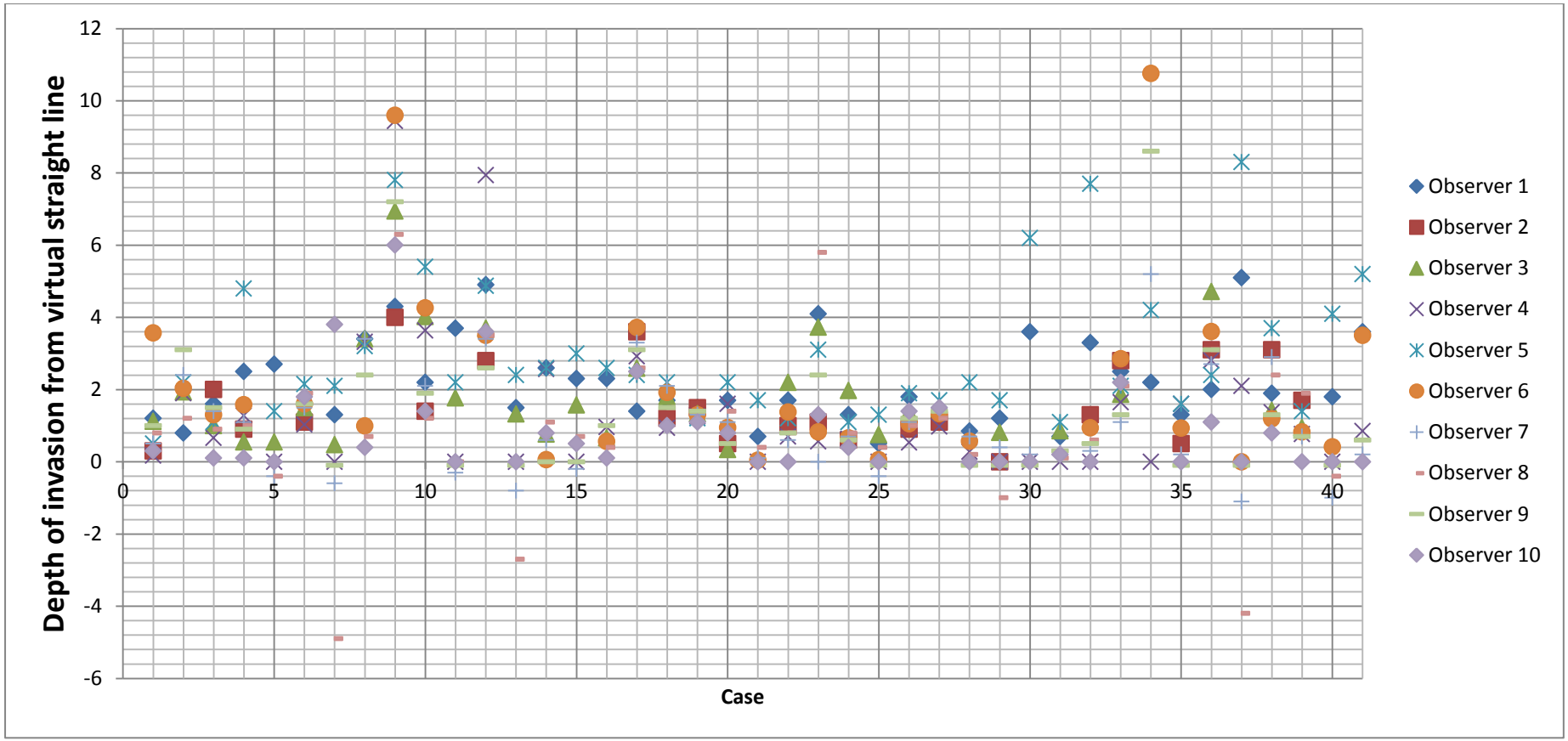


Figure 5.27: Overall results for the measurements of the depth of invasion from virtual straight line in round 2.

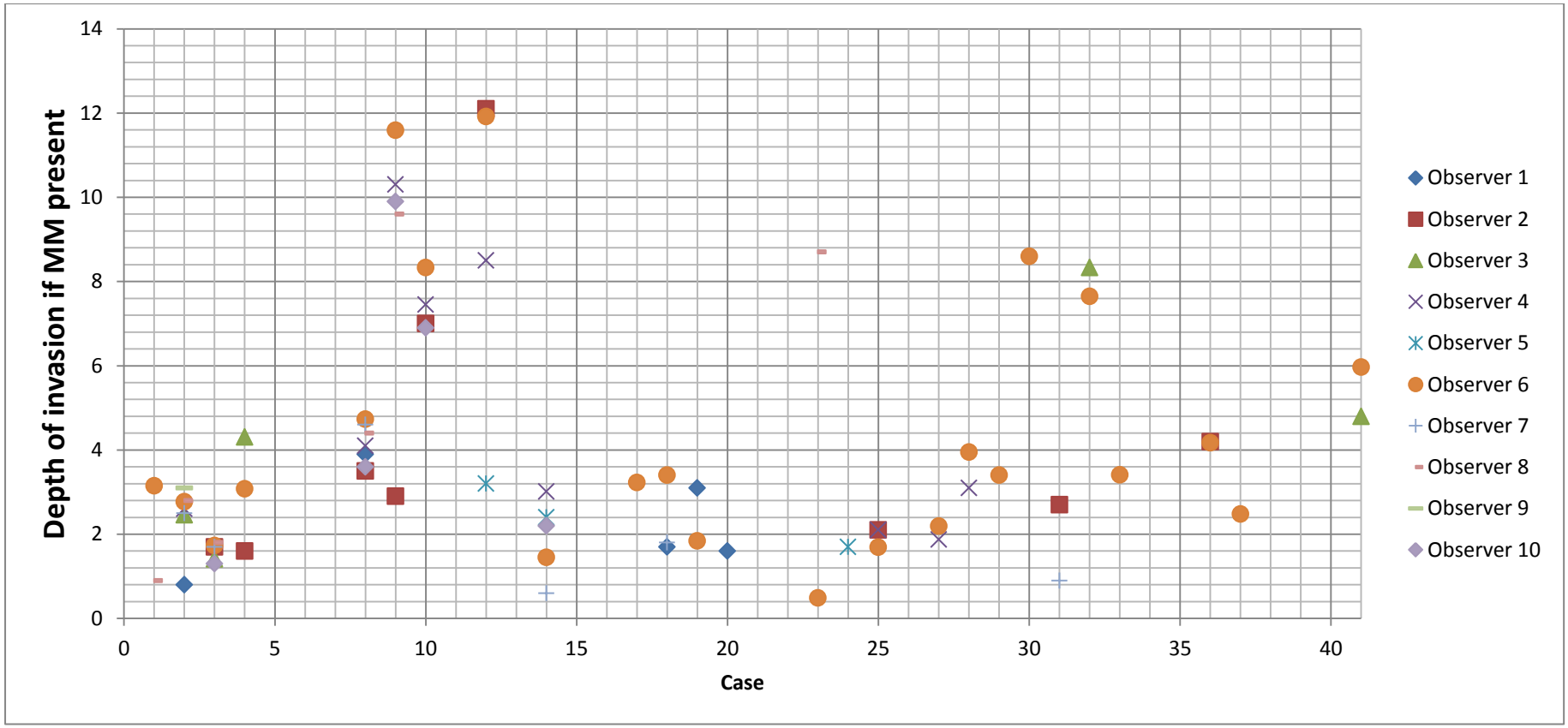


Figure 5.28: Overall results for the measurements of the depth of invasion if the muscularis mucosae was present.

5.6.2.2.4 Area of submucosal invasion

In round 2, we had asked the pathologist to measure the area of submucosal invasion. The ICC for the area of submucosal invasion was 0.59 (95% CI 0.47 – 0.72, $p < 0.001$). Figure 5.29 demonstrates the measurements of the individual cases by the pathologists.

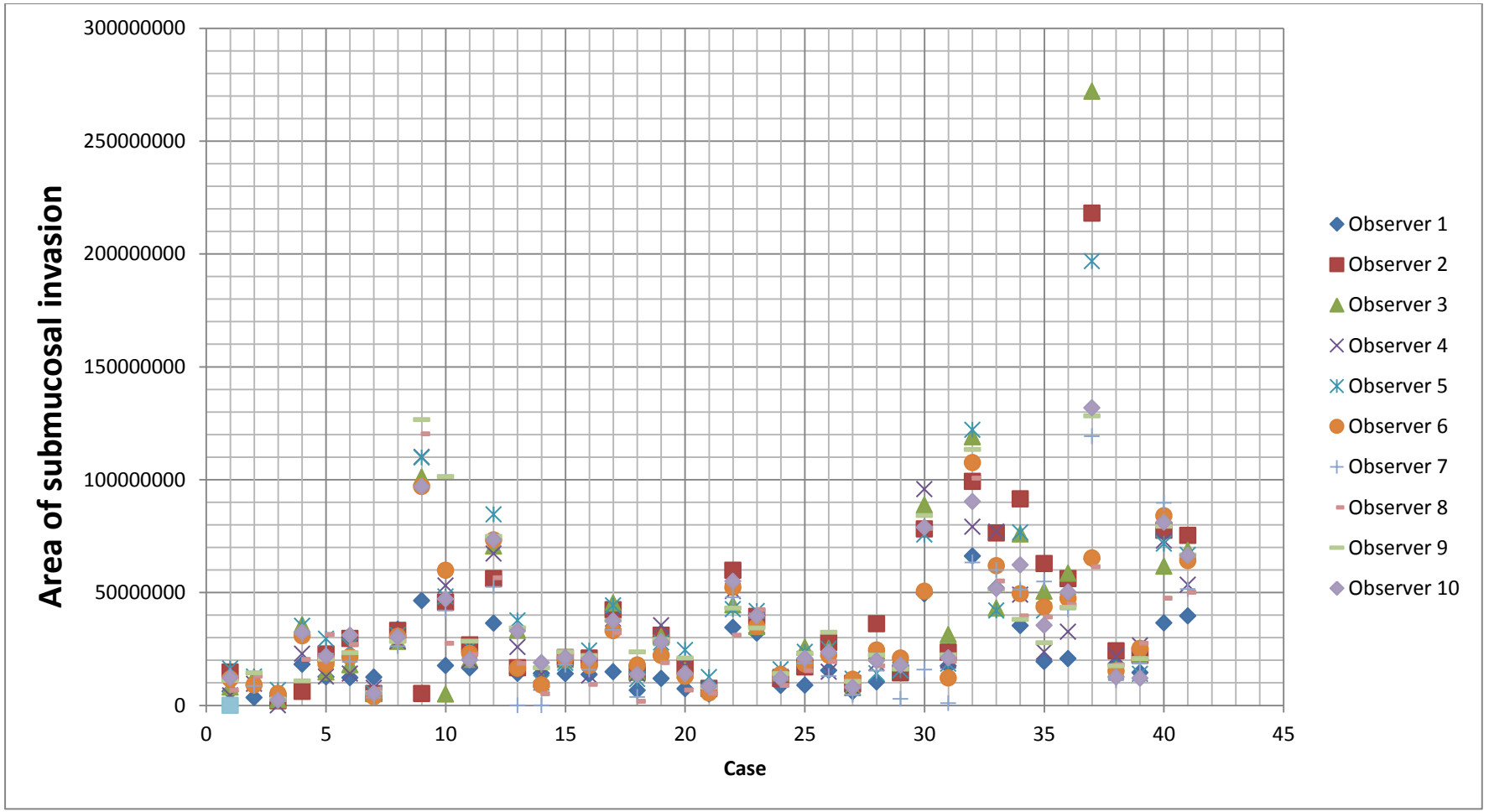


Figure 5.29: Overall results for the area of submucosal invasion in round 2.

Table 5.3 summarizes the results from both round 1 and 2.

Qualitative/ Quantitative factors		Round 1	Round 2
Shape of lesion [κ (95% CI)]		0.33 (0.30 – 0.36)	0.21 (0.17 – 0.25)
Grade of differentiation [κ (95% CI)]		0.13 (0.09 – 0.18)	0.19 (0.15 – 0.24)
Lymphatic invasion [κ (95% CI)]		0.08 (0.03 – 0.12)	0.12 (0.08 – 0.17)
Vascular invasion [κ (95% CI)]		0.08 (0.03 – 0.19)	0.19 (0.14 – 0.22)
Haggitt Level [κ (95% CI)]		0.17 (0.15 – 0.19)	0.15 (0.13 - 0.17)
Kikuchi Level [κ (95% CI)]		0.05 (0.03 – 0.08)	0.07 (0.04 – 0.10)
Lesion fully excised? [κ (95% CI)]		0.49 (0.44 – 0.53)	
For full resection? [κ (95% CI)]		0.18 (0.14 – 0.22)	0.32 (0.28 – 0.36)
Width of lesion [ICC (95% CI)]		0.73 (0.63 – 0.82)	0.95 (0.92 – 0.96)
Width of carcinoma [ICC (95% CI)]			0.01 (-0.03 – 0.08)
Distance to margin [ICC (95% CI)]	Own method	0.58 (0.45 – 0.69)	0.79 (0.69 – 0.88)
	Based on recommendations		0.88 (0.81 – 0.93)
Depth of invasion [ICC (95% CI)]	Epithelia surface	0.67 (0.55 – 0.78)	0.86 (0.79 – 0.91)
	Virtual muscularis mucosae - Curve		0.78 (0.67 – 0.87)
	Virtual muscularis mucosae - Straight		0.40 (0.25 – 0.62)
	Visible muscularis mucosae		0.29 (0.04 – 0.95)
Area of submucosal invasion (95% CI)			0.59 (0.47 – 0.72)

Table 5.3: Summary of the results from both round 1 and 2 of the interobserver variation studies.

