The diagnostic molecular pathology of colorectal carcinoma using automated PCR

A thesis submitted for the degree of

Doctor of Medicine

by research

Presented by

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February 2017

Declaration

I, Richard Colling confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Richard Colling

23rd of February 2017

Abstract

Background: Diagnostic molecular testing in colorectal cancer (CRC) offers a number of benefits including predicting prognosis, directing targeted therapies and screening for hereditary cancer syndromes. Molecular testing however is expensive, requires specialist facilities and staff and is time consuming, limiting its widespread availability. The Idylla System is an automated testing platform that could overcome these issues.

Aims: To appraise the suitability of the Idylla System for use in clinical practice by evaluating the system's accuracy and financial impact.

Hypothesis: The Idylla System has high accuracy for detecting mutations in *BRAF*, *KRAS* and *NRAS* genes in CRC resection tissue and is a cost-effective alternative to current testing platforms.

Methods: Ethical approval was granted by Oxfordshire Research and Ethics Committee A (reference: 04/Q1604/21). Diagnostic accuracy was determined for the Idylla System in detecting *BRAF* and *KRAS* mutations with a comparison against conventional polymerase chain reaction (PCR). Further validations were also performed for *BRAF*, *KRAS* and *NRAS* mutation testing against NGS and IHC methods. An audit of the molecular diagnostics workload was carried out and a cost-analysis performed.

Results: The Idylla system had a sensitivity of 100.0% (95% CI: 88.3% to 100.0%) and a specificity of up to 100.0% (95% CI: 94.7% to 100.0%) for detecting *BRAF* mutations and a sensitivity of 100.0% (95% CI: 79.6% to 100.0%) and a specificity of up to 92.9% (95% CI: 68.5% to 98.7%) for detecting *KRAS* Mutations. There was 100% concordance for *NRAS* testing. A cost-analysis estimated that the Idylla System could save from around £12,000 to anywhere up to £40,000 per year in some centres.

Conclusions: The results support the hypothesis that the Idylla System is an accurate system for detecting relevant mutations in CRC and demonstrate the system to be cost-effective. The Idylla system is therefore suitable for use in routine clinical practice.

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Acknowledgments

This project and thesis could not have been completed without the help and advice of a number of dedicated mentors. I would firstly like to thank my UCL supervisors, Manuel Rodriguez-Justo and Marco Novelli, for their support and guidance in carrying out this work. I would also like to thank the Divisional Graduate Tutors, Julie Olszewski and John Hartley, and my thesis examiners. I would like to thank my local Oxford supervisors, Lai Mun Wang and Elizabeth Soilleux, for all their support and guidance during this project.

I would like to thank all of the staff at the Department of Cellular Pathology in the John Radcliffe Hospital, Oxford for their help and support in accommodating my work in the laboratory and wider department. I would like to thank in particular the clinical lead Derek Roskell and laboratory manager Sharon Roberts-Gant for supporting me and allowing me to undertake my work within the institution. I also thank Helene Euston-Mellor who assisted me with the slide scanning to produce the images in Figures 1 and 2. I would again like to thank the lead clinician for the laboratory, Elizabeth Soilleux for supervising me while in the laboratory and for collaborating to carry out the project under her ethics provision.

I would like to thank the all the staff in the Department of Oncology at the University of Oxford, in particular Anna Schuh, for supporting me as a Molecular Diagnostics Fellow whilst carrying out this work. I would like to thank the staff at Health Education Thames Valley and my Educational Supervisor Ioannis Roxanis for their support in my pursuit of this thesis whilst an Academic Clinical Fellow and Specialty Registrar in Histopathology in Oxford, and later whilst Out Of Programme. I would like to thank the Oxford Centre for Histopathology Research for their assistance in providing and handling patient tissue. I would like to thank the Oxford Molecular Diagnostics Centre for carrying out the comparison molecular tests. I would like to thank the Oxfordshire Health Services Research Committee for assisting in funding part of this project with a research grant. I would also like to thank Biocartis for providing the Idylla System on trial and their support in completing the project.

Finally, I would like to my family and friends who have shown great support, patience and guidance over the course of the work carried out herein.

Publications arising from this work

Some of the data and ideas in this thesis have been (or are planned to be) presented in published works or posters. This is indicated in all relevant sections as far as possible and to the best of the author's knowledge. Any minor omissions, if present, are unintentional. A copy of each of these publications and posters is present at the end of this thesis.

Publications arising from this work:

Colling R, Church D, Carmichael J, Murphy L, East J, Risby P, Kerr R, Chetty R, Wang LM. Screening for Lynch Syndrome & Referral to Clinical Genetics By Selective Mismatch Repair Protein Immmunohistochemistry Testing: An Audit and Cost Analysis. *J Clin Pathol* 2015;68(12):1036-9. doi:10.1136/jclinpath-2015-203083.[1]

Colling R, Wang LM, Soilleux E. Automated PCR detection of *BRAF* mutations in colorectal adenocarcinoma: a diagnostic test accuracy study. *J Clin Pathol* 2015;69:398-402. doi:10.1136/jclinpath-2015-203345.[2]

Solassol J, Vendrell J, Märkl B, Haas C, Bellosillo B, Montagut C, Smith M, O'Sullivan B, D'Haene N, Le Mercier M, Grauslund M, Melchior LC, Burt E, Cotter F, Stieber D, Schmitt FL, Motta V, Lauricella C, Colling R, Soilleux E, Fassan M, Mescoli C, Collin C, Pagès JC, Sillekens P. Multi-Center Evaluation of the Fully Automated PCR-Based Idylla™ *KRAS* Mutation Assay for Rapid *KRAS* Mutation Status Determination on Formalin-Fixed Paraffin-Embedded Tissue of Human Colorectal Cancer. PLoS ONE 2016 11(9): e0163444. doi: 10.1371/journal.pone.0163444.[3]

Colling R, Wang LM, Soilleux E. Validating automated PCR for use in the molecular diagnostics of colorectal carcinoma. Accepted for publication in *J Clin Pathol* (in press).[4]

Poster presentations:

Colling R, Church D, Carmichael J, Murphy L, East J, Risby P, Kerr R, Chetty R, Wang L. Screening for Lynch Syndrome in the UK: An Audit and Cost Analysis. Mod Pathol 2016;28(Suppl 2):497A (Abstract 1998). doi:10.1038/modpathol.2015.29.[5]

Colling R, Wang LM, Soilleux E. Validating a Novel Automated PCR System for use in the Molecular Diagnostics of Colorectal Adenocarcinoma. J Pathol 2016;240(Suppl 1):S31. doi: 10.1002/path.4818.[6]

Colling R, Wang LM, Soilleux E. Sensitivity and Specificity of Automated PCR (Idylla) in the Detection of *BRAF* Mutations in Colorectal Carcinoma. Pathol 2016;240(Suppl 1):S31. doi: 10.1002/path.4818.[7]

Colling R, Wang LM, Soilleux E. Sensitivity and Specificity of Automated PCR (Idylla) in the Detection of *KRAS* Mutations in Colorectal Carcinoma. Pathol 2016;240(Suppl 1):S31. doi: 10.1002/path.4818.[8]

Chapter 1: Introduction

1.1 Introduction to the thesis

This thesis is focused on the diagnostic molecular pathology of bowel cancer. This is a disease which is a significant health problem in the United Kingdom (UK) and much improvement is still needed.[9] Genetic testing of cancer tissue is one way in which the outcome for patients is hoped to improve. Mutations in three key genes, *BRAF*, *KRAS* and *NRAS* play a significant role, not only in the development of bowel cancer, but also in guiding the course of treatment for these patients. Testing for mutations in these genes has been recommended for several years now and is therefore increasingly common in National Health Service (NHS) laboratories.[10-14] However despite guidelines, testing is still not universal for some of these genes and this is probably due to a lack of resources and facilities.[15] The problem could be addressed by any number of the new technologies that are constantly being developed in the field of molecular diagnostics, but proper evaluation of such new systems is vital if we are to provide valuable and meaningful diagnostic information. The aim of this thesis is to investigate a new molecular diagnostics platform which could help address the problem of under-testing in bowel cancer across the NHS. This work will assess the accuracy of this new technology and the potential for it to overcome many of the barriers to universal testing.

1.2 Bowel cancer

1.2.1 The epidemiology and aetiology of bowel cancer

Bowel cancer is overall the fourth most common cancer in the UK (excluding non-melanoma skin cancer). Just over 40,000 cases are diagnosed each year, accounting for 10-15% of all new cancers. Bowel cancer mortality in the UK is around 30% and causes almost one in 10 cancer-related deaths.[9] Internationally, there are 1.3 million cases per year and these result in over 600,000 deaths.[16] The incidence of bowel cancer is slightly higher in men, but for both men and women individually it is the third most common type of cancer. The incidence rises sharply after the age of 50 years and the mean age of presentation is 70.[9]

The aetiology of bowel cancer is poorly understood but is likely to be a combination of genetic and environmental factors.[10] There is good evidence that family history is a strong risk factor for developing bowel cancer. This familial risk is largely due to polygenic inheritance, but a number of inherited cancer syndromes are also directly linked to developing bowel cancer.[10,17,18] Life-style probably plays a significant role in the development bowel cancer as well, with obesity, poor diet (high in fat and red meat, low in fiber) and a high intake of alcohol all associated with an increased risk.[19-25] Another

important group at risk of bowel cancer are those with other pre-existing disorders of the gut, such as inflammatory bowel disease.[26]

1.2.2 Relevant anatomy, histology, and physiology of the large bowel

The gastrointestinal tract develops from the endoderm layer of the embryonic trilaminar disc. From around week four of gestation, the disc undergoes folding during which the endoderm forms a tube running the length of the embryo. From this tube the mouth, pharynx, oesophagus, stomach, small and large intestine and anus form.[27] The gut is embryologically divided into the foregut (mouth to duodenum), midgut (duodenum to the distal third of the transverse colon) and hindgut (distal transverse colon to anus). Many of the accessory glands of the gut (the liver, pancreas etc.) develop from out pouchings of the endoderm early in organogenesis.[28,29]

The bowel is a tube-like structure that comprises the mid and hindgut structures of the small intestine (duodenum, jejunum, ileum) and large intestine (caecum and vermiform appendix, colon, rectum and anal canal).[30] The large bowel begins at the caecum, which is around 10cm in length and sits in the right iliac fossa. Here, the small bowel (ileum) empties through the ileocecal valve into the large bowel. From the caecum the bowel extends retroperitoneally for about 20cm superiorly as the ascending colon into the right upper quadrant and, at the level of L2, forms the hepatic flexure. The bowel then extends across the body, intraperitoneally, for about 45cm as the transverse colon and, at the level of T12, forms the splenic flexure in the left upper quadrant. Next, the bowel extends retroperitoneally and inferiorly for around 25cm as the descending colon, into the left side of the pelvis.[31] Once the bowel enters the pelvis it forms a 40cm section known as the sigmoid colon, due the S-shape course it takes. The anatomical boundaries of the sigmoid colon are not defined consistently however.[32] At approximately the level of S3, just above the reflection of the peritoneum, the bowel forms a straight 12cm section known as the rectum.[31,33,34] At the level of the levator ani muscle the bowel is known as the anal canal, a portion that extends for around 12cm to the anus.[31,35]

The colon is attached to the abdominal wall by a fold of fatty connective tissue, known as the mesentery. Blood vessels, lymphatics and nerves all traverse the mesentery centrally, to supply the wall of the bowel.[36] The midgut portions of the large bowel receive a blood supply from branches of the superior mesenteric artery and drain by the superior mesenteric vein into the portal system. Hindgut structures are largely supplied by the inferior mesenteric vessels, with the middle and lower thirds of the rectum supply from the internal iliac vessels. The lymphatic system of the bowel is rich and drains in a course that follows the vasculature, with lymph nodes scattered at junctional points along this course.[37] Within the bowel wall is a complex network of autonomic nerves, known as the enteric nervous system. This system regulates bowel wall muscle tone and the secretions of the

bowel during digestion. Although the enteric nervous system functions somewhat autonomously, it receives spinal cord input from higher centers which influence digestion.[38]

The colon is a main site for the absorption of water in the body and, in doing this, functions to form and store faecal matter.[39] The luminal surface of the bowel is lined by mucosa and this comprises a layer of simple (one cell thick) columnar epithelium sitting on a basement membrane, overlying lamina propria (loose connective tissue) and a thin layer of smooth muscle known as the muscularis mucosae. The epithelium is arranged in test-tube shaped intestinal glands that increase the surface area for absorption. This is shown in Figure 1. Scattered between epithelial cells are mucous secreting goblet cells, occasional neuroendocrine cells which play a role in modulating the enteric nervous system and (in the right side of the colon) Paneth cells, which may function as part of the innate immune system.[40,41] At the junction between the upper two-thirds and lower one-third of the anal canal is the dentate or pectinate line, which marks a transition from glandular epithelium to squamous epithelium.[42] Hilton's white line marks the progression to keratinisation of the squamous epithelium,[43] which is continuous with the epidermis of the anal margin skin.[35,44]

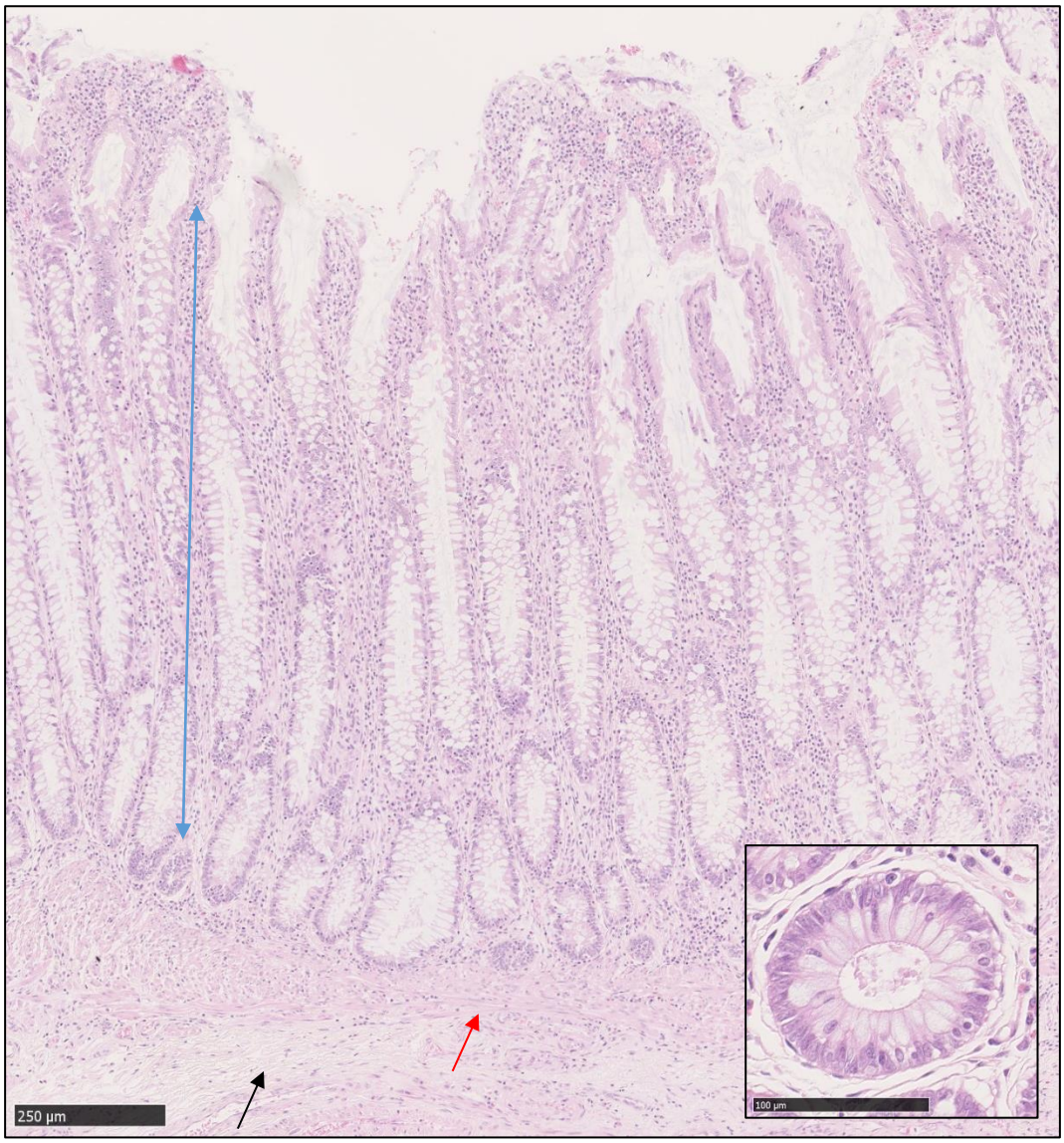

Figure 1. Photomicrograph of normal colorectal histology at low power. The mucosa is seen side on showing the intestinal glands arranged vertically (blue arrow), often said to resemble test tubes in a rack. The underlying pink fibers of the muscularis mucosae are also seen (red arrow), along with the lighter underlying connective tissue of the submucosa (black arrow). The deeper muscularis propria is not seen in this view. Inset: high power view of a normal intestinal gland as seen from above (cross cut) and surrounded by lamina propria. The epithelial cells are neatly arranged in a single layer with small regular basal nuclei. The gland lumen in seen in the center. H&E staining scanned at x400. *H&E = haematoxlyin and eosin*

Below the mucosa is a connective tissue layer known as the submucosa that extends to the underlying circular smooth muscle layer known as the muscularis propria.[41] This muscle layer is involved in the contractile peristalsis that propels bowel contents along its route. A second, deeper layer of longitudinal muscularis propria also exists and this is continuous with three bands of muscle that extend along the length of the intra-abdominal surface of the colon, known as the taenia coli.[41] The contractile nature of the taenia causes shortening of the overall length of the large bowel, causing the wall to fold in on itself and form pouched areas known as haustra. In the distal parts of the rectum, the muscularis propria transitions into to skeletal muscle as part of the rectal and anal sphincters.[31] In various locations, along the bowel length are aggregates of mucosal-associated lymphoid tissue (MALT) present. MALT plays an important role in the adaptive immune system defenses in the bowel and helps regulate the commensal population of bacteria normally present in the colon.[45]

1.2.3 The classification of large bowel tumours

'Tumour' is a Latin word originally used to describe any swelling in the body (similarly the term 'oncology' is from the Greek 'oncosis' meaning swelling). Over time this has evolved and its use today usually refers to an abnormal, neoplastic (new growth) expansion of cells forming a tissue mass. Such tumours that remain localised to the tissue of origin are generally regarded as benign. Tumours comprising cells that develop the ability to infiltrate and invade surrounding tissues, as well as travel to distant parts of the body, are usually referred to as malignant. Although benign tumours can result in fatal consequences through local compression (such as benign meningiomas of the skull compressing the brain stem), in general it is malignant tumours that disseminate, take over the normal structure of the body and lead to death. The word 'cancer' is a general term used to refer to tumours that are malignant. When considering cancers of any site in the body, it is generally helpful to consider these in the context of all tumour types found in that tissue.[46]

Bowel cancer most commonly affects the large intestine; small bowel tumours are rare and account for only tiny burden (<3%) of the disease.[47] Therefore, the focus of this work is on large bowel tumours, referred to in the literature as 'colorectal'. Most colorectal tumours, benign and malignant, arise from and distend the mucosal lining of the bowel.[48] This usually forms a structure protruding into the luminal space, referred to as a polyp. Polyps in the traditional description are pedunculated (connected to the bowel with a stalk) but often they appear as a more gradually raised (sessile) area. Flat lesions are relatively uncommon but almost any of the tumours described here could theoretically present as flat lesion. Polypoidal lesions of the colorectum due to mucosal inflammation (inflammatory polyps, lymphoid aggregates) are not usually regarded as tumours and thought to have no malignant potential.[49]

In the UK, bowel cancers are reported following guidelines set out by the Royal College of Pathologists (RCPath) and the NHS. These guidelines largely follow the classification of bowel cancer given by the World Health Organization (WHO), summarised in Table 1. The WHO classification includes benign and malignant tumours.[13,48,50] Primary epithelial tumours of the colorectum are subdivided into non-invasive and invasive lesions. Non-invasive epithelial lesions include 'conventional' adenomas, serrated (saw-toothed appearance of the mucosa) lesions and hamartomas (non-neoplastic, benign masses of abnormally arranged tissue). Each is further sub-classified based on the histological architecture. Non-invasive lesions are benign and their prognosis is excellent if completed removed. However these lesions may or may not contain dysplasia.[48] Dysplasia is a form of neoplasia and is said to be a 'pre-malignant' feature because there is an increased risk of malignant progression (invasion) of the tumour. Dysplastic tumours share morphological features with malignant tumours (atypical morphology such as a disordered proliferation of glands and cells showing nuclear enlargement, irregularity and hyperchromasia) but lack invasion of surrounding tissues (malignancy is by strict definition invasion through a basement membrane).[46] The degree of dysplasia, where present, is graded as low or high.[48]

Epithelial Tumours
Non-invasive
Adenoma (with low or high-grade dysplasia)
Tubular
Villous
Tubulovillous
Serrated lesions
Hyperplastic polyp
Sessile serrated adenoma/polyp (sessile serrated lesion)
Traditional serrated adenoma
Hamartomas
Cowden-associated polyp
Peutz-Jeghers polyp
Carcinomas (invasive)
Adenocarcinoma
Cribriform comedo-type adenocarcinoma
Medullary carcinoma
Mucinous adenocarcinoma
Micropapillary carcinoma
Serrated adenocarcinoma
Signet-ring cell carcinoma
Adenosquamous carcinoma
Spindle cell carcinoma
Squamous cell carcinoma
Undifferentiated carcinoma
Neuroendocrine neoplasms
Neuroendocrine tumour (NET): grade1/2 (carcinoid)
Neuroendocrine carcinoma (NET grade 3): large/small cell
Mixed adenoneuroendocrine carcinoma
Enterochromaffin cell, serotonin-producing NET
L cell, glucagon-like peptide-producing & other peptide-producing NETs
Mesenchymal tumours
Leiomyoma
Lipoma
Angiosarcoma
Gastrointestinal stromal tumour
Kaposi sarcoma
Leiomyosarcoma
Lymphoma
Secondary/metastasis

Table 1. The WHO histological classification of tumours of the colorectum.[48]

Conventional adenomas are very common, most showing a tubular architecture that appears to recapitulate the normal tube like structure of the bowel mucosa. Villous adenomas form finger-like projections and are slightly less common. All conventional adenomas have at least low-grade dysplasia by definition and have the potential to develop high-grade dysplasia or invasive carcinoma. Serrated lesions include hyperplastic polyps (HP), which are probably by far the most common tumour of the colorectum.[48] Hyperplasia refers to a non-neoplastic, overgrowth of normal tissue.[51] The malignant potential of HPs (and hamartomas) has been debated for some time, but in the non-syndromic setting this is likely to be very low.[46,52,53] Sessile serrated adenomas/polyps lesions (SSL) are a less common type of serrated lesion and these have similar appearances to HPs. In the United States (US), SSLs are known as sessile serrated adenoma/polyps (SSA/P). Unlike conventional adenomas, SSLs are not dysplastic by definition but may harbor foci of low or high-grade dysplasia and as such do have malignant potential.[34,49,54] The final type of serrated lesion is the traditional serrated adenomas (TSA). TSAs are relatively uncommon but were described before SSLs and hence were designated 'traditional' in recognition of more recently described SSLs. TSAs look somewhat different in appearance to SSLs or HPs and they are considered to have at least low-grade dysplasia by definition.[34,48] Polyps with overlapping appearances of many of the various categories are also recognised. Occasionally, small areas of early invasion (by adenocarcinoma) are seen in polyps and these cases are termed 'polyp cancers'.[48,55,56]

There are several subtypes of invasive tumour (cancer) of the colorectum in the WHO classification. The most common are those of epithelial origin (carcinomas). Carcinomas include adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, spindle cell carcinoma. This study will focus on the most common (90%) subtype of carcinoma, adenocarcinoma, and therefore from herein 'colorectal cancer' (or 'CRC') will be taken to mean this subtype. Most conventional adenocarcinomas display no specific features, however around 5% of tumours display a discrete morphological subtype. The subtypes include mucinous (rich in mucin and mucin producing cells), signet-ring cell, medullary (very hyperchromatic cells) and serrated (saw-tooth) carcinomas.[10,48,57,58] An example of the typical morphology of a conventional colorectal adenocarcinoma is shown in Figure 2.

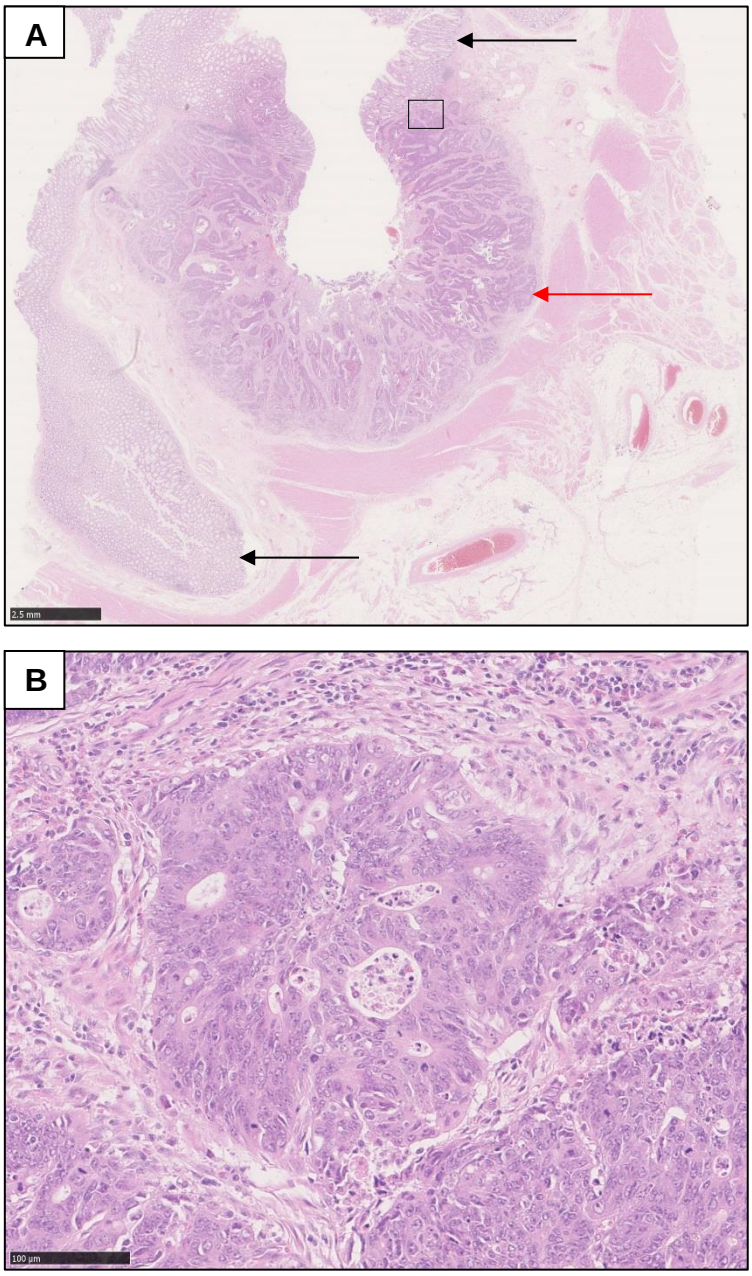

Figure 2. Photomicrograph of colorectal adenocarcinoma histology. ***A.*** The typical low power morphological appearances of a moderately differentiated adenocarcinoma of the colorectum (red arrow) with background normal epithelium present either side (black arrows). The dark pink band of muscularis propria can be seen here (running from top right to bottom left) lying deep to the mucosa and overlying serosal blood vessels and fat. ***B.*** A high power view of a malignant tumour nest within the submucosa (area from square in *A*), showing an attempt at glandular formation (as seen in moderately differentiated tumours), crowded and disorganised cells, nuclear enlargement and hyperchromasia, mitoses and central/luminal necrosis. Compare with the normal glandular structure demonstrated in the Figure 1 insert. H&E staining scanned at x400. *H&E = haematoxlyin and eosin*

Neuroendocrine tumours and non-epithelial tumours (including lymphomas and mesenchymal tumours) are uncommon and are not considered further in this thesis. Tumours arising in the appendix and the squamous lined portions of the anal canal and anus are usually considered separately from colorectal tumours and will also not be considered further in this work. Although metastasis to the colorectum is rare, extension of local tumours (e.g. of the bladder) is relativity common – this study however focuses on tumours arising from the colorectum and therefore secondary tumours will not be considered further in this thesis.[10,48,57]

1.2.4 The pathogenesis of colorectal cancer

Cancer is a genetic disease. Knudson described cancer development as an accumulation of mutations in key regulatory genes.[46] Genetic dysregulation results in tumour cells that are phenotypically characterised by several key hallmarks including the, propensity to proliferate, resistance to cell death, evasion of growth suppression, evasion of the immune system, an alteration of cell metabolism, the induction of blood vessel formation (angiogenesis) and the ability to invade and migrate.[59] The pathogenesis of CRC is one of the well-established models of cancer development and demonstrates many of these hallmarks.[10,48] Table 2 shows an overview of the major genetic abnormalities present in sporadic CRCs[60] that are discussed throughout this Subsection.

Gene	Lesion	Consequence	Clinical Significance
APC	Deleted or mutated in 90% of CRCs	Wnt signaling dysregulation and β-catenin accumulation	Little prognostic significance, not routinely tested (germline mutations in FAP)
KRAS	Point mutation in 30-40% of CRC	Over-activation of EGF/MAPK signaling and proliferation	Predicts prognosis and response to some treatments, routinely tested in clinical practice
NRAS	Point mutation in 5% of CRC	Over-activation of EGF/MAPK signaling and proliferation	Predicts prognosis and response to some treatments, often tested in clinical practice
18q	LOH in 70% of CRCs, results in SMAD4 deletion	Increased proliferation and progression to cancer, common in CIN	Little prognostic significance, not routinely tested
TP53	Mutation or deletion due to LOH 17p in 50% of CRCs	Increased proliferation and progression to cancer	Little prognostic significance, not routinely tested
BRAF	Point mutation in 10% of CRC	Over-activation of EGF/MAPK signaling and proliferation	Predicts prognosis and response to some treatments, used in Lynch syndrome screening routinely tested in clinical practice
MLH1	Hypermethylation (80%) or point mutation in 10% to 15% of CRC	MMR deficiency and MSI	Predicts prognosis and response to some treatments, used in Lynch syndrome screening routinely assessed by IHC in clinical practice

Table 2. An overview of the common major genetic abnormalities present in sporadic colorectal cancer.[48,60] Underlined are the clinically significant genes that are recommended for testing in routine practice. These include *KRAS*, *NRAS*, *BRAF* and *MLH1*.[11-14,61] *CRC = colorectal cancer, LOH = loss of heterozygosity, EGF = epidermal growth factor, MAPK = mitogen-activated protein kinase, CIN = chromosomal instability, MMR = mismatch repair, MSI = microsatellite instability, FAP = familial adenomatous polyposis, IHC = immunohistochemistry.*

The first model of CRC was described by Vogelstein and presents carcinoma as occurring in a step-wise sequence from normal tissue.[60,62,63] This sequence is demonstrated Figure 3. The first step in the adenoma-carcinoma sequence is the development of a conventional adenoma with low-grade dysplasia, arising from normal tissue due to mutations in various genes. A key gene identified early on was *APC* (adenomatous polyposis coli).[60,62] APC is involved in the Wnt signaling pathway that regulates β-catenin concentrations in the cytosol. β-catenin is a protein which, when translocated to the nucleus, results in the activation of a wide range of proliferation and anti-apoptotic pathways. β-catenin in turn is regulated by APC, the latter forming a destruction complex with other mediators to target β-catenin for ubiquitiniation. Wnt signaling inactivates the destruction complex, resulting in accumulation of β-catenin and increased cell survival.[64,65] Loss-of-function mutations in the *APC* gene are found in over 90% of CRCs and are one of the first steps in the adenoma-carcinoma sequence.[60,66]

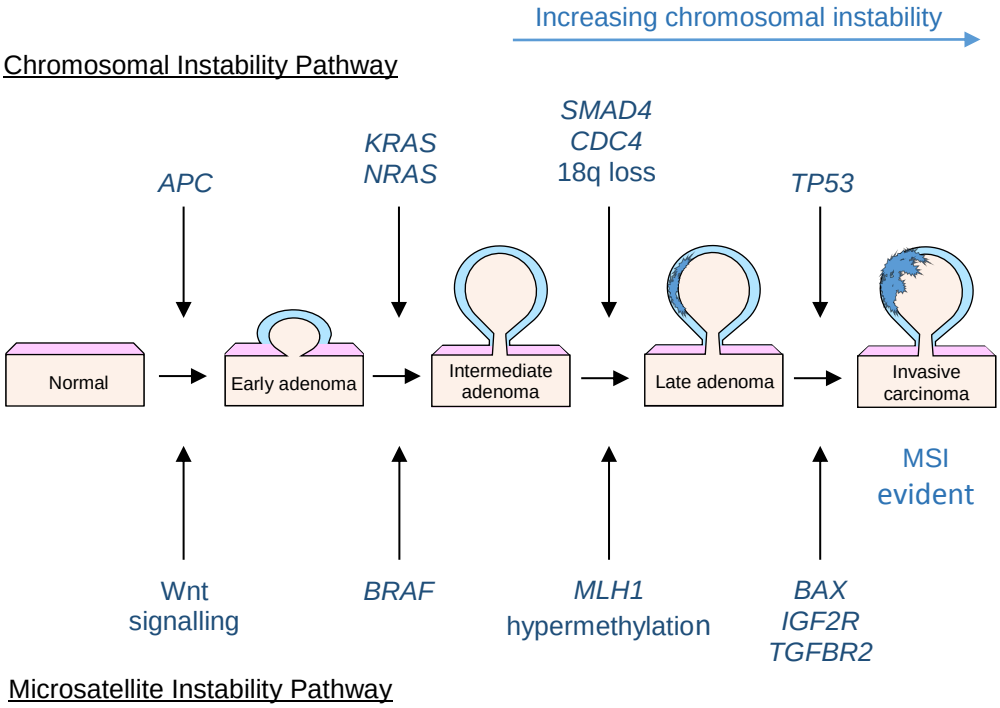

Figure 3. The adenoma-carcinoma sequence. In this model, colorectal adenocarcinoma develops from normal colonic mucosa (shown in light pink) in a step-wise progression. This take place classically via the chromosomal instability pathway (genetic events shown along the top)**.** Initially an *APC* mutation results in an early adenoma harboring low-grade dysplasia (light blue). Mutations in *KRAS* or *NRAS* result in enlargement of the polyp to an 'intermediate' adenoma. Later events include loss of 18q and p53 (*TP53*), with the adenoma progressing through high-grade dysplasia (dark blue) to invasion into the sub-epithelial tissue (light yellow). The microsatellite instability pathway demonstrates a similar step-wise progression (genetic events shown along the bottom). This is characterised by hypermethylation of *MLH1*. Tumours from either pathway may show a CpG island methylation phenotype (CIMP), where by hypermethylation downregulates tumour suppressor genes.[60,67,68] *CIMP = CpG island methylation phenotype (High/Low), LOH = loss of heterozygosity, MSI = microsatellite instability.*

The next step in this sequence is a mutation in one of the Ras (rat sarcoma) genes, resulting in enlargement of the adenoma (so called intermediate adenoma).[60,67,68] Around 30–40% of sporadic CRCs have mutations in *KRAS*[69-71] and around 5% have mutations in *NRAS*.[71,72] RAS proteins are mediators in the mitogen-activated protein kinase (MAPK) pathway, which regulates cell proliferation in response to external growth factors such as epidermal growth factor (EGF). Binding of epidermal growth factor (EGF) to its receptor, epidermal growth factor receptor (EGFR), results in activation of KRas and subsequent activation of a cascade, as shown in Figure 4. This results in the up-regulation of a number of transcription factors, such as c-myc, that eventually result in increased cell division and survival.[69,73] Mutations in *KRAS* or *NRAS* can result in constitutive expression and activation of KRas, leading to an over-activation of the MAPK pathway and uncontrolled cell proliferation and neoplasia. Ras mutations are clinically significant because there are therapies targeted at the MAPK pathway.[69,70,74] This is discussed in further detail in Chapter 4.

