Developing biomarker-driven treatments for oesophagogastric cancer

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Declaration:

I declare that the work presented in this thesis is my own. I have stated the contribution from other parties within the following acknowledgements section and at relevant points in the text.

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Finally to my wife.

Abstract:

Introduction

Despite recent advances in both genetic characterisation and development of novel targeted agents the outlook for advanced oesophagogastric (OG) cancers remains poor and there is a need for improved biomarker-driven treatment approaches. The work presented in this thesis details the design, set-up and oversight of the i*MYC* trial, a biomarker-driven phase 2 clinical trial in advanced OG cancer incorporating a novel prospective screening programme, as well as results from the application of differing methodologies of biomarker identification including single gene analysis and whole genome sequencing techniques, applied to both tumour and circulating tumour (ct)DNA.

Methods

1. i*MYC* is an open label, phase II non-randomised trial assessing the efficacy of ibrutinib monotherapy in advanced pre-treated *MYC* and/or *HER2* amplified OG cancer. As part of the trial a screening programme utilising a fluorescent in-situ hybridisation (FISH) assay for assessment of tumour *MYC* amplification was instituted, and a further digital droplet (dd)PCR assay developed and applied to both tumour and ctDNA.

2. Low coverage whole genome sequencing (lcWGS) was applied to ctDNA extracted from blood samples taken as part of a prior translational research study. Samples were taken at both baseline and on progression after comparable first-line systemic chemotherapy in advanced OG adenocarcinoma patients. Analysis of

somatic copy number alterations (SCNAs), genomic instability and evolution of genomic changes over the course of chemotherapy was assessed.

Results

1. One hundred and thirty five archival tumour specimens have undergone successful FISH analysis as part of the i*MYC* trial, with 23% displaying evidence of *MYC* amplification, most commonly in the presence of polysomy. Inter-tumour heterogeneity was observed, with the percentage of cancer cells harbouring *MYC* amplification ranging widely between samples (median 51%, range 11-94%). Intra-tumoural clonal diversity of *MYC* amplification was also observed, with a significant degree of variance in amplification ratios (Bartlett's test for equal variance $p<0.001$), and an association between greater variance in *MYC* amplification and improved outcome with first-line chemotherapy. The ddPCR assay was most accurate in quantifying *MYC* amplification in tumour-derived DNA from cases with a high proportion (>70%) of amplified cells within the tumour specimen, but was not reliable in samples containing a low proportion of amplified cells or in ctDNA from this cohort of patients. To date eight patients have undergone treatment within the main component of the trial

2. Thirty samples underwent lcWGS analysis of ctDNA at a baseline time-point. In 23/30 (76.7%) cases an analysable SCNA profile was found. The presence of liver metastases, primary tumour in-situ or of oesophageal or junctional tumour location predicted for a high circulating tumour DNA fraction, and concordance was seen with prior targeted tumour sequencing results as well as additional further amplification events identified. SCNA profiles changed during chemotherapy, indicating that

cancer cell populations evolved during treatment, however no recurrent SCNA changes were acquired at progression.

Conclusions

The i*MYC* trial represents the first attempt to prospectively identify and target *MYC* amplifications in OG cancer. *MYC* amplifications has been found to be a commonly occurring event, and heterogeneity of amplification patterns has been observed. The sensitivity of a novel ddPCR assay appears limited to tumour samples displaying a high degree of *MYC* amplification only.

The successful analysis of SCNA profiles in ctDNA, as well as the detection of clinically significant amplification events, suggest that lcWGS may be a promising technology to assess the genomic landscapes of metastatic OG cancers. Tracking the evolution of OGA cancer cell populations in ctDNA is feasible during chemotherapy and the observation of genetic evolution warrants investigation in larger series and with higher resolution techniques to reveal potential genetic predictors of response and drivers of chemotherapy resistance. The presence of liver metastasis is a potential biomarker for the selection of patients with high ctDNA content for such studies.

Abbreviations:

BSC	Best Supportive Care
BTK	Bruton's Tyrosine Kinase
CCR	Committee for Clinical Research
cfDNA	Circulating free Deoxyribonucleic Acid
ctDNA	Circulating tumour Deoxyribonucleic Acid
CI	Confidence Interval
CIN	Chromosomal Instability
CR	Complete Response
ddPCR	Digital droplet polymerase chain reaction
DNA	Deoxyribonucleic Acid
DVT	Deep Vein Thrombosis
EBV	Epstein Barr Virus
ECOG	Eastern Cooperative Oncology Group
EMA	European Medicines Agency
FFPE	Formalin-Fixed Paraffin-Embedded
FISH	Fluorescent in-situ hybridisation
FOrMAT	Feasibility of a Molecular Characterisation Approach to Treatment
GC	Guanine-Cytosine
GI	Gastrointestinal
GORD	Gastro-Oesophageal Reflux Disease
HR	Hazard Ratio
HRQoL	Health-Related Quality of Life
IDMC	Independent Data Monitoring Committee

IHC	Immunohistochemistry
lcWGS	Low coverage Whole Genome Sequencing
MHRA	Medicines and Healthcare Regulatory Authority
mRNA	Messenger RNA
MSI	Microsatellite Instability
MSI-H	Microsatellite Instability High
OG	Oesophagogastric
OGJ	Oesophagogastric junction
ORR	Objective Response Rate
OS	Overall Survival
PCR	Polymerase Chain Reaction
PD	Progressive Disease
PFS	Progression Free Survival
PR	Partial Response
PS	Performance Status
RCT	Randomised Controlled Trial
RNA	Ribonucleic Acid
SCC	Squamous Cell Cancer
SCNA	Somatic Copy Number Alteration
SD	Stable Disease
SSNV	Somatic Single Nucleotide Variant
TCGA	The Cancer Genome Atlas
TKI	Tyrosine Kinase Inhibitor
TMG	Trial Management Group

UGI	Upper Gastrointestinal
WGii	Whole Genome Instability Index
WGS	Whole Genome Sequencing

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Hypotheses:

MYC amplification can be assessed prospectively in advanced OG cancer patients and ibrutinib has clinical activity in a molecularly enriched cohort of *MYC* and/or *HER2* amplified advanced OG cancer patients who have progressed after first-line treatment.

lcWGS sequencing can be used for longitudinal analysis of blood-derived ctDNA in advanced OG cancer, and can determine predictive genetic biomarkers and treatment resistance mechanisms.

Aims:

To assess frequency and patterns of *MYC* amplification in primary OG samples and ctDNA as part of the prospective screening component of the i*MYC* clinical study.

To determine the utility of using ddPCR analysis in detecting *MYC* amplification status from primary OG samples and ctDNA.

To assess the mechanism of action and efficacy of ibrutinib in pre-treated, advanced OG cancer through the design and execution of the i*MYC* clinical trial.

To investigate the feasibility of ctDNA analysis using lcWGS as a tool for genetic biomarker investigation in OG cancer.

To identify sequential changes in ctDNA as a consequence of therapy exposure in order to investigate novel drivers of acquired resistance.

1 Introduction: current standards and changing treatment trends in advanced oesophagogastric cancer

1.1 Introduction to oesophagogastric cancer

Oesophageal and gastric cancers are a challenging health issue, representing the sixth and third leading causes of global cancer mortality respectively [1]. There is substantial geographic variation in incidence, with the highest incidence reported in Asia, South America and Eastern Europe [2, 3]. Worldwide, squamous cell carcinoma (SCC) is the predominant histological subtype however in North America and Europe the incidence of oesophageal adenocarcinoma has increased in the last 20 years, whereas that of SCC has decreased [3]. This is likely to reflect the distinct aetiological factors implicated in the development of the two histological subtypes. SCC is strongly correlated with excessive alcohol consumption, cigarette smoking and poor socioeconomic status, whereas adenocarcinoma is associated with obesity and gastroesophageal reflux disease (GORD) [4, 5]. Thus the rise in adenocarcinoma subtype may be in part due to changing lifestyle factors in Western populations [5].

Partial or complete gastrectomy with lymphadenectomy remains the only potentially curative therapy for early stage oesophagogastric adenocarcinoma, and the addition of adjunctive chemotherapy and radiotherapy treatments further improve survival outcomes [6, 7]. As a result of increased sensitivity to radiotherapy, radical chemoradiotherapy approaches can be curative for a subset of locally advanced oesophageal SCC tumours [6]. Even with optimal multimodality therapy more than 60% of patients will develop locally recurrent or metastatic disease, and in Western

countries the majority of patients are either diagnosed at an advanced unresectable stage or relapse within 5 years after initial curative-intent treatment [8, 9].

Despite recent advances in both the genetic characterisation of the disease and development of novel treatment agents, the outlook for advanced OG cancer remains poor, with median overall survival (OS) not extending beyond 12 months in the majority of clinical trials. Proof of concept for the utilisation of targeted therapies in the disease has been shown in the first line setting for the approximately 20% of *HER2* amplified adenocarcinomas through the use of trastuzumab alongside chemotherapy [10]. The anti-*VEGFR2* monoclonal antibody ramucirumab has been established both as monotherapy and in combination with paclitaxel in biomarker unselected second line settings [11, 12]. Further trials of molecularly targeted agents have in the main proved disappointing, with trials of *HER2* dual targeting, *EGFR*, *MET*, *P13K/mTOR* and *PARP*-inhibition all yielding negative results [13–19].

The use of high throughput whole genome sequencing has allowed for an improved appreciation of common genetic alterations in OG cancer. The Cancer Genome Atlas (TCGA) described a landmark analysis of 295 primary gastric adenocarcinomas, proposing a molecular classification into four subtypes: Epstein Barr Virus (EBV) positive tumours, microsatellite unstable tumours, genomically stable tumours and tumours with chromosomal instability (CIN); with each subtype showing distinct genomic features with potentially important clinical implications [20]. Genomic technology has similarly been applied to oesophageal cancer in an effort to improve stratification on a genetic and molecular level. The genomic profiles of 71 SCC and 231 oesophageal adenocarcinomas have been compared, focusing on the

identification of therapeutically relevant genomic alterations in both groups [21]. Similarly high frequencies of clinically relevant genomic alterations were found in both histological subtypes; however the profiles of genomic alterations in the two diseases differed substantially. *KRAS* and *HER2* were more frequently altered in adenocarcinoma, while *MTOR* pathway genes (*PIK3CA*, *PTEN*) and *NOTCH1* were more frequently altered in SCC. Exploitation of the molecular differences between the two histological sub-types may help direct optimal application of targeted therapies in this disease. This growing knowledge of genetically distinct subtypes of OG cancer along with the use of emerging genomic assays to identify clinically relevant mutations suggest that a significant proportion of these cancers may be amenable to targeted therapies in the near future.

1.2 Overview of current treatment landscape

1.2.1 Chemotherapy

Patients with advanced OG cancer whose performance status (PS) is adequate would normally be offered systemic chemotherapy with the aim of improving both cancer-related symptoms and extending life [22]. There are a wide variety of regimens used and no universal standard for first-line chemotherapy has been established. Doublet chemotherapy is common, with the ‘benchmark’ combination being a fluoropyrimidine combined with a platinum agent [23]. Triplet regimens have also been investigated, and in the UK a fluoropyrimidine and platinum with the addition of the anthracycline epirubicin has been widely used [24, 25]. Epirubicin/platinum/fluoropyrimidine triplets have not been directly compared to a

fluoropyrimidine/platinum doublet in a phase III trial, and conflicting data from meta-analyses with regards to benefit means variation in uptake remains [22, 26].

A 2013 meta-analysis evaluated second-line chemotherapy versus best supportive care, finding a significant overall reduction in the risk of death with chemotherapy (hazard ratio (HR) = 0.64; $p<0.0001$) [27]. Randomised trials have confirmed survival advantages for both irinotecan and taxane single agent chemotherapy versus best supportive care (BSC) alone in this setting [28–30]. Again no standard second line chemotherapy regimen has been established. A Japanese phase III study of irinotecan versus weekly paclitaxel failed to demonstrate superiority of irinotecan (median OS 8.4 vs 9.5 months) [31], therefore either agent is a reasonable treatment choice.

Compelling level one evidence for chemotherapy benefit in the third line space has recently been presented for the chemotherapy drug conjugate trifluridine-tipiracil (TAS102), with a recent randomised controlled trial (RCT) reporting a median OS vs placebo of 5.7 vs 3.6 months (HR 0.69; $p=0.0003$) [32].

1.2.2 Targeted therapy

The landmark ToGA trial evaluated the combination of trastuzumab with a cisplatin/ fluoropyrimidine chemotherapy doublet in patients with previously untreated, advanced HER2 positive disease [10]. *HER2* status was assessed using both immunohistochemistery (IHC) and fluorescence in-situ hybridisation (FISH), and tumours were considered *HER2* positive on the basis of either an IHC3+ result or FISH amplification showing a *HER2*/CEP17 ratio of ≥2. The combination of

trastuzumab significantly improved the primary endpoint of median OS from 11.1 to 13.8 months. A pre-planned exploratory analysis according to *HER2* status suggested that OS was improved in patients with high expression of HER2. Patients with the strongest expression (IHC3+) with concomitant FISH gene amplification received the greatest benefit, with an absolute improvement in OS of over 5 months (17.9 compared to 12.3 months). IHC expression also appeared to incrementally predict for benefit: trastuzumab was most effective in prolonging survival in IHC3+ tumours, less effective in patients with IHC 2+ tumours, and ineffective in those with *HER2* gene-amplified (FISH-positive) but non-protein-expressing (IHC 0 or 1+) tumours. The combination of pertuzumab, a monoclonal antibody directed at the extracellular domain of HER2, with chemotherapy and trastuzumab has returned promising results in the treatment of *HER2* positive breast cancer; however a phase III evaluation of pertuzumab in combination with first-line trastuzumab/ chemotherapy in advanced OG cancer failed to meet its primary OS endpoint [13, 33]. Similarly, the trastuzumab/ chemotherapy drug conjugate trastuzumab emtansine was found to be non-superior to standard second line taxane chemotherapy [34]. There are some non-randomised evidence to suggest that continuation of trastuzumab beyond first-line progression results in improved survival outcomes, however there is currently no established role for *HER2* targeting beyond the first line [35, 36]. The anti-*VEGFR2* monoclonal antibody ramucirumab has been shown to improve survival in the second line both as monotherapy and in combination with paclitaxel, although reimbursement issues mean that there is variability in its uptake globally, and it is not routinely available within the UK [11, 12]. More recently it was shown not to improve survival when combined with chemotherapy in the first line setting [37].

Building on the success of *HER2* and anti-angiogenic targeting, a number of further targeted approaches have been trialled. The phase II placebo-controlled INTEGRATE study investigated regorafenib, a dual *VEGFR2-TIE2* tyrosine kinase inhibitor (TKI), on patients with refractory gastric adenocarcinoma in the third line. Regorafenib demonstrated a significant improvement in progression free survival (PFS) (11.1 vs 3.9 weeks, HR 0.40; $p<0.001$) and a trend towards improved OS, and has now progressed to a phase III trial [38][NCT02773524]. Further promising results have been achieved with the *VEGFR2* TKI apatinib, which has shown a survival benefit in a Chinese phase III placebo-controlled trial [39]. Both PFS and OS were significantly improved and the drug is now licenced for use in China as a third line treatment option. A subsequent phase III trial in a mixed European and Asian population in third line or beyond however has shown a significant PFS benefit but no associated OS benefit [40]. Further important data such as the pre- and- post trial lines of therapy received are awaited, and the role of the drug globally remains to be established. When considering the potential use of antiangiogenics such as regorafenib and apatinib it is important to consider the safety profiles of such drugs, particularly their known potentiation of bleeding risk. This is of particular relevance to upper GI tract tumours, which have a higher baseline population incidence of GI bleeding [41]. A large phase III study evaluating bevacizumab in the early disease setting noted an increased incidence of anastamotic leak and bleeding in the junctional/ oesophageal adenocarcinoma subgroup, highlighting potential challenges of these drugs in OG cancer [42].

Further trials of molecularly targeted agents have in the main proved disappointing. Antibody inhibitors of *EGFR* cetuximab and panitumumab have been investigated in

large phase 3 trials, failing to show a survival advantage [15, 43]. Similarly, phase III trials agents targeting the *MET*, and *P13K/mTOR* pathway have also yielded negative results [16, 18]. PARP-inhibitors have been shown to have synergistically-enhanced activity in tumours with impaired DNA repair mechanisms, such as those associated with *BRCA* mutations or ATM deficiency [44, 45]. A randomised phase II study compared paclitaxel plus or minus olaparib in a study population enriched for patients with low or undetectable ATM levels, with an OS benefit seen [45], however the subsequent larger phase III study evaluating the same combination was negative for its primary survival endpoint [19].

1.2.3 Immunotherapy

As with other solid organ tumours there has been an increasing interest in immunotherapeutic treatment approaches in advanced OG cancer, primarily focused on the use of checkpoint inhibitors to induce immune-mediated cytotoxic responses. The landmark ATTRACTION-2 trial has investigated the effect of the anti-PD-1 agent nivolumab vs placebo in 493 East Asian advanced gastric cancer patients in the third line setting, and is the first phase III trial to demonstrate a statistically significant survival benefit for immunotherapy in OG cancer [46]. Median OS was 5.3 months for nivolumab and 4.1 months for placebo (HR 0.63; $p<0.0001$), and 12 month OS rates were 26.2% and 10.9% respectively. The phase II KEYNOTE-059 study enrolled 259 patients treated with at least 2 lines of prior therapy for pembrolizumab treatment [47]. ORR was 11.6%, with median OS and PFS of 5.6 and 2.0 months respectively for the whole group. Response rates were higher in PD-L1 positive (combined positive score (CPS)≥1) patients (15.5 vs 6.4%).

The phase III KEYNOTE-062 study has evaluated pembrolizumab in a first line, PD-L1 positive (CPS≥1) population as both monotherapy and in combination with standard platinum/ 5FU chemotherapy [48]. The complex statistical design of the study initially evaluated non-inferiority of pembrolizumab compared to chemotherapy alone, followed by superiority of the pembrolizumab + chemotherapy combination vs chemotherapy. The trial met its primary endpoint of non-inferiority of first line pembrolizumab as compared to chemotherapy in this PD-L1 enriched population, with a notable improvement in OS with pembrolizumab for highly PD-L1 positive (CPS≥10) patients compared with chemotherapy (17.4 vs 10.8 months). However the combination of pembrolizumab + chemotherapy did not confer a significant survival advantage as compared to chemotherapy alone in either the CPS≥1 or CPS≥10 groups as per pre-specified statistical boundaries.

Further trials of checkpoint inhibitor immunotherapy have also failed to show superiority when compared to existing standard treatments. The phase 3 JAVELIN Gastric 300 trial compared the anti-PD-L1 antibody avelumab with physician's choice of paclitaxel or irinotecan in patients who had progressed beyond second-line therapy, and did not meet its primary endpoint, reporting median OS of avelumab vs chemotherapy of 4.6 vs 5.0 months (HR 1.1; 95% CI 0.9–1.4; p= 0.81), although the safety profile of avelumab was found to be more favourable [49]. Similarly, the KEYNOTE-061 study which compared pembrolizumab with paclitaxel in second-line PD-L1-positive patients also did not meet its OS and PFS endpoints [50]. Thus in trials where chemotherapies have been used as a comparator arm the results from anti-PD1/ PD-L1 approaches have been less compelling.

A number of further immunotherapy combinations are also being trialled. These include checkpoint inhibitor combinations of PD-1 and *CTLA4* inhibitors and immunotherapy plus further targeted therapy combinations. Combined PD-1 and *CTLA4* blockade with nivolumab and ipilimumab has been shown to be more effective than single-agent ipilimumab in advanced melanoma, however at a cost of significantly more toxicity. The CHECKMATE-032 phase Ib/2 trial has investigated the activity of nivolumab alone vs nivolumab plus ipilimumab in 2 differing dosing schedules in OG cancer irrespective of PD-L1 status. They have reported higher response and disease control rates in the lower dose nivolumab/ higher dose ipilimumab cohort, although this combination was associated with a greater burden of toxicity [51]. Combinatorial approaches investigating checkpoint inhibitor and anti-angiogenic targeting are ongoing. Scientific rationale for such an approach is provided by a number of preclinical studies describing pro-immunogenic effects of anti-angiogenic drugs [52, 53]. A cohort of 41 advanced OG adenocarcinoma patients were treated with ramucirumab in combination with pembrolizumab as part of the JVDF study, of whom 59% had at least 2 prior line of systemic therapy, showing favourable response and survival data in this pretreated cohort [54]. Further combinations under investigation include checkpoint inhibitors with targeted agents such as trastuzumab, and other microenvironment and immune modulating agents such as epacadocostat, andecaliximab and *LAG3* [55, 56].

PD-L1 expression status was not a requirement for entry into CHECKMATE-032, KEYNOTE-059 or ATTRACTION-2, and PD-L1-positivity varied across studies. Trends towards improved response in PD-L1 positive patients have been noted, however a significant challenge to interpretation lies in differences in determination

of PD-L1-positivity. The antibodies used for IHC assessment, and the criteria for defining PD-L1 positivity has differed between studies, with pembrolizumab studies counting tumour and stromal immune cells toward total PD-L1-positivity using CPS, while nivolumab/ ipilimumab studies use PD-L1 staining on tumour cells only. Heterogeneity of biomarker expression may also pose a problem, as PD-L1 expression status may be heterogeneously expressed in gastric cancer [57]. In their OG cancer analysis the TCGA group reported 22% of tumours as microsatellite instability high (MSI-H), displaying characteristic genomic instability resulting in high mutational burden and neoantigen expression, a characteristic known to potentiate the effect of immune checkpoint inhibition [20]. The TCGA cohort included both early and late stage tumours, and as it is known that MSI-H conveys favourable prognosis in early-stage radically treated tumours, the incidence in advanced disease is likely to be much lower. This is borne out in further analyses such as that of Memorial Sloan Kettering Cancer Centre, which found that only 3% tumours in their advanced cohort were MSI-H [58]. In both KEYNOTE-059 and -062 MSI-H was an infrequent occurrence, but associated with improved outcomes with pembrolizumab [47][59]. Further biomarker data to predict response, including gene expression profiling may aid in selecting patients for checkpoint blockade immunotherapy moving forwards.

1.3 Evaluating local treatment patterns and outcomes

1.3.1 Rationale

A number of groups have published on their own institutional experience of advanced OG cancer management, although included numbers of patients have generally been small [60–62]. As the treatment landscape for OG cancer continues

to evolve it would be expected that a greater proportion of patients will go on to receive sequential lines of therapy, and targeted and immunotherapeutic agents will become increasingly utilized. The colorectal cancer treatment paradigm has been instructive as an exemplar of rational sequencing of multiple lines of therapy to incrementally improve survival outcomes in the advanced disease setting. At present however it is a challenge for clinicians treating OG cancer to identify appropriate patients and rationally sequence treatments in such a way as to provide optimal benefit. In order to guide treatment decisions and plan relevant clinical trials in the field it is important to understand current treatment patterns and outcomes in 'real world' populations, as well as to inform trial design in the era of immunotherapy and third line treatment studies.

1.3.2 Methods

I undertook a retrospective analysis of consecutively treated patients receiving at least one cycle of chemotherapy for OG adenocarcinoma in the advanced disease setting at the Royal Marsden Hospital between April 2009 and November 2015. Potential patients were identified through use of hospital diagnostic coding. Data was collected via review of the electronic patient record. Demographic, treatment and survival outcomes were recorded. Statistical analysis was performed using Stata 13 statistical software.

1.3.3 Results

1.3.3.1 Demographics

Five hundred and eleven patients were identified; 384 (75%) male, 127 (25%) female; median age at diagnosis 66 years (range 24- 90). The performance status of patients at cycle 1 of first line treatment was ECOG PS 0 in 64 (13%); PS1 in 276 (54%); PS2 in 87 (17%); PS3 in 1 and not recorded in 83 (16%) patients. Site of primary tumour was oesophageal in 148 (29%), oesophageogastric junction (OGJ) in 173 (34%) and stomach in 90 (37%) patients. Disease extent at commencement of first line treatment was locally advanced (unresectable) in 68 (13%), de-novo metastatic disease at presentation in 335 (66%) and relapsed metastatic after previous radical treatment in 108 (21%) patients. HER2 status was positive in 73 (20%), negative in 296 (80%) and not recorded in 142 patients. Patient characteristics are summarised in table 1.

Total number of patients	511
Sex:	
Male	384 (75%)
Female	127 (25%)
Median age at diagnosis (yrs, range)	66 (24-90)
Performance status:	
0	64 (13%)
1	276 (54%)
2	87(17%)
3	1
Not recorded	83 (16%)
Site of primary tumour:	
Oesophagus	148 (29%)
GOJ	173 (34%)
Stomach	190 (37%)
Disease extent at commencement of first line treatment:	
Locally advanced (unresectable)	68 (13%)
De novo metastatic	335 (66%)
Relapsed metastatic after radical treatment	108 (21%)
HER2 status:	
Positive	73 (14%)
Negative	296 (58%)
Not recorded	142 (28%)

Table 1. Patient characteristics of Royal Marsden advanced OG cancer cohort.

