ctDNA Guided Diagnosis and Management of Gastrointestinal Cancers

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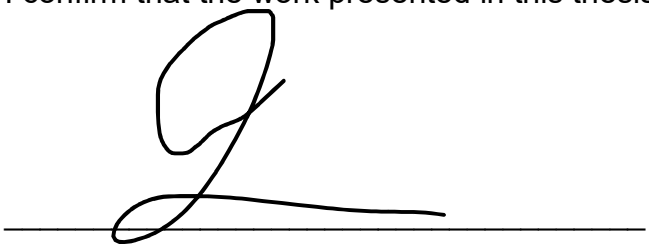
Author's Declaration

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



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Abstract

Gastrointestinal (GI) cancers including pancreatic (PC), biliary tract (BTC) and colorectal cancers (CRC) rely on a tissue biopsy through invasive procedures such as endoscopies and colonoscopies. Timely diagnosis of these cancers is critical to facilitate access potentially life prolonging treatments. The COVID-19 pandemic highlighted the fragility of the current invasive diagnostic pathways for upper and lower GI cancers. In the UK, there was a reduction in capacity of colonoscopies and endoscopies as resources were re-directed to focus on the pandemic. There is a high unmet need for non-invasive biomarkers to support a diagnosis of cancer in patients with suspected PC, BTC and CRC.

My thesis focuses on the following work I have completed;

- Designing and reporting on a prospective study of circulating tumour DNA (ctDNA) to support a diagnosis of PC/BTC and to triage colonoscopies in patients with suspected CRC
- Designing and implementing a prospective, real-world programme assessing ctDNA as a supportive diagnostic biomarker in patients with suspected advanced PC and BTC

I developed and reported on the PREVAIL ctDNA study, assessing the use of ctDNA as a supportive diagnostic tool in patients with suspected cancer across multiple tumour types including PC, BTC and CRC. This is the first prospective study assessing the use of a multi-gene ctDNA assay, in a tumour-agnostic approach to support a diagnosis of PC/BTC and inform treatment decisions.

My thesis reports on the rationale, design, and final results from the PC, BTC and CRC cohorts of the PREVAIL ctDNA study.

Following the PREVAIL ctDNA study, I developed and implemented a larger, real-world programme assessing ctDNA as a supportive diagnostic biomarker in patients with suspected advanced PC/BTC (the ACCESS programme). The programme is actively recruiting at the time of writing my thesis. My final chapter will describe the rationale, design and interim results from the ACCESS programme.

Abbreviations

1L	First line
2L	Second line
2WW	Two-week wait
AA	Advanced adenoma
ACC	Acinar cell carcinoma
ADC	Adenocarcinoma
AE	Adverse event
AIP	Autoimmune pancreatitis
AJCC	American Joint Committee on Cancer
AMP	Association for Molecular Pathology
ARCH	Age-related clonal haematopoiesis
ASC	Active symptom control
ASCO	American Society of Clinical Oncology
BBD	Benign biliary disease
BEAMing	Beads, emulsion, amplification, magnetics
BSC	Biological specimen coordination
BTC	Biliary tract cancer
CA 19.9	Cancer antigen 19-9
CAP	College of American Pathologists
CBD	Common bile duct
CCC	Cholangiocarcinoma
cfDNA	Circulating free DNA
CHIP	Clonal Haematopoiesis of Indeterminate Potential
CI	Confidence interval
CMP	Centre of Molecular Pathology
CONSORT	Consolidated Standards of Reporting Trials
CORD	Combined restriction digital PCR
CpG	5'-C-phosphate-G-3'
CR-mCRC	Chemo refractory metastatic CRC
CRC	Colorectal cancer
CRF	Case report form
CRM	Circumferential resection margin
CRUK	Cancer research UK
CSO	Cancer signal origin
CT	Computerised tomography
CTC	CT colonoscopy
CTCs	Circulating tumour cells
ctDNA	Circulating tumour DNA
ddPCR	Digital droplet PCR techniques

DDR	DNA damage repair
DISH	Dual colour silver in situ hybridisation
ECCC	Extra-hepatic cholangiocarcinoma
ERCP	Endoscopic retrograde cholangiopancreatography
ESCAT	ESMO Scale for Clinical Actionability of molecular Targets
EUS	Endoscopic ultrasound
FIT	Faecal immunohistochemical test
FDG-PET	Fluorodeoxyglucose positron emission tomography
FDS	Faster diagnostic standard
FNA	Fine needle aspiration
FNB	Fine needle biopsy
FOLFOX	Fluoropyrimidine and oxaliplatin chemotherapy
GBC	Gallbladder cancer
gFOBT	Guaiac faecal occult blood test
GI	Gastro-intestinal
GTAB	Genomic tumour advisory board
HCL	Hairy cell leukaemia
HGD	High grade dysplastic polyps
HRD	Homologous recombination deficiency
ICI	Immune-checkpoint inhibitors
ICR	Institute of Cancer Research
IGV	Integrated genomics viewer
IHC	Immunohistochemistry
IHCC	Intra-hepatic cholangiocarcinoma
IPMN	Intraductal papillary mucinous neoplasm
IVD	In-vitro diagnostics
KYT	Know your tumour initiative
LAPC	Locally advanced pancreatic cancer
LGD	Low grade dysplasia
lcWGS	Low coverage whole genome sequencing
LOD	Limit of detection
MCED	Multi-cancer early detection test
MDM	Multi-disciplinary meeting
mPC	Metastatic pancreatic cancer
MRCP	Magnetic resonance cholangiopancreatography
MRD	Minimal residual disease
MSI	Microsatellite instability
MSP	Methylation-specific PCR
MTB	Molecular tumour board
mVAF	Maximum VAF
NCCN	National comprehensive cancer network
NGS	Next generation sequencing

NHSE	National Health Service England
NIHR	National Institute for Health and Care Research
OS	Overall survival
panNET	Pancreatic neuroendocrine tumour
PASC	Pancreatic adenosquamous carcinoma
PDSA	Plan do study act
PC	Pancreatic cancer
PCN	Pancreatic cystic neoplasm
PDAC	Pancreatic ductal adenocarcinoma
PIS	Patient information sheet
PPIE	Patient and public involvement and engagement
PROs	Patient reported outcomes
PTC	Percutaneous transhepatic cholangiography
QOL	Quality of life
R&D	Research and Development
REC	Research ethics committee
RMH	Royal Marsden hospital
RMP	Rm partners
SAP	Statistical analysis plan
SBRI	Small busines research initiative
SIV	Site initiation visit
SMA	Superior mesenteric artery
SMV	Superior mesenteric vein
Sn	Sensitivity
SNPs	Single nucleotide polymorphisms
SNV	Single nucleotide variant
Sp	Specificity
SV	Structural variant
T-DXd	Trastuzumab deruxtecan
TME	Total mesorectal excision
UICC	Union for International Cancer Control
UK	United Kingdom
UMI	Unique molecular identifier
US	Ultrasound
VAF	Variant allelic frequency
VUS	Variant of uncertain significance
WBC	White blood cells
WES	Whole exome sequencing
WGM	Whole genome methylation
WGS	Whole genome sequencing
WHO	The world health organisation
XR	X-ray

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1. Background

1 Pancreatic Cancer

Pancreatic cancer (PC) can arise from exocrine or neuroendocrine cells. Approximately 95% of all PC are exocrine PC, the most frequent subtype being pancreatic ductal adenocarcinoma (PDAC). I will refer to PDAC as PC throughout this thesis unless otherwise stated.

1.1.1 Diagnostic Approach in Suspected Pancreatic Cancer

In those individuals presenting with symptoms suggestive of PC, imaging is performed to identify the primary lesion and establish the extent of disease (and staging). Histological confirmation is required through an invasive biopsy to achieve a formal diagnosis.

The use of CT imaging in the identification of PC carries a high Sn (~80%) and Sp (~100%) and is used in determining likelihood of resectability and the present of distant metastases. However, similarly to US, the Sn of CT is lower in the identification of smaller tumours (<2cm in size) (1-4).

Endoscopic ultrasound (EUS) is an invasive technique used to identify lesions in the pancreas through direct visualisation of the pancreas during endoscopy. It can be combined with fine needle aspiration (FNA) or biopsy (FNB). EUS and FNB/FNA combined has a high Sn (between 80-90%) and Sp (between 90-99%) for the detection of PC (3). However, reports have shown that Sn

varies and may be as low as 77% (5). A large meta-analysis showed that FNB provided slightly higher diagnostic accuracy compared to FNA (87 vs 80%), owing to the larger tissue samples obtained from FNB (6). The addition of molecular markers (such as KRAS) to histopathological diagnosis improves the Sn.

Endoscopic retrograde cholangiopancreatography (ERCP) is an invasive procedure that uses endoscopy to cannulate the biliary tree and, at the same time, fluorescent X-ray (XR) to visualise the ductal system. It enables examination and treatment of the biliary system for diverse biliary and pancreatic conditions including malignant and non-malignant disease (i.e. biliary stone, non-malignant strictures). ERCP with contrast has good Sn and Sp in the diagnosis of PC (92% and 96% respectively) (3). However, ERCP alone is not sufficient for a diagnosis of PC in the absence of a biopsy and has largely been replaced by EUS +/- FNB in most diagnostic centres. ERCP can be combined with tissue sample techniques, such as cytological brushings to obtain a histological diagnosis. Cytology from brushings taken at the time of ERCP has a relatively low diagnostic yield for PC, with reported Sn between 20-60% and Sp of ~90%, and generally used for the diagnosis of malignant biliary strictures as outlined below (7, 8). ERCP is an invasive procedure which carries a risk of pancreatitis (3-4%), cholangitis (1.5%), bleeding (1-2%), and mortality (0.1-0.2%) (9, 10). Therefore, MRCP may be preferred over ERCP in those not requiring intervention (such as treating biliary obstruction), as well as in those with gastric outlet obstruction, duodenal strictures, active pancreatitis.

In those with metastatic disease amenable to biopsy (i.e., liver metastases), a percutaneous needle biopsy has a good Sn (90-100%) and Sp (83-100%) for PC (11). However, the diagnostic yield of an invasive biopsy (percutaneous or EUS/ERCP guided) may be as low as 80% and often requires repeated procedures to obtain a histopathological diagnosis (5, 11, 12). In addition, the genomic sequencing to support access to appropriate targeted therapies/immunotherapy requires additional tissue to provide adequate DNA for sequencing.

Hence, there is growing interest in the use of non-invasive, diagnostic tests for PC. The tumour marker carbohydrate antigen 19-9 (CA19.9) is a glycoprotein complex found on many types of cells, including PC, which can be detected and quantified in the circulation. Measurement of CA19.9 is currently recommended by NCCN as part of the diagnostic and staging work-up of patients with PC. Pre-operative elevation in CA19.9 can predict prognosis and likelihood of resectability in those with potentially resectable PC, and can be used in the metastatic setting to monitor response to treatment (13). The use of CA19.9 in the diagnosis of PC is limited, given its relatively low Sn (70%) and Sp (68%) (14). The use of a biomarker such as CA19.9, which has low Sp and a high rate of false positive results, often leads to the need for additional diagnostic procedures, causing increased anxiety in patients and potentially causing harm. The level of CA19.9 is dependent on tumour burden and can be falsely negative in those with Lewis-negative antibody phenotype. CA19.9 can be elevated in several non-malignant conditions (such as cholangitis, liver

cirrhosis, gallstone disease) and non-pancreatic related malignant conditions (i.e., neuroendocrine tumours, BTC, hepatocellular carcinoma, gastric, ovarian, colorectal, lung and breast cancers), limiting its use as a screening and diagnostic tool in PC. In asymptomatic patients, the positive predictive value (PPV) of CA19.9 as a screening tool for PC is only 0.9% (15). The optimal cut off for CA19.9 elevation is ≥37 units/ml, however, even at higher cut-offs such as >300 units/ml, CA19.9 is not completely accurate in distinguishing between benign and malignant pancreatic conditions (16). The various invasive diagnostic procedures and their associated diagnostic yield is summarised in table 1.

Table 1- Diagnostic yield of various methods in the diagnosis of PC

	Sn (%)	Sp (%)	PPV (%)	NPV (%)	Ref(s)
Trans-abdominal US	89	99	93	98	(17)
CT	70-80	43-100	60	56	(1, 3, 4)
MRCP	84	97	91	93	(18)
ERCP	70-92	96	NR	NR	(3)
ERCP + brushings	20-60	90	98	61	(7, 8)
EUS + FNA	77-90	68-80	100	43-77	(5)
EUS + FNB	83	100	NR	NR	(3)
Trans-cutaneous biopsy	91-100	83-100	91-100	83-100	(11)

Sn- Sensitivity; Sp- specificity; PPV- positive predictive value; NPV- negative predictive value; US- ultrasound; CT- computed topography; MRCP- Magnetic resonance cholangiopancreatography; ERCP- Endoscopic retrograde cholangiopancreatography; EUS- Endoscopic ultrasound; FNA- Fine needle aspiration; FNB- Fine needle biopsy; NR- not reported; Ref- references

1.1.2 Molecular Landscape of Pancreatic Cancer

2 Frequently Aberrated Genes in Pancreatic Cancer

The molecular landscape of PC has been extensively investigated in large studies, predominantly based on tissue sequencing (19). The development and widespread use of next generation sequencing (NGS), including whole exome (WES) and genome (WGS) sequencing, has expanded the knowledge of PC molecular biology in characterisation of driver and targetable aberrations in this disease. *KRAS* mutations are seen in majority of patients with PC, however there are several other mutated genes seen at relatively low frequencies which contribute to the high level of intra-tumoural heterogeneity in PC.

Single nucleotide variants (SNV) represent the most common genomic aberration in PC, with an average mutational burden in PC of 2.64 per Mb (19). The most commonly mutated genes in PC are *KRAS* (~90%), *TP53* (~70%), *CDKN2A* (~30%) and *SMAD4* (~30%). Waddel et al. assessed the molecular landscape of PC in a large cohort of patients using WGS, with SNV point mutations in *KDM6A* (18%), *PREX2* (10%) and *RNF43* (10%) present at a relatively high frequency (19). RNF43 mutations are potentially sensitive to WNT inhibition and the subject of ongoing clinical trials (NCT04907851). The most common (>5%) aberrations seen in PC are shown in figure 1 based on the Cancer Genome Atlas Research Network group (20).

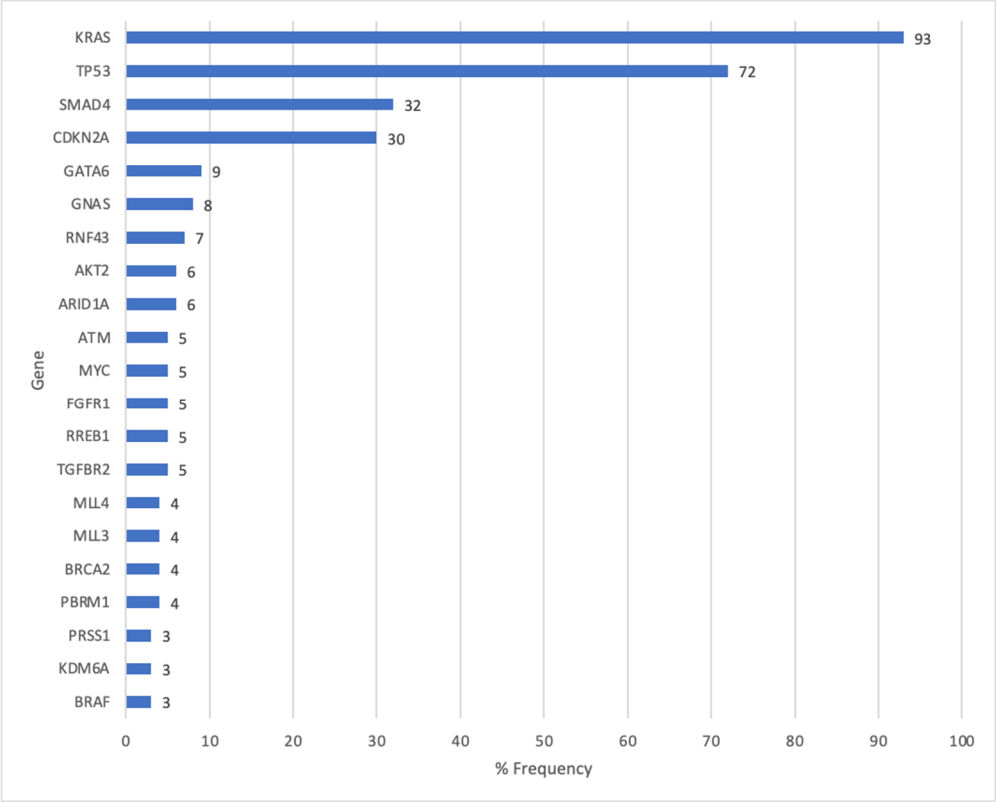

Figure 1- Common occurring aberrations in PC (≥3 %)

Somatic *KRAS* mutations are seen in most patients with PC, with frequency reported between 66% and 93% (20). A large whole-exome sequencing (WES) study involving 150 patients with PDAC revealed *KRAS* mutations occur in 93% of samples, most commonly at codons G12D (44%), G12V (29%) and G12R (20%) (20). Majority of these mutations are clonal, however sub-clonal *KRAS* mutations can be detected a greater sequencing depth.

KRAS wildtype tumours are seen in approximately 8-10% of PC, and associated with favourable prognosis compared with *KRAS* mutant tumours (21). *KRAS* wildtype tumours are associated with other driver mutations including *TP53* (44%), *ATM* (26%), *CDK2NA* (13%), *BRCA1*/2(12%), *EGFR* (11%), *FGFR1*/2 (9%), *BRAF* (8%), *PIK3CA* (7%), *MET* (7%) and *HER2* (6%)

(22, 23). *KRAS* wildtype tumours were also enriched with *ATM*, *JAK3* and *NOTCH1* mutations compared to *KRAS* mutant tumours. A recent presentation at ESMO revealed *KRAS* wildtype tumours are associated with detection of a potentially targetable alteration in 38% of patients including *RET* fusion, *BRAF* V600E, MSI-H, *FGFR2* fusion, *PALB2* mutation and NTRK fusions (21). Majority of *KRAS* wildtype tumours have been shown to increase signalling through the RAS-MAPK pathway, demonstrating the importance of this pathway in the pathogenesis of PC in both *KRAS* mutant and wildtype tumours (20, 24).

TP53 gene encodes for tumour suppressor protein p53 which is important in regulating cell division. Inactivating *TP53* mutations occur in approximately 70% of PDAC, and commonly occur with *KRAS* mutations (20, 25). These mutations occur relatively late in PC pathogenesis in promoting cell invasion and metastasis through epithelial-mesenchymal transition (26, 27).

SMAD4 mutations are seen in approximately 30% of PC and is a key somatic oncogenic driver which activates ERK/p38/AKT pathways in PC (20, 28). Loss of *SMAD4* in PC cell lines are associated with chemo-resistance in in vitro studies, and the dual blockade of EGFR and HER2 could overcome this resistance to gemcitabine in *SMAD4* mutant PC (29).

Structure variants (SV) are also seen commonly in PC, with an average of 119 per individual (19). Waddell et al. investigated the molecular landscape of patients with PDAC and sub-typed tumours into 4 categories based on the

pattern of structural re-arrangements. These subtypes have potential clinical importance, particularly subtype 2 (locally re-arranged) which harboured potentially targetable amplifications in *HER2*, *MET*, *CDK6*, *PIK3CA* and *PIK3R3* in approximately 1-2% of patients (19). Other common SV in PC include amplifications of 1q (33%) and deletion of 6p (41%), 6q (51%) and 9p (harbouring *CDK2NA*) (48%) (20). Amplification of the 8q24 locus containing the *MYC* gene can be detected in 14% of PC, and is associated with adeno-squamous histology and aggressive disease (24). *MYC* amplifications occur early in PC development and may drive rapid progression (24).

Gene fusions are enriched in *KRAS* wildtype PC and can be seen in 20-30% of cases, most commonly occurring in *FGFR2* (7.7%), *MET* (7.7%), *NRG1* (2.1%), *RAF1* (1%), and *NTRK* (1%) (30). Some of these oncogenic fusions have potential therapeutic relevance (see section 1.1.5.3).

Gene expression can also be dysregulated in PC, with several implicated genes contributing to PC pathogenesis. Abnormal DNA methylation markers are frequently seen in PC and often occur early in PC pathogenesis. DNA methylation expression analyses have revealed that several affected genes are critically important in PC development, including abnormal methylation of *UCHL1*, *NPTX2*, *ZFP82*, *PARP6 BRCA1* and *MGMT* (20, 31, 32). *IGF2BP2* is often affected in PanIN and may provide a tool for differentiating malignant versus non-malignant pancreatic masses through non-invasive ctDNA analysis (33).

3 Tumour Heterogeneity in Pancreatic Cancer

PC is characterized by the presence of multiple, co-existing clonal mutations, leading to a high degree of intra-tumour heterogeneity. This heterogeneity is a major contributor to therapeutic resistance, particularly in the case of targeted therapies. Therapeutic resistance is primarily seen in resistant clones which harbour resistance mutations, and can contribute to differential responses even when present at low allelic frequencies (34). Heterogeneity can be seen within the primary lesion (2 cells with differing mutation), within the metastatic lesion, or between the primary tumour and the metastasis. In PC, there is high concordance between metastatic sites for clonal driver mutations, with passenger, non-driver mutations accounting for most intra-tumour heterogeneity (35). The challenges of heterogeneity in tissue biopsies can be overcome when using ctDNA sequencing.

4 Molecular Landscape of Non-Ductal Pancreatic Cancer

The molecular landscape of non-ductal PC such as pancreatic neuroendocrine (panNET) and acinar cell carcinoma (ACC) have also been studied and outlined in table 2.

Table 2- Molecular and histological landscape of non-ductal pancreatic cancer subtypes

Non-ductal subtype	Frequency	Histological features	Molecular features

Acinar cell carcinoma	1%	Granular cells, stain positive for trypsin	Fewer recurrent SVs (SMAD4 30%; TP53 13%; APC 10%; KRAS 9%) Chromosome structural changes including amplifications and deletions Targetable alterations (BRCA, PALB2, ATM, BRAF V600E)
Pancreatic adeno-squamous carcinoma	<1%	Malignant squamous cells	Recurrent SNVs similar to PDAC including *TP53* (87%), *KRAS* (73%), *MYC* amplifications (47%), *CDKN2A* deletions (40%) and *SMAD4* (20%).
Sporadic neuroendocrine tumour	<5%	Round/oval nuclei, eosinophilic granular cytoplasm	*MEN1* (70%), *DAXX* (25%) and *ATRX* (17%). Mutations within PI3K/mTOR pathway (15%)

4.1.1 Management of Pancreatic Cancer

5 Neoadjuvant and Adjuvant Treatment in Early-Stage Pancreatic Cancer

In those with upfront resectable disease (10-15%), adjuvant chemotherapy following surgical resection with mFOLFIRINOX is associated with an improvement in overall survival (OS) compared to gemcitabine alone (36). Table 3 below describes the adjuvant chemotherapy trials in PC with corresponding survival benefit. However, in those with borderline resectable disease (10-15%), neoadjuvant chemotherapy to treat micro-metastatic disease has been shown to improve resectability rate. Neoadjuvant FOLFIRINOX is widely adopted as a standard treatment for borderline resectable tumours (37). Table 4 below describes the neoadjuvant chemotherapy trials in resectable, borderline resectable and unresectable PC.

Table 3- Adjuvant chemotherapy trials in pancreatic cancer

	N=	Experimental arm	Control arm	mOS	HR (p value)	Ref
ESPAC-1	143	5-FU/LV	Observation	19.7 v 14.0	0.66 (0.0005)	(38)
CONKO-001	368	Gemcitabine	Observation	22.8 v 20.2	NR (0.005)	(39)
ESPAC-3	1,088	Gemcitabine	5-FU/LV	23.6 v 23.0	0.95 (0.39)	(40)
ESPAC-4	732	Gemcitabine + Capecitabine	Gemcitabine	27.7 v 26.0	0.82 (0.032)	(41)
PRODIGE-24	493	mFOLFIRINOX	Gemcitabine	54.4 v 35.0	0.64 (0.003)	(36)
APACT	866	nab-paclitaxel + gemcitabine	Gemcitabine	40.5 v 36.2	0.82 (0.045)	(42)

mOS- median overall survival; Ref- reference; 5FU/LV- leucovorin plus 5-fluorouracil; HR- hazard ratio; NR- not reported

Table 4- Neoadjuvant chemotherapy trials in pancreatic cancer

Study	Phase	n	Resectability definition	Primary End Point	Arm	Resection rate (%)	R0 (%)	mOS (mo)	ORR (%)	Ref
NEOLAP (2019) *	II	165	BR or UR [1]	Resection rate	AG -> Surgery vs Sequential AG->FOLFIRINOX	35.9 43.9	65 69	18.5 20.7	22.6 21.7	(43)
SWOG S1505 (2021) *	II	102	R and BR (no arterial involvement; <180 SMV/PV)	2yr OS	FOLFIRINOX -> Surgery-> FOLFIRINOX vs AG-> Surgery-> AG	73 70	85 85	23.2 23.6	9 21	(44)
PACT-15 (2018)	II	88	R without arterial or venous involvement	1yr EFS	Surgery -> adjuvant gemcitabine (A) or PEXG (B) vs PEXG -> surgery -> PEXG (C)	85 (A) 90(B) 84(C)	27 (A) 37 (B) 63 (C)	20 (A) 26.4 (B) 38.2 (C)	NR	(45)
JSAP-05 (2019)	II/III	364	Resectable without arterial involvement	OS	Surgery -> adjuvant S1 Gem/S1-> Surgery-> S1	82 93	NR	26.6 36.7	NR	(46)
FOLFIRINOX (2018)	II	48	BR as per local MDT	R0 resection rate	FOLFIRINOX + CRT [2]	67	97	37.7	44	(47)
ESPAC5F (2020) *	II	90	BR [1]	Resection rate	Surgery-> Gem/Cap (A) Gem/Cap or FOLFIRINOX or CRT -> Surgery -> Adjuvant chemo	62 55	15 23	42% [3] 77% [3]	NR	(48)
PREOPANC (2020)	III	248	Resectable (no contact with SMA, coeliac trunk, CHA and <90 degrees with SMV)	OS	Gem-> Gem + RT -> surgery -> gem Surgery-> adjuvant gem	61 72	71 40	16.0 14.3	NR	(49)

[1]definition as per NCCN guidelines; [2] Did not have adjuvant therapy; [3] 1-yr survival rate

* Not significant for primary end point

BR- borderline resectable; UR- unresectable; R- resectable; SMV- superior mesenteric vein; PV- portal vein; SMA- superior mesenteric artery; CHA- common hepatic artery; SMV- superior mesenteric vein; OS- overall survival; EFS- event free survival; AG- nab-paclitaxel, gemcitabine; PEXG- cisplatin, epirubicin, capecitabine, gemcitabine; ORR- objective response rate; Gem- gemcitabine.

6 Systemic Anti-Cancer Therapies in Metastatic Pancreatic Cancer

For most patients with locally advanced unresectable (30%) and metastatic disease (50%), chemotherapy to prolong life expectancy, alleviate symptoms and improve quality of life (QOL) can be used. Combination chemotherapy with FOLFIRINOX or gemcitabine/nab-paclitaxel chemotherapy has shown superior efficacy over single agent chemotherapy with gemcitabine. The decision on a specific regimen is dependent on patient fitness and comorbidities. The mean time to progression following first line (1L) chemotherapy is between 5-6 months, with the majority progressing at 1-year. Only one third of patients who progress on 1L chemotherapy are suitable for second line (2L) chemotherapy (50). Fluoropyrimidine alone or in combination with oxaliplatin, gemcitabine monotherapy, or liposomal irinotecan can be used in the 2L setting. However, most patients will progress within 3 months with responses seen in less than 20% of patients (51). Table 5 outlines the various 1L chemotherapy treatment regimens in patients with advanced PC and their associated efficacy.

Table 5- Efficacy of chemotherapy in 1st line advanced pancreatic cancer (positive trials)

Chemotherapy regimen	**ORR (%)**	**PFS (months)**	**OS (months)**	**Ref**
Gemcitabine monotherapy	9.4	3.3	5.7	(52, 53)
Fluorouracil monotherapy	9	1	4.4	(52, 54)
S1 monotherapy	21	3.8	9.7	(55)
Cisplatin + gemcitabine	10.1	3.9	8.3	(56)
Gemcitabine + capecitabine	19.1	5.3	7.1	(57)
Gemcitabine + S1	29.3	5.7	9.9	(55, 58)
Gemcitabine + erlotinib	8.6	3.7	6.2	(59)
Fluorouracil + oxaliplatin	27.6	4.0	7.5	(60)

Fluorouracil, oxaliplatin + irinotecan (FOLFIRINOX)	31.6	6.4	11.1	(53)
Nab-paclitaxel + gemcitabine	23	5.5	8.5	(61)

7 Targeted Therapies in Metastatic Pancreatic Cancer

The increasing application of NGS in the molecular profiling of PC has led to the identification of specific targetable genomic aberrations therefore the use of targeted agents. Molecular profiling to detect somatic aberrations in BRCA 1/2 and NTRK fusions are currently funded by the NHS. Table 6 summarises studies assessing targeted drugs in PC and their associated efficacy.

DNA damage repair (DDR) mutations are seen in approximately 25% of PC. Majority involve *BRCA1/2 gene*, however other markers of homologous recombination deficiency (HRD) such as mutations in PALB2 can also be seen (19, 20). Germline *BRCA2* mutations are seen in approximately 3-5% of patients with PC and associated with defective DNA damage repair responses (62, 63). Responses to platinum-based chemotherapy in the 1L setting are higher in those with *BRCA1/2* mutations, and the use of olaparib (PARP inhibitor) as maintenance therapy has been shown to improve PFS (but not OS) in germline BRCA1/2 mutant PC following 1L chemotherapy (64). Other markers of HRD including somatic and germline mutations in *ATM*, *STK11*, *ATR*, *CHECK2*, *PALB2*, and *RAD51C* are seen in 4%, 1.5%, 1%, <1%, <1% and <1% respectively, and may be predictive of response to platinum-based chemotherapy (25, 65, 66).

EGFR overexpression can be seen in up to 45% of PC, which can potentially be targeted with anti-EGFR agents (67). Erlotinib was studied in a large phase III trial of patients with mPC in a biomarker unselected population. Patients were randomised to either erlotinib with gemcitabine or gemcitabine with placebo. The combination of erlotinib and gemcitabine was associated with a prolonged mOS (6.24m v 5.91m), and was granted FDA approval in 2005 as 1L treatment of mPC (59). However, this combination has largely been replaced by FOLFIRINOX in the 1L treatment. EGFR inhibition in *KRAS* wildtype mPC has also been studied in a large, phase IIb randomised trial using nimotuzumab (anti-EGFR monoclonal antibody) in combination with gemcitabine (versus gemcitabine + placebo) as 1L therapy (68). Nimotuzumab with gemcitabine was associated with higher mOS (8.6m v 6.0m), with similar responses observed between the two groups. Those with *KRAS* wildtype status and/or EGFR overexpression had a significant OS benefit at 12 months in the combination arm. However, this combination is currently not standard of care in the 1L setting.

The use of precision therapeutics in PC is rapidly evolving. Importantly, to identify targetable aberrations, sufficient tissue at the time of diagnostic biopsy is required for NGS.

Table 6- Molecularly targeted therapies in PC

Molecular target	**Prevalence in PC**	**n=**	**Therapy**	**ORR (%)**	**DCR (%)**	**Median survival, m (vs control arm)**	**FDA approval**	**ESCAT tier**	**Ref**
ALK fusions	0.16%	4	Alectinib, lorlatinib, ceritinib	NR	75	NR	No	IIIA	(69)
BRAF V600E mutation	3%	2 1	Vemurafenib Dabrafenib + trametinib	0 100	50 100	NR	No	IIIA	(24, 70, 71)
BRCA 1/2 germline mutation	3-5% (BRCA2) <1% (BRCA1)	154	Olaparib[1]	NR	NR	19.0 (v 19.2[2])	Yes	IA	(72)
BRCA1/2 somatic mutation	4%	3 2	Rucaparib Rucaparib	66.7 50	66.7	NR	No	IIIB	(73-75)
FGFR2 fusions	<1%	1	Pemigatinib	100	100	NR	No	NR	(76)
HER2 amplification	11%	17	Trastuzumab + capecitabine	NR	NR	6.9	No	IIIA	(77)
KRAS G12C mutation	90%	38 21	Sotorasib Adagrasib	21 33.3	84 81	6.9 8.0	No No	IIIA	(78, 79)
MDM2 amplification	<1%	2	AMG 232	0	100	NR	No	IIIA	(20)
MSI/MMRd	1-2%	22	Pembrolizumab	18.2	NR	4.0	Yes	IC	(80, 81)
NRG1 fusions	<1%	2 1	Afatinib Erlotinib + pertuzumab	100 100	100 100	NR	No	IIIA	(82-84)
NTRK fusions	0.34%	3 1	Entrectinib Larotectinib	33 100	67 100	12.8m[3] NR	Yes	IC	(85-88)

Germline PALB2 mutation	1%	6	Rucaparib	50	50	NR	No	NR	(20, 75)
PIK3CA	4%	24 15	Buparlisib + trametinib[4] Alpelisib + gemcitabine/nab-paclitaxel[4]	0 62	50 87.5	5 8.7	No	IIIA	(89, 90) (24)
RET fusions	<1%	4 11	Pralsetinib Selpercatinib	100 54%	100 100	NR NR	No Yes	IIIA	(91, 92)
ROS1 fusions	<1%	1	Entrectinib	0	100	NR	No	IIIA	(93)

1- maintenance treatment; 2- versus placebo; 3- median PFS; 4- biomarker unselected population

See appendix A for ESCAT score.

8 Biliary Tract Cancer

8.1.1 Anatomical Classification of Biliary Tract Cancers

Biliary tract cancers (BTC) can be classified based on their anatomic primary site into intra-hepatic cholangiocarcinoma (IHCC), extra-hepatic cholangiocarcinoma (ECCC) or gallbladder cancer (GBC). ECCC is further divided into either hilar/perihilar cholangiocarcinoma (Klastkin tumours) or distal cholangiocarcinoma, according to the relationship between the tumour and the cystic duct-common bile duct confluence. BTC can be further classified according to their molecular biology, with recurrent aberrations corresponding to the anatomical classification (see section 1.2.4). Ampullary cancers are a rare GI malignancy which arise from the ampullar of Vater (the confluence of the common bile and pancreatic ducts). Ampullary cancers can be categorised based on the epithelial site of origin, either arising from intestinal or pancreato-biliary epithelia. Figure 2 outlines the classification of BTC.

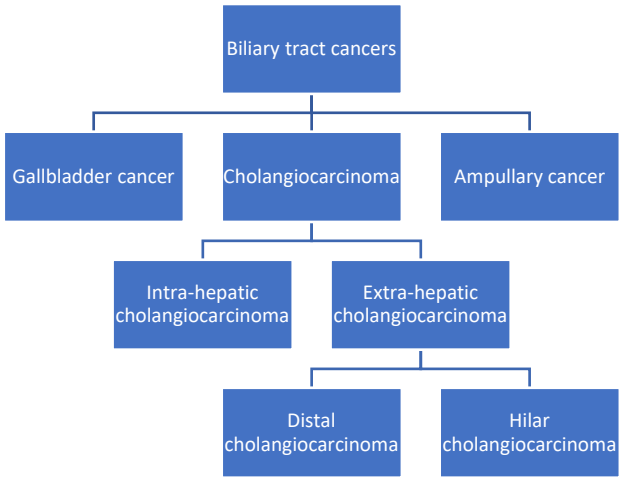

Figure 2- Classification of biliary tract cancers

8.1.2 Diagnostic Approach to Suspected Biliary Tract Cancer

The diagnostic approach in a patient with symptoms suggest of BTC generally consists of single or combination modality imaging (US, CT, MRCP, and/or PET scan) to determine the site(s) of disease and an invasive diagnostic procedure (endoscopic or percutaneous biopsy) to inform a histological diagnosis.

In patients with suspected IHCC, MRI or CT imaging is often performed to identify the primary lesion and detect extra-hepatic metastases. In those with, predominantly distal EHCC, ERCP or EUS is often used to facilitate both therapeutic intervention (i.e. stenting) and tissue biopsy to obtain a histological diagnosis. CT and MRI imaging of suspected EHCC may fail to identify a mass lesion, and common findings of intra- and extra-hepatic duct dilatation may be the only apparent radiological finding.

Obtaining a histological diagnosis in patients with BTC is critical to inform appropriate treatment, particularly in those who require neoadjuvant chemotherapy prior to surgery, or systemic chemotherapy in the setting of advanced, unresectable disease. There are various methods to obtain a tissue diagnosis in BTC as outlined in table 7 below.

Bile cytology has a relatively low diagnostic yield. A study investigating the diagnostic accuracy of bile cytology in BTC showed a low Sn of 26%. The use of brush cytology during ERCP improved Sn (69%), however does miss a

significant proportion of malignant cases (94). Samples collected through ERCP have higher diagnostic yield than during percutaneous transhepatic cholangiography (PTC) (94).

The use of EUS with FNA has a greater Sn compared to ERCP/brushings (~70-80%) (95). However, the use of EUS/FNA is limited to predominantly distal cholangiocarcinoma, or sampling suspicious regional lymph nodes in those with hilar and IHCC. EUA/FNA has a lower Sn in the diagnosis of proximal BTC (59%) (96). The diagnostic yield of all invasive biopsy sampling procedures in BTC is relatively lower compared to other tumour types, such as non-HPB, luminal GI cancers such as CRC, with one in four biopsies reported as non-diagnostic (12). The unfavourable location of these tumours presents challenges when attempting to obtain a histological diagnosis, which impacts the speed at which these difficult to biopsy tumours are diagnosed and subsequently treated.

Table 7- Diagnostic yield of various methods in the diagnosis of BTC

	Sn	**Sp**	**Ref**
Bile cytology	26	100	(94)
Brush cytology	69	100	(94)
Bile + brush cytology	69	100	(94)
EUS + FNA	77	100	(95)

8.1.3 Molecular Landscape of Biliary Tract Cancer

BTC are a heterogenous group of malignancies and can be classified by certain molecular signatures largely represented by their anatomical location

and more recently by their aetiology (infection versus non-infective) and geographical location.

Potentially targetable genomic aberrations can be detected in up to 40% of BTC (97). Germline mutations also with potential therapeutic relevance can be seen in 11% of patients, mostly affecting *BRCA1*/2, *MLH1*, *MSH2* and *RAD51D* (98). Germline *BRCA1*/2 mutations are seen in 3-5% of BTC, with frequencies consistent across all subtypes of BTC without enrichment for an anatomical location (99, 100). In addition, germline *BRCA1*/2 mutations are associated with a higher frequency of MSI-H (19.5 vs 1.7%) (100).

A large, multi-national study using an integrated approach of whole-exome sequencing and epigenomic DNA methylation analysis identified 4 distinct subtypes of cholangiocarcinoma (101). Cluster 1 is associated with a high burden of recurrent SNVs, enriched for mutations in *TP53*, *ARID1A* and *BRCA1*/2, as well as *HER2* amplifications. This subtype was associated with a high frequency of CpG island hypermethylation, and predominately seen in Asian populations. Cluster 2 is associated with both fluke-positive and negative disease with a high proportion of recurrent SNV in *TP53*, and *HER2* amplification. This mixed cluster had no geographical nor anatomical association. Cluster 3 exhibited a high level of copy number alterations, particularly in 2p, 2q, and high expression of immune pathway genes aberrations. These tumours are often fluke-negative and predominately intrahepatic. Finally, cluster 4 are enriched with recurrent SNVs in *BAP1*, *IDH1*/2 and *FGFR* alterations. These tumours are predominately intra-hepatic

and not associated with fluke-positive disease. This subtype is associated with more favourable prognosis compared to other clusters. Clusters 3 and 4 are more commonly seen in non-Asian populations.

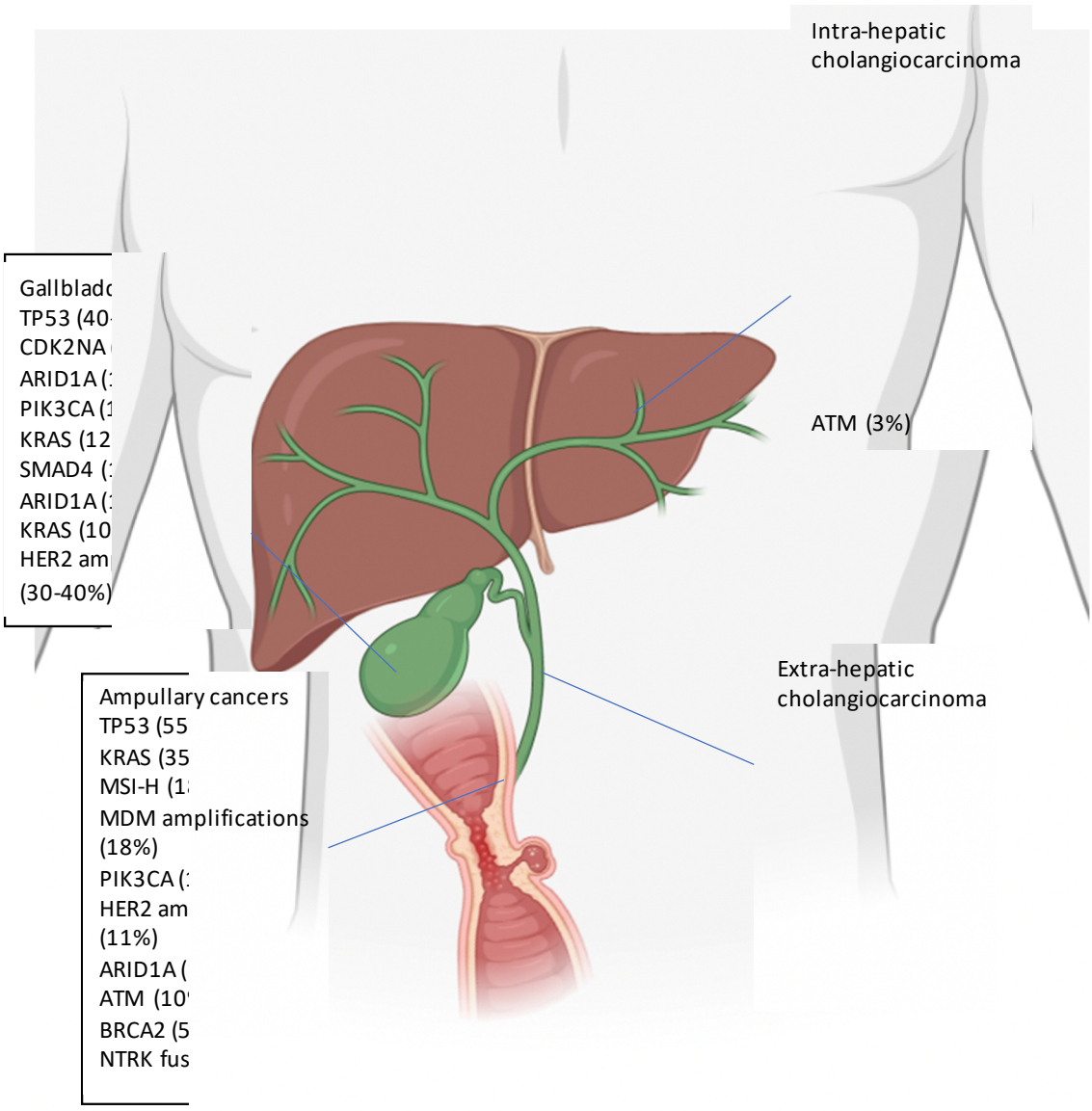

Figure 3- Molecular landscape of BTC according to anatomical site

References- (85, 97, 98, 102-115)
Data extracted from- COSMIC Website: cancer.sanger.ac.uk/cosmic

8.1.4 Management of Biliary Tract Cancer

9 Adjuvant Treatment of Early-Stage Biliary Tract Cancer

The treatment of BTC is dependent on location and stage of disease, with surgical resection the only curative treatment.

In patients with distal cholangiocarcinoma who are suitable for a pancreaticoduodenectomy (Whipple's procedure), survival is dependent on the tumour size, lymph node involvement and involvement of surgical margins. Patients with IHCC and proximal hilar cholangiocarcinoma often require liver resection.

Adjuvant chemotherapy is recommended for all patients with BTC following surgical resection. The optimal treatment is 6 months of chemotherapy with capecitabine alone for 6 months duration based on the BILCAP trial (116).

10 Systemic Chemotherapy in Metastatic Biliary Tract Cancer

In those with metastatic disease, chemotherapy forms the mainstay of treatment. 1L chemotherapy for BTC consists of combination of platinum-gemcitabine. The ABC-02 trial provided evidence of the use of cisplatin in combination with gemcitabine, showing improved mOS (11.7m v 8.1 m) compared to gemcitabine alone (117). More recently, durvalumab (anti-programmed cell death ligand 1 [PD-L1]) in combination with cisplatin and gemcitabine chemotherapy was evaluated in the TOPAZ-1 study, compared to chemotherapy alone (118). The addition of durvalumab was associated with higher responses (ORR 26.7% v 18.7%) and longer OS (HR 0.80, 95% CI

0.66-0.97, p=0.021). Durvalumab was subsequently granted FDA approval in the 1L treatment of metastatic BTC.

In patients who progress on 1L chemotherapy, between 40-50% will be suitable for 2L therapy (118). The ABC-06 study compared fluoropyrimidine and oxaliplatin (FOLFOX) chemotherapy (with active symptom control [ASC]), to ASC alone (119). FOLFOX chemotherapy was associated with prolonged survival (mOS 6.2m vs 5.3m, HR 0.69, 95% CI 0.50-0.97, p=0.031), with only slightly higher rates of grade 3-5 adverse events (AE) (69% vs 52%). This study included patients with GBC and ampullary cancers. The ABC-06 study created a new treatment paradigm in the 2L setting of metastatic BTC with FOLFOX chemotherapy.

Targeted agents are defining new treatment paradigms for BTC, given the increased use of tumour genomic sequencing and development of novel targeted therapies.

11 Targeted Therapies in Metastatic Biliary Tract Cancer

There are several genetic alterations commonly observed in BTC that can be targeted, as summarised in table 8.

FGFR2 fusion positive tumours are seen in approximately 15-20% of IHCC and can be targeted using inhibitors such as pemigatinib and infigratinib, both FDA approved in the later line treatment of metastatic or locally advanced unresectable cholangiocarcinoma. The FIGHT-202 trial was a single arm

study of pemigatinib in the 2L treatment of *FGFR2* aberrated (fusion, amplification, SNV and indel) advanced cholangiocarcinoma (120). Pemigatinib was associated with a mOS of 21.1m and mPFS of 6.9m in those with *FGFR2* fusion/rearrangements. There were no responses in those with other, non-*FGFR* fusion alterations, with mPFS and mOS of 6.7 and 4.0 months respectively. A similar benefit was seen with infigratinib in patients with advanced cholangiocarcinoma with *FGFR2* fusion or rearrangement (121). The FIGHT-302 trial (pemigatinib) (NCT03656536) and QED PROOF (infigratinib) (NCT03773302) are ongoing phase III randomised studies in 1L treatment of FGFR2 fusions/rearrangement positive BTC involving FGFR inhibition versus cisplatin with gemcitabine.

*I*DH mutated tumours are also seen in approximately 20% of IHCC which can be targeted by ivosidenib, an oral small molecular inhibitor of *IDH1*. The ClarIDHy trial was a phase 3 randomised, double-blind study of ivosidenib versus placebo in the 2/3rd line setting of *IDH1* mutant cholangiocarcinoma (122, 123). Ivosidenib was associated with improved PFS (mPFS 2.7m versus 1.4m), with numerically higher survival (mOS 10.3m versus 7.5m; HR 0.79 [95% CI 0.56-1.12], p=0.09) which did not reach statistical significance owing to the crossover design of the study (57% crossover). Ivosidenib was granted FDA approval for use in the later line setting of *IHD1*-mutant cholangiocarcinoma.

Table 8 describes completed trials of molecularly targeted treatment in BTC.

Table 8-Molecularly targeted therapies in BTC

Molecular target	**Prevalence in BTC (%)**	**n=**	**Therapy**	**ORR (%)**	**Median survival (months)**	**FDA approval**	**ESCAT**	**Ref**
BRAF V600E mutation	5	43	Dabrafenib + trametinib	51	14	No	IIB	(124)
BRCA 1/2 (germline/somatic)	3	4	PARP inhibitor[1]	NR	11-64.76m	No	IIIA	(125)
FGFR2 fusion	15	146 108 103	Pemigatinib Infigratinib Futibatinib	35·5 23.1 42	21.1[2] 12.2 21.7	Yes Yes Yes	IB	(120, 121, 126)
HER2 amplification	10	39 32	Pertuzumab + trastuzumab Trastuzumab deruxtecan	23 36.4	10.9 7.1	No No	IIIA	(127, 128)
IDH1 mutation	20	187	Ivosidenib	2	10.3 (vs 7.5m[3])	Yes	IA	(122, 129)
KRAS G12C	<1%	12	Adagrasib	41.7	15.1	No	N/A	(79)
MSI-H	2	22 2	Pembrolizumab Nivolumab	40.9 50	24.3 NR	Yes Yes	IC	(80, 130)
NTRK fusion	<1	1 2	Entrectinib Larotrectinib	100 50	NR NR	Yes	IC	(85, 86, 131, 132)
PIK3CA	12	1	TAS-117	0	NR	No	IIIA	(103, 133)
RET Fusion	1	1	Selpercetinib	100	NR	Yes	N/A	(92)
ROS1 Fusion	1-9	1	Crizotinib	0	NR	No	N/A	(134)

1- PARP inhibitor not named in study; 2 in *FGFR2* fusion/rearrangement positive patients; 3- versus placebo; N/A- not available; See appendix A for ESCAT score.

12 Early-Stage Colorectal Cancer

12.1.1 Screening of Early-Stage Colorectal Cancer

Almost 10% of CRC diagnoses are made through screening in asymptomatic patients (135). Early detection of CRC is critical to improving outcomes, and colonoscopic screening has been shown to reduce CRC-related mortality (136). The natural history of CRC development from adenoma to invasive carcinoma occurs over several years, and so colonoscopy screening at regular intervals is important in preventing CRC, which can facilitate removal of pre-cancerous lesions. However, the invasive nature of colonoscopies requires adequate patient fitness, bowel preparation and can be associated with complications, including perforation (0.04%) and major bleeding (0.08%) (137). Furthermore, the use of invasive procedures such as colonoscopies in the screening of asymptomatic patients is associated with added costs and place a burden on the NHS.

Non-invasive methods to screen for early-stage CRC are attractive. CT colonoscopy (CTC) is a non-invasive imaging tool which can be used in the diagnosis of suspected CRC, often in patients who are not fit for colonoscopy. Although CTC does not require sedation, bowel preparation similar to that used prior to colonoscopies are still needed, and may not be suitable in some patients (history of congestive heart failure, dialysis). The evidence to support CTC in detection of CRC has been investigated in large multi-centre trials showing a Sn to detect large adenomatous polyps >1cm of ~67% and Sp ~89% (137). Another study showed CTC (with follow up colonoscopy) in

comparison to upfront colonoscopy was associated with similar detection of CRC and high-grade dysplastic polyps, suggesting its use in the screening for CRC (138). CTC however are less Sn in detection of small adenomas <5mm and given they are non-invasive, do not facilitate polyp removals (138, 139). Importantly, the use of CTC in screening asymptomatic people is associated with extra-colonic findings of non-importance requiring further investigations 11% of patients, adding to heightened patient anxiety and NHS costs (137).

Faecal immunochemical testing (FIT) relies on antibody based detection of haemoglobin in faeces. The Sn of FIT testing in detection of CRC ranges from 73-88% and Sp between 91-96% (137). gFOBT testing also relies on detection of haemoglobin through a guaiac reaction on a chemical indicator. FIT has a slightly higher Sn in detecting CRC compared to FOBT (140). A large RCT involving >50,000 asymptomatic patients aged 50-69 years compared a one-off colonoscopy with FIT testing every 2 years on CRC-related mortality. Those in the FIT group were more likely to participate in screening (34.2% vs 24.6%), however, similar rates of CRC were detected between the two groups (0.1%) (141). Importantly, advanced adenomas (AA) (defined as high grade dysplastic polyps [HGD], or adenomatous polyps >10mm in size or with predominant villous histology, i.e. high risk pre-malignant lesions) were more likely to be detected through colonoscopy than FIT testing (OR 2.30 P<0.001), highlighting the limitation of FIT testing in screening asymptomatic patients in the detection of pre-cancerous lesions. The use of a multiple, repeated FIT testing has been shown to be more effective in detection of CRC compared to

a single test, and is currently recommend by NICE (142). Table 9 describes the diagnostic accuracy of CRC screening methods.

Table 9- Diagnostic accuracy of CRC screening methods

	Colonoscopy		**FIT (≥10ug/g)**		**gFOBT**		**CT Colonoscopy**	
	Advanced adenoma >10mm	CRC	Advanced adenoma	CRC	Advanced adenoma	CRC	Advanced adenoma	CRC
Sensitivity	89-95	18-100	28	78.6	6-17	50-75	89	86-100
Specificity	89		93		96-99		94	
CRC mortality	HR 0.32		RR 0.90		RR 0.84		NR	
Reference	(137, 143)		(144, 145)		(137, 146)		(137)	(137)

FIT testing is currently used in the UK for triaging patients in primary care settings with low risk symptoms, who do not meet the criteria for referral on a 2WW pathway. A retrospective study assessed FIT testing in those with low risk symptoms of CRC (symptomatic) across multiple primary care centres in England. The prevalence of CRC in FIT positive (≥10ug/g) was 1.3% (147). The Sn and Sp to detect CRC at this cut-off was 91.1% and 80.7% respectively. The level of FIT positivity increases the PPV, with FIT level of ≥100 ug/g giving a PPV of 17%. However, the NICE recommended threshold is ≥10ug/g in determining FIT positivity.

During the COVID19 pandemic, the British Society of Gastroenterologist recommended colonoscopies for urgent cases given the risk of spreading COVID-19 infection to other patients and staff (148). The guidance recommended the use of FIT testing to triage CRC into urgent and non-urgent pathways. A single centre observation study assessed the use of FIT in

triaging colonoscopy during the pandemic. The use of FIT testing in the triaging of diagnostic colonoscopies reduced use of colonoscopy and was not associated with a reduced CRC detection rate and or additional burden on NHS services (149). Importantly, the uptake for FIT testing is only 70.7% in those aged 60-74 who were invited within 6 months across England between 2020-2021, highlighting additional measures are needed to enhance update (135).

At present, the use of FIT testing to triage colonoscopic screening of CRC is currently recommended by NICE and incorporated into the National Bowel Cancer Screening Programme, at a threshold of ≥10ug/g.

12.1.2 Molecular Landscape of Colorectal Cancer

The knowledge of the molecular characteristics has defined genetic predisposing syndromes and driven precision therapeutics in CRC. Common aberrations seen in CRC have been shown to predict response to therapies and prognosis, and alert to the likelihood of a genetic predisposition syndrome (informing screening regimens for patients and their families).

13 Adenoma-Carcinoma Sequence and Accumulation of Genomic Driver Mutations in Colorectal Cancer

The pathogenesis of adenomatous-CRC can be described by the adenoma-carcinoma sequence. A normal colonic epithelial cell transforms into an invasive malignancy through a step-wise accumulation of multiple genomic

aberrations (germline and/or somatic). An alternative pathway to invasive malignancy is through a serrated polyp pathway. CRC is defined by specific, somatic aberrations which begin in a single cell, and acquired secondary aberrations which drives clonal expansion, contributing to invasion and metastasis. The adeno-carcinoma sequence is outlined in figure 4 which describes the process of clonal expansion seen in the majority of CRC.

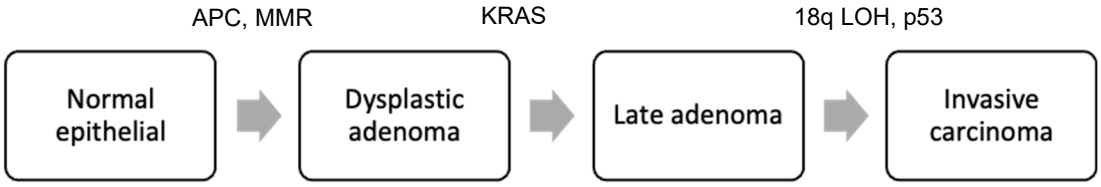

Figure 4- Adenoma-carcinoma sequence

14 Specific Aberration in Colorectal Cancer and Associated Therapeutic Implications

The most common genomic aberrations in CRC are SNV in *APC*, *RAS* and *TP53*. However, DNA methylation, gene fusions, insertions/deletions (Indels) and amplifications have also been described as driver aberrations in CRC. There are currently several potentially targetable aberrations in mCRC, some of which have an established role in the treatment of mCRC (such as ICI in dMMR mCRC, *BRAFV600E* mutations, *HER2* amplifications, and *NTRK* and *RET* fusions), while others are still being investigated in clinical trials (SNVs *KRAS* G12C, *PIK3CA* and *MAP2K1*, and amplifications in *MET* and *FGFR*). However, the availability of agents to target common aberrations seen in mCRC is limited (i.e. *KRAS*, *TP53*, *APC*), with several promising trial still

ongoing. Figure 5 describes the common aberrations seen in CRC according to the COSMIC database.

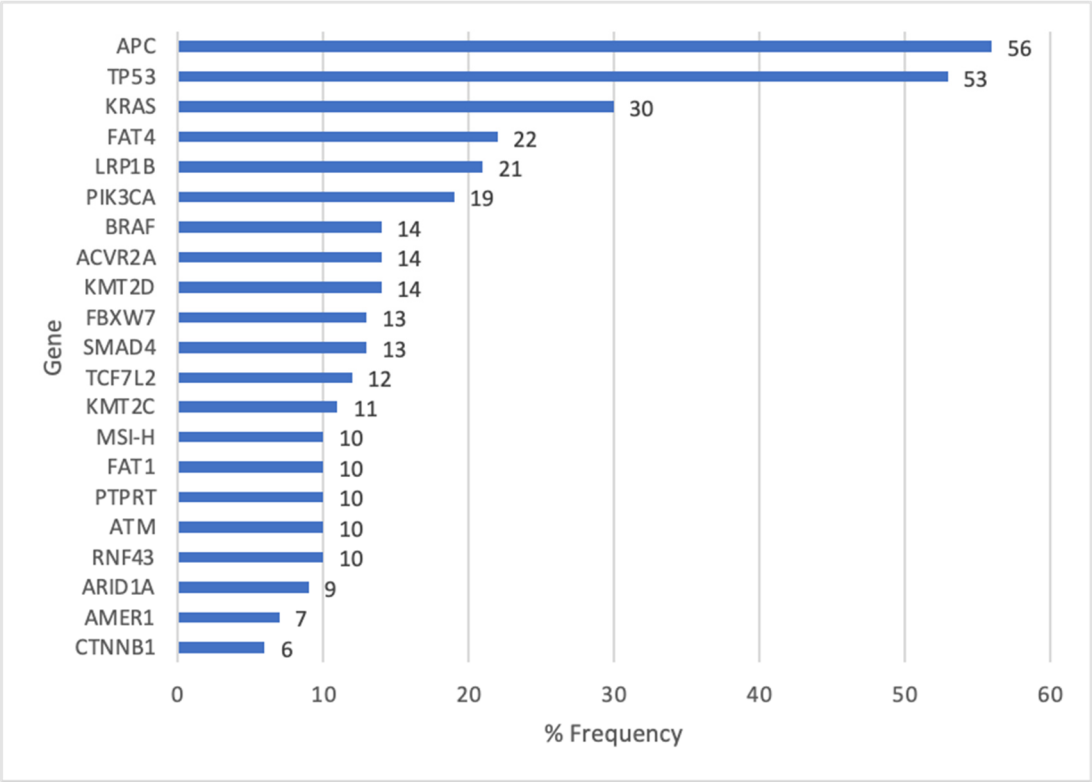

Figure 5- Common aberrations seen in CRC

Reference: COSMIC Website: cancer.sanger.ac.uk/cosmic

15 RAS Mutations in Colorectal Cancer

Mutations in the *RAS* oncogene are seen in approximately 50% of mCRC. Mutations in *KRAS*, *NRAS* and *HRAS* occur in 40%, 4.3% and 1.7% of mCRC respectively (150). *RAS* SNV point mutations have been implicated in the late adeno-carcinoma sequence, contributing to invasion and metastasis (151). Most *KRAS* mutations occur in exon 12, specifically G12D, which accounts for 30% of all *KRAS* mutations (152). The G12C variant is seen in 7% of *KRAS* mutations and characterised by an amino acid substitution of glycine-to-cysteine at position 12 (152, 153). Detection of RAS mutations have several clinical implications in mCRC, including in predicting a lack of response to

EGFR inhibition (154, 155). Studies have also suggested detection of *KRAS* mutation in faecal DNA may be used in CRC screening which enhances the PPV of FOBT, however the clinical utility of this has not be established (156).