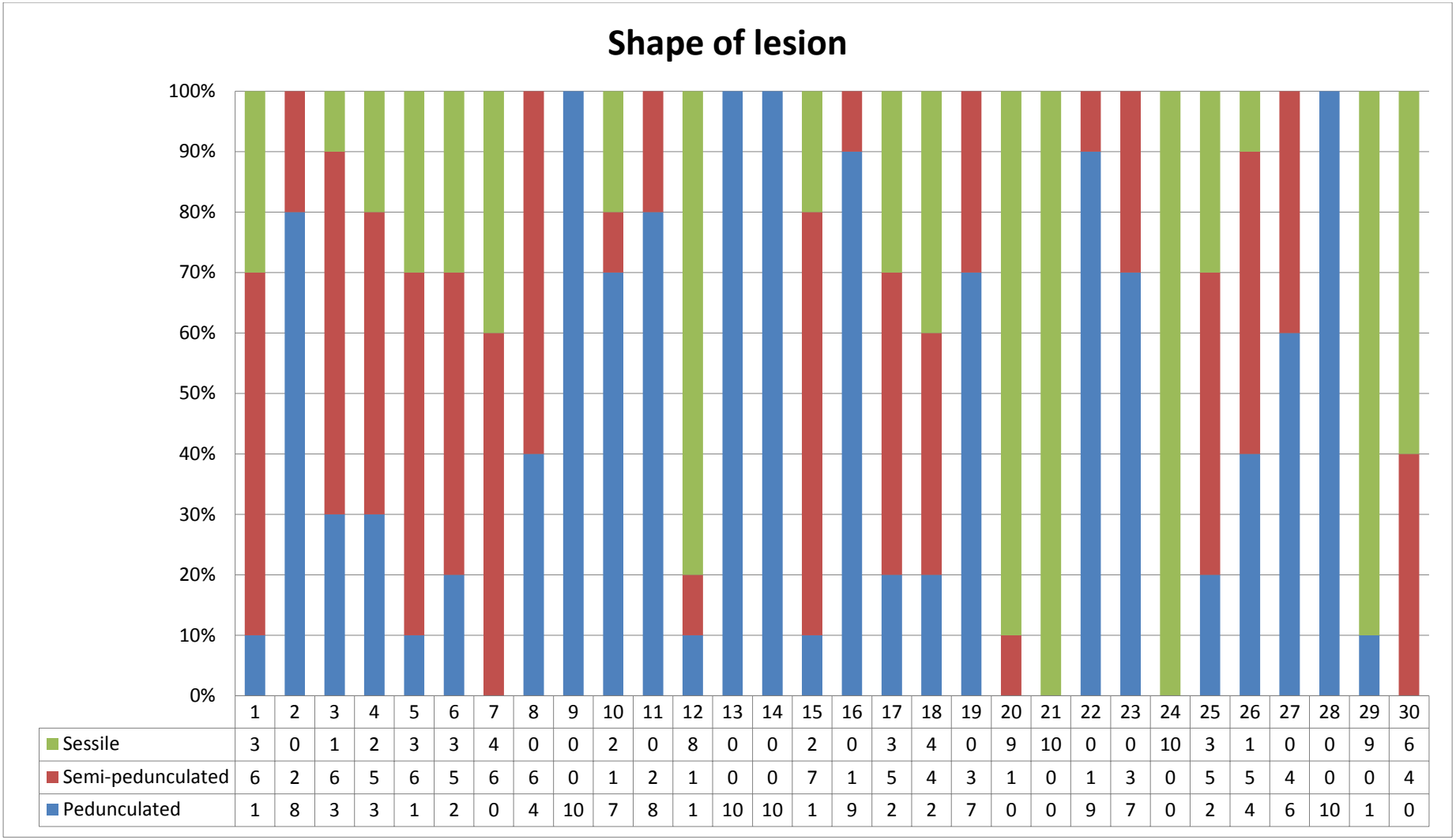
5.6.3 Further analysis of round 1 and 2 without non-assessable cases

A further analysis had been carried out with the non-assessable cases omitted. This was done to see whether there was an improvement in the kappa scores of the qualitative analysis of the lesions when the difficult lesions were omitted. Tables 5.4 and 5.5 show the comparisons between the cases in round 1 and 2 and the non-assessable cases that were omitted.

5.6.3.1 Round 1 result

Shape of lesion

In assessing the shape of the lesion, 30 out of the original 41 cases were included (11 non-assessable cases omitted). The inter-observer agreement in deciding the shape of the lesion generated a κ value of 0.39 (95% CI 0.30 – 0.46, $p < 0.001$). Figure 5.30 demonstrates the breakdown of the shape of the lesion that the pathologist had decided on each case.

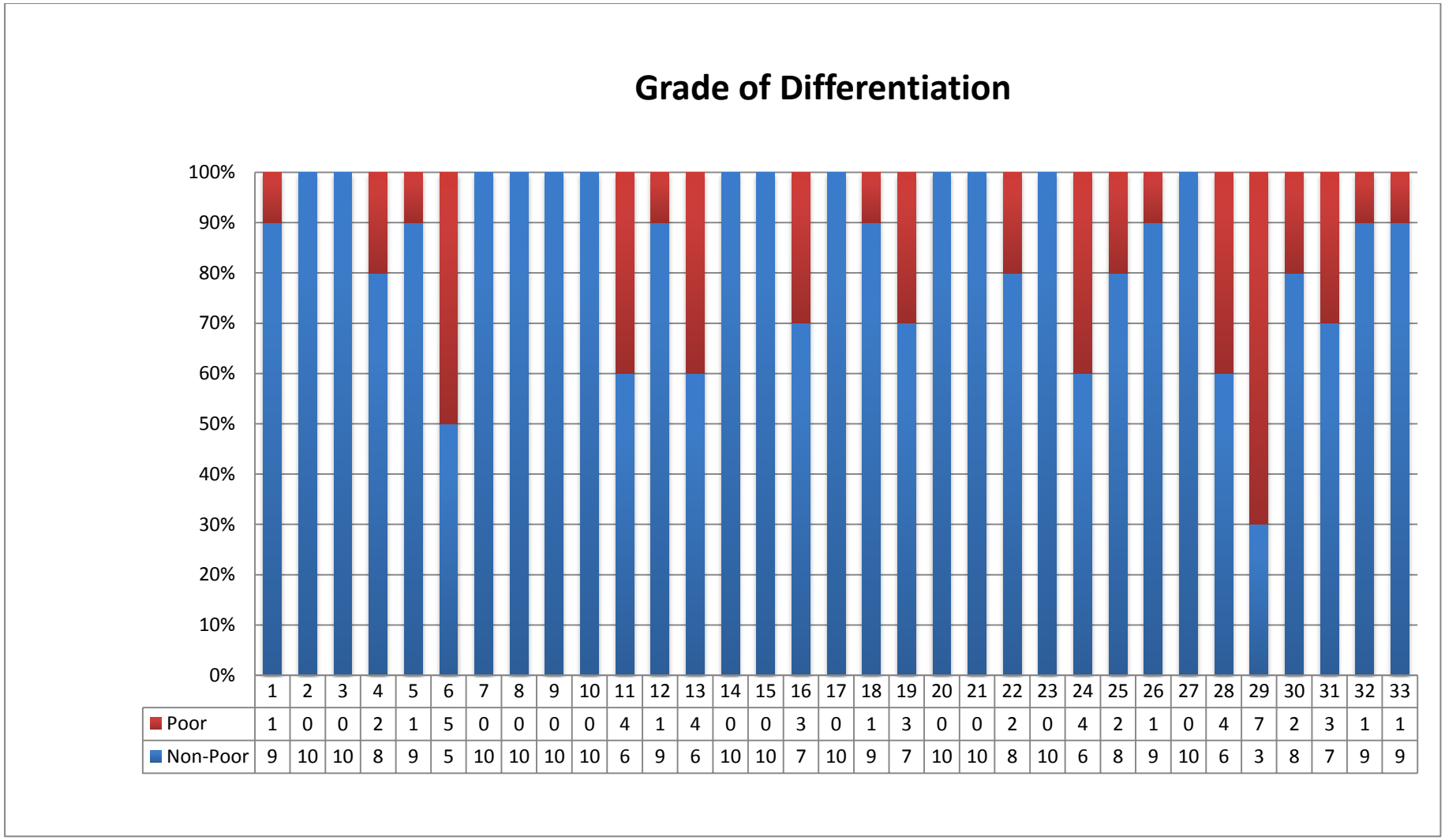


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Sessile	3	0	1	2	3	3	4	0	0	2	0	8	0	0	2	0	3	4	0	9	10	0	0	10	3	1	0	0	9	6
Semi-pedunculated	6	2	6	5	6	5	6	6	0	1	2	1	0	0	7	1	5	4	3	1	0	1	3	0	5	5	4	0	0	4
Pedunculated	1	8	3	3	1	2	0	4	10	7	8	1	10	10	1	9	2	2	7	0	0	9	7	0	2	4	6	10	1	0

Figure 5.30: Overall results for each case in deciding the shape of the lesion in round 1 excluding the non-assessable cases.

Grade of differentiation

For grade of differentiation, 33 cases were used (8 non-assessable cases omitted). The overall κ score for the reporting of the grade of differentiation was 0.16 (95% CI 0.11 – 0.21, $p < 0.001$). Figure 5.31 demonstrates the breakdown of the grade of differentiation for each case.

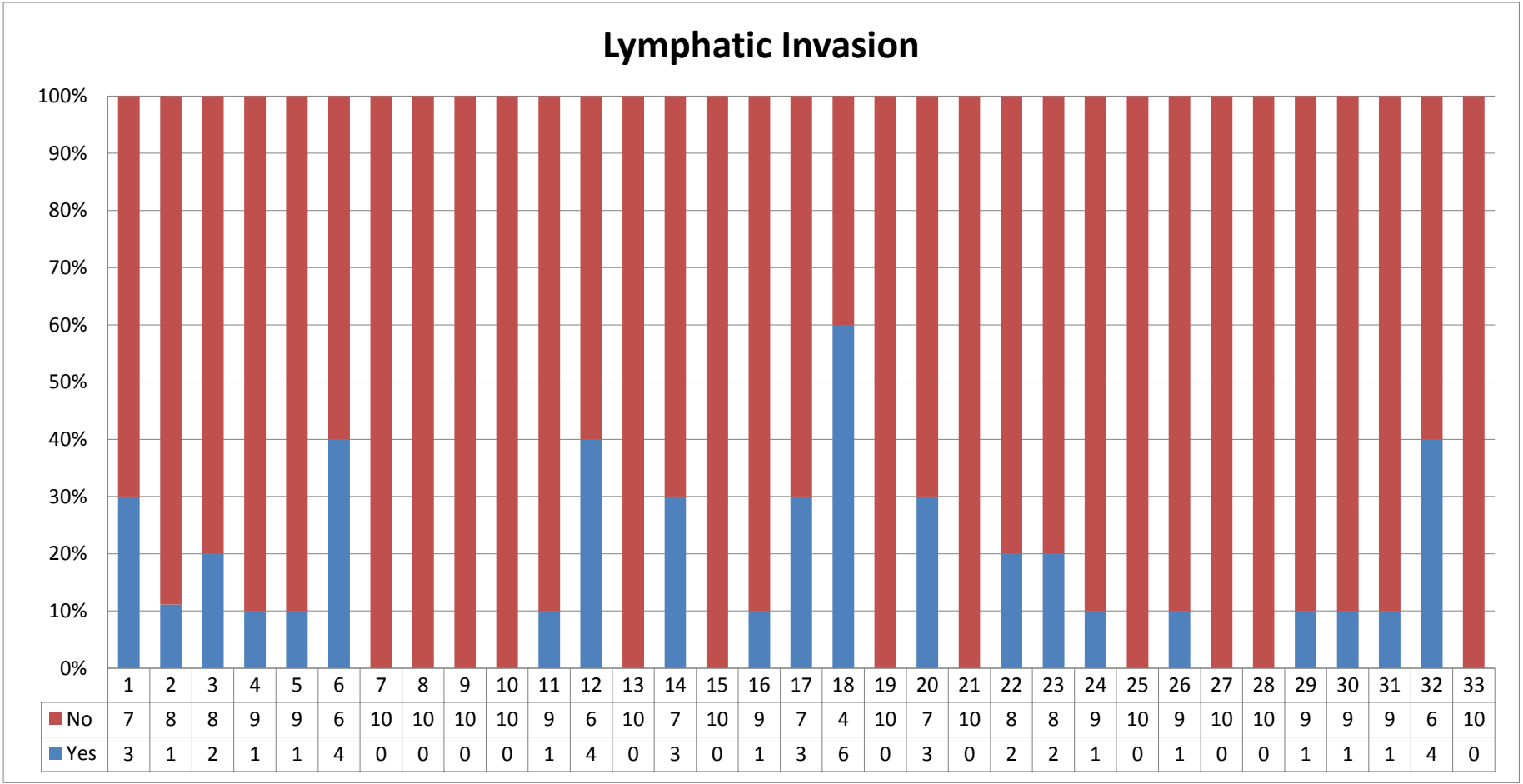


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
Poor	1	0	0	2	1	5	0	0	0	0	4	1	4	0	0	3	0	1	3	0	0	2	0	4	2	1	0	4	7	2	3	1	1
Non-Poor	9	10	10	8	9	5	10	10	10	10	6	9	6	10	10	7	10	9	7	10	10	8	10	6	8	9	10	6	3	8	7	9	9

Figure 5.31: Overall results for each case in deciding the grade of differentiation in round 1 excluding the non-assessable cases.