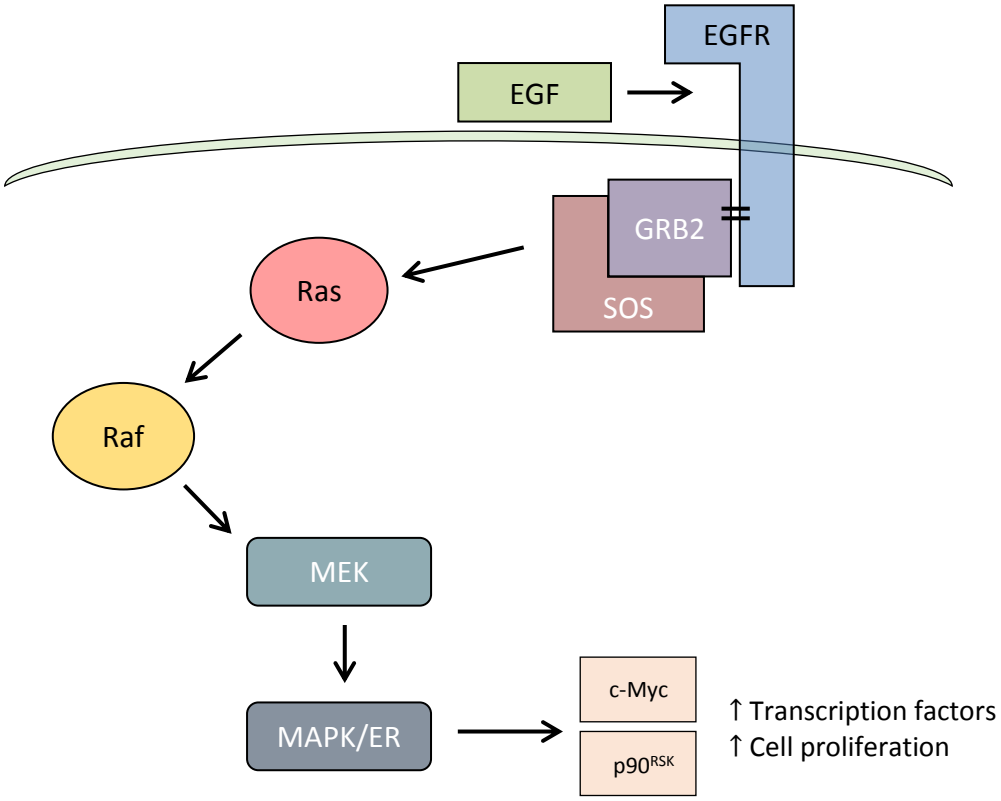

Figure 4. An overview of the MAPK signaling pathway. Growth factors (e.g. EGF) activate the EGFR receptor resulting in downstream activation via the receptor's intrinsic tyrosine kinase action. Downstream molecules such as Ras proteins (e.g. Kras, Nras), Raf proteins (predominantly Braf) and eventually other MAPKs that up-regulate cell proliferation are in turn activated in a casecase. Mutations in Ras or Raf genes result in constitutively activated proteins in this pathway, even in the absence of EGFR signaling.[73,75] *MAPK = mitogen-activated protein kinase, EGF = epidermal growth factor, EGFR = epidermal growth factor receptor, Raf = rapidly accelerating fibrosarcoma family proteins, Ras = rat sarcoma family proteins*

Loss of heterozygosity (LOH; loss of a gene containing chromosomal region) at chromosome 18q is the next major event in the adenoma-carcinoma sequence and is the most common large-scale chromosomal event seen in CRC. This is accompanied by a loss of expression of genes located in this region, such as SMAD4 a mediator in the TGF-β driven proliferation pathway. It is probably around this time that the adenoma develops high-grade dysplasia. From this point on, large-scale chromosomal abnormalities begin to accumulate.[60,67,68] LOH is also seen across a large number of chromosomes,[76] commonly this results in a loss of the *TP53* gene (located in a LOH region of chromosome 17). The gene product p53 is a well-known tumour suppressor involved in apoptotic and cell-arrest pathways that are initiated by cell stress and deoxyribonucleic acid (DNA) damage. Loss of p53 function seems to be a gateway event between dysplasia and invasion.[60,77]

The marked chromosomal abnormalities in the classical adenoma-carcinoma sequence described above has led to the use of the term 'chromosomal instability' (or 'CIN') pathway. 'CIN' differentiates the sequence from other more recently described pathways (discussed below), however CIN probably accounts for around 70% of sporadic CRCs. The pathway is shown along the top of Figure 3.[60,67,68]

The CIN pathway may be more common in patients with a history of inflammatory bowel disease (IBD), whose overall risk of CRC is much higher than the background population. IBD is a spectrum of chronic inflammatory disorders with an autoimmune basis. Predominantly IBD includes Crohn's disease and ulcerative colitis.[26,78] IBD patients that develop CRC show somatic mutations in classical CIN genes such as *APC*, *KRAS* and *TP53* as well as a number of inflammation pathways such as *COX-2*.[79,80] In contrast to non-IBD CRCs, these tumours appear to develop more rapidly (driven by inflammatory cytokines) and may have an altered sequence of event; loss of p53 seems to occur much earlier and may be present in over 50% of biopsies from patients with inflamed non-neoplastic epithelium. These changes seem to affect a wide area of colonic mucosa (known as a field effect) and dysplasia may be multi-focal and non-polypoid in morphology.[81,82]

A second major pathway of CRC pathogenesis is associated with mismatch repair (MMR) deficiency and *BRAF* mutations.[60,67,68] MMR proteins are involved in the repair of point mutations and insertion/deletions (indels) which occur during DNA replication. Each dividing cell is estimated to gain 10,000 DNA replication errors per day and therefore the MMR pathway is a vital cell survival mechanism.[83] The MMR pathway was originally described in *E.coli*.[84] During DNA replication, errors are initially recognised (sensed) by the Mutator S (MutS) protein which then in-turn recruits the Mutator L (MutL) protein. The MutS/MutL complex then forms and this recruits endonucleases to the site to initiate excision and repair. The MMR system in eukaryotes is demonstrated in Figure 5. In eukaryotes there are two major MutS homologs involved in mitotic MMR. These homologs are heterodimers comprised of either MSH2 and MSH6 (MutSα – senses base mispairing)

or MSH3 and MSH6 (MutSβ – senses indels). Other homologs containing MSH4 and MSH5 may exist and are thought to be involved in meiotic MMR. The major MutL machinery homologs include an MLH1/PMS2 (MutLα) dimer, an MLH1/PMS1 (MutLβ) dimer and an MLH1/MLH3 (MutLγ) dimer. The MLH1/PMS2 dimer is thought to be the complex most dominant in mitosis, with the MutLβ complex probably more important in meiosis. The function of MutLγ is unknown.[85,86]

MMR proteins are encoded by corresponding MMR genes. The expression of some MMR genes seems to influence the expression of others. In this respect, MLH1 and MSH2 can be thought of as dominant proteins in the heterodimer complexes because a lack of either protein usually leads to the loss of the respective heterodimer binding partners. For example, the loss of MLH1 will almost always lead to the loss of PMS2. This does not happen the other way around however, i.e. the loss of PMS2 does not usually result in the loss of MLH1. Similarly, the loss of MSH2 leads to the loss of MLH6.[87-89]

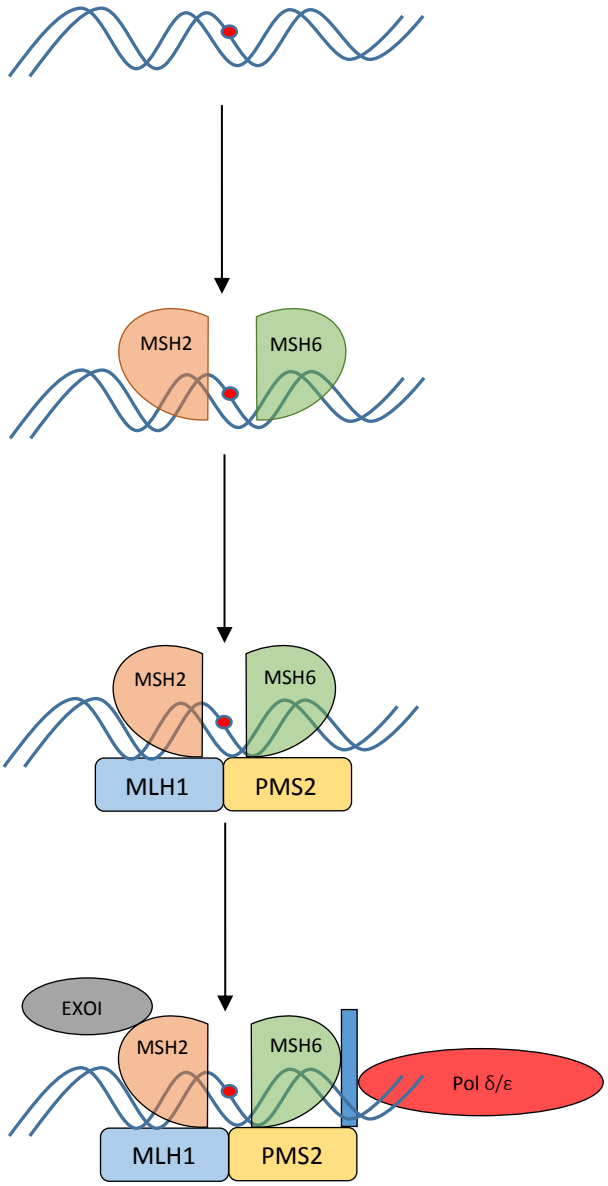

Figure 5. DNA mismatch repair (MMR) mechanisms. The process begins with a mismatched base (demonstrated as a red dot in the double helix) and this is sensed by the MSH2/MSH6 (mispairs) or MSH2/MSH3 (indels) sensing heterodimer complex. Sensing is followed by MLH1 recruitment and the formation of a second heterodimer with PMS2 (predominantly in mitosis), PM1 (unknown function) or MLH3 (predominantly in meiosis). The MMR complex then recruits endonucleases such as EXO1 to excise the lesion and DNA polymerase δ or ε (via the linker protein PCNA) to re-replicate the segment.[84-86]

Point mutations and indels are common (especially the former) in DNA replication and areas of the genome where there are long repeats of a short sequence of bases are particularly prone. These areas are known as microsatellites and they comprise short tandem repeats (a 2-5 base sequence repeated up to 50 times). Microsatellites are scattered throughout the genome, many within gene coding areas, and mutations in these areas result in lengthening of the segment – known as microsatellite instability (MSI).[90-92] Around 15% of sporadic CRCs demonstrate MSI and this is now recognised as a second major pathway for the development of CRC (demonstrated along the bottom of Figure 3).[93,94] MSI is easily detected by examining the DNA sequence and the degree of MSI can be graded as MSI-H (high) or MSI-L (low), depending upon the degree of instability.[95] MSI is almost always due to MMR deficiency, most commonly MLH1 epigenetic silencing by hypermethylation (see later), and various methods of assessing the function of MMR proteins is also now available.[96]

Despite the name, the instability of microsatellites and associated MMR protein dysfunction are actually not detected until late in the progression of MSI tumours. Like the CIN pathway, initial genetic events in the MSI pathway are probably alterations in Wnt signaling. In contrast to CIN cancers however, MSI tumours rarely have mutations in Ras genes or show large scale chromosomal abnormalities. Instead, MSI tumours commonly show mutations in *BRAF* (an early event), *CDC4* and *BAX*.[60] *BRAF* mutations are particularly prevalent, present in 10–15% of sporadic CRCs. Like Ras proteins (Kras and Nras), Braf is a mediator in the MAPK pathway (see Figure 4).[60,71,73,97] Interestingly, Ras and *BRAF* mutations however are almost mutually exclusive in colorectal tumours.[98,99] Clinically, identifying MSI, MMR deficiency or *BRAF* mutations is useful[11,13] and this is discussed further in Section 1.4 and Chapter 3.

A third major group of tumours now recognised belong to the CpG island methylation phenotype (CIMP) category.[60,67,68] CpG islands are segments of DNA around 1000 base pairs (bp) in length, showing an enrichment of CG repeats (the 'p' in CpG represents a phosphate bond). These regions are very common in the genome and many are found in gene promotor regions.[100,101] Methylation of cytosine bases in these areas results in a cascade of events that cause histone modification and local chromatin remodeling. Ultimately, this leads to reduced transcription at the methylation site.[102] It is thought that around 60% to 80% of CpG islands are methylated and this allows the genome to be modified by a mechanism other than classical transcription regulation, a mechanism known as epigenetics. Mutations in genes that regulate methylation pathways are thought to in turn lead to changes in the methylation patterns of key cell cycle regulatory genes.[101] Many CRCs (CIN and MSI tumours) show global, non-specific hypomethylation, which is thought to probably lead to an over expression of certain proliferative oncogenes.[60,103] In CIMP CRC however, there is hypermethylation and reduced expression of tumour suppressor genes and these seem to be a distinct subset of tumours. Depending on the degree of

hypermethylation, such cancers may be designated as CIMP-H (high) or CIMP-L (low).[67] The relationship of CIMP tumours to the CIN and MSI pathways is demonstrated in Figure 6. Although CIMP is described as a third pathway for the adenoma-carcinoma sequence, as can be seen there is much overlap of CIMP with the CIN and MSI pathways.[60,68] Some serrated lesions are more associated with CIMP than others however. SSLs and serrated cancers arising from SSLs are usually CIMP-H, have MSI and show *BRAF* mutations. In contrast, TSAs and serrated cancers arising from TSAs are usually CIMP-L, microsatellite stable (MSS) or MSI-L and have *KRAS* mutations. Hyperplastic polyps are suspected as a precursor lesion in the development of both SSLs and TSAs as these polyps usually have either a *BRAF* or a *KRAS* mutation.[34,60,68,104-106]

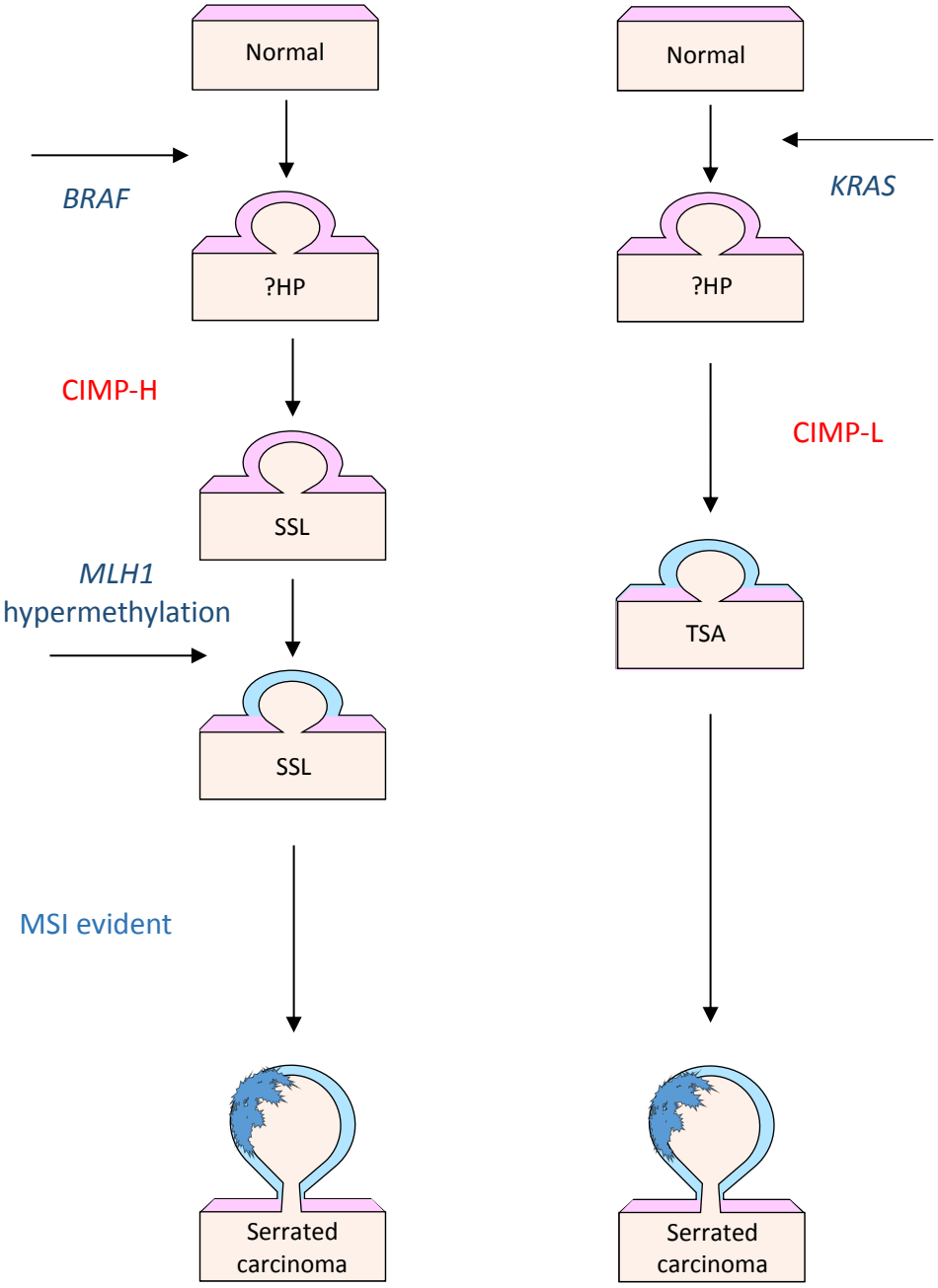

Figure 6. Proposed pathways of serrated carcinoma development. In this model dysplasia (shown in light blue) and serrated adenocarcinomata (dark blue) develop from normal colonic mucosa (light pink) in a step-wise progression via distinct events. Sessile serrated lesions (SSL) show early *BRAF* mutations and arise from a possible hyperplastic polyp (HP) precursor. These tumours are CIMP-H and consequently show *MLH1* hypermethylation and microsatellite instability (MSI). Traditional serrated adenomas (TSA) may also develop via a HP precursor but by contrast show early *KRAS* mutations and are CIMP-L.[34,60,67,68,105] *HP = hyperplastic polyp, CIMP = CpG island methylation phenotype (High/Low), SSL = sessile serrated lesion, TSA = traditional serrated adenoma, MSI = microsatellite instability.*

The pathogenesis of CRC after the development of invasion includes mechanisms by which the tumour is able to disseminate throughout the body. In order to do this, the tumour must traverse the sub-epithelial connective tissue and access the lymphovascular space. Epithelial-mesenchymal transition (EMT) describes the process by which carcinoma cells take on the phenotype of mobile stromal cells (such as fibroblasts).[46,107,108] Many pathways are involved in this process, including Wnt.[109] Once the tumour has accessed the lymphatics or vasculature, cells are able to disseminate to lymph nodes and distant organs – the latter causing the most disruption to normal physiology. Metastases in vital organs and carcinomatosis (generalised, wide-spread metastases) results in end-organ failure and death, commonly due to opportunistic infection.[46]

Most CRCs are sporadic, however around 3–5% of occur in the setting of a directly inheritable cancer syndrome.[18] Table 3 gives a summary of the major inherited colorectal tumour syndromes. Lynch syndrome (LS) is the most common of these in CRC and is caused by germline monoallelic mutations in MMR genes, resulting in MSI tumours. LS is discussed in detail in Chapter 3.[11] Constitutional mismatch repair-deficiency syndrome is a rare and aggressive cancer syndrome that is caused by the biallelic MMR gene mutation counterpart to LS.[110] Many of the other inherited CRC syndromes present with polyposes (numerous benign and malignant polyps).[111] The most famous of these is familial adenomatous polyposis (FAP), which is characterised by thousands of conventional adenomas in the colon and an extremely high risk of malignancy. FAP is due to autosomal dominant germline mutations in *APC,* the function of which was discussed above. Some APC mutations are less pathogenic and an attenuated FAP syndrome also exists.[112] *MUTYH*-associated polyposis is characterised by numerous conventional adenomas and is caused by mutations in the *MYH* base-excision repair gene.[113] Polymerase proofreading-associated polyposis (PPAP) is caused by germline mutations in *POLE* and *POLD*, which encode DNA polymerases. PPAP patients generally develop numerous conventional adenomas. DNA polymerases have a proofreading function alongside their role in DNA replication and cancers which arise from this syndrome (or those with somatic *POLE*/*POLD* mutations) show a very high rate of mutations (the hyper/ultramutator phenotype).[114,115] The hyperplastic or serrated polyposis syndrome presents with numerous serrated lesions and these patients have an increased risk of serrated carcinoma; no germline mutation has yet been identified.[116] Finally, hereditary mixed-polyposis is caused by *GREM1* mutations and presents with various types of neoplastic polyps.[117] There are also syndromes which present with numerous hamartomatous polyps, including Cowden's syndrome (germline *PTEN* mutation),[118] juvenile polyposis (germline *SMAD4* or *BMPR1A* mutation)[119] and Peutz-Jeghers syndrome (*STK-11* mutation).[120]

Syndrome	Gene(s) affected	Characteristics
Lynch syndrome[11]	*MLH1, PMS2, MSH2, MSH6*	Non-polyposis CRC, MSI, cancers at other sites (e.g. endometrium)
Familial adenomatous polyposis (FAP)[112]	*APC*	Thousands of colonic polyps , high risk of CRC
MUTYH-associated polyposis[113]	*MUTYH*	Similar to FAP
Polymerase proofreading-associated polyposis[114,115]	*POLE, POLD1*	Multiple adenomas with hypermutated cancer development
Hyperplastic/serrated polyposis[116]	Unknown	Multiple serrated lesions with increased cancer risk
Hereditary mixed polyposis[117]	*GREM1*	Mixed polyp types, probably cancer risk
Cowden syndrome[118]	*PTEN*	Hamartomas of the colon, skin tumours, high risk of various cancers
Juvenile polyposis[119]	*SMAD4, BMPR1A*	Hamartomas (more commonly in stomach), high cancer risk
Peutz-Jeghers syndrome[120]	*STK-11*	Hamartomas (more commonly small bowel) and mucocutaneous pigmentation, increased cancer risk

Table 3. A summary of the major colorectal tumour syndromes, the genes affected and the clinical characteristics. *CRC = colorectal cancer, MSI = microsatellite instability, FAP = familial adenomatous polyposis.*

1.2.5 The clinical presentation of colorectal cancer

Most patients with CRC are initially asymptomatic but symptoms when present are generally non-specific, including weight loss, abdominal pain and rectal bleeding. Two thirds of CRCs present as left sided (distal) tumours and the remaining third are found in the transverse and right colon. 20% of patients present with distant metastases.[10] Bowel obstruction or perforation at presentation is uncommon but, when this occurs, it imparts a poor prognosis.[60]

1.2.6 The investigation and diagnosis of colorectal cancer

The investigation and diagnosis of CRC in the UK is carried out following the National Institute for Health and Care Excellence (NICE) and the British Society of Gastroenterology (BSG) guidelines.[12,121,122] Early CRC is often asymptomatic and therefore the UK NHS Bowel Cancer Screening Program (BCSP) is now offering both a one-off bowel scope (flexible sigmoidoscopy) screening for persons aged 55 and then after biannual faecal occult blood testing (FOB) for persons aged 60 to 74. FOB testing is based on the principle that CRC undergoes microscopic haemorrhage into the faecal stream.[121,123] Patients with suspected cancer are referred for urgent investigation within two weeks. Patients with symptoms or positive screening results are usually investigated by colonoscopy (whole bowel endoscopy). Alternatively, where colonoscopy has failed or is not possible, other options include flexible sigmoidoscopy and computerised tomography (CT) colonoscopy. Small tumours (polyps) identified by colonoscopy will usually be excised, larger lesions are biopsied. Histopathological evaluation (see later in Subsection 1.2.9) of colonoscopy acquired tissue samples is the gold standard test for CRC. The diagnosis of colorectal lesions is based on the WHO classification given in Table 1 and reported following the guidelines for the BCSP.[10,12,50,122,123] The reporting process may involve the use of immunohistochemistry (IHC; also see later in Subsection 1.2.9) to aid diagnosis or detect MMR deficiency (MLH1 IHC for example). Molecular testing is becoming increasingly common in CRC as this can help guide management (see later Section 1.4). In particular, molecular testing for *BRAF*, *KRAS* and *NRAS* mutations is common.[11-14]

Patients with a strong family history of colorectal cancer, polyposis syndromes or inflammatory bowel disease are offered screening with primary colonoscopy at varying internals depending on risk stratification.[12,121,123,124]

1.2.7 The Staging of colorectal cancer

Staging is an assessment of how far a cancer has invaded and spread throughout the body. This is preliminarily carried out clinically on the patient by using various imaging techniques. The final definitive Stage is given later by a pathologist and is derived from examination of the surgical resection specimen. In general, Staging involves determining the local spread

of the primary tumour, the presence of metastases in regional lymph nodes and the spread of metastases to other distant sites in the body.[46]

There are two Staging systems recommended by the RCPath for CRC reporting in the UK.[13] The Dukes' Staging system is the older of the two and still preferred by some surgeons and oncologists.[125] Dukes' Staging has undergone a number of modifications since the original A to C categories were described. The Dukes and Bussey modification first split category C into C1 and C2, and the Turnbull modification added a D category. The current system is shown in full in Table 4.[13,125,126] The more recent Union for International Cancer Control (UICC) Tumour Node Metastasis (TNM) classification (given in Table 5) streamlines a system for Staging across sites in the body and is increasingly becoming the gold standard.[127] Although the TNM system categories emulate the same groups as the traditional Dukes' system (see a comparison in Table 6), it is more detailed and therefore offers greater patient sub-classification. Therefore, Dukes' is being largely replaced by the TNM system in clinical practice.[128]

Dukes' Stage	**Criteria**
A	No invasion beyond muscularis propria, no metastases
B	Invasion beyond muscularis propria, no metastases
C1	Regional lymph nodes positive, highest resected node negative
C2	Regional lymph nodes positive, highest resected node positive
D	Distant metastases

Table 4. The Dukes' staging system for colorectal cancer. The approximate equivalent TNM classification is given in brackets. The Stage D group and split of C into C1 and C2 were later developments from the original A to C system.[13,129]

TNM Designation	Criteria
T	**Primary Tumour**
TX	Cannot be assessed
T0	No invasive tumour
T1	Tumour invades the submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades through muscularis propria into serosa or pericolic/perirectal tissue
T4a	Tumour directly invades other organs or structures
T4b	Tumour perforates the visceral peritoneum
N	**Regional Lymph Nodes**
NX	Cannot be assessed
N0	No regional lymph node metastases
N1	Metastases in 1 to 3 regional lymph nodes
N2	Metastases in 4 or more regional lymph nodes
M	**Distant Metastases**
MX	Cannot be assessed
M0	No distant metastases
M1	Distant metastases identified

Table 5. The Union for International Cancer Control 5th TNM classification of colorectal cancer.[13,129]

Groupings	UICC Groupings	Dukes Stage Equivalent
T0 N0 M0	0	N/A
T1 or T2 and N0 M0	I	A
T3 or T4 and N0 M0	II	B
T1, T2, T3 or T4 and N1 or N2 and M0	III	C1 or C2
T1, T2, T3 or T4 and N1 or N2 and M1	IV	D

Table 6. A comparison of the Union for International Cancer Control (5th edition) and Dukes' Staging systems.[13]

Although TNM is replacing the older Dukes' system, there are problems with using TNM. The system is currently up to its 8th edition,[127] however the RCPath guidelines (not updated since the 7th edition) mandate the use of the 5th edition, citing a lack of high-level evidence for the changes made in the TNM 6th and 7th editions. The RCPath guidelines also reject the 5th TNM's designation of Tis to tumours that have breached the epithelial basement membrane (BM) and invaded the lamina propria.[13] Although a BM breach would satisfy the definition of malignancy, (invasion of the surrounding tissues)[46] the RCPath designates these as T0 adenomas with high-grade dysplasia and does not recognise the Tis designation (therefore Tis is not given in Table 5); only tumours that invade the submucosa are designated T1. The reason for this disagreement is because the traditional view is that lamina propria invasion has no metastatic potential due to a lack of lymphovascular spaces.[13] It has been shown however that the lamina propria does contain lymphatics[130] and that intramucosal carcinoma may be clinically important to differentiate from adenoma.[50,131,132] The issue remains an area of contention in the UK. There are advantages and disadvantages to both the UICC and Dukes' Staging systems then and disagreement over which should be used in practice. Therefore, the RCPath guidelines recommend that pathologists report the Stage with both systems currently.[13] Internal RCPath and NHS BCSP discussions (unpublished) suggest the 8th TNM will be recommended from 2018.