1.3.3.2 Treatment

In the first line treatment setting 320 (63%) of patients received a triplet chemotherapy regimen. These were predominantly platinum/ fluoropyrimidine doublets with the addition of either trastuzumab or an anthracycline. One hundred seventy one (33%) of patients received doublet therapy, predominantly a platinum/ fluoropyrimidine doublet, and 20 (4%) of patients received single agent treatment. Of the 511 patients, 200 (39%) proceeded to receive second line treatment and of these 24 (12%), 68 (34%) and 108 (54%) received triplet, doublet or single agent treatment respectively. Seventy out of 511 patients (14%) went on to receive third line treatment and of these 2 (3%), 26 (37%) and 42 (60%) received triplet, doublet or single agent therapy respectively. Twenty percent of patients treated in the first line participated in a clinical trial, compared to 29% and 36% of those receiving second line and third line treatment respectively. Clinical trials in the first line setting predominantly involved standard chemotherapy with the addition of a targeted agent, such as REAL-3 (EOX +/- panitumumab, 60 participants), RILOMET (ECX +/- rilotumumab, 12 participants) and JAGUAR (FOLFOX +/- ipatasertib, 9 participants), whereas the most common trials in the third line setting were phase 1 trials (9 participants). Figure 1A-C illustrates the combinations of chemotherapy received and a breakdown of the clinical trials in the first, second and third lines respectively.

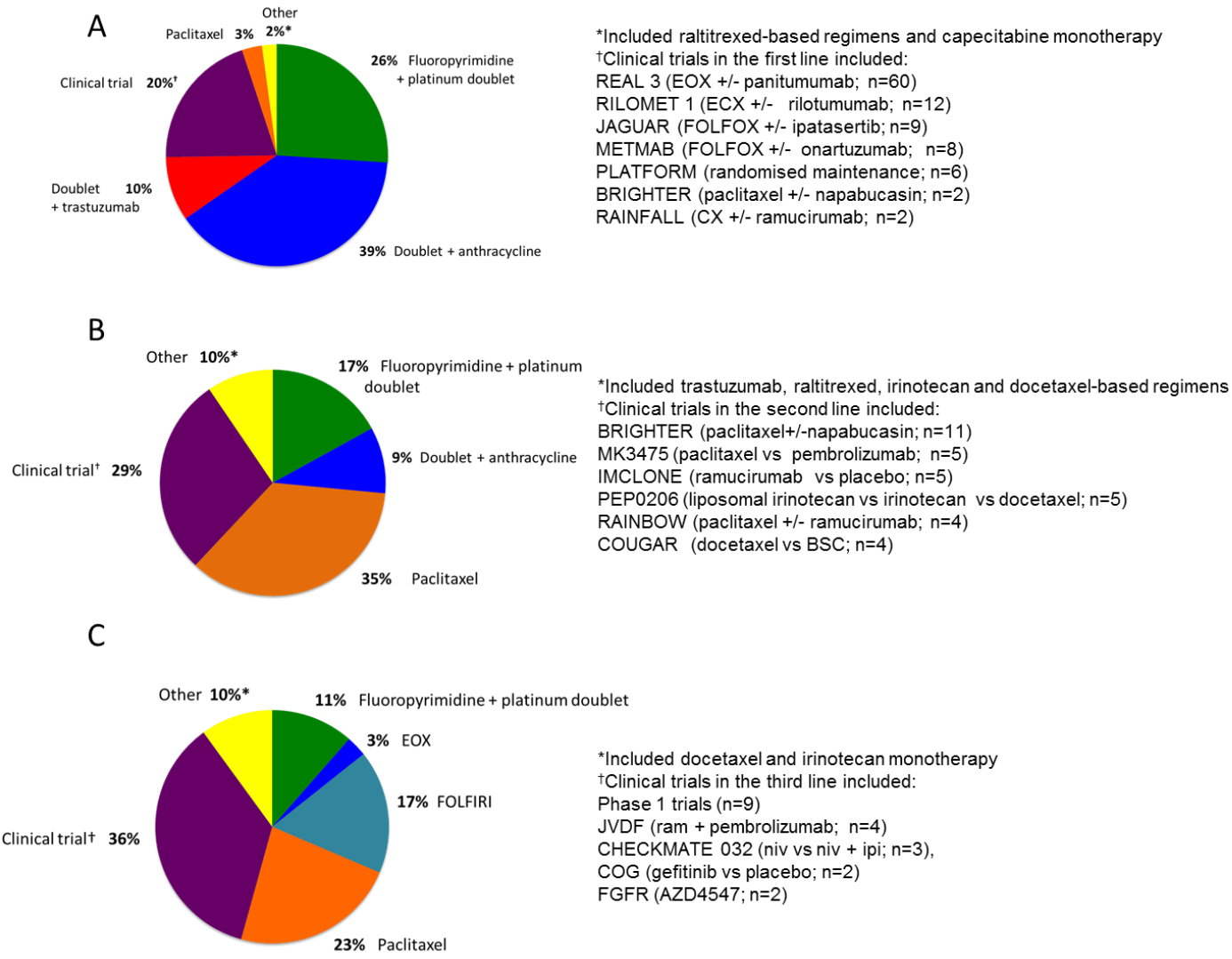


Figure 1. Breakdown of treatments received in the (A) first line setting (n=511) (B) second line setting (n=200) and (C) third line setting (n=70).

An increase in the proportion of patients continuing onto second line was observed over the time period of data collection. In the first quarter of the study period 45/136 (33%) of patients proceeded to second line therapy, whereas in the fourth quarter this increased to 63/135 (47%) (figure 2). The uptake of HER2 testing over time also increased, with 74% cases prior to April 2010 not reported compared to no cases from April 2015 onwards.

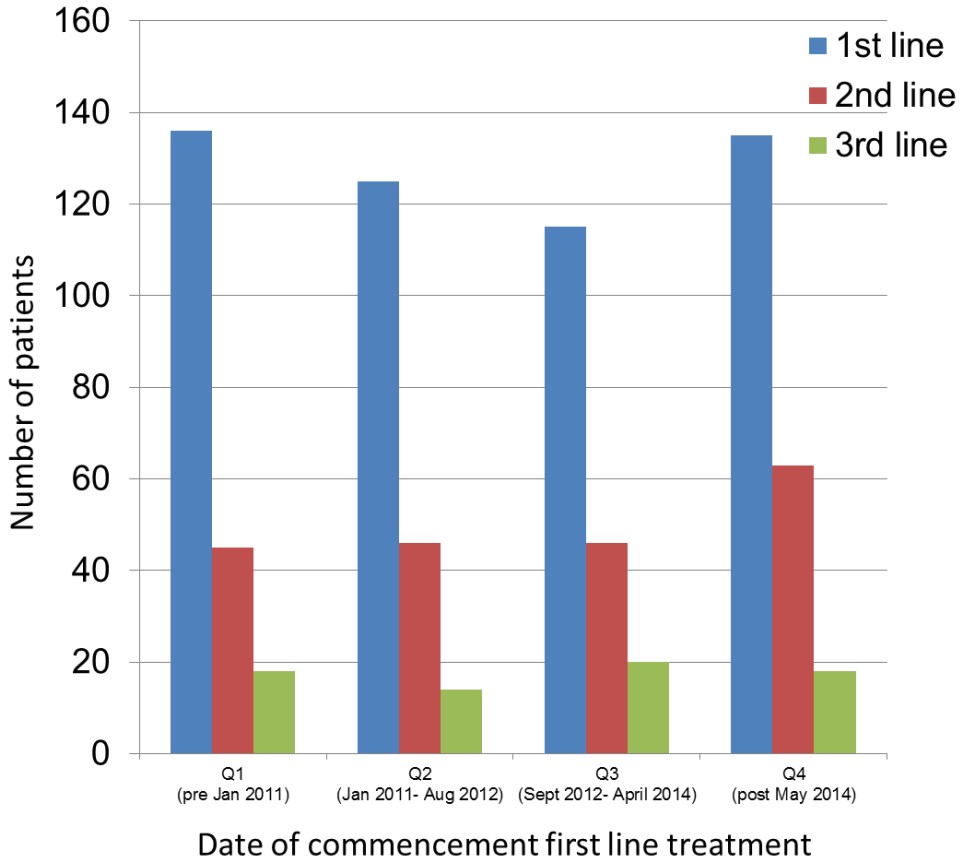


Figure 2. Changes in uptake of sequential treatment over time.

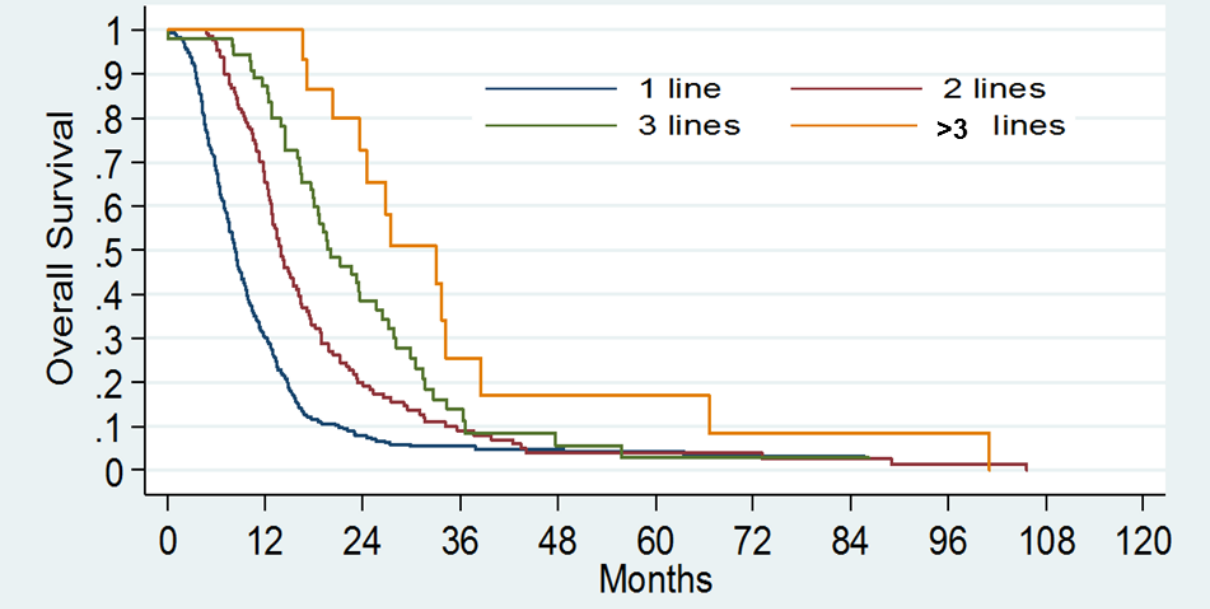
1.3.3.3 Treatment efficacy

In the first line setting the overall best response was complete response (CR) in 2%, partial response (PR) in 47%, stable disease (SD) in 29%, and progressive disease (PD) in 22% (table 2). In the second line setting overall best response was PR 21%, SD 34% and PD 45%. In the third line overall best response was PR 19%, SD 24% and PD 57%. Median OS for the whole cohort from the date of diagnosis of advanced disease was 11.5 months. OS from the commencement of second and third line treatment respectively were 6.0 and 4.6 months respectively. Survival was significantly correlated with number of treatment lines received (p<0.001 log rank) with median OS from diagnosis of 8.3, 14.0, 20.1 and 33.0 months depending on whether patients received 1, 2, 3 or >3 lines of treatment respectively. Progression

free survival (PFS) from initiation of first, second and third line treatment was 5.5, 3.0, and 1.9 months respectively (table 2; figure 3).

	1st line	**2nd line**	**3rd line**	**>3 lines**
N	511 (100%)	200 (39%)	70 (14%)	15 (3%)
Treatment	Triplet 63% Doublet 33% Single 4%	Triplet 12% Doublet 34% Single 54%	Triplet 3% Doublet 37% Single 60%	-
Clinical trial participation	103 (20%)	57 (29%)	25 (36%)	5 (33%)
Median number of cycles	6	3	3	-
Overall best response	CR 2% PR 47% SD 29% PD 22%	CR 0% PR 20% SD 34% PD 41%	CR 0% PR 19% SD 24% PD 57%	-
PFS (m)	5.5	3.0	1.8	
OS (m)	11.5 (whole cohort)			
OS (m) According to lines of treatment received	8.3 (received 1st line only)	14.0 (received 1st and 2nd line)	20.1 (received 1st, 2nd and 3rd line)	33.0 (received >3 lines)

Table 2. Variables according to treatment line.



Chemotherapy line	No at risk	No of events	Median Survival (m)	95% C.I.
1 only	311	286	8.32	7.57-9.05
2 only	130	119	13.95	12.90-15.89
3 only	55	48	20.10	17.60-25.76
>3	15	13	33.03	20.30-38.55

Figure 3. Overall survival by number of treatment lines received.

There was no significant difference in OS in the advanced setting between patients with relapsed disease after previous radical treatment and those with metastatic disease at diagnosis (12.6 vs 11.3m; *p*=0.10 log rank). OS was significantly improved for confirmed HER2 +ve patients compared to HER2 -ve (15.0 vs 11.9m; *p*=0.02 log rank) (figure 4), and in those patients treated within a therapeutic clinical trial at any line of treatment compared with those who were not (13.5 vs 10.1m; *p*=0.02 log rank).

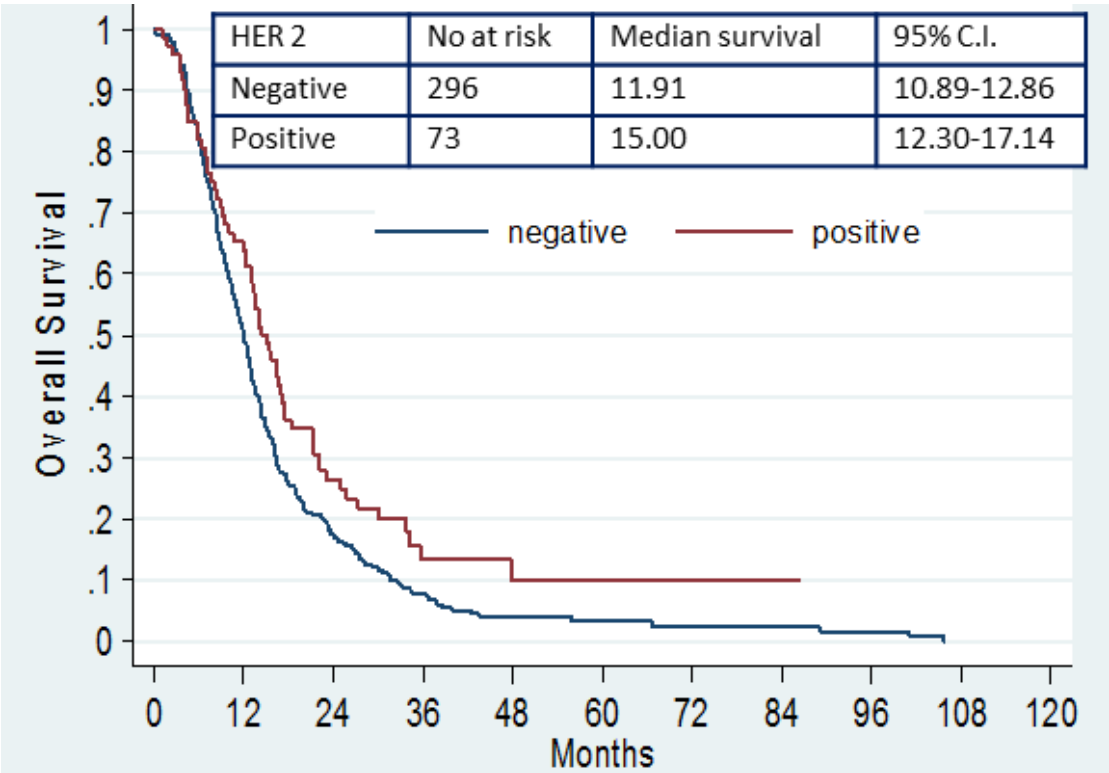


Figure 4. Overall survival by HER2 status

1.3.4 Discussion

This comprehensive analysis of treatment and survival for advanced OG adenocarcinoma patients treated within my own institution reflects both the current landscape and developing trends in the treatment of this condition. The patient population of predominantly men (75%), with a median age of 66 is typical of the demographics for this disease. Unlike most of the large trial populations however the distribution of performance status showed that 17% of patients had a recorded PS >2 at baseline, who would typically be excluded from most clinical trials. A platinum doublet plus or minus an additional third drug was most commonly used in the first line, and single-agent paclitaxel in the second line, however a substantial proportion of second line patients were treated with doublet or triplet combination therapies. The majority of these cases represented rechallenges of a regimen that was

previously efficacious. This reflects what is often seen in day-to-day practice, where a minority of patients maintain sensitivity to platinum/ fluoropyrimidine combinations and thus benefit from multiple rechallenges. Emerging data from the field of colorectal cancer has shown the utility of using sequential circulating tumour DNA analysis to investigate dynamic mechanisms of sensitivity and resistance during therapy [63]. Whether such approaches have a role in OG cancer in identifying patients with more intrinsically chemotherapy-sensitive disease suitable for multiple lines of therapy remains an ongoing research question.

Participation in clinical trials was high, with 20%, 29% and 36% of patients participating in first, second and third line trials respectively. This exceeds the reported 14% rate of trial participation in the UK as a whole, which is in itself purported to exceed that of other countries, with as few as 3% of US cancer patients participating in clinical trials [64, 65]. The growth in the use of subsequent lines of treatment is reflective of the accumulating level-1 evidence base over the time period of the analysis. Many of the major second line gastric cancer studies were published between 2011 and 2014, giving clinicians a greater selection of evidence-based options for later line treatment. Similarly HER2 testing trends changed considerably over the analysis period. This coincides with both the landmark TOGA trial results and the funding of trastuzumab in the UK for HER2 positive gastric cancer patients which was approved in November 2010: prior to this date 24% of patients commencing 1st line treatment underwent HER2 testing of their tumour, whereas after this date 89% underwent testing.

Overall response rates are comparable with published trial data, however when considering these it is important to note that a significant proportion of patients did not maintain a response for the duration of their treatment. Of 349 patients who had SD or better at the time point of their initial response assessment scan on first line therapy, 119 (34%) had documented radiological PD by the end of treatment whilst 38 (11%) had no further scans due to either unacceptable toxicity, clinical progression or death curtailing completion of treatment. So, although initial disease control rates are reasonable, this is often followed by rapid radiological progression or clinical deterioration during the same treatment line. There is increasing interest in utilizing maintenance therapies in OG cancer, with a number of ongoing trials using either de-escalated chemotherapy, targeted or immunotherapeutic agents to try and augment and maintain response to first line chemotherapy [66, 67]. Given that disease control rates fall substantially during the course of chemotherapy this presents a challenge to investigators trialing such maintenance strategies.

Median OS and PFS are again similar to those reported in landmark trials in predominantly European patient populations, however an OS of 15.0 months for HER2 positive patients was higher than the 13.8 months reported in the TOGA trial, and also interestingly exceeded that of the chemotherapy plus trastuzumab arm of the JACOB study (14.2 months) [13]. This may be explained by the fact that UK NICE guidelines allow funding for trastuzumab only for patients who display HER2 immunohistochemistry (IHC) 3+ staining, a subgroup which was shown in TOGA subgroup analysis to have superior survival times. More recent studies have investigated the relationship between *HER2* gene amplification and clinical benefit from trastuzumab, showing that the level of *HER2* amplification significantly predicts

sensitivity to therapy and overall survival, and that the optimal *HER2* amplification ratio predicting for trastuzumab benefit is likely to be considerably higher than currently mandated definitions of positivity [68, 69]. Further refinement of biomarker selection may be necessary to optimise benefit in this group of patients moving forwards.

As expected, survival was significantly correlated with the number of treatment lines received. There is obvious selection bias, as patients suitable for sequential treatment are likely to represent a self-selecting fitter group. There may also be underlying tumour-biological factors rendering them more sensitive to existing chemotherapies, and selecting patients suitable for such sequential treatment will become a more relevant clinical challenge moving forwards. There have been previous attempts to define relevant prognostic factors in advanced OG cancer as well as ongoing work to identify genetic signatures predictive of response to both standard chemotherapies and immunotherapeutic agents [70–72]. The 70 patients who underwent third line or beyond treatment in this cohort were slightly younger (median age 55 years) and had a longer PFS with first and second line treatment (9.3 and 4.2 months respectively) than the cohort as a whole, factors which have previously been associated with favourable response in the third line [73, 74]. It is striking that only 39% of the overall cohort went on to receive second line therapy. Even within a modern clinical trial such as the 2018 RAINFALL study the rate of second line treatment uptake reached only 51% [37], underlining the importance of improving first line interventions for patients who will often only have one opportunity for the application of systemic therapy. The improved survival seen in patients who went on to receive further lines of treatment highlights the positive impact sequential

lines of therapy may have for carefully selected patients, and should encourage physicians to pursue this approach where appropriate.

There are known variations in treatment and outcome in advanced OG cancer globally, with greater uptake of sequential lines of therapy and improved survival in East Asian compared to European populations. Analyses of treatment patterns in non-trial East Asian populations have reported rates of uptake of second line chemotherapy ranging from 54% to as high as 80%, and such disparities have important implications for research in the field [60, 61, 75]. Subgroup analysis of the RAINBOW trial evaluating second line paclitaxel with or without ramucirumab revealed only a non-significant OS benefit for ramucirumab in East Asian patients, despite significant improvements in both response rate and PFS [76]. This was likely to have been driven by a substantially higher uptake of post-progression treatment lines in this population, and the effect of differential uptake of further treatment must be considered when interpreting survival outcomes in first and second line trials involving both Eastern and Western patient cohorts [77].

As we move into an era where immunotherapeutics and targeted agents become more closely integrated into advanced OG cancer treatment pathways it is hoped further survival improvements will be seen, with multiple lines of therapy exposure utilising these novel agents becoming more commonplace, particularly in European practice where such sequential treatment approaches remain relatively uncommon. It is important to understand current and evolving trends in order to tailor ongoing research effectively within existing treatment frameworks. Combining clinical and biological characteristics to refine prognostic and predictive models moving forwards

may identify patients suitable for more prolonged and rationally-sequenced treatments in the advanced disease setting.

2 The i*MYC* trial: pre-clinical rationale, trial development, and results from prospective screening

2.1 Targeting *MYC*

The *MYC* proto-oncogene is one of the most commonly dysregulated genes in human cancer [78]. It functions as a transcription factor controlling the transcriptional output of discrete sets of genes involved in key carcinogenic processes including cell growth, cell cycle control, energy production, anabolic metabolism and DNA replication [79–84]. Abnormal activation can be due to transcriptional overexpression via gene amplification, translocation or alteration in upstream signalling pathways, and *MYC* abnormalities play a number of important diagnostic and prognostic roles in haematological malignancies [85]. Burkitt's lymphoma is characterised by a *MYC* chromosomal translocation which drives constitutive expression, whilst in diffuse large B cell lymphoma translocations are seen in 5-15% of cases and confer a worse prognosis [86, 87]. Although it is implicated in OG carcinogenesis, reported frequencies of *MYC* amplification from massively parallel sequencing and high density SNP arrays have ranged widely in this tumour type [88–90]. In breast and ovarian cancer a correlation between tumour *MYC* status and response to chemotherapy has been described; and it has been observed that cells driven by *MYC* may be sensitised to chemotherapy-induced apoptosis [91–93]. This association was seen for fluoropyrimidine, platinum and taxane- containing chemotherapies, all of which are commonly used in the management of OG cancer.

Given the ubiquity and importance of *MYC* alterations in human cancer effective methods for targeting *MYC* represent an attractive potential therapeutic avenue;

although one that has traditionally been regarded as challenging. *MYC* is a transcription factor localised in the cell nucleus which lacks enzymatic activity or a specific structural target for small molecule inhibitors, and is inaccessible to antibody-based therapies [94]. Techniques explored to date have included targeting of the *MYC*-regulatory BET bromodomain proteins, and use of small molecule inhibitors to disrupt the interaction of *MYC* and its oligodimerization partner MAX [95–99]. Strategies based on the concept of synthetic lethality, whereby disruption of two or more genes in combination result in a deleterious phenotype, have also been investigated to indirectly target tumours displaying *MYC* over-activity [100, 101]. Prior work carried out at the Gene Function Laboratory of the Institute of Cancer Research used interference screen data for oesophageal cell lines to identify candidate genetic dependencies that could potentially be exploited as therapeutic targets. Selectively decreased cell viability was found following silencing of Bruton's Tyrosine Kinase (BTK) in *MYC*-amplified cell lines, which was further validated using ibrutinib, a clinical BTK inhibitor that is also known to target *HER2* [102]. In *MYC* amplified cells, ibrutinib down-regulated levels of MYC protein and downstream effectors, and elicited G1 cell cycle arrest and apoptosis. BTK signalling occurs partly via the canonical RAS-RAF-MEK-ERK pathway. ERK is a known mediator of MYC phosphorylation, and a putative mechanism of action for *MYC* and BTK interaction was identified through observation of BTK-dependent, ERK-mediated, MYC phosphorylation [103].

2.2 i*MYC* trial development

On the basis of the preclinical work described, a trial evaluating the efficacy of ibrutinib in *MYC* and/or *HER2* amplified advanced OG cancer was considered. The

research question was addressed through development of a phase 2 proof-of-concept, non-randomised study of ibrutinib in advanced pre-treated OG cancer, with patients recruited on the basis of biomarker selection of *MYC* and/or *HER2* positivity. Initial meetings with the commercial partner Janssen took place over the early part of 2015. I managed the trial set-up process including protocol development and ethical and regulatory submission, and worked with the cytogenetics department at the Royal Marsden Hospital in order to establish a testing protocol to diagnostic standard for the assessment of *MYC* amplification in advanced OG cancer through the use of a fluorescent in-situ hybridisation (FISH) assay (figure 5). The resulting i*MYC* trial (NCT 02884453) opened in May 2016, and represents the first attempt at targeting *MYC* amplification via synthetic lethal gene interactions through the novel application of an existing anti-cancer drug.

