Markers predictive of response, resistance and relapse in colorectal cancer

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AUTHOR'S DECLARATION

I can confirm that the work presented in this thesis is my own.

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PUBLICATIONS AND AWARDS DURING MD(Res) DEGREE

Relevant peer-reviewed publications

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3. Battersby NJ, Dattani M, Rao S, Cunningham D, Tait D, Adams R, Moran B.J, **Khakoo S**, Tekkis P, Rasheed S, Mirnezami A, Quirke P, West NP Nagtegaal I, Chong I, Sadanandam A, Valeri N, Thomas K, Frost M, Brown G. A rectal cancer feasibility study with an embedded phase III trial design assessing magnetic resonance tumour regression grade (mrTRG) as a novel biomarker to stratify management by good and poor response to chemoradiotherapy (TRIGGER): Study protocol for a randomised controlled trial. Trials 2017;18:394.

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ABSTRACT

Response to neo-adjuvant chemo-radiotherapy (CRT) in locally advanced rectal cancer varies. Circulating tumour DNA (ctDNA) has emerged as a surrogate marker of the tumour genome. I hypothesised that ctDNA could be an early indicator of response to enable therapy adaptation. Therefore tumour tissue and blood samples were collected pre, mid, post CRT and post-surgery using a single centre translational research protocol. A tumour informed approach was used to design up to 3 droplet digital polymerase chain reaction (ddPCR) assays per patient. ctDNA status after CRT was associated with MRI tumour regression grade response. ctDNA persistence or detection post CRT was an indicator of poor prognosis.

ctDNA detection following curative surgery could predict relapse or indicate minimal residual disease. As pre-analytical factors can affect ctDNA detection rates, I wrote a translational protocol to assess the feasibility of a tumour-informed, multi-centre approach for ctDNA analysis from 48 CRC patients without distant metastases at diagnosis. Using ddPCR, ctDNA was detectable in 67% (n=32/48) pre-surgery and 19% (n=9/48) post-surgery. Pre-surgery ctDNA detection rates increased with stage. All 5 patients that relapsed had detectable ctDNA post-surgery and at the time of confirmed relapse. Of the patients with no evidence of relapse, 39 (91%) had undetectable ctDNA post-surgery.

ctDNA identified patients at risk of developing metastases during CRT or after surgery and could be used to tailor treatment from as early as mid CRT or guide adjuvant chemotherapy decisions post-surgery in an attempt to prevent the development of metastases.

Development of metastases represents a major cause of mortality. Comparing tumour tissue from primary CRC and corresponding lung metastases may be informative to understand the metastatic process and identify novel therapeutic targets. In matched samples from 13 patients;

analysis of the transcriptome showed that only 944 out of 19,374 genes analysed (4.9%) were differentially expressed between primary CRC and respective lung metastases. Genes that were upregulated in metastases compared to primary tumours were most likely to be involved in the immune response pathway. Consensus molecular subtype 4 in lung metastases emerged as a poor prognostic marker. Findings require confirmation in a larger cohort.

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LIST OF KEY ABBREVIATIONS

CEA: Carcino-embryonic antigen

cCR: Complete clinical response

cfDNA: Cell free DNA

CMS: consensus molecular subtype

CR: Complete response

CRC: Colorectal cancer

CRCSC: Colorectal cancer subtyping consortium

CRM: Circumferential resection margin

CRT: Chemo-radiotherapy

CT: Computed tomography

ctDNA: Circulating tumour DNA

DFS: Disease free survival

EGFR: Epidermal growth factor receptor

EMT: Epithelial mesenchymal transition

EMVI: Extramural venous invasion

FA: Fractional abundance

FDR: false discovery rate

FFPE: Formalin fixed paraffin embedded

HR: Hazard ratio

IQR: Interquartile range

LARC: Locally advanced rectal cancer

LRFS: local recurrence free survival

MFS: Metastases free survival

MRD: Minimal residual disease

MRI: Magnetic resonance imaging

mrTRG: MRI tumour regression grade

NGS: Next generation sequencing

OS: Overall survival

pCR: Complete pathological response

PD: Progressive disease

PR: Partial response

RECIST: Response evaluation criteria in solid tumours

RFS: Recurrence free survival

ROC: Receiver operator characteristic

SD: Stable disease

TME: Total mesorectal excision

TNT: Total neoadjuvant approach

TRG: Tumour regression grade

UK: United Kingdom

VAF: Variant allele frequency

1. INTRODUCTION

1.1 Epidemiology

Colorectal cancer (CRC) remains a significant public health issue globally. In 2018, it was the 3rd most common malignancy and 2nd leading cause of cancer-related mortality with over 1.8 million new cases and approximately 881,000 deaths worldwide.[1] Similarly, in the United Kingdom (UK), with an average of 41, 042 new diagnoses between 2014 and 2016, it is the 4th most common cancer and 2nd most common cause of cancer related deaths with 16, 384 deaths being attributable to the disease in 2016.[2] CRC is associated with age. Between 2014-2016, on average, over 40% of new cases were diagnosed in patients over the age of 75 each year.[2]

Rectal cancer accounts for around 30% of patients with CRC. There is striking variation in the anatomical distribution by both age and sex with elderly women being more likely to have a proximal tumour and younger men being more likely to have a rectal tumour (***Figure 1.1***).[3]

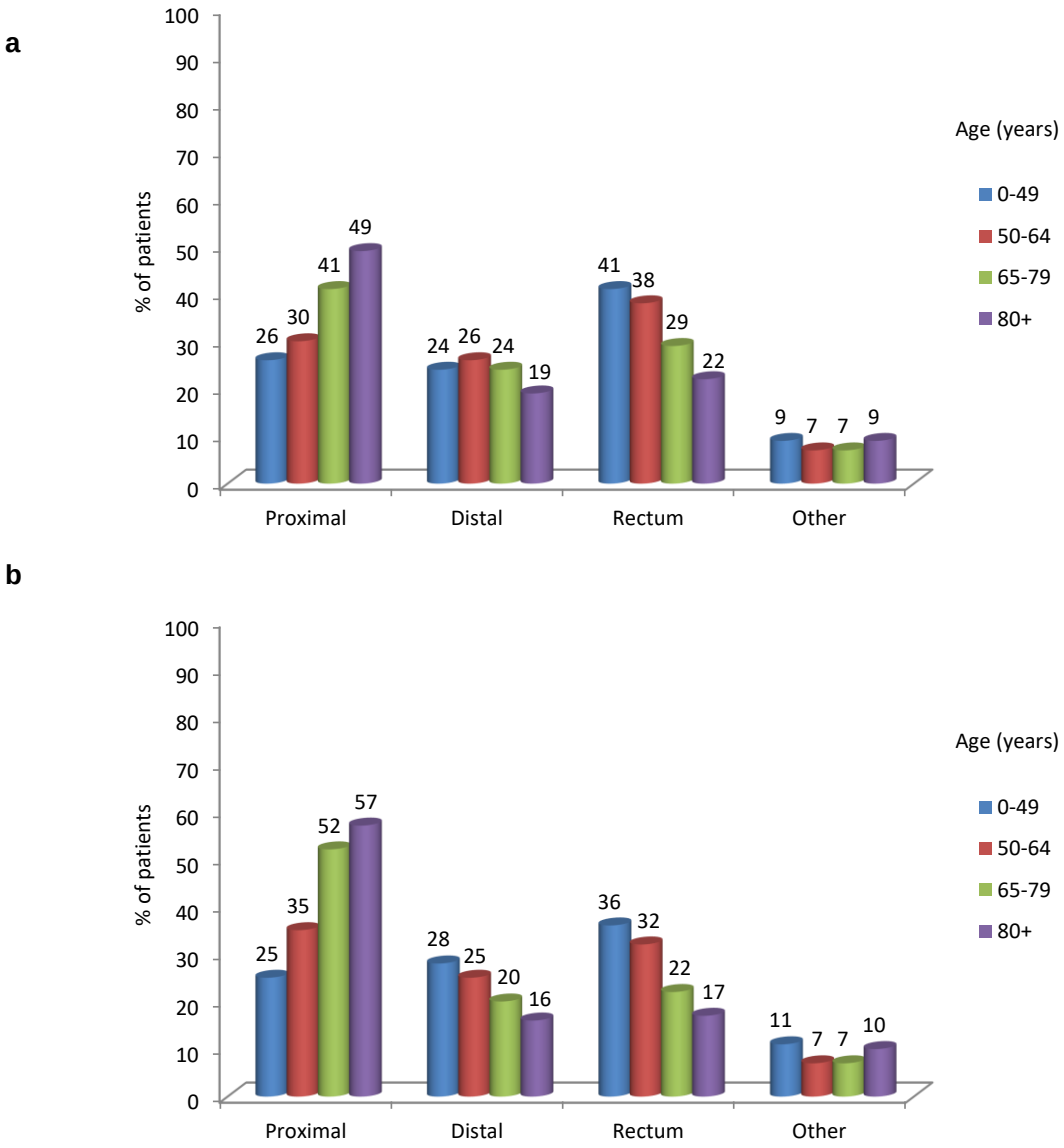


Figure 1.1 Distribution of colorectal cancer location by age in (a) men and (b) women

(modified from Siegel et al, CA Cancer J Clin 2017)

In 2014, in England, 15% of patients were diagnosed with stage I disease, 23% stage II disease, 26% stage III disease, 22% stage IV disease and 13% had an unknown stage.[2] Prognosis is strongly related to stage at diagnosis and 5-year relative survival has shown steady improvement over the years for both colon and rectal cancer (***Figure 1.2***).

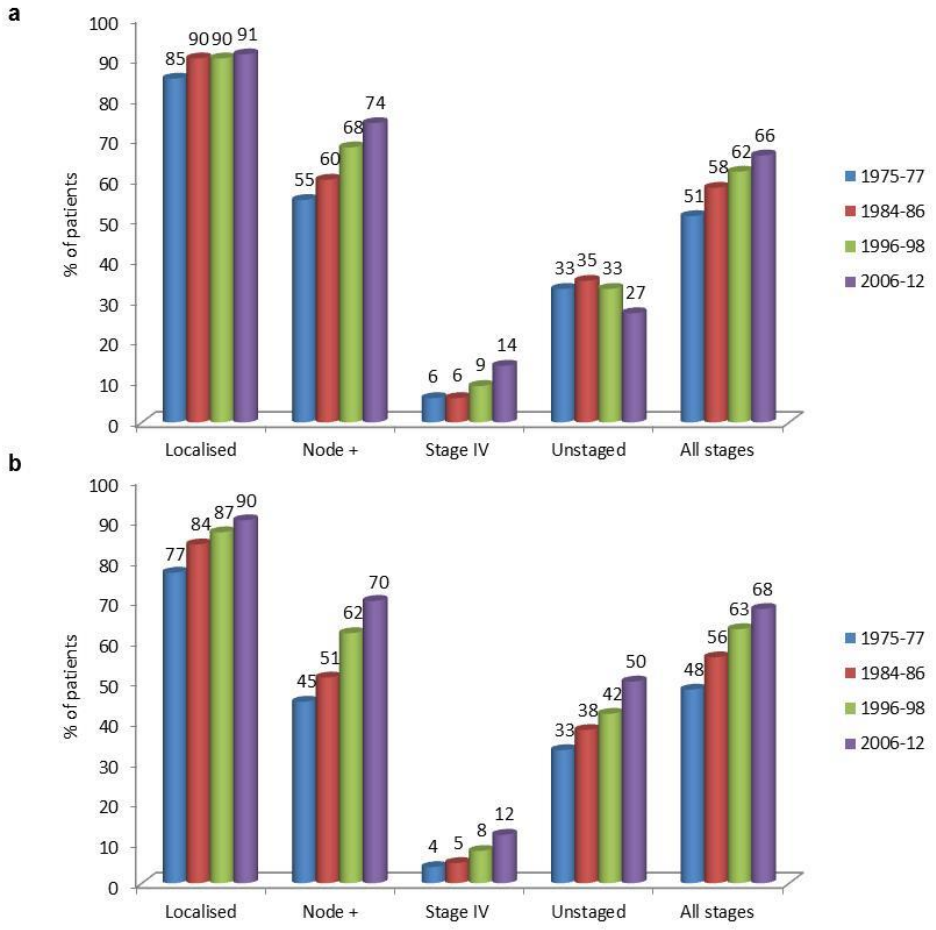


Figure 1.2 Trends in 5-year relative survival rate by stage in the United States from 1975 to 2012 in (a) colon and (b) rectal cancer

(modified from Siegel et al, CA Cancer J Clin 2017, legend represents year of diagnosis)

Improvement in survival is likely to reflect the widespread adoption of screening and surveillance programmes as well as advances in surgical and imaging techniques.

1.2 Treatment of stage I-III CRC

CRC patients with no evidence of distant metastases (stage I-III disease) are treated with surgery. Surgery alone will cure approximately 90%, 80% and 65% of patients with stage I, II and III disease respectively.[4] A 3-6 month course of adjuvant chemotherapy is typically offered to patients with high risk stage II disease and all patients with stage III disease in order to minimise the risk of relapse. Stage II disease considered to be at significant risk of relapse includes patients with: T4 tumours, perforated tumour or bowel obstruction at the time of presentation, less than 12 lymph nodes removed during surgery, poorly differentiated histology and the presence of extramural venous invasion (EMVI). In stage II patients, regardless of risk, the absolute survival benefit from adjuvant chemotherapy is small at 3.6%.[5] In patients with stage III (node positive) disease, the absolute improvement in survival due to adjuvant chemotherapy after surgery is approximately 14% with 7-10% of the benefit being derived from fluorouracil or its prodrug (capecitabine) and the remainder (4%) being attributable to oxaliplatin but at the expense of increased side effects.[6] It is recognised that a proportion of patients that are currently offered adjuvant chemotherapy based on conventional risk factors, have already been cured by surgery alone. Therefore a better marker to differentiate the patients that are most likely to benefit from adjuvant chemotherapy compared to those that can be spared from its associated toxicity is needed. Currently, absence of mismatch repair deficiency and more recently, lack of CDX2 expression in colon cancer patients are the only known biomarkers that predict for a higher likelihood of benefit from adjuvant chemotherapy in stage II patients.[7]

Despite surgery and adjuvant chemotherapy, around 15-20% of patients with stage II and 30% of patients with stage III disease relapse.[4, 8] Early identification of patients that will inevitably relapse may allow treatment intensification or alternative treatment strategies as an attempt to improve outcome. However, the benefit of such an approach has yet to be proven.

Following adjuvant chemotherapy, patients undergo a period of surveillance with computed tomography (CT) scans and serial tumour marker measurements of carcino-embryonic antigen (CEA) to detect relapse. Current surveillance strategies have limitations as CEA can be elevated with benign conditions and it is less sensitive for lung metastases, peritoneal or local recurrences and not all patients are CEA-secretors.[9, 10] CT scans cannot detect micro-metastatic disease and the frequency of imaging has to be rationalised due to concerns over radiation exposure. Early detection of relapse may still allow curative resection in the presence of limited disease. Therefore better markers to reliably predict relapse are needed.

The role of adjuvant chemotherapy in rectal cancer is less clear. Clinicians administering adjuvant chemotherapy have largely extrapolated evidence from colon cancer studies. Although the QUASAR study included rectal cancer patients and demonstrated a survival benefit in patients with stage II CRC receiving adjuvant chemotherapy with fluorouracil, only 6% of patients received pre-operative radiotherapy.[5] More recently, a large meta-analysis with a median follow up of 7 years, included 4 phase III, randomised

controlled studies (n=1196) and compared fluorouracil based chemotherapy to observation after neoadjuvant CRT and surgery in patients with stage (y)p stage II and III rectal cancer. They found no improvement in distant relapse rate (HR: 0.94, 95% CI: 0.78-1.14, *P*=0.52), DFS (HR: 0.91, 95% CI: 0.77-1.07, *P*=0.23) or OS (HR: 0.97, 95% CI: 0.81-1.17, *P*=0.78) with adjuvant chemotherapy.[11] However in sub-group analyses, patients with a tumour 10-15cm from the anal verge appeared to benefit from adjuvant chemotherapy with an improvement in DFS and fewer distant recurrences. The results of this meta-analysis are limited by the fact that: 2 of the included studies closed to recruitment early and are therefore underpowered,[12, 13] only 43-74% of patients completed adjuvant chemotherapy across the studies and patients that were good responders to CRT as evidenced by (y)pTNM stage 0-1 were not included. Interestingly, another meta-analysis which addressed the same question found that adjuvant chemotherapy improves 5-year OS (OR=0.64, 95% CI: 0.46-0.88, *P*=0.006) and 5-year DFS (OR=0.71, 95% CI: 0.6-0.83, P<0.0001) and improvement in 5-year OS is more pronounced in downstaged tumours.[14] This is perhaps unsurprising as patients showing evidence of a good response to CRT may be more likely to be chemosensitive. In support of this, a recent meta-analysis concluded that adjuvant chemotherapy significantly improved OS in patients with rectal cancer achieving a pCR after neoadjuvant treatment and surgery (HR=0.5, 95% CI: 0.43-0.59, *P*<0.001).[15]

1.3 The Evolution of Rectal Cancer Management

1.3.1 Optimising surgery

One of the most significant breakthroughs for improving outcomes in the management of rectal cancer was the incorporation of total mesorectal excision (TME) surgery which was first described by Heald in 1979.[16] Its implementation led to a decrease in local recurrence rates from approximately 24% with conventional surgical techniques[17] to 3-8% following TME surgery.[17–20] Similarly, survival also improved from 40-50% for 5 year disease free survival in the pre-TME era[17] to 70-85% with the addition of TME surgery.[17–20]

The addition of radiotherapy to TME surgery resulted in further improvement in local control with a 10 year local relapse rate of 5% in those receiving short course radiotherapy prior to TME surgery compared to 11% in those receiving TME surgery alone (p<0.0001).[21] However, there was no statistically significant difference in 10 year distant relapse rates or 10 year overall survival rates between the two treatment arms.

1.3.2 Establishing the current standard of care in LARC

In 2004, the randomised, phase III German CAO/ARO/AIO-94 study established neo-adjuvant chemo-radiotherapy as standard of care for patients with clinical T3-4 or node positive tumours due to improved local control rates if CRT was given before TME surgery compared to after surgery (5 year local relapse rate of 6% vs. 13%, p=0.006).[22] Importantly, the study also showed that toxicity was significantly less if CRT was administered before surgery (grade 3 or 4 acute toxic effects 27% vs. 40%,

p=0.001 and long term toxic effects 14% vs. 24%, *p*=0.01). Updated results from this study showed no statistically significant difference in 10 year distant metastasis rates or disease free survival rates but showed that a significant improvement in local control rate persisted in favour of the pre-operative CRT arm (10 year local relapse rate 7.1% vs. 10.1%, *p*=0.048).[23] The benefit of adding concomitant neo-adjuvant chemotherapy to radiotherapy to minimise local recurrence was established by the Federation Francophone Cancerologie Digestive (FFCD) 9203 trial[24] and European Organisation for Research and Treatment of Cancer (EORTC) 22921 trials.[25, 26] In both trials, pre-operative CRT was also associated with a significantly higher complete pathological response (pCR) rate than pre-operative long-course radiotherapy (***Table 1.1***).[24, 27]

Table 1.1 Key studies involved in establishing the standard of care treatment for cT3-4 or node positive rectal cancer

Study name	Comparator arms	N	Treatment	Key Eligibility	Primary Endpoint	pCR (%)	5-year Local Recurrence (%)	5-year DFS (%)	5-year OS (%)
CAO/ARO/AIO-94[22]	Neo-adjuvant CRT	421	50.4 Gy with 5-FU 1000 *mg/m² d*1-5 *q*28x2→TME surgery→5-FU 500*mg/m² bolus d*1-5 *q*28x4	cT3-4 or N+ Age≤75	5-year OS	8^	5^	68	74
	Adjuvant CRT	402	TME surgery→55.8 Gy with 5-FU 1000 mg/m² *d*1-5 *q*28x2→5-FU 500*mg/m² bolus d*1-5 *q*28x4			0^	9.7^	65	76
FFCD 9203[24]	Neo-adjuvant CRT	375	45 Gy with 5-FU 350*mg/m² d*1-5 *q28x2*→surgery→adj chemox4	cT3-4 Age<75	5-year OS	11.4^	8.1^	59.4	67.4
	Neo-adjuvant RT	367	45 Gy→ surgery→adj chemox4			3.6^	16.5^	55.5	67.9
EORTC 22921[25, 27]	Neo-adjuvant CRT	506	45 Gy with 5-FU 350*mg/m² d*1-5 *q28x2*→surgery→+/- adj chemox4	cT3-4 Age≤80	5-year OS	13.7^	7.6-8.7^	56.1	65.8
	Neo-adjuvant RT	505	45 Gy→ surgery→+/-adj chemox4			5.3^	9.6-17.1^	54.4	64.8

^ Statistically significant

Abbreviations: CRT=chemo-radiotherapy, 5-FU=5-fluorouracil, TME=total mesorectal excision, adj chemo=adjuvant chemotherapy, cT3-4=clinical stage T3-4, N+=nodal involvement, pCR=complete pathological response

Two randomised controlled trials have compared short course radiotherapy and long-course CRT.[28, 29] In the Polish study, which included cT3-T4 tumours irrespective of nodal status, the primary endpoint was sphincter preservation rate which was not significantly different between the two treatment arms (61.2% for short course radiotherapy vs. 58% for long course CRT, *p*=0.57).[28] The 4-year local recurrence rate (10.6% for short course radiotherapy vs. 15.6% for long course CRT, *p*=0.21) and 4-year OS rate (67.2% for short course radiotherapy vs. 66.2% for long course CRT, *p*=0.96) were not significantly different between the two treatment arms. Similarly, in the Trans-Tasman Radiation Oncology Group Trial 01.04, which also recruited patients with cT3-4 tumours irrespective of nodal status, 3 year local recurrence rate which was the primary endpoint, was not significantly different between the two treatment arms (7.5% for short course radiotherapy vs. 4.4% for long course CRT, *p*=0.24) and 5-year OS was also similar (74% for short course radiotherapy vs. 70% for long course CRT, *p*=0.62).[29] Additionally, in both trials, there was no statistically significant difference in late toxicity for either treatment strategy. Both short course radiotherapy and long course CRT are therefore considered acceptable standards of care. However, based on the results of these trials, if down staging is required, long course CRT is generally the preferred option.

1.3.3 Treatment intensification during CRT with chemotherapy

Although the optimisation of neo-adjuvant treatment strategies and the incorporation of TME surgery resulted in lower local recurrence rates, development of distant metastases remains the main cause of morbidity and mortality. Several studies examined whether treatment intensification during

radiotherapy would improve outcomes. Oxaliplatin was seen as a prime candidate to investigate due to its radio-sensitising properties[30] along with its demonstrated activity in the advanced disease setting. With the exception of the 3-year DFS benefit in the German CAO/ARO/AIO-04 study, there was no DFS or OS benefit demonstrated in the investigational oxaliplatin containing arm across the studies and toxicity was higher (***Table 1.2***). Capecitabine was found to be non-inferior to fluorouracil and given its convenience of administration, has largely replaced 5-fluorouracil as a radiosensitiser.[31] No phase III studies have reported regarding the role of irinotecan as a radiosensitiser in this setting. The results of the phase III, ARISTOTLE trial (ISRCTN09351447) which is investigating the addition of irinotecan to capecitabine based CRT should address this.

Table 1.2 Studies investigating treatment intensification with the addition of oxaliplatin to CRT

Trial	N	Key eligibility	Treatment arms	Primary Endpoint	pCR rate (%)	5-year DFS (%)	5-year OS (%)	Overall Grade 3-4 toxicity (%)
STAR-01[32, 33]	747	cT3-4 and/or cN+ (resectable), within 12cm from the anal verge, age≤75	5-FU+RT 50.4Gy	5-year OS	16	66.3	77.6	8^
			5-FU+Ox60+RT 50.4Gy		16	69.2	80.4	24^
ACCORD 12/0405-PRODIGE 02[34–36]	598	T2 (anterior and lower rectum), T3 and T4 (resectable), age≤80	Cape+RT 45Gy	pCR	13.9	63.1	73	10.9^
			CAPOX50+RT 50Gy		19.2	66.1	82	25.4^
NSABP-R04[37]	1608	cT3-4 and/or cN+, within 12cm from the anal verge	5-FU or Cape+RT 45-55.8Gy	3-year local control	17.8	64.2	79	5-FU:26.2, Cape:28.8
			5-FU/Ox50 or CAPOX50+RT 45-55.8Gy		19.5	69.2	81.3	5-FU/Ox50:39.8, CAPOX50:40.3
CAO/ARO/AIO-04[38, 39]	1265	cT3-4 or cN+, within 12cm from the anal verge	5-FU+RT 50.4Gy	3-year DFS	13.0^	71.2^*	88.0*	21
			5-FU/Ox50+RT 50.4Gy		17.0^	75.9^*	88.7*	25
PETACC-6[40, 41]	1094	cT3-4 or cN+, within 12cm of the anal verge, resectable or potentially resectable	Cape+RT 45-50.4Gy	3-year DFS	11.3	71.3	83.1	15.1
			CAPOX50+RT 45-50.4Gy		13.3	70.5	80.1	36.7
FORWARC[42, 43]	312	cT3-4 or cN+, within 12cm from the anal verge, age≤75	5-FU+RT 46-50.4Gy	3-year DFS	14.0^	76.4*	93.7*	N/A
			mFOLFOX+RT 46-50.4		27.5^	77.8*	92.0*	N/A

^ Statistically significant, * 3-year outcome

Abbreviations: RT=radiotherapy, 5-FU=5-fluorouracil, cT3-4=clinical stage T3-4, N+=nodal involvement, Cape=capecitabine, CAPOX=capecitabine/oxaliplatin, Ox50=oxaliplatin 50mg/m^2, *Ox60*=oxaliplatin 60mg/m^2, *mFOLFOX=modified FOLFOX (5-fluorouracil, leucovorin, oxaliplatin), pCR=complete pathological response, DFS=disease free survival, OS=overall survival*

1.3.4 Total neoadjuvant therapy

Given that chemotherapy was given alongside radiotherapy at radiosensitising doses, it is perhaps unsurprising that attempts at reducing the risk of metastatic disease developing have remained unsuccessful. Total neoadjuvant therapy (TNT) refers to the strategy of giving all treatment upfront, therefore CRT is administered with a course of systemic chemotherapy either prior to or after completion of CRT but prior to surgery. The rationale is to give systemic chemotherapy early in the treatment paradigm in an attempt to eradicate any micrometastases early and potentially increase the chance of an R0 resection. Additionally, there is the possible advantage of increasing the pCR rate and gaining some knowledge regarding the chemo-sensitivity of the tumour. Administering the chemotherapy prior to surgery may improve compliance which can be affected particularly if recovery from surgery is prolonged. A recent meta-analysis which included 28 studies (n=3579 patients) showed that the pooled pCR rate for TNT was 22.4% (95% CI: 19.4%-25.7%).[44] In the 10 comparative studies with data available, TNT increased the odds of pCR by 39% (OR 1.4, 95% CI: 1.08-1.81, *P*=0.01). In the 7 comparative studies with available data, median OS was longer in patients treated with TNT compared to CRT alone (HR=0.73, 95% CI: 0.59-0.9, *P*=0.004). Although the data is encouraging, given the limited number of randomised studies included, the results of large confirmatory trials will be required to draw any firm conclusions. Moreover, in order to understand how best to sequence existing therapies and prevent the over-treatment of patients that may not require

such an intensive treatment strategy, better ways to risk stratify our patients are clearly needed.

1.3.5 Omitting radiotherapy

In recognition of the need to avoid the toxicity associated with pelvic radiotherapy, there have been some efforts to assess whether neoadjuvant radiotherapy can be omitted all together. A small pilot study addressing this question included 32 patients with stage II-III rectal cancer from a single centre. Patients with T4 disease and/or ≥4 pelvic lymph nodes >2cm were excluded. 30 patients were treated with neoadjuvant chemotherapy (mFOLFOX6+bevacizumab) and surgery alone. 2 patients were unable to complete their course of neoadjuvant chemotherapy and were subsequently treated with radiotherapy. Of the patients treated with systemic chemotherapy without radiotherapy, oncological outcomes were not compromised: pCR was 25%, all patients achieved an R0 resection, 4 year local recurrence rate was 0%, 4 year DFS was 84% and 4 year OS was 91%.[45] However, post-operative fistulas were observed in 15-28% of patients, most likely due to the use of bevacizumab. Other small, single centre studies have shown that it might be feasible to omit radiotherapy in carefully selected patients.[46–48] More recently, the phase II FORTUNE study reported on the use of a triplet chemotherapy regimen (mFOLFOXIRI) and demonstrated a 20.4% pCR rate and tumour downstaging rate of 42.7%.[49] The phase III, randomised controlled PROSPECT study (NCT01515787) will hopefully be able to provide more definitive evidence regarding the option of omitting radiotherapy in a sub-population of patients with LARC.

1.3.6 Avoiding surgery

Surgery for rectal cancer is associated with 1-2% peri-operative mortality and long term functional morbidity is common with potential for a permanent stoma and over 60% of patients experiencing urinary and sexual dysfunction.[50, 51] In 2004, Habr-Gama et al. first reported on the feasibility of an organ preservation approach with a 5-year OS of 100% and a 5-year DFS of 92% for patients deemed to have a complete clinical response (cCR) that did not proceed with surgery.[52] Since then, organ preservation has continued to receive significant research interest with cCR acting as a surrogate for pCR. The largest pooled dataset comprising 1009 patients from 47 participating centres in 15 different countries was recently published.[53] Outcome data was available for 880 patients. The 5-year OS rate was 84.7% and the re-growth rate at 2 years was 25.2%. Of the patients who had a recurrence, 87.8% were successfully salvaged by surgery. Although the data appears to be promising, the patient population in this dataset was heterogeneous and varied definitions of cCR were used. The results of large, prospective clinical trials will determine whether this strategy can be routinely implemented safely. Molecular and imaging biomarkers are needed to better define the patient population that may or may not benefit and also to pick up recurrence at an early stage when still salvageable by surgery.

1.4 Circulating tumour DNA

1.4.1 The discovery of circulating tumour DNA (ctDNA)

Since its initial discovery in 1948,[54] circulating cell free DNA (cfDNA), which refers to extra-cellular fragmented DNA, was found to be present in higher

concentrations in cancer patients than in healthy volunteers.[55] However, increased levels have also been reported in other conditions associated with cellular injury or necrosis such as myocardial infarction.[56]

In healthy individuals, haematopoietic cells are thought to be the main source of cfDNA.[57] In patients with cancer, tumour derived DNA (ctDNA) contributes to a proportion of the cfDNA. Genetic and epigenetic changes that can be detected in ctDNA include mutations, methylation, copy number variations and structural changes or re-arrangements.

Apoptosis and necrosis along with active secretion and inefficient phagocytosis have all been suggested to be the main mechanisms explaining how the DNA enters the blood stream.[58] The proportion of ctDNA can be as low as 0.01%, particularly in early stage disease. The evolution of highly sensitive digital genomic technologies and improvement in sequencing technologies has allowed ctDNA research to progress.

1.4.2 ctDNA detection methods and clinical applications

There are a number of different techniques that can be used to detect ctDNA (***Table 1.3***).[59] Studies using different methodologies have confirmed that ctDNA can also be detected in patients with non-metastatic CRC where the overall volume of disease is lower with detection rates ranging from 40-77% for stages I-III respectively.[60–63]

In order to maximise the chance of detecting ctDNA when present, particularly when only likely to be present at low levels in the early disease setting, it is imperative that pre-analytical factors are taken into consideration and optimised. Such factors include: using a large gauge diameter needle (≤21G) for blood draw and opting for cell stabilising tubes for collection if processing is likely to be delayed beyond 4-6 hours, ensuring an adequate volume of blood is collected for downstream analysis and isolating ctDNA from plasma rather than serum which contains larger quantities of DNA released from immune cells during the clotting process. Additionally, shaking of samples should be avoided with mixing being restricted to inverting and interim storage temperatures should be determined by blood collection bottle utilised (4°C for K_2EDTA tubes and 10-30°C for cell stabilisation tubes). Sample processing should involve double centrifugation to allow for separation into layers and prevent contamination from lymphocyte DNA (pellets fall to the bottom of the tube with double centrifugation). In order to avoid multiple freeze thaw cycles which could compromise sample quality, appropriate volumes of plasma should be aliquoted into single use tubes and stored at -80°C. Finally, patient related factors such as: co-morbidities, time of sampling with relation to treatment and type of treatment received as well as whether the patient was fasting or had recently exercised will influence the interpretation of results and should be accounted for. The recently published whitepaper from the Colon and Rectal-Anal Task Forces of the United States National Cancer Institute has provided a helpful summary of these key factors.[64]

Broadly speaking, ctDNA detection techniques are polymerase chain reaction (PCR) or next generation sequencing (NGS) based and either strategy can be tumour informed whereby prior knowledge of genomic alterations from sequencing tumour tissue guides future ctDNA analysis. A tumour informed strategy offers the advantages of increased sensitivity and a lower likelihood of false positives. False positives generally occur due to sequencing errors or non-tumour related mutations such as clonal haematopoiesis of indeterminate potential (CHIP). However, the additional expense and potential delays due to tumour sequencing should not be overlooked especially if the results of ctDNA analysis will be used to determine the initiation of time-sensitive treatment. A tumour naïve strategy is viable with an NGS approach and offers the advantage of a faster workflow and the potential to capture treatment emergent mutations. However, given that the sensitivity is not as high as for a tumour informed strategy, consideration should be given to incorporating methylation in addition to mutation tracking if the clinical application requires low level detection. Additionally, although ultra-sensitive NGS techniques allow the analysis of larger target regions, they are prone to false positive results which can be minimised by the incorporation of random molecular barcodes for error reduction.[65, 66] Sequencing of peripheral blood cell DNA can mitigate against false positives arising due to CHIP by allowing for differentiation from somatic tumour mutations. Whilst there are clear advantages associated with NGS, it should be noted that higher DNA input, lower sensitivities for rare variants and more extensive data interpretation with bioinformatics support is required compared to droplet digital (ddPCR). Whilst ddPCR remains a

highly sensitive technique, it only allows a few genes to be interrogated at any given time and therefore the clinical application should determine which analysis technique would be most appropriate.

Aside from pre-analytical factors, at the point of analysis, quality control is also vital. Extracted DNA concentration should be quantified by fluorometry, mass spectrometry or quantitative PCR. The cfDNA content should also be assessed using electrophoresis. cfDNA is highly fragmented and the fragment length typically peaks at 167bp which is consistent with the length of DNA wrapped around a single nucleosome along with a short linker DNA bound to a histone. Therefore in the absence of significant genomic contamination from other sources, it is expected that electrophoresis should demonstrate a peak at 167bp. Interestingly, exploiting cfDNA properties by fragment size analysis whereby size selection for analysis to enrich for the presence of ctDNA has been used as a strategy to increase detection sensitivity.[67] Moreover, size distribution and fragmentation patterns have also been associated with tissue of origin and may be useful for early diagnostics.[67, 68]

Table 1.3 Summary of key ctDNA detection techniques

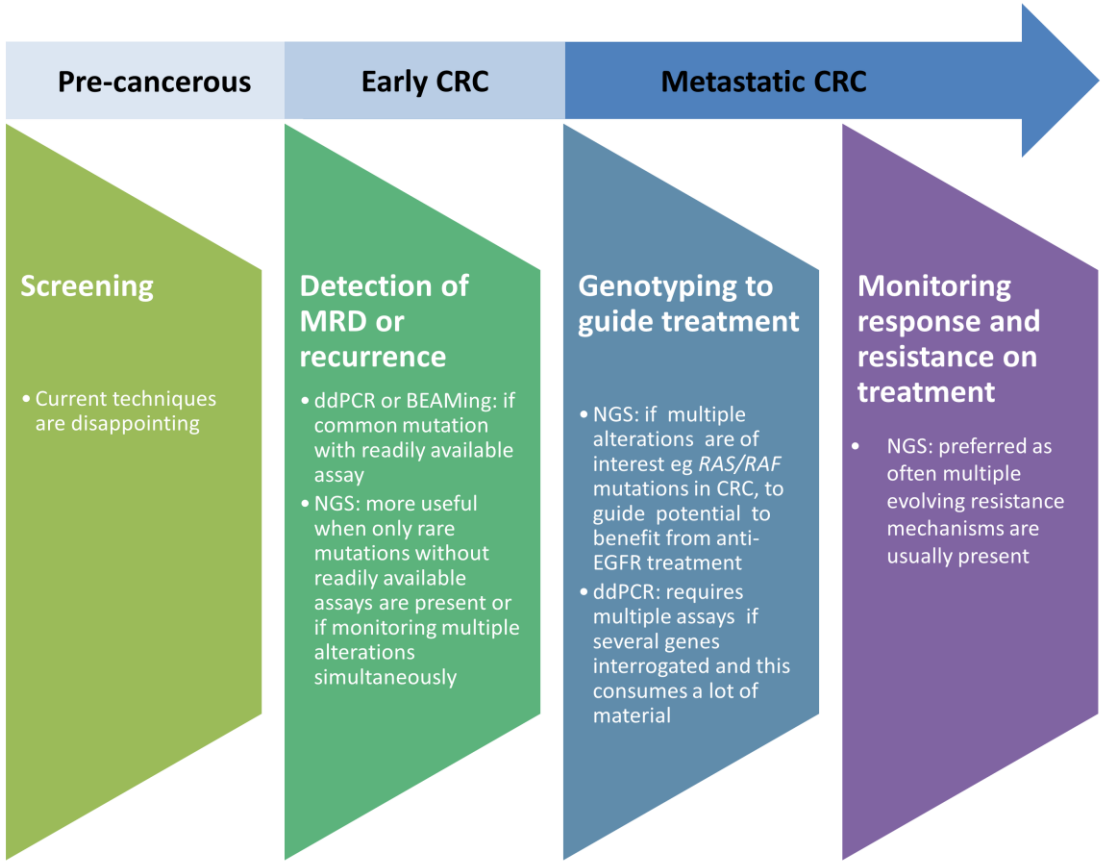
Technique	**Limit of detection**	**Advantages**	**Disadvantages**
Allele specific PCR E.g. COLD-PCR, PNAs, ARMS	0.1-1%	Low cost Easy to perform	Low sensitivity Limited number of genes interrogated at a time Genes need to be pre-determined
Digital PCR E.g. ddPCR and BEAMing	0.05% or less	High sensitivity and specificity Reasonable cost Easy to perform	Limited number of genes interrogated at a time Genes need to be pre-determined
NGS E.g. amplicon based such as TAM-Seq, Safe-SeqS	0.01-2%	Allows for more genes to be interrogated at a time than dPCR	Wide range of sensitivity depending on NGS platform used e.g. PCR amplicon strategies more sensitive and less expensive than whole genome or exome sequencing

(extracted from Khakoo et al, Crit Rev Oncol Hematol 2018)

Abbreviations: COLD-PCR=co-amplification at lower denaturation temperature, *PNAs= peptide nucleic acids*, *ARMS= amplification refractory mutation system*, *BEAMing= bead emulsion amplification and magnetics*, *dPCR=digital PCR*, *ddPCR= droplet digital PCR*, *NGS=next generation sequencing*, *TAM-Seq= tagged amplicon deep sequencing*, *Safe-SeqS= safe sequencing system*

There are a number of potential clinical applications for ctDNA in CRC including: early diagnosis and screening, guiding therapy adaptation in rectal cancer, detecting minimal residual disease (MRD) and relapse in patients having curative surgery, genotyping to guide therapy and identifying early emergence of resistance to therapy in advanced disease to allow therapy adaptation.[59] In patients on anti-epidermal growth factor receptor (EGFR) therapy, mutated *KRAS* alleles have been demonstrated in the ctDNA of patients at the time of progression.[69] These decline upon discontinuation of therapy thereby suggesting a role for re-challenge with anti-EGFR therapy which has been the subject of on-going research.[70] The technique used to detect ctDNA should be determined by the downstream application (***Figure 1.3***).