16 TP53 Mutations in Colorectal Cancer

TP53 gene encodes p53 protein which acts as a regulator of multiple cellular processes including activating cell cycle checkpoints, inducing apoptosis, inducing senescence and regulating gene expression of multiple genes (including DNA damage repair genes, *MDM2*, *PTEN*, *CDK4*/6). Inactivating point mutations in TP53 occur in 50% of CRC and require inactivation of both alleles to drive tumorigenesis. *TP53* mutations occur late in the adeno-carcinoma sequence. Their implication of prognosis is mixed, with most studies suggesting no impact on survival compared to wildtype tumours (157).

17 APC Mutations in Colorectal Cancer

APC gene mutations occur early in the development of CRC and occur in a high proportion of sporadic CRC (80%). The *APC* gene controls Wnt/b-catenin signalling in cells, with loss of *APC* driving Wnt signalling and subsequently uncontrolled cellular growth (158). It has been shown that *APC* mutant adenomas can regress to cells with normal, non-malignant pathways when *APC* function is restored, demonstrating the critical nature of *APC* mutations in the development of CRC (159). Point mutations in *RNF43* (20% of CRC) and R-spondin (*RSPO*) fusions (10% of CRC) can also trigger abnormal Wnt pathway signalling which may respond to Wnt targeting therapies such as porcupine inhibitors. Inhibiting this pathway is subject to ongoing clinical trials (NCT04907539) (160, 161).

18 BRAF Mutations in Colorectal Cancer

BRAF is a human gene encoding for serine/threonine-protein kinase B-Raf (BRAF). *BRAF* is a member of the RAF kinase family and plays an important role in the epidermal growth factor receptor (EGFR)-mediated mitogen-activated protein kinase (MAPK) pathway (162). *BRAF* not only activates the MAPK pathway that profoundly affects cell growth, proliferation, and differentiation but also affects other key cellular processes, such as cell migration (through RHO small GTPases), apoptosis (through the regulation of Bcl-2), and survival (through the HIPPO pathway) (162). In mCRC, BRAF mutations occur in 8-12%, and are mutually exclusive with RAS mutations (163, 164). The most frequent mutation is *BRAFV600E* (1799T-A nucleotide change), accounting for 80% of all *BRAF* mutations (165). *BRAF* V600E mutated mCRC have common clinical and pathological characteristics, with higher frequency in elderly, females, right-sided tumours with mucinous histology and microsatellite instability. In addition, mCRC harbouring a *BRAF* mutations has a higher rate of nodal and peritoneal metastases and a lower rate of lung involvement (163).

19 MMR Pathway Aberrations in Colorectal Cancer

The MMR pathway encompasses several proteins that form complexes which identify and repair DNA replication errors. Aberrations in MMR genes (MSH2, MSH6, MLH1, PMS2) therefore increase the mutational rate of coding and non-coding microsatellites, which result in micro-satellite instability (MSI) and consequently contributes to tumour development.

Deficiency of MMR proteins can result from either germline or sporadic alterations. In germline cases, an inherited mutation in one of the MMR genes (usually MLH1 or MSH2) through somatic inactivation of the corresponding wild-type allele either through a point mutation or hypermethylation, which triggers tumorigenesis. Sporadic cases are most commonly due to hypermethylation of the promotor region of the *MLH1* gene (166). In these hypermethylated tumours, 70% of cases are due to mutations in the *BRAF* V600E gene.

dMMR/MSI-H is seen in approximately 20% in stage II, 10-15% stage III and 5% of patients with stage IV CRC (167, 168). In stage II disease, MMRd is associated with a lack of response to fluoropyrimidine chemotherapy (169-171).

20 The COVID-19 Pandemic and Impact on Diagnostic and Treatment Pathways in Difficult to Diagnose Cancers

The recent coronavirus pandemic (COVID-19) has led to nearly 200,000 deaths in the UK as of the end of December 2022 (172). The initial impact on the UK healthcare system has been significant as resources were re-directed to manage the pandemic. The NHS experienced overwhelming in-patient and intensive care capacity constraints, while majority of elective services were stopped. The “collateral damage” from the COVID-19 pandemic is estimated to be significant, with an excess of deaths from other non-COVID-19 related causes including cardiovascular disease and cancers. During the pandemic,

Cancer Research UK (CRUK) estimated an additional 2,700 cancers will be missed every week as a result of delays in primary care presentations (173). A national population-based modelling study predicted a 4.8-16.6% increase in the number of deaths from breast, colon, lung, and oesophageal cancers over the next 5 years (equivalent to 3621 additional deaths) (174).

The reasons for excess collateral deaths secondary to the COVID19 pandemic are multifactorial. However, the biggest impact is on delays in the diagnostic pathway from GP referral, delays in hospital-based diagnostic pathways and subsequently delays in commencing treatment. Other causes include

1. Reduction in capacity for diagnostic services due to the prioritisation of COVID19 testing and treatment
2. Reduction in cancer screening update
3. Disruption to supply chain of diagnostic tests
4. Staffing shortages with limited capacity and increased pressure on staff

The pandemic has had a significant impact on NHS Cancer Targets which measure the performance of diagnostic and treatment wait times for patients with cancer. There are currently 3 key targets which are measured. The older two-week wait (2WW) standard has largely been replaced by the faster diagnostic standard (FDS) as a more meaningful target, however 2WW data is still used as referenced in this thesis. As of November 2022, no NHS cancer target is being met in England for suspected upper (including gastric, HPB cancers) and lower GI cancers as outlined in table 11.

Table 10- NHS Cancer Wait Targets in England as of November 2022

	Definition	**Operation target**	**Upper GI Cancer**	**Lower GI Cancer**
28-day FDS	Referral to cancer ruled out or receive a diagnosis within 28 days	75%	68.58	48.73
31-day decision to treat	Treatment should be given within 1 month of deciding to treat	96%	N/A	89.89
62-day treatment	Treatment should be given within 62 days of initial referral	85%	N/A	40.37
2WW	Specialist appointment within 2 weeks of an urgent referral	93%	86.8%	80.66

The pandemic resulted in significant disruption in cancer diagnostics and procedures, with an impact on the 2WW pathway for suspected CRC. To mitigate the delays in the 2WW pathway, FIT testing was prioritised as a triaging tool prior to colonoscopy throughout the UK. NICE and BSG recommended FIT testing for patients with signs or symptoms of suspected CRC, with all patients with FIT ≥10ug/Hb being referred for an urgent colonoscopy through an urgent pathway. Data suggested that using this cut-off, approximately 89% of deaths attributable to diagnostic delay would be avoided, with an additional reduction in requirement for colonoscopies by over 80% (175). In addition, this cut off has been shown to identify 89% of CRC (Sn 89.4%), however with a relatively low pre-test probability Therefore, additional non-invasive tools to screen for CRC and diagnose CRC in those with symptomatic disease are needed.

For upper GI cancers, the 62-day wait is currently not being met by any UK country for PC/BTC (176). The pre-existing delay in commencing treatment for

PC/BTC due to pressures in the current diagnostic pathway has been amplified by the COVID-19 pandemic. These tumours are difficult to biopsy given their deep location in the body, and associated with the need for repeated invasive biopsies in up to 25% of patients (12). There is a resource need for endoscopy services and histopathology services (including tissue collection, processing, and reporting). These tumours are aggressive, and timely initiation of treatment is critical. Therefore, delays in diagnosis have a significant impact on accessing potentially lifesaving/extending treatments.

The fragility of the infrastructure for traditional tumour diagnostics has been highlighted by the COVID-19 pandemic. There is therefore a strong imperative for non-invasive, accessible, and reliable tests to support diagnose of GI cancers and facilitate cancer treatment to reduce the constraints on the cancer diagnostic pathways and improve patient outcomes. There is a potential for genomic medicine to support not only treatment decisions, but also faster diagnosis in cancer. The use of circulating tumour DNA (ctDNA) may have a role in speeding diagnosis of GI cancers, to cut through bottle necks in the diagnostic pathway and facilitate access to personalised treatments.

21 Introduction to Circulating Tumour DNA

ctDNA refers to DNA released from tumours into the blood circulation. Circulating free DNA (cfDNA) is a broader term which includes tumour derived DNA (i.e. ctDNA) but also DNA from non-tumour sources, such as in those from certain physiological conditions (i.e. pregnancy, strenuous exercise), pathological conditions (normal cellular necrosis and apoptosis), and from

lysis of white blood cells (WBC) (i.e. from clonal haematopoiesis of indeterminant potential [CHIP] or germline mutations).

In healthy individuals, cfDNA is present at relatively low average concentrations (between <1 to 30 ng/ml) (177). In individuals with cancer, the average concentration of cfDNA is higher (178). However, DNA of tumour origin (i.e. ctDNA) represents a small fraction of total cfDNA in the blood, which usually ranges between <0.1 and 10% (179).

The mechanisms by which ctDNA is released into the circulation is not fully understood. However, certain hypotheses have been suggested including secretion of ctDNA from active tumour cells (primary, metastatic or from circulating tumour cells [CTCs]), or release from necrotic tumour cells. ctDNA concentrations can vary over time within an individual, with higher concentrations seen in those with a larger tumour burden and higher stage of disease (180). There is also inter-patient variability in ctDNA concentrations across different tumour types, with certain tumours more likely to secrete DNA (i.e. pancreatic, colorectal, breast) and others less likely (including brain, prostate, renal) (180).

The advantages and challenges of using ctDNA and tissue biopsies is outlined in table 12.

Table 11- Advantages and Challenges of Tissue and Liquid Biopsies in Cancer

Tissue Biopsy	Liquid Biopsy
Requires invasive procedure	Minimally invasive
Unable to capture tumour heterogeneity	Overcomes challenges of tumour heterogeneity
Unable to assess temporal genomic changes	Real time genomic monitoring and cancer evolution monitoring
Very low risk of false positives (CHIP)	Risk of false positives (CHIP)
Risk of non-diagnostic sample	Variable detection rate (dependant on stage, site of metastases, type of cancer)
Technical consideration for tissue processing required (storage of tissue, cutting, histopathological review, fixatives)	Pre-analytical variable requirements (plasma collection, storage, isolation, and processing)
Higher DNA quantity availability for broad sequencing panels (including WES/WGS)	Variable DNA yields (possible limitations for WES/WGS)
Inadvertent identification of germline variants possible	Paired analysis of plasma and buffy coat with germline subtraction possible to minimise inadvertent identification of germline variants

21.1.1 Technical Considerations of ctDNA Assays

The implementation of a robust ctDNA based assay fit for clinical utility requires consideration of multiple technical variables. These include the pre-analytical consideration, specific features of the ctDNA based assay, variant calling and reporting. The pre-analytical considerations will be discussed in this section. Figure 6 describes the process in development of a ctDNA assay.

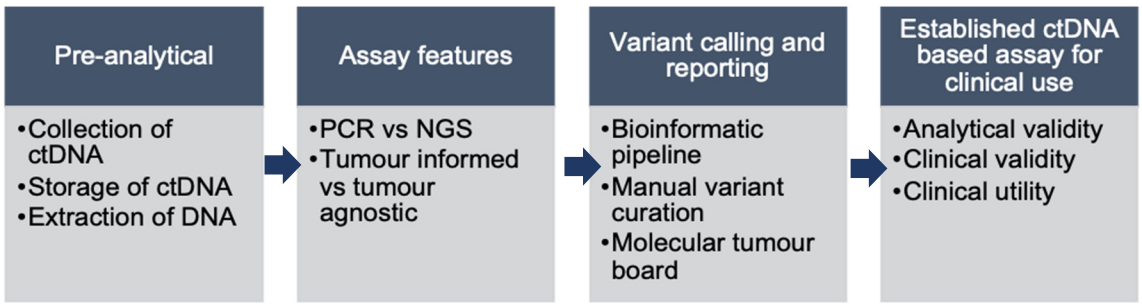

Figure 6- Consideration in developing ctDNA assays for clinical utility

To ensure a high level of analytical Sn, the detection of ctDNA requires optimisation of multiple variables in the pre-sequencing phase. Authors of the NCI Colon and Rectal-Anal Task Forces Whitepaper have previously outlined the pre-analytical variables needing consideration when developing a ctDNA assay (181). The importance of considering these factors prior to the sequencing of ctDNA is to ensure that contamination of non-tumour derived DNA (including CHIP and germline variants) are excluded. Therefore, important storage and blood collection guidelines have been developed to reduce the risk of false positive results derived from contaminated DNA. These variables are outlined in table 13. Importantly, genomic DNA from WBC occur at higher concentration in serum compared to plasma due to clotting of blood, therefore it is recommend that plasma is isolated and subsequently used when isolating DNA prior to sequencing (182). Figure 7 describes the components of whole blood.

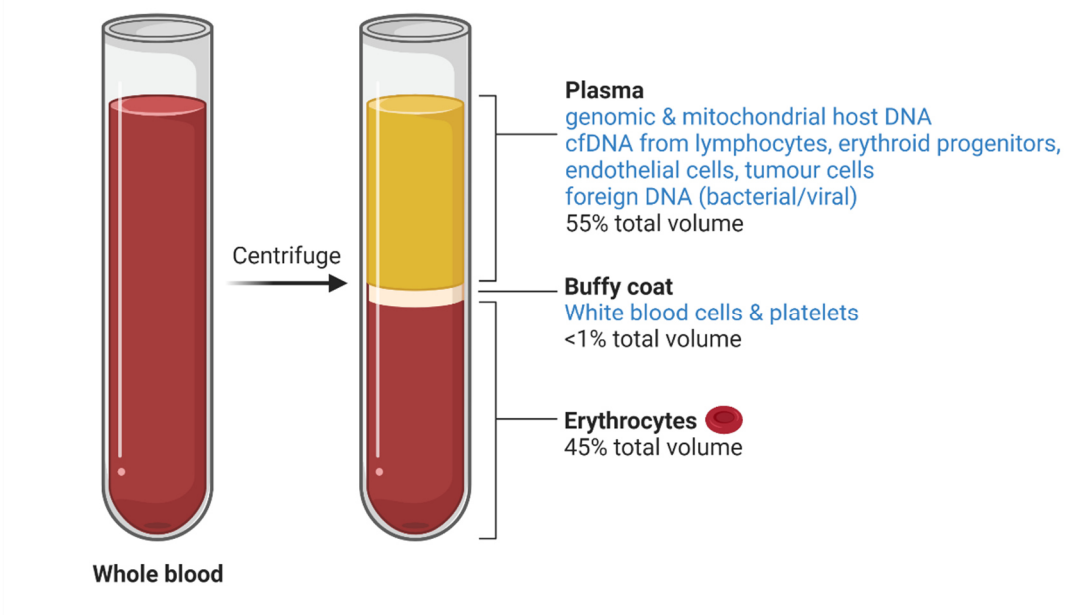

Figure 7- Components of whole bloods

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Table 12- Pre-analytical Variables

Pre-analytical Variable	**Recommendation**	**Rationale**
Blood collection	Large bore needle (≤21G)	Reduced lysis of leukocytes
	Use of EDTA or cell stabilising tubes (i.e. Streck©)	EDTA tubes contain anticoagulant however may contain contaminated DNA from leukocytes
Plasma isolation and storage	Collection of sufficient blood volume for specific use and assay characteristics	To avoid low plasma volume and low DNA concentrations
	Do not freeze unspun blood	To prevent lysis of leukocytes
	Do not expose plasma to multiple cycles of freezing and thawing (at most 3 freeze thaw cycles)	Avoid by using single use tubes of smaller volume through multiple aliquoting
	Isolate plasma within 24 hours for EDTA or 7 days for cell stabilising tubes	To prevent lysis of leukocytes
cfDNA extraction	Extract DNA from plasma rather than serum using centrifugation and filtration	Fewer leukocytes in plasma compared to serum
Transportation and DNA storage	Store extracted DNA at -20C or -80C for up to 10 years	To minimise nuclease activity

Adapted from Meddeb et al. Guidelines for the Preanalytical Conditions for Analyzing Circulating Cell-Free DNA, Clinical Chemistry, 2019, Reference (182)

There are also certain biological factors needing consideration which affect the release of cfDNA. These include biological/physiological process (i.e. age, sex, smoking, pregnancy, exercise) and pathological process (i.e. inflammation, auto-immunity, organ failure). Fig 8 describes the association between these processes and cfDNA concentrations. Pregnancy is also associated with elevated levels of cfDNA, however the majority of this is foetal in origin (183). Therefore, dilution of tumour derived cfDNA by foetal cfDNA reduces the ability to detect ctDNA in the plasma of a pregnant patient,

increasing the false negative rate. It is important to consider these biological factors when interpreting cfDNA results.

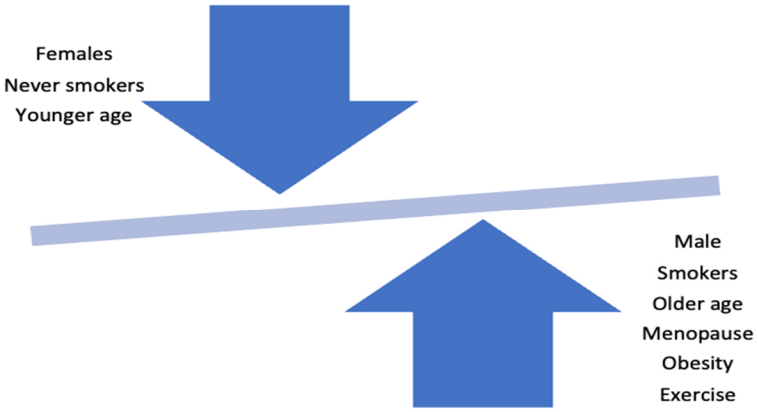


Figure 8- Biological Factors Associated with cfDNA Release

21.1.2 DNA Sequencing of Circulating Tumour DNA

ctDNA sequencing approaches include either a targeted or non-targeted. Targeted techniques such as single variant droplet digital PCR [ddPCR] or BEAMing (beads, emulsion, amplification, magnetics) or multi-gene panels (targeted next generation sequencing [NGS]). Non-targeted approaches include whole genome sequencing (WGS), whole exome sequencing (WES) and low coverage whole genome sequencing (lcWGS). The advantage of targeted over non-targeted techniques is the greater sequencing depth when using targeted approaches, which can detect variants present at very low variant allelic frequencies (VAF) in ctDNA. However, non-targeted approaches increase the breadth of coverage across the exome/genome, albeit usually at lower read depths.

PCR-based genotyping often requires an a-priori knowledge of the patient's tumour genomic sequence (i.e. tumour-informed approach) or in tumour types with known common variants (such as *KRAS* mutation in PC). PCR-based techniques can be allele-specific, targeting a known mutation with very high Sn. This technique uses an allele specific primer to target and subsequently amplify only certain small genomic region. These include the Cobas© assay to detect certain recurrent *EGFR* mutations in patients with non-small cell lung cancer. Although this technique is highly specific for particular allele(s), it is less sensitive compared to other types of PCR-based sequencing such as BEAMing and ddPCR. Emulsion is a technique which employs water-in-oil emulsion droplets to separate individual DNA molecules from each other and allows amplification to occur independently for each droplet. The physical separation of DNA reduces the risk of artefactual amplification and formation of unwanted chimeric DNA (184). BEAMing utilising this technique with specific beads bound to DNA prior to amplification. Amplification of DNA results in multiple copies of the original DNA molecule being attached to the compartmentalised bead. These beads are then magnetically removed, and the resulting DNA can then be quantified and sequenced using PCR-based methods. The use of beads in the amplification process ensures the resulting amplified DNA population ratio is a reflection of the original template, and so quantification of DNA can be made (185).

ddPCR-based genotyping was developed to improve the Sn over other PCR based-DNA techniques. It utilises a similar water-in-oil emulsion principle to separate DNA into isolated droplets (up to 20,000) with each droplet containing

only 1 copy of template DNA. Primers and fluorescent probes are then used to facilitate PCR amplification of target wildtype and mutant DNA droplets. The proportion of mutant DNA droplets is then estimated using a Poisson distribution to determine an absolute mutant allelic count in the sample. This process allows for detection of SNV, but also copy number variants. ddPCR offers highest Sn and precision in the amplification and sequencing of DNA, with multiple molecular diagnostics clinical uses including in cancer diagnostics.

PCR techniques are considered highly sensitive at detecting mutant DNA at relatively low levels VAF, as low as <0.01%. However, these PCR techniques are limited as they are only capable of detecting a single, specific variant allele. NGS assays can also target specific alleles, however, can perform high-throughput sequencing of multiple alleles at a single time. These assays however can detect VAF as low as <0.1% and are less sensitive compared to PCR-based assays.

Targeted NGS-based assays are often categorised based on their method of target enrichment. Amplicon sequencing enriches target DNA sequences by creating amplicons, which are subsequently indexed and pooled (i.e. multiplexed) prior to amplification and sequencing. This technique allows for a high coverage sequencing of multiple targets in a single reaction. This technique offers fast turnaround times, but due to uneven coverage does not offer reliable CNV calling.

Hybridisation capture-based sequencing is another targeted NGS method which does not use a PCR primer design like amplicon sequencing but enriches regions of interest using panel baits hybridizing to libraries. Unwanted, uncaptured fragments are subsequently washed away. Hybridisation method is longer, but more suitable for identifying various mutation classes, including large indels, CNV and SVs. It involves the use DNA fragmentation and repair with the addition of specific adaptors containing a specific barcode unique to the sample. The sample is then sequenced and then the barcodes are separated during the bioinformatic analysis to separate individual sample DNA. This allows for multiple samples to be processed in a single run (parallel sequencing).

Sequencing DNA at a high depth is associated with an increased risk of detecting sequencing errors, PCR errors, and artefacts at the same time. This is known as background genomic noise and gives rise to low-frequency variants (i.e. variants are low VAF <0.01%). The use of unique molecular identifiers (UMIs) can reduce background genomic noise during sequencing by unmasking these low- frequency variants. This process involves molecular tagging of DNA fragments before amplification. Following sequencing, the UMI reads are aligned and grouped into families, to provide enhanced Sn and accuracy when calling variants. Table 14 describes the variants ctDNA sequencing techniques with associated limitations and benefits.

Table 13- ctDNA Sequencing Techniques

Method	Example	Molecular Targets	Detection limit	Limitations	Benefits
Emulsion PCR Assays	ddPCR BEAMing	Known mutations	<0.01%	Less specific. Only detects CNV/fusions using one assay per set up	Fully quantitative
Targeted NGS Assays					
Amplicon based	TAM-Seq	Hotspot SNV and CNV	<0.1%	Less sensitive and limited variant analysis compared to capture based	Fast and cost effective
Capture based	Guardant360©	SNV, CNV, fusions	<0.1%	Lower specificity compared to amplicon-based assays, complex and slower.	Higher sensitivity compared with amplicon sequencing
Non-targeted NGS Assays	Whole genomic sequencing Whole exome sequencing	All variants	<1%	Less sequencing depth compared with NGS, costly	Genomic wide analysis

Adapted from Mencel et al The Role of ctDNA in Gastric Cancer. Cancers (Basel). 2022. Reference (186)

The approach to ctDNA sequencing analysis can be classified as tumour-informed or tumour-agnostic. A tumour informed approach requires pre-existing knowledge of the genomic aberrations of the tumour, based on prior tissue sequencing. PCR-based methods of DNA sequencing can be targeted to specific alleles, at high sequencing depths to detect plasma-based variants are very low VAF. This is particularly useful in the detection of minimal residual

disease (MRD). However, in some settings, genomic sequencing of tissue is not feasible, such as in difficult to access tumours, samples with insufficient DNA quantity and quality for high-throughput NGS sequencing, and patient factors such as preference and co-morbidities. In this setting, a tumour agnostic approach using a plasma-only assay can be employed. This approach does not require knowledge of the tumour's genome (i.e. tissue-based sequencing not needed) and utilises either a targeted or non-targeted approach to sequence ctDNA. This tumour agnostic, plasma-alone approach is optimal for ctDNA-based screening programmes.

Sequencing of ctDNA can include multiple genomic aberrations, similar to tissue-based sequencing. These include SNV, copy number variants (CNV), insertions and deletions (Indels), gene fusions and rearrangements, and methylation. These aberrations are defined in table 15.

Table 14- Definition of Genomic Aberrations Detected using ctDNA

Genomic aberration	**Definition**
Single nucleotide variant (SNV)	Single nucleotide change (A, C, G, T) in the DNA sequence - Non-synonymous (change in codon leads to change in amino acid product) - Synonymous (Silent) (nucleotide substitution has no impact on amino acid encoded by codon)
Copy number variant (CNV)	Change in the number of copies of a gene or region of the genome - Gains - Losses Includes amplifications, deletions and duplications involving several base pairs to an entire chromosome and therefore changes the total amount of DNA (and protein product) compared to the reference genome.

Insertion and deletions (Indels)	One or more nucleotides are inserted or removed. In frame: deletion/insertion of N nucleotides where N is divisible by 3. Frameshift: deletion/insertion of N nucleotides where N is not divisible by 3, leading to a change in the reading frame
Gene fusions	Two separate genes join into a hybrid gene due to chromosomal re-arrangement
Chromosomal rearrangements	Swapping of material between or within chromosomes. May lead to production of a fusion gene or may disrupt relationship between gene and regulatory element

DNA methylation can also be assessed in ctDNA. Changes in patterns of DNA methylation is an important driver of carcinogenesis, and often occurs early in the development of cancer. It therefore has potential use in cancer screening, monitoring for MRD and in early cancer diagnosis. DNA methylation is an epigenetic change, involving the addition of a methyl group to a cytosine group, mostly occurring in CpG dinucleotides. CpG are abundant in the human genome, and most are methylated. In cancer, methylation patterns are generally homogenous, with consistent patterns seen within tumour cells of a similar origin. Therefore, tissue-of-origin can often be identified using DNA methylation signatures for cancers of unknown primary and in screening programmes. The methods of ctDNA methylation analyses are outlined in table 16.

Table 15- Methods of DNA methylation Analysis using ctDNA

Method	Technology examples	Cost	Cancer type	Coverage	Limitation
Bisulfite conversion based	Use of bisulfite salts to deaminate unmethylated cystosine residues and methylated cystosine remain methylated. PCR sequencing reveals methylated status of DNA				
	Whole genome bisulfite sequencing (WGBS)	High	Multiple	Most comprehensive	Low sequencing depth
	Methylated CpG tandem amplification and sequencing (MCTA-seq)	Moderate	HCC	High coverage (25,000 sequences)	Limited to CGIs and may miss other methylated changes
	Microarray	Low	CRC	Covers hotspot methylation (850,000 CpG sites)	Low coverage of CpGs
Bisulfite-free methods	Antibody enrichment methods rather than using bisulfite (i.e. bisulfite free method) to limit degradation of DNA. Uses methyl CpG binding proteins to enrich for methylated regions.				
	Restriction enzyme based ddPCR	Low	RCC	Ultra-low depth however limited coverage to only a few loci	Limited coverage
	Enrichment based	Moderate	Multiple	Specific to 5-hydrosymethylcytosine to cover whole genome	Low resolution and unable to provide single methylation information

Adapted from Luo et al. Liquid Biopsy of Methylation Biomarkers in Cell-Free DNA. Trends Mol Med. 2021. Reference (187)
Abbreviations: HCC- hepatocellular carcinoma; CRC- colorectal cancer; RCC- renal cell carcinoma. CpG- 5'-C-phosphate-G-3'

My thesis focuses on the use of ctDNA through liquid biopsy. However, there are other types of liquid biopsy not centred on ctDNA. These include the use of circulating tumour cells (CTCs), exosomes and nucleosomes, and circulating miRNA sequencing. Although these will be reference in the introduction, they are not covered in my results chapters.

21.1.3 Origin of cfDNA, and Clonal Haematopoiesis of Indeterminate Potential

cfDNA can originate from multiple sources from either physiological or pathological conditions. Non-tumour derived cfDNA containing genomic aberrations can be seen in the following scenarios

- cfDNA from physiological conditions (pregnancy, exercise) and non-malignant conditions (inflammatory)
- cfDNA from normal, benign cells (188)
- Clonal haematopoiesis on indeterminate potential (CHIP)
- Germline mutations

CHIP is a condition characterised by the clonal expansion of haematopoietic stem cells which contain a somatic mutation. It is seen in the circulation of patients without a haematological disorder, with incidence increasing with age. Approximately 20% of healthy individuals aged over 60 years old have evidence of CHIP. Common CHIP-related mutations include variants in genes associated with haematological malignancies, such as *TET2, DNMT3A and ASXL1* (189). However, *TP53* and *KRAS* mutations related to CHIP can also be detected in 0.2% and 0.02% of healthy individuals, and are also commonly seen in CRC, PC and BTC (190). These CHIP mutations could give rise to a false positive when detected in the ctDNA, and are of concern when using a

tumour agnostic approach. However, this can be mitigated by sequencing the buffy coat (WBC) in parallel to plasma and subtracting SNVs identified in DNA from WBC. This approach has been validated in a randomised control study of perioperative treatment in patients with gastric cancer (CRITICS Trial) using ctDNA as a predictive biomarker compared with matched buffy coat controls (191). In addition, sequencing plasma rather than serum is considered the standard approach to reduce the risk of false positives from CHIP which are more commonly seen when sequencing serum samples.

The variant allele frequency of variants detected in cfDNA in the plasma can be indicative of germline origin when matched buffy coat control is not available for sequencing. Generally, heterozygous germline variants detected in the plasma are present at VAF of approximately 50%.

Somatic tumour derived variants are present at relatively higher concentration than non-tumour derived somatic variants given they are often clonal (and sometime sub-clonal) in origin. Therefore, understanding an assay's limit of detection (LOD) and a validated bioinformatic pipeline is critical in the interpretation of ctDNA results. However, non-tumour derived variants at low frequency can be mis-interpreted for somatic, tumour derived variants. Therefore, assay fit for clinical use must have an appropriate level of analytical and clinical Sn (see section 1.5.5).

21.1.4 ctDNA Sequencing Analysis Reporting

The reporting and subsequent clinical interpretation of ctDNA data requires multiple additional steps.

22 Bioinformatic Analysis

A robust bioinformatic analysis is critical in variant detection and calling. Through the use of UMIs and molecular barcoding, it can reduce background noise and remove PCR errors, a bioinformatic pipeline can be tailored to remove these errors. Bioinformatic pipelines use complex, multi-step process involving the identification of somatic variants by comparing allelic frequencies in normal samples to tumour samples.

The first step is examination of sequencing quality by FastQC. Then, the sample tumour genome is aligned to the human reference genome (GRCh37) using a pre-specified algorithm. Reads with multiple alignments are then discarded. All read group are then alignments merged using software (e.g. Picard). Following this, co-cleaning of aligned reads is performed which re-calibrates BAM files (i.e. with software such as GATK). This step enhances the alignment of bases. Aligned reads are then processed through a variant calling pipeline for variant identification (i.e. MuSE, VarScan, VarDict). The aim of this variant calling pipeline is to (1) identify germline mutations, (2) identify somatic variants at appropriate depth, supportive reads and VAF, by comparing sample data to TCGA normal genome. This latter step flags variants present in the dbSNP and 1000 Genome Project to eliminate common, non-pathogenic single nucleotide polymorphisms (SNPs). The final process involves annotation of somatic variants by describing the gene

affected, aberration location, protein-level consequence (i.e. using GBrowse, CooVar).

The goal of bioinformatics is to correctly identify true tumour aberrations, annotate their functional consequences and impact on gene expression and remove sequencing artefacts.

23 Manual Curation of Variants

Depending on the ctDNA approach used (i.e. tumour agnostic or tumour informed), further curation of variants detected is often required. In addition, germline variant with a frequency of >0.0001% in the general population (on Genome Aggregation database) are often removed given the likelihood of a germline variant. Germline variants are often removed through bioinformatic analysis, however manual checking of rare, ethnicity specific is often required, by checking the matched patients buffy coat sequencing.

24 Molecular Tumour Board in the Clinical Interpretation of Reports

With recent advancements in molecular profiling of tumours, the detection of variants with potential actionability is of increasing importance. There are a growing number of targeted therapies in clinical use and clinical trials, and selection patients with specific aberrations which predict benefit of targeted therapies is critical. The interpretation of actionability is complex, and often requires knowledge of precision therapeutics. Currently, there are several online databases of cancer specific genomic drivers and their associated

actionability, including COSMIC (Catalogue of Somatic Mutations in Cancer), My Cancer Genome, and OncoKB. In addition, there are several guidelines for the interpretation of reporting of variants in cancer, including the AMP-ASCO-CAP and ESMO ESCAT guidelines (82, 192).

However, there are risks when using these guidelines in isolation. Variant classification may differ in different clinical contexts, and between online databases and clinical guidelines. It is challenging to provide a tailor-based treatment specific to an individual patient need. However, a molecular tumour board (MTB), comprising of medical oncologist, genomic specialists, clinical scientists and pathologists can facilitate this. The role of an MTB is in the clinical interpretation of variants, with a focus on determining a variants functional relevance and targetability. Functional relevance comprises an assessment of whether the gene variant affects protein structure and function. The assessment of targetability can provide tailor-based treatment recommendations including enrolment in active clinical trials.

24.1.1 Analytical Validity, Clinical Validity and Clinical Utility of ctDNA Based Assays

The application of ctDNA based assays in clinical practice requires the generation of evidence to establish analytical validity, clinical validity, and subsequently clinical utility. The Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group have published guidance on the integration of genomic test use in clinical practice (193).

Analytical validity of a ctDNA assay is defined as the assay's ability to measure the specific genotype, often in comparison to tissue-based sequencing (181, 193, 194). An alternative "gold-standard" end point is in comparison to reference mutant DNA with pre-specified serial dilutions to establish the assays performance. This alternative approach in establishing analytical validity may overcome intra-tumoural heterogeneity seen in tumour samples. Importantly, the analytical Sn (i.e. ctDNA detection rate), analytical Sp (true negative rate) and reliability must be robust to provide sufficient analytical validity prior to testing clinical validity.

Clinical validity of a ctDNA assay is defined as the assay's ability to detect the clinical phenotype or particular disease it is intended to detect. Often, the outcome is binary (i.e. disease confirmed or not) by defining a pre-specified cut-off which determines a "positive" and "negative" result. Clinical utility is the ctDNA assay's ability to improve clinical outcomes by adding value to the management (193). This provides a level of support to make management decisions based on the results from the ctDNA assay. Figure 9 summaries these definitions with specific reference to the use of ctDNA in the diagnosis of cancer.

Figure 9- Analytical Validity, Clinical Validity and Clinical Utility Of ctDNA Based Assays in the Diagnosis of Cancer

There are several commercial ctDNA based assays which are available for use with varying levels of clinical validity. A list of current commercial ctDNA based assays currently in use are shown in table 17. There are also several assays developed in different genomic laboratory hubs including the CMP (as described in results chapters 1 and 2).

Table 16- Commercially Available ctDNA Assays in Solid Tumour (Pan-Cancer and GI-focused assays)

Assay name	Company	Utility	Method of sequencing	Variants called	Gene panel	Tumour type	FDA approved indication
Guardant 360©	Guardant Health	Comprehensive genomic profiling	Hybridization enrichment NGS	SNV, Indels, CNV, fusions, MSI	73 gene	Multi	Yes
FoundationOne Liquid biopsy	Foundation medicine	Comprehensive genomic profiling	Hybridization enrichment NGS	SNV, Indels, CNV, fusions, MSI	324	Multi	Yes
Signatera	Natera	MRD	WES + multiplex PCR based NGS	SNVs, Indels	Tumour informed	Multi	Yes
Guardant Reveal	Guardant Health	MRD	Hybrid capture based NGS	SNV, Indels, Methylation	Tumour agnostic	CRC	No
Galleri	GRAIL	Screening	Bisulfite sequencing	Methylation	>100,000 methylation regions	Multi	No
PlasmaRESOLVE	PDGx	Comprehensive genomic profiling	Hybrid capture based NGS	SNV, Indel, Amplifications, fusions	33 genes	Multi	No
Freenome CRC screening	Freenome	Screening	NR	Genome, transcriptomics, proteomics	Multi	CRC	No
FirstSightCRC™	CellMaxLife	Screening	NGS and circulating epithelial cell detection	NR	Multi	CRC	No
Infinity	Guardant Health	Comprehensive genomic profiling, screening	Hybridization enrichment NGS	NGS and methylation	800 gene panel; TMB, MSI, 15MG methylation panel	Multi	No
DETECT-A (CancerSEEK)	Exact Sciences	Screening	Hybrid capture based NGS	SNVs, Indels and protein analytes	16 gene and 9 protein biomarkers	Multi	Yes

There is evolving and established clinical utility for ctDNA across cancer medicine, including in screening, faster diagnosis, detection of minimal residual disease (MRD) and in advanced tumours (see Fig 10). Below describes the analytical validity, clinical validity and utility of ctDNA in PC, BTC and CRC.

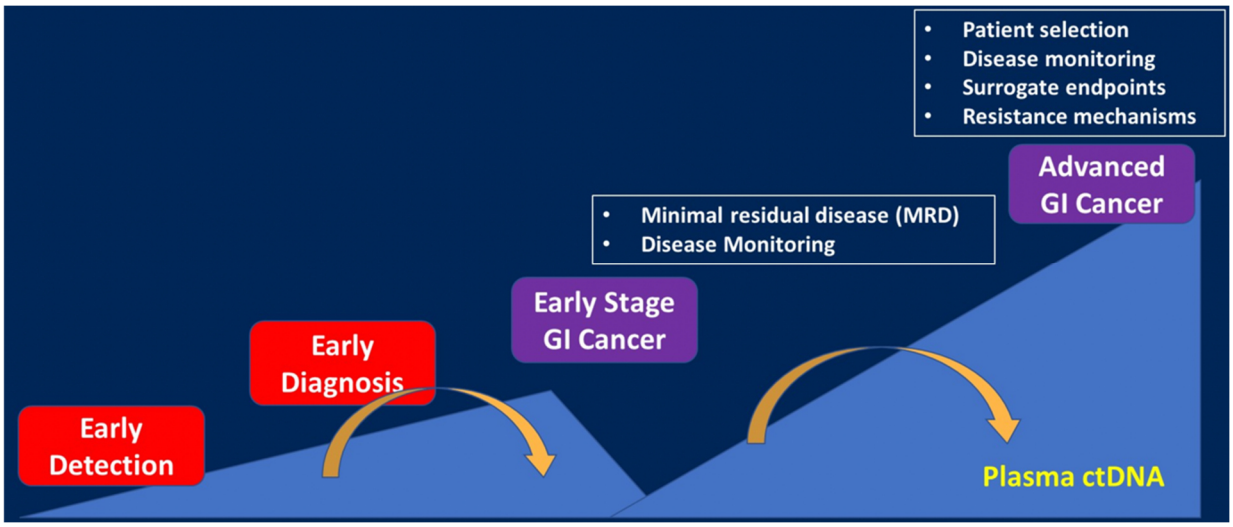


Figure 10- applications of ctDNA in GI cancers

Reference: (195)

25 Circulating Tumour DNA in Pancreatic Cancer

There are several potential applications of ctDNA in PC, including in

- Screening asymptomatic patients
- Facilitating diagnosis in symptomatic patients
- Detection of MRD and monitoring disease recurrence
- Molecular profiling of advanced disease
- Disease response assessment

These applications of ctDNA in PC, including the clinical validity and utility will be discussed here.

25.1.1 Analytical Validity of Detecting ctDNA in Pancreatic Cancer

ctDNA can be detected in the circulation of patients with PC. Early studies have evaluated ctDNA in PC using a single gene ddPCR approach targeting the *KRAS* gene. As 90% of patients with PC have a KRAS mutation, it is a suitable target for ddPCR testing using ctDNA (196).

A seminal study using PCR-based methodology demonstrated a ctDNA detection rate of 27% in plasma from patients with histologically proven PC (197). The detection rate increases with advancing stage of disease, with 0%, 9%, 20% and 43% detection rates seen in stages I, II, III, and IV disease respectively (197). Another similar study of 34 patients with PC, also revealed a ctDNA detection rate of 90% in those with stage IV and 50% with non-metastatic disease, using a ddPCR technique targeting *KRAS* (180). Other studies have shown a lower ctDNA detection rate when using PCR-based methods in early-stage PC compared with higher stage disease (198, 199). Overall, ctDNA rates range between 20-100% when using ddPCR methods to detect KRAS mutations. The broad range of detection rates seen within these studies is multi-factorial and related to stage of disease, presence of liver metastases and assay analytical Sn (200). In addition, these studies have shown a relatively broad range of concordance between KRAS ddPCR plasma and tissue-based sequencing, ranging between 27-100%.

To enhance the Sn of ctDNA in PC, multi-gene sequencing approaches can be used which target other commonly mutated genes seen in PC (such as

TP53, *CDKN2A*, *SMAD4*) which can be sequenced simultaneously using a multi-gene NGS assay (19, 201). There are several commercial assays including Guardant360© and FoundationOne available for molecularly profiling advanced tumours using ctDNA based NGS sequencing (see table 17). The Guardant360© assay is a plasma-based NGS test analysing 74 genes. It is approved for molecular profiling any solid tumour, including stage III/IV PC and BTC with a detection rate of 85% in PC (202). In 2019, Patel et al showed a 70% ctDNA detection rate in 112 patients with predominantly metastatic PC, however full tissue concordance was only 6% (47% partial concordance). Recent data from a single centre used the Guardant360© assay to sequence 104 patients with PC showing a similar ctDNA detection rate of 91% in metastatic PC (mPC) and 74% in locally advanced PC (LAPC). However, KRAS mutations were only detected in 74% of mPC and 44% of LAPC. Interestingly, 85% of plasma-detected KRAS mutant PC had liver metastases, suggesting higher ctDNA detection rate in those with liver metastases. Nonetheless, this highlights the relatively low concordance between tissue and plasma-based sequencing in PC (203).

A large meta-analysis of 14 studies involving 369 patients with PC showed a pooled Sn and Sp of plasma molecular profiling of 70% and 86% respectively, with lower Sn seen in studies using ddPCR assays compared with multi-gene assays (204). In this meta-analysis, the pooled concordance between plasma ctDNA and tissue sequencing was 32%.

The ctDNA detection rate in PC according to stage of disease and sequencing technique used is summarised in table 18.

Table 17- ctDNA Detection Rates in Pancreatic Cancer

Stage of disease	ddPCR assay (KRAS)	NGS assay
I	0-10% (197, 205)	50% (206)
II	10-30% (197, 205)	
III	25-40% (197, 207, 208)	62.5-85% (202, 208)
IV	70-90% (207, 209, 210)	80-90% (211-213)

25.1.2 ctDNA in Screening for Pancreatic Cancer

There is emerging evidence assessing the use of ctDNA in cancer screening using ctDNA methylation signatures, including in PC. The Galleri blood test is a targeted methylation based ctDNA assay used to detect multiple cancers at an early stage. Using a tumour agnostic approach, the Galleri methylation test has shown a high level of Sp (99.5%) and moderate Sn (51.5%) for detecting a cancer signal across multiple tumour types across 4077 participants. This multi-cancer early detection (MCED) test has a very low false positive rate of 0.48% (214).

In this study, 135 patients were found to have PC. Of these, 113 were detected using the MCED test (i.e. overall Sn 83.7%). Sn increased with advancing stage of disease, with stage I, II, III and IV sensitivities of 61.9% (13/21), 60.0% (12/20), 85.7% (18.21) and 95.9% (70/73). The study also assessed the MCED test's ability to predict the cancer signal origin (CSO). In the 113 patients with a pancreatic methylation signature detected on the Galleri assay,

104 were diagnosed with PC (i.e. prediction of 92.0%). The relatively good Sn of this assay to detect early-stage PC shows promise as a convenient screening tool.

Following this, the PATHFINDER study assessed the Galleri test in 7 US health care systems (n= 6662) to evaluate the clinical experience of MCED testing. Objectives included an assessment of the extent of further diagnostic testing required to achieve diagnostic resolution, including time to diagnostic resolution and the number and types of diagnostic tests. Secondary objectives including the diagnostic accuracy (i.e. Sp, PPV, NPV, number needed to screen and CSO accuracy), in addition to patient reported outcomes (PROs) (anxiety and distress). A cancer signal was detected in 1.4% of patients overall, with the median days to diagnostic resolution of 79 days. In those with a cancer signal detected but without evidence of cancer (i.e. false positive, 63% rate), the median time to diagnostic resolution was 162 days, with 30% requiring an invasive diagnostic procedure to exclude malignancy. The test performance in detecting cancer was relatively poor overall, with a PPV of 38.0%. A refined MCED assay was used to re-evaluate the test characteristics which improved the PPV. This refined assay is being investigated in the PATHFINDER2 study in screening 20,000 patients (NCT05155605).

Futures studies assessing the use of MCED on cancer related survival and optimisation of the false positive rate is needed. The Galleri-NHS randomised trial (ISRCTN 91431511) is assessing the clinical utility of the yearly MCED test (over 3 years) compared to usual screening, in 140,000 asymptomatic

patients in the NHS. This study also includes 6238 patients with symptomatic disease (SYMPLIFY). The GuardantINFINITY assay by Guardant Health is a large pan-cancer NGS test of >800 genes, incorporating DNA genomic analysis with exome-wide methylation analysis. This approach may have a role in screening and diagnosis and directing targeted therapy approaches.

The Galleri test is currently being investigated several prospective, multicentre observational studies as outlined in table 19.

Table 18- Ongoing Prospective Trials using the Galleri MCED Test

Study name (NCT number)	**N=**	**Tumour type**	**Target population**	**Study end date**
STRIVE (NCT03085888)	120,000	Breast cancer	Average risk female breast cancer undergoing screening mammogram	June 2022
SUMMIT (NCT03934866)	13,000	Lung cancer	High risk lung cancer patients undergoing low dose CT screening	August 2023
PATHFINDER2 (NCT05155605)	20,000	Multi cancer	Asymptomatic patients	June 2023
Galleri-NHS	140,000	Multi cancer	Asymptomatic patient (including 6238 symptomatic-SYMPLIFY)	June 2023

25.1.3 ctDNA Detection of Minimal Residual Disease

ctDNA has emerged as a useful non-invasive tool to detect MRD in CRC, and may have a future role in resectable PC. Detection of MRD has several potential benefits. A summary of prognostic ctDNA MRD studies in early stage PC is outlined in table 20.

Table 19- Prognostic ctDNA MRD Studies in Early Stage Pancreatic Cancer

Author, year	N=	Sample collection timepoint	Approach	DFS MRD positive (months)	OS MRD positive (months)	DFS MRD negative (months)	OS MRD negative (months)
Hadano, 2016 (215)	105	Post-op	ddPCR KRAS	NR	13.6	NR	27.6
Pietrasz, 2017 (216)	31	Post-op	ddPCR KRAS	4.6	19.3	17.6	32.2
Groot, 2019 (217)	59	Post-op	ddPCR KRAS	5.0	17.0	15.0	NR
Lee, 2019 (218)	81	Post-op	ddPCR KRAS	10.3	13.6	NR	NR
Yamaguchi, 2021 (219)	97	Post-op	ddPCR KRAS	6.9	16.4	19.2	44.3
Watanabe, 2019 (220)	39	Post-op	ddPCR KRAS	NR	15.8	NR	33.7

25.1.4 ctDNA-Based Molecular Profiling, Selection of Personalised Therapy in Advanced Pancreatic Cancer

The molecular profiling of PC allows for identification of driver mutations, some of which have therapeutic relevance, offering patients a personalised approached in their management. Tissue-based NGS provides an opportunity to identify these targetable alterations, however there is a growing body of evidence to support ctDNA in the molecular profiling of advanced PC for this purpose. ctDNA offers multiple advantages over tissue based NGS and can be collected non-invasively at serial time points with greater ease (including prior to subsequent lines of therapy). ctDNA also overcomes the issue of heterogeneity seen with tissue based NGS, and may capture variants not detected when using tissue biopsies from a single site. ctDNA may also offer

other value in addition to the molecular profiling of tumours, including in in prognostication and monitoring disease response.

The SCRUM-Japan GI-SCREEN and GOZILA are large observation studies that aimed to compare molecular tumour profiling using ctDNA with tissue genotyping. Comprehensive plasma-only genomic profiling using the Guardant360© assay performed on 363 patients with mPC and 188 with metastatic cholangiocarcinoma revealed a ctDNA detection rate of 83.4% in PC and 90% in BTC (using a VAF cut off >0.03) (23). *KRAS* mutations were detected in 49% of PC, with *TP53* mutations in only 5%. Interestingly, 3% of patients with PC and 4.3% of BTC had pathogenic *BRCA2* mutations which were not detected with tissue sequencing. Although correlation with tissue sequencing was not described by tumour type, overall tissue concordance was between 85-100%. An important aim of this study was to assess the clinical utility of ctDNA genotyping in comparison to tissue to detect targetable mutation for clinical trials. A separate cohort of patients (n=5,621) were enrolled into the GI-SCREEN study (an equivalent tissue-based NGS study), and outcomes were compared with the ctDNA based study (GOZILA). Compared with tissue based sequencing, ctDNA genotyping was associated with improved analytical success (99.9% vs 89.4%), shorter duration of screening (11 days vs 33 days) and higher rates of enrolment in clinical trials using targeted agents (9.5% vs 4.1%). This study highlights the advantages of ctDNA based molecular profiling across all GI cancers. Other studies have similarly assessed the use of ctDNA based genotyping in detection of targetable agents.

However, other studies have not shown similar moderate-high levels of concordance of tissue and plasma-based sequencing (NGS and ddPCR) (221, 222). Majority of studies have shown variability in the shedding of KRAS mutations in plasma, with contributing factors related to;

- Non-pancreatic head primaries (223)
- Lower tumour volume (197)
- Low VAF (222)
- Sample of primary versus metastatic site (206)
- Non-liver metastatic disease (200)

The intertumoral heterogeneity seen in PC has been well described. Tissue molecular profiling frequently fails to adequately capture the genomic profile of the tumour. ctDNA sequencing to capture a complete molecular profile of PC may overcome the heterogeneity seen with tissue biopsies and several studies have shown superiority in capturing important, potentially targetable therapies. However, the detection of *KRAS* in the plasma of patients with PC seems to vary across studies and with the development of *KRAS* G12C inhibitors, the detection of this mutation is important to ensure these patients are correctly identified. Table 21 summaries ctDNA genotyping studies in advanced PC which assessed tissue-plasma concordance.

Table 20- Studies of ctDNA-Tissue Concordance in Pancreatic Cancer

	N=	Sequencing technique	Target mutations	KRAS Tissue concordance	NGS Full concordance	NGS Partial concordance
Adamo, 2017 (223)	26	ddPCR	KRAS	0%	N/A	N/A
Berger, 2018 (224)	20	NGS	TP53, SMAD4, CDKN2A, KRAS, APC, ATM, FBXW7	NR	NR	82%

Bernard, 2019 (200)	194	ddPCR	KRAS	95%	N/A	N/A
Bettegowda, 2014 (180)	34	PCR	KRAS	87%	N/A	N/A
Brychta, 2016 (225)	50	ddPCR	KRAS	36%	N/A	N/A
Castells, 1999 (197)	44	ddPCR	KRAS	32%	N/A	N/A
Dianxu, 2002 (226)	58	PCR	KRAS	76%	N/A	N/A
Kim, 2018 (227)	106	PCR	KRAS	77%	N/A	N/A
Kinugasa, 2015 (228)	141	PCR	KRAS	77%	N/A	N/A
Ko, 2016 (229)	32	NGS	Guardant360 (73 genes)	100%	NR	66%
Marchese, 2006 (230)	30	ddPCR	KRAS	0%	NR	NR
Mulcahy, 1998 (231)	10	PCR	KRAS	100%	NR	NR
Okada, 2020 (232)	96	ddPCR	KRAS	81%	NR	NR
Husain, 2022 (222)	105	NGS	FoundationOne (324 genes)	58% KRAS	NR	NR
Patel, 2019 (206)	112	NGS	Guardant360 (73 genes)	52% KRAS	6%	47%
Pishvaian, 2016 (233)	34	NGS	Guardant360 (73 genes)	25%	NR	NR
Sefrioui, 2017 (234)	52	PCR	KRAS	75%	N/A	N/A
Sivapalan, 2022 (235)	7	WES	Multi-gene	NR	NR	55%
Strijker, 2020 (236)	58	ddPCR	KRAS	10%	N/A	N/A
Uemura, 2004 (237)	28	PCR	KRAS	44%	N/A	N/A
Wu, 2014 (238)	24	PCR	KRAS	100%	N/A	N/A
Yamada, 1998 (239)	21	ddPCR	KRAS (codon 12)	60%	N/A	N/A
Vietsch, 2018 (221)	5	NGS	Custom panel (56 genes)	NR	NR	28%
Wang, 2022 (240)	57	NGS	Oncoscreen Plus (520 genes)	NR	NR	66.7%

25.1.5 ctDNA and other Circulating Biomarkers to Support Diagnosis in Pancreatic Cancer

The evidence to support ctDNA as a diagnostic analyte in GI cancers is limited. Several studies have shown potential clinical utility of ctDNA to support a diagnosis of PC with varying degrees of Sn and Sp. However, majority of these studies are retrospective and used a single gene targeted approach (ddPCR *KRAS*). In addition, no study has reported the use of ctDNA in the diagnosis and subsequent treatment decision making in PC. To date, there is no diagnostic ctDNA study in PC using a multi-gene panel, used in a diagnostic pathway which informs treatment decisions.

A tumour agonistic approach is the only method to prospective assess ctDNA as a diagnostic analyte in patients with suspected cancer without a tissue biopsy. Criteria for using a pre-specified VAF threshold to determine ctDNA detection requires robust analytical Sn, and is largely dependent on the assay's characteristics. In addition, identification of false positives including CHIP variants, germline variants, and variants from non-malignant tissue including pancreatitis and cysts may also confound results and should be mitigated against.

This section will summarise the data to support the use of ctDNA (including genotyping, methylation) and other circulating biomarkers in the diagnosis of PC.

26 ctDNA in the Diagnosis of Pancreatic Cancer

The median concentration of total cfDNA is higher in those with cancer compared to those without cancer, and therefore has potential for use to diagnose cancer in those with symptoms and signs suggestive of cancer.

The largest study assessing ctDNA in the diagnosis of PC is from LeCalvez-Klem et al. This retrospective analysis of 437 patients with PC (across all stages) compared PCR based sequencing (of *KRAS*) and compared to patients with chronic pancreatitis and healthy controls. Detection of *KRAS* was low (19%), with Sn and Sp only 17.5% and 98.2% respectively as a diagnostic tool in PC. However, ctDNA detection increased with stage of disease (10% localised, 17.5% LAPC and 33% mPC). Interestingly, *KRAS* was also detected in 3.7% of healthy controls and 4.3% of chronic pancreatitis. In addition, 3.5% of non-PDAC PC had *KRAS* mutations and these were predominantly NET histology.

To date, there is no data to support the use of a ctDNA approach using a multi-gene assay in the diagnosis of PC/ BTC. A summary of all diagnostic ctDNA studies in PC is summarised in table 22.

Table 21- Studies of ctDNA in the Diagnosis of Pancreatic Cancer

Author Study, year	N=	Controls, n	Stage 4 (%)	Sequencing method	Approach (Agnostic, informed)	Target mutation(s)	Detection rate (overall)	Detection rate KRAS	Detection rate TP53	Sn (%)	Sp (%)	Sample type
Berger, 2016	24	21 IPMN, 38 HC	100	Targeted ddPCR	Informed	GNAS codon 201 KRAS codon 12	42%	42%	N/A	81	84	Serum
Castells, 1999	44	37 CP, 10 non PDAC 9 AP 4 HC	52	Targeted, ddPCR	Informed	KRAS codon 12	27%	27% Stage I- 0% Stage 2- 9% Stage 3- 20% Stage 4- 43%	NR	27	100	Plasma
Cohen, 2017	221	182 HC	0	Targeted, ddPCR	Agnostic	KRAS	30% stage I- 10% Stage II- 33%	30%	20%	60	99.5	Plasma
Dianxu, 2002	58	21 HC	45	Targeted, ddPCR	Agnostic	KRAS	71%	71%	N/A	71		Plasma
Eissa, 2019	39	95 HC 8 CP	0	Targeted methylation	Agnostic	2 gene promotors (ADAMTS1 and BNC1)	NR	NR	NR	97.3	91.6	Plasma
LeCalvez-Kelm, 2016	437	141 CP 394 HC	40	Targeted, ddPCR	Agnostic	KRAS	19%	19%	NR	17.5	98.2	Plasma
Liggett, 2010	30	30 CP 30 HC	NR	Targeted, methylation	Agnostic	17 gene promoters	NR	NR	NR	91.2	90.8	Plasma
Maire, 2002	47	31 CP	49	Targeted, ddPCR	Agnostic	KRAS codon 12 (only G12D)	47%	47%	NR	47	85	Serum
Marchese, 2006	30	CP	5	Targeted, ddPCR	Agnostic	KRAS	0%	0%	NR	0	0	Serum

Sefrioui, 2017	52	16 HC	42	Targeted, ddPCR	Agnostic	KRAS	56%	56%	NR	65	75	Plasma
Wang, 2002	149	44 HC		Targeted, ddPCR	Agnostic	KRAS	N/A	35% Stage I/II- 28.6% Stage III/IV- 42.0%	N/A	35	88	Plasma
Wu, 2014	24	25 HC	NR	Targeted, PCR	Agnostic	KRAS	72%	72%	NR	80.6	87.5	Plasma
Zhu, 2020	507	265 HC	NR	Targeted, ddPCR	Mixed	Mixed	NR	NR	NR	64	92	Mixed

27 ctDNA Methylation in the Diagnosis of Pancreatic Cancer

More recently, DNA methylation analysis using ctDNA has been studied in the diagnosis of PC as outlined in table 23.