Lymphatic invasion

For lymphatic invasion, 33 cases were used (8 non-assessable cases omitted). The overall κ score for the reporting of lymphatic invasion was 0.11 (95% CI 0.06 – 0.16, $p < 0.0001$). Figure 5.32 demonstrates the breakdown of lymphatic invasion for each case.

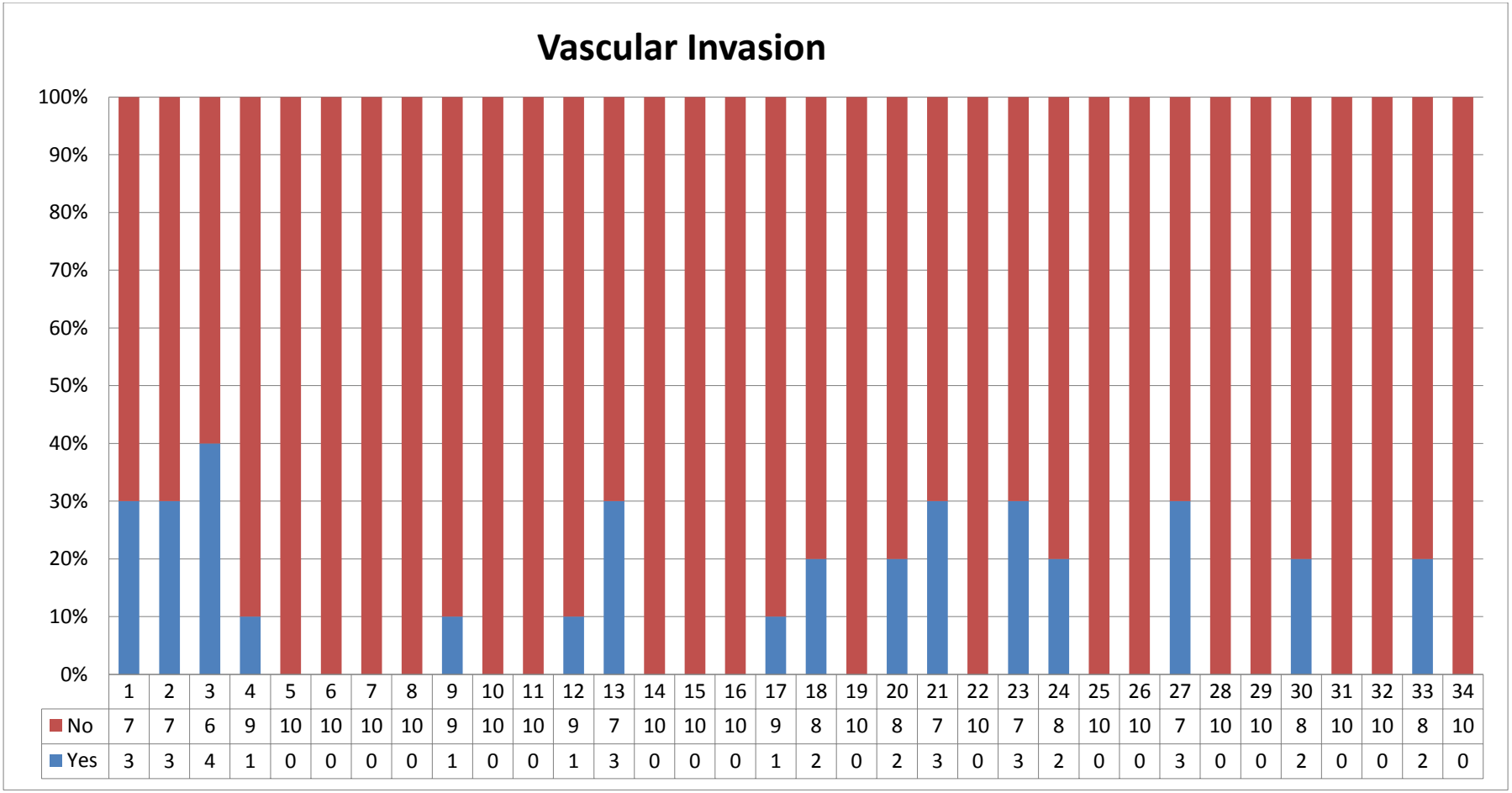


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
No	7	8	8	9	9	6	10	10	10	10	9	6	10	7	10	9	7	4	10	7	10	8	8	9	10	9	10	10	9	9	9	6	10
Yes	3	1	2	1	1	4	0	0	0	0	1	4	0	3	0	1	3	6	0	3	0	2	2	1	0	1	0	0	1	1	1	4	0

Figure 5.32: Overall results for each case in deciding the presence of lymphatic invasion in round 1 excluding the non-assessable cases.

Vascular invasion

For vascular invasion, 34 cases were used (7 non-assessable cases omitted). The overall κ score for the reporting of vascular invasion was 0.08 (95% CI 0.03 – 0.13, $p < 0.00001$). Figure 5.33 displays the breakdown of each case.

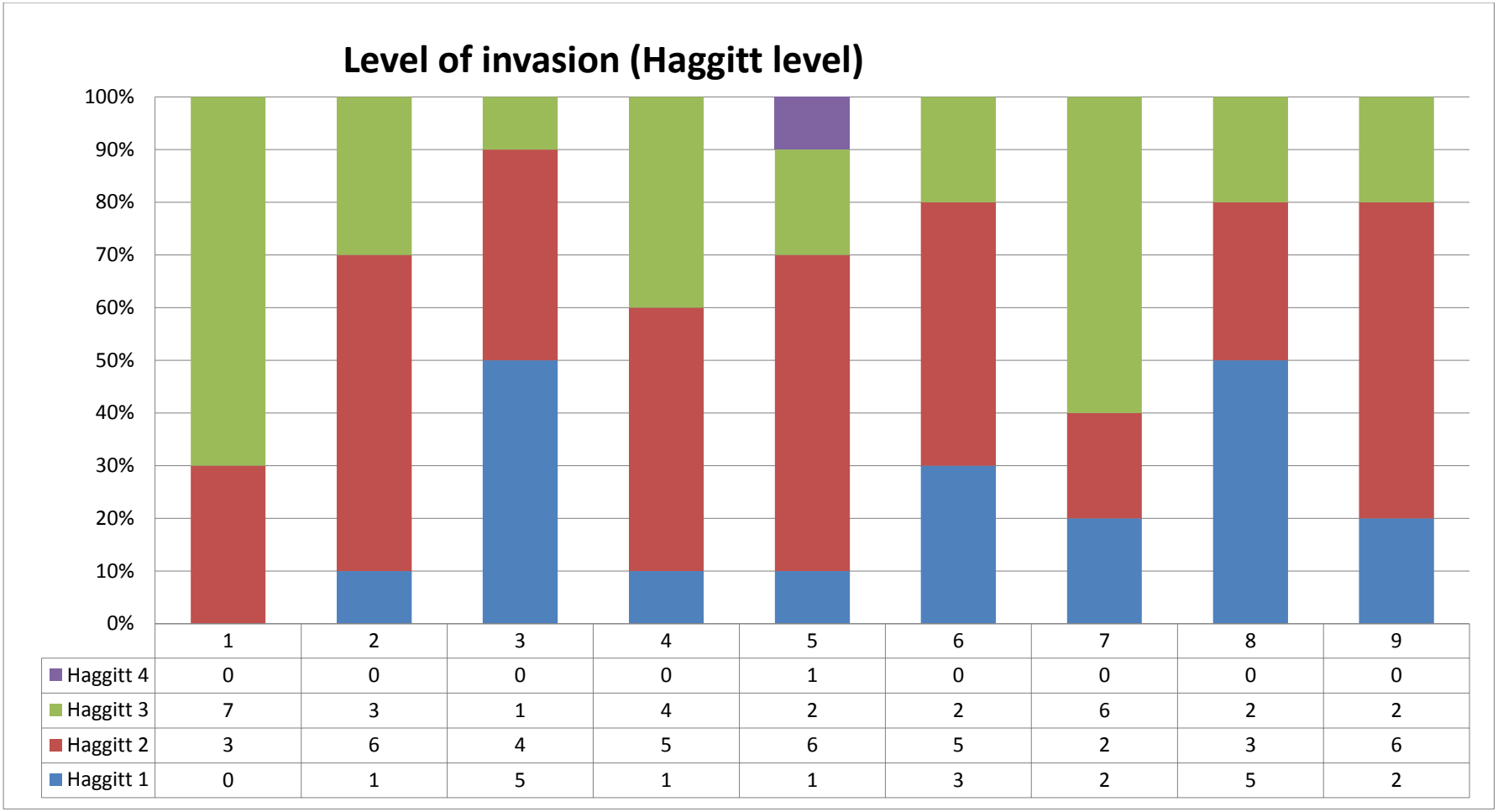


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
No	7	7	6	9	10	10	10	10	9	10	10	9	7	10	10	10	9	8	10	8	7	10	7	8	10	10	7	10	10	8	10	10	8	10
Yes	3	3	4	1	0	0	0	0	1	0	0	1	3	0	0	0	1	2	0	2	3	0	3	2	0	0	3	0	0	2	0	0	2	0

Figure 5.33: Overall results for each case in deciding the presence of vascular invasion in round 1 excluding the non-assessable cases.

Haggit level

For Haggit level of invasion, 9 cases were used (32 non-assessable cases omitted). The overall κ score for the reporting of Haggitt levels was 0.04 (95% CI -0.03 – 0.10, $p < 0.0001$). Figure 5.34 displays the breakdown of each case.



	1	2	3	4	5	6	7	8	9
Haggitt 4	0	0	0	0	1	0	0	0	0
Haggitt 3	7	3	1	4	2	2	6	2	2
Haggitt 2	3	6	4	5	6	5	2	3	6
Haggitt 1	0	1	5	1	1	3	2	5	2

Figure 5.34: Overall results for each case in deciding the Haggitt level of invasion in round 1 excluding the non-assessable cases.

Kikuchi level

For Kikuchi level of invasion, only 2 cases were used (39 non-assessable cases omitted). The overall κ score for the reporting of Kikuchi levels was 0.12 (95% CI -0.06 – 0.30, p <0.0001). Figure 5.35 displays the breakdown of both the cases.

