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ORIGINAL ARTICLE

Investigating the feasibility of tumour molecular profiling in gastrointestinal malignancies in routine clinical practice

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Background: Targeted capture sequencing can potentially facilitate precision medicine, but the feasibility of this approach in gastrointestinal (GI) malignancies is unknown.

Patients and methods: The FOrMAT (Feasibility of a Molecular Characterisation Approach to Treatment) study was a feasibility study enrolling patients with advanced GI malignancies from February 2014 to November 2015. Targeted capture sequencing (mainly using archival formalin-fixed paraffin-embedded diagnostic/resection samples) was carried out to detect mutations, copy number variations and translocations in up to 46 genes which had prognostic/predictive significance or were targets in current/upcoming clinical trials.

Results: Of the 222 patients recruited, 215 patients (96.8%) had available tissue samples, 125 patients (56.3%) had $\geq$16 genes successfully sequenced and 136 patients (61.2%) had $\geq$1 genes successfully sequenced. Sample characteristics influenced the proportion of successfully sequenced samples, e.g. tumour type (colorectal 70.9%, biliary 52.6%, oesophagogastric 50.7%, pancreas 27.3%, $P = 0.002$), tumour cellularity (high versus low: 78.3% versus 13.3%, $P \leq 0.001$), tumour content (high versus low: 78.6% versus 27.3%, $P = 0.001$) and type of sample (resection versus biopsy: 82.4% versus 47.6%, $P \leq 0.001$). Currently, actionable alterations were detected in 90 (40.5%) of the 222 patients recruited (66% of the 136 patients sequenced) and 2 patients subsequently received a targeted therapy. The most frequently detected currently actionable alterations were mutations in *KRAS*, *BRAF*, *TP53* and *PIK3CA*. For the 205 patients with archival samples, the median time to obtain sequencing results was 18.9 weeks, including a median of 4.9 weeks for sample retrieval and 5.1 weeks for sequencing.

Conclusions: Targeted sequencing detected actionable alterations in formalin-fixed paraffin-embedded samples, but tissue characteristics are of critical importance in determining sequencing success. Routine molecular profiling of GI tumours outside of clinical trials is not an effective use of healthcare resources unless more targeted drugs become available.

ClinicalTrials.gov identifier: NCT02112357

Key words: gastrointestinal malignancies, molecular profiling, precision medicine, sequencing, tissue characteristics

Introduction

Recent technological advances have resulted in an explosion of precision medicine trials and genomic research. For example, in the United States, the National Cancer Institute (NCI) MATCH trial is screening patients for suitability for targeted therapies [1]. Similarly, the UK Government is aiming to sequence the genomes from 25 000 cancer patients and the UK National Lung Matrix

trial is exploring multiple targeted therapies in lung cancer [2, 3]. It is hoped that these strategies will facilitate drug development and that molecular profiling will become part of routine clinical practice.

The Feasibility of a Molecular Characterisation Approach to Treatment (FOrMAT) study (ClinicalTrials.gov identifier NCT02112357) was a prospective study conducted at the Royal Marsden (RM) which aimed to investigate the feasibility of molecular profiling to Clinical Pathology Accreditation (CPA)-certified standards in gastrointestinal (GI) malignancies in routine clinical practice (see trial protocol in supplementary material S1, available at *Annals of Oncology* online).

Methods

Patients

Eligibility criteria included locally advanced/metastatic oesophagogastric (OG), pancreatic, biliary tract or colorectal cancer (CRC), performance status (PS) ≤2 and an archival tumour specimen [formalin-fixed paraffin-embedded (FFPE)] from the primary tumour or non-bony metastasis or a biopsiable site of disease. Patients could be at any stage of treatment of advanced disease. The study was approved by a research ethics committee and our institutional review board and all patients provided written consent.