Table 22- Studies of ctDNA Based DNA Methylation as a Diagnostic Tool in Pancreatic Cancer

Author(s)	Cases N=	Controls	Gene(s)	Methods	Sn	Sp
PDAC vs HC						
Eissa, 2019 (241)	39	HC 95	ADAMTS1, BNC1	MSP	97.3	91.6
Melnikov, 2009 (242)	30	HC 30	5 promotor regions	Microarray-mediated methylation	76	59
Majumder, 2021 (243)	170	HC 70	13 gene panel	MSP	90	92
Shinjo, 2020 (244)	47	HC 14	ADAMTS1 HOXA1 PCDH10 SEMA5A SPSB4	MSP	49	86
Yi, 2013 (245)	173	HC 4	BNC1 and ADAMTS1	MSP	81	85
Suehiro, 2022 (246)	91	82 HC	HOXA1, SST + CA19.9	CORD	89	85.7
PDAC vs PanC						
Vrba, 2022 (247)	19	PanC 44	10 gene panel	MSP	100	95
PDAC vs CP						
Park, 2012 (248)	104	CP 60	NPTX2	MSP	80	76
PDAC vs non-PDAC (HC/CP)						
Fujimoto, 2021 (249)	55	CP 12 HC 80	RUNX3	CORD	50.9	93.5
Henriksen, 2016 (250)	95	CP 97 SN 27	28 gene panel	MSP	76	83
Liggett, 2010 (251)	30	CP 30 HC 30	8 promotor regions	Microarray-mediated methylation	90.8[1] 81.7[2]	91.2[1] 78[2]

MSP- methylation-specific PCR; PanC- pancreatic cyst; PDAC- pancreatic ductal adenocarcinoma; CORD- combined restriction digital PCR

Those studies who examined multiple genes, overall Sn/Sp was used.

1- PDAC vs non PDAC

2- 2- CP vs HC

28 Other Blood Based Biomarkers in the Diagnosis of Pancreatic Cancer

Other blood-based circulating biomarkers have also been studied as a potential diagnostic tool in PC. A summary of studies assessing other circulating biomarkers in the diagnosis of PC are reported in table 24 below.

Table 23- Studies Assessing Circulating Biomarkers in the Diagnosis of Pancreatic Cancer (with reported sensitivity and specificity)

Study	Biomarker(s	Cases	Control	Sn	Sp
Proteins/Cytokines					
Berger, 2019 (252)	thrombospondin-2	52	15 IMPN 32 CP	50*	96*
Nam, 2022 (253)	Asprosin	347	209 HC	95.7	92.4
Balasenthil, 2017 (254)	Tissue factor pathway inhibitor, tenascin C + CA19.9	206	86 CP 31 BBD 108 HC	75^	82^
Capello, 2017 (255)	TIMP1, LRG1, and CA19.9	75	27 HC 19 CP	84.9^	66.7^
Kaur, 2017 (256)	MUC5AC	53	22HC	83	80
Koopmann, 2006	MIC-1	50	50 HC	90	94
miRNA					
Deng, 2016 (257)	miR-25	303	600 HC	75.6	93.0
Zhou, 2018 (258)	6 gene panel (miR-122-5p, miR-125b-5p, miR-192-5p, miR-193b-3p, miR-221-3p, miR-27b-3p)	112	116 HC	88.7	89.1
Zou, 2019 (259)	6 gene panel (et-7b-5p, miR-192-5p, miR-19a-3p, miR-19b-3p, miR-223-3p, miR-25-3p)	129	107 HC	95.3	76.7
Shao, 2021 (260)	miR-483-3p	107	22 HC	74.6	77.3
Exosomes					
Yu, 2020, 89	mRNA d signature (FGA, KRT19, HIST1H2BK, ITIH2, MARCH2,	121	76 HC + CP	93.4	85.1

	CLDN1, MAL2 and TIMP1)				
Melo, 2015 (261)	GPC1+ crExos (protein)	190	100 HC	100	100
Lai 2017 (262)	miR-10b miR-21 miR-30c miR-181a miR-let7a	29	6 HC	100 100 100 100 100	100 100 100 100 100
Lewis, 2018 (263)	2 protein markers (Glypican-1 and CD63)	20	11 HC	99	82
Goto, 2018 (264)	ExmiR-191, ExmiR-21 ExmiR-451a	32	22 HC 29 IPMN	71.9^ 80.7 65.8	84.2^ 81 85.7
Lux 2019 (265)	cMET cMET + PDL1	55	26 CP 10 IPMN	70 72.4	85 89.5

* reported Sn/Sp for chronic pancreatitis (CP); ^ reported Sn/Sp for healthy control (HC)
Sn/Sp for multiple biomarkers reported as total if not listed individually

29 Circulating tumour DNA in Biliary Tract Cancer

29.1.1 Detection of ctDNA in Biliary Tract Cancer

ctDNA can also be detected in the circulation of patients with BTC. ctDNA detection correlates with higher stages of disease and tumour volume (266, 267). Most studies have assessed multi-gene NGS panels given the broad range of mutations detected in BTC. Several studies have used ctDNA in those with early stage BTC, however individual detection rates have not been reported (206, 266-268). ctDNA detection rate is similar between gallbladder and cholangiocarcinoma (222, 240). Table 25 outlines several ctDNA studies in BTC according to ctDNA detection rate and tissue-plasma concordance.

Table 24- ctDNA Based Studies in Biliary Tract Cancer

Author Study	N=	Controls, n	M1 (%)	Utility	Sequencing method	Commercial assay	Target mutation(s)	Detection rate (overall)	Sen for diagnosis	Sp for diagnosis	Sample type	Tissue Concordance (%)
Andersen, 2016	8	N	NR	Molecular profiling	PCR	N/A	4 genes (KRAS, NRAS, BRAF, PIK3CA)	100%	NR	NR	Plasma	100%
Berchuck, 2022	1671	N	100	Molecular profiling	NGS	Guardant360	Muti-gene (73 genes)	84%	NR	NR	Plasma	87% IDH1 100% BRAF V600E 18% FGFR2 fusions 1% KRAS G12C, 5% KRAS G12D 7% PIK3CA HER2 amplification 5%
Csoma, 2022	25	N	20	Molecular profiling	NGS	Archer VariantPlex	Multi-gene (67 genes)	85%	NR	NR	Plasma	62%
Ettrich, 2019	24	N	88	Molecular profiling	NGS	N/A	15 gene panel	45%	NR	NR	Plasma	74%
Han, 2021	16	4	NR	Molecular profiling, diagnostic	ddPCR	N/A	KRAS	19% in plasma; (50% in tissue; 48% in bile)	NR	NR	Plasma, bile	80% bile and tissue 42.9% plasma and tissue
Husain, 2022	546 CCC 109 GB	N	NR	Molecular profiling	NGS	FoundationOne	Muti-gene (324 genes)	92% CCC 94% GB	NR	NR	Plasma	NR
Lamarca, 2020	112	N	89	Molecular profiling	NGS	FoundationOne	324 genes	88%	NR	NR	Plasma	100% for FGFR2 and IDH1
Mody, 2019	124	N	52	Molecular profiling	NGS	Guardant360	74 genes	76%	NR	NR	Plasma	NR
Nakamura , 2020	363 PC 188 BTC	N	1	Molecular profiling	NGS	Guardant360	Muti-gene (73 genes)	90%	NR	NR	Plasma	NR
Odegaard, 2018	NR	N	NR	Molecular profiling	ddPCR, NGS	Guardant360	Muti-gene (73 genes)	80%	NR	NR	Plasma	NR
Okamura, 2020	71	N	94	Molecular profiling; prognosis	NGS	Guardant360	Muti-gene (73 genes)	76%	NR	NR	Plasma	67-90%
Wang, 2022	57 PC 47 BTC	N	NR	Molecular profiling	NGS	Oncoscreen Plus	520 cancer related genes	CCC- 88.9 GB- 85%	NR	NR	Plasma	CCC 81.5% GB 80%

Wintachai , 2021	62	33 BBD 30 HC	58	Diagnosis	NGS	NR	60 genes	87%	88.7	96.7	Plasma	56.3%
Zill, 2018	PC 1122 BTC 422	N	NR	Molecular profiling	NGS	Guardant360	Muti-gene (73 genes)	80%	NR	NR	Plasma	NR
Zill, 2015	18 PC 8 BTC	N	23	Molecular profiling	NGS	Guardant360	Muti-gene (73 genes)	92%	NR	NR	Plasma	12%

HC- healthy controls; BBD- benign biliary disease; NGS- next generation sequencing; NR- not reported; GB- gallbladder cancer; CCC- cholangiocarcinoma; PC- pancreatic cancer; BTC- biliary tract canc

29.1.2 ctDNA in Screening for Biliary Tract Cancer

The use of ctDNA in the screening for BTC is an evolving field. Detection of BTC at an early stage is advantageous given the highly aggressive nature of disease. Currently, there are no screening programmes for the early detection of BTC in average risk patients.

As previously discussed, the Galleri MCED assay has been assessed for use in the screening of BTC. This study included patients who were later diagnosed with gallbladder (n=17) and liver and bile duct cancers (n=46), which also included HCC (214). The MCED assay had impressive Sn in the diagnosis of GBC (70.6%) and liver/bile duct cancers (93.5%). In liver/bile duct cancers, Sn for stage I (6/6), II (7/10), III (9/9) and IV (20/20) disease was 100%, 70%, 100%, and 100% respectively. In addition, the accuracy in identifying the CSO was 92% for pancreatic and gallbladder cancers (grouped together) and 81.6% for bile duct and liver cancers. Approximately 20% of ‘cancer signal detected’ suspected bile duct/liver cancer were due to a different primary tumour (predominantly driven by PC and GBC).

29.1.3 ctDNA Detection of Minimal Residual Disease in Biliary Tract Cancers

Monitoring disease recurrence in BTC is often performed using tumour markers (CA 19.9) and imaging, however with limited Sn and Sp. There are no recommended guidelines with robust evidence to support post-operative surveillance following curative treatment for cholangiocarcinoma. However, several guidelines including NCCN recommend surveillance tumour markers

and imaging as indicated. Therefore, there is a need for non-invasive predictive biomarkers to predict disease recurrence in patients with resectable BTC.

A study using the Signatera multi-gene NGS assay as tumour informed approach assessed ctDNA in detection of MRD in cholangiocarcinoma (n=35) and GBC (n=14) using serial longitudinal measurements (269). This study demonstrated MRD detection with ctDNA in 20% of BTC, however the impact of MRD on DFS was not assessed. Further studies assessing the clinical validity of this approach in relation to DFS is critical in establishing benefit of ctDNA based MRD detection. In future, MRD detection in BTC may be incorporated in adjuvant chemotherapy trials to assess those at highest at risk of relapse and guiding adjuvant treatment decisions (i.e. intensifying treatment for MRD positive, while de-escalating treatment in MRD negative).

29.1.4 ctDNA-Based Molecular Profiling and Selection of Personalised Therapy in Advanced Biliary Tract Cancer

The use of molecular profiling has grown significantly in the field of BTC with the advent of specific targeted treatments with regulatory approvals. ctDNA can be used to identify targetable mutations and determine appropriate personalized therapies in a non-invasive manner, eliminating the need for repeated tissue biopsies. The application of ctDNA for molecular profiling in BTC has proven clinical utility.

As previous described, the large SCRUM-Japan GI-SCREEN and GOZILA observation studies compared ctDNA-based genotyping to tumour tissue sequencing (270). The Guardant360© assay was used, with detection rate in BTC of 90% (all metastatic disease). Interestingly, potentially targetable mutations including germline *BRCA* mutation were detected in 4.3% (conferring sensitivity to PARP and platinum based chemotherapy) and MSI-H in over 3% (conferring sensitivity to ICI).

The genomic landscape of BTC have also been described by ctDNA based sequency. A ctDNA sequencing study of 1671 patients with advanced BTC (91% cholangiocarcinoma, 9% GBC) assessed the Guardant360©, and included a subset for concordance tissue-plasma analysis (271). The overall ctDNA detection rate was 84%, most common detected variants in *TP53* (50%), *KRAS* (17%), *PIK3CA* (14%) and *HER2* (10%). Potentially targetable alterations were seen in 44% of patients. This included *FGFR2* fusions (1.4%), *IHD1* mutations (9.3%), *BRAF* V600E mutations (1.3%) and *HER2* amplifications (4.9%). Interestingly, concordance was relatively low for *FGFR2* fusions (18%), with majority of *FGFR2* fusions detected in tissue (4.3%) not seen in plasma. Concordance was high for *IDH2* (9.5% in tissue) and *HER2* amplifications (5.6% in tissue). The low concordance between tissue-plasma sequencing for *FGFR2* fusions may be related to the analytical design of the Guardant360© assay in detecting alternative gene fusions partners. The Sn is higher for *BICC1* fusion (most common fusion partner) compared to non-*BICC1* fusion partners. This is an inherent problem with hybrid capture based

NGS panels in detection of promiscuous genes, and alternative target enrichment methods may improve ctDNA based detection of fusion partners.

A study by Lamarca et al. assessed the sample failure rate and concordance between paired tissue and ctDNA sequences using the FoundationOne liquid biopsy in 104 patients with metastatic BTC, including IHCC (n=71), EHCC (n=24), GBC (n=3) and ampullary cancer (n=6) (272). The sample failure rate was higher for tissue (26.8%) compared with plasma sequencing (15.4%), with a significant proportion of samples with insufficient tumoural DNA content for tissue-based sequencing (<20%). The overall ctDNA detection rate was 88.4%, with targetable mutation detected in 40.2%. These include *IHD1* mutations (19.1%) and *FGFR2* fusions (5.6%). Tissue-plasma sequencing concordance was 100%, however this analysis only included 6 patients with paired samples. This important study highlights the need for non-invasive molecular profiling tools in BTC, considering the relatively high failure rate of tissue based sequencing and the need for adequate tumour content.

29.1.5 ctDNA as a Diagnostic Tool in Biliary Tract Cancer

To date, only one study has assessed the use of ctDNA as a diagnostic tool in BTC. Unlike in PC and given the complex genomic landscape of BTC, the use of a single gene using a targeted ddPCR approach is not suitable as a diagnostic tool in BTC. Therefore, multi-gene NGS panels are best suited for use in the diagnosis of BTC.

A study of 62 patients with CCA, which included 33 benign biliary disease and 30 healthy controls assessed the use of ctDNA (custom 60 gene panel) in the diagnosis of PC (273). This included patients with stage I (9.6%), II (12.9%), III (18.8%) and IV (58.1%) disease. This retrospective study demonstrated a Sn and Sp in the diagnosis of CCA compared with health controls of 88.7% and 82.3% respectively. However, when discriminating CCA from benign biliary disease, the Sp was lower (57.6%). ctDNA was also found to be superior compared to tumour markers including CEA and CA19.9. Interestingly, the most common variants detected were *ARID1A* (30%), *PBRM1* (30%) and *FGFR3* (30%). This retrospective study was limited to assessing the diagnostic use of ctDNA in BTC and did not involve treatment decisions based on ctDNA result.

29.1.6 Other Circulating Biomarkers in the Diagnosis of Biliary Tract Cancers

There are other circulating non-invasive biomarkers which have been studied in the diagnosis of BTC. These include circulating miRNA, circulating proteins, extracellular vesicles, and metabolites. However, these biomarkers do not have established clinical utility. Table 26 summaries the diagnostic studies of circulating biomarkers for BTC with reported diagnostic parameters.

Table 25- Diagnostic Studies of Circulating Biomarkers in Biliary Tract Cancer (with reported diagnostic parameters)

Author	Biomarker(s)	Cases	Control	Sn	Sp
MiRNA					
Wang 2015, (274)	miR-21	74	74 HC	87.8	90.5

Jiang, 2020 (275)	miR-21, miR-221	31	40 BBD	77.4[1]	97.5[1]
Cheng, 2015 (276)	miR-106a	103	35 BBD 20 HC	81.6	85
Silakit, 2014 (277)	miR-192	11	9 HC	74	72
Loosen, 2019 (278)	miR-122l miR-192, miR-29b, miR-155	94	40	98.3	100
Voigtländer , 2015 (279)	miR-1281, miR-126, miR-26a, miR-30b miR-122,	31	12 HC 40 BBD[2]	55 68 52 52 32	90 93 93 88 90
Wang 2015 (280)	miR-150	15	15 HC	80.6	58.1
Wang, 2015 (281)	miR-26a	66	66 HC	84.8	81.8
Cytokines and Proteins					
Huang, 2014 (282)	CYFRA 21-1	134	52 HC	74.6	84.6
Leelawat, 2010 (283)	MMP7	59	128 BBD	68	87
Loosen, 2017 (284)	Osteopontin	107	55 HC	87.5	100
Cheon, 2007 (285)	IL-6	26	23 HC 26 HCC	73	92
Onsurathum, 2018 (286)	S100A6	29	22 HC 15 BBD	86.2	90.9
Shen, 2012 (287)	SSP411	35	13 BBD[3] 23 HC[3]	90.0	83.3
Loosen, 2020 (288)	suPAR	117	76 HC	78.3	72.7
Shi, 2013 (289)	DKK1	37	50 HC	75.7	100
Kimawaha, 2020 (290)	TGF-b1	45	45	71.1	68.9
Metabolites					
Liang, 2016 (291)	4 metabolite panel (21-Deoxycortisol, Bilirubin, LysoPC (14:0), LysoPC (15:0))	225	101	98.5	99.2
Banales, 2019 (292)	*Bile acids* GCA GCDCA *Steroids* Androsterone sulfate II DHEAS ChoE (22:6) ChoE (20:4) *Monohexosylceramides (*CMH(d18:1/16:0) Phosphatidylcholines (PC16:0/16:0) Sphingomyelins (SMd18:2/20:0)	20	20 HC[4] 20 HCC 20 PSC	 65 70 95 65 65 80 75 55 65	 90 90 58 85 85 75 80 100 75

EV					
Julich-Haertel, 2017 (293)	AnnexinV, EpCAM, ASGPR1 taMPs[1]	26	18 HC	81.4	46.9
Arbelaiz, 2017 (294)	AMPN VNN1 PIGR IGHA1 CRP GGT1 IGHA2 FIBG NEP GPC5C	43	32 HC	90.7 72.1 83.7 81.4 79.1 72.1 72.1 79.1 65.1 65.1	65.6 87.5 71.8 75.0 68.7 87.5 78.1 75.0 81.2 75.0

1- combined biomarker Sn/Sp; 2- Sn/Sp just for BBD; 3- Sn/Sp for CCA vs BBD and HC together. 4- Sn/Sp for CCA vs HC. GCA; GCDCA- Glycocholic acid, Glycochenodeoxycholic acid, DHEAS- Dehydroepiandrosterone; taMPs- tumour associated microparticles.

30 Circulating Tumour DNA in Colorectal Cancer

30.1.1 Detection of ctDNA in Colorectal Cancer

CRC has a high level of ctDNA shedding which can be detected for multiple clinical uses. The ctDNA detection rate in CRC increases with advancing stage of disease (see table 27) (180). ctDNA can be detected in up to 90% of patients with stage IV disease, and in 75% in non-metastatic, early stage disease (180). ctDNA VAF has also been shown to be a surrogate for disease burden in metastatic disease, with higher mVAF associated with a shorted OS (295). The detection of ctDNA is also higher in those with liver metastases (compared with non-liver metastases), and elevated CEA and CA19.9 (296). The concordance between plasma NGS and tissue sequencing has been shown to be high for RAS mutations (>93%) (297). Concordance between plasma KRAS and tissue KRAS also is influenced by the site of metastases, with

concordance between plasma and liver, peritoneal and lung metastases of 91%, 88% and 64% respectively (298).

Table 26- ctDNA detection rate in colorectal cancer according to stage of disease

	ctDNA detection rate	Ref
Stage I	77%	(299)
Stage II	95%	(299)
Stage III	96%	(299)
Stage IV	84-96%	(211, 299)

30.1.2 ctDNA in Screening and Diagnosis of Colorectal Cancer

There have been several studies assessing the use of ctDNA in screening for CRC. The concentration of ctDNA in those with early stage disease is lower than metastatic disease, however ddPCR can detect mutations with Sn as high as 0.001% and can be used as a screening tool in CRC.

A study by Junca et al of 155 patients who underwent colonoscopy had ctDNA collected for ddPCR *KRAS* and *BRAF* mutation analysis (300). Colonoscopy was performed for previous history of polyps or family history, and for those with symptoms in only 21%. Overall, CRC was detected in 20 patients, with 39 having an AA. The median cfDNA was highest in those with CRC (13.5 ng/ml), compared with the AA group (10.03ng/ml) and lowest for the no-lesion group (9.04ng/ml). *KRAS*/*BRAF* mutations were detected in 7.7% of CRC and 2.5% of AA group using a tumour agnostic approach. No ctDNA was detected in the normal no-cancer group. The Sn and Sp for detection of CRC was 45% and 75%, respectively. For AA, Sn and Sp were 2.6% and 4.3% respectively. This

study was limited to sequencing of only two variants which are commonly seen in CRC. However, demonstrated a low Sn and Sp in detection of pre-cancerous lesions, which is critical issue in screening programmes.

A multi-gene ctDNA assay has been studied in patients with adenomatous polyps and involved 71 patients with low-risk adenomas. Sequencing for mutations in APC, CTNNB1, BRAF and KRAS was performed using ddPCR methods (301). ctDNA detection was 39.4% (APC [28.2%], CTNNB1 [9.9%], KRAS [5.6%] and BRAF [2.9%]). As APC mutations are an early genomic even in adenomatous polyps, the use of assays which cover the APC gene is important when using ctDNA as a screening tool in CRC.

More recently, DNA methylation analysis has been studied in the screening for CRC. To date, the Epi proColon is the only blood-based DNA methylation with FDA approval for use in screening CRC. This assay is based on the detection of Septin9 (*mSEPT9*) methylated DNA which is a main regulator of cell division. The Sn of m*SEPT9* in the detection of CRC ranges between 40-90%, which is superior to CEA, CA19-19 and FOBT. The assay also has high detection rates for high-grade dysplastic adenoma (62.5%).

A large retrospective multicentre cohort study evaluated ctDNA methylation patterns in 1138 patients including those with AA, CRC and health controls using the ColonES assay. The ColonES assay is a ctDNA methylation assay which detected methylated patterns in 191 regions, with high Sn for detecting AA (79%) and CRC (86.6%), and Sp of 88.1% (302).

The Galleri MCED assay demonstrated an overall Sn for the detection of CRC of 82% (214). Sn was lower for stage I (43.3%), and higher for stages II (85%), III (87.9%) and IV (95.3%). The accuracy of predicting the CSO was very high, with 92.7% of CRC signal calls identifying a CRC primary lesion. A larger clinical validation study assessing the use of these MCED assays in the detection of early CRC and adenomatous polyps is critical in for use in screening programmes.

The combination of multiple markers in the screening of CRC is important to enhance the Sn of these assays, especially in the detection of early stage and high risk polyps. The AI-EMERGE study assessed the use of a multi-omic assay incorporating methylation, proteomics and RNA transcription analysis in screening for CRC (303). This analytical validation study compared the multi-omic assay to single gene methylation analysis (*mSEPT9*) and FIT testing and demonstrated highest Sn for detection of AA using a multi-omic approach (Sn 41%), and lower for mSEPT9 (Sn 22%) and FIT (Sn 24%). The assay could also detect polyps <10mm with 25% Sn. The PREEMPT CRC study is an ongoing, prospective multi-centre study aiming to validate this multi-omic assay in average risk patients undergoing screening colonoscopy which is the largest prospective study in ct-DNA based CRC screening (n= 25,000) (304). The LUNAR-2 assay (Guardant Health) has been developed which detects somatic genomic variants, methylation and fragmentomic signals in ctDNA for use in CRC screening. Recent data demonstrated a high Sn (91%) and Sp (94%) in detecting early stage CRC (stages I-III) (305). The ECLIPSE study,

evaluating this assay in 8,000 patients undergoing colonoscopy showed a Sn of 83% and Sp of 90% for detecting CRC, with Sn of 13% for AA (306).

Studies assessing methylation in screening for CRC are outlined in table 28.

Table 27- Studies Assessing ctDNA Based Methylation in Colorectal Cancer Screening

Author	Gene	N=	Technology	Sn for AA	Sn for CRC	Sp
Ma, 2019 (307)	mSEPT9	117 CRC 96 AA 70 HC	RT-PCR	23.3	71.8	66.7
Yuan, 2016 (308)	mSEPT9 mOSMR	187 CRC 25 AA 109 HC	RT-PCR	12 20*	62.6 74.9*	91.7 86.2*
Luo, 2020 (309)	cg10673833	801 CRC 1021 HC	ddPCR	NR	89.7	86.8
Young, 2021 (310)	BCAT1, IKZF1 and IRF4	184 CRC 616 AA 820 HC	MSP	15.7^	73.9^	90.1^
Song, 2017 (311)	mSEPT9	85 CRC 364 AA 324 HC	MSP	31.8	NR	NR
Fu, 2018 (312)	mSEPT9	98 CRC 101 AA 253 HC	RT-PCR	7.9	61.2	93.7
Lofton-Day, 2008 (313)	TMEFF2 NGFR SEPT9	133 CRC 179 HC	RT-PCR	NR	30* 33* 52*	NR* NR* 95*

* Sn/Sp for individual methylation markers; ^ Sn/Sp for combined methylation markers; AA- advanced adenoma; CRC- colorectal cancer. HC- healthy controls.

30.1.3 ctDNA-Based Molecular Profiling, Selection of Personalised Therapy and Disease Response Assessment in Colorectal Cancer

ctDNA has several potential uses in the treatment of metastatic CRC, in disease response assessment, identifying mechanisms of resistance, and molecular profiling to select targeted therapies.

The use of ctDNA in monitor response to treatment in mCRC has been extensively studied. Diaz et al. showed that ctDNA detection of resistance *KRAS* sub-clonal mutations in patients receiving panitumumab was the primary driver of acquired resistance to EGFR inhibitors (314). Following this, the use of ctDNA in detecting resistant *KRAS* subclones and re-challenging with cetuximab was investigated in a phase 2 study (315). Patients with *RAS*/*RAF* wildtype status having prior irinotecan and cetuximab chemotherapy and then progressed, were re-challenged. At baseline, *RAS* mutations were detected in 48% of patients, and none of these patients responded to rechallenge. Those with *RAS* wildtype status on ctDNA had an ORR of 21%, with longer mPFS compared to *RAS* mutant disease (4.0m vs 1.9m). This demonstrated that ctDNA guided re-challenge strategies can select patients who may benefit from EGFR inhibitors. This was also seen in the CHRONOS trial using panitumumab re-challenge (316).

Longitudinal monitoring of ctDNA in patients with GI cancers may predict radiological response. A study of 101 patients with GI cancers (including 69 with CRC, 26 with PC and 18 with BTC) had serial ctDNA measurements to assess the relationship between VAF and radiological response (317). A dynamic ctDNA VAF change at 4 weeks following initiation of treatment predicted responses, with a 90% change in ctDNA concentration at 4 weeks predicting partial/complete responses and stable disease with higher Sn compared with tumour markers (Sn 60% vs 24%). Larger, comprehensive

studies are needed to fully assess the appropriate ctDNA threshold in predicting response.

Molecular profiling of mCRC has been studied in several large cohort studies including the SCRUM-Japan GI-SCREEN and GOZILA observation studies (270). This included 654 with mCRC with a ctDNA detection rate of 96.0%. The most common aberrations seen were *KRAS*, *APC*, and *TP53*. A separate analysis of 658 patients with metastatic GI cancer including 347 with mCRC assessed MSI analysis using Guadrant360©, comparing tissue-based and ctDNA PCR-based MSI methods (318). In mCRC cases, the overall agreement was 99.4%, with 3 ctDNA-only detected MSH-H patients receiving an anti-PD1 drug with good response. This study confirmed the clinical validity of ctDNA MSI assessment given the high concordance with tissue.

TruSight Oncology ctDNA is a pan-cancer, NGS assay approved for comprehensive plasma based molecular profiling. This broad panel covers 523 genes, including TMB and MSI. This panel has been evaluated in 20 patients with mCRC showing high concordance with tissue (77%) and with ddPCR (94%), demonstrating utility in the genomic profiling of mCRC (319).

31 Hypotheses

- Liquid biopsies can be used to support a diagnosis and triage screening colonoscopies in patients with suspected colorectal cancer
- Liquid biopsies can be used to support a diagnosis and treatment in patients with suspected pancreatic and biliary tract cancer
- Liquid biopsies can be implemented into the routine diagnostic pathway for patients with suspected pancreatic and biliary tract cancer

32 Aims

- To assess the use of ctDNA in the diagnosis and triaging of colonoscopies in patients with suspected early-stage colorectal cancer
- To assess the use of ctDNA to support a diagnosis and facilitate management of patients with suspected pancreatic and biliary tract cancer
- To implement ctDNA into the routine diagnostic pathway for patients with suspected pancreatic or biliary tract cancer

33 PREVAIL ctDNA Study- Early Stage Colorectal Cohort

34 Abstract

Background: There are approximately 20,000 new cases of early stage CRC in the UK each year. Colonoscopic screening to detect and treat early invasive disease and pre-malignant adenomatous polyps is associated with improved CRC-related mortality. FIT is currently used as a non-invasive tool in triaging screening colonoscopies. However, the diagnostic accuracy in the detection of CRC and adenomatous polyps is low, with a relatively high false positive rate (10-30%). The recent COVID19 pandemic significantly impacted endoscopy services with a reduction in capacity for screening colonoscopies. The prevalence of early stage CRC, the high false positive rate with current screening tools and the COVID19 impact on diagnostic services, highlights an urgent need for additional non-invasive tools to screen for CRC to reduce the number of unnecessary colonoscopies and rationalise resources appropriately. ctDNA can be detected in 77-95% and 39.4% of patients with CRC and adenomatous polyps, respectively. My hypothesis is that ctDNA can be used in addition to FIT testing to triage colonoscopies in patients with suspected CRC. My secondary hypothesis is that ctDNA can be detected in patients with CRC and adenomatous polyps. **Methods**: This single centre prospective study conducted at the RMH from June 2020 to February 2023 included patients with suspected CRC based on FIT positivity. ctDNA was collected at baseline for plasma-only next generation sequencing (NGS) using a custom multi-gene panel. An MTB reviewed results for interpretation and

clinical context. The primary end point was the proportion of patients with detectable ctDNA. Secondary end points including correlation of ctDNA detection with colonoscopy findings and concordance analysis between tissue based NGS and ctDNA NGS. **Results**: 32 patients were recruited and eligible for the primary end point. ctDNA detection rate was 12.2%, with 2 patients having ctDNA result consistent with a diagnosis of CRC based on MTB discussion. In 30 patients who subsequently underwent post-registration colonoscopy, the Sn and Sp of ctDNA in the diagnosis of CRC was 33.3% and 85.2% respectively. The Sn and Sp for ctDNA in the diagnosis of advanced colorectal neoplasia (tubular adenoma >10mm, villous features, HGD or invasive cancer) was 33.3% and 87.5%. **Conclusion**: ctDNA can be detected in CRC and advanced colorectal neoplasia in FIT positive patients, however with low Sn and Sp highlighting the need for additional tests to further refine urgency for colonoscopy testing.

35 Background and Rationale

There are approximately 42,000 new cases of CRC in the UK each year, with 50% presenting with potentially curable early-stage disease (320). The mortality rates for CRC in the UK have been declining for several decades, largely related to nation-wide colonoscopic screening programmes in the detection of early-stage disease and treatment of pre-malignant adenomatous polyps. Average-risk population based screening colonoscopies have been shown to reduce CRC-related mortality (136). These procedures however are invasive, require specialist gastroenterology and histopathology services, and

are associated with complications including perforation and bleeding. More recently, FIT testing to triage screening colonoscopies reduces the use of colonoscopies without a detriment in CRC detection, owing to the low rate of false negatives (149). However, the prevalence of CRC following a positive FIT test (≥10ug/g) is only 1.3%. Given this low pre-test probability, there is a large impact on NHS cancers diagnostic services with an additional burden of unnecessary colonoscopies. Furthermore, the Sn and Sp for the detection of AA (defined as high grade dysplastic polyps [HGD], or adenomatous polyps >10mm in size or with predominant villous histology, i.e. high risk pre-malignant lesions) with FIT is relatively low, between 25-50% and 70-95% respectively, providing a false positive rate of 10-30% (321).

The COVID19 pandemic had a major impact on cancer diagnostic services, especially aerosol generating procedure such as colonoscopies. Given relatively low diagnostic accuracy in the detection of CRC and AA using FIT testing, and the reduced capacity for screening colonoscopies, highlighted a high unmet need for an additional, non-invasive tool to help triage screening colonoscopies in patients with suspected CRC.

ctDNA can be detected in the circulation of 77-96% of early stage, non-metastatic CRC and in up to 39.4% of AA (299, 301). There is evolving evidence for the use of ctDNA methylation in screening for CRC (214). ctDNA can be detected in asymptomatic patients undergoing screening colonoscopies (300, 322, 323). However, the use of ctDNA for faster diagnosis of symptomatic patients, triaging of colonoscopies, and facilitating treatment

in patients with suspected CRC has not been previously investigated in a prospective study.

My research aim was to pilot ctDNA in triaging patients with suspected CRC for colonoscopy. My secondary aims were to assess the relationship between ctDNA results and colonoscopy findings, including the correlation between plasma and tissue based molecular profiling of CRC and adenomatous polyps.

36 Methods

36.1.1 Overall Study Set Up

The PREVAIL Part 1 ctDNA study (**PRE**venting **V**iral pandemic **A**ssociated risk of cancer death using less **I**nvasive diagnostic tests– **L**iquid biopsies) at the beginning of the COVID19 pandemic. From March to May 2020, capacity for invasive diagnostic services at the Royal Marsden Hospital (RMH) were reduced due to the re-allocation of resources focusing on managing the pandemic. As such, we saw an unmet need for non-invasive tools to facilitate a diagnosis in patients with suspected cancer. This was critically important for tumour types which require aerosol-generating procedures to obtain tissue including gastroscopy, colonoscopy, cystoscopy.

At the time, our NIHR Clinical Genomics Translational Research Laboratory at RMH (also serves as one of the regional Genomic Laboratory Hubs for somatic genomic profiling) had an established, clinically validated ctDNA assays and

genomic expertise, and was able to develop the infrastructure to deploy this into our diagnostic pathway.

This study was in response to a COVID-19 related funding call from the Royal Marsden Cancer Charity where we were successfully awarded a research grant. A research protocol was written under the supervision of Dr Naureen Starling (chief investigator) in collaboration with all co-investigators. The protocol development involved an extensive literature review covering the diagnostics in several tumour types (colorectal, pancreatic, biliary tract, gastrointestinal stromal tumour [GIST], breast, urothelial, and lung cancer) including the associated molecular genetic landscape and ctDNA detection encompassing a range of analytic techniques in these cancers as summarised in my introduction. The research hypotheses, their corresponding aims and objectives and study end points were defined and used for the statistical analysis plan (SAP).

Patient and public representative at the time (Pete Wheatstone) were engaged to discuss several aspects of the protocol. This included discussions on the research question and importance to patients/public, framing the research question, the consent process, the sample collection and schedule of assessments, data information and storage of samples. Pete reviewed the patient information sheet (PIS) and provided important feedback regarding the information given to patients in the document and general communication about the study.

The SAP defined the end points and statistical analysis methods based on a case report form (CRF) to systematically capture patient level data which included patient demographics, molecular diagnostic results, molecular tumour board outcome and treatment outcomes. This was used for data collection and entry onto an electronic database (MACRO).

The logistics of sample collection were defined by Ruwaida Begum (Lead Translational Research Manager) and the Clinical Genomics Department at RMH (Paul Carter, Paula Proszek). This included the development of the laboratory manual which defined standard operating procedures regarding plasma collection, transport, plasma sequencing and storage of samples.

We considered it essential to engage an MTB in the interpretation of genomic results, particularly in a new indication of ctDNA for faster diagnosis. As such, the West London Genomic Tumour Advisory Board (GTAB) were engaged with the study. A PREVAIL specific proforma was approved by the GTAB.

Following local research and development (R&D) approval at the RMH, we presented the study protocol to the Research Ethics Committee (REC) (Northeast (Tyne & Wear South) Research Ethics Committee (approval number 20/NE/0152)). The committee feedback was positive and provided us with a favourable outcome without any changes needed to the protocol or PIS. This was approved within a fast turnaround given the urgent need considering the COVID19 pandemic.

Following REC approval, we received local approval to open the PREVAIL study at RMH (Sutton and Chelsea). Prior to opening for recruitment, we ensured all sites and staff were appropriately trained. This involved creating a site initiation visit (SIV) presentation outlining the study and presented this to all research study staff. This included GI unit (early stage colorectal, pancreatic, biliary tract cohorts), sarcoma unit (GIST cohort), and urology unit (urothelial cohort). Following subsequent protocol amendments, SIVs were performed for the breast unit (breast cancer cohort) and for the GI unit (metastatic/relapsed CRC).

PREVAIL Part 1 opened to recruitment at the RMH in June 2020 as a single centre study. The study opened within 3 months from study concept, funding approval, local and national REC approvals. An overview of my role in the study management is discussed in section 4.3.13.

36.1.2 Overall Study Design

The PREVAIL ctDNA Part 1 trial (NCT04566614) is a pilot study assessing the use of ctDNA in the diagnosis of patients with suspected cancers, including PC, BTC, CRC, breast, gastrointestinal stromal tumour (GIST), and urothelial cancers. My MD(Res) thesis will focus on the early stage CRC (chapter 4), and the pancreatic and biliary tract cohorts (chapter 5). The relapsed/metastatic CRC cohort is not included in this thesis as it is still actively recruiting as of March 2023.

The current eligibility for inclusion in PREVAIL Part 1 include;

- Participants aged ≥18 years old.
- Patients with suspected malignancy without a definitive histological diagnosis (including those with inconclusive biopsy results or awaiting invasive biopsy for diagnosis) of the following tumour type
    - Early stage colorectal cancer (FIT intermediate and high risk)
    - Metastatic colorectal cancer (Relapsed or de novo)
    - Pancreatic cancer (early and late stage)
    - Biliary tract cancer (early and late stage)
    - Gastrointestinal stromal tumours (early and late stage)
    - Lung cancer (early and late stage)
    - Urothelial cancer (early and late stage)
    - Breast cancer (de novo metastatic or relapsed)

      Or patients with histological diagnosis of metastatic colorectal cancer, GIST, lung cancer or breast cancer without adequate tissue (or awaiting) for NHS genomic test directory predictive biomarker testing results

- Ability to provide informed consent
- Patients with performance status suitable for oncological treatments

Patients with an established histological diagnosis which is adequate to support standard of care treatment were excluded.

Patients had 50mls of whole blood collected for ctDNA prior to any invasive diagnostic procedure. Baseline data were collected including past medical and family history, imaging results (including TNM stage) and tumour markers. ctDNA analysis included multi-gene targeted NGS for single nucleotide variants (SNVs), copy number variants (CNVs), insertion and deletions (Indels) and lcWGS. This was performed using the ct-GI panels (for both CRC

cohorts), ct-PAED (for PC/BTC, urothelial and GIST cohort) and ct-BREAST (for breast cohort).

Following ctDNA analysis, data on genomic aberrations detected, MTB recommendations, and treatment outcome were collected and entered in an online database (MACRO). Any patient who subsequently underwent an invasive diagnostic procedure had tissue NGS performed for concordance analysis.

The clinical trial process from patient recruitment, sample analysis, reporting, discussion at MTB and feedback of results to clinicians is outlined in Fig 11 below. All experiments were performed at the CMP, NIHR Biomedical Research Centre. This is a UKAS/ISO accredited laboratory within Clinical Genomics at the Royal Marsden NHS Trust. The laboratory is part of the North London Genomic Laboratory Hub and links into the West London Genomic Tumour Advisory Board (GTAB). As this is an accredited diagnostics laboratory, my role within the laboratory experimental work was observation. The laboratory ctDNA workflow (including DNA isolation, quantification, library preparation, capture and sequencing) and tissue sequencing was performed by trained scientists who were critical in performing at a high level. The scientists who were involved in this study include Paula Proszek, Paul Carter, Michaela Smalley, with support from Dr Andy Feber and Professor Michael Hubank. The validation of the assay was previously established by my colleagues (doctoral students) Dr Gayathri Anandappa, Dr Shelize Khakoo (ct-GI) and Dr Reda Stankunaite (ct-PAED panel).

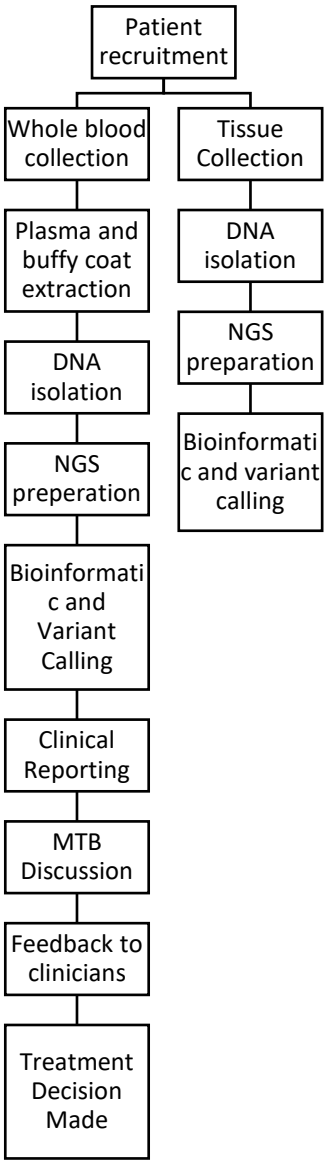


Figure 11- PREVAIL Clinical-Laboratory Workflow

The primary end point of the study is “the proportion of patients with a ctDNA result consistent with a diagnosis of malignancy based on MTB discussion”.

The secondary and exploratory endpoints include

- The association of ctDNA result with histological specimens (for sensitivity, specificity, and concordance analyses)
- Proportion of patients who commenced treatment based on ctDNA result
- The association of ctDNA result with clinico-radiological characteristics, and lcWGS
- The detection of targetable mutations

36.1.3 Early Stage Colorectal Cancer Cohort Study Design

Patients with suspected early-stage CRC were considered suitable for this study if they were referred for a screening or diagnostic colonoscopy and were FIT positive (≥10ng/ml). Patients could be included regardless of whether imaging was performed, provided they had a positive FIT test.

FIT tests were performed in primary care and a referral made to the RMH colorectal triage assessment unit following a positive FIT test. Criteria for an immediate colonoscopy at RMH include

- Positive FIT test or mixed/dark blood in stool
- Change in bowel habit ≥6 weeks
- Iron deficiency anaemia (Hb <11g/dl in men; <10g/dl)
- Abdominal and/or rectal mass
- Family history (2 fist degree relatives with CRC or first degree relative <40 years)

However, regardless of other symptoms, all patients needed a positive FIT test to be eligible for the study. This was decided to maximise the pre-test probability. Availability for colonoscopies, as with other aerosol generating procedures, were reduced during the COVID pandemic in early 2020, and so the original plan was to use ctDNA to stratify the timing of colonoscopies. Patients with detectable ctDNA supportive of a diagnosis of CRC could be

offered management based on this result in lieu of tissue diagnosis, in conjunction with other investigations (including tumour markers and imaging) and after discussion at the MTB. This could include definitive surgery or neoadjuvant chemo-radiotherapy for rectal tumours. Patients without detectable ctDNA continued through the standard diagnostic pathway and proceeded to a colonoscopy as part of a COVID19 adjusted diagnostic pathway. My original research hypothesis was that ctDNA could be used to triage colonoscopies at the peak of the pandemic when capacity for invasive procedures was reduced. A subset of patients who may not undergo a colonoscopy due to the pandemic could access treatment on ctDNA alone as part of the study.

However, as capacity for colonoscopies was stepped-up, and the 2-week wait for RMH colonoscopy diagnostics improved, potentially suitable patients were able to access colonoscopy within an appropriate timeframe and so the primary aim to use ctDNA as a triaging tool was not tested in a real-world setting. However, I continued to identify patients to address the secondary objective.

Patients were flagged to me and the research team by the colorectal triage telephone clinic staff and endoscopy suite nurses. I also screened colonoscopy lists regularly. Following identification of a suitable patient, I performed a pre-screen check to ensure suitability to the study. Patients were subsequently approached for consideration of PREVAIL and a PIS was sent via email or mail. Patients could be consented on the same day as receiving

the PIS after agreement with our PPIE group and REC. The rationale for this was to minimise patient visits during the COVID-19 pandemic. However, wherever possible patients were given at least 24 hours to read the PIS. The study was discussed with the patient and if agreeable, the consent form was signed only after receiving informed consent to participate.

36.1.4 Blood Sample Collection and DNA Isolation

Whole blood samples were collected prospectively from patients at baseline prior to an invasive procedure/biopsy and treatment. Whole blood was collected in blood stabilisation tubes (Streck®) to reduce the risk of WBC lysis and release of non-tumour derived DNA. Each patient had up to five Streck© tubes collected (i.e. 50mls of whole blood) and transported to the GI Unit Biological Specimen Coordination (BSC) team for plasma separation. Phlebotomy staff were trained previously on the appropriate methods of sample collection including complete filling and gentle inversion of each Streck© tube.

Plasma was isolated from blood by a member of the GI BSC team (led by Ruwaida Begum and Isma Rana) using centrifugation at 1,600g for 10 minutes at room temperature. Plasma was collected into 15ml falcon tubes. The 5mm of plasma layer on top of the buffy coat was not collected to minimise WBC contamination. Repeat centrifuge (i.e. double) was performed at 1,600g for 10 minutes at room temperature and the final supernatant plasma was collected

into 1.8ml cryovials. The buffy coat was subsequently extracted from each Streck© tube and stored separately in 1.8ml cryovials. All samples were labelled with trial ID, patient initials, date of collection, timepoint and sample type. Samples were then stored at -80°C and transferred to the Translational Research Laboratory at the RMH. Plasma isolation occurred within average of 2 days of whole blood collection.

cfDNA was extracted from the plasma using the QIAsymphony Circulating DNA Kit (Qiagen) from 5.5 – 8.0 ml of plasma. The cfDNA extracts were quantified using Qubit dsDNA HS Assay Kit and, if genomic DNA contamination was suspected, it was checked for DNA size distribution using cfDNA ScreenTape (Agilent Technologies).

36.1.5 Validation of ct-GI Targeted Next Generation Sequencing Panels

The ct-GI panel covers 22 genes including SNV and Indels. The genes covered in the ct-GI panel are commonly aberrated in CRC, including APC, TP53, KRAS (exon 2, 3), BRAF (exons 11, 14, 15, 16) and NRAS (exon 2, 3). The gene coverage of the ct-GI panel is outlined in appendix B below.

The ct-GI panel has been validated against ddPCR, tissue and the ct-PAED panel. This was previously validated by Drs Paul Carter and Paula Proszek by the CMP and the ICR (Institute of Cancer Research) as part of the TRACC study (NCT04050345).

Briefly, the validation included 32 plasma samples from patients with early stage CRC. All samples had matching tissue-based sequencing and 21 samples had matching plasma based ddPCR sequencing performed (across 41 variants). The analytical Sn of the assay is 85% (i.e. ctDNA detection rate) using a VAF cut-off of >0.1% as a tumour agnostic approach. Concordance with ddPCR improved with higher VAF cut-off, with concordance of 73.2%, 82.9% and 92.7% with VAV >0%, >0.1% and >0.2% respectively. Overall, the correlation between ddPCR and NGS was relatively good across all VAF (R^2= 0.8619). However, the panel is not validated for CNV calling due to the lack of CNV probes in the panel design.

36.1.6 Library Preparation and Targeted Plasma Based Next Generation Sequencing

NGS libraries were prepared using the Cell3™ Target Library Preparation kit (Nonacus, Birmingham, UK). Dual-index Unique Molecular Identifiers (UMI) adapters (Nonacus) were utilised to allow for PCR and sequencing error suppression. DNA was inputted into libraries (25-50ng).

Up to 16 NGS libraries were pooled and captured using a custom targeted panel (Nonacus) and cleaned using the Cell3™ Hybridisation and Wash Kit (Nonacus). The hybridisation step was followed by non-target regions being washed away, to reduce off target capture.

The captures were then amplified using 12 or 13 cycles of PCR depending on which panel was used for the ct-GI panel and ct-PAED panel respectively.

Library clean-up was performed using Target Pure NGS clean up beads and quantified using Qubit. The captures were then quality controlled, combined and diluted to a final concentration of 0.8 nM for sequencing to >20,000x on a NovaSeq 6000 (Illumina, San Diego, USA) using 2 x 100 bp paired-end reads. Buffy coat DNA was sequenced using the same method, with lower sequencing depth of ~5000x (consensus depth of 500x) to exclude CHIP in ctDNA. Figure 12 describes the plasma ctDNA workflow.

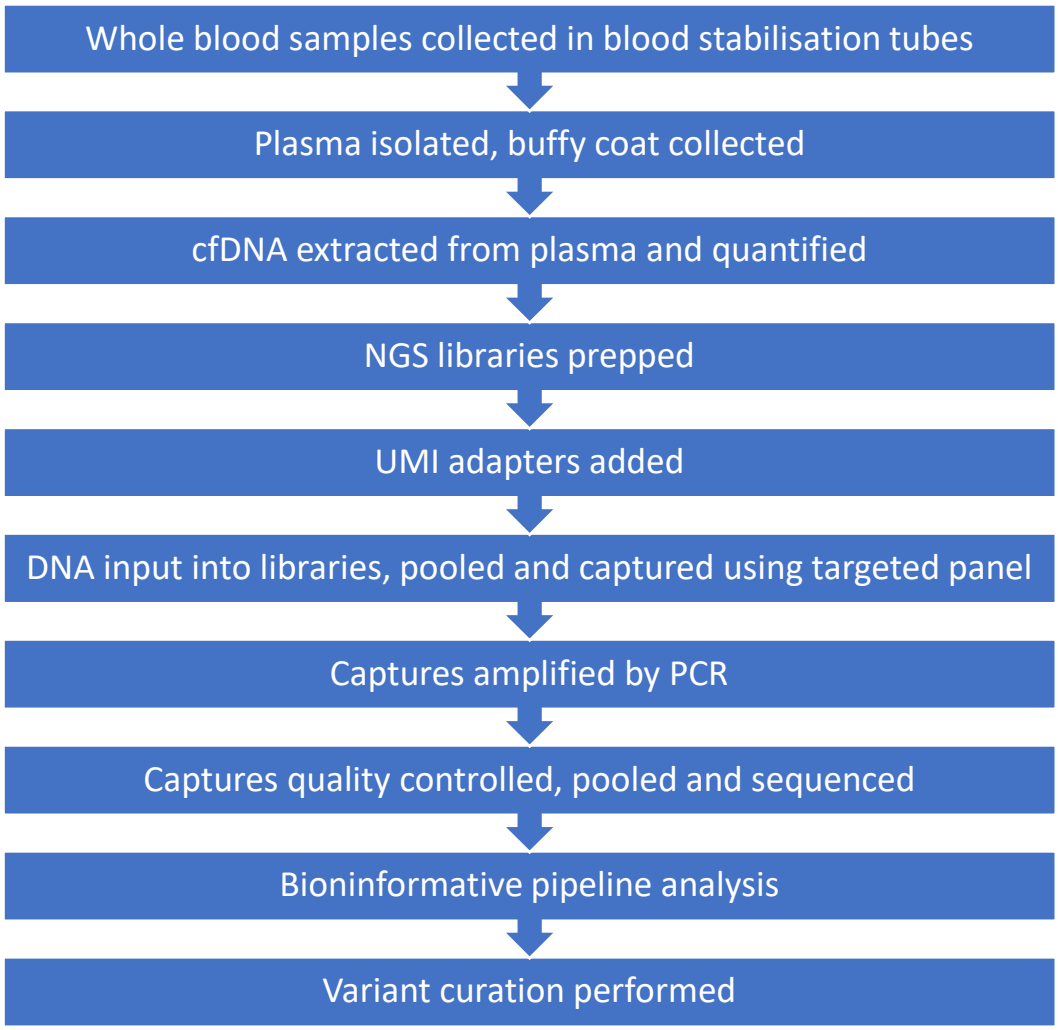


Figure 12- Plasma Sequencing Workflow

36.1.7 Bioinformatic Analysis and Variant Calling (shared methodology)

A bespoke pipeline (MDIMSv4) developed in-house by the Translational Research Group, RMH was used to analyse ctDNA data.

Base calls were demultiplexed using bcl2fastq2 (v2.20.0). Reads had adapters removed with Picard (v2.23.8) and were aligned to the reference genome (GRCh37/Hg19) using BWA-MEM (v0.7.17). Reads were then collapsed from the same original molecule using UMIs by fgbio (v1.3.0). These UMIs are

specific to the ctDNA pipeline as an error correction to reduce PCR amplification bias. The reads were grouped by the UMI sequence with the same UMI and given a molecular identifier. Consensus calls were generated by finding the most likely base at each position within a UMI family. At a sequencing depth of >10,000x, a minimum UMI consensus reads (families) was defined as containing 3 molecules with the same UMI. UMI families containing <3 reads were discarded from further analysis by filtering these out. Overlapping forward and reverse reads were hard clipped with ClipBam (fgbio v1.3.0). Picard was used for QC. BAM files were base recalibrated using GATK (v4.1.9.0) and the filtered consensus BAM file was aligned to the human reference genome again. Finally variant calling was performed with VarDict (v1.8.2) without germline subtraction. VarDict platform allows for calling variants at low VAF which is important for cfDNA assays. QC files were generated using Picard CollectTargetedPcrMetrics.

Common in healthy population and benign germline variants were removed during the bioinformatic pipeline and not through parallel buffy coat sequencing and subtraction. Likely germline calls based on a VAF in general population in the Genome Aggregation Database (https://gnomad.broadinstitute.org/) >0.0001 were removed from the dataset. Private SNP present in buffy coat were removed from analysis by checkers using data from buffy coat and subtraction. Putative variants were manually checked using IGV (Integrated Genomics Viewer).

36.1.8 Defining a Threshold for Tumour Agnostic Variant Calling and Clinical Reporting for ct-GI Panel

The ct-GI panel was previously used on samples for the TRACC study as a tumour informed approach to detect MRD in early stage CRC. Therefore, it was important to establish a threshold for variant detection in plasma with the assay in a tumour agnostic approach given the design of the PREVAIL study.

The ctDNA workflow at the CMP had established methods to reduce false positives, including from background sequencing errors (using molecular barcoding, robust bioinformatic analysis for variant calling and quality filters), buffy coat sequencing and germline calls extraction (to mitigate CHIP and germline variants). The implications of a false positive in this study could lead to a patient without cancer being treated for a malignancy with systemic therapy. Therefore, a higher VAF cut off for determining variant detection as a tumour agonistic approach was used.

The LOD of the ct-GI assay was 0.125% with at least 3 consensus reads. For de novo (i.e. tumour agnostic) variant calling, we used the following threshold to define ctDNA detected

- VAF 0.1-0.3% with ≥5 consensus reads of a variant strongly linked with the cancer type
- VAF 0.1-0.3% with ≥3 consensus reads for a common hotspot – KRAS/BRAF in CRC
- VAF 0.3-0.49% with ≥5 consensus reads for variants linked with cancer type or pathogenic mutations in other genes
- VAF ≥0.5% with ≥6 consensus reads of any variant
- A pseudogene with VAF ≥0.4% and >7 consensus reads
- Homopolymers >4 bases in length with VAF ≥0.4% and >10 reads
- Strand biases with VAF ≥0.3% and ≥7 reads

Using a VAF cut off of 0.1% and higher, the accuracy of the ct-GI panel in detecting variants (as validated against tissue NGS and ddPCR) is 90.5%. The tumour informed concordance analysis used a VAF threshold of ≥0.05% (based on the TRACC study).

36.1.9 Tissue Collection, DNA Extraction, Targeted Tissue Based Next Generation Sequencing and Variant Calling

At registration, consent was also given to collect and sequence future biopsies or surgical specimens following ctDNA collection. Post-registration tumour samples were only collected in patients who subsequently underwent a biopsy or resection and were found to have a histopathology diagnosis of cancer (or adenomatous polyps for CRC cohort). However, a biopsy was not mandatory on study.

Formalin-fixed paraffin-embedded (FFPE) biopsies from patients had 5 unstained slides cut at 10 µm and one 3 µm H&E stained slide with tumour area(s) marked and assessed for tumour content, cellularity and necrosis by a histopathologist. Tumour areas were micro-dissected from unstained slides to enrich for tumour cell content. Tumour DNA extraction was performed using the Maxwell RSC FFPE Plus DNA Kit. Tumour DNA was quantified using the Qubit dsDNA HS or BR assay kit.

NGS libraries were constructed using the KAPA HyperPlus Prep Kit and HyperCap baits (Roche) DNA input was 50-200ng (for mean fragment size of

>1000bp) or 400ng (if <1000bp). NGS libraries were then amplified using 6 or 10 cycles of PCR depending on the DNA input. The libraries were then cleaned using SeqCap EZ purification beads and quantified by Qubit, and captured using the RMH200 Solid Tumour NGS Panel. This custom panel was developed by the CMP as a diagnostic NGS assay used for molecular profiling tumours in a clinical setting. This panel consists of 233 genes, with the ability to detect SNV, Indels, CNV, and SV including some gene translocations. See appendix C for the gene coverage list. DNA sequencing was performed on a the NovaSeq6000 (Illumina).

Analysis was performed using the in-house bioinformatic pipeline (MDIMSv4) as previously described. Briefly, de-multiplexing was performed using bcl2fastq and reads were aligned using BWA to the human reference genome. SNV and Indels were called using Mutect2/GATK. Variants were then manual checked using IGV. A somatic variant with a VAF of 5% and minimum supportive reads of 10 or more was considered sufficient for somatic variant calling in tissue.

36.1.10 Plasma Based ddPCR Sequencing

Plasma ddPCR was performed on samples collected as part of the PREVAIL study retrospectively depending on the ctDNA NGS and tissue NGS results in specific circumstances.

Plasma previously collected and stored had cfDNA extracted using the QIAsymphony Circulating DNA Kit (Qiagen) and quantified using the Qubit dsDNA HS Assay Kit. The ddPCR TaqMan assays were custom designed by Clinical Genomics team and manufactured by Thermo Fisher. Variant probes were labelled with 6-FAM and wildtype probes labelled with VIC/HEX. Assays were optimised using gradient PCR using positive control samples. ddPCR was performed using the QX200 Droplet Digital PCR System (Bio-Rad). Following amplification of ddPCR, the reactions were read on a QX200 Droplet Reader (Bio-Rad). Mutant copies per droplet (Mcd) were calculating from the Poisson distribution. Samples were set up in duplicates and minimum 2 positive droplets in each replicate were required to call sample positive. At least 20000 total droplets and 600 wild type droplets in merged wells were required to call sample negative. Criteria to define positive ddPCR was a minimum of 2 positive drops in at least one variant with a minimum of half of the total droplets that could be generated being valid.

36.1.11 Molecular Tumour Board

A weekly MTB was set up to discuss all patients registered on PREVAIL. For all patient, a PREVAIL specific MTB proforma and presented each patient in the presence of clinical genomic experts.

Baseline characteristics, symptoms, family history, tumours markers and imaging results were discussed in conjunction with the ctDNA molecular diagnostic report. The MTB was blind to any subsequent biopsy results.

The MTB was asked to interpret molecular results in the context of the following

- Clinical history, including symptoms, imaging findings, tumour markers and clinical index of suspicion
- Suspected tumour type
- Specific aberration detected, pathogenicity and VAF

Variants were classified on the following 3 outcomes;

- Likelihood of being tumour derived: low, moderate, high
- Supportive of a cancer diagnosis: yes or no
- Targetability based on ESCAT guidelines- class 1, 2, 3 (see appendix A).

Initially I planned to create a guidance form for the MTB to ensure consistency across all patients. However the MTB advised individualised discussions given the complex and iterative nature of multi-disciplinary meeting discussions also incorporating genomics. At the end of recruitment, some patients were re-discussed due to inconsistencies in the MTB outcomes.

An outline of the iterative nature including re-discussions at the MTB are outlined in section 5.4.14.