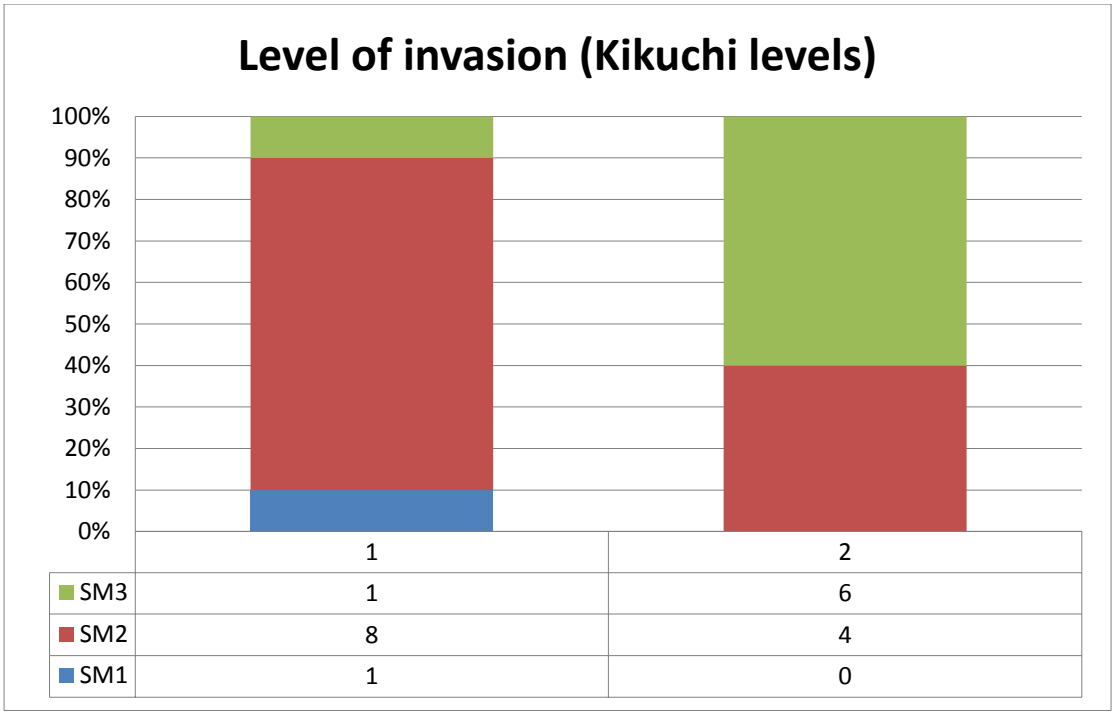
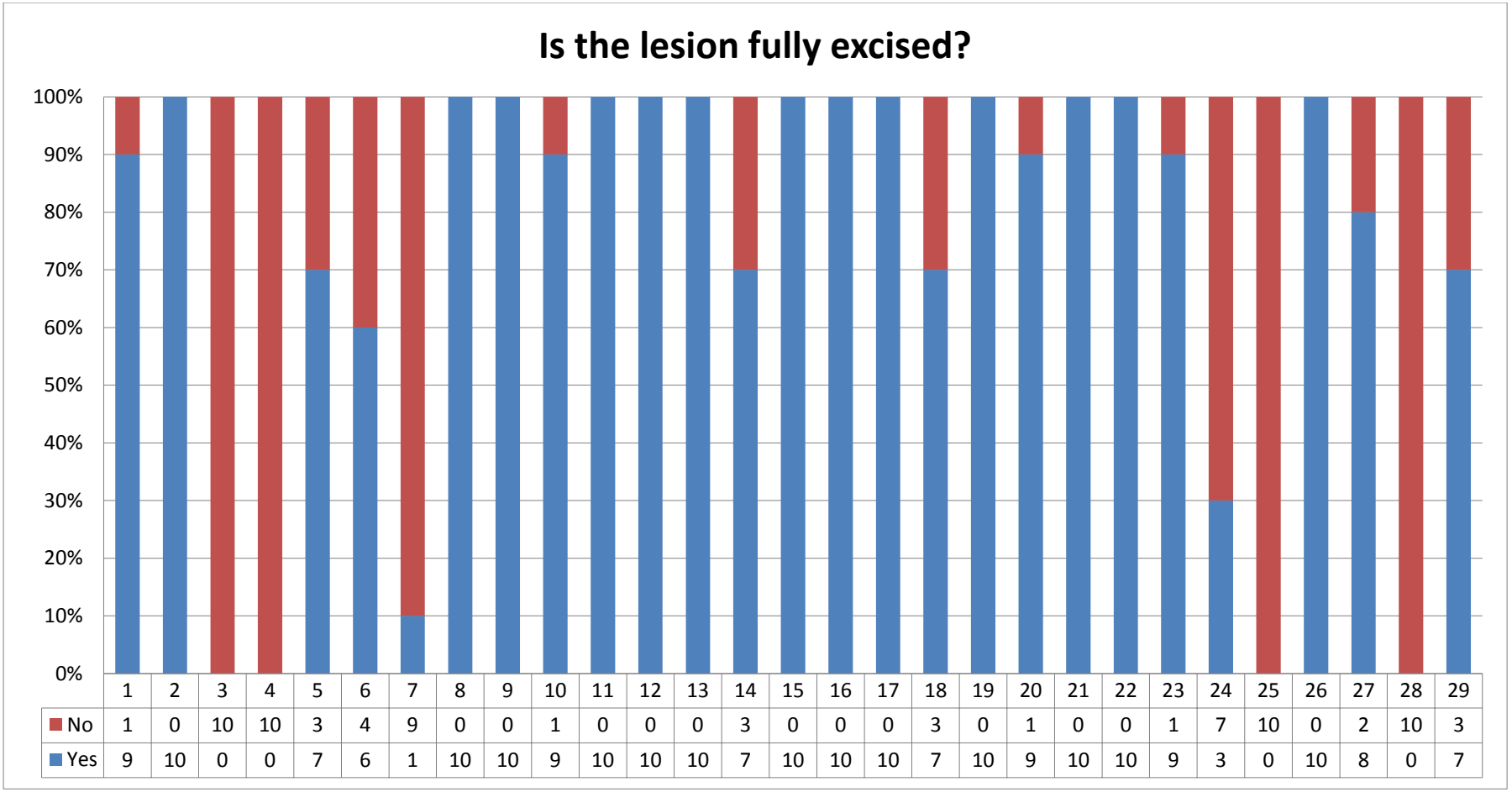


Figure 5.35: Overall results for each case in deciding the Kikuchi level of invasion in round 1 excluding the non-assessable cases.

Is the lesion fully excised?

In this section, 29 cases were used (12 non-assessable cases omitted). The overall κ score for that was generated by the question posed was 0.12 (95% CI -0.06 – 0.30, $p < 0.0001$).

Figure 5.36 demonstrates the breakdown of each case in relation to the question posed.

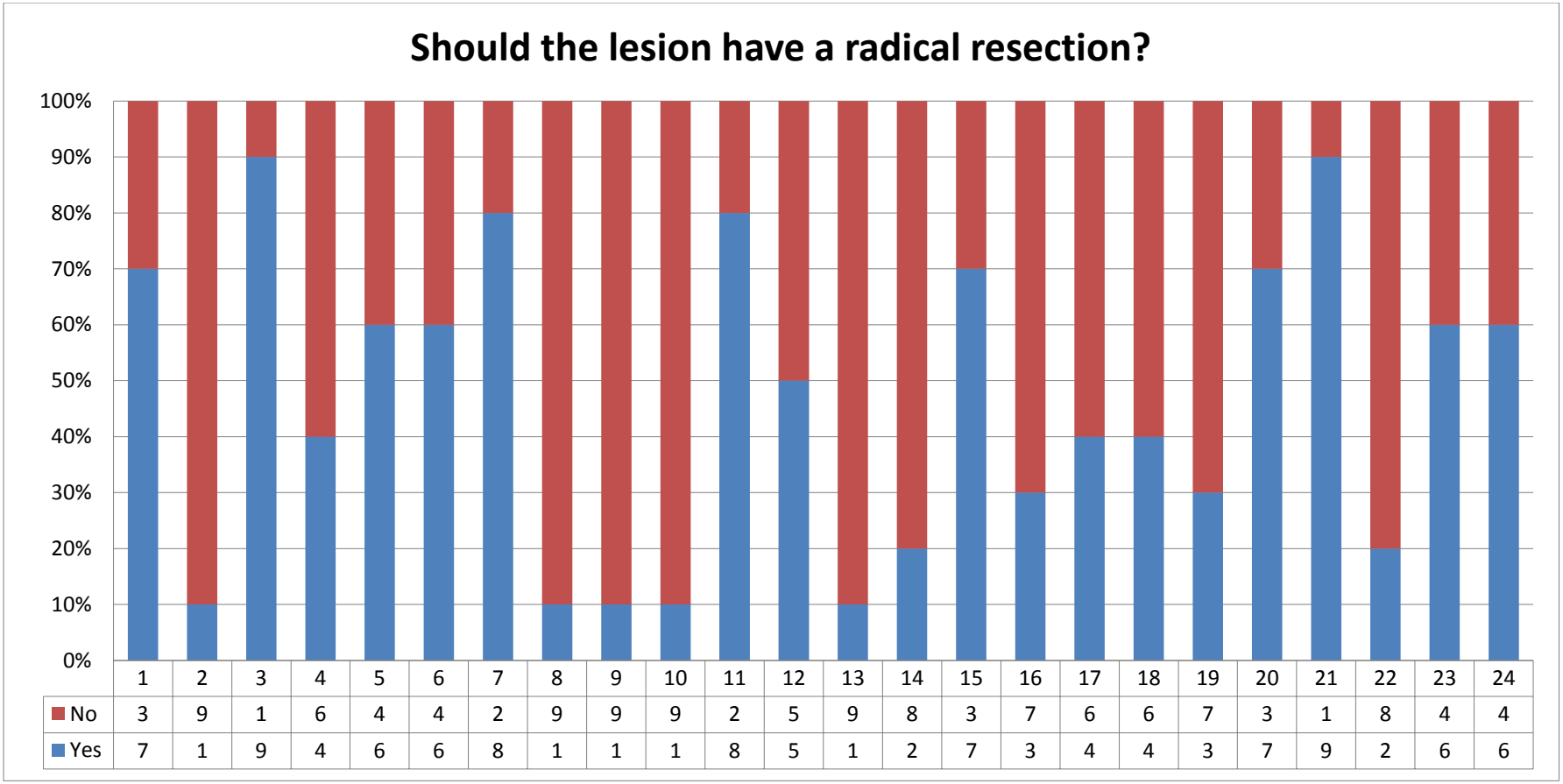


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
No	1	0	10	10	3	4	9	0	0	1	0	0	0	3	0	0	0	3	0	1	0	0	1	7	10	0	2	10	3
Yes	9	10	0	0	7	6	1	10	10	9	10	10	10	7	10	10	10	7	10	9	10	10	9	3	0	10	8	0	7

Figure 5.36: Overall results for each case in deciding whether the lesion is fully excised in round 1 excluding the non-assessable cases.

Should the lesion have a radical resection?

In this section, 24 cases were used (17 non-assessable cases omitted). The overall κ score that was generated by this question posed was 0.24 (95% CI 0.18 – 0.30, $p < 0.0001$). Figure 5.37 demonstrates the breakdown of each case in relation to the question posed.



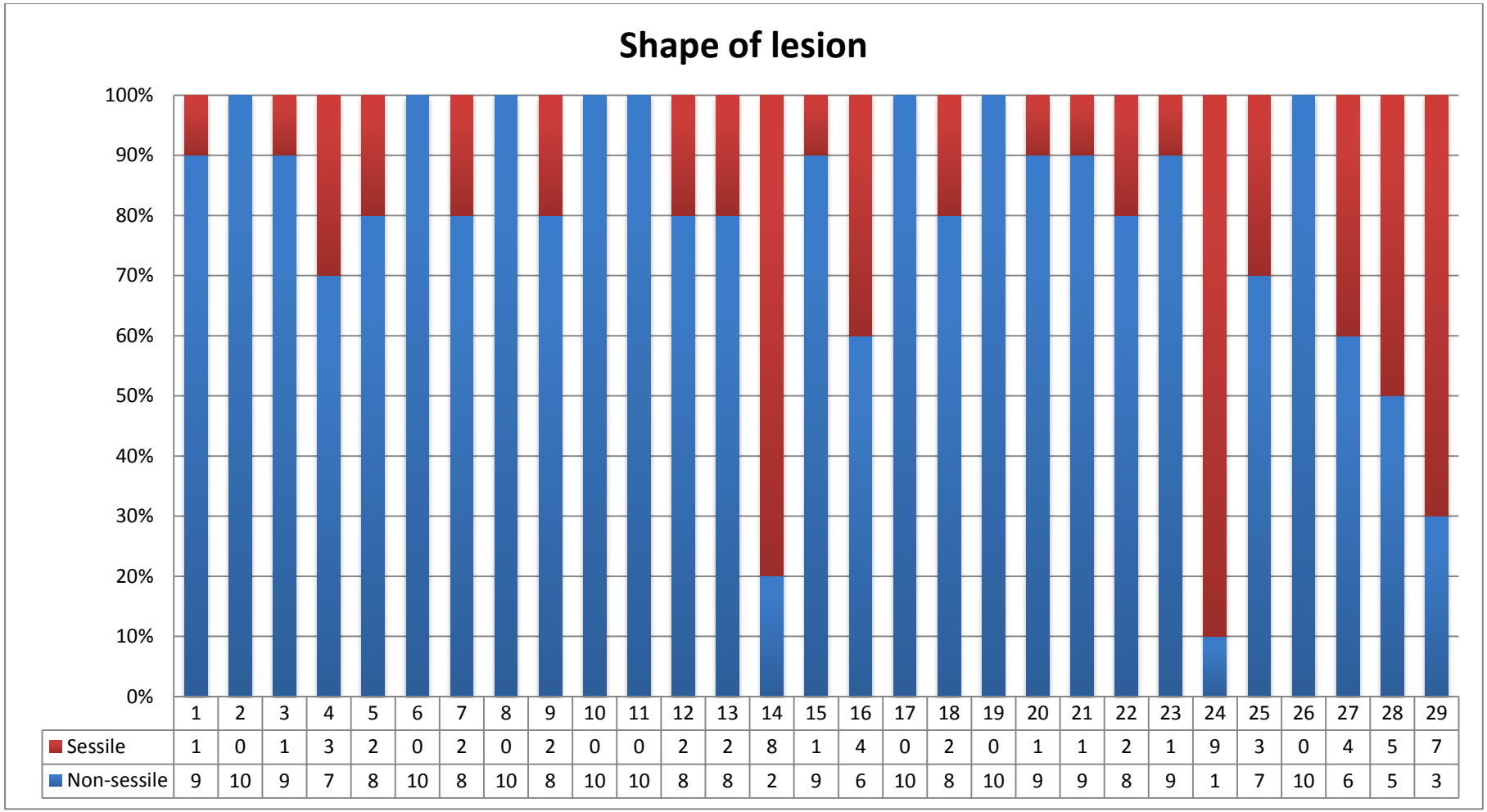
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
No	3	9	1	6	4	4	2	9	9	9	2	5	9	8	3	7	6	6	7	3	1	8	4	4
Yes	7	1	9	4	6	6	8	1	1	1	8	5	1	2	7	3	4	4	3	7	9	2	6	6

Figure 5.37: Overall results for each case in deciding whether the lesion should receive a radical resection in round 1 excluding the non-assessable cases.

5.6.3.2 Round 2 results

Shape of lesion

For the second round, 29 cases were used (12 non-assessable cases omitted). The inter-observer agreement in deciding the shape of the lesion generated a κ value of 0.30 (95% CI 0.25 - .035, $p < 0.001$). Figure 5.38 demonstrates the breakdown of the shape of the lesion that the pathologist had decided on each case.