The landmark study suggesting that ctDNA may have a role as a marker of MRD in CRC mainly included stage IV patients that were undergoing hepatic metastasectomies.[71] ctDNA was detectable in all pre-surgery samples and remained detectable in 16 out of the 20 cases with plasma available at the first follow-up visit (range 13-56 days). Recurrences occurred in all but one of these cases whereas no recurrences occurred in any of the cases where ctDNA was undetectable (p=0.006). Since then, further supporting evidence for ctDNA as a surrogate for MRD has been provided in other studies which included patients with stage II and III CRC and more recently also in LARC (***Table 1.4***).[62, 63, 72–78]



(extracted from Khakoo et al, Crit Rev Oncol Hematol 2018)

Figure 1.3 Suggested techniques for ctDNA analysis in different clinical scenarios
Abbreviations: MRD=minimal residual disease, *ddPCR=droplet digital polymerase chain reaction*, *BEAMing= bead emulsion amplification and magnetics*, *NGS=next generation sequencing, EGFR=epidermal growth factor receptor*

Table 1.4 Summary of key published studies of ctDNA as a surrogate for minimal residual disease which included colon and rectal cancer or only colon cancer patients

Study	**Population**	**Genomic alterations investigated (and techniques utilised for ctDNA detection)**	**Blood sampling time-points**	**Key Results**
Diehn[73] 2018	*N*=145 Stage II=86 Stage III=59	NGS based to identify SNVs present in tumour tissue to track in plasma. 197 genes recurrently mutated in CRC were interrogated. Median of 4 SNVs /sample	Mean of 10 days post-surgery	-Patients with detectable ctDNA (n = 12) had a shorter 2-year relapse-free survival (17% vs. 88%; HR 10.3; 95% CI 2.3-46.9; p < 0.00001), time to recurrence (HR 20.6; 95% CI 3.1-139.0; p < 0.00001) and overall survival (OS; HR 3.4; 95% CI 0.5-25.8; p = 0.041) than patients with undetectable ctDNA (n = 132). -11 (92%) patients with detectable ctDNA developed recurrence compared to 9 (7%) patients with undetectable ctDNA.
Reinert[74] 2016	*N*=14 Stage I=1 Stage II=6 Stage III=3 Stage IV=4	Patient specific somatic structural variants identified by NGS of tumour tissue and up to 6 assays designed per patient (ddPCR)	Day 0 (Pre-op) Day 8 Day 30 Then 3-monthly	-2 to 15 (mean 10) months lead time on detection of metastatic recurrence when compared to conventional follow up. -The sensitivity and specificity of ctDNA in terms of detecting post-surgery relapse was 100%

Reinert[63] 2019	*N*=125 Stage I=5 Stage II=39 Stage III=81	16 patient specific somatic SNVs and short indels identified by whole exome sequencing of tumour were selected. Ultradeep multiplex PCR based NGS of plasma	Day 0 (Pre-op ≤14 d prior to surgery) Day 30 Then 3-monthly	-Median follow up was 12.5 months (range1.4-38.5 months) -Pre-operative ctDNA was detected in 88.5% patients and detection rates were 40% for stage I, 92% for stage II and 90% for stage III -Day 30: 84/94 (89.4%) had undetectable ctDNA and 10/94 (10.6%) had detectable ctDNA -7/10 (70%) of those with detectable ctDNA had a recurrence compared to 10/84 (11.9%) of the patients with undetectable ctDNA -RFS was significantly shorter in ctDNA positive patients compared to ctDNA negative patients (HR 7.2; 95% CI 2.7-19.0, *P*<0.001)
Scholer[75] 2017	*N*=45 Stage I=5 Stage II=8 Stage III=8 Stage IV=24	Somatic structural variants and somatic point mutations identified in tumour tissue by mate pair sequencing and/or to *KRAS* hotspot mutation profiling Assays were designed for ctDNA profiling (ddPCR)	Day 0 (Pre-op) Day 8 Day 30 Then 3-monthly	-The ctDNA detection rate in pre-operative samples was 74%. -CEA was elevated in 55.5% of cases pre-operatively -No ctDNA was detected post-operatively in relapse free patients -All patients who relapsed had multiple post-operative plasma samples with ctDNA detectable -Post-operative CEA was elevated in 78.6% of cases with a relapse -The median ctDNA lead time was 9.4 months compared to radiological detection of relapse

Tarazona[76] 2019	*N*=94 Stage I=14 Stage II=41 Stage III=39	Somatic mutations were identified in tumour tissue using a custom targeted NGS panel which included 29 genes. ddPCR was used to track ctDNA in plasma	Pre-operatively 6-8 weeks Post-op 4 monthly up to 5 years	-Median follow up was 24.7 months (range 1-45.2 months) -The pre-operative ctDNA detection rate was 63.8% - Baseline ctDNA concentration was significantly higher in stage II-III compared to stage I (*P*=0.018) -14/69 (20.3%) had detectable ctDNA 6-8 weeks post-op -8/14 (57.1%) of those with detectable ctDNA post-operatively had a recurrence compared to 9/55 (16.4%) of the patients with undetectable ctDNA - DFS was significantly shorter in ctDNA positive compared to ctDNA negative patients (HR 6.96; *P*=0.0001) -ctDNA positivity after chemotherapy was associated with a significantly shorter DFS (HR 10.02; 95% CI 9.2-307.3, *P* < 0.0001)
Tie[72] 2016	*N*=230 All stage II colon cancer patients	TP53, APC, KRAS, NRAS, BRAF, PIK3CA, CTNNB1, SMAD4, and FBXW7 (massively parallel sequencing platform, Safe-SeqS)	Post-operatively at 4-10 weeks and then 3 monthly for a subset of 167 patients for up to 2 years	-In patients that had adjuvant chemotherapy (n=52), 6 had detectable ctDNA. Detectable ctDNA after completion of chemotherapy was associated with an inferior recurrence-free survival (P= 0.001). -In patients not treated with adjuvant chemotherapy (n=178), ctDNA was detected in 14 (7.9%) patients, 11 (79%) of whom had a recurrence at a median follow-up of 27 months. -The sensitivity and specificity of postoperative ctDNA in predicting recurrence at 36 months were 48% and 100% respectively. -ctDNA was more frequently positive at the time of radiological recurrence than CEA (*P*=0.002)

Tie[78] 2019	*N=96* All stage III colon cancer patients	1 mutation identified in the tumour by targeted sequencing of 15 genes was assessed in the plasma for the presence of ctDNA (massively parallel sequencing platform, Safe-SeqS)	Post-operatively at 4-10 weeks After completing treatment within 6 weeks of the final chemotherapy cycle	-Median follow up was 28.9 months (range 11.6-46.4 months) -ctDNA was detected in 20/96 (21.0%) of patients post-operatively -24 patients had a recurrence and ctDNA was detectable in 10 (42.0%) post-operatively - Patients with detectable ctDNA after surgery had an increased risk of recurrence (HR, 3.8; 95% CI, 2.4- 21.0, *P*<0.001) - 72 patients completed all 24 weeks of chemotherapy and post-chemotherapy plasma was available in 66 of these patients. ctDNA was detectable in 10/66 (15.2%) - The ctDNA status of the post-chemotherapy sample was strongly associated with recurrence free interval (HR, 6.8; 95% CI, 11.0-157.0, *P* <0.001)
Wang[77] 2019	*N*=58 Stage I=9 Stage II=21 Stage III=28	Targeted sequencing of 15 genes in tissue (massively parallel sequencing platform, Safe-SeqS)	1 month after surgery then 3-6 monthly	-10/13 (77%) patients with detectable ctDNA had a recurrence compared to 0/45 with undetectable ctDNA

1.5 Thesis hypothesis and aims

The hypothesis of the research conducted in this MD(Res) project is that blood and tissue-based biomarkers can be used to predict clinical outcome in patients with CRC.

-Chapter 2: The aim was to investigate whether ctDNA is a marker of response or progression in patients with localised rectal cancer being treated with long course CRT using a single centre, pre-existing translational research protocol (REC number 08/H0714/59) where bloods were collected prospectively. Blood samples for this analysis were collected between February 2015 and November 2016 and results are presented from page 62.

-Chapter 3: The aim was to evaluate the feasibility of ctDNA analysis as assessed by detection rates in samples collected from multiple UK centres both pre-operatively and longitudinally post-operatively by developing a translational research protocol (REC number 15/LO/1576) which enabled prospective blood and tissue collection from patients with stage II and III CRC. Blood samples for this analysis were collected from December 2016 until November 2018 for the analyses included in this thesis. Results are presented from page 106.

-Chapter 4: The aim was to compare the transcriptomic profiles of matched tissue from primary CRC and corresponding secondary lung metastases using the pre-existing lung resection translational protocol (REC number 12/SC/0158). Tissue from lung metastasectomies occurring between 1997 and 2012 at the Royal Brompton and Harefield Foundation Trust was sought for this analysis. Results are presented from page 130.

2 ctDNA TO GUIDE THERAPY ADAPTATION IN RECTAL CANCER

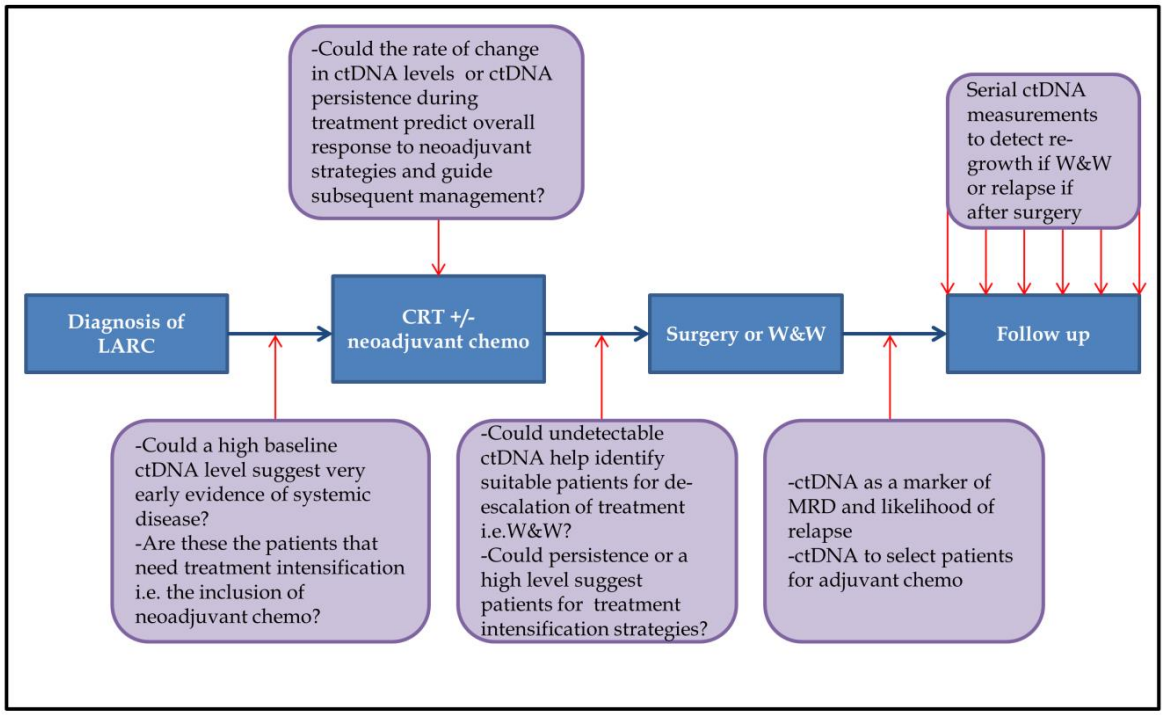
2.1 Background

Response to long course CRT for LARC can vary quite considerably. This has led to an interest in trying to identify prognostic and predictive biomarkers to allow treatment strategies to be adapted and where possible, spare patients from unnecessary toxicity without compromising oncological outcome.

15-20% of patients have pCR to standard of care neo-adjuvant CRT and such patients have better survival rates.[79, 80] Recognition of this and the need to avoid the functional morbidity associated with surgery has led to increased interest in organ preservation.[51] The main challenge is appropriate patient selection. As discussed in the introduction, studies have included heterogeneous populations and this has led to difficulty with standardisation and data interpretation.[81]

In patients that show a poor response to CRT, a trial of neo-adjuvant chemotherapy may be offered in an attempt to downsize the tumour and enable less invasive surgery. Early identification of patients with radio-resistance or those at risk of developing systemic disease is essential, as these patients may benefit from treatment intensification or alternative treatment strategies.

Response assessment typically relies on review of the resected tumour tissue following CRT. The post-treatment T and N stage has been shown to predict local recurrence, DFS and OS.[82] The pathological tumour regression grade (pTRG) which assesses the degree of fibrosis in relation to remaining tumour has also been shown to be a predictor of OS and DFS.[83–85] Before surgery, response to CRT can be assessed by conventional methods such as Response Evaluation Criteria in Solid Tumours (RECIST) which measures the change in size of the target lesion. However, fibrosis arising from the treatment can make the accuracy of RECIST measurements more challenging. More recently, MRI derived TRG (mrTRG) which most closely resembles the Mandard pTRG system, was evaluated in the MERCURY trial and found to predict DFS and OS.[86] mrTRG provides an accurate means to assess response to CRT pre-operatively and as such, has the potential to allow therapy adaptation whilst also providing prognostic information.[86] Although mrTRG is a measure of local response, it may not be fully representative of systemic disease status. Therefore, integration of longitudinal monitoring with a blood-based biomarker such as ctDNA, with response assessment by imaging, is attractive. As a non-invasive, surrogate for the tumour genome, incorporation of ctDNA analysis may be complementary to imaging by providing information at the molecular level (***Figure 2.1***).



(modified from Khakoo et al, Crit Rev Oncol Hematol 2018)

Figure 2.1 Potential time-points for ctDNA analysis to guide management of locally advanced rectal cancer
Abbreviations: LARC=locally advanced rectal cancer, W&W=watch and wait, MRD=minimal residual disease

However to date, there has been limited published research addressing its value in an exclusively rectal cancer population.[87, 88] Given the relative ease of analysing total cfDNA quantity, some studies assessed its prognostic role in this population group (***Table 2.1***). All 3 studies used different analysis techniques.[89–91] However, all 3 studies consistently found that baseline cfDNA levels were not associated with response to CRT. Only 1 of the 3 studies evaluated the relationship between cfDNA and survival outcomes and found that a higher baseline cfDNA was associated with a significantly shorter DFS.[91]

Whilst the data regarding the potential prognostic role of baseline cfDNA is interesting, as a marker, it is affected by multiple factors such as other co-morbidities, exercise and inflammation which would limit its clinical value. Identifying tumour-specific alterations in plasma, whilst more technically challenging, is likely to be a more accurate marker of clinical outcome. Four studies have published data on ctDNA in localised rectal cancer and these are summarised in ***Table 2.2***.

Table 2.1 Key published studies of cfDNA in patients with localised rectal cancer

Study	N	Patient characteristics	Methodology	Key results
Zitt et al.[89] (2008)	26	-Mean age in years (range) 63.1 (34-83) -All patients cT3 or cT4 -CRT with 45Gy combined with 350mg/m^2/day 5-FU on treatment days	-Blood samples were collected before CRT, after CRT (i.e. 5th week) and post-operatively at the end of treatment -Patients with ypT stage 0,1 and 2 tumours were classified as responders and those with no change (ypT3 and ypT4) as non-responders - DNA was extracted from plasma and quantified by real time PCR	-There was no statistically significant difference in the quantity of cfDNA between responders and non-responders at baseline or at the end of CRT -At the end of treatment (post-operatively) responders showed a further decrease in cfDNA whereas cfDNA increased in the non-responders (P=0.0006)
Agostini et al.[90] (2011)	67	-Median age in years (range) 61 (20-79) -Tumour ≤7cm from anal verge in 63% -69% had cT3-T4 disease -88% node positive -Total RT dose ≥50 Gy in 94% -13% had concomitant capecitabine -84% had concomitant 5-FU (continuous or bolus) -40% received Oxaliplatin	-Blood samples were collected before and after CRT - Patients were classified as responders (pathological TRG 1-2) or non-responders (TRG 3-5) - cfDNA levels were analysed by qPCR of β-globin. cfDNA integrity index was calculated.	-Baseline levels of cfDNA were not associated with tumour response -The post-CRT levels of cfDNA integrity index were significantly lower in responsive compared to non-responsive disease (P=0.0009)
Schou et al.[91] (2018)	123	-Median age 67 -cT3 if CRM involved/threatened or node positive All cT4	-Blood collected at baseline, after induction chemotherapy, after CRT and after surgery -Total cfDNA was measured by direct fluorescent assay of plasma samples	-There was no significant difference in baseline cfDNA levels between patients who achieved pCR and the poor responders -Patients with baseline cfDNA levels above the 75th quartile had a higher risk of local or distant recurrence and a shorter time to recurrence compared to patients with levels below the 75th quartile (HR 2.48, 95% CI:1.3-4.8, *P*=0.007)

Table 2.2 Key published studies of ctDNA in patients with localised rectal cancer

Study	N	Patient characteristics	Methodology	Key results
Sun et al.[92] (2014)	34	-Age range 29-73 -LARC	-Blood collected 7days prior to and after CRT -Concentration, KRAS mutation and MGMT promoter methylation status were measured by PCR	-ctDNA as measured by *KRAS* mutation, decreased in both good and poor responders to CRT -High MGMT promoter methylation status at baseline was associated with a good response to CRT
Carpinetti et al.[93] (2015)	4	-Age not specified -cT3N0-1 -rectal cancer located up to 7cm from the anal verge -CRT with 50.4-54 Gy and 5-FU based chemo	-Blood collected at diagnosis, after CRT (between weeks 3 and 9), at the time of response assessment (week 13) and during follow up -Patient specific chromosomal rearrangements identified by whole genome sequencing of the tumour - DNA was extracted from plasma and ddPCR was used to detect ctDNA	- A patient with pCR and 2 patients with incomplete pathological response had undetectable ctDNA after completion of CRT - Patients who developed metastatic disease during follow up had detectable ctDNA
Sclafani et al.[94] (2018)	97	-high risk LARC as defined by at least one of the following: cT3 with threatened/involved CRM or evidence of EMVI, cT3c, cT3d or cT4	-Baseline blood sample analysed -ddPCR for common *KRAS* and *BRAF* mutations and any additional patient specific *KRAS* mutation detected in tissue	-ctDNA detection rate in the *KRAS* mutant population was 66% -Detection of *KRAS* mutation in ctDNA did not predict prognosis or refine patient selection for cetuximab
Tie et al.[62] (2019)	159	-LARC (T3/4 or node positive)	-Blood collected: prior to CRT, 4-6 weeks post CRT, 4-10 weeks post-surgery -Targeted sequencing of 15 genes in tissue -Massively parallel sequencing platform, Safe-SeqS	-ctDNA detection rates were 77%, 8.3% and 12% for each chronological time-point -Significantly worse recurrence-free survival was seen if ctDNA was detectable after CRT (HR 6.6; P<0.001) or after surgery (HR 13.0; P<0.001). -19 patients had ctDNA detectable post-surgery of which 11 (58%) experienced recurrence -140 patients were ctDNA negative post-surgery and 128 (91%) did not experience recurrence. -Post-operative ctDNA detection was predictive of recurrence irrespective of adjuvant chemotherapy use

Abbreviations: MGMT=06-methylguanine-DNAmethyltransferase

The landmark observational study which was recently published by Tie et al. provided compelling evidence for ctDNA as both a prognostic and predictive biomarker in LARC patients receiving treatment with long course CRT followed by surgery.[62] However, they did not collect plasma during CRT, include any patients proceeding with organ preservation or assess CRT response according to the mrTRG. The relationship between ctDNA status and response according to mrTRG therefore remains unknown. Additionally, the prognostic value of blood collection for ctDNA analysis during CRT is unclear but could allow the early tailoring of treatment if proven. The potential for ctDNA to select patients for organ preservation or identify local re-growth early has not been investigated.

With this in mind, the screening study for genetic changes in colorectal, gastrointestinal and hepatobiliary cancers (SSGCC-1), sponsored by the Royal Marsden NHS Foundation Trust, was used to recruit and analyse ctDNA dynamics and response in patients with rectal cancer being treated with long course CRT. The main study aims were:

1. To assess whether detection of ctDNA before, during or after CRT is associated with MRI defined response of the primary by RECIST and mrTRG

2. To assess whether detection of ctDNA before, during, after CRT and post-surgery is associated with clinical characteristics and outcome

3. To evaluate whether the quantity of detectable ctDNA during and after CRT is associated with the development of metastases and establish a ctDNA threshold if a relationship exists

4. To determine whether ctDNA could be used to identify local re-growth in organ preservation patients

2.2 Methods

2.2.1 Study design, participants and procedures

This was a single centre study (NCT00825110) where I co-ordinated the prospective recruitment and collection of blood and tissue samples from consecutive patients meeting the eligibility criteria. All patients aged 18 years or older, with a diagnosis of LARC (cT3-4 and/or node positive) confirmed on histology and absence of metastases on imaging, scheduled to undergo long course chemo-radiotherapy (CRT) at the Royal Marsden Hospital between February 2015 and November 2016, were eligible. During this time-period, patients that did not meet the criteria for LARC but met all other eligibility criteria and had a low rectal tumour and/or an adverse risk feature where the multi-disciplinary team (MDT) recommended long course CRT, were also eligible. CRT consisted of capecitabine 1650mg/m^2/day for 6 weeks alongside 50.4-54 Gy radiotherapy. Treatment decisions following CRT were made following MDT discussion and took into account tumour response and patient related factors. In the absence of disease progression with metastatic disease, treatment options included: surgery, organ preservation or neo-adjuvant chemotherapy (if the risk of R1 resection was high due to poor response to CRT). The study was approved by a human research ethics

committee and all patients provided written informed consent prior to their participation.

Serial blood samples were collected: pre-treatment (within 4 weeks prior to commencing CRT), mid-CRT (3-4 weeks from the start of CRT), after completion of CRT (4-12 weeks from completion) and post-surgery (within 4-12 weeks). For patients pursuing organ preservation, bloods were collected 3-6 monthly from the end of CRT until within 3 months of re-growth.

CT to confirm the absence of metastatic disease was carried out at baseline in conjunction with MRI for local staging. MRI was used to assess response 3-6 weeks following completion of CRT and CT evaluated systemic disease status. Radiologists assessing response to CRT were blinded to ctDNA results. All ctDNA analyses were conducted by individuals blinded to the clinical status of patients. Response of the primary tumour was assessed using RECIST version 1.1 and an independent radiologist provided the mrTRG.

Responders were defined as patients achieving a complete response (CR) or partial response (PR) according to RECIST or mrTRG1-2. Patients with stable disease (SD) or progressive disease (PD) by RECIST or mrTRG 3-5 were classed as poor-responders. The mrTRG definitions of response were consistent with that currently being used within the TRIGGER study (NCT02704520) which is evaluating the role of mrTRG as a biomarker to stratify the management of LARC patients according to mrTRG determined response.[95]

For patients proceeding directly to surgery, resected specimens were also assessed by pathological Mandard TRG which most closely resembles the mrTRG (***Table 2.3***).

Table 2.3 Comparison between MRI and Mandard's pathological tumour regression grading system

Tumour Regression Grade	MRI	Mandard
1	complete radiological response (linear scar only)	complete regression (=fibrosis without detectable tissue of tumour)
2	good response (dense fibrosis, no obvious tumour signal)	fibrosis with scattered tumour cells
3	moderate response (>50% fibrosis and visible intermediate signal)	fibrosis and tumour cells with preponderance of fibrosis
4	slight response (mostly tumour)	fibrosis and tumour cells with preponderance of tumour cells
5	no response/regrowth of tumour	tissue of tumour without changes of regression

2.2.2 Identification of somatic mutations in tumour tissue

Tumour tissue was available for sequencing from 52 patients. DNA was extracted from formalin fixed paraffin embedded (FFPE) tumour tissue and NGS libraries were prepared as previously described.[96] DNA extracts with a concentration <6.7 ng/µl were concentrated using the DNA Clean and Concentrate Kit (Zymo Research, Irvine, USA), re-quantified and subjected to an additional four cycles of PCR during library preparation.

Target enrichment was performed on the multiplexed DNA pools using a gastrointestinal-specific custom capture panel (Roche) utilised in clinical trials at the Royal Marsden NHS Hospital.[96] The captured DNA pools were amplified for 11 PCR cycles and quantified using the KAPA Library

Quantification Kit (Roche) on a 7500 Fast Real-Time PCR instrument (Thermo Fisher) prior to sequencing on either a NextSeq or MiSeq platform (Illumina, San Diego, USA).

For five biopsies and one resection, where either the sample failed capture NGS, or the histopathologist deemed the FFPE sections to have insufficient tumour content for capture NGS, a custom TruSight CRC amplicon NGS protocol (Illumina) targeting hotspots in *KRAS*, *NRAS*, *BRAF*, *PIK3CA* and *TP53* was performed as an alternative.

Sequencing data was de-multiplexed and aligned to the human reference genome build GRch37 using either MiSeq Reporter software v2.5.1 or a combination of Bcl2fastq v.2.19 (Illumina) and BWA-mem v.0.7.12 depending on the platform used for sequencing. The resulting variant call files (VCF) were annotated using Illumina Variant Studio v2.2 and filtered using a bed file to concentrate on the genes of interest (*KRAS*, *NRAS*, *BRAF*, *PIK3CA*, *APC* and *TP53*). A somatic variant was considered to be present if it had a variant allele frequency (VAF) ≥5%.

For organ preservation patients experiencing local re-growth, the full Gastrointestinal panel was used for the analysis.[96] Variants were manually visualised in Integrated Genomics Viewer (IGV) v2.3. The variant with the highest VAF in tumour tissue was tracked in the corresponding cfDNA by ddPCR, with up to two additional variants tracked per patient where discovered.

The sequencing results from the diagnostic biopsy were used to determine suitable variants to track in plasma in all but 2 cases. The resection was used when there was insufficient biopsy tissue or sequencing failure of the biopsy. In eight cases, where paired results from the diagnostic biopsy and the resection were available, the genomic profile exhibited strong concordance suggesting that it would be acceptable to use the resection tissue of treated patients where the diagnostic biopsy is unsuitable.

2.2.3 Ki-67 analysis in tumour tissue

Immunohistochemical staining of Ki-67 was performed using an autostainer (Ventana Ultra, Ventana Medical Systems, AZ, USA). Each section was cut, dry baked, deparaffinised, underwent heat-induced antigen retrieval and incubated with the MIB-1 clone (Agilent) according to a standard protocol. An experienced pathologist interpreted results with the Ki-67 labeling index (defined as number of cancer cell nuclei showing positive staining/total number of cancer cell nuclei × 100%).

2.2.4 ctDNA analysis

Blood samples were collected in Cell-Free DNA blood collection tubes (Streck, LaVista, USA) and centrifuged twice at 1,600 x g for 10 min. The plasma was then aliquoted and stored at -80°C until cfDNA purification. cfDNA was purified from plasma using the QIAamp Circulating Nucleic Acid Kit or QIAsymphony Circulating DNA Kit (Qiagen). The cfDNA extracts were quantified using the Qubit dsDNA HS Assay Kit, and cfDNA content was assessed using Genomic DNA Screen Tape. ddPCR assays were ordered

as Custom TaqMan SNP Genotyping Assays (Thermo Fisher) and validated by ddPCR on either tumour tissue or Tru-Q mutant blends (Horizon, Cambridge, UK) containing the variant of interest as well as fragmented Male Human Genomic DNA (Promega, Madison, USA) prior to running on the cfDNA extracts (***Figure 2.2***).

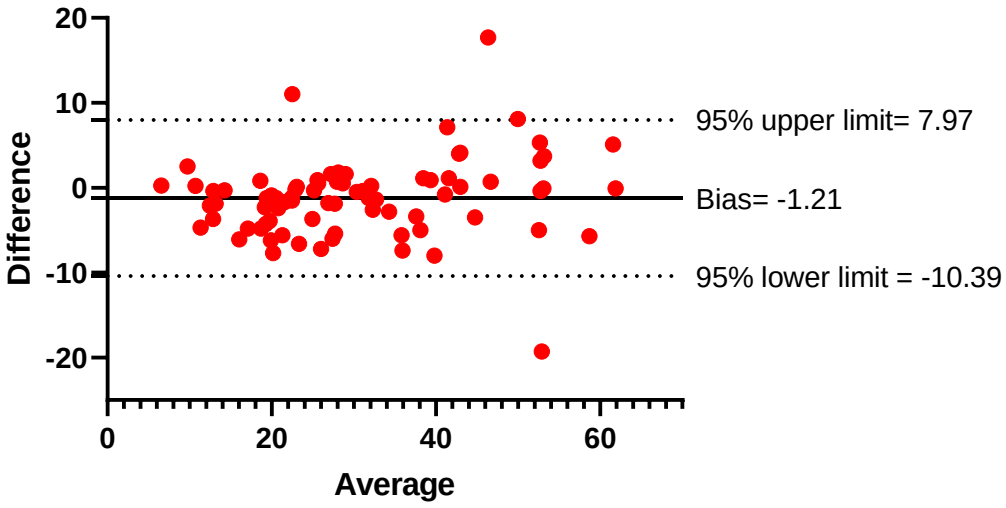


Figure 2.2 Trueness: Bland Altman plot to compare ddPCR assays on tumour tissue with NGS results for the same variants in tumour tissue
*Difference refers to the difference in results yielded by NGS and ddPCR for the same variant and this is plotted against the average of the two values.

31/74 assays were tested for limit of detection by spiking tumour DNA or Tru-Q blends into 10-fold serial dilutions of Promega DNA followed by ddPCR. The limit of detection ranged from 0.00116 – 2.66 % (median 0.0426%).

ddPCR was performed using the QX200 Droplet Digital PCR System (Bio-Rad, Hercules, USA) as per the manufacturer's instruction. The ddPCR mixes were partitioned into a median ~40,000 droplets per variant (Bio-Rad) prior to PCR on a C1000 Touch (Bio-Rad) or Veriti (Thermo Fisher) thermal cycle using the protocol: 95 °C for 10 min; 94 °C for 30 sec/variable annealing and extension temperature for 1 min for 40 cycles; 98 °C for 10

min. A median of 2.5 ml of plasma equivalent was screened per plasma time-point collected. Every run contained a positive control, negative control and a no template control for each variant being analysed. The majority of cfDNA extracts were run in duplicate on ddPCR and this data was used to calculate the precision of the method in 12 baseline samples selected at random (***Figure 2.3***). In instances where the sample had to be diluted prior to ddPCR because of high DNA concentration, the cfDNA was run in additional wells. For these samples, the precision data was calculated using the first two replicates run.

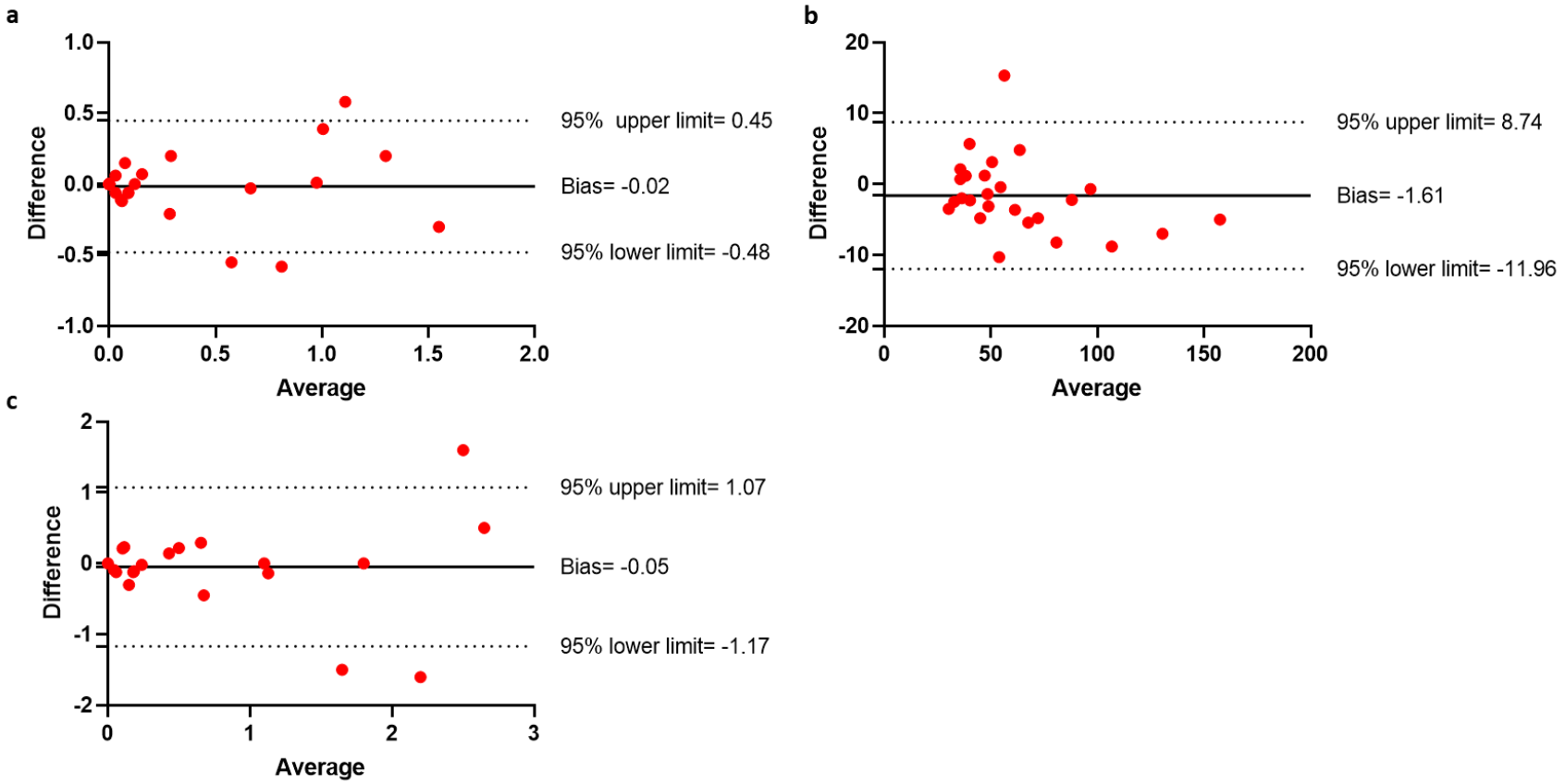


Figure 2.3 Precision: Bland Altman plots of replicates for (a) mutant concentration in copies/µl, (b) wild-type concentration in copies/µl and (c) mutant fractional abundance (%)

*Difference refers to the difference in results yielded by the replicates and this is plotted against the average of the two values.