36.1.12 Statistical Considerations and Sample Size Calculation

In this pilot study, an *a priori* threshold to establish the feasibility of ctDNA as a diagnostic tool in suspected cancers (across all tumour types) was defined as a 45% ctDNA detection rate. The sample size was calculated using an Ahern single stage design, defining a detection rate of P0 as 20% and aiming

for P1 of 45%. With 80% power, and a one-sided 10% significance level, at least 16 patients per tumour cohort were needed. With 16 patients in each tumour cohort, the positivity rate could be estimated to within +/-24.5%. The sample size for the PC and BTC cohorts was set at 16 based on this. If the colorectal cohort met the threshold of 6/16 patients having a detected ctDNA result, then recruitment could continue up to 32 patients.

The analysis was performed by cohort. Patient characteristics were described including ctDNA detection rate according to clinico-pathological characteristics.

The concordance analysis was performed in patients with (1) histological diagnosis of PC/BTC, (2) adequate DNA quantity and quality for tissue NGS sequencing, (3) less than 12 weeks between plasma and tissue samples collection and (4) both samples collected prior to initiation of treatment. Only single nucleotide variants (SNVs) in exons covered by both assays were included. Indels, copy number variants (CNVs) and structural variants (SV) were not included in this analysis. A concordance analysis was performed by-patient (total number of patients with at least 1 concordant SNV by the total number of patients) and by-variant (total number of concordant SNVs by the total number of variants detected) as a tumour agnostic (using a pre-defined threshold for calling plasma ctDNA detection) and a tumour-informed approach. The Sn was defined as the proportion of tumour tissue mutations that were detected by both assays.

The study end points with corresponding analysis method and inclusion population are listed in table 29 below.

Table 28- PREVAIL ctDNA study endpoints

End point/Objective	**Definition**	**Method of analysis**	**Population included**	**Performed by**
ctDNA detection rate	Proportion of patients with a ctDNA result consistent with a diagnosis of malignancy	Reported by cancer type with 90% CI	All enrolled patient who had ctDNA collected and successfully sequenced	Sofia Sardo
Treatment outcomes	Proportion of patients with a ctDNA result consistent with a diagnosis of malignancy and commenced treatment	Reported by cancer type with 90% CI	All patients with positive ctDNA result who commenced treatment (either on ctDNA alone or in combination with tissue biopsy) in comparison to all patients enrolled who had ctDNA collected and successful sequenced	Sofia Sardo
Association of ctDNA result with histological specimens	Diagnostic accuracy (sensitivity, specificity analysis)	Chi-squared (or Fisher's exact test for cell frequencies <5)	All patients with a histological biopsy with definitive result (i.e. benign, malignant) in comparison to ctDNA results	Sofia Sardo
	Concordance analysis	Descriptive only	All patients who had successful tissue and plasma NGS, of concordant somatic SNVs which are covered by both panels	Justin Mencel, Paula Proszek
ctDNA genomic landscape	Description of ctDNA and tissue based genomic landscape	Descriptive only	All patients who had a positive ctDNA for the ctDNA landscape all patients who had a tissue biopsy which confirmed the diagnosis of PC/BTC for the tissue arm.	Justin Mencel
Association of ctDNA results with clinical and	Cross-tabulation of ctDNA detection by	Descriptive only	All enrolled patient who had ctDNA collected	Sofia Sardo

radiological characteristics	baseline characteristics (e.g. staging, tumour markers)		and successfully sequenced.	
Association of ctDNA results with retrospective lcWGS in detection of CNV	Descriptive comparison between the results from the initial ctDNA analysis and lcWGS	Descriptive only	All enrolled patient who had ctDNA collected and successfully sequenced	Sofia Sardo
Test turnaround time	Difference in time taken between liquid and tissue biopsy and a cancer diagnosis	Descriptive only	All enrolled patient who had ctDNA collected and reported, and had at least one tissue biopsy. Patients with biopsied performed outside of RMH without knowledge of dates were excluded. Defined as ctDNA - time enrolled on PREVAIL to MTB discussion tissue- time from referral for biopsy to histopathology reporting	Sofia Sardo

37 Results

37.1.1 Patient Recruitment and Tissue Collection

The CRC cohort recruited 33 patients from June 2020 to Feb 2023. Based on the sample size calculation, at least 16 patients were needed for sufficient power to assess the primary end point. However, the threshold of 6/16 patients having detectable ctDNA was met at an interim analysis, we continued to recruit up to 32 patients. The 32nd patient recruited (CRC110) however was deemed ineligible following ctDNA collection (unable to undergo colonoscopy,

further investigations or treatment due to acute concurrent co-morbidity), and so was replaced by a 33rd patient.

When the study opened, recruitment was relatively slow, as capacity for colonoscopy service within the Royal Marsden Partners Cancer Alliance had been stepped up quickly, however there were fewer referrals for 2WW colonoscopies at the RMH compared to other sites (see figure 13 for recruitment). For that reason, fewer patients than expected who were suitable for the study were identified. In early 2022, the RMH received more referrals for screening colonoscopies of FIT positive patients, as such recruitment increased as demonstrated in the recruitment graph below.

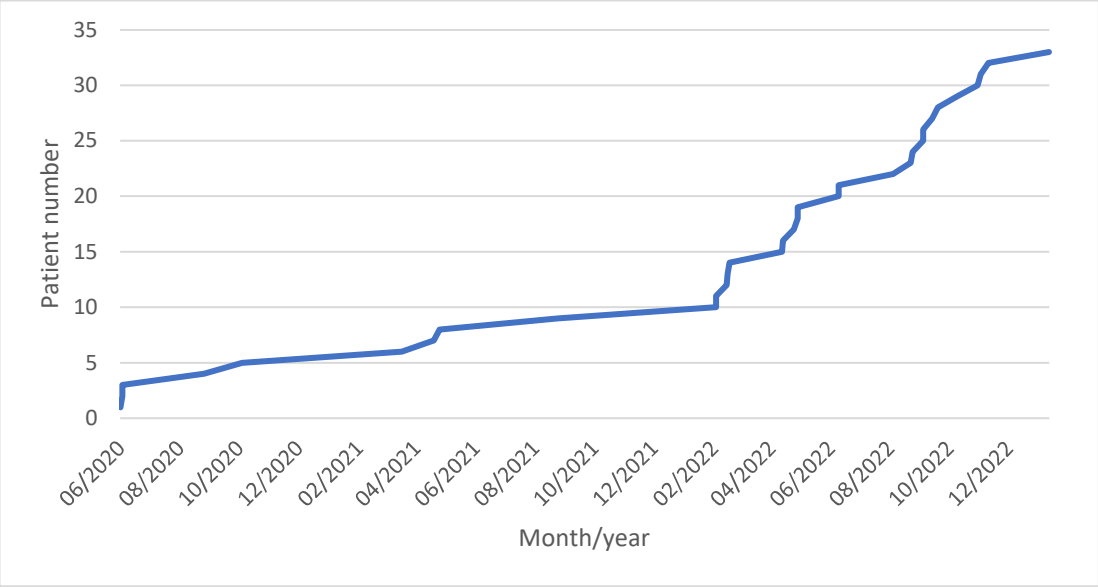


Figure 13- Recruitment to early stage CRC cohort

All patients had blood collected at baseline prior to definitive histological or colonoscopic diagnosis. Following ctDNA collection, patients with a confirmed

histological diagnosis of adenocarcinoma, high grade and low grade adenomatous polyps had tissue collected retrospectively for tissue-based NGS. 2 patients had a non-diagnostic biopsy prior to registration but did not undergo a repeat colonoscopy following registration. 1 patient (C102) had ctDNA collected following a non-diagnostic rectal biopsy showing HGD, and a repeat colonoscopy post-ctDNA collection also demonstrated HGD. However, we only collected the tissue from the first colonoscopy rather second as the two biopsies were considered the same pathology.

37.1.2 Patient Characteristics

32 eligible patients were registered, including 18 (56.3%) males and 14 (43.8%) females. The median age was 66 years (range 36-90 years). The most common reason for enrolment was through a routine 2WW suspected CRC pathway awaiting colonoscopy (84.4%). The remaining 5 patients (15.2%) had a non-diagnostic pre-registration biopsy. All patients had an elevated FIT test, with the mean FIT level of 155.4 (range 10-1400).

Only 6 patients had imaging prior to ctDNA collection, including 1 patient with suspected stage II and 5 patients with suspected stage III CRC. The other 27 patients were not staged prior to ctDNA collection as the suspected lower GI cancer pathway does not routinely include imaging.

A past history of cancer was reported in 3 patients (C62 had active metastatic ocular melanoma, C78 had localised treated prostate cancer and C106 had

suspected localised prostate cancer on imaging). C90 was 32 weeks pregnant at registration.

Baseline characteristics are described in table 31.

Table 29- Baseline characteristics of CRC cohort

	Colorectal (n=32)
Age, years	
median	66
range	36-90
Sex	
Female	14 (43.8)
Male	18 (56.3)
Ethnicity	
Asian	2 (6.2)
Caucasian (White)	29 (90.6)
Other	1 (3.1)
Reason for enrolment	
Inconclusive biopsy	5 (15.6)
Routine 2WW pathway	27 (84.4)

A post ctDNA colonoscopy was performed in 30 patients. 3 patients who underwent colonoscopy were subsequently diagnosed with localised colorectal ADC. Two patients (C19, C37) did not undergo post ctDNA colonoscopy as a pre-registration colonoscopy revealed HGD. CONSORT diagram of all enrolled patients are shown in Fig 14.

Figure 14- CONSORT Diagram of all Registered Patients in CRC cohort

37.1.3 ctDNA Detection in Suspected Colorectal Cancer

The overall ctDNA detection rate in patients with suspected early-stage CRC was 21.2% (7/32) using a tumour agnostic approach. All ctDNA detected aberrations were SNVs. The mean number of somatic variants detected by patient was 1.42 in ctDNA detected patients (range 1-3).

Factors associated with ctDNA detection are outlined in table 32. Patients with ADC and HGD had a relatively higher rate of ctDNA detection (33.3-50%).

Patients with low grade dysplastic (LGD) polyps had ctDNA detection of 14.3%. In patients with a high FIT level (>100ng/l), ctDNA detection was 37.5%. However, no formal statistical analyses were performed to detect differences between the two groups given the low numbers in individual groups.

Table 30- Clinico-pathological factors associated with ctDNA detection in CRC cohort

	ctDNA positive		**ctDNA negative**	
	N	%	N	%
All patients	7	21.9%	25	78.1%
Adenocarcinoma	1	33.3%	2	66.7%
Advanced adenoma	1	25%	3	75%
High grade dysplasia	1	50%	1	50%
Low grade dysplasia	1	12.5%	7	87.5%
Advanced Neoplasia	2	33.3%	4	66.7%
FIT				
<100	4	16.7%	20	83.3%
=>100	3	37.5%	5	62.5%

The mean cfDNA yield was 90.45ng (range 30.4-395.6ng), with a mean maximum VAF (mVAF) of 4.41% (range 0.1-40%). A mean consensus depth of >1500x (aim 2000x) and duplicate percentage of >85% was considered optimal for sequencing quality. Optimal DNA input was between 25 to 50ng with a minimum of 10ng required. Table 33 describes the plasma-based cfDNA sequencing QC of all registered patients in the CRC cohort.

Table 31- Quality control and analytical performance of ct-GI plasma based NGS panel

Patient ID	**cfDNA yield [ng]**	**DNA Input in library prep**	**duplicates**	**on target**	**Mean consensus depth**

5	197.2	50.0	94.9	97.32	2313
6	116	50.0	94.33	97.56	2354
7	83.6	50.0	92	97.2	1863
14	40	35.0	93.82	96.42	1969
19	59.6	50.0	61.23	86.18	1832
37	172.4	50.0	91.24	96.05	1942
44	65.2	50.0	90.85	86.14	2276
46	48.8	42.7	89.46	86.85	1888
62	38.8	33.8	91.35	95.98	2096
67	30.4	26.4	92.84	96.16	1817
68	32.4	28.3	92.29	96.05	1706
69	58	50.0	93.93	96.36	1698
70	38	33.4	93.99	96.03	1552
71	62	50.0	93.95	96.56	1807
74	96.4	50.0	94.42	96.75	1252
75	51.6	45.2	94.87	97.06	1401
76	76.4	50.0	94.26	97.05	1835
77	59.2	50.0	94.39	97.12	1869
78	150.4	50.0	94.62	97.14	2103
82	395.6	50.0	91.57	88.99	1151
83	113.6	50.0	89.18	85.61	717
90	70.8	50.0	85.38	95.16	3023
92	82	50.0	88.86	92.11	1407
94	78	50.0	87.05	93.51	2061
96	63.6	50.0	86.44	94.61	2375
97	249.2	50.0	86.07	94.07	2009
100	56	49.0	88.94	92.95	2137
102	39.2	34.4	89.92	92.09	1621
104	58	50.0	92.4	93.78	1917
106	59.6	50.0	92.48	93.96	2669
107	62.4	50.0	66.38	92.56	1942
114	197.2	50.0	94.9	97.32	2313

37.1.4 Clinical Significance of Variants Detected

All molecular results were discussed at an MTB for clinical context. Of the 10 variants detected across the 7 patients, the MTB determined only 4 variants were consistent with a diagnosis of CRC. This included patients C14, C37 and

C46. Other variants were deemed not supportive of a diagnosis of CRC alone for several reasons (i.e. possibility of CHIP, no clinical features to support a diagnosis of CRC, and variants present at low VAF). SNVs were classified into VUS (n=7 variants) or pathogenic (n=4) based on MTB discussion. Only 1 potentially targetable mutation was detected in plasma (BRAF V600E in C46).

Table 34 describes the ctDNA results and corresponding MTB discussion in the CRC cohort.

Table 32- ctDNA NGS and Molecular Tumour Board Outcome Results in CRC Cohort

ID	Age	T	N	M	Pre-registration biopsy	Post-ctDNA Colonoscopy	Colonoscopy Result	Histology	Advanced adenoma	Advanced neoplasia	ctDNA result	Gene	HGVSp	VAF	Tier	VUS	MTB supportive of CRC
5	62	Tx	Nx	Mx	No	Yes	Normal	N/A	N	N	Positive	NOTCH1	Ala1707Thr	0.1%	3	Yes	No
6	69	Tx	Nx	M0	No	Yes	Abnormal	HGA	Y	Y	Positive	ATM	Ser49Tyr	0.2%	3	Yes	No
												DOCK2	Val1448Met	0.2%	4	Yes	No
7	64	Tx	Nx	Mx	No	Yes	Abnormal	HP	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
14	62	T3	N1	M0	HGA	Yes	Abnormal	ADC	N	Y	Positive	APC	Glu1374Ter	0.2%	3	No	Yes
												TP53	Arg175His	0.2%	2	No	Yes
												ATM	Arg1882Gln	0.2%	3	Yes	No
19	60	T3	N1	M0	HGA	No	N/A	N/A	Y	Y	Positive	APC	1 958+1G>T*	0.3%	3	Yes	No
37	67	T3	N1	M0	HGA	No	N/A	N/A	Y	Y	Positive	TP53	Arg273His	1.8%	2	No	Yes
44	77	Tx	Nx	Mx	No	Yes	Normal	N/A	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
46	80	Tx	Nx	Mx	No	Yes	Abnormal	LGA	N	N	Positive	BRAF	Val600Glu	0.9%	2	No	Yes
62	57	T3	N0	M0	HGA	Yes	Abnormal	ADC	N	Y	Negative	N/A	N/A	N/A	N/A	N/A	N/A
67	78	Tx	Nx	Mx	No	Yes	Abnormal	HP	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
68	74	Tx	Nx	Mx	No	Yes	Normal	N/A	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
69	56	Tx	Nx	Mx	No	Yes	Normal	N/A	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
70	66	Tx	Nx	Mx	No	Yes	Normal	N/A	N	N	Positive	RNF43	Asn167Ser	0.4%	3	Yes	No
71	62	Tx	Nx	Mx	No	Yes	Abnormal	LGA	Y	Y	Negative	N/A	N/A	N/A	N/A	N/A	N/A
74	66	Tx	Nx	Mx	No	Yes	Normal	N/A	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
75	53	Tx	Nx	Mx	No	Yes	Normal	N/A	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
76	72	Tx	Nx	Mx	No	Yes	Abnormal	LGA	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
77	82	Tx	Nx	Mx	No	Yes	Abnormal	LGA	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
78	76	Tx	Nx	Mx	No	Yes	Abnormal	LGA	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
82	69	Tx	Nx	Mx	No	Yes	Abnormal	LGA	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
83	53	Tx	Nx	Mx	No	Yes	Normal	N/A	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
90	36	T3	N1	M0	No	Yes	Abnormal	ADC	N	Y	Negative	N/A	N/A	N/A	N/A	N/A	N/A
92	66	Tx	Nx	Mx	No	Yes	Abnormal	LGA	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
94	66	Tx	Nx	Mx	No	Yes	Normal	N/A	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
96	65	Tx	Nx	Mx	No	Yes	Normal	N/A	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A

97	73	Tx	Nx	Mx	No	Yes	Normal	N/A	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
100	78	Tx	Nx	Mx	No	Yes	Normal	N/A	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
102	51	T4	N2	M0	HGA	Yes	Abnormal	HGD	Y	Y	Negative	N/A	N/A	N/A	N/A	N/A	N/A
104	87	Tx	Nx	M0	No	Yes	Normal	N/A	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
106	90	Tx	Nx	M0	No	Yes	Abnormal	LGA	Y	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
107	61	Tx	Nx	Mx	No	Yes	Normal	N/A	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A
114	41	Tx	Nx	Mx	No	Yes	Normal	N/A	N	N	Negative	N/A	N/A	N/A	N/A	N/A	N/A

HGVSp- Human Genome Variation Society protein classification; LG – low grade adenomatous polyp; HGA- high grade adenomatous polyp; HP- hyperplastic polyp; ADC- adenocarcinoma; N/A- not available; VAF- variant allele frequency; VUS- variant of unknown significance; MTB- molecular tumour board; Advanced adenoma- high grade dysplasia, adenomatous polyps >10mm and predominate villous histology; Advanced neoplasia- advanced adenomas and adenocarcinomas.

* HGVS coding sequence

37.1.5 Correlation of ctDNA Detection with Colonoscopy Findings

The diagnostic accuracy of ctDNA detection in the diagnosis of patients with suspected CRC was assessed. The binary variable (ctDNA detected or not detected) was compared to the histological confirmation of CRC through colonoscopy. This included 30 of 32 patients who underwent a colonoscopy. C19 and C37 did not undergo post-registration colonoscopy and excluded from this analysis. For all patients, there was an interval of between 12 weeks between ctDNA collection and colonoscopy.

The Sn and Sp of ctDNA in the diagnosis of CRC was 33.3% (90% CI 1.7-86.5) and 85.2% (90% CI 69.2-94.8) respectively (see table 35). The PPV was 20% (90% CI 1.0-65.7) and NPV was 92% (90% CI 76.9-98.6). There were 2 false negatives, with C62 and C90 having a diagnosis of ADC on colonoscopy with no detectable ctDNA. However, C90 was pregnant, and the false negative was possibly related to high levels of cfDNA due to late stage pregnancy and dilution of ctDNA.

There were 4 false positives including C5, C6, C46 and C70. C5 had a *NOTCH1* variant detected at VAF 0.1% on ctDNA with a normal colonoscopy. The MTB deemed this variant a VUS and not supportive of a diagnosis of CRC. Sequencing of the buffy coat was negative for *NOTCH1* and therefore unlikely to be CHIP related. C6 had ATM and *DOCK2* variants, both detected at VAF 0.2%. Similarly, the MTB deemed these variants both VUS and not supportive of a diagnosis of CRC. Buffy coat sequencing was wildtype for both *ATM* and *DOCK2* mutations. Colonoscopy revealed HGD, however both variants were

not detected on retrospective tissue NGS. C46 was of interest; they had a *BRAF* V600E mutation detected in plasma, and colonoscopy revealed LGD. Tissue based NGS revealed no *BRAF* V600E mutation, with subsequent investigations including routine complete blood examination showing pancytopenia. Peripheral blood flow cytometry showed a clonal B cell population expressing CD25, CD103, CD123 and CD11c, in keeping with a diagnosis of hairy cell leukaemia (HCL), which was the likely origin of the pathogenic plasma detected *BRAF* mutation. Finally, C70 had a normal colonoscopy with an *RNF43* mutation at VAF 0.4% on ctDNA. The MTB deemed this not consistent with a diagnosis of CRC, however recommended further investigations including an endoscopy and abdominal imaging which revealed no clear source of the *RNF43* mutation. The patient is currently undergoing surveillance for an occult malignant through the GI/early diagnosis team at RMH.

Table 33- Diagnostic Accuracy of ctDNA in the Diagnosis of Suspected Colorectal Adenocarcinoma

	Adenocarcinoma on colonoscopy		**Total**
ctDNA detected	**Yes**	**No**	
Yes	1	4	5
No	2	23	25
Total	3	27	30
Sensitivity	33.3 (1.7, 86.5)		
Specificity	85.2 (69.2, 94.8)		
PPV	20.0 (1.0, 65.7)		
NPV	92.0 (76.9, 98.6)		

Given the importance of the MTB in the interpretation of variants detected, we performed an MTB-adjusted diagnostic accuracy analysis which included the

MTB interpretation of variants. ctDNA detection which the MTB considered not supportive of a diagnosis of CRC was considered as negative, while ctDNA variants detected which the MTB considered supportive of a diagnosis of CRC was considered positive. Therefore, C5, C6 and C70 were re-classified as true negatives.

The Sn was similar at 33.3% however Sp increased to 96.3% when considering the interpretation of the MTB (see table 36). The only false positive was C46 (*BRAF* V600E positive HCL) which allowed appropriate referral to the haematology team.

Table 34- Diagnostic Accuracy of MTB-adjusted ctDNA Results in the Diagnosis of Suspected Colorectal Adenocarcinoma

	Adenocarcinoma on colonoscopy		**Total**
ctDNA detected and MTB consistent with diagnosis of CRC	**Yes**	**No**	
Yes	1	1	2
No	2	26	28
Total	3	27	30
Sensitivity	33.3 (1.7, 86.5)		
Specificity	96.3 (83.6, 99.8)		
PPV	50.0 (2.5, 97.5)		
NPV	92.9 (79.2, 98.7)		

We then assessed the diagnostic accuracy of ctDNA detection for advanced neoplasia (including AA and invasive colorectal ADC). The definition of AA included all HDG, and any adenomas >10mm or with predominant villous histology. All adenomatous polyps which did not meet these criteria were not

considered AA. Similarly the analysis excluded patients who did not undergo post-registration colonoscopy. The Sn and Sp of ctDNA in the detection of advanced neoplasia was 28.6% and 87.0% respectively (see table 37).

Table 35- Diagnostic Accuracy of ctDNA in the Diagnosis of Suspected Colorectal Advanced Neoplasia

	Advanced neoplasia on colonoscopy		**Total**
ctDNA detected	**Yes**	**No**	
Yes	2	3	5
No	5	20	25
Total	6	24	30
Sensitivity	28.6 (5.3, 65.9)		
Specificity	87.0 (69.6, 96.3)		
PPV	40.0 (7.6, 81.1)		
NPV	80.0 (62.5, 91.8)		

There were 3 false positives. These included C5, C46, and C70. There were 5 false negatives including 2 with adenocarcinoma (C62, C90) and 3 with AA (C71, 102 and C106).

37.1.6 Concordance Between Tissue and Plasma Based Next Generation Sequencing

Of all patients who underwent a colonoscopy (pre and post- ctDNA collection), 15 patients had a histological diagnosis ADC, high grade or low grade adenoma and underwent tissue-based NGS to define the tissue genomic landscape in comparison to plasma NGS results. This included the 2 patients who underwent a pre-registration colonoscopy (C19, C37).

ADC was diagnosed in 3 patients. Common SNVs detected were *TP53* and *APC* mutations in all patients. *KRAS* mutation was found in 1 patient. HGD adenomas were detected in 4 patients. Common SNVs were *APC* mutations in all patients, with *TP53* and *KRAS* mutations in only 2 patients. LGD adenomas were found in 8 patients. Common SNVs were in *APC* mutations in 88% with lower frequency of *KRAS* and *TP53* mutation as expected (1 patient). The frequency of tissue based NGS variants detected are outlined in Fig 15.

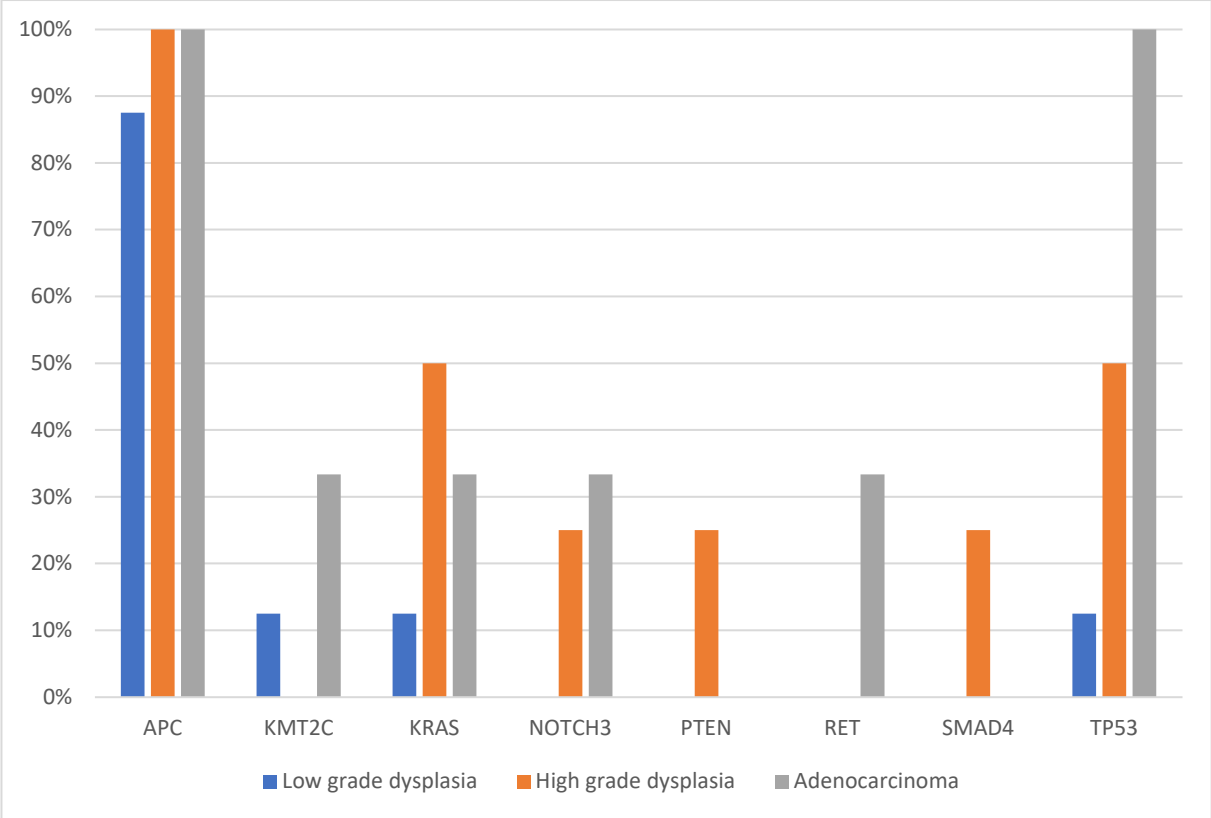


Figure 15- Tissue based genomic landscape of adenocarcinoma and adenomatous polyps in CRC cohort

Tissue NGS and corresponding plasma-NGS results are shown in fig 16. Germline variants detected in tissue as highlighted in blue were not called in plasma.

Tissue														ctDNA									
Trial ID	Date taken	Diagnosis	Procedure	Cellularity	Tumour content	Necrosis	Gene	Position	Protein	HGVS	Dep	Alt Dep	Alt Freq	Date taken	% Dup	% on target	Mean dep	Gene	Position	Protein	HGVS	Dep	Alt Dep	Alt Freq
							APC	chr5:112175754	p.(Leu1488PhefsTer19	c.4464del	357	197	55%					APC	chr5:112175754	p.(Leu1488PhefsTer19	c.4464del	4110	0	0.0%
							FBXW7	chr4:153258983	p.(Arg278Ter	c.832C>T	706	224	32%					FBXW7	chr4:153258983	p.(Arg278Ter	c.832C>T	2863	0	0.0%
							TP53	chr17:7578388	p.(Arg181His	c.542G>A	403	120	30%					TP53	chr17:7578388	p.(Arg181His	c.542G>A	4767	2	0.0%
							ATM	chr11:108098576	p.Ser49Tyr	c.146C>A	1141	1						ATM	chr11:108098576	p.Ser49Tyr	c.146C>A	3165	5	0.2%
6	30/07/2020	HGD	Biopsy	High	70%	No	DOCK2	chr5:169483734	p.Val1448Met	c.4342G>A	0	0		25/06/2020	94.33	97.56	2354	DOCK2	chr5:169483734	p.Val1448Met	c.4342G>A	4189	8	0.2%
							BRIP1	chr17:59924485	p.(Ile202Val	c.604A>G	581	301	52%					BRIP1	chr17:59924485	p.(Ile202Val	c.604A>G	0	0	
							FAT1	chr4:187525005	p.(Glu3558del	c.10672_10674del	476	146	31%					FAT1	chr4:187525005	p.(Glu3558del	c.10672_10674del	0	0	
							PPP2R2A	chr8:26196417	p.(Ser42Ala	c.124T>G	767	235	31%					PPP2R2A	chr8:26196417	p.(Ser42Ala	c.124T>G	0	0	
							SMARCA4	chr19:11134230	p.(Arg966Trp	c.2896C>T	432	106	25%					SMARCA4	chr19:11134230	p.(Arg966Trp	c.2896C>T	0	0	
							APC	chr5:112175411	p.Glu1374*	c.4120G>T	934	339	36%					APC	chr5:112175411	p.Glu1374*	c.4120G>T	3132	7	0.2%
							TP53	chr17:7578406	p.(Arg175His)	c.524G>A	549	301	55%					TP53	chr17:7578406	p.(Arg175His)	c.524G>A	3420	7	0.2%
							APC	chr5:112174152	p.Leu954*	c.2861T>A	993	357	36%					APC	chr5:112174152	p.Leu954*	c.2861T>A	3000	2	0.1%
							FBXW7	chr4:153251954	p.Trp351*	c.1052G>A	633	145	23%					FBXW7	chr4:153251954	p.Trp351*	c.1052G>A	3222	0	0.0%
							ATM	chr11:108175550	p.Arg1882Gln	c.5645G>A	798	0						ATM	chr11:108175550	p.Arg1882Gln	c.5645G>A	2949	5	0.2%
							APC	chr5:112174669	p.Ser1126Arg	c.3378C>G	1049	489	47%					APC	chr5:112174669	p.Ser1126Arg	c.3378C>G	3271	1547	47.3%
14	08/10/2020	ADC	Biopsy	Intermediate	30 - 50%	No	ARID1A	chr1:27100181	p.Gln1332_Gln1333dup	c.3996_4001dupGCAGCA	397	275	69%	17/09/2020	93.82	96.42	1969	ARID1A	chr1:27100181	p.Gln1332_Gln1333dup	c.3996_4001dupGCAGCA	3538	966	27.3%
							ATM	chr11:108098555	p.His42Arg	c.125A>G	870	342	39%					ATM	chr11:108098555	p.His42Arg	c.125A>G	2644	1324	50.1%
							CEBPA	chr19:33792731	p.His195_Pro196dup	c.584_589dupACCCGC	655	259	40%					CEBPA	chr19:33792731	p.His195_Pro196dup	c.584_589dupACCCGC	0	0	
							KMT2C	chr7:151875096	na	c.7443-1->A	425	68	16%					KMT2C	chr7:151875096	na	c.7443-1->A	0	0	
							NOTCH3	chr19:15271820	p.Arg2207Trp	c.6619C>T	358	167	47%					NOTCH3	chr19:15271820	p.Arg2207Trp	c.6619C>T	0	0	
							PHOX2B	chr4:41747889	p.Val294Ile	c.880G>A	438	192	44%					PHOX2B	chr4:41747889	p.Val294Ile	c.880G>A	0	0	
							RET	chr10:43606815	p.Arg475Gln	c.1424G>A	625	360	58%					RET	chr10:43606815	p.Arg475Gln	c.1424G>A	1	0	0.0%
							APC	chr5:112170863	na	c.1958+1G>T	579	102	18%					APC	chr5:112170863	na	c.1958+1G>T	2557	7	0.3%
							KRAS	chr12:25398284	p.(Gly12Val)	c.35G>T	612	113	18%					KRAS	chr12:25398284	p.(Gly12Val)	c.35G>T	2599	4	0.2%
							SMAD4	chr18:48584513	p.Leu229fs	c.687delG	669	148	22%					SMAD4	chr18:48584513	p.Leu229fs	c.687delG	3005	16	0.5%
19	15/10/2020	HGD	Biopsy	Intermediate	30 - 50%	No	SMAD4	chr18:48591925	p.Cys363Phe	c.1088G>T	866	181	21%	17/11/2020	61.23	86.18	1832	SMAD4	chr18:48591925	p.Cys363Phe	c.1088G>T	3226	5	0.2%
							ATM	chr11:108203565	p.Ala2622Gly	c.7865C>G	654	322	49%					ATM	chr11:108203565	p.Ala2622Gly	c.7865C>G	2567	1277	49.7%
							NOTCH3	chr19:15299069	p.Gly490Ala	c.1469G>C	400	200	50%					NOTCH3	chr19:15299069	p.Gly490Ala	c.1469G>C	0	0	
							PIN1	chr19:9959765	na	c.383-1G>A	410	75	18%					PIN1	chr19:9959765	na	c.383-1G>A	0	0	
							TP53	chr17:7577120	p.(Arg273His	c.818G>A	763	119	16%					TP53	chr17:7577120	p.(Arg273His	c.818G>A	3366	62	1.8%
							APC	Chr5:112174469-112174527			1362	231	17%					APC	Chr5:112174469-112174527			3602	33	0.9%
							ATRX	chrX:76909656	p.(Arg1417Trp	c.4249C>T	1313	171	13%					ATRX	chrX:76909656	p.(Arg1417Trp		0	0	
							AXIN1	chr16:338196	p.(Glu839Lys	c.2515G>A	934	143	15%					AXIN1	chr16:338196	p.(Glu839Lys	c.2515G>A	0	0	
							BRIP1	chr17:59820468	p.(Arg762Leu	c.2285G>T	700	99	14%					BRIP1	chr17:59820468	p.(Arg762Leu	c.2285G>T	0	0	
37	01/04/2021	HGD	Biopsy	Intermediate	30%	No	EZH2	chr7:148526826	p.(Asp160Asn	c.478G>A	951	131	14%	08/04/2021	91.24	96.05	1942	EZH2	chr7:148526826	p.(Asp160Asn	c.478G>A	0	0	
							GPR161	chr1:168066352	p.(Gly185Ser	c.553G>A	954	75	8%					GPR161	chr1:168066352	p.(Gly185Ser	c.553G>A	0	0	
							POLD1	chr19:50919743	p.(Glu971Lys	c.2911G>A	714	41	6%					POLD1	chr19:50919743	p.(Glu971Lys	c.2911G>A	0	0	
							PTCH1	chr9:98229452	p.(Glu836Lys	c.2506G>A	1081	530	49%					PTCH1	chr9:98229452	p.(Glu836Lys	c.2506G>A	0	0	
							PTEN	chr10:89720811	p.(Asn323LysfsTer2	c.968dup	1028	244	24%					PTEN	chr10:89720811	p.(Asn323LysfsTer2	c.968dup	0	0	
46	17/05/2021	LGD	Biopsy	Intermediate	30%	No	BRAF	chr7:140453136	p.Val600Glu	c.1799T>A	2386	3	0%	17/05/2021	89.46	86.85	1888	BRAF	chr7:140453136	p.Val600Glu	c.1799T>A	2762	26	0.9%
							KMT2D	chr12:49446006	p.(Arg487Gln	c.1460G>A	1301	71	5%					KMT2D	chr12:49446006	p.(Arg487Gln	c.1460G>A	0	0	
							APC	chr5:112175216	p.(Glu1309Ter	c.3925G>T	1340	404	30%					APC	chr5:112175216	p.(Glu1309Ter	c.3925G>T	3493	0	0.0%
							APC	chr5:112155031	p.(Asn436IlefsTer18	c.1307del	873	276	32%					APC	chr5:112155031	p.(Asn436IlefsTer18	c.1307del	3358	0	0.0%
62	10/11/2021	ADC	Biopsy	High	70%	No	FBXW7	chr4:153249385	p.(Arg465Cys	c.1393C>T	1281	761	59%	24/09/2021	91.35	95.98	2096	FBXW7	chr4:153249385	p.(Arg465Cys	c.1393C>T	3207	1	0.0%
							TP53	chr17:7579860	p.(Thr18HisfsTer26	c.52del	856	359	42%					TP53	chr17:7579860	p.(Thr18HisfsTer26	c.52del	110	0	0.0%
							RAD50	chr5:131923258	p.(Arg254His	c.761G>A	2049	583	28%					RAD50	chr5:131923258	p.(Arg254His	c.761G>A	0	0	
							APC	chr5:112164646	p.(Glu574Ter	c.1720G>T	884	228	26%					APC	chr5:112164646	p.(Glu574Ter	c.1720G>T	2556	0	
							APC	chr5:112175457	p.(Val1390GlnfsTer4	c.4168_4169del	1612	450	28%					APC	chr5:112175457	p.(Val1390GlnfsTer4	c.4168_4169del	2833	0	
71	14/03/2022	LGD	Biopsy	High	70%	No	APC	chr5:112178625	p.(Lys2445Arg	c.7334A>G	1874	918	49%	11/03/2022	93.95	96.56	1807	APC	chr5:112178625	p.(Lys2445Arg	c.7334A>G	3006	1499	49.9%
							GNAS	chr20:57484599	p.(Arg871His	c.2612G>A	2414	566	23%					GNAS	chr20:57484599	p.(Arg871His	c.2612G>A	0	0	
							TP53	chr17:7579358	p.(Arg110Leu	c.329G>T	813	346	43%					TP53	chr17:7579358	p.(Arg110Leu	c.329G>T	0	0	
							FGFR1											FGFR1	Exons 2,2a-3,3a-18 Gain					

76	16/05/2022	LGD	Biopsy	Intermediate	30%	No	APC	chr5:112170683	p.(Trp593Ter	c.1779G>A	1353	122	9%	16/05/2022	94.26	97.05	1835	APC	chr5:112170683	p.(Trp593Ter	c.1779G>A	2745	0	0.0%
							APC	chr5:112175174	p.(Glu1295Ter	c.3883G>T	1229	127	10%					APC	chr5:112175174	p.(Glu1295Ter	c.3883G>T	2709	0	0.0%
							KMT2D	chr12:49434669	p.(Ser2295Phe	c.6884C>T	571	282	49%					KMT2D	chr12:49434669	p.(Ser2295Phe	c.6884C>T	0	0	
							PDCD1LG2	chr9:5549400	p.(Cys143Arg	c.427T>C	947	478	50%					PDCD1LG2	chr9:5549400	p.(Cys143Arg	c.427T>C	0	0	
77	23/05/2022	LGD	Biopsy	Intermediate	30%	No	APC	chr5:112174631	p.(Arg1114Ter	c.3340C>T	1456	145	10%	20/05/2022	94.39	97.12	1869	APC	chr5:112174631	p.(Arg1114Ter	c.3340C>T	3130	2	0.1%
							KRAS	chr12:25398285	p.(Gly12Arg)	c.34G>C	1050	97	9%					KRAS	chr12:25398285	p.(Gly12Arg)	c.34G>C	2425	4	0.2%
							APC	chr5:112102927	p.(Ser89GlnfsTer36	c.264del	1546	162	10%					APC	chr5:112102927	p.(Ser89GlnfsTer36	c.264del	2646	0	0.0%
							APC	chr5:112175943	p.(Glu1552GlyfsTer6	c.4655_4656del	1342	145	11%					APC	chr5:112175943	p.(Glu1552GlyfsTer6	c.4655_4656del	3168	0	0.0%
							EP300	chr22:41533667	p.(Ser545Gly	c.1633A>G	851	403	47%					EP300	chr22:41533667	p.(Ser545Gly	c.1633A>G	0	0	
							PTCH2	chr1:45293136	p.(Leu770Arg	c.2309T>G	860	79	9%					PTCH2	chr1:45293136	p.(Leu770Arg	c.2309T>G	0	0	
78	23/05/2022	LGD	Biopsy	Intermediate	50%	No	AMER1	chrX:63411291	p.(Arg626Ter	c.1876C>T	1101	364	33%	20/05/2022	94.62	97.14	2103	AMER1	chrX:63411291	p.(Arg626Ter	c.1876C>T	0	0	
							APC	chr5:112116592	p.(Arg213Ter	c.637C>T	1585	458	29%					APC	chr5:112116592	p.(Arg213Ter	c.637C>T	2810	0	0.0%
							APC	chr5:112175890	p.(Asn1535MetfsTer30	c.4602del	2079	635	31%					APC	chr5:112175890	p.(Asn1535MetfsTer30	c.4602del	3142	0	0.0%
							ATR	chr3:142215351	p.(Asn1917Ile	c.5750A>T	1177	74	6%					ATR	chr3:142215351	p.(Asn1917Ile	c.5750A>T	0	0	
							STAG2	chrX:123195094	p.(Tyr479Ter	c.1437C>A	1160	765	66%					STAG2	chrX:123195094	p.(Tyr479Ter	c.1437C>A	0	0	
82	04/07/2022	LGD	Biopsy	Intermediate	50%	No	APC	chr5:112175798	p.(Ser1503TyrfsTer4	c.4508del	1357	370	27%	01/07/2022	91.57	88.99	1151	APC	chr5:112175798	p.(Ser1503TyrfsTer4	c.4508del	1946	0	0.0%
							APC	chr5:112175216	p.(Glu1309Ter	c.3925G>T	1314	385	29%					APC	chr5:112175216	p.(Glu1309Ter	c.3925G>T	1908	0	0.0%
							APC	chr5:112162803	NA	c.1409-2A>T	1184	372	31%					APC	chr5:112162803	NA	c.1409-2A>T	1428	0	0.0%
							ARID1A	chr1:27094445	p.(Tyr1052LeufsTer53	c.3154dup	827	67	8%					ARID1A	chr1:27094445	p.(Tyr1052LeufsTer53	c.3154dup	1628	0	0.0%
							FBXW7	chr4:153247366	p.(Arg479Leu	c.1436G>T	883	104	12%					FBXW7	chr4:153247366	p.(Arg479Leu	c.1436G>T	1643	0	0.0%
90	27/09/2022	ADC	Resection	High	30 - 50%	Unknown	APC	chr5:112128143	p.(Arg216Ter	c.646C>T	957	224	23%	26/08/2022	85.38	95.16	3023	APC	chr5:112128143	p.(Arg216Ter	c.646C>T	3660	3	0.1%
							APC	chr5:112175057	p.(Gln1256Ter	c.3766C>T	838	403	48%					APC	chr5:112175057	p.(Gln1256Ter	c.3766C>T	5318	8	0.2%
							KRAS	chr12:25398284	p.(Gly12Asp)	c.35G>A	989	451	46%					KRAS	chr12:25398284	p.(Gly12Asp)	c.35G>A	4452	3	0.1%
							TP53	chr17:7577085	p.(Glu285Ter	c.853G>T	1089	282	26%					TP53	chr17:7577085	p.(Glu285Ter	c.853G>T	4919	3	0.1%
							TP53	chr17:7577121	p.(Arg273Cys	c.817C>T	1006	338	34%					TP53	chr17:7577121	p.(Arg273Cys	c.817C>T	4607	2	0.0%
							C11orf30	chr11:76174966	p.(Val225Ile	c.673G>A	1091	365	33%					C11orf30	chr11:76174966	p.(Val225Ile	c.673G>A	0	0	
92	13/09/2022	LGD	Biopsy	Intermediate	50%	No	APC	chr5:112162825	p.(Glu477Ter	c.1429G>T	1384	102	7%	13/09/2022	88.86	92.11	1407	APC	chr5:112162825	p.(Glu477Ter	c.1429G>T	2287	0	0.0%
							APC	chr5:112175475	p.(Glu1397Ter	c.4188dup	1343	102	8%					APC	chr5:112175475	p.(Glu1397Ter	c.4188dup	2112	0	0.0%
							APC	chr5:112176516	p.(Arg1742His	c.5225G>A	1462	726	50%					APC	chr5:112176516	p.(Arg1742His	c.5225G>A	2282	1077	47.2%
							ASXL1	chr20:31022427	p.(Thr638Ala	c.1912A>G	1538	128	8%					ASXL1	chr20:31022427	p.(Thr638Ala	c.1912A>G	1	0	0.0%
							BARD1	chr2:215645546	p.(Thr351Met	c.1052C>T	1859	948	51%					BARD1	chr2:215645546	p.(Thr351Met	c.1052C>T	0	0	
							F2R	chr5:76028826	p.(Asn259Ser	c.776A>G	1720	853	50%					F2R	chr5:76028826	p.(Asn259Ser	c.776A>G	0	0	
							KMT2C	chr7:151860806	p.(Pro3286Ser	c.9856C>T	1343	672	50%					KMT2C	chr7:151860806	p.(Pro3286Ser	c.9856C>T	0	0	
							POLE	chr12:133226392	p.(His1222Gln	c.3666C>A	1502	734	49%					POLE	chr12:133226392	p.(His1222Gln	c.3666C>A	1	0	0.0%
							PTCH1	chr9:98231178	p.(Pro702Arg	c.2105C>G	1371	658	48%					PTCH1	chr9:98231178	p.(Pro702Arg	c.2105C>G	0	0	
102	30/09/2022	HGD	Biopsy	High	70%	Yes	APC	chr5:112175211	p.(Glu1309AspfsTer4	c.3927_3931del	1052	257	24%	13/10/2022	89.92	92.09	1621	APC	chr5:112175211	p.(Glu1309AspfsTer4	c.3927_3931del	2678	0	0.0%
							APC	chr5:112175109	p.(Arg1273SerfsTer15	c.3819del	984	303	31%					APC	chr5:112175109	p.(Arg1273SerfsTer15	c.3819del	2219	0	0.0%
							PIK3CA	chr3:178936082	p.(Glu542Lys)	c.1624G>A	1071	345	32%					PIK3CA	chr3:178936082	p.(Glu542Lys)	c.1624G>A	1042	0	0.0%
							CIC	chr19:42794869	p.(Ala650Val	c.1949C>T	463	92	20%					CIC	chr19:42794869	p.(Ala650Val	c.1949C>T	0	0	
							FGFR3	chr4:1807896	p.(Thr654Ser	c.1961C>G	630	290	46%					FGFR3	chr4:1807896	p.(Thr654Ser	c.1961C>G	0	0	
							IGF1R	chr15:99434635	p.(Ala241Val	c.722C>T	898	436	49%					IGF1R	chr15:99434635	p.(Ala241Val	c.722C>T	0	0	
							KRAS	chr12:25378562	p.(Ala146Thr)	c.436G>A	1304	564	43%					KRAS	chr12:25378562	p.(Ala146Thr)	c.436G>A	1	0	0.0%
							TERT	chr5:1295228	na	g.1295228G>A	481	135	28%					TERT	chr5:1295228	na	g.1295228G>A	0	0	
106	21/11/2022	LGD	Biopsy	Intermediate	50%	No	APC	chr5:112175347	p.(Phe1354IlefsTer15	c.4060_4076del	1686	414	25%	21/11/2022	92.4	93.78	1917	APC	chr5:112175347	p.(Phe1354IlefsTer15	c.4060_4076del	3454	0	0.0%
							TSC2	chr16:2129333	p.(Gly1063Val	c.3188G>T	1133	150	13%					TSC2	chr16:2129333	p.(Gly1063Val	c.3188G>T	0	0	

Figure 16- FFPE Tissue NGS using RMH-200 Panel for CRC cohort

Blue: Germline variant in tissue, Grey: Variant in tissue not covered by ct-GI panel; Red: Variant in tissue not detected in plasma; Yellow: Variant in tissue detected in plasma, but not called blind and below the tumour agnostic cut-off; Green: Variant in tissue detected in plasma and reported blind; Purple: Variant reported in plasma, but not detected in tissue above the cut-off; White: variant in tissue

A concordance analysis was performed on all patients with a histological diagnosis of ADC, HGD and LGD, with successful tissue NGS and variants detected across both tissue and plasma assays.

The partial concordance by patient was 20% using a tumour agnostic approach, which increased to 31% when retrospectively called using a tumour informed approach as the variant calling threshold is lower. Concordance by variant between somatic tissue and plasma alterations was 9.3% for variants called as a tumour-agnostic approach. This increased to 37.2% when calling additional variants retrospectively using a tumour informed approach.

Noting low numbers in individual groups, the partial concordance by histology was also assessed. The partial concordance for CRC, high grade and low grade adenomas was 15.4%, 16.7% and 0% for tumour agnostic variant calling, and 61.5%, 50% and 11.1% for tumour informed variant calling, respectively. Table 38 outlines the concordance between tissue and plasma based NGS which is further represented in figure 17 below.

Table 36- Concordance between tissue and plasma based NGS

Concordance	**Tumour agnostic**	**Tumour informed**
By patient	20% (3/15)	33.3% (5/15)
By variant overall	9.3% (4/43)	37.2% (16/43)
By variant ADC	15.4% (2/13)	61.5% (8/13)

By variant HGD	16.7% (2/12)	50.0% (6/12)
By variant LGD	0.0% (0/18)	11.1% (2/18)

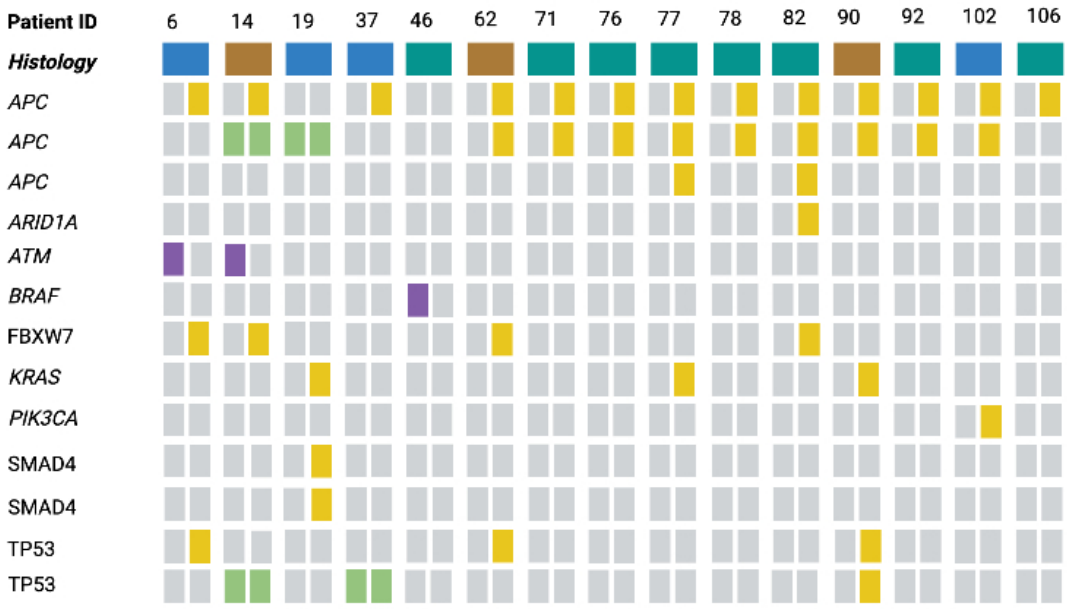


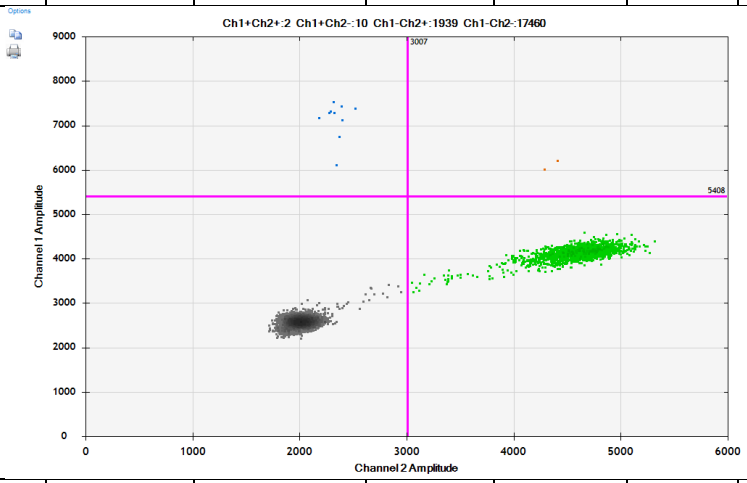
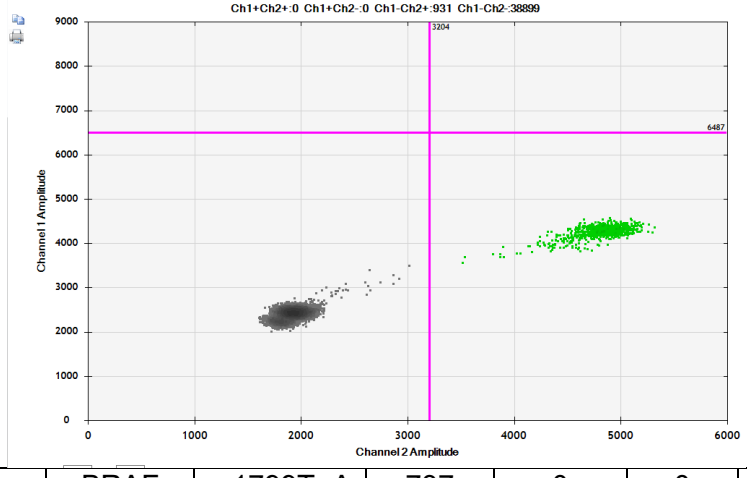
Figure 17- Tumour agnostic concordance between tissue and plasma based NGS in CRC cohort; Histology: blue (High grade dysplasia), brown (adenocarcinoma), turquoise (low grade dysplasia).

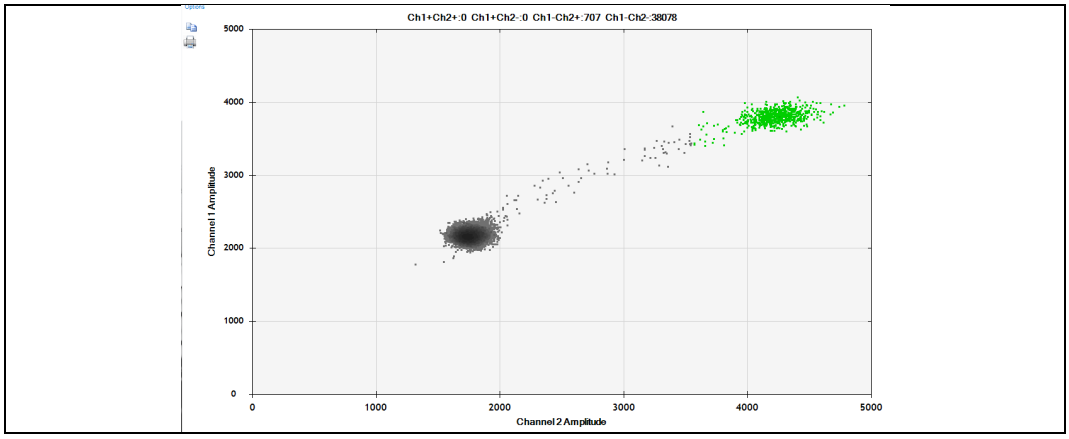
Mutations: concordant (green), tissue only (yellow), plasma only (purple).

C6 had both *ATM* and *DOCK2* variants in plasma which were not detected in the tissue. *ATM* is covered by the RMH200 panel, however *DOCK2* is not covered and could have been present in the polyp. These mutations were not detected in the buffy coat and therefore unlikely to be CHIP related. C14 was diagnosed with ADC with several tissue-based variants detected, however only two tissue based variants matched plasma (*APC* and *TP53* mutations). The plasma detected *ATM* mutation was not detected in tissue. Similarly this was not detected in the buffy coat and so unlikely to be CHIP related.

Finally, the *BRAF* V600E mutation detected in C46 was not detected in tissue using RMH200 panel and additionally on ddPCR (see table 39). We confirmed the *BRAF* V600E mutation in the plasma using ddPCR. We subsequently performed ddPCR targeted sequencing of the buffy coat which revealed no *BRAF* V600E mutation.

Table 37- ddPCR targeting BRAF gene in patient C45 plasma, buffy coat and tissue

Sample	GENE NAME	HGVS	Ref pos	Mut pos	VAF (%)	ddPCR positive
Plasma	BRAF	c.1799T>A	1941	12	0.73	Yes
						
Buffy coat	BRAF	c.1799T>A	931	0	0	No
						
FFPE	BRAF	c.1799T>A	737	0	0	No



As previously described, we performed further investigations to assess for the origin of this variant including flow cytometry of peripheral lymphocytes and ddPCR on plasma. Flow cytometry revealed a B cell population positive for markers of HCL (See Fig 18). ddPCR genotyping of the BRAFV600E variant (exon 11, 15) was performed on the peripheral blood DNA to confirm the plasma-based NGS sequencing results. This revealed no BRAF mutation. However, the clonal B cell represented <1% of the total nucleated cells on flow cytometry, and therefore the ddPCR result is likely a false negative given the high likelihood of low-level BRAF variants in the blood (Sn 97.6% for ddPCR).

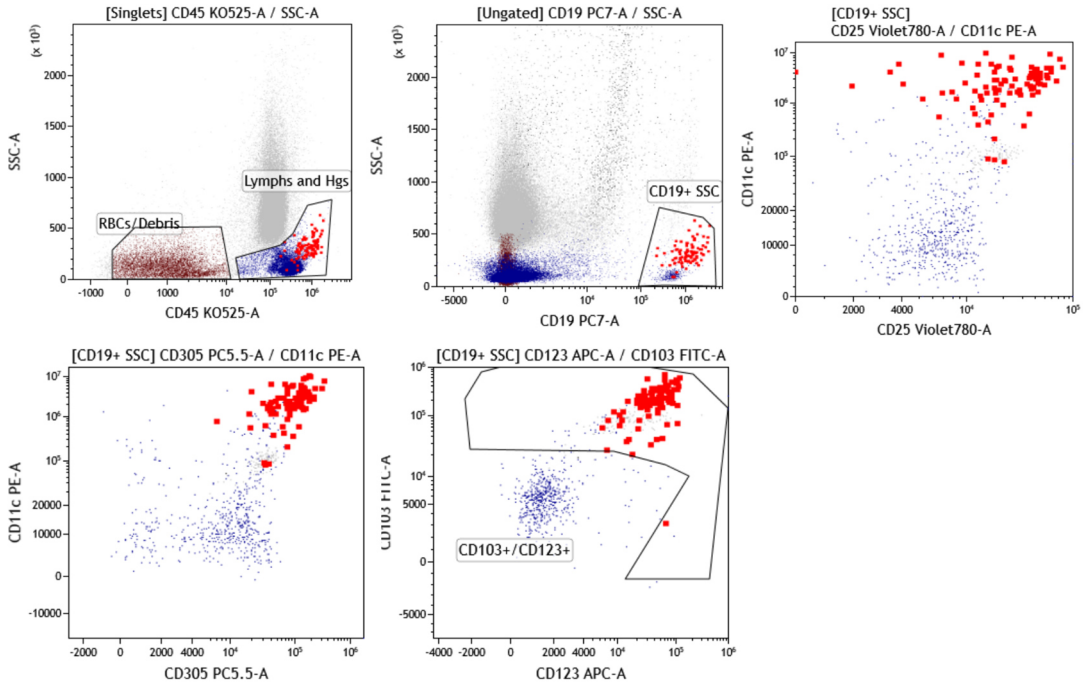


Figure 18- A small population of B cells (highlighted in red) show a distinct immunophenotype in keeping with hairy cell leukaemia, positive for CD19, CD123, CD103, CD11C, CD25 and CD305 with increased side scatter

38 Discussion

The COVID19 pandemic highlighted the urgent need for non-invasive tools to screen, triage and diagnose for suspected CRC given the significant impact on endoscopy services. CRUK reported that the number of invitations for screening for suspected CRC during the pandemic was 14% lower than pre-pandemic numbers, with 650,000 fewer patients participating in screening. In addition, the cancer referrals wait times increased, with only 48.7% of patients meeting the FDS (cancer diagnosed or ruled out within 28 days) despite a national target of 75%. Following the pandemic, the number of patients awaiting a colonoscopy for screening and diagnosis of CRC was approximately 200,000 in the UK as of November 2022 (324).