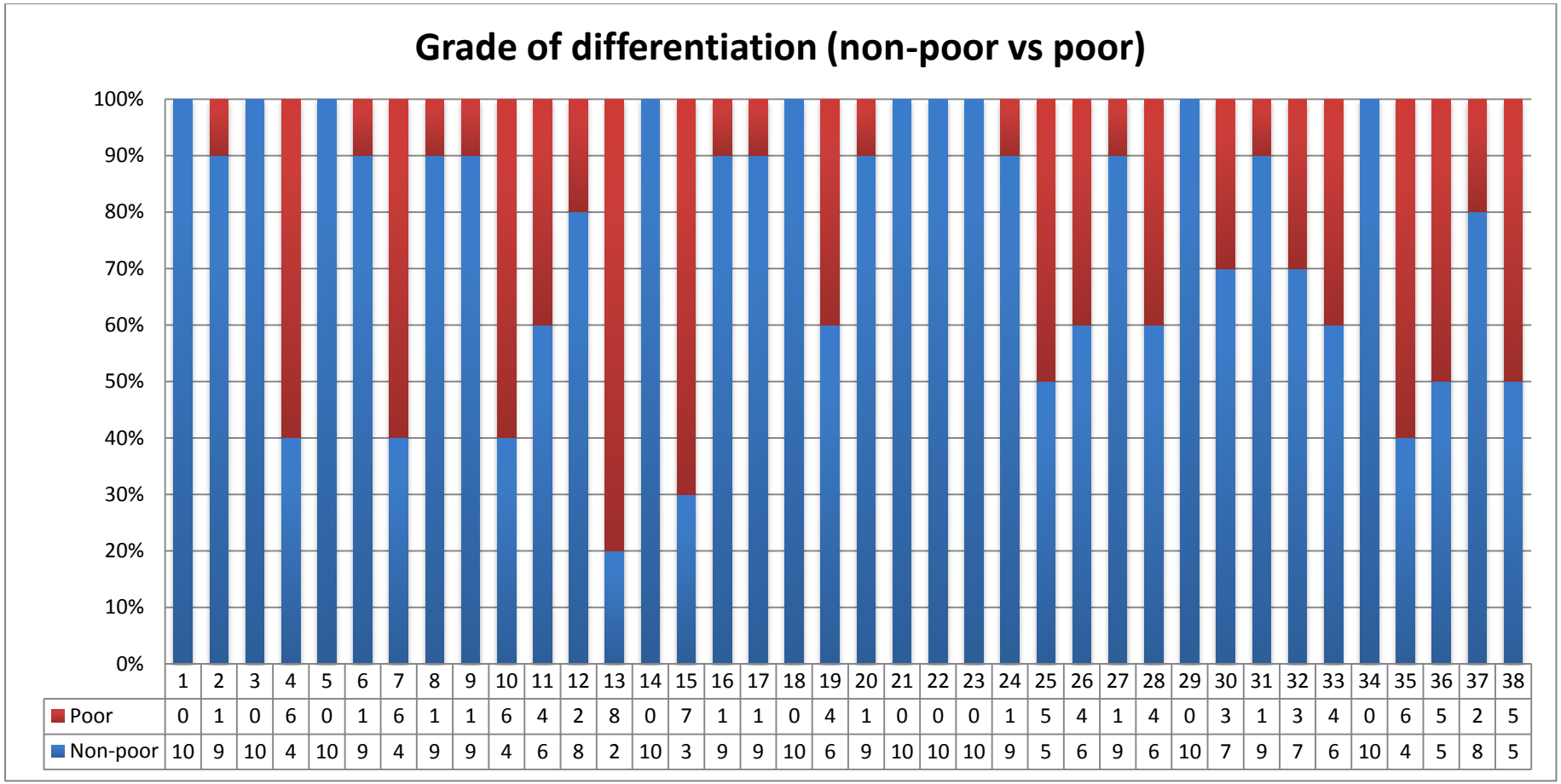


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Sessile	1	0	1	3	2	0	2	0	2	0	0	2	2	8	1	4	0	2	0	1	1	2	1	9	3	0	4	5	7
Non-sessile	9	10	9	7	8	10	8	10	8	10	10	8	8	2	9	6	10	8	10	9	9	8	9	1	7	10	6	5	3

Figure 5.38: Overall results for each case in deciding the shape of the lesion in round 2 excluding the non-assessable cases.

Grade of differentiation

For grade of differentiation, 38 cases were used (3 non-assessable cases omitted). The overall κ score for the reporting of the grade of differentiation was 0.20 (95% CI 0.15 – 0.25, $p < 0.001$). Figure 5.39 demonstrates the breakdown of the grade of differentiation for each case.

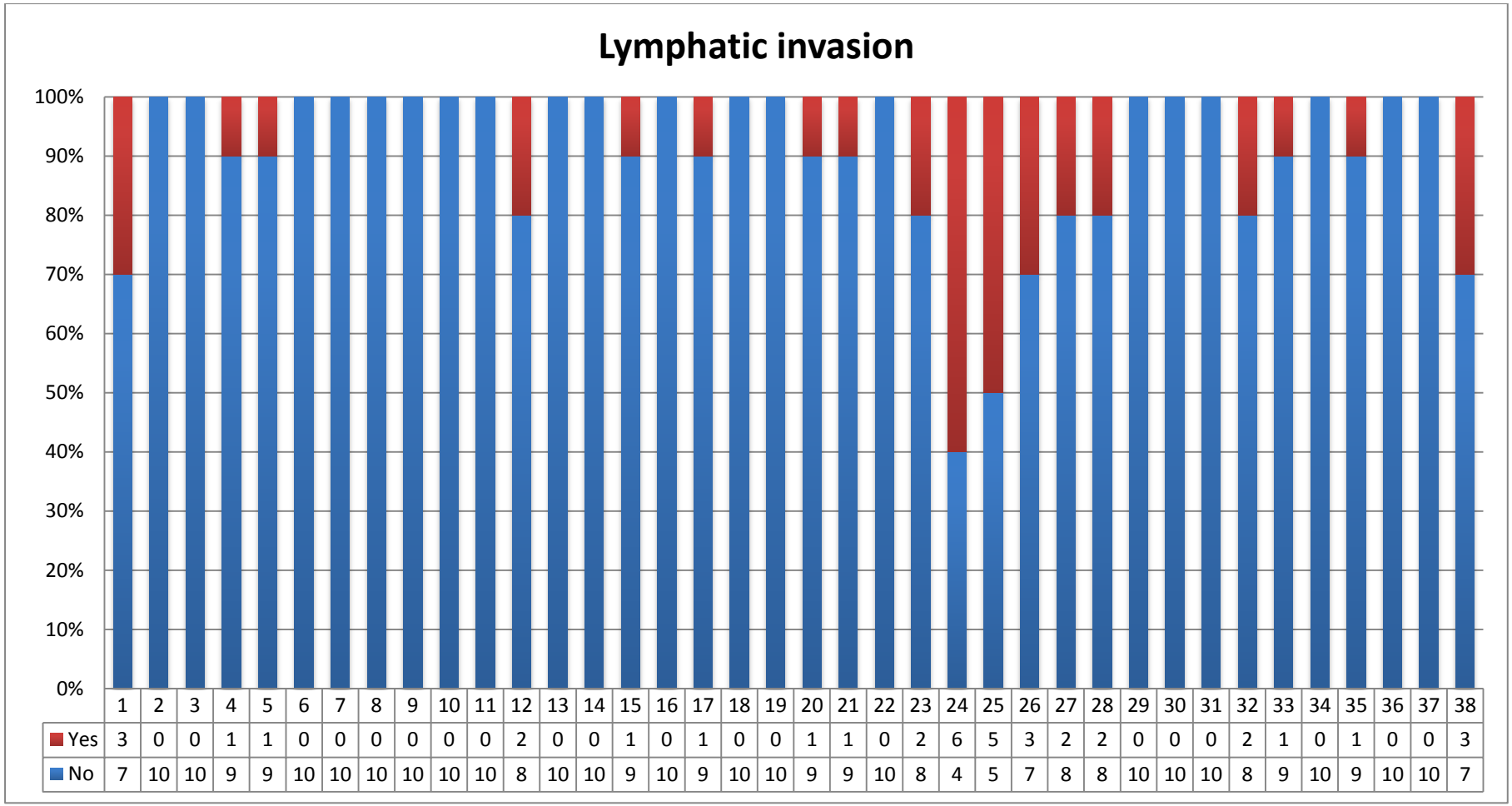


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Poor	0	1	0	6	0	1	6	1	1	6	4	2	8	0	7	1	1	0	4	1	0	0	0	1	5	4	1	4	0	3	1	3	4	0	6	5	2	5
Non-poor	10	9	10	4	10	9	4	9	9	4	6	8	2	10	3	9	9	10	6	9	10	10	10	9	5	6	9	6	10	7	9	7	6	10	4	5	8	5

Figure 5.39: Overall results for each case in deciding whether the grade of differentiation of the pT1 CRC in round 2 excluding the non-assessable cases.

Lymphatic invasion

For lymphatic invasion, 38 cases were used (3 non-assessable cases omitted). The overall κ score for the reporting of lymphatic invasion was 0.14 (95% CI 0.09 – 0.18, $p < 0.0001$). Figure 5.40 demonstrates the breakdown of lymphatic invasion for each case.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Yes	3	0	0	1	1	0	0	0	0	0	0	2	0	0	1	0	1	0	0	1	1	0	2	6	5	3	2	2	0	0	0	2	1	0	1	0	0	3
No	7	10	10	9	9	10	10	10	10	10	10	8	10	10	9	10	9	10	10	9	9	10	8	4	5	7	8	8	10	10	10	8	9	10	9	10	10	7

Figure 5.40: Overall results for each case in deciding the presence of lymphatic invasion in round 2 excluding the non-assessable cases.

Vascular invasion

For vascular invasion, 37 cases were used (4 non-assessable cases omitted). The overall κ score for the reporting of vascular invasion was 0.23 (95% CI 0.18 – 0.28, $p < 0.0001$). Figure 5.41 displays the breakdown of each case.

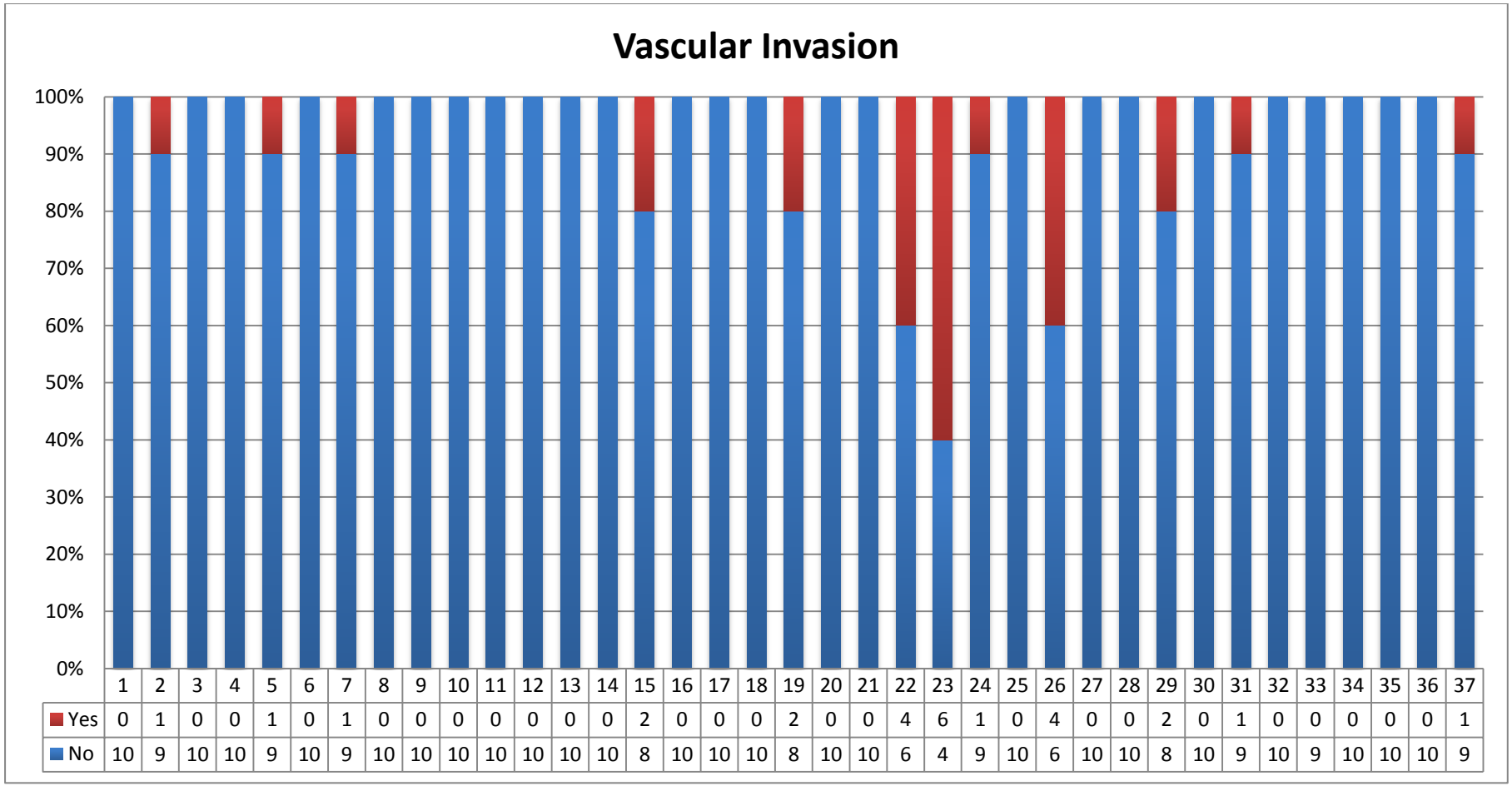
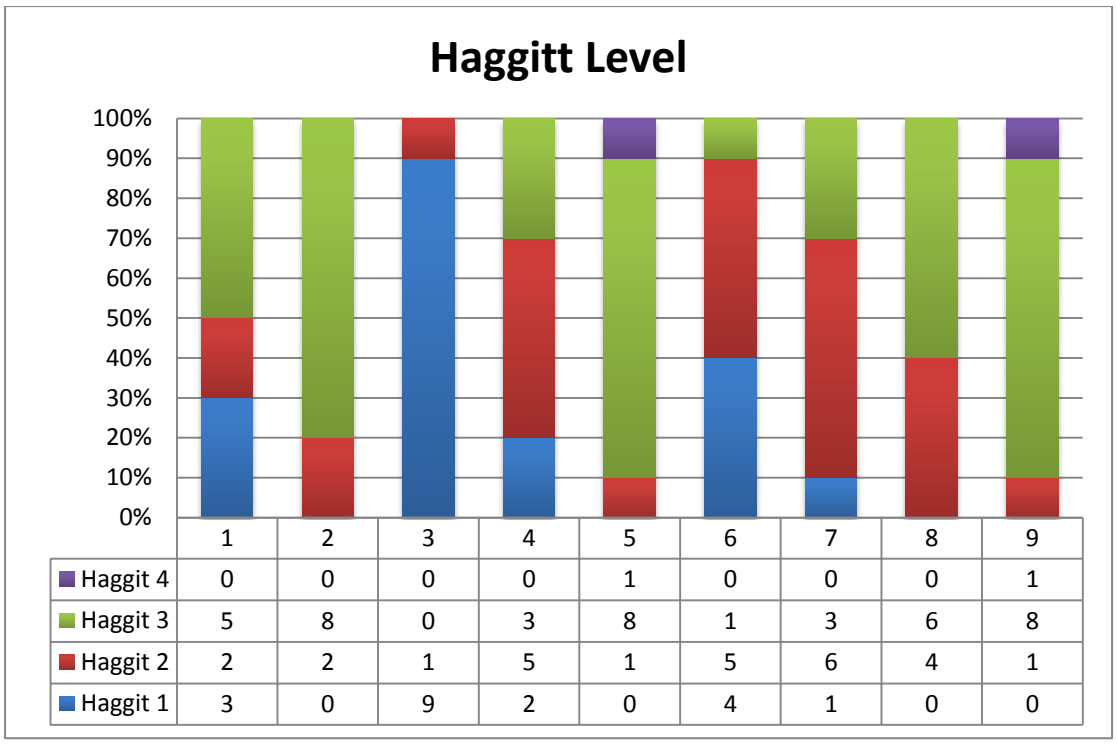