Sequencing

Five 10-μm unstained slides and two haematoxylin and eosin-stained slides were cut from FFPE blocks. Experienced pathologists assessed tumour content and cellularity. Unstained slides were macrodissected and DNA extracted using the QIAamp DNA FFPE Tissue kit (Qiagen). Non-cancerous control DNA was extracted from whole blood using the QIAamp DNA Blood Mini kit (Qiagen). DNA (200 ng) with an average molecular weight >1000 bp or 400 ng of DNA with an average molecular weight <1000 bp (both tumour and control) was used in the HyperPlus kit (KAPA Biosystems) to generate sequencing libraries. Regions of interest were captured using SeqCap EZ Choice Enrichment kit (Nimblegen) designed against 46 genes (see supplementary Table S1, available at *Annals of Oncology* online). Libraries were sequenced on a MiSeq (Illumina) with 76 bp paired-end reads using v3 chemistry.

The panel was validated (using 50 samples previously analysed for *KRAS, NRAS, BRAF* and *PIK3CA* in a previous study) and carried out in a CPA-certified laboratory. Sequencing processes were continuously improved. Only mutation data (not CNV and translocations) were reported for the first 54 patients and an optimised version of the panel was used for 33% of patients. Amplifications were only reported where multiple consecutive regions within the gene were amplified and were assessed using log2 ratio of tumour coverage/normal coverage in a capture region.

Analysis of sequencing data

The primary analysis was carried out through MiSeq Reporter (v2.5.1; Illumina) and the secondary analysis used an in-house Molecular Diagnostics Information Management System to generate QC, variant annotation, data visualisation and a clinical report, which was uploaded to patients' medical records. Variants detected in ≥5% reads with five variant supporting reads were reported. The depth required varied according to tumour content (see supplementary Table S2, available at *Annals of Oncology* online). Confidence levels for wild-type status for each gene were determined based on the percentage of bases sequenced at a minimum depth corresponding to the neoplastic cell content (>85% of bases per gene covered at minimum depth = passed gene). The model for determining wild-type was initially based on achieving sufficient depth to detect variants occurring in 10% of the tumour clone but was subsequently amended to detect variants occurring in 50% of the tumour clone.

Collection of clinical data and review of sequencing results

Clinical and demographic data were prospectively collected from patients' medical notes. Sequencing results were discussed at a Sequencing Tumour Board (STB), which comprised oncologists, pathologists, bioinformaticians and molecular and clinician scientists. The STB made recommendations on the clinical significance of the results and whether an alteration was currently actionable ('actionable' was defined as a known alteration indicating potential suitability for a targeted therapy available at RM, including within other clinical trials). Any changes to patients' management were at the discretion of their treating physicians.

Statistical analysis

The primary end point was the percentage of patients in whom a currently actionable molecular alteration was detected. Secondary end points included concordance of results with standard clinical tests, the proportion of patients in whom genetic sequencing was successfully carried out [sub-divided into levels for analysis of mutations: level 1: all genes at >85% coverage, level 2: ≥16 genes (i.e. ∼50% of the 33 genes analysed for mutations in version 1 of the panel) at >85% coverage, level 3: 1–15 genes at >85% coverage or detection of a mutation] and the time taken to obtain sequencing results. Univariate and multivariate binary logistic analyses investigated whether sample characteristics were associated with the sequencing success level.

The recruitment target was 204 patients. Patients for whom a tissue sample could not be obtained were replaced. Pre-specified interim analyses were planned after 50 and 100 patients and were carried out after 54 and 107 patients had been discussed at the STB.

Results

Patient characteristics

Between February 2014 and November 2015, 222 patients were recruited [CRC: 107 patients (48.2%), OG: 73 patients (32.9%), pancreatic/biliary: 42 patients (18.9%)]. The majority of patients had metastatic disease (96.4%) and had a PS of 0–1 (90.6%). Patient characteristics are shown in supplementary Table S3, available at *Annals of Oncology* online and details of patients' previous treatment in supplementary Table S4, available at *Annals of Oncology* online.

After a median follow-up of 9.3 months, 91 patients (41.6%) were deceased and 4 patients had been lost to follow-up. The median overall survival from time of study registration was 15.3, 11.1 and 2.9 months for patients with a PS of 0, 1 and 2, respectively ($P < 0.001$).