When setting up the PREVAIL study, my initial hypothesis was that ctDNA can be used to triage patients with suspected early stage CRC. At the beginning of the COVID19 pandemic, referrals to the RMH for suspected lower GI cancer were reduced and majority of patients had screening colonoscopies performed at other diagnostic centres. With a significant decline in referrals for colonoscopy, the ability to answer this primary hypothesis was limited. As referrals increased in the recovery period and at the end of the pandemic, capacity for endoscopy services and subsequent wait times at the RMH returned to normal. Therefore, my secondary hypothesis became more relevant in this setting; that ctDNA results correlates with colonoscopy. My primary aim was to assess the ctDNA detection in patients with suspected CRC.

The ctDNA detection rate in patients with suspected CRC with FIT positivity was 21.2% overall, 33.3% in those with ADC and 20% in those with adenomatous polyps. The detection rate may be slightly higher than previous reports given the high pre-test probability in our cohort as we only included those with FIT positivity. The Sn and Sp of ctDNA in in our cohort of 32 patients was 33.3% and 96.3% based on MTB discussion, respectively. Our study is limited due to the low number of CRC diagnoses on colonoscopy which could be mitigated by adjusting the inclusion criteria to include those with a higher FIT cut off, those with imaging suspicious of CRC only, or selecting those with a hereditary pre-disposition syndrome to enhance the pre-test probability. Fleshner et al. assessed the use of ctDNA in the diagnosis of CRC and pre-

cancerous lesions involving 200 patients undergoing screening and diagnostic colonoscopies. A ctDNA detection of 6% was reported when using a multi-gene assay, and 13.2% in those with colonic polyps (4.7% false positive rate) (322). However, 80% of patients had a normal colonoscopy, no patient having adenocarcinoma and only 20% having a polyp detected, thereby limiting the capacity to assess ctDNA in the diagnosis of CRC.

The use of non-invasive tests to detect AA is of interest given their pre-malignant potential. Studies have demonstrated FIT testing provides a low Sn and Sp to detect AA, with colonoscopy screening considered the gold standard in diagnosis and therapeutic management. The use of ctDNA in the detection of AA has been studied, showing similar low diagnostic accuracy owing to the low tumour DNA shedding of these pre-cancerous lesions in the circulation. Junca et al assessed the use of a ddPCR targeted approach (targeting *BRAF* and *KRAS* genes) in 130 patients undergoing screening colonoscopy (300). The ctDNA detection rate in those with AA was low (2.6%) with limited Sp of 16.9%. In our cohort, the ctDNA detection rate in those with AA was 25%. It is worth noting that our study used a multi-gene assay with greater genome coverage which may explain the higher Sn.

False positive results represent a major concern for screening particularly with ctDNA currently being investigated in several large scale population based studies (NCT05611632, NCT05155605, NCT03085888). Although ctDNA analysis in the molecular profiling of advanced tumours is often performed in

those with histologically confirmed malignancy, its use in screening asymptomatic patients and in early diagnosis of symptomatic patients in a tumour-agnostic approach using a plasma-only assay is being explored in these studies. The risk of detecting false positives may lead to over-investigation and heightened patient anxiety. When using a plasma-based ctDNA NGS assay, false positives may arise from non-tumour derived DNA, including age-related clonal haematopoiesis (ARCH), CHIP, germline aberrations, and other somatically driven non-tumour derived DNA. CHIP can be seen in 9.5% of individuals aged over 70 years old, with the incidence rising with age (325). Discrimination of CHIP from variants of somatic origin is possible by using buffy coat DNA as a matched control which was performed in our study to reduce this risk. Germline variants can also be detected by sequencing lymphocyte-derived DNA. Circulating somatic mutations can also be seen in a small proportion of non-malignant related conditions such as in inflammatory diseases, and can arise from benign tissue at low allelic frequencies (188). However, even relatively low rates of false positives results can have significant implications for large, population based screening programmes including increased use of resources and costs to the NHS. Recently, the GRAIL Pathfinder study assessed the use of ctDNA methylation (using the Galleri test) to detect a cancer signal across multiple tumour types. Although the false positive rate was very low (0.8%), there is a potential to detect non-tumour derived variants in the plasma of asymptomatic patients when using ctDNA as a screening tool (326). This could add to the burden on the NHS and colonoscopy services if used as a non-targeted approach across the large UK population.

The false positive rate for patients with advanced neoplasia (i.e. ctDNA detection in those without CRC or adenomatous polyps) was 12% in our study. We detected 4 false positives (C5, C14, C46 and C70). C5 had a plasma *NOTCH* mutation detected with a normal colonoscopy. *NOTCH1* mutations can be seen in the majority of T-cell acute lymphoblastic leukaemias (327). However, this patient had normal blood counts and buffy coat sequencing revealed no *NOTCH1* mutation, which is therefore unlikely to be CHIP related. C6 had a HDG polyp resected with plasma NGS revealing *DOCK2* and *ATM* mutations, both of which were not detected in the tissue. *DOCK2* mutations are seen in approximately 10% of inflammatory bowel disease (IBD) related CRC (328). *DOCK2* is also involved in the proliferation of T lymphocytes, and theoretically could be CHIP-related. However, this patient did not have a history of IBD and buffy coat sequencing did not reveal *DOCK2* or *ATM* mutations. This patient was also found to have a LGD polyp which was not sequenced, and so the origin of these mutations may be from the non-sequenced colonic polyp. C46 had a detected BRAF mutation however further investigations revealed the origin was from HCL rather than CRC. C70 had an *RNF43* mutation detected in the plasma with a normal colonoscopy. This patient had extensive investigations without a clear cause for the ctDNA result. These mutations for these patients however were correctly identified at MTB as being not supportive of a cancer diagnosis. The role of the MTB is critical in the interpretation of variants detected, to give clinical context to the genomic report and provide a level of certainty to whether a mutation is likely tumour

derived or not. The Sp of ctDNA in the diagnosis of CRC increased when incorporating the MTB interpretation by reducing the false positive rate.

Sn for CRC diminished to 33.3% (false negative rate 66.7%). We detected 2 false negatives in patients who were histologically diagnosed with invasive ADC. C90 was 32 weeks pregnant at registration, with no ctDNA detected despite being diagnosed with stage III colon cancer. Pregnancy is associated with higher levels of cfDNA and dilution of tumour derived ctDNA was likely the contributing factor to the false negative. C62 had multiple variants detected on tissue NGS which were not detected on the ctGI panel, even at high sequencing depth. This patient had stage II adenocarcinoma (T3N0M0), with the early stage of disease probably contributing to false negative. The reported false negative rate of the Galleri test in the detection of CRC was 18%, with Sn for stages I, II, III and IV disease of 43.3%, 85.0%, 87.9% and 95.3% (214). However, the Galleri assay incorporates methylation analysis which is associated with higher diagnostic accuracy compared with qualitative DNA genotyping alone (329). Low detection rate of ctDNA in CRC and adenomatous polyps is likely related to the genomic only analysis used in our patient cohort.

The concordance between plasma and tissue was also investigated in 15 patients who were diagnosed with ADC or adenomatous polyps. The concordance was generally low, with concordance by variant of 9.3%. Partial concordance by patient was 20%. The low concordance seen in this cohort is likely represented by the high proportion of LGA polyps in the study which is

associated with lower levels of ctDNA shedding. The DNA shedding of polyps is generally low and therefore the use of a qualitative, multi-gene sequencing panel to screen for these polyps may be of limited use compared to methylation-based assays.

ctDNA methylation assays have shown promise in the detection AA. AI-EMERGE and the ECLIPSE study have assessed ctDNA based assays incorporating DNA genotyping with methylation and shown sensitivities in the detection of AA of 41% and 13% respectively. In our study, a Sn of 25% for the detection of AA was shown. Future studies incorporating DNA methylation, genotyping and other blood based biomarkers could provide a higher level of Sn in the detection of AA.

The PREVAIL study has several limitations. Firstly, the original hypothesis could not be answered given the dynamic changes of the COVID19 pandemic and the diversion of screening colonoscopies from the Royal Marsden. Ideally, I planned on using ctDNA as a colonoscopy triaging tool, to expedite colonoscopies in those with detectable ctDNA and patients with un-detectable ctDNA would continue along a COVID-19 adjusted pathway. Although this hypothesis could not be answered, my secondary hypothesis of assessing the use of ctDNA in predicting colonoscopy findings could be answered. However, the small number of patients with abnormal colonoscopies did impact the diagnostic accuracy analyses. A larger study may provide a more accurate Sn and Sp of ctDNA in screening for CRC. Secondly, we did not exclude patients with a previous or concurrent cancer diagnosis and the cohort included several

patients with melanoma and prostate cancer. As this study was set up during the COVID19 pandemic when capacity for invasive diagnostics were limited, we needed to ensure patients with suspected cancer across all cohorts had access to a diagnostic test and therefore were not excluded. The MTB however was aware of the past history of malignancy in their interpretation of ctDNA molecular results. Thirdly, we did not calculate the test turnaround time in this cohort, and this may add support for the use of ctDNA as a triaging tool. Several other studies ctDNA based tumour profiling studies have shown quicker turnaround time with ctDNA versus tissue sequencing methods (211, 330).

Our study has shown a lower than expected ctDNA detection rate in those with suspected CRC based on a positive FIT test. The use a multi-oemic assay which incorporates methylation and genomics may improve Sn and is the subject of ongoing programmes. With the increase of ctDNA based research studies and screening programmes, it is important to reflect on the many challenges of this technology. The detection of ctDNA in an asymptomatic patient with normal imaging, in particular requires a focused diagnostic approach. Targeted investigations should be stratified depending on the aberration detected and the patient symptoms. This could help to avoid unnecessary patient anxiety and reduce the risk of over-investigation or a surge in additional burden on diagnostic services, particularly in the setting of large, population based ctDNA screening trials. It is increasingly recognised that such patients can benefit from discussion in an MTB involving genomic and clinical experts, to interpret reported variants (particularly when using a

plasma-only assay without prior knowledge of the tumour genome). The MTB's role in the interpretation of these ctDNA aberrations can give clinical context and provide recommendations for further targeted investigations. Given the future increased uptake of plasma-only assays across cancer medicine, it is imperative that we then also develop robust genomic and diagnostic infrastructure, incorporating MTBs, and downstream services such as rapid diagnostic centres that will help to enable better, and earlier cancer diagnosis and treatment.

39 PREVAIL ctDNA Pilot Study- Pancreatic and Biliary Tract Cohort

40 Abstract

Background: Pancreatic (PC) and biliary tract cancers (BTC) often present with advanced disease, with ctDNA detected in 80-90% of cases. Obtaining a tissue biopsy is challenging, requires complex invasive endoscopies and may not be feasible to due comorbidities. The recent COVID19 constraints on endoscopy and histopathology services highlighted the urgent unmet need for non-invasive diagnostic tools such as ctDNA to facilitate a diagnosis in patients with suspected PC/BTC. My hypothesis is that ctDNA can be used in the diagnosis of patients with suspected PC/BTC. I therefore piloted liquid biopsies to detect ctDNA in the diagnostic pathway for patients with suspected PC/BTC to support a cancer diagnosis. **Methods**: This single centre prospective study conducted at the Royal Marsden Hospital from June 2020 to August 2021 included patients with radiologically suspicious PC/BTC without histological diagnosis. ctDNA was collected plasma-based next generation sequencing (NGS) using a custom multi-gene panel as a tumour-agnostic approach. A molecular tumour board (MTB) reviewed results for interpretation and clinical context. The primary end point was the proportion of patients with a ctDNA result consistent with a diagnosis of malignancy following MTB discussion. **Results**: 32 patients were recruited across both cohorts (n=16 PC, n=16 BTC). ctDNA detection rate was 69% and 56% in patients with suspected PC and BTC respectively. In 27 patients who subsequently underwent a tissue diagnosis, the Sn and Sp of ctDNA in the diagnosis of PC was 80% and 100%,

and in BTC was 70% and 75% respectively. **Conclusion**: ctDNA can be used to support a diagnosis of cancer in patients with suspected PC/BTC.

41 Background and Rationale

The diagnosis and management of PC/BTC relies on histological confirmation through an invasive procedure. These procedures (such as EUS/FNA) are performed through specialist endoscopy, require sedation, with potential complications including infection/bleeding, and require an appropriate level of patient fitness. Tissue biopsy through EUS/FNA can also be challenging due tumour related factors given the location of PC and BTC. Given the necrotic and fibrotic nature of these tumour, obtaining adequate tissue for histological confirmation of invasive malignancy can be difficult and a repeat procedure invasive procedure is needed in a quarter of patients (5, 331). The challenges of using tissue in the diagnose of PC/BTC contributes to the complexity of the diagnostic pathway in these patients, and often leads to delays in diagnosis and treatment. The pandemic highlighted the urgent need for non-invasive diagnostic tools to speed diagnosis and facilitate access to treatment in PC/BTC.

ctDNA can be detected in 80-90% of patients with histologically confirmed locally advanced and metastatic PC/BTC, and in up to 20-40% in those with early-stage disease (202). The use of ctDNA as a diagnostic biomarker in patients with suspected PC/BTC is limited, however several studies have

shown variable diagnostic accuracy for ctDNA in histologically confirmed PC, ranging from 20-80% Sn and 60-90% Sp. These studies used targeted ddPCR techniques (targeting KRAS mutations). The Sn and Sp of ctDNA in the diagnosis of BTC using a multi-gene panel has been reported as 88.6% and 96.7% respectively (273). To date, there is no data to support the use of ctDNA as a diagnostic biomarker, using a multi-gene assay, as a tumour agnostic approach in a prospective study to facilitate treatment in patients with suspicious PC/BTC without histological evidence of cancer. To address this, I set up the PREVAIL ctDNA pilot trial (NCT04566614) to assess ctDNA to support a diagnosis of patients with suspected cancers across 6 cancer types. Here I present data on the PC/BTC cohorts.

My hypothesis is that ctDNA can be used to support a diagnosis of cancer in patients with suspected PC/BTC. My aim was to investigate the use of ctDNA to support a cancer diagnosis in patients with radiologically suspected PC/BTC. The primary objective was to assess the number of patients with a ctDNA result consistent with a diagnosis of malignancy.

42 Methods

42.1.1 Overall Study Set Up

The PREVAIL ctDNA overall study set up has been previously described in section 4.3.1.

42.1.2 Overall Study Design

The PREVAIL ctDNA overall study design has been previously described in section 4.3.2.

42.1.3 Pancreatic and Biliary Tract Cohort Study Design

Patients with radiologically suspicious PC and BTC were considered suitable for this study if they had no prior histological diagnosis. All patients had radiological suspicious disease either on CT imaging or through ERCP/MRCP (i.e. biliary strictures). Patients with suspicious PC/BTC of any stage were considered suitable.

Majority of patients were recruited whilst waiting for an invasive procedure (including some having ctDNA collected on the day of the procedure). However, no patient had blood collected after the invasive procedure was performed. Also, no patient who commenced systemic anti-cancer treatment were included.

All patients had a discussion at the MTB. Patient with detectable ctDNA could be offered treatment based on the ctDNA result, in lieu of tissue diagnosis as part of this study. Patients without detectable ctDNA continued through the standard diagnostic pathway.

Patients were identified in GI clinics and upper GI endoscopy lists. Patients were then pre-screened and called to assess willingness to participate. Patients could provide consent and have blood collected on the day of receiving the PIS.

42.1.4 Blood Sample Collection and DNA Isolation

The blood sample collection and DNA isolation for the PC/BTC cohort has been previously described in section 4.3.4 which is the same across all cohorts in the PREVAIL ctDNA study.

42.1.5 Validation of ct-PAED Targeted Next Generation Sequencing Panels

For the PC/BTC cohorts, we use the ct-PAED panel. This 67-gene panel was designed to detect clinically relevant mutations from children with solid tumours using an NGS capture based method. The gene coverage of the ct-PAED panel is outlined in appendix D. The ct-PAED panel was used for the PC/BTC cohorts given its applicability to PC and BTC.

Most of the commonly mutated genes seen in PC are covered by the ct-PAED panel, including *KRAS*, *TP53*, *CDK2NA*. However, this panel does not cover *SMAD4*, or *MET* genes which are also relatively common in PC. It also does not cover potentially actionable mutations including SNV in *BRCA1*/2 and microsatellite genes. This assay also does not detect actionable gene fusions or translocations such as *NTRK*.

In BTC, the most common aberrations include SNVs in *p53*, *IDH1/2*, FGFR, *ARID1A*, and *KRAS* are covered by the ct-PAED panel. Similarly, however, *SMAD4* is not covered by this panel, nor are fusions in *FGFR*. The inability of this panel to detect fusions was considered unlikely to impact the diagnostic potential of ctDNA in the PREVAIL study for BTC which was the primary aim of the study.

This custom panel was developed by the CMP and has established clinical validation in a tumour agnostic approach (332). The rationale for developing the ct-PAED assay was to avoid needle biopsies in paediatric cancer patients, to offer a non-invasive method to molecular profile these tumours. This pan-paediatric cancer solid tumour panel uses molecular barcoding and background error suppression to reduce PCR sequencing errors, same as for ct-GI panel. The panel has been validated using (1) ctDNA control cancer specific SNVs at known VAFs, (2) FFPE samples with known SNVs and (3) clinical ctDNA samples with SNVs confirmed by ddPCR. The panel covers SNV, small indel and CNVs and not fusions or large structural variants.

The established LOD is 0.125% VAF, providing good Sn and Sp to detect SNVs of 96.5% and 81.5% respectively. The panel has good Sn/Sp for short Indels of a few bases. However, the panel is not validated for larger Indels of 25-50 bases as these do not reference to the human genome and are removed in the bioinformatic pipeline. These long Indels are called by a separate SV bioinformatic pipeline which is not currently validated. A tumour agnostic

approach for variant calling was performed using a threshold of >0.3% VAF with ≥5 consensus reads. This threshold was trained using UMI error suppression on matched buffy coats. All variants were detected in the VAF range 0.18-47.1%. Validation of the RM ct-PAED panel with known SNVs and Indels were also matched to tumour FFPE samples. In those with extra-cranial disease, the tissue-plasma SNV concordance was 94.4%. Table 39 shows the diagnostic accuracy of the ctPAED panel across different aberrations at a LOD 0.125%.

Table 38- Sensitivity and Specificity of ctPAED Panel Across different aberrations at a LOD 0.125%

Aberration type	**Sn (95% CI)**	**Sp**
SNV	96.5 (90.1-99.3)	81.5 (68.6-90.8)
Short indels	97.3 (90.7- 99.7)	97.9 (93.7-100%)

Based on this study, the PREVAIL study used the following criteria for variant calling using a tumour agnostic approach (de-novo variant calling)

- **VAF 0.3%:** ≥5 “unique” reads of variant strongly linked with cancer type, or ≥3 reads of very common hotspot, e.g. KRAS, BRAF in CRC
- **VAF 0.49%:** ≥5 reads, only call variants in genes strongly linked to cancer type, or pathogenic mutations in other genes
- **VAF ≥0.5%:** ≥6 reads, any variant

In addition, homopolymer reads at the start of a sequence are often interpreted as PCR sequencing errors in the bioinformatic pipeline, leading to false positive results. Therefore, a stricter threshold for calling homopolymers was

used. For homopolymers of >4 bases in length, the requirement for variant calling was >10 reads and 0.4% VAF.

Pseudogenes are segments of DNA that are similar to a functional gene, but do not code for proteins. These genes are ubiquitous in the human genome and include PTENP1 (PTEN) and BRAFP1 (BRAF). Target enrichment can reduce the risk of pseudogenes contributing to false positives during NGS. However, high level of certainty for variant calling of pseudogenes is necessary to avoid these false positives. The CMP variant calling cut off for SNVs in pseudogenes is higher than for SNVs in other genes, with criteria for variant calling of >7 reads with VAF >0.4%.

For the concordance analysis, a tumour informed approach was performed retrospectively on plasma samples. The ctPAED panel has a LOD of 0.05% with 2 reads for tumour informed variant calling.

42.1.6 Library Preparation and Targeted Plasma Based Next Generation Sequencing

The library preparation and targeted NGS for the PC/BTC cohort is similar across all cohorts in the PREVAIL ctDNA study, the details of which are described in section 4.3.6.

42.1.7 Bioinformatic Analysis and Variant Calling

The bioinformatic analysis and variant calling for the PC/BTC cohort has been previously described in section 4.3.7 which is the same across all cohorts in the PREVAIL ctDNA study.

42.1.8 Tissue Collection, DNA Extraction, Targeted Tissue Based NGS Sequencing and Variant Calling

The tissue collection and sequencing workflow for the PC/BTC cohort has been previously described in section 4.3.9 which is the same across all cohorts in the PREVAIL ctDNA study.

42.1.9 Plasma Based ddPCR Sequencing

The plasma sample ddPCR sequencing for the PC/BTC cohort has been previously described in section 4.3.10 which is the same across all cohorts in the PREVAIL ctDNA study.

42.1.10 Plasma Based Low Coverage Whole Genome Sequencing

lcWGS was performed for samples within the PC/BTC cohort to assess for copy number alterations not detected using the ct-PAED panel. The lcWGS has been validated using paediatric solid tumour samples which was previously published (332). lcWGS was able to detect all variants seen in tissue, in plasma samples with a ctDNA purity of >10%. Samples with ctDNA purity of <10% were associated with a high rate of false negatives. The lcWGS

sequences the genome at 1 base per location (1x depth) at a minimum to detect high level amplification and large chromosome deletions.

cfDNA libraries constructed using Nonacus Cell3™ kit, were used for lcWGS. They were pooled by equal mass and sequenced to the depth of 1-5x on a NovaSeq 6000 (Illumina, San Diego, USA) using 2 x 100 bp paired-end reads. The base call (bcl) files were de-multiplexed using bcl2fastq and reads then aligned to the human reference genome. The lcWGS data was analysed using an R package: ichorCNA (Adalsteinsson et. Al. 2017), a package designed to calculate copy number status and tumour fraction in a sample.

42.1.11 Molecular Tumour Board

The MTB discussion for the PC/BTC cohort has been previously described in section 4.3.11 which is the same across all cohorts in the PREVAIL ctDNA study.

42.1.12 Statistical Considerations and Sample Size Calculation

The statistical considerations and sample size calculation was similar across all cohorts, however for PC/BTC further explanation is described in section 4.3.12.

43 Results

43.1.1 Patient Recruitment

The PC and BTC cohorts completed recruitment in June 2021 and Sept 2021 respectively (see Fig 19). The analysis for the primary end point was undertaken in September 2021 and presented at the 2022 ASCO GI Symposium. The analysis for the secondary endpoints were undertaken in January 2023 and included in this thesis.

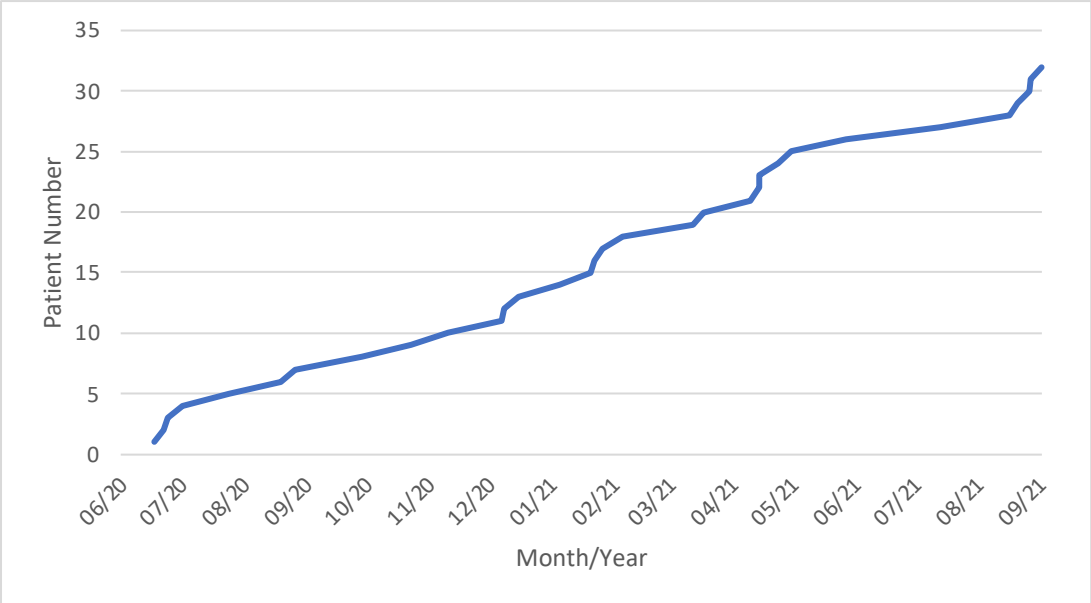


Figure 19- Recruitment to PC/BTC cohorts from Jun 20 to Sept 21

43.1.2 Tissue Collection

The PC and BTC cohorts recruited 16 patients each (total 32). All patients registered had blood collected for ctDNA NGS at baseline. Only patients who had a subsequent histological diagnosis of malignancy (regardless of ctDNA result and type of cancer diagnosed) had tissue collected retrospectively and

sent for tissue based NGS. Table 40 outlines the tissue collection log for patients in PC/BTC cohorts. BTC 59 did not have sufficient tumour DNA for tissue-NGS.

Table 39- Tissue and sample collection for all registered patients

ID	Blood collection	Date blood collected	ddPCR	lcWGS	Tissue collected	Successful tissue NGS	Date tissue taken
1	Y	17/06/2020	N	Y	N	N/A	N/A
2	Y	22/06/2020	N	Y	Y	Y	21/9/20
4	Y	24/06/2020	N	Y	Y	Y	7/9/20
8	Y	01/07/2020	Y	Y	Y	Y	8/7/20
10	Y	24/07/2020	N	Y	N	N/A	N/A
11	Y	19/08/2020	N	Y	Y	Y	14/10/20
12	Y	26/08/2020	Y	Y	Y	Y	10/9/20
15	Y	28/09/2020	Y	Y	Y	Y	8/10/20
18	Y	23/10/20*	Y	Y	Y	Y	29/7/20
20	Y	10/11/2020	Y	Y	Y	Y	3/12/20
22	Y	07/12/2020	N	Y	N	N/A	N/A
23	Y	08/12/2020	Y	Y	Y	Y	11/12/20
24	Y	15/12/2020	N	Y	N	N/A	N/A
26	Y	05/01/2021	N	Y	N	N/A	N/A
27	Y	20/01/2021	N	Y	Y	Y	2/2/21
28	Y	26/01/2021	Y	Y	Y	Y	29/1/21
29	Y	22/01/2021	N	Y	Y	Y	01/03/2021
31	Y	05/02/2021	N	Y	Y	Y	05/02/2021
34	Y	12/03/2021	N	Y	Y	Y	13/05/2021
35	Y	17/03/2021	N	Y	N	N/A	N/A
38	Y	09/04/2021	Y	Y	Y	Y	22/4/21
39	Y	14/04/2021	N	Y	Y	Y	06/05/2021
40	Y	14/04/2021	N	Y	N	N/A	N/A
42	Y	23/04/2021	N	Y	N	N/A	N/A
43	Y	30/04/2021	N	Y	N	N/A	N/A
48	Y	27/05/2021	N	Y	N	N/A	N/A
53	Y	13/07/2021*	N	Y	Y	Y	12/05/2021
54	Y	16/08/2021	N	Y	N	N/A	N/A
55	Y	20/08/2021	N	Y	Y	Y	27/09/2021
56	Y	26/08/2021	N	Y	Y	Y	29/09/2021
58	Y	02/09/2021	N	Y	Y	Y	27/09/2021
59	Y	03/09/2021	N	Y	Y	N	N/A

* Patients 18 and 53 had tissue samples taken outside RMH which were reported as non-diagnostic but suspicious for malignancy (therefore fulfilling

eligibility criteria). ctDNA was taken subsequent to this. Following a second RMH pathology review, these tumours were classified as malignant (with invasive cancer seen on biopsy) and so were collected for tNGS analysis.

43.1.3 Patient Characteristics

32 patients recruited across both cohorts included 15 males and 17 females with a median age of 73 years (range 49 - 90). The most common reason for enrolment was due to tumour-related factors (i.e. technically challenging, inconclusive initial biopsies) in 22 (67%) patients, with 18 (56%) having a non-diagnostic pre-registration biopsy. In all registered patients, most had early stage or locally advanced disease on baseline imaging, with 4 (25%) patients with suspected PC and 1 (6.2%) patient with suspected BTC presenting with metastatic disease.

In both cohorts, 27 (84.3%) of patients had a post-registration biopsy, with 19 having histological confirmation of PC or BTC. Baseline characteristics are shown in table 41.

Table 40- Patient baseline characteristics

	PC n= (%)	**BTC n= (%)**
Age, years		
Median	73	74
Range	55-84	49-90
Sex		
Male	10 (62.5)	5 (31.2)
Female	6 (37.5)	11 (68.8)
Ethnicity		
African	1 (6.3)	0 (0)
Asian	2 (12.5)	1 (6.25)
White	11 (68.8)	14 (87.5)
Other	2 (12.5)	1 (6.3)

Anatomical Location (BTC cohort only)		
Intrahepatic	-	4 (25)
Extra-hepatic (hilar/distal)	-	7 (43.7)
Gallbladder	-	5 (31.3)
Ampullary	-	0
Reason for Enrolment		
COVID19 related delays	1 (6.3)	0 (0)
Inconclusive biopsy	10 (62.5)	8 (50.0)
Technical challenging	1 (6.3)	3 (18.8)
Patient preference	1 (6.3)	2 (12.5)
Co-morbidities/Other	3 (18.7)	3 (18.7)
UICC/AJCC Staging (for confirmed malignancy only)		
Local/Early stage	4 (25)	3 (18.7)
Locally Advanced	4 (25)	6 (37.5)
Metastatic	2 (12.5)	1 (6.3)
Not staged/no confirmed malignancy	6 (37.5)	6 (37.5)
Local lymph node metastases (for confirmed malignancy only)	6 (37.5)	4 (25)
Sites of metastases (for confirmed malignancy only)		
Distant lymph node	2 (12.5)	0 (0)
Lung	0 (0)	1 (6.3)
Liver	1 (6.3)	0 (0)
Peritoneal	1 (6.3)	0 (0)
CA 19.9		
Elevated (≥38)	8 (50)	8 (50)
Normal (<38)	6 (37.5)	6 (37.5)
Not performed	2 (12.5)	2 (12.5)
Elevated CEA		
Elevated (≥4)	8 (50)	1 (6.3)
Normal (<4)	5 (31.2)	12 (75)
Not performed	3 (18.7)	3 (18.7)

Some patients had a past or current history of malignancy or significant risk factor. BTC10 had a history of early stage breast cancer in 2015 (but was disease free at registration). BTC58 had stable but active, bilateral early stage breast cancer on letrozole at enrolment. BTC31 had a resected stage III melanoma immediately prior to registration. BTC53 had a known history of Lynch syndrome.

All patients registered (n=32) had plasma collected and successful ctDNA targeted-NGS sequencing, analysis and reporting (i.e. 100% analytical success). See CONSORT diagram of enrolled patients (Fig 20).

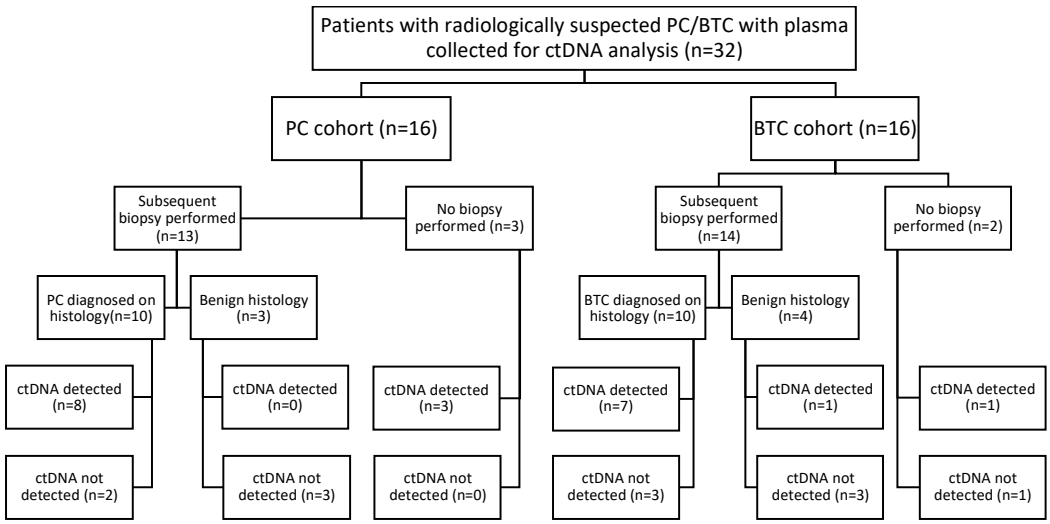


Figure 20- CONSORT Diagram of all Enrolled Patients

43.1.4 ctDNA Detection in Suspected Pancreatic and Biliary Tract Cancers

The ctDNA detection rate in patients with suspected PC and BTC (primary end point) was 69% (11/16) for PC and 56% (9/16) for BTC, using a tumour-agnostic approach. All ctDNA detected aberrations were SNVs, with no CNV, SV or Indels being detected.

The mean number of somatic variants detected by patient was 1.73 (19 variants/11 patients) (range 1-3) in PC and 2.89 (26 variants/9 patients) (range 1-8) in BTC cohorts.

The factors associated with ctDNA detection across both cohorts are outlined in table 42. This included the presence of regional nodal involvement (80%) metastatic disease (100%), liver metastases (100%), elevated CA 19.9 (75%), and elevated CEA (78%). Given the low numbers in individual groups, no formal statistical analysis was performed to detect differences between the two groups.

Table 41- Clinico-pathological factors associated with ctDNA detection in PC/BTC

	ctDNA absent (negative)		**ctDNA present (positive)**		**N=**
	N	%	N	%	
Metastatic*					
Non-Metastatic	5	29.4	12	70.6	17
Metastatic	0	0	3	100	3
Regional nodal involvement*					
Node negative	3	30	7	70	10
Node positive	2	20	8	80	10
Stage of Disease*					
Early	1	14.3	6	85.7	7
Locally Advanced	4	40	6	60	10
Metastatic	0	0	3	100	3
Liver metastases+					
Non-Liver Metastases	0	0	2	100	2
Liver Metastases	0	0	1	100	1
CA19.9					
>=38	4	25	12	75	16
<38	6	50	6	50	12
Not performed	2	50	2	50	4
CEA					
>=4	2	22	7	78	9
<4	8	47	9	53	17
Not performed	2	50	2	50	4
Not available	0	0	2	100	2

* staging only performed in patients with confirmed malignancy (n=20)

+ only in patients with confirmed malignancy and metastatic disease (n=3)

The mean cfDNA yield was 119.46ng (range 37.4-604ng), with a mean maximum VAF (mVAF) of 1.22% (range 0.1 – 5.2%). A mean consensus depth of >1500x (aim 2000x) and duplicate percentage of >85% was considered optimal for sequencing quality. Optimal DNA input 10ng (min), optimal 25-50ng. Table 43 describes the plasma-based cfDNA sequencing QC of all registered patients.

All samples had optimal QC however PC35 and BTC59 mean consensus depth were lower than the optimal cut off. This may have contributed to BTC59 false negative result.

Table 42- Quality control and analytical performance of ct-PAED plasma based NGS panel

PC Cohort					
Patient ID	**cfDNA yield [ng]**	**DNA Input in library prep**	**duplicates**	**on target**	**Mean consensus depth**
1	116.4	50.0	94.3	97.05	2239
2	79.6	50.0	71.17	95.76	1567
8	59.6	50.0	93.75	93.76	1122
12	113.6	50.0	89.05	95.02	1183
15	77.2	50.0	84.47	92.54	1505
18	39.76	34.79	86.18	95.11	1752
20	39.84	34.86	91.84	95.1	1363
23	184	50.0	83.3	92.45	2312
24	95.2	50.0	91.6	94.59	2085
27	66.8	50.0	90.8	89.56	1898
28	129.2	50.0	88.17	94.46	1700
35	334.4	50.0	89.69	89.12	881
38	66	50.0	88.15	95.36	1996
40	70.4	50.0	89.87	95.08	1798
42	87.2	50.0	68.27	93.84	2312
43	37.4	32.73	70.96	93.3	1855
BTC Cohort					

Patient	cfDNA yield [ng]	DNA Input	duplicates	on target	Mean consensus depth
4	96.8	50.0	69.78	95.8	1444
10	64.8	50.0	91.76	95.73	1878
11	160	50.0	89.75	95.38	1404
22	115.6	50.0	83.32	92.37	2273
26	140.4	50.0	89.61	94.92	2042
29	49.6	43.4	89.33	90.15	1537
31	136.4	50.0	70.79	91.51	1777
34	175.6	50.0	71.89	95.95	1746
39	79.2	50.0	88.46	95.02	1930
48	77.6	50.0	75.92	93.41	2257
53	69.2	50.0	79.55	90.25	1035
54	229.6	50.0	78.79	98.29	2059
55	91.6	50.0	78.92	98.31	2231
56	604	50.0	74.36	97.01	1742
58	52	50.0	61.42	97	2059
59	84	50.0	66.89	97.3	597

43.1.5 Clinical Significance of Variants Detected

All molecular results were discussed at an MTB for clinical context. All patients with detectable ctDNA had at least 1 variant which was consistent with a diagnosis of malignancy based on MTB discussion.

In the PC cohort (n=11 patients with detected ctDNA), 19 SNVs were identified, of which 11 (58%) were considered variants of unknown significance (VUS) and 8 variants (42%) were considered pathogenic.

In 9 patients with detected ctDNA in BTC cohort, 26 variants were identified, of which 13 (50%) were considered VUS and 13 (50%) were considered pathogenic.

Targetable genomic aberrations as per ESCAT guidelines were detected in plasma in 37.5% (6/16) and 6.3% (1/16) of patients in PC and BTC cohorts respectively, including in *ERBB2*, *PIK3CA*, and *KRAS*. However, all variants were classified as tier IIIA (hypothetical target) which requires clinical benefit to be demonstrated in patients with a specific alteration but in a different tumour type. As such, efficacy in PC/BTC is hypothetical and not proven. In this cohort, no actionable variants were detected which can be targeted with an FDA approved drug. The ESCAT potentially targetable variants include two *KRAS* G12D (PC1 and BTC53), two *KRAS* G12V (PC18 and PC28), and a *KRAS* K1117N (PC12). None of these *KRAS* mutations are current targetable with clinically effective drugs in PC/BTC. In addition, *PICK3CA* R88Q and V344M were detected in BTC58 which are also not druggable in BTC. SNV in *ERBB2* (Gln329Lys in PC 8; V477M in PC38; L755S in BTC58) were detected in 3 patients. These SNVs are currently not druggable in PC or BTC.

Table 44 describes the ctDNA results in both cohorts.

Table 43- ctDNA NGS and Molecular Tumour Board Outcome Results in PC/BTC Cohorts

ID	Age	CA 19.9	Cohort	Stage	T	N	M	Site of mets	ctDNA detected	Mutation(s) detected	VAF (%)	Tier	VUS (Y/N)	HGVSp	Targetable (ESCAT Tier)	Tissue biopsy	Tissue biopsy result	Treatment based on
1	75.4	2	P	M	3	2	1	Lung, liver, lymph node	Y	KRAS	0.6	1	N	Gly12Asp	IIIA	N	N/A	Other[1]
										TP53	0.3	2	N	Arg175His	NR			
2	80	2	P	LA	4	2	0	lymph node	N	N/A	N/A	N/A	N/A	N/A	N/A	Y	ADC	Tissue
4	50	762	B	E	4	1	0	N/A	Y	ATRX	1.00	4	Y	Glu2015Asp	NR	Y	ADC	Both
8	75	2471	P	E	2	0	0	N/A	Y	ERBB2	0.30	3	Y	Gln329Lys	IIIA	Y	ADC	Both
										FGFR1	0.10	3	Y	Val425Phe	NR			
										TSC2	0.20	4	Y	Gln1118His	NR			
10	67.8	1325	B	E	2	0	0	N/A	Y	ALK	0.20	3	Y	Ala1251Asp	NR	N	N/A	Other[2]
11	79	32	B	E	2	0	0	N/A	Y	STAG2	0.90	4	Y	Asp663Gly	NR	Y	ADC	Both
										TP53	0.30	3	N	Arg282Gln	NR			
12	54	966	P	LA	2	1	0	N/A	Y	NF1	0.30	3	Y	Ala2028Ser	NR	Y	ADC	Both
										KRAS	0.30	3	N	Lys117Asn	IIIA			
15	71	64	P	LA	4	1	0	N/A	N	N/A	N/A	N/A	N/A	N/A	N/A	Y	ADC	Tissue
18	73	1173	P	M	2	1	1	Peritoneum, lymph nodes	Y	KRAS	0.30	2	N	Gly12Val	IIIA	Y	ADC	Both
										TP53	0.10	3	N	Pro152Gln	NR			
20	80	682	P	E	2	0	0	N/A	Y	ATM	1.10	3	Y	Val203Met	NR	Y	ADC	Both
										TP53	0.30	3	Y	Glu2014Ala	NR			
22	48.8	10	B	LA	4	1	0	N/A	N	N/A	N/A	N/A	N/A	N/A	N/A	Y	Benign (inflam)	N/A
23	72	NR	P	E	2	0	0	N/A	Y	TP53	0.10	3	Y	Ser33Pro	NR	Y	ADC	Both
24	55.2	726	P	M	4	1	1	Lung, liver, lymph node	Y	PTEN	0.2	3	Y	Val45Ile	NR	N	N/A	Other[3]
26	63.9	331	B	LA	2	1	0	N/A	N	N/A	N/A	N/A	N/A	N/A	N/A	Y	Benign (inflam)	N/A
27	84	53	P	E	3	0	0	N/A	Y	SMARCA4	0.10	3	Y	Arg99Trp	NR	Y	ACC	Both
										CREBBP	0.10	3	Y	Ala2049Thr	NR			
28	61	16930	P	M	4	1	1	Liver	Y	KRAS	1.3	1	N	Gly12Val	IIIA	Y	ADC	Both
										TP53	0.4	1	N	Arg175His	NR			
29	77	9	B	E	2	0	0	N/A	Y	SMARCA4	0.2	3	Y	Arg377His	NR	Y	ADC	Both
31	83	253	B	LA	2	1	0	N/A	Y	IDH2	0.6	2	N	Arg172Trp	NR	Y	ADC	Both

										TP53	0.4	2	Y	Arg273Cys	NR			
34	53	59	B	M	4	1	1	Lung	Y	ARID1A	3.0	3	Y	Pro703Leu	NR	Y	ADC	N/A
35	59	32	P	NR	NR	NR	NR	N/A	N	N/A	N/A	N/A	N/A	N/A	N/A	Y	Benign (AIP)	N/A
38	54	2	P	LA	4	1	0	N/A	Y	ERBB2	0.50	3	Y	Val477Met	IIIA	Y	ADC	Both
39	83	15	B	LA	3	0	0	N/A	Y	TP53	0.60	3	Y	Pro36Leu	NR	Y	OSCC, benign GB biopsy	Tissue
										TP53	1.80	3	N	Arg282Trp	NR			
										PTCH1	0.40	3	Y	Val1173Met	NR			
40	81.7	NR	P	LA	3	1	0	N/A	Y	ATM	1.1	3	Y	Leu295Pro	NR	N	N/A	Other[2]
42	78.2	17	P	NR	NR	NR	NR	N/A	N	N/A	N/A	N/A	N/A	N/A	N/A	Y	Benign (SCA)	N/A
43	59.3	14	P	E	2	0	0	N/A	N	N/A	N/A	N/A	N/A	N/A	N/A	Y	Benign (Inflam)	N/A
48	83.6	NR	B	NR	NR	NR	NR	N/A	N	N/A	N/A	N/A	N/A	N/A	N/A	Y	Benign (PanC cytology negative)	N/A
53	67	5226	B	LA	3	1	0	N/A	Y	ARID1A	2.70	3	N	Glu896Ter	N/A	Y	ADC	Both
										BCOR	4.00	4	N	Gly677Ter	N/A			
										CDKN2A	2.00	3	N	Arg80Ter	N/A			
										KRAS	1.40	2	N	Gly12Asp	IIIA			
										NF1	1.00	3	N	Cys2371IlefsTer20	N/A			
										PDGFRA	2.60	3	Y	Tyr676His	N/A			
										SMARCA4	1.50	3	Y	Arg849Gln	N/A			
54	90	248	B	E	2	0	0	N/A	N	N/A	N/A			N/A	N/A	N	N/A	N/A
55	81	1	B	E	2	0	0	N/A	N	N/A	N/A			N/A	N/A	Y	ADC	Tissue
56	59	NR	B	LA	3	0	0	N/A	N	N/A	N/A			N/A	N/A	Y	ADC	Tissue
58	86	2	B	LA	3	0	0	N/A	Y	ARID1A	3.0	3	Y	Pro877Ser	NR	Y	ADC	Other[2]
										ERBB2	1.9	3	N	Leu755Ser	IIIA			
										PIK3CA	0.7	3	N	Arg88Gln	IIIA			
										PIK3CA	5.2	3	N	Val344Met	IIIA			
										PTCH1	0.7	3	Y	Ala563Val	NR			
										TP53	4.1	3	N	Tyr234His	NR			

										TSC2	0.3	3	N	Gly1344_Lys1345delinsGlu	NR			
										FGFR1	2.8	3	Y	Arg501His	NR			
59	70	2530	B	LA	3	0	0	N/A	N	N/A	N/A			N/A	N/A	Y	ADC	Tissue

N/A- not available; NR- not reported; B biliary tract; P- pancreatic; E- early stage, LA- locally advanced; M metastatic; VAF- variant allele frequency; VUS- variant of unknown significance; HGVSp- Human Genome Variation Society protein classification.
ADC; adenocarcinoma; ACC; acinar cell carcinoma; OSCC- oesophageal SCC; SCA- serous cystadenoma; PanC- pancreatic cyst

1 treatment based on pre-registration biopsy suspicious of malignancy and imaging
2 Not fit for treatment
3 treatment commenced outside of institution

43.1.6 Clinical Accuracy of ctDNA in the Diagnosis of Pancreatic and Biliary Tract Cancers

The Sn and Sp of ctDNA detection in the diagnosis of patients with suspected PC and BTC was assessed. The binary variable (ctDNA detected or not detected) was compared to the histological confirmation of PC or BTC on subsequent biopsy. This included 13 of 16 patients in PC cohort and 14 of 16 patients in the BTC cohort. Importantly, all patients had an interval of less than 12 weeks between tissue and plasma collection. Of note, 37% of patients across both cohorts had more than 1 biopsy to obtain a tissue diagnosis following an initial non-diagnostic result.

In the PC cohort, the Sn and Sp of ctDNA in the diagnosis of PC was 80% (90% confidence interval [CI] 49.3 – 96.3) and 100% (90% CI 36.8 – 100) respectively, with a positive predictive value (PPV) of 100% (90% CI 68.8 - 100) and negative predictive value (NPV) of 60% (90% CI 18.9 – 92.4). There were 2 false negatives (i.e. ctDNA not detected in those with a histological diagnosis of PC) using a tumour agnostic approach. PC2 had tissue confirmation of PDAC, and tissue-NGS revealed a somatic *BRCA2* mutation which is not covered by the ct-PAED panel, and a germline *MAP2KA* mutation which is removed through the bioinformatic pipeline and not reported in the plasma. PC15 also had histological confirmation of PDAC, and tissue-NGS revealed somatic KRAS variant not detected in plasma.

There were no false positive results (i.e. ctDNA detected in those without a histological diagnosis of PC). 3 of 16 patients did not have a subsequent

biopsy performed and not included in the diagnostic accuracy analysis, however all 3 patients had detectable ctDNA. This included PC1 (*TP53* and *KRAS* mutations detected in plasma) who was treated with systemic chemotherapy based on a pre-registration biopsy showing intra-mucosal adenocarcinoma and radiological features suggestive of PC; PC24 (*PTEN* mutations detected in plasma) treated with systemic chemotherapy without a tissue biopsy (at different institution); and PC40 (*ATM* mutation detected in plasma) deemed unfit for systemic therapy or biopsy. The diagnostic accuracy results for the PC cohort are described in table 45.

Table 44- Diagnostic Accuracy of ctDNA in the Diagnosis of Suspected Pancreatic Cancer

		Histological diagnosis of PC		**Total**
		Yes	**No**	
ctDNA detected	**Yes**	8	0	8
	No	2	3	5
Total		10	3	13
Sensitivity: 80 (90 CI 49.3-96.3) Specificity: 100 (95 CI 36.8-100**) PPV: 100 (95 CI 68.8-100**) NPV: 60 (90 CI 18.9-92.4)				

* 95% confidence interval

The Sn and Sp of ctDNA in the diagnosis of suspected BTC was 70% (90% CI 39.3-91.3) and 75% (90% CI 24.9-98.7) respectively, with 87.5% (90% CI 52.9-99.4) PPV and 50% (90% CI 15.3-84.7) NPV. There was 1 false positive (BTC39) having detectable ctDNA (*PTCH1* and *TP53* Arg282Trp and *TP53* Pro36Leu) and a gallbladder mass biopsy demonstrating benign histology. Further investigations including an FDG-PET revealed a localised

oesophageal mass which was biopsy proven oesophageal squamous cell carcinoma (OSCC), contributing to the ctDNA detected in plasma.

There were 3 false negative results (histological confirmation of BTC with negative ctDNA). BTC 55 and 56 had adequate tumour DNA for tissue NGS. In BTC 56, tissue variants in *APC* and *FANCI* were detected. However, these are not covered, were recorded as a false negative. BTC 55 had mutations in *ARD1A*, *CTNNB1*, *FGFR1* and *TP53* (all covered by ct-PAED panel), however these mutations were not detected in plasma.

A post-registration biopsy was not performed in 2 of 16 patients with suspected BTC, with 1 (BTC 10) having ctDNA detected (*ALK* mutation) and radiological suspicious disease however deemed unfit for treatment. BTC 54 had no ctDNA detected with likely cholangiocarcinoma based on imaging, however, was deemed unfit for a tissue biopsy. The diagnostic accuracy results for the BTC cohort are described in table 46.

Table 45- Diagnostic Accuracy of ctDNA in the Diagnosis of Suspected Biliary Tract Cancer

		Histological diagnosis of cancer		**Total**
		Yes	**No**	
ctDNA detected	**Yes**	7	1	8
	No	3	3	6
Total		10	4	14
Sensitivity: 70 (90 CI 39.3-91.3) Specificity: 75 (90 CI 24.9-98.7) PPV: 87.5 (90 CI 52.9-99.4) NPV: 50 (90 CI 15.3-84.7)				

43.1.7 Genomic Landscape of Plasma and Tissue NGS in PC

Following enrolment, 27 patients underwent invasive biopsy, of which 21 were found to have a diagnosis of cancer including 10 (62.5%) patients in PC cohort and 11 (56.2%) in BTC cohort. All 21 patients had tissue-based NGS testing requested to define the tissue tumoural genomic landscape of patients with suspected PC/BTC. One patient with BTC (BTC 59) had insufficient tumour DNA for tissue based NGS. The ctDNA genomic landscape is described across all ctDNA detected patients (with BTC 39 diagnosed with OSCC was not included in this analysis).

In PC, only patients with histological confirmed malignancy were included in this analysis. This included 10 patients with tNGS of which 8 had plasma variants detected, and a histological diagnosis of PC were included in this analysis. A total of 15 somatic SNVs were detected in the plasma and 35 detected in tissue, with a mean number of somatic alterations detected per patient was 1.9 (15/8) with ctDNA and 3.5 (35/10) with tNGS. No CNV or SV were detected in tissue or plasma.

The most common variants detected were

- *TP53* - 50% in plasma, 50% in tissue
- *KRAS* - 38% in plasma, 80% in tissue
- *ERBB2*- 25% in plasma and 20% in tissue
- *SMAD4*- 20% in tissue (not covered on the ct-PAED panel)

The most common variants detected in both plasma and tissue are shown in Fig 21.

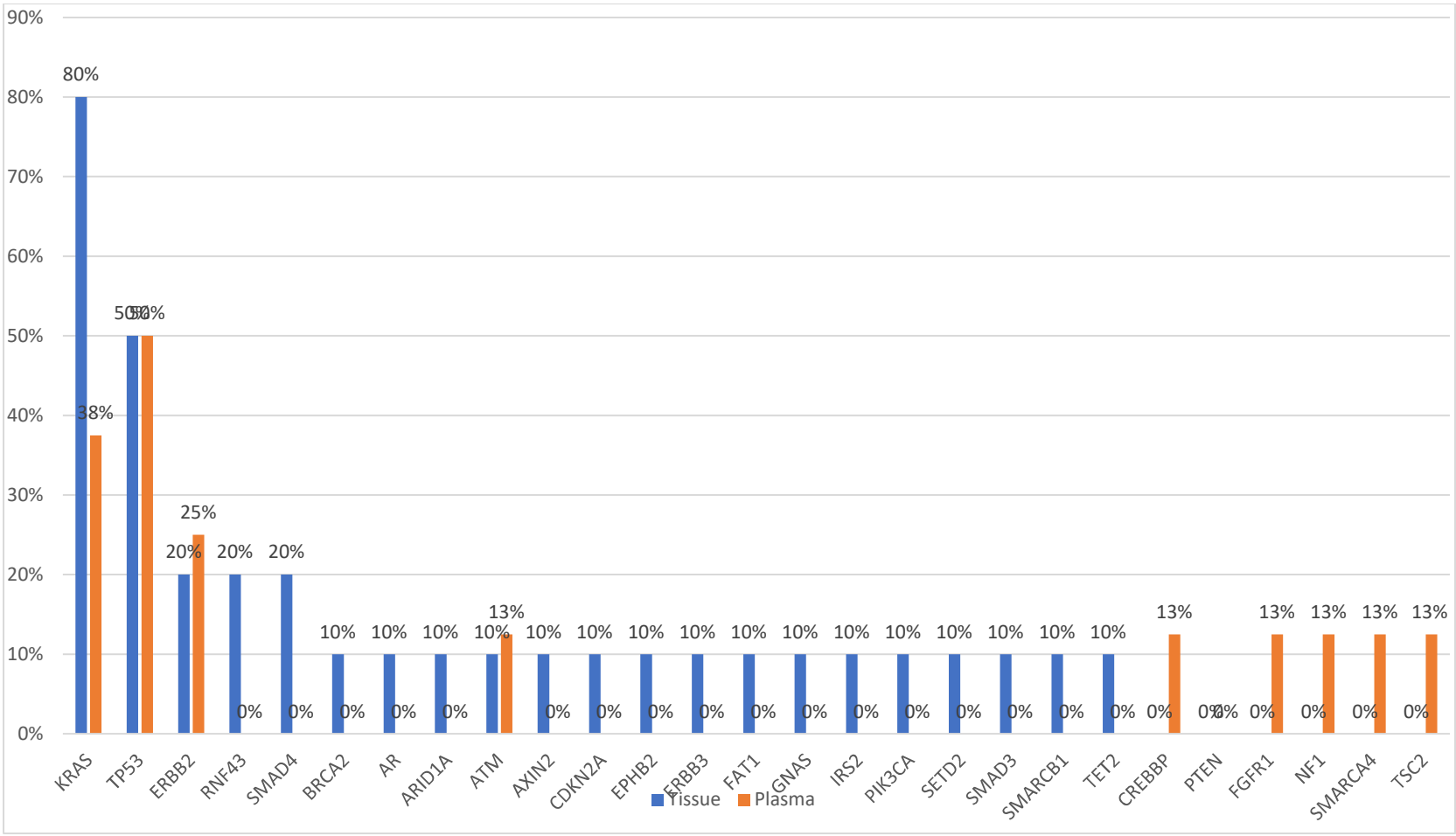


Figure 21- Genomic landscape of suspected PC in ctDNA and Tissue (blue- tissue; orange- plasma).

The results from tissue NGS and corresponding plasma NGS in the PC cohort are shown in table 47. Germline variants detected in tissue were reported (highlighted in blue) but not called in plasma. We detected *KRAS* mutations in 8 samples (all 8 were PDAC). The two *KRAS* wildtype tumours were PC2 (metastatic PDAC). This patient had a somatic *BRCA2* mutation which was a likely driver mutation. PC27 was also KRAS wildtype, with acinar cell carcinoma (ACC) on histology. As expected, tissue NGS revealed a *SMAD4* mutation.

According to ESCAT classification of targetable variants, 90% of patients had at least one targetable mutation detected through tissue NGS (compared to 62.5% in plasma in the corresponding 8 patients). These include *KRAS* and *PIK3CA* somatic variants and *BRCA1* germline variant (in PC23). However, this *BRCA* mutation is considered a benign polymorphism without pathogenicity. As previously described, no tissue harboured a targetable *KRAS* G12C mutation, as such these non-G12C variants are not considered druggable. An important finding of an *RNF43* variant in PC20 may confer sensitivity to Porcupine inhibition.