Figure 5.41: Overall results for each case in deciding the presence of vascular invasion in round 2 excluding the non-assessable cases.

Haggit level

For Haggit level of invasion, 9 cases were used (32 non-assessable cases omitted). The overall κ score for the reporting of Haggitt levels was 0.24 (95% CI 0.17 – 0.31, $p < 0.0001$). Figure 5.42 displays the breakdown of each case.



	1	2	3	4	5	6	7	8	9
Haggit 4	0	0	0	0	1	0	0	0	1
Haggit 3	5	8	0	3	8	1	3	6	8
Haggit 2	2	2	1	5	1	5	6	4	1
Haggit 1	3	0	9	2	0	4	1	0	0

Figure 5.42: Overall results for each case in deciding the Haggitt level of invasion in round 2 excluding the non-assessable cases.

Kikuchi levels

For Kikuchi levels of invasion, all 41 cases were non-assessable and hence omitted from analysis.

Should the lesion be resected?

In this section, 26 cases were used (15 non-assessable cases omitted). The overall κ score that was generated by this question posed was 0.46 (95% CI 0.39 – 0.51, $p < 0.0001$). Figure 5.43 demonstrates the breakdown of each case in relation to the question posed.

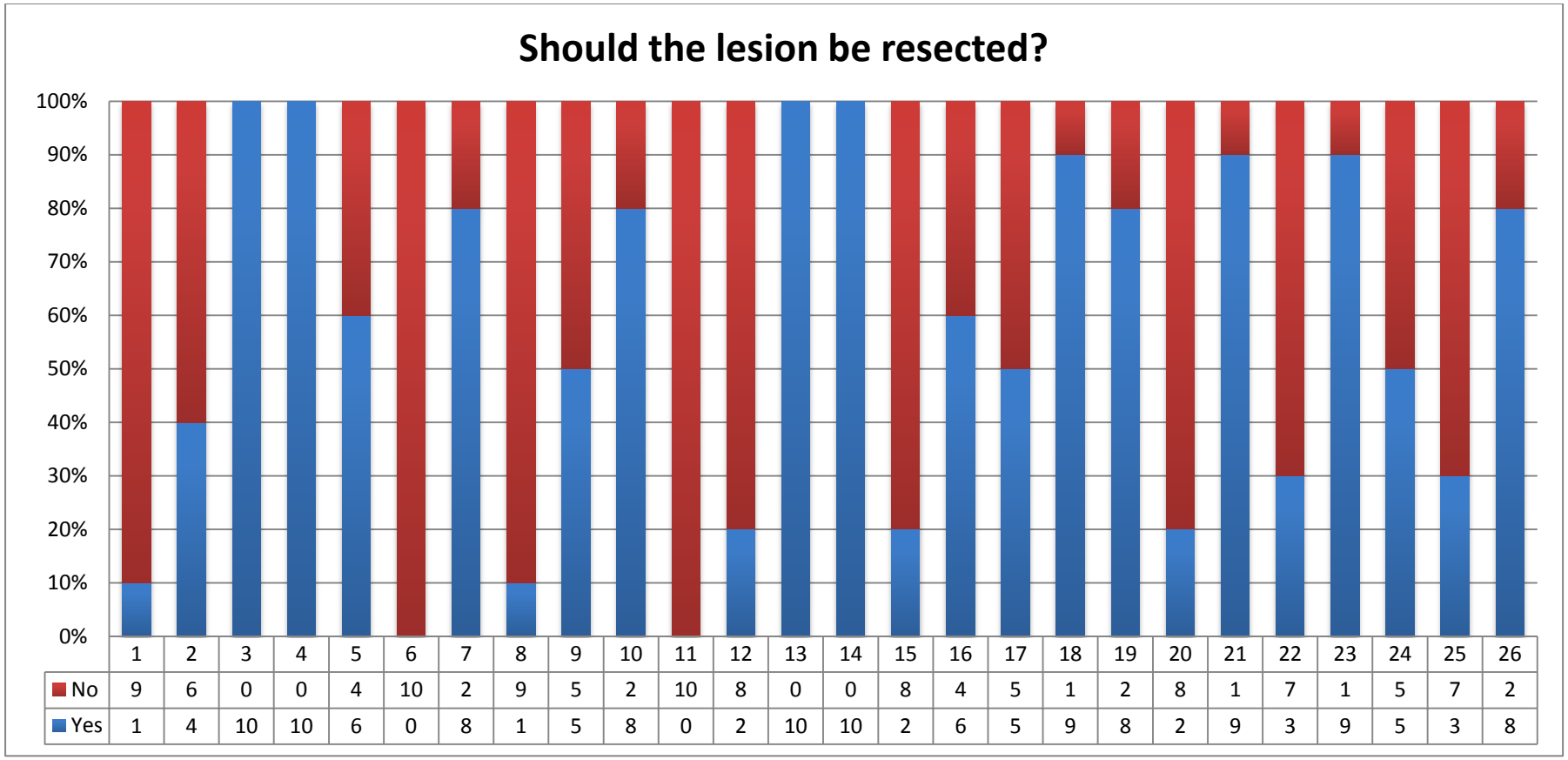


Figure 5.43: Overall results in round 2 for each case in deciding whether the lesion should receive a radical resection excluding the non-assessable cases.

Tables 5.4 and 5.5 summarize the results from both rounds 1/2 and modified rounds 1/2.

Qualitative/ Quantitative factors	Round 1	Modified Round 1	Number of cases excluded
Shape of lesion [κ (95% CI)]	0.33 (0.30 – 0.36)	0.39 (0.30 – 0.46)	11
Grade of differentiation [κ (95% CI)]	0.13 (0.09 – 0.18)	0.16 (0.11 – 0.21)	8
Lymphatic invasion [κ (95% CI)]	0.08 (0.03 – 0.12)	0.11 (0.06 – 0.16)	8
Vascular invasion [κ (95% CI)]	0.08 (0.03 – 0.19)	0.08 (0.03 – 0.13)	7
Haggitt Level [κ (95% CI)]	0.17 (0.15 – 0.19)	0.04 (-0.03 - 0.10)	32
Kikuchi Level [κ (95% CI)]	0.05 (0.03 – 0.08)	0.12 (0.06 – 0.30)	39
Lesion fully excised? [κ (95% CI)]	0.49 (0.44 – 0.53)	0.12 (0.06 – 0.30)	12
For full resection? [κ (95% CI)]	0.18 (0.14 – 0.22)	0.24 (0.18 – 0.30)	17

Table 5.4: Summary of the results from both round 1 and modified round 1 (exclusion of non-assessable cases).

Qualitative/ Quantitative factors	Round 2	Modified Round 2	Number of cases excluded
Shape of lesion [κ (95% CI)]	0.21 (0.17 – 0.25)	0.30 (0.25 – 0.35)	12
Grade of differentiation [κ (95% CI)]	0.19 (0.15 – 0.24)	0.20 (0.15 – 0.25)	3
Lymphatic invasion [κ (95% CI)]	0.12 (0.08 – 0.17)	0.14 (0.09 – 0.18)	3
Vascular invasion [κ (95% CI)]	0.19 (0.14 – 0.22)	0.23 (0.18 – 0.28)	4
Haggitt Level [κ (95% CI)]	0.15 (0.13 – 0.17)	0.24 (0.17 - 0.31)	32
Kikuchi Level [κ (95% CI)]	0.07 (0.04 – 0.10)		41
For full resection? [κ (95% CI)]	0.32 (0.28 – 0.36)	0.46 (0.39 – 0.51)	15

Table 5.5: Summary of the results from both round 2 and modified round 2 (exclusion of non-assessable cases).

5.6.4 Results of intra-observer study

Nine out of the 10 pathologists had participated in the intra-observer study. Tables 5.6 and 5.7 summarises the results of the intra-observer study that was conducted in between both rounds of the inter-observer studies. The kappa scores for quantitative factors appeared to be more reproducible when compared to the qualitative factors.

Kappa Scores (intra-observer) – Qualitative analysis								
Observer	Shape of lesion	Grade of differentiation	Lymphatic invasion	Vascular invasion	Haggitt level	Kikuchi level	Lesion fully excised?	For resection?
1	0.46	0.18	0.64	0.48	0.30	0.42	0.13	-0.09
2	0.38	0.25	0.22	0.17	0.17	-0.01	0.27	0.31
3	0.37	0.26	0.38	0.44	0.17	0.58	0.54	0.16
4	0.62	0.32	0.43	-0.05	0.27	0.28	0.58	0.56
5	0.73	0.87	0.75	0.75	0.91	1.00	0.90	0.92
6	0.35	0.42	0.43	0.43	0.47	0.64	0.61	0.54
7	0.79	0.51	0.87	0.78	0.78	0.77	0.89	0.92
8	0.48	0.37	0.23	0.08	0.41	0.65	0.69	0.49
10	0.50	0.05	-0.03	0.5	0.51	-0.47	0.77	0.50

Table 5.6: Summary of the results for the intra-observer variation study on the qualitative factors.

Intra-class correlation co-efficient [ICC] (95% confidence interval) – Quantitative analysis			
Observer	Distance to nearest margin	Depth of invasion	Width of invasion
1	0.88(0.79 – 0.92)	0.90 (0.83 – 0.94)	0.76 (0.46 – 0.89)
2	0.66 (0.45 – 0.81)	0.60 (0.36 – 0.76)	0.37 (0.02 – 0.59)
3	0.81 (0.66 – 0.89)	0.83 (0.69 – 0.91)	0.78 (0.59 – 0.88)
4	0.86 (0.77 – 0.92)	0.78 (0.66 – 0.87)	0.79 (0.64 – 0.88)
5	0.99 (0.98 – 1.00)	0.97 (0.95 – 1.00)	0.99 (0.97 – 0.99)
6	0.96 (0.91 – 0.98)	0.87 (0.62 – 0.94)	0.80 (0.64 – 0.89)
7	0.98 (0.97 – 0.99)	0.97 (0.95 – 0.98)	0.72 (0.56 – 0.83)
8	0.95 (0.91 – 0.97)	0.58 (0.37 – 0.73)	0.80 (0.68 – 0.87)
10	0.89 (0.81 – 0.93)	0.61 (0.41 – 0.75)	0.63 (0.45 – 0.76)

Table 5.7: Summary of the results for the intra-observer variation study on the quantitative factors.

5.7 Discussion

The main aim of this study was to assess the inter- and intra-observer agreement in reporting features of malignant colorectal polyps by regional lead histopathologists participating in the NHSBCSP within the asymptomatic population. However, effective clinical management of these malignant polyps is dependent on the quality of the histological assessment and diagnosis of these lesions.