Polyp cancers by definition will tend to fall into the T1/Dukes' A stage, however providing additional information on how deep within the polyp the cancer has invaded (Haggitt level for pedunculated polyps or Kikuchi level for sessile polyps) may be prognostic and so is recommended by the RCPath.[13,50,129]

1.2.8 The management of colorectal tumours

The management of colorectal tumours in the UK is also based on NICE and BSG Guidelines. The discussion below is derived from these guidelines, however local practices and individualised treatment plans may vary.[12,121,122,133-135]

Patients with isolated non-malignant and non-dysplastic polyps (e.g. hyperplastic polyps), or negative colonoscopies, in general require no further intervention. Small, non-invasive pedunculated or sessile dysplastic lesions are usually treated by simple snare polypectomy. Larger polyps can be removed by piecemeal endoscopic mucosal resection (EMR). Distal polyps that cannot be easily removed at endoscopy can be removed using a more extensive technique known as transanal endoscopic microsurgery (TEM). Polyps are categorised into low (one or two small adenomas, both ≤10mm), moderate (three to four small adenomas or one adenoma ≥10mm) and high (more than five small adenomas or more than three small adenomas, one of which is ≥10mm) risk. Low risk polyps can essentially be discharged from screening whereas follow-up colonoscopy is recommended for moderate risk (at three years) and high-risk (at one year) polyps up to the age of 75 years. Large sessile polyps are at risk of incomplete excision at endoscopy and therefore resection site tattooing and re-examination after two to three months is advised. Polyp cancers are often discovered incidentally within clinically benign appearing polyps and these are managed similar to high-risk benign polyps, with follow-up shortly after surgery if initial excision margins were clear of tumour. Patients diagnosed with polyposis syndromes at this stage are offered more intensive colonoscopy follow-up, depending upon their risk.[12,121,123,135]

Patients with invasive disease will undergo CT scanning as part of a pre-operative assessment for tumour Stage (see earlier) and resectability (technical ease of surgery). Rectal tumours are further assessed for resectability with magnetic resonance imaging (MRI).[12,133] Pre-operative blood carcinoembryonic antigen levels are predictive of outcome, but likely just reflect tumour burden and are not often used routinely.[60]

Radiologically resectable Stage I-III colonic tumours are generally amenable to surgical resection, either traditional open or preferably where possible laparoscopic surgery. EMR is sometimes considered for Stage I colon tumours. Small resectable stage I rectal tumours may be treatable by TEM or EMR. Further management is based on histopathology of the resection specimen.[12,133]

Radiologically unresectable Stage I-III colonic tumours may be offered neoadjuvant (prior to surgery) chemotherapy to try to optimise the tumour into a resectable state. Treatment would otherwise be palliative. Chemotherapy options include capecitabine monotherapy or oxaliplatin with 5-flurouracil (5-FU) and folinic acid combinations.[12,133]

Radiologically resectable Stage I-III *rectal* tumours deemed to be high-risk (tumours with venous space invasion or resection specimens likely to result in positive surgical resection margin, as predetermined by imaging) may be offered short-course preoperative radiotherapy (SCPRT) and/or neoadjuvant chemotherapy to optimise (minimize the risk of) the tumour for surgery.[12]

Patients with Stage IV disease by imaging may be offered surgery if both the primary and distant metastases can be resected, otherwise resection may be limited to the primary tumour with further chemo-radiotherapy palliative treatment or palliative care alone. Monoclonal antibody (mAb) therapy with cetuximab or panitumumab may be indicated in some patients with advanced metastatic disease (see Chapter 4).[12,133]

The type of surgery offered for CRC depends on the location of the tumour. Most are left sided cancers and require either a left hemicolectomy (removal of most of the distal large bowel), sigmoidectomy, anterior resection (excision of the lower sigmoid and upper rectum) or an abdominoperineal resection (excision of the rectum and anal canal). Right-sided tumours will usually be excised as a right hemicolectomy and occasional tumours may require transverse colectomy. A pancolectomy (excision of the entire colon) is rare for cancer management but may be used where synchronous tumours (separate primary CRC tumours arising at the same time in different sites) affect distal and proximal parts of the bowel. Further management is planned post-operatively, based upon the histopathological evaluation of resected tissue and molecular testing (see Subsection 1.2.9 below).[136]

1.2.9 The histopathological assessment of colorectal cancer

The histopathological assessment and reporting of CRC in the UK must follow RCPath guidelines in order to meet UK Government laboratory accreditation policy, overseen by the UK Accreditation Service (UKAS).[13,137,138] Traditional histopathology involves both a macroscopic (gross) and a microscopic examination of tissues removed from the body. For preservation and easy of preparing microscopy slides, tissues are formalin-fixed and paraffin-embedded (FFPE). The resulting blocks of tissue are thinly sectioned using a microtome, placed on a glass slide and stained for contrast. The routine histological stain is the haematoxylin and eosin (H&E) preparation, as demonstrated in Figure 1. Most diagnoses are made by a morphological examination of the microscopic tissue with H&E. The development of immunostaining has seen histopathology expand into the assessment of cell protein expression. In IHC, a specific primary antibody (Ab) is applied to tissue sections and this binds to the target protein (if present) of interest. A secondary Ab labeled with a chromogen is then applied and this secondary Ab in turn binds to the primary Ab. Finally, the chromogen is enzymatically converted into a visible dye that stains the tissue, highlighting the presence and location of the target protein by light microscopy (immunoperoxidase staining). Alternatively, a fluorescent dye is used

(immunofluorescence staining). Now largely automated, both morphological and protein expression analysis of tissue sections has remained the focus of diagnostic histopathology for some 30 plus years.[139-141]

The initial histopathological diagnosis of CRC is usually made on a small tissue biopsy taken during the screening or diagnostic process. Definitive histological diagnosis of the tumour is considered a gold standard test and is necessary to predict outcome and direct therapy. This initial diagnosis will be later confirmed on the resection specimen. Once a diagnosis of adenocarcinoma is made, a number of other features must also be assessed. The grade of the cancer gives an indication of the aggressiveness of the disease. This is based upon the morphological appearances (the degree of glandular differentiation) as is given as well, moderately or poorly differentiated – the latter carrying the worst prognosis if considered independently of other factors.[10,48,57] Of less prognostic significance, but still helpful and in common use, is noting the presence or absence of lymphovascular space invasion or perineural invasion.[142]

Following the biopsy diagnosis, the pathologist will receive the surgically resected specimen. The specimen should be fixed, examined macroscopically, dissected and examined microscopically to confirm the biopsy findings. Confirmation of the diagnosis and other features on the resection specimen is considered definitive and therefore should always be carried out. The specimen will generally include a long segment of bowel that contains the tumour and a safe distance of normal bowel to either side, to ensure complete removal of the cancer. Attached to the bowel will be a portion of mesentery, blood vessels, lymphatics and draining lymph nodes. Usually the mesentery is resected as a triangular wedge-shaped segment along with its corresponding vascular branch from the superior or inferior mesenteric artery, the apex of which is tied off at the vessel ligation site. Also at the apex is the highest (most proximal) draining lymph node. The pathologist will assess how cleanly the specimen has been dissected from the body by the surgeon, as the plain of excision can in some circumstances influence outcome. The pathologist will then ink the specimen (see later) and open the bowel to examine the tumour. It is important to note the relationship between rectal tumours and the peritoneal reflection.[13] Rectal tumours above the peritoneal reflection actually behave like colon tumours and have a better prognosis, so this is important information to relay to the surgeon and oncologist.[33] The pathologist will then make cuts through the tumour to see the extent of its spread. Samples of the invasive area will be taken for microscopic confirmation. The extent that the tumour has spread locally is important as this gives an indication of how advanced the disease is and predicts outcome for the patient. This is referred to as Staging and was discussed earlier in Subsection 1.2.7. As part of the Staging process, assessment of tumour spread to lymph nodes is required and so these nodes will be dissected out from the mesentery and examined microscopically.[13]

The completeness of excision is obviously very important, as residual tumour in the patient must be followed up with further treatment. Therefore, the tissue margins must be assessed microscopically and the status reported to the surgeon ('involved by' or 'clear of' tumour). As part of this process the pathologist will ink the margin surfaces during dissection, to aid with identification during microscopy. The highest draining node and the root of the apical blood vessels also give an indication of possible residual tumour left in the body and so these structures are always examined microscopically.[13]

Distant metastases are also very important as these indicate a very poor prognosis (Stage IV disease). In general, metastases are easily detected on imaging and biopsy confirmation is not usually necessary. The treatment at that point is likely to be palliative and as such, resection of metastatic tissue is also uncommon. It is unusual then for the pathologist to examine metastatic tissue and so the M category of Staging is usually assessed by a radiologist.[10,13,57]

The findings of the pathologist are reported to the surgical and oncological teams and this is used to determine the prognosis and any follow-up therapy needed. Follow-up management is based on NICE and BSG guidance. Pathologically (histologically) Stage III and high-risk (tumours with venous space invasion or resection specimens with positive surgical resection margins) pathologically Stage II rectal tumours are followed up with post-operative adjuvant chemotherapy. Pathologically Stage II colonic tumours deemed high-risk (poorly differentiated, obstructed, with perineural invasion or low surgical lymph node yield, as determined by histological assessment) and pathological stage III tumours are also offered post-operative adjuvant chemotherapy.[10,12,57]

1.2.10 The prognosis of colorectal cancer

The prognosis of CRC is largely based upon Staging, which is carried out as described above (Subsection 1.2.7) by combining pathology and radiology findings. In developed regions, the overall 5-year survival for CRC is around 65% and has remained static for some years. Just over half of patients present with low-staged (Stage I/II, limited to the bowel wall) tumours and have relatively good prognoses when treated by surgical resection, with a 5-year survival of around 90%. Tumours that have spread to local lymph nodes (Stage III) have a poorer prognosis and this group makes up the majority of patients with higher-staged disease. The 5-year survival in this group is around 70% and these patients usually require adjuvant chemotherapy. The survival figures fall sharply to around 10% for patients who present with, or later develop, distant (e.g. liver/lung) metastases (Stage IV). In some instances, these patients are fit and tumour metastases are amenable to resection. However, for those with inoperable metastases the management is palliative.[10]

1.3 Diagnostic molecular pathology

1.3.1 Overview of molecular diagnostics

So far, this thesis has discussed the traditional approach to cancer management based upon histopathological and radiological tumour assessment. However, there is now an understanding that the genetic make-up of a tumour can significantly inform and guide clinical management. Testing normal tissues (such as blood) for germline mutations and inherited syndromes is not new and is a well-established practice that takes place in Clinical Genetics laboratories in most hospitals. It is becoming increasingly recognised however that testing abnormal tissues and tumours is also clinically useful. This recognition has led to a new branch of pathology known as 'molecular diagnostics', or 'diagnostic molecular pathology'. The two main types of lesion encountered in this practice are translocations and point mutations. Testing for these lesions may aid the diagnostic process, guide treatment and influence prognosis.[18,112,143]

Translocations and other chromosome level abnormalities have been detected using cytogenetic approaches for many decades but these are now becoming increasingly viewed as a part of diagnostic molecular pathology testing in cancer management. Traditionally karyotyping, a technique whereby the chromosomes were stained and viewed directly, was used. The most common method of detecting translocations in the diagnostic setting today is by *in situ* hybridisation (ISH) techniques. ISH uses a labeled nucleic acid probe to bind to denatured DNA within cells to allow visual recognition. Similarly to IHC, probes may be labeled with a chromogen (CISH) visible by light microscopy or a fluorophore (FISH) visible with fluorescent microscopy. Probes are designed to bind to specific regions of the chromosome which will allow the microscopist to determine the status of chromosomes. Probes are usually designed for two different locations on a chromosome with two different colours, allowing the spatial analysis of chromosomal components. Classically, two 'break-apart' probes target DNA regions close together on the genome and in the non-translocated setting the two colours blend (for example green and red become yellow). In the translocated scenario, the colours are seen as separate and distinct (red and green), indicating a separation of the chromosomal regions. So-called 'fusion' probes are designed in the opposite manner, to bind to areas of DNA at loci of chromosome fusion (thus translocated is yellow and non-translocated is red and green). FISH can also be used to assess gene copy number variation relative to ploidy within cells, in a technique known as comparative genomic hybridisation.[143]

Mutation analysis is really the main area of emerging genetic testing in diagnostic molecular pathology. Mutation testing relies upon two main techniques – polymerase chain reaction (PCR) and gene sequencing, both of which will be discussed in turn in the Subsections below.[143]

1.3.2 Sample preparation

Before any mutational analysis can be carried out, nucleic acid (most commonly DNA) needs to be extracted from the tissue. DNA extraction requires physical disruption of the cell and nuclear membranes, and digestion of DNA-binding proteins. Extraction can be carried out on fresh tissue, frozen tissue (fresh tissue which was frozen and processed at a later time after thawing), FFPE tissue and blood. Blood is generally only used for germline sequencing or for testing haematological disorders. Sequencing germline DNA is useful for molecular tumour testing because the germline is subtracted from the tumour DNA sequence and this leaves only somatic changes. PCR or sequencing for solid tumour molecular diagnostics is usually carried out on either fresh frozen (FF) or FFPE tissue. The general pathway for tissue handling in molecular diagnostics is shown in Figure 7.[144]

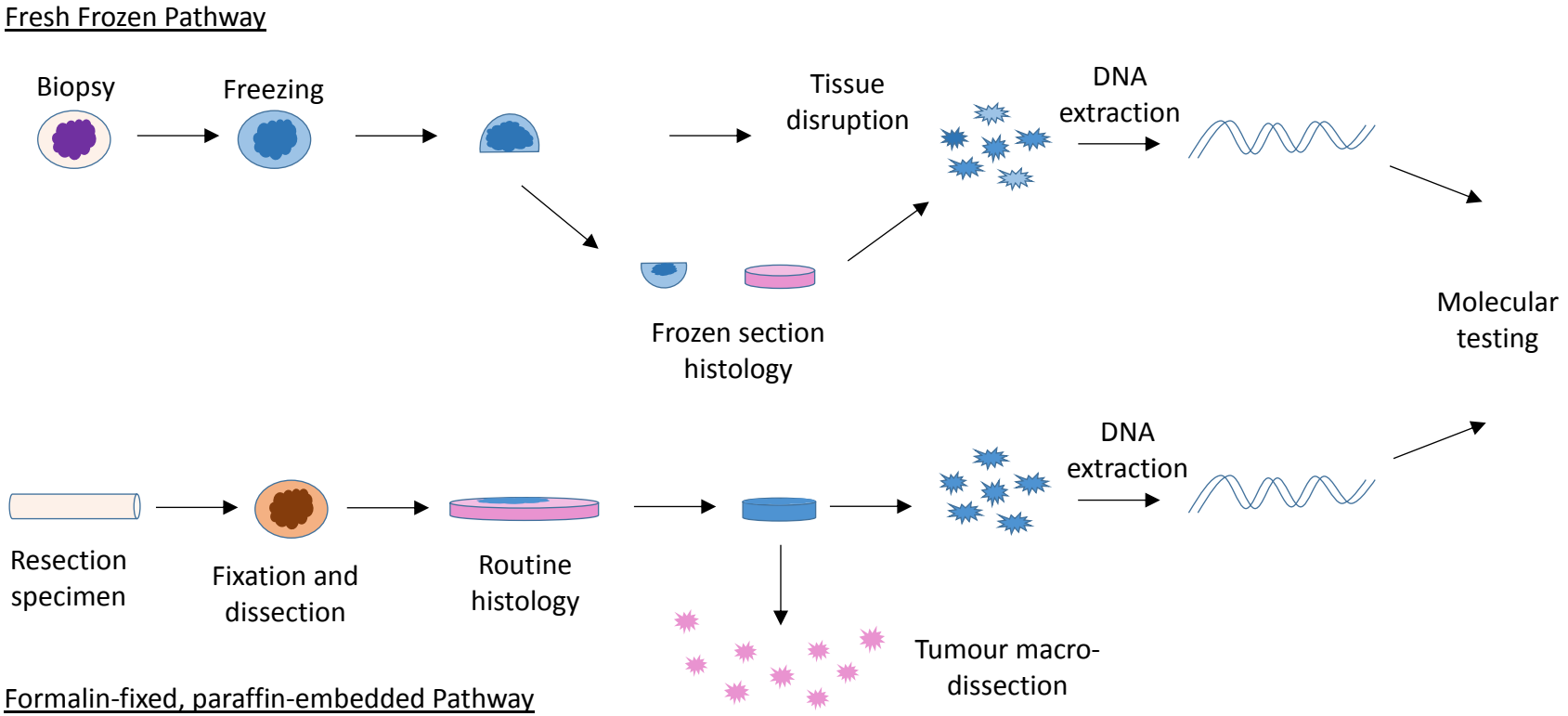

Figure 7. An overview of the tissue handling workflow in diagnostic molecular pathology. Small biopsies taken from the patient for molecular testing are generally processed in the Fresh Frozen (FF) pathway (along the top). After freezing in liquid nitrogen, a small sample is taken for frozen section histology to confirm the presence of tumour. For large resection specimens FF tissue may be taken before fixation, however the need for molecular testing is often only realised at a later stage and so must be carried out on the fixed tissue. In this circumstance the Formalin Fixed, Paraffin Embedded (FFPE) pathway (along the bottom) is needed. In the FFPE pathway, the tumour is identified by routine histology and thin sections from that tissue block are matched against the slides. The DNA content is optimised by using macro-dissection to discard non-tumour tissue. In either pathway, DNA (or RNA) is extracted from the sample following histology and this is stored for molecular testing.[144] *DNA = deoxyribonucleic acid, RNA = ribonucleic acid*

The first step in molecular testing is to confirm that the tissue sample contains viable (non-necrotic) tumour cells and if so to make an estimate of the number of nuclei (i.e. the amount of DNA) present that are of tumour (somatic) or background normal (germline) cells. Each molecular test has its own limit of detection (LOD; also called analytical sensitivity) of mutation frequencies within the extracted DNA pool. It is therefore best practice to check the tissue sample meets the minimum tumour DNA content (usually given as a percentage tumour nuclei content) required in order to avoid diluting the sample with the germline and masking somatic mutations (causing false negative results). Checking the sample tumour content can easily be carried out using H&E histology. To do this, a piece of the fresh tissue biopsy is frozen and a frozen microtomy section is taken for histology. If suitable for testing the remaining tissue is processed for DNA extraction. For cases being tested from FFPE tissue (tissue samples not taken specifically for molecular testing, e.g. resection specimens), histology sections will already have been prepared for diagnostic examination. From the H&E slides, a suitable area of the tumour (area high in tumour cell content, non-necrotic and with little DNA contamination by lymphocytes) will be selected and marked with ink. The corresponding paraffin-embedded tissue block will then be used for molecular testing. From that tissue block, either microtomy sections will be cut or a small tissue punch will be taken for DNA extraction[144]

DNA extraction is a time-consuming process requiring specialist skill and equipment. To ensure the DNA is of high quality, the cold ischaemia time (time from when biopsy was taken to fresh extraction or fixation) should be kept to under 24 hours. Data on warm ischaemia time (while blood flow to sampled area is disrupted but tissue is still in the body) are lacking. Fresh or fresh-frozen samples are the ideal for molecular testing as the extracted DNA is usually of high quality and is not altered by fixation.[145] It is technically difficult to acquire samples from soft, unpreserved material (i.e. from the patient or fresh resection specimen), so these tend to be large pieces of tissue. In comparison, FFPE samples are easy to take from the tissue block and are usually small. In order to extract the DNA, the sample for testing first requires disruption (dividing into smaller pieces, cells separated from each other and the connective tissue matrix, cell and nuclear membranes lysed) and homogenisation (cells and cellular components evenly distributed in the suspension). These are achieved using a combination of physical (rotator-blade) and chemical (denaturing enzyme buffer) methods; disruption and homogenisation usually occur simultaneously.[144,146-148]

Tissue fixation results in DNA fragmentation, DNA base changes (C>T and G>A) and DNA-DNA or DNA-protein cross-linking, all of which can introduce artifacts when sequencing or amplifying.[145] This makes molecular testing less reliable when carried out on FFPE tissue and so these samples are discouraged where possible. However, routine diagnostic molecular pathology tests are carried out on only a small proportion of cases and this it is usually only realised that molecular testing is needed after a histological diagnosis is made.

Therefore, testing FFPE tissue is difficult to avoid in practice. The method of formalin preparation and fixation may however influence the extent of DNA damage and so fixation protocols can be optimised for molecular testing.[144,149] There are a large number of available tissue fixatives, each with specific advantages and disadvantages. The most widely used in the clinical setting for histology are based on formaldehyde. Traditionally, formaldehyde fixatives were prepared as either 10% formalin (40% formaldehyde aqueous stock solution diluted to 4% formaldehyde with distilled water) or 10% formal saline (10% formalin buffered with sodium chloride and sodium phosphate).[150] Modern formalin protocols buffer 10% formalin (40% formaldehyde aqueous stock solution that may contain up to 12% methanol, diluted with distilled water) to a neutral pH with sodium dihydrogen phosphate, monophosphate (4g per 100ml formalin) and disodium hydrogen phosphate, anhydrous (6.5g per 100ml formalin).[151] Neutral-buffered formalin appears to be superior for preservation of DNA than older buffered or unbuffered formalin fixatives, and therefore it is now the recommended fixative for all clinical histology laboratories. Data on non-formaldehyde fixatives is beginning to surface and may show future promise.[152] Fixation should be for no longer than 72 hours and carried out at 4°C. Fixed tissue should then be embedded in plain paraffin wax. As mentioned above, FFPE specimens are generally small because they are easier to acquire from relatively solid preserved material.[144,149] The samples are often already partially disrupted (small punches or thin microtomy sections) and therefore require little physical disruption. However, FFPE tissue requires deparaffinisation, re-hydration, cell separation and homogenisation; this is often achieved using ultrasonication. The cells can then be disrupted (lysed) using an enzyme buffer and homogenised with a vortex mixer.[146,148] Once extracted, DNA is purified using a number of filtration methods. Extracted nucleic acids are generally of variable quality and quantity and therefore a quality control step using fragment size analysis and/or total nucleic acid quantitation (UV-absorbance or fluorescence) is usually reccomended.[146,148,153,154]

1.3.3 Conventional PCR-based tests

The PCR reaction is a simple process of amplifying fragments of DNA, as demonstrated in Figure 8. Double stranded DNA (dsDNA) is heated until the strands denature and separate (melt). Specific DNA sequences known as primers are then added. The sample temperature is next lowered, allowing the primers to anneal (by hybridisation) to the target DNA. DNA polymerase and G, T, A and C nucleotide bases are then added and the temperature is raised slightly to allow the polymerases to extend the primers across each DNA strand until the whole region of interest is replicated. The result is two new molecules of dsDNA. The reaction can be cycled over and over again, each time doubling the DNA content.[155]

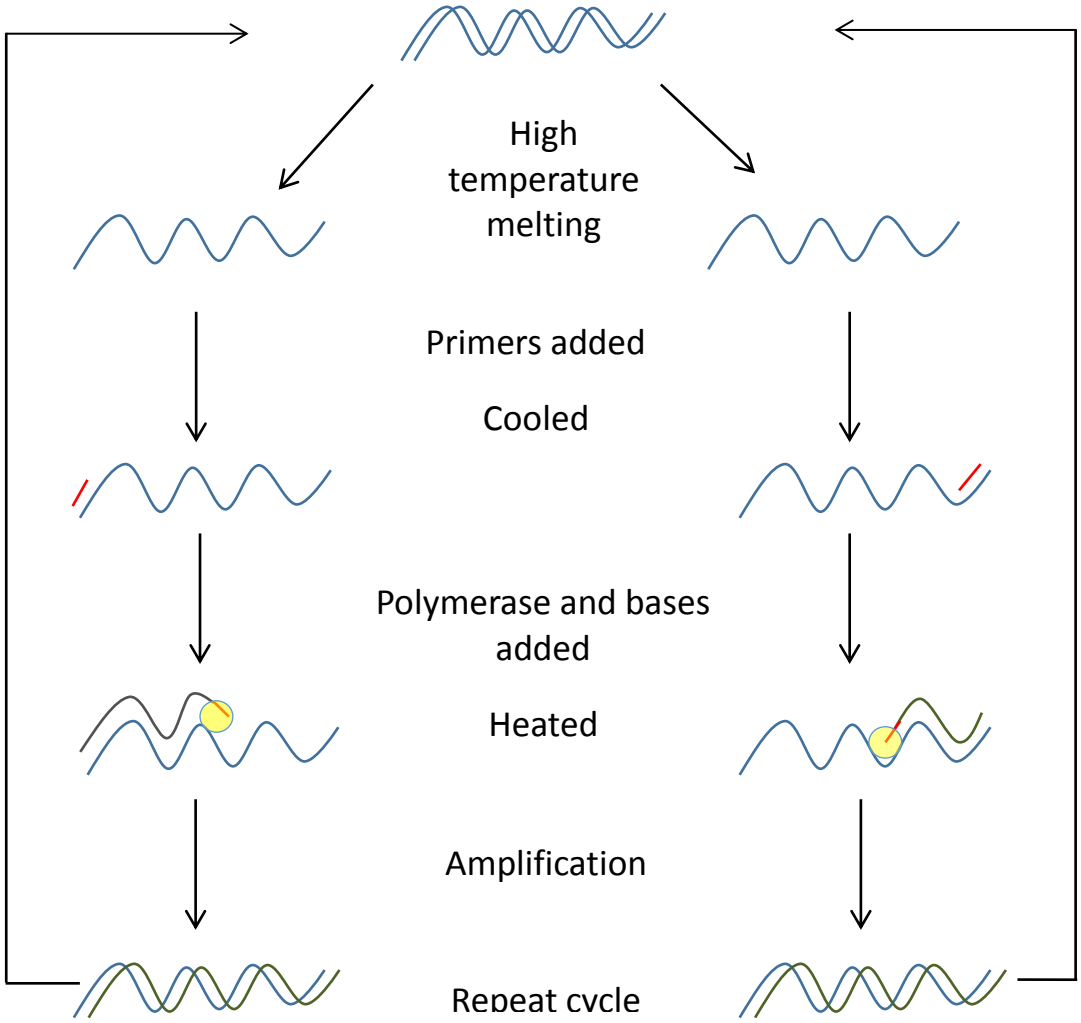

Figure 8. The basic principle of the polymerase chain reaction (PCR). First, the DNA is denaturised by heating to around 95°C for up to 30 seconds. This causes hydrogen bonds to break between the strands, allowing separation. The temperature is then lowered to around 50°C for 30 seconds, primers are added and these anneal through hybridisation to specific DNA sequences of interest. Next, nucleotide bases are added along with DNA polymerase (only added once in the first cycle) and the sample is heated to around 80°C. The heating time is dependent on the number of bases being amplified (around 1000 bases per minute). The process is cycled over and over until detectable levels of DNA have been amplified.[155] *DNA = deoxyribonucleic acid*

Traditionally, PCR products were run out on gel electrophoresis in order to identify whether a specific product was present. The advent of real-time quantitative PCR (qPCR) saw a move to semi-automated analysis of the DNA product. In qPCR a fluorescent signal is produced in the presence of the DNA product in solution. This originally was carried out by the reporter-dye method, whereby a dye that produced luminescence upon binding to double-stranded DNA, would accumulate during the reaction. This was later replaced by florescent reporting probes (e.g. TaqMan), which contain a luminescent reporter molecule and an anti-luminescence quencher probe in close proximity. In the resting state no signal is produced and during the hybridisation stage the probe will anneal to single stranded DNA (ssDNA) fragments of specific sequences. Upon extension by DNA polymerase, the quencher is released allowing the reporter to fluoresce and be detected by the PCR machine.[155,156]

The detection of fluorescent a signal enables the quantification of the amplified DNA with reasonable accuracy. The point at which the fluorescent signal intensity reaches above the background noise threshold (known as the cycle quantification or Cq value) can be used to calculate (known as a ΔΔCq calculation) the end product concentration. The quantification process involves calibrating the system against a known concentration of DNA beforehand. The PCR test is considered positive once the product exceeds the Cq value. The ΔΔCq method of identifying a DNA sequence is diagnostically useful when detecting for example vial DNA, however this method has less utility in gene mutation detection because designing primers which specifically target mutated DNA (such as point mutations) is challenging and thus specifically amplifying only mutated DNA above the Cq value is difficult. Many conventional PCR-based mutations tests instead use a melting point analyses. In melting point analyses, the rate at which the amplified dsDNA product melts over a range of temperatures is compared a known reference standard for the target sequence in question. Once again a florescent probe (various types available) is used to detect the amount of DNA product which has melted. Subtle changes in the nucleotide base sequence can significantly alter the melting curve profile of a DNA product (G-C bonds are more thermostable than A-T bonds) and therefore reporting a mutation with this method becomes highly specific.[155,156]

An advancement of traditional PCR, known as droplet digital PCR (ddPCR), is becoming popular for validation work. This technique uses extracted DNA dispersed into oil nandroplets, each droplet containing one molecule of DNA. The PCR reaction then takes place within the droplet, which is then either positive or negative by fluorescent signaling. This is a very sensitive method of detecting tiny quantities of a DNA segment of interest and using Poisson statistics, exact quantification is possible. The downside of this technology however is the high cost and need for highly skilled staff, meaning it is yet to be used in routine clinical practice.[157]

1.3.4 Sequencing-based tests

Sequencing refers to a technique whereby the actual sequence of bases is determined in a molecule of RNA or DNA. The traditional chain-termination technique was developed by Frederick Sanger in the late 1970's.[158] In so-called 'Sanger sequencing' the DNA is extracted and amplified using PCR. The DNA samples are then split into four aliquots. All four DNA bases are added to the aliquots along with DNA polymerase and a primer to start the reaction. In addition, a dideoxynucleotide form of one of the bases is added; a dideoxynucleotide of a different base to each of the four aliquots. The dideoxynucleotide when randomly incorporated to the extending DNA molecule (instead of a regular deoxynucleotide) halts the chain reaction at that base. This results in a sample comprising DNA molecules terminated at different lengths, each one representing a position where that base is present in the sequence. When all four aliquots are run out next to each other on an electrophoresis gel, the radiolabeled dideoxynucleotide bases will be seen at each position in the DNA fragmentation ladders, allowing the sequence to be determined. Later the technique evolved into what is known as capillary sequencing, where by different fluorescent tags in the sequence are read by a laser and the sequence is determined by an automated process.[159]

Sanger sequencing was a revolution in molecular biology and much of the human genome project was carried out using this technology. There are practical limitations to Sanger sequencing however, the method is slow, expensive and sometimes of poor quality, which means the diagnostic utility of this technology has remained limited. Despite these issues, Sanger sequencing is still considered a gold standard by many, however next-generation sequencing (NGS) attempts to overcome the issues with Sanger's methodology.[159] NGS is a high-throughput method of sequencing with a fast turnaround time and low cost. Many different platforms and versions of the principle are available, however the underlying principle is massive sequencing of small DNA fragments in parallel. DNA is first extracted and then fragmented randomly. The fragments are ligated at each end with adaptors. The adaptors allow the DNA fragments to bind to a matrix for sequencing and can incorporate a unique base sequence barcode so that multiple patient samples can be processed together. The two main platforms available for clinical diagnostic use are the Illumina (Illumina, Inc.)[160] and Ion Torrent systems (Thermo Fisher Scientific Inc.).[161] The Illumina platform uses a glass slide 'flow cell' matrix that is covered by a 'lawn' of complimentary adaptors. The complimentary adaptors allow sample DNA fragment adaptor binding. The sample is washed over the slide and the DNA fragments bind, becoming spatially fixed at one co-ordinate on the slide. In contrast, Ion Torrent uses a solution of microbeads, each with a similar complimentary adaptor. Once the DNA fragment attaches to the microbead, each microbead is centrifuged into a single microwell. In both Illumina and Ion Torrent systems, DNA fragments are amplified *in situ* using conventional PCR methods, during which each of the four bases is washed over the samples in turn in a continuous cycle. The

incorporation of a base into the extending fragment is detected – 'sequencing by synthesis'. In the Illunima platform, a fluorescent signal is produced as a base is incorporated and a high-resolution digital image capture and analysis system acquires the signal at each co-ordinate on the glass slide lawn after each base addition. In the Ion Torrent platform an ion sensitive field-effect transistor semiconductor chip detects changes in the pH in the overlying microwell as hydrogen ions are released during base incorporation. In both systems, the DNA fragment is read as it is replicated and because many copies of the same genome are present in the sample, there are multiple random DNA fragments covering and overlapping any one area of the genome. Furthermore, each DNA fragment is replicated (read) numerous times in the sequencing process. Reads (the sequence from each replication) are combined, and overlapped using bioinformatic software, by comparing with a reference genome, so the entire sequence can be presented. The reference genome may either be the same person's germline from their blood or an internationally standardised reference genome (currently GRCh38). As mentioned, any one area of the genome is present in multiple DNA fragments that are read numerous times, therefore every base of DNA will have been sequenced many times. The number of times each base has been read is known as the depth of coverage and this gives an indication of how accurate the sequencing is likely to have been. There are many applications of NGS, targeted genes or gene segments can be sequenced or the entire genome can be read (whole genome sequencing; WGS). Software can be used to annotate regions where base changes (compared to reference sequence or germline) are present. Any base change identified can be crosschecked with reference databases and the wider literature, to determine if the base change has a known pathological or clinical consequence.[159-161]

Traditional Sanger sequencing showed initial success in the research, forensic and some clinical settings, but the application to diagnostic pathology has been limited. NGS is now emerging as a technology that offers fast and cheap detection of gene mutations and can compete with conventional PCR in molecular diagnostics. Most prominently, the use of WGS is being promoted with the 100,000 genomes project, however understanding this data and integrating it into clinical care is some way off. Targeted gene panels though, which use NGS to sequence specific known sites of deleterious gene mutations, has the most clinical potential at present and is becoming popular. Gene panels offer mutation analyses at speeds and costs comparable to conventional PCR, while offering a much wider range of mutation detection in one assay than a PCR-based test can.[159,162]

In this Subsection, the two main types of NGS methodologies in widespread use have been discussed. There are other types of NGS systems available however and all have quite different properties and test limitations. It is important to note which type of NGS methodology is being discussed when evaluating any system because each is quite different. In this thesis, the Ion Torrent system has been focused upon and therefore the

term ‘NGS’ from herein, unless otherwise stated, will be used to mean sequencing specifically on this platform.