Table 46- Tissue NGS and ctDNA NGS for PC

FFPE Tissue														ctDNA										ddPCR					
Trial ID	Date taken	Procedure	Cellularity	Tumour content	Necrosis?	Gene	Position	Protein	HGVS	Dep	Alt Dep	Alt Freq	Comments	Date taken	% Dup	% on target	Mean dep	Gene	Position	Protein	HGVS	Dep	Alt Dep	Alt Freq	Comments	Gene	HGVS	Ref pos	Mut pos	FA (%)	ddPCR +VE?
2	21/09/2020	Primary biopsy	Low	<30%	No	BRCA2	chr13:32905159	p.(Ala262Val	c.785C>T	788	70	8.9%		22/06/2020	71.2	95.8	1567	BRCA2	chr13:32905159	p.(Ala262Val	c.785C>T				Not covered						
						MAP2K2	chr19:4123817	p.(Ala19Val	c.56C>T	421	201	48%	Likely germline					MAP2K2	chr19:4123817	p.(Ala19Val	c.56C>T				Not covered						
8	08/07/2020	Primary biopsy	Low	<30%	No	ARID1A	chr1:27105553	p.(Arg1722Ter)	c.5164C>T	511	117	22.9%		01/07/2020	93.8	93.8	1122	ARID1A	chr1:27105553	p.(Arg1722Ter)	c.5164C>T	1178	4	0.34%							
						DAXX	chr6:33287527	p.(Lys524Glu)	c.1570A>G	983	479	48.7%	Likely germline					DAXX	chr6:33287527	p.(Lys524Glu)	c.1570A>G				Not covered						
						KRAS	chr12:25398284	p.(Gly12Asp))	c.35G>A	705	129	18.3%	Defined hotspot					KRAS	chr12:25398284	p.(Gly12Asp))	c.35G>A	1203	0	0.00%		KRAS	c.35G>A	1714	0	0	N
						NOTCH3	chr19:15284887	p.(Glu1576Asp)	c.4728G>C	685	334	48.8%	Likely germline					NOTCH3	chr19:15284887	p.(Glu1576Asp)	c.4728G>C				Not covered						
						TCF3	chr19:1623951	p.(Ser183Leu)	c.548C>T	739	384	52.0%	Likely germline					TCF3	chr19:1623951	p.(Ser183Leu)	c.548C>T				Not covered						
						TP53	chr17:7578265	p.(Ile195Thr)	c.584T>C	604	145	24.0%	Pathogenic variant					TP53	chr17:7578265	p.(Ile195Thr)	c.584T>C	2292	0	0.00%							
																		ERBB2	chr17:37868264	p.(Gln328Lys)	c.985C>A	2325	6	0.26%	No reads in tissue						
																		FGFR1	chr8:38277155	p.(Val425Phe)	c.1273G>T	3565	5	0.14%	No reads in tissue						
																		TSC2	chr16:2129627	p.(Gln1118His)	c.3354G>T	2444	5	0.21%	No reads in tissue						
12	10/09/2020	Primary biopsy	Intermediate	<30%	No	CDKN2A	chr9:21971111	p.(His83Tyr)	c.247C>T	1107	161	14.5%		26/08/2020	89.1	95.0	1183	CDKN2A	chr9:21971111	p.(His83Tyr)	c.247C>T	2327	3	0.13%							
						EPHB2	chr1:23191576	p.(Arg392Cys)	c.1174C>T	769	82	10.7%						EPHB2	chr1:23191576	p.(Arg392Cys)	c.1174C>T				Not covered						
						FAT1	chr4:187509922	p.(Ala4531Thr)	c.13591G>A	1090	530	48.6%	Likely germline					FAT1	chr4:187509922	p.(Ala4531Thr)	c.13591G>A				Not covered						
						KRAS	chr12:25398284	p.(Gly12Asp))	c.35G>A	794	103	13.0%	Defined hotspot					KRAS	chr12:25398284	p.(Gly12Asp))	c.35G>A	1329	1	0.08%	<LOD	KRAS	c.35G>A	1540	0	0	N
						TP53	chr17:7578290	NA)	c.560-1G>T	898	134	14.9%						TP53	chr17:7578290	NA)	c.560-1G>T	2198	2	0.09%							
																		KRAS	chr12:25378647	p.(Lys117Asn))	c.351A>T	1910	6	0.31%	0.3% in tissue						
																		NF1	chr17:29663426	p.(Ala2028Ser)	c.6082G>T	1915	5	0.26%	No reads in tissue						
15	08/10/2020	Unknown	Intermediate	<30%	No	BARD1	chr2:215632255	p.(Val507Met	c.1518_1519inv	198	109	55%	Likely germline	28/09/2020	84.5	92.5	1505	BARD1	chr2:215632255	p.(Val507Met	c.1518_1519inv				Not covered						
						FGFR4	chr5:176520455	p.(Arg434Gly	c.1300C>G	74	46	62%	Confirmed germline					FGFR4	chr5:176520455	p.(Arg434Gly	c.1300C>G	634	321	51%	Confirmed germline variant						
						KRAS	chr12:25398284	p.(Gly12Asp)	c.35G>A	157	9	6%						KRAS	chr12:25398284	p.(Gly12Asp)	c.35G>A	1523	0	0%	negative by ddPCR in buffy coat	KRAS	c.35G>A	290	0	0	N
						PTCH1	chr9:98200387	p.(Pro1384Leu	c.4151C>T	91	47	52%	Confirmed germline					PTCH1	chr9:98200387	p.(Pro1384Leu	c.4151C>T	3510	1680	48%	Confirmed germline variant						
18	29/07/2020	Metastatic biopsy	Low	<30%	No	ARID1B	chr6:157099872	p.(Ser270Cys)	c.809C>G	622	317	51.0%	Likely germline	23/10/2020	86.2	95.1	1752	ARID1B	chr6:157099872	p.(Ser270Cys)	c.809C>G				Not covered						
						ATM	chr11:108198409	p.(Leu2338Pro)	c.7013T>C	1023	68	6.6%						ATM	chr11:108198409	p.(Leu2338Pro)	c.7013T>C	3300	4	0.12%							
						ATM	chr11:108200944	p.(Tyr2437Ter)	c.7311C>A	1118	534	47.8%	Confirmed germline					ATM	chr11:108200944	p.(Tyr2437Ter)	c.7311C>A	1878	892	47.50%	Rare germline; unknown significance						
						FAT1	chr4:187630587	p.(Arg132Pro)	c.395G>C	1396	665	47.6%	Likely germline					FAT1	chr4:187630587	p.(Arg132Pro)	c.395G>C				Not covered						
						IRS2	chr13:110437162	p.(Gly411_Gly413dup)	c.1230_1238dup	672	238	35.4%	Likely germline					IRS2	chr13:110437162	p.(Gly411_Gly413dup)	c.1230_1238dup				Not covered						
						KRAS	chr12:25398284	p.(Gly12Val))	c.35G>T	1146	75	6.5%	Defined hotspots					KRAS	chr12:25398284	p.(Gly12Val))	c.35G>T	1791	5	0.28%	ddPCR negative in buffy coat, 7% in FFPE	KRAS	c.35G>T	1710	5	0.285	Y
						RAD51D	chr17:33428288	p.(Asp299Asn)	c.895G>A	1002	493	49.2%	Likely germline					RAD51D	chr17:33428288	p.(Asp299Asn)	c.895G>A				Not covered						
						SETD2	chr3:47164792	p.(Arg445His)	c.1334G>A	853	358	42.0%	Likely germline					SETD2	chr3:47164792	p.(Arg445His)	c.1334G>A				Not covered						
						SMAD3	chr15:67473708	p.(Pro263Arg)	c.788C>G	889	115	12.9%						SMAD3	chr15:67473708	p.(Pro263Arg)	c.788C>G				Not covered						
						SMAD4	chr18:48575160	p.(Phe119LysfsTer3)	c.355_362del	1247	71	5.7%						SMAD4	chr18:48575160	p.(Phe119LysfsTer3)	c.355_362del				Not covered						
																		TP53	chr17:7578475	p.Pro152Gln	c.455C>A	4288	5	0.12%	Low confidence. 0% in tissue						
20	03/12/2020	Primary biopsy	Low	<30%	No	KMT2D	chr12:49433722	p.(Arg2611Cys)	c.7831C>T	615	298	48.5%	Likely germline	10/11/2020	91.8	95.1	1363	KMT2D	chr12:49433722	p.(Arg2611Cys)	c.7831C>T				Not covered						
						KRAS	chr12:25398284	p.(Gly12Asp))	c.35G>A	845	113	13.4%	Defined hotspots					KRAS	chr12:25398284	p.(Gly12Asp))	c.35G>A	1526	1	0.07%	<LOD	KRAS	c.35G>A	2000	2	0.1	N
						PIK3CA	chr3:178936082	p.(Glu542Lys))	c.1624G>A	1044	109	10.4%	Defined hotspots					PIK3CA	chr3:178936082	p.(Glu542Lys))	c.1624G>A	804	0	0.00%							
						RNF43	chr17:56440922	p.(Phe139Leu)	c.415T>C	357	40	11.2%						RNF43	chr17:56440922	p.(Phe139Leu)	c.415T>C				Not covered						
																		ATM	chr11:108186584	p.(Glu2014Ala)	c.6041A>C	2233	24	1.08%	0.3% in tissue. 0.7% in blood						
																		TP53	chr17:7578242	p.(Val203Met)	c.607G>A	2632	8	0.30%	No reads in tissue						
23	11/12/2020	Primary biopsy	Intermediate	<30%	No	AR	chrX:66942714	p.(Arg832Gln)	c.2495G>A	352	48	13.6%		08/12/2020	83.3	92.5	2312	AR	chrX:66942714	p.(Arg832Gln)	c.2495G>A				Not covered						
						BRCA1	chr17:41256153	p.(Glu143Lys)	c.427G>A	501	263	52.5%	Likely germline					BRCA1	chr17:41256153	p.(Glu143Lys)	c.427G>A				Not covered						
						KRAS	chr12:25398284	p.(Gly12Val))	c.35G>T	591	60	10.2%	Defined hotspots					KRAS	chr12:25398284	p.(Gly12Val))	c.35G>T	2589	0	0.00%		KRAS	c.35G>T	3475	3	0.08	N
						NOTCH1	chr9:139399458	p.(Ala1562Val)	c.4685C>T	274	135	49.3%	Likely germline					NOTCH1	chr9:139399458	p.(Ala1562Val)	c.4685C>T				Not covered						
						TP53	chr17:7577548	p.(Gly245Ser)	c.733G>A	395	42	10.6%	Pathogenic variant					TP53	chr17:7577548	p.(Gly245Ser)	c.733G>A	3082	0	0.00%							
																		TP53	chr17:7579590	p.(Ser33Pro)	c.97T>C	4286	5	0.12%	No reads in tissue						
27	02/02/2021	Primary biopsy	Low	<30%	No	ERBB3	chr12:56495091	p.(Gly1150Arg)	c.3448G>C	354	24	6.8%		20/01/2021	90.8	89.6	1898	ERBB3	chr12:56495091	p.(Gly1150Arg)	c.3448G>C				Not covered						
						GNAS	chr20:57484420	p.(Arg844Cys)	c.2530C>T	471	62	13.2%						GNAS	chr20:57484420	p.(Arg844Cys)	c.2530C>T				Not covered						
						SMAD4	chr18:48593406	p.(Gly386Val)	c.1157G>T	328	28	8.5%						SMAD4	chr18:48593406	p.(Gly386Val)	c.1157G>T				Not covered						
						SMARCB1	chr22:24133968	p.(Arg40Gln)	c.119G>A	384	22	5.7%						SMARCB1	chr22:24133968	p.(Arg40Gln)	c.119G>A	3324	3	0.09%							
																		CREBBP	chr16:3778903	p.(Ala2049Thr)	c.6145G>A	3754	5	0.13%	1 read in tissue						
																		SMARCA4	chr19:1096021	p.(Arg997Trp)	c.2995C>T	4299	5	0.12%	No reads in tissue						
28	29/01/2021	Primary biopsy	Intermediate	30-50%	No	CHEK2	chr22:29121337	p.(Tyr156Cys)	c.467A>G	493	254	51.5%	Likely germline	26/01/2021	88.2	94.5	1700	CHEK2	chr22:29121337	p.(Tyr156Cys)	c.467A>G				Not covered						
						KRAS	chr12:25398284	p.(Gly12Val))	c.35G>T	681	196	28.8%	Defined hotspots					KRAS	chr12:25398284	p.(Gly12Val))	c.35G>T	1833	23	1.26%		KRAS	c.35G>T	2196	10	0.44	Y
						MAP2K4	chr17:11924249	p.(Ser16Arg)	c.46A>C	268	147	54.9%	Likley germline					MAP2K4	chr17:11924249	p.(Ser16Arg)	c.46A>C				Not covered						
						RNF43	chr17:56448310	p.(Arg113Ter)	c.337C>T	297	80	26.9%						RNF43	chr17:56448310	p.(Arg113Ter)	c.337C>T				Not covered						
						RUNX1	chr21:36259204	p.(Asn96Ser)	c.287A>G	517	249	48.2%	Likely germline					RUNX1	chr21:36259204	p.(Asn96Ser)	c.287A>G				Not covered						
						TET2	chr4:106164779	p.(Arg1216Gln)	c.3647G>A	857	138	16.1%						TET2	chr4:106164779	p.(Arg1216Gln)	c.3647G>A				Not covered						
						TP53	chr17:7578406	p.(Arg175His)	c.524G>A	463	99	21.4%	Pathogenic variant					TP53	chr17:7578406	p.(Arg175His)	c.524G>A	3939	16	0.41%							
38	22/04/2021	Primary biopsy	Intermediate	30-50%	No	AXIN2	chr17:63554686	p.(Arg18His)	c.53G>A	160	19	11.9%		09/04/2021	88.2	95.4	1996	AXIN2	chr17:63554686	p.(Arg18His)	c.53G>A				Not covered						
						KRAS	chr12:25398284	p.(Gly12Asp))	c.35G>A	250	41	16.4%	Defined hotspots					KRAS	chr12:25398284	p.(Gly12Asp))	c.35G>A	1977	1	0.05%	<LOD	KRAS	c.35G>A	1870	1	0.05	N
						MAP3K1	chr5:56177587	p.(Arg854Cys)	c.2560C>T	211	130	61.6%	Likely germline					MAP3K1	chr5:56177587	p.(Arg854Cys)	c.2560C>T				Not covered						
						TP53	chr17:7579546	p.(Pro47ArgfsTer76)	c.140del	105	22	21.0%	Pathogenic variant					TP53	chr17:7579546	p.(Pro47ArgfsTer76)	c.140del	4168	4	0.10%							
																		ERBB2	chr17:37872108	p.(Val477Met)	c.1429G>A	4535	23	0.51%	No reads in tissue						

Blue: Germline variant in tissue, unlikely to be a somatic variant; Confirmed germline in buffy coats sequencing; likely germline >0.00001% on gnomAD. Grey: Variant in tissue not covered by ctPAED panel; Red: Variant in tissue not detected in plasma; Yellow: Variant in tissue detected in plasma, but not called blind and below the tumour agnostic cut-off; Green: Variant in tissue detected in plasma and reported blind; Purple: Variant reported in plasma, but not detected in tissue above the cut-off; White: pathogenic variant in tissue

43.1.8 Concordance Between Tissue and Plasma Based Next Generation Sequencing in PC

A concordance analysis was performed on patients who had a histological diagnosis of PC, successful tissue NGS and variants detected across both assays. The by-patient analysis included all patients with a histological diagnosis of PC with successful tissue NGS analysis, with tissue variants detected covered by the ct-PAED panel. All germline variants and mutations not covered by both panels were removed from the by variant analysis. Out of 10 patients with a histological diagnosis of PC who underwent tissue-based NGS, 9 fulfilled these criteria (PC2 excluded as tissue variants detected were not covered by ctPAED panel).

In this cohort, 18 variants were detected by tissue (83.3% [n=15] tissue only) and 15 variants detected by plasma (80% [n=12] plasma only). Partial concordance by patient was 22% (2/9) using a tumour agnostic approach, and 67% (6/9) using a tumour informed approach. Concordance by variant between somatic tissue and plasma alterations was 16.7% (3/18) for variants called blind, which increased to 50% (9/18) when calling additional variants retrospectively using a tumour informed approach. 40% (12/30) of all variants detected were only detected in plasma, suggesting genomic heterogeneity in tissue.

8 of 9 patients had a detectable *KRAS* mutation in tissue, of which 2 were also detected in plasma (25%). Noting low numbers, concordance for *KRAS* mutation was 100% in those with metastatic disease, and 0% in early stage

and locally advanced disease. PC12 had a *KRAS* G12D mutation detected in tissue (not detected in plasma), however a plasma detected *KRAS* K1117N was not detected in tissue.

The poor concordance between plasma and tissue for *KRAS* mutations was further assessed. I looked at differences in the sequencing depth between tissue-NGS using the RMH200 panel and plasma NGS using the ct-PAED panel. The mean sequencing depth of all genes was 651.3 with tNGS and 2563.3 with ctDNA sequencing. Interestingly, the mean KRAS gene sequencing depth was 646.1 with tNGS and 1742.3 with ctDNA, suggesting the ct-PAED panel sequencing depth of *KRAS* is lower than the depth in tissue NGS.

Concordance between tissue and plasma NGS in the PC cohort is described in Fig 22.

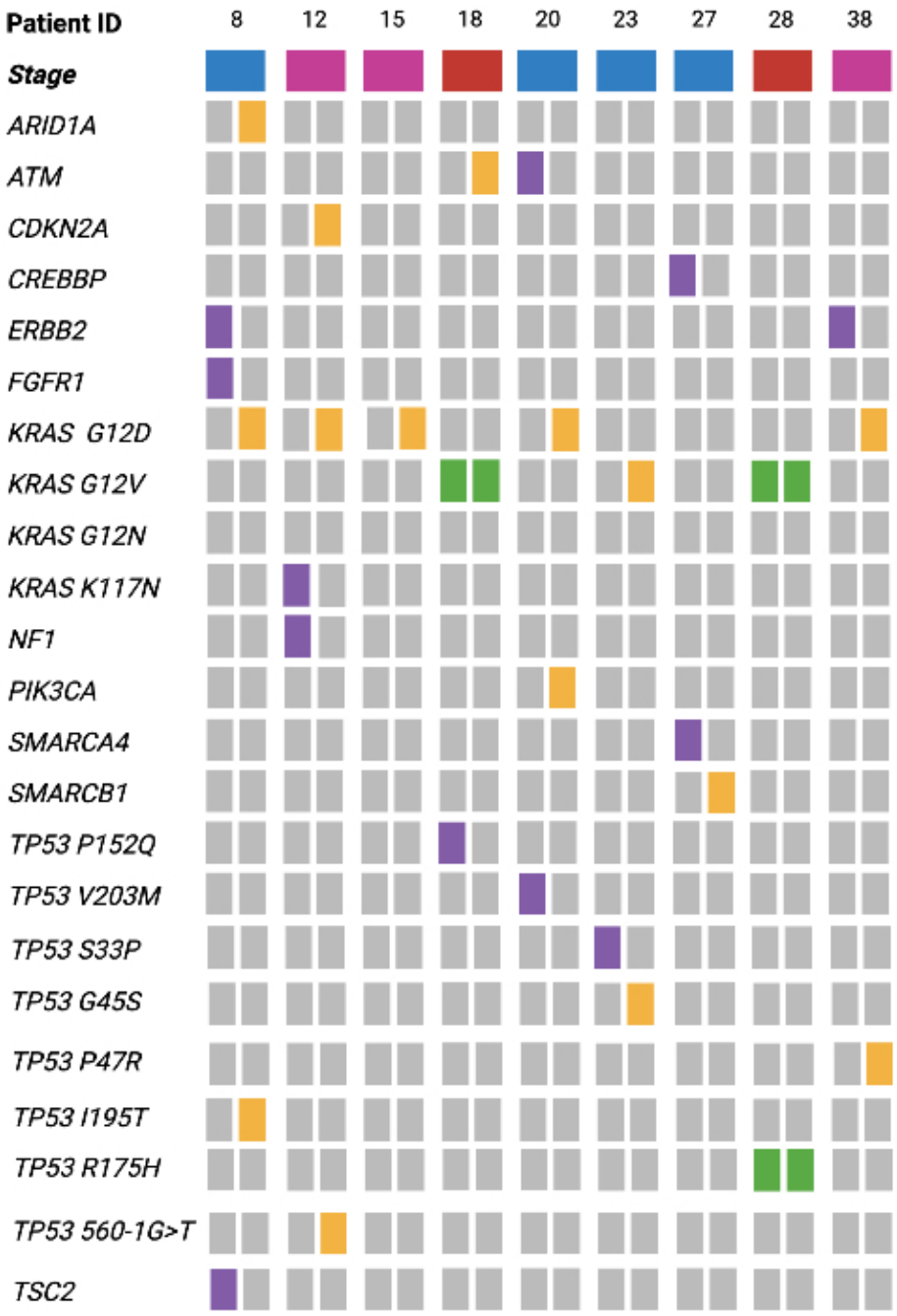


Figure 22- Concordance between tissue and plasma based NGS in PC cohort

Stage: blue (early stage), pink (locally advanced), red (metastatic)
Mutations: concordant (green), tissue only (yellow), plasma only (purple).

43.1.9 KRAS ddPCR Results

To further explore the low concordance between tissue and plasma-based NGS in detecting *KRAS* mutations, we performed ddPCR targeting the specific KRAS gene locus on the cfDNA library preparations. Concordance was 100% between cfDNA NGS and cfDNA ddPCR (2/2 positive by both ddPCR and plasma NGS and 6/6 negative by both ddPCR and plasma NGS). Even at high sequencing depths, KRAS concordance between tissue and plasma was 25%. Table 48 describes the KRAS concordance between tissue NGS, plasma NGS and plasma ddPCR. Figure 23 shows the KRAS variant detected in plasma using ddPCR and tissue on FFPE, but negative in the buffy coat for PC18.

Table 47- Concordance between tissue NGS, plasma NGS and plasma ddPCR for KRAS Mutation

	KRASm Detected		
ID	**Tissue**	**Plasma (NGS)**	**Plasma (ddPCR)**
8	+	-	-
12	+	-	-
15	+	-	-
18	+	+	+
20	+	-	-
23	+	-	-
28	+	+	+
38	+	-	-

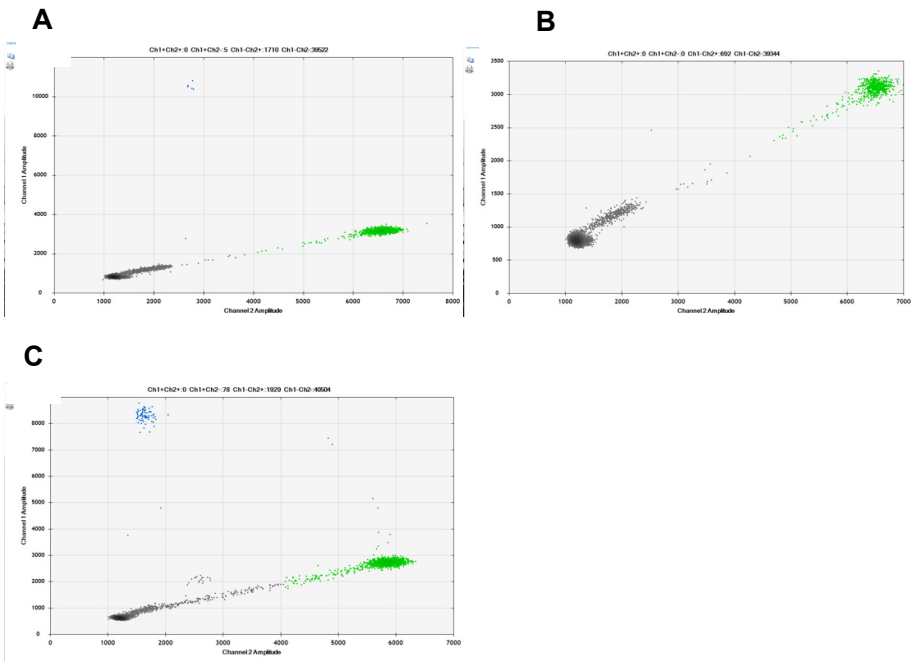


Figure 23- ddPCR for PC18 (A) Plasma ddPCR KRAS G12V positive (B) ddPCR of buffy coat showing no KRAS mutant copies detected (C) ddPCR on FFPE positive for KRAS G12V

43.1.10 Genomic Landscape of Plasma and Tissue NGS in BTC

In BTC, 11 patients were found to have a histological diagnosis of cancer. BTC 59 had insufficient tumour DNA for tissue based NGS. BTC 39 was diagnosed with oesophageal SCC and not included in this analysis. Therefore, 9 patients with tNGS and 7 plasma detected variants and a histological diagnosis of BTC were included in this analysis.

A total of 22 somatic variants were detected in plasma and 66 somatic variants detected in tissue, with a mean number of somatic variants detected per patient of 3.1 (22/7) in ctDNA and 7.3 (66/9) in tissue.

The most common variants detected were

- *ARID1A* - 33% in plasma, 67% in tissue
- *TP53* - 33% in tissue and plasma

The variants detected in both plasma and tissue are shown in fig 24.

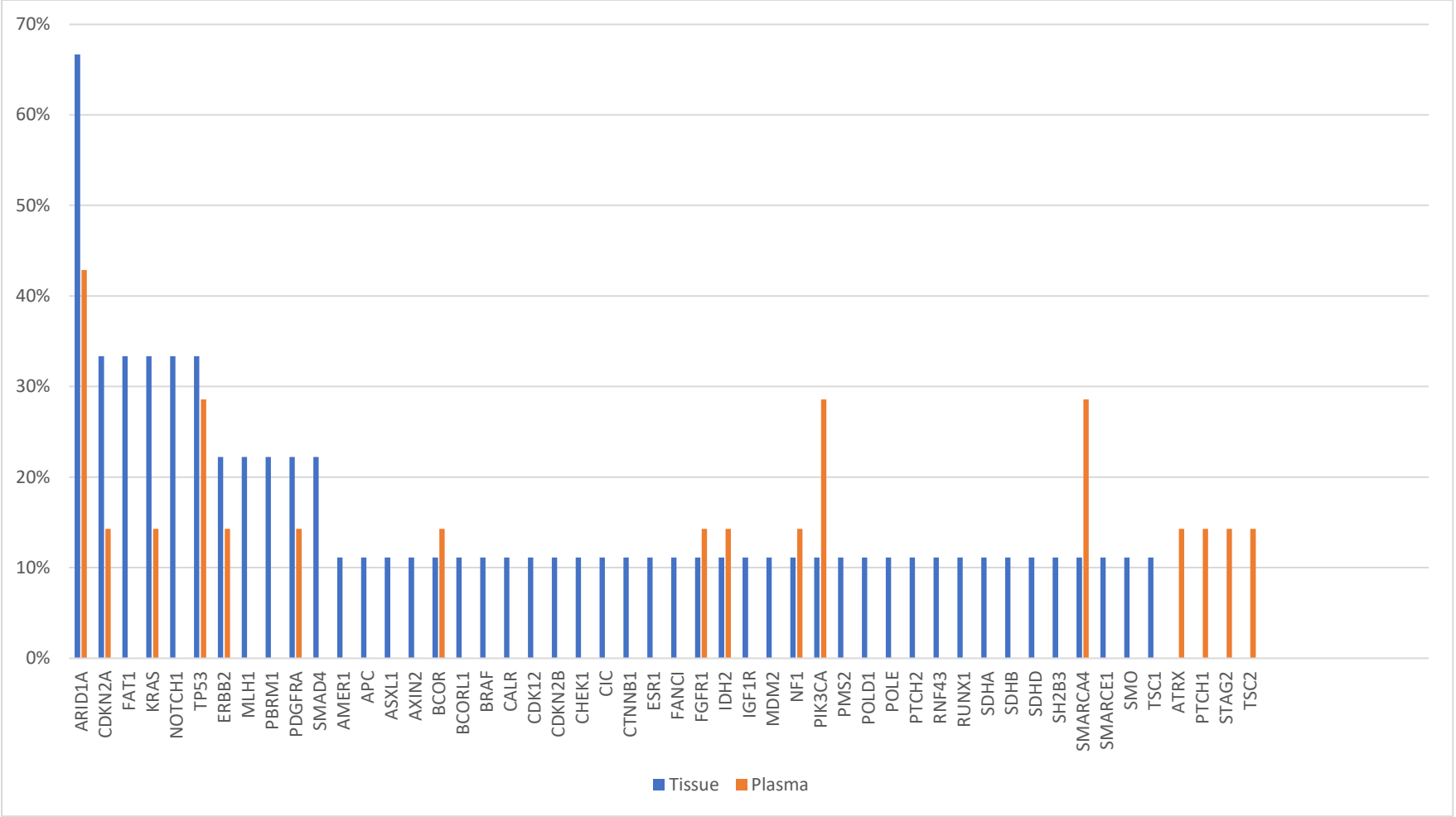


Figure 24- Genomic landscape of suspected BTC in ctDNA and Tissue (blue- tissue; orange- plasma)

The results from tissue NGS and corresponding plasma NGS in the BTC cohort are shown in fig 25. Germline variants detected in tissue were reported (highlighted in blue) which were not called in plasma.

5 structural variants and 5 amplifications were detected in tissue, which were not detected in plasma. This included SV in *CDK2NA* (BTC11), *KRAS* (BTC31), *MDM2* and *KRAS* amplifications (BTC53), amplifications in *CDK12*, *ERBB2* and *SMARCE1* (BTC58), and deletions in *CDK2NA*/B and *TSC1* (BTC55).

We detected the following potentially targetable variants in tissue;

- *BRAF* V600E (BTC4) ESCAT IIB
- *HER2* SNV (BTC34, BTC 39) IIIA
- MSI-H- BTC 53 (*MLH1*), BTC58 (*MLH1*, *PMS2*)- IC
- *HER2* amplification (BTC58)- IIIA

Overall, 11.1 (1/9) and 55% (5/9) of patients had at least one targetable mutation detected based on ESCAT in plasma and tissue, respectively. We detected MSH-H using tissue NGS in 2 patients including mutation in *MLH1* (n=2) and *PMS2* (n=1) which are not covered by the ct-PAED panel. BTC 53 had loss of *MLH1*/*PMS2* on IHC and was treated with nivolumab. BTC 58 had normal expression of MLH1 and PMS2 on IHC, but mutations detected on tissue NGS.

Two of three patients without ctDNA detected but a histological diagnosis of BTC had successful tissue NGS performed. One patient (BTC 56) had SNVs

in *APC* and *FANCI* which are not covered by the ct-PAED panel. Another patient (BTC 55) had SNVs in *ARID1A*, *CTNNB1*, *FAT1*, *FGFR1*, *IGR1*, *SMAD4*, and *TP53* detected in tissue but not plasma. The FGFR1 seen at VAF of 52% in plasma was detected in the buffy coat and not reported as a somatic SNV.

Figure 25- Tissue NGS and ctDNA NGS for BTC

Tissue

Trial ID	Date taken	Diagnosis	Procedure	Cellularity	Tumour content	Necrosis?	Gene	Position	Protein	HGVS	Dep	Alt Dep	Alt Freq	Comments
4	7/9/20	Hilar CCC	Biopsy primary	Intermediate	30-50%	No	AMER1	chrX:63411179	p.(Met656Ter)	c.1966_1987del	414	44	10.63%	
							BRAF	chr7:140453136	p.(Val600Glu))	c.1799T>A	897	52	5.80%	Defined hotspots
							CHEK1	chr11:125503118	p.(Arg162His)	c.485G>A	648	327	50.46%	Likely germline
							RUNX1	chr21:36171704	p.(Gln283_Tyr287delinsHis)	c.849_860del	568	51	8.98%	
11	14/10/20	Distal CCC	Resection primary	Intermediate	30-50%	No	SDHD	chr11:111959690	p.(Ala90Val)	c.269C>T	733	44	6.00%	
							TP53	chr17:7578442	p.(Tyr163Cys)	c.488A>G	500	29	5.80%	Likely pathogenic variant
							CDKN2A	chr9:21970962	delCGCGCGCAGGTACCGTGCGACATCGCGATG					Potential structural variant
29	1/3/21	Intra-hepatic CCC	Resection primary	Intermediate	30-50%	No	FLT3	chr13:28631516	p.(Asn151Ser)	c.452A>G	2004	978	48.80%	Likely germline
31	5/2/21	Intra-hepatic CCC	Primary biopsy	High	>50%	No	IDH2	chr15:90631839	p.(Arg172Trp))	c.514A>T	503	106	21.07%	Defined hotspots
							PDGFRA	chr4:55156495	p.(His966Asn)	c.2896C>A	756	156	20.64%	
							KRAS	chr12:25403701	insGAGCCGCTGAGCCTCTGGCC					Potential structural variant
34	13/5/21	Gallbladder ADC	Primary biopsy	Intermediate	<30%	No	ATM	chr11:108196216	p.(Lys2253LeufsTer2)	c.6757_6763del	469	212	45.20%	Likely germline
							FAT1	chr4:187521157	p.(Ile3999MetfsTer13)	c.11997del	343	27	7.87%	
							POLD1	chr19:50902154	p.(Lys16Glu)	c.46A>G	198	113	57.07%	Likely germline
39	6/5/21	Oesophageal SCC	Primary biopsy	Intermediate	30-50%	No	BCORL1	chrX:129154981	p.(Pro1155Thr)	c.3463C>A	1132	524	46.29%	Likely germline
							ERBB2	chr17:37883750	p.(Ser1122Ter)	c.3365_3366del	592	81	13.68%	
							ERBB3	chr12:56478902	p.(Met120Val)	c.358A>G	851	77	9.05%	
							IDH2	chr15:90628267	p.(Arg382Trp)	c.1144C>T	714	71	9.94%	
							KMT2C	chr7:151900066	p.(Arg1349Ter)	c.4045C>T	1760	262	14.89%	
							KMT2D	chr12:49431721	p.(Lys3140ArgfsTer2)	c.9417del	786	101	12.85%	
							MDM4	chr1:204518259	p.(Glu308Lys)	c.922G>A	1034	265	25.63%	
							MLH1	chr3:37083790	p.(Asp567ValfsTer24)	c.1700del	844	151	17.89%	
							MSH6	chr2:48030590	p.(Gly1070ValfsTer9)	c.3209del	962	136	14.14%	
							MSH6	chr2:48030639	p.(Phe1088LeufsTer5)	c.3254dup	1072	133	12.41%	
							NF1	chr17:29552221	p.(Arg652Cys)	c.1954C>T	874	421	48.17%	Likely germline
							NFE2	chr12:54686430	p.(Arg284Cys)	c.850C>T	1143	148	12.95%	
							PAX5	chr9:37002716	p.(Ser178Leu)	c.533C>T	728	92	12.64%	
							SDHA	chr5:236541	NA)	c.1261-2A>G	485	67	13.81%	
							TET2	chr4:106196213	p.(Arg1516Ter)	c.4546C>T	776	150	19.33%	
							TP53	chr17:7577094	p.(Arg282Trp)	c.844C>T	1086	325	29.93%	
							TP53	chr17:7579580	p.(Pro36Leu)	c.107C>T	745	113	15.17%	

Trial ID	Date taken	% Dup	% on target	Mean dep	GENE_NAME	POSITION	PROTEIN	HGVS	TUMOR. DEPTH	TUMOR.ALT DEPTH	TUMOR.ALT FREQ	Comments
4	24/6/20	69.8	95.8	1444	AMER1	chrX:63411179	p.(Met656Ter)	c.1966_1987del	1443	0	0.00%	
					BRAF	chr7:140453136	p.(Val600Glu))	c.1799T>A	2160	2	0.09%	
					CHEK1	chr11:125503118	p.(Arg162His)	c.485G>A				Not covered
					RUNX1	chr21:36171704	p.(Gln283_Tyr287delinsHis)	c.849_860del				Not covered
					ATRX	chrX:76849231	p.(Glu2015Asp)	c.6045G>T	869	9	1.04%	0.9% in tissue
11	19/8/20	89.8	95.4	1404	SDHD	chr11:111959690	p.(Ala90Val)	c.269C>T				Not covered
					TP53	chr17:7578442	p.(Tyr163Cys)	c.488A>G	3436	0	0.00%	
					CDKN2A	chr9:21970962		delCGCGCGCAGGTACCGTGCGACATCGCGATG	3359	0	0.00%	
					STAG2	chrX:123197864	p.(Asp663Gly)	c.1988A>G	2556	24	0.94%	0.08% in tissue
					TP53	chr17:7577093	p.(Arg282Gln)	c.845G>A	2986	9	0.30%	0.3% in tissue
29	22/1/21	89.3	90.2	1537	FLT3	chr13:28631516	p.(Asn151Ser)	c.452A>G				Not covered
					SMARCA4	chr19:11100004	p.(Arg377His)	c.1130G>A	2737	5	0.18%	0.08% in tissue
31	5/2/21	70.8	91.5	1777	IDH2	chr15:90631839	p.(Arg172Trp))	c.514A>T	3698	24	0.65%	
					PDGFRA	chr4:55156495	p.(His966Asn)	c.2896C>A				Not covered
					KRAS	chr12:25403701		insGAGCCGCTGAGCCTCTGGCC	1500	>153	>10%	Not called by structural variant caller
					TP53	chr17:7577121	p.(Arg273Cys)	c.817C>T	3573	13	0.36%	0.3% in tissue
34	12/3/21	71.9	96.0	1746	ATM	chr11:108196216	p.(Lys2253LeufsTer2)	c.6757_6763del	1652	649	39.29%	Confirmed likely germline
					FAT1	chr4:187521157	p.(Ile3999MetfsTer13)	c.11997del				Not covered
					POLD1	chr19:50902154	p.(Lys16Glu)	c.46A>G				Not covered
					ARID1A	chr1:27087534	p.(Pro703Leu)	c.2108C>T	3417	102	2.99%	3.5% in tissue
39	14/4/21	88.5	95.0	1930	BCORL1	chrX:129154981	p.(Pro1155Thr)	c.3463C>A				Not covered
					ERBB2	chr17:37883750	p.(Ser1122Ter)	c.3365_3366del	4560	0	0.00%	
					ERBB3	chr12:56478902	p.(Met120Val)	c.358A>G				Not covered
					IDH2	chr15:90628267	p.(Arg382Trp)	c.1144C>T				Not covered
					KMT2C	chr7:151900066	p.(Arg1349Ter)	c.4045C>T				Not covered
					KMT2D	chr12:49431721	p.(Lys3140ArgfsTer2)	c.9417del				Not covered
					MDM4	chr1:204518259	p.(Glu308Lys)	c.922G>A				Not covered
					MLH1	chr3:37083790	p.(Asp567ValfsTer24)	c.1700del				Not covered
					MSH6	chr2:48030590	p.(Gly1070ValfsTer9)	c.3209del				Not covered
					MSH6	chr2:48030639	p.(Phe1088LeufsTer5)	c.3254dup				Not covered
					NF1	chr17:29552221	p.(Arg652Cys)	c.1954C>T	2776	1310	47.19%	Rare germline; unknown significance
					NFE2	chr12:54686430	p.(Arg284Cys)	c.850C>T				Not covered
					PAX5	chr9:37002716	p.(Ser178Leu)	c.533C>T				Not covered
					SDHA	chr5:236541	NA)	c.1261-2A>G				Not covered
					TET2	chr4:106196213	p.(Arg1516Ter)	c.4546C>T				Not covered
					TP53	chr17:7577094	p.(Arg282Trp)	c.844C>T	3715	65	1.75%	
					TP53	chr17:7579580	p.(Pro36Leu)	c.107C>T	3401	19	0.56%	
					PTCH1	chr9:98212155	p.(Val1173Met)	c.3517G>A	2977	12	0.40%	0% in tissue

53	12/5/21	Hilar CCC	Primary biopsy	Intermediate	30-50%	Yes	ARID1A	chr1:27089730	p.(Glu898Ter)	c.2686G>T	1299	254	19.55%		13/7/21	79.6	90.3	1035	ARID1A	chr1:27089730	p.(Glu898Ter)	c.2686G>T	2094	58	2.67%	
							ARID1A	chr1:27101416	p.(Pro1568LeufsTer44)	c.4703del	811	131	16.15%						ARID1A	chr1:27101416	p.(Pro1568LeufsTer44)	c.4703del	521	4	0.77%	Homopolymer >4
							ARID1A	chr1:27102070	p.(Glu1668GlyfsTer30)	c.5000dup	1151	80	6.95%						ARID1A	chr1:27102070	p.(Glu1668GlyfsTer30)	c.5000dup	1809	9	0.50%	
							ARID1B	chr6:157522359	p.(Pro1544Leu)	c.4631C>T	985	595	60.41%	Likely germline					ARID1B	chr6:157522359	p.(Pro1544Leu)	c.4631C>T				Not covered
							AXIN2	chr17:63533761	p.(Arg465Cys)	c.1393C>T	1082	227	20.98%						AXIN2	chr17:63533761	p.(Arg465Cys)	c.1393C>T				Not covered
							BCOR	chrX:39932570	p.(Gly677Ter)	c.2029G>T	606	266	43.89%						BCOR	chrX:39932570	p.(Gly677Ter)	c.2029G>T	1119	45	4.02%	
							CALR	chr19:13054649	p.(Glu398_Asp400del)	c.1191_1199del	1238	200	16.16%						CALR	chr19:13054649	p.(Glu398_Asp400del)	c.1191_1199del				Not covered
							CDKN2A	chr9:21971120	p.(Arg80Ter)	c.238C>T	911	266	29.20%						CDKN2A	chr9:21971120	p.(Arg80Ter)	c.238C>T	2092	41	1.96%	
							KRAS	chr12:25398284	p.(Gly12Asp))	c.35G>A	1523	324	21.27%	Defined hotspots					KRAS	chr12:25398284	p.(Gly12Asp))	c.35G>A	1275	18	1.41%	
							MLH1	chr3:37045935	p.(Thr117Met)	c.350C>T	1183	741	62.64%	Likely germline					MLH1	chr3:37045935	p.(Thr117Met)	c.350C>T				Not covered
							NF1	chr17:29670073	p.(Cys2371IlefsTer20)	c.7111_7126del	1689	283	16.76%						NF1	chr17:29670073	p.(Cys2371IlefsTer20)	c.7111_7126del	1692	17	1.01%	
							NOTCH1	chr9:139396277	p.(Arg1854His)	c.5561G>A	810	206	25.43%						NOTCH1	chr9:139396277	p.(Arg1854His)	c.5561G>A				Not covered
							PBRM1	chr3:52682399	p.(Asn258MetfsTer25)	c.773del	1158	296	25.61%						PBRM1	chr3:52682399	p.(Asn258MetfsTer25)	c.773del				Not covered
							PDGFRA	chr4:55144552	p.(Tyr676His)	c.2026T>C	1556	297	19.09%						PDGFRA	chr4:55144552	p.(Tyr676His)	c.2026T>C	1167	30	2.57%	
							POLE	chr12:133226040	p.(Arg1286His)	c.3857G>A	945	372	39.37%						POLE	chr12:133226040	p.(Arg1286His)	c.3857G>A				Not covered
							PTCH2	chr1:45297429	p.(Leu189SerfsTer50)	c.565del	1050	55	5.24%						PTCH2	chr1:45297429	p.(Leu189SerfsTer50)	c.565del				Not covered
							SMARCA4	chr19:11130307	p.(Arg849Gln)	c.2546G>A	1143	224	19.60%						SMARCA4	chr19:11130307	p.(Arg849Gln)	c.2546G>A	2211	33	1.49%	
							MDM2			Amplification				MDM2 amplified					MDM2			Amplification					Not covered
							KRAS			Gain				Possible KRAS gain					KRAS			Gain					Not detected (not validated)
55	27/9/21	Gallbladder ADC	Resection primary	Intermediate	50%	Yes	ARID1A	chr1:27099089	p.(His1170GlnfsTer22	c.3510_3511del	574	444	77.35%		20/8/21	78.9	98.3	2231	ARID1A	chr1:27099089	p.(His1170GlnfsTer22	c.3510_3511del	4248	0	0%	
							CTNNB1	chr3:41266137	p.(Ser45Phe	c.134C>T	1066	488	45.78%						CTNNB1	chr3:41266137	p.(Ser45Phe	c.134C>T	3957	1	0%	
							FAT1	chr4:187524780	p.(Val3634Ile	c.10900G>A	1036	523	50.48%						FAT1	chr4:187524780	p.(Val3634Ile	c.10900G>A	0	0		Not covered
							FGFR1	chr8:38271208	p.(Glu834Lys	c.2500G>A	552	204	36.96%	Confirmed germline					FGFR1	chr8:38271208	p.(Glu834Lys	c.2500G>A	4383	2282	52%	Germline, confirmed in blood
							IGF1R	chr15:99434850	p.(Arg313Ser	c.937C>A	784	398	50.77%						IGF1R	chr15:99434850	p.(Arg313Ser	c.937C>A	0	0		Not covered
							SMAD4	chr18:48604764	p.(His530ThrfsTer47	c.1587dup	853	689	80.77%						SMAD4	chr18:48604764	p.(His530ThrfsTer47	c.1587dup	0	0		Not covered
							TP53	chr17:7579528	p.(Trp53Ter	c.159G>A	392	309	78.83%						TP53	chr17:7579528	p.(Trp53Ter	c.159G>A	5157	0	0%	
							CDKN2A							Exons 1,1a-3,3a Deletion					CDKN2A								No deletion
							CDKN2B							Exons 1-2 Deletion					CDKN2B								No deletion
							TSC1							Exons 3-23 Deletion					TSC1								No deletion
56	29/9/21	Distal CCC	Resection primary	Low	<30%	No	APC	chr5:112173949	p.(Gln888His	c.2658G>T	1142	564	49.39%		26/8/21	74.4	97.0	1742	APC	chr5:112173949	p.(Gln888His	c.2658G>T	0	0		Not covered
							FANCI	chr15:89801943	p.(Leu33del	c.96_98del	990	460	46.47%						FANCI	chr15:89801943	p.(Leu33del	c.96_98del	0	0		Not covered
58	17/9/21	Gallbladder ADC	Primary biopsy	Low	<30%	Yes	ARID1A	chr1:27024001	p.(Gln372AlafsTer28)	c.1113dup	158	66	41.77%		27/8/21	61.4	97.0	2059	ARID1A	chr1:27024001	p.(Gln372AlafsTer28)	c.1113dup	2098	55	2.62%	Homopolymer
							ARID1A	chr1:27089673	p.(Pro877Ser)	c.2629C>T	360	149	41.39%						ARID1A	chr1:27089673	p.(Pro877Ser)	c.2629C>T	4679	142	3.04%	
							ASXL1	chr20:31022441	p.(Gly645ValfsTer58)	c.1934del	392	164	41.84%						ASXL1	chr20:31022441	p.(Gly645ValfsTer58)	c.1934del				Not covered
							BCORL1	chrX:129190010	p.(Pro1681GlnfsTer20)	c.5042del	282	110	39.01%						BCORL1	chrX:129190010	p.(Pro1681GlnfsTer20)	c.5042del				Not covered
							CHEK1	chr11:125507434	p.(Lys270Arg)	c.809A>G	426	206	48.36%						CHEK1	chr11:125507434	p.(Lys270Arg)	c.809A>G				Not covered
							CIC	chr19:42796906	p.(Val1122Met)	c.3364G>A	237	194	81.86%						CIC	chr19:42796906	p.(Val1122Met)	c.3364G>A				Not covered
							ERBB2	chr17:37880220	p.(Leu755Ser)	c.2264T>C	816	113	13.85%						ERBB2	chr17:37880220	p.(Leu755Ser)	c.2264T>C	3295	61	1.85%	
							ESR1	chr6:152415650	p.(Gln500His)	c.1500G>C	363	144	39.67%						ESR1	chr6:152415650	p.(Gln500His)	c.1500G>C				Not covered
							FAT1	chr4:187549379	p.(Val1580Ala)	c.4739T>C	383	138	36.03%						FAT1	chr4:187549379	p.(Val1580Ala)	c.4739T>C				Not covered
							FGFR1	chr8:38275767	p.(Arg501His)	c.1502G>A	287	106	36.93%						FGFR1	chr8:38275767	p.(Arg501His)	c.1502G>A	4087	130	2.77%	
							MLH1	chr3:37090416	p.(Cys672MetfsTer3)	c.2013dup	237	160	67.51%						MLH1	chr3:37090416	p.(Cys672MetfsTer3)	c.2013dup				Not covered
							NOTCH1	chr9:139390966	p.(Gln2409Ter)	c.7225C>T	439	100	22.78%						NOTCH1	chr9:139390966	p.(Gln2409Ter)	c.7225C>T				Not covered
							NOTCH1	chr9:139391170	p.(Ser2341ProfsTer7)	c.7020del	357	96	26.89%						NOTCH1	chr9:139391170	p.(Ser2341ProfsTer7)	c.7020del				Not covered
							NTRK2	chr9:87563405	p.(Arg598His)	c.1793G>A	252	82	32.54%	Likely germline					NTRK2	chr9:87563405	p.(Arg598His)	c.1793G>A				Not covered
							PBRM1	chr3:52584654	NA)	c.4360-3_4360-2del	210	134	63.81%						PBRM1	chr3:52584654	NA)	c.4360-3_4360-2del				Not covered
							PIK3CA	chr3:178921548	p.(Val344Met)	c.1030G>A	376	283	75.27%						PIK3CA	chr3:178921548	p.(Val344Met)	c.1030G>A	1660	86	5.18%	
							PMS2	chr7:6026820	p.(Asp526Asn)	c.1576G>A	401	199	49.63%						PMS2	chr7:6026820	p.(Asp526Asn)	c.1576G>A				Not covered
							POLD1	chr19:50905323	p.(Arg180GlyfsTer3)	c.537del	333	122	36.64%						POLD1	chr19:50905323	p.(Arg180GlyfsTer3)	c.537del				Not covered
							RNF43	chr17:56435160	p.(Pro660ValfsTer41)	c.1975_1976dup	280	89	31.79%						RNF43	chr17:56435160	p.(Pro660ValfsTer41)	c.1975_1976dup				Not covered
							SDHA	chr5:226101	p.(His187Arg)	c.560A>G	410	155	37.81%						SDHA	chr5:226101	p.(His187Arg)	c.560A>G				Not covered
							SDHB	chr1:17380510	p.(Ala2Val)	c.5C>T	145	44	30.35%						SDHB	chr1:17380510	p.(Ala2Val)	c.5C>T				Not covered
							SH2B3	chr12:111856588	p.(Ser213Arg)	c.639C>A	364	321	88.19%						SH2B3	chr12:111856588	p.(Ser213Arg)	c.639C>A				Not covered
							SMAD4	chr18:48604787	p.(Asp537Tyr)	c.1609G>T	282	188	66.67%						SMAD4	chr18:48604787	p.(Asp537Tyr)	c.1609G>T				Not covered
							SMO	chr7:128845163	p.(Asn219Lys)	c.657C>A	363	133	36.64%						SMO	chr7:128845163	p.(Asn219Lys)	c.657C>A				Not covered
							TP53	chr17:7577581	p.(Tyr234His)	c.700T>C	167	113	67.67%						TP53	chr17:7577581	p.(Tyr234His)	c.700T>C				Not covered
							CDK12			Amplification				CDK12 amplified					CDK12			Amplification					Not detected (not validated)
							ERBB2			Amplification				ERBB2 amplified					ERBB2			Amplification					Not detected
							SMARCE1			Amplification				SMARCE1 amplified					SMARCE1			Amplification					Not detected (not validated)
																			PIK3CA	chr3:178916876	p.Arg88Gln	c.263G>A	3327	23	0.69%	0% in tissue
																			PTCH1	chr9:98233556	p.Ala563Val	c.1688_1689delinsTGT	6170	41	0.67%	0.15% in tissue
																			TSC2	chr16:2134252	p.Gly1344_Lys1345delinsGlu	c.4031_4033del	7199	20	0.28%	0% in tissue

43.1.11 Concordance Between Tissue and Plasma Based Next Generation Sequencing in BTC

In the BTC cohort, 8 of 16 patients with histopathological diagnosis of BTC, who underwent successful tNGS analysis, and had variants detected which were covered by both panels were included in this analysis. BTC 39 diagnosed with OSCC and BTC 56 with variants not covered by both panels were excluded from this analysis. Large Indels, aberrations within homopolymer genes and amplifications not validated by the ctPAED assay were excluded from this analysis. This analysis included only SNVs and small Indels.

In this cohort, 22 variants were detected by tissue (9 tissue only) and 22 detected by plasma (9 plasma only). Partial concordance by patient was 37.5% (3/8) using a tumour agnostic approach, which increased to 50% using a tumour informed approach. By variant, concordance between somatic tissue and plasma alterations was 59.1% (13/22) for variants called blind, which increased to 72.7% (16/22) when calling additional variants retrospectively using a tumour informed approach. Overall, 29% (9/31) of mutations detected were only detected in plasma and not tissue. All germline variants detected in tissue were removed using buffy coat variants subtraction, therefore not reported in plasma with only somatic variants being reported. BTC39 with oesophageal cancer was not included in the concordance analysis. However, tissue NGS revealed SNVs in *ERBB2*, *MLH1*, *MSH6* and *TP53* mutations (Arg282Trp and Pro36Leu). Both *TP53* mutations were detected in plasma, while the ERBB2 mutation was not detected in plasma. Fig 26 shows the concordance between tissue and plasma sequencing in the BTC cohort.

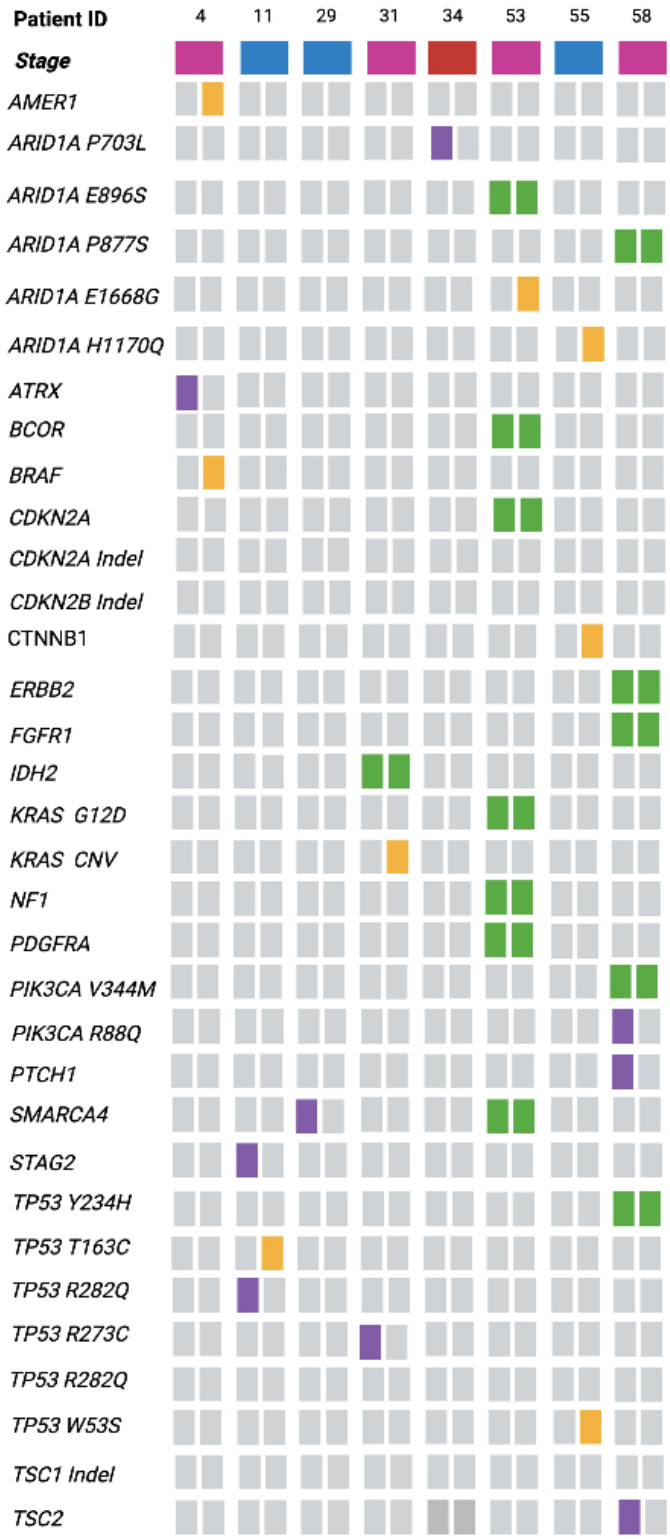


Figure 26- Concordance between tissue and plasma based NGS in BTC cohort

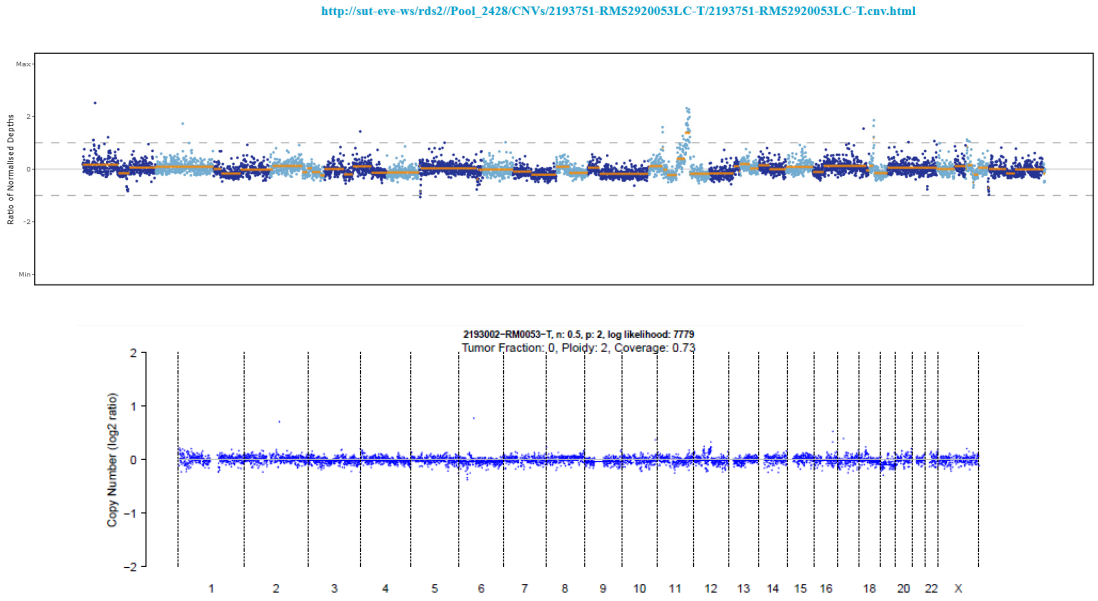
Stage: blue (early stage), pink (locally advanced), red (metastatic)
Mutations: concordant (green), tissue only (yellow), plasma only (purple).

43.1.12 lcWGS Data

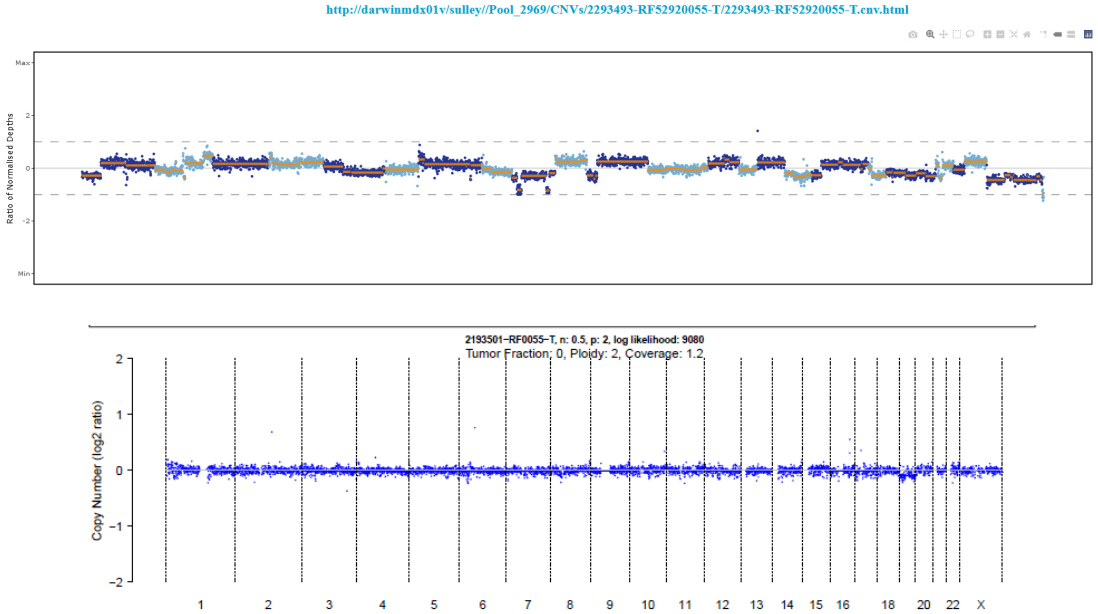
lcWGS was performed on all patients in both cohorts to assess for copy number changes. The aim of lcWGS was to detect large copy number alterations which the ct-PAED assay is unable to detect. lcWGS also allows determination of ctDNA fraction of the total cfDNA.

The hypothesis was that given copy number alterations occur in 30-40% of PC/BTC, the addition of lcWGS may enhance the Sn of ctDNA as a diagnostic tool in PC/BTC in detecting alterations not detected using the ctPAED panel. This was particularly important in the ctDNA negative cohort. lcWGS analysis requires tumour DNA purity of >10%. All samples had <10% ctDNA fraction of the cfDNA. As such, all lcWGS on plasma demonstrated no large copy number alterations, including no high level amplifications or chromosome arm deletions.