The amplified ddPCR reactions were kept at 4 °C before being read on a QX200 Droplet Reader (Bio-Rad) (***Figure 2.4***). The ddPCR data was analysed using QuantaSoft v1.7.4 software to calculate the mutant copies per droplet, fractional abundance (FA) and mutant copies per millilitre of plasma.[97] FA % was expressed as the proportion of mutant alleles in the total cfDNA (mutant and wild-type DNA).

Mutant copies per droplet (Mcd) were calculated from the Poisson distribution using the formula:

$$Mcd = -\ln[1 - (Md/t)]$$

where Md is the number of mutant-positive droplets and t is the total number of droplets generated. To calculate the mutant copies per millilitre of plasma (Mcmp) the number of mutant-positive droplets was adjusted for the number of wells run, the volume of plasma equivalent run, the total number of droplets generated, and the median size of droplet (0.89 nl) using the formula:

$$Mcmp = \frac{[Md \times 20{,}000 \times \text{No. PCR replicates})/VPE]}{(t \times 0.89)}$$

As defined by others, a plasma time-point was designated positive if a minimum of two mutant-positive droplets were present for at least one variant with a minimum of half of the total droplets that could theoretically be generated per variant screened being valid.[97] Additional plasma equivalent was run if less than half of the total droplets that could be generated per variant screened were valid.

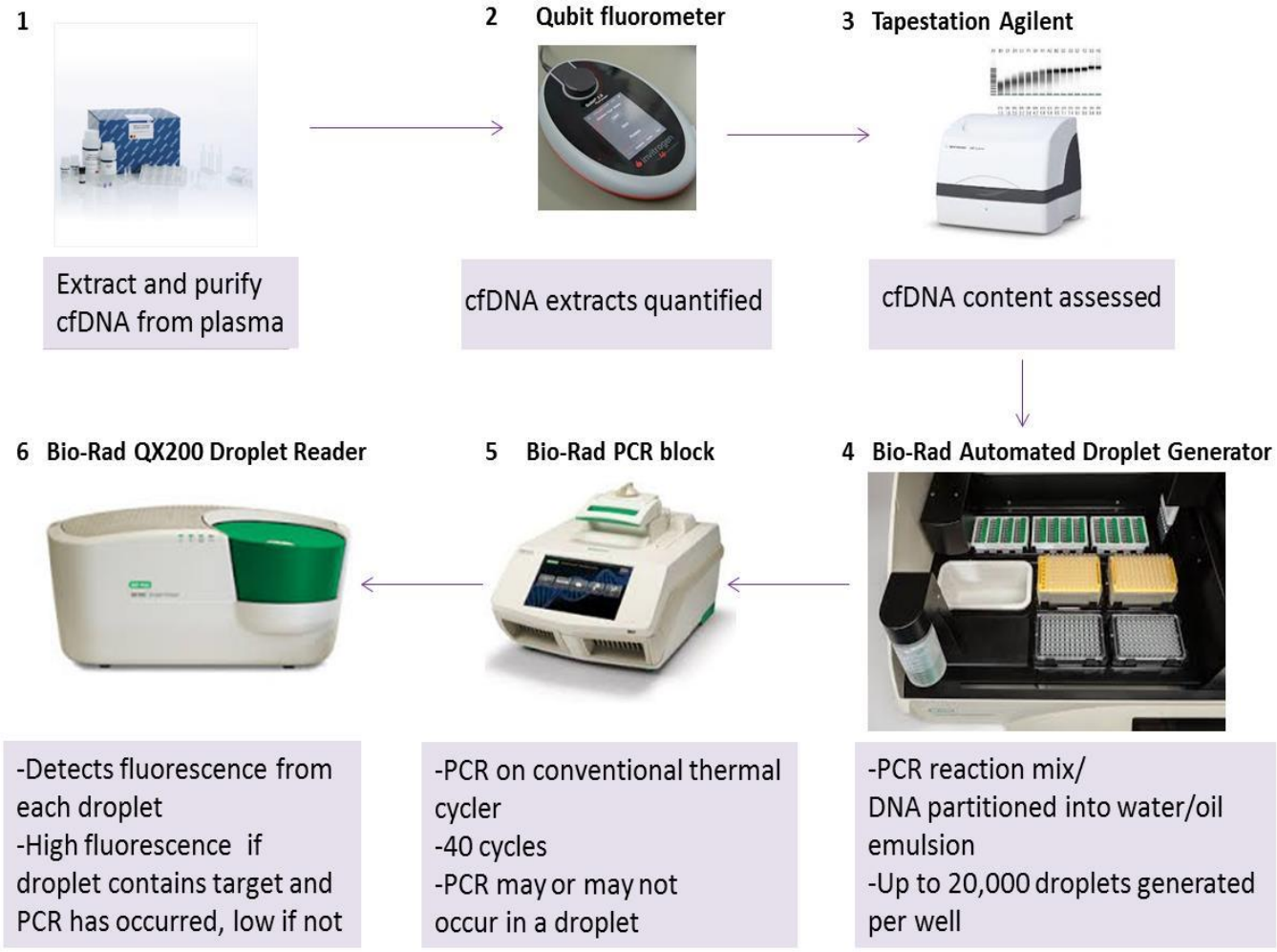


Figure 2.4 Summarising the droplet digital PCR workflow

2.2.5 Statistical analysis

Fisher's exact test was used to assess differences in clinical characteristics or radiological response between patients with undetectable and detectable ctDNA at each time-point. The Mann Whitney test was used for continuous variables. Spearman's rank correlation was used to check for an association between ctDNA level pre-treatment and Ki-67 or pre-treatment radiological lesion size. If ctDNA from more than one variant was present, the highest detectable value for all analyses was used.

Primary outcome was radiological response by ctDNA detectability per time-point. Other outcome measures were metastases free survival (MFS), DFS, local recurrence free survival (LRFS), and OS. MFS was measured from study entry to development of metastases or death from any cause and was censored at the last follow up. DFS was measured from date of surgery until relapse or death from any cause and was censored at the last follow up. LRFS was measured from end of CRT for patients proceeding with an organ preservation approach until local tumour re-growth or death from any cause and was censored at the last follow up. Re-growth was determined by date of histological confirmation from biopsy or when unavailable or non-confirmatory, date of MRI suggestive of re-growth was used if re-growth was subsequently confirmed on histopathology after surgery. OS was measured from study entry to death from any cause or censored by last follow up if alive.

The Kaplan-Meier method was used for the survival estimates whilst Cox proportional hazards models were used to compare the survival rates between groups and to estimate the hazard ratios. All analyses were performed using Stata software (version 13.1) where *P* values <0.05 (2-sided) were considered significant.

2.3 Results

2.3.1 Patient characteristics

47 patients were evaluable for ctDNA and primary tumour response analysis on MRI by RECIST and mrTRG (***Figure 2.5***). Baseline characteristics are summarised in ***Table 2.4***. Median age was 59 years (range 30-83). Forty-six patients (98%) were CDX2 positive and 45 (96%) were proficient for mismatch repair.

On completion of CRT, 8/47 (17%) patients had developed metastases with a further 3 developing metastases after surgery. 32% of patients (n=15/47) proceeded with an organ preservation approach following evidence of mrTRG 1 or 2. A post-surgery blood sample (prior to any adjuvant chemotherapy) was available in 23 patients.

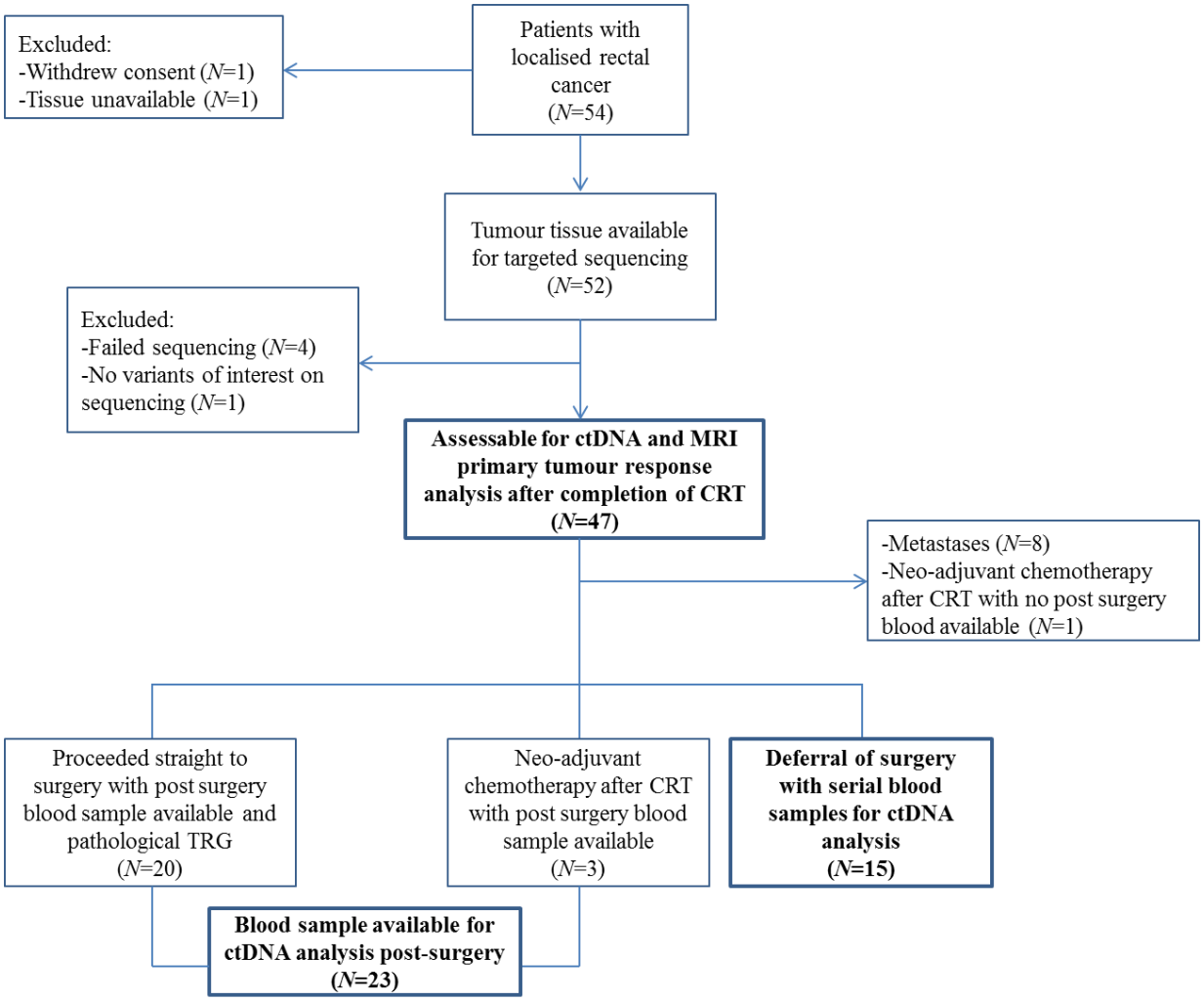


Figure 2.5 Flow of patients for each analysis from the SSGCC cohort.

The bold boxes highlight distinct populations with specific treatment pathways where analysis was conducted at key time-points.

Table 2.4 Clinical characteristics and MRI response by ctDNA status

	Pre-CRT ctDNA (n=47)			**Mid CRT ctDNA** (n=47)			**End of CRT ctDNA** (n=47)			**Post-surgery ctDNA** (n=23)		
Variable	+ve (*N*=35)	-ve (*N*=12)	*P*	+ve (*N*=10)	-ve (*N*=37)	*P*	+ve (*N*=10)	-ve (*N*=37)	*P*	+ve (*N*=3)	-ve (*N*=20)	*P*
Age, years												
Median	62	57.5	0.37	57	59	0.42	57.5	59	0.69	50	59	0.52
IQR (p25-p75)	50-66	47-60		40-64	51-66		48-64	51-66		37-66	49-65.5	
Gender, n (%)												
Male	22 (63)	7 (58)	1.00	8 (80)	21 (57)	0.28	8 (80)	21 (57)	0.28	2 (67)	11 (55)	1.00
Female	13 (37)	5 (42)		2 (20)	16 (43)		2 (20)	16 (43)		1 (33)	9 (45)	
Baseline MRI EMVI status, n (%)												
Positive	29 (83)	9 (75)	0.67	10(100)	28 (76)	0.17	10(100)	28 (76)	0.17	3(100)	17(85)	1.00
Negative	6 (17)	3 (25)		0	9 (24)		0	9 (24)		0	3 (15)	
Baseline MRI CRM status, n (%)												
Involved	23 (66)	6 (50)	0.48	7 (70)	22 (59)	0.34	6 (60)	23 (62)	0.76	3 (100)	12 (60)	0.64
Threatened	5 (14)	1 (8)		2 (20)	4 (11)		2 (20)	4 (11)		0	2 (10)	
Safe	7 (20)	5 (42)		1 (10)	11 (30)		2 (20)	10 (27)		0	6 (30)	
Distance from anal verge in cm, n (%)												
≤5	9 (26)	3 (25)	1.00	3 (30)	9 (24)	0.70	2 (20)	10 (27)	1.00	1 (33)	4 (20)	0.54
>5	26 (74)	9 (75)		7 (70)	28 (76)		8 (80)	27 (73)		2 (67)	16 (80)	
Stage, n (%)												
I-II	3 (9)	3 (25)	0.16	1 (10)	5 (14)	1.00	1 (10)	5 (14)	1.00	0	1 (5)	1.00
III	32 (91)	9 (75)		9 (90)	32 (86)		9 (90)	32 (86)		3 (100)	19 (95)	

cT stage, n (%)												
0-2	2 (6)	2 (17)	0.27	0	4 (11)	0.56	0	4 (11)	0.56	0	0	-
3-4	33 (94)	10 (83)		10(100)	33 (89)		10(100)	33 (89)		3(100)	20(100)	
c N stage, n (%)												
0	3 (9)	3 (25)	0.16	1 (10)	5 (14)	1.00	1 (10)	5 (14)	1.00	0	1 (5)	1.00
≥1	32 (91)	9 (75)		9 (90)	32 (86)		9 (90)	32 (86)		3 (100)	19 (95)	
pT stage, n (%)												
0-2	7 (39)	2 (40)	1.00	2 (33)	7 (41)	1.00	0	9 (45)	0.25	0	9 (45)	0.25
3-4	11 (61)	3 (60)		4 (67)	10 (59)		3 (100)	11 (55)		3 (100)	11(55)	
pN stage, n (%)												
0	12 (67)	4 (80)	1.00	5 (83)	11 (65)	0.62	1 (33)	15 (75)	0.21	0	16 (80)	0.02
≥1	6 (33)	1(20)		1 (17)	6 (35)		2 (67)	5 (25)		3 (100)	4 (20)	
Baseline CEA in ug/l, n (%)*												
<5	19 (56)	11 (100)	0.008	7 (70)	23 (66)	1.00	6 (67)	24 (67)	1.00	2 (67)	14 (70)	1.00
≥5	15 (44)	0		3 (30)	12 (34)		3 (33)	12 (33)		1 (33)	6 (30)	
MRI response by RECIST												
Good responders (CR and PR)	27 (77)	10 (83)	1.00	7 (70)	30 (81)	0.42	8 (80)	29 (78)	1.00	3 (100)	15 (75)	1.00
Poor responders (SD and PD)	8 (23)	2 (17)		3 (30)	7 (19)		2 (20)	8 (22)		0	5 (25)	
MRI TRG response												
Good responders (TRG 1-2)	14 (40)	6 (50)	0.74	3 (30)	17 (46)	0.48	1 (10)	19 (51)	0.03	0	7 (35)	0.53
Poor responders (TRG 3-5)	21 (60)	6 (50)		7 (70)	20 (54)		9 (90)	18 (49)		3 (100)	13 (65)	
Developed Metastases, n (%)												
No	25 (71)	11 (92)	0.24	6 (60)	30 (81)	0.21	3 (30)	33 (89)	<0.001	0	20 (100)	0.001
Yes	10 (29)	1 (8)		4 (40)	7 (19)		7 (70)	4 (11)		3 (100)	0	

* Baseline CEA was unavailable for 2 patients
Abbreviations: IQR=interquartile range, +ve=ctDNA detectable, -ve=ctDNA undetectable

2.3.2 Mutation Analysis in tissue

The sequencing failure rate (where no results were available from the diagnostic biopsy or the resection) was 4/52 (8%).

At least one somatic mutation was identified in the genes of interest of 47/48 (98%) of successfully sequenced cases. The median number of mutations in tissue was 2 (range 0-5). A list of all the mutations in the genes of interest and detection rates is listed in the appendix (***Table 7.1***).

2.3.3 ctDNA detectability in blood

A median of 2 variants (range 1-3) were tracked in the plasma of each patient. Blood plasma was collected a median of: 6 days prior to commencing CRT (IQR 4-13), 21 days from the start of CRT (IQR 20-22), and 37 days from completion of CRT (IQR 34-41.5). The post-surgery blood sample was collected a median of 47 days from surgery (IQR 39.5-60.5). The detection rate for ctDNA was: 35/47 (74%) pre-treatment, 10/47 (21%) mid CRT, 10/47 (21%) at the end of CRT and 3/23 (13%) post-surgery.

Amongst the 15 CEA secretors, pre-treatment ctDNA was detectable in all cases compared with 19/30 (63%) non-CEA secretors, (P=0.008). No other baseline characteristics were significantly different between patients with detectable or undetectable ctDNA at any time-point (***Table 2.4***). However, pathological node positive patients on the resection specimen were more likely to have detectable ctDNA post-surgery than node negative patients (P=0.02).

2.3.4 ctDNA detection and CRT response assessment

Radiological response to CRT was assessed by MRI, a median of 31 days (interquartile range, IQR; 29-33.5) following completion of treatment. RECIST measurement of the primary tumour demonstrated that 37/47 (79%) of patients had a good response to treatment (CR n=6, PR n=31). The remaining patients had a poor response to treatment (SD n=9, PD n=1). There was no difference in response determined by RECIST between patients with detectable ctDNA and undetectable ctDNA at any time-point.

Pre-treatment, ctDNA was detectable in 3/5 (60%), 11/15 (73%), 10/13 (77%), 8/11 (73%), and 3/3 (100%) of patients with mrTRG 1, 2, 3, 4 and 5 respectively. Poor responders were more likely to have detectable ctDNA on completion of CRT than good responders (33%, n=9/27 compared with 5%, n=1/20, p=0.03). There was no difference in mrTRG response between patients with detectable and undetectable ctDNA at any other time-point (***Table 2.4***).

Pathological complete response (pCR) was reported in 3/23 (13%) patients and in all 3 of these cases, ctDNA was detectable pre-treatment and became undetectable from mid CRT onwards (***Figure 2.6***). All 3 of these cases had mrTRG2 and consistent with the literature, likely had on-going regression during the interval between MRI assessment and surgery which subsequently resulted in pCR.[98, 99]

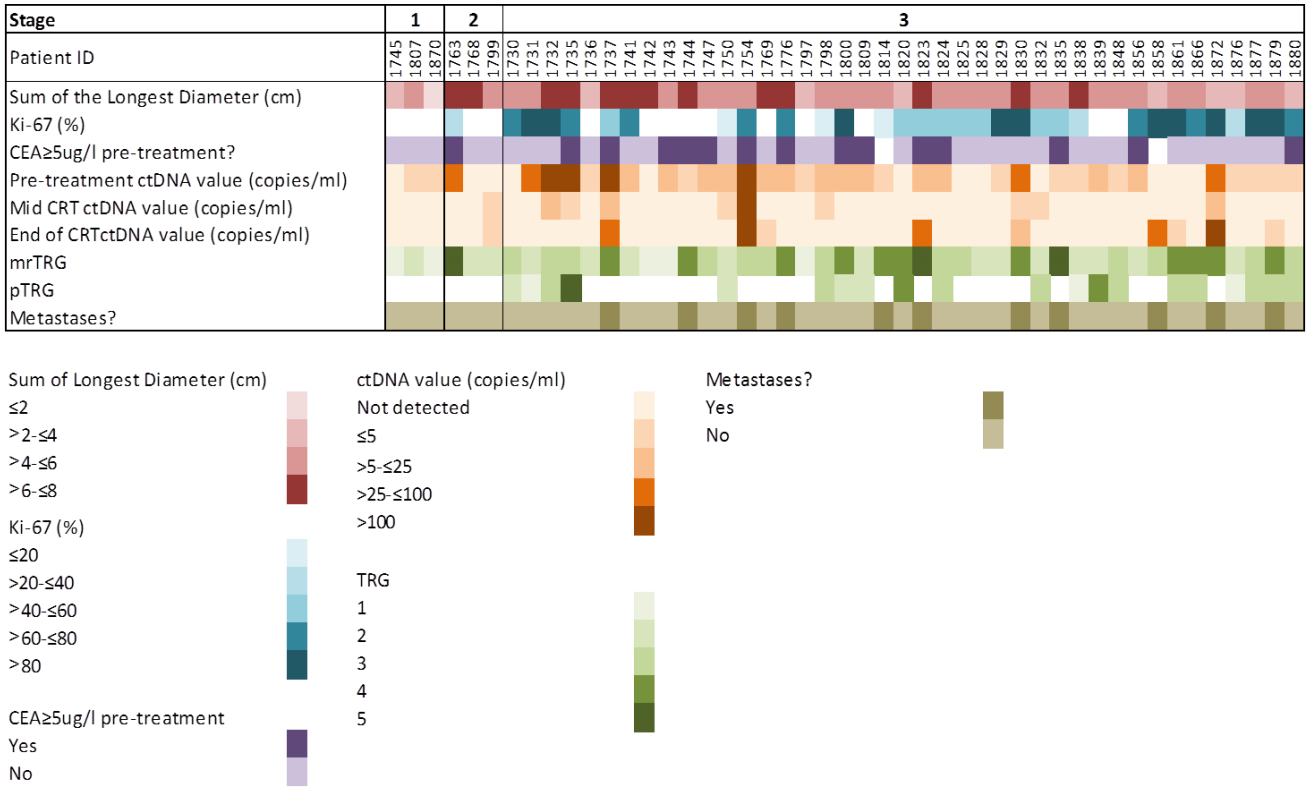


Figure 2.6 Heat map showing the relationship between stage, lesion size, Ki-67, ctDNA value and clinical outcome

There was adequate diagnostic biopsy tissue for Ki-67 analysis in 32 cases. There was no significant difference in the mean Ki-67 value between patients with detectable ctDNA pre-treatment (64%, SD 26.4) and those with undetectable ctDNA (66%, SD 18.4). Ki-67 results were available in 24 (69%) of the 35 patients that had detectable ctDNA pre-treatment. Spearman's rank correlation revealed no association between Ki-67 and baseline ctDNA level in copies/ml (r_S=0·20; p=0·35). Of the 20 patients with both mrTRG and pathological TRG by Mandard available, 11 were poor responders (TRG 3-5) by both methods and 5 were good responders (TRG 1-2) by both methods leading to a concordance rate of 80%. Of the 4 discrepant cases, mrTRG underestimated the degree of response in 2 cases and over-estimated it in the other 2 cases.
Blank boxes= no data available

2.3.5 Baseline ctDNA value and primary lesion size

There was no significant difference in the pre-treatment lesion size by RECIST between those with detectable and undetectable ctDNA (z= -0.78, *P*=0.45). Receiver operator characteristic (ROC) analysis was unable to find an optimal pre-treatment lesion size threshold to predict ctDNA detectability pre-treatment (Area under the curve; AUC 0.58, 95% CI 0.39-0.77). In patients with detectable ctDNA pre-treatment (n=35), there was a weak positive correlation between pre-treatment lesion size and quantity of ctDNA in copies/ml (r_S=0.51) and FA (r_S=0.42, ***Figure 2.7***).

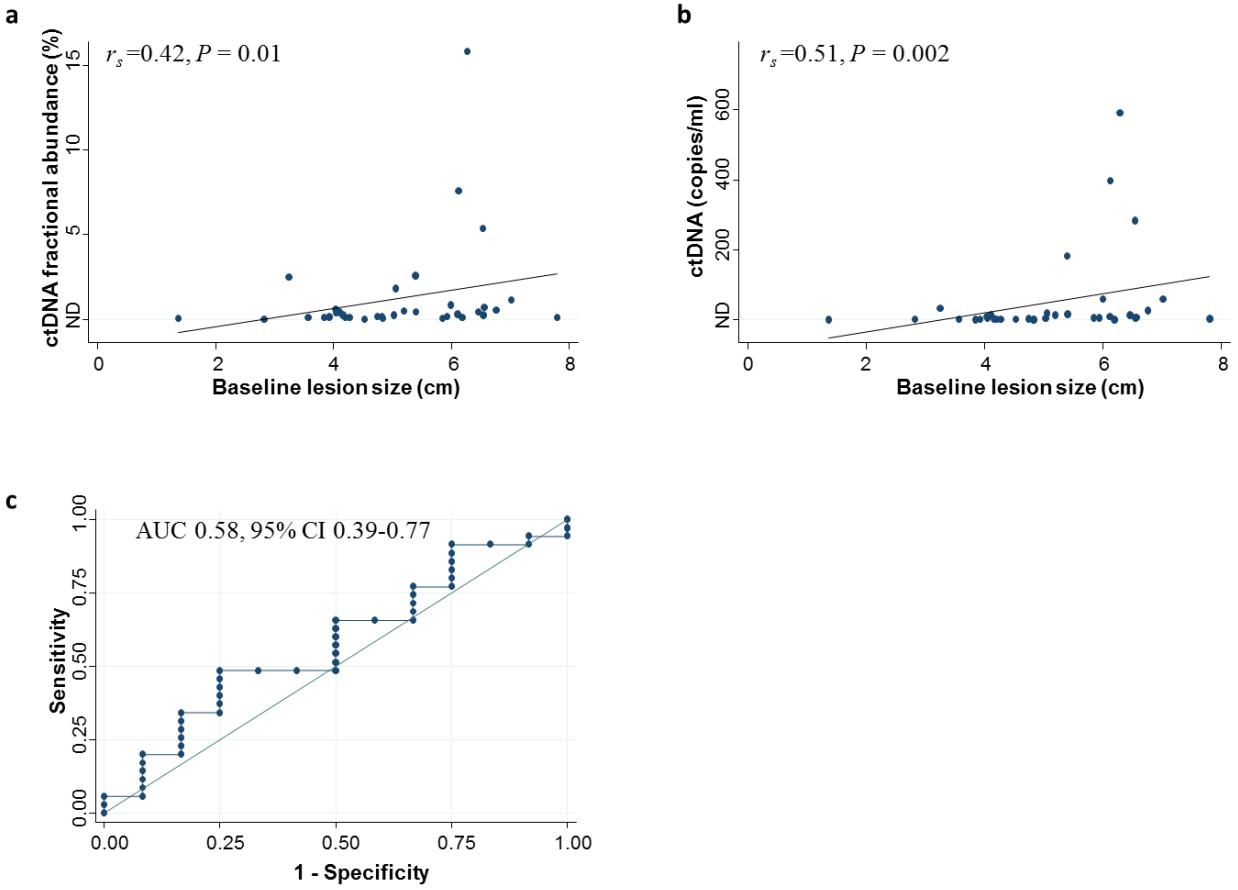
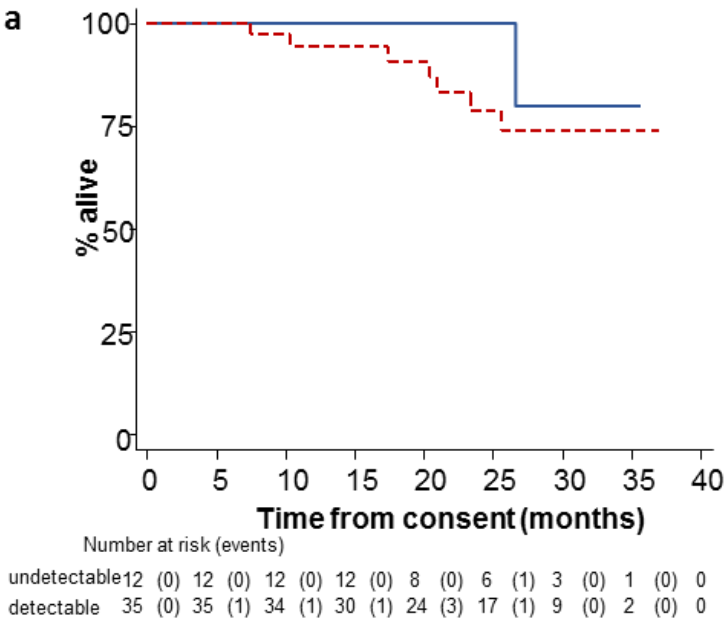


Figure 2.7 The relationship between pre-treatment lesion size and quantity of pre-treatment ctDNA in (a) fractional abundance (b) copies/ml and (c) ROC analysis to assess whether pre-treatment lesion size is a predictor of ctDNA detectability

2.3.6 ctDNA detectability and survival analyses

At the time of analysis, median follow up was 26·4 months (IQR 19.7-31.3) and 8/47 (17%) patients had died. OS data are immature but preliminary data can be seen in (***Figure 2.8***).

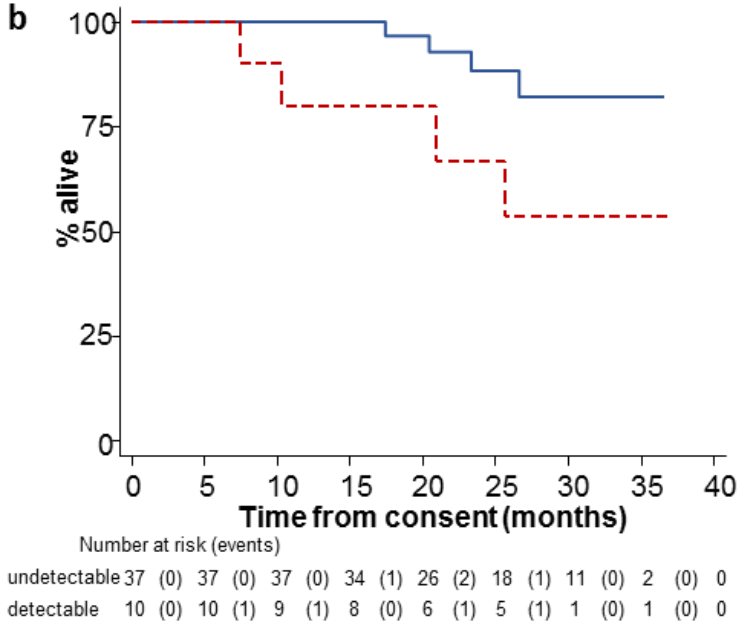
Of the 11 patients that developed metastases, ctDNA detection at the end of CRT was higher (n=7, 64%) compared with those that did not (n=3/36, 8%, *P*<0.001). Detection of ctDNA pre-treatment that persisted at the mid CRT time-point was also higher in patients that developed metastases (n=4/11, 36%) compared to those that did not (n=4/36, 11%; *P*=0.07).



***P* = 0.42 HR 2.3 (95% CI: 0.3-19.1)**

Number of events / Total:
undetectable: 1 / 12
detectable: 7 / 35

ctDNA undetectable
ctDNA detectable



***P* = 0.06 HR 3.8 (95% CI: 0.9-15.4)**

Number of events / Total:
undetectable: 4 / 37
detectable: 4 / 10

ctDNA undetectable
ctDNA detectable

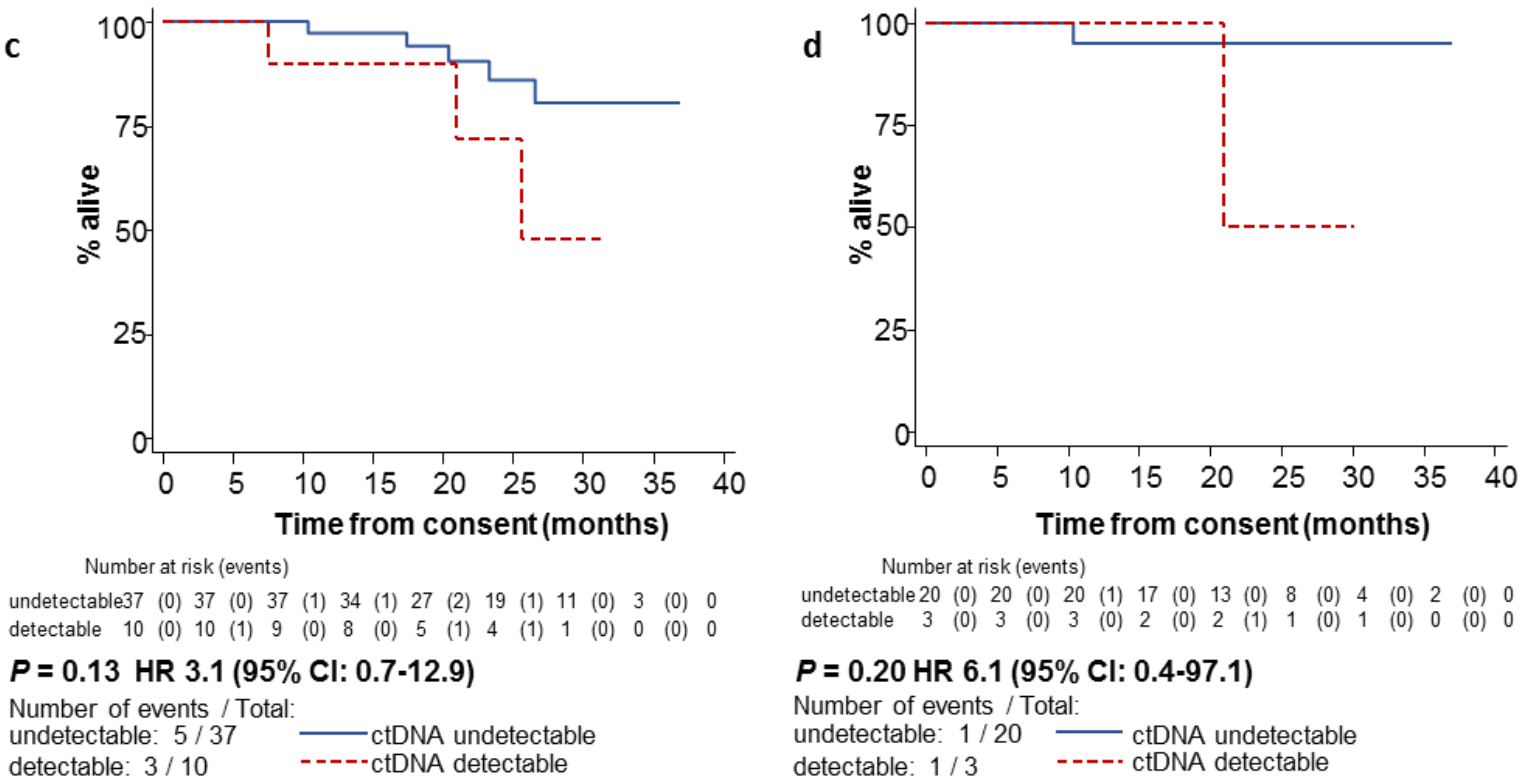


Figure 2.8 Kaplan-Meier estimates of overall survival by ctDNA status (a) pre-treatment, (b) mid CRT (c) on completion of CRT and (d) post-surgery

In line with previous observations, there was no difference in MFS by patients with detectable or undetectable ctDNA pre-treatment (HR 2.1; 95% CI 0.5-9.6, *P*=0.33; ***Figure 2.9***).[94] This was also true for the mid CRT time-point (HR 2.6; 95% CI 0.9-8.1, *P*=0.09; ***Figure 2.9***). MFS was significantly shorter in patients with detectable ctDNA on completion of CRT compared with patients with undetectable ctDNA (HR 7.1; 2.4-21.5, *P*<0.001; ***Figure 2.9***).