1.3.5 RNA and protein expression profiling

Finally, a remaining major area of molecular diagnostics, which is worth noting, are molecular technologies that investigate RNA and protein expression. Detecting and profiling the RNA content of cells (transcriptomics) allows for a direct measurement of cell gene expression. The main techniques have involved extracting RNA, converting this to complimentary DNA (cDNA) and using a number of hybridisation techniques for detecting the levels of expression. Although these techniques proved promising initially, there seems to have been little translation into routine diagnostics as of yet. This could change in the future as these techniques become more streamlined.[163] Protein expression using IHC may be surpassed in the future by emerging proteomics techniques – the examination of the whole protein content of the cell using mass spectrometry. Once again, this has yet to make it into routine diagnostic practice but once the cost and time restraints are resolved this could be a powerful clinical tool.[164]

1.4 The molecular diagnostics of colorectal cancer

Routine diagnostic molecular pathology tests for patients with CRC generally include targeted identification of specific gene mutations and determining the MSI status. The most common tests in routine practice are for mutations in *BRAF*, *KRAS* and *NRAS*. Whilst most centers use PCR-based tests for targeted mutation identification, a few centers use targeted NGS panels. Whole genome sequencing (WGS) is generally not used in routine practice yet.[165]

Microsatellite status is prognostic (MSI indicates a better survival), may indicate poor response (if MSI present) to 5-FU therapy and most importantly is useful for LS screening (MSI is present in LS-associated tumours; see Chapter 3).[60,93,166] MSI status can be determined with PCR-based assaying using multiple markers of microsatellite length. Depending on the degree of microsatellite expansion, tumours are designated as microsatellite stable (MSS), MSI low (MSI-L) or MSI high (MSI-H) – as mentioned earlier in Subsection 1.2.4. Alternatively, many centers use MMR protein IHC in conjunction with *BRAF* targeted mutation testing (see below). Loss of expression in one or more of the four MMR proteins (MMR deficiency), MLH1, PMS2, MSH2 or MSH6, is associated with MSI.[11,167] The method of determining MSS vs. MSI/MMR deficiency is probably not important[167] and IHC evaluation of MMR protein expression in conjunction with *BRAF* PCR is the most commonly used approach to LS screening in the UK and US. MMR gene methylation is also used in some institutions.[11,13,167,168]

Targeted mutation detection in current routine practice focuses on the *BRAF, KRAS* and *NRAS* genes. A detailed discussion of each of these will follow in Chapters (3 & 4), but a brief overview will be considered here. *BRAF* mutation status is prognostic for patients with Stage II or above CRC and is also useful in screening for LS in conjunction with MMR IHC (as mentioned above). *BRAF* testing in this context is carried out on those tumours with a loss of MLH1 IHC expression. This is a common event in sporadic as well as hereditary tumours and causes many false positives in LS screening. *BRAF* mutations are almost never present in LS (see Chapter 3) and so the detection of such a mutation helps rule out LS. *BRAF* testing has therefore been a routine PCR-based test for some time in many pathology laboratories.[11,13,167,168] Recently, an IHC method of detecting *BRAF* mutations has gained popularity and is used in a number of institutions, although accuracy data have been conflicting.[169,170]

KRAS (and probably *NRAS*) mutation status is useful in directing anti-EGFR monoclonal antibody (mAb) therapy in Stage IV CRC and may be prognostic. Therefore *KRAS* (and increasingly *NRAS*) testing has now become routine in many pathology laboratories. Commercial PCR-based tests are available for *KRAS*, however there are few widely used, commercially available and approved *NRAS* tests. Generally, *NRAS* testing is carried out with NGS.[69,70,133,134,171,172] Routine *EGFR* testing for CRC is not common, however recent data suggest that the S492R codon change may predict response to therapy in Stage IV disease. *EGFR* S492R testing in CRC may become increasingly used in the future, however no commercial test is available yet.[173,174]

Although this thesis focuses on current clinically routine testing, it is worth mentioning other areas of molecular testing that have been, or are currently under investigation. Unsurprisingly, somatic *APC* testing has been evaluated over the years. An *APC* mutation appears to impart a poor prognosis, however this mutation is found in over 90% of sporadic tumours and so the clinical utility of testing for these mutations has not be easy to prove. A similar story exists for *TP53* mutations and the loss of 18q, although some specific losses in the latter (i.e. *'deleted in colorectal carcinoma'* and *SMAD4* gene mutations) are predictive of poor outcome and reduced response to 5-FU therapy. Despite this, 18q testing has yet to make it into clinical practice due to some conflicting data on the issue.[60]

The effectiveness of 5-FU is also predicted by a number of polymorphisms in several enzyme pathways, including thymidylate synthetase, dehydropyrimidine dehydrogenase and methylenetetrahydrofolate reductase. Similarly, oxaliplatin therapy may be predicted by polymorphisms in glutathione-*S*-transferases. These have yet to make it into clinical practice, but this may be a significant area of personalised medicine in the future, once WGS is more widely available.[60]

HER-2 amplification (common in breast cancer) is seen in around 13% of rectal cancers and may be associated with poor outcome in those treated with 5-FU. HER-2 amplification is also thought to impart anti-EGFR mAb therapy resistance in a small number of *KRAS* wild type cases.[175,176] The data is early on these findings, however testing may be important in the future. Likewise, some early data suggest *PIK3CA* mutations may predict anti-EGFR mAB and aspirin therapy, and direct inhibitors of the gene product (PI3K) look to become important in the future of CRC therapy.[177]

Finally, an emerging area of cancer treatment is immunotherapy. Strictly, immunotherapy could be said to include any of the above Ab-based therapies, but the term is more commonly used to mean vaccine or checkpoint inhibitor drugs[178]. Vaccine therapy in CRC has been focused around anti-carcinoembryonic antigen (CEA) immunisation strategies[179] and this could be an indication for routine CEA testing (on tumours or blood) in the future. Checkpoint inhibitors aim to alter the T-cell immune response to tumours. The alteration can be by enhancing the immune surveillance of T-cells or can be by overcoming the mechanisms by which tumours avoid cell-mediated death. The most intensively investigated drug targets involved in immune surveillance are PD-1/PDL-1 and CTLA-4. It is likely that testing of these or other related targets (such as markers of microsatellite or MMR status), to stratify patients for checkpoint therapies, will be a significant part of future routine molecular diagnostics for CRC.[178]

1.5 Developments in colorectal molecular diagnostics

The current (and probably the future) focus of research in CRC surrounds the molecular (genetic) basis of the disease and how this can inform the diagnostic and management pathways. This will likely include molecular screening tests, non-invasive (blood-based) diagnostics and monitoring, as well as personalised medicine. For histopathology, the implications are likely to be further molecular scrutiny of tumours and the role of molecular diagnostics in CRC will become increasingly important.[180] Aside from this, the main movement of molecular diagnostics now is driven towards building and integrating PCR and NGS-based tests into routine practice to allow wider access to targeted testing across the NHS. The main issue is related to service provision. PCR and NGS-based technologies require expensive specialist laboratories and skilled staff, and so availability is fragmented across the UK. Therefore, testing is not universal and is lacking in many NHS hospitals.[15] There is a continual drive to streamline testing and reduce costs in order to address these problems. At the same time, new technologies are evolving at a fast pace. One such new technology is fully automated PCR. This approach is promoted as cheap, fast and requiring no specialist facilities or staff. In this sense, automated PCR may address some of the current issues with diagnostic molecular pathology and help widen testing availability. The technology however has not been rigorously validated or studied to date. The focus of this thesis in the following Chapters is to address this lack of validation data. The work will be

carried out within the context of CRC, because this comprises a significant proportion of the histopathology and molecular testing workload in most clinical departments. The investigation will focus on the use of automated PCR for routine molecular testing in CRC. This includes *BRAF*, *KRAS* and *NRAS* mutation testing.[155,165,167,180,181]

1.6 Automated PCR

1.6.1 The Idylla System

Many of the commercially available PCR and NGS systems offer partial or semi-automated processing. Fully automated PCR however (referred to from hereon as 'automated PCR') is novel and as yet there is only one platform commercially available that offers end-to-end automation in one setup: the Idylla System (Biocartis). Idylla is a fully automated real-time PCR system that can detect mutations in *BRAF*, *KRAS*, *NRAS* and *EGFR*, as well as the presence of some respiratory viruses, in a variety of tissue sample types. The system has an on-demand (i.e. non-batched) turn-around time of between 90 and 150 minutes (depending on the test), including pre-test preparation and post-test analysis. It is suitable for use by staff not trained in molecular biological techniques and can be carried out in any setting (including the potential for point-of-care testing with blood in the future). The system is a small, stand-alone platform which can be placed in any histopathology (i.e. non-specialist/non-molecular) laboratory (see Figure 9) and comprises a computer console and up to four processing units. Each processing unit can test one patient sample per run and each unit can run different tests with different samples, at the same time and on-demand, independent of the other units. The system provides end-to-end processing without the need for additional molecular equipment. The cost per test is variable and based on the caseload, but is competitive at around £100 per test (based on the manufacture quotation). Tissue preparation (section cutting) adds no additional burden beyond that of conventional PCR.[161,182,183]

Figure 9. The Idylla computer console (above) with a single processing unit (below).

The Idylla technology is cartridge-based (see Figure 10) and uses microfluidic (capillary action-based pumping) processing with all the reagents on-board. This allows almost all of the pre-test sample preparation to be automated. The cartridges require a user only to input a small volume of FFPE tissue and the remaining processes, including nucleic acid extraction, are fully automated – carried out onboard the processing unit (Figure 9). The system does this using high-frequency ultrasound focused at the sample (dissolved in a buffer and enzyme solution) to disrupt the cell membranes.[182]

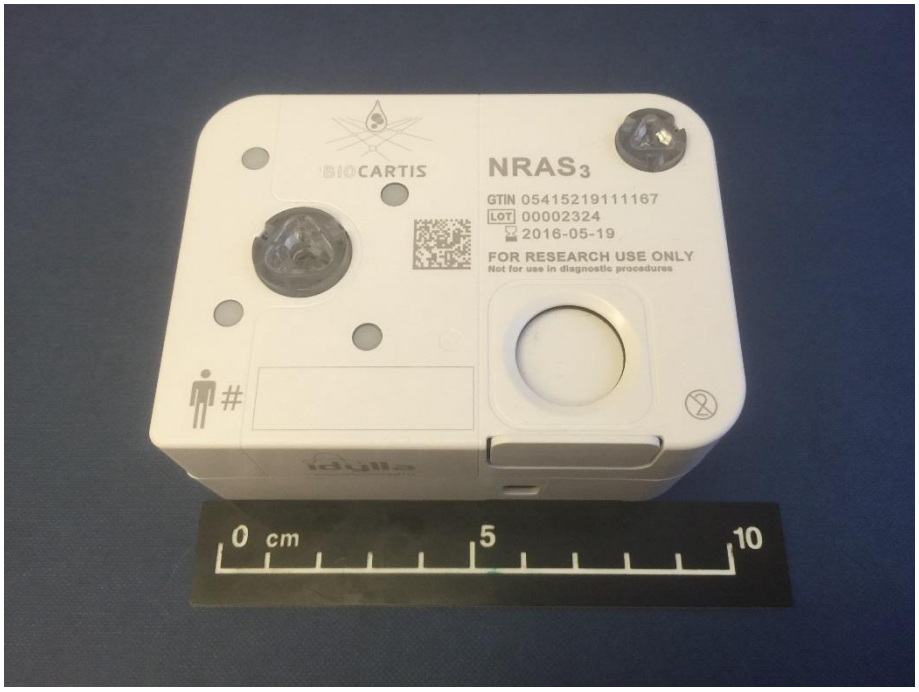

Figure 10. An Idylla test cartridge (an *NRAS-BRAF-EGFR*S492R Mutation Assay cartridge shown here).

At the time of planning this thesis, the Idylla System had two test cartridges for commercial use in diagnostic settings. The Idylla *BRAF* Mutation Test[184] and the *KRAS* Mutation Test[185] are both Conformité Européene *in vitro* diagnostics (CE-IVD marked) approved (legally required and marks completion of a quality control process before sale as a diagnostic medical device in the European Economic Area)[186,187] and were the first Idylla assays on sale for diagnostic use.[182,184,185,188] Further prototype tests were also available at the time. The Idylla *NRAS-BRAF-EGFR*S492R Mutation Assay[189] and the Idylla ct*BRAF* Mutation Assay[190] were available for Research Use Only (RUO; not CE-IVD marked). The Idylla Respiratory (IFV-RSV) Panel (for infectious disease) was also available but this test was not clinically relevant for this study.[191]

The Idylla PCR amplification takes place onboard the console and the system uses real-time fluorophore-based detection with a highly specific system of novel primers, probes and signaling molecules – conventional TaqMan-based methodology (discussed earlier in Subsection 1.3.3) for the Idylla *BRAF* Mutation Test and a novel PlexPCR-based (SpeeDx Pty Ltd) methodology for the Idylla *KRAS* and *NRAS-BRAF-EGFR* assays.[185,189,192] PlexPCR uses a system of primers (PlexPrime) that create amplicons containing a small region with a sequence different from that of the target DNA. This works together with a specific amplicon sequence-matched reporter probe (PlexZyme). This detection method significantly increases the overall target specificity and allows multiplexing of numerous gene mutations in one assay.[193,194]

A Cq value is calculated onboard the Idylla computer console for each mutation target and this is automatically compared against a wild type standard curve to give a ΔCq value. If this ΔCq value falls within a validated range, a positive (mutant) result is given; if the value falls outside of the validated range, a negative (wild type) result is given. Because the Idylla assay probes have highly specific binding properties (unlike those used by conventional PCR systems), the signal curve produced during the PCR reaction can itself be used for detection of the mutation with the Cq method, unlike most conventional platforms which use the melting technique as discussed in Subsection 1.3.3. This means that the Idylla results are ready at the end of the PCR amplification process, making the whole process quicker and able to detect low levels of tumour mutation burden with high specificity. The curve analysis is automated on-board the console and the results are presented on screen as either 'No mutation detected' or '*X* mutation detected'.[182]

1.6.2 Potential benefits of Idylla

There are essentially two basic methodologies for detecting gene mutations in clinical practice, those based on PCR and those based on gene sequencing. A comparison of the tests available for *BRAF*, *KRAS* and *NRAS* testing on the Idylla with the most commonly used PCR system (Cobas) and NGS (Ion Torrent) assays for these targets are given in

Table 7. Tests that are based on PCR are by far the most commonly used in practice as this is a fast, robust (low test failure rate) and cost effective technique. Sequencing technologies, at present, are only available to a small number of UK NHS clinical laboratories in specialist centres. Sequencing technology is much slower and it is far more expensive than PCR. PCR is therefore preferential. There are several potential benefits of the Idylla System over conventional PCR approaches or NGS technology. First, the Idylla System is fully automated, meaning that no specialist training is needed and no specialist setting is needed to house the system. The typical NHS histopathology laboratory does not have the specialist training or facilities needed in order to run molecular tests such as PCR or NGS gene panels. The Idylla System is unique in that all the processes are automated and carried out on-board the system. This means that it can be placed in any histopathology laboratory and tissue does not have to be sent elsewhere for testing. This eliminates a whole range of practical, legal and administrative issues around sending tissue out of the department. The Idylla tests are also much faster than most molecular tests, providing same day results when current tests can take on average a week or longer. Finally, the Idylla claims to need far less tissue than most PCR or NGS platforms, with adequate DNA obtained from a single tissue section (cf. up to eight tissue sections often needed for PCR or NGS). Therefore, the Idylla System could prove to be superior to many other technologies available for *BRAF*, *KRAS* and *NRAS* testing in CRC. The potential advantages could remove the barriers to testing in some centers and help to provide the wider availability of these tests that is needed.[182]

Gene	Idylla[182]	Cobas z 480 (PCR)[183]	Ion PGM (NGS)[161]
BRAF	**1) *NRAS-BRAF-EGFR*S492R Mutation Assay**[189] **Coverage:** V600D/E/E2/K/R **PCR method:** PlexPrime/PlexZyme Cq curve **Detection limit:** ≤5% **Turn-around time:** 2 hours (approx.) **2) *BRAF* Mutation Test**[184] **Coverage:** V600D/E/E2/K/M/R **PCR method:** TaqMan **Detection limit:** 1% **Turn-around time:** 1.5 hours	**1) *BRAF* V600 Mutation Test**[195] **Coverage:** V600E **PCR method:** TaqMan Cq curve **Detection limit:** >5% **Turn-around time:** <8 hours	**AmpliSeq Cancer Hotspot Panel version 2**[196] **Coverage†:** R444W, P453T, R462I, G464E/V/R, G466R/V/A/E, G469R/S/V/A/E, V471F, N581S, I582M, F583F, L584L/F, E586E/K, D587E/A, I592M/V, D594E/N/V/G, F595S/L, G596D/R, L597L/Q/S/V/R, A598V, A598_T599insV, T599I, T599_V600insTT, T599_V600insT, V600D/E/G/A/K/R/M/L/Q, K601N/E, V600_K601>E, K601del, R603*, W604G, W604del S605F/N/G, G606E, H608R, **Detection limit:** 98% detection rate for 5% variant frequency at positions with average sequencing coverage from 1,000X to 4,000X **Turn-around time:** Single day
KRAS	***KRAS* Mutations Test**[185] **Coverage:** G12C/R/S/A/D/V, G13D, A59E(G&T), Q61H(H2)/K(K2)/L(R), K117N1(N2), A146P(T&V) **PCR method:** PlexPrime/PlexZyme Cq curve **Detection limit:** ≤5% **Turn-around time:** 2 hours (approx.)	***KRAS* Mutation Test**[197] **Coverage:** G12C/R/S/A/D/V, G13C/S/R/D/V/A, Q61K/E/P/R/L/H/H2 **PCR method:** TaqMelt¶ melting curve **Detection limit:** <5% **Turn-around time:** <8 hours	**AmpliSeq Cancer Hotspot Panel version 2**[196] **Coverage†:** V8V, G10_A11insG, G12_G13insG, A11P/V, A11_G12insGA, G12F/C/L/V/S/R/E/V/D/A/G/Y/I/W, G13R/C/S/R/V/D/A/V/G/E, G13_V14insG, V14I , G15S, A18D, L19F, Q22K, T58I ,A59G/T/E, Q61K/E/P/R/L/H/K, K117N, A146T/V/P **Detection limit:** see above **Turn-around time:** Single day
NRAS	***NRAS-BRAF-EGFR*S492R Mutation Assay**[189] **Coverage:** G12C/S/D/A(V), G13D/V(R), A59T, Q61K/L/R/H(H2), K117N(N2), A146T(V) **PCR method:** PlexPrime/PlexZyme Cq curve **Detection limit:** 1-5% **Turn-around time:** 2 hours (approx.)	No relevant test available on this platform	**AmpliSeq Cancer Hotspot Panel version 2**[196] **Coverage†:** A11T, G12N/R/C/S/D/A/V/G, G13R/C/S/V/D/A/G, A18T, G60E, Q61L/K/R/E/P/L/R/H/Q, S65C, A146T **Detection limit:** see above **Turn-around time:** Single day

*Terminating codon notation. †Coverage given is for the codon changes that are likely to be relevant in CRC. ¶TaqMelt probes are proprietary detection probes used by Roche in melting point assays. () indicate codon changes covered by Idylla but not distinguishable by the system from the preceding codon

Table 7. *BRAF, NRAS and KRAS* assays on the Idylla System compared with common PCR (Cobas) and NGS (Ion Torrent) platforms. Turn-around times and detection limits are quoted from manufacturers. Permission to reproduce this table from a similar published table has been granted by the British Medical Journal Publishing Group Ltd.[4] *PGM = personal genome machine, PCR = polymerase chain reaction, NGS = next generation sequencing, X = average number of times base/DNA segment has been sequenced*

1.6.3 Literature review for the Idylla System

The published literature on the Idylla System and its technology is extremely limited. When this thesis was conceived and planned there were no studies with Idylla System published. Biocartis did provid access to a number of posters and a conference abstracts on their website.[198] These appeared to be internal to or sponsored by Biocartis and contain data from small validation style studies mostly using the Idylla *BRAF* Mutation Test. These posters quote the concordance of Idylla with PCR and various sequencing methodologies as between 97% and 100%. The cases analysed in these studies appear to be highly selected and the results probably only serve as a proof-of-principle. It is extremely unlikely this data had been peer-reviewed and is therefore of uncertain reliability. However, if taken on face value, the data did suggest a high level of concordance with conventional testing methods and so the diagnostic accuracy was expected to be high.[199-207] No attempt to formally investigate the diagnostic accuracy of the Idylla System had been made before this thesis work was started though. Some of the data from the initial posters and abstracts appears to have been included in later publications by the same authors. These papers and other literature published since this thesis work was carried out, is discussed in Section 5.5.

1.6.4 Research questions unanswered by the literature

The evidence for the Idylla System was extremely scanty and so there remained a number of unanswered research questions. There was a need to carry out full, unbiased and unselected diagnostic test accuracy studies for the Idylla System in detecting *BRAF*, *KRAS* and *NRAS* mutations with comparisons against routine standard tests currently used (conventional PCR and NGS). There was also a need to compare the *BRAF* Mutation Assay with IHC methods that are becoming popular in many laboratories.

The aim of the study described in the following Chapter was to address these unanswered research questions as far as possible within the limits of time and funding.

1.7 Chapter summary

CRC is a significant health problem in the UK and results in a large number of deaths each year. The aetiology of CRC is not well established but the pathogenesis is thought to occur via an intermediate adenoma stage and progress along a number of well-defined molecular pathways. The traditional approach to managing patients with CRC is largely based on the prognosis, determined by Staging. Staging involves initial clinical Staging using radiological findings and final definitive Staging based on a histopathological assessment of the resected tumour. The mainstay of treatment for CRC is surgery, but histological features may predict the likelihood of disease progression and death. Based on the histolopathological findings, further chemo-radiotherapy may be offered. In recent years, a

number of therapies have been developed that are aimed at altering the significant molecular pathways in CRC pathogenesis. Mutations in the MAPK pathway are major drivers of CRC pathogenesis and as such detecting these mutations has prognostic significance for patients. Mutations in these pathways also render anti-EGFR mAb therapies ineffective and are therefore very clinically relevant. The most clinically informative mutations in the MAPK pathway are found in *BRAF*, *KRAS* and *NRAS* and so these are the most commonly tested genes in routine clinical practice. Despite testing recommendations by many prominent organisations, there is a shortfall in availability of testing in many centers, probably in large part due to funding limitations. Newer testing technologies to the market however could address these issues. One such technology that could overcome this problem is an automated PCR platform called the Idylla System. This thesis aims to assess the potential of the Idylla tests for use in clinical practice by evaluating the accuracy and cost-effectiveness of the system.

1.8 Hypothesis

The hypothesis for this thesis is that the Idylla System is highly accurate in detecting *BRAF*, *KRAS* and *NRAS* mutations in CRC FFPE tissue and that the system is superior to other technologies currently available.

1.9 Aims and objectives of the thesis

This study aimed to test the hypothesis, set out above, that the Idylla System is accurate and superior to other technologies available for *BRAF*, *KRAS* and *NRAS* testing in CRC. Such a study would address the lack of evidence for the Idylla System in the literature, validate the various assays where possible and conduct clinical assessments of test diagnostic accuracy. The study also aimed to evaluate the system in terms of placement in the diagnostic pathway, financial implications and practicalities of use.

The objectives were:

- An audit of the potential workload and financial burden of the Idylla System
- A validation and diagnostic test accuracy study of the Idylla *BRAF* Mutation Test
- A validation and diagnostic test accuracy study of the Idylla *KRAS* Mutation Test
- A validation of the Idylla *NRAS-BRAF-EGFR*S492R Mutation Assay

Some of the ideas and information presented in this Chapter are also presented in publications and posters arising from this work.[1-8]

Chapter 2: Materials and Methods

2.1 Introduction

To test the hypothesis and address the aims of this thesis, a number of experiments were conducted as part of a wider study. The study design and experiments are described in this Chapter. A description of the specific materials and protocols are given first followed by a discussion of the overall design and conduct of the study.

2.2 Materials

2.2.1 Tissue

- Diagnostic FFPE tissue blocks
- Corresponding H&E histology slide

2.2.2 Reagents

- 4% hydrogen peroxide (Sigma-Aldrich, Inc.)
- Absolute ethanol (Sigma-Aldrich, Inc.)
- 10M Molecular biology grade NaOH (Sigma-Aldrich, Inc.)
- Haematoxylin II (Ventana Medical Systems, Inc.)
- Nuclease free water (QIAGEN)
- Molecular biology grade water (Sigma-Aldrich, Inc.)
- Distilled water (Thermo Fisher Scientific Inc.)
- Tap water
- DPX mountant (Sigma-Aldrich, Inc.)
- Laboratory grade mineral oil (Sigma-Aldrich, Inc.)
- Deparaffinization Solution (QIAGEN)[208]
- EZ Preparation Solution (Ventana Medical Systems, Inc.)[209]
- Cell Conditioning 1 (high pH Tris-Borate-EDTA; Ventana Medical Systems, Inc.) Buffer[210]

2.2.3 Consumables

- Microtomy blades (Leica Biosystems)
- Electrostatically charged glass microscopy slides 25 x 75mm, for IHC cases only (Thermo Fisher Scientific Inc.)
- Uncharged glass slides 25 x 75mm (Thermo Fisher Scientific Inc.)
- Cover slips 25 x 50 mm (Thermo Fisher Scientific Inc.)
- Razor blades (Agar Scientific Ltd)

- Scalpels (Sigma-Aldrich, Inc.)
- 1.5ml microcentrifuge (eppendorf) tubes (Sigma-Aldrich, Inc.)
- 0.2ml PCR tubes (Sigma-Aldrich, Inc.)
- 96 well optical plates (Thermo Fisher Scientific Inc.)
- Clear adhesive plate film (Thermo Fisher Scientific Inc.)
- 5mm blotting paper discs (supplied with Idylla by Biocartis)
- Filtered universal pipette tips, range of volumes (Thermo Fisher Scientific Inc.)
- QIAamp MinElute Columns (QIAGEN)[211]

2.2.4 Assay kits

- dsDNA HS Assay Kit (Thermo Fisher Scientific Inc.)[212]
- Cobas 4800 *BRAF* V600 Mutation Test (Roche Molecular Systems Inc.)[195]
- Cobas *KRAS* Mutation Test (Roche Molecular Systems Inc.)[197]
- Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific Inc.)[213]
- Ion Xpress Barcode Adapters (Thermo Fisher Scientific Inc.)[214]
- Ion AmpliSeq Cancer Hotspot Panel version 2 kit (Thermo Fisher Scientific Inc.)[196]
- Axygen Axyprep Mag PCR Clean-up Kit magnetic beads (Thermo Fisher Scientific Inc.)[215]
- Ion Library TaqMan Quantitation Assay (Thermo Fisher Scientific Inc.)[216]
- Rox Reference Dye (Thermo Fisher Scientific Inc.)[217]
- Custom TaqMan Probes with FAM/MGB reporter molecules (Thermo Fisher Scientific Inc.)[218]
- Ion One Touch 2 200 Template Kit (Thermo Fisher Scientific Inc.)[219]
- Ion 318 Chip Kit version 2 (Thermo Fisher Scientific Inc.)[220]
- QIAamp FFPE Tissue Kit (QIAGEN)[221]
- Anti-*BRAF* V600E (VE1) Mouse Monoclonal Primary Antibody (Ventana Medical Systems, Inc.)[222]
- OptiView Amplification Kit (Ventana Medical Systems, Inc.)[223]
- OptiView DAB IHC Detection Kit (Ventana Medical Systems, Inc.)[224]
- Idylla *BRAF* Mutation Test (Biocartis)[184]
- Idylla *KRAS* Mutation Test (Biocartis)[185]
- Idylla *NRAS-BRAF-EGFR*S492R Mutation Assay (Biocartis)[189]
- Dynabeads MyOne Streptavidin C1 Beads (Thermo Fisher Scientific Inc.)[225]

2.2.5 Equipment

- Rotary microtone (Leica Biosystems)
- Microtomy water bath (Leica Biosystems)
- Microcentrifuge (Thermo Fisher Scientific Inc.)

- Plate centrifuge (Thermo Fisher Scientific Inc.)
- Vortex mixer (Thermo Fisher Scientific Inc.)
- Ultraviolet steralisation cabinet (Thermo Fisher Scientific Inc.)
- Thermal cycler (Biometra)
- Compound light microscope (Olympus)
- Slide etching marker (Leica Biosystems)
- Slide marker pen (Leica Biosystems)
- Magnetic tube rack (Thermo Fisher Scientific Inc.)
- P10, P20, P200 & P1000 micropipettors (Sigma-Aldrich, Inc.)
- Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc.)[226]
- Qubit 2.0 Fluorometer (Thermo Fisher Scientific Inc.)[227]
- Heating incubation block (Thermo Fisher Scientific Inc.)
- Cobas z 480 Analyzer (Roche Molecular Systems Inc.)[183]
- Applied BioSystems 7500 Real Time PCR System (Thermo Fisher Scientific Inc.)[228]
- Ion OneTouch 2 Instrument and Enrichment System (Thermo Fisher Scientific Inc.)[229]
- Ion Chip Minifuge (Thermo Fisher Scientific Inc.)[230]
- Ion Torrent PGM System (Thermo Fisher Scientific Inc.)[231]
- Idylla System (Biocartis) [182]
- QX100 Droplet Digital PCR System (Bio-Rad Laboratories, Inc.)[232]
- Benchmark ULTRA (Ventana Medical Systems, Inc.)[233]

Some suppliers varied depending on laboratory stock ordering and availability; where no specific citation is given, example suppliers that are used locally are quoted

2.3 Methods

2.3.1 Overview

In order to test the hypothesis, a series of experiments were carried out on retrospective diagnostic tissue from patients with CRC. The overall study design is explained below in Section 2.4, but first the methods of individual experiments are given. Firstly, tissue on glass slides was acquired from the existing archival bank. This was used directly for IHC but was removed (by macro-dissection) from the slide for molecular testing. Macro-dissected tissue was used for a range of PCR techniques and for NGS. DNA was extracted prior to conventional PCR and NGS. For NGS a sample library was prepared and amplified before being sequenced. Each of these steps are discussed in detail in the following Subsections.

2.3.2 Slide preparation

Tissue tested in the study was taken from the same tissue blocks that were used in the comparison tests originally carried out for molecular diagnostics. Tissue sections were cut from FFPE blocks using a microtome and floated on warm water in a water bath containing tap water at 30°C. Sections were collected on glass slides and left to dry. Molecular testing was carried out on 5µm sections placed on uncharged slides. H&E sections were prepared with 4µm sections placed on uncharged slides. IHC testing was carried out on 4µm tissue sections place on charged slides. Water baths and microtome blades were changed between cases for molecular testing to avoid contamination. FFPE slides were stored at room temperature until ready for use.

2.3.3 Immunohistochemistry

VE1 IHC was performed on unstained 4µm FFPE tissue whole sections that were placed on positively electrostatically charged glass slides and baked in a 60°C oven for two hours. Sections were then loaded onto the Benchmark ULTRA (Ventana Medical Systems, Inc.)[233] for automated IHC processing using the pre-installed protocol for VE1 (Protocol 7). Sections were dewaxed in EZ Preparation Solution (Ventana Medical Systems, Inc.)[209] at 72°C for 20 minutes. Antigen retrieval was carried out by incubating the sections in Cell Conditioning 1 (high pH Tris-Borate-EDTA; Ventana Medical Systems, Inc.) Buffer[210] at 95°C for 64 minutes. Slides were then washed with 4% hydrogen peroxide for pre-primary Ab peroxidase inhibition. Sections were incubated with anti-*BRAF* V600E (VE1) Mouse Monoclonal Primary Antibody[222] for 16 minutes at 36°C. Amplification was carried out with the OptiView Amplification Kit[223] by incubating with kit reagent for 8 minutes. Detection was carried out with the OptiView DAB IHC Detection Kit[224] reagent**.** Tissue was counterstained with Haematoxylin II for four minutes and blued for a further four minutes in tap water. Slides were mounted in DPX and a glass cover slip was applied. The slides were left overnight to dry.

2.3.4 Tissue macro-dissection

In general, tissue blocks contain tumour with adjacent non-neoplastic tissue which can potentially dilute the tumour nucleic acid content if processed together in the same sample. Tissue used in molecular tests is ideally acquired from sections placed on slides rather than used directly from the cut microtome sections. Placing tissue on slides allows comparison of the unstained section with the stained histology slide and then non-tumour tissue can be selectively removed (scraped away) and discarded prior to the assay. This is a technique known as macro-dissection.[144]

To carry out macro-dissection, the unstained slide was compared and matched against the corresponding H&E stained sections using light microscopy. The tumour was marked on

the H&E slides with a pen by the reporting pathologist at the time of diagnosis. Tissue was then approximated on the unstained sections. Where possible the same area of tissue that had undergone PCR or NGS originally was selected for Idylla testing and IHC.[144] Tumour identified on the unstained section was marked using an etching slide marker. Macro-dissection was carried out using a razor blade or scalpel. Tissue to be discarded was first scraped from the glass slide and disposed of. The tissue of interest was then scraped from the slide and set aside ready for testing. Macro-dissected tissue was stored at room temperature in 1.5ml Eppendorf tubes if not tested immediately.

Each assay had minimum tissue requirements to be met by macro-dissection. For conventional PCR testing, a tissue area of up to 250mm^2 on up to eight sections at a maximum thickness of 10um was used with a minimum of 50% tumour nuclei content (*BRAF* Mutation Test)[195] or 10% (*KRAS* Mutation Test)[197] in line with Cobas manufacturer (Roche Molecular Systems Inc.) directions and internally validated quality assurance (QA) protocols. For NGS, a tissue area of up to 250mm^2 on up to eight sections at a maximum thickness of 10um was used at a minimum of 40% tumour nuclei content, in line with Ion Torrent manufacturer (Thermo Fisher Scientific Inc.) directions[196] and internally validated QA protocols. For Idylla testing a tissue area of 50-600mm^2 from a single 5µm section containing ≥10% (*KRAS* Mutation Test), 25% (*NRAS-BRAF-EGFR*S492R Mutation Assay) or 50% (*BRAF* Mutation Test) tumour nuclei content was used as directed by Biocartis.[184,185,189]

All Idylla testing was carried out using the same FFPE tissue block that underwent original molecular testing (PCR or NGS). In a small minority of cases, 1mm punch tissue was taken from FFPE blocks for original molecular testing. Where this occurred, tissue for macro-dissection was selected surrounding the punched area.

2.3.5 Nucleic acid extraction

Nucleic acid extraction was carried out for conventional PCR and NGS by the Oxford Molecular Diagnostics Centre (OMDC). Manual extraction was not carried out for Idylla testing as this was automated internally within the cartridge. Nucleic acid was extracted from macro-dissected FFPE tissue. The QIAamp FFPE Tissue Kit (QIAGEN) was used for extraction.[221] FFPE was incubated in Deparaffinization Solution (QIAGEN)[208] on a heat block at 56°C for three minutes. Lysis buffer and proteinase K were added and the solution was incubated at 56°C for 16 hours and then 90°C for one hour. An ethanol-based buffer was then added and the sample transferred to a QIAamp MinElute Column (QIAGEN)[211] for centrifuge filtration, followed by elution with the elution buffer.

QA was carried out in line with manufacture instructions for Cobas (Roche Molecular Systems Inc.) PCR and Ion Torrent (Thermo Fisher Scientific Inc.) NGS. For all samples,

the extracted nucleic acid content was quantified to calculate volumes required in PCR and sequencing. For PCR testing, samples were quantified using the Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc.).[226] A minimum of 125ng of DNA at a minimum concentration of 5ng/µl (*BRAF* Mutation Test)[195] and a minimum of 50ng of DNA at a concentration of 4ng/µl (*KRAS* Mutation Test)[197] were required as per Cobas (Roche Molecular Systems Inc.) manufacturer instructions. For NGS-based tests, samples were quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific Inc.)[227] with the dsDNA HS Assay Kit (Thermo Fisher Scientific Inc.)[212] and a minimum of 10ng of DNA at a concentration of 3ng/µl was required for the Ion AmpliSeq Cancer Hotspot Panel version 2 (v2) kit (Thermo Fisher Scientific Inc.) in line with manufacturer requirements.[196] If the concentration of DNA was lower than the minimum required, where possible further FFPE tissue underwent extraction to enhance the concentration. An estimate of purity (DNA:protein ratio; the acceptable range was 1.8-2.0) was acquired for both PCR and NGS, carried out using the Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc.).[226]

Extracted nucleic acid was stored at room temperature for up to 24 hours, at 4°C for up to 14 days and up to 60 days at -20°C. A maximum of three freeze-thaw cycles were permitted. Samples not adherent to QA protocols were discarded.

2.3.6 Conventional PCR

Conventional PCR testing was carried out on extracted DNA, prepared at a concentration of 2ng/µl. The Cobas *BRAF* V600 Mutation Test and Cobas *KRAS* Mutation Test (Roche Molecular Systems Inc.) were used for conventional PCR.[195,197] A master mix containing primers, probes, DNA polymerase and nucleotides was prepared from the kit reagents. Samples were added to a 96-well plate along with the master mix, wild type and mutant gene controls. The plates were covered by an adhesive film and centrifuged. Plates were run on the Cobas z 480 Analyzer (Roche Molecular Systems Inc.).[183]

2.3.7 Droplet digital PCR

Droplet digital PCR (ddPCR) was performed on discordant cases, for confirmation of results by Biocartis. Full details of this protocol were not disclosed. DNA was extracted from unstained tissue section slides provided to Biocartis. Tests were run on a QX100 Droplet Digital PCR System (Bio-Rad Laboratories, Inc.).[232]

2.3.8 Automated PCR

Automated PCR was carried out on the Idylla System[182] directly on macro-dissected FFPE tissue. DNA extraction was carried out automatically by the Idylla System during the assay. FFPE tissue was placed between two 5mm discs of blotting paper, wetted with nuclease

free water, and placed inside an Idylla test cartridge (Biocartis). The cartridge was loaded onto the Idylla System (Biocartis) for processing. The Idylla *BRAF* Mutation Test, the Idylla *KRAS* Mutation Test and the Idylla *NRAS-BRAF-EGFR*S492R Mutation Assay (Biocartis) cartridges were used.[184,185,189] All results from comparison tests were blinded at the time of Idylla testing.

2.3.9 Sequencing library preparation

The library was prepared from extracted DNA under an ultraviolet lit hood using the Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific Inc.).[213] and the Ion AmpliSeq Cancer Hotspot Panel v2 primers (Thermo Fisher Scientific Inc.).[196] The concentration was standardised to 10ng/µl**.** Following this, unique specimen barcodes were added (Ion Xpress Barcode Adapters; Thermo Fisher Scientific Inc.),[214] along with DNA ligase and adaptors and the samples were again loaded in a thermal cycler for the ligation reaction. Purification and clean up was carried out using Axygen Axyprep Mag PCR Clean-up Kit magnetic beads (Thermo Fisher Scientific Inc.).[215] Ligated samples were incubated with Axyprep beads then washed three times in ethanol on a magnetic block. Purified DNA was then eluted from the Axyprep beads, the beads separated on the magnetic block and then removed.