The secondary aim of this study was to assess the reliability of measuring proven and novel qualitative factors of colorectal polyp cancers. The proven high risk quantitative and qualitative factors and new novel quantitative factors such as width of invading carcinoma were tested between lead consultant histopathologists who participated in the NHS BCSP. This study is different from other previous inter-observer studies, as the novel quantitative factors mentioned above have not been previously assessed.

5.7.1 Grade of differentiation

The grading of CRC is often considered a simple task. However, studies have shown that this is not the case and there is often poor to fair inter-observer agreement in grading of CRC (Chandler's study yielded a Kappa value of 0.351 and 0.358 for a three tiered and two tiered grading system respectively (Chandler et al 2008); Komuta's study yielded a Kappa value of 0.163 for a two tiered grading system) (Komuta et al 2004). This was apparent in this study where the Kappa value for the grading of pT1 CRC was 0.13 and 0.19 in round 1 and 2 respectively. There was no

improvement in the inter-observer agreement despite omitting the non-assessable cases in the modified round 1 and 2.

One of the main reasons that CRC grading produces a large degree of inter-observer variation is that grading is very subjective. The subjectivity of grading occurs because of the creation of artificial boundaries across a continuum and tumour heterogeneity. The intra-tumour variation that arises in a CRC makes the application of the simplest grading system difficult (Compton 2003). For pT1 CRC, pathologists have been advised to grade the CRC based on the area of poorest differentiation (Loughrey, Quirke and Shepherd 2014). Here, subjectivity may arise, as the areas of poorest differentiation may be small and inconspicuous and often missed during microscopic examination.

There are two recognized methods of grading CRC. The first method that is recommended by the WHO is to grade a tumour as a poorly differentiated CRC if the predominant proportion of the cancer displays poor differentiation (Halvorsen and Seim 1988). This method is currently employed for pT2 CRCs and above in the UK (Loughrey, Quirke and Shepherd 2014). The second method is that a grade of poor differentiation should be applied when any area of the cancer is considered to show poor differentiation. The NHS BCSP recommends the second method for pT1 cancers (NHS BCSP 2016).

In this study, the group of pathologists did not feel that there was an optimal method in determining the grade of differentiation based on the three methods as described above. In conclusion, they agreed to continue using the standard method of grading the pT1 CRC based on the area of poorest differentiation in order to ensure that no poorly differentiated cancer was missed.

5.7.2 Haggitt and Kikuchi Classification

The inter-observer agreement in classifying pT1 CRC in both Haggitt and Kikuchi classifications were poor in this study. The Kappa values for Haggitt classification were 0.17 and 0.14 in rounds 1 and 2 respectively. The Kappa values for Kikuchi classification were lower with 0.05 and 0.07 in rounds 1 and 2 respectively.

One of the main issues that contributed to the poor inter-observer agreement was the shape of the lesion. The shape of the lesion normally dictates the use of either the Haggitt or Kikuchi classification: Haggitt for polypoid lesions and Kikuchi for sessile lesions. We found high inter-observer variation with defining the shape with Kappa scores of 0.33 and 0.21 in both round 1 and 2 respectively. Interestingly, the Kappa scores did not improve in round 2 despite reducing the rating categories (4 rating categories in round 1 compared to 3 rating categories in round 2). The kappa scores did improve but only to a small degree when the non-assessable cases were omitted in both rounds (0.39 for round 1 and 0.30 for round 2).

In the real world, colonic polyps come in a variety of shapes ranging from sessile to pedunculated and also a combination of both sessile and pedunculated (semi-pedunculated). This will only further complicate the Haggitt or Kikuchi classifications and increase the inter-observer variability.

Another factor that may contribute to low Kappa values within the Kikuchi classification is the fact that pathologists were unable to give a Kikuchi class to lesions that lacked a muscularis mucosae. Again, the lack of a muscularis mucosae means that the reporting pathologist is unable to subdivide the submucosa since they do not know where to start measuring from. Even when the non-assessable

lesions were excluded, Kappa values did not improve. In this study, we also studied the inter-observer variation of pathologists reporting in the presence of a muscularis mucosae within the lesion and the results still generated a kappa score of 0.014.

5.7.3 Lymphatic and vascular invasion

Lympho-vascular invasion in CRC is considered a strong indicator for a poorer outcome and often influences the post-surgical treatment of the patient. This is the reason that the detection of lympho-vascular invasion is vital in providing a prognosis and also confirming the use of adjuvant therapy after cancer resection. However, the detection of lympho-vascular invasion is often difficult on a standard H & E slide. This was clearly evident from this study with poor inter-observer agreement for both lymphatic and vascular invasion in the two rounds. Previous studies (Harris et al 2008, Kojima et al 2013) have shown better inter-observer agreements but overall, the kappa values were still moderate at best.

The use of immunohistochemical stains such as CD31 (used in identifying vascular endothelial cells) and D2-40 (used in identifying lymphatic endothelial cells) have aided the pathologist in identifying lymphovascular invasion in many malignancies. However, these immunohistochemical stains are not being used routinely in the identification of lymphovascular invasion in pT1 CRC and the literature is not supportive of substantial benefit for immunohistochemistry (Harris et al 2008, Kojima et al 2013). There are a number of reasons why these stains are not used on a routine basis. Firstly, these stains add cost and are labour intensive for laboratories and the pathologist. Secondly, applying these special stains adds at a minimum 1

day and may delay the result. Thirdly, special lymphovascular stains such as CD31 and D2-40 have not been shown to improve the inter-observer agreement in the identification of lympho-vascular invasion when compared to the standard H & E stains (Harris et al 2008, Kojima et al 2013).

In performing this study, the general consensus between the pathologists was that they did not think the immunohistochemical stains for lymphatic and vascular invasion would be more beneficial or more predictive for LNM. It was agreed that these stains need further evaluation and to show significant benefit before they can be used routinely in the NHSBCSP.

5.7.4 Depth of invasion

Depth of invasion should ideally be measured from the bottom of the muscularis mucosae (the most superficial part of the lamina propria) to the deepest part of the tumour mass/ cell. This quantitative measurement should be reproducible and simple to perform. However, this is not always the case.

The invading tumour often destroys the muscularis mucosae making it difficult or impossible to measure the depth of invasion from the muscularis mucosae. The other option in measuring the depth of invasion is to measure from the luminal surface to the deepest site of invasion. However, this may introduce two factors that can decrease the reproducibility of this method of measurement. Firstly, ulceration of the surface in some of the cancers may lead to an apparent thinning of the thickness of the lesion and may lead to an under estimation of the depth of invasion.

Conversely, some cancers exhibit exophytic adenomatous growth and this increases the tumour thickness and would lead to an overestimation of the tumour depth.

In this study, we have investigated the reproducibility of the measurement of depth of invasion. The depth of invasion had produced moderate and almost perfect agreement in both round 1 and 2 respectively. The reason behind the high levels of agreement in measuring depth of invasion in this study is that the participating pathologists mainly measured the depth from the epithelial surface of the lesion, as recommended by the Japanese, ignoring the muscularis mucosae (which was absent in most cases). Adopting this would inappropriately increase the resection rate using the Japanese definition of resection if invasion is >1mm.

In this study we also analysed the inter-observer variation of measuring the depth of invasion by generating different types of virtual muscularis mucosae. The first consisted of creating an imaginary muscularis mucosae which would have been present if it had not been destroyed by the cancer. This measurement performed nearly as well as measurement from the surface. The second type of virtual muscularis mucosae is a straight line that connects the edges of the residual muscularis mucosae. This method of creating a virtual muscularis mucosae has not been proven to be prognostic for lymph node metastasis and did not improve the degree of observer variation. However, the measurement of depth from the different types of virtual muscularis mucosae was undertaken to explore the different ways to overcome the problem that arises when measuring depth of invasion when a muscularis mucosae is not present.

5.7.5 Distance to margin

The measurement of the distance from the invasive carcinoma to the resection margin produced reasonable inter-observer agreement. In round 1, an ICC of 0.58 was obtained and this had increased to 0.77 in round 2 when the pathologists had been provided with further guidance.

A perfect or almost perfect agreement is difficult to achieve in measuring the distance to the resection margin. This is due to the method by which the polyps are removed endoscopically within the bowel. The majority of polyps are removed by a diathermy snare or with a similar device. The diathermy produces a considerable amount of heat that burns and coagulates the resection margin of the polyp specimen. This introduces a number of artefactual changes. These changes include retraction of the resection margin into the tissue sample thereby reducing the distance of the resection margin. The heat that the diathermy produces also causes the resection margin to be “jagged”. The blood vessels and connective tissue within the submucosa coagulate at different temperatures and causes the coagulated blood vessels to stand proud of the rest of the tissue of the retracted diathermy margin. There may also be marked clefting along the sides of the coagulated blood vessels because of the differential shrinkage of the vessels and stroma leading to the splitting or fragmentation of the resection margin.

The improvement in inter-observer agreement between the two rounds was achieved after a senior pathologist had made a simple but important recommendation after round one, which was then implemented, in round 2. The recommendation suggested was to adopt a conservative approach when measuring the distance to the margin, ignore retraction artefact and only measure to the margin that they are

confident on. This recommendation has now been introduced into routine practice within the NHSBCSP and confirmed in the latest revision of the pathology guidelines (NHSBCSP 2016).

5.7.6 Width of lesion/carcinoma

In this study, we have defined the width of a lesion as both the width of the carcinoma and adenoma component together. The ICC value for the width of lesion in both round 1 and 2 were in almost perfect agreement (0.728 and 0.949 respectively).

However, when the group of pathologists were requested to measure the width of the carcinoma only in round 2, the ICC value showed poor agreement (ICC value of 0.01). The strong agreement of the ICC values in both rounds for width of the lesion could be attributed to the pathologist easily identifying the boundaries between the normal colonic mucosa and the carcinoma/ adenoma component. In contrast, the poor agreement obtained in measuring the width of carcinoma could be due to the difficulty in identifying the transition from the carcinomatous to the adenomatous component. This might be improved by better guidance and definitions. This is important to test as this measurement performed well in predicting LNM as described in Chapter 2.

5.7.7 Decision to resect

Pathologists currently play a major role in deciding the type of treatment a patient receives for their early colorectal cancer. The decision to resect the bowel containing the early colorectal cancer has important implications for the patient, surgeons and the NHS. Therefore, it is very important that the pathologist gets the decision right.

There are several histopathological factors that can influence and guide a pathologist into making the correct decision. Examples of these histopathological factors are the grade of differentiation of the cancer, lymphatic and vascular involvement, the depth of invasion, the distance of the cancer from the excision margins and the size of the cancer. There are other clinical decisions that may affect the decision of resect the bowel containing the early colorectal cancer such as the pre-morbid state of the patient. However, the treatment decision is never straightforward requiring discussion in the multidisciplinary team.

In this study, we aimed to assess the degree of inter-observer variability in making the decision to resect the bowel containing the early colorectal cancer. This was an artificial process as they were required to make a decision on only one slide and they did not have to define the basis of that decision. In both rounds of the study, only a fair level of agreement could be achieved (0.265 and 0.30 in round 1 and 2 respectively).

The overall fair level of agreement in making the decision for resection could be due to summation of a number of different high risk factors that individually demonstrated poor or moderate inter-observer agreement and others such as incomplete excision that had better inter-observer variation helping to lift the agreement.

The fair level of agreement could also be down to the individual pathologist regarding different histopathological factors as high risk. In this study, there were no standardized criteria implemented in determining the decision for resection, however one would expect individual risk factors such as incomplete excision, poor differentiation, vascular and lymphatic invasion as well as sm3 invasion to lead to such a decision. The multiplicity of potential factors and their inter-observer variation must contribute to this issue. If there is inter-observer variation in differentiation, lymphatic and vascular invasion then the ultimate decision to resect will vary widely. This is most likely the reason for only a fair level of agreement.

5.7.8 Exclusion of non-assessable cases

The decision was made to exclude the non-assessable cases in an attempt to reduce the number of cases when a feature might not be used e.g Haggitt vs Kikuchi in each qualitative analysis and thus, hopefully improve the inter-observer agreement. Re-analysis of the inter-observer variation studies without the non-assessable cases showed slight improvements or no change in the kappa scores with the decision for full resection in both round 1 and 2 showing the greatest improvement in kappa scoring (table 5.4 and 5.5). However, for the classification of Haggitt levels and the assessment of the completeness of excision of the lesion in the modified round 1 showed a decreased kappa score.

Despite the reduction in both the cases and categories in each qualitative analysis, no major improvement in inter-observer agreement was achieved. This highlights the subjectivity of qualitative analysis of these lesions. Therefore, either better

qualitative definitions and guidelines are needed in assessing these lesions to help reduce the inter-observer variability, however this may be in vain due to the subjective nature of many of the definitions or assessments. If possible we need to move to reproducible quantitative assessments.

5.7.9 Prospects of novel quantitative factors

The findings of width of the invading carcinoma and area of submucosal invasion as a predictor for LNM is very promising as these two quantitative factors can be quickly and easily measured on standard H & E slides. Additionally, there is no extra cost in measuring these quantitative factors.