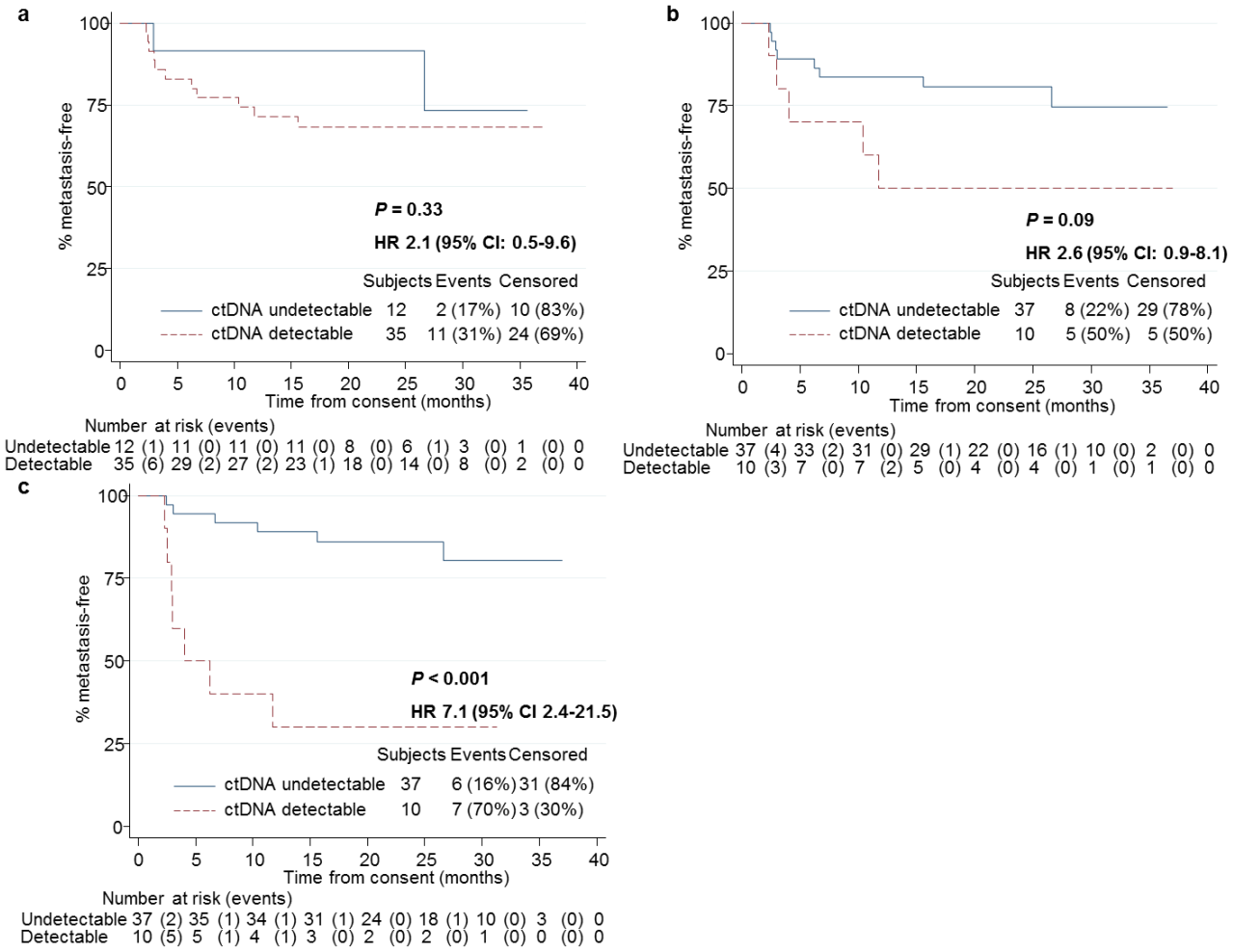


Figure 2.9 Kaplan-Meier estimates of metastases-free survival by ctDNA status: (a) pre-treatment, (b) mid CRT and (c) on completion of CRT

Persistence of detectable ctDNA from pre-treatment to the mid CRT time-point was associated with worse MFS compared with cases where ctDNA was not detected both pre-treatment and at the mid CRT time-point (HR 3.8; 95% CI 1.2-11.7, *P*=0.02; ***Figure 2.10***). Similarly, persistence of detectable ctDNA from pre-treatment to the end of CRT was also associated with worse MFS compared with cases where ctDNA did not persist throughout (HR 11.5; 95% CI 3.3-40.4, *P*<0.001; ***Figure 2.10***).

Two examples of patients developing metastases by the end of CRT are depicted below along with their ctDNA levels, CEA and radiological images (***Figure 2.11***).

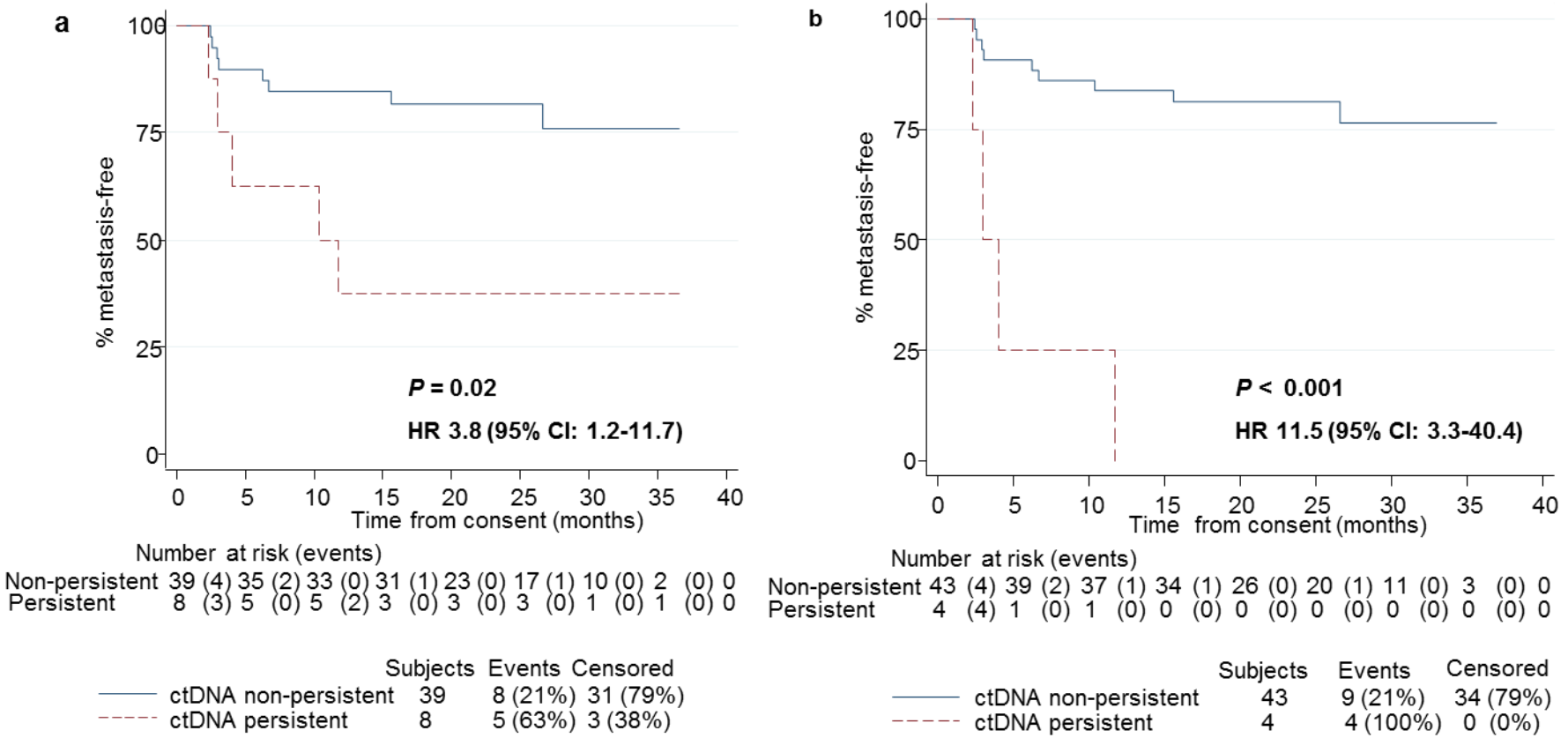
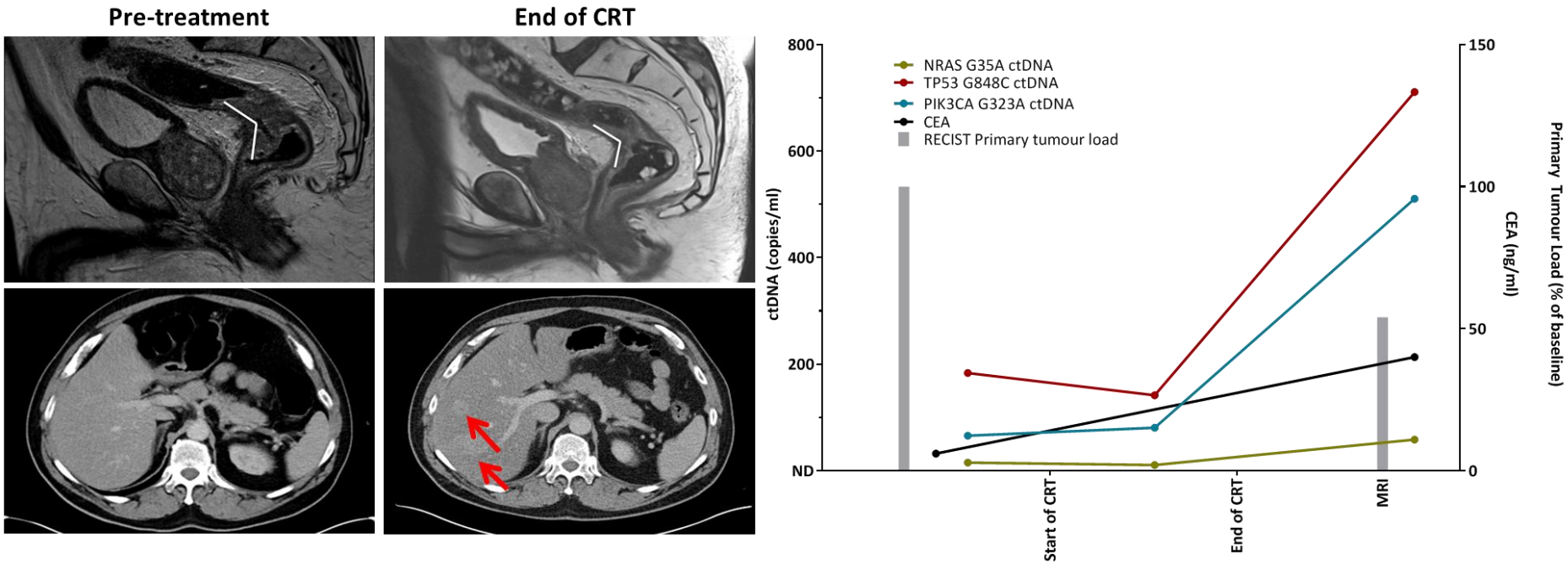


Figure 2.10 Kaplan-Meier estimates of metastases-free survival by ctDNA persistence (a) pre-treatment and mid CRT (b) pre-treatment, mid CRT and end of CRT compared to non-persistence

a


Pre-treatment
End of CRT
NRAS G35A ctDNA
TP53 G848C ctDNA
PIK3CA G323A ctDNA
CEA
RECIST Primary tumour load
ctDNA (copies/ml)
800
600
400
200
ND
150
100
50
0
CEA (ng/ml)
Primary Tumour Load (% of baseline)
Start of CRT
End of CRT
MRI

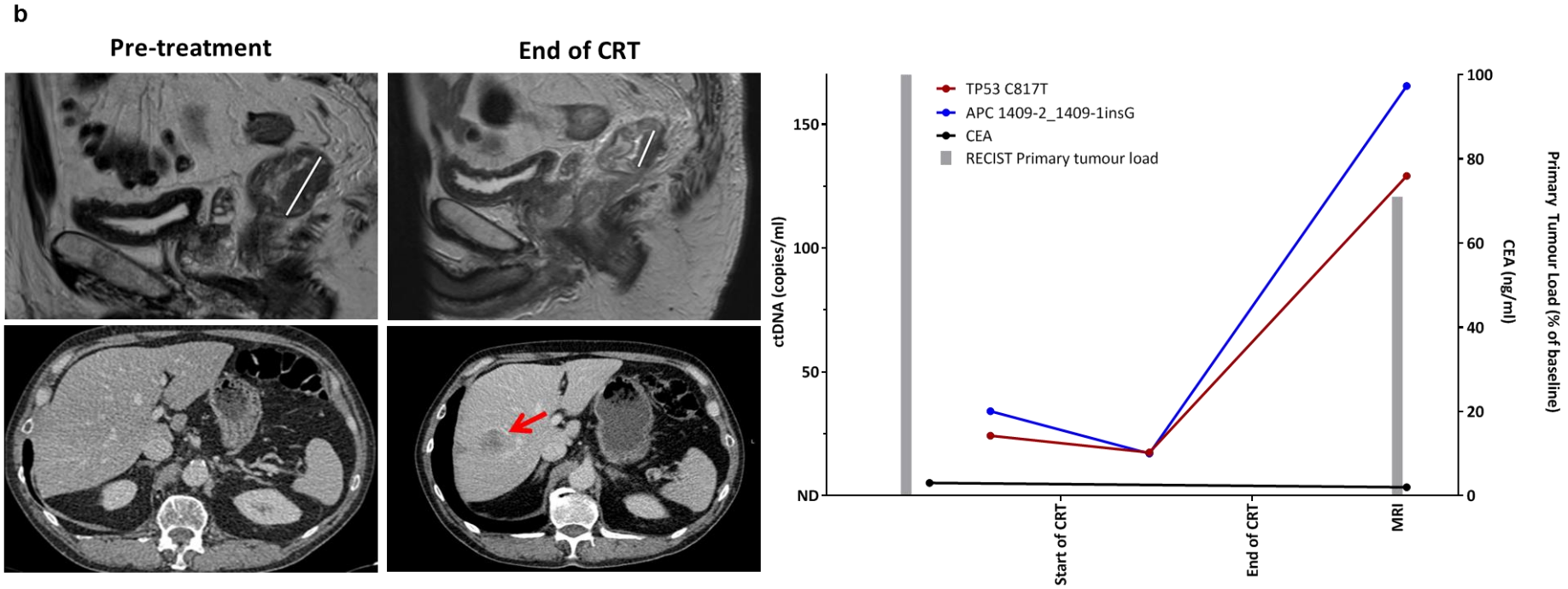


Figure 2.11 Graphs showing ctDNA detectability and CEA over time in 2 patients developing metastases on completion of CRT along with corresponding radiological images (a) the patient had a partial response in the primary tumour but developed liver metastases. The ctDNA levels dropped slightly at the mid CRT time-point and then showed a dynamic increase to levels higher than pre-treatment reflecting the development of metastases. The CEA also increased in this patient. (b) this patient had stable disease in the primary tumour but developed liver metastases. The ctDNA levels showed a slight drop by mid CRT followed by a sharp rise reflecting the development of liver metastases. The CEA was unhelpful in this patient.

For the patients proceeding straight to surgery, the post-surgery sample had detectable ctDNA in all 3 patients that relapsed following surgery and was undetectable in the 20 patients who did not relapse (*P*=0·001). Of the 20 patients with undetectable ctDNA following surgery, 1 patient died during follow up but the cause of death was unknown. Patients developed metastases a mean of 128 days from surgery (standard deviation, SD 82.4). ctDNA was detected a mean of 78 days (SD 53.0) prior to confirmation of relapse on imaging in the post-surgery sample of these patients. DFS was significantly shorter in the 3 patients with detectable ctDNA post-surgery compared to the 20 patients with undetectable ctDNA (HR 39.9; 95% CI 4.0-399.5; *P*=0.002; ***Figure 2.12***). Of the 3 patients with detectable ctDNA, 2 patients had an R1 resection compared to 1 patient amongst the 20 ctDNA negative patients. Most patients (21/23) received adjuvant chemotherapy following surgery.

In patients deemed suitable for an organ preservation approach (n=15), 10 patients had local re-growth of their tumour a median of 11 months from the end of CRT (IQR 6.25-13.5). LRFS was shorter in patients with detectable ctDNA at the end of CRT compared with patients with undetectable ctDNA (HR 5.8; 95% CI 0.9-35.3; *P*=0.06; ***Figure 2.12***).

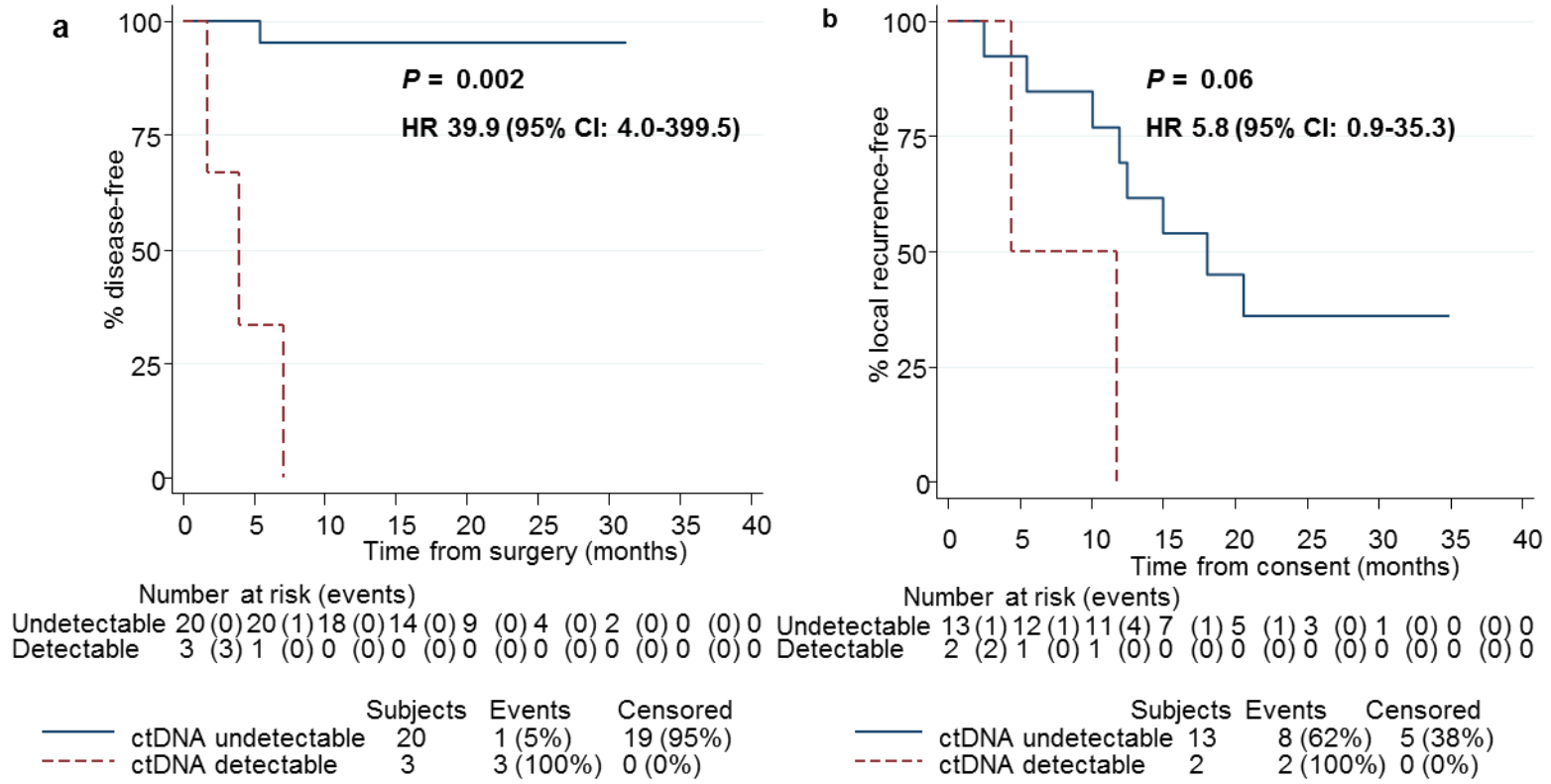


Figure 2.12 Kaplan-Meier estimates of (a) disease free survival and (b) local recurrence free survival in organ preservation patients by ctDNA status

2.3.7 ctDNA quantification to predict development of metastatic disease

In patients with detectable ctDNA, the FA was compared between patients who went on to develop metastases with those who did not using the Mann Whitney U test (***Figure 2.13***). There was no difference in the distribution of FA pre-treatment for patients that developed metastases (median 0.48%, IQR 0.12-2.5%) from those that did not (median 0.27%; IQR 0.1-0.5%; z = -1.24, *P*= 0.21). However, the mid CRT FA was significantly higher in patients that developed metastases (median 0.8%, IQR 0.4-4.6%) compared to those that did not (median 0.03%, IQR 0.02-0.07%; z = -2.46, *P*=0.01). Similarly, the FA at the end of CRT was also significantly higher in patients that developed metastases (median 2.3%, IQR 0.2-7.4%) compared to those that did not (median 0.08%, IQR 0.01-0.2%; z = -2.17, *P*=0.03). A ROC analysis was performed to assess whether FA is a good predictor for developing metastases (***Figure 2.13***). The pre-treatment FA was not a good marker to discriminate between patients that developed metastases from those that did not (AUC= 0.64, 95% CI: 0.42 to 0.85). However, with a sensitivity of 100% and a specificity of 83.3%, a FA threshold of ≥0.07% at the mid CRT time-point correctly predicted 90% of the patients that went on to develop metastases (AUC= 0.98, 95% CI: 0.92 to 1). Similarly, with a sensitivity of 100% and a specificity of 66.7%, a FA threshold of ≥0.13% at the end of CRT correctly predicted 90% of the patients that developed metastases (AUC= 0.95, 95% CI: 0.82 to 1).

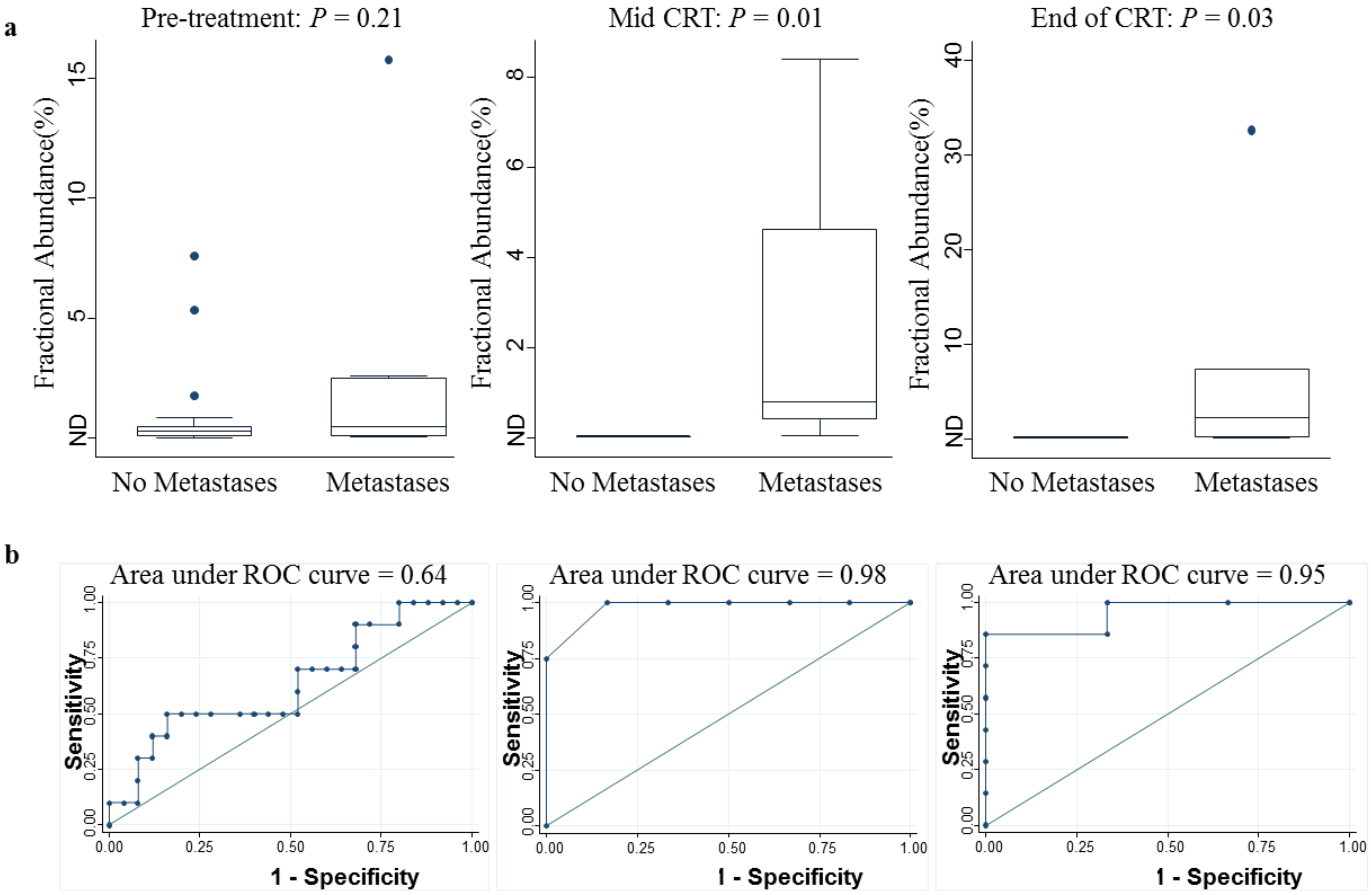


Figure 2.13 (a) Distribution of fractional abundance in patients with detectable ctDNA by whether they developed metastases at each time-point (b) ROC analysis for ctDNA fractional abundance to predict development of metastases at each time-point
Abbreviations: ND=not detected, ROC=receiver operator characteristic

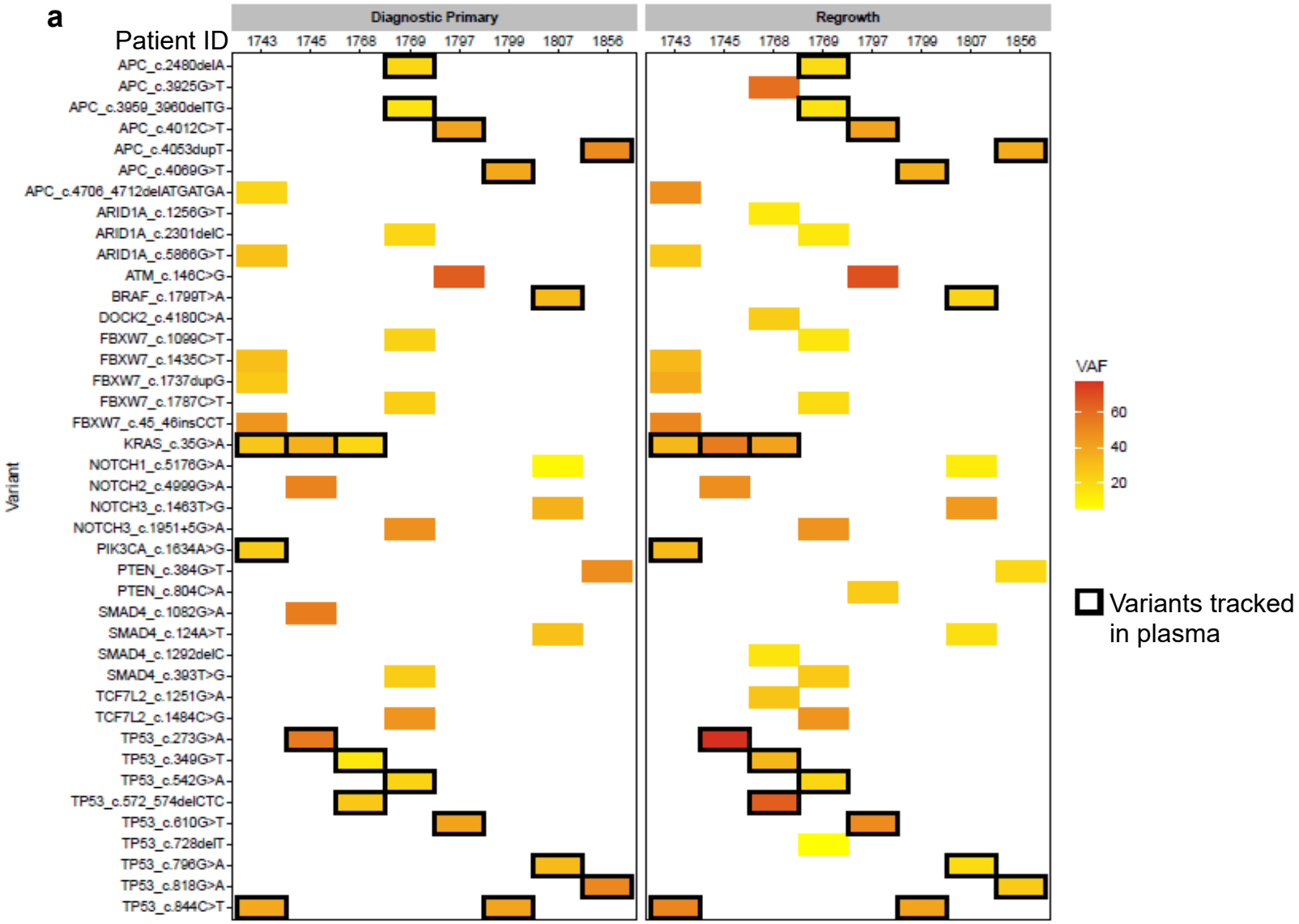
2.3.8 ctDNA and detection of local re-growth in organ preservation patients

Plasma was available a median of 4.5 days (IQR -15.5-18) from the point of confirmed re-growth (n=10). ctDNA was only detectable in one case of re-growth (***Table 2.5***).

The tissue from re-growth was therefore sequenced and compared to the diagnostic biopsy to assess whether the mutation profile had changed to account for the low ctDNA detection rate (***Figure 2.14***). 8 matching tissue pairs were available. Although some new variants emerged, variants that were originally selected to be tracked in plasma still persisted in the re-growth tissue at a sufficiently high VAF in all the patients.

Table 2.5 Clinical characteristics of organ preservation patients and association with ctDNA status

Trial ID	Baseline clinical TNM			Baseline ctDNA detectable?	Re-growth	ctDNA detectable at re-growth?	Pathological stage post-surgery	
	T	N	M				T	N
1736	3c	1c	0	Yes	No			
1741	3c	2	0	Yes	Yes	No	2	0
1742	2	1	0	No	No			
1743	3b	1	0	Yes	Yes	No	3	0
1745	1	0	0	No	Yes	No	3	0
1747	3c	1	0	Yes	No			
1768	4	0	0	No	Yes	No	3	0
1769	4	1c	0	Yes	Yes	No	Unfit for surgery	
1797	3	1	0	Yes	Yes	No	3	0
1799	3d	0	0	No	Yes	No	3	1
1807	2	0	0	Yes	Yes	No	3	0
1825	3b	1c	0	No	Yes	No	3	1
1828	3c	1c	0	No	No			
1856	3c	1c	0	Yes	Yes	Yes	3	2
1870	2	0	0	Yes	No			


a
Patient ID
Diagnostic Primary
Regrowth
1743
1745
1768
1769
1797
1799
1807
1856
Variant
APC_c.2480delA
APC_c.3925G>T
APC_c.3959_3960delTG
APC_c.4012C>T
APC_c.4053dupT
APC_c.4069G>T
APC_c.4706_4712delATGATGA
ARID1A_c.1256G>T
ARID1A_c.2301delC
ARID1A_c.5866G>T
ATM_c.146C>G
BRAF_c.1799T>A
DOCK2_c.4180C>A
FBXW7_c.1099C>T
FBXW7_c.1435C>T
FBXW7_c.1737dupG
FBXW7_c.1787C>T
FBXW7_c.45_46insCCT
KRAS_c.35G>A
NOTCH1_c.5176G>A
NOTCH2_c.4999G>A
NOTCH3_c.1463T>G
NOTCH3_c.1951+5G>A
PIK3CA_c.1634A>G
PTEN_c.384G>T
PTEN_c.804C>A
SMAD4_c.1082G>A
SMAD4_c.124A>T
SMAD4_c.1292delC
SMAD4_c.393T>G
TCF7L2_c.1251G>A
TCF7L2_c.1484C>G
TP53_c.273G>A
TP53_c.349G>T
TP53_c.542G>A
TP53_c.572_574delCTC
TP53_c.610G>T
TP53_c.728delT
TP53_c.796G>A
TP53_c.818G>A
TP53_c.844C>T
VAF
60
40
20
Variants tracked in plasma

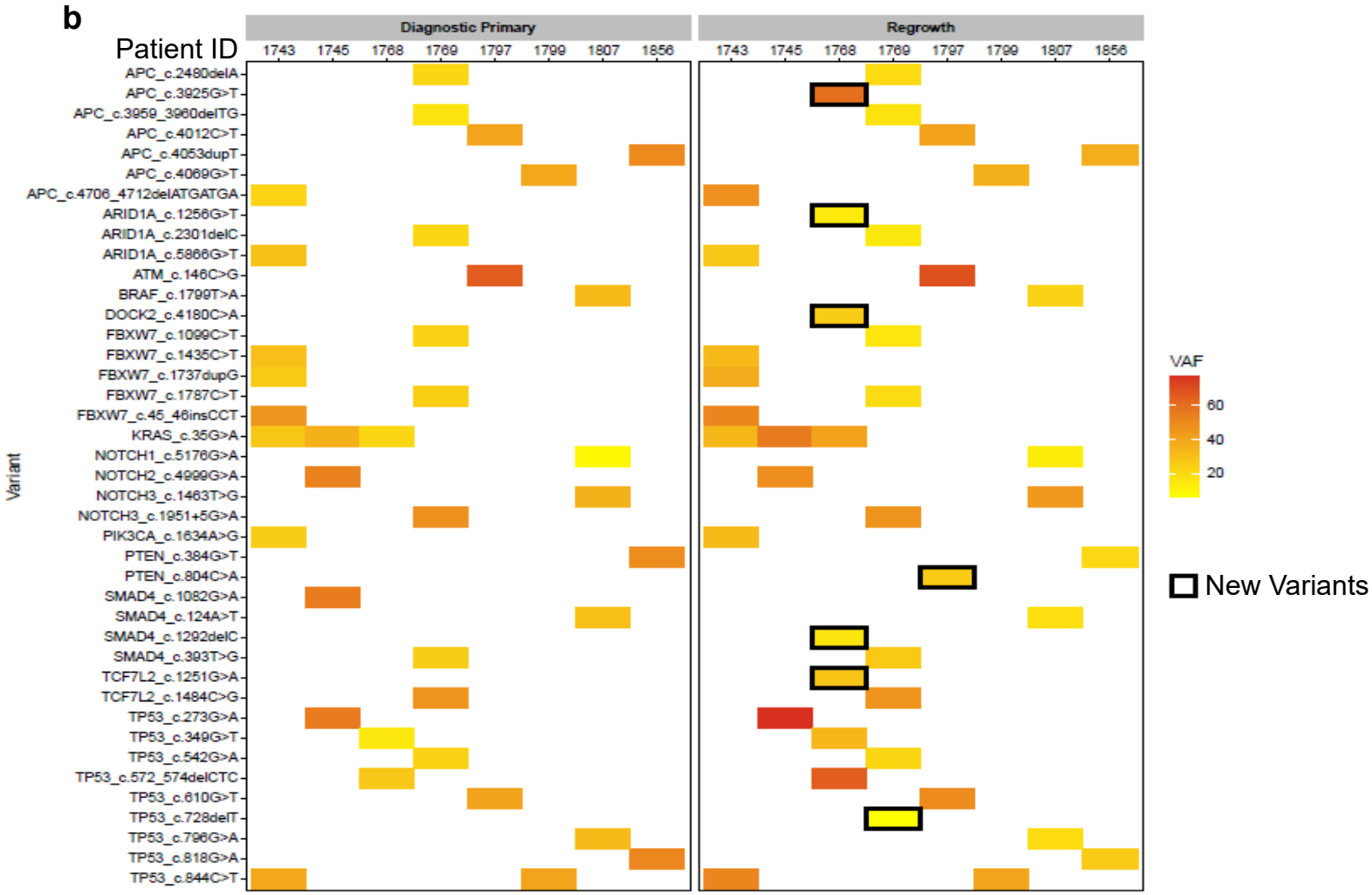


Figure 2.14 Full 46 gene panel sequencing tissue results comparing the diagnostic biopsy and re-growth tissue. VAF=variant allele frequency. (a) The tracked variants determined from the diagnostic biopsy were still present in the re-growth sample at a high enough VAF to be detectable in plasma. (b) New variants emerged in 3 out of 8 re-growth samples but the original variants being tracked were still present and should have been detectable in plasma.

2.4 Discussion

Both mrTRG and ctDNA have attracted a significant amount of research interest although as yet, both are unvalidated biomarkers. Here, we are the first to report that in localised rectal cancer, there is an association between ctDNA status after completing CRT and response determined by mrTRG(*P*=0.03). This is primarily driven by the fact that patients with a poor response to CRT were much more likely to have detectable ctDNA following completion of CRT.

Additionally, our results confirm that ctDNA is a good surrogate for the emergence of systemic disease with 70% of patients with detectable ctDNA at the end of CRT and all patients with detectable ctDNA post-surgery developing metastatic disease.[62, 72, 93] We also found that MFS was significantly shorter in patients with persistently detectable ctDNA in the neo-adjuvant period or in patients with detectable ctDNA at the end of CRT irrespective of other time-points. Our data suggests that in patients with detectable ctDNA at the mid or end of CRT time-points, the FA quantity should also be assessed to predict the patients that will go on to develop metastases. This strategy may help select patients that could benefit from treatment intensification or alternative treatment strategies in an attempt to prevent the development of metastases. Most patients with rectal cancer require surgery after CRT which implies that there is still some tumour activity present, even if at low levels. This may explain why patients with low FA do not have metastases. Our results are consistent with a previous

report in localised lung cancer patients where a threshold of >0.1% during radiotherapy was associated with progression.[100]

There were 3 patients with detectable ctDNA on completion of CRT that have not developed metastases within the specified follow up period. Two of these patients proceeded with an organ preservation approach and both had local re-growth of their tumours. Our data, although limited by numbers, suggests that detection of ctDNA at the end of CRT in patients otherwise presumed to be good candidates for organ preservation, may predict local re-growth. Therefore ctDNA could be a complementary tool to MRI and endoscopic evaluation and detect patients at risk of re-growth amongst those initially felt to be suitable for an organ preservation strategy. However, undetectable ctDNA at the end of CRT was unhelpful in this regard as 8 out of the 13 patients with undetectable ctDNA had evidence of local re-growth (although it should be noted that ctDNA was not detected at any time-point in 3 of these cases). Larger prospective studies will be needed to confirm these findings.