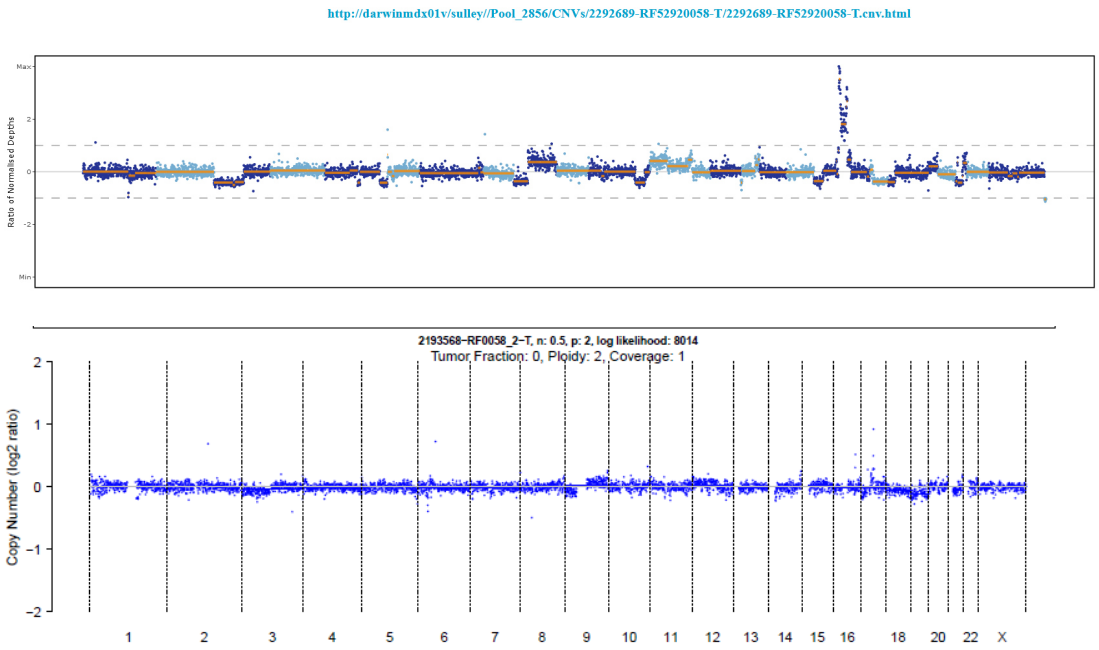
However, using the RMH200 sequencing on tissue samples, we detected gene amplifications in BTC53 and BTC 58. However, these were low level copy number changes which were not detected on the plasma based lcWGS. These were unlikely to be detected in plasma given the cfDNA fraction was <10%. BTC53 had MDM amplification and KRAS gain which were detected on FFPE Solid panel CNV plot but not on lcWGS in plasma.



For BTC55, deletions of TSC1 and CDK2NA were detected on FFPE Solid panel CNV plot which were not detected on lcWGS in plasma.



Patient 58 had CDK12, SMARCE and ERBB2 amplifications detected on FFPE Solid panel CNV plot but not on lcWGS in plasma.



Other missed Indels and CNV including 11 (CDK2NA deletion) and 31 (KRAS amplification) were not called on structural variant caller due to low tumour fraction.

43.1.13 Test Turn Around Results

The median time from PREVAIL consent to diagnosis being made on ctDNA was 20 days. The median time from referral for an invasive procedure to diagnosis being made on histopathology was 29 days. See fig 27 for test turn around results.

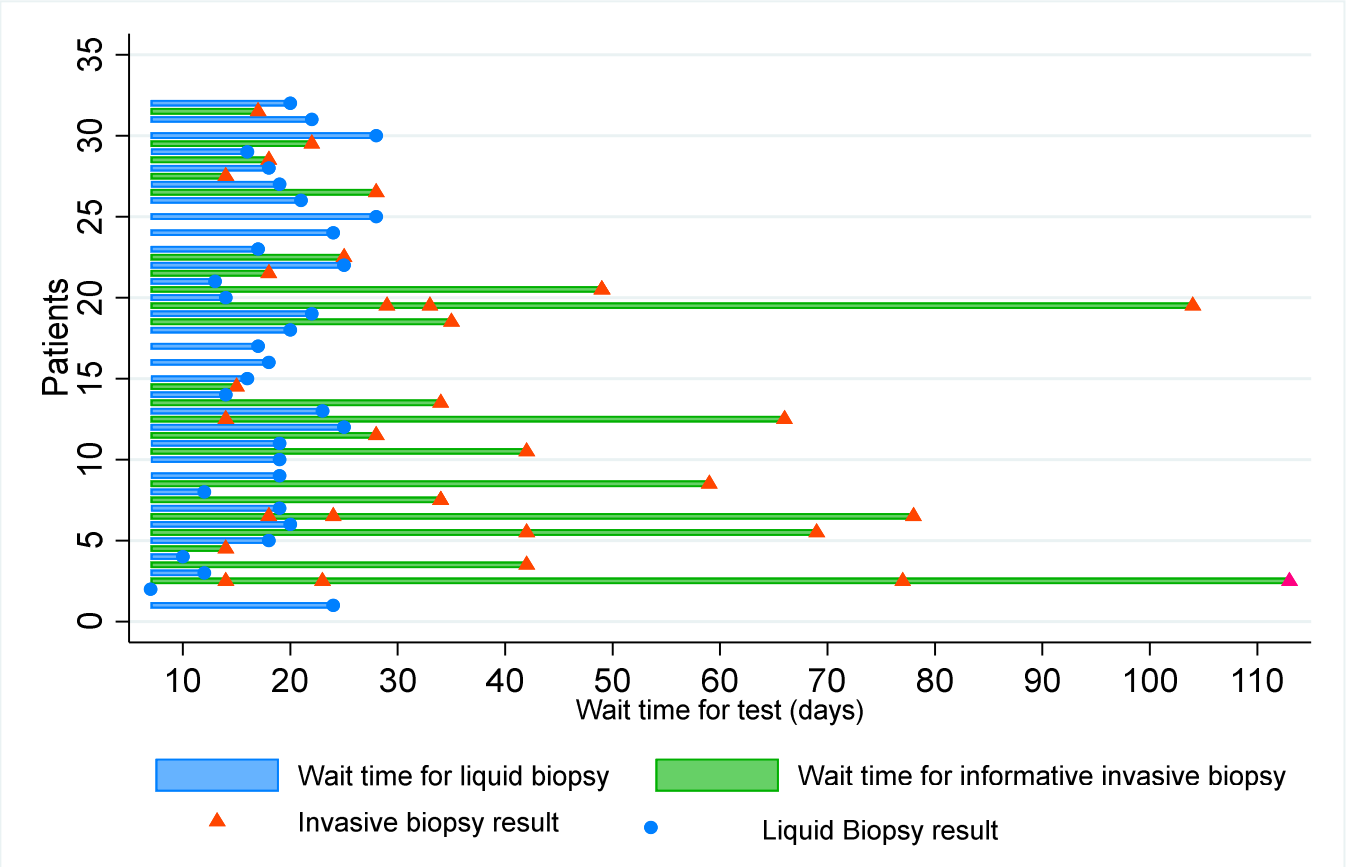


Figure 27- Test turnaround time for PC/BTC cohort

Time to diagnosis on liquid biopsy pathway (blue) and tissue biopsy pathway (green). Results are highlighted by indicators at end of the diagnostic pathway (blue dot liquid biopsy result, red triangle tissue biopsy result). Wait time for tissue biopsy pathway is cumulative for patients undergoing repeated biopsies.

43.1.14 Iterative Process and Learning Points from a Pilot ctDNA Study

This pilot study was set up with the intent of collecting descriptive data to inform a larger implementation programme (ACCESS programme- results chapter 6). The results from the PREVAIL ctDNA PC/BTC cohort support the use of a ctDNA supplemented diagnostic pathway to be trialled in routine care routine care. However, there were several learning points from this pilot study which fed into the set-up of the ACCESS programme. Describing these are important in any pilot study to give precedence to other, future studies.

Firstly, the MTB discussions initially were an iterative process. Patients were discussed immediately after their ctDNA molecular results were sent to me from the CMP at the next weekly MTB. The plan to discuss all cases in this manner and the initial MTB discussion would decide if a patient who had ctDNA detected was informative (supportive of cancer diagnosis) or non-informative (ctDNA detected but not supportive of cancer diagnosis). The plan was then to re-discuss the non-informative cases at the end of recruitment for consistency and following several months of MTB iterative learning from the PREVAIL study. The patients who were re-discussed are outlined in table 49.

More recently, several patients had ctDNA molecular diagnostic reports re-issued. This was due to the CMP laboratory reporting some variants initially as somatic, however on a subsequent review these variants were re-classified as either germline or CHIP related. Although these variants were later re-

classified and a new molecular report was provided retrospectively, no patients were affected as a result.

I learnt the importance of iterative learning from pilot studies. This subsequently fed into the ACCESS programme which was designed with NHSE PDSA methodology. Importantly, no patients were affected by the re-discussion that took place retrospectively.

The results presented in the results chapter are the final and most accurate data set.

Table 48- Iterative process of MTB discussions

Patient ID	Initial molecular report	Initial MTB discussion outcome	Rationale for reclassification	Change in molecular report	Second MTB discussion Outcome	Outcome for patient
PC1	ATM KRAS V14I KRAS G12D	Supportive of cancer diagnosis	Initial ctDNA panel was ct-GI and not ct-PAEDs. Sample re-tested using ct-PAEDs	KRAS G12D TP53	Same as initial	Treated based on pre-registration tissue; not affected by change in report
BTC4	ATRX	Not supportive of cancer diagnosis	Re-discussion at end of recruitment	Nil	Consistent with cancer	Treated on tissue biopsy; not affected
PC8	ERBB2 FGFR1 TSC2	Not supportive of cancer diagnosis	Re-discussion at end of recruitment	Nil	Consistent with cancer	Treated on tissue biopsy; not affected
BTC10	ALK	Not supportive of cancer diagnosis	Re-discussion at end of recruitment	Nil	Consistent with cancer	Not treated due to poor PS; not affected by re-classification
BTC11	TP53 STAG2	Not supportive of cancer diagnosis	Re-discussion at end of recruitment	Nil	Consistent with cancer	Treated on tissue biopsy; not affected
PC12	NF1 KRAS	Not supportive of cancer diagnosis	Re-discussion at end of recruitment	Nil	Consistent with cancer	Treated on tissue biopsy; not affected
PC20	ATM TP53	Not supportive	Re-discussion at end of recruitment	Nil	Consistent with cancer	Treated on tissue biopsy; not affected

		of cancer diagnosis				
PC23	TP53	Not supportive of cancer diagnosis	Re-discussion at end of recruitment	Nil	Consistent with cancer	Treated on tissue biopsy; not affected
PC24	NF1 PIK3R1 PTEN	Not supportive of cancer diagnosis	Re-discussion at end of recruitment	Nil	Consistent with cancer (MTB identified PIK3R1 as germline)	Treated for mPC outside of RMH
	NF1 PIK3R1 PTEN	Consistent with cancer	Re-discussion as CMP found NF1 CHIP and PIK3R1 were germline variants as buffy coat wasn't sequenced at the time	PTEN	Not performed as no change in MTB cancer diagnosis made	As above
PC27	SMARCA4 CREBBP	Not supportive of cancer diagnosis	Re-discussion at end of recruitment	Nil	Consistent with cancer	Treated on tissue biopsy; not affected
PC29	SMARCA4 CREBBP	Not supportive of cancer diagnosis	Re-discussion at end of recruitment	Nil	Consistent with cancer	Treated on tissue biopsy; not affected
PC38	ERBB2 TP53	Supportive of cancer diagnosis; TP53 germline	TP53 identified as germline mutation (removed from report)	ERBB2	No change	Not affected
BTC39	NF1 TP53 P36L TP53 R282W PTCH1	Supportive of cancer diagnosis; NF1 germline	NF1 identified as germline mutation (removed from report)	TP53 P36L TP53 R282W PTCH1	No change	Not affected
PC40	ATM	Not supportive of cancer diagnosis	Re-discussion at end of recruitment	Nil	Consistent with cancer	Not treated due to poor PS; not affected by re-classification
BTC54	TP53 ATM	Supportive of cancer diagnosis	Found to be CHIP variants in buffy coat a week after MDT discussion	Negative	Not re-discussed and reported as ctDNA not detected	Patient never treated for cancer

44 Discussion

The ctDNA detection in patients with suspicious PC and BTC was 69% and 56% respectively. Although the ctDNA detection rate in histologically confirmed PC ranges from 20% to 90%, and 40%-90% in BTC, this is largely

dependent on the stage of disease, ctDNA assay and approach used (198, 205, 206, 208, 211, 266, 273, 333, 334). ctDNA detection rates are higher with multi-gene panels (approximately 80-90%) compared with single gene sequencing (i.e. 20-50% with ddPCR for KRAS mutations in PC) (208, 216, 236). The detection rate from our pilot study for PC and BTC is in keeping with the current literature and supports ctDNA in the diagnosis of patients with suspected PC/BTC.

To assess the use of ctDNA as a diagnostic biomarker in PC/BTC, we conducted a Sn and Sp analysis, and showed good diagnostic accuracy for PC (Sn 70%; Sp 100%) and BTC (Sn 70%; Sp 75%). Previous studies have reported slightly lower diagnostic accuracy in PC. A meta-analysis assessing ctDNA as a diagnostic biomarker in PC showed a pooled Sn of 64% and Sp of 92% (335). However, these studies used a single gene PCR assay (targeting *KRAS*). In BTC, a study by Wintachai et al confirmed a Sn and Sp of 88.7% and 96.7% for the diagnosis of BTC using a custom multi-gene assay (273). However, our pilot study is the first to prospectively assess ctDNA using a multi-gene assay in a tumour agnostic approach, in those with suspected PC/BTC to inform treatment decisions.

The use of ctDNA as a diagnostic biomarker in a tumour agnostic approach requires careful consideration to reduce false positives and enhancing Sp. False positives may arise from PCR sequencing errors, CHIP (*TP53* and *KRAS* CHIP related-mutations can be detected in 0.2% and 0.02% of healthy individuals, respectively), non-malignant conditions (*KRAS* mutations can be

detected in up to 5% of chronic pancreatitis), detection of non-tumour derived somatic mutations (generally seen at low VAF in healthy population), and germline mutations (190, 199, 223, 336). When using a tumour agnostic approach, certain pre-analytic considerations should be made. These include sequencing plasma and buffy coat sequencing and subtraction to exclude CHIP/germline mutations, using UMI barcoding (to reduce artefact PCR and sequencing errors), and establishing a validated VAF cut-off for a tumour agnostic assay to detect somatic tumour derived variants (181, 337, 338). The assay we used for this pilot study was most fit for purpose for PC/BTC for several reasons. Firstly, it covered common aberrations seen in these two tumours. Secondly, it had established clinical validity with validated LOD for use in molecularly profiling tumours (as a tumour agnostic approach). Thirdly, given the impact of COVID19, there was an urgency to deploy this test into the diagnostic pathway for faster diagnosis and facilitate treatment in patients with suspected PC/BTC who were unable to access invasive EUS/FNA. The importance of an MTB to determine the likelihood of a ctDNA based aberration being tumour derived is critical in providing an additional level of support and governance to molecular results. The MTB helped to identify several missed CHIP and germline variants including for patients 24 and 39.

In our pilot study, we detected no false positive results in the PC cohort and one in the BTC cohort who was subsequently diagnosed with OSCC. The use of ctDNA to detect SNVs in the diagnosis of BTC is relatively non-specific, as *TP53* mutations (and other co-occurring mutations) can be seen in both

diseases. Therefore, the MTB considers the specific SNV detected and the clinical history to provide a recommendation.

A recent study assessing the performance of several ctDNA based assays (including targeted sequencing of SNVs, and whole genome methylation [WGM]) on the accuracy in determining CSO across multiple cancer types, showed relatively poor Sn for ctDNA (339). The overall accuracy for determining CSO was 74.8% for WGM, and 34.6% for SNV with buffy coat extraction. Specifically for BTC, the accuracy using 22.2% and 44.4% for SNV and WGM respectively; and 35.3% and 64.7% for SNV and WGM respectively in PC. The study concluded that WGM was more sensitive than SNV detection and did not require WBC sequencing to predict CSO with the highest accuracy.

However, no single diagnostic test should be used in isolation, and the use of ctDNA in the context of other clinical findings, such as presentation, tumour markers, and imaging findings is critical. Although tissue biopsy remains the gold standard in cancer diagnosis, the false positive rate is between 1.1-3.8% which was shown in a large retrospective diagnostic study involving EUS/FNA assessment of solid pancreatic lesions (n=367) (340). Similarly, Sefrioui et al. assessed ctDNA and CTC in the diagnosis of patients with a solid pancreatic mass undergoing EUS-FNA biopsy. The study included 68 patients undergoing EUS/FNA, with 58 malignant tumours being identified following definitive surgery. The Sn and Sp of EUS/FNA was 73% and 88% respectively, with two patients classified as PDAC on EUS/FNA histology subsequently diagnosed with non-PDAC malignant tumours on surgical resection (one

metastatic renal cell carcinoma, one pancreatic NET) (234). This highlights the importance of using a range of clinical and pathological tools in the diagnosis of PC/BTC to avoid false positives.

The false negative rate of ctDNA in the diagnosis of PC and BTC was 15.3% and 21.4% respectively in our pilot study. All had highly suspicious disease with imaging suggestive of PC/BTC. Therefore the pre-test probability of enrolled patients was higher in our pilot study population in comparison to a screening, asymptomatic population. False negatives impact the Sn of a diagnostic test, and so increasing the Sn can be performed through several methods. Firstly, studies have shown that a multi-parametric approach including tumour markers can enhance Sn of the assay. An updated Sn analysis incorporating CA19.9 however was not performed due to the low numbers in each cohort. For example, of the two false negatives in the PC cohort, only one had an elevated CA19.9. This could be considered in a larger study such as the ACCESS programme (see results chapter 6). Enhancing Sn can also be performed through addition of ctDNA methylation analysis. ctDNA based DNA methylation has been studied in PC and BTC, including the Galleri targeted methylation based ctDNA assay to detect multiple cancer types. This methylation assay has a Sn and Sp of 61.9%, 60%, 85.7% and 95.9% in stage I, II, III and IV PC, and 100%, 70%, 100%, and 100% in stage I, II, III, and IV BTC, respectively (214). Plasma based methylation signatures can also distinguish between pancreatic cancer and benign pancreatic conditions (251). These assays may have a role in screening for early stage disease, however the limited Sn in patients with stage I PC requires further

investigation. In addition, methylation-only assays lack DNA genomic analyses which are used to support precision therapeutics.

An initial objective of mine was to assess the Sn and Sp of a multi-parametric analysis incorporating ctDNA detection of SNVs with methylation in the diagnosis of PC/BTC. My hypothesis was that a plasma methylation assay will enhance the Sn and Sp in the diagnosis in PC/BTC. I had planned to have all samples (n=132 across all PREVAIL cohorts) analysed using the Guardant Infinity methylation assay. However given delays in recruitment I was not able to report on this for this results chapter. This analysis will be conducted and reported later this year. The benefit of adding methylation analysis to SNV can help identify a CSO. It may have differentiated the OSCC from a BTC (in BTC39). Determining CSO would also be useful in those with a history of malignancy, such as BTC 58 (current breast cancer) and BTC35 (resected stage III melanoma). We detected several concordant and non-concordant mutations between plasma and gallbladder biopsy in BTC58, and the non-concordant mutations may be derived from breast cancer tissue. This was only evidence through sequencing of tumour, and so a tumour agnostic, plasma only methylation analysis may provide this additional information.

Another measure I took to enhance the Sn and reduce false negatives was assessing lcWGS on all plasma samples to detect high level gene amplifications and large chromosomal deletions which were not detected using the ctPAED panel. Although copy number alterations (CNA) are seen in 30-40% of PC and BTC, we detected no alterations in both cohorts. The low

tumour fraction in samples (all <10%) limited a reliable evaluation of CNAs using lcWGS. This was because most patients had non-metastatic disease and therefore low levels of ctDNA. To mitigate for this, we could perform higher coverage of the whole genome (i.e. >1x coverage). However this required additional costs and as the lcWGS missed 5 patients with CNA, 4 patients had detectable SNV on ctDNA and so deeper coverage on lcWGS would not significantly impact the Sn or Sp.

False negatives are also seen in tissue biopsies, and in our cohort, 16% of patients had more than one invasive procedure for definitive diagnosis. Similar rates of false negatives with EUS/FNA have also been described in the literature (5, 331). The time to diagnosis using tissue versus liquid biopsy was also assessed in our study. We showed that the time to diagnosis using liquid biopsy was shorter than tissue biopsy. This has been shown in previous studies comparing tissue and liquid based NGS (211, 330). This is predominantly related to the complex invasive diagnostic pathway for PC/BTC which requires triaging referrals, appropriate staffing for endoscopy and histopathology, and tissue collection and processing. The liquid biopsy pathway was less complex, involved less steps but was confounded by batch testing of samples which likely exaggerated the time to diagnosis in this pathway. In the future, this liquid biopsy pathway may shorten with higher number of samples being tested simultaneous.

In keeping with other studies, we identified a relatively low concordance between tissue and plasma based NGS (212, 213, 236, 273, 334). However,

in our cohort, the average number of aberrations detected per patient was fewer in plasma than tissue. This is related to the different assays used, with the ctPAED panel sequencing 67 genes, compared to the RMH200 tissue assay which sequences 200 genes. Nevertheless, 40% and 28.1% of mutations detected were in plasma only in the PC and BTC cohort respectively and demonstrates the use of ctDNA to overcome heterogeneity seen in tissue biopsies. We detected 16.7% mutations in PC and 56.5% mutations in BTC in plasma which matched tissue and were called blind based on our established VAF threshold (reported without prior knowledge of tumour sequencing data).

A contributing factor for the low plasma-tissue concordance seen in the PC cohort may be relate to the lower detection of *KRAS* mutations in the plasma compared to tissue. *KRAS* mutations can be seen in 90% of patients with histologically confirmed PC (196). Previous studies assessing ctDNA in PC have shown relatively low detection of *KRAS* mutations using ddPCR and multi-gene ctDNA assays of 15-50% (205, 211, 222, 334, 336, 341). In our study, 80% of PC tumours had a *KRAS* mutation detected on tissue-NGS, with only 2 having *KRAS* mutations detected in the plasma when analysed using the ct-PAED panel and confirmed through plasma-based ddPCR.

There are several factors which may explain the low shedding of *KRAS* into the circulation of patients with PC. Firstly, studies have shown that *KRAS* detection in plasma is higher in those with metastatic disease, and patients with early stage disease having lower concentrations of KRAS in plasma (206). Cohen et al. showed that 38% of patients with detectable *KRAS* by ddPCR

had <2 mutant templates per millilitre of plasma, with the average number of 5.3 (205). This included mostly early stage PC, suggesting ultra-sensitive techniques are needed to detect KRASm at such low VAF. In our study, concordant *KRAS* mutations were found in those with metastatic disease (liver and peritoneal). The remainder of the plasma-negative, tissue-positive *KRAS* mutant PC had either localised or locally advanced disease. Secondly, liver predominate disease may also contribute to shedding of *KRAS*. A study by Patel et al. demonstrated the highest rates of concordance between cfDNA and tNGS if the tissue was from a liver metastasis (compared to primary tumour) (206). In the PREVAIL study, all patients had a tissue biopsy and NGS performed on a primary tumour which may have contributed to the low concordance. Although majority of primary pancreatic tumour harbour KRAS mutations, their shedding of KRAS into the circulation is less compared to liver metastases. There are several theories which may explain this, including the possibility that KRAS and all cfDNA is metabolised by the liver (and kidneys) (342). Alternatively, the desmoplastic nature of primary pancreatic tumours might limit the secretion of DNA into the circulation. Pancreatic tumours are comprised of neoplastic cells (often <20% of the tumour), with majority of cells arising from dense fibrotic stroma (343). This fibrotic stroma may limit the secretion of KRASm in the plasma and account for the low concordance between tissue and plasma.

Thirdly, technical factors may also contribute to the low tissue-plasma KRAS concordance. In the KYT initiative study, plasma based NGS sequencing depth was lower compared with tissue NGS. Although we showed the KRAS

sequencing depth using the ctPAED panel was slightly less than using the RMH200 panel, confirmatory ddPCR on plasma samples demonstrated 100% concordance. Therefore, technical assay factors did not contribute to the low detection rate of KRAS in plasma.

The use of ctDNA in the diagnosis of PC was not significantly affected by the low *KRAS* detection. There was only one false negative (PC15) who was overall ctDNA negative (across all mutations) and had a tissue *KRAS* mutation. However, the low *KRAS* shedding is important when using ctDNA in the molecular profiling of advanced PC to detect targetable mutations, including *KRAS* G12C. This may not be relevant in those with metastatic disease with large volume liver disease as the plasma-*KRAS* detection rate in these patients is often high. However, false negative ctDNA results present challenges in those with locally advanced, unresectable tumours in detecting KRAS G12C.

Despite the low concordance rate between tissue and plasma sequencing, an important use of ctDNA in PC/BTC in the molecular profiling of tumours is to overcome limitations of tissue biopsies, including failed NGS analysis and tumour heterogeneity. The failure rate for NGS in PC is approximately 10-15% which is often due to the low quality of tumour DNA (344). In addition, most studies have assessed sequencing on tumours with a higher proportion of tumour cellularity (>40% in some analyses) (19). There is a paucity of data on the genomic landscape on PC in tumours with lower tumour cellularity. ctDNA

may overcome the challenge and describe a complete genomic landscape of PC.

Our study has several limitations. Firstly, we did not exclude patients with a previous or concurrent cancer diagnosis (BTC50 and BTC 58 had history of breast cancer, BTC31 had a history of melanoma). As this study was set up during the COVID19 pandemic when capacity for invasive diagnostics were limited, we needed to ensure patients with highly suspicious PC/BTC had access to a diagnostic test and therefore were not excluded. To mitigate the risk of false positives from this inclusion, any history of malignancy was considered during the MTB interpretation of the ctDNA molecular diagnostic results. However, as discussed in chapter 6, the ACCESS programme excludes all patients with a history of malignancy within the past 3 years.

Secondly, although our ct-PAED plasma based panel did include the most common aberrations in PC (*KRAS*, *TP53* and *CDK2NA*), and BTC (*TP53*, *IHD1*/2, *KRAS*), the panel does not cover *SMAD4*, *MET* or *BRCA1*/2 genes, which can be detected in 50%, 40%, and 3% of PC respectively. The panel also does not cover potentially actionable aberrations in BTC, including *FGFR2* fusions (15%), *NTRK* fusions (3%) or *HER2* amplifications (30-40% of GBC). Although the primary aim of the study was to assess the use of ctDNA to facilitate a diagnosis rather than detect actionable aberrations, an important secondary aim was to facilitate treatment using ctDNA.

Lastly, the study did not collect data on survival or treatment responses in those treated based on ctDNA. This will however be further assessed in a larger, ongoing validation study (PREVAIL ctDNA Part 2) as outlined in chapter 6.

This pilot study is the first to assess the use of a plasma-only ctDNA assay as a tumour agnostic approach in the diagnosis of PC/BTC to inform treatment decision making. Although this study was initially set up during the COVID-19 pandemic to facilitate treatment in patients with radiologically suspicious PC/BTC unable to undergo invasive biopsies, the results and impact of this study may have a role in the future diagnosis of PC/BTC. These tumours are difficult to diagnosis given their location, require trained personnel to perform specialised invasive procedures, often require multiple invasive procedures owing to the necrotic nature of these tumours. There is a strong imperative for non-invasive tools in the diagnosis of PC/BTC, to circumvent bottlenecks in the diagnostic pathway and facilitate a diagnosis and treatment for these difficult to diagnose tumours. Our study supports the addition of ctDNA to the diagnostic pathway in patients with suspected PC/BTC, with a defined and structured framework for a liquid biopsy pathway, from sample collection, analysis and reporting of molecular results, facilitating an MTB discussion, and providing outcome results to clinicians to inform treatment decisions. Our subsequent, larger validation ACCESS programme set up across several London hospitals aims to implement ctDNA in the diagnostic pathway for patients with suspicious PC/BTC, aiming to improve efficiencies in the diagnostic pathway. This implementation programme deployed a ctDNA

diagnostic approach in PC/BTC using a commercial provider on a large scale, thereby providing a cost-effective service with a fast test turnaround time.

45 ACCESS Implementation Programme

46 Abstract

Introduction: PC/BTC have the worse prognosis of any solid cancer. Majority of patients present with inoperable locally advanced or metastatic disease, owing to the aggressive nature of the disease. Timely diagnosis is critical to ensuring patients receive treatment early, to reduce risk of progression and symptomatic disease. The diagnosis of PC/BTC in secondary care is challenging and relies on an invasive biopsy using specialist endoscopy and histopathology. The invasive diagnostic pathway is complex, with ¼ patients undergoing a repeated invasive procedure due to a non-diagnostic biopsy. This is reflected in the poor NHS cancer diagnostic targets across the UK for upper GI cancers. There is an unmet need for faster and safer diagnosis in patients with suspected PC/BTC. We hypothesise that ctDNA can be implemented into the hospital-based diagnostic pathway for patients with suspected stage III/IV PC/BTC and speed diagnosis and facilitate access to treatment. **Methods**: This multi-centre prospective implementation programme is currently being conducted at 6 RM Partners (RMP) hospitals from October 2022, aiming to recruit up to 650 patients over 12 months. Eligibility includes patients with radiologically suspicious stage III/IV PC/BTC

without a histological diagnosis. Eligible patients are referred in parallel for an invasive diagnostic procedure and ctDNA collected for plasma-NGS using the Guardant360© multi-gene panel. An MTB reviewed results and classified ctDNA detected variants as supportive or not of a cancer diagnosis, in the context of other clinical, imaging and biochemical markers. Patients with a diagnosis made on ctDNA could proceed to treatment through an ethically approved prospective study (PREVAIL ctDNA Part 2). The primary end point at this interim analysis was the implementation of a ctDNA augmented diagnostic pathway across 6 hospitals. Secondary end points include the proportion of patients who undergo a repeated invasive procedure and NHS cancer wait times. **Results**: The Guardant360© assay was successfully implemented into the routine diagnostic pathway for suspected PC/BTC cancer in 6 RMP hospitals. At the time of data cut off, 50 patients were registered, with 42 patients having ctDNA collected and reported (analysable population). ctDNA was detected in 78.6% of suspected PC and 81.3% in suspected BTC. The repeated invasive procedure rate in all patient's following enrolment was 16.1%. Overall, 66.7% had a diagnosis made, with 50% having a diagnosis on both ctDNA and tissue, and 14.3% on liquid biopsy alone. 2 patients were treated on liquid biopsy alone without a tissue diagnosis. **Discussion**: A ctDNA augmented diagnostic pathway can be implemented into the routine invasive diagnostic pathway for patients with suspected stage III/IV PC/BTC. Ongoing recruitment will address secondary end points including the impact on repeated investigations and NHS cancer wait times.

47 Background

PC and BTC have the worst prognosis of any solid cancer. Although survival rates for most cancers in the UK have increased over the past 50 years (average 52.5% increase in 5-year survival), PC and BTC 5-year survival has only improved by 6.9% and 12.1% respectively, much lower than average. There is significant variation in 10-year survival rate across the UK, ranging from 4.8% to 10.6% depending on the geographical location. The UK reports the lowest 5 year survival for PC compared to other regions including Asia, America and Europe. It is important to understand the factors contributing to the poor survival rates seen in PC/BTC in comparison to other cancer types, healthcare settings worldwide, and variations within the UK.

PC/BTC are highly aggressive tumours and often present with locally advanced unresectable or metastatic disease at diagnosis (80-85%). Only 50% of patients with metastatic disease will receive systemic palliative chemotherapy due to poor performance status and rapid decline owing to the aggressive nature of the disease (345). Early detection is critical to ensuring patients receive treatment early as diagnostic delays result in disease progression.

The time between onset of symptoms and diagnosis for patients with PC/BTC in secondary care is slow. Delays in diagnosis lead to delays in initiating treatment, worsening symptoms, poor patient experience and a negative impact on survival. The diagnostic pathway for suspected PC/BTC in

secondary care is complex in comparison to other tumour types and depends on obtaining a suitable tissue biopsy through an invasive procedure. This can be challenging, due to tumour factors (i.e. deep location, non-diagnostic necrotic samples) or patient factors (i.e. infection, comorbidities, older age). Patients may experience significant delays within the diagnostic pathway, including (1) at the point of entry (i.e. patients requiring optimisation prior to invasive procedures due to sepsis or comorbidities), (2) at critical bottlenecks within the pathway (i.e. delays in specialist endoscopy services and histopathology), (3) the need for repeated invasive procedures from inconclusive biopsy results. The latter contributes to approximately 25-40% of patients requiring a repeated invasive procedure to confirm a diagnosis (12, 331, 346, 347). Our PREVAIL pilot study showed that 56% of patients with suspected PC/BTC had a pre-registration non-diagnostic biopsy.

This complex diagnostic pathway leads to delays nationally, as reflected by the current NHS upper GI cancer wait times which are not currently met across the UK. We therefore urgently need a faster, safer, cost effective streamlined diagnostic pathway for patients with suspected PC/BTC. The use of non-invasive diagnostic biomarkers such as ctDNA may address this unmet need.

ctDNA can be detected in 80-85% of patients with histologically detected locally advanced and metastatic PC/BTC (accounting for the majority of newly diagnosed cases). The PREVAIL ctDNA pilot study support the use for a ctDNA supplemented diagnostic pathway using an in-house custom NGS panel in 32 patients with suspicious PC/BTC. The Guardant360© assay is an

FDA approved and CE marked test for comprehensive genomic profiling using plasma-based NGS of ctDNA across any stage III/IV solid cancer, including in PC/BTC. This 73-gene test sequences DNA for SNVs, Indels, CNV, and fusions, including commonly mutated genes in PC/BTC (*KRAS*, *CDKN2A*, *TP53* and *SMAD4*) and important targetable mutations in *BRCA1/2*, *NTRK* fusions, *BRAF V600E*, *IDH1*, *HER2 amplifications*, *FGFR fusions*, and MSI. See appendix E for Guardant360© gene panel. The assay has robust evidence with a high level of analytical Sp and Sn (~100%), and a ctDNA detection rate of 85% in stage III/IV PC/BTC (213, 333).

The seminal analytical validation paper of the Guardant360© assay confirmed high level of analytical Sp (>99.99%) at a low VAF of 0.1% without false positives (348). The clinical Sn (i.e. ctDNA detection rate) was 85% across multiple solid tumours (stage III/IV). A subsequent paper by Odegaard et al. confirmed these findings with a high level of analytical Sp at a VAF of 0.02% (333). Clinical validation using orthogonal plasma and tissue-based NGS in 10,593 patients showed high level of agreement (>99%) and clinical Sn (85.9%), confirming the high level of concordance between the Guardant360© assay and tissue-based NGS (333). A larger clinical validation of the Guardant360© assay in 21,807 patients reported high level of clinical Sn in PC (80%, n=870) and BTC (85%, n=430). In addition to the published evidence, real world data from routine use of the Guardant360© assay in over 11,000 PC showed the ctDNA rate of 85% in stage III/IV disease (unpublished data). The analytical performance of the Guardant360© assay is outlined in Fig 28.

PERFORMANCE SPECIFICATIONS

Alteration Type	Reportable Range	Allelic Fraction/ Copy Number	Analytical Sensitivity	Analytical Specificity*
SNVs	≥0.04%	>0.5%	100%	100%
		0.1 - 0.5%	88.3%	98.9%
Indels	≥0.04%	>0.5%	99.8%	100%
		0.1 - 0.5%	73.9%	
Fusions**	≥0.04%	≥0.3%	100%	100%
		0.05 - 0.3%	90.8%	
CNAs***	≥2.18 copies	2.3 copies****	100%	100%
MSI	MSI-H Detected	> 0.1%	95%	100%

Figure 28- Analytical Performance of Guardant360© Panel

Our hypothesis is that adding ctDNA using the Guardant360© assay can be implemented into the routine diagnostic pathway for patients with suspected stage III/IV PC/BTC. Our secondary hypothesis is that ctDNA can speed diagnosis and facilitate access to treatment in patients with suspected stage III/IV PC/BTC. Our primary aim is to implement ctDNA into the routine diagnostic pathway for patients with suspected PC/BTC. Our secondary aim was to assess the impact of implementing ctDNA into the routine diagnostic services on repeated investigations and NHS cancer wait times.

48 Methods

48.1.1 Overall Programme Design

ACCESS (F**A**ster diagnosis in pan**C**reatic and bile duct **C**anc**E**rs using liquid bipo**S**ie**S**) is a multi-centre, implementation programme (not considered to be research) to implement ctDNA into the routine diagnostic pathway of patients with suspected stage III/IV PC/BTC in 6 hospitals within the RMP Cancer

Alliance network. This is following a successful grant awarded in February 2022 by the Small Busines Research Initiative (SBRI)/NHSE (programme ID: SBRIC01P3008) in collaboration with Guardant Health. The programme will run over 12 months and include up to 650 patients with suspected stage III/IV PC/BTC (i.e. highly suspicious on imaging) awaiting an invasive diagnostic procedure within the secondary care setting.

Patients will be referred for an invasive diagnostic procedure in parallel to a liquid biopsy. These patients will proceed through the invasive diagnostic pathway as would be standard of care, with Guardant360© as an additional supplemental test. Plasma for ctDNA will be collected at individual sites and transported to Guardant Health (Redwood City, California) to detect and sequence ctDNA. The result will be sent back to the referring team and discussed at an MTB and multi-disciplinary meeting (MDM).

Most patients will undergo both invasive tissue and liquid biopsies. These patients will benefit from earlier molecular profiling to direct precision therapeutics. For patients with inconclusive invasive tissue biopsy result but with informative liquid biopsy result, the MTB and HPB MDM may recommend treatment based on the liquid biopsy result alone, rather than repeating an invasive procedure. These patients will be offered and enrolled into the PREVAIL Part 2 study (an extension of PREVAIL Part 1 for PC/BTC cohorts; REC amendment no. AM2206-21) which will allow treatment based on a liquid biopsy result alone, provided the MTB interpret the variants and clinical data to be potentially diagnostic.

This results chapter will report on an interim analysis of the first 50 patients registered to the ACCESS programme. As the programme is still open and actively recruiting, the subsequent objectives will be reported once recruitment is complete. Data from the PREVAIL Part 2 study (i.e. treatment and survival) will be reported once recruitment is complete.

48.1.2 My Role in Programme Set Up

In February 2021, we prepared a grant submission for the ACCESS programme. The scope of work we were involved in included staff engagement, diagnostic pathway mapping, patient and public involvement and engagement (PPIE), and site level training. In addition, we developed a service evaluation (receiving institutional review board approval on 14/7/2022) to define the programmes endpoints and an SAP in collaboration with the programme statisticians.

In March 2022 we were award a £1.45 million to implement liquid biopsies into the routine diagnostic pathway. The programme opened in October 2022 and is planned to close to recruitment in June 2023. Below is an outline of my roles in the ACCESS programme.

48.1.3 Patient and Public Involvement and Engagement

The ACCESS programme was supported by our PPIE representatives, Jackie Edgeller (Pancreatic Cancer UK) and Helen Morement (AMMF-Cholangiocarcinoma Charity). With support from our PPIE representatives, we was able to deliver several tasks to set up the ACCESS programme.

We initially conducted a focus group including 6 patient representatives (including those with PC and carers of patients with PC). In this 2-hour focus group we presented the ACCESS programme to the group. The PPIE group gave valuable insights into their lived experiences during the diagnostic stage of their illness including highlighting the delays in receiving a diagnosis and subsequently starting treatment. The feedback regarding the ACCESS programme was positive and the group agreed this is a large area of unmet clinical need. The group recommended the use of PROs and patient experience questionnaires. The focus group was critical in framing our research question(s) prior to the final programme framework.

Following this meeting, Jackie and Helen joined the steering meeting, and were heavily involved in the set up and now management of the programme. A PIS was approved by the steering meeting following input by Jackie and Helen.

48.1.4 Diagnostic Pathway Mapping and Defining an Augmented Pathway

We subsequently defined the current invasive diagnostic pathway for patients presenting to RMP diagnostic centres with suspected stage III/IV PC/BTC based on imaging (see fig 29), and mapped the new, liquid biopsy augmented pathway (see fig 30).

This current pathway is complex, and the figure highlights critical bottlenecks in the diagnostic pathway. Firstly, patients may have difficulty entering the pathway, often related to co-morbidities, sepsis, the need for biliary decompression and stenting and current illnesses which require optimisation prior to invasive diagnostics. Those with poor performance status not suitable for systemic therapies often do not undergo an invasive diagnostic procedure and referred for palliative care. The second bottleneck is within the invasive diagnostic pathway itself, such as limited capacity of endoscopy and staffing shortages (including anaesthetic, specialist endoscopy and histopathology staff). Finally, the third major delay is due to patients who require repeated invasive biopsies following a non-diagnostic initial biopsy (25-40%). These patients with highly suspicious disease on imaging, but without a definitive histopathological diagnosis often require repeat biopsy to inform treatment decisions. The additional burden of these repeated invasive biopsies on the current, diagnostic pathway is unique to PC/BTC given their necrotic nature and high frequency of non-diagnostic initial biopsies.

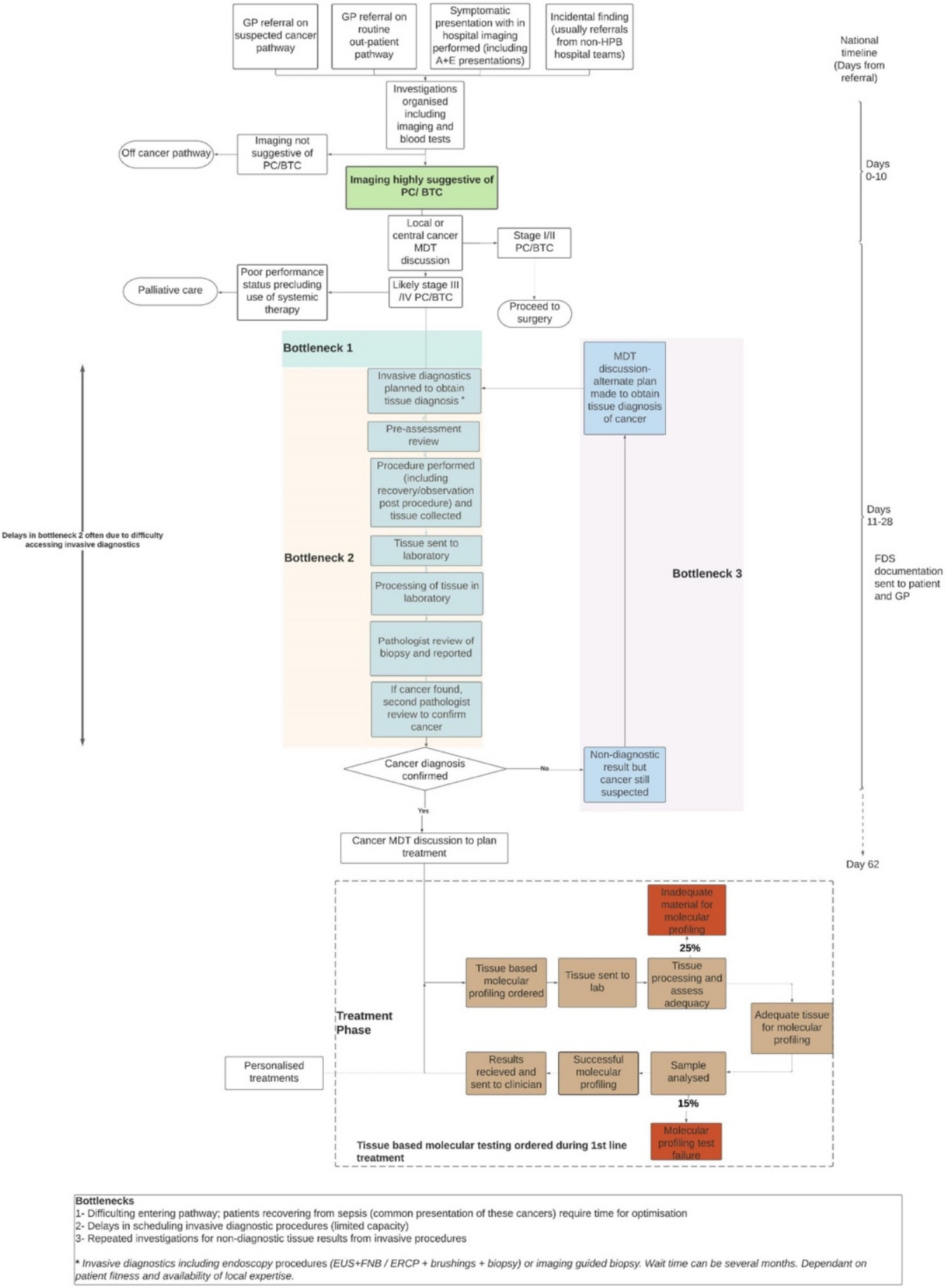
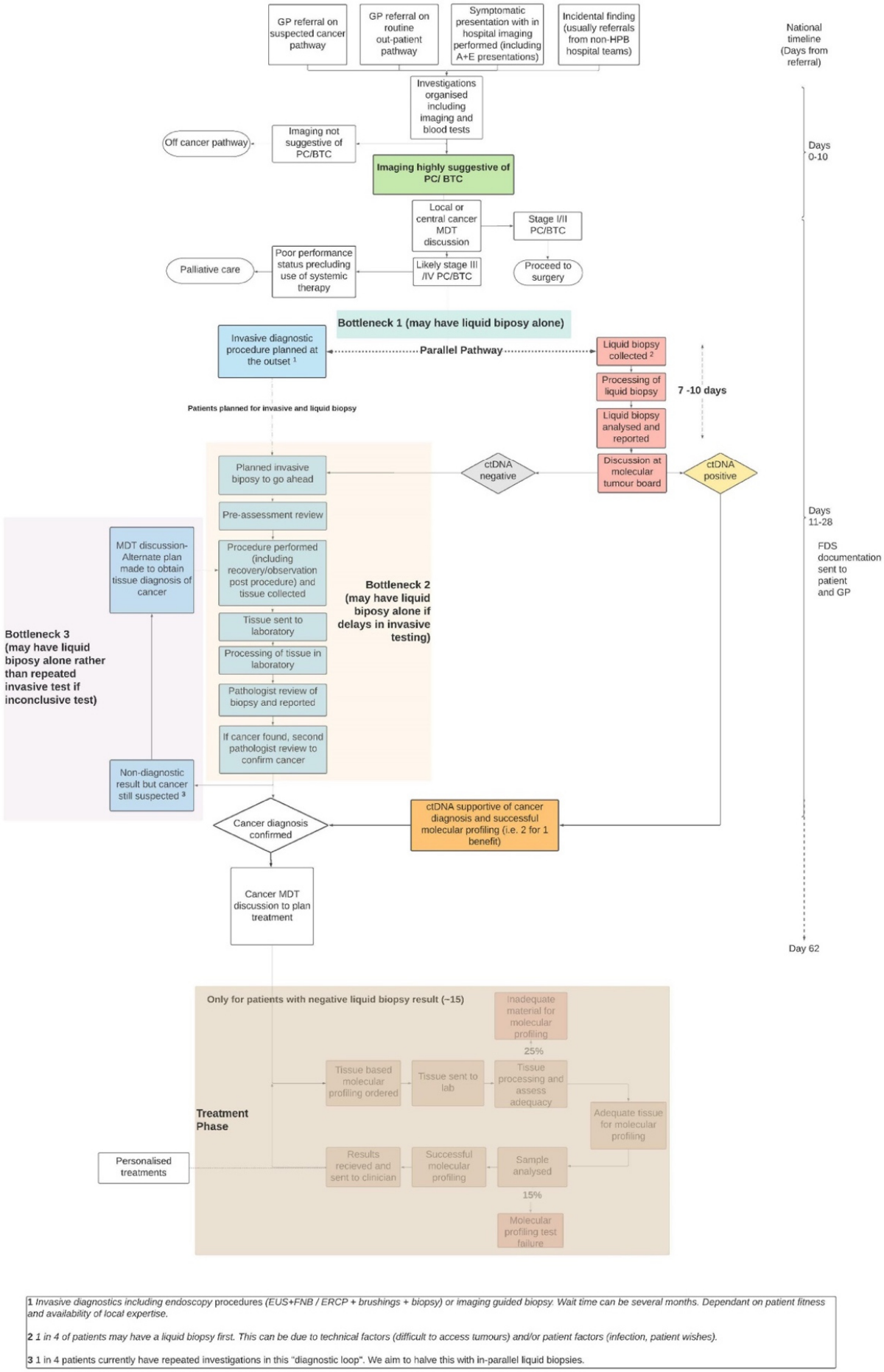


Figure 29- Current hospital-based diagnostic and treatment pathway

To mitigate these three contributing delaying factors in the current invasive diagnostic pathway, we designed an augmented pathway. This new pathway adds a parallel, liquid biopsy pathway in addition to an invasive biopsy pathway. Thereby, we envisage that patients with suspicious PC/BTC may enter the pathway with more ease given the parallel nature of the design. Those who enter the pathway, but encounter delays due to capacity (i.e. bottleneck 2) may have an expedited diagnosis through ctDNA and may not need to proceed to a tissue diagnosis (with sanctioning by the MTB/HPB MDM). In addition, those with a non-diagnostic initial biopsy but an informative liquid biopsy may exit the invasive diagnostic pathway if a diagnosis is made on liquid biopsy. Patients with both tissue and liquid biopsy supportive of PC/BTC will benefit from up-front molecular profiling to support use of precision therapies.



1 *Invasive diagnostics including endoscopy* procedures *(EUS+FNB / ERCP + brushings + biopsy) or imaging guided biopsy. Wait time can be several months. Dependant on patient fitness and availability of local expertise.*

2 *1 in 4 of patients may have a liquid biopsy first. This can be due to technical factors (difficult to access tumours) and/or patient factors (infection, patient wishes).*

3 1 in 4 patients currently have repeated investigations in this "diagnostic loop". We aim to halve this with in-parallel liquid biopsies.

Figure 30- Hospital-based diagnostic and treatment augmented pathway

48.1.5 Site Engagement and Training

We engaged with multiple clinical sites across the RMP cancer alliance to determine

- Their willingness to participate in the ACCESS programme
- Each site's current diagnostic pathway for suspected PC/BTC including named personnel involved in the pathway to ensure this aligns with the new augmented pathway
- Expected 12 month recruitment targets based on estimated figures for invasive diagnostics for PC/BTC

Training of staff included producing online material for staff at each site including slides on the programme outlining the new diagnostic pathway and genomics training slides. ACCESS staff included a (1) clinical administrator such as a clinical nurse, (2) gastroenterologists and (3) medical oncologists within each site were appropriately trained on the programme.

48.1.6 ACCESS Programme Patient Eligibility

<u>Inclusion criteria</u>

- Participants aged 18 years or above
- Radiologically suspicious
    - Pancreatic cancer- Stage III and IV
    - Biliary tract cancer (including ampullary, gallbladder and bile duct cancer)- Stage III/IV
- Performance status suitable for oncological treatment

Exclusion criteria

- Previously diagnosed invasive or haematological malignancy within the past 3 years

48.1.7 ctDNA Workflow- Specimen Collection, Guardant 360© Next Generation Sequencing, and Molecular Reporting

All eligible patients had 2 Streck© tubes taken for plasma-based NGS using the Guardant360© assay. Samples were collected at each site following information provided to the patient (verbally and written information sheet). Verbal consent to take the sample was provided by patients, and clinicians signed a test requisition as part of this process. No written consent was needed for the programme as this was not considered research; however patients were able to opt out of the programme if they wish. Given this programme is not a clinical study, and following consultation with HRA, local R&D, our PPIE and clinical geneticist, the decision for verbal consenting for the Guardant360© assay as is standard of care was sufficient. All data collected through the programme was performed through a locally approved service evaluation in an anonymised fashion.

Streck tubes were subsequently sent to Guardant Health for plasma isolation and DNA sequencing on the 73-gene NGS panel. The panel covers 73 genes for SNVs, 23 genes for Indels, 18 genes for CNV and 6 genes for fusion detection. It also has clinical validation for detection of MSI (349).

The Guardant 360© ctDNA workflow has been reported (348). Briefly, plasma samples were processed using QIAmp Circulating Nucleic Acid Kit (Qiagen) according to manufacturer's instructions. cfDNA was then extracted from plasma and quantified using Qubit 2.0 fluorometer. DNA was extracted and ends repaired prior to ligation of adaptors for sequencing. The digital sequencing workflow involves ligation of non-unique oligonucleotide barcodes to each half of individual double stranded cfDNA to generate libraries. The resulting library is a self-referenced digital sequence library of a coded single-strand half of the original double-stranded cfDNA sample. Theoretically, this method has higher efficiency in converting DNA molecules to analysable material. The digital sequence libraries are then amplified and enriched for target genes using custom RNA probes.

The robust bioinformatic pipeline is then used to analyse sequencing data results. Each strand of double-stranded cfDNA molecule is tagged and a custom built software is used to assess whether a mutation has been acquired due to a sequencing error, library preparation error, or DNA damage during sample processing. Paired end reads are then aligned to the reference genome. Custom scripts are used to remove sequencing background error noise, identify germline SNPs, and subsequently call somatic SNVs by removing the erroneous variants.

A key difference between the Guardant360© and the ct-PAED workflow is in the identification of CHIP variants. The Guarsant360© does not sequence the

buffy coat for subtraction of CHIP variants and relies on the bioinformatic pipeline to identify and remove CHIP. Therefore, CHIP variants need consideration when interpreting Guardant360© reports (considered during MTB discussions).

48.1.8 Molecular Tumour Board

The North-West London MTB were involved in the interpretation of molecular diagnostic reports from patients included in the ACCESS programme. All patients with detectable ctDNA were discussed at the next weekly MTB, while those without detectable ctDNA were not discussed and continued along the routine diagnostic pathway.

The role of the MTB in the ACCESS programme was to determine the significance of variants detected in the context of clinical presentation, imaging and tumour markers. Given this was an implementation programme with the aim of speeding diagnosis, the MTB were not blind to histopathology results if available. The MTB were asked to provide the following outputs for each patient;

- Whether the collection of variants detected were supportive of a diagnosis of cancer, and if so, supportive of a diagnosis of PC/BTC
- The level of diagnostic support
    - Diagnostic of
    - Consistent with
    - Possibly consistent with

- Not consistent with

An MTB proforma was produced to provide these outputs (attached as appendix F), which was reviewed and approved by the MTB chair.

Based on learnings from the PREVAIL pilot study, to ensure consistency in the MTB diagnosis, a general guidance for reporting variants detected were produced and described below in table 50. However, given the iterative nature of the programme these were not strict and there was a degree of clinical interpretation provided by members of the MTB during discussions.

Table 49- General MTB guidance on interpreting variants in the diagnosis of PC/BTC

Level of diagnostic support	**Example**
Diagnostic*	Pathogenic mutation(s) detected (i.e. KRAS, TP53, SMAD4, IDH, BRCA, FGFR fusion) commonly seen in PC/BTC with clinical features supportive of PC/BTC.
Consistent*	Mutation(s) detected which are commonly seen in PC/BTC which may not be pathogenic (i.e. VUS). However, the clinical features are in keeping with PC/BTC.
Possibly consistent	Mutations(s) detected which can be seen in other cancers, with clinical features not typical of PC/BTC.
Not consistent	Suspected CHIP variant or mutation detected typical of alternative cancer.

* diagnostic and consistent with term “informative”; while possibly consistent, not-consistent and non-detectable ctDNA was considered “non-informative”.

Following patient discussion at the MTB, the completed MTB proforma was sent back to the referring clinicians. The MTB provided an additional level of

interpretation and diagnostic support to the Guardant360© genomic report provided by Guardant Health.

Once the MTB results were fed back to the clinicians, there were 6 possible scenarios that describe patient flow within the diagnostic pathway (see table 51). This augmented ACCESS pathway allowed patients to be treated on a liquid biopsy with ctDNA alone (i.e. without tissue diagnosis) following discussion at the MTB and through the PREVAIL part 2 study.

Table 50- Scenarios for patient flow to treatment in previous diagnostic pathway and augmented pathway depending on histology and ctDNA result

Invasive biopsy performed	**Histopathology report**	**ctDNA MTB result**	**Previous Diagnostic Pathway**	**Augmented Diagnostic Pathway**
Yes	Cancer diagnosis	Cancer diagnosis	Treatment	Treatment
Yes	Cancer diagnosis	Non-diagnostic	Treatment	Treatment
Yes	Non-diagnostic	Cancer diagnosis	Repeat biopsy	Treatment (PREVAIL part 2)
No	N/A	Cancer diagnosis	Invasive biopsy	Treatment (PREVAIL part 2)
Yes	Non-diagnostic	Non-diagnostic	Repeat biopsy	Repeat biopsy
No	N/A	Non-diagnostic	Invasive biopsy	Invasive biopsy

48.1.9 PREVAIL Part 2 Protocol

For patients with informative ctDNA result (i.e. cancer diagnosis made by MTB) who either did not undergo an invasive procedure or had a non-diagnostic invasive biopsy, treatment could be offered based on a diagnosis made from ctDNA (in lieu of tissue). This was done through the PREVAIL Part

2 study given treatment for PC/BTC without a histological diagnosis is not considered standard practice. It is important to note that ctDNA results were not considered in isolation, and an informative ctDNA result based on MTB discussion in the context of other clinical features (including symptoms, tumour markers and imaging) were used to form a diagnosis.

PREVAIL Part 2 protocol required a substantive amendment to the existing PREVAIL Part 1 study and a new PREVAIL Part 2 PIS. The amendment was approved by the same REC involved in PREVAIL Part 1.

PREVAIL ctDNA Part 2 is a multicentre, prospective study assessing the role of using Guardant360© in the diagnosis and treatment of patients with radiologically suspicious, stage III/IV PC/BTC without a definitive histological diagnosis of invasive malignancy (either because of prior inconclusive invasive biopsy result or an invasive procedure not being performed). All patient needed an informative ctDNA results (collected as part of the ACCESS programme) which the MTB deemed diagnostic or consistent with PC/BTC diagnosis. In addition, all patients were discussed at the HPB MDM to discuss recommendations on treatment based on the ctDNA result before enrolling onto PREVAIL Part 2.

As part of this study, treatment may be recommended based on the ctDNA result. Patients with an informative liquid biopsy result but awaiting an invasive procedure could enrol in PREVAIL Part 2 and have a planned invasive procedure cancelled following informed consent onto PREVAIL Part 2.

Patients with an invasive biopsy result which is suitable to guide treatment were not eligible for the PREVAIL ctDNA part 2 study and may have treatment or further investigations off study. Treating clinicians had access to the MTB outcome and patients may be treated based on the ctDNA result in the context of symptoms, tumour markers, and imaging results as a complete diagnostic package. Treatment was decided by the treating physician following HPB MDM discussion, and treatment decisions were not dictated by this trial.

As part of PREVAIL Part 2, additional data was captured to assess the efficacy of treatment based on ctDNA alone. These include baseline characteristics, ctDNA result, MTB discussion outcome, treatment decision, imaging responses and survival.

My MD will not report on patients within PREVAIL Part 2 as recruitment up to March 2023 is ongoing and not fully accrued. I will report on the preliminary results of the ACCESS programme and therefore describe the clinical evaluation and statistical consideration of the entire ACCESS programme rather than the PREVAIL Part 2 Study.