For the width of the invading carcinoma, unfortunately, there was great inter-observer variation (ICC value – 0.01) between the pathologists in the inter-observer study. This is a contrast to the inter-observer variation analysis (to assess the reproducibility of the quantitative factors in chapter 2) done between the main and senior author that produced an ICC value of 0.76. Two issues could explain the discrepancy in the ICC values. Firstly, the caseload for the inter-observer variation analysis between the main/senior author and the group of pathologists were different. Secondly, the number of cases also varied: 10 cases were examined in the inter-observer variation analysis of the main/senior author compared to the inter-observer variation analysis between the pathologist where 41 cases were examined. Despite the poor ICC values obtained in the inter-observer variation analysis between the pathologists for the width of the invading carcinoma, we feel that this quantitative factor is still a simple and valuable factor in the prediction of LNM and as

mentioned earlier, a better inter-observer agreement might be achieved in measuring this factor with better definitions and guidance. Also, we need further studies since the width of carcinoma only missed only 1 case with a LNM that was identified by the area of submucosal invasion.

The area of submucosal invasion had fared much better than the width of invasion in terms of inter-obsever agreement. In this study, the area of submucosal invasion had produced a moderate agreement between the pathologists (ICC value – 0.59). One of the reasons that a better agreement could not be achieved in measuring this quantitative factor is the difficulty in identifying the muscularis mucosae if the muscularis mucosae had been destroyed by the CRC.

It is worth mentioning that the ICC value obtained between the main and senior author is very encouraging (ICC value – 0.95). This shows that with better definitions and adequate training, the inter-observer agreement for the area of submucosal invasion can be improved. We feel that this is necessary as the area of submucosal invasion is an important quantitative factor in predicting LNM.

5.8 Conclusion

The overall results from this study emphasises the urgent need to improve the level of agreement in reporting early CRCs especially the early CRCs identified within the NHS BCSP. The areas that that needed improvement or replacement were mainly qualitative factors such grade of differentiation, lymphovascular invasion and level of invasion based on Haggitt and Kikuchi definitions. Despite the quantitative factors faring slightly better than the qualitative factors in terms interobserver agreement, there is still a lot of work to be done to improve both these factors.

The creation and implementation of the NHS BCSP EQA scheme has helped improve the quality of pathology and maintains a high quality level of reporting within the screening programme. However, the problematic areas must be further addressed and improved. A move to quantitative factors, a robust multivariate model that gives recurrence risk for an individual patient, combined with better reporting guidelines and clarification of the diagnostic criteria paired with training and educational workshops are required. We also agree with Turner et al that further development of pathology networks specialising in the assessment of complex or unusual lesions from the NHSBCSP along with further support via tele-consultation would help improve interobserver agreement (Turner et a 2013).

We hope that these suggestions may improve the risk assessments and ensure a higher standard level of reporting and thus, reducing inter-observer variations between pathologists. Nevertheless, the search for better predictors of metastasis must go on.

Chapter 6 Overall conclusions and future work

6.1 The future of the NHS BCSP

The introduction of the NHS BCSP has led to an increase in detection of CRC within the screened population. The NHS BCSP has also increased the diagnosis of early and favourable staged CRC. As of May 2016, 25,528 CRCs have been detected via FOBt and 248,109 high risk polyps were identified (P Quirke 2016, personal communication). This has resulted in an increase in both local procedures and radical resections.

The numbers of radical resections and local procedures are expected to further increase in the near future with the replacement of FOBt with faecal immunochemical testing (FIT). FIT is an important technical improvement to the standard FOBt. FIT works principally by the detection of the human globin protein by specific antibodies. Whilst the haem component of haemoglobin is common to all species, globin is species specific and so FIT should not be subjected to interference by blood from normal dietary intake. The Japanese have been using FIT in their bowel cancer screening schemes since the 1980s (Saito et al 2000, Ross 2010). In the Netherlands, studies have shown that participation and detection rates for advanced adenomas and CRC were significantly higher for FIT when compared to FOBt (van Rossum et al 2008, van Rossum et al 2009). Therefore, we may expect and increase detection of early CRC with the introduction of FIT.

The UK Flexible Sigmoidoscopy Screening Trial has shown that flexible sigmoidoscopy is a safe and practical screening test that offers substantial and long term benefits (Atkinet al 2010). Together with the NHS BCSP, it is hoped that in the future, CRC would be discovered at an earlier stage and thus reducing stage 4 diseases and improving outcomes in the treatment of CRC.

6.2 The challenges of diagnosing and treating pT1 CRCs

The management of patients with pT1 CRCs is highly dependent on the quality of the histological reporting of these CRCs. One of the challenges faced by pathologists is the inter-observer variability in the histological assessment of pT1 CRCs. There is still a substantial degree of inter-observer variability in the histological assessments of high risk features such as grade of differentiation, lymphatic and vascular invasion and this was evident in this study. These findings are consistent with those of previous studies (Foss et al 2012, Turner et al 2013) and highlights that there is still room for improvement in the histological assessment and reporting of pT1 CRCs. This study also highlights the need for development of better definitions and clarification for the current histopathological reporting criteria of these high risk features and inclusion of these in NHS BCSP reporting guidelines leading to fewer discrepancies in histopathological reporting and improved patient management.

Another challenge faced by the multi-disciplinary team is choosing the correct treatment for patients with early CRCs. With the NHS BCSP in place, the huge shift towards early CRCs has had a major impact on the treatment that is provided for these cancers. The main goal of treatment in early colorectal cancer is to provide a

cure with no or minimal morbidity and mortality risk. Therefore, histopathological analysis plays an important role in deciding the type of treatment that can be offered to patients with pT1 CRCs. Known subjective high risk histopathological features such as poor grade of differentiation, lymphatic and vascular invasion have already helped dichotomize patients into two main treatment groups; local procedure or radical resection. In this present study, we have identified three further novel quantitative histopathological features that may be useful in the therapeutic decision making. These three quantitative features are the width of invasion of the cancer, area of submucosal invasion and proportion of tumour stroma (PoTS). Both the area of submucosal invasion and PoTS are independent prognostic markers for LNM in our studies.

The width of invasion and area of submucosal invasion have been shown to be reproducible in this study (width of invasion: ICC score of 0.728 and 0.949 in round 1 and round 2 of the inter-observer variation study; area of submucosal invasion: ICC score of 0.95 in the reproducibility test between main and senior author). These two quantitative features are also applicable to all pT1 CRCs, even those of different shapes and therefore, could hopefully be used as a substitute for the Haggitt and Kikuchi classifications which require different assessment methods dependent on whether flat, raised or polypoid. Another benefit of using these quantitative factors is that there will less subjectivity in assessing pT1 lesions compared to the well-known high risk qualitative factors such as grade of differentiation, lymphatic and vascular invasion.

The PoTS is an interesting and new quantitative feature. Our studies have shown that a high PoTS is a prognostic factor for LNM and could be useful in therapeutic

decision making. However, this novel quantitative factor will need to be tested and validated in a larger series of pT1 CRC compared to other suggested factors.

As shown in this study, screened pT1 CRC are smaller in size compared to symptomatic pT1 CRC. Therefore, the treatment options for screened pT1 CRC will either consist of conservative or surgical management. The conservative management includes watchful waiting and monitoring with radiological analysis (MRI). Surgical management on the other hand includes local procedures (TEMS and EMR) or radical resection the cancer. The main determinant for either treatment options is whether the pT1 CRC is at risk of LNM. Thus, we hope that the findings of these new and novel quantitative features will help stratify screened pT1 CRC patients into the correct treatment groups and thereby minimizing mortality and morbidity.

6.3 Future works

Further studies are needed in screened and non-screened populations to validate these novel quantitative features. The cut-off values of the novel quantitative factors were obtained by the ROC curves are a hypothesis and will need validating in multiple larger independent series of pT1 CRC.

In regards to improving inter-observer variation between pathologists, clearer definitions and guidelines need to be developed, tested and validated ideally by a group of pathologist who are part of the NHSBCSP. If possible grading should be automated or replaced, as this is an important feature predicting LNM. Poorly differentiated tumours must also be tested for deficient mismatch repair to exclude

this as a cause of the poor differentiation due to their lower metastatic potential (Braun et al 2008, Hutchins et al 2011, Smith et al 2013)

Another phenotypic feature in CRC that needs further evaluation is tumour budding. Tumour budding has been recognized as an important additional prognostic factor. Tumour budding in CRC has been associated with poorer overall survival and increased risk of LNM and/or distant metastasis (Hase et al 1995, Nakamura et al 2005, Lugli, Karamitopoulou and Zlobec 2012, Koelzer, Zlobec and Lugli 2014, Koelzer, Zlobec and Lugli 2016). Tumour budding may help identify pT1 CRC that are high risk for LNM (Wang et al 2005, Yamauchi et al 2008, Tateishi et al 2010, Nakadoi et al 2012). Tumour budding may also prove to be an additional indicator for neoadjuvant therapy in early rectal cancers (Morodomi et al 1989, Rogers et al 2014).

However, at this present time, tumour budding is not routinely used in the reporting of CRC. The two main reasons are because there is no standardized method or definition in measuring tumour budding and the inter-observer agreement in tumour budding is still variable (Koelzer, Zlobec and Lugli 2016, Okamura et al 2016). Efforts have already been made to improve inter-observer agreement in assessing tumour budding but results are conflicting. Kai et al (2016) showed that the use of cytokeratin immunohistochemistry (AE1/AE3 immunostain) improved the evaluation of tumour budding and reduced inter-observer variability by less experienced pathologists. However, Okamura et al (2016) showed that tumour budding detected by cytokeratin immunohistochemistry (CAM 5.2 immunostain) was not superior to the standard H & E stain. The results of Okamura's study is promising as there is no

need for extra staining of the specimen and the standard H & E staining would suffice.

Hence, the assessment of the possibility of using tumour budding as a prognostic factor should be undertaken. In particular, a standardized method and optimal scoring method should be established. This has just been achieved at a recent consensus conference allowing the place of budding to be effectively tested (P Quirke 2016, personal communication) and compared to other factors and this is now on-going in our laboratory.

6.4 Conclusion

These are exciting times for everyone involved in CRC screening and treating early CRCs. The results of the NHS BCSP are very encouraging with the increased detection of early CRCs and improved outcomes. Similarly, the goal of providing a more personalised treatment for patients based on the phenotypic features of their early CRC appears achievable with the promising novel high risk features identified in this study. This will not only be advantageous to patients in terms of reducing morbidity/ mortality and increasing cure rates but it will also reduce healthcare costs.

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Appendices

Appendix I: Invitation letter to 1st round of interobserver study

Dear Colleague,

As you are aware from the committee meeting we are keen to investigate the reproducibility of the factors and measurements we are asking our colleagues to make on pT1 cancers. We would like you to undertake 10 key tasks on this occasion and we will ask you to repeat this in 6 months time.

These tasks are:

1. To define the shape of the lesion (Pedunculated / Semi-pedunculated/ Sessile)
2. To define the grade of differentiation
3. To identify the distance of carcinoma to the nearest margin
4. To identify the presence of lymphatic invasion
5. To identify the presence of vascular invasion
6. To state whether lesion can be assessed by Haggitt's levels and identify the level of invasion(Level 1/ 2/ 3/ 4)
7. To state whether lesion can be assessed by Kikuchi's levels and identify the level of invasion (SM1/ SM2/ SM3)
8. To measure the width of carcinoma
9. To measure the depth of invasion of the carcinoma
10. To state whether the lesion/ carcinoma is fully excised and/or should be resected

The cases can be accessed on the website as below:

http://www.virtualpathology.leeds.ac.uk/demo/et.php

The findings will be anonymised and your data fed back against everybody else after the second round. All participants will be included as authors. If you do not

have time to undertake this exercise, then we will have to exclude you from the authorship but we would like a high level of participation so as inform recommendations to the NHS Bowel Cancer Screening Programme.

If you have any technical issues please contact Eu-Wing Toh (euwing@gmail.com) and if you have any matters of principle please contact me p.quirke@leeds.ac.uk

Yours sincerely,

Professor Phil Quirke

Appendix II: Invitation letter to 2nd round of interobserver study

Dear Colleague,

Following on from the recent NHS BCSP committee meeting and the revised diagnostic criteria that we redeveloped we continue to be keen to investigate the reproducibility of the factors and measurements that we undertook on the last series of pT1 cancers. We would like you to undertake 11 key tasks on this occasion.

These tasks are:

1. To define the shape of the lesion (Non-sessile/Sessile) and also measurements of the width and base of lesion in defining the shape of the lesion (appendix I). The width of the lesion in this context is the width of the adenoma and carcinoma component together
2. To measure the width of carcinoma (carcinoma component only)
3. To identify the distance of lesion to the nearest margin based on your methods and the new method described by Dr Da Costa(see appendix II)
4. To measure the depth of invasion of the carcinoma based on 4 methods (see appendix II)
5. Area of invasion of carcinoma below the muscularis mucosae (see apendix III)
6. To define the grade of differentiation (Non-poor/ Poor)
7. To identify the presence of lymphatic invasion
8. To identify the presence of vascular invasion
9. To state the Haggitt's levels (Level 1/ 2/ 3/ 4)
10. To state the Kikuchi's levels (SM1/ SM2/ SM3)
11. To state whether the lesion/ carcinoma should be resected and state why it should be resected

We would like to request that all the tasks be answered to the best that is possible and please try not to give answers as 'non-applicable'. Similar to the previous studies, the findings will be anonymised and your data fed back against everybody in an upcoming meeting. All participants will be included as authors. If you do not have time to undertake this exercise, then we will have to exclude you

from the authorship but we would like a high level of participation so as inform recommendations to the NHS Bowel Cancer Screening Programme.

If you have any technical issues please contact Eu-Wing Toh euwing@gmail.com and if you have any matters of principle please contact me p.quirke@leeds.ac.uk

Best wishes,

Eu-Wing Toh and Phil Quirke