We also assessed whether ctDNA was detectable around the time of local re-growth in organ preservation patients. Only 1 patient had detectable ctDNA out of the 10 patients experiencing a local re-growth. The low detection rate could not be attributed to a change in the mutation profile from the diagnostic biopsy to the re-growth. It is likely to be related to the sensitivity of our current technique for the presence of very limited disease. In support of this, ctDNA is less likely to be detectable in cases of lower tumour volume or loco-regional recurrence and detection rates appear to

decrease with earlier disease stages.[60–62, 101] Our data also suggests that ctDNA is more likely to be detectable in node positive disease on pathology (*P*=0·02) and this association has also reported by others.[62] Interestingly, the one case where ctDNA was detectable at the time of re-growth had the highest nodal burden (N2 disease).

Our pre-treatment ctDNA detection rate of 74% was comparable to the published literature.[60–62] However, the incorporation of universal methylation methods as described by others may improve sensitivity.[102–104] Our end of treatment detection rate of 21% was however higher and likely reflects the fact that we had several patients that developed systemic progression soon after completing CRT. We speculate that the high progression rate may be due to the fact that a significant proportion of our patients had poor prognostic features at baseline. For example, 81% were MRI detected-EMVI (mrEMVI) positive and this has previously been associated with a higher rate of development of distant metastases compared to mrEMVI negative cases.[105]

It remains unclear why ctDNA consistently remains undetectable in approximately 25% of treatment naïve patients with localised disease. While difficulty detecting pre-treatment ctDNA may reflect a less aggressive or smaller tumour, our results do not support this as patients with undetectable ctDNA pre-treatment did not have prolonged survival or smaller pre-treatment lesions compared to those with detectable ctDNA. Moreover, unlike in early lung cancer, the level of the proliferation marker Ki-67 did not

differentiate between ctDNA detectable and undetectable cases in our patients (***Figure 2.6***).[101] Our data is limited in that we did not consider the ratio of proliferation to apoptosis or necrosis and both mechanisms of cell death have been implicated in the pathophysiology for ctDNA release.[58, 106] Failure to detect ctDNA might in part be related to technical issues despite using ddPCR, a highly sensitive technique. Indeed, we tracked up to 3 variants in plasma, of which one had the highest VAF in tissue. If only the variant with the highest VAF in tissue had been tracked in plasma, our pre-treatment detection rate would have dropped to 64% (n=30). Our data, as well as the findings of others, suggests that at least 2 variants should be tracked in plasma where possible.[76] Another possible explanation for the inability to detect ctDNA pre-treatment in some cases may be that ddPCR assays were only based on mutations in 6 genes of interest as they are known to be common in colorectal cancer.[107–110] It is possible that the driver mutation may not have been in one of these genes. Our ctDNA analysis was restricted to the known mutation profile of the primary tumour which could be considered a limitation as clonal evolution cannot be studied. However, in colorectal cancer, primary tumours and metastases exhibit high genomic concordance and focusing on the known mutation profile in the tumour may minimise the risk of false positives particularly in light of recent reports identifying somatic mutations in cfDNA arising from clonal haematopoiesis.[111–114]

In order to implement the routine analysis of ctDNA into clinical practice, it is essential to establish in which patients it will be a useful marker and ensure

adequate turn-around times for reporting results. Our results showed that ctDNA was detectable in all CEA secretors pre-treatment as well as an additional 19 patients who were non-CEA secretors suggesting that it may be a better marker for serial monitoring. Our methodology required the design of multiple assays that were unique to individual patients particularly for variants in large genes such as *APC*. This was time-consuming and may limit incorporation of the methodology used here into routine clinical practice. However, improvements in sequencing technology with the ability for ultra-deep sequencing with error correction techniques to reduce false positives might be a viable alternative.[65]

Our study has several limitations, some of which are inherent to the observational design of the study, as well as the small sample size and the limited number of post-surgery samples which could be collected. However, this is also reflective of the real world setting where the potential treatment options for LARC following CRT can vary depending on response. Our study is strengthened by the fact that: patients were prospectively recruited for the purpose of these analyses and given that this was a single centre study, there was consistency in identifying suitable patients for recruitment as the same members of the MDT were involved. Additionally, all analyses were conducted in the same laboratory with uniform methodology and laboratory staff were blinded to the clinical status of patients whilst radiologists were blinded to the ctDNA results. Although the data should be interpreted with caution, the results presented here should be regarded as hypothesis

generating and may guide the direction of future study particularly given that risk adapted treatment strategies continue to be a research priority.

In conclusion, our findings support the notion that mrTRG and ctDNA are complementary tools to both assess local response and systemic disease status to guide therapy adaptation in rectal cancer. Prospective trials are in progress and will address the true value of incorporating such strategies into routine clinical practice. This work has been published in Clinical cancer research.[115]

3 DETECTION of ctDNA IN STAGE II AND III CRC

3.1 Background

The treatment of CRC incorporates the tumour-node-metastasis (TNM) status which takes into account the depth of tumour wall invasion and lymph node involvement and is associated with prognosis.[116] Several biomarkers including Immunoscore and multi-gene assays such as Oncotype DX, ColoPrint and ColDX have also demonstrated prognostic potential but are not routinely recommended due to their high cost and uncertainty regarding their value in predicting treatment benefit. There is a clinical unmet need for biomarkers that are both prognostic and predictive. ctDNA is emerging as one such candidate biomarker.[117–126]

3.1.1 TRACC study development

The Tracking mutations in cell free tumour DNA to predict relapse in early colorectal cancer (TRACC) study was conceived in 2014 (***Figure 3.1***) as data regarding the detection of ctDNA in a primarily stage II and III CRC population was limited at the time. Published studies had incorporated small numbers of heterogeneous populations and used varying ctDNA collection, processing and analysis methodologies which made data interpretation difficult. For example, in some cases serum had been collected although it is now recognised that plasma is a better medium, as it is subject to less contamination by genomic DNA.[127]

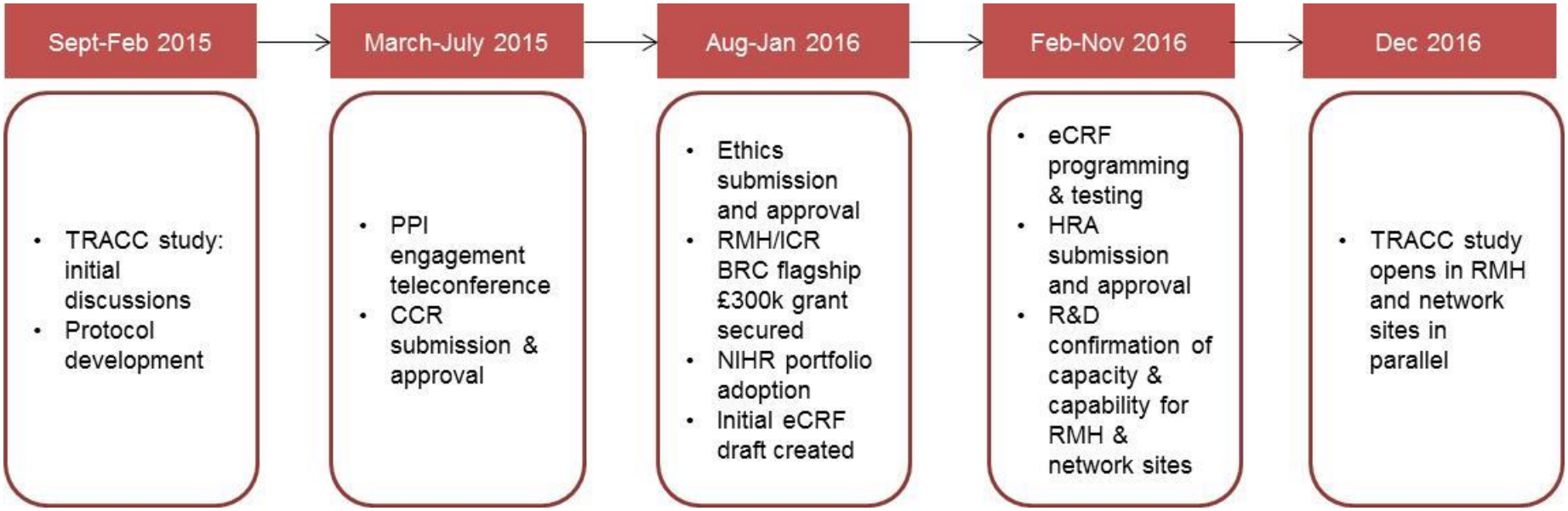


Figure 3.1 Key milestones in the set-up of the TRACC study
Abbreviations: PPI=patient and public involvement, CCR=committee for clinical research, RMH/ICR BRC=Royal Marsden Hospital/ Institute of Cancer Research biomedical research centre, NIHR=national institute for health research, eCRF=electronic case report form, HRA=health research authority, R&D= research and development

I wrote the TRACC study protocol and electronic case report forms, gathered patient and public involvement, took the study through ethical approval and national institute for health research (NIHR) adoption and have been running the study with the support of the TRACC study team and chief investigator. We achieved a flagship BRC grant and additional funding through Professor Cunningham's NIHR senior investigator award. I have set-up a network of 20 liquid biopsy centres and supported the development of standard operating procedures to ensure quality for the routine acquisition of plasma samples and tumour tissue to allow centralised processing at the centre for molecular pathology (***Figure 3.2***).

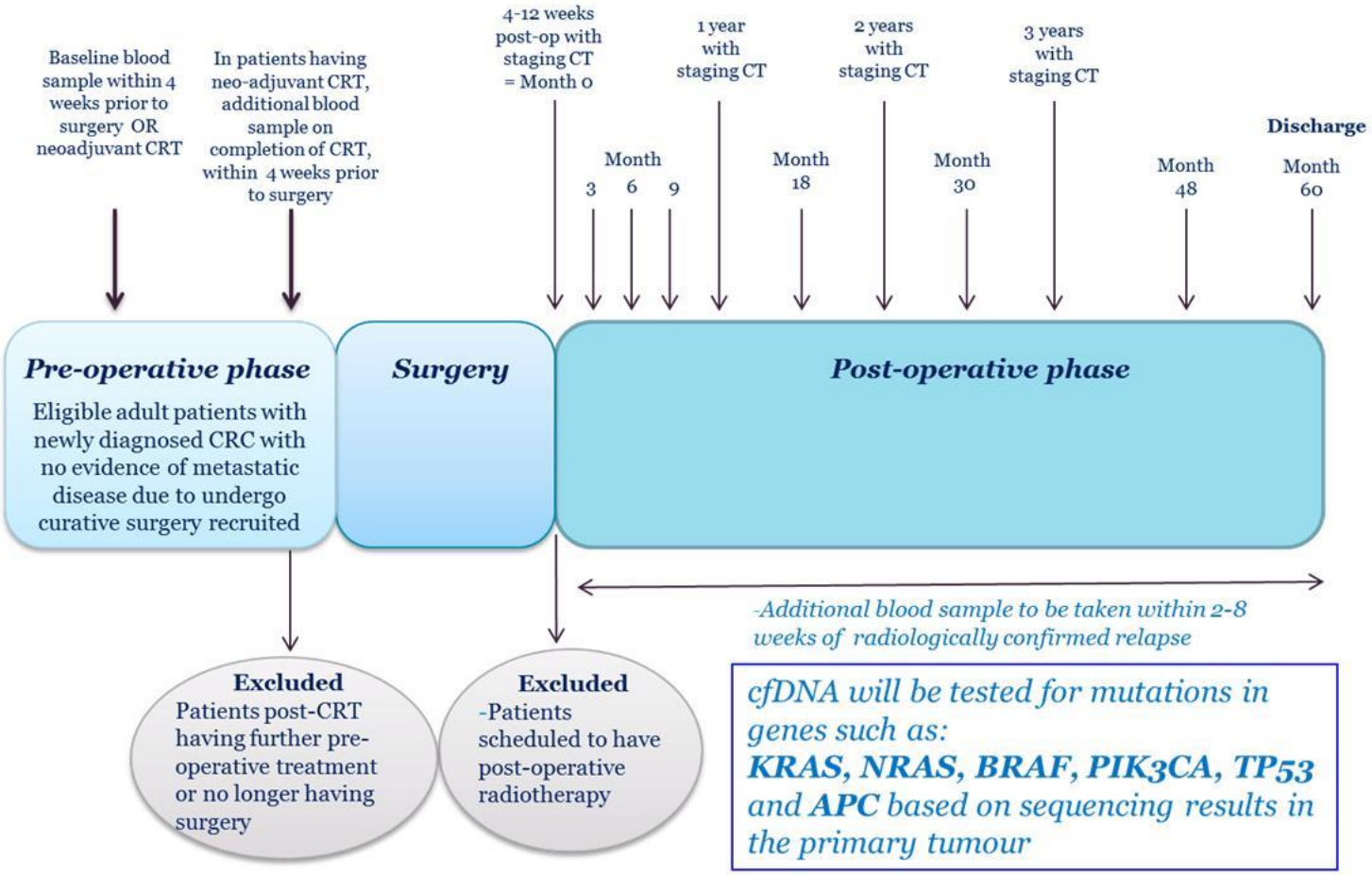


Figure 3.2 TRACC study trial schema with time-points for blood collection and CT scans

The TRACC protocol encompasses a feasibility component (part A) which relates to the first 48 patients (24 stage II and 24 stage III) that were evaluable for the presence of ctDNA pre-operatively and within 4-12 weeks post-operatively and is the focus of this chapter. The main aims of the feasibility study were:

1. To assess the detectability of ctDNA pre-operatively and post-operatively in patients with stage II and stage III CRC and if present, relate it to disease stage

2. To assess whether detection of ctDNA pre and post-surgery is associated with clinical characteristics and outcome

3. To evaluate whether the quantity of pre-treatment ctDNA is associated with the development of metastases and establish a ctDNA threshold if a relationship exists

The key differences between the TRACC feasibility study and the work already described in the previous chapter include the multi-centre recruitment of patients and acquisition of samples, as well as the inclusion of patients with colon cancer in addition to rectal cancer. The role of the feasibility study was to verify that the pre-operative ctDNA detection rate is sufficient for on-going recruitment into part B of the study which is a 1000 patient, UK, multi-centre study where the association between detectable ctDNA at the first post-operative visit and DFS is being investigated.

3.2 Methods

3.2.1 Study design, participants and procedures

From December 2016 when the study opened to recruitment, patients aged ≥18 years, with a new diagnosis of histologically confirmed CRC, scheduled to undergo surgery with no radiological evidence of metastatic disease from were eligible. Patients with another active or prior invasive malignancy within 5 years were not eligible. Patients receiving neoadjuvant chemotherapy, those with stage I disease, patients with a synchronous primary CRC or those not proceeding to surgery following CRT were excluded from the feasibility analysis. For patients being treated with CRT, staging was based on baseline radiological stage. In all other cases, pathology was used to derive stage.

Blood samples were collected at baseline within 4 weeks of surgery or within 4 weeks prior to the start of CRT, on completion of CRT, 4-12 weeks post-surgery and within 2-8 weeks of relapse. Blood samples were collected at additional time-points (***Figure 3.2***) but were not the focus of the feasibility study.

CT to confirm the absence of metastatic disease was carried out at baseline and post-operatively. In patients with rectal cancer, an MRI scan was used for local staging and to assess response if CRT was given. All ctDNA analyses were conducted by individuals blinded to the clinical status of patients. All radiologists reporting scans on patients in the TRACC study were blinded to the ctDNA results.

3.2.2 DNA extraction and quantification from tumour tissue

2 x 3 µM sections and 5 x 10 µM sections were cut from formalin-fixed paraffin embedded (FFPE) biopsies and surgical re-sections and fixed onto microscope slides. The 3 µM sections were stained with haematoxylin and eosin and assessed for tumour content, cellularity and necrosis by a histopathologist. The unstained slides were deparaffinised in xylene and washed in 100% and then 70% ethanol. The tumour areas were macrodissected for tumour cell enrichment. Tumour DNA was extracted using the QIAamp FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was quantified using the Qubit dsDNA HS or BR Assay Kit (Thermo Fisher, Waltham, USA) and the degree of fragmentation assessed using a 2200 Tapestation (Agilent, Santa Clara, USA). DNA extracts with a concentration <6.7 ng/µl were concentrated using the DNA Clean and Concentrate Kit (Zymo Research, Irvine, USA) and re-quantified prior to NGS library preparation. Patients' matching blood controls were extracted using the QIAsymphony DSP DNA Midi Kit and quantified using the Qubit dsDNA BR Assay Kit.

3.2.3 Processing of plasma and extraction and quantification of cfDNA

Blood samples were collected in Cell-Free DNA BCT tubes (Streck, LaVista, USA) and centrifuged twice at 1,600 x g for 10 min. Plasma was aliquoted and stored at -80°C until DNA extraction. cfDNA was extracted from 3.25-4 ml (mean 3.98 ml) of plasma using the QIAsymphony Circulating DNA Kit (Qiagen) according to the manufacturer's instructions. The cfDNA extracts

was quantified using the Qubit dsDNA HS Assay Kit and cfDNA content assessed using a 2200 Tapestation instrument (Agilent, Santa Clara, USA).

3.2.4 Preparation of NGS libraries and sequencing

NGS libraries were prepared for matched tumour and blood control samples using the Kapa HyperPlus Kit (Roche, Basel, Switzerland). DNA input per was 200 ng if the average peak size was >1000 bp, or 400 ng if <1000 bp. Library clean-up and dual size selection were performed using AMPure XP clean-up beads (Beckman Coulter, Brea, USA). Libraries were amplified using 6 cycles of PCR for samples with a DNA input of 200 ng or 400 ng, or 10 cycles for samples with <200 ng input. The amplified libraries were cleaned up using SeqCap EZ Purification beads (Roche) and quantified by Qubit with the size distribution evaluated using High Sensitivity D1000 Screen Tape (Agilent). Target enrichment was performed on the multiplexed DNA pools using a gastrointestinal-specific custom capture panel (Roche) utilised in clinical trials at the Royal Marsden NHS Hospital. The SeqCap EZ Hybridization and Wash Kit (Roche) was used for panel hybridisation and clean-up. The captured DNA pool was amplified for 11 cycles followed by clean-up using SeqCap EZ Purification beads (Roche). The amplified DNA pool was quantified using Qubit and the size distribution assessed using Tapestation. Samples were sequenced on a NextSeq 500 (Illumina, San Diego, USA) according to the manufacturer's instructions.

3.2.5 NGS analysis

Analysis was performed on matched tumour and blood control samples using an in-house developed pipeline MDIMSv4 (Molecular Diagnostic Information

Management System v4.0) using the following bioinformatics software: demultiplexing was performed using bcl2fastq 2.17.1.14, reads were aligned using BWA *0.7.12* to the human reference genome build GRch37 (Hg19), single nucleotide variants and indels were called using Mutect2 as part of GATK 4.0.5.1, and variants were annotated with Oncotator v1.5.1.0. Variants were manually visualised in Integrated Genomics Viewer (IGV) v2.3. A somatic variant was accepted as being present if it had a VAF≥5%.

For patients being treated with neoadjuvant CRT, sequencing of the biopsy tissue was attempted in the first instance and if unsuccessful, the resection specimen was used. This approach was chosen in order to eliminate the possibility of CRT induced tissue alterations from compromising the results and also as some patients have a complete response to CRT which would leave no viable tumour tissue in the resection specimen. For all patients proceeding straight to surgery, the resection tissue was used.

3.2.6 ddPCR assay design and optimisation

ddPCR TaqMan assays were custom designed by Thermo Fisher or Integrated DNA Technologies (Coralville, Iowa, United States). Variant and wild-type probes were labelled with 6-FAM and VIC/HEX respectively. Assays were optimised using gradient PCR on a tumour sample confirmed to contain the variant of interest by NGS, and the false-positive droplet rate estimated using fragmented Human Genomic DNA: Male (Promega, Madison, USA).

3.2.7 Droplet Digital PCR

ddPCR was performed using the QX200 Droplet Digital PCR System (Bio-Rad, Hercules, USA). Two variants with the highest VAF discovered by NGS in the corresponding tumour sample were tracked in the cfDNA of all patients unless an assay could not be designed. In these 3 cases, the variant with the next highest VAF was chosen. In 1 case when there were 2 variants with equal 2nd highest VAF, the variant that was reported at a higher frequency in the Catalogue of Somatic Mutations In Cancer (COSMIC) was chosen to be tracked in plasma.

A median 1.9 ml volume of plasma equivalent was screened per variant per patient. cfDNA was added to a total volume of 22 µl in a ddPCR reaction containing 11 µl ddPCR Supermix for Probes (Bio-Rad) and 0.55 µl of 40x concentrated ddPCR assay. The final concentration of the primers and probes was 900 nM and 200 nM respectively. The ddPCR mixes were partitioned into a median ~100,000 droplets per variant on an Automated Droplet Generator (Bio-Rad) prior to PCR on a 96-well C1000 Touch (Bio-Rad) or Veriti (Thermo Fisher) thermal cycle using the protocol: 95 °C for 10 min; 94 °C for 30 sec/variable annealing and extension temperature for 1 min for 40 cycles; 98 °C for 10 min. The temperature ramp rate was set to 2°C/sec for all steps. Every run contained a positive control, negative control and a no template control for each variant being analysed. The amplified ddPCR reactions were kept at 4 °C before being read on a QX200 Droplet Reader (Bio-Rad).

All variants tracked in plasma were confirmed in the corresponding tumour DNA or diluted NGS library prep by ddPCR. The ddPCR FA showed good agreement with the NGS VAF with a limited bias and narrow agreement interval on a Bland Altman plot (***Figure 3.3***).

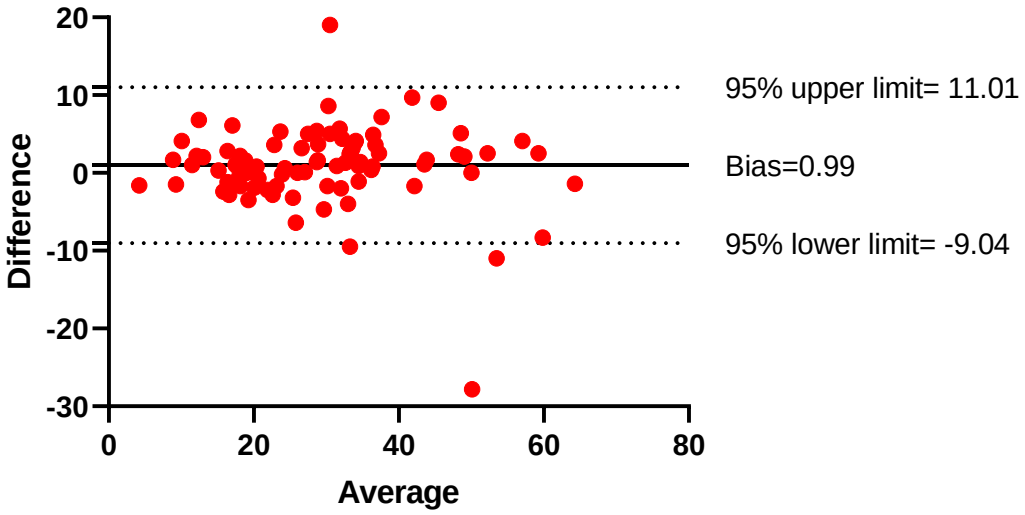


Figure 3.3 Trueness: Bland Altman plot to compare ddPCR assays on tumour tissue with NGS results for the same variants in tumour tissue.

*Difference refers to the difference in results yielded by NGS and ddPCR for the same variant and this is plotted against the average of the two values

The cfDNA extracts were run in triplicate for each variant being tracked and this data was used to calculate the precision of the method. In some cases, e.g. because of high concentration, the cfDNA was diluted and run in additional wells to ensure that the same volume of plasma was screened. For these samples, the precision data was calculated using the first two replicates run (***Figure 3.4***). As described in chapter 2, the ddPCR data was analysed using QuantaSoft v1.7.4 software and quantity of ctDNA was determined in copies/ml and FA. As described in chapter 2, 15 out of 66 assays were tested for limit of detection. The limit of detection ranged from 0.0012 – 0.19 % (median 0.038%).

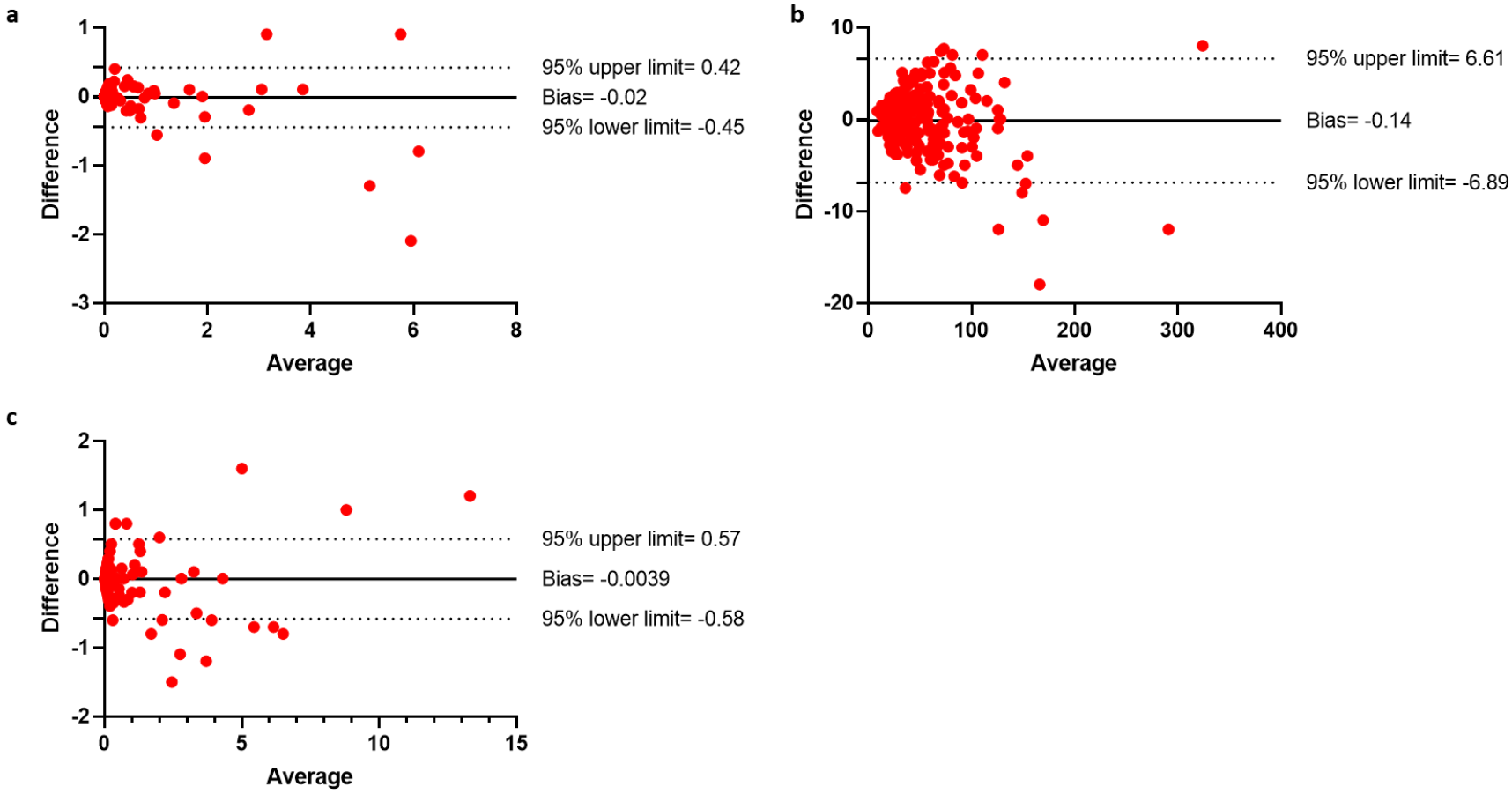


Figure 3.4 Precision: Bland Altman plots of replicates for (a) mutant concentration in copies/µl, (b) wild-type concentration in copies/µl and (c) mutant fractional abundance (%)

*Difference refers to the difference in results yielded by the replicates and this is plotted against the average of the two values.

3.2.8 Statistical analysis

The following premise was used to determine the sample size for the feasibility study: if 12 or more of the 48 patients have trackable mutations detected in the cfDNA pre-operatively then this would rule out a 15% detection rate in favour of a 30% rate, with 80% power and 5% significance level. This was a guide as to whether to continue to the main study or not. Statistical analyses were intended to be mainly descriptive. Fisher's exact test was used to assess differences in clinical characteristics between patients with undetectable and detectable ctDNA both pre and post-surgery whilst the Mann Whitney test was used for continuous variables. The pre-treatment quantity of ctDNA in patients that developed metastases was compared with those that did not using the Mann Whitney test. If ctDNA from more than one variant was present, the highest detectable value for all analyses was used. Graph Pad Prism version 8 was used for all analyses and *P* values <0.05 were considered statistically significant.

3.3 Results

3.3.1 Patient characteristics

56% of patients were male, median age 64, range 31-83. Included patients were representative of 10 participating centres. Baseline characteristics are summarised in ***Table 3.1***.

Table 3.1 Baseline characteristics of patients in the TRACC feasibility study

		N	%
Age (yrs)	Median (IQR)	64	57 – 75
	Mean (SD)	65.0	10.6
	Minimum - Maximum	31	83
Gender	Female	21	44
	Male	27	56
ECOG PS	0	39	81
	1	7	15
	2	2	4
Race	White British	33	69
	White Irish	3	6
	White other	3	6
	Black African	1	2
	Black Caribbean	3	6
	Asian Other	1	2
	Not available	4	8
Primary Site	Caecum	7	15
	Ascending Colon	4	8
	Hepatic Flexure	2	4
	Transverse Colon	6	13
	Splenic Flexure	1	2
	Descending Colon	1	2
	Sigmoid Colon	11	23
	Recto-Sigmoid	6	13
	Rectum	10	21

3.3.2 Mutation analysis in tissue

In order to reach the target of 48 patients with ctDNA results, as required by the feasibility, analysis of tumour tissue from 55 patients was required. Reasons for excluding patients and their respective samples are summarised in ***Figure 3.5***. The sequencing failure rate (where no results were available from the diagnostic biopsy or the resection) was 5/55 (9%). At least one somatic mutation was identified in the genes of interest of 48/50 (96%) of successfully sequenced cases (***Figure 3.5***). The median number of mutations in the genes of interest in tissue was 2 (range 0-5). A full list of all mutations detected in the genes of interest in tissue and their corresponding detection rates if tracked in plasma is listed in the appendix (***Table 7.2***).

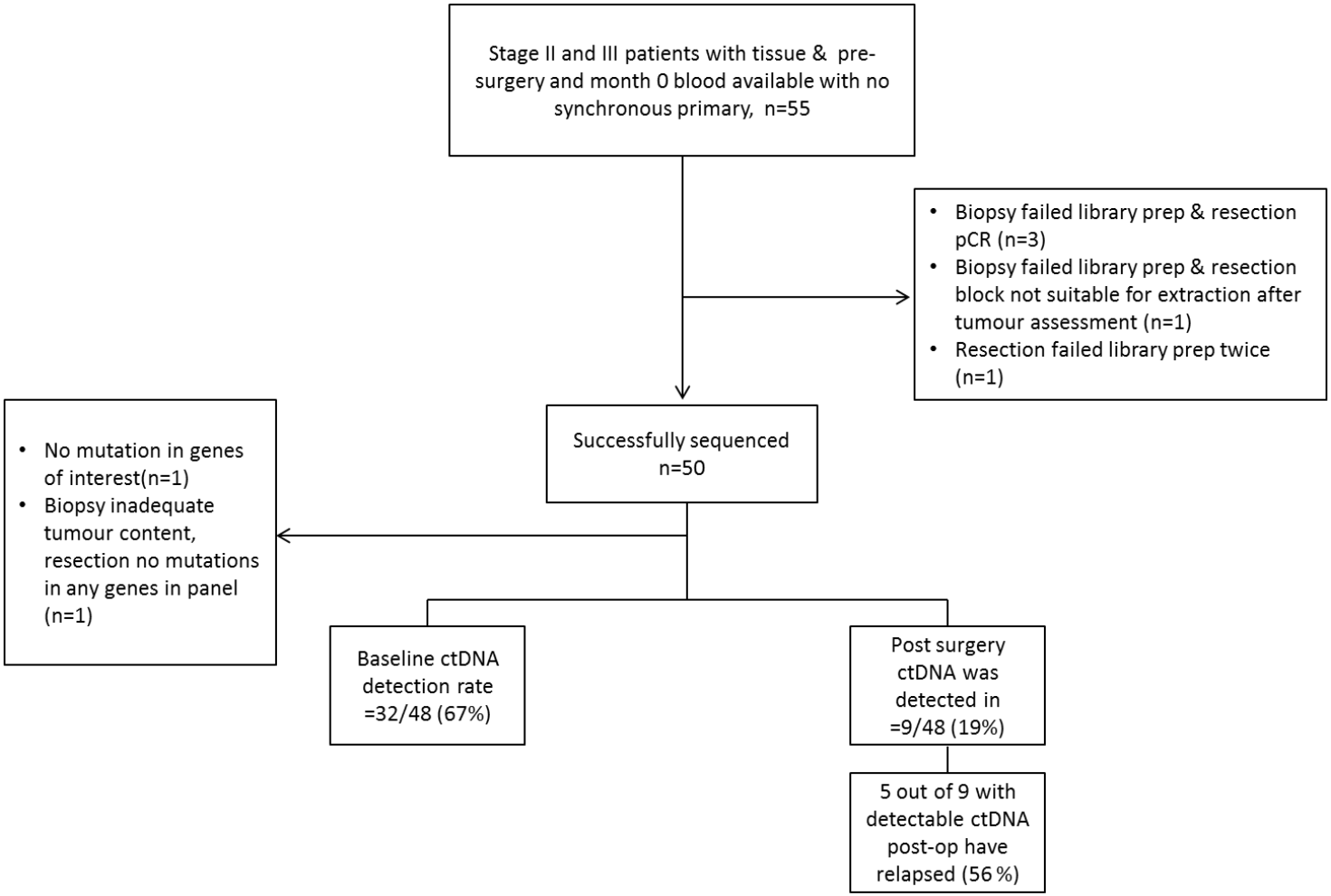


Figure 3.5 TRACC study feasibility analysis and reasons for exclusion of patients at each stage

The percentage of patients with a mutation in each of the genes of interest was largely as expected (***Figure 3.6***) with the exception of a higher number of patients with a mutation in *PIK3CA*.[107–110]

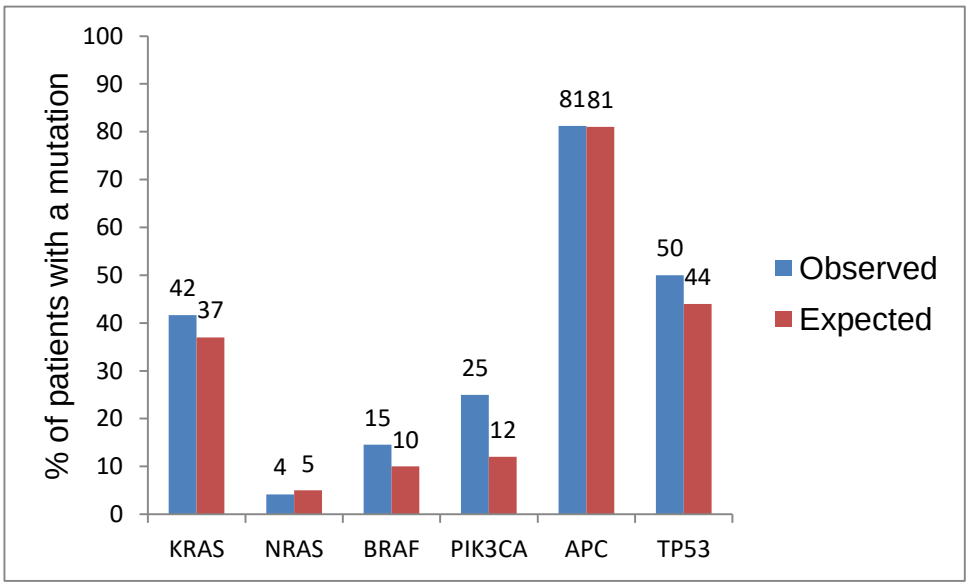


Figure 3.6 Tissue sequencing results comparing observed with expected frequency

The diagnostic biopsy was successfully used for sequencing in 8/10 (80%) of the patients that were treated with CRT. In the 2 unsuccessful cases, this was due to a failure of library preparation and depletion of the sample for any further attempts. The resection tissue was therefore used for sequencing in 40/48 (83%) cases.