Technical success

Of the 222 patients recruited, 215 patients had available tissue samples (96.8%), 136 patients (61.2%) had ≥1 genes successfully sequenced (see Figure 1) and 125 patients (56.3%) had a level 1–2 sequencing result. The proportion of patients with a level 1 result improved during the study [12.5% for samples processed before interim analysis 1 (cohort 1), 31.3% for samples processed

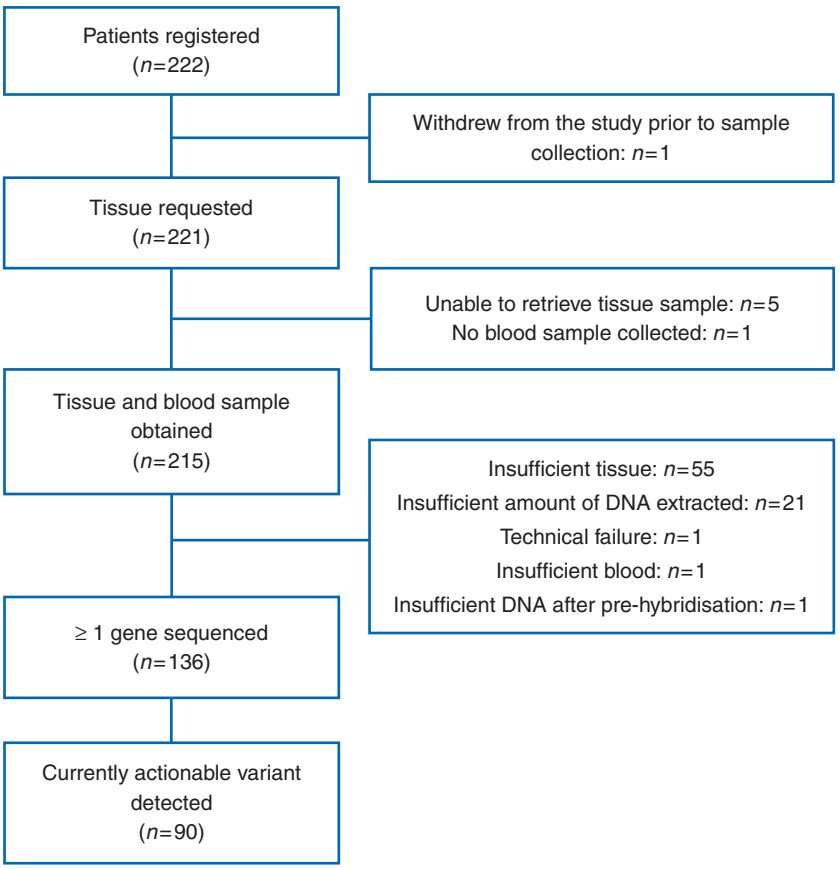

Figure 1. Flow diagram for the FOrMAT (Feasibility of a Molecular Characterisation Approach to Treatment) study.

between interim analyses 1 and 2 (cohort 2) and 57.6% for samples processed after interim analysis 2 (cohort 3); see supplementary Figure S1, available at *Annals of Oncology* online]. The optimised version of the panel was used for cohort 3 samples and a level 1–2 sequencing result was obtained for 84.8% of resection and 54.2% of biopsy specimens in this cohort.

Table 1 and Figure 2 show the effects of tissue sample characteristics on the sequencing success level. In a multivariate analysis, only higher DNA quantities were significantly associated with successful generation of level 1–2 sequencing results ($P < 0.001$). Level 1–2 results were obtained for 3 of the 10 patients who underwent a repeat biopsy (three OG, two liver and two colorectal biopsies failed). Samples were from the primary tumour in 158 patients and from metastatic sites in 56 patients. Molecular characteristics may potentially differ between primary tumours and metastatic sites, but this was not routinely assessed in this study.

Concordance with standard molecular tests

Concordance rates for patients with results from both a standard-of-care molecular diagnostics test (five-gene targeted NGS colorectal panel, COBAS *KRAS* or CE-SSCA *BRAF* tests) and the FOrMAT panel were 98.5% for *KRAS* (67/68), 100.0% for *NRAS* (59/59), 100.0% for *BRAF* (64/64), 93.5% for *PIK3CA* (43/46) and 84.4% (38/45) for *TP53*. Discordant results were due to the use of different tissue blocks and the FOrMAT panel covering more exons at a greater depth.