48.1.10 Quality of Life Assessment

As part of the ACCESS programme, we incorporated an assessment of QOL. Following discussion with our PPIE group and health economists, we opted for the EQ-5D-5L questionnaire.

This questionnaire covers 5 domains including mobility, self-care, activities of daily living, pain level and anxiety/depression. It also includes patients' self-assessment on their current health. The questionnaire has been validated in several studies including patients with PC/BTC (122, 350-352). The questionnaire can also allow for calculation of quality adjusted life years and used for cost-utility and effectiveness analysis.

2 questionnaires were given to patients to complete at 2 timepoints. This included one at registration (i.e. prior to biopsy) and for patients who were offered treatment, within 7 days of commencing treatment. Initially we planned to have an additional questionnaire at the time of diagnosis however concerns were raised by our PPIE groups regarding the burden for patients in adding additional questions.

However, with two time points used, we can assess the QOL in patients who proceed through the following pathways

- No diagnostic procedure (ctDNA only pathway)
- Single diagnostic procedure (Tissue and ctDNA pathway)
- Repeated diagnostic procedure (Tissue positive, ctDNA negative pathway)

QOL comparisons will be made between patients involved in the ACCESS programme and historical data collected by the RMP. This will provide insights into the QOL and patient experience through different journeys from initial presentation to diagnosis.

48.1.11 Clinical Evaluation and Statistical Consideration

The ACCESS implementation will use PDSA (plan, do, study, act) methodology as part of NHS improvement methodology to assess the outputs and adjust accordingly given the iterative nature of the programme.

My hypothesis is that ctDNA can be implemented into the routine diagnostic pathway for patients with suspected PC/BTC. My secondary hypotheses are that a ctDNA augmented diagnostic pathway will improve efficiency in this pathway and reduce the need for repeated invasive procedures.

The primary and secondary objectives are outlined in table 52.

Table 51- ACCESS Programme Objectives and Corresponding Endpoints

Primary Objective	**Primary end point**
To implement ctDNA into the routine diagnostic pathway for patients with suspected PC/BTC	An implemented ctDNA augmented diagnostic pathway across 6 hospitals
Secondary objectives	**Secondary end points**
To determine if implementation of Guardant360© into the routine diagnostic pathway for PC/BTC reduces the number of repeated invasive procedures	Proportion of patients who undergo a repeated invasive procedure
To assess the impact of Guardant360© in the routine diagnostic pathway for PC/BTC on RMP NHS Cancer Targets	RMP NHS Cancer Targets (Faster Diagnostic Standard (FDS) and 62 day wait)
To assess the invasive procedure-related morbidity and mortality following implementation of Guardant360©into the routine diagnostic pathway for PC/BTC	Type, frequency, and severity of complications from invasive diagnostic procedures

To understand the efficiency gains in diagnostic services following implementation of Guardant360©into the routine diagnostic pathway for PC/BTC	Number and type of invasive procedures performed, histopathology reviews, tissue based NGS testing, hospital visits and length of stay
To assess the cost effectiveness of implementing Guardant360©into the routine diagnostic pathway	Healthcare costs and cost per-quality adjusted-life-year
To understand the impact of implementing Guardant360©into the routine diagnostic pathway on quality of life (QOL).	QOL as measured by EQ-5D-5L

The minimum sample size was calculated based on an estimation of a reduction of 50% (from 25% to 12.5%) in the repeated invasive procedures rate. With a 1% two-sided significance level and 90% power, a sample size of 152 is needed.

However, we aimed to recruit 650 patients over 12 months based on the expected referrals for suspected HPB cancer (RMP data) over 12 months; for the following reasons

- To more accurately predict the proportion spared of repeated invasive procedures
- To ensure 12-month coverage through PDSA cycle to facilitate adoption, business case, and health economic analysis
- To allow adequate assessment of improved NHS targets over longer time period
- To provide sufficient numbers across 6 hospitals (site level)

As part of PDSA methodology, a clinical evaluation of outcome measures will be reported as described in table 53.

Table 52- PDSA outcome assessment timeframe

Number of patients recruited	Analysis/analyses to perform
50	Primary end point (compare proportion of repeat invasive biopsies to 25% historical proportion)
160	Primary end point; secondary end points (except healthcare costs)
325	Futility analysis (stopping a programme early)
485	Primary end point; secondary end points (except healthcare costs)
650	Final analysis of all end points (including healthcare costs)

A futility analysis is planned when 50% of patients (i.e. 325 patients) have proceeded through the pathway. The steering committee will assess the following as a criterion to stop (1) <30% ctDNA detection rate using Guardant 360©.

As of March 2023, 50 patients had been recruited and so the first PDSA outcomes have been reported in my thesis. The other endpoint will be reported following sufficient accrual.

Data was entered by individual sites and cleaned prior to database lock at 50 patients enrolled (March 2023). The statistical analysis report provided data for this thesis chapter.

48.1.12 Human Factors

As part of this implementation programme, an in-depth analysis of human factors was performed. The primary aim was to assess the practicalities of implementing an augmented liquid biopsy pathway into the diagnostic pathway of patients with suspected PC/BTC. This included (1) stakeholder mapping (2) a description of the HPB diagnostic pathway, (3) identification of existing gaps in the diagnostic pathway and (4) an understanding of barriers to adoption and potential scenarios of use. This was to ensure the implementation programme design was adequate for successful implementation into the NHS diagnostic pathway at the end of the programme.

The human factors work was carried out by Massimo Micocci, Melody Ni and Peter Buckle from NIHR In-vitro diagnostics (IVD) at Imperial College London.

Once stakeholders were identified, one-to-one interviews using semi-structured questions were performed in 2 phases. The first phase was to (1) define the current HPB pathway and (2) identify existing gaps in the pathway. The second phase was to understand barriers to adoption and potential scenarios of use. These are still ongoing at the time of writing and are not included in my thesis.

49 Preliminary Results

49.1.1 Existing Gaps in the pathway

Stakeholders identified 6 major opportunities for where ctDNA could mitigate bottlenecks within the invasive diagnostic pathway as outlined in table 54.

Table 53- Existing gaps in the current invasive diagnostic pathway for PC/BTC

Gaps	Description	Research questions
1.Referral criteria	The current criteria for GPs to refer patients onto the 2WW pathway leads to a high volume of patients entering secondary care, placing significant strain onto clinicians, and contributing to long delays for standard out-patient referrals. Also, most GPs do not have access to imaging to detect PC/BTC.	Could ctDNA be used to streamline patient flow in cases of pathways entry delays including for referrals on non-2WW pathways?
2.High demand for imaging	Capacity constraints on imaging services can contribute to diagnostic delays. Those referred into the non-2WW outpatient pathway typically do not receive their first diagnostic test for several weeks.	Could ctDNA be used to streamline patient flow in cases of pathways bottlenecks?
3.Low availability of specialist staff	Low availability of specialists who can perform invasive diagnostic tests (such as EUS and ERCPs).	Could ctDNA be used to streamline patient flow in cases of pathways bottlenecks?
4.Low availability of reliable rapid screening tools	Limited screening tools in primary care	Could ctDNA be used as a screening tool in primary care setting?
5.Repeated testing	Some patients will undergo multiple invasive procedures in order to obtain a viable tissue sample which contributes to delays in the patient's diagnostic pathway.	Could ctDNA be used to confirm a diagnosis in the absence of confirmatory tissue diagnosis?
6.High demand on pathology	The high demand for pathological services cause delays in the processing of tissue samples.	Could ctDNA be used to confirm a diagnosis in the absence of confirmatory tissue diagnosis?

This programme focuses on rapid diagnosis (gaps 1-3) and reducing the need for repetitive invasive diagnostic and reducing demand on histopathology (gap 5, 6). The programme is not assessing gap 4 in screening for PC/BTC.

49.1.2 Patient Recruitment

The programme opened across 6 RMP hospitals at different times as listed below in table 55.

Table 54- Recruitment across RMP sites

Site	Date opened	Recruited as of March 2023
Kingston	13/10/22	11
Royal Marsden	14/10/22	17
Chelsea and Westminster	18/10/22	4
Croydon	18/10/22	9
Epsom & St Hellier	28/10/22	6
West Middlesex	19/01/23	3

A 12 month recruitment period between Jul 22 to Jul 23 was planned. However, given delays in contractual agreements between Guardant Health and RMP, the programme did not open until Oct 22 (3 months late). Delays in staff recruitment to support the programme also impacted the start date. In addition, we were advised by NHSE to limit the inclusion criteria to ensure patients who are not suitable for an invasive procedure are not considered eligible, and only patients with stage III/IV disease were considered given the assay is CE marked and FDA approved only for this indication (criteria for funding). Therefore, the initial plan to recruit 650 patients across 6 centres in 12 months (which was based on RMP number for referrals for HPB cancers) was an overestimate, as such recruitment has been slow and mitigation discussions are ongoing with the funder. The current recruitment figure is shown in 35.

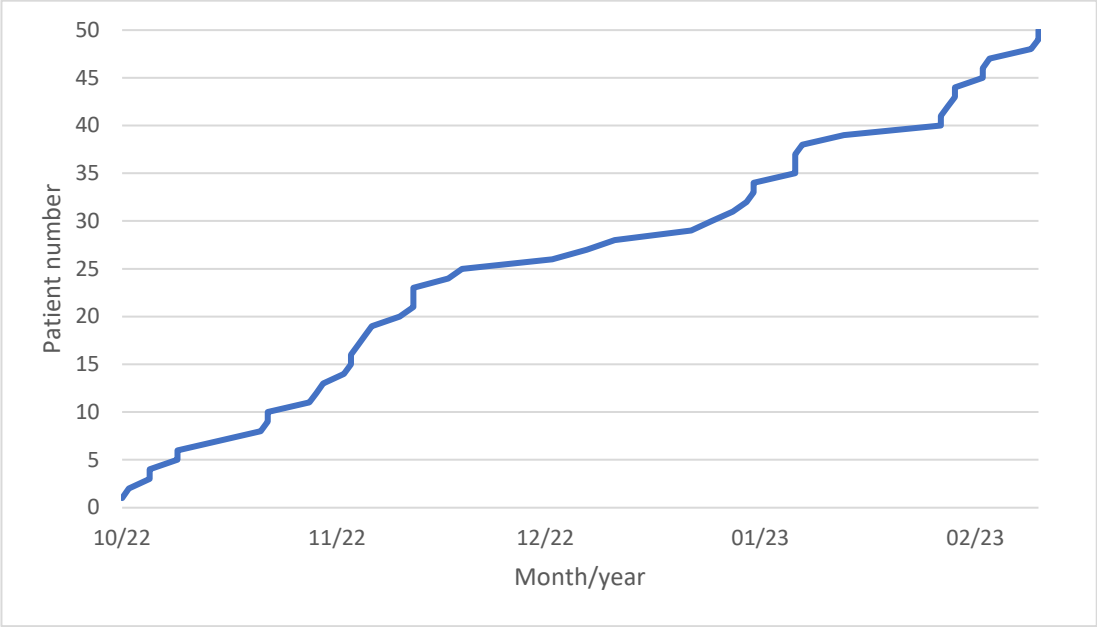


Figure 31- ACCESS recruitment graph until March 2023

49.1.3 Patient Characteristics

As part of the PDSA methodology, the first 50 patients recruited were analysed for assessment of several endpoints as part of an iterative learning process to ensure implementation issues were identified and addressed. This was performed at data cut off on 10th March 2023.

A CONSORT diagram of all registered patients is shown in fig 36. Of 50 registered patients, 1 withdrew immediately following registration. 49 patients were subsequently enrolled and referred for ctDNA collection. 7 were deemed ineligible due to rapid deterioration (including 3 patient deaths), lost to follow up prior to ctDNA collection and pending ctDNA collection prior to data cut-off.

42 patients were registered and had ctDNA collected for inclusion in this interim analysis.

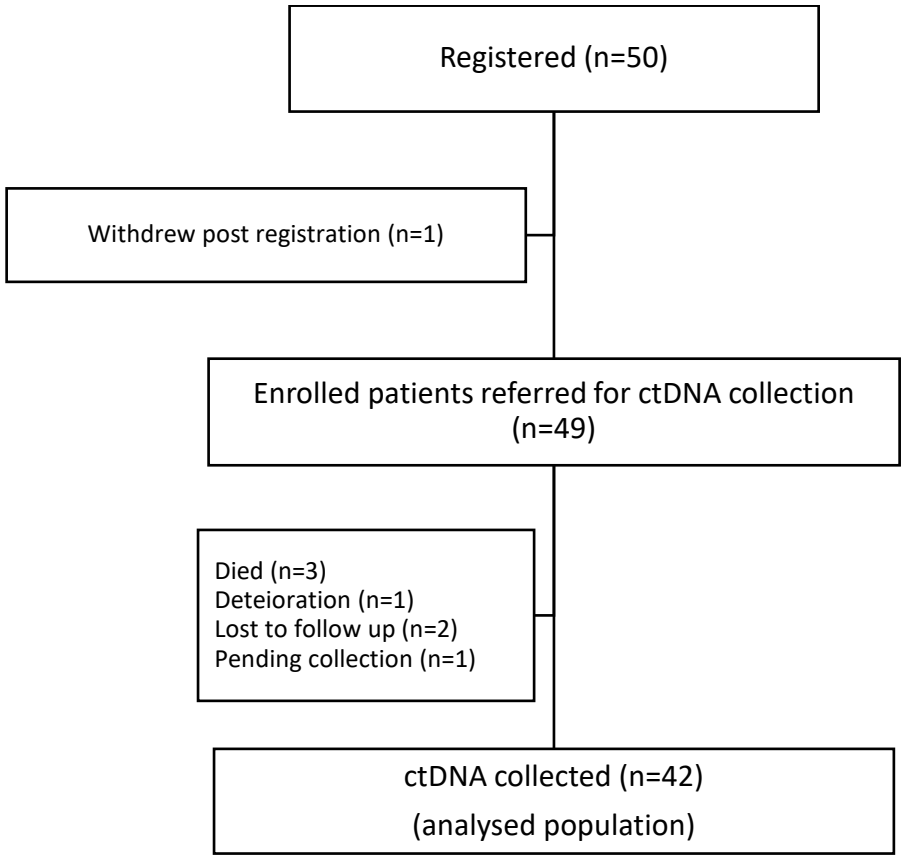


Figure 32- CONSORT diagram of registered patients within ACCESS

Baseline characteristics of all enrolled patients (n=49) in the programme are outlined in table 56. The median age was 73 years old, ranging from 43 to 94 years. Majority were male (55.1%) and of Caucasian ethnicity (71.4%). Patients were recruited across all sites; however majority were recruited from RMH (34.7%). 34.7% were referred into the diagnostic pathway through a 2WW pathway.

Baseline imaging was performed in all patients. 75.5% had suspected PC with 24.5% had suspected BTC. Most patients had suspected stage IV disease (55%).

Table 55- Baseline characteristics of all enrolled patients in ACCESS

Variable	N=49 (%)
Age	
Median	73
Range	43-94
Sex	
Male	27 (55.1)
Female	22 (44.9)
Ethnicity	
Caucasian	35 (71.4)
African/Caribbean	1 (2)
Mixed	2 (4.1)
Other	11 (22.5)
Recruitment site	
C&W	4 (8.2)
WMSH	3 (6.1)
RMH	17 (34.7)
KH	11 (22.5)
ESH	5 (10.2)
CUH	9 (18.3)
Referral pathway	
2WW	17 (34.7)
Routine	11 (22.5)
A&E	15 (30.6)
Inpatient	4 (8.1)
Other	2 (4.1)
Suspected cancer site	
PC	37 (75.5)
BTC	12 (24.5)
Suspected Cancer stage	
III	22 (45)
IV	27 (55)

49.1.4 ctDNA Detection in Pancreatic and Biliary Tract Cancer

All eligible patients had ctDNA collected (n=42). The ctDNA detection rate overall was 78.6% (n=33), and 81.3% (n=26) in suspected PC and 70% (n=7) in suspected BTC.

MTB discussion of all ctDNA detected cases was performed (n=33). The MTB deemed 54.5% (n=18) of results supportive of a diagnosis of PC/BTC, with 13 diagnostic and 5 consistent with PC/BTC. Of the 15 ctDNA results which were deemed not consistent with a diagnosis of PC/BTC, 13 were considered possibly consistent and 2 not consistent with a diagnosis of PC/BTC. For eligibility in PREVAIL part 2 (i.e. to be treated on liquid biopsy alone), a level of diagnostic confidence of “diagnostic” or “consistent with” was required and therefore used as a category of supportive of diagnosis of PC/BTC.

An outline for the ctDNA results and MTB outcome is listed by patient in table 57.

Table 56- Molecular diagnostic results from ACCESS

ID	Tumour	Stage	ctDNA result	Gene/ Biomarker	Alteration	VAF%/Copy number	EMA Approved Therapies	MTB level of diagnostic support	Tissue Biopsy	Tissue biopsy result
RM001	BTC	3	N/C	N/A	N/A	N/A	N/A	N/A	No	N/A
RM0002	PC	3	+	MET	E34K	0.14%	No	Possibly	Yes	Non-diagnostic
RM0004	PC	3	+	BRCA1	T293I	0.12%	No	Possibly	Yes	PDAC
				CDK4	P302L	0.22%	No			
RM0003	PC	3	+	KRAS	Amplification	2.48	No	Diagnostic	Yes	PDAC
				MYC	Amplification	2.37	No			
				CDKN2A	L16fs	2.12%	No			
				KRAS	G12V	23.84%	No			
				TP53	K132N	4.24%	No			
CY0001	PC	4	+	NRAS	G12D	0.07%	No	Consistent	Yes	Non-diagnostic
KI001	PC	4	-	N/A	N/A	N/A	N/A	N/A	Yes	Pancreatitis
RM0005	PC	4	+	ATM[1]	W164	0.25%	Olaparib[2]	Consistent	Yes	PDAC
				EGFR	G343D	0.74%	No			
				ERBB2	K684T	4.55%	No			
				KRAS	G12V	9.08%	No			
				RB1	W681	3.95%	No			
				TP53	Q144	4.85%	No			
RM0006	PC	3	+	KRAS	G12V	0.06%	No	Consistent	Yes	PDAC
KI002	PC	3	N/C	N/A	N/A	N/A	N/A	N/A	No	N/A

RM007	BTC	3	N/C	N/A	N/A	N/A	N/A	N/A	No	N/A
RM0009	PC	3	+	ARID1A	P225L	0.16%	No	Possibly	Yes	PDAC
RM010	PC	4	N/C	N/A	N/A	N/A	N/A	N/A	No	N/A
KI0003	PC	4	+	BRCA2	L1227fs	53.24%	Olaparib, rucaparib, talazoparib	Diagnostic	Yes	PDAC
				KRAS	G12V	2.5%	No			
				TERT	c.-124C>T	3.87%	No			
				TP53	I254fs	0.15%	No			
				TP53	P190fs	1.57%	No			
CY0002	PC	3	+	CCNE1	Amplification	2.45	No	Consistent	No	N/A
				EGFR	Amplification	2.61	Yes			
				PIK3CA	Amplification	2.62	No			
				ATM[1]	R3008C	0.2%	Olaparib[2]			
				CDKN2A	T18del	10.55%	No			
				EGFR	R72K	0.19%	No			
				KRAS	G12D	19.64%	No			
				MTOR	V122I	0.11%	No			
				TP53	Q38	15.28%	No			
RM0012	PC	4	+	KRAS	G12V	5.2%	No	Consistent	Yes	PDAC
				TP53	H179R	1.41%	No			
RM011	PC	4	-	N/A	N/A	N/A	N/A	N/A	Yes	Non-diagnostic
CY0003	BTC	4	+	CDK12	R661K	2.11%	No	Possibly	No	N/A
				ERBB2	S310F	8.25%	T-DXd[2]			

				FGFR2	R579Q	0.15%	No			
				TP53	E294	0.69%	No			
RM0013	PC	3	+	KRAS	G12V	0.24%	No	Diagnostic	Yes	PDAC
				PTEN	L265L	0.42%	No			
				TP53	G108fs	0.14%	No			
CY0004	BTC	4	+	ARID1A	P1326Q	0.67%	No	Possibly	Yes	Gallbladder adenocarcinoma
				ATM[1]	S2394	7.66%	Olaparib[2]			
				KIT	S24C	2.94%	No			
				PIK3CA	M1043I	12.31%	Alpelisib[2]			
				TERT	c.-124C>T	1.65%	No			
				TP53	E180K	8.28%	No			
RM0014	PC	4	+	CDKN2A	R80	12.59%	No	Diagnostic	Yes	PDAC
				KRAS	G12D	13.34%	No			
				SMAD4	L98F	19.39%	No			
				TP53	S96fs	18.34%	No			
KI004	PC	4	-	N/A	N/A	N/A	N/A	N/A	Yes	PDAC
RM0015	BTC	4	+	CCND1	A205A	0.22%	No	Possibly	Yes	Non-diagnostic
				NF1	c.3974+2T>A	0.13%	Selumetinib[2]			
CY0005	PC	4	+	ARID1A	P279fs	1.45%	No	Diagnostic	Yes	Pancreatic squamous cell carcinoma
				CDKN2A	D14fs	0.91%	No			
				KRAS	G12D	0.96%	No			
				TP53	R175H	1.2%	No			
CY0006	PC	4	+	CDK4	R55C	0.34%	No	Diagnostic	Yes	PDAC

				KRAS	G12V	0.56%	No			
				TP53	R273H	0.37%	No			
RM016	PC	3	N/C	N/A	N/A	N/A	N/A	N/A	No	N/A
CY007	BTC	3	-	N/A	N/A	N/A	N/A	N/A	Yes	Non-diagnostic
RM0017	PC	4	+	KRAS	Amplification	7.29	No	Possibly	Yes	PDAC
				MYC	Amplification	4.68	No			
				AR	G636D	0.57%	No			
				CDKN2A	S7fs	3.43%	No			
				PTEN	G165E	0.54%	No			
				PTEN	R130	0.13%	No			
				RET	R844L	2.52%	No			
				TP53	c.75-1G>A	4.35%	No			
KI005	PC	4	-	N/A	N/A	N/A	N/A	N/A	Yes	PDAC
CY0008	BTC	4	+	APC	K2052R	1.63%	No	Diagnostic	No	N/A
				APC	R2237	2.97%	No			
				AR	A404A	4.18%	No			
				BRCA2	N2189fs	0.14%	Olaparib, talazoparib[2]			
				PIK3CA	L443S	0.41%	No			
				MSI-High	N/A	N/A	Pembrolizumab			
KI0006	PC	3	+	EGFR	G719G	0.44%	No	Not consistent	Yes	PDAC
CE0001	PC	4	+	CDKN2A	H83Y	1.15%	No	Diagnostic	Yes	PDAC
				KRAS	G12V	1.99%	No			

				TP53	G245S	1.38%	No			
EP0001	PC	3	+	CDK2NA	c1047-2A>G	44.67%	No	Diagnostic	Yes	PDAC
				FGFR2	R84K	0.6%	No			
				KRAS	G12D	0.28%	No			
				TP53	c783-1G>A	0.14%	No			
KI0008	PC	3	+	FBXW7	E489K	0.18%	No	Possibly	Yes	Non-diagnostic
				NRAS	G12D	0.06%	No			
				TP53	I251V	0.11%	No			
KI0007	PC	4	+	PIK3CA	Amplification	2.42	No	Diagnostic	Yes	PDAC
				ATM[1]	c.3285-1G>T	21.22%	Olaparib[2]			
				KRAS	G12R	42.8%	No			
				TP53	Y220C	16.51%	No			
EP0003	PC	3	+	TP53	S106R	0.12%	No	Possibly	Yes	Non-diagnostic
CE0002	PC	3	+	VHL	Q96fs	0.27%	No	Not consistent	Yes	Non-diagnostic
EP002	N/C	N/C	N/C	N/A	N/A	N/A	N/A	N/A	No	N/A
KI0009	PC	4	+	KRAS	G12V	16.97%	No	Possibly	No	N/A
				MAPK3	L138L	0.82%	No			
				MAPK3	Q149H	0.61%	No			
				PIK3CA	E542K	0.25%	Alpelisib[2]			
				PIK3CA	E545K	0.53%	Alpelisib[2]			
				PIK3CA	E545Q	0.25%	Alpelisib[2]			
				TP53	E339	8.19%	No			
WD0001	BTC	3	+	ERBB2	K937K	0.17%	No	Possibly	Yes	Non-diagnostic

				FGFR2	D321N	0.2%	No			
				NTRK1	Q660Q	1.22%	No			
				RB1	G423	0.9%	No			
				TP53	H168fs	0.07%	No			
				VHL	Q96fs	0.27%	No			
WD002	BTC	3	-	N/A	N/A	N/A	N/A	N/A	No	N/A
CE003	PC	4	-	N/A	N/A	N/A	N/A	N/A	Yes	PDAC
RM008	PC	4	-	N/A	N/A	N/A	N/A	N/A	Yes	Pancreatic NET
KI011	PC	4	N/C	N/A	N/A	N/A	N/A	N/A	No	N/A
KI010	PC	4	P	N/A	N/A	N/A	N/A	N/A	No	N/A
EP005	BTC	4	+	APC	E2463K	0.29%	No	Possibly	Yes	Non-diagnostic
				ATM	E292D	0.13%	No			
				BRAF	N581I	0.40%	No			
				ERBB2	R354R	0.31%	No			
				RAD1	Indel	0.05%	No			
EP0004	PC	4	+	KRAS	G12D	4.52%	No	Diagnostic	Yes	PDAC
				TERT	R672S	0.18%	No			
				TP53	P153fs	1.31%	No			
CY0009	PC	3	+	ALK	A1168T	0.39%	No	Diagnostic	No	N/A
				ARID1A	Q372fs	25.25%	No			
				NRAS	Q61R	19.73%	No			
				TP53	I255F	0.86%	No			
				TP53	M246R	25.52%	No			

EP006	PC	3	+	TP53	R273P	0.13%	No	Possibly	Yes	Non-diagnostic
CE004	PC	4	+	KRAS	G12R	0.23%	No	Diagnostic	Yes	PDAC
				STK11	P217fs	0.31%	No			
WD003	BTC	3	-	N/A	N/A	N/A	N/A	N/A	Yes	Non-diagnostic

1- germline mutation reported only; 2- EMA approved therapy for other indication; P- pending result; N/C- not collected; N/A- not applicable; BTC- biliary tract cancer; PC- pancreatic cancer; NET- neuroendocrine tumour; PDAC- pancreatic ductal adenocarcinoma; T-DXd- Trastuzumab deruxtecan

49.1.5 The Augmented Diagnostic Pathway

Mapping of the liquid biopsy augmented pathway was performed to assess the impact of the new pathway on patient flow. Of 42 patient who had ctDNA collected, 36 (85.7%) underwent both liquid and tissue biopsy, while 6 (14.3%) proceeded through a liquid biopsy alone pathway. Of the 36 patients who underwent at least one invasive diagnostic procedure, 5 had an invasive procedure performed prior to enrolment. Of the 31 patients who had at least 1 invasive procedure following enrolment, 5 had a repeated invasive biopsy with a repeated invasive biopsy rate of 16.1% in all patients following enrolment.

In those undergoing both tissue and liquid biopsies, 24 (66.7%) patients had an informative tissue biopsy (exiting the diagnostic pathway) while 12 (33.3%) had a non-informative biopsy. Of the informative tissue biopsies, 20 (83%) had PDAC, 1 (4.2%) had pancreatic squamous cell carcinoma (pSCC), 1 (4.2%) had pancreatic NET, 1 (4.2%) had cholangiocarcinoma, and 1 (4.2%) had pancreatitis. In these patients, ctDNA was detected in 85% of PDAC, 100% cholangiocarcinoma and pSCC and not detected in the patient with panNET or pancreatitis.

In the 12 patients with non-informative tissue biopsies, 9 had ctDNA detected. The MTB deemed 8 of these not consistent/possibly consistent with a diagnosis of cancer, while 1 had an informative ctDNA result consistent with BTC and commenced treatment on ctDNA alone.

Overall, 28 (n=66.7%) had a diagnosis made, with 14 (50%) diagnosis made on both ctDNA and tissue biopsy, 10 (35.7%) on tissue biopsy alone and 4 (14.3%) in liquid biopsy alone. Of the 28 patients who had a diagnosis made, 17 patients commenced treatment, while 11 did not due to death (n=4), awaiting chemotherapy at time of data cut off (n=4), patient choice (n=2), and non-cancer histology (n=1).

In the 6 patients who had a liquid biopsy alone, 3 had negative or non-informative ctDNA results (1 died post ctDNA, 2 were awaiting tissue biopsy at data cut off). In the 3 patients with informative liquid biopsy results and no invasive procedure performed, 1 commenced treatment on liquid alone, while 1 deteriorated rapidly prior to chemotherapy and 1 patient still awaiting chemotherapy at data cut off.

Fig 37 describes the ACCESS patient's pathway through the augmented diagnostic pathway showing diagnostic and treatment outcomes.

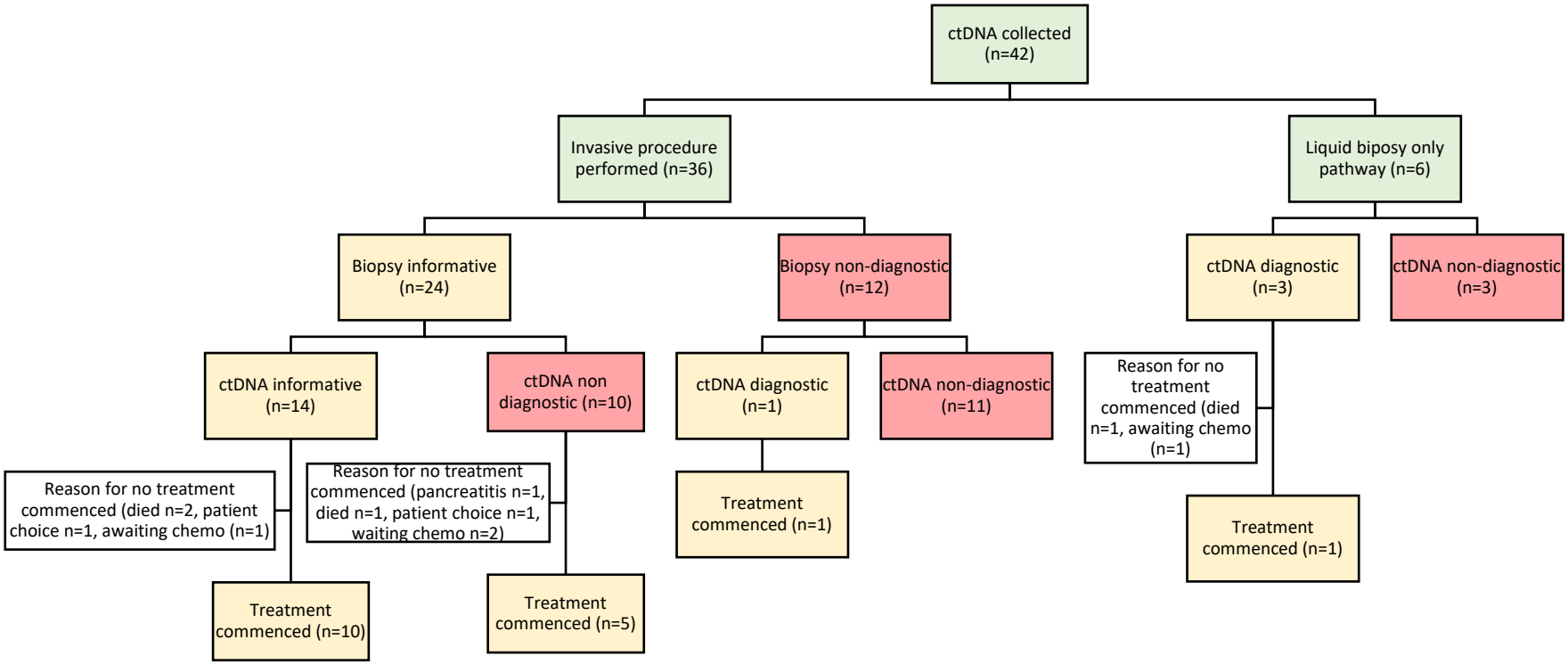


Figure 33- Patient flow in the ACCESS programme

50 Discussion

The invasive diagnostic pathway for patients with suspected PC/BTC is complex, with delays in diagnosis and initiation of treatment contributing to poor survival and patient experience. We implemented a parallel non-invasive diagnostic pathway for patients with suspected PC/BTC.

The challenges in the diagnosis of PC/BTC are largely related to the anatomy and the resultant complexity of the invasive diagnostic pathway. Stakeholder interviews performed by NIHR IVD identified several important challenges within this pathway, including delays in entering the pathway (especially for non-2WW referrals), delays within the diagnostic pathway (high demand for imaging, invasive diagnostic procedures and histology), and delays due to repeated invasive procedures. These represent the major gaps in the current invasive diagnostic pathway for patients with suspected PC/BTC.

Approximately 25-40% of patients with PC/BTC require a repeated invasive biopsy for diagnosis. By implementing a parallel ctDNA pathway into the routine invasive pathway for patients with suspected PC/BTC, we showed a repeated invasive biopsy rate of 16.1%. Our hypothesis is that an augmented ctDNA pathway will reduce the invasive procedure rate by 50% (i.e. 25% to 12.5%). Noting small numbers, the preliminary result from this interim analysis is promising. However, ongoing recruitment is needed to fully assess the primary end point.

The delays within the diagnostic pathway are multi-factorial, these include limited capacity for endoscopic services and histopathology processing. A recent workforce survey showed that only 3% of histopathology departments are fully staffed, with outsourcing services costing the NHS an estimated £27 million per year (353). We showed that 7.1% of patients had a diagnosis made on liquid biopsy alone, without confirmative tissue biopsy, indicating a possible reduction in histopathology requests. A report on the use of histopathology diagnostic requests will be produced at the completion of the programme.

Importantly, 66.7% of patients in the programme received a definitive diagnosis, with 50% having a diagnosis on both tissue and liquid, 35.7% on tissue alone and 14.3% on liquid biopsy alone. The 4 patients diagnosed on liquid biopsy alone would have required a definitive biopsy performed, thereby reducing the burden on endoscopic and histology services. Overall, 65.4% with histologically confirmed PC/BTC initiated treatment. However, 15.4% of patients were still awaiting treatment at data cut off and would likely have initiated chemotherapy. Historically, 50% of patients with metastatic PC will commence 1L chemotherapy (345). The COVID19 pandemic had a significant impact on NHS cancer wait times with NHS upper GI performing below operation targets. The current 28-day FDS (time from referral to cancer diagnosis) for upper GI cancers across all UK trusts 68.6% (target 75%). Further assessment of the 28-day FDS and other cancer outcomes will be performed at the completion of the programme.

The analytical Sn of Guardant360© in patients with stage III/IV PC/BTC has established evidence. We detected ctDNA in 78.6% of patients overall (including 81.3% with PC and 70% with BTC). A large validation study included patients with histologically confirmed stage III/IV PC and BTC, demonstrating ctDNA detection rate of 80% and 85% using the Guardant360© assay, respectively. The slightly lower ctDNA detection in our cohort is likely related to the lower number of histologically confirmed PC/BTC, with some patients having benign conditions, inflammatory (i.e. pancreatitis), or non-cholangiocarcinoma or PDAC histologies.

The PREVAIL Part 2 study allowed patients to be treated on ctDNA alone. This included CY0001 who was referred following 3 non-diagnostic invasive biopsies for suspicious BTC. ctDNA analysis revealed an *NRAS* mutation which the MTB deemed consistent with a diagnosis of BTC. This patient commenced chemotherapy with cisplatin and gemcitabine through the PREVAIL Part 2 study. Patient CY0008 had ctDNA collected and reported prior to an invasive diagnostic procedure being performed. ctDNA revealed MSI-H status and he subsequently commenced pembrolizumab without a confirmatory histological diagnosis. This study will report on objective responses and survival outcomes in patients who are treated on liquid biopsy alone. Although the primary objective of this programme is to speed diagnosis in patients with suspected PC/BTC, the use of ctDNA in the comprehensive genomic profiling to detect actionable mutations provides an additional benefit. We detected potentially actionable mutations in 9 patients overall (21.4%).

There are several limitations of the ACCESS implementation programme. Firstly, given the natural history of PC/BTC, 16% of patients who were initially registered were not suitable for ctDNA collection or an invasive procedure, predominantly driven by rapid deterioration. Providing an assay in primary care at the initial onset of the 2WW pathway could mitigate for this rapid decline. Secondly, we enrolled a patient with dual primary cancers (RM0017) who had a localised oesophageal adenocarcinoma diagnosis following ctDNA collection. A liver biopsy revealed a poorly differentiated adenocarcinoma of likely pancreatic origin. ctDNA revealed several aberrations (including CDK2NA, PTEN, RET and TP53 mutations) which were not specific for PC and so the MTB deemed this possibly consistent with PC given the dual primary lesions. This is largely a limitation of the assay in determining of CSO. The addition of DNA based methylation assessment may provide this detail. Thirdly, this programme excluded patients with stages I/II also may benefit from faster diagnosis as highlighted by the stakeholder evaluation. However, as the Guardant 360© assay is not indicated in stage I/II disease (programme requirement), these patients could not be included in the programme.

An implementation programme of a new diagnostic pathway requires appropriate pathway mapping, understanding barriers to adoption and stakeholder engagement. The final data analysis will feed into a cost effectiveness analysis. Human factors are critical in understanding barriers to adoption to successfully deploy this technology into the NHS. A cost effectiveness model which will be performed by NIHR IVD. The first phase includes a systemic review to gather evidence to support liquid biopsy as an

adjunct or in lieu of tissue diagnosis. A theoretical cost-consequence model will be developed which will be informed by the stakeholder analysis. This model will be refined by incorporating data from the implementation programme final analysis to generate a health economic evidence for future NICE submission. Importantly, QOL data will be collected and feed into the health economic analysis.

The ACCESS programme aims to speed diagnosis in patients with suspected PC/BTC. There are other ct-DNA based diagnostic studies currently ongoing. The PATHFINDER study includes patients with symptomatic disease (including BTC/PC), assessing the diagnostic accuracy of ctDNA-based methylation MCED (the Galleri assay). Our programme however offers unique outputs, additive to the PATHFINDER study. The evidence generated from this programme (including impact on faster diagnosis), DNA genotyping (to identify targetable mutations), the development of genomic infrastructure (including an MTB) and the implementation of a ctDNA-based analyte into the diagnostic pathway are some of the additional benefits and insights provided by the ACCESS programme. ctDNA based diagnostic tools in screening and faster diagnosis will revolutionise the cancer diagnostic pathways across the NHS. We have shown that these tools can be implemented into the routine diagnostic pathway.

51 Conclusions

The diagnosis of cancer relies on a considered approach, incorporating clinical presentation and history, tumour markers, imaging and histological diagnosis. The latter is considered the gold standard in cancer diagnosis. The COVID19 pandemic highlighted the fragility of the current cancer diagnostic pathway for GI cancers, which rely on invasive procedures to obtain a histological diagnosis. Delays in within the invasive diagnostic pathway, and the ongoing histopathology workforce shortage, necessitates additional non-invasive biomarkers to support a cancer diagnosis for these difficult to diagnose tumours.

Liquid biopsies using ctDNA-based genotyping are increasingly used as a non-invasive biomarker in molecular profiling advanced tumours to select personalised therapies. However, the evidence to support liquid biopsies as a supportive non-invasive diagnostic biomarker to speed diagnosis of suspected PC, BTC and CRC is encouraging. The PREVAIL ctDNA study showed support for the use of ctDNA in the diagnosis of patients with suspected PC/BTC with good sensitivity and specificity. These assays can be safely implemented into the routine diagnostic pathway as we have shown in the ACCESS programme, with the aim of improving NHS cancer wait times and facilitating access to potentially life-saving treatments. The role of an MTB in providing clinical context to detected variants is critical in reducing false positive results, and direct personalised therapies. implementation of ctDNA assays into the routine diagnostic pathway require appropriate genomic

training and specialist MTB staff. With multi-parametric platforms incorporating ctDNA genotyping and methylation analyses being investigated in large, prospective cancer screening studies. These non-invasive, highly sensitive, multi-cancer early detection tests have a promising role in the future of cancer screening. However, these commercial assays require further refinement to ensure adequate diagnostic accuracy and successful implementation of such tests within the NHS is critical in order achieve the NHS Long Term Plan.

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Appendices

Appendix A- ESMO Scale of Clinical Actionability for Molecular Targets

ESCAT: ESMO Scale of Clinical Actionability for molecular Targets

	ESCAT evidence tier		Required level of evidence	Clinical implication
Ready for routine use	I Alteration-drug match is associated with improved outcome in clinical trials	I-A	Prospective, randomised clinical trials show the alteration-drug match in a specific tumour type results in a clinically meaningful improvement of a survival end point	Access to the treatment should be considered standard of care
		I-B	Prospective, non-randomised clinical trials show that the alteration-drug match in a specific tumour type, results in clinically meaningful benefit as defined by ESMO MCBS 1.1	
		I-C	Clinical trials across tumour types or basket clinical trials show clinical benefit associated with the alteration-drug match, with similar benefit observed across tumour types	
Investigational	II Alteration-drug match is associated with antitumour activity, but magnitude of benefit is unknown	II-A	Retrospective studies show patients with the specific alteration in a specific tumour type experience clinically meaningful benefit with matched drug compared with alteration-negative patients	Treatment to be considered "preferable" in the context of evidence collection either as a prospective registry or as a prospective clinical trial
		II-B	Prospective clinical trial(s) show the alteration-drug match in a specific tumour type results in increased responsiveness when treated with a matched drug, however, no data currently available on survival end points	
Hypothetical target	III Alteration-drug match suspected to improve outcome based on clinical trial data in other tumour type(s) or with similar molecular alteration	III-A	Clinical benefit demonstrated in patients with the specific alteration (as tiers I and II above) but in a different tumour type. Limited/absence of clinical evidence available for the patient-specific cancer type or broadly across cancer types	Clinical trials to be discussed with patients
		III-B	An alteration that has a similar predicted functional impact as an already studied tier I abnormality in the same gene or pathway, but does not have associated supportive clinical data	
	IV Pre-clinical evidence of actionability	IV-A	Evidence that the alteration or a functionally similar alteration influences drug sensitivity in preclinical *in vitro* or *in vivo* models	Treatment should "only be considered" in the context of early clinical trials. Lack of clinical data should be stressed to patients
		IV-B	Actionability predicted *in silico*	
Combination development	V Alteration-drug match is associated with objective response, but without clinically meaningful benefit		Prospective studies show that targeted therapy is associated with objective responses, but this does not lead to improved outcome	Clinical trials assessing drug combination strategies could be considered
Lack of Evidence	X Lack of evidence for actionability		No evidence that the genomic alteration is therapeutically actionable	The finding should not be taken into account for clinical decision

Reference: (82)

Appendix B- Gastrointestinal ctDNA (ct-GI) panel

Gene	Gene ROI
ACVR2A	Exon 10
AKT1	Exon 3
APC	Exons 1-16
ARID1A	Exons 1-20
ATM	Exons 1-63
BRAF	Exons 11,14-16
CTNNB1	Exons 3,7-8
DOCK2	Exons 1-52
EGFR	Exons 18-21
FBXW7	Exons 1a,1b,1c-12
KRAS	Exons 2-3
NOTCH1	Exons 1-34
NRAS	Exons 2-3
PIK3CA	Exons 2-21
PTEN	Exons 5,7
QC	Exons 1-20
RET	Exons 10-11,15-16
RNF43	Exons 2-10
SMAD2	Exons 2-11
SMAD4	Exons 2-12
TCF7L2	Exons 1-14
TGFBR2	Exon 7

TP53	Exons 4-10,10a-11

Appendix C – RMH tissue based NGS panel

Gene	Gene ROI
ABL1	Exons 1-11
ACVR1	Exons 3-11
AKT1	Exons 2-14
AKT2	Exon 3
AKT3	Exon 2
ALK	Exons 19-29 Introns 18-19
AMER1	Exon 2
ANTXR2	Exons 1-17
APC	Exons 1-16
AR	Exons 1-8
ARAF	Exons 2-16
ARID1A	Exons 1-20
ARID1B	Exons 1-20
ARID2	Exons 1-21 Intron 20
ASXL1	Exons 1-13
ATM	Exons 1-63
ATR	Exons 1-47 Intron 31
ATRX	Exons 1-35
AURKA	Exons 2-9
AXIN1	Exons 1-11
AXIN2	Exons 1-11
B2M	Exons 1-3
BAP1	Exons 1-17
BARD1	Exons 1-11 Intron 9

BBC3	Exons 2-4
BCL2	Exon 2
BCOR	Exons 1,1a-15 Intron 14
BCORL1	Exons 1-13
BIRC3	Exons 2-9
BRAF	Exons 1-10,10a-18 Introns 8:1,8:2-10
BRCA1	Exons 2-13,13a-23
BRCA2	Exons 2-27
BRIP1	Exons 2-20
BTG1	Exons 1-2
BTK	Exon 15
CALR	Exon 9
CASP8	Exons 3-10
CBL	Exons 8-9
CCND1	Exons 1-5
CCND2	Exons 1-5
CCNE1	Exons 2-12
CCNE2	Exons 2-12
CD79B	Exons 1-6
CDH1	Exons 1-16
CDK12	Exons 1-14
CDK2	Exons 1-7
CDK4	Exons 2-8
CDK6	Exons 2-8
CDKN1A	Exons 2,2a-3
CDKN1B	Exons 1-2
CDKN2A	Exons 1,1a-3,3a
CDKN2B	Exons 1-2
CDKN2C	Exons 1-2
CEBPA	Exon 1
CHEK1	Exons 1-13
CHEK2	Exons 2-16
CIC	Exons 1-20
CKS1B	Exons 1-3
CREBBP	Exons 1-31
CRLF1	Exon 7
CTNNB1	Exons 2-15
CXCR4	Exon 1
DAXX	Exons 2-8
DDR2	Exon 14
DDX3X	Exons 1-17
DICER1	Exons 2-27
DPYD	Exons 11,13-14,22 Intron 10

DROSHA	Exons 3-35
EGFR	Exons 1-16,16a,16b-28
ELP1	Exons 1-37
EMSY	Exons 2-21
EP300	Exons 1-31
EPHB2	Exons 1-16
ERBB2	Exons 1-27
ERBB3	Exons 1-28
ESR1	Exons 2-9
ETV6	Exons 1-8
EZH2	Exons 1,1a-6,8-20
F2R	Exons 1-2
FADD	Exons 1-2
FAM46C	Exon 2
FANCI	Exons 2-38
FANCL	Exons 1-14
FAT1	Exons 2-27
FBXW7	Exons 1,1a,1b,1c-3,3a-12
FGF10	Exons 1-3
FGFR1	Exons 2,2a-3,3a-18
FGFR2	Exons 2-8,8a-17,17a,17b-18
FGFR3	Exons 2-8,8a-18
FGFR4	Exons 2-18
FH	Exons 1-10
FLT3	Exons 1-20
FOXL2	Exon 1
FOXO1	Exons 1-2
GATA1	Exons 1-6
GATA3	Exons 2-6
GNA11	Exon 5
GNAQ	Exon 5
GNAS	Exons 1,1a,1b-13
GPR161	Exons 4-8
H3F3A	Exons 2-4
H3F3B	Exons 2-4
HIST1H3B	Exon 1
HIST1H3C	Exon 1
HIST2H3A	Exon 1
HIST2H3C	Exon 1
HRAS	Exons 2-4,4a-5
ID3	Exons 1-2
IDH1	Exons 1-10
IDH2	Exons 1-11

IGF1R	Exons 1-21
IRF4	Exons 2-9
IRS2	Exons 1-2
JAK2	Exon 14
KBTBD4	Exons 2-4
KDM6A	Exons 1-29
KDR	Exons 6-26
KEAP1	Exons 2-6
KIT	Exons 8-11,13-14,17-18
KLF2	Exons 1-3
KMT2A	Exons 1-36
KMT2C	Exons 1-59
KMT2D	Exons 1-54
KRAS	Exons 1-4
LIN28B	Exons 1-4
LZTR1	Exons 1-21
MAP2K1	Exons 1-11
MAP2K2	Exons 1-11
MAP2K4	Exons 1-12
MAP3K1	Exons 1-20
MAPK1	Exons 1-8
MCL1	Exons 1-3
MDM2	Exons 1-11
MDM4	Exons 2-11
MEN1	Exons 2-10
MET	Exons 1-21
MLH1	Exons 1-19
MN1	Exons 1-2
MPL	Exon 10
MRE11A	Exons 2-20 Intron 7
MSH2	Exons 1-16
MSH6	Exons 1-10
MTOR	Exons 28-58
MYC	Exons 1-3
MYCL	Exons 1-3
MYCN	Exons 2-3
MYD88	Exon 5
MYOD1	Exons 1-3
NF1	Exons 1-58
NF2	Exons 1-16,16a Intron 7
NFE2	Exons 2-3
NFE2L2	Exons 1-5
NOTCH1	Exons 1-34

NOTCH2	Exons 1-2,5,20,30
NOTCH3	Exons 1-33
NPM1	Exons 10-11
NRAS	Exons 1-4
NTRK1	Exons 8-10
NTRK2	Exons 18-19
NTRK3	Exons 16-17
OTX1	Exons 3-5
PALB2	Exons 1-13
PAX5	Exons 1-9
PBRM1	Exons 2-30
PDCD1LG2	Exons 2-7
PDGFRA	Exons 1-23
PHOX2B	Exons 1-3
PIK3CA	Exons 2-21
PIK3CD	Exons 3-24
PIK3R1	Exons 2-16
PIN1	Exons 1-4
PMS1	Exons 2-13
PMS2	Exons 1-15
POLD1	Exons 2-3,5-27
POLE	Exons 1-49
POT1	Exons 2-15,15a-19
PPM1D	Exons 1-6
PPP2R2A	Exons 1-10
PRKAR1A	Exons 2-11
PTCH1	Exons 1,1a,1b-23
PTCH2	Exons 1-22
PTEN	Exons 1-9
PTPN11	Exons 2-3,5,7-15
RAD21	Exons 1-14
RAD50	Exons 1-25
RAD51B	Exons 2-11,11b-12 Intron 10
RAD51C	Exons 1-9
RAD51D	Exons 1-10
RAD54L	Exons 1-18
RAF1	Exons 2-17
RB1	Exons 1-27
RBM10	Exons 2-24
RET	Exons 2-20 Intron 11
RICTOR	Exon 14
RIT1	Exons 1,1a-6
RNF43	Exons 2-10

ROS1	Exons 31-43 Introns 31-34
RUNX1	Exons 1-5,5a-9
RhoA	Exons 2-5
SDHA	Exons 1-15
SDHB	Exons 1-8
SDHC	Exons 1-6
SDHD	Exons 1-4
SETBP1	Exons 1,1a-4,4a-6
SETD2	Exons 1-21
SF3B1	Exons 1-5,5a-25
SH2B3	Exons 2-8
SMAD2	Exons 2-11
SMAD3	Exons 1-9
SMAD4	Exons 2-12
SMARCA4	Exons 2-36
SMARCB1	Exons 1-9
SMARCE1	Exons 2-11
SMO	Exons 1-12
SOX2	Exon 1
SRSF2	Exons 1-2
STAG2	Exons 1,1a-35
STK11	Exons 1-9
SUFU	Exons 1-11,11a-12
TCEB1	Exons 2-4
TCF3	Exons 2-19
TERT	Exons 1-16
TET2	Exons 1,1a-11
TFE3	Exons 1-10
TG	Exon 18
TP53	Exons 1-10,10a-11
TP63	Exon 11
TSC1	Exons 3-23
TSC2	Exons 2-42
U2AF1	Exons 1-3,3a-8
VHL	Exons 1-3
WT1	Exons 1,1a-10
YAP1	Exons 1-9 Introns 5-8

Appendix D- Paediatric ctDNA (ct-PAED) panel

Gene	Gene ROI
ACVR1	Exons 6-9
AKT1	Exon 3
ALK	Exons 19-29 Intron 19
AMER1	Exon 2
ARID1A	Exons 1-20
ATM	Exons 1-63
ATRX	Exons 1-35
BCOR	Exons 2-15
BRAF	Exon 15
CCND1	Exons 1-5
CCND2	Exon 5
CCNE1	Exon 7
CDKN2A	Exons 1,1a-3,3a
CDKN2B	Exons 1-2
CREBBP	Exons 1-31
CTNNB1	Exon 3
EGFR	Exons 1-28
ERBB2	Exons 1-4,6-27
EWSR1	Exons 7-12 Introns 7a,7a:1,7a:2-9,11
EZH2	Exons 1,1a-6,8-20 Intron 1
FBXW7	Exons 8-12
FGFR1	Exons 1-2,2a-18
FGFR2	Exons 2-17,17a,17c-18
FGFR3	Exons 2-19

FGFR4	Exons 11-18
H3F3A	Exons 2-4
HIST1H3A	Exon 1
HIST1H3B	Exon 1
HIST1H3C	Exon 1
HIST2H3C	Exon 1
HRAS	Exons 2-6
IDH1	Exons 4,8
IDH2	Exon 4
KIT	Exons 9-11,13-14,17-18
KRAS	Exons 1-4
MAP2K1	Exons 2-3
MYC	Exons 1-3
MYCN	Exon 2
MYOD1	Exons 1-3
NF1	Exons 1-58
NRAS	Exons 2-4
PDGFRA	Exons 9-15,18
PIK3CA	Exons 2-21
PIK3R1	Exons 2,9-11,13
PPM1D	Exons 1-6
PTCH1	Exons 1,1a,1b-23
PTEN	Exons 1-9
PTPN11	Exons 3,8,13
RB1	Exons 1-27
SMARCA4	Exons 2-29,31-36
SMARCB1	Exons 1-9
SMO	Exons 6,9
STAG2	Exons 1,1a-35
SUFU	Exons 1-11,11a-12
TERT	Exon upstream
TP53	Exons 1-10,10a-11
TSC1	Exons 3-23
TSC2	Exons 2-42
WT1	Exons 6-9

Appendix E- Guardant 360© Assay

Point Mutations (SNVs) *(73 Genes)*							Indels *(23 Genes)*		Amplifications (CNVs) *(18 Genes)*		Fusions *(6 Genes)*
AKT1	*ALK*	*APC*	*AR*	*ARAF*	*ARID1A*	*ATM*	*ATM*	*APC*	*AR*	*BRAF*	*ALK*
BRAF	*BRCA1*	*BRCA2*	*CCND1*	*CCND2*	*CCNE1*	*CDH1*	*ARID1A*	*BRCA1*	*CCND1*	*CCND2*	*FGFR2*
CDK4	*CDK6*	*CDKN2A*	*CTNNB1*	*DDR2*	*EGFR*	*ERBB2*	*BRCA2*	*CDH1*	*CCNE1*	*CDK4*	*FGFR3*
ESR1	*EZH2*	*FBXW7*	*FGFR1*	*FGFR2*	*FGFR3*	*GATA3*	*CDKN2A*	*EGFR*	*CDK6*	*EGFR*	*NTRK1*
GNA11	*GNAQ*	*GNAS*	*HNF1A*	*HRAS*	*IDH1*	*IDH2*	*ERBB2*	*GATA3*	*ERBB2*	*FGFR1*	*RET*
JAK2	*JAK3*	*KIT*	*KRAS*	*MAP2K1*	*MAP2K2*	*MAPK1*	*KIT*	*MET*	*FGFR2*	*KIT*	*ROS1*
MAPK3	*MET*	*MLH1*	*MPL*	*MTOR*	*MYC*	*NF1*	*MLH1*	*MTOR*	*KRAS*	*MET*	
NFE2L2	*NOTCH1*	*NPM1*	*NRAS*	*NTRK1*	*NTRK3*	*PDGFRA*	*NF1*	*PDGFRA*	*MYC*	*PDGFRA*	
PIK3CA	*PTEN*	*PTPN11*	*RAF1*	*RB1*	*RET*	*RHEB*	*PTEN*	*RB1*	*PIK3CA*	*RAF1*	
RHOA	*RIT1*	*ROS1*	*SMAD4*	*SMO*	*STK11*	*TERT***	*SMAD4*	*STK11*			
TP53	*TSC1*	*VHL*					*TP53*	*TSC1*			
						*** includes TERT promoter region*	*VHL*				

**Exons selected to maximize detection of known somatic mutations. List available upon request*

Appendix F- ACCESS MTB proforma

ACCESS PROGRAMME- MOLECULAR TUMOUR BOARD (MTB) REFERRAL

Meeting date	
Staff attending (to be completed by clinician on day)	

PATIENT DETAILS

ACCESS Participant ID		**Hospital ID**	
Date of birth		**NHS number**	
Hospital site	☐Chelsea and Westminster ☐Epsom/St Hellier Hospital ☐Kingston hospital	☐West Middlesex Hospital ☐Croydon University Hospital ☐Royal Marsden Hospital	
Tumour cohort	☐Pancreatic ☐Biliary tract ☐Either pancreatic or biliary tract		

Clinical Details

Age/Gender	
Presentation	
Comorbidities	
Known somatic/germline genomic findings (including MMR)	
Family history	
Tumour markers	
Imaging findings	
Suspected clinical stage	T _____ N ___ M _____ ☐III ☐IV

CTDNA SAMPLE

Date of blood draw	
Date of report	
Guardant360© ID (eg A0123456)	
Gene panel	Guardant360©

CTDNA RESULTS

PLEASE EMAIL coordinator.mdtgel@rmh.nhs.uk **FOR REFERRAL INTO WEEKLY MTB MEETING**

MTB DIAGNOSIS

Supportive of cancer diagnosis (Y/N)	
Supportive of diagnosis of pancreatic or biliary tract cancer (Y/N)	
Level of diagnostic support	☐ **Diagnostic** ☐ **Consistent** ☐ **Possibly consistent**

	☐ **Not consistent**

MTB SUMMARY & RECOMMENDATION

Publications, Posters and Presentations

- "The prognostic factors in early stage BRAF mutant colorectal cancer: Experience from a large volume UK tertiary centre". ESMO 2021. Mencel J, Lamont H, Rao S, Watkins D, Fribbens C, Cunningham D, Chau I, Starling N.
- "Liquid biopsy for diagnosis in patients with suspected pancreatic and biliary tract cancers: PREVAIL ctDNA pilot trial". ASCO GI 2022. Justin Mencel, Andrew Feber, Ruwaida Begum, Paul Carter, Michaela Smalley, Elli Bourmpaki, Josh Shur, Sameer Zar, Darina Kohoutova, Sanjay Popat, Angela George, Terri Patricia McVeigh, Michael Hubank, Clare Peckitt, Charlotte Victoria Fribbens, David J. Watkins, Sheela Rao, Ian Chau, David Cunningham, Naureen Starling.
- ASCO 2021 Conference "Shifting the dial for localized pancreatic cancer". Co-author with Dr Naureen Starling
- Turkes F, Mencel J, Starling N. Targeting the immune milieu in gastrointestinal cancers. J Gastroenterol. 2020 Oct;55(10):909-926. doi: 10.1007/s00535-020-01710-x. Epub 2020 Aug 3. PMID: 32748171; PMCID: PMC7519898.
- Mencel J, Slater S, Cartwright E, Starling N. The Role of ctDNA in Gastric Cancer. Cancers (Basel). 2022;14(20):5105. Published 2022 Oct 18. doi:10.3390/cancers14205105
- Mencel J, Chau I. Optimizing Immunotherapy Combinations in Mismatch Repair Proficient/Microsatellite-Stable Metastatic Colorectal Cancer. ASCO Daily News. Jan 2022.
- Mencel J, Chau I. Targeting KRAS G12C Is a Significant Milestone in Metastatic Colorectal Cancer, But Work Remains. ASCO Daily News. Jan 2023.

Awards

- ASCO 2022 Conquer Cancer Merit Award (2022)