Table 3.2 Number of patients with mutations and their frequency amongst the genes of interest in tissue

		Number of subjects	**% subjects**	***Number of specific mutations***	***Number of subjects***	***% subjects***
Type of samples	Biopsy Resection	8 40	17 83			
Number of mutations overall in genes interest	1 2 3 4 5	6 20 14 4 4	13 42 29 8 8			
KRAS		20	42	*1*	*20*	*100*
NRAS		2	4	*1*	*2*	*100*
BRAF		7	15	*1*	*7*	*100*
PIK3CA		12	25	*1*	*12*	*100*
APC		39	81	*1* *2* *3*	*23* *14* *2*	*59* *36* *5*
TP53		24	50	*1* *2*	*22* *2*	*92* *8*

3.3.3 ctDNA detectability in blood

Baseline plasma was collected a median of: 5 days (range 0-20 days) prior to surgery in patients proceeding straight to surgery (n=38) and a median of 6.5 days (range 0-27 days) prior to CRT (n=10) in patients having neoadjuvant treatment. Post-surgery plasma was collected a median of 40 days (range 18-82 days) from surgery.

The overall ctDNA detection rate was 67% (n=32/48) at baseline and 19% (n=9/48) post-surgery. Baseline ctDNA detection increased with stage: 63% (n=15/24) for stage 2 and 71% (n=17/24) for stage 3. Concordance between tissue and plasma amongst the genes of interest was highest for mutations detected in *NRAS (***Figure 3.7***)* however tissue mutations in *NRAS* were uncommon (***Table 3.2***).

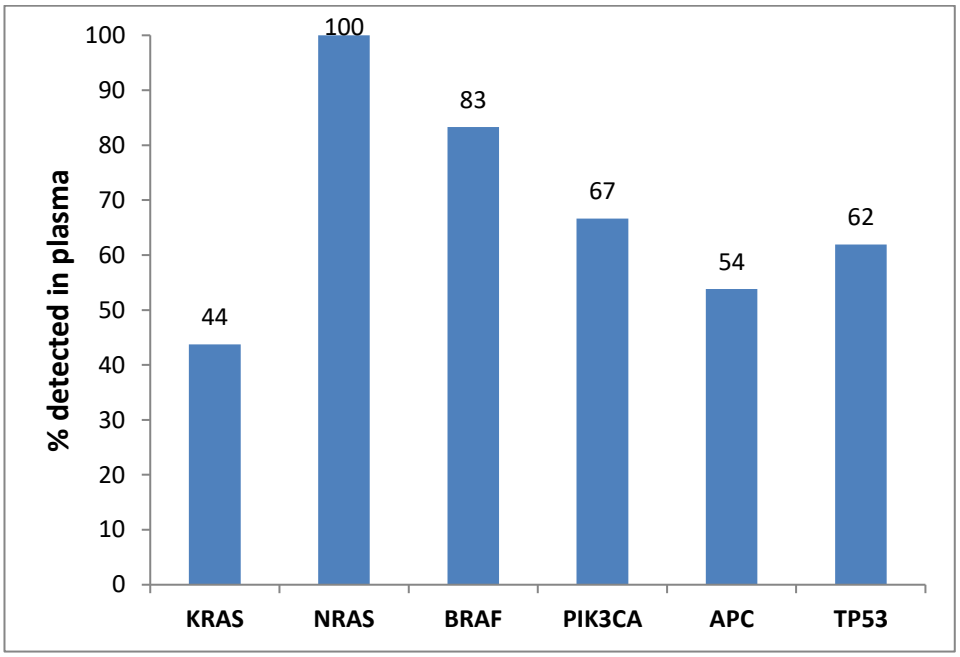


Figure 3.7 Baseline detection rates in plasma for mutations tracked amongst the genes of interest

*2 variants with the highest VAF among the genes of interest (*KRAS*, *NRAS*, *BRAF*, *PIK3CA*, *APC* and *TP53*) were chosen to be tracked in plasma per patient per time-point. The graph shows detection rates in baseline plasma samples across the genes of interest if chosen to be tracked.

In 6 cases, only 1 variant was tracked in plasma as no other variants were detected in the genes of interest in tissue. The pre-treatment ctDNA detection rate in these cases was 67% (n=4). Of the 32 patients with detectable mutations in tumour tissue and plasma cfDNA pre-operatively, 7 (22%) had detectable mutations in plasma cfDNA postoperatively. Of the 16 patients without detectable mutations in plasma cfDNA pre-operatively but evidence of a mutation in tissue, 2 had detectable mutations in plasma cfDNA post-operatively (***Table 3.3***).

Table 3.3 Contingency table summarising pre and post-operative ctDNA detection

Pre-treatment ctDNA detected	**Post-surgery ctDNA detectable**		
	No	**Yes**	**Total**
No	14	2	16
Yes	25	7	32
Total	39	9	48

With a median follow up of 12.6 months (range 1-18 months), 5 patients have relapsed since surgery and all of these patients had detectable ctDNA post-surgery and at the time of confirmed relapse and 3 examples are shown in ***Figure 3.8***.

Of the patients with no evidence of relapse, 39 (91%) had undetectable ctDNA post-surgery. The characteristics of patients that relapsed are summarised in table ***Table 3.4***.

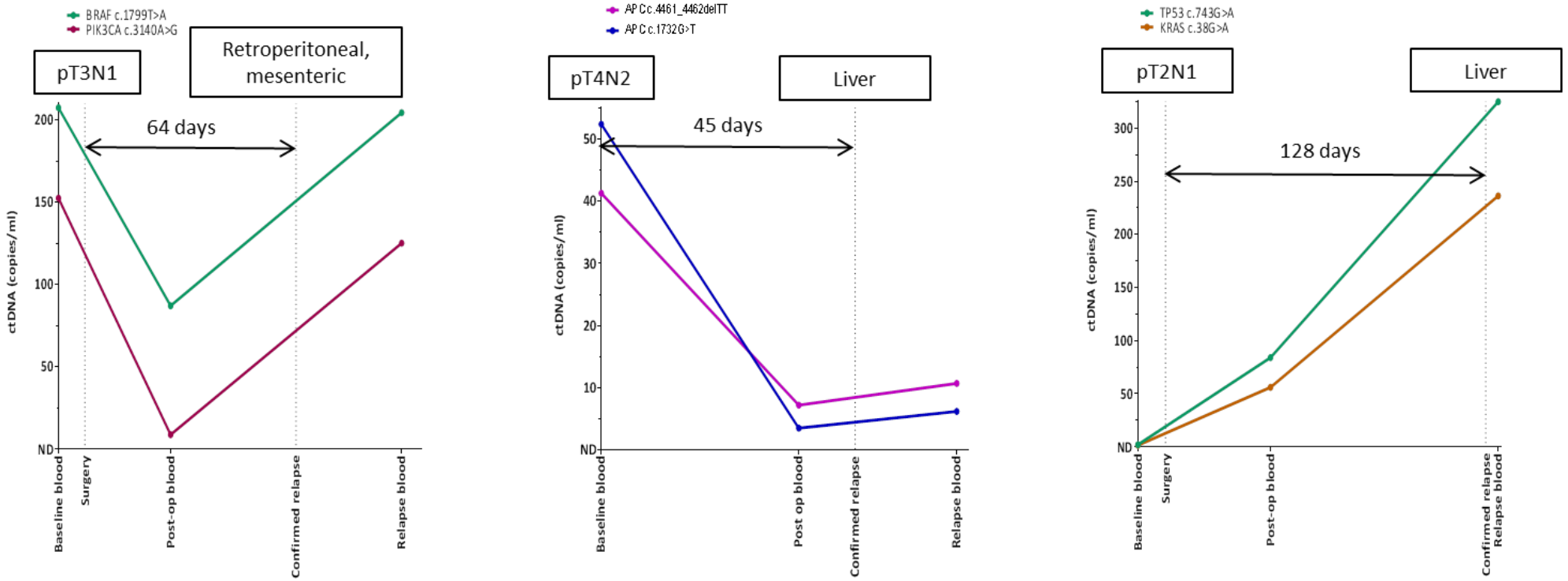


Figure 3.8 Graphs showing ctDNA detectability over time in 3 patients experiencing relapse

Table 3.4 Clinical characteristics of the patients experiencing relapse

Patient ID	Baseline Radiological Stage	Pathological Stage	Pathological EMVI status	Resection status	Histology differentiation	Total lymph node yield	Sites of disease at relapse	CEA value at relapse if available
EP0033	T3N1cM0	T3N1	Positive	Rx	Poor	≥12	Retroperitoneal, mesenteric	2
EP0004	T3N1cM0	T4N2	Positive	R0	Moderate	≥12	Liver	1
NM0091	T2N1bM0	T2N1	Unknown	R0	Moderate	≥12	Liver	27.4
EP0035	T3dN2M0	T3N2b	Positive	R0	Moderate	≥12	Mediastinal mass	1
CY0025	T3N1cM0	T4bN1	Positive	R0	Moderate	≥12	Omentum, peritoneum, ascites	15

3.3.4 ctDNA status pre and post-surgery by clinical characteristics

With the exception of ctDNA detectability after surgery and a statistically significant association with the development of metastases (*P*<0001), ctDNA status did not appear to be associated with any other clinical characteristics. Consistent with the findings of the SSGCC-1 study in the previous chapter, there was a trend towards statistical significance (*P*=0.05) for an association between ctDNA status after surgery and pathological nodal status (***Table 3.5***).

Table 3.5 Clinical characteristics by ctDNA status

Variable	Baseline ctDNA (n=48)			Post-surgery ctDNA (n=48)		
	Positive (*N*=32)	Negative (*N*=16)	*P*	Positive (*N*=9)	Negative (*N*=39)	*P*
Age, years						
Median	65	63	0.85	61	64	0.72
IQR (p25-p75)	57-75	60-74		53-75	60-74	
Gender, n (%)						
Male	19(59)	8(50)	0.56	5(56)	22(56)	1.00
Female	13(41)	8(50)		4(44)	17(44)	
ECOG Performance status, n (%)						
0	25(78)	14(88)	0.70	8(89)	31(79)	1.00
1-2	7(22)	2(12)		1(11)	8(21)	
Tumour side, n (%)						
Right	14(44)	5(31)	0.54	5(56)	14(36)	0.45
Left (including rectum)	18(56)	11(69)		4(44)	25(64)	
cT stage, n (%)						
0-2	8(25)	3(19)	0.73	2(22)	9(23)	1.00
3-4	24(75)	13(81)		7(78)	30(77)	
cN stage, n (%)						
0	9(28)	3(19)	0.73	1(11)	9(23)	0.66
≥1	23(72)	13(81)		8(89)	30(77)	
pT stage, n (%)						
0-2	4(12)	3(19)	0.67	2(22)	5(13)	0.60
3-4	28(88)	13(81)		7(78)	34(87)	
pN stage, n (%)						
0	19(59)	12(75)	0.35	3(33)	28(72)	0.05
≥1	13(41)	4((25)		6(67)	11(28)	
pEMVI status, n (%)*						
Positive	15(47)	3(20)	0.11	5(56)	13(33)	0.27
Negative	17(53)	12(80)		4(44)	25(64)	
Overall Stage, n (%)†						
II	15(47)	9(56)	0.76	2(22)	22(56)	0.14
III	17(53)	7(44)		7(78)	17(44)	
Developed metastases, n (%)						
Yes	5	0	0.15	5(56)	0(0)	<0.0001
No	27	16		4(44)	39(100)	

* the pEMVI status was unknown in 1 case, † overall stage was derived from pathology for those proceeding straight to surgery and from baseline imaging in those being treated with CRT.

Abbreviations: cT=clinical T stage, *cN=clinical nodal stage, pT=pathological T stage, pN=pathological nodal stage, pEMVI status=pathological extramural venous invasion*

3.3.5 Pre-treatment ctDNA quantity to predict the development of metastases

Amongst patients with detectable ctDNA pre-treatment, the median ctDNA FA was 1.24% (IQR 0.15-4.07%) in patients that developed metastases (n=5) compared to 0.18% (IQR 0.10-0.48%) in the patients that have not developed metastases (n=27). In copies/ml, the median quantity was 52.4 (IQR 2.1-207.6 copies/ml) in patients that have developed metastases compared to 4.6 (IQR 2.5-10.85 copies/ml) in the patients that have not developed metastases. Although the pre-treatment ctDNA quantity was higher in patients that developed metastases, this was not statistically significant (***Figure 3.9***).

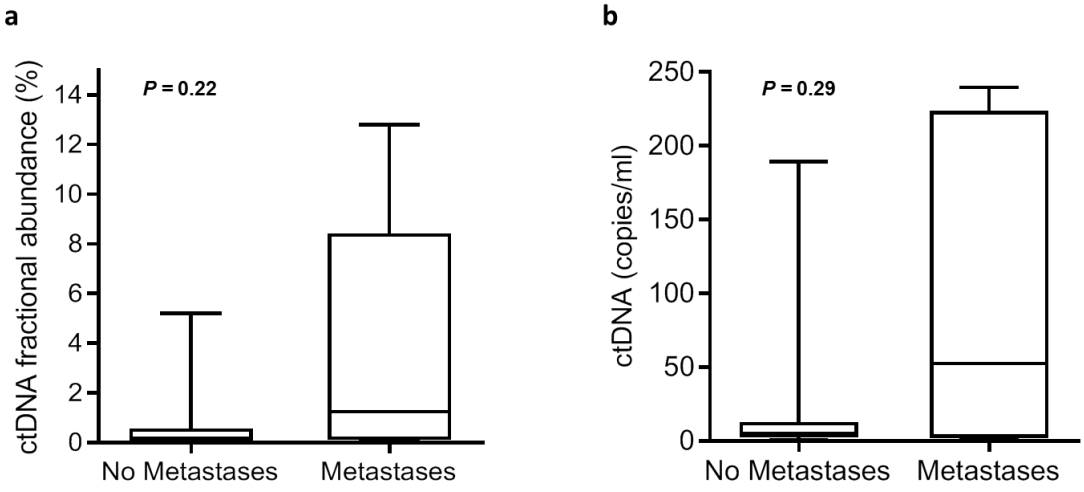


Figure 3.9 Pre-treatment ctDNA quantity in patients that developed metastases compared to those that did not in (a) fractional abundance (%) and (b) copies/ml

3.4 Discussion

The TRACC feasibility study confirmed that when blood samples are collected in Streck cell free DNA blood collection tubes from multiple centres, they can still remain viable for ctDNA analysis. Pre-analytical factors such as

sample collection, transportation, processing and storage are important as they can affect the likelihood of detecting ctDNA due to contamination from leukocyte genomic DNA.[128] Although no universally accepted guidelines currently exist, standardisation will be needed in order to understand how best to interpret the data emerging from different research groups. Validation of the ddPCR assays by assessing trueness and precision provided increased confidence over the results yielded here.

Our overall baseline ctDNA detection rate was 67%, which is lower than that seen in the SSGCC-1 study (74%). The lower detection rate could be attributable to the proportion of patients with stage III disease which was higher in SSGCC-1 (87%) compared to the TRACC feasibility study (50%). As expected, in TRACC, the baseline ctDNA detection rate was indeed lower for stage II (63%) compared to stage III disease (71%) and although in this small dataset, this did not reach statistical significance (P=0.76), the published literature has widely reported on more advanced disease stages being associated with higher ctDNA detection rates.[60, 61, 101] The lower detection rate is unlikely to be a result of the primary tumour location, where SSGCC-1 only included rectal cancer patients and the TRACC feasibility study predominantly included colon cancer patients (79%), as there was no significant difference in baseline ctDNA detection by right vs. left sided tumours (P=0.54).

To date, 5 patients within the feasibility study have relapsed and all of these patients had ctDNA detectable at baseline, post-operatively and at the time

of relapse. The quantity of ctDNA pre-operatively was unable to differentiate between patients that would and would not go on to relapse. Relapse occurred a median of 2 months from surgery (range 1-8 months) and 4 of these patients were deemed to have had a complete macroscopic resection on histology, (resection status was not specified in one case). ctDNA predicted relapse with a median lead time of 1 month over radiological relapse. Interestingly, of the 5 patients that relapsed, only 2 had an elevated CEA at the time of relapse. Of note, 4 patients with no evidence of relapse also had detectable ctDNA post-operatively and of these 4 patients, only 2 had detectable ctDNA pre-operatively when the tumour was still in situ. With such a short median follow-up time of 12.6 months, we cannot be certain whether these represent false positive results or an early indicator of likely future relapse due to MRD. Of the 39 patients who did not have ctDNA detectable post-operatively, none have relapsed. Whilst taking into consideration the short follow up time, limited number of patients and events, the current sensitivity for the post-operative blood time-point to predict relapse is 100%, the specificity is 91%, the negative predictive value is100% and the positive predictive value is 56%.

Our data provides further supporting evidence to the existing literature that ctDNA may be an indicator of MRD, with its absence post-operatively determining a good prognostic group that could be spared from adjuvant chemotherapy and its associated toxicity. Indeed, the notion of limiting adjuvant chemotherapy has been welcomed following data from the International Duration Evaluation of Adjuvant therapy (IDEA) pooled analysis

of 6 studies comprising over 12 000 patients.[129] Adjuvant chemotherapy with 3 months of Capecitabine and Oxaliplatin was found to be non-inferior to the standard duration of 6 months in terms of survival and these results have been practice changing. Although shortening the duration of adjuvant chemotherapy is certainly an important advance in the field, by incorporating molecular biomarkers such as ctDNA, there is potential to completely omit adjuvant chemotherapy in a subgroup of patients and reserve it only for patients where there is unequivocal evidence of likely benefit. The impact of chemotherapy on a patient's quality of life or financial well-being should not be under-estimated. Additionally, reserving chemotherapy for biomarker-selected cases is likely to be cost-saving and have a positive impact on healthcare resources. Internationally, the value of such a chemotherapy sparing approach has been recognised and this has led to the initiation of global trials where the results of ctDNA following surgery are being used to determine the need for adjuvant chemotherapy. Within our own group, the TRACC study protocol (NCT 04050345) has been further developed to include an interventional part (Part C). However, based on this feasibility study which used a tumour informed approach to design ddPCR assays, the turnaround times would not be rapid enough for clinical decision making. Moving forward, direct sequencing of plasma will be required and further work will be needed to assess the accuracy of moving away from a tumour informed approach. Alternatively, a universal biomarker such as methylated ctDNA may be an option and data implementing its use has shown great promise.[103, 104]

4 COMPARISON BETWEEN PRIMARY CRC TUMOURS AND THEIR CORRESPONDING LUNG METASTASES

4.1 Background

The previous chapters have assessed ctDNA detectability as a marker of predicting relapse in CRC. Integration of ctDNA with other forms of liquid biopsy such as RNA based markers and consensus molecular subtyping (CMS), might improve sensitivity. As a first step towards this, a better understanding of metastasis promoting pathways and differences between primary CRC tumours and their metastases is essential. This would also pave the way for improving therapeutics.

The formation of metastases requires the initiation of a series of sequential intricate processes to enable cells from the primary tumour to disseminate to a distant site and successfully adapt to a different microenvironment where they can colonise. This series of events is termed the invasion-metastasis cascade (***Figure 4.1***).[130–133]

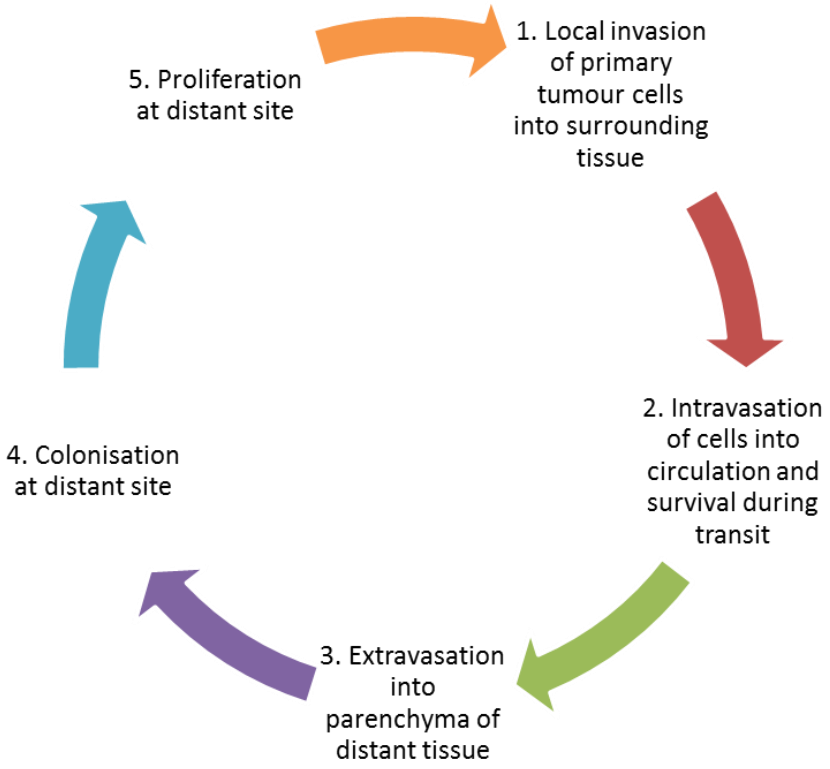


Figure 4.1 The invasion-metastasis cascade

Local invasion of primary tumour cells into surrounding tissue may occur by epithelial-mesenchymal transition (EMT) whereby epithelial cells may acquire mesenchymal properties such as increased motility, invasiveness and the ability to degrade extra-cellular matrix proteins. Local invasion may also occur by collective migration where cell clusters rather than single cells are involved and requires a more co-ordinated process.

In 1889, the 'seed and soil' hypothesis was first coined by Stephen Paget whereby the development of metastasis is determined by the interaction between the cancer cells or 'seeds' and the host microenvironment or 'soil'.[134] This concept was later contested by James Ewing who postulated that metastases occur due to mechanical factors including anatomical differences in the vasculature affecting blood flow.[135] In reality, both of these theories may influence the propensity to develop metastases.

Another important contribution in the field came from Fidler who proposed that tumour cells have differing degrees of metastatic potential.[136] Further work by Talmadge et al. showed that metastases are clonal and arise from a single proliferating cell.[137, 138] The organ specificity of tumours was later noted across several studies.[139–141]

Efforts to target metastases have included directing therapies against host factors such as angiogenesis. However, results have been somewhat disappointing and most patients still succumb to their metastatic disease.

In CRC, 23-30% of patients present with metastatic disease with the liver being the most common site of metastases, followed by lung metastases which occur in 10-20% of patients.[2, 142–145] Lung metastases are more common in patients with rectal cancer. It is thought that this might be due to the fact that in rectal cancer, unlike colon cancer, the vascular drainage bypasses the portal system and first encounters the central circulation.[146–148]

Whilst most patients with lung metastases are treated with palliative intent, a subset of patients with oligometastatic disease may benefit from resection of their lung metastases with curative intent. Evidence from 21 series including a total of 8361 patients with surgically resected lung metastases demonstrated 5 year survival rates after the first lung metastasectomy of 24-82% with mOS ranging from 35-70 months.[149] Although resection of lung metastases has been less extensively studied compared to resection of liver metastases, it is an accepted treatment option in carefully selected cases

and has been recommended in guidelines.[150] There is no agreed consensus on selection criteria for this approach. This is due to the lack of data from randomised trials[151] and the fact that lung metastases can arise in various clinical scenarios: synchronous with the primary CRC, as the first site of metachronous metastatic disease, after management of a liver metastasis or after a previous lung metastasis has been managed. However, a short disease free interval between resection of the primary tumour and the development of lung metastases, multiple lung metastases, an elevated CEA prior to thoracotomy and positive hilar and/or mediastinal nodes have all been associated with poor prognosis.[152, 153]

Aside from clinical factors, genomic and transcriptomic parameters have been studied to elucidate inherent markers of prognosis and identify novel therapeutic targets. Six independent groups developed molecular classification systems for CRC based on gene expression data.[117, 154–158] The consensus molecular subtyping consortium led an initiative to unify the classification systems and identified 4 distinct groups (***Figure 4.2***) with CMS2 being the most prevalent (37%) followed by CMS4 (23%), CMS1 (14%) and CMS3 (13%).[159] The remaining 13% could not be classified due to mixed features.

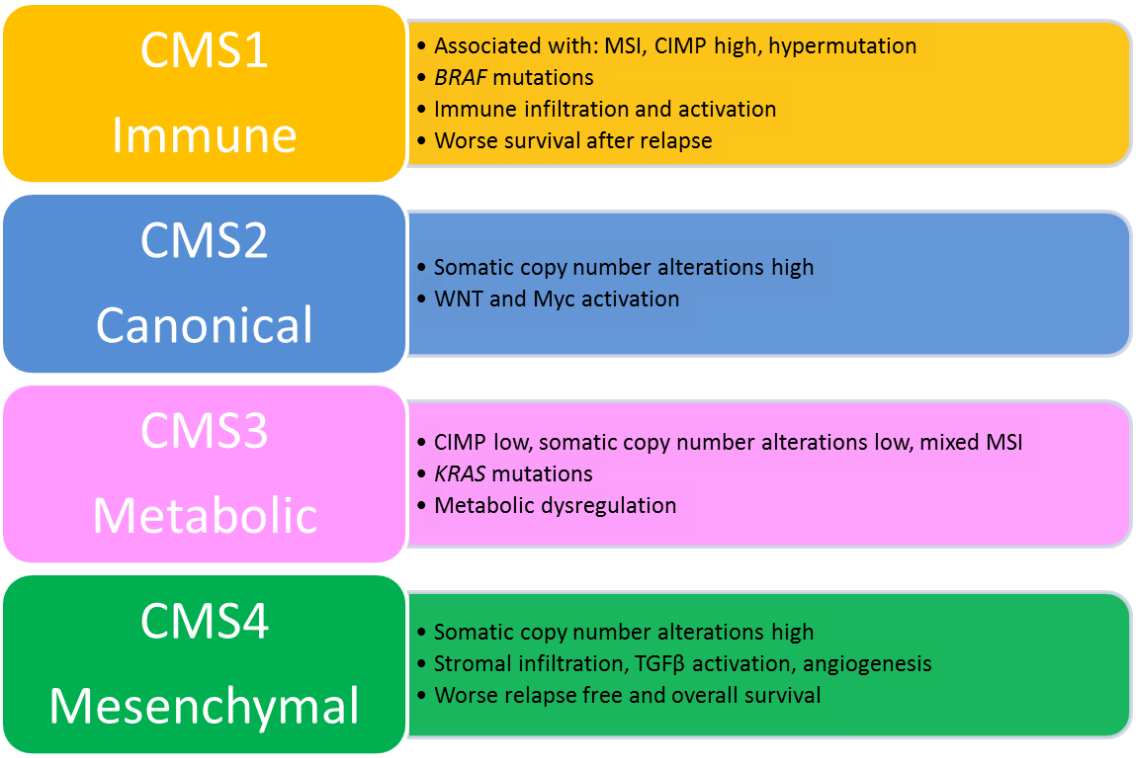


Figure 4.2 The consensus molecular subtypes of CRC

(modified from Guinney et al, Nat Med 2015)

The CMS subtypes were predominantly derived from treatment naïve primary tumour samples in the early stage colon cancer, off-trial setting. Stage IV disease at diagnosis and rectal cancer were underrepresented accounting for 8% and 15% of all samples respectively.[160] There have since been retrospective analyses from clinical trials in the metastatic disease setting.[161–163] However, these analyses were conducted in the primary tumour of patients with metastatic disease rather than from the metastatic site.

Interestingly, consistent with the CRC subtyping consortium (CRCSC) dataset in a predominantly early stage disease setting, the CMS2 subtype remained the most prevalent and CMS3 subtype remained the least prevalent in these 1st line advanced disease studies (***Figure 4.3***). However,

the results should be interpreted with caution as analysis of samples from clinical trials is likely to have introduced selection bias towards fitter patients. Poor performance status can be related to aggressive tumour biology and high tumour burden and such patients are typically excluded from clinical trials.

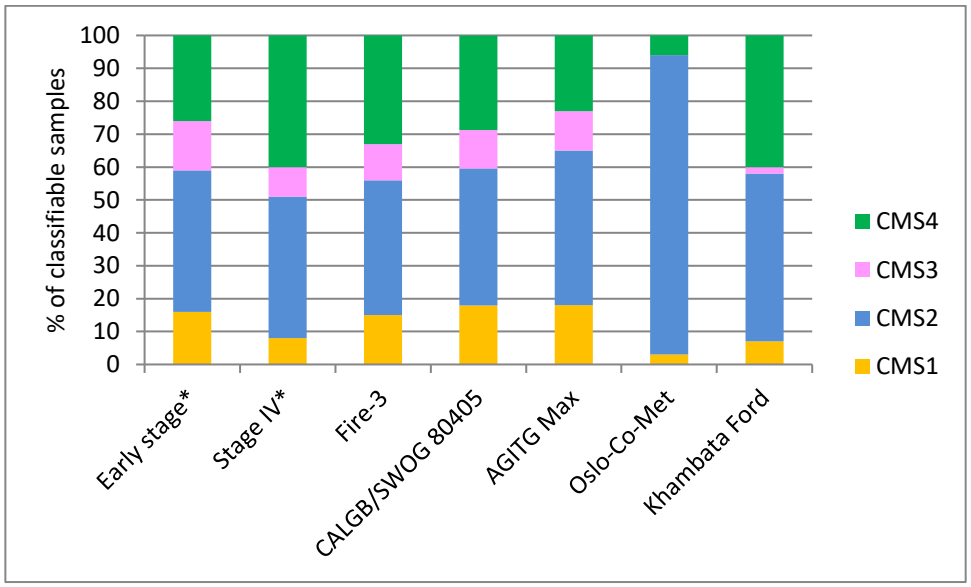


Figure 4.3 CMS subtype distribution across different studies or data sets

*refers to stage at diagnosis in the CRCSC dataset[159]
Fire-3[163]: compared 1st line therapy with FOLFIRI plus cetuximab or bevacizumab in exon 2 *KRAS* wild type mCRC patients
CALGB/SWOG 80405[162]: compared the addition of cetuximab or bevacizumab to FOLFOX or FOLFIRI in 1st line advanced CRC
AGITG Max[161]: compared the addition of bevacizumab to capecitabine+/-mitomycin to capecitabine alone in 1st line mCRC
Oslo-Co-Met[164]: compared open to laparoscopic resection of colorectal liver metastases
Khambata-Ford[165]: transcriptional profiling study from pre-treatment biopsies in patients with mCRC planned to commence cetuximab monotherapy (70% were liver metastases, CMS subtyping data here relates to these liver metastases samples only)

The CMS subtypes have been used to classify liver metastases samples in the publicly available Khambata Ford dataset and within the Oslo-Co-Met trial.[160, 164, 165] Although the CMS2 subtype remains the most prevalent, in the Khambata Ford dataset, there appears to be enrichment for the CMS4,

mesenchymal subtype with a decrease in the proportion of CMS1 and CMS3 when compared to the early stage disease setting.

To date there has been no published work evaluating the CMS subtypes exclusively in a cohort of patients with lung metastases and matched corresponding primary CRC tumours. Similarly, gene expression data, which could more accurately define phenotypic heterogeneity between primary tumours and their lung metastases, is limited in this context. The main aims were therefore:

1. To compare the transcriptomic profiles of matched tissue from primary CRC and corresponding secondary lung metastases

2. To compare CMS subtypes of matched tissue from primary CRC and corresponding secondary lung metastases and relate them to prognosis

4.2 Methods

Patients who underwent lung metastasectomy for CRC with curative intent between 1997 and 2012 at the Royal Brompton and Harefield Foundation Trust were identified by searching surgical records. Clinical data which included patient demographics, treatment details, histopathology reports and outcomes were collected. Patients were excluded if lung nodules were found to be unrelated to CRC or if they did not have any treatment at the Royal Marsden Hospital. Data, samples and analyses were conducted within the retrospective lung resection translational protocol which was approved by a human research ethics committee (REC reference 12/SC/0158).

FFPE tissue that was surplus to clinical requirements was collected from matching primary CRC tumour, lung metastases and resection from any other metastases (if applicable). Representative areas from normal colorectal and lung tissue were collected as a source of germline DNA. DNA was isolated as previously described and NGS was performed using a targeted 46 gene capture panel, the results of which have already been published.[114] RNA was extracted from the same samples using Ambion's Recoverall Nucleic Acid Purification Kit according to manufacturer's instructions and quantified using nanodrop. The samples were sent to Illumina/Quintiles to profile the global transcriptome using TruSeq RNA Access RNA Sequencing.

I led regular teleconferences to guide further analyses which were conducted by bioinformaticians (Dr Alain Pacis and team, McGill University). Adaptor sequences and low quality score bases (Phred score < 30) were first

trimmed using Trimmomatic as previously described.[166] The resulting reads were aligned to the GRCh37 human reference genome assembly, using STAR.[167] Read counts were obtained using HTSeq and were represented as a table which reported, for each sample (columns), the number of reads mapped to a given gene (rows).[168] For all downstream analyses, lowly-expressed genes with an average read count lower than 10 across all samples were excluded, resulting in 19,374 genes in total. The R package limma was used to identify differences in gene expression levels between different groups.[169] Nominal p-values were corrected for multiple testing using the Benjamini-Hochberg method. Differentially expressed genes were identified using the cutoffs |log2 fold change (FC)| > 1 and false discovery rate (FDR) < 0.05.

The R package CMS caller was used to classify tumour samples based on 530 gene predictors.[170] I then worked with bioinformatics team to interpret the data arising from these analyses when taking the clinical context into consideration. The Kaplan-Meier method was used for the survival estimate. Graph Pad Prism version 8 was used for survival analysis.

4.3 Results

81 patients undergoing a total of 121 pulmonary metastasectomies were recruited. Of these patients, 15 had matching tumour tissue from their primary CRC and secondary lung metastases with targeted NGS results available. The previously published results by Sing Yu et al. showed that 11 out of 15 patients (73%) had a concordant molecular profile.[114]

Of these 15 patients, 11 had sufficient material for RNA-sequencing. There were 2 further patients without sufficient material for targeted NGS but with adequate material for RNA sequencing. The work presented here relates to the transcriptomic analysis in the cohort of 13 patients and their clinical characteristics are provided in (***Table 4.1***). Detailed individual clinical histories are provided in the appendix (***Table 7.3***). In 2 patients, tissue from more than 1 lung metastases were available and these are referred to as lung metastases 1 and lung metastases 2 as an indication of chronological sequence of events in relation to each other.

Table 4.1 Clinical characteristics of n=13 patients included in transcriptomic analysis

		N	%
Age* (yrs)	Median	64	
	Minimum-Maximum	38	70
Gender	Male	9	69
	Female	4	31
Smoking status*	Smoker	1	8
	Ex-smoker	4	31
	Never-smoker	7	54
	Unknown	1	8
Primary site	Ascending colon	2	15
	Sigmoid colon	5	38
	Rectum	6	46
Primary surgery	Anterior resection	6	46
	Left hemi-colectomy	1	8
	Right hemi-colectomy	2	15
	Sigmoid colectomy	2	15
	Recto-sigmoidectomy	1	8
	Resection NOS	1	8
Histology: Differentiation	Moderate	11	85
	Unknown	2	15
Histology: EMVI	Yes	5	38
	No	4	31
	Unknown	4	31
T stage*	2	1	8
	3	10	77
	4	2	15
N stage*	0	5	38
	1	6	46
	2	2	15
Overall Stage*	I	1	8
	II	4	31
	III	6	46
	IV	2	15
Metastatic site*	Liver	1	8
	Lung	1	8
	None	11	85
Time from diagnosis to development of first metastasis any site (months)	Median	17	
	Minimum-Maximum	0	36
Site of first metastasis	Liver	3	23
	Lung	9	69
	Liver and lung	1	8
Time from diagnosis to development of first lung metastasis (months)	Median	19	
	Minimum-Maximum	0	48
Location of first lung metastasis	Bilateral	6	46
	Unilateral	7	54
Total number of lung resections with malignancy confirmed	Median	1	
	Minimum-Maximum	1	3

*Refers to: at diagnosis

4.3.1 RNA sequencing gene expression analysis

Principal component analysis was conducted in 40 samples from 13 patients (***Figure 4.4***). Primary tumour samples segregated away from normal lung and colorectal tissue. Similarly, primary tumour samples and metastases samples aggregated into 2 groups although they remained in close proximity to each other.

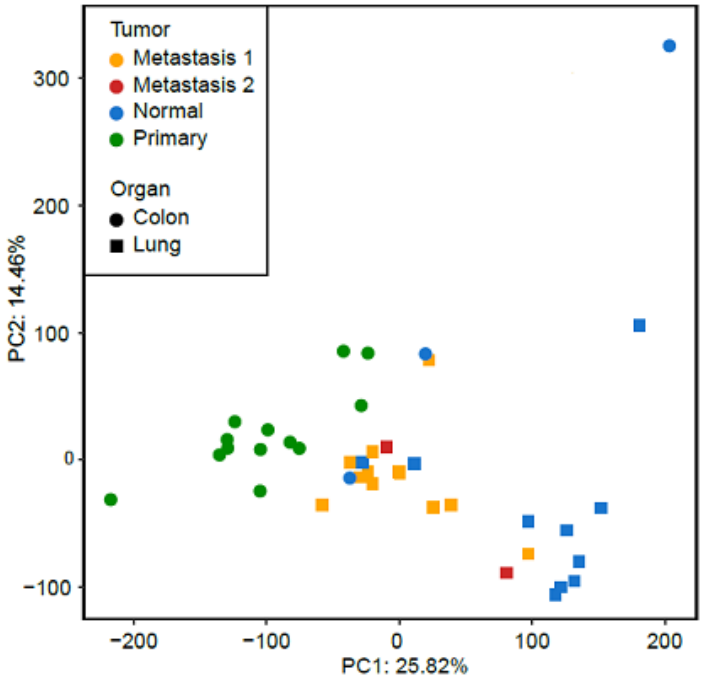


Figure 4.4 Principal component analysis of gene expression data from all samples n=40

*Colon refers to both colon and rectum samples. 2 cases with a second lung metastasis with available tissue are included

As expected, and summarised below, there were a number of genes that were differentially expressed in tumour tissue compared to normal tissue (***Table 4.2***).