The library was quantified using the Ion Library TaqMan Quantitation Assay (Thermo Fisher Scientific Inc.)[216] on a 96-well plate. Each specimen was tested in duplicate. Master mix, primers and probes were added to each well. Standardisation was carried out against the *E. coli* DH10B reference samples included in the kit. Rox Reference Dye (Thermo Fisher Scientific Inc.)[217] was used as the passive reference and Custom TaqMan Probes with FAM/MGB reporter molecules (Thermo Fisher Scientific Inc.)[218] were used. The qPCR reaction was run on the Applied BioSystems 7500 Real Time PCR System (Thermo Fisher Scientific Inc.).[228] Quantification was calculated for each sample based on the qPCR curves and the samples were diluted to 100 pM for NGS.

2.3.10 Library amplification and enrichment

Library samples were amplified and enriched using the Ion OneTouch 2 200 Template Kit (Thermo Fisher Scientific Inc.)[219] and run on the Ion OneTouch 2 System, which comprises the OneTouch Instrument and Enrichment System (ES).[229] Samples were combined and the kit Reagent Mix, PCR Reagent, Enzyme Mix and Ion Sphere Particles were added. The samples were then loaded on to the Ion OneTouch Plus Reaction Filter Assembly (included in Template kit; Thermo Fisher Scientific Inc.), sealed with oil and run on the Ion OneTouch 2 Instrument (Thermo Fisher Scientific Inc.) for templating and amplification. Following this, the filtration tubes were emptied and the pellets re-suspended in recovery solution and combined. Enrichment was carried out using Dynabeads MyOne Streptavidin C1 Beads[225]

on the Ion OneTouch ES (Thermo Fisher Scientific Inc.). Control sphere particles were added to the samples following enrichment and thermal cycling was carried out.

2.3.11 Sequencing

PGM 200 Sequencing Polymerase (from the OneTouch 2 200 kit; Thermo Fisher Scientific Inc.) was added to the amplified and enriched DNA library samples. The libraries were then loaded on to an Ion Torrent Chip (Ion 318 Chip Kit v2; Thermo Fisher Scientific Inc.).[220] The chip was spun on an Ion Chip Minifuge (Thermo Fisher Scientific Inc.)[230] and then loaded and run on an Ion Torrent PGM System (Thermo Fisher Scientific Inc.).[231] Analysis was carried out using the web-based Ion Reporter Software (Thermo Fisher Scientific Inc.),[234] using the most recent international reference genome.

2.4 Study design

2.4.1 Overview

The experiments described above were brought together in an overall study design to test the hypothesis. Initially, an audit of the case workload was carried out to identify relevant archival cases and to also assess the financial impact of introducing the Idylla System into routine practice. Next a series of comparison tests were carried out to assess the accuracy of the Idylla System. It is important at the outset to highlight the difference between a test's performance under controlled laboratory conditions using preselected positive and negative cases, and a test's diagnostic accuracy in the clinical setting for detecting a disease state (here a *BRAF*, *KRAS* or *NRAS* mutant tumour). The former is a technical validation and often serves as a proof-of-concept for a new test or to verify the successful implementation of an established test. These validation studies are designed to determine the new (index) test's concordance with existing standard (reference) tests. A validation study in some settings can also be used to determine the LOD (or analytical sensitivity). These measures should not be confused with the sensitivity and specificity of test derived from a diagnostic trial in clinical settings. Diagnostic studies are far more informative for using a test in clinical practice and therefore a preferable study design.[235-237]

The main study in this thesis comprised two arms. The first arm followed a diagnostic test accuracy study design with the aim of estimating the accuracy of the Idylla *BRAF* and *KRAS* Mutation Tests on the Idylla System under clinical conditions. There was already some limited validation data from Biocartis for these tests and a diagnostic trial would be the best model for testing the hypothesis.[184,185] The comparisons were made with the Cobas *KRAS* and *BRAF* Mutation Tests run on the Cobas 4800 System z 480 analyzer (Roche Molecular Systems Inc.).[183]

In order to fully investigate diagnostic accuracy (sensitivity, specificity etc.) an unbiased, unselected population of cases that reflect genuine practice is required.[237,238] This was not always possible for all Idylla tests due to funding, tissue or cartridge availability limitations. For these reasons, the second arm of the study then was limited to a series of more technical validations. Although this is less informative, it still allows some assessment of the test's performance to be made. Also, in some circumstances where an Idylla test had been assessed in the first arm of the study by comparing with PCR, it was probably enough to just make a more technical validation with NGS and a full study was unnecessary. For other Idylla tests not assessed in the first arm, a technical validation would serve as a proof-of-principle for a future diagnostic accuracy study. In this second arm, validation studies aimed to investigate concordance. The LOD was not assessed as accuracy was the primary focus of this thesis. The *BRAF* Mutation Test was validated against the anti-*BRAF* V600E (VE1) Mouse Monoclonal Primary Antibody IHC assay (Ventana Medical Systems, Inc.) as this is now a commonly used test[169,222]. The *KRAS* Mutation Test was validated against NGS Ion Torrent methodology (Thermo Fisher Scientific Inc.)[161] using the Ion AmpliSeq Cancer Hotspot Panel v2 (Thermo Fisher Scientific Inc.)[196] gene panel assay (which includes *BRAF, KRAS* and *NRAS* coverage) on the Ion Torrent PGM System (Thermo Fisher Scientific Inc.).[231] Finally, the *NRAS-BRAF-EGFR*S492R Mutation Assay (Biocartis)[189] was validated against the same NGS panel.

As well as test accuracy, an important indicator of the value of a test is reproducibility. This is a third type of study design for evaluating tests. As accuracy was the focus of this thesis, reproducibility was not specifically measured in this work. Automated molecular test reproducibility is expected to be very high anyway and resources were limited in this thesis to investigate this matter specifically. However, the reproducibility (agreement) of the IHC scoring was assessed using Cohen's kappa, because IHC agreement is a known problem in diagnostic histopathology. This problem is discussed further in Section 5.4.

Full validation of the *NRAS-BRAF-EGFR*S492R Mutation Assay was not possible as no comparison tests are commercially available for the S492 codon change in EGFR.

2.4.2 Timeline

The data collection began in spring 2015 with the audit. Cases from the year 2013 were selected to ensure a full calendar year could be audited and that a long enough time period had passed before auditing to avoid missing molecular and supplementary results (which could take several months to be transcribed to the system). Data for the *BRAF* Mutation Assay clinical study were also collected in the spring of 2015 and cases with full molecular reports were selected from 2013-2014 in the diagnostic archival database. *KRAS* data were collected in part over the summer of 2015 and in part during late 2015/early 2016 when funding was available. Cases were selected from 2014-2015. Later cases (than those used

for *BRAF* testing) were selected because during 2015 there was a transition to NGS and data from both PCR and NGS were required for the thesis. *NRAS-BRAF-EGFR*S492R Mutation Assay validation took place in early 2016 following delays with cartridge availability. Cases from a long period between 2013 and 2016 were needed to maximise number of *NRAS* mutant tumours to be included (see Subsection 4.1.3). Results are presented in a logical order for convenience in the following Chapters, however this obviously may not always reflect the true timeline of the study.

2.4.3 Study setting

The study was based in the Department of Cellular Pathology at the John Radcliffe (JR) Hospital, part of the Oxford University Hospitals NHS Foundation Trust (OUH).[239] Where applicable, work was carried out within Clinical Pathology Accreditation (CPA) / UKAS accredited laboratories to the International Organization for Standardization (ISO) 15189:2012 specifications and in accordance with the Care Quality Commission (CQC) regulations.[137,240,241]

2.4.4 Ethics approval and local regulations

Ethical approval for the study was granted by the National Research and Ethics Service (Oxfordshire Research and Ethics Committee A; reference 04/Q1604/21), see Appendix 1.[242] The Oxford Centre for Histopathology Research (OCHRe) approved the thesis work to be carried out under local regulations (Application number 14/A209 and amendment numbers 15/A041 and 16/A002), see Appendix 2.[243]

2.4.5 Funding

Consumables, some Idylla tests and costs from OCHRe were funded from a grant provided by the Oxfordshire Health Services Research Committee (Fund 8262).[244] Salary was provided by the Department of Oncology, University of Oxford via the National Institute for Health Research Biomedical Research Centre, Oxford.[245] The Idylla System was made available for the project by Biocartis free of charge and on loan for a trial period. Biocartis provided a proportion of the Idylla tests for free. Biocartis did not directly fund any of the work, provide any salary or provide any financial incentive. Biocartis has no involvement in the production of this thesis. There were no competing interests of any of the parties involved in the study.

2.4.6 Risk assessment

A Control of Substances Hazardous to Health (COSHH) risk assessment was carried out prior to Idylla data collection, see Appendix 3.

2.4.7 Contributions

The author devised and designed the work in this thesis, secured the funding, selected and prepared tissue, carried out all Idylla testing and carried out all statistical analyses. Original diagnostic specimen preparation – including dissection, processing, slide preparation and tissue preparation for molecular testing – was carried out by members of the Department of Cellular Pathology, John Radcliffe Hospital. Tissue for the study was provided anonymised by OCHRe. Original diagnostic molecular testing used for comparisons was carried out by OMDC.[246] IHC testing was carried out with assistance from members of the Department of Cellular Pathology, John Radcliffe Hospital. IHC scoring was carried out by consultants in the department.

2.4.8 Participants and inclusion criteria

Cases were selected from the departmental database at OUH. Inclusion criteria used for the database search were: reported cases from any patient with microscopic findings of a primary adenocarcinoma in a colorectal specimen. Such specimens included endoscopic biopsies, colectomy, hemicolectomy, anterior resection, abdominoperineal (including low) resections, transanal endoscopic mucosal resections (TEMs) and metastasis excisions. For technical validations only primary tumour tissue from resections were tested; all specimen types were included in clinical studies.

Participants from whom cases were selected were all NHS patients undergoing treatment for colorectal adenocarcinoma within OUH. All diagnoses were authorised by a qualified consultant histopathologist. The majority of these patients had undergone their surgery at the JR or the neighbouring Churchill Hospital (also OUH). NHS patients were asked to provide or withhold consent for their tissue to be used in teaching and research as standard practice before any type of biopsy/surgery.[247]

For the audit part of the thesis, all cases of CRC reported through the OUH department over a 12-month period were identified and the number of molecular tests ordered were recorded. A consecutive series of cases meeting the inclusion criteria over a three-month period were selected out from the search results for the clinical diagnostic accuracy study of *BRAF* and *KRAS* testing. For *BRAF, KRAS* and *NRAS* validation studies, a sample was selected out from these search results to include positives and negatives, in line with recommendations.[248] There was some overlap in the patients participating in some parts of the study.

2.4.9 Exclusion criteria

Referred cases and cases from patients lacking documented consent were excluded. Referred cases were included for the audit however as this provided relevant information about genuine workloads. Internal cases (not referred) where the tissue blocks were not available or lost (i.e. cannot therefore be tested) were excluded. Cases from other tumour sites (non-colorectal) or tumour types (non-adenocarcinomas or metastases) were also excluded. Cases where using tissue for research would have left no diagnostic tissue remaining or would otherwise encroach on the usual, safe diagnostic pathway were excluded in line with OCHRe policy.[243]

2.4.10 Sample size estimations

For the technical validation studies, 20 cases were selected in line recommendations.[248] Sample size estimations and calculations were carried out for the *BRAF* and *KRAS* clinical studies prior to data collection, in line with guidelines on clinical diagnostic test accuracy study design.[249] These are described in relevant sections. General advice about sample size calculations was given by a medical statistician. The sample size calculations presented in this thesis were carried out manually by the author.

2.4.11 Interpretation

Determining IHC results is discussed later in Chapter 3. Any mutation detected by Idylla, Cobas or NGS was considered to be a positive result. Wild type genes were considered negative results.

2.5 Statistical Analysis

Statistical analysis was carried out using standard formulae. Diagnostic accuracy (test accuracy) was analysed using sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and likelihood ratios. Diagnostics accuracy was only assessed for comparisons with unselected cohorts of patients with minimal bias. The technical validations (selected positive/negative cases) were analysed using concordance (agreement) only. Inter-observer agreement (test reproducibility) was analysed with Cohen's kappa. Where appropriate, values were calculated with confidence intervals (CI) of 95%. The calculations presented in this thesis were carried out manually by the author with the aid of Microsoft Excel. See Appendix 4 for details and formulae.[235,250,251]

Some of the methods and information presented in this Chapter are also presented in publications and posters arising from this work.[1-8]

Chapter 3: *BRAF* Testing with Automated PCR

3.1 Introduction

3.1.1 Chapter overview

BRAF is a key marker in CRC and mutations in the gene are commonly tested for in routine clinical practice. This is predominantly because detecting *BRAF* mutations aids in the screening for Lynch Syndrome.[97] However, testing is lacking in many centers due to poor access to molecular laboratories or a lack of resources.[15] This Chapter aims to test the hypothesis that the Idylla System is highly accurate for *BRAF* testing in CRC and is superior to other systems available. This could then potentially allow the Idyla system to be used to overcome the under-testing seen in many areas of the NHS. *BRAF* was the first gene target for which testing was offered on the Idylla System and as such this part of the study took place first, influencing how the Ras testing part of the study was later designed. Therefore, the *BRAF* results will be discussed first. This will begin with consideration of the gene and its diagnostic utility. This is followed with an audit of the clinical service locally in order to evaluate the financial impact of *BRAF* testing with the Idylla System. Next, a full clinical study of the *BRAF* Mutation Test compared with conventional PCR is presented in order to establish the diagnostic accuracy of Idylla to detect *BRAF* mutations. Funding did not cover a full clinical study for the system compared with NGS or IHC, however basic technical validations of these are presented to give some indication accuracy in this context. This Chapter will specifically evaluate the Idylla *BRAF* Mutation Test and the *BRAF* component of the Idylla *NRAS-BRAF-EGFR*S492R Mutation Assay.

3.1.2 The *BRAF* gene

Rapidly accelerated fibrosarcoma (Raf) kinases are downstream mediators of EGFR signaling in the MAPK pathway (see Figure 4). Three members of the Raf kinase family have been identified: *ARAF*, *BRAF* and *CRAF*. As already discussed in Chapter 1, the primary function of Raf kinases is the regulation of cell proliferation in response to external growth factors such as epidermal growth factor (EGF). Each Raf kinase probably has different targets within the cell and Braf (Raf b homolog encoded by *BRAF*) and Craf (Raf c homolog encoded by *CRAF*) appear to be more significant in EGF signaling than Araf (Raf a homolog encoded by *ARAF*). In addition, Braf is known to directly activate Craf.[75,252,253] Mutations in Raf kinases result in constituent activation of the MAPK proliferation pathway in the absence of EGF. These mutations have been identified in numerous cancers, such as melanoma, papillary thyroid carcinoma, and some lymphomas.[254] *BRAF* mutations are the most widely implicated in carcinogenesis; this may explain why *BRAF* is prognostic and the gene target of most clinical utility. *ARAF* and *CRAF* mutations are less common and appear to have less pathogenic or clinical relevance.[75]

However, given the action of Braf, mutant *BRAF* may also increase the activity of Craf and this could be one mechanism for resistance to anti-*BRAF* therapy (see below).[255]

BRAF mutations are detected in around 10% of CRC resection specimens. Clinically, *BRAF* status is useful for prognosis and screening for Lynch syndrome (LS).[97] Although targeted cancer therapies against *BRAF* mutations, such as vemurafenib, have been successful in improving patient outcomes in metastatic malignant melanoma,[256] results have been disappointing in colorectal cancer.[257-260] On the other hand, anti-EGFR mAb therapies in metastatic colorectal cancer have proven to be of significant benefit to patients and therefore testing for mutations which may render the MAPK pathway constituently active (in Ras or Raf proteins) has been explored. Whilst the benefit of identifying Ras mutations has been demonstrated, stratifying treatment based on Raf (i.e. *BRAF*) mutations is less clear. Some data suggest *BRAF* mutations may be of use in directing treatment,[60,261,262] but testing is not currently recommended.[260,263,264] *BRAF* mutations are used as a marker or poor prognosis however. *ARAF* and *CRAF* testing are not thought to be clinically useful and are not carried out in routine practice.

3.1.3 Lynch syndrome and *BRAF* testing

The main clinical indication for *BRAF* testing in CRC patients currently is for LS screening. Most cases of CRC are sporadic, but around 3-5% of cases occur in patients with LS. LS is an inherited, autosomal dominant cancer syndrome caused by mutations in MMR genes. A number of genes have been implemented in LS over the years but many have subsequently been shown not to be associated with increased cancer risk. The genes now thought to cause LS are *MLH1, PMS2, MSH2, MSH6* (discussed in Chapter 1) and, rarely, epithelial cell adhesion molecule (*EPCAM*).[265-267] *EPCAM* encodes EpCAM is a transmembrane cell-cell adhesion molecule. The gene directly precedes *MSH2* on chromosome 2 and mutant *EPCAM* expression results in transcriptional read-through silencing of *MSH2* expression. In colorectal epithelium, where there is generally high *EPCAM* expression, this can lead to a loss of MSH2 function and CRC.[268] LS can also lead to, endometrial, ovarian, stomach and other cancers. Identifying cancer patients who may have LS is important for preventing future tumours.[11] Therefore, screening of CRC tumour resection tissue for LS is now endorsed by the Royal College of Pathologists, NICE and others.[11,167,168,266,267,269]

The terminology and diagnostic criteria surrounding LS are complex and so the history of this syndrome warrants some brief discussion. LS was possibly first described by Warthin in 1913 in a group of families with a predisposition to develop gastrointestinal and gynaecological cancers. Henry T. Lynch later described similar features in other families in the 1960's. Following this, the term 'Lynch syndrome' became popular to refer to these patients.[270] The syndrome presents as a non-polyposis (although there is an increased

tendency for patients to develop polyps) associated cancer, hence the term 'hereditary non-polyposis colorectal cancer' (HNPCC), proposed by Lynch, later became the agreed name. The clinical criteria for diagnosing HNPCC, known as the Amsterdam criteria (see Table 8) were agreed upon in the in 1980s. The original Amsterdam criteria (Amsterdam I) were later felt to be too stringent and so were refined (Amsterdam II) to encompass more patients at-risk.[271,272] As the genetic basis of the disease was uncovered however, some began to again use the term 'Lynch Syndrome' (or 'LS') to refer to a subset of HNPCC patients with proven MMR gene mutations. With this new molecular understanding, criteria for genetic testing, known as the Bethesda criteria, were introduced. These criteria have also been revised several times and currently the Revised Bethesda criteria are in use (see Table 9). Today, all HNPCC cases are thought to be caused by MMR gene mutations, making LS and HNPCC synonymous and the clinical diagnostic Amsterdam criteria largely redundant.[168,265-267,271,273-277]

Amsterdam I
At least three family members with histologically confirmed colorectal cancer
One of which must be a first degree relative of the other two
At least two consecutive generations must be affected
At least one of the cancer cases must have diagnosed before age 50
Familial adenomatous polyposis must be excluded
Amsterdam II
At least three family members must have a cancer associated with HNPCC (colorectal, endometrial, urothelial, small bowel etc.)
One of which must be a first degree relative of the other two
At least two consecutive generations must be affected
At least one of the HNPCC related cancers must have been diagnosed before age 50
Familial adenomatous polyposis must be excluded

Table 8. The Amsterdam criteria for HNPCC. Patients meeting the criteria for either Amsterdam I[278] or the revised (the original criteria were deemed to be too narrow) Amsterdam II[271,272] are designated as HNPCC. When a patient had a proven genetic aetiology, in around 60% of HNPCC cases, they were said to have LS.[265,271] *HNPCC = hereditary non-polyposis colorectal cancer, LS = Lynch syndrome.*

Revised Bethesda Criteria
1. CRC diagnosed at younger than 50 years
2. Presence of synchronous or metachronous CRC or other LS-associated tumours
3. CRC with MSI-high pathological-associated features (Crohn's-like lymphocytic reaction, mucinous/signet cell differentiation, or medullary growth pattern) diagnosed in an individual younger than 60 years old
4. Patient with CRC and CRC or LS-associated tumour diagnosed in at least 1 first-degree relative younger than 50 years old
5. Patient with CRC and CRC or LS-associated tumour at any age in 2 first-degree or second-degree relatives

Table 9. The revised Bethesda guidelines for LS screening. Patients meeting one or more criteria should be screened. LS-associated tumours include colon, rectum, stomach, ovary, endometrium, pancreas, uterus, kidney, biliary tract, brain, small bowel and some skin tumours. These supersede the Amsterdam criteria (I and II) for genetic screening purposes, as all HNPCC patients would be included by default within these Bethesda criteria.[11,74,168,266,267,271,274] Permission to reproduce this table from a similar published table has been granted by the British Medical Journal Publishing Group Ltd.[1,2] *LS = Lynch syndrome, CRC = colorectal carcinoma, MSI = microsatellite instability*

There continues to be disagreement and conflicting use of the terms 'hereditary non-polyposis colorectal cancer' (or 'HNPCC') and 'Lynch syndrome' (or 'LS') but, as mentioned, most now agree that LS and HNPCC should be considered the same entity. There are a number of alternative terms for patients fulfilling the Amsterdam criteria without proven genetic abnormalities, such as 'Lynch-like' tumours (somatic MSI and MMR deficiency but no germline mutation) and familial cancer syndrome X (patients fulfilling the Amsterdam criteria but have no germline mutation) and these probably should be used instead of 'HNPCC'. The distinction still made by some between HNPCC and LS becomes a moot point anyway because the Revised Bethesda criteria *de facto* indicate genetic testing for all traditional HNPCC patients and as such, supersede the Amsterdam criteria for identifying at-risk patients. Therefore, 'HNPCC' is probably best regarded as an outdated term. It is for these reasons that 'Lynch syndrome' (or 'LS') shall be preferred and used in this thesis as a synonym for 'hereditary non-polyposis colorectal cancer' (or

'HNPCC') and will mean any patient with a germline mutation in *MLH1*, *PMS2*, *MSH2*, *MSH6* or *EPCAM*.[168,265-267,271,273,274]

Most guidelines (including the RCPath) recommend all patients meeting the Revised Bethesda criteria (Table 9) should be screened for LS (i.e. germline mutations in MMR genes), however recent NICE guidelines suggest screening all patients with CRC. The most common screening approach uses IHC evaluation of MMR protein expression and this is supported by NICE guidance. Alternatively direct is MSI testing may be used (also supported by NICE). Following MMR or MSI testing the screening pathway also incorporates *BRAF* mutation testing (see Figure 11) and *MLH1* promoter region hypermethylation testing. The loss of MLH1 expression alone has low specificity for detecting LS (in part due to *MLH1* hypermethylation and somatic mutation in sporadic tumours), but this is improved by incorporating tests to rule out *MLH1* hypermethylation and by including *BRAF* mutation analysis in to the algorithm. *BRAF* mutations are rare in non-sporadic tumours and thus identifying a mutation virtually excludes LS.[11,13,167,168,265-267,269,274,279] The *BRAF* 1799 nucleotide point mutation substitution of thymine to adenine (c.1799T>A) which results in an amino acid change from valine to glutamate at the codon position 600 (V600E, first incorrectly designated at V599E) is the most common lesion found and so many tests target this base change, however mutations in the E2, D, K, M and R codons have all been shown to be clinically relevant, including in LS screening.[74,279-282]

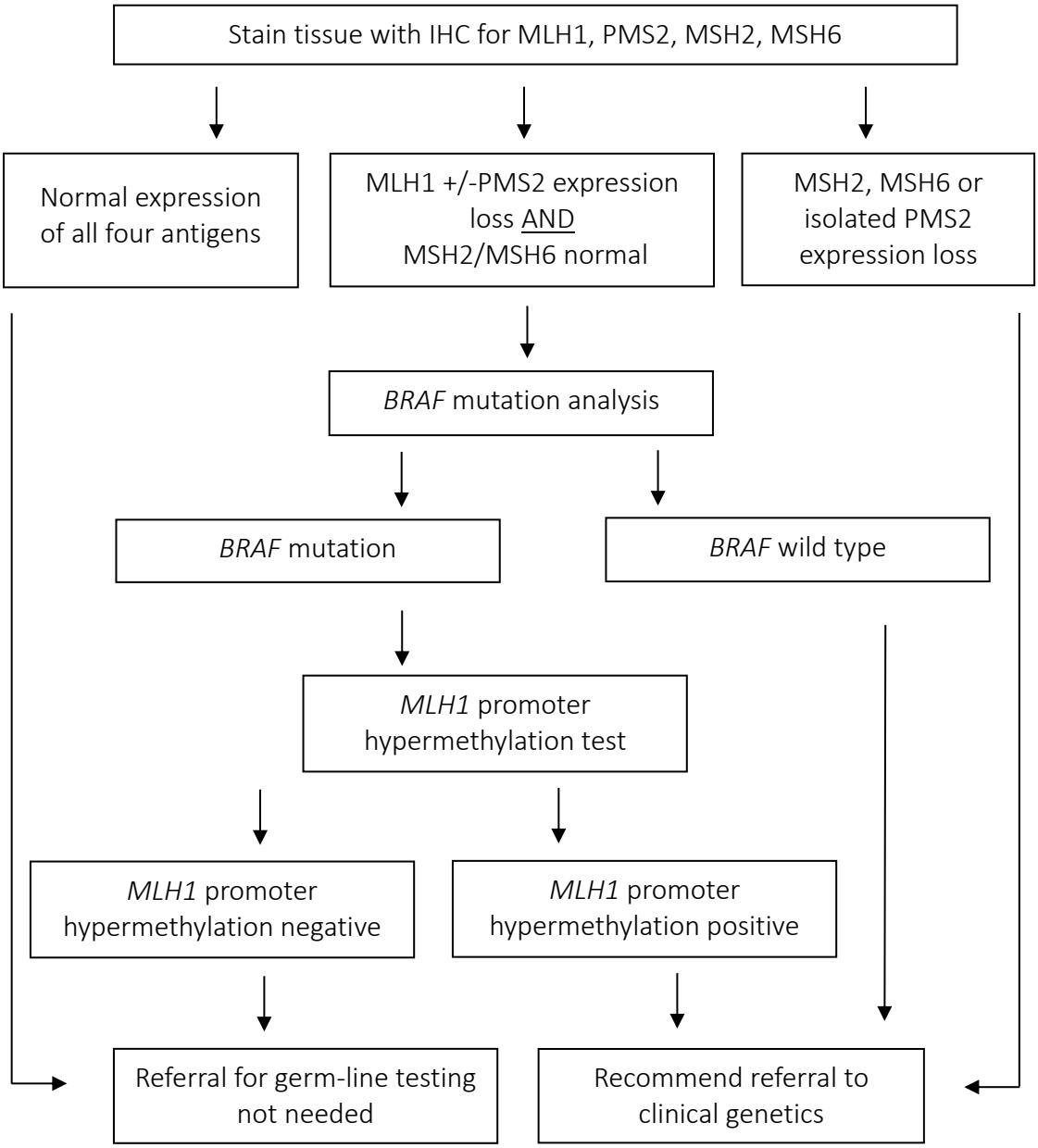

Figure 11. A flow diagram of the NICE recommended LS screening algorithm in CRC. Older guidelines indicate LS screening for patients meeting Bethesda criteria, new NICE guidelines call for all CRC patients to be screened. This figure is adapted from similar images presented in publications arising from this work and others and is based on LS screening guidelines.[11,266,267,269] Permission to reproduce this figure from a similar published figure has been granted by the British Medical Journal Publishing Group Ltd.[1,2] *NICE = National Institute for Health and Care Excellence, CRC = colorectal cancer, LS = Lynch syndrome*

There are alternative methods for LS screening. LS patients have MSI CRC and as mentioned above (and supported by NICE) MSI can be detected directly by using PCR.[269] In this method, specific tandem repeat sequences are amplified and the fragment lengths are visually compared to a control reference curve of the distributions of fragment lengths. MSI tumours contain expanded microsatellites due to mutations and thus amplicons of the tandem repeats in these microsatellite will have fragment lengths that are different than expected. When compared with the reference curve, these fragments will be observed as additional peaks in the graph. The US National Cancer Institute (NCI) standardised the reference panel of markers used for MSI testing to include two mononucleotide repeats (*BAT25* and *BAT26*) and three dinucleotide repeats (*D5S346*, *D2S123* and *D17S250*). MSI-H tumours demonstrate expansion in two or more markers, MSI-L in only one. MSI testing by PCR correlates well with IHC, but is more expensive and technically challenging than IHC screening.[283,284] Some have suggested another alternative is testing for *BRAF* mutations alone in LS screening in those patients meeting the revised Bethesda criteria, however this approach is much less common.[285] In reality, most of the testing options offer similar accuracy and overall similar costs and because this is a screening process and not a definitive test for LS, it probably doesn't really matter which method is being used. Recommendations based on health economics assessments back this up and state that as long as some form of screening is used, the method is not important.[167]

There are other reasons why *BRAF* testing is common in practice, other than directly for LS screening. Many centers are beginning to carry out MMR IHC testing as routine to establish MSI status because MSI-H carries a poorer prognosis. Consequently *BRAF* testing is needed on a greater number of cases because more MLH-1 loss is being detected and LS needs to be excluded as a duty of care.[93,166] *BRAF* testing is also recommended by some in stage II or greater CRC as a prognostic marker.[74,279-282]

There are conflicting data over whether *BRAF* mutations are predictive of resistance to anti-EGFR mAb therapy (see Chapter 4). This is not a current indication for testing therefore. There are also some early data which indicate that non-V600 mutations may impart a good prognosis, again this is not a routine indication for testing yet however.[262,286,287]

3.1.4 Rationale for *BRAF* testing with automated PCR

Most *BRAF* testing is currently carried out using conventional PCR-based tests, the most common being the Cobas platform.[183,281] Unlike Cobas, which only detects the V600E amino acid change,[195] the Idylla *BRAF* Mutation Test cartridge covers the full range (D, E, E2, K, M and R)[184] of clinically relevant and actionable V600 mutations in CRC and has a turn-around time of 90 minutes. The test cannot however distinguish between E, E2 and D or between K, R or M substitutions.[279] The *NRAS-BRAF-EGFR*S493R Mutation Assay also

covers a larger range of *BRAF* mutations than most conventional PCR tests (E, D, K and R), but similarly cannot distinguish between E and D or K and R substitutions. This assay has a turn-around time of two hours. The Ion AmpliSeq Cancer Hotspot Panel v2 covers all of the *BRAF* codon changes covered by Idylla and Cobas, however the test turn-around time is at least 24 hours.[196] The review of the literature identified some deficiencies in the evidence and the aim of this part of the study was to technically validate the Idylla System for the detection of *BRAF* mutations in CRC and to clinically assess the diagnostic accuracy in patients for whom *BRAF* testing is carried out as standard care. This includes LS screening for patients meeting the revised Bethesda criteria and for prognostication in stage II (pT3N0 & pT4N0) or above CRC patients.

3.2 Experimental design

3.2.1 *BRAF* workload and costings audit

All cases of CRC from a 12-month period were assessed for the number of tumours undergoing MMR IHC. This method was chosen to capture all patients who may need *BRAF* testing; testing is only indicated in CRC for patients with MLH1 loss by IHC. *BRAF* testing is not routine locally however due to funding shortages, but this method identified all patients who *should* be eligible for *BRAF* testing, see Figure 11. Note however, the audit preceded NICE guidance for testing all patients and here was based on Bethesda criteria only, in line with RCPath guidance at the time.[13,269]

3.2.2 *BRAF* automated PCR diagnostic test accuracy study

The Idylla *BRAF* Mutation Test was compared against the standard-care test, the Cobas *BRAF* V600 Mutation Test performed on the Cobas 4800 System. A consecutive series of cases was selected from the departmental database search results (see Chapter 2). Selection criteria in addition to those set out in Chapter 2 were used to limit cases selected from patients for whom *BRAF* was to be carried out on clinical grounds (LS screening, Stage III or above, at the request of clinicians for prognostication).

At this stage no reliable data existed on how well the Idylla was likely to perform under clinical conditions for a formal sample size calculation. Therefore, a basic nomogram method of estimating the same size was carried out in line with recommendations (see Appendix 4).[288] This method is semi-formal and allows an easy visual representation of the various likely sample sizes needed for a range of test sensitivities or specificities, as well as varying prevalence and confidence intervals. For *BRAF* testing, the aim is to screen out and reduce the number of patients for Clinical Genetics testing by obtaining a positive result. Therefore, high specificity is probably more important in this setting and so the specificity nomogram was used. The specificity was estimated to be high based on the

poster abstracts discussed in Subsection 1.6.3. This was expected to be around 90 to 100% and using the nomogram method, a range of possible sample sizes were estimated. The results of this suggested that a sample size of 100 cases was probably a reasonable and achievable estimate.

3.2.3 Validating *BRAF* automated PCR with IHC

The Idylla *BRAF* Mutation Test was validated against IHC with the VE1 Ab on the Ventana Benchmark ULTRA autostainer platform.[233] 18 cases were selected out from the clinical study cohort results (Subsection 3.3.2) and two new cases selected required additional Idylla testing: 10 positive and 10 negative by conventional PCR (Cobas *BRAF* V600 Mutation Test). Only cases with V600E mutations were selected as the VE1 Ab does not bind with other amino acid changes. The cases were processed in line with the methods outlined in Chapter 2.

IHC staining was not scored by the author due to a possible risk of bias (lack of blinding), but instead was independently assessed by two consultant (fully qualified) histopathologists who were blinded to any molecular test results. Recommendations suggest only one pathologist is needed for scoring, however the study here aimed to investigate agreement (reproducibility) for VE1, so two pathologists were used. No consensus on how to score the VE1 Ab exists so a sensible scoring system was devised based on that used by others (including the original laboratory which produced the Ab) and based on recommended H-scoring methodologies.[169,170,289,290] Only cytoplasmic staining was considered positive as non-cytoplasmic staining is reported to be non-specific.[169] Cases were scored for intensity (1 to 3, 0 = no staining) and percentage of cells stained (0 = <20%, 1 = 20-50%, 2 = >50%). For each case, the pathologists' scores were multiplied together (intensity x %) and the mean of the two assessors' scores was taken. A final score of >1 was considered positive. Agreement between pathologists was assessed using Cohen's kappa as described in Section 2.5.

3.2.4 Validating *BRAF* automated PCR with NGS

The Idylla *NRAS-BRAF-EGFR*S492R Mutation Assay was validated for BRAF mutation detection against NGS with the Ion AmpliSeq Cancer Hotspot Panel v2. 18 cases separate from the clinical cohort (Subsection 3.3.2) were selected (this cohort did not have NGS testing at the time so different cases needed selecting): 9 positive and 9 negative by NGS. Only 18 cases were selected due to the limited supply of prototype assays available and limited funding. Cases were processed in line with the methods outlines in Chapter 2.