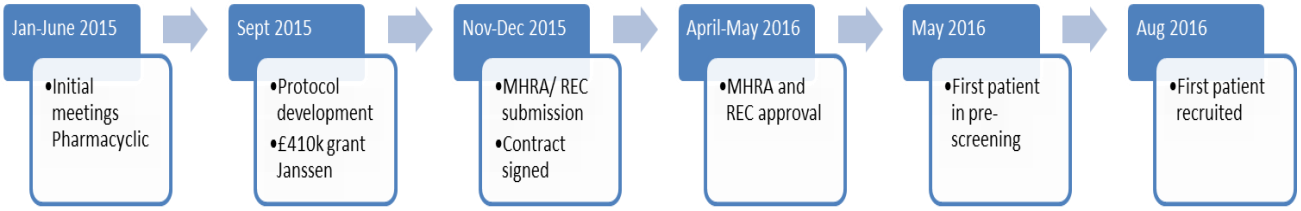


Figure 5. Timeline of i*MYC* clinical trial set-up detailing key milestones: preliminary meetings with representatives from Pharmacyclics took place at the beginning of 2015. Janssen pharmaceuticals had previously acquired the rights to co-develop Ibrutinib and expressed an interest in financially supporting the trial. Protocol development, costings and commercial funding support were coordinated from September 2015 onwards prior to final regulatory and ethical approval in May 2016. Patient recruitment into pre-screening began in May 2016, with first patient recruited onto the main study in August 2016.

2.3 Clonal diversity of *MYC* amplification evaluated by FISH and ddPCR: results from prospective screening

Patients treated at the Royal Marsden Hospital with locally advanced or metastatic OG cancer of either SCC or adenocarcinoma histological subtype being considered for or undergoing systemic anti-cancer therapy were eligible for screening for the i*MYC* trial. At the time of consent an archival diagnostic formalin-fixed paraffin-embedded (FFPE) tumour sample was obtained for analysis and blood samples were collected in Streck Cell-Free DNA blood collection tubes. Here I give the first report of the frequency and patterns of tumour *MYC* amplification from a prospective screening study in OG cancer. I describe the development of a novel digital droplet (dd)PCR assay using two independent reference probes, and assess the correlation of *MYC* amplification detected by FISH with the PCR assay in both primary tumour tissue and ctDNA samples.

2.3.1 Rationale for MYC amplification assessment in OG cancer using FISH and ddPCR

Fluorescent in-situ hybridisation (FISH) is a cytogenetic technique that uses customised fluorescent probes which bind to specific DNA sequences with a high degree of sequence complementarity, and is considered one of the most specific and sensitive methods for detection of oncogene amplifications in human tumour samples [104, 105]. At the time of trial development the only commercially available probes available were for the purpose of detecting *MYC* translocations, the most commonly occurring and clinically significant variant in haematological malignancies. A dual probe FISH assay for tumour *MYC* amplification in OG cancer using probes from Vysis (Abbott Molecular, Maidenhead, UK) was developed and validated for the purposes of the study; with sample preparation and analysis being performed by the cytogenetics department at the Royal Marsden Hospital. Screening was performed using a combination of a reference probe mapping to the centromere of chromosome 8 (CEP8) and a *MYC* probe mapping to chromosome 8q24, covering the entire coding region of the *MYC* gene from exons 1-3. The dual probe approach was used to distinguish between increased copies of chromosome 8 and true *MYC* amplification.

Digital polymerase chain reaction has the potential to accurately quantify the concentration of nucleic acids in a sample to a much greater degree than traditional quantitative PCR through the counting individual DNA molecules [106]. ddPCR is a method that utilises a water-oil emulsion droplet system generating up to 20,000 droplets within which the DNA segment of interest and background DNA are

randomly distributed. Once the droplets have been generated they are then transferred to a 96 well plate and PCR amplification takes place in each of the droplets simultaneously. Following PCR amplification the plate is transferred to a droplet reader and each individual droplet is analyzed. Each of the reactions produces a fluorescent signal, positive or negative depending on whether or not the target DNA is present. The procedure requires small concentrations of DNA for accurate analysis and is thus well suited to identifying genetic amplifications in ctDNA, an area under considerable study across multiple tumour types [107]. In the field of OG cancer ddPCR has been used to evaluate *HER2* amplification in both biopsy and ctDNA specimens, with a high concordance between ddPCR and established IHC/FISH methods [108]. A further study has evaluated the ability of ctDNA ddPCR to reflect dynamic changes in *HER2* during gastric cancer treatment and follow-up [109]. Emerging data such as this suggests some promise for the use of ddPCR as a repeatable and non-invasive approach for evaluation of clinically relevant single-gene amplifications.

2.4 Methods

2.4.1 Sample preparation and DNA extraction

For tumour biopsy samples, FFPE tissue was prepared by the translational histopathology lab at the Royal Marsden Hospital by cutting into 5-8 micron sections, de-paraffinised and stained with nucleofast red. I then performed tumour macrodissection on prepared sections to ensure >70% tumour content. I performed DNA extraction with the QIAamp DNA FFPE tissue kit (Qiagen) as per manufacturer's instructions, briefly: the sample was lysed and incubated at 90°C;

DNA was then bound, washed and eluted from a membrane; plasma samples were isolated by centrifugation at 1,600g for 10 minutes at room temperature, followed by a second centrifugation at 1,600g for 10 minutes before storing at -80°C until required.

I isolated circulating free (cf)DNA from 4-5mL of plasma using the QIAamp Circulating Nucleic Acid kit (Qiagen) as per manufacturer's instructions, briefly: the sample was lysed and incubated followed by adsorbtion from a large volume onto a membrane by drawing the lysate through with vacuum pressure using a vacuum manifold, before washing and elution. Tumour and cfDNA was quantitated using the Qubit dsDNA HS assay (Thermo Fisher Scientific); DNA was stored at -20°C.

2.4.2 FISH

Dual-label FISH was performed on each tissue section using standard techniques by the cytogenic laboratory at the RM Hospital. Tissue slides were deparaffinised in Histo-Clear solution (National Diagnostics/ Thermo Fisher Scientific) followed by dehydration in absolute alcohol. Slides were treated with 1% Antigen Retrieval Buffer (TCS Biosciences) for 5 minutes in boiling pressure cooker followed by Digest-ALL pepsin (Invitrogen) treatment at 37°C and standard dehydration series prior adding probe mix. Probe and target DNA were co-denaturated by incubating at 75°C for 2 minutes followed by hybridisation at at 37°C overnight. Post hybridisation slides were washed in 0.4 x SSC/ 0.3% Igepal at 72°C for 2 minutes followed by 2 x SSC/ 0.1% Igepal at room temperature for 1 minute. Nuclei were counterstained with VECTASHIELD® Antifade Mounting Media (Vector Laboratories) and covered with a coverslip. Microscopy was done blinded by two individual analysts on a fluorescent

microscope equipped with imaging software. The section was screened and a minimum of 100 nuclei counted for assessment of amplification status within any abnormal region. The FISH results were reported in a standardised manner, recording both the range and modal ratio of CEP8 and *MYC* signals and, in the case of amplification, the proportion of cells displaying an amplified signal. The results were deemed to be amplified if *MYC*:CEP8 ratio ≥ 2.5. As there are no established definitions of *MYC* 'positivity' in solid organ tumours, previous studies investigating *MYC*-amplification have used FISH-based techniques which defined positivity based upon established cut-offs derived from *HER2* FISH testing in breast cancer [110].

2.4.3 Real-time PCR

RNA was extracted from ESO26, an oesophageal cell line, and 1µg was converted to complementary (c)DNA using the iScript™ cDNA synthesis kit (Bio-Rad) as per manufacturer's instructions. A 5-fold serial dilution of cDNA was made and this was combined with ddPCR™ Supermix for Probes (No dUTP) (Bio-Rad) and each of the ddPCR probes (*MYC*, *CEBPD*, *RPPH1*) individually. MicroAmp™ optical 384 well plates (Thermo Fisher) were used and plates were run on a QuantStudio™ 6 Flex (Applied Biosystems) at 50ºC for 2 minutes, 95ºC for 2 minutes and 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. Analysis was performed using QuantStudio™ Real-Time PCR software version 1.3.

2.4.4 ddPCR

FAM-labelled *MYC* (Thermo Fisher Scientific), VIC-labelled *RPPH1* (Thermo Fisher Scientific) and HEX-labelled *CEBPD* (Bio-Rad) probes were used in a multiplex

reaction to detect *MYC* amplification in a ratio of 1:0.6:1.5. PCR reactions were prepared with 2x ddPCR™ Supermix for Probes (Bio-Rad) and 2.5ng DNA (ctDNA or FFPE) was used per well. A total of 4 to 8 wells were run per sample in order to generate sufficient positive droplets for each control gene. DNA was separated into individual droplets using the QX200™ Droplet Generator (Bio-Rad). This was followed by PCR with cycling conditions of 95°C for 10 minutes, 40 cycles of 95°C for 15s and 60°C for 1 minute with a temperate ramp of 2.5°C per second, followed by 98°C for 10 minutes. Plates were read on a QX200™ Droplet Reader (Bio-Rad) in the FAM/HEX channel and analysed using QuantaSoft v1.7.4 software (Bio-Rad). Two wells containing no DNA were run as negative controls on all plates.

The following quality control criteria were applied: at least 12,000 total droplets per well and at least 300 droplets positive for *RPPH1* and for *CEBPD* for each sample. Any wells not meeting the first criterion were excluded from analysis and any samples not meeting the second criterion were deemed to have failed. Data were analysed with respect to each control gene individually and the average ratio of *MYC*:*RPPH1* and *MYC*:*CEBPD* reported for each sample.

2.5 Results

2.5.1 Prospective evaluation of MYC amplification by FISH

2.5.1.1 Patient cohort

Between July 2016 and January 2018, 162 patients were consented for recruitment to the prospective screening programme as part of the *iMYC* study. Patients were predominantly male (131/162; 81%), with a median age at diagnosis of 61.2 years

(range 36.4–85.5 years). The majority of cases comprised either oesophageal or OGJ tumours (127/162; 78%). Eighty six percent (139/162) of cases were of adenocarcinoma histological subtype and 92% (149/162) had evidence of distant metastases at the time of enrolment. Of these, 88% of patients had received platinum-based first line chemotherapy treatment. The demographic and clinical data for this cohort are summarised in table 3.

Total number of patients	N= 162
Age (median, range): At diagnosis At time of ctDNA analysis	 61.2 (36.4- 85.5) 62.2 (37.1- 85.8)
Sex (%): Male Female	 131 (81%) 31 (19%)
Site of primary tumour: Oesophagus/ OGJ Stomach	 127 (78%) 35 (22%)
Histological subtype: Adenocarcinoma Squamous cell carcinoma	 139 (86%) 23 (14%)
HER2 status: Positive Negative	 27 (17%) 135 (83%)
Disease status at enrolment: Locally advanced Metastatic	 13 (8%) 149 (92%)
First line treatment received (metastatic patients only): Platinum based Taxane based Other No treatment	 131 (88%) 9 (6%) 7 (5%) 2 (1%)
ctDNA analysis performed	127 (78%)

Table 3. Patient characteristics of i*MYC* pre-screening population.

Given that *MYC* amplification had not previously been assessed in a prospective manner in this way before, I wished to assess the range in amplifications patterns seen in this tumour type. For the purposes of assessing heterogeneity, samples were categorized as: ‘diploid, copy number neutral’ (displaying 2 *MYC* and 2 CEP8 signals per cell), ‘polysomatic’ (additional signals of both *MYC* and CEP8), ‘*MYC*

amplified' (increased ratio of *MYC* to CEP8 signals) +/- polysomy (figure 6A-C). Where a range of signal patterns was seen, the most prevalent (modal) pattern within the sample was recorded.

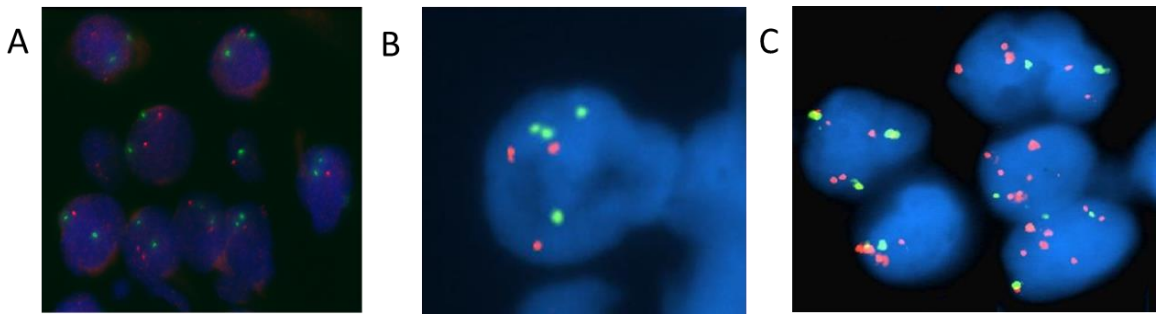


Figure 6. FISH signalling patterns observed in primary OG samples (red= *MYC* signal; green= CEP8 signal). (A) 'diploid, copy number neutral' (2 *MYC* and 2 CEP8 signals per cell); (B) 'polysomatic' (additional signals of both *MYC* and CEP8); (C) '*MYC* amplified' (increased ratio of *MYC* to CEP8 signals).

As of November 2018 162 patient samples had been analysed, of which analysis was successful in 135 (83%; figure 7). Twenty-seven cases (17%) failed testing due to inadequate tumour material or fixation issues. Of the 135 samples that underwent successful analysis, a 'diploid, copy number neutral' signal pattern was seen in 24 (18%) cases. The most commonly observed pattern was polysomy without *MYC* amplification, seen in 80 (59%) cases. Amplification with no evidence of polysomy was seen in 16 (12%) samples and amplification with polysomy was seen in 15 (11%) samples.

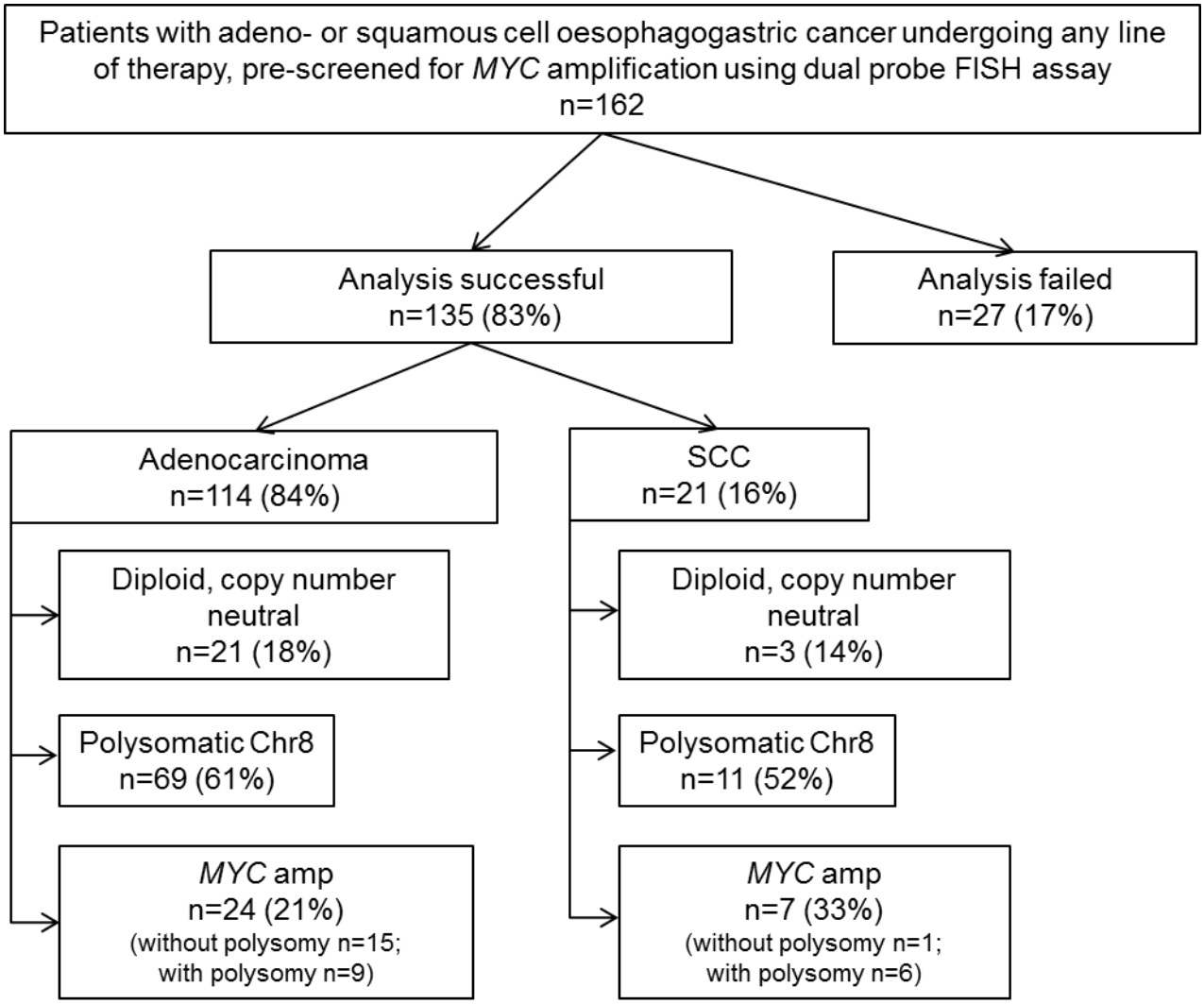


Figure 7. Primary results of FISH screening for *MYC* amplification.

When considering these patterns of *MYC* amplification, inter-tumour heterogeneity was observed, with the percentage of cancer cells harbouring *MYC* amplification ranging widely between samples (median 51%, range 11–94%) (figure 8A). I also wished to assess the extent of intratumoural clonal diversity manifested by the range of *MYC* amplification within each tumour: significant intra-tumour heterogeneity was observed, with 22/31 (71%) amplified samples showing a range of amplification ratios within the evaluated tumour specimen (Figure 9B-C) and a significant variance in amplification ratios (Bartlett's test for equal variance $p<0.001$). Taken together,

these results show that increased copies of *MYC* occur most commonly in the presence of polysomy and there is considerable inter- and intra-tumoural heterogeneity of *MYC* amplification in this prospective OG cancer cohort.

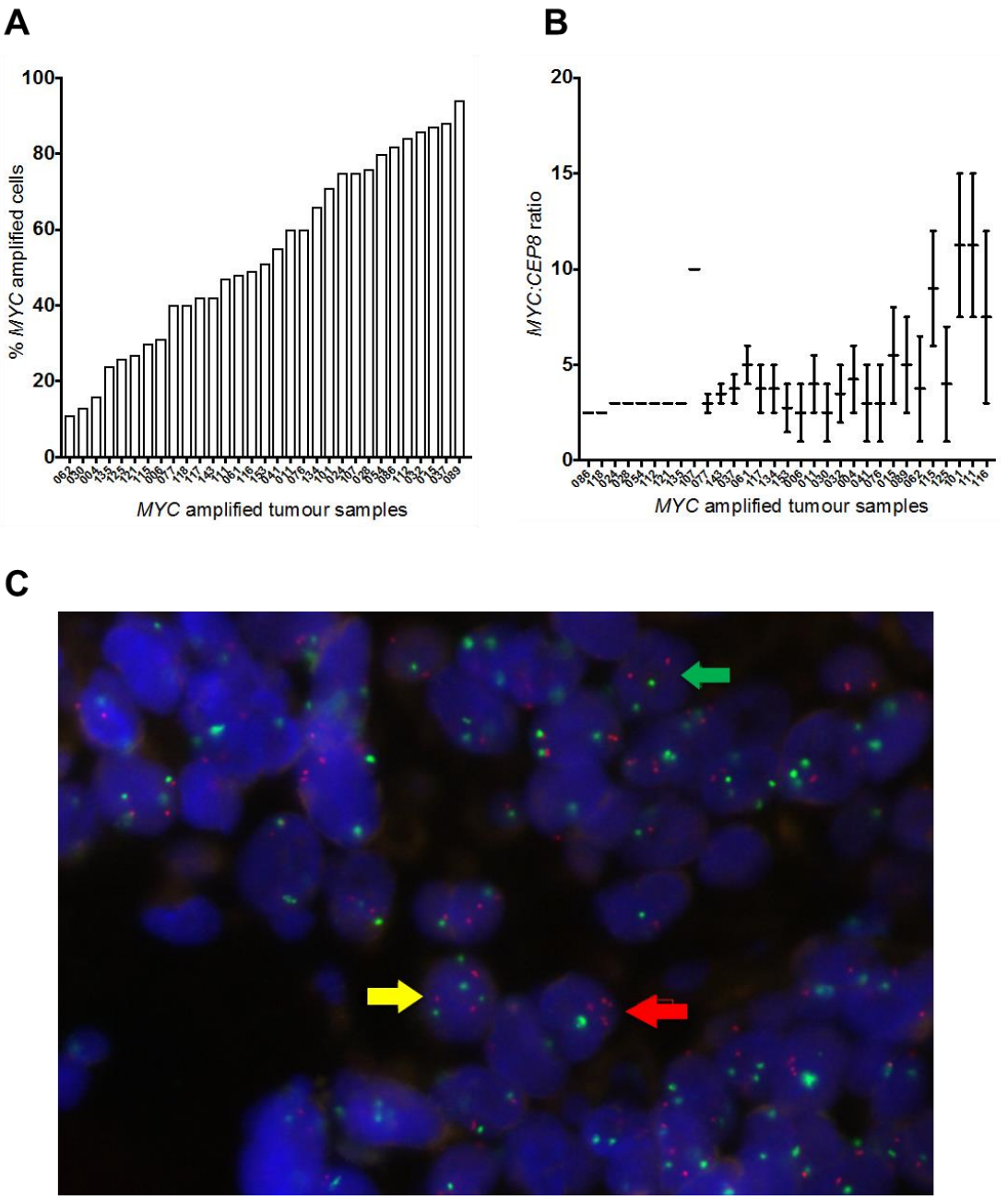


Figure 8. Inter- and intra- tumoural heterogeneity of *MYC* amplification. (A) bar chart illustrating proportion of cells displaying *MYC* amplification within each amplified specimen; (B) line chart demonstrating range of *MYC* amplification ratios seen within each amplified specimen; (C) individual sample showing intra-tumoural heterogeneity of signalling patterns; green arrow= normal diploid; yellow arrow= polysomatic; red arrow= *MYC* amplified.

2.5.1.2 Correlation of MYC amplification status with response and outcome

After defining the degree of *MYC* amplification within each tumour, I wished to assess whether there was any correlation between *MYC* amplification status and clinical correlates of response to prior systemic therapy and outcome. I observed that although *MYC* amplification was identified in a higher percentage of SCC histological subtypes (7/21; 33%) than adenocarcinoma (24/114; 21%), the difference was not statistically significant (p=0.22 χ^2). There was no difference in HER2 status, tumour differentiation or presence of signet cells between *MYC* amplified and non-amplified tumours (table 4).

	***MYC* amplification status**		***p*-value**
	Amplified	Non-amplified	
Histological subtype:			
AC	24	90	0.22
SCC	7	14	
HER2 status:			
Positive	2	19	0.11
Negative	29	85	
Tumour differentiation:			
Poor	15	45	0.61
Moderate- high	16	59	
Presence of signet cells:			
Yes	2	4	0.53
No	29	100	

Table 4: Contingency tables showing distribution of clinico-pathological variables between *MYC* amplified and non-amplified samples (*p*-values χ^2 test).

When considering the potential influence of *MYC* amplification on clinical outcome in patients with advanced disease treated with any first line systemic therapy (N=125), I found no significant difference in response rates (68 vs 55%; p=0.22 χ^2), median PFS (22.9 vs 22.1 weeks; p=0.55 log-rank; figure 9A) or OS (61.6 vs 63.3 weeks; p=0.13 log-rank; figure 9B) between *MYC* amplified and non-amplified tumours. Given the marked differences within the screening population in terms of first line treatment received, I then selected the patient group who had received a standard chemotherapy combination comprising a fluoropyrimidine and platinum agent with or

without the addition of an anthracycline (N=84). For these patients, there was an increased trend towards improved overall response rates in the *MYC*-amplified cohort (64% vs 45%, p=0.09 χ^2) but *MYC* amplification status did not affect median PFS (21.9 vs 18.7 weeks; p=0.76 log-rank) or overall survival (61.6 vs 62.0 weeks; p=0.33 log-rank). In a further exploratory analysis, I assessed the impact of the degree of clonal diversity in *MYC* amplification on survival outcomes for these patients by undertaking tertile analysis based on the range of *MYC:*CEP8 ratios observed within the amplified tumour specimens. I observed that tumour samples with the highest variance in *MYC* amplification were associated with a significantly longer progression free survival following first line treatment (34.1 versus 18.7 weeks; p=0.0182 log-rank; figure 9C) but no difference in overall survival was seen (68.7 vs 50.7 weeks; p=0.24 log-rank; figure 9D). The proportion of amplified cells within the each tumour sample did not appear to influence survival.