Table 4.2 Number of differentially expressed genes identified at a |log2FC| > 1 and False discovery rate (FDR) < 0.05

	Primary tumour n=13	Lung metastasis 1 n=12	Lung metastasis 2 n=2
Number of differentially expressed genes (compared to normal colon/rectum)	4049	2867	56

*No data was available for one lung metastasis 1 sample

There was strong correlation between the primary tumour and lung metastases with regards to changes in gene expression from normal colorectal tissue (***Figure 4.5***).

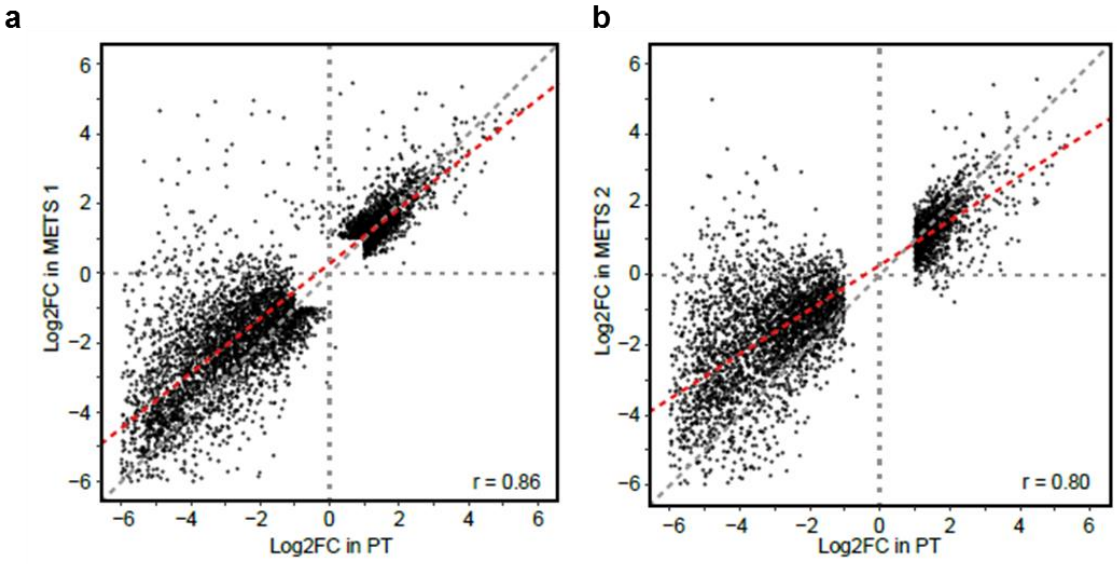


Figure 4.5 Scatterplots depicting the correlation between changes in gene expression in primary tumour (x-axis) or metastasis (y-axis), against normal colon or rectum samples**.**

*Scatterplot a: includes metastasis 1 samples (n=12), scatterplot b includes metastasis 2 samples (n=2). The scatterplots show the union of the differentially expressed genes in primary tumour and metastasis 1 or 2.

Abbreviations: PT=primary tumour and METS=metastasis

Out of the 19,374 genes analysed, only 944 (4.9%) were differentially expressed when the primary tumour was compared to the first lung metastasis (n=13) (***Figure 4.6***). There were fewer differentially expressed genes when the primary tumour was compared to the second chronological lung metastasis. However, this is likely to be due to the small number of samples with tissue from a 2nd lung metastasis (n=2). The full data set is visually represented in a heat map (***Figure 4.7***).

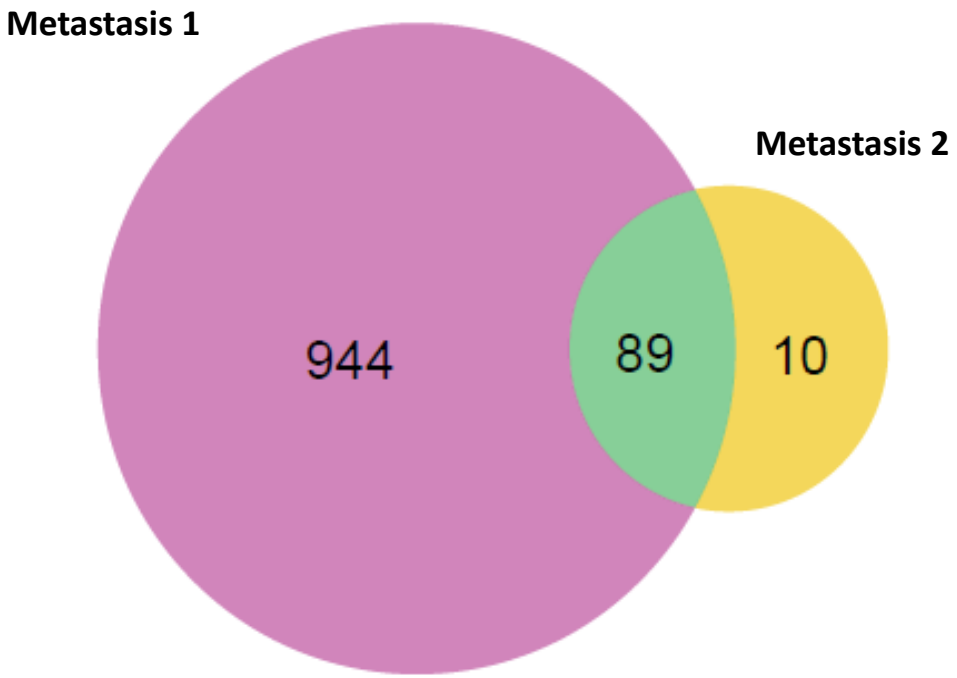


Figure 4.6 Venn Diagram showing the number of differentially expressed genes* (|log2FC| > 1 and FDR < 0.05) contrasting metastasis 1 or 2 samples to primary tumour samples.

*Tissue-specific genes (genes changing between normal colorectum and lung were removed)

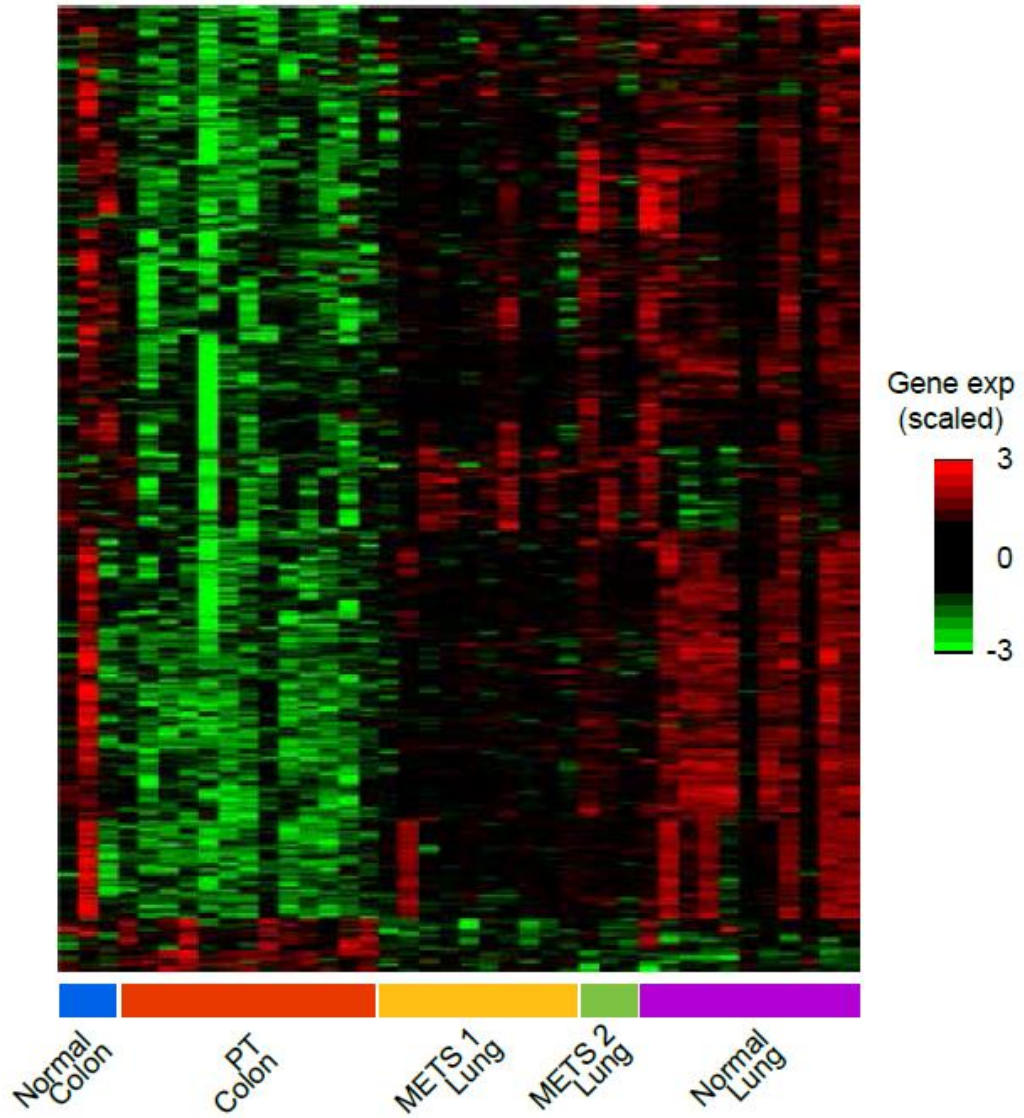


Figure 4.7 Heatmap of patterns of gene expression constructed using unsupervised hierarchical clustering

The 1,043 differentially expressed genes identified in Figure 4.5 have been included.

Abbreviations: Normal colon= normal colorectal tissue, PT colon= primary tumour tissue in the colon or rectum, METS= metastasis

The genes that were differentially expressed between the metastases samples and the primary tumour sample were most likely to be involved in the immune system process. Other pathways identified are summarised in ***Figure 4.8***.

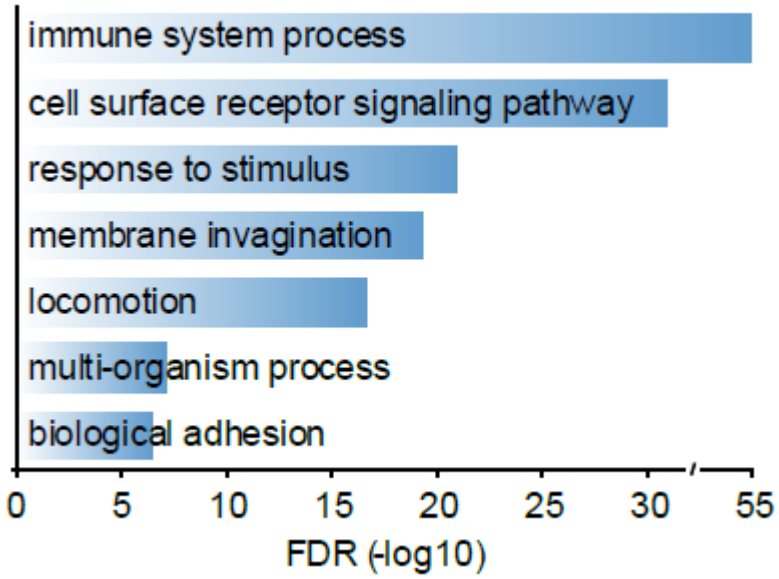


Figure 4.8 Gene ontology enrichment analyses among all genes differentially expressed between metastasis 1 or 2 compared to primary tumour samples

Further analysis was then conducted using hierarchical clustering in order to identify two separate clusters with genes that were either over-expressed or under-expressed in the metastases compared to the primary tumour (***Figure 4.9***). Genes were most likely to be over-expressed in the metastases samples compared to the primary tumour (n=990). Genes that were over-expressed were most likely to be involved in immune response. On the other hand, genes that were under-expressed in the metastases were likely to be involved in maintaining the composition and organisation of the extra-cellular matrix.

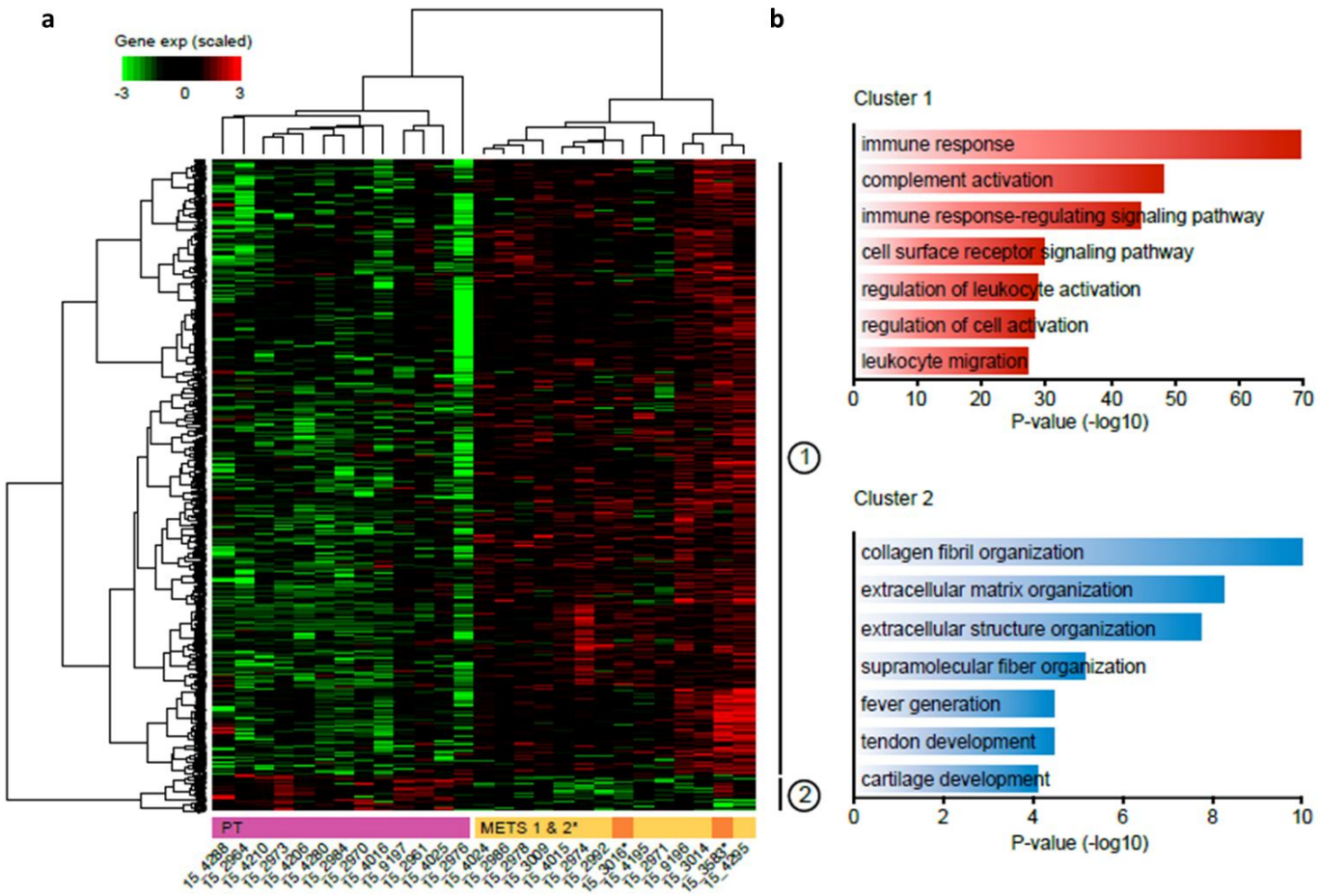


Figure 4.9 (a) Heat map identifying 2 different clusters of differentially expressed genes using hierarchical clustering (b) Gene ontology analysis in the 2 different clusters
Cluster 1= genes over-expressed in metastasis compared to primary tumour samples (n = 990), Cluster 2=genes under-expressed in metastasis compared to primary tumour samples (n = 59). The 2 cases with a 2nd lung metastasis are highlighted in orange on the heat map.

4.3.2 Distribution of CMS subtypes among primary CRC tumours and their corresponding lung metastases

Amongst the primary CRC tumour samples (n=13), CMS4 was the most prevalent subtype (n=5), followed by CMS2 (n=4), CMS3 (n=2) and CMS1 (n=1). In one case, the CMS subtype could not be predicted (***Figure 4.10***).

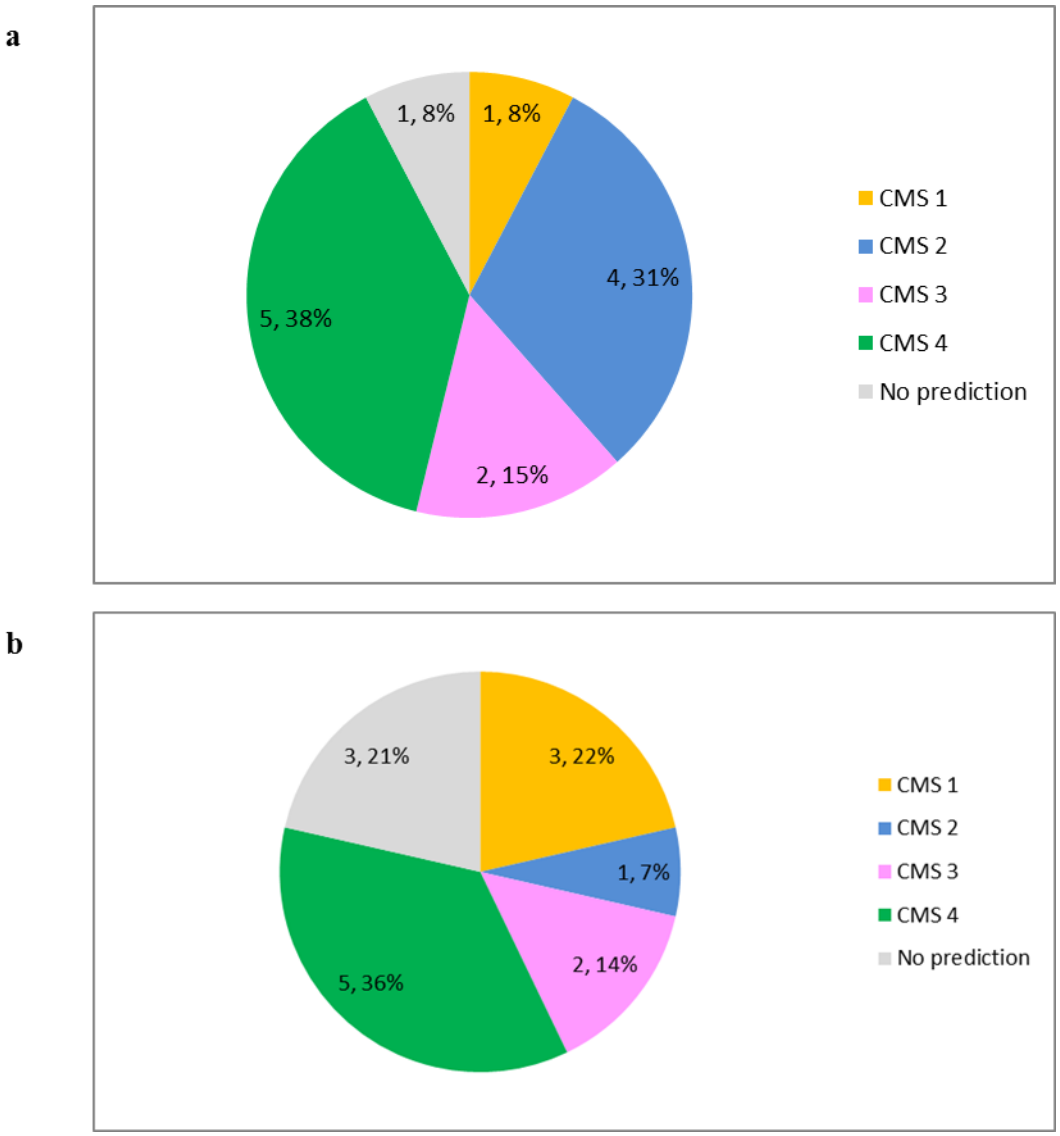


Figure 4.10 Distribution of the CMS subtypes among (a) primary CRC tumours and (b) lung metastases

Amongst all available lung metastases samples (n=14), CMS4 remained the most prevalent subtype (n=5) followed by CMS1 (n=3), CMS3 (n=2) and CMS2 (n=1). In 3 cases, no prediction was possible.

Of the 9 cases where a CMS subtype could be predicted in both the primary tumour tissue and corresponding lung metastasis, there was a switch in subtype in 5 cases (56%, ***Table 4.3***). Of these 5 cases where a switch in subtype was observed, the most common switch was between CMS1 and CMS4, in either direction in 3 cases (60%). In the other 2 cases of CMS switching, this was from CMS2 in the primary tumour tissue to CMS4 in the lung metastasis. Out of all the cases of CMS3 in the primary tumour (n=2), none switched subtype in the lung metastasis. Similarly, there was 1 case of CMS2 and 1 case of CMS4 which did not switch subtype from the primary tumour to the lung metastasis.

Table 4.3 CMS subtype and genomic alterations in primary tumours and their corresponding lung metastases

Trial ID	Stage	Primary site	CMS subtype			Genetic alterations			Chemo pre-resection lung metastasis 1	Chemo pre-resection lung metastasis 2	Outcome
			Primary tumour	Lung metastasis 1	Lung metastasis 2	Primary tumour	Lung metastasis 1	Lung metastasis 2			
2	II	Left	CMS4	CMS1	CMS4	*APC, KRAS, PIK3CA*	*APC, KRAS, PIK3CA*	No variants	CAPOX+Bev	FOLFIRI+Bev	Deceased
5	II	Rectum	CMS2	CMS4		*FBXW7*	*FBXW7*		CAPIRI		Deceased
7	I	Left	CMS2	CMS2		*TP53, ARID1A*	*TP53, ARID1A*		None		Survivor
11	III	Rectum	CMS3	CMS3		*APC, KRAS, TP53, FBXW7*	*APC, KRAS, TP53, FBXW7*		Raltitrexed/ Oxaliplatin		Deceased
12	III	Rectum	CMS4	No prediction		Not available	Not available		CAPOX		Lost to f/u
17	IV	Rectum	CMS4	CMS1		Not available	Not available		None		Survivor
19	II	Left	CMS3	CMS3		*NRAS, PIK3CA, PTEN, SMAD4*	*NRAS, PIK3CA, PTEN, SMAD4*		None		Survivor
25	III	Left	No prediction	CMS1		*APC, KRAS*	*APC, KRAS*		None		Survivor
32	III	Rectum	CMS4	No prediction		*APC, KRAS, TP53, CTNNB1, NOTCH3*	*APC, KRAS, TP53, CTNNB1, NOTCH3*		None		Deceased
45	IV	Left	CMS1	CMS4		*APC*	No variants		CAPOX		Deceased
50	III	Right	CMS2	Not available		*APC, KRAS, TP53, TCF7L2*	*APC, KRAS, TP53, TCF7L2*		FOLFIRI		Deceased
51	II	Rectum	CMS4	CMS4		*APC, KRAS, PIK3CA, TP53, AKT1, SMAD4, TCF7L2*	*APC, KRAS, TP53, TCF7L2*		FOLFIRI		Deceased
56	III	Rectum	CMS2	CMS4	No prediction	No variants	No variants	No variants	FOLFIRI/ Sunitinib	None	Deceased

Abbreviations: CAPOX=capecitabine/oxaliplatin, Bev=bevacizumab, CAPIRI=capecitabine/irinotecan, FOLFIRI=fluorouracil/irinotecan, f/u=follow up

4.3.3 CMS subtypes as prognostic markers

With a median follow up 111 months (range 28-135 months), mOS in our cohort of patients (n=13) was 74 months from diagnosis of metastatic disease (***Figure 4.11***). CMS1 and CMS2 in lung metastases were associated with the best prognosis whereas CMS4 was associated with the worst prognosis.

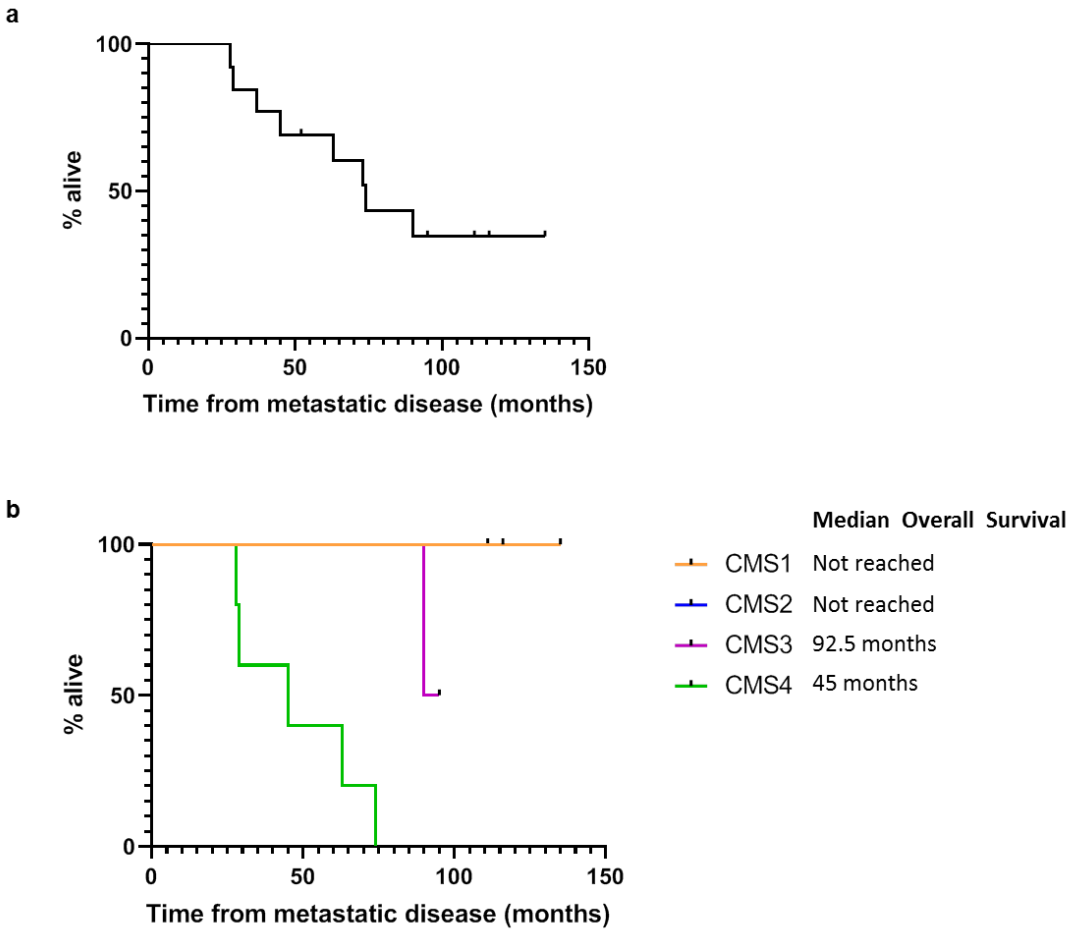


Figure 4.11 Kaplan-Meier estimates of overall survival (a) in all patients (n=13) (b) by CMS subtype in the lung metastasis

*Where more than 1 metastasis was present, the CMS subtype of the 2nd metastasis was used. CMS2 overlaps with CMS 1 and therefore cannot be seen.

4.4 Discussion

In our small but unique dataset, we found that CMS4 and CMS2 were the most prevalent subtypes amongst primary CRC tumour samples. This is consistent with the CRCSC data and other studies profiling the primary tumour in the 1st line advanced disease setting.[159, 161–163]

By virtue of the fact that rectal tumours have a predilection to metastasise to the lung, our cohort included a higher proportion of tumours from rectal cancer patients (46%) than the CRCSC dataset (15%). In the CRCSC data set, CMS4 was more prevalent in rectal tumours (31%) compared to left (27%) or right sided tumours (24%). CMS1 on the other hand was much more common in right sided tumours (31%) compared to left sided (7%) or rectal tumours (3%). The high relative proportion of rectal tumours in our cohort may have contributed to the high proportion of CMS4 and low proportion of CMS1 compared to the overall CRCSC data set. The influence of tumour location on CMS subtype is not surprising given that it is now widely established that there are clear differences in molecular biology, prognosis and response to therapy between right and left sided tumours.[171] Right sided tumours refer to tumours arising anywhere from the caecum to the proximal two thirds of the transverse colon whereas left sided tumours encompass tumours arising anywhere from the distal one third of the transverse colon until the rectum. A large meta-analysis including samples from almost 1.5 million participants demonstrated that left sided tumours have a significantly reduced risk of death independent of stage, race and adjuvant chemotherapy (HR, 0.82; 95% CI, 0.79-0.84; P< 0.001).[172] Left

sided, *RAS* wild type tumours are also more likely to respond to anti-*EGFR* antibodies.[173, 174] The differences in clinical behaviour are likely to be multi-factorial and include underlying molecular characteristics with right sided tumours being more likely to have a mucinous histology and harbour *BRAF* mutations whereas left sided tumours are more likely to have a gene expression profile consistent with activation of the *EGFR* pathway.

Amongst our small lung metastases sample set, as one might expect in the advanced stage disease setting, the CMS4 mesenchymal subtype was most prevalent. However, in the Khambata Ford dataset and Oslo-Co-Met trial where liver metastases were profiled, the CMS2 subtype remained the most prevalent although there appeared to be enrichment for the CMS4 subtype in the Khambata Ford dataset when compared to the early stage primary tumours from the CRCSC data set.

Interestingly, the aggressive phenotype of CMS4 was previously mainly attributed to genes expressed by stromal cells rather than epithelial cells.[175, 176] Isella et al. attempted to assess the true contribution of cancer cell intrinsic features by exploiting patient derived xenografts where stromal components of the tumour are substituted by murine counterparts and avoided with the use of human specific arrays.[177] They identified 5 CRC intrinsic subtypes (CRIS) and found that the CMS4 subtype was distributed across all CRIS subtypes with enrichment for CRIS-B which is associated with TGF-β pathway activity, EMT and poor prognosis. Prior to CRIS assignment, they evaluated both primary CRC tumours and liver metastases

using 3 published CRC signatures[155, 156, 158] which have now been reconciled into the CMS subtypes by the CRCSC. They found that transcriptional traits were largely similar between primary CRC and liver metastases.

In a more recent study which included CMS subtyping of primary CRC and matched metastases (from any site, n=71) a discordance rate of 40% was identified which is similar to the 44% seen in our dataset.[178] CMS 2 and 4 discordance was the most common whereas in our cohort of patients, discordance between CMS1 and 4 was most frequent. However, it is worth noting that in their dataset only 4 patients (6%) had matched primary CRC and lung metastases. This may account for the different trends observed. In the case of discordance between CMS2 and 4, the authors demonstrated that tumour intrinsic gene expression is largely maintained and any discordance is likely to be due to differences in the stromal composition. Indeed, stromal composition can be affected by treatments such as chemotherapy and radiotherapy.[179] CMS subtype switching following treatment has previously been described.[176, 180, 181]

In paired pre and post radiotherapy biopsies from patients with rectal cancer, a switch in subtype was observed in most cases (8/11, 73%) with the commonest switch being from CMS2 to CMS4 in all but 1 case (where a switch from CMS3 to CMS4 was present). Of the cases with a switch to CMS4, 2 cases had a complete response to treatment thereby further confirming the contribution of the stroma to the subtype derived.[176] More recently, data from our wider group demonstrated that a switch from CMS2

to CMS4 was present in patients progressing on cetuximab monotherapy in the absence of other identifiable genetic resistance mechanisms thereby supporting the importance of integrating analysis of the transcriptome.[181] Our own results also concur with this concept as although genomic alterations were largely concordant in primary CRC and corresponding metastases, when a limited NGS panel was used, analysis of the transcriptome revealed that there had been a switch in CMS subtypes. Woolston et al. demonstrated that selection pressure applied by cetuximab treatment resulted in CMS subtype switching and was associated with an increase in immune infiltrates and upregulation of immune checkpoint proteins such as LAG3 and PD-L1 in patients that initially responded but later progressed on cetuximab. Such changes may have therapeutic implications and recognition of this has led to the initiation of a clinical trial (iSCORE NCT03867799).[181]

In our dataset, 4 out of 5 patients (80%) with a switch in subtype had received neo-adjuvant chemotherapy prior to lung resection compared to 2 out of 4 (50%) without a switch in subtype. This suggests that treatment alone cannot account for subtype switching which may also be a reflection of prognosis or metastasis mechanisms. In our dataset, we had 4 patients that were discharged from follow up in the oncology clinic due to a long disease-free interval of at least 5 years which was suggestive of cure. None of these cases had metastatic samples with CMS4 subtype. Instead in tumours where CMS4 was maintained throughout or a switch to CMS4 subtype was observed, all patients are currently deceased. Indeed, patients with CMS4 lung metastasis samples had the worst mOS. In patients with lung

metastases from primary CRC, CMS4 subtype may be of particular relevance as a marker of poor prognosis but further work will be needed to confirm these findings. In a recent publication by Piskol et al., the CMS1 subtype in metastatic samples was associated with the worst overall survival.[178] However lung metastases were less prevalent in this study. Intriguingly, in our dataset the opposite pattern seemed to be observed which may signify that prognosis inferred by CMS subtypes might be specific to metastatic sites. The prognosis was good for 2 out of 3 patients with CMS1 lung metastases as they were discharged from oncology follow up. In the 1 case where the patient is deceased despite the 1st lung metastasis having a CMS1 subtype, the 2nd lung metastasis was CMS4 which may explain why prognosis was not as favourable. Indeed, this case confirms the heterogeneity that is present between different lung metastases in the same patient.

The higher prevalence of CMS1 subtype in metastatic samples (22%) compared to primary CRC samples (8%) was also reported by Piskol et al. where CMS1 was present in 16.9% of metastases samples and 9.34% of primary CRC tumours.[178] The CMS1 subtype is associated with immune infiltration and activation. Our RNA sequencing data revealed upregulation of genes involved in the immune response in metastases samples compared to primary CRC samples which is consistent with the increase in CMS1 subtype amongst metastatic samples that we observed. However, it remains uncertain whether upregulation of genes involved in the immune response is due to treatment or due to the metastatic process itself as most patients

received neo-adjuvant chemotherapy prior to resection of their lung metastases. Indeed there is significant mounting evidence that chemotherapy can affect the immunogenicity of tumour cells with both oxaliplatin and 5-fluorouracil (5-FU) being associated with an increase in tumour infiltrating cytotoxic T lymphocytes.[182–184] In CRC patients treated with 5-FU, tumour infiltrating CTLs were associated with a favourable prognosis.[185] Similarly, analysis of the intra-metastatic immune infiltrates in patients that had CRC metastases resected showed that response to treatment was associated with increased immune densities and a high immunoscore was associated with longer DFS and OS.[186] Upregulation of immune infiltrates and checkpoint inhibitor proteins in patients with acquired resistance to cetuximab with an associated switch in transcriptomic profile has been reported.[181] There may be potential to exploit this therapeutically and this is an area of active research.

Interestingly, when the transcriptomic profiles of matched primary CRC tumours and metastases (mainly liver) were compared in another study (n=13), the adrenergic and Reelin pathways were found to be upregulated in metastases whereas in our lung metastases specific data, upregulation of genes involved in the immune response pathway was the most significant.[187] These different findings support the importance of studying individual metastatic sites separately in an attempt to better understand the metastatic process and develop better treatments.

The data presented here is subject to a number of limitations including the small number of matched samples available for analysis and the lack of CMS prediction in some cases. Nevertheless, the results are hypothesis-generating and provide the basis for future work. The notion of intra-tumour heterogeneity is well described and in the context of CMS subtyping, has been demonstrated when multi-region tissue analysis was conducted.[188] We did not analyse multiple regions and this could have influenced our results. Moreover, the tissue samples analysed reflect a static time-point in a patient's clinical history with only 2 cases having tissue from more than 1 lung metastasis that were separated in time. Therefore integration of serial ctDNA analysis (as described in chapters 2 and 3) could be useful to study tumour evolution and this strategy has already been successfully embraced in other tumour types.[189] It also has to be recognised that patients included here had resectable metastatic disease and therefore the findings may not be applicable to cases with more extensive disease distribution and potentially more aggressive disease biology. Warm autopsies may provide an opportunity to secure tissue from multiple metastatic sites in patients with aggressive disease biology and the potential for this has already been implemented in the trial setting in studies.[190] The Posthumous Evaluation of Advanced Cancer Environment (PEACE) study (NCT 03004755) has already provided some valuable insights into patterns of metastatic spread by using cases sampled at post-mortem. High primary tumour heterogeneity appeared to be associated with a lower chance of metastasizing.[191]

Finally, there are potential technical limitations pertaining to the CMS subtyping which should be considered when interpreting and comparing results across studies. The initial CRCSC developed the CMS classifiers using fresh frozen samples rather than FFPE tumour tissue although work by our wider group and others has now been conducted in archival tumour samples.[178, 192] Additionally, we used the newest available CMS classifier (CMS caller) which has been optimised for use in pre-clinical samples but also showed 83% prediction accuracy in primary CRC tumour samples and has been utilised by others.[160, 170, 181] However, its accuracy for subtyping samples from metastatic sites has yet to be determined.