Detection of mutations, amplifications and translocations

Supplementary Figure S2, available at *Annals of Oncology* online, shows the mutations detected in the 136 patients in whom sequencing was successfully carried out. The most commonly mutated genes were *TP53* (80.9%), *APC* (46.3%) and *KRAS* (28.7%). Mutations occurring at a frequency of $>5\%$ were detected in 12 genes, of which five were classed as currently actionable (*TP53, KRAS, BRAF, PIK3CA* and *CDKN2A*). Amplifications were detected in 18 patients and the most commonly occurring amplifications were *KRAS* (4.4%), *ERBB2* (3.7%) and *CCND1* (2.2%). No translocations were reported and potential deletions were not reported due to insufficient validation data.

A currently actionable molecular alteration was detected in 90 (40.5%) of the 222 patients recruited (66% of the 136 patients sequenced). Sixty-one patients had CRC, 18 OG, 6 biliary tract and 5 pancreatic cancer and details of these alterations are shown in supplementary Table S5, available at *Annals of Oncology* online. The majority of actionable alterations conferred eligibility for a clinical trial, but 21 patients had alterations that may have been actionable via off-label use of a targeted therapy (mainly *BRAF* mutations and HER2 amplifications). In patients with CRC, *RAS* mutations were defined as actionable as clinical trials were available for these patients (*RAS* wild-type was not classed as actionable) and 26 patients had a currently actionable alteration in a gene other than *RAS/BRAF*.

Table 1. Univariate binary logistic analysis of factors influencing the success of sequencing in patients with available tissue samples

Variable	*N*	Sequencing level 1–2		Odds ratio	95% CI	*P* value
		N	%			
Tumour type ($n = 215$)						
Colon	103	73	70.9	1.0	–	0.002
Pancreas	22	6	27.3	0.17	0.06–0.49	0.001
Biliary	19	10	52.6	0.47	0.18–1.22	0.121
OG	71	36	50.7	0.45	0.25–0.84	0.011
Specimen type ($n = 213$)						
Resection	68	56	82.4	1.0	–	<0.001
Biopsy	145	69	47.6	0.22	0.11–0.42	
Site of sample ($n = 214$)						
Colorectal	76	59	77.6	1.0	–	0.001
OG	65	36	55.4	0.39	0.20–0.79	0.009
Biliary/pancreas	17	7	41.2	0.24	0.08–0.71	0.010
Liver	31	12	38.7	0.20	0.08–0.49	<0.001
Other	25	11	44.0	0.27	0.10–0.68	0.006
Cellularity ($n = 174$)						
High	106	83	78.3	1.0	–	<0.001
Intermediate	53	23	43.4	0.21	0.10–0.43	<0.001
Low	15	2	13.3	0.04	0.01–0.20	<0.001
Tumour content ($n = 173$)						
High (> 50%)	28	22	78.6	1.0	–	0.002
Intermediate (30%–50%)	123	80	65.0	0.51	0.19–1.35	0.173
Low (< 30%)	22	6	27.3	0.10	0.03–0.38	0.001
Amount of DNA ($n = 193$)						
<200 ng	58	1	1.7	0.001	0.0001–0.01	<0.001
200–1000 ng	35	30	85.7	0.38	0.11–1.34	0.134
>1000 ng	100	94	94.0	1.0	–	<0.001
DNA integrity number ($n = 140$)						
0–1.9	24	17	70.8	0.33	0.07–1.47	0.147
2.0–4.5	91	86	94.5	2.35	0.52–10.6	0.267
4.6–10	25	22	88.0	1.0	–	0.010

The proportion of samples that were resection specimens were 16.4% for OG cancer, 20.0% for pancreatic cancer, 25.0% for biliary tract cancer and 46.2% for CRC.

CI, confidence interval; OG, oesophagogastric.