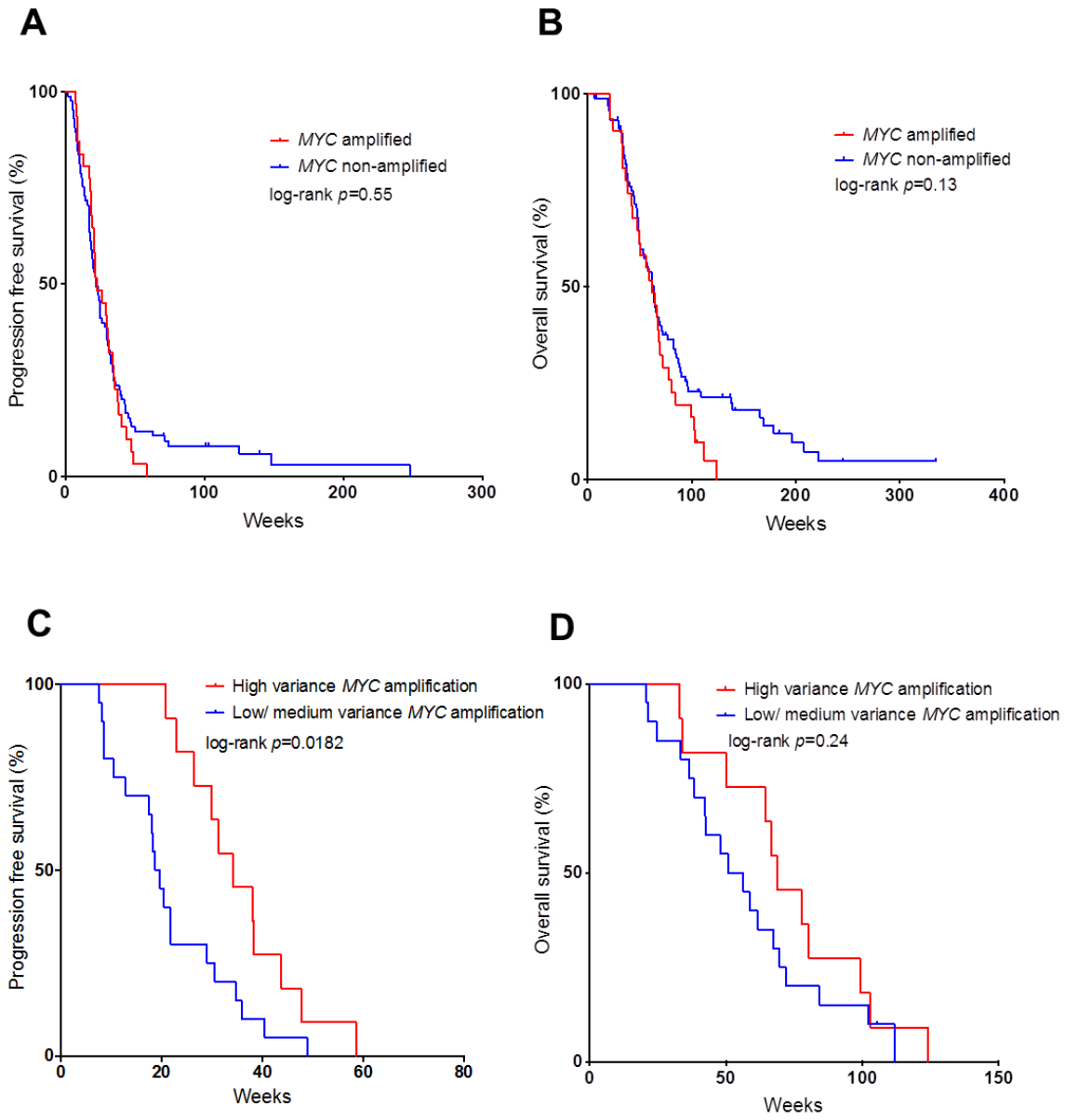


Figure 9. Influence of *MYC* amplification status on survival. (A) Progression free survival on first line systemic treatment for *MYC* amplified versus non-amplified tumours by FISH (22.9 vs 22.1 weeks; *p*=0.58 log-rank); (B) Overall survival for *MYC* amplified versus non-amplified tumours by FISH (61.6 vs 61.6 weeks; *p*=0.21 log-rank); (C) Progression free survival for high variance (defined as top tertile of tumours by range of *MYC* amplification observed within specimen) versus low/ medium variance (low and mid tertile tumours by range of *MYC* amplification observed within specimen) tumours (34.1 vs 19.2 weeks; *p*=0.0182 log-rank); (D) Overall survival of high variance versus low/ medium variance *MYC* amplified tumours (68.7 vs 53.6 weeks; *p*=0.24 log-rank).

2.5.1.3 Patterns of amplification

To further evaluate patterns of *MYC* amplification that may account for ibrutinib sensitivity I undertook analysis of metaphase spreads derived from *MYC* amplified oesophageal tumour cell lines. Chromosomal translocations at 8q were observed, a known frequent alteration in OG cancer. This is consistent with recent next generation sequencing studies that report the frequent occurrence of chromosomal instability particularly within the context of the most commonly-occurring CIN subtype [20][89]. Extra-chromosomal oncogenic amplification of *MYC* has also been reported, whereby amplification occurs in nuclear extra-chromosomal DNA. Such extra-chromosomal amplifications have recently been identified as driving intra-tumoural heterogeneity, with potentially important clinical implications in terms of clinical behaviour and drug sensitivity [111]. Cytogenetic analyses such as FISH can localise such extra-chromosomal amplicons, and based on the amplification patterns seen in our cohort extra-chromosomal amplifications appeared to be occurring events in selected highly amplified cases, however to date FISH analysis of metaphase spread cell line data has not confirmed this (figure 10).

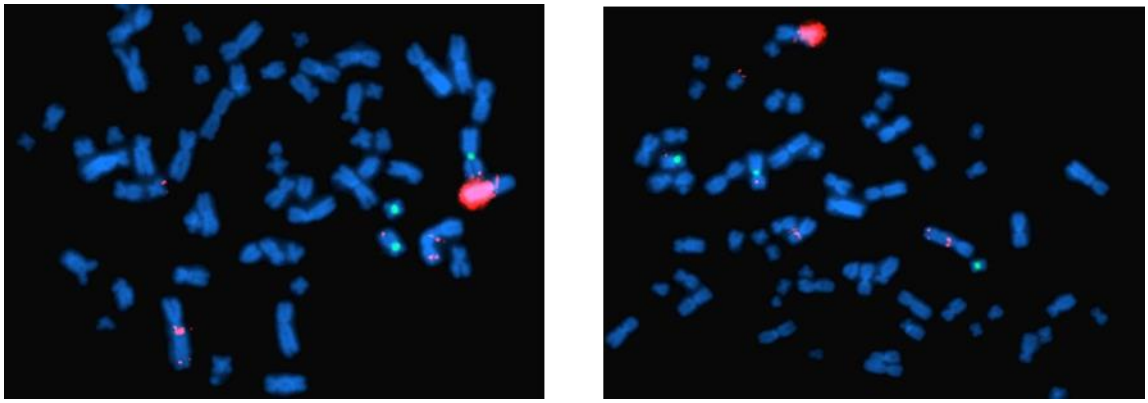


Figure 10. FISH analysis of metaphase spread with MYC and CEP8 probes as previous, demonstrating intra-chromsomal amplification of MYC, with translocation of 8q.

2.5.2 Identification of ddPCR reference genes and assay optimisation

A challenge in using ddPCR for the detection of gene amplifications is the reliable quantification of changes in the abundances of germline reference loci [112]. Taking *MYC* as an example, an optimal reference region is one that in *MYC*-amplified cancer is never co-amplified, and in non-amplified cancer robustly has the same stable copy number as *MYC*. To find potential on-chromosome 8 reference genes for *MYC*, publically available copy number data (both log 2 ratios and thresholded copy number calls) for tumour samples from gastric and oesophogeal cancers were obtained from cBioPortal using the CGDS-R package (available at http://www.cbioportal.org/cgds_r.jsp) [113]. This resulted in 443 gastric and 186 oesophageal tumours which were then divided into *MYC* amplified (59 gastric and 17 oesophageal) and *MYC* non-amplified (384 gastric and 169 oesophageal) tumours as categorised by cBioPortal copy number calls (figure 11A). The copy number ratio of *MYC* versus all possible reference genes on chromosome 8 was calculated and the difference between the *MYC*-amplified and non-amplified tumours was assessed using a Student's t-test (figure 11B). The sensitivity of each putative reference gene

was assessed as the proportion of *MYC*-amplified tumours where the copy number ratio was higher than the maximum ratio in *MYC* non-amplified cancers. Additionally, the correlation between the copy number of non-amplified *MYC* tumours with each potential reference probe was calculated to find references with a similar copy number profile in non-amplified tumours. Putative reference regions along chromosome 8 showing low *p*-value, high sensitivity and high correlation were identified by graphical plots (figure 11C-D). Candidate genes in these regions were then considered and copy number values were plotted to confirm the validity of the selected references. For the final analysis, two reference genes were chosen in order to aid in distinguishing between chromosome 8 polysomy and true *MYC* amplification. A chromosome 8 reference gene, *CEBPD*, was selected on the basis of high sensitivity for *MYC* amplification and the chromosome 14 reference gene, *RPPH1*, which is frequently used in assessment of copy number variation (figure 11D).

Once optimal reference genes were established we set out to determine the parameters for successful ddPCR amplification. Probe-based detection methods use fluorescently labelled probes positioned between two PCR primers, with the measured fluorescence signal directly proportional to the amount of target DNA present. Using fluorophore dye combinations where target and reference genes are assigned different fluorescent labels within dual probe assays enables the ability to quantify and detect multiple targets per sample. The proprietary fluorophore probes used were FAM-labelled *MYC* (Thermo Fisher Scientific), VIC-labelled *RPPH1* (Thermo Fisher Scientific) and HEX-labelled *CEBPD* (Bio-Rad) in a multiplex reaction to detect *MYC* amplification. VIC and HEX have similar spectral properties,

and were used in differing ratios in order to be distinguished. ddPCR reaction plates could be read using either FAM/VIC or FAM/HEX channel. We tested both HEX and VIC detection channels on the Bio-Rad machine in the multiplex reaction and noted that detection of either fluorescence pairings generated comparable data (figure 12 A-B).

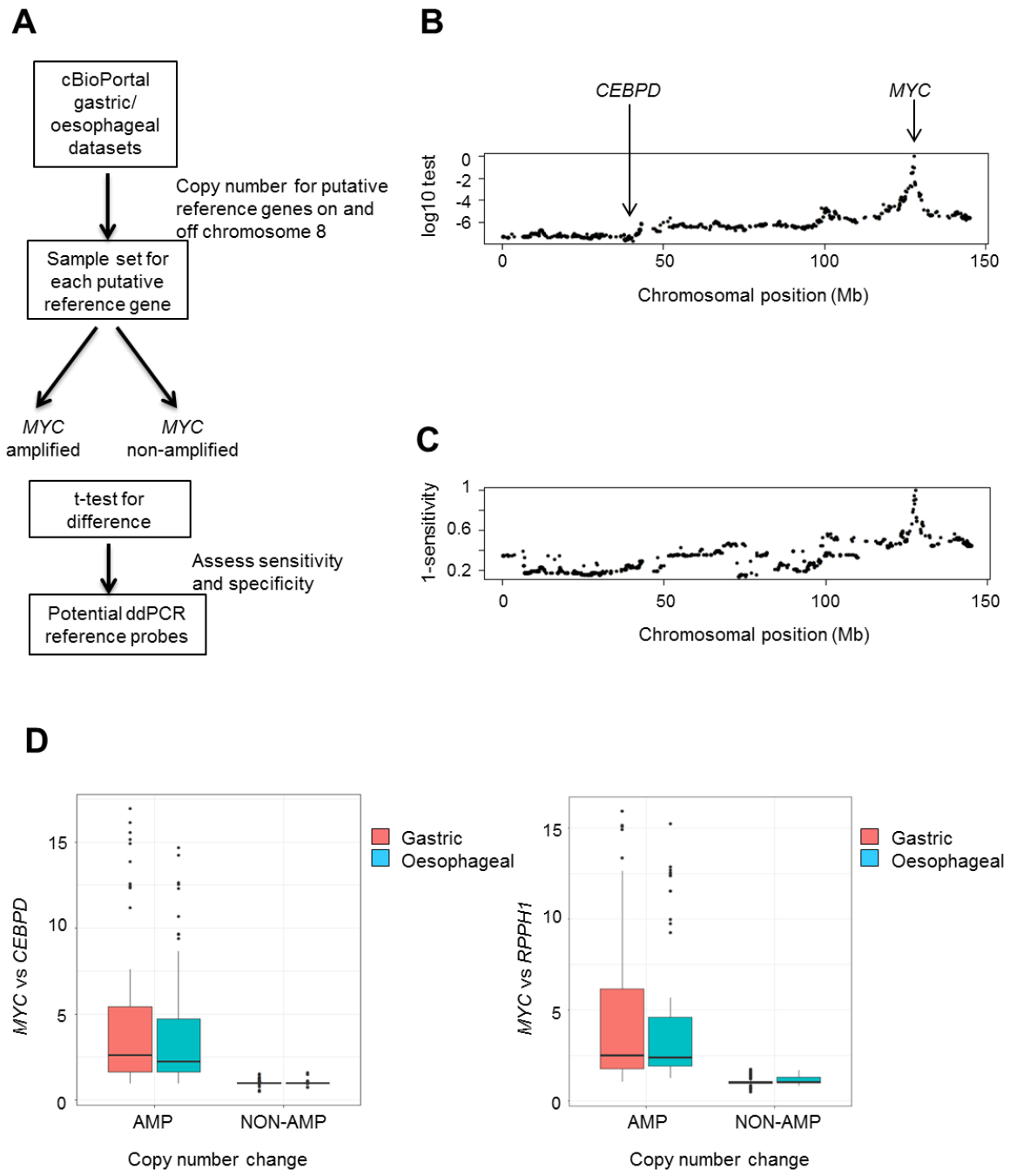


Figure 11. Identification of suitable reference genes for probes in ddPCR assay. (A) Workflow for identifying ddPCR reference probe; (B) Analysis of copy number data from 443 gastric and 186 oesophageal primary cancers. The ratio of *MYC* to each gene on chromosome 8 was calculated and this was compared in *MYC* amplified and non-amplified cancers by Student's t test. Arrows indicate the position of *CEBPD* and *MYC*. (C) The corresponding sensitivity was assessed for each gene along chromosome 8. (D) Comparison of *MYC*:*CEBPD* and *MYC*:*RPPH1* copy number ratios in *MYC* amplified and non-amplified cancers from the same datasets.

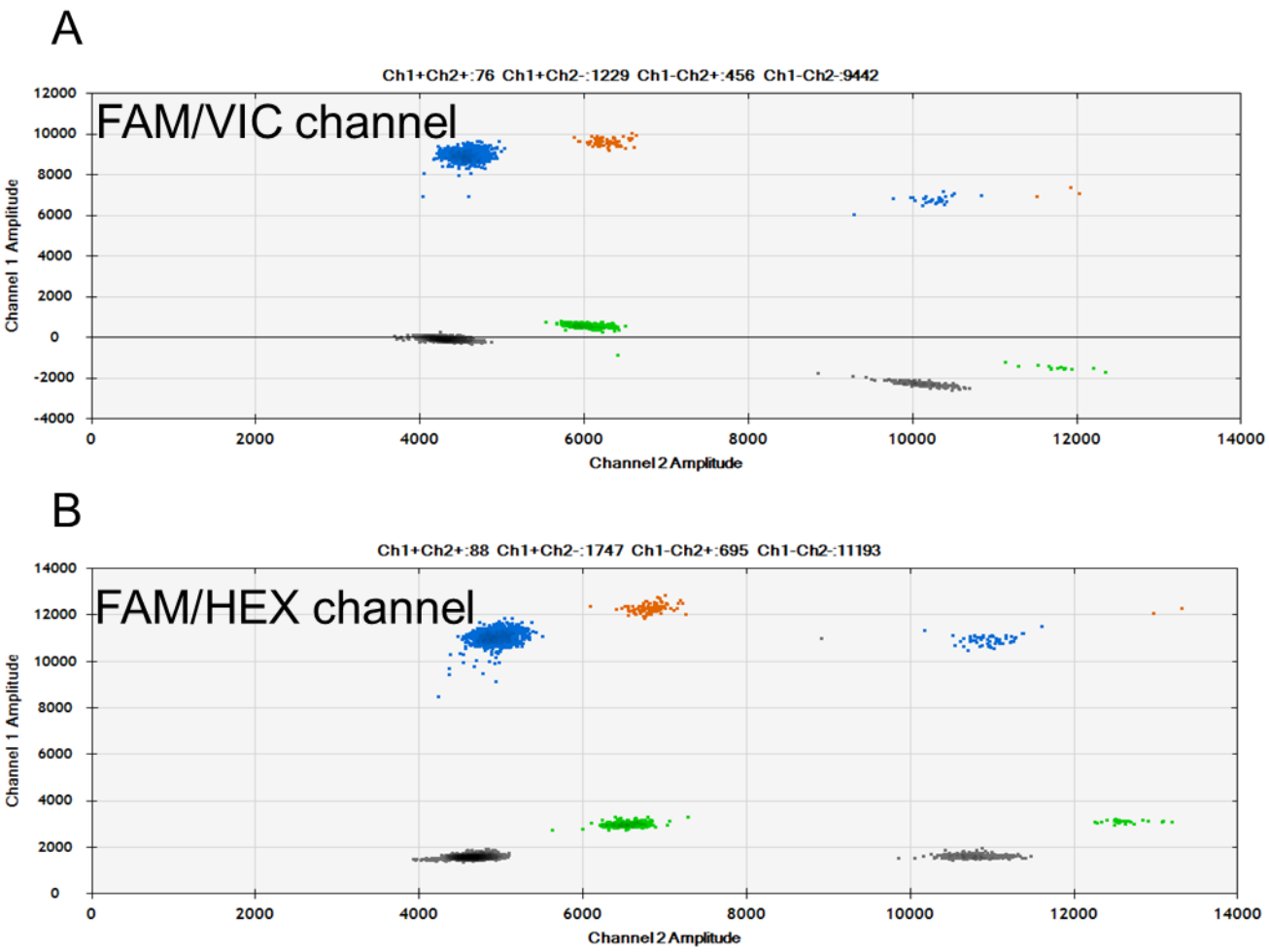


Figure 12. 2D plots of ddPCR data showing fluorescence from channel 1 (*MYC*) plotted against fluorescence from channel 2 (reference gene) for each droplet using the (A) FAM/VIC channel or (B) FAM/HEX channel. Comparable results are generated when ddPCR multiplex reactions read using either the HEX or VIC channels.

All further analyses were performed in the FAM/HEX channel as the HEX fluorophore was more abundant in the reaction mix, and real-time quantitative PCR confirmed the reaction efficiency for each probe to be highly comparable (within 10%) (figure 13A). ddPCR testing was initially carried out in a development set of 73 cancer cell lines, 18 of which were oesophageal cell lines with matched available in-

house array comparative genomic hybridisation (aCGH) data. Where ddPCR was successful and copy number data were available, *MYC* ddPCR ratio was plotted against log2 *MYC* copy number data from https://portals.broadinstitute.org/ccle (figure 13B). This analysis demonstrated a strong correlation (r=0.7991) between *MYC* ddPCR ratio and *MYC* copy number, suggesting that our ddPCR assay was able to correctly identify cell lines harbouring *MYC* amplification. Given that our predominant interest is in OG histology, we considered the oesophageal tumour cell lines separately and grouped the data according to *MYC* amplification status. Despite limited numbers of these cell lines, we observed a trend towards distinguishing between these groups (p=0.0732, Mann-Whitney U; figure 13C).

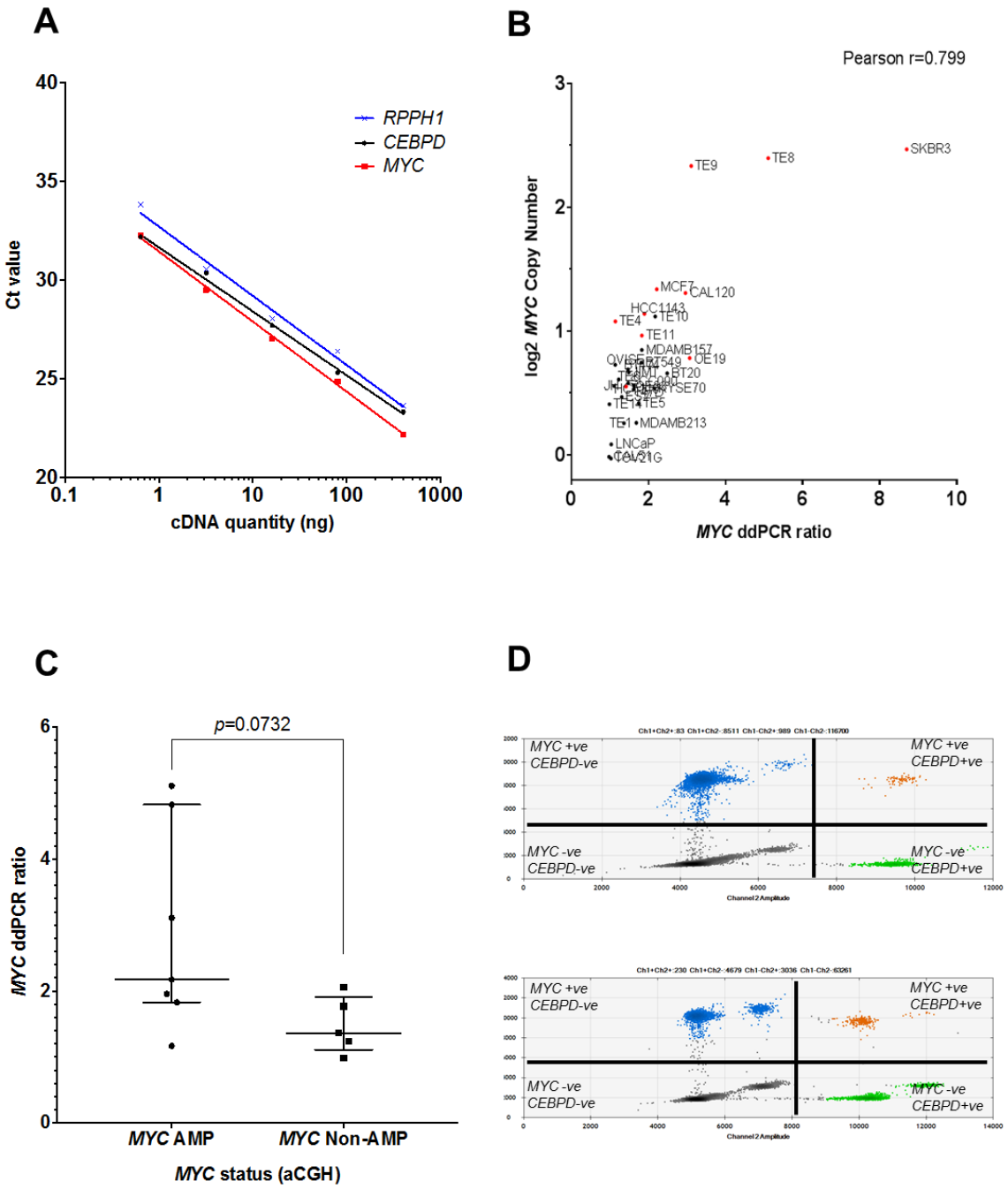


Figure 13. Development of ddPCR assay; (A) Real-Time quantitative PCR demonstrates equal amplification from all three probes used in the ddPCR assay with the slope and reaction efficiency for each standard curve similar, indicative of unbiased amplification; (B) Correlation between cell line *MYC* copy number (CN) as determined by CCLE and *MYC* copy number as determine by ddPCR. Cell lines with known *MYC* amplifications are highlighted in red (Pearson r coefficient= 0.7991; two-tailed $p<0.0001$); (C) aCGH data used to classify oesophageal cell lines

into either amplified (AMP) or non-amplified (NON AMP) groups (p=0.0732, Mann Whitney U); (D) 2D plot of ddPCR data showing fluorescence from channel 1 (*MYC*) plotted against fluorescence from channel 2 (reference gene) for each droplet, with the drops clustering into groups as shown. Data are displayed for a *MYC* amplified ctDNA sample (ratio= 11.96; upper plot) and *MYC* non-amplified ctDNA sample (ratio= 1.34; lower plot).

2.5.3 Digital PCR evaluation of iMYC trial samples

We then analysed patient samples retrieved as part of the i*MYC* study, and examples of the raw data generated are illustrated in figure 13D. As of October 2018, 105 archival FFPE tumour samples have undergone DNA extraction. Of these, ddPCR analysis was successful in 98 (93%) (figure 14).