3.3 Results

3.3.1 *BRAF* workload and costings

In a 12-month period, 284 CRC resections were reported in the department. 209 cases (74%) were screened for LS. Cases were tested for *BRAF* mutations in line with the LS pathway as set out in Figure 11. Around half of those cases undergoing IHC were because they met the Revised Bethesda criteria and *BRAF* was only carried out on those with loss of MLH1 expression. The other half of cases were evaluated with IHC for prognostication in Stage II and above cancer. Consequently, MLH1 loss in this cohort raised the question of LS and so cases were also tested for *BRAF* as part of the full LS screening. In total 44 cases were eligible for *BRAF* testing, however NHS funding was in place for only 28. The cost of the Cobas *BRAF* V600 Mutation Test is around £180 per case and therefore the total annual cost was estimated at £7,920. The list price of the Idylla *BRAF* Mutation Test given by Biocartis is £110 per case, or £4,840 per year. The potential saving of introducing the Idylla System for *BRAF* testing therefore was estimated to be £3,080. Had all 284 CRC cases been screened for LS in line with new NICE guidelines,[269] the number of *BRAF* tests would probably have been much higher and the saving greater. Further discussion of these findings is presented later in Subsection 4.3.1.

3.3.2 *BRAF* automated PCR diagnostic accuracy

100 consecutive CRC cases from 97 patients were retrieved, representing around 30% of CRC resection workload. A summary of the patient characteristics is given in Table 10. 96 cases were resection specimens and four cases were biopsies. All cases were invasive adenocarcinoma covering the clinical spectrum from well to poorly differentiated tumours and ranging from Stage I to IV tumours. All cases met the minimum tissue requirements for testing. The four biopsy cases and two of the resection cases required multiple sections to meet the tissue area requirements; the maximum number of sections used in any case was six. Of the 98 cases with results (i.e. excluding failed tests), 30 (31%) cases were MLH1 negative on IHC and therefore tested for *BRAF* mutations within the context of LS screening. The remaining 68 cases had been tested for *BRAF* mutations for prognostication.

Characteristic	**Values** n=97
Age*	72 (12) years
Male	43%
Adenocarcinoma	100%
Site	
Right colon	52%
Transverse colon	7%
Left/sigmoid colon	14%
Rectum	27%
Specimen type	
Biopsy	4%
Polypectomy	1%
Right hemicolectomy	53%
Transverse colectomy	1%
Left Hemi/sigmoidcolectomy	9%
Anterior resection	26%
Abdominoperineal resections	3%
Pancolectomy	1%
TEM	2%
Metastatic resections	0%
Grade	
Well differentiated	4%
Moderately differentiated	76%
Poorly differentiated	20%
Stage at surgery	
Stage I	20%
Stage II	44%
Stage III	31%
Stage IV	1%
Unknown (biopsy only)	4%
Neo-adjuvant chemotherapy given	8%

** = variables expressed as a Mean (Standard Deviation)*

Table 10. Summary of the patient characteristics for the patients tested with the Idylla *BRAF* Mutation Test in the diagnostic accuracy study. *TEM = transanal endoscopic mucosal resection.*

All cases had both the Idylla test and the Cobas PCR. Two cases (both resections) contained insufficient DNA in the tissue available to the study to produce a result, thus these were excluded from the statistical analysis. Three initial tests failed due to an Idylla machine fault and the processing unit was replaced (tests were repeated). Two tests failed due to initial user error (these were repeated). One test failed under normal conditions (also repeated).

BRAF mutations were found in 29 cases by Cobas making the reference test prevalence 29.5% (95% CI: 21.5% to 39.3%) and in 30 cases by Idylla making this prevalence 30.6% (95% CI: 22.4% to 40.3%). This is a little higher than predicted by the literature, although was representative of the detection levels usually seen in local practice. All mutations detected by both tests were V600E. In total 30 cases had tested positive for *BRAF* mutations on the Idylla System. All but one case showed agreement between the Idylla System and Cobas, making the concordance 98.98% (95% CI: 94.4% to 99.8%). All 29 Cobas positive cases tested positive with Idylla and 68 of the 69 Cobas negative cases tested negative with Idylla. Therefore, the one discordant case was Idylla positive and Cobas negative. A summary of the results is given in Table 11.

	Cobas Positive	**Cobas Negative**	Totals
Idylla Positive	29	1	30
Idylla Negative	0	68	68
Totals	29	69	98

Table 11. Summary of the diagnostic accuracy study comparing Idylla (index test) with Cobas (reference test) PCR for *BRAF* mutations Permission to reproduce this table from a similar published table has been granted by the British Medical Journal Publishing Group Ltd.[2]

The sensitivity of the Idylla System therefore was 100.0% (95% CI: 88.3% to 100.0%) and the specificity was 98.6% (95% CI: 92.2% to 99.7%). The positive predictive value was 96.7% (95% CI: 83.3% to 99.4%) and the negative predictive value was 100% (95% CI: 94.7% to 100.0%). The positive likelihood ratio was 69 and the negative likelihood ratio was zero.

The case was adjudicator tested by ddPCR for *BRAF* which found the case to be positive for the V600E mutation, making the Idylla result a true positive and the original Cobas result a false negative. Including these findings in the statistical analysis improves the results: the concordance then was 100.0% (95% CI: 96.2% to 100.0%), the specificity 100.0% (95% CI: 94.7% to 100.0%) and the PPV 100.0% (95% CI: 88.7% to 100.0%). A summary of the statistical analysis is given in Table 12.

Statistic	**Value**
Prevalence (defined by Idylla)	30.6% (95% CI: 22.4% to 40.3%)
Prevalence (defined by Cobas)	29.5% (95% CI: 21.5% to 39.3%)
Concordance	98.98% (95% CI: 96.8% to 97.5%)
Concordance including ddPCR results	100.0% (95% CI: 96.2% to 100.0%)
Sensitivity	100.0% (95% CI: 88.3% to 100.0%)
Specificity	98.6% (95% CI: 92.2% to 99.7%)
Specificity including ddPCR results	100.0% (95% CI: 94.7% to 100.0%)
PPV	96.7% (95% CI: 83.3% to 99.4%)
PPV including ddPCR results	100.0% (95% CI: 88.7% to 100.0%)
NPV	100% (95% CI: 94.7% to 100.0%)
LH+	69
LH+ including ddPCR results	Undefined (a/0)*
LH-	0

* = division by zero is mathematically undefined

Table 12. Summary of the calculated statistics from the Idylla data for the *BRAF* diagnostic test accuracy study. *PPV = positive predictive value, NPV = negative predictive value, LH+ = likelihood ratio positive, LH- = likelihood ratio negative, ddPCR = droplet digital PCR*

3.3.3 *BRAF* automated PCR validation with IHC

There were 12 cases which were scored overall >1 and called positive with IHC. An example of VE1 staining is shown in Figure 12 and the raw data of the *BRAF* IHC scoring are shown in Table 13.

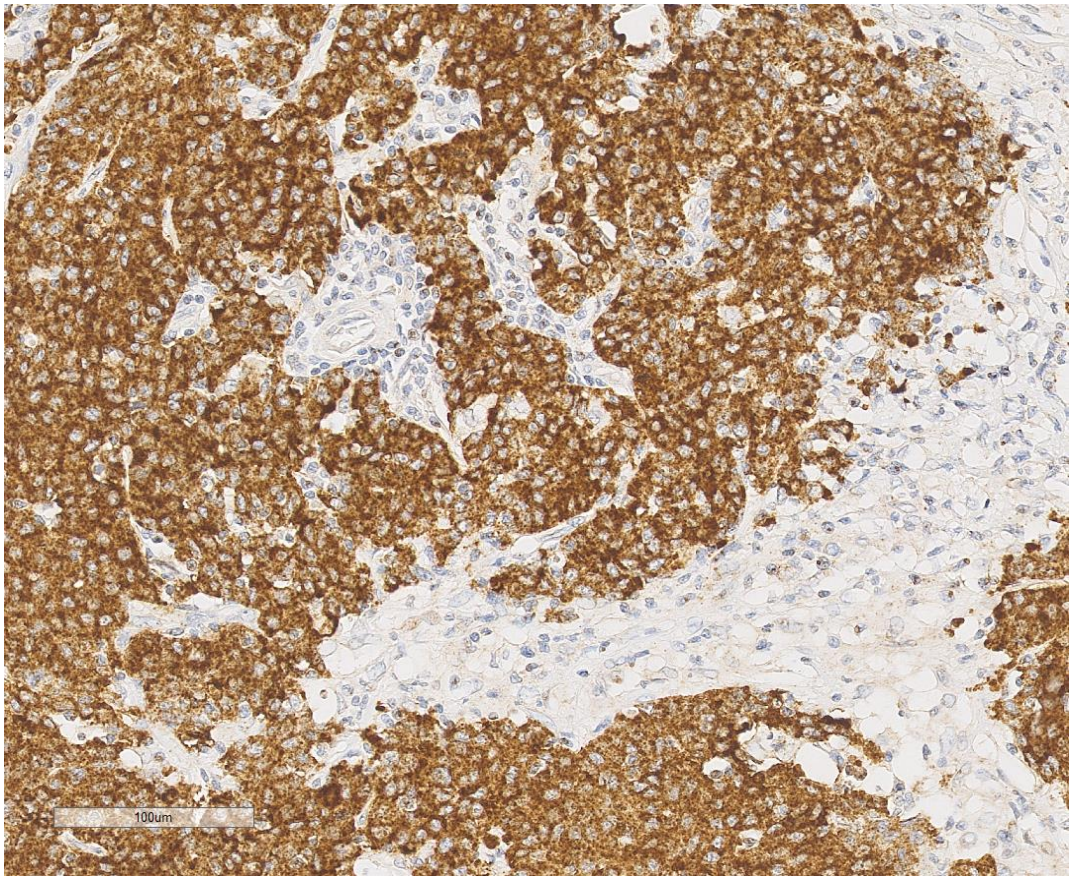

Figure 12. Photomicrograph of a poorly differentiated colorectal adenocarcinoma stained with the VE1 antibody by IHC. This is case 1 from Table 13 and shows predominantly strong brown IHC cytoplasmic staining (intensity scored 3 by both histopathologists) in all tumour cells (% tumour scored 2 by both histopathologists). The background stroma is negative for IHC staining and seen as light blue haematoxylin-stained cells. DAB chromogen staining and counterstained with haematoxylin, scanned at x400. Permission to reproduce this figure from the same published image has been granted by the British Medical Journal Publishing Group Ltd.[4] *DAB = 3,3'-Diaminobenzidine, IHC = immunohistochemistry*

Case	Pathologist 1: cell percentage	Pathologist 1: intensity	Pathologist 1: combined score	Pathologist 2: cell percentage	Pathologist 2: intensity	Pathologist 2: combined score	Average score	IHC Result
1	2	3	6	2	3	6	6	+
2	2	2	4	2	2	4	4	+
3	0	0	0	0	0	0	0	-
4	0	0	0	0	2	0	0	-
5	0	0	0	0	0	0	0	-
6	2	2	4	2	2	4	4	+
7	0	0	0	0	0	0	0	-
8	1	1	1	2	1	2	1.5	+*
9	2	3	6	2	3	6	6	+
10	2	3	6	2	3	6	6	+
11	2	2	4	2	2	4	4	+
12	0	0	0	0	1	0	0	-
13	2	3	6	2	3	6	6	+
14	2	3	6	2	3	6	6	+
15	0	0	0	0	1	0	0	-
16	0	0	0	1	1	1	0.5	-
17	1	1	1	2	1	2	1.5	+
18	0	0	0	0	0	0	0	-
19	1	2	2	2	1	2	2	+*
20	0	0	0	2	2	4	2	+

* = discordant case with molecular result (Idylla/Cobas)

Table 13. Results of the VE1 immunohistochemistry scoring.

In this part of the study, accuracy of Idylla and reproducibility of VE1 were assessed. For assessing accuracy, there was agreement between IHC and Idylla in 18 cases making the concordance 90% (95% CI: 69.9% to 97.2%). There was however 100.0% (95% CI: 83.9% to 100.0%) concordance between Idylla and Cobas in these cases and all mutations detected were V600E (VE1 is V600E specific). In both discordant cases (case 8 and case 19 in Table 13) there were minor disagreement between the two pathologists scoring the slides and both felt the staining was ambiguous. A summary of these results is given in Table 14.

	IHC Positive	**IHC Negative**	Totals
Idylla Positive	10	0	10
Idylla Negative	2	8	10
Totals	12	8	20

Table 14. Summary of the *BRAF* validation study comparing Idylla with IHC. Permission to reproduce this table from a similar published table has been granted by the British Medical Journal Publishing Group Ltd.[4]

For assessing reproducibility, there was agreement between the two pathologists in 17 of the 20 cases. There were three cases was called positive by Pathologist 2 but negative by Pathologist 1 (cases 8, 17 and 20 in Table 13). This gave a kappa value of 0.71 (95% CI: 0.41 to 0.99). The results are summarised in Table 15.

	Pathologist 2 positive	**Pathologist 2 negative**	Totals
Pathologist 1 positive	9	0	9
Pathologist 2 negative	3	8	11
Totals	12	8	20

Table 15. Agreement between pathologists scoring the VE1 IHC.

3.3.4 *BRAF* automated PCR validation with NGS

The results of the *BRAF* NGS validation study are shown in Table 16. These results are derived from the same Idylla run (*NRAS-BRAF-EGFR*S492R Mutation Assay) carried out from which the *NRAS* results in Subsection 4.3.4 were derived. There was one failed test due to a technical error with the Idylla software. As this was the same cartridge testing for *NRAS*, as such it represents one failed assay but two failed tests. A replacement assay to repeat the sample could not be obtained. 12 of these same cases were also tested with a different assay run (the Idylla *KRAS* Mutation Test), the results of which are presented in Subsection 4.3.3.

There was 100.0% (95% CI: 81.6% to 100.0%) concordance between Idylla and Ion Torrent. All mutations detected were V600E.

	NGS Positive	**NGS Negative**	Totals
Idylla Positive	8	0	8
Idylla Negative	0	9	9
Totals	8	9	17

Table 16. Summary of the *BRAF* validation study comparing Idylla with NGS. Permission to reproduce this table from a similar published table has been granted by the British Medical Journal Publishing Group Ltd [4]

3.4 Conclusion

The results from the diagnostic study show that the Idylla *BRAF* Mutation Test has a very high level of diagnostic accuracy. The Idylla *NRAS-BRAF-EGFR*S492R Mutation Assay showed high accuracy by complete concordance with the reference test for detecting *BRAF* mutations. This was the first study to demonstrate the accuracy of these tests in CRC and these findings support the hypothesis.

The *BRAF* Mutation Test showed only moderate concordance with IHC. However, Idylla showed 100% concordance with Cobas in these cases, highlighting the problem being with the VE1 Ab and not a weakness of Idylla. IHC is therefore not a suitable test for routine clinical practice.

In the current clinical context, there is a need for faster, more cost effective *BRAF* testing. Screening for LS is now increasingly recommended by many professional bodies[11,13] and

the most recent guidance suggests LS screening for *all* patients with CRC.[269] Furthermore, emerging treatment options are providing more indications for establishing MMR status. Thus requests for *BRAF* testing are only going to increase.[291] The need for a rapid test which is far more widely available to smaller centers, at lower cost, can be fulfilled by the Idylla System.

3.5 Discussion

There was only one discrepant case in the *BRAF* Mutation Test diagnostic study. This was shown by ddPCR to be a true positive result by Idylla and a false negative by Cobas. Initially, this was suspected to be due to lack of D, E2, K or R codon coverage by Cobas, however ddPCR confirmed the mutation was the V600E change and was therefore a lack of sensitivity of Cobas. In this case, conventional PCR would have led to an unnecessary referral to clinical genetics for LS assessment with significant patient anxiety and significant financial cost. In this regards, Idylla was demonstrated to be a superior test and again this supports the hypothesis. There were six failed Idylla *BRAF* Mutation Tests. All but one of these were actual failures under normal working conditions and therefore the failure rate was acceptably low (~1%).

The Idylla *NRAS-BRAF-EGFR*S492R Mutation Assay was shown to be accurate in a concordance validation but the test would ideally need further evaluation in a diagnostic accuracy study before routine use could be recommended with high confidence. Only one test failed, but this was due to a cartridge software error rather than an intrinsic issue with the test.

IHC with VE1 failed to produce satisfactory results. IHC is a cheaper test (Around £30) by comparison to either Idylla (£110) or Cobas (£180), however given the fact that significant management decisions could be made upon this result (such as referral for genetic screening), these results would not give enough confidence in VE1 for it to be used in routine practice.[170] Ab-based tests are generally very cheap and very fast ways of detecting the presence of a protein of interest, commonly in tissue sections (IHC) or on the electrophoresis gels of cell lysis products (Western blot). The idea that some cells preferentially express certain proteins or combinations of proteins is the basis for using these tests in clinical practice.[292] There are some drawbacks to this approach however. Firstly, the validation of Abs produced commercially or in private laboratories is not always robust and there is often no guarantee that a particular Ab purchased will specifically target the protein in question. Furthermore, the protocol for using Abs is rarely fully optimized or validated. When validation data are available, it is often difficult to obtain and commonly not checked by the user (who may not even know how to check).[293,294] Beyond this, there is often a lack of unbiased clinical data on the diagnostic accuracy of many of the antibodies used in clinical practice. Indeed, many IHC tests are used in combinations and there is

rarely any validation of such 'panels'.[292] This problem can only be tackled by enforcing strict guidelines for Ab validation.[294]

A second major issue is how such tests are interpreted. Interpretation to date is predominantly performed visually.[292] IHC tests rely on staining with chromogen dyes and this is a continuous variable, not a discrete positive negative result. For most IHC stains, the protocol can be changed to allow for greater or lesser staining depending on the user preference. What's more, false positive staining due to endogenous peroxidase or other artefacts is a very common pitfall for the observer. Therefore, there is potentially a great deal of inter-observer variation and subjectivity introduced into these tests, and any scorer agreement is not often assess empirically.[294,295] The potential for digital image analysis to overcome the subjectivity of IHC is starting to emerge as more reliable however and this could be a potential solution to reproducibility in the future.[296]

Despite these limitations, IHC is very widely used in histopathology practice and viewed by many as robust.[292,295] The potential to use IHC for molecular targets is obviously therefore very attractive to many histopathologists and oncologists. An example of probably the most commonly used IHC marker of a molecular target is the Ab for p53, marking the expression of the *TP53* gene. In this example, a mutation in *TP53* either renders the protein stable or results in truncation, and consequently is detected as an over expression (strong staining in all cells) or a total absence of staining.[297,298] In the context of CRC, like most targeted molecular testing, genetic lesions are generally focused on small point mutations or indels and engineering an Ab for a subtle base change is challenging. The VE1 Ab was one of the first developed against a single amino acid change (*BRAF* V600E),[290] although similar Abs for IHC detection of *KRAS* mutations have also been developed.[299,300] The limitation of these Ab tests however is that they are only targeted for one mutation at a time as the Ab structure is specific and there is a very limited range of chromogen colours (and secondary Ab combinations) which can be applied to a single tissue section for marking multiple antigens. For this reason, it would be necessary to use a great number of tissue sections to get the full coverage of clinically relevant mutations in several gene targets, such as is required in *KRAS* for example.[14,260,292] For routine clinical use this is not practical and for basic science work the information about the underlying biology available from IHC staining is rather limited when compared with other available technologies, such as NGS or other sequencing methods.[301,302]

In this study VE1 showed only moderate concordance with molecular testing and this is not surprising given the mixed findings of recent reviews of the Ab. These have mentioned difficulties with stain interpretation, false positive pitfalls, wide ranging diagnostic accuracy rates and variable inter-observer agreement, but have come to somewhat differing conclusions.[169,170,290,303] In this study the inter-observer agreement was good with a kappa value of 0.7, demonstrating that the rather simplistic scoring system used was quite useful.

However, despite good scoring agreement, the Ab still performed too weakly for confident routine clinical use and in line with some of the mentioned recent literature, it is not recommended for use based on the findings in this study.

The methods, data, statistical analysis and some points of discussion presented in this Chapter are also presented in publications and posters arising from this work.[1-8] *Non-integer values are rounded to one decimal point. For all statistical analysis formulae, see Appendix 4.*

Chapter 4: Ras Testing with Automated PCR

4.1 Introduction

4.1.1 Chapter overview

KRAS and *NRAS* genes are key molecular targets in CRC and mutations in these are commonly tested for in clinical practice. This is predominantly because mutations in these genes can predict response to targeted therapies. This Chapter aims to test the hypothesis that the Idylla System is accurate for detecting *KRAS* and *NRAS* mutations in CRC. The Chapter will begin with an overall discussion of Raf signaling and its diagnostic utility. This is followed with an audit of the clinical service locally to establish the financial impact of using the Idylla System for Ras testing. Next, a full clinical study of the Idylla *KRAS* Mutation Test compared with conventional PCR is presented to evaluate the diagnostic accuracy of the test. Funding did not cover a full clinical study for the test compared with NGS, however a technical validation is presented to give some indication of this. This Chapter also includes a validation of the *NRAS* component of the Idylla *NRAS-BRAF-EGFR*S492R Mutation Assay in order to give an indication of the accuracy of the system for detecting *NRAS* mutations.

4.1.2 Ras genes

The Rat sarcoma (Ras) subfamily of GTPase proteins are downstream mediators EGF signaling in the MAPK pathway and belong to the larger Ras superfamily of related proteins. These proteins act upstream of Raf kinases, such as *BRAF*. As discussed, binding of EGF to its receptor EGFR results in activation of Ras proteins and subsequent activation of a cascade which results in the up-regulation of a number of transcription factors, such as c-myc (Figure 4). This eventually results in increased cell division. Mutations in Ras proteins can result in constitutive activation of KRas, leading to an over-activation of the MAPK pathway, uncontrolled cell proliferation and neoplasia. There are three members of the Ras subfamily: *KRAS*, *NRAS* and *HRAS*. Each Ras member has similar actions within the cell, but may be expressed at different levels in different tissues and may have different downstream targets. [69,70,74,304]

The Kirsten Ras viral oncogene homologue (KRas) is encoded by the proto-oncogene *KRAS* and appears to be the dominant member of the Ras group most widely expressed. *KRAS* has been implicated in the development of numerous malignant and pre-malignant (dysplastic) tumour types, including those of the lung, pancreas, breast and, as mentioned, are driver mutations in the adenoma-carcinoma sequence in CRC. Around 30-40% of CRCs harbor *KRAS* mutations, most importantly in exons 2, 3 and 4.[10,14,69,70] *KRAS* mutations in CRC may impart a poorer prognosis, especially those in exon 2 (which are the most common), and in those patients with wild type *BRAF*.[305,306]

The Neuroblastoma Ras viral oncogene homolog (Nras) is encoded by the proto-oncogene *NRAS*. Mutations in *NRAS* can also result in constitutive activation of Nras, similarly leading to an over-activation of the MAPK pathway and uncontrolled cell proliferation and neoplasia.[72,307] *NRAS* mutations are uncommon though and only detected in around 5% of CRCs. *HRAS* appears to be of less clinical significance in CRC.[72,308]

4.1.3 Ras testing in CRC

Ras testing is important clinically as it is used to guide anti-EGFR therapy. Two types of anti-EGFR therapy exist: tyrosine kinase inhibitors (TKi), small molecule drugs that inhibit the tyrosine kinase activity of EGFR, and monoclonal antibodies (mAb) that specifically bind to and block EGFR receptor activity (receptor antagnostism). Anti-EGFR mAb therapies such as cetuximab and panitumumab have proven effective in colorectal adenocarcinoma; TKis are not thought to be useful. Patients with non-adenocarcinoma subtypes of CRC or metastases to the colorectum have not been shown to respond to therapy. These anti-EGFR mAb drugs are not curative but can extend remission times. Given this, along with their cost and side-effect profiles, these therapies are generally reserved for Stage IV CRC for patients with unresectable metastases. Around 20% of patients who have metastases with wild type somatic *KRAS* show a good response to anti-EGFR mAb therapy. However, CRC patients with somatic mutations in almost any *KRAS* codon show autonomous stimulation of the MAPK pathway and do not respond to treatment.[10,14,69,70,133,134,171,260,261] Interestingly, one mutation, the G13D codon change, seems to impart a better response to anti-EGFR mAb therapy. The mechanism behind this is uncertain.[309] It is clinically prudent to identify individuals with any of these mutations in order to avoid exposing non-responding patients to potentially harmful side effects and wasting financial resources. Therefore, testing CRC tissue for mutations in *KRAS* is becoming routine with UK, European and US guidelines recommending *KRAS* testing in all primary CRC tumours following surgical excision.[14,70,260,261,310,311]

Initially, only mutations in *KRAS* were thought to predict response to anti-EGFR mAb therapy, however it is becoming increasingly recognised that *NRAS* mutations are predictive and hence it is becoming increasingly common to test for both. Patients with *NRAS* mutations, via a similar mechanism to that proposed with *KRAS* mutations, are probably resistant to anti-EGFR mAb therapies. The role of *HRAS* in CRC is unclear and this is rarely tested in clinical practice.[14,70,260,261,264,310,311]

There is no high-profile guidance for Ras testing in the NHS and what is available focuses on *KRAS* testing. NICE guidelines state that anti-EGFR mAb therapy is effective in patients with wild type *KRAS* metastases but do not explicitly recommend Ras testing or comment on proposed diagnostic algorithms or methods.[133] The development of further NICE guidance on testing has been discontinued[312] and an update to anti-EGFR mAb therapy in

metastatic CRC has been pending publication for some time.[134] Some pathologists suggest that testing all primary CRC resection specimens at initial reporting would avoid treatment initiation delays should they be found to develop metastases at a later date. This so-called 'reflexive testing' is controversial and often funding will only cover testing patients with proven metastases, however in some centers reflexive testing is a popular approach. Therefore, because no consensus on which CRC patients should have Ras testing exists, testing is fragmented across the UK and often performed *ad hoc* at the request of oncologists. It is extremely likely that some patients are started on therapy without testing and many others are tested unnecessarily.[10,14,133,260,310]

There is also no consensus on which tissue the Ras testing should be carried out, but this is often the primary tumour. The reasons for this may be historical, with unwarranted worries about low concentrations of DNA in biopsy material (metastases are not usually resected) being prevalent for some time.[313] There is also now a general feeling that there is good concordance between primary and secondary tumour genotypes and that testing metastatic tissue is not needed.[145,314] The phenomenon of tumour heterogeneity between populations of cells in a tumour (intra-tumoral) and between metastatic and primary tumours (inter-tumoral) is well documented however[315-320] and it is uncertain how genotype of the metastatic tumour affects anti-EGFR mAb therapy; most of the clinical drug trials were probably carried out with primary tumour *KRAS* testing.[321] Given this confusing situation, there is no consensus on which tissue should be tested and pathology guidance suggests primary or secondary tumour tissue can be tested, with only a 'preference' for metastatic tissue where available. Extracting DNA from resected metastases is easy but these patients are not considered palliative, are not offered anti-EGFR mAb therapy and so these specimens should probably not be tested.[14,314]

4.1.4 Rationale for Ras testing with automated PCR

One of the most widely used *KRAS* tests is run on the Cobas platform. The test is now quite old and can only detect changes in exon 2 (codons 12 & 13) and exon 3 (codon 61).[183,197] However, recent evidence suggests testing for targets in exon 4 (codons 117 and 46), therefore so-called 'extended Ras testing' now being called for in routine practice.[322] The Idylla *KRAS* Mutation Test has recently been launched for use in CRC in Europe. The assay has a turn-around time of two hours and covers the detection of a similar range (to Cobas) of clinically relevant *KRAS* mutations that can be used in the clinical setting for guiding anti-EGFR mAb therapy, see Table 7. The substitutions which can be detected by the test are G12C/R/S/A/D/V, G13D, A59E/G/T (exon 3; the test cannot distinguish between different mutations in codon 59), Q61H/H2/K/K2/L/R (the test cannot distinguish between H and H2, between K and K2 or between L and R changes), K117N1/N2 (the test cannot distinguish between N1 and N2 changes) and A146P/T/V (the test cannot distinguish between mutations in codon 146). Despite the limitations of differentiating

codon changes, determining the mutation type is not currently necessary for directing therapy. Testing for *KRAS* mutations with the Idylla System may also offer faster results than Cobas and may do so at a lower cost. The Ion AmpliSeq Cancer Hotspot Panel v2 covers all of the *KRAS* codon changes covered by Idylla and Cobas, however the test turn-around time is at least 24 hours.[196] There are no published data on the Idylla *KRAS* Mutation Test as discussed, therefore this part of the study aimed to address this lack of evidence.

There are few widely available and approved commercial PCR-based tests for detecting *NRAS* mutations. Most centers use NGS gene panel approaches for detecting these mutations. This approach is slow and costly as discussed.[264,323] The Idylla *NRAS-BRAF-EGFR*S492R Mutation Assay is currently available for research only and exists in a prototype format for testing. The Idylla assay has a similar mutation coverage to NGS, see Table 7. The assay has a turn-around time of two hours and detects the G12C/S/D/A/V (the test does not distinguish between A and V), G13D/V/R (the test does not distinguish between V and R), A59T, Q61K/L/R/H/H2 (the test does not distinguish between H and H2), K117N/N2 (the test does not distinguish between N and N2) and A146T/V (the test does not distinguish between T and V) substitutions. Although there is little data available for *NRAS* testing, as with *KRAS* mutations, the type of *NRAS* mutation is probably not important and so these limitations are unlikely to be inhibitory to clinical use.[189] The Ion AmpliSeq Cancer Hotspot Panel v2 covers most of the *NRAS* codon changes covered by Idylla (it does not include coverage of A59T or A146V), however the test turn-around time is at least 24 hours.[196] The Idylla assay then potentially offers a cheaper and much faster testing method over NGS approaches, but requires validation and clinical evaluation. There was only a limited supply of prototype assays available for testing and therefore a full clinical study was not possible at this time. The test however requires a basic validation and this was possible within these limitations.

4.2 Experimental design

4.2.1 Ras workload and costings audit

In line with local and national/international guidelines already mentioned, *KRAS* testing is carried out on all CRC excisions.[14,70,260,261,310,311] Therefore the total number of annual CRC cases was used in the costing audit for *KRAS* testing. These methods are similar to those described in Chapters 2 and 3. The number of cases requiring *NRAS* testing would essential be the same (284 patients) as those requiring *KRAS* testing as the indication is the same in both therapies.[14,70,260,261,264,310,311]

4.2.2 *KRAS* automated PCR diagnostic accuracy study

The Idylla *KRAS* Mutation Test was compared against the standard-care Cobas *KRAS* Mutation test. All cases were tested for *KRAS* mutations at the time of original reporting. A consecutive series of cases was selected from the diagnostic archival database search results (see Chapter 2). Selection criteria in addition to those set out in Chapter 2 were used to limit cases selected from patients for whom *KRAS* testing was performed.

At this point in the study there was more information available to make a sample size calculation possible. The results of the *BRAF* diagnostic study carried out in Chapter 3 suggested the sensitivity and specificity of Idylla is 100%. In the intervening time a number studies were published and further poster abstracts were presented that also suggested the Idylla System had high diagnostic accuracy (see Chapter 5).[324-328] For *KRAS* mutations the sensitivity of a test is probably more important as it is used to rule out patients from targeted therapy. A conservative estimate of 99% was taken based on the findings in Chapter 3 and other published data. Furthermore, the standard formulae do not function well at values of 100% (numerator of zero) so a lower value had to be used. This is in line with standard practice. The sample size was estimated for a confidence interval of 95% and was carried out using standard formulae (see Appendix 4).[235,329]

The estimated sample size was calculated as 30 cases for a prevalence of *KRAS* mutations at 50% (based on local rates):

$$\mathrm{DP} = 1.96^2 \frac{0.99\ (1 - \ 0.99)}{0.05^2} = 15$$

$$n\ (Sn) = \frac{15}{0.5} = \mathbf{30}$$

4.2.3 Validating *KRAS* automated PCR with NGS

The Idylla *KRAS* Mutation Test was validated against NGS with the Ion Torrent Gene Panel. Test. 18 cases were selected rather than 20 as funds were limited and prioritised for the clinical study. Nine positive and nine negative cases by NGS were selected. Cases were processed in line with the methods outlined in Chapter 2.

4.2.4 Validating *NRAS* automated PCR with NGS

The Idylla *NRAS-BRAF-EGFR*S492R Mutation Assay was validated for *NRAS* mutations against NGS with the Ion Torrent gene panel. 18 cases were selected for the validation as only a limited number of prototype testing cartridges were available and these were supplied in packs of six per unit. Five positive and 13 negative cases by conventional NGS were selected. Only five positive cases had been identified from the entire hospital database of *NRAS* testing (not unexpected as these mutations are uncommon). The cases were processed in line with the methods outlines in Chapter 2.

4.3 Results

4.3.1 Ras testing workload and costings

As presented in Subsection 3.3.1, 284 CRC cases were reported in the department over a 12-month period; all would have been eligible for *KRAS* testing. Only around half had actually undergone testing due to funding shortfalls. The Cobas *KRAS* Mutation Test costs around £180 and therefore the total cost would be estimated at £51,120. The list price of the Idylla *KRAS* Mutation Test given by Biocartis is £149 per test, or £42,316 per year. Therefore, the potential saving by introducing the Idylla System for *KRAS* testing is £8,804. There was obviously some overlap of the actual cases audited here with those for *BRAF*.

NRAS testing has only just been introduced in Oxford, however all 284 *KRAS* eligible cases would also have been eligible for *NRAS* testing within the same time period. No conventional PCR test is available therefore NGS testing on the gene panel, which is around £300 per case, would be needed. For *NRAS* alone this would cost an estimated £85,200. The list price of the Idylla *NRAS/BRAF/EGFR*S492R Mutation Assay given by Biocartis is £212, or £60,208 per year. This would give a potential saving of £22,992 if the Idylla System was introduced into routine practice for *NRAS* testing alone.

4.3.2 *KRAS* automated PCR diagnostic accuracy

30 CRC samples from 30 patients were processed with the Idylla System. A summary of the patient characteristics is given in Table 17. All cases were invasive adenocarcinoma covering the clinical spectrum from well to poorly differentiated tumours and ranging from Stage I to III tumours. There were no biopsy specimens in this cohort. All cases met the minimum tissue requirements for testing and no case required more than one tissue section for Idylla testing.