Of the 90 patients with currently actionable alterations, 58 patients had a PS of 0–1 at the time of the STB discussion, 6 had a PS of 2–3, 12 were deceased and PS was unknown for 13 patients (e.g. discharged to palliative care). At the time of analysis, two patients (0.9%) had received targeted treatment in a clinical trial as a result of this study.

Sample processing timelines

Samples were retrieved from multiple different institutions and processed at RM (a tertiary centre with a widespread referral base). Archival tissue samples could not be located for five patients. For the 205 patients with available archival samples, the median time from study registration to date of sequencing result was 18.9 weeks, including a median of 4.9 weeks for sample retrieval and 5.1 weeks for sequencing. Turnaround times improved over the course of the study (see Figure 3).

Discussion

There has been huge investment in precision medicine. For example, the US Precision Medicine Initiative includes US$70 million in funding for precision medicine in oncology [4]. However, genetic sequencing is technically challenging, with failure rates of 10%–42% reported by other molecular profiling studies [5–10]. For example, in the MATCH trial, sequencing was not completed for 13% of submitted samples due to poor sample quality (e.g. insufficient tumour cores and low tumour content) [10]. Similarly, 27% of biopsied patients in the SHIVA trial did not have suitable tissue for analysis (even though fresh biopsies were mandated), mainly due to insufficient tumour cellularity or no tumour cells in the biopsy [8].

In our study, a level 1–2 sequencing result was not obtained for 44% of recruited patients (improved to 35% for cohort 3). This high failure rate was due to a number of factors, including the gene panel being carried out to CPA standards (which are more

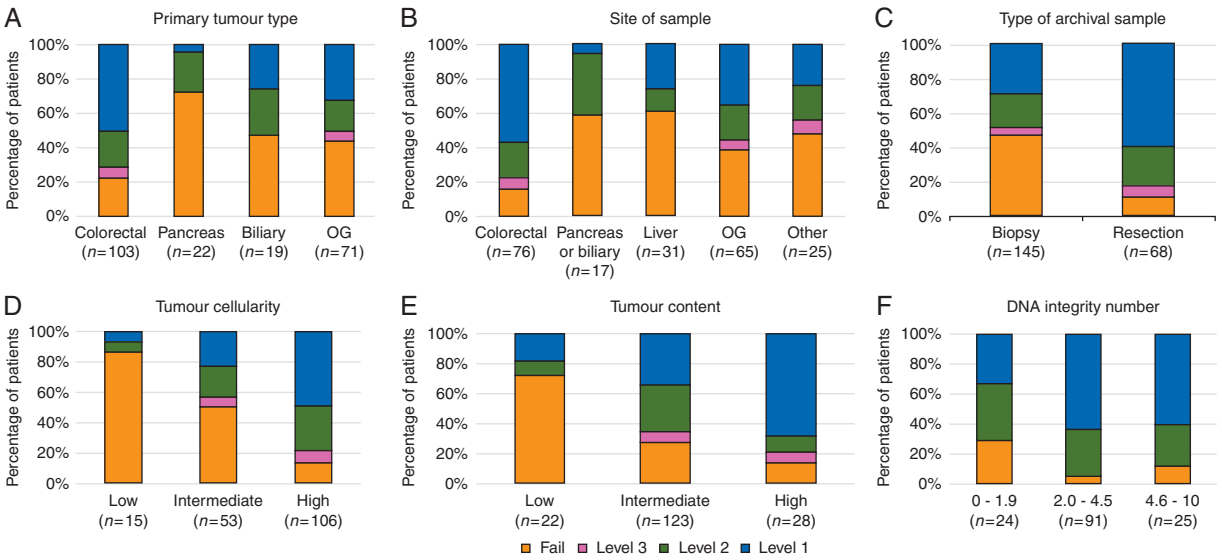

Figure 2. Tumour cellularity is defined as the amount of cells in the sample. Tumour content is the proportion of the cells that are tumour cells. Influence of sample characteristics on the technical success of sequencing.