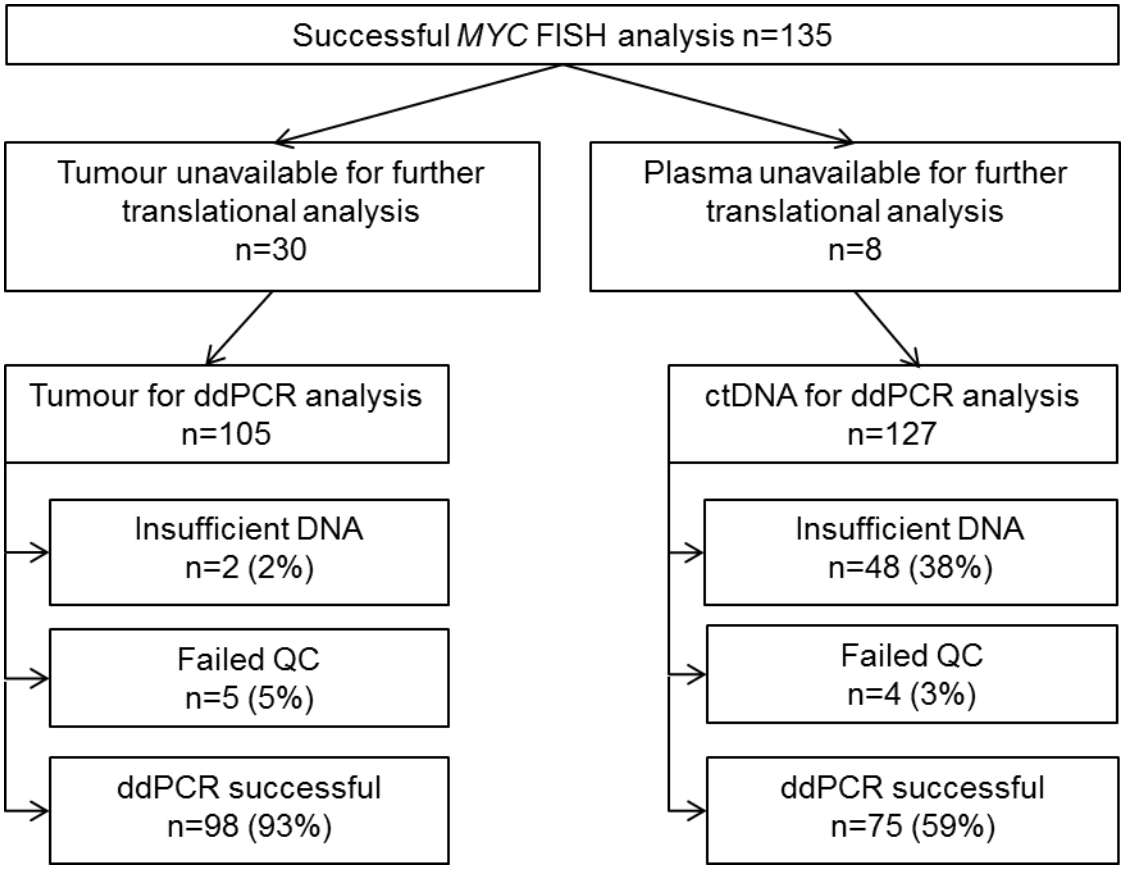


Figure 14. *MYC* amplification assessment by ddPCR in patient samples from the i*MYC* trial.

When I compared *MYC* amplification status assessed by FISH and ddPCR, I observed that the median tumour ddPCR ratio was higher than for non-amplified samples in the *MYC* amplified OG tumours (1.417 vs 1.246; *p*=0.017 Mann-Whitney U; figure 15A). However, the ddPCR ratios detected in OG tumours were lower than expected compared with cell line data (figure 13C), highlighting the difference between cell line and primary samples. Considering that significant heterogeneity of *MYC* amplification patterns as detected by FISH had been noted in this patient cohort, I hypothesised that ddPCR analysis would have greater sensitivity in more highly-amplified cases. To address this, I undertook a tertile analysis based on the proportion of amplified cells within the sample. Consistent with the hypothesis, I observed no significant difference in ddPCR ratio between amplified samples containing greater or less than 35% *MYC* amplified cells (1.432 vs 1.387; *p*=0.3111 Mann-Whitney U; figure 15B). In contrast, for samples containing greater or less than 70% amplified cells, the ddPCR ratio was found to be significantly higher in these more amplified samples (2.512 vs 1.396; *p*=0.0008 Mann-Whitney U; figure 15C), with a receiver operator area under the curve of 0.8958 (95% CI 0.7026-1.089; *p*=0.0015). Using a ddPCR cut-off ratio of 2.0 resulted in a sensitivity of 87.5% and specificity of 100% in identifying these highly (>70% cells) amplified cases.

ddPCR analysis using ctDNA was only possible in 75/127 (59%) of plasma samples, as the remainder had insufficient DNA for analysis (figure 14). I observed no difference in plasma ddPCR ratios between *MYC* amplified and non-amplified tumours (1.547 vs 1.450; *p*=0.5705 Mann-Whitney U), and the percentage of cells harbouring *MYC* amplification in the primary tumour did not influence the ctDNA

ddPCR ratio. Taken together, my analyses reveal that ddPCR is most accurate in detecting *MYC* amplification in tumour-derived DNA from OG cases with a high proportion of amplified cells in the tumour specimen while it was not reliable in samples containing a low proportion of amplified cells or in ctDNA extracted from this patient cohort.

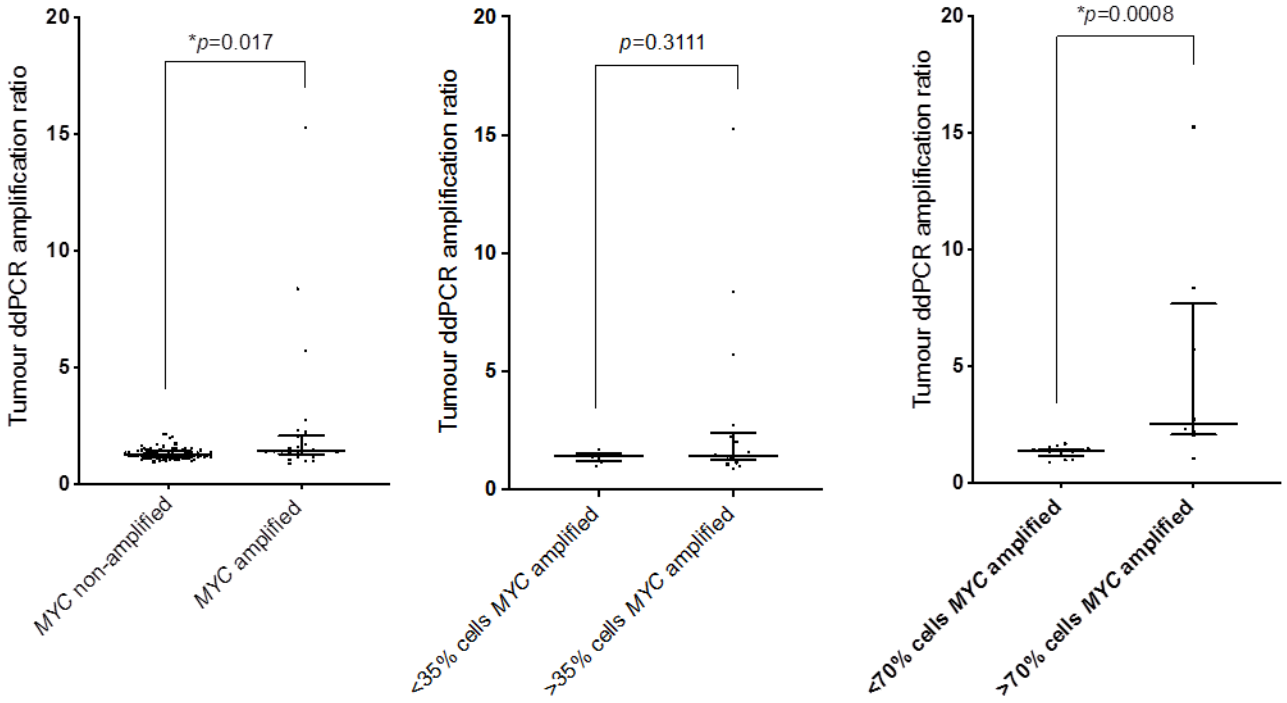


Figure 15. Relationship between tumour ddPCR ratio and *MYC* amplification status by FISH. Box and whisker plots showing (A) a significant difference in tumour ddPCR ratio between *MYC* amplified and non-amplified samples assessed by FISH (p=0.017, Mann Whitney U); (B) no difference in tumour ddPCR ratio between *MYC* amplified samples with< 35% and >35% amplified cells, respectively (p=0.3111, Mann Whitney U); (C) a significant difference in tumour ddPCR ratio between *MYC* amplified samples with <70% and >70% amplified cells, respectively (p=0.008 Mann Whitney U).

2.6 Discussion

MYC alterations are among the most common genetic changes in human cancer and great efforts are being made to develop effective *MYC*- targeting treatments. This trial is the first time *MYC* amplification has been prospectively assessed by a diagnostic FISH assay in this tumour type for the purposes of therapeutic targeting. My results illustrate the utility of cytogenetic analysis by FISH to assess *MYC* amplification prospectively for the purposes of a biomarker-selected trial by providing reliable and reproducible results in real-time. I found OG tumour *MYC* amplification assessed by FISH to be a relatively common event, occurring in 33% of SCC and 21% of AC tumours respectively. This is consistent with *MYC* amplification frequencies reported in a recent analysis of oesophageal adenocarcinoma and SCC undertaken by the Cancer Genome Atlas Research Network [89]. A high degree of heterogeneity of *MYC* amplification was observed, with a wide range of both percentage of amplified cells and amplification ratios seen within these trial specimens. Heterogeneity of *MYC* amplification has previously been described in this tumour type, with results from a retrospective FISH analysis of 109 gastric cancer specimens reporting a *MYC* amplification rate of 25%, of which 85% of amplified samples showed evidence of spatial heterogeneity of expression based on tissue microarrays, markedly higher than the 47% of *HER2* amplified samples which demonstrated heterogeneity [114]. Intra-tumoural heterogeneity in OG cancer, comprising both spatial heterogeneity in different tumour areas and temporal heterogeneity along progression from primary to recurrent or advanced disease, is increasingly recognised [115], and heterogeneity of expression of *HER2* and *FGFR* genes have been associated with differential responses to targeted therapies in gastric cancer [116, 117]. Whether the heterogeneity of *MYC* amplification will

impact upon the efficacy of its targeting within our current trial remains to be seen. However, this will be an important consideration in future efforts to target *MYC* from a clinical perspective.

In our current study, a trend was observed towards improved response to first line platinum/ fluoropyrimidine-based chemotherapy in *MYC*-amplified tumours, consistent with previously described associations between *MYC* amplification and favourable chemotherapy response in breast and ovarian cancer [118, 119]. Although *MYC* status did not influence survival outcomes overall, an association with improved survival was seen in cases with a higher variance in amplification ranges within the OG tumour specimen. *MYC* amplification has been associated with a tumour-hypoxic molecular signature indicating increased underlying genomic instability [120]. Genomic instability has also been correlated with improved outcomes to platinum-based chemotherapy [121] and it is possible that clonal diversity of *MYC* amplification may be a surrogate for this. However further work is necessary to clarify the role of *MYC* in mediating platinum response in this disease, and validation of our findings in a larger independent dataset will be required.

Given the heterogeneity of *MYC* amplification seen, identifying amplifications from pooled DNA as compared to single cell based-analysis such as FISH is likely to be challenging. Our novel ddPCR assay was developed based upon robust identification of suitable reference genes. Although a statistically significant difference in tumour ddPCR ratio was seen between *MYC* amplified and non-amplified tumours assessed by FISH, the absolute difference was small. However, this ddPCR platform was able to identify highly amplified tumour samples (those

containing >70% *MYC* amplified cells). Thus the ability of the ddPCR assay to detect *MYC* amplifications may be limited to those tumour samples displaying a homogenous high-level pattern of *MYC* amplification, and is not optimal in detecting small clonal subpopulations of amplified cells in primary OG tumour specimens. Its application to ctDNA was limited by the relatively small numbers of samples where adequate DNA could be extracted and successfully analysed. The high failure rate encountered is likely to be due, in part, to the nature of the i*MYC* pre-screening cohort, in that patients had bloods taken at the time of screening which could be before, during or after a line of systemic treatment. ctDNA has been posited as a marker of response to both cytotoxic and targeted treatments, with absolute reductions in fraction of ctDNA observed during treatment [122]. Thus, the yield of extracted ctDNA from plasma was potentially influenced by the clinical context of the patient at the time point when the blood was taken. In addition, the accuracy of ctDNA ddPCR in detecting amplifications in other genes such as *HER2* is known to be lower than tumour ddPCR when comparing with FISH assessment of primary tumour samples [108], and the increased heterogeneity of *MYC* as compared to *HER2* [123] could further contribute to the lower level of concordance seen here. Furthermore, *MYC* amplification has been associated with both aneuploidy and increased intra-tumoural heterogeneity in other solid tumours, and may be an acquired event in tumour evolution from primary to metastatic disease, thus potentially effecting equivalence of results between primary tumour specimens and ctDNA from blood samples taken at different times of disease progression [124, 125].

As research into effective *MYC*-targeting treatment continues, robust and reproducible methods of biomarker detection will be necessary. The clinical *MYC* FISH assay used in this study has revealed a high degree of heterogeneity of amplification in primary OG cancer. Whilst I show that ddPCR can also be used to detect *MYC* amplifications in tumour samples with a high proportion of amplified cells, further work is necessary to optimise this technique in ctDNA.

3 i*MYC* trial enrolment, treatment and safety monitoring

3.1 Trial overview

For the purposes of entry into the main component of the i*MYC* trial at least 4 of the first 9 recruited patients must demonstrate *MYC* amplification and the remaining 5 show either *MYC* or *HER2* amplification, or co-amplification of both. Patients are treated with ibrutinib monotherapy until progression or unacceptable toxicity. A schematic overview of the trial is shown in figure 16. As of September 2019, 182 patients have undergone screening tumour analysis, and 8 patients have been treated on the main component of the study.

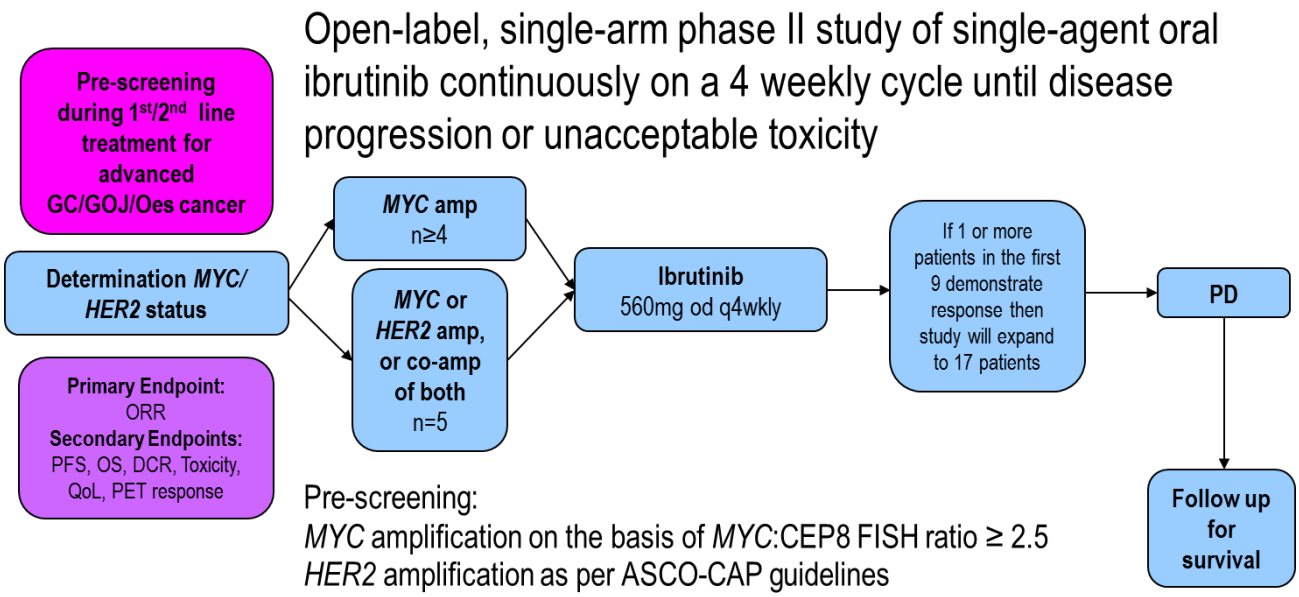


Figure 16. Schematic overview of the i*MYC* trial. Patients with a diagnosis of locally advanced inoperable or metastatic oesophageal or gastric cancer are eligible for screening, whereby archival tumour tissue is assessed for presence of *MYC* and *HER2* amplification. Potentially eligible who have failed at least one prior line of systemic therapy can be considered for the main component of the study, consisting of single-agent ibrutinib treatment until progression or unacceptable toxicity.

There are a number of translational endpoints incorporated within the trial. A mandatory baseline biopsy, as well as optional on-treatment and progression biopsies are collected to examine molecular pathways altered by ibrutinib in correlation with clinical response, including high throughput RNA sequencing to assess changes in *MYC* target genes. Fresh biopsy specimens will be considered for ex-vivo propagation to generate immortalized cells to undergo functional genomic and drug screening. Blood sampling for translational testing including ctDNA analysis and circulating tumour cell enumeration is taken at baseline, cycle 1 day 15, cycle 3 day 1 and then every 8 weeks until cessation of treatment.

3.2 Investigational medicinal product: Ibrutinib

Ibrutinib (PCI 32765) is a first-in-class oral small molecule inhibitor of BTK which has European Medicines Agency (EMA) approval for the treatment of adult patients with refractory mantle cell lymphoma, chronic lymphocytic leukaemia and Waldenström's macroglobulinaemia [126].

BTK plays an key role in B-cell trafficking, chemotaxis, and adhesion via signalling through B-cell surface and chemokine receptors [127]. It is essential for migration of B-cells into lymphoid tissues, and its inhibition results in the shifting of malignant cells into peripheral blood [128]. In addition to its effects on BTK, further mechanisms have been identified which may contribute to ibrutinib anti-cancer effect in solid organ tumour indications: (1) direct anti-tumour effect via inhibition of both *EGFR* and *HER2* [102, 129]; (2) changes in the tumour microenvironment via modulation of mast cell and macrophage function [130] and (3) changes in immune cytokine profiles through interleukin-2-inducible kinase (ITK) inhibition, resulting in the skewing of Th1/Th2 polarity [131]. Thus the putative mechanisms of action for ibrutinib in solid organ tumours are a complex combination of direct anti-tumour effect and both micro-environmental and immune modulation.

Prior to our study, early translational data had identified BTK expression in gastric cancer, and noted that BTK-inhibition with ibrutinib in cell line and mouse models had direct cytoxic and chemo-sensitizing effects [132]. A commercial phase 2 trial combining ibrutinib with the chemotherapy agent docetaxel in the second line treatment of advanced gastric and junctional adenocarcinomas opened subsequent to our study (*A Phase 1b/2 Study of Ibrutinib Combination Therapy in Selected*

Advanced Gastrointestinal And Genitourinary Tumors; EudraCT Number: 2015-003656-40).

Safety warnings of note for ibrutinib treatment include bleeding events incorporating both minor haemorrhagic events such as contusion, epistaxis, and petechiae as well as major events including gastrointestinal bleeding, intracranial haemorrhage and haematuria; as well as infection risk, cytopaenias, interstitial lung disease and atrial fibrillation [133].

3.3 Patient selection

The study population of i*MYC* consists of patients aged 18 years or older with locally advanced, unresectable or metastatic oesophagogastric carcinoma showing *MYC* and/or *HER2* amplification, who have an ECOG performance status of 0-2; adequate hepatic, renal and haematologic function; and who have been treated with one or more prior chemotherapies for advanced metastatic disease.

Key inclusion and exclusion criteria are as follows:

Key inclusion criteria:

- Histologically proven metastatic or locally advanced inoperable squamous or adeno carcinoma of the oesophagus, stomach or oesophago-gastric junction.

- Documented progression after at least 1 prior line of chemotherapy for advanced disease. For *HER2* positive tumours documented progression after at least 1 line of chemotherapy with or without *HER2* directed therapy. Patients who have progression of disease at any point during neoadjuvant/ adjuvant chemotherapy or definitive

chemoradiotherapy, or <6 months after the last dose of neoadjuvant/adjuvant chemotherapy or definitive chemoradiotherapy may be enrolled.

- *MYC* or *HER2* gene amplification

- At least one measurable target lesion, as per RECIST criteria 1.1

Key exclusion criteria:

- Clinically significant cardiovascular disease such as uncontrolled or symptomatic arrhythmias, congestive heart failure, or myocardial infarction within 6 months of screening

- ECG abrnormalities considered by the investigator to be clinically significant, or repeated baseline prolongation of the rate-corrected QT interval (QTc).

- Actively bleeding oesophagogastric tumours.

- History of stroke or intracranial haemorrhage within 6 months prior to enrolment.

- Known brain metastases.

- Prior or current therapeutic anticoagulant treatment with low-molecular weight heparin, vitamin k antagonists or oral direct factor Xa inhibitors.

3.4 Endpoints and statistical considerations

The primary objective of the study is to assess the activity of ibrutinib in *MYC* and/or *HER2* amplified advanced oesophagogastric cancers. Secondary objectives include assessment of the safety and tolerability of ibrutinib in patients with oesophagogastric cancers.

This is assessed through a primary endpoint of objective response rate (ORR) in patients with *MYC* and *HER2* amplified advanced pre-treated oesophagogastric carcinomas treated with ibrutinib. Secondary endpoints include PFS, OS, patient reported outcome of health related quality of life (HRQoL), and correlation of metabolic changes on FDG-PET with changes in gene expression, circulating tumour cells and structural changes on CT scanning (figure 17).

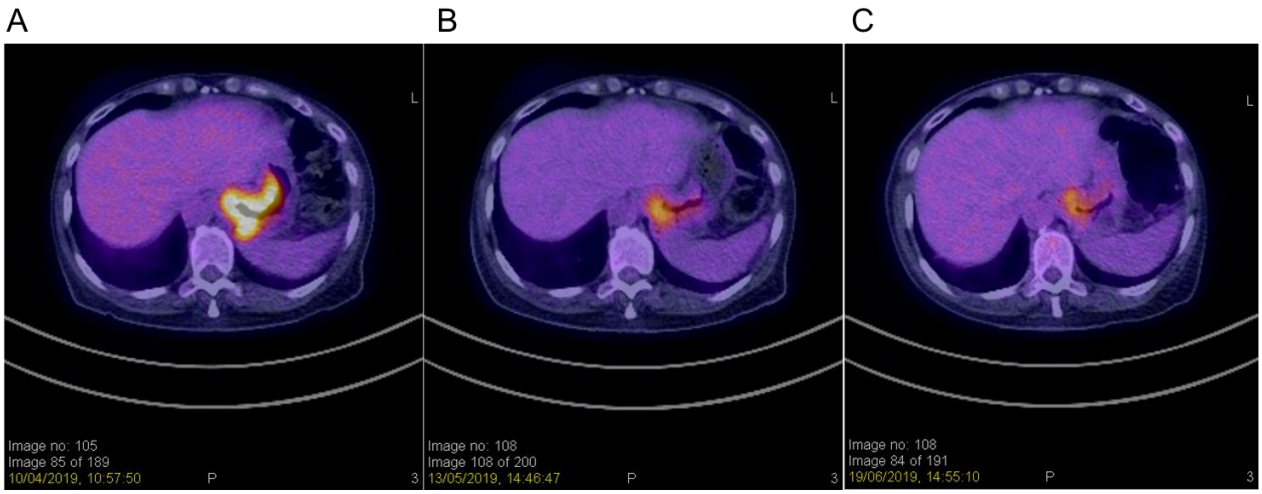


Figure 17. Images of sequential PET-CT scanning of i*MYC* patient at protocol-mandated timepoints of (A) screening, (B) day 14 and (C) week 8 ibrutinib treatment.

ORR is based upon best response seen and an interim analysis will take place once the first 9 patients have been on treatment for a minimum of 8 weeks in order to decide on expansion to 17 patients in total. Assuming ibrutinib is active with a true underlying response rate of >20%, the chance of seeing no responses in a cohort of 17 patients would be low (approximately 3%, calculated based on binomial probabilities). A Simon two-stage design is incorporated for the primary endpoint as follows: if 1 or more patients in the first 9 demonstrate a response then the study may expand to 17 patients. If no patient in the first 9 demonstrates a response then study closure will be considered. If 3 or more patients out of 17 demonstrate a response then further research is indicated. This gives 80% power with an alpha of 5% to detect a 25% response rate whilst ruling out a 5% activity rate.

3.5 Study conduct and emergent safety concerns

3.5.1 Upper gastrointestinal bleeding episodes observed on study

During the initial treatment phase of the study an emergent safety issue was identified regarding an apparent increased incidence of upper gastrointestinal (UGI) bleeding risk for treated patients, which impacted upon the ongoing conduct of the trial. Out of the first 5 patients treated on the study we observed 3 episodes of UGI bleeding, 2 of which resulted in death. It is relevant to consider the clinical circumstances of these cases as it influenced my ongoing management of the trial:

Patient 1 had a diagnosis of HER2 positive metastatic OGJ adenocarcinoma, previously treated with chemotherapy plus trastuzumab. The patient had been commenced on therapeutic dalteparin (low molecular weight heparin, LMWH) anticoagulation for deep vein thrombosis (DVT) and pulmonary embolism (PE) during first line chemotherapy, and was on this anti-coagulation treatment at the time of trial commencement. After 8 days of ibrutinib treatment the patient noted increased oozing from dalteparin injection sites. One week later the patient was admitted to their local hospital with a single episode of small volume haematemesis. UGI endoscopy showed bleeding from primary tumour and Ibrutinib was discontinued. The patient subsequently received urgent palliative radiotherapy after which treatment they recommenced on a prophylactic dose of dalteparin and proceeded through the remainder of his treatment uneventfully.

Patient 2 had a diagnosis of HER2 positive metastatic poorly differentiated adenosquamous carcinoma of the distal oesophagus previously treated with first line

ECX and second line paclitaxel chemotherapy. This patient had an oesophageal stent in situ prior to commencement on trial and was also on dalteparin for a PE. During their first cycle of ibrutinib they attended local services for new leg swelling, and ultrasound (USS) confirmed new DVT. The dalteparin dose was increased, and the patient was then readmiited to their local hospital with significant UGI bleeding and anaemia. An endoscopy showed significant ingrowth of tumour over stent, with oozing blood loss. Both ibrutinib and dalteparin treatment were held and the patient was transfused 7 units of blood over the course of their inpatient stay. They commenced palliative radiotherapy, although shortly after the first of five planned treatments experienced a further episode of profound UGI bleeding leading to death as an inpatient.