Characteristic	**Values** n=30
Age*	71 (9) years
Male	57.0%
Adenocarcinoma	100.0%
Site	
Right colon	30.0%
Transverse colon	3.3%
Left/sigmoid colon	30.0%
Rectum	36.6%
Specimen type	
Right hemicolectomy	33.3%
Transverse colectomy	3.3%
Left Hemi/sigmoidcolectomy	13.3%
Anterior resection	36.6%
Abdominoperineal resections	6.6%
TEM	6.6%
Grade	
Well differentiated	3.3%
Moderately differentiated	83.3%
Poorly differentiated	13.3%
Stage at surgery	
Stage I	16.6%
Stage II	40.0%
Stage III	43.3%
Stage IV	0.0%
Neo-adjuvant chemotherapy given	2 cases (6.6%)

* Variables expressed as a Mean (Standard Deviation)

Table 17. Summary of the patient characteristics for cases tested with the Idylla *KRAS* Mutation Test. *TEM = transanal endoscopic mucosal resection.*

All cases had the Idylla test and conventional PCR. No cases failed testing by Idylla. In keeping with the expected findings from the literature, *KRAS* mutations were found in 15 cases by Cobas making the reference test prevalence 50.0% (95% CI: 33.2% to 66.9%) and in 17 cases by Idylla making this a prevalence of 56.7% (95%CI: 39.2% to 72.6%). There were no failed tests.

Of the mutations found by Idylla, 88.2% (15/17 mutations) were in codons 12 and 13 (see Table 18) and over a range of specific amino acid changes. None of the additional mutation types covered by the Idylla test (mutations in codons 59, 117 and 146) were detected in this study.

Mutation type	Frequency
G12C	1
G12R	1
G12A	1
G12S	0
G12V	3 (incl. 1 discordant)
G12D	9
G13D	0
A59E/G/T*	0
Q61K/K2*	0
Q61L/R*	2 (incl. 1 discordant)
Q61H/H2*	0
K117N1/N2*	0
A146P/T/V*	0

*The Idylla System cannot distinguish between these codon changes[185]

Table 18. Summary of the *KRAS* mutation types detected in the clinical study. The table includes the full list of detectable mutations by the Idylla *KRAS* Mutation Test. Mutation types here are given by Idylla; Cobas reports only the exon of the mutation. Codon changes not detected by Idylla are not shown; no such codon changes happened to be present in the cohort tested here. One of the five G12V mutations was not concordant with Cobas and this was later shown to be mutant by ddPCR. The one Q61L/R mutations was Cobas negative and later confirmed to be wild type with ddPCR. Frequencies are given in absolute numbers. Mutations are given at the protein level with amino acid change notation. Numbers represent codon/amino acid position. *G =glycine, C = cysteine, R = arginine, A = alanine, S = serine, V = valine, D = aspartate, E = glutamate, T = threonine, K = lysine, L = leucine, H = histidine, N = asparagine, P = proline, ddPCR = droplet digital polymerase chain reaction*

There was agreement between the Idylla System and Cobas in 28 of the 30 cases, making the concordance 93.3% (95% CI: 78.7% to 98.2%). Both discordant cases were Idylla positive but Cobas negative. A summary of the results is given in Table 19.

	Cobas Positive	**Cobas Negative**	Totals
Idylla Positive	15	2	17
Idylla Negative	0	13	13
Totals	15	15	30

Table 19. Summary of the diagnostic accuracy study test results comparing Idylla (index test) with Cobas (reference test) PCR for *KRAS* mutations.

The sensitivity of the Idylla System was 100.0% (95% CI: 79.6% to 100.0%) and the specificity was 86.7% (95% CI: 62.1% to 96.3%). The positive predictive value was 88.2% (95% CI: 65.7% to 96.7%) and the negative predictive value was 100.0% (95% CI: 77.2% to 100.0%). The positive likelihood ratio was 7.5 and the negative likelihood ratio was zero. A summary of the statistical analysis is given in Table 20.

Statistic	Value
Prevalence (by Idylla)	56.7% (95%CI: 39.2% to 72.6%)
Prevalence (by Cobas)	50.0% (95% CI: 33.2% to 66.9%)
Concordance	93.3% (95% CI: 78.7% to 98.2%)
Concordance including ddPCR results	96.7% (95% CI: 83.3% to 99.4%)
Sensitivity	100.0% (95% CI: 79.6% to 100.0%)
Specificity	86.7% (95% CI: 62.1% to 96.3%)
Specificity including ddPCR results	92.9% (95% CI: 68.5% to 98.7%)
PPV	88.2% (95% CI: 65.7% to 96.7%)
PPV including ddPCR results	94.1% (95% CI: 73.0% to 99.0%)
NPV	100.0% (95% CI: 77.2% to 100.0%)
LH+	7.5
LH+ including ddPCR results	14.0
LH-	0.0

Table 20. Summary of the calculated statistics from Idylla data for the *KRAS* diagnostic test accuracy study. *PPV = positive predictive value, NPV = negative predictive value, LH+ = likelihood ratio positive, LH- = likelihood ratio negative, ddPCR = droplet digital PCR*

The discordant cases tested both met the minimum tissue requirements for Idylla and therefore were adequate samples. Both discordant cases were disagreements between Idylla and Cobas. One discordant case was a G12V codon change and one was a Q61L/R codon change. The two cases were adjudicator tested by ddPCR for *KRAS.* The G12V codon change was confirmed by ddPCR making the Idylla results a true positive. The Q61L/R case however was confirmed to be wild type by ddPCR making the Idylla result a false positive. Including these findings in the statistical analysis improves the results: the concordance then was 96.7% (95% CI: 83.3% to 99.4%), the specificity 92.9% (95% CI: 68.5% to 98.7%), the PPV 94.1% (95% CI: 73.0% to 99.0%) and the positive likelihood ratio 14.0.

4.3.3 *KRAS* automated PCR validation with NGS

The results of the *KRAS* PCR validation study are shown in Table 21. 13 of these cases were from the same cohort of patients tested for *BRAF* and *NRAS* mutations in Subsections 3.3.4 and 4.3.4 but represent unique assay runs. Five cases were a unique set of patients not tested elsewhere.

	NGS Positive	**NGS Negative**	Totals
Idylla Positive	9	0	9
Idylla Negative	0	9	9
Totals	9	9	18

Table 21. Summary of the *KRAS* validation study comparing Idylla with NGS. Permission to reproduce this table from a similar published table has been granted by the British Medical Journal Publishing Group Ltd.[4]

There was 100.0% (95% CI: 82.4 to 100.0%) concordance between Idylla and Cobas. The mutation types detected are given in Table 22.

Mutation type	Frequency
G12C	1
G12R	0
G12A	0
G12S	0
G12V	2
G12D	5
G13D	0
A59E/G/T*	0
Q61K/K2*	1
Q61L/R*	0
Q61H/H2*	0
K117N1/N2*	0
A146P/T/V*	0

*The Idylla System cannot distinguish between these codon changes[185]

Table 22. Summary of the *KRAS* mutation types detected in the validation study with NGS. The table includes the full list of detectable mutations by the Idylla *KRAS* Mutation Test. Codon changes not detected by Idylla are not shown; no such codon changes happened to be present in the unselected cohort tested here. Frequencies are given in absolute numbers. Mutations are given at the protein level with amino acid change notation. Numbers represent codon/amino acid position. *G =glycine, C = cysteine, R = arginine, A = alanine, S = serine, V = valine, D = aspartate, E = glutamate, T = threonine, K = lysine, L = leucine, H = histidine, N = asparagine, P = proline.*

4.3.4 *NRAS* automated PCR validation with NGS

The results of the *NRAS* validation study are shown in Table 23. The mutations detected are given in Table 24. These results are derived from the same Idylla run (*NRAS-BRAF-EGFR*S492R Mutation Assay) carried out from which the *BRAF* results in Subsection 3.3.4 were derived. 12 patients from this cohort were also tested with a separate assay for *KRAS* mutations (see result of this in Subsection 4.3.3).

	NGS Positive	**NGS Negative**	Totals
Idylla Positive	5	1	6
Idylla Negative	0	11	11
Totals	5	12	17

Table 23. Summary of the *NRAS* validation study comparing Idylla with NGS. Permission to reproduce this table from a similar published table has been granted by the British Medical Journal Publishing Group Ltd. [4]

Mutation type	Frequency
G12C	1
G12S	0
G12D	3
G12A/V*	0
G13D	0
G13V/R*	0
A59T	0
Q61K	0
Q61L	1 (discordant, NGS wild type)
Q61R	1
Q61H/H2*	0
K117N/N2*	0
A146T/V*	0

*The Idylla System cannot distinguish between these codon changes[189]

Table 24. Summary of the *NRAS* mutation types detected in the validation study with NGS. Codon changes not detected by Idylla are not shown; no such codon changes happened to be present in the unselected cohort tested here. Frequencies are given in absolute numbers. Mutations are given at the protein level with amino acid change notation. Numbers represent codon/amino acid position. *G =glycine, C = cysteine, R = arginine, A = alanine, S = serine, V = valine, D = aspartate, E = glutamate, T = threonine, K = lysine, L = leucine, H = histidine, N = asparagine, P = proline, NGS = next generation sequencing.*

The concordance between Idylla and Ion Torrent was 94.1% (95% CI: 73.0% to 99.0%). There was one discordant case that was Idylla positive (Q61L) but wild type by Ion Torrent (Q61L is covered by the Ion Torrent panel). The case met the minimum tissue requirements for the Idylla processing. ddPCR confirmed the presence of the Q61L mutation making the concordance 100%. There was one failed test due to an Idylla software error; this was the same assay failure as in Subsection 3.3.4 and thus represents overall one failed cartridge but two failed tests. A replacement assay to repeat the sample could not be obtained.

4.4 Conclusion

The results of the diagnostic study show the Idylla System has a high level of accuracy for detecting *KRAS* mutations. The results also show a high concordance of Idylla with NGS for *NRAS* testing. These finding support the hypothesis and are the first study to evaluate the system for *KRAS* or *NRAS* testing.

4.5 Discussion

There were two discordant test results in the Idylla *KRAS* Mutation Test diagnostic study, lowering the specificity slightly. In addition, the CIs were wider than anticipated. This likely reflects the fact that, despite a formal sample size calculation, in hindsight a bigger sample size would have improved the analysis. The discordant tests were further analysed with ddPCR, which showed one to be a true result by Idylla and the other to be a false positive call. Including these data in the analysis improves the CIs, the specificity is greater and the accuracy is within the realms of acceptability. These results were also reassured by the perfect concordance between Idylla and NGS for *KRAS* testing.

The discordant results were not affected by which mutations were covered in either assay as the codon changes which were detected are covered by both Idylla and Cobas. In the case of the false positive Idylla result, this could have been due to contamination (although highly unlikely given the protocol) or the mutation being present in only a small clonal population of cells within the tumour. Although ddPCR is far more analytically sensitive (detects a lower mutation burden within the DNA pool) than Idylla, there is a possibility that a small clone could have been cut out in the later sections tested with ddPCR. This is extremely unlikely however. The clinical significance of a false positive or negative test in this circumstance is uncertain. The treatment with anti-EGFR mAb therapy is palliative anyway and small clonal populations would eventually be selected for once exposed to therapy. Therefore, while either inaccurate result would not be clinically ideal, neither offers greater accuracy over any reasonably available commercial assay used for standard testing. On balance then, the accuracy between the two systems (Idylla and Cobas) is probably comparable enough for the Idylla System to be safe for clinical use. There were

no failed Idylla tests in the *KRAS* part of the study and this probably reflects some of the improvements made on the system from the feedback given after the *BRAF* testing.

The Idylla *NRAS-BRAF-EGFR*S492R Mutation Assay showed complete concordance but a further diagnostic study of the assay should be undertaken before taking the test fully into clinical practice. This is a crucial step for the Idylla System for use in CRC, as *NRAS* testing could become routine. There was one failed assay due to an initial software issue. This was fully resolved with a system update and really only reflected that the fact that the *NRAS* assay had only just been released.

An important limitation of Ras tests on the Idylla system is the difference in coverage of some rare mutations when compared with Cobas or NGS (see Table 7). For example, the Idylla *KRAS* Mutation Test lacks coverage of the *KRAS* G13C mutation. This is a rare mutation and the clinical implications of this are not fully known,[330] but this might be shown to be an important mutation in CRC in the future as more data become apparent. Similarly, there are some *NRAS* mutations not covered by Idylla but are by NGS. The converse is also true; some Ras codon changes are covered by Idylla but not the other conventional tests investigated here. The clinical implications of this issue are not currently known but again, this could be important in the future.

Similar methods, data, statistical analysis and some points of discussion presented in this Chapter are also presented in publications and posters arising from this work.[1-8] Non-integer values are rounded to one decimal point. For all statistical analysis formulae, see Appendix 4.

Chapter 5: Discussion

5.1 Main findings

In this thesis the results have shown that the Idylla System is able to detect mutations in *BRAF*, *KRAS* and *NRAS* with a very high degree of accuracy, confirming the hypothesis set out in Chapter 1. Only one Idylla result out of 180 molecular assay comparisons carried out in this thesis was shown to be a false result (one *KRAS* Mutation Test result). The Idylla *BRAF* Mutation Test was shown to be specific and potentially more sensitive that the Cobas equivalent test. The Idylla *KRAS* Mutation Test was shown to be at least as sensitive and specific as the Cobas equivalent test and have other benefits which are discussed below. The *NRAS-BRAF-EGFR*S492R Mutation Assay had a high level of concordance with standard care testing and further clinical evaluation would be useful.

5.2 Potential benefits of automated PCR

There are several potential benefits of the Idylla System over conventional PCR or NGS approaches. First of all the Idylla System is fully automated and was found to be very easy to use in this study. No specialist training is needed and no specialist setting is required to house the system. In contrast, the typical NHS histopathology laboratory does not have the specialist training or facilities needed in order to run molecular tests such as conventional PCR or NGS. The Idylla System is unique in that all the processes are automated and carried out on-board the system; no other commercially available system can do this. These facts eliminate a whole range of practical, legal and administrative issues around sending tissue out of the department. It also streamlines the diagnostic process, reduces the risk of losing cases to follow-up and cuts down the turnaround time.

The speed of the Idylla System is also an attractive benefit. Standard tests can take at least a week to get a result and often this can be so variable and unpredictable that several attempts to check for results are often needed. The Idylla System will give a result within three hours of the histopathologist requesting it. Therefore the turnaround time is cut significantly. This has several knock–on benefits. Firstly, should patients meet the criteria at time of diagnosis for Ab therapy, the system can minimise any delay in treatment. This may potentially be of clinical benefit as well as help improve patient satisfaction. Cutting the turnaround time also has benefits for the department as well. One single report (histological and molecular result) can be signed out by a consultant on the same day, eliminating the need to wait, continually chase results and authorise additional reports (which could be missed by the clinician). A prospective clinical follow-up study would be needed to confirm these hypotheses however.

The Idylla *BRAF* assays cover a greater range of clinically relevant targets than most conventional tests and the Idylla Ras assays cover some codon changes not covered by

the comparison tests. Although none of these rare mutations were encountered in this study, the extra coverage offed by Idylla could increase the number of mutant cancers identified in routine practice and this could help direct therapy towards those patients who are most likely to benefit. More clinical data however are needed to support this particular hypothesis.

In a publically funded healthcare system, the financial implications of any new technology introduced is of great interest. This study suggests that The Idylla System is more cost-effective than standard care tests in current use. The audit and cost estimates showed significant savings for each individual test. In the real-life clinical scenario combinations of tests change the actual overall costings. It is likely that most centers at the moment would want to use the Idylla *BRAF* Mutation Test and the Idylla *KRAS* Mutation Test. In this study (an average-sized cancer centre) 284 CRC cases were reported each year. From these 44 were eligible for *BRAF* testing and all 284 were eligible for *KRAS* testing. From calculations in Chapters 3 and 4, annual Cobas testing was estimated to cost £59,040 overall. The Idylla in contrast was projected to cost £47,156 overall, thus offering a potential saving of £11,884. In centres where there is limited funding for molecular testing despite recommendations, such savings could be very clinically impactful. Alternatively, if Ion Torrent NGS is being used in a centre (around £300 per case, each assay covering both *KRAS* and *BRAF* thus all 284 cases are tested but only once) the total annual cost could be estimated at £85,200, offering a far greater potential saving of £38,949 with Idylla in this scenario. Testing numbers may change however, based on new NICE guidelines to screen all CRC patients for LS.[269]

Some centers are now routinely testing for *NRAS* mutations and this is usually with NGS. In this scenario the estimated cost for Ion Torrent NGS remains the same at £85,200 per year. In contrast, testing with the *KRAS* Mutation Test in combination with the *NRAS-BRAF-EGFR*S492R Mutation Assay (calculated costs in Chapter 4) to cover all three targets would be £102,524 per year, potentially an additional expense of £13,324 per year with the Idylla System. This is not cost effective and the other benefits of the test would need to be weighed when deciding on whether to use the system. The figures here are based on list prices however, and the cost per test falls with larger orders.

Another factor which is difficult to assess is the overall cost of the system, which for Idylla with one processing unit is around £40,000 purchased outright; subscription deal on a test-by-test basis are also possible. NGS and Cobas cost many times this, but most molecular centres will already have such systems – meaning to introduce Idylla testing may incur an additional outlay cost. A center may decide however that being able to offer a both detailed NGS-based technologies and rapid on-demand PCR testing may cater for a wider range of clinical scenarios. There are also roles the Idylla may play in the future and therefore acquiring the system in the short-term could be useful for new developments in molecular

diagnostics in the long run. There are some cartridges in development that will allow testing of tiny amounts of unfixed cytological (loose single cells) material. This would allow very rapid genetic testing of tumours which are amenable to fine needle aspiration (FNA), such as thyroid and lung cancers,[331,332] and, as will be mentioned later in Section 5.5, the existing cartridge may work with tissue other than FFPE.[333] Minimally invasive molecular testing could be carried out in the clinic while the patient waited and any treatment started immediately without delay. There are also test cartridges in development that will be able to test blood samples for circulating tumour cell DNA (ctDNA) or free plasma DNA. One such cartridge was released prior to this thesis as already discussed in Chapter 1, the Idylla ct*BRAF* Mutation Assay,[190] and a further similar assay, the Idylla ct*KRAS* Mutation Assay, has been released since.[334] Testing for ctDNA mutations is an increasingly popular method of monitoring patients for relapse but also could be a potential method for detecting mutations within the tumour, faster and with less invasive procedures.[335,336] The most significant benefit of this testing method in CRC would probably be in detecting *KRAS*/*NRAS* mutations in patients who later develop metastatic disease. In this scenario, because of inter-tumour heterogeneity, original testing results may not reflect the true genotype of the metastatic cell population.[320] These patients often do not undergo biopsy of their secondary tumour, either because it wrongly assumed not to be diagnostically useful or because it is technically challenging and risky (e.g. brain metastases). Also, once a patient has developed metastases, there is an urgent need to start targeted therapy and standard molecular testing takes too long. Therefore, *KRAS*/*NRAS* mutations are often excluded only on the primary tumour tissue.[337] Some data have suggested there is a reasonably high (92%) rate of genotype concordance between primary and secondary tumours,[316] however other studies have mixed findings.[315,317-319] In the envisioned future of precision medicine, testing secondary tumours will surely be insisted upon,[338] and will allow the clinical impact of this to be evaluated in drug trials based on metastatic tumour tissue Ras testing. If the Idylla ct*KRAS* Mutation Assay proves to be accurate, this would potentially be more informative that current primary tumour testing and facilitate better-informed treatment decisions while causing very minimal delays in starting treatment.

5.3 Potential drawbacks of automated PCR

There are several potential drawbacks to the Idylla System. While the cost of the PCR in our institution would be lower with the Idylla System, in other institutions this may not be the case. The Cobas system is batched and the cost per test is variable depending upon the number of cases being tested. Therefore the Cobas system may be cheaper in some institutions. In addition, the introduction of Idylla may result in a fall in use of the Cobas system and therefore this may result in underutilisation of an valuable system that is expensive to maintain (and can't be decommissioned as it is needed for other tests in the Trust). There may also be a risk of increasing the waiting times and cost per test for other

Cobas tests as the numbers of cases for batching falls. There is also the risk of de-skilling staff who run the Cobas system.

The Cobas system is not so much of a 'black box' set up as the Idylla System is. With Cobas, the user can examine the processes of the analyser and the sample preparation and can adjust the protocol if needed (for example testing different kinds of tissues may need slight changes in the system settings). This cannot be done with the Idylla System. Also the results of the Cobas system can be interrogated and many values such as the Cq value (discussed in Chapter 1) can be read and the melting curve can be manually examined. Interrogating these variables can be useful when verifying results and can give an indication of how confident the user should be of a mutation. In contrast NGS can give additional information such as an indication of the frequency of mutated cells within the tissue tested. None of these can be easily achieved with the Idylla System and while this makes the test less subjective, it can potentially limit the ability to quality control the results produced.

An important point to consider with any new technology is the potential for hardware or software failure. There were several failed Idylla tests but these however appeared to be teething problems. Only one cartridge failed under normal working conditions (true failure rate <1%), which is probably within the margin of acceptability. Commonly, molecular tests may fail because of poor quality samples. Biocartis state that they Idylla assays can be run on just a single FFPE section. For the majority of cases in this thesis this was true. Only two tests were reported as insufficient material and these were paucicellular (<10% tumour cells) mucinous tumours where the recommended single section was obviously not enough for sufficient DNA yield. Had further tissue sections been available (in clinical practice this would have been the case, however repeating the tests was not possible in the study setting due to limited funding), these cases could have been tested with multiple tissue sections and would probably have been successful. This is an interesting point in general for molecular testing of mucinous tumours, which although not highlighted in the literature, is anecdotally a known problem amongst laboratory staff. Mucinous tumours by nature secrete huge amounts of mucin-like material and the cells often sit singly or in small islands within a sea of acellular stroma. Mucinous tumours then need many sections or tissue punches in order to get enough DNA for testing. In contrast, the percentage of cells within these samples that are tumour is usually very high as there is little inflammatory or stromal cell content.

Already discussed (see Section 4.5) earlier was the issue about mutation coverage. There are some mutations not covered by Idylla and others that are *only* covered by Idylla (see Table 7). These are in general very rare codon changes and no such mutation was encountered in this study, however the clinical implications of these may end up being important in the future and so any lack of coverage by the Idylla is a potential drawback of

the system. Also important to note is that the Idylla does not always distinguish between particular types of codon changes and often just reports a mutation from a group of several related changes (also see Table 7). For example, the *KRAS* Q61L mutation cannot be distinguished from the *KRAS* Q61R mutation and is reported as a '*KRAS* Q61L/R' mutation. This is probably due to a limitation with the primers and reporting probes. It is generally not clinically useful to know which particular codon change has taken place but, once again, the clinical implications for targeted or other therapy of rare mutations may not be fully known and so the ideal would be to know exactly which codon change has taken place. Therefore, this is also a potential limitation of the Idylla system.

As alluded to in the earlier discussion about cost-effectiveness, to be useful in CRC molecular diagnostics the Idylla System needs to be able to offer the complete package of tests needed in clinical practice. Most guidelines mentioned call for *BRAF* and *KRAS* testing currently and the Idylla has CE-IVD marked assays for both of these. *NRAS* is however likely to play a greater role in the future of CRC targeted therapy guidance. At the time of planning this thesis the only assay which covered *NRAS* was the Idylla *NRAS-BRAF-EGFR*S492R Mutation Assay and this was RUO, not CE-IVD marked. Since this thesis work was undertaken, a CE-IVD approved Idylla assay for *NRAS* and *BRAF* testing has been released, the Idylla *NRAS-BRAF* Mutation Test.[339] This appears to be a final version of the *NRAS-BRAF-EGFR*S492R Mutation Assay, but without the *EGFR* testing component, probably as the clinical use for this was not warranted. This thesis has validated the *BRAF* and *NRAS* components of this test as a proof-of-concept and a full clinical evaluation of its accuracy would now be helpful. This is crucial for the system and if this cannot be demonstrated clinicians will probably opt for NGS gene panel testing because all three gene targets are easily covered by NGS and this technology has been extensively validated.

As mentioned, the Idylla *NRAS-BRAF-EGFR*S492R Mutation Assay also tests for mutations in the *EGFR* gene. Currently, management guidelines do not recommend routine testing for this mutation, however this may change in the future and this part of the assay could be released in a CE-IVD approved test at some point. Therefore, technical validation of this component would still be useful and this should also be followed up by a clinical study.[259-261,340,341] At this time, no commercial PCR or NGS-based tests are available for the S492R mutation but gene sequencing could be used to validate any findings. It may be difficult to identify positive cases though in the short-term.

The rivalry of NGS with PCR-based platforms has been mentioned already several times but this should not be underestimated. The cost of whole genome and targeted NGS is rapidly falling, but only a limited number of such tests are clinically validated and approved so far; those that have been approved remain expensive (around £1000 per sample). The situation is very fluid though. It is probably reasonable to anticipate that the cost of targeted

panels and indeed WGS will fall in coming years. In addition, many newer assays are in development and while not generally clinically approved or validated yet, may in the near future offer superior turnaround times and at a lower cost. Therefore, Biocartis will need to be quick to market and offer flexible pricing in order to make the test viable in the long-term. In the short-term, for centres only requiring *BRAF* and *KRAS* testing, the system could be acquired on a licence arrangement and be cost effective.

5.4 Findings in the context of the recent literature

When this project was conceived there were no publications for the Idylla System in the literature. Since this work was started however, a number of publications have arisen in and it is worth considering the results of this thesis along side those publications.

The first study appear in the literature was presented online as an epublication initially in mid 2015, when the *BRAF* work of this thesis was well underway. This evaluated the Idylla System in a Respiratory Panel for blood testing. The paper presents a technical feasibility study which attempts to derive Idylla Cq values from conventional PCR comparisons. The study has little relevance to mutation testing in cancer however and no data on clinical diagnostic accuracy was presented.[328]

The next study published with Idylla was a detailed and thorough technical evaluation of the Idylla *BRAF* Mutation Test, published in late 2015 when the *KRAS* data started to be collected for this thesis. The authors test the Idylla System with a number of known commercially available control sample cells lines and show a high degree of validity when compared with a range of PCR and NGS-based tests. The system is further evaluated in a set of commercially available FFPE control tumour samples. The final stage in the paper was a clinical evaluation at a major teaching hospital. 100 patients were selected with differing tumour sites (including skin, colorectum, ovary, thyroid) and the Idylla was compared with an in-house PCR. A detailed discussion of how the cases were selected was not provided and although it appears that an attempt to replicate the true clinical setting made, the potential for selection bias cannot be excluded. Overall the authors quote concordance rates between 95% and 100% and diagnostic accuracy is not established for any tumour type. Although there are limitations to this study, the findings are in keeping with the results of this thesis.[326]

The next study published compared the Idylla *BRAF* Mutation Test with ‘reference laboratory results’ (this is not defined) in 148 patients with melanoma. This was published just after the previous study discussed in late 2015. The cases were said to be from those with ‘left over FFPE’ tissue and it is likely there was significant selection bias with this approach. The concordance was quoted by the authors as between 97% and 100% but the sensitivity and specificity were not calculated. Despite these limitations, the findings are

overall in keeping with the results presented in this thesis. The reference list in the paper suggests there are two pending studies which are in press, however further details of these could not be found.[327]

At around the same time as the above publications in late 2015, there were a number of Biocartis sponsored posters presented. One of these investigated the role of Idyla in detecting *BRAF* mutations in circulating tumour DNA (the Idylla ct*BRAF* Mutation Assay already mentioned), finding a high level of concordance with standard methods.[324] A second poster investigated the same assay and found similar results.[342] Although in keeping with the results of this thesis, this is of less relevance as this work was carried out on blood samples. Another poster was the first to present *KRAS* and *NRAS* data with comparisons of the Idylla *KRAS* Mutation Test and the Idylla *NRAS-BRAF-EGFR*S492R Mutation Assay with Cobas PCR and NGS. This demonstrated high concordance, again in keeping with the findings of this thesis.[325]

The first study for *KRAS* mutations to be published on the Idylla was the next to appear in the literature. In this study pancreatic cancer samples were tested using the Idylla *KRAS* Mutation Test. A common test to diagnose lesions in the pancreas is with cytology is a FNA. This is a challenging test to interpret however and detecting *KRAS* mutations in FNA specimens (indicating malignancy) is clinically helpful in difficult cases. The study actually used extracted DNA inserted directly into the cartridge, as FFPE material is not easily derived from FNA specimens. This worked in 49 of 52 cases and showed a specificity of 100% when compared with Sanger sequencing and NGS, very much in keeping with the *KRAS* assay findings in this thesis. The sensitivity however was much lower at 55.1%, but this was similar to NGS and is probably explained by the low cellularity of cytology specimens rather than a direct result of poor Idylla performance. These results demonstrate a novel use of the system in a different tissue type that can reliably rule out the presence of *KRAS* mutations (i.e. almost rule out malignancy) in a challenging area of diagnostic pathology.[333] Around the same time of this publication, appeared a study investigating the *BRAF* Mutation Test in melanoma patients, comparing with conventional PCR, NGS and IHC. This found high concordance with molecular testing but interestingly like this thesis also ran into difficulties with IHC comparisons, especially with disagreements between scorers of VE1 Ab staining.[343]

A large multicenter study looking at *BRAF* mutations in a variety of tissue (52 CRC cases) types was the next to be published. This found concordance of the Idylla *BRAF* Mutation Test with pyrosequencing and NGS at 96.6%.[344] This was shortly followed up by a publication of the Idylla ct*BRAF* Mutation Assay[345] and a publication of a prototype Idylla *EGFR* assay, [346] both showing high concordance. A further study with the Idylla *BRAF* Mutation Test was next published, this time looking at thyroid tumours. The presence of *BRAF* mutations in thyroid tumours can aid the diagnosis of papillary carcinoma, are

prognostic and may direct therapy for these patients. The study used 110 highly selected cases and this may have introduced bias, however they found that the sensitivity was 98.8% and a specificity of 100% when compared with a combination of PCR and pyrosequencing, very much in keeping with the findings of this thesis.[347]

The data from this thesis comparing the Idylla *KRAS* Mutation Test with PCR (Subsection 4.3.2) was combined with similar data for CRC cases from other centers across Europe and published as one large cohort (375 cases). The overall combined study design was not that of a diagnostic accuracy study and the publication appropriately therefore did not report sensitivity and specificity etc. However the concordance with a combination of other methods, such as PCR, ddPCR, pyrosequencing and NGS, was found to be 98.9%, very much in keeping with the overall results (including other validations herein) for *KRAS* testing found in this thesis.[3]

Recent publications for Idylla included an evaluation of a prototype Ebola virus assay that showed 95.9% concordance with standard testing methods[348] and an evaluation of the Idylla EGFR Mutation Assay in lung cancer, finding 87.5% concordance with NGS but poorer results compared with ddPCR (27.8% concordance) for one particular codon change (T790M).[349] Other recent evidence available is from several poster presentations. The first was an AstraZeneca sponsored poster abstract, co-authored by Biocartis, of a small study of 56 samples (cell lines) comparing the performance of 12 different technologies. This included conventional PCR and NGS, for detecting *KRAS* mutations. In this study Idylla detected the correct result in 96% of cases, outperforming many of the existing technologies, including Cobas and Ion Torrent.[350] The second poster was also co-authored by Biocartis and evaluated the Idylla *NRAS-BRAF-EGFR*S492R Mutation Assay. The results showed agreement with sequencing in 98.9% (*NRAS*), 99.6% (*BRAF*) and 100% (*EGFR*, n=1) of cases, again very much in keeping with the results of this project.[351] The third poster evaluated the Idylla *EGFR* Mutation Assay and found this to have 99.4% agreement with NGS.[352] Although the results from both of these posters are likely not peer-reviewed and there are potential conflicts with the authors, the results are in keeping with the findings of this thesis.

Since this thesis was started a wide range of publications and posters have become available presenting evidence for the Idylla System. The data from this thesis has also been published and presented in posters, although (other than the *KRAS* data) these publications and posters have been omitted from the above discussion of the recent literature, for obvious reasons.[1-8] Although some of the evidence discussed here is in poster format and sponsored by Biocartis, the majority of it has been later published in peer-reviewed journals. Furthermore, the overall findings for Idylla are consistent throughout all the evidence identified. In this thesis, the findings match the overall picture that the Idylla

can accurately detect *BRAF*, *KRAS* and *NRAS* mutations and is in some respects superior to the other systems used in routine practice.

5.5 Study limitations and potential future work

The results presented in this thesis have addressed the hypothesis. The primary outcome has been addressed and the accuracy of the Idylla System has been demonstrated to be high. The study was carried out and reported following conventional evidence-based medicine principles and satisfies the Standards for Reporting Diagnostic Accuracy (STARD) statement.[237,249] The secondary outcomes and research questions have been partially addressed and this thesis has demonstrated the system to be easy to use and potentially cost effective. There are a number of limitations of this work however and there are further issues to be addressed.

Firstly, a Biomedical Scientist would carry out testing in the real-life context. While this does not pose any issue with technical ability, there may be issues with workflow, staff time to run the test and actual space in the working laboratory (although small the only space available for this study was in a storage area) for the machine and cartridges. In this sense the feasibility of using the system in daily practice has only been partially assessed. Furthermore, the cost analysis set out is an estimate and an actual prospective cost-impact study has not been carried out fully. This may be an area of further work. Likewise, the expected impact upon turn-around time is speculative and an audit or similar study of use would be needed to provide empirical evidence of this.

A criticism of this study could be that retrospective cases were assessed. Although bias was minimised by blinding and consecutive/unselected case testing, a prospective study will always have a lower risk of bias. In addition, the use of the system in the long term may see test failures once the full spectrum of cases with variable tissue qualities have been attempted with the machine over a sustained period of use. Several failed tests were encountered early on, due to a combination of user error and design faults. Once these were overcome, no further failed tests were encountered – however once placed in a busy laboratory and under constant use by numerous staff, further failed tests are likely to be seen. Therefore, a sensible next step is to trial the system for a sustained period of time and subject the service to audit. A further step could be to assess the actual clinical outcome in these patients and determine how testing with the Idylla System impacted upon clinical care and patient outcome.

There are some specific problems with the use of Idylla in CRC. In CRC the routine molecular targeted testing is focused on *BRAF*, *KRAS* and *NRAS* and therefore any new system introduced will need to be able to cater for all three targets. The Idylla System has been demonstrated to be valid for use in *BRAF* and *KRAS* here, however there remains

work to be carried out for *NRAS*. More work is needed to clinically evaluate the accuracy of Idylla *NRAS* testing.