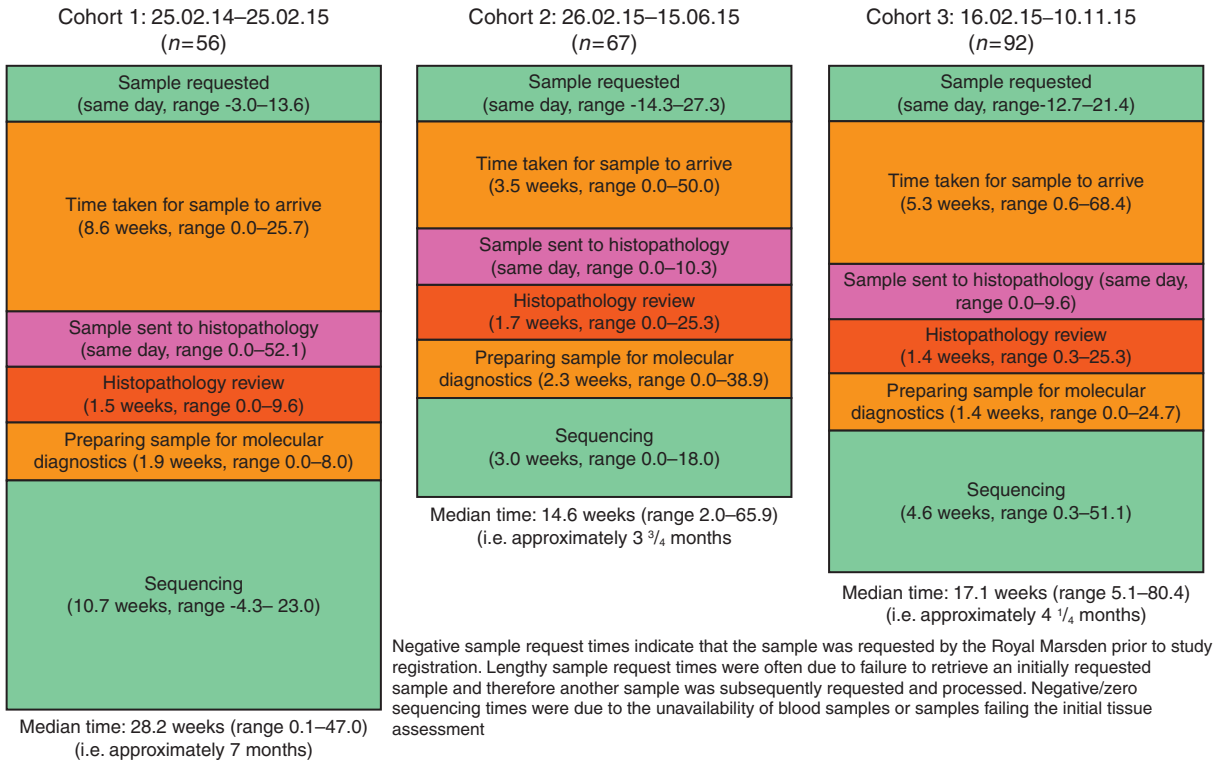

Figure 3. Timelines for processing of archival samples during the course of the FOrMAT (Feasibility of a Molecular Characterisation Approach to Treatment) study.

stringent than analysis carried out for purely research purposes) and in particular was driven by frequent failures in upper GI patients (54%) compared with CRC patients (27%). It was difficult to obtain the 200 ng of DNA required from our tissue samples. This may have been partly due to the pathological characteristics of GI tumours as we demonstrated that tumour content and cellularity are associated with DNA yield. Eighty-four percent of our samples had a tumour content of <50% and 39% of samples had a low or intermediate cellularity and therefore if we had mandated a tumour content of >50% (as in the SAFIR and SHIVA studies [5, 8]), the majority of our samples would not have been analysed. These results are of importance for genomic trials in GI cancers, especially upper GI tumours.