Patient 3 had a diagnosis of MYC amplified locally advanced inoperable oesophageal SCC previously treated with CX chemotherapy and concomitant radical radiotherapy with susequent disease progression. There was no history of prior stenting or previous anticoagulation therapy. On cycle 5 day 18 of ibrutinib the patient was admitted to their local hospital with haematemesis and found to be anaemic. Ibrutinib was stopped prior to an endoscopy which showed no active bleeding but a structuring, impassable oesophageal lesion. A repeat endoscopy with oesophageal stent insertion was undertaken. The patient then had a further admission with a lower respiratory tract infection. Shortly after discharge patient collapsed at home and died with evidence of UGI blood loss. The last dose of ibrutinib had been 5 weeks previously.

During this time two further patients had been recruited and proceeded through trial treatment uneventfully. The latter two bleeding episodes happened in close proximity to one another.

3.5.2 Actions taken in response to safety issues

I initially discussed the episodes with the chair of the RM Trust Committee of Clinical Research (CCR) and convened an urgent meeting of the Trial Management Group (TMG). The discussion within the TMG considered bleeding risk directly attributable to ibrutinib as well as other possible relevant factors in the above cases, particularly concomitant administration of low molecular weight heparin in two cases and instrumentation in the form of stent placement in two cases. Immediately following this meeting I instituted an urgent protocol amendment to halt recruitment to the study. The Medicines and Healthcare Regulatory Authority (MHRA) was informed and a detailed report was sent to them.

Following this an urgent meeting of the trial Independent Data Review Committee (IDMC) was set up. In anticipation of this I liaised with the Ibrutinib development team at Janssen regarding provision of safety data from recently completed or ongoing trials that may have been of relevance to the discussion. A number of large phase 2/3 trials had been evaluating the use of ibrutinib in solid organ indications concurrent to our study. We were particularly interested in two ongoing studies of ibrutinib for GI cancer indications (the RESOLVE and PCYC1128 trials), and requested an update from the manufacturer with regards any adverse safety signals noted. The RESOLVE trial is a phase 3 randomised trial of ibrutinib 560mg or placebo with gemcitabine/ nab-paclitaxel in first line advanced pancreatic

adenocarcinoma [134]. Of 424 randomised patients no unexpected safety signal with regard to bleeding was reported. The previously mentioned PCYC1128 trial is an ongoing phase 1/2 study of ibrutinib in combination with standard chemotherapies in renal, urothelial, colorectal and gastric/OGJ adenocarcinoma. We noted that 11 patients to date at that time had been treated with ibrutinib 560mg in conjunction with docetaxel for second line gastric/ OGJ adenocarcinoma. Similarly, no unexpected safety signal with regard to bleeding was reported for any of the four tumour cohorts in this trial. During the IDMC meeting the clinical and treatment details of all patients enrolled within i*MYC* were considered in detail. The known published risk profile and safety signal from relevant ongoing trials of ibrutinib were highlighted.

The summary of product characteristics for ibrutinib notes an increased incidence of haemorrhagic events, including both minor and, rarely, major events [133]. It also states warfarin or other vitamin-K antagonists should not be administered concomitantly with ibrutinib. Gastrointestinal bleeding is not mentioned specifically, and the reference safety information specifically lists intracranial haemorrhage only. At the outset of the trial the drug manufacturers Janssen advised on safety and recommended that concomitant vitamin-K antagonists were excluded. Other anti–platelet and anticoagulants were allowed in-keeping with the standard safety recommendations for the drug.

After reviewing the above the IDMC made the following recommendations:

- Any patient with a history of prior or current anticoagulation treatment with low-molecular weight heparin, vitamin-K antagonist or equivalent should be excluded from the trial

- For patients with primary tumour in-situ, previous oesophageal stenting procedures should be excluded from the trial

- For patients on trial where an oesophageal stenting procedure is deemed necessary, ibrutinib should be discontinued a minimum 7 days pre- procedure

The above changes were incorporated into a substantial amendment to the trial, which subsequently reopened to recruitment in September 2017.

3.6 Discussion

My involvement in i*MYC* trial has provided real-world experience in the set-up and oversight of a clinical trial in OG cancer. I managed the set-up process from protocol development through to ethical and regulatory submission. This incorporated a substantial amount of multi-disciplinary team working, particularly with regard to the development and implementation of a novel FISH biomarker screening test. Throughout this process I maintained regular contact with our commercial partner Janssen to provide relevant clinical and logistical updates. Once screening and recruitment were under way a further challenge arose in that a number of episodes of bleeding were observed on the trial. Although bleeding risk had been described with ibrutinib, this was not classed as a major safety concern within the licensed haematological indications where the majority of existing clinical safety data was drawn from. A rapid escalation through the appropriate trial management structures from the TMG, local CCR and, ultimately, IDMC was required. After this comprehensive review a protocol amendment was instituted. Undertaking this process provided further experience of trial oversight and, more importantly, the practical measures required in order to ensure ongoing patient safety during the conduct of a clinical trial.

4 Somatic copy number alteration in OG cancer

4.1 Introduction

The most common subtype of gastric cancer as described by the TCGA analysis is the CIN subtype, characterised by chromosomal instability, aneuploidy and, in many cases, focal amplification of receptor tyrosine kinases [20]. The genomes of these cancers harbour multiple DNA somatic copy number alterations (SCNAs), defined as deviations in the number of whole chromosomes, chromosome arms or fragments from the normal number of two copies per cell. With the exception of *p53* mutations which occur in 70-80% of OG adenocarcinomas of the CIN subtype, mutations in cancer driver genes are relatively rare in these cancers and SCNAs are considered the predominant type of genetic driver alterations [20, 89]. Common SCNAs identified in CIN tumours in these landmark sequencing studies include amplifications of chromosomal regions harbouring genes encoding for receptor tyrosine kinases or their ligands such as *HER2*, *EGFR* and *VEGFA*; as well as those involved pathways regulating proliferation (*MYC*), and cell cycle (*CCNE1*, *CCND1* and *CDK6*). These SCNAs have been implicated as key and, in the case of *HER2*, clinically actionable drivers in OGA [135, 136].

The CIN subtype is common among gastric cancers arising proximally from the OGJ or cardia [20] and in oesophageal adenocarcinomas [89]. The ‘genomically stable’ subtype is characterised by few SCNAs and associated with the diffuse histological subtype of gastric cancer that commonly arises more distally from the stomach body [20]. The incidence of non-cardia gastric adenocarcinomas is declining in Western populations, whilst that of junctional and oesophageal tumours is increasing [137].

These tumours are predominantly of the CIN subtype, and thus detection of SCNAs, in particular the clinically and biologically relevant driver events within these complex profiles, are important for the ongoing development of new biomarkers and therapies.

4.2 SCNA analysis by low coverage whole genome sequencing

SCNAs have traditionally been analysed through microarray-based techniques, although more recently improved sensitivity for SCNA detection has been achieved through exome or whole genome sequencing (WGS). However due to cost, long turnaround times and intensive bioinformatics analysis requirements, such large scale genomics analyses are often not feasible. Low coverage WGS (lcWGS), using a coverage of only 0.1- 0.5x (i.e. where only 10-50% of the genome is sequenced), has been shown to be sufficient for reliable detection of SCNAs, with recent data showing superior SCNA calling compared to older array hybridisation-based standards [138]. Crucially, lcWGS can also be applied to analyse tumour-derived cfDNA extracted from the plasma of cancer patients [139]. Such liquid biopsies offer clear practical advantages over conventional biopsies, including the minimally invasive nature of sample acquisition, relative ease of standardisation of sampling protocols, and the ability to obtain repeated samples over time. The latter is of particular interest, as changes in SCNA profiles over the course of treatment may shed light on response and resistance mechanisms to existing chemotherapy agents as well as to novel targeted agents and immunotherapies.

Intratumour heterogeneity is recognised as a major challenge in the delivery of effective molecular targeted treatment in OGA [115, 140]. Copy number variation of

molecular targets, as assessed in both tumour and ctDNA, has been shown to impact on therapeutic targeting of *HER2*, *FGFR* and *EGFR,* with high level amplifications being associated with more favourable responses [117, 141, 142]. Application of targeted genomic sequencing to ctDNA analysis has been shown to allow the detection of mutations which are more heterogeneous within OGA [143, 144]. Such liquid biopsy techniques may also facilitate tracking of genetic profile changes over time, but this has not been applied to OGAs undergoing systemic therapy as yet. IchorCNA is a validated bioinformatics analytical approach to lcWGS data which can be used to quantify tumour fraction from cfDNA without prior knowledge of specific variants present in the primary tumour sample [145]. This allows for an estimation of absolute tumour-derived content in a sample and, when applied to sequential samples, an evaluation of changes in circulating tumour content over time.

I applied lcWGS to ctDNA from 30 patients with advanced OGA to investigate whether SCNA analysis can predict responses to first-line chemotherapy, and how these profiles may evolve during chemotherapy treatment, both in terms of absolute tumour content and potential changes in genetic profiles. This was an application of a relatively novel technique of circulating DNA analysis which had not been applied in this context before, however the small sample size meant that any results would be hypothesis-generating only, and would require further prospective exploration in larger patient sample sets.

4.3 Methods

4.3.1 Design and sample collection

The FOrMAT (Feasibility of a Molecular Characterisation Approach to Treatment) study enrolled patients with advanced GI malignancies treated at the Royal Marsden from February 2014 to November 2015 [146]. The primary endpoint of the trial involved targeted capture-based DNA sequencing using archival FFPE diagnostic or resection specimens in order to detect mutations, SCNAs and translocations in up to 46 genes which had prognostic or predictive significance, or were potential targets in existing or upcoming clinical trials (table 5). As part of the translational tissue collection component of the trial blood samples were also taken for enrolled patients at trial entry and at the timepoint of response assessment CT scans during treatment. The trial recruited 71 advanced OG cancer patients in total. I interrogated FOrMAT clinical trial databases to identify suitable patients with a diagnosis of advanced OG adenocarcinoma who had undergone baseline research blood sampling prior to the start of first-line chemotherapy for advanced disease, and whom had sequential bloods spanning at least one full course of comparable first-line systemic chemotherapy, consisting of a platinum/fluoropyrimidine doublet in all cases, plus or minus anthracyline or, in the case of HER2 positive tumours, trastuzumab.

Mutations						
AKT	*APC*	*ARID1A*	*ATM*	*BRAF*	*CDK4*	*CDKN2A/B*
CTNNB1	*DOCK2*	*EGFR*	*ELMO1*	*ERBB2/4*	*FBXW7*	*HRAS*
IDH1/2	*JAK3*	*KIT*	*KRAS*	*MAP2K1/2*	*NOTCH1/2/3*	*NRAS*
PDGFRA	*PIK3CA*	*PTEN*	*RET*	*ROS1*	*SMAD4*	*TCF7L2*
TP53	*UGT1A1*	*VHL*				
Copy number variations						
ALK	*CCND1*	*CDK4/6*	*EGFR*	*ERBB2*	*FGFR2*	*IGF1/2*
KRAS	*MET*	*MST1R*	*PIK3CA*	*TRIM44*		
Translocations						
ALK	*FGFR2*	*RET*	*ROS1*			

Table 5. Genes included in the FOrMAT targeted capture panel.

4.3.2 DNA extraction and quantification

Plasma was separated within 2 hours of blood draw and frozen at -80C. I used QIAamp Circulating Nucleic Acid Kit (Qiagen) to isolate cfDNA from 3 to 4 ml plasma according as per manufacturer's instructions and as described in section *2.4.1.* ctDNA is naturally fragmented with a predominant fragment size of approximately 160bp, thought to correspond to the length of DNA wrapped around one nucleosome or multiples thereof [147]. cfDNA within a size range of 100 to 700bp was quantified

using a Bioanalyzer High Sensitivity chip (Agilent), encompassing the predominant three cfDNA fragment peaks (figure 18).

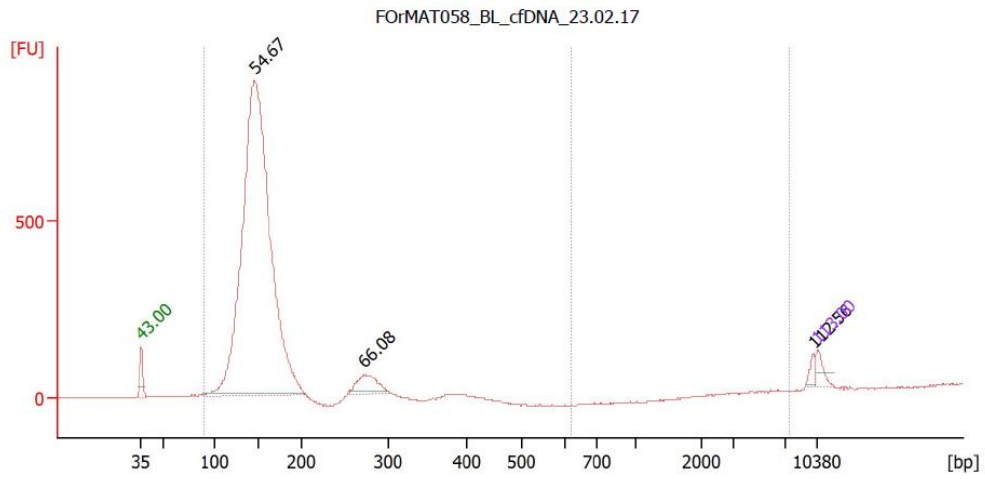


Figure 18. Example of a cfDNA sample Bioanalyzer (Agilent) profile showing relative DNA quantity plotted against DNA fragment size (bp). cfDNA was quantified across a window from 100bp to 700bp.

4.3.3 Low coverage whole genome sequencing

For the majority of cases 10ng of input DNA was used for sequencing, although it has been reported that lower quantities can be successfully used for analysis, and in some cases of limited yield 5ng was used [148]. Libraries were prepared using the NEBNext Ultra DNA Library Prep kit, pooled and sequenced using an Illumina HiSeq2500, a benchtop high-throughput sequencing instrument. The method is based on the sequencing technique described by Baslan et al for lcWGS of DNA from single cells, adapted for analysis of ctDNA [149].

4.3.4 Somatic copy number alteration analysis

The central principle of SCNA analysis is based on the presumption that the sequenced DNA molecules will be a random sample of the entire genome. Thus approximately the same number of sequencing reads should align to different segments of the genome provided these segments have the same length. If a specific segment of DNA has been duplicated within cancer-derived DNA and is present at a higher copy number than the remainder of the genome, an excess of reads would be found to align to this genome segment. Conversely if a DNA segment has been lost by the cancer, fewer than expected sequencing reads would be aligned that that genomic segment (figure 19).

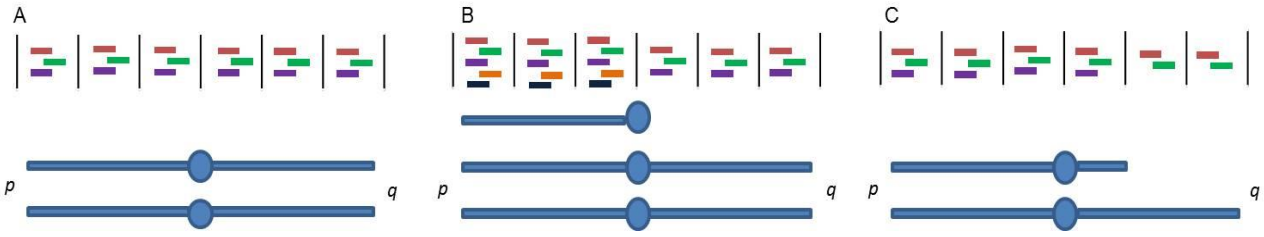


Figure 19. (A) genomic segments containing equal numbers of reads; (B) chromosomal gain resulting in excess of aligned reads; (C) chromosomal loss resulting in fewer than expected reads.

By computing the local read density relative to the expected read density it is possible to infer somatic copy number gains and losses across cancer genomes. The method applied to this data automatically splits the genome into non-overlapping segments (bins), to each of which the same number of sequencing reads is expected to align. The number of reads aligned to each bin is further normalized for guanine-cytosine (GC) content and the tumour log ratio (where values above 0 indicate a

copy number gain and below 0 a copy number loss) is computed by comparing against the mean log ratio per bin across nine gender-matched healthy donors. The resultant 'z-score' represents the observed versus expected ratio of reads per bin normalised by healthy donor. These normalised z-scores are segmented in order to combine adjacent genomic bins that have a similar copy number, and then median centred in order to convert read numbers into a comparable output. Sequencing reads were aligned to the human reference genome (hg19) using Bowtie (v1.2.9) [150] and resultant bam files were deduplicated using Picard *MarkDuplicates* (http://picard.sourceforge.net; v.2.1.0). Reads were subsequently assigned to non-overlapping 500 kb bins and normalized to correct for GC-content and mappability bias using the HMMcopy suite (http://compbio.bccrc.ca/software/hmmcopy/) [151].

A challenge in prior ctDNA sequencing efforts has been quantification of the true tumour-derived DNA fraction within the sample. Previous approaches have often focused on targeted detection of somatic single nucleotide variants (SSNVs) in recurrently mutated cancer genes identified from prior primary tumour analysis [152]. LcWGS of circulating DNA presents analytical challenges due to the low coverage of sequencing performed, absence of matched normal germline DNA and low tumour content of many cfDNA samples [153]. IchorCNA is an analytical approach used to quantify tumour fraction in cfDNA without prior knowledge of SSNV or SCNAs present in the primary tumour sample [145]. IchorCNA segmented data was normalised using the best-fit tumour content and ploidy solution in order to compare samples from the same individual taken at different timepoints. To compare multiple samples, data was uniformally segmented using interpolate.pcf, part of the copynumber package in R (http://bioconductor.org/packages/copynumber/)[154].

Seg files were viewed as a heat map using the Integrated Genome Viewer software (Broad Institute; v.2.3.97), allowing comparison of genomic SCNA profiles across multiple samples with the ability to zoom in to areas of interest in order to investigate genes located within this genomic region [155]. Focal SCNAs were identified by assigning mapped reads to 50kb bins using the method described by Baslan [149]. SCNAs were assessed in IGV by two independent observers and recorded for all patients.

4.4 Results

4.4.1 Clinical characteristics and DNA yield

cfDNA was extracted from 3-4 mL of plasma from each of 30 patients with advanced OGA prior to starting first line palliative chemotherapy, as per criteria stipulated above, with clinical and pathological characteristics summarized in table 6.

Histopathological variable		
Number of Cases:		30
Anatomic site of primary:	Gastric	6 (20%)
	OGJ/ oesophageal	24 (80%)
Histological subtype:	Intestinal	28 (93%)
	Diffuse	2 (7%)
Clinical stage at presentation:	Locally advanced	3 (10%)
	Metastatic	27 (90%)
HER2 status*:	Positive	6 (20%)
	Negative	24 (80%)
First line chemotherapy:	Platinum/fluoropyrimidine doublet	9 (30%)
	Doublet + anthracycline	15 (50%)
	Doublet + trastuzumab	6 (20%)
Metastatic sites: Liver	Yes	16 (53%)
	No	14 (47%)
Peritoneal	Yes	6 (20%)
	No	24 (80%)
Lung	Yes	8 (27%)
	No	22 (73%)
Number of metastatic organ sites:	0 - 1	22 (73%)
	≥ 2	8 (27%)
Primary tumour in situ:	Yes	23 (77%)
	No	7 (23%)
CA19-9 secretor:	Yes	15 (50%)
	No	15 (50%)

*defined as HER2 IHC +++ on baseline diagnostic specimen from patient clinical records; OGJ-oesophagogastric junction

Table 6: Clinical characteristics of included patients.

From each of the 30 cases, a median of 8.88 ng of cfDNA was extracted per ml of plasma (25th percentile: 5.36ng per ml; 75th percentile: 19.72ng per ml) with a minimum of 1.37ng per ml and maximum of 74.04ng per ml (figure 20). A higher cfDNA yield was seen in the 23 cases where the primary tumour was in-situ, as compared to the 7 cases which had undergone surgical resection (9.66 vs 4.81ng per ml; table 7). cfDNA yield was also higher in patients with liver metastatic disease (10.09 vs 6.6ng/ml). No other clinical or pathological parameters appeared to be associated with measured pre-treatment cfDNA concentration.

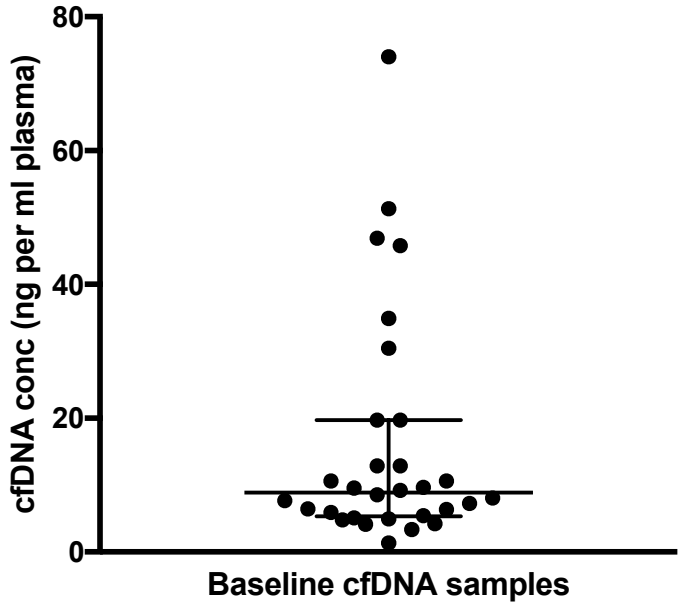


Figure 20. Baseline cfDNA yields (ng per ml plasma) obtained from 30 patients with metastatic OG cancer.

Histopathological variable		N	Median cfDNA concentration (ng/mL plasma)	Median ctDNA fraction (%)	Median ctDNA concentration (ng/mL plasma)
Primary tumour in situ	Yes	23	9.66	9.10	2.14
	No	7	4.81	0.00	0.00
Liver metastases present	Yes	16	10.09	18.01	2.18
	No	14	6.80	7.23	0.35
Primary tumour anatomic site	Gastric	6	8.65	3.33	0.24
	Non-gastric	24	9.05	9.31	0.84
No. of metastatic organ sites	0-1	22	8.31	7.77	0.47
	≥2	8	1.22	14.47	0.58
HER2 status	Positive	6	11.22	8.81	2.25
	Negative	24	8.32	8.22	0.47
CA19-9 secretion	Yes	15	9.21	8.10	0.61
	No	15	8.54	9.02	0.78

Table 7. Correlation of cfDNA concentration, median ichorCNA ctDNA fraction and ctDNA concentration with clinical and laboratory variables (*p*-values Mann-Whitney U).

The ichorCNA bioinformatics package [145] was used to reconstruct copy number profiles from sequencing data and to estimate the fraction of cfDNA derived from tumour cells (henceforth denoted as circulating *tumour* (ct)DNA fraction). Based on ichorCNA analysis, 7/30 cases (23.3%) had ctDNA fraction of zero, leaving 23 cases (76.7%) in which SCNA analysis could be performed. The seven cases with zero tumour content included all three tumours that were only locally advanced rather than metastatic in this cohort (Cases 2, 152, 195). The other four (57.1%) cases with zero tumour fraction had metastatic disease involving only a single organ site (cases 52, 66, 119, 144). The ctDNA fraction showed a poor correlation with the total cfDNA concentration in the plasma (Pearson correlation r^2=0.2312), suggesting that the release of ctDNA from tumour cells and the total amount of cfDNA, which is a mix of DNA from malignant and non-malignant cells, are largely independent from each other.

The presence of the primary tumour in situ (9.1% vs 0% median ctDNA fraction), of liver metastases (18.0% vs 7.2%), of oesophageal and junctional as compared to gastric primary tumour (9.3% vs 3.3%), and number of metastatic sites involved (0-1 vs ≥2; 7.8% vs 14.5%) all resulted in higher ctDNA fraction (table 7). Taken together, copy number profiles could be analysed from ctDNA in 76.7% of cases and within this data set there appeared to be some association of clinical characteristics with ctDNA yield, although numbers were too small within these subgroups to meaningfully assess significance. Cases of liver metastases showed the highest baseline tumour fraction of 18%.

I next investigated whether any baseline ctDNA metrics correlate with OS by splitting into three equal sized groups. Neither the total cfDNA concentration extracted from plasma (figure 21A), nor the ctDNA fraction estimated by ichorCNA (figure 21B) correlated with OS. However, the absolute ctDNA concentration in the plasma, calculated by multiplying the total cfDNA concentration with the ichorCNA ctDNA fraction, revealed a significant OS difference (figure 21C). The third of patients with the lowest absolute ctDNA concentration (mean 0.09 ng/mL) had a median OS of 19.5 months whereas those with intermediate (mean 0.92 ng/mL) and high (mean 10.12 ng/mL) absolute ctDNA concentration had a median OS of 11.3 and 12.8 months, respectively.