In the current state, at least two test runs/cartridges are needed to provide a full assessment for CRC and in this sense a single cartridge which covered all the mutations for CRC would be far more preferential. Biocartis have communicated that this is planned in the future and this could be a further area for research in terms of validation and estimating accuracy. Microsatellite status is also increasingly being requested in clinical practice. For most centers this involves MMR protein expression evaluation by IHC but some centers use molecular testing with conventional PCR. To improve the Idylla System and to make it more appealing to those institutions with a large molecular preference, the inclusion of MSI testing within a cartridge would be very beneficial. Biocartis have communicated that such a prototype is in development and this would be an area of further work for validation and accuracy assessment.

This thesis does not evaluate the reproducibility of the Idylla System, neither by repeating samples nor by carrying out assessments at different testing sites. This is an important piece of work which should be carried out.

Finally, since this thesis was undertaken a number of new assays have been released on the Idylla. As mentioned the Idylla *NRAS-BRAF* Mutation Test[339] and the Idylla *EGFR* Mutation Assay (with a wider coverage than just the S492R codon change and mainly focused at lung cancer testing)[353] have been developed and in addition an Idylla ct*KRAS* Mutation Assay (aimed at identifying circulating tumour *KRAS* mutations in various cancers, including CRC) has now been released.[334] It would be of value to investigate the accuracy of these new tests, especially the ct*KRAS* test in CRC for reasons discussed earlier about circulating tumour DNA in Section 5.2

5.6 Recommendations for practice and closing remarks

Based on the evidence presented here, the Idylla System can be recommended for clinical use in detecting *BRAF* and *KRAS* mutations in FFPE tissue from colorectal adenocarcinomas. For centres only requiring *KRAS* and *BRAF* testing this system would be as accurate or more accurate that current tests and would offer significant potential patient benefits such as reduced turn-around time and costs. For centres requiring *NRAS* testing the system in principle is valid but not yet fully evaluated. Therefore, it is recommended the Idylla System is used cautiously for *NRAS* mutation detection in the clinical setting.

Similar conclusions and points of discussion presented in this Chapter are also presented in publications and posters arising from this work.[1-8]

APPENDIX 1 – Ethics Approval

Study Title: Expression pattern of C-type lectins in Health and Disease.
Internal Reference No: 04/Q1604/21
Ethics Ref: 04/Q1604/21
Date and Version No: 07.01.2015 Version 12

Chief Investigator: Dr Elizabeth Soilleux
Consultant Pathologist and Honorary Senior Clinical Lecturer
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Oxford OX3 9DU
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Fax. 01865 220519
E-mail: **Elizabeth.soilleux@ndcls.ox.ac.uk**

Sponsor: Oxford University

Funder (if applicable): Oxford University Medical Research Fund

Signatures: The approved protocol should be signed by author(s) and/or person(s) authorised to sign the protocol

Chief Investigator: Dr Elizabeth Soilleux
Consultant Pathologist and Honorary Senior Clinical Lecturer
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Fax. 01865 220519
E-mail: Elizabeth.soilleux@ndcls.ox.ac.uk

APPENDIX 2 – Tissue Regulations

Oxford Centre for Histopathology Research application (14/A209)

Section 1 Applicant details		
Research group / department		Cellular Pathology & NDCLS
Lead applicant *(e.g. Head of Department or Group, or clinical trial PI)*	Name	Dr Elizabeth Soilleux
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	Address	Dept of Cellular Pathology, OUH Trust
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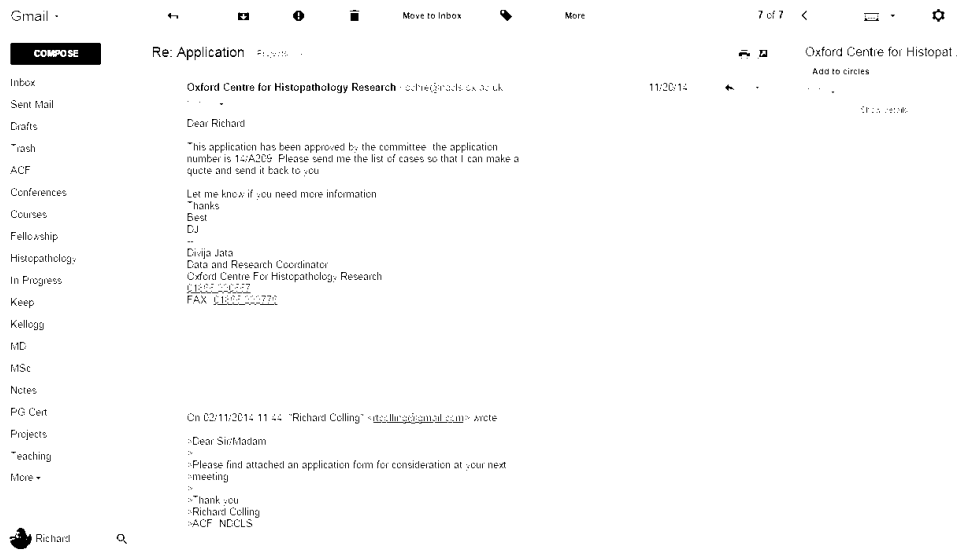
	Email	elizabeth.soilleux@ndcls.ox.ac.uk
Contact person *(if different from above) (i.e. the person who will coordinate the request(s) with OCHRe)*	Name	Dr Richard Colling
	Job Title	Academic Clinical Fellow
	Address	Dept of Cellular Pathology, OUH Trust
	Tel No.	01865 220581
	Email	rtcolling@gmail.com
Shipping details *(if different from above)*	Name	Dr Richard Colling
	Address	
	Tel No.	
Section 2 Funding details		
Research funder (*e.g. commercial company, NHS or University)*		Oxfordshire Health Services Research Committee
Contact person for quotation / funding questions		Dr Richard Colling
Section 3 Approval details		
Ethical approval details *(if applicable) A copy of the approval letter should be submitted with this application*	Reference No.	04lQ1604l21
	Title	Expression pattern of C-type lectins in Health and Disease
	Approval date	04.06.2004
	Expiry date	04.06.2019
R&D approval details *A copy of the approval letter should be submitted with this application*	Approval body	Oxford University
	Reference no. (if applicable)	04lQ1604l21
	Other information	Confirmatory paperwork/ e-mails available from Dr Elizabeth Soilleux
Sponsor	Organisation	University of Oxford
Section 4 Project details		
Research project title		Immunohistochemical testing of BRAF mutation for Lynch Syndrome screening in colorectal carcinoma
Lay summary *(this may be made available on the OCHRe website, please advise if confidential)*		Colon cancer is the fourth most common cancer in the UK and a leading cause of cancer death. Whilst most cancers are sporadic, there are a group of patients for whom cancer runs in the family. Lynch syndrome accounts for a large proportion of these inherited colon cancers and is also known to cause other tumours, for example of the uterus and stomach. In Lynch syndrome there are inherited mutations in genes which control the repair of damaged DNA; this puts carriers at an increased risk of developing cancers.. A number of centers in the UK and internationally have begun to screen for Lynch syndrome in those patients with colon cancer that are at high risk. This can be done using a test which is carried out in hospital pathology departments on tissue samples from tumours removed surgically. Patients with a positive test are then followed up in

	clinical genetics departments for further diagnostic tests. More recently, the screening process has been refined by testing for a mutation in a gene called BRAF. This gene is unrelated to Lynch syndrome and patients with this mutation almost never have Lynch syndrome, meaning they do not need follow up in the genetics clinic. This has helped streamline the screening process. However, the standard BRAF test requires specialist techniques, is time consuming and expensive. Recently, new simpler and faster BRAF tests have been developed. There are currently two different new tests commercially available and only one has been tested on colon cancer. This proposed study would compare the accuracy of both of these tests in colon cancer and evaluate the practical and financial feasibility of introducing this in to routine practice.
Aims and objectives *(Scientific background, plan of investigation, methodology and any pilot data)*	**Background:** Colorectal adenocarcinoma is the fourth most common malignancy in the UK and around 25% of cases occur in an inherited context. Lynch syndrome is the most common inherited cancer syndrome leading to colorectal carcinoma and is caused by loss-of-function germ line mutations in mismatch repair (MMR) genes. Currently patients with colorectal carcinoma at high risk for Lynch syndrome are screened by pathologists for MMR mutations using immunohistochemistry (IHC) on resection tissue. Those patients with loss-of-expression of the MLH1 or PMS2 type MMR proteins on IHC are then screened for BRAF mutations using PCR. The patients without BRAF mutations are at highest risk of Lynch syndrome and are referred to specialist clinical genetics follow up. Many centers do not have the funding or facilities to carry out PCR analysis (including Oxford) and so the potential for IHC detection of BRAF mutations needs investigation. There are currently two commercial antibodies, only one of which has been evaluated in colorectal carcinoma. **Aims:** The aim of this study is to validate the two commercially available antibodies in detecting BRAF V600E mutations by immunohistochemistry (IHC) against standard polymerase chain reaction (PCR) mutational analysis within the context of Lynch syndrome (LS) screening. Furthermore, the practical and financial feasibility of these tests will be investigated in order to inform practice locally. **Methodology:**

	Cases of colorectal carcinoma with MSI and known BRAF V600E mutation status (as determined by PCR) will be drawn from the diagnostic archive. In total, 20 cases will be selected – 10 with BRAF mutations on PCR and 10 without. Cases will be anonymised and two x 4 micron sections will be cut from each. The anti-BRAF antibodies (Roche and NewEast Bioscience) will be optimized for immunostaining on an Ultra Discovery Immunostainer (Roche-Ventanna) using sections of colorectal carcinoma known on the basis of PCR results to be either positive or negative for the BRAF V600E mutation. Immunostaining for BRAF V600E will be performed using the Ultra Discovery Immunostainer (Roche) and results will be assessed by two observers blind to the PCR results.
External Peer Review *(confirm if project has been subject to external peer review)*	Reviewed by the Oxfordshire Health Services Research Committee
NHS Pathologist *(advise if you have discussed this with an NHS pathologist – if applicable)*	Dr Elizabeth Soilleux (ES) and Dr Richard Colling (RC) are both NHS pathologists. Dr Lai Mun Wang (LMW) is also collaborating.
Section 5 Samples, service and data	
Sample requirements (*Sample numbers if known or description of samples required)*	We require 10 cases of BRAF V600E mutated colorectal carcinoma and 10 unmutated cases. 20 appropriate cases will be selected from the Cellular Pathology diagnostic archive by RC and ES. One block from each case will be selected for histology. There will be 20 blocks in total, 1 per case. A list will be provided to OCHRe once application is approved.
Full details of histology services *(e.g. number of sections required, staining, processing)*	OCHRe to check consent status of cases requested. OCHRe to provide 10 unstained FFPE sections of a colorectal carcinoma case with known BRAF V600E mutation for optimization. Subsequently, we will require 40 unstained FFPE sections (i.e. two sections of each of the 20 blocks) on coated slides suitable for immunostaining. Therefore 50 unstained sections are required in total. Immunostaining will be performed by RC and ES once the Ventana Discovery Ultra Immunostainer arrives (17.11.14).
How will material be used? *(How will you use the materials requested in this application?)*	To validate two anti-BRAF V600E mutation antibodies for subsequent clinical diagnostic use.
Data requirements *(Specify any accompanying data you require e.g. copy of pathology reports)*	Cases will be anonymous. BRAF V600E mutation status by PCR will be requested on each case.

Thank you for completing this OCHRe Part 2 application form. Please remember to provide electronic or paper copies of ethics and R&D approval documents when you submit it to OCHRe.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MD
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Notes
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Teaching
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Richard
Re: Application
Oxford Centre for Histopathology Research
11/20/14
Oxford Centre for Histopat
Add to circles
Dear Richard
This application has been approved by the committee the application number is 14/A209. Please send me the list of cases so that I can make a quote and send it back to you
Let me know if you need more information
Thanks
Best
DJ
--
Divija Jata
Data and Research Coordinator
Oxford Centre For Histopathology Research
FAX
On 02/11/2014 11:44 "Richard Colling"
wrote
>Dear Sir/Madam
>
>Please find attached an application form for consideration at your next
>meeting
>
>Thank you
>Richard Colling
>ACF NDCLS

Oxford Centre for Histopathology Research application (15/A041)

Section 1 Applicant details		
Research group / department		Cellular Pathology & NDCLS
Lead applicant *(e.g. Head of Department or Group, or clinical trial PI)*	Name	Dr Elizabeth Soilleux
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	Email	elizabeth.soilleux@ndcls.ox.ac.uk
Contact person *(if different from above) (i.e. the person who will coordinate the request(s) with OCHRe)*	Name	Dr Richard Colling
	Job Title	Academic Clinical Fellow
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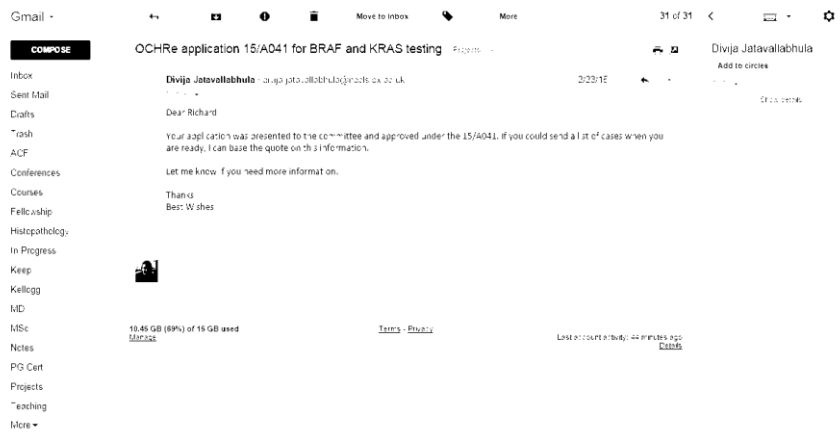
	Tel No.	01865 220581
	Email	rtcolling@gmail.com
Shipping details *(if different from above)*	Name	Dr Richard Colling
	Address	
	Tel No.	
Section 2 Funding details		
Research funder *(e.g. commercial company, NHS or University)*		Oxfordshire Health Services Research Committee
Contact person for quotation / funding questions		Dr Richard Colling
Section 3 Approval details		
Ethical approval details *(if applicable)* *A copy of the approval letter should be submitted with this application*	Reference No.	04IQ1604I21
	Title	Expression pattern of C-type lectins in Health and Disease
	Approval date	04.06.2004
	Expiry date	04.06.2019
R&D approval details *A copy of the approval letter should be submitted with this*	Approval body	Oxford University
	Reference no. (if applicable)	04IQ1604I21
	Other information	Confirmatory paperwork/ e-mails available from Dr Elizabeth Soilleux

applicatio n		
Sponsor	Organisation	University of Oxford
Section 4 Project details		
Research project title		Automated PCR testing of BRAF and KRAS mutation in colorectal carcinoma
Lay summary *(this may be made available on the OCHRe website, please advise if confidential)*		Colon cancer is the fourth most common cancer in the UK and a leading cause of cancer death. It is not routine practice to test for the presence of BRAF and KRAS mutations in some patients with these tumours. BRAF mutations have important prognostic value and targeted therapies against this mutation are in development. In addition, BRAF mutation testing is recommended in the screening of Lynch Syndrome in these patients. KRAS mutations hold important prognostic value and are also used now clinically to guide therapy. The standard BRAF and KRAS mutation tests require specialist techniques and for most small district general hospitals this poses a problem. The tests are time consuming and expensive. Recently, a new simple, fast and cheap automated machine has become available for use in BRAF and KRAS testing. The machine offers a potential benefit for those smaller and remote sites where standard testing methods are difficult. This proposed study would compare the accuracy of this new testing method in colon cancer and evaluate the practical and financial feasibility of introducing this in to routine practice in smaller hospitals.
Aims and objectives *(Scientific background, plan of investigation, methodology and any pilot data)*		**Background:** Colorectal adenocarcinoma is the fourth most common malignancy in the UK and around 25% of cases occur in an inherited context. It has become routine practice to investigate these tumours for BRAF and KRAS mutations. BRAF mutations give prognostic information and while anti-BRAF therapy is not currently used in colon cancer, this may be a possibility in the future. BRAF mutational analysis is also used as routine in the screening for Lynch Syndrome in colorectal cancer patients. KRAS mutations also impart prognostic information and are used to guide the therapy given in current oncology practice. Current practice is to carry out mutational analysis using real-time polymerase chain reaction (PCR). Many small, district general centres do not have the facilities to carry out traditional PCR analysis and so the potential for other options for detection of BRAF mutations needs investigation. One such solution for these centres is an automated PCR approach. **Aims:** The aim of this study is to validate the Idylla automated PCR in detecting BRAF mutations against standard PCR analysis, within the context of Lynch syndrome (LS) screening. Furthermore, the practical and financial feasibility of these tests will be investigated in order to inform practice locally. **Methodology:** Cases of colorectal carcinoma with known BRAF and KRAS mutation status (as determined by Cobas z480 PCR) will be drawn from the diagnostic archive. In total, around 100 cases will be selected for BRAF and KRAS; 50 BRAF/KRAS + mutations on PCR and 50 BRAF/KRAS -. In many instances, these will be the same cases for both

	BRAF and KRAS. Cases will be anonymised and unstained sections will be cut from each. We will use scrolls cut at 10 microns where possible. Otherwise we will request 4 micron sections on glass slides for macrodissection. Automated PCR will be performed using the Idylla platform (BioCartis) BRAF and KRAS cartridges. This platform is being provided on loan with all the reagents for free by BioCartis. This will be placed in Cellular Pathology on Level 1 (permission already granted by Sharon Roberts-Gant). A loan agreement will be in place via Clinical Engineering, who will carry out a system electrical check. A health and safety assessment will be carried out by RC and ES.
External Peer Review *(confirm if project has been subject to external peer review)*	Reviewed by the Oxfordshire Health Services Research Committee
NHS Pathologist *(advise if you have discussed this with an NHS pathologist – if applicable)*	Dr Elizabeth Soilleux (ES) and Dr Richard Colling (RC) are both NHS pathologists. Dr Lai Mun Wang (LMW) is also collaborating.
Section 5 Samples, service and data	
Sample requirements (*Sample numbers if known or description of samples required)*	Cases will be selected from the Cellular Pathology diagnostic archive by RC and ES. One block from each case will be selected for PCR. There will be 50 cases in total. A list will be provided to OCHRe once application is approved.
Full details of histology services *(e.g. number of sections required, staining, processing)*	OCHRe to check consent status of cases requested. OCHRe to provide an unstained FFPE sections from each block (one or two depending on if the case can be used for both KRAS and BRAF testing. As this is for PCR the usual molecular precautions will be needed (cleaning of the blade/water bath between blocks as appropriate etc.) Where possible we will request 10 micron scrolls. Where macrodissection is needed we will request sections on uncoated slides. The automated PCR will be performed by RC on the Idylla System within the Cellular Pathology laboratory on Level 1.
How will material be used? *(How will you use the materials requested in this application?)*	To validate automated Idylla BRAF/KRAS PCR.
Data requirements *(Specify any accompanying data you require e.g. copy of pathology reports)*	Cases will be anonymous. BRAF and KRAS mutation status by Cobas z480 PCR (already performed) will be requested on each case. We would like to use the same anonymised system OCHRe have used for our previous BRAF IHC application so we can match cases and compare while not breaking the anonymisation process if this is possible.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Projects
Teaching
More
OCHRe application 15/A041 for BRAF and KRAS testing
Divija Jatavallabhula
Add to circles
Divija Jatavallabhula
2/23/15
Dear Richard
Your application was presented to the committee and approved under the 15/A041. If you could send a list of cases when you are ready, I can base the quote on this information.
Let me know if you need more information.
Thanks
Best Wishes
10.46 GB (69%) of 15 GB used
Manage
Terms - Privacy
Last account activity: 44 minutes ago
Details

Oxford Centre for Histopathology Research application (16/A002)

Section 1 Applicant details		
Research group / department		Cellular Pathology & NDCLS
Lead applicant *(e.g. Head of Department or Group, or clinical trial PI)*	Name	Dr Elizabeth Soilleux
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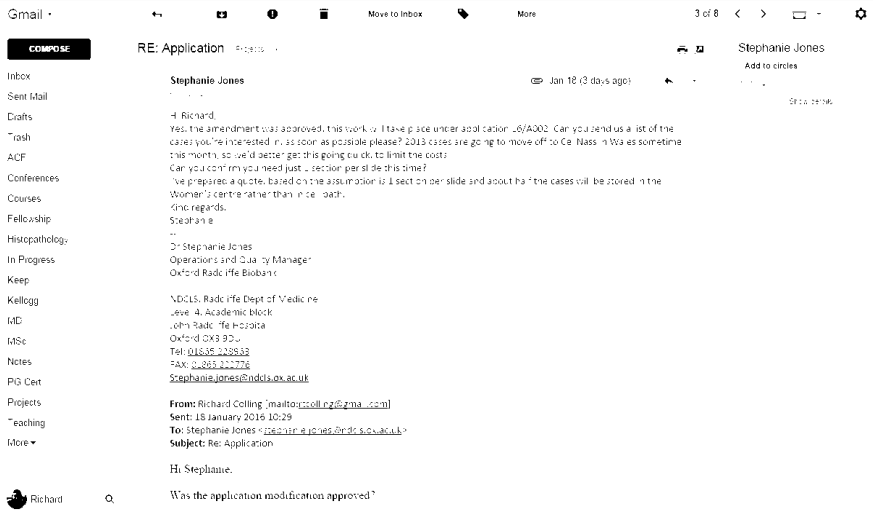
	Address	Dept of Cellular Pathology, OUH Trust
	Tel No.	01865 220535
	Email	elizabeth.soilleux@ndcls.ox.ac.uk
Contact person *(if different from above) (i.e. the person who will coordinate the request(s) with OCHRe)*	Name	Dr Richard Colling
	Job Title	Academic Clinical Fellow
	Address	Dept of Cellular Pathology, OUH Trust
	Tel No.	01865 220581
	Email	rtcolling@gmail.com
Shipping details *(if different from above)*	Name	Dr Richard Colling
	Address	
	Tel No.	
Section 2 Funding details		
Research funder (*e.g. commercial company, NHS or University)*		Oxfordshire Health Services Research Committee
Contact person for quotation / funding questions		Dr Richard Colling
Section 3 Approval details		
Ethical approval details *(if applicable) A copy of the approval letter should be submitted with this application*	Reference No.	04lQ1604l21
	Title	Expression pattern of C-type lectins in Health and Disease
	Approval date	04.06.2004
	Expiry date	04.06.2019
R&D approval details *A copy of the approval letter should be submitted with this application*	Approval body	Oxford University
	Reference no. (if applicable)	04lQ1604l21
	Other information	Confirmatory paperwork/ e-mails available from Dr Elizabeth Soilleux
Sponsor	Organisation	University of Oxford
Section 4 Project details		
Research project title		Automated PCR testing of KRAS, NRAS and EGFR mutations in colorectal carcinoma
Lay summary *(this may be made available on the OCHRe website, please advise if confidential)*		This is an amendment of the previous application (15/A041) for a small extension of the project to include NRAS and EGFR mutation testing. Previous work in this study has validated the Idylla System for the detection of BRAF and KRAS mutations. Lay Summary: Colon cancer is the fourth most common cancer in the UK and a leading cause of cancer death. It is routine practice to test for the presence of NRAS/EGFR mutations in some patients with these tumours. NRAS/EGFR mutations hold important prognostic value and are also used now clinically to guide therapy.

	The standard NRAS/EGFR mutation tests require specialist techniques and for most small district general hospitals this poses a problem. The tests are time consuming and expensive. Recently, a new simple, fast and cheap automated machine has become available for use in NRAS/EGFR testing. The machine offers a potential benefit for those smaller and remote sites where standard testing methods are difficult. This proposed study would compare the accuracy of this new testing method in colon cancer and evaluate the practical and financial feasibility of introducing this in to routine practice in smaller hospitals.
Aims and objectives *(Scientific background, plan of investigation, methodology and any pilot data)*	**Background:** Colorectal adenocarcinoma (CRC) is the fourth most common malignancy in the UK. It has become routine practice locally and increasingly, nationally, to investigate these tumours for NRAS/EGFR mutations as these impart prognostic information and are used to guide the therapy given in current oncology practice. Until recently KRAS mutation status was sufficient to predict response with anti-EGFR therapy in these patients, however it is increasingly recognised that mutations in NRAS and EGFR contribute to anti-EGFR therapy resistance; therefore testing of KRAS with NRAS and EGFR is becoming routine. Current practice is to carry out mutational analysis using NGS gene panels for KRAS/NRAS/EGFR. Many small, district general centres do not have the facilities to carry out NGS analyses and so the potential for other options for detection of mutations needs investigation. One such solution for these centres is an automated PCR approach. The previous work in this study validated a new platform called 'Idylla' which provides automated and simple standalone PCR which can be run in any lab. We validated this for BRAF mutations in CRC paitents (as part of the Lynch syndrome screening pathway) and KRAS mutations (for reasons mentioned above). The system has now launched a new assay which tests KRAS/NRAS/EGFR in combination. There is no evidence or validation data published for this system as yet. **Aims:** This is an amendment to the previous application for an additional small validation study for the Idylla KRAS/NRAS/EGFR test. **Methodology:** Cases of colorectal carcinoma with known KRAS/NRAS/EGFR mutation status (as determined by Ion torrent NGS cancer panel) will be drawn from the diagnostic archive. In total, 30 cases will be selected (estimate). Cases will be anonymised and unstained sections will be cut from each. Automated PCR will be performed using the Idylla platform (BioCartis) KRAS/NRAS/EGFR cartridges.

	This platform is being provided on loan with all the reagents for free by BioCartis. This is already placed in Cellular Pathology on Level 1 (permission already granted by Sharon Roberts-Gant). A loan agreement will be in place. Clinical Engineering, have already carried out a system electrical check. A health and safety assessment has been carried out by RC.
External Peer Review *(confirm if project has been subject to external peer review)*	Reviewed by the Oxfordshire Health Services Research Committee
NHS Pathologist *(advise if you have discussed this with an NHS pathologist – if applicable)*	Dr Elizabeth Soilleux (ES) and Dr Richard Colling (RC) are both NHS pathologists. Dr Lai Mun Wang (LMW) is also collaborating (NHS Consultant Histopathologist at OUH).
Section 5 Samples, service and data	
Sample requirements (*Sample numbers if known or description of samples required)*	Cases will be selected from the Cellular Pathology diagnostic archive by RC. One block from each case will be selected for PCR. There will be 30 cases in total (estimate). A list will be provided to OCHRe once application is approved. Extra sections will be provided for discordant results if needed.
Full details of histology services *(e.g. number of sections required, staining, processing)*	OCHRe to check consent status of cases requested. OCHRe to provide a single 5 micron unstained FFPE sections from each block on uncoated glass slides for macrodissection. We will need sections cut with PCR protocol (cleaning blade and water bath between cases). The automated PCR will be performed by RC on the Idylla System within the Cellular Pathology laboratory on Level 1.
How will material be used? *(How will you use the materials requested in this application?)*	To validate automated Idylla KRAS/NRAS/EGFR PCR.
Data requirements *(Specify any accompanying data you require e.g. copy of pathology reports)*	Cases will be anonymous. We would like to use the same anonymised system OCHRe have used for our previous applications (15/A041) i.e. case number only on slide.

Thank you for completing this OCHRe Part 2 application form. Please remember to provide electronic or paper copies of ethics and R&D approval documents when you submit it to OCHRe.

NDCLS, Level 4, Academic Block, University of Oxford, John Radcliffe Hospital Headington, Oxford, OX3 9DU Email: ochre@ndcls.ox.ac.uk Tel: 01865 220557 Fax: 01865 222776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Move to Inbox
More
3 of 8
COMPOSE
Inbox
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Trash
ACF
Conferences
Courses
Fellowship
Histopathology
In Progress
Keep
Kellogg
MD
MSc
Notes
PG Cert
Projects
Teaching
More
Richard
RE: Application
Stephanie Jones
Add to circles
Stephanie Jones
Jan 18 (3 days ago)
Hi Richard,
Yes, the amendment was approved, this work will take place under application 16/A002. Can you send us a list of the cases you're interested in as soon as possible please? 2013 cases are going to move off to Cardiff in Wales sometime this month, so we'd better get this going quick, to limit the costs.
Can you confirm you need just 1 section per slide this time?
I've prepared a quote, based on the assumption is 1 section per slide and about half the cases will be stored in the Women's centre rather than in Headington.
Kind regards,
Stephanie
--
Dr Stephanie Jones
Operations and Quality Manager
Oxford Radcliffe Biobank
NDCLS, Radcliffe Dept of Medicine
Level 4, Academic block
John Radcliffe Hospital
Oxford OX3 9DU
Tel: 01865 222532
FAX: 01865 222776
Stephanie.jones@ndcls.ox.ac.uk
From: Richard Colling [mailto:rcolling@gmail.com]
Sent: 18 January 2016 10:29
To: Stephanie Jones <stephanie.jones@ndcls.ox.ac.uk>
Subject: Re: Application
Hi Stephanie,
Was the application modification approved?
Thanks,

APPENDIX 3 – Risk Assessment (COSHH)

CELLULAR PATHOLOGY CSU
Oxford University Hospitals

COSHH ASSESSMENT FORM

Risk Category

archive ○ Yes

Section	CSU			Room No	EM
Personnel	Richard Colling/Idylla user				
No. of Staff exposed per activity	1	Length of Time	10min	Frequency of exposure	Daily
Original Assessor	Richard Colling			Current Assessor	
Procedure	Use of Idylla automated PCR machine (on loan,			SOP Ref No	N/A
Original Date Assessed	14/04/2015	Date Re-assessed		Next Assessment Date	

Name of Chemical	Risk Ass. Cat.	Required Protection No.
Liquid buffers		1/4b
Solubilizing agent		1/4b
Preserving agent		1/4b
Primer		1/4b
Probe		1/4b
Nucleotides		1/4b
Enzyme mix		1/4b

Name of Organism	Risk Ass. Cat.	Required Protection No.

Protection

1. Open Bench (GLP)
2. Safety Cabinet Class 1
3. Fume Cupboard / ventilated bench
4. Gloves
   ○ a) Vinyl ○ c) Nitrile
   ◉ b) Latex ○ d) Cryo
5. Face / eye protection
6. Respiratory protection
7. Other protection (specify)

Reference used MDS provided by Biocartis (RTC has a copy of this).

Storage of reagents Reagents are received pre-prepared and remain within sealed cartridges throughout the protocol. Store at room temp.

Disposal procedures	Cartridges are discarded, unopened as routine clinical waste.
Accident procedures	None should be required as risk is close to zero however standard precautions include: **EYES**-All-irrigate with water 15mins. **SKIN**- All- wash with soap and water - then obtain medical advice. **INGESTION**- All- wash mouth , drink water, do not induce vomiting - then obtain medical advice. **INHALATION**- All-remove from exposure - then obtain medical advice.
Additional hazards eg electrical, explosive	None

	Name	Signature	Date
Current Assessor	R Colling		14/4/2015

Approval	Name	Signature	Date
Supervisor/ Safety Officer/ Deputy Safety Officer			

APPENDIX 4 – Statistical Formulae

In this appendix below the formulae used in this work are given. These are well established methods documented elsewhere. [235,250,251,288]

Sample Size Estimations:

The nomogram and instructions can be found at:

URL:http://emj.bmj.com/content/22/3/180/suppl/DC1?ck=nck [Accessed 21/04/2016]

The sample size (*n*) is estimated by:

$$n = \frac{\mathrm{DP}}{\mathrm{P}}$$

Where P is the prevalence of disease, DP (disease positive) is calculated as:

$$\mathrm{DP} = z^2 \frac{Sn\ (1 - \ Sn)}{\mathrm{W}^2}$$

Where *Sn* is the predicted sensitivity, W is the desired confidence interval (0.05 for 95%) and *z* (representing $z_{1-\alpha/2}$) is taken from the standard Normal distribution (1.96 at the 95% CI).

Statistical Analysis (accuracy):

Prevalence (P):

$$P = \frac{Disease\ positive}{Population}$$

Accuracy data are presented in 2 x 2 tables:

	Disease present	**Disease absent**
Test Positive	True Positive test result (TP)	False Positive test result (FP)
Test Negative	False Negative test result (FN)	True Negative test results (TN)

Concordance (*C*) / accuracy:

$$C = \frac{(TP + TN)}{(TP + FP + TN + FN)}$$

Sensitivity (*Sn*):

$$Sn = \frac{TP}{(TP + FN)}$$

Specificity (*Sp*)

$$Sp = \frac{TN}{(FP + TN)}$$

Positive predictive value (*PPV*):

$$PPV = \frac{TP}{(TP + FP)}$$

Negative predictive value (*NPV*):

$$NPV = \frac{TN}{(TN + FN)}$$

Likelihood ratio positive (*LH*+) and negative (*LH*-):

$$LH+ = \frac{Sn}{(1 - Sp)}$$

and

$$LH- = \frac{(1 - Sn)}{Sp}$$

Statistical Analysis (reproducibility):

Reproducibility data are presented in 2 x 2 tables:

	Reviewer 2 positive	**Reviewer 2 negative**
Reviewer 1 positive	Positive agreement (PA)	Positive/negative disagreement (PND)
Reviewer 2 negative	Negative/positive disagreement (NPD)	Negative agreement (NA)

Firstly, the proportion of cases in which the observers agree (Ag):

$$Ag = \frac{A + D}{Total\ cases}$$

Probability (R) of the observer (O_1 or O_2) having produced the results randomly (i.e. by guessing), for each observer:

$$R_{O1} = \frac{A+B}{Total\ cases}$$

$$R_{O2} = \frac{A+C}{Total\ cases}$$

Probability of both randomly agreeing the result is positive (R_+) and both randomly agreeing the result is negative (R_-):

$R_+ = \quad RO1 \quad \times \quad RO2$

$R_- = \quad (1 - RO1) \quad \times \quad (1 - RO2)$

The overall probability of agreeing (R_a):

$R_a = \quad R_+ \quad + \quad R_-$

Cohen's kappa (k) statistic:

$$k = \frac{Ag - Ra}{1 - Ra}$$

0.0 → agreement only by chance
0.0 – 0.2 → poor agreement
0.2 – 0.4 → fair agreement
0.4 – 0.6 → moderate agreement
0.6 – 0.8 → good agreement
0.8 – 1.0 → very good agreement

Confidence intervals (CI):

Wilson's method:

$$CI = \quad \frac{(A - B)}{C} \quad to \quad \frac{A + B}{C}$$

Where

$$A = 2r + z^2$$
$$B = z\sqrt{z^2 + 4rq}$$
$$C = 2(n + z^2)$$

And

$$q = 1 - p$$
$$p = \frac{r}{n}$$

Given that *n* is the sample size and *r* is the proportion in question.

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