Our study analysed the feasibility of molecular profiling in a real-world setting, and therefore our samples were mainly routine FFPE biopsy or resection samples from either the primary

tumour or a metastasis and initial tissue processing was usually carried out at referring, non-specialist hospitals. FFPE samples are more challenging to sequence than fresh-frozen samples as DNA yield and quality are influenced by characteristics of the fixation and paraffin-embedding process [11, 12]. In addition, some samples had been exhausted by standard-of-care tests or use in other competing clinical trials and some resection specimens were affected by response to previous neoadjuvant treatment [13, 14]. We decided not to mandate a fresh biopsy as this would not reflect routine clinical practice and an objective was to implement molecular medicine based on the tissue procured in a routine diagnostic setting.

Experiments are ongoing to reduce the amount of DNA required for the gene panel and thereby improve the proportion of patients who are successfully sequenced. However, one reason for the high quantity of DNA required and the high sequencing 'failure' of some genes was that (unlike most other molecular profiling studies) we aimed to achieve sufficient depth to confidently determine patients' wild-type status. Wild-type status is clinically relevant for some genes [15], but it may not have been necessary to highly accurately ascertain wild-type status for all genes.

Despite being a tertiary centre with dedicated resources, our sample retrieval times were significantly longer than the 7 days reported by the MATCH trial, and our overall turnaround times were also substantially longer than the 26 days reported by MD Anderson [6, 10]. A major challenge was obtaining archived diagnostic samples (which were frequently stored at the referring hospital or off-site storage facility) [16], and often there were multiple competing demands on a single tissue block (e.g. blocks may have been sent for use in other clinical trials). Our turnaround times initially improved (e.g. due to closer liaison with referring centres), but as also seen in the MATCH trial the more rapid accrual in the latter part of the study impacted on laboratory capacity [10]. Significant consideration must be given to the workflows required to deliver precision medicine, especially in universal healthcare systems, and changes have already been made (e.g. guidelines updated to permit cancer centres to store patients' tissue samples for future analysis rather than returning samples to referring centres).

This feasibility study focused on optimising sequencing procedures and turnaround times, and was not therefore designed to maximise the number of patients receiving targeted therapies. However, ultimately one aim of molecular profiling is to identify patients who are suitable for targeted therapies. Only two patients in our study and 3%–13% of patients in other studies subsequently received a targeted therapy [5, 6]. Although molecular profiling may identify small sub-groups of patients who may benefit from specific therapies, these patients might not be suitable for treatment or may not be able to access targeted drugs. In the UK, access to targeted therapies is mainly through clinical trials as the health service does not fund off-label use of targeted therapies. In our study many patients were not suitable for clinical trials at the time sequencing results were obtained (patients with a PS of 2 had a particularly poor prognosis), some patients are currently undergoing standard-of-care therapies and we were dependent on our clinical trial portfolio (which recently became more focused on immunotherapeutic agents) as our study was not linked to specific therapeutic options.

Molecular profiling is expensive and resource intensive and although a potentially actionable alteration was found in 40.5% of recruited patients it is also important to consider whether precision medicine is likely to be clinically effective in GI cancers. Unlike in other tumour types, single driver mutations are uncommon and therefore administering a targeted drug for one specific alteration is unlikely to lead to an improvement in outcomes. In the future, the use of more comprehensive sequencing data to provide information on mutational signatures may help identify larger sub-groups of patients who benefit from specific treatment approaches [17]. However, based on the current availability of targeted therapies, only a small proportion of patients are likely to benefit which raises the question for healthcare systems of cost/benefit ratio.

Our study highlights some of the challenges of molecular profiling in GI tumours and is informative for other those involved in other studies, such as the 100 000 Genomes Project and the CRUK Stratified Medicine Program [3, 18]. If molecular profiling is to be incorporated into routine clinical care, guidance should be developed on the workflows for tissue collection and processing (including considering creating multiple tissue blocks and/or extracting DNA upfront or considering the role of other tissue preservation methods, including the collection of fresh tissue as part of routine diagnostic work-up), education/training in molecular medicine should be improved and samples should be analysed in specialist centres which process a large volume of samples to maximise expertise and workflow times. However, although selected patients with a good PS may benefit from targeted therapies (e.g. in phase I or umbrella trials), routine molecular profiling of patients with GI tumours is not currently an effective use of resources.

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Disclosure

The authors have declared no conflicts of interest.

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