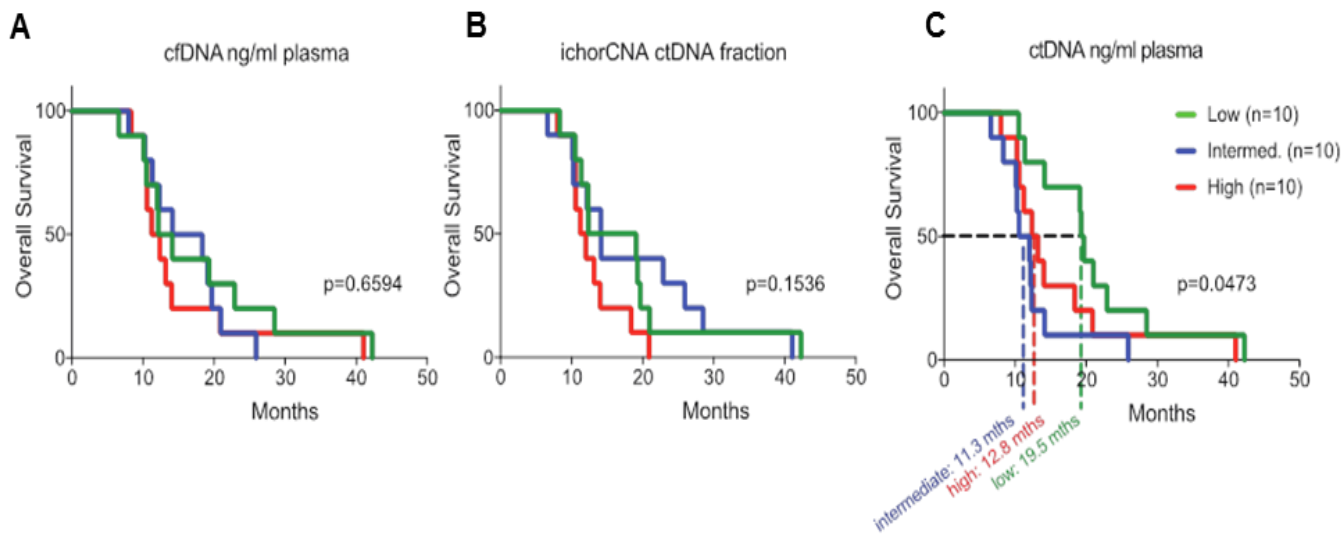


Figure 21 (A) Kaplan-Meier survival analyses of pre-treatment samples grouping by high/intermediate/low cfDNA yield ng/mL plasma, (B) ichorCNA ctDNA fraction, and (C) ctDNA concentration ng/mL plasma (*p*-values Log-rank (Mantel-Cox) test).

4.4.2 Correlation of overall copy number profile and chromosomal instability metrics with treatment response

We next used the ichorCNA-derived ctDNA fraction and ploidy estimates to convert SCNA data into integer copy number profiles to investigate whether any specific copy number aberrations or chromosomal instability metrics correlated with subsequent responses to chemotherapy (figure 22A-B). The frequency of copy number gains or losses in 13 responders (based on best radiological response assessment with serial CT scans during treatment; figure 22C) was compared to those in 10 non-responders who had stable or progressive disease as best response (figure 22D). Frequency plots showed an overall similar appearance in both groups. Several chromosomes showed alterations that were unique to the responder group (figure 22E) and not present in the non-responder group (figure 22F). Gains of chromosomes 2q and 8p were the most frequent (>1/3 of cases) unique aberrations observed only among responders (figure 22E).

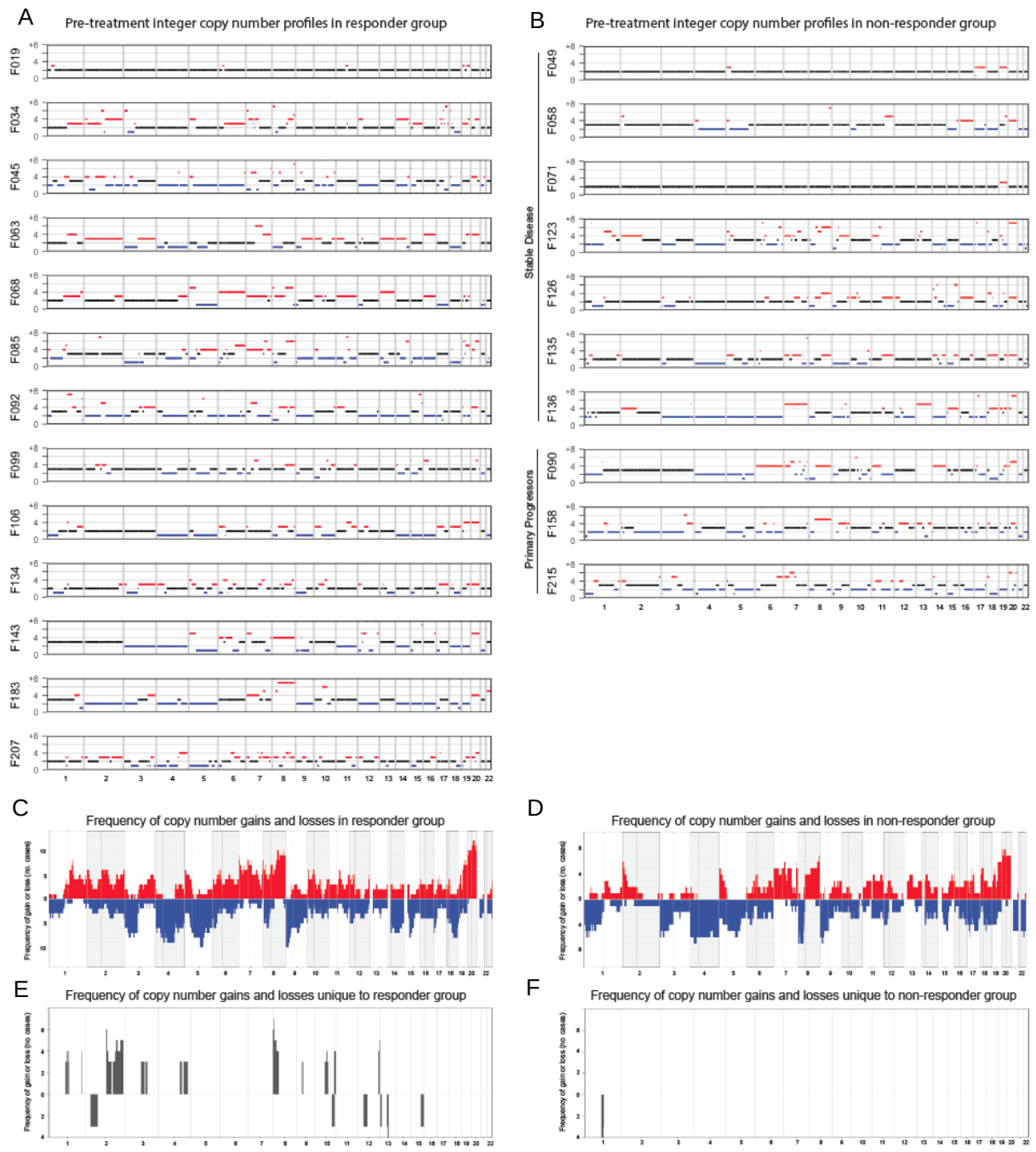


Figure 22 (A) Integer copy number profiles (500 kb bins) for pre-treatment samples, grouped by subsequent response or (B) non-response to treatment. Red= gain, Blue= loss, Black= ploidy. (C) Frequency plots showing the number of cases that show segment gains (red) or losses (blue) in the responder and (D) non-responder groups. (E) Frequency plots showing segment gains and losses that are unique to the responder group or (F) non-responder group.

A minimal consistent region of 28 Mb on Chr2q encompassing 182 genes was observed in 5/13 responders (34, 63, 68, 134 and 207). These 2q gains were, in four cases, a single copy number gain relative to ploidy. A 7.5 Mb minimal consistent region on Chr8p encompassing 17 genes (table 8) was detected in six cases (34, 45, 68, 99, 143, 183), four of which as multiple copies above ploidy. Of the uniquely gained genes, MCPH1 (microcephalin) is notable as a key regulator of DNA damage response and a repressor of human telomerase reverse transcriptase function [156], and gains of MCPH1 have been implicated in increased platinum sensitivity in non-small cell lung cancer (figure 24) [157]. Chr8p also harbours GATA4 which is frequently gained or amplified in OGA [89, 158], but this was located outside the unique region as gains of GATA4 were observed in both responders and non-responders (figure 23). Other uniquely altered regions were less frequent and hence difficult to assess (figure 22E). In contrast, only a single loss of a 12 Mb minimal consistent region encompassing 117 genes on Chr1p in four cases (123, 126, 90 and 158) was unique to the non-responder group (figure 22F). None of these changes were seen consistently across all responder or non-responder groups, and the small numbers of cases do not allow for any inference of clinical significance in terms of potential predictors of treatment response.

CSMD1	*ERI1*	*LINC00599*
LOC100287015	*MIR4660*	*MIR124-1*
MCPH1	*PPP1R3B*	*MSRA*
ANGPT2	*LOC157273*	*PRSS55*
CLDN23	*TNKS*	*RP1L1*
MFHAS1	*MIR597*	

Table 8. Genes in frequently gained region of chromosome 8p in responders

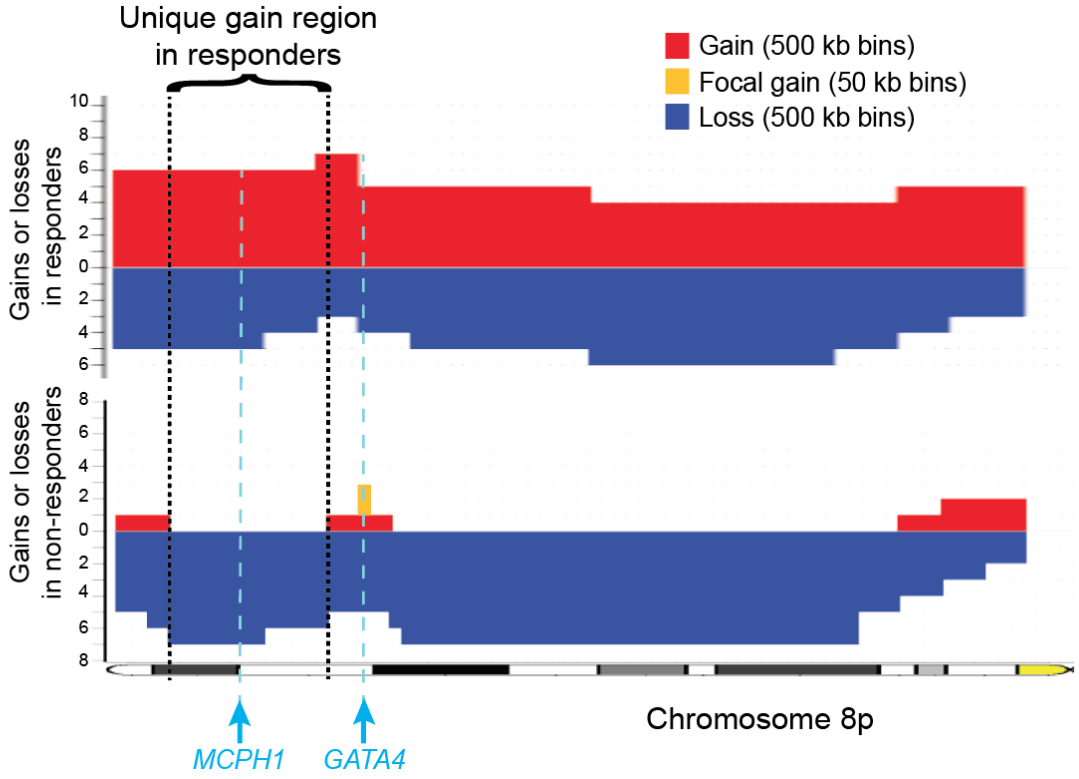


Figure 23. Frequency of gain (red) and loss (blue) segments of chromosome 8p in the responder group (top) and non-responder group (bottom). The most frequent region of unique 8p gain is indicated, bounded by dotted lines. The locations of *MCPH1* and *GATA4* are delineated with a dashed line. Two additional non-responder cases showed focal amplifications (orange) of *GATA4,* which were identifiedwith the 50 kb bin method but not the 500 kb ichorCNA analysis.

Chromosomal instability (CIN) has been associated with poor survival outcomes in a number of solid tumour cancer types, although its role in treatment response and tumour evolution is complex [159, 160]. We went on to assess whether CIN-metrics including the weighted genomic instability index (wGII) [161, 162] (figure 24A), the number of gained or lost chromosomal segments (figure 25B) or ploidy (figure 24C) associated with responses or could predict survival in our cohort. None of these metrics showed a significant difference in responders vs non-responders or an association with progression-free (figure 24D-F) or overall survival (figure 24G-I). Taken together, the presence of Chr2q and 8p gains in pre-treatment ctDNA showed an association with chemotherapy responses. In contrast, we could not identify a role of CIN metrics to predict patient outcomes in this cohort.

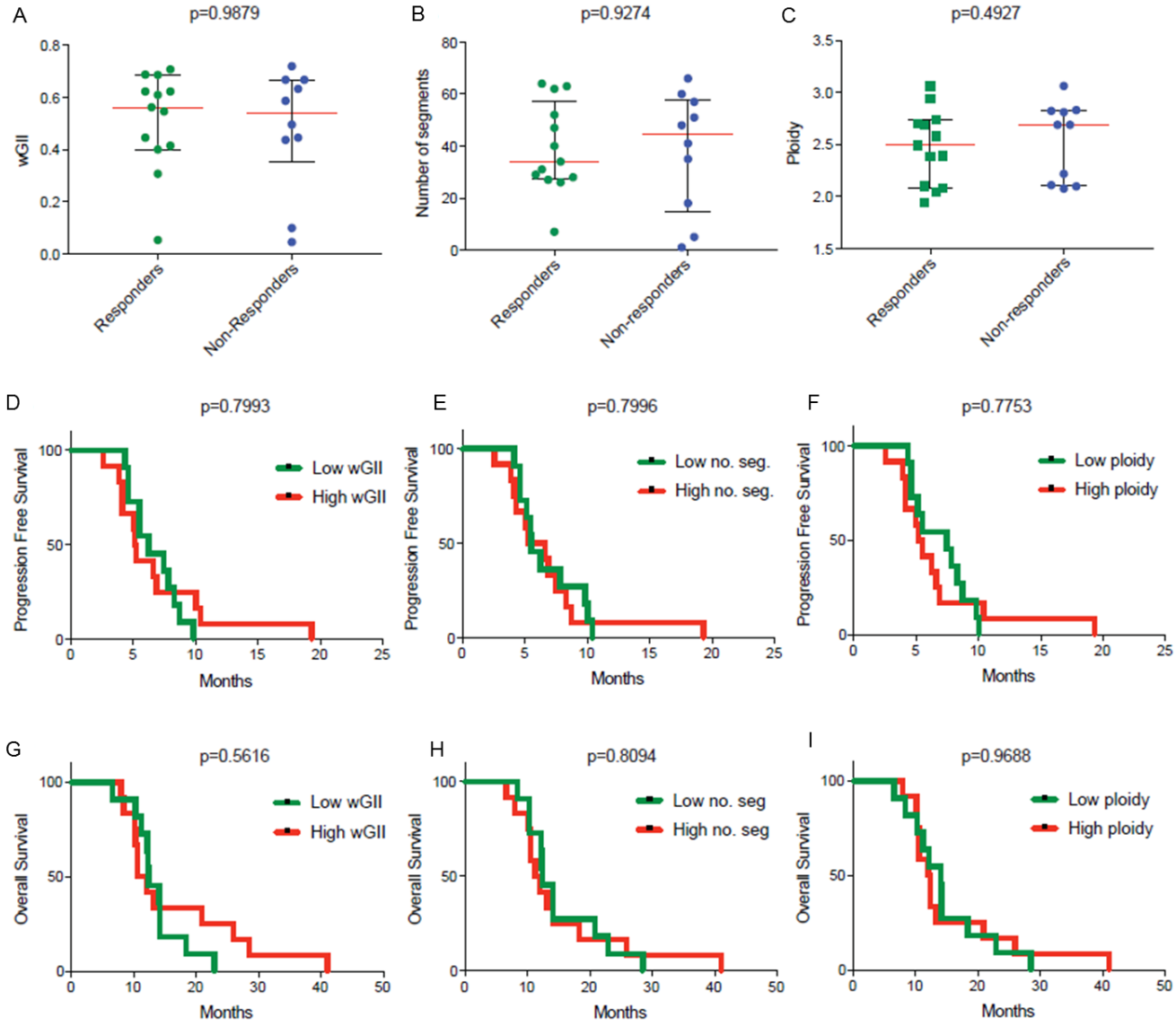


Figure 24. Association of pre-treatment CIN metrics with subsequent treatment response by comparing analysis of genomic change relative to ploidy using (A) weighted Genomic Instability index (wGII), (B) non-ploidy segment number, and (C) ploidy between responder and non-responder groups (line denotes median and interquartile range; *p*-value Mann Whitney U). (D) Kaplan Meier progression free survival analyses grouping by high/low wGII, (E) non-ploidy segment number and (F) ploidy. (G) Kaplan Meier overall survival analyses grouping by high/low wGII, (H) non-ploidy segment number and (I) ploidy.

4.4.3 Presence of focal SCNAs, correlation with treatment response, and comparison with prior targeted sequencing results

The ichorCNA analysis divides chromosomes into 500kb large bins in order to assess the copy number state of these segments. Focal genomic amplifications are often narrow (down to a few dozen kbps) [163] and may have been overlooked as a consequence. Therefore, to further interrogate whether focal amplifications could be detected in the lcWGS data, we applied a 50kbp bin approach [149]. This revealed narrow high-level amplifications of several OGA driver genes (figure 25) [20, 89]. Any of the high level amplifications (*EGFR, HER2, KRAS, MET, MYC, MAPK1/ERK2, CCND1* and *GATA4*) that were observed in two or more cases were detected in both responders and in non-responders. Several others were only observed once and were hence too rare to draw any conclusions. Thus, high-level amplifications detected at pre-treatment did not associate with chemotherapy responses in this cohort.

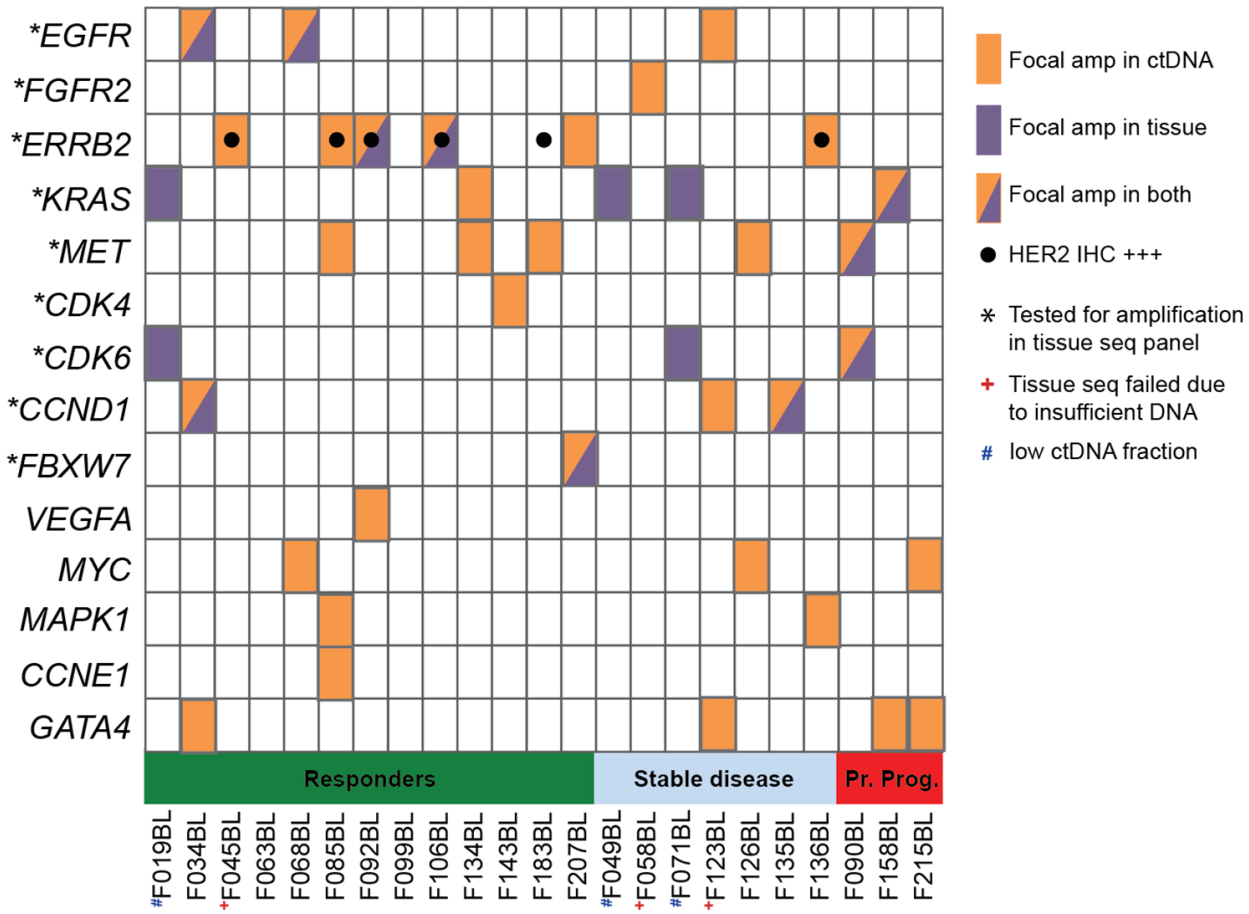


Figure 25. Heatmap showing focal gene amplifications (50 kb bins) detected by ctDNA lcWGS at pre-treatment (orange) or by archival target sequencing (purple) in each case. Black dots indicate cases classed as HER2 positive by immunohistochemistry. Green= responder group, blue= stable group, red= primary progressor group.

As part of the FOrMAT clinical trial, archival FFPE diagnostic or resection samples were sequenced with a custom panel targeting 46 genes that had prognostic or predictive significance, or were potential targets in existing or upcoming clinical trials (table 5) [146]. Amplifications of *EGFR*, *CCND1*, *CDK6*, *MET*, *HER2*, *KRAS*, and *FBXW7* had been identified in tissue samples from 11 cases (19, 34, 49, 68, 71, 90, 92, 106, 135, 158, 207). No amplifications were observed in nine cases and archival target sequencing failed in three cases (45, 58, 123). ctDNA lcWGS of pre-treatment

plasma re-identified all of the gene amplifications found by archival tumour sequencing in eight cases (figure 25). Compared to tissue sequencing, ctDNA analysis could not detect *CDK6* and/or *KRAS* amplifications in three cases that had low ctDNA content (Case 19: 9.1%; Case 49: 7.3%; Case 71: 8.1%). Importantly, in seven cases, ctDNA lcWGS identified additional amplifications of genes that were included in the FOrMAT sequencing panel but for which no amplification was detected in the archival tissue analysis: Case 85 (*MET* and *ERBB2* amplification in plasma), Case 126 (*MET*), Case 134 (*MET, KRAS*), Case 136 (*ERBB2*), Case 143 (*CDK4*) and Case 183 (*MET*), Case 207 (*ERBB2*). In addition, ctDNA sequencing identified 11 amplifications (in nine cases) of genes that were not covered by the FOrMAT panel, including *GATA4*, *VEGFA* and *MYC*.

Of six cases (45, 71, 85, 92, 106 and 136) that had been classified as HER2 positive based on standard IHC testing of archival baseline tissue, ctDNA sequencing detected *HER2* amplifications in five cases. Archival tissue sequencing had identified *HER2* amplifications in only two of five successfully sequenced cases (figure 26). In one case (71) IHC analysis of archival tissue had identified HER2 positivity, but no amplification was detected by either archival tissue sequencing or ctDNA lcWGS. Three of the *HER2* amplified cases (85, 92, 136) had concurrent amplifications in *MAPK1*, *MET*, or *VEGFA* in the cfDNA (figure 25).

MYC amplifications were seen in 3/23 (13%) of cases within this cohort using lcWGS of ctDNA. As reported in chapter 2, my application of FISH analysis to primary OG adenocarcinoma tumour samples within the i*MYC* screening dataset resulted in a detected *MYC* amplification rate of 24%. However when applying ddPCR

assessment to ctDNA samples within this dataset *MYC* amplification appeared to be a less common event. As described, no established cut off for ddPCR evaluation of *MYC* amplification exists, but when applying a commonly-used ddPCR cut-off ratio of 2.0 only 7/75 (9%) of analysed plasma samples were suggestive of ctDNA *MYC* amplification. Thus my results from both whole genome and single-gene analysis approaches suggest that *MYC* amplification in ctDNA is a potentially rarer event than tumour amplification. This may be as a result of the recognised intratumoural heterogeneity seen in OG cancer [115] and discussed in chapter 2, and the implications for treatment and prognosis for this small group of patients who display high-level *MYC* amplification within circulating DNA remains unknown.

4.4.4 Evolution of SCNA profiles on treatment

To assess the evolution of SCNA through treatment, lcWGS was applied to ctDNA collected at the time of radiological progression during or after first line platinum/ fluoropyrimidine based combination chemotherapy from 20 patients that had detectable ctDNA pre-treatment profiles and had a post-treatment sample available. Twelve of these had an initial radiological response with subsequent disease progression (primary responders). Eight showed stable disease or primary progression during chemotherapy (primary non-responders). There was no change in ichor CNA ctDNA fraction between baseline and progression for all matched samples (table 9; figure 26A). In the primary responder group, the ichorCNA ctDNA fraction at progression was significantly lower than pre-treatment (17% vs 7.6%; p=0.02; table 9; figure 26B) whereas no significant change was observed in the primary non-responder group (table 9; figure 26C). Only three out of twenty samples

taken at progression had a ctDNA content of zero (Cases 68, 99, 183), showing that ctDNA remains detectable in the majority of tumours.

		N	Median ctDNA fraction (%)	*p*-value
All paired cases	Pre-treatment	20	15.18	0.1567
	Progression	20	8.72	
Initial radiological response followed by progression to chemotherapy: 'primary responders'	Pre-treatment	12	17.00	0.0200
	Progression	12	7.59	
Stable disease or primary radiological progression to chemotherapy: 'primary non-responders'	Pre-treatment	8	11.27	0.7984
	Progression	8	13.58	

Table 9. Comparison of ichorCNA estimated ctDNA fraction at pre-treatment and progression of first line chemotherapy (*p*-values Mann-Whitney U).

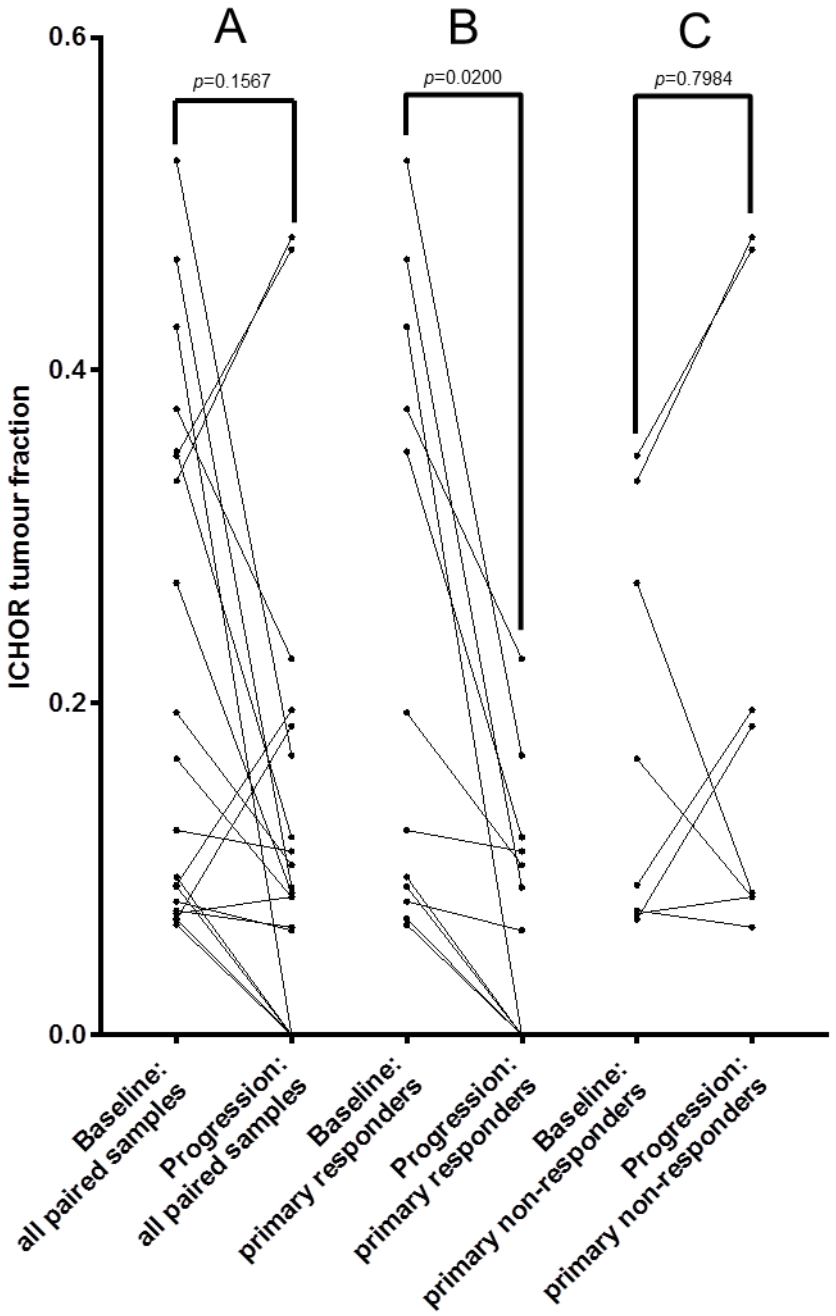


Figure 26 (A) Change in IchorCNA ctDNA tumour fraction between all matched baseline and progression samples; (B) between primary responder group and (C) between primary non-responder group (*p*-values Mann-Whitney U).

The copy number profiles of the remaining 17 cases (figure 27A-B) were assessed for changes over the course of chemotherapy treatment (figure 27C). Using the 50kb

bin approach, all focal amplifications present before treatment were re-identified at progression, with no new focal amplifications identified at progression.

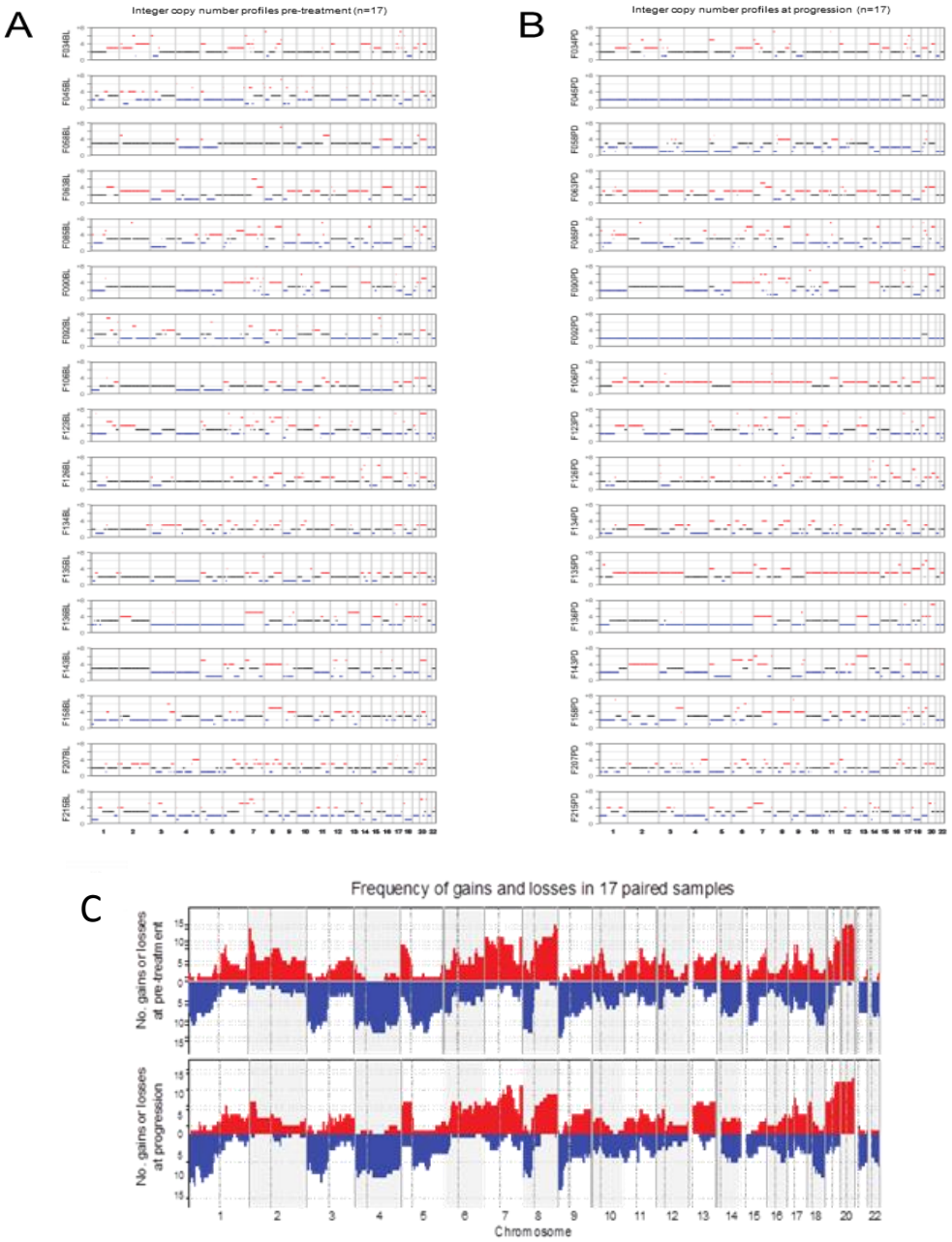


Figure 27. Integer copy number profiles for the 17 paired non-zero ctDNA cases at (A) baseline and (B) progression. (C) Frequency plots showing the number of cases (n=17) that show segment gains (red) or losses (blue) at pre-treatment (top) and at progression (bottom).

4.5 Discussion

Through use of liquid biopsy I successfully analysed the SCNA profiles of 23/30 (76.7%) advanced OGAs. Serial analysis before and after first line chemotherapy was feasible in 17/20 (85%) of cases that had detectable ctDNA prior to treatment. This demonstrates proof-of-concept that lcWGS of ctDNA can reveal genome wide SCNA profiles in the majority of patients with advanced OGA, for example to investigate novel prognostic or predictive biomarkers.

A number of clinical characteristics were observed that may support the selection of patients with a predictably higher ctDNA analysis success rates in future studies: the presence of liver metastases was associated with the highest baseline ctDNA content, whilst the ctDNA content was also higher if the primary tumour was in-situ. This may be the result of more aggressive tumours presenting with synchronous metastatic disease at baseline compared to those with metachronous metastases following resection. All seven cases with zero ctDNA pre-treatment either only had locally advanced disease or low metastatic burden. A more recent study has reported on clinical correlates of ctDNA content through use of a targeted NGS panel, using maximal tumour somatic variant allelic frequency (maxVAF) as a proxy to estimate ctDNA content [164]. In a cohort of 144 untreated advanced gastric cancer patients within this study, higher baseline ctDNA content was significantly associated with primary tumour in-situ and presence of liver and lung metastases [164]. The use of such clinical characteristics as biomarkers to guide selection of OG cancer patients for ctDNA sequencing analysis requires further validation, but may allow prioritisation of such patients for liquid biopsy-based genotyping over sequencing of OGA tumour tissue, which has had moderate reported success rates

due to technical challenges such as frequent low tumour content in endoscopic biopsies [146, 165]. With readily assessable clinical characteristics to identify suitable patients, ctDNA analysis could be used to assess amplifications for molecular stratification and particularly to longitudinally investigate SCNA evolution.

Neither baseline cfDNA concentration nor ctDNA fraction correlated with survival, however, a low absolute plasma ctDNA concentration was associated with better OS. Again, small numbers do not allow inference of clinical significance and larger prospective studies are needed to validate the clinical utility of circulating DNA metrics for optimisation of treatment and surveillance strategies [166].

High CIN has been linked to poorer prognosis and drug sensitivity across a range of cancer types [167, 168]. Application of several CIN metrics could not identify a correlation with chemotherapy response or survival in our cohort. This could indicate that CIN metrics may perform less well when generated from ctDNA, as this samples a summative copy number profile of the entire cancer population. Alternatively, these metrics may only weakly correlate with aggressiveness and treatment sensitivity and specific genetic aberrations, acquired as a consequence of CIN, may be more relevant in determining the response and outcome of individual tumours. Although studies of larger cohorts may be able to reveal an association in the future, my results suggest that analysis of these CIN metrics in ctDNA is unlikely to be useful to predict individual patient outcomes in unselected patients undergoing first line chemotherapy.

For patients with evaluable ctDNA, multiple SCNAs could be identified in genes that are currently clinically relevant, or may become relevant to future practice. In samples with detectable ctDNA all amplifications that had been found by previous targeted sequencing of matched FFPE tissue samples within the FOrMAT study were identified [146]. In seven cases lcWGS found additional focal amplifications in genes that had been analysed by targeted sequencing in tissue and where no amplification had been called. Furthermore, lcWGS revealed multiple additional amplifications of potentially targetable driver genes such as *VEGFA*, highlighting the advantage of whole genome approaches over predetermined targeted sequencing gene sets. Concurrent baseline amplifications of *MAPK1*, *MET*, or *VEGFA* with *HER2* were seen in 3/6 HER2 positive cases. These may potentially influence variability of outcomes to HER2 targeted therapy, as amplifications of *MET* and *MAPK1* have previously been implicated in trastuzumab resistance [169, 170], however the limited numbers in this cohort precluded meaningful survival analyses.

Comparison of pre-treatment SCNA profiles revealed gains of chromosomes 2q and 8p in cases that subsequently responded to treatment, and these gains were absent in non-responders. These could be investigated in larger cohorts to assess their potential role as predictive biomarkers. The uniquely gained region on chromosome 8p harbours the DNA damage regulator *MCPH1,* which has been suggested to increase sensitivity to platinum chemotherapy [171]. This is therefore a candidate gene for further investigation. Identifying predictive biomarkers of chemotherapy response is an unmet need, but has been challenging. To date the most extensive study of genetic predictors of therapy response using targeted sequencing of tumour

tissue in advanced OGA failed to identify any biomarkers of response to platinum based chemotherapy [165].

Both ctDNA detection and lcWGS was possible from plasma samples taken at the timepoint of progression on first line chemotherapy, with 17/20 (85%) cases having detectable ctDNA. SCNA profiles were relatively stable between the pre-treatment and progression samples. The lack of recurrent copy number change events at progression in this study may be a result of the small evaluable cohort, but it is also feasible that chemotherapy resistance may be driven by point mutations. Use of a higher resolution technique that will allow the combined analysis of SCNA and mutations (such as whole exome ctDNA sequencing) may be warranted, with patient selection based on the presence of liver metastases to maximise successful sequencing rates and cost efficiency. Longitudinal ctDNA analysis has become a favoured method to interrogate resistance mechanisms during treatment, such as the tracking of known oncogenic *RAS* mutations in colorectal cancer [172]. Additionally lcWGS has been applied to larger cohorts of lung [173] and prostate cancer [174], finding longitudinal treatment-related changes in both tumour content and SCNA profiles. Notably in the recent lung cancer study ctDNA fraction was dynamic during treatment, with increases in tumour fraction and corresponding SCNAs detected before radiographic progression. Furthermore recurrent SCNAs were enriched in post treatment ctDNA compared to baseline, suggesting potential resistance mechanisms [173]. Dynamic ctDNA testing could be equally applicable to monitor resistance to therapy in OGA.

The potential clinical application for this technique lies in the feasibility of biomarker stratification on the basis of lcWGS ctDNA sequencing, circumventing some of the limitations related to tumour heterogeneity in OGA [117]. Furthermore, sequential lcWGS of ctDNA is a low cost method for continuing to investigate genetic changes associated with chemotherapy response in larger series or for early detection of resistance mechanisms to novel agents in clinical trials. Preliminary proof of concept for the use of longitudinal ctDNA analysis in OG cancer to predict response and resistance to HER2-targeting treatment has already been described [175]: *HER2* copy number alterations detected by targeted sequencing were found to be associated with both innate and acquired trastuzumab resistance. Additionally, mutations in genes including *PIK3CA*, *HER2* and *ERBB4* were also associated with resistance, highlighting the benefit of combined mutation identification and SCNA analysis in interrogating drug resistance mechanisms. Detection of relevant gene amplifications in ctDNA has been already shown to be clinically important for patient selection and therapeutic targeting of *FGFR* in gastric cancer [117].

As novel targeted and immune-modulating therapies are introduced into clinical management of OGA, there will be a need for stratification of patients in order to guide personalised treatment. The use of genome-wide analysis techniques to interrogate key driver events and genomic evolution over time will be important in refining the effective biomarker stratification of such treatments moving forwards. Ultimately this may support precision medicine approaches in both trial and routine clinical practise settings by avoiding the cost, delay and clinical complications of repeated invasive biopsy procedures.

The results described in this analysis demonstrate proof-of-concept for the use of lcWGS for sequential dynamic biomarker investigation in OG cancer. There was considerable variability in the yields of cfDNA extracted from plasma samples taken at a pre-chemotherapy baseline timepoint. However the use of ICHOR tumour fraction analysis allowed for quantification of tumour DNA and in 23/30 cases lcWGS of circulating DNA extracted from baseline pre-treatment plasma samples resulted in an evaluable profile with measurable ctDNA content.

5 Overall discussion and conclusions

The work included in this thesis incorporates an evaluation of the current therapeutic landscape of OG cancer and the results of projects exploring biomarker-driven investigation and treatments in this disease type. The data generated by my review of current treatment patterns and outcomes presented in Chapter 1 demonstrates the unmet clinical need for more varied and efficacious treatments in this field. Beyond trastuzumab for *HER2* positive patients and ramucirumab in unselected second line populations there are no further clinically validated biomarkers or effective targeted treatments despite large scale efforts for over a decade. As with other solid organ tumours there is a growing interest in the application of immunotherapy, however their effectiveness appears limited to a subset of patients only. Again this is relevant to the field of biomarker development, as efforts are now being made to identify and stratify patients for whom these treatments will be effective. Putative predictive biomarkers to enrich for immunotherapy response include both assessment of relevant single candidate genes such as *PD-L1*, or genome-wide assessments of aggregate changes such as tumour mutational burden. The techniques described in this work are therefore relevant to the developing field of biomarker investigation in OG cancer.

Specific to the screening work detailed in chapter 2, the *MYC* gene has previously proved challenging as a therapeutic target for a number of reasons, including variability of expression and lack of amenability to direct therapeutic drug targeting. The indirect targeting approach via synthetic lethality with the BTK-mediated pathway within the i*MYC* trial is based upon robust pre-clinical rationale. Prospective screening of *MYC* is not something which had been attempted before in solid organ

cancers, and the work illustrates the challenges of biomarker identification, quantification and application in a 'real-world' patient screening program. I found a high degree of heterogeneity in the assessment of *MYC* amplification by our FISH assay. Application of a ddPCR technique to both tumour and blood-derived ctDNA showed that the technique was potentially effective in identifying *MYC* amplifications within DNA from the most highly amplified tumours only. Tumoural heterogeneity of biomarker expression is an increasingly recognised problem, with the potential to impair efforts towards effective biomarker driven treatment. As research into effective *MYC*-targeting treatment continues, robust and reproducible methods of biomarker detection will be necessary and the work presented here is an important first evaluation of this important cancer gene in solid tumour oncology.

The design, set-up and execution of a phase II trial gave me experience of the practical challenges entailed in the instigation and oversight of a clinical trial incorporating biomarker screening and targeted treatment. Some of this experience can be generalised to the research process: protocol development, ethical and regulatory approval and liaison with industry partners are transferable skills applicable to most oncology clinical research. As outlined in chapter 3, the unexpected safety events which occurred on the trial and the resultant pause in recruitment has meant that clinical data presented in this thesis is limited. However the rapid evaluation of clinical safety data and involvement of relevant regulatory authorities to ensure patient safety within the trial has provided further important clinical research experience.

An alternative method of biomarker investigation is through genome-wide sequencing approaches. Chapter 4 describes the application of lcWGS to circulating DNA extracted from sequential blood samples in advanced OG cancer patients. This approach allowed for correlation of ctDNA metrics with baseline clinical characteristics and treatment response, as well as evaluation of both genome wide and focal copy number alterations. Sequential analysis of SCNA profiles during treatment has not been performed in this tumour type and again this work points towards another future refinement for the use of 'liquid biopsy' for biomarker identification and tracking in OG cancer. The presence of liver metastases and of a primary tumour in-situ predicted for detectable SCNA profiles in baseline ctDNA: clinical characteristics which could potentially guide investigators to suitable cases in which to apply such techniques in the future. Although little change was observed in gain or loss of individual focal amplifications, genome aberration increased with time on treatment, with SCNA profiles changing substantially in the majority of patients during treatment, indicating major subclonal shifts in the tumour cell population. This warrants further investigation in larger series in order to assess potential drivers of chemotherapy resistance and the clinical relevance of clonal shifts.

As the therapeutic landscape in OG cancer changes it is expected that targeted and immunotherapeutics will become more closely integrated into clinical treatment pathways. Their successful ongoing development will require the application of molecular and genomic techniques to the identification of predictive biomarkers and tracking of drug response, as well as the evaluation of targeted treatments in early phase clinical trials. The work reported in this thesis encompasses development of a

novel biomarker-driven treatment, and describes the use of ctDNA sequencing as a promising avenue of future biomarker investigation in OG cancer.

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7 Appendix: publications, presentations and awards related to this work

7.1 Publications

Davidson M, Aronson L, Howard-Reeves J et al. Clonal diversity of *MYC* amplification evaluated by fluorescent in situ hybridisation and digital droplet polymerase chain reaction in oesophagogastric cancer: Results from a prospective clinical trial screening programme. *European Journal of Cancer* 2019; 122: 12-21

Davidson M, Barber L, Woolston A et al. Detecting and tracking circulating tumour DNA copy number profiles during first line chemotherapy in oesophagogastric adenocarcinoma. *Cancers* 2019; 11(5). pii: E736. doi: 10.3390/cancers11050736

Davidson M, Edwards P, Calamai V, Cunningham D, Starling N. Third line treatment of advanced oesophagogastric cancer: A critical review of current evidence and evolving trends. *Cancer Treatment Reviews* 2018; 71: 32-38

Davidson M, Cafferkey C, Goode E et al. Survival in advanced oesophagogastric adenocarcinoma improves with the use of multiple lines of therapy: results from an analysis of over 500 patients. *Clinical Colorectal Cancer* 2018; 17(3): 223-230

Davidson M & Chau I. Multimodality treatment of operable gastric and oesophageal adenocarcinoma: evaluating neoadjuvant, adjuvant and perioperative approaches. *Expert Review of Anticancer Therapy* 2018; 18(4): 327-338

Mansukhani S, Barber L, Moorcraft SY, Davidson M et al. Ultra-sensitive mutation detection and genome-wide DNA copy number reconstruction by error corrected circulating tumour DNA sequencing. *Clinical Chemistry* 2018; 64(11): 1626-1635

Davidson M, Chau I, Cunningham D et al. Impact of tumour histological subtype on chemotherapy outcome in advanced oesophageal cancer. *World J Gastrointest Oncol* 2017; 9(8): 333-340

Chong I, Aronson L, Bryant H, Gulati A, Campbell J, Elliott R, Pettitt S, Wilkerson P, Lambros M, Reis-Filho J, Ramessur A, Davidson M et al. Mapping genetic vulnerabilities reveals BTK as a novel therapeutic target in oesophageal cancer. *Gut* 2017; 67: 1780-1792

Davidson M & Starling N. Trastuzumab in the management of gastroesophageal cancer: patient selection and perspectives. *Oncotargets and Therapy* 2016; 16(9): 7235-7245

Davidson M & Chau I. Immunotherapy for oesophagogastric cancer. *Expert Opinion on Biological Therapy* 2016; 16(10): 1197-207

Davidson M & Chau I. Variations in outcome for advanced gastric cancer between Japanese and Western patients: a subgroup analysis of the RAINBOW trial. *Translational Gastroenterology and Hepatology* 2016; 1(46)

Davidson M, Smyth E & Cunningham D. Clinical role of ramucirumab alone or in combination with paclitaxel for gastric and gastro-oesophageal adenocarcinoma. *Oncotargets and Therapy* 2016; 25(9): 4539-48

Davidson M, Okines A & Starling N. Current and future therapies for advanced gastric cancer. *Clinical Colorectal Cancer* 2015; 14(4): 239-50

7.2 Presentations

EACR + ESMO Joint Conference on Liquid Biopsies (2019). Poster presentation: Circulating tumour DNA analysis reveals genome wide copy number profile evolution during first line chemotherapy in oesophagogastric adenocarcinoma patients

ESMO Asia (2017). Oral presentation: Clonal diversity of *MYC* amplification evaluated by FISH and digital droplet polymerase chain reaction (ddPCR) in oesophagogastric (OG) cancer
(part of ESMO Asia Congress official press release programme: https://www.esmo.org/Conferences/Past-Conferences/ESMO-Asia-2017-Congress/News/MYC-Amplification-in-Oesophagogastric-Cancer-Investigated)

ESMO (2017). Poster presentation: Survival in advanced oesophagogastric adenocarcinoma improves with the use of multiple lines of therapy: Results from an analysis of over 500 patients

ESMO World Congress on Gastrointestinal Cancer (2017). Poster discussion: Heterogeneity of *MYC* amplification in oesophagogastric carcinoma: results from a prospective screening study

ASCO Gastrointestinal Cancers Symposium (2017). Poster presentation: i*MYC*: Proof-of-concept study of ibrutinib in *MYC* and *HER2* amplified oesophagogastric carcinoma

7.3 Awards

Patricia McGregor Travel Fellowships grant to support attendance at 2019 EACR/ ESMO Liquid Biopsy Conference

ESMO travel grant: registration, travel and accommodation for 2017 ESMO Asia Congress, Singapore

7.4 Selected published manuscripts

Survival in advanced oesophagogastric adenocarcinoma improves with the use of multiple lines of therapy: results from an analysis of over 500 patients. *Clinical Colorectal Cancer* 2018; 17(3):223-230

Clonal diversity of *MYC* amplification evaluated by fluorescent in situ hybridisation and digital droplet polymerase chain reaction in oesophagogastric cancer: Results from a prospective clinical trial screening programme. *European Journal of Cancer* 2019; 122: 12-21

Detecting and tracking circulating tumour DNA copy number profiles during first line chemotherapy in oesophagogastric adenocarcinoma. *Cancers* 2019; 11(5). pii: E736. doi: 10.3390/cancers 11050736

Survival in Advanced Esophagogastric Adenocarcinoma Improves With Use of Multiple Lines of Therapy: Results From an Analysis of More Than 500 Patients

Michael Davidson, Catherine Cafferkey, Emily Frances Goode, Kyriakos Kouvelakis, Daniel Hughes, Pablo Reguera, Eleftheria Kalaitzaki, Clare Peckitt, Sheela Rao, David Watkins, Ian Chau, David Cunningham, Naureen Starling

Abstract

We report on the treatment and survival of 511 