Implementation of CMS subtypes in routine clinical use has been challenging. This is partly due to lack of simple, cost effective assays suitable for FFPE tumour tissue although there are increasing efforts to rectify this by developing classifiers based on protein markers by immunohistochemistry or gene expression signals using ncounter platform (Nanostring technologies).[192, 193] The predictive value of CMS subtypes still remains uncertain. Previous findings suggested that CMS subtyping might be predictive of benefit from FOLFIRI and cetuximab.[158, 194] However, results have been inconsistent across studies assessing the predictive value of CMS subtypes for standard of care treatment.[162, 163] In the absence of proven value as a predictive tool, the role of CMS subtyping in the clinical setting may be limited. However, it still remains a useful method to better understand CRC biology.

As previously discussed, there has been limited work in metastatic samples particularly in the context of matched samples with primary CRC tumours. Our data from a cohort of patients with tissue from lung metastases samples suggests that the prognostic value of CMS subtypes may be different to previously published data which included multiple metastatic sites. Further work with a larger sample size will be needed to assess whether there are true differences which are specific to lung metastases.

5 CONCLUSIONS

In conclusion, the work presented here has shown that ctDNA is likely to be a valuable tool to enable risk-stratified treatment strategies in both the neo-adjuvant setting for rectal cancer and the post-operative setting in patients with CRC. As defined in the Cancer Research UK biomarker roadmap, the ctDNA work presented here has ensured that the stages of biomarker discovery and assay development as well as the first step of biomarker qualification have been met.[195] The first stage of biomarker development requires an accurate and reproducible assay to measure the biomarker. In both chapter 2 and 3, assays were evaluated for trueness by comparing ddPCR to NGS of tumour tissue DNA containing the variant of interest where NGS was considered the gold standard. Additionally, analyses were conducted at least in duplicate thereby allowing the precision and reproducibility of the method to be confirmed. Assay performance was evaluated as the next step of biomarker development by assessing the limit of detection of assays by spiking tumour DNA into 10-fold serial dilutions of Promega DNA. Whilst this could not be done for every assay due to the time, cost and limited tumour DNA available, when the analysis was conducted, the results that emerged were satisfactory and generally the limit of detection was ≤0.05% and in keeping with the published literature on ddPCR.[59] In order to mitigate for the risk of a sub-optimal assay and increase sensitivity, at least 2-3 assays were used per patient per time-point when multiple variants in the genes of interest were identified and assays were available. Knowledge that the tumour was in situ for the baseline sample in both the SSGCC rectal cohort and also within the TRACC feasibility study provided a

valuable opportunity to assess true ctDNA detection rates with the current methodology and relate these to disease stage. Both the TRACC feasibility study and the SSGCC rectal cohort had prospectively collected samples for the purposes of ctDNA analysis to enable correlation with clinical outcomes. The work presented here has demonstrated the role of ctDNA as a prognostic biomarker. In the rectal cohort of patients in chapter 2, in line with the study published by Tie et al, detection of ctDNA on completion of radiotherapy was associated with a worse prognosis.[62] In addition, I have shown that persistence of ctDNA in the neo-adjuvant setting is another marker of poor prognosis. In support of my findings two more recently published studies have also confirmed the value of serial ctDNA monitoring in the neo-adjuvant setting in patients with locally advanced rectal cancer.[196, 197] The data in the post-operative setting presented in the rectal cohort and TRACC feasibility study is consistent with the findings of others and demonstrates that detection of ctDNA is associated with relapse whereas undetectable ctDNA is associated with a good prognosis.[62, 63, 72, 75, 76, 78, 104, 198, 199] Furthermore, as in the neo-adjuvant setting, the literature has also shown the importance of serial ctDNA monitoring in the post-operative setting with serial monitoring being a better means of increasing sensitivity to predict relapse rather than sampling at a single-timepoint.[63, 200] Detection of ctDNA after completion of adjuvant chemotherapy has also been associated with an increased risk of recurrence when compared to patients who have undetectable ctDNA after treatment.[63, 78] Therefore, ctDNA clearance following adjuvant chemotherapy is also of interest as a real time marker of treatment efficacy which if proven as a surrogate for survival outcomes, may

have the potential to shorten the duration of trials in the adjuvant setting which usually take many years to report. Additionally, it may offer the opportunity for novel drug development aimed at targeting MRD that appears to be resistant to standard chemotherapy treatments. Indeed, treatment for persistent MRD following standard adjuvant chemotherapy is an area under investigation in ongoing clinical trials. The ACT-3 trial (NCT04259944) will be using the tumour-uninformed Guardant LUNAR-1 ctDNA analysis platform and comparing standard of care surveillance in this population to FOLFIRI in *BRAF* wild-type, microsatellite stable patients with other experimental treatment options including Encorafenib, Binimetinib and Cetuximab or Nivolumab reserved for *BRAF* mutant and microsatellite high patients respectively. Similarly, the non-randomised, interventional PEGASUS trial (NCT04259944) is also assessing the role of FOLFIRI in patients who continue to have detectable ctDNA following adjuvant chemotherapy. The trial uses the Guardant LUNAR-1 platform and initial adjuvant chemotherapy decisions are determined by the results of ctDNA analysis.[201] Also in the setting of persistent ctDNA detection despite adjuvant chemotherapy, the Japanese ALTAIR trial (UMIN000039205) will be using the tumour informed Signatera ctDNA analysis platform and randomising patients to placebo/surveillance or treatment with trifluridine/tipiracil.[201]

For ctDNA to be incorporated into routine clinical practice as a marker of MRD and hence an adjuvant chemotherapy decision making tool, randomised clinical trials are needed and represent the final step in the Cancer Research UK biomarker roadmap. The TRACC observational study

has recruited over 700 patients. It has provided the foundation for an interventional £3 million NIHR funded interventional study which opened to recruitment in January 2020.[202] The TRACC feasibility study provided a valuable platform to ensure that pre-analytical factors such as sample collection, transportation, processing and storage did not compromise ctDNA detectability when multiple recruiting sites were involved. Analysis of samples from 450 patients (including those that formed the feasibility analysis) has validated a tumour agnostic NGS approach for the interventional TRACC study to enable more realistic turnaround times for clinical utility. Globally, prospective clinical trials are underway to ascertain the true value of integrating ctDNA analysis as a method for determining the need for adjuvant treatment in the early disease setting and are well summarised in a recent review.[201] In general, trial designs include: a ctDNA guided strategy design where patients are randomised to receive standard of care or a ctDNA guided approach or alternatively, a ctDNA by treatment interaction design where all patients are randomised after the ctDNA result is known.[203, 204] Our own interventional TRACC study follows a ctDNA guided strategy design and findings will contribute to international collaboration within the IDEA-CIRCULATE initiative and support international consensus in this developing field. Randomised studies have incorporated different treatment options for the ctDNA guided interventional arm with both treatment escalation and de-escalation strategies being explored. In our interventional TRACC study, in the ctDNA guided arm, the primary focus will be a de-escalation strategy in patients that have undetectable ctDNA within 8 weeks of surgery. In patients that have their treatment de-escalated to either

no chemotherapy or to single agent chemotherapy (rather than doublet chemotherapy), detection of ctDNA at 3 months will require treatment escalation either to commencing adjuvant chemotherapy or to doublet chemotherapy from single agent. Patients who have ctDNA detected within the first 8 weeks of surgery will not have their treatment escalated. Adjuvant chemotherapy is normally commenced within 12 weeks. The TRACC study will provide a unique platform to assess the impact of delayed initiation of adjuvant chemotherapy in a select group of patients. Additionally, collection of blood and tumour tissue within the observational TRACC protocol will provide a well annotated clinical dataset to enable further translational correlative analyses to explore disease biology.

As work in the field progresses, we will also gain further insight into the limitations of ctDNA analysis. From the metastatic setting, there is already valuable data showing that ctDNA may be less likely to be detected in patients with metastases in the lung or peritoneum and in patients with a mucinous histology.[205, 206] This clearly has implications in the early disease surveillance setting. Similarly, there is a subset of patients in whom ctDNA is never detectable despite the primary tumour being in situ. At the current time it remains unclear whether there are biological differences which differentiate shedders from non-shedders or whether lack of ctDNA detection is simply a reflection of inadequate sensitivity of currently available analysis techniques. For the purposes of the interventional TRACC study, only patients with detectable ctDNA pre-operatively are being recruited as the reliability of

ctDNA as a biomarker is less certain if it is undetectable when the primary is in situ.

Although several randomised ctDNA interventional trials are underway and need to demonstrate clinical utility prior to routine clinical implementation, there are still likely to be several challenges and unanswered questions remain. Firstly, different platforms are being used for analysis and this may make overall data interpretation more difficult. Most observational studies have largely reported on ctDNA being either detectable or undetectable. There is now emerging evidence that the ctDNA level itself may have prognostic significance both in the work presented here from the rectal cohort and also the work of others.[198] With the use of different analysis platforms, clinically meaningful ctDNA levels might differ. Secondly, the turnaround times will need to be reliable to be able to make clinical decisions. While the tumour informed approach currently remains the most sensitive if multiple variants are tracked, it requires extra time and cost for tumour sequencing. Thirdly, the optimal timing of blood draws is uncertain. In the immediate post-operative period, increased release of cfDNA secondary to trauma from the surgery can make the tumour derived DNA more difficult to detect and therefore lead to false negatives. Waiting may increase the chance of detecting ctDNA as tumour burden may increase but this should not compromise the standard of care timelines to initiate adjuvant chemotherapy. It has been proposed that serial sampling in patients with undetectable ctDNA at 4 weeks might be an option to mitigate against false negatives.[203] Finally, pre-analytical variability between studies remains a

concern. The whitepaper from the Colon and Rectal-Anal Task Forces of the United States National Cancer Institute has made recommendations to standardise this and the timing of optimal blood draw.[64] Adherence to the recommendations will likely facilitate easier data interpretation across studies.

The role of ctDNA in surveillance following surgery is supported by data demonstrating genomic concordance between primary CRC and corresponding metastases. However, even in cases of genomic concordance using a limited NGS panel, we showed that transcriptomic profiles may differ between primary CRC and their corresponding lung metastases. This highlights the value of integrating analysis of the transcriptome to better understand metastasis promoting pathways and identify targets for future therapeutic intervention. Our preliminary findings suggest that gene expression profiles are largely conserved with only 4.9% of the analysed genes being differentially expressed when the primary tumour was compared to the first lung metastasis. Despite this, there was evidence of a switch in transcriptome-based CMS subtype between the primary tumour and matched lung metastasis sample in 5 out of 9 cases (56%) where CMS subtype prediction was possible. Although most patients (80%) with a switch in subtype had received neo-adjuvant chemotherapy, patients also switched subtypes in the absence of neoadjuvant treatment suggesting that reasons for CMS subtype switching may be multi-factorial and reflect metastatic mechanisms and changes in response to selection pressure from treatment. Unique to our dataset, which is the largest collection of matched primary

tumours with metastases arising exclusively in the lung; metastases with CMS1 appeared to be associated with a favourable prognosis. This suggests that the prognostic value of CMS subtypes may be metastatic site specific and further work will be needed to confirm these findings. Aside from its prognostic role, the predictive role of a switch in CMS subtype remains unclear. Further prospective work investigating the role of CMS switch in predicting response to specific therapy, such as immunotherapy is warranted as this is of clinical interest.

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7 APPENDIX

Table 7.1 Frequency of mutations detected in tissue and their detection rate in plasma in the SSGCC-1 study

	Tissue		Plasma		
Gene	**Variant**	**Frequency**	**Tracked in plasma?**	**Frequency**	**Pre-treatment detection rate**
KRAS	c.35G>A	7	Yes	7	4/7 (57%)
	c.38G>A	4	Yes	3	1/3 (33%)
	c.35G>C	2	Yes	2	0/2 (0%)
	c.35G>T	2	Yes	2	2/2 (100%)
	c.34G>A	1	Yes	1	1/1 (100%)
	c.34G>T	1	Yes	1	0/1 (0%)
	c.40G>A	1	No	0	N/A
NRAS	c.35G>A	1	Yes	1	1/1 (100%)
	c.182A>G	1	Yes	1	0/1 (0%)
BRAF	c.1799T>A	1	Yes	1	0/1 (0%)
PIK3CA	c.1633G>A	2	Yes	2	1/2 (50%)
	c.323G>A	1	Yes	1	1/1 (100%)
	c.1624G>A	1	Yes	1	1/1 (100%)
	c.1634A>G	1	Yes	1	0/1 (0%)
	c.3140A>G	1	Yes	1	1/1 (100%)
	c.1093G>A	1	No	0	N/A
TP53	c.524G>A	5	Yes	5	3/5 (60%)
	c.844C>T	4	Yes	4	2/4 (50%)
	c.818G>A	2	Yes	2	2/2 (100%)
	c.147delT	1	Yes	1	1/1 (100%)
	c.273G>A	1	Yes	1	0/1 (0%)
	c.329G>T	1	Yes	1	1/1 (100%)
	c.349G>T	1	Yes	1	0/1 (0%)
	c.376T>G	1	Yes	1	0/1 (0%)
	c.380C>T	1	Yes	1	0/1 (0%)
	c.395A>G	1	Yes	1	1/1 (100%)
	c.404G>T	1	Yes	1	1/1 (100%)
	c.482C>A	1	Yes	1	1/1 (100%)
	c.517G>T	1	Yes	1	0/1 (0%)
	c.528C>G	1	Yes	1	1/1 (100%)
	c.542G>A	1	Yes	1	1/1 (100%)
	c.550_551insC	1	No	0	N/A
	c.550G>C	1	No	0	N/A
	c.559+1delG	1	Yes	1	0/1 (0%)
	c.572_574delCTC	1	Yes	1	0/1 (0%)
	c.577C>T	1	Yes	1	0/1 (0%)
	c.610G>T	1	Yes	1	0/1 (0%)
	c.637C>T	1	Yes	1	0/1 (0%)
	c.644G>A	1	Yes	1	1/1 (100%)
	c.672+2T>C	1	No	0	N/A
	c.711G>A	1	Yes	1	1/1 (100%)
	c.712T>A	1	Yes	1	0/1 (0%)
	c.734G>A	1	Yes	1	0/1 (0%)
	c.743G>A	1	Yes	1	1/1 (100%)
	c.772G>C	1	Yes	1	1/1 (100%)
	c.782+1G>A	1	Yes	1	1/1 (100%)
	c.796G>A	1	Yes	1	1/1 (100%)
	c.817C>T	1	Yes	1	1/1 (100%)
	c.848G>C	1	Yes	1	1/1 (100%)
	c.916C>T	1	Yes	1	1/1 (100%)
	c.920-2A>G	1	Yes	1	1/1 (100%)
APC	c.3921_3925delAAAAG	3	Yes	2	1/2 (50%)
	c.3907C>T	2	Yes	2	1/2 (50%)
	c.472T>C	1	Yes	1	0/1 (0%)
	c.637C>T	1	Yes	1	1/1 (100%)
	c.667C>T	1	No	0	N/A
	c.835-1G>A	1	Yes	1	0/1 (0%)
	c.904C>T	1	No	0	N/A

	c.933+1G>A	1	Yes	1	1/1 (100%)
	c.1409-2_1409-1insG	1	Yes	1	1/1 (100%)
	c.1504G>T	1	No	0	N/A
	c.1660C>T	1	Yes	1	1/1 (100%)
	c.1690C>T	1	No	0	N/A
	c.1707_1708insA	1	Yes	1	1/1 (100%)
	c.2006_2007insA	1	No	0	N/A
	c.2336T>A	1	No	0	N/A
	c.2413C>T	1	Yes	1	0/1 (0%)
	c.2479delA	1	Yes	1	1/1 (100%)
	c.2563G>T	1	Yes	1	1/1 (100%)
	c.2589C>G	1	Yes	1	1/1 (100%)
	c.2626C>T	1	Yes	1	0/1 (0%)
	c.2861_2874delTAGAATACAAGAGA	1	No	0	N/A
	c.3173A>G	1	No	0	N/A
	c.3325delG	1	Yes	1	1/1 (100%)
	c.3386T>C	1	No	0	N/A
	c.3544A>T	1	Yes	1	1/1 (100%)
	c.3862delG	1	Yes	1	1/1 (100%)
	c.3890_3897delATTCTGCT	1	No	0	N/A
	c.3902delC	1	Yes	1	1/1 (100%)
	c.3916G>T	1	No	0	N/A
	c.3949G>T	1	Yes	1	1/1 (100%)
	c.3956_3957delCT	1	Yes	1	0/1 (0%)
	c.3957_3958delTG	1	Yes	1	1/1 (100%)
	c.3958delG	1	No	0	N/A
	c.3960_3964delGAGCG	1	No	0	N/A
	c.4012C>T	1	Yes	1	1/1 (100%)
	c.4052_4053insT	1	Yes	1	1/1 (100%)
	c.4069G>T	1	Yes	1	0/1 (0%)
	c.4132C>T	1	No	0	N/A
	c.4216C>T	1	No	0	N/A
	c.4233delT	1	Yes	1	1/1 (100%)
	c.4296delA	1	No	0	N/A
	c.4360delA	1	No	0	N/A
	c.4385_4386delAG	1	Yes	1	1/1 (100%)
	c.4587G>C	1	Yes	1	0/1 (0%)
	c.4660_4661insA	1	Yes	1	1/1 (100%)
	c.4706_4712delATGATGA	1	No	0	N/A
	c.5270C>G	1	No	0	N/A
	c.5270C>T	1	No	0	N/A

Table 7.2 Frequency of mutations detected in tissue and their detection rate in plasma in the TRACC feasibility study

	Tissue		Plasma		
Gene	**Variant**	**Frequency**	**Tracked in plasma?**	**Frequency**	**Pre-treatment detection rate**
KRAS	c.35G>T	7	Yes	5	1/5 (20%)
	c.38G>A	4	Yes	4	2/4 (50%)
	c.35G>A	3	Yes	2	0/2 (0%)
	c.34G>A	2	Yes	2	1/2 (50%)
	c.35G>C	2	Yes	2	2/2 (100%)
	c.34G>T	1	No	0	N/A
	c.436G>A	1	Yes	1	1/1 (100%)
NRAS	c.181C>A	1	Yes	1	1/1 (100%)
	c.34G>T	1	Yes	1	1/1 (100%)
BRAF	c.1799T>A	5	Yes	5	4/5 (80%)
	c.1385G>T	1	No	0	N/A
	c.1860+4G>A	1	Yes	1	1/1 (100%)
PIK3CA	c.3140A>G	4	Yes	4	2/4 (50%)
	c.1633G>A	4	Yes	1	1/1 (100%)
	c.1624G>A	1	Yes	1	1/1 (100%)
	c.1634A>G	1	No	0	N/A
	c.3067C>T	1	No	0	N/A
	c.1031_1051dupTGAATGTAAATATTCGAGACA	1	No	0	N/A
APC	c.3340C>T	3	Yes	3	2/3 (67%)
	c.694C>T	3	Yes	2	1/2 (50%)
	c.847C>T	3	Yes	3	1/3 (33%)
	c.1495C>T	2	Yes	1	1/1 (100%)
	c.3856G>T	2	Yes	2	1/2 (50%)
	c.3944C>A	2	Yes	2	1/2 (50%)
	c.4348C>T	2	Yes	1	0/1 (0%)
	c.1732G>T	1	Yes	1	1/1 (100%)
	c.2206A>T	1	Yes	1	1/1 (100%)
	c.2248C>T	1	No	0	N/A
	c.2365C>T	1	Yes	1	0/1 (100%)
	c.2497delA	1	Yes	1	0/1 (100%)
	c.2626C>T	1	Yes	1	1/1 (100%)
	c.2938delA	1	No	0	N/A
	c.3119delG	1	Yes	1	1/1 (100%)
	c.1138C>T	1	Yes	1	1/1 (100%)
	c.3396dupA_3402delC	1	Yes	1	1/1 (100%)
	c.3921_3925delAAAAG	1	Yes	1	0/1 (100%)
	c.3925G>T	1	No	0	N/A
	c.3944C>G	1	Yes	1	0/1 (0%)
	c.3970delC	1	Yes	1	1/1 (100%)
	c.3982C>T	1	Yes	1	0/1 (0%)
	c.4012C>T	1	Yes	1	1/1 (100%)
	c.4077dupA_4078T>A	1	Yes	1	1/1 (100%)
	c.4135G>T	1	Yes	1	0/1 (0%)
	c.4216C>T	1	Yes	1	0/1 (0%)
	c.4307delG	1	Yes	1	0/1 (0%)
	c.4360delA	1	Yes	1	1/1 (100%)
	c.4463_4464dupTA	1	Yes	1	1/1 (100%)
	c.4666dupA	1	Yes	1	0/1 (0%)
	c.568G>T	1	Yes	1	0/1 (0%)
	c.637C>T	1	Yes	1	0/1 (0%)
	c.646-1G>A	1	No	0	N/A
	c.4668_4669delTA	1	No	0	N/A
	c.1847dupT	1	No	0	N/A
	c.2378_2379delAA	1	Yes	1	1/1 (100%)
	c.3883G>T	1	Yes	1	1/1 (100%)
	c.3915dupA_3916G>A	1	No	0	N/A
	c.4067C>G	1	No	0	N/A
	c.4099C>T	1	No	0	N/A
	c.4385_4386delAG	1	No	0	N/A
	c.4385_4388delAGAG	1	No	0	N/A

	c.4461_4462delTT	1	Yes	1	1/1 (100%)
	c.4661delA	1	No	0	N/A
	c.526G>T	1	No	0	N/A
TP53	c.742C>T	2	Yes	1	0/1(0%)
	c.112C>T	1	Yes	1	0/1 (0%)
	c.319T>G	1	Yes	1	1/1 (100%)
	c.375+1G>A	1	Yes	1	1/1 (100%)
	c.375G>A	1	Yes	1	1/1 (100%)
	c.395A>G	1	Yes	1	1/1 (100%)
	c.456dupC	1	No	0	N/A
	c.473G>A	1	Yes	1	1/1 (100%)
	c.517G>T	1	Yes	1	0/1 (0%)
	c.524G>A	1	Yes	1	1/1 (100%)
	c.574C>T	1	Yes	1	1/1 (100%)
	c.584T>C	1	No	0	N/A
	c.595G>T	1	Yes	1	1/1 (100%)
	c.659A>G	1	Yes	1	0/1 (0%)
	c.701A>G	1	Yes	1	1/1 (100%)
	c.707_709delACA	1	Yes	1	1/1 (100%)
	c.712T>G	1	Yes	1	1/1 (100%)
	c.743G>A	1	Yes	1	1/1 (100%)
	c.817C>T	1	No	0	N/A
	c.818G>A	1	Yes	1	0/1 (0%)
	c.820dupT	1	Yes	1	0/1 (0%)
	c.993+1G>A	1	No	0	N/A
	c.993+1G>C	1	Yes	1	0/1 (0%)
	c.528C>G	1	Yes	1	1/1 (100%)
	c.321C>A	1	Yes	1	0/1 (0%)

Table 7.3 Detailed clinical histories of patients included in the transcriptomic analysis (n=13)

Trial ID	Clinical information
2	**01st Oct 05:** Sigmoid colectomy for T4, N0 (0/16) moderately differentiated adenocarcinoma of the sigmoid colon with no extra mural venous invasion **14th Nov 05:** Post-operative scan: Two synchronous PET avid lung metastasis (left upper lobe and right lower lobe) **Dec 05:** Reversal of ileostomy **09th Jan 06-13th March 06**: 4 Cycles CAPOX +Bevacizumab with stable disease on CT **16th May 06:** Right lung metastasectomy with a metastasis in the **right lower lobe** (and granulomas in the right lower lobe and right upper lobe) **29thJune 2006:** Left lung metastasectomy with a metastasis in the left upper lobe (and 2 granulomas in the left lower lobe). New lesion in the apical segment of the left lower lobe on post-operative imaging **18th Sept-19th Feb 07:** completed FOLFIRI and Bevacizumab x12 cycles with an initial reduction then stable disease of the lung nodule. **26 March 07: Completion left upper lobe lobectomy** **August 07:** Probable new solitary metastasis in right apex and anastomotic recurrence. **Nov 07:** Completed Pelvic radiotherapy, 54 Gy in 30# with concomitant Capecitabine, completed to a good partial response **February 08:** Anterior resection with colostomy with post-operative complications **June 2008:** Metastasectomy right apical region followed by watch and wait **July 09:** Pelvic recurrence and possible lung recurrence. Capecitabine and Bevacizumab x3 cycles with a favourable response in the lung recurrence but developed an entero-vesical fistula. **December 09**: Ileostomy **March 2010:** left endo-bronchial stent **April 2010:** Re-staging PET shows progressive disease in left main bronchus, commenced capecitabine/bevacizumab on 26th April, with general then general decline after 3 cycles **September 2010:** completed palliative radiotherapy to origin of left main bronchus/mediastinal nodes around carina **March 2011:** Deceased
5	**Jan 2000:** Anterior resection for T3, N0 (0/10) adenocarcinoma of the rectum. **June 2000:** Completed adjuvant chemotherapy with 12 weeks of protracted venous infusion 5FU in a trial. **Feb 2001:** Recurrent solitary liver metastasis **April 2001:** right hemi-hepatectomy, pathology confirmed 2 metastases. **August 2001**: metastasis in the left lower lung resected September 2001. **March 2002:** further metastasis in the left lobe of the liver resected 26th April 2002. Treatment with Capecitabine and Oxaliplatin for 4 cycles. **December 2003**: Further nodule noted at the apex of the left lung **March 2004:** completed 4 cycles of Capecitabine and Oxaliplatin **19th April 2004:** Left pulmonary metastasectomy. **August 2004:** completed post-operative Capecitabine and Oxaliplatin x4 cycles **January 2005:** recurrence with 2 new lung metastases at the left apex confirmed on PET scanning. Local recurrence in the pelvis on MRI. **February-April 2005:** Capecitabine and Irinotecan x4 cycles a reduction in the size of the 2 lung metastases and a fall in CEA from 16 to 7. **June 2005:** completed pelvic radiotherapy 54Gy with concomitant Capecitabine. MRI pelvis shows stable disease. Most recent CT shows some increase in the size of one of the lung metastasis. **31st Aug 2005: Completion upper lobectomy.** **January to April 2006:** Capecitabine and Irinotecan x4 cycles. CT and MRI scan show stable disease but elevated tumour marker. PET scan demonstrated active disease in the right side of the rectum extending to the prostate and seminal vesicles. **July 2006:** Radical pelvic extenteration and cystectomy with residual disease involving the prostatic urethra. Post-operative imaging demonstrated liver and lung metastases. **Aug 2006:** post-operative period complicated by abscess **27th April 2007:** deceased
7	**1998:** Stage III adenocarcinoma followed by adjuvant chemotherapy with 5-FU **Aug 2000:** Pulmonary metastases (right and left) noted **16th October 2000: Left lower lobe metastasectomy** **17th November 2000:** right upper lobe metastasectomy **Feb 2001:** commenced adjuvant 5-FU with early termination due to toxicity **27th Nov 09:** Discharged from f/u

11	**November 1998:** Stage III rectal cancer treated with resection and post-operative chemotherapy with Raltitrexed. Subsequent liver and lung metastases treated with chemotherapy: Capecitabine and Mitomycin C for 24 weeks with stable disease. **July 2002:** Liver metastasectomy. **September 2002:** Raltitrexed and Oxaliplatin completed with progressive disease in the lung. **21st November 2002: Left Lung metastasectomy** (with adenocarcinoma in apical tumour and basal segments of left lower lobe) to a disease-free state. **February 2005:** Pre-sacral recurrence **5th June- 14th July 2006:** pelvic radiotherapy 45Gy in 25# followed by 9Gy in 5#, with concurrent Raltitrexed with stable disease. **March 2007:** Bilateral small volume lung nodules. Raltitrexed and Irinotecan x3 cycles with a favourable response in the lung metastasis. Completed 8 cycles of Raltitrexed and Irinotecan to stable disease (but with a minor increase in a few of the nodules by a few millimetres). **3rd Jan 2009:** Deceased
12	**August 2001:** Anterior resection of T3N1 mid rectal moderately differentiated adenocarcinoma with adjuvant chemotherapy **May 2004:** pulmonary metastases on surveillance scan **Sept 2004:** completed 4 cycles CAPOX **2nd Nov 2004:** pulmonary metastasectomy for **left lower lobe adenocarcinoma** **09th Dec 04:** pulmonary metastasectomy for right upper lobe tumour **20.6.07:** progressive disease, 2 new nodules in the right lower lobe and a large solitary deposit in the liver on MRI. **Aug 07:** single agent Irinotecan **Feb 08:** progressive disease after 8 cycles in lung and liver **March-Aug 08:** 8 cycles CAPOX with progressive disease in lung and liver on September CT scan (Lost to follow/up as went to Argentina)
17	**February 2005:** Mid rectal poorly differentiated adenocarcinoma with three liver metastases Capecitabine, Oxaliplatin chemotherapy x 4 cycles. Good partial response in the liver and in the primary tumour. **June 2005:** Completed chemotherapy/radiation to the primary tumour. MRI imaging showed further response in both the liver and the primary tumour. **August 2005:** Anterior resection. No evidence of residual tumour in the resected specimen. **October 2005:** Right hepatectomy **February 2006:** Post-operative Capecitabine and Oxaliplatin x4 cycles completed **March 2009:** Recurrence with a right apical nodule and superior mediastinal lymph node **24th March 09: Wedge resection of the right upper lobe nodule and removal of the pre-vascular lymph node** **March-October 2009:** Capecitabine, Oxaliplatin and Bevacizumab x8 cycles completed **16th May 2016:** discharged from f/u
19	**July 2008:** Surgical resection of T3 N0 well/moderately differentiated sigmoid carcinoma. Post-operative CT scan revealed lung nodule suggestive of metastasis, positive on PET scan. **13th Nov 2008:** Underwent resection of lung metastasis, **right upper lobe adenocarcinoma** **September 2009:** Completed 12 cycles of FOLFOX chemotherapy. On follow up since then **5th Sept 2016:** discharged from f/u
25	**October 2007:** anterior resection for T3 N1 (2/16) sigmoid colon adenocarcinoma **July 2008:** completed 12 cycles of Adjuvant FOLFOX chemotherapy (Oxaliplatin omitted after 10 cycles due to peripheral neuropathy) **15th September 2009:** Recurrence 1 lung metastasis, **treated with metastasectomy (right lower lobe lung)** **August 2011:** Recurrence 2 right upper lobe metastases; one of them treated with stereotactic radiotherapy, 60Gy in 8# completed 2.6.11. **November 2011:** progression of right upper lobe metastasis treated with radiofrequency ablation **24th September 2018:** D/C from f/u

32	**Jul - Aug 2010:** T3c N1 M0 EMVI +ve moderately differentiated adenocarcinoma of the upper rectum, Chemoradiotherapy 54Gy in 30# completed followed by resection **Jan - Jul 2011:** Adjuvant FOLFOX chemotherapy. **Nov 2011:** Reversal of stoma. **Jan 2012:** Relapse in the liver and lung. **10th Feb 2012: <u>Metastasectomy of left lower lobe lung metastasis.</u>** **Jun 2012:** 4 cycles of neo-adjuvant CAPOX chemotherapy completed **Jul 2012:** Surgical resection of a solitary liver lesion in segment VIII. **Sep - Dec 2012:** 4 cycles of adjuvant CAPOX chemotherapy. **Feb 2013:** Right thoracotomy lung resection for the right lung metastases. **Sep 2014:** Right lung metastasis, stable liver metastasis. Chemotherapy with FOLFIRI + Aflibercept followed by consideration of RFA to the lung metastasis. **May 2015:** RFA to liver **Jun 2015:** Post-RFA CT scan shows evidence of disease progression in the lungs with new bilateral pulmonary nodules. **Feb 2016:** CT scan showed mild progression in the lung metastases but disease was not biopsiable. **May 2016:** CT showed further disease progression with relapse of liver metastatic disease measuring 52mm in size along with progression in lung metastases. **Jun 2016:** Commenced treatment with Regorafenib, stable disease after 2 and 4 cycles. Progressive disease after 6 cycles of Regorafenib. **Mar - May 2017:** Stable disease after 3 cycles of Trifluridine/Tipiuracil, further dose reduction of chemotherapy in view of grade 4 neutropenia, followed by progressive disease. **Aug 2017:** Commenced Phase I clinical trial with progressive disease **November 2017 - January 2018:** phase 1 trial **January 2018:** Disease progression **3rd Feb 2018:** Deceased
45	**June 2008:** Left hemi-colectomy for a T4N0M1 metastatic sigmoid colon adenocarcinoma with synchronous lung involvement **December 2008:** Capecitabine and Oxaliplatin x 5 cycles completed. **16th December 2008: <u>Left thoracotomy and pulmonary metastasectomy for mucinous adenocarcinoma in lingular nodule and lateral basal segment.</u>** **9th February 2009:** Right thoracotomy and pulmonary metastasectomy for right upper lobe mucinous adenocarcinoma. **April 2009:** Capecitabine and Oxaliplatin x 2 cycles stopped due to nausea. Capecitabine x 1 cycle completed May 2009 to progressive disease. **June 2009:** FOLFIRI commenced. Progressive disease after 6 cycles **Oct 2009:** third line Capecitabine/Mitomycin chemotherapy with progressive disease in liver and peritoneum after 2 cycles **Jan 2010:** Phase I clinical trial with progressive disease on cycle 2 day 24 in March **Nov 2010:** deceased
50	**February 2005:** Right hemicolectomy for ascending colon cancer, pT3N2(3/15) and adjuvant chemotherapy with Oxaliplatin and 5FU completed eleven cycles. **February 2007:** developed liver metastases and treated with staged liver resection as three new metastases found in the left lobe at laparotomy. **July 2007-December 2007:** lung metastases on CT, treated with FOLFIRI **29th February 2008: <u>right thoracotomy with multiple areas of adenocarcinoma in lower and upper lobe</u>** **April to November 2008:** FOLFIRI and Cetuximab. **February 2009:** progressive disease in lungs and liver. **July 2009:** commenced further chemotherapy with Capecitabine and Oxaliplatin. **October 2009:** evidence of bone metastases on bone scan. Received palliative radiotherapy to her right pelvis and ribs. **December 2009**: disease progression on CT scan and tumour markers. **24th March 2010:** Deceased

51	**February to July 2008:** adjuvant Capecitabine x 8 cycles for pT3b N0 M0 (0/14) EMVI positive rectal adenocarcinoma. **August 2010:** Re-staging CT shows bilateral upper lobe lung metastases. **September 2010:** consideration of peri-operative FOLFIRI (Oxaliplatin relatively contra-indicated due to peripheral neuropathy from diabetes) plus lung resection. **December 2010:** Completed 6 cycles of neoadjuvant FOLFIRI **16th January 2011:** Patient underwent resection of lung metastases with central upper lobe adenocarcinoma **10th February 2011: <u>Patient underwent resection of lung metastases</u>** with left subsegmental resection posterior upper lobe containing adenocarcinoma. Post-operative course complicated by acute myocardial infarction and multiple VF arrests requiring insertion of coronary artery stenting and implantable defibrillator. **December 2011:** disease progression in lung. **January 2012:** commenced on FOLFIRI chemotherapy. Bevacizumab added in from Cycle 4, but 1 cycle only as found to have ejection fraction of between 15 to 20%. This was subsequently stopped. **April 2012 until February 2013**: Cycle 5 to 18 FOLFIRI with small volume lung metastases and essentially stable disease. **March 2013:** Treatment break. **July 2013:** Disease progression in lung metastases. **August 2013-March 2014:** FOLFIRI x 11 cycles with disease progression. **April 2014:** For consideration of FOLFOX (patient agrees that there is a relative complication due to pre-existing diabetic neuropathy), deteriorated before starting **9th June 2014:** Deceased
56	**2005:** diagnosed with T3 N1 EMVI positive adenocarcinoma of ascending colon treated with right hemicolectomy and six months adjuvant 5-FU. **December 2006:** developed metastatic pulmonary nodules treated in trial with FOLFIRI and Sunitinib. Only received 4 cycles (poorly tolerated), partial response in lung nodules. **17th April 2007: <u>Metastasectomy (left upper lobe adenocarcinoma)</u>** followed by adjuvant chemotherapy, initially 5-FU, poorly tolerated so changed to Capecitabine, completed September 2007. **21st February 2008: <u>right thoracotomy for RUL subsegmental solitary pulmonary metastases.</u>** Rising markers with enlarging left upper lobe lesion. **17 September 2008:** RFA to lung nodule **March 2009:** new brain metastases, completed WBRT 23rd March 2009 **27th April 2009:** deceased

*The lung metastasis samples that were used to profile the transcriptome have been highlighted in bold underlined in each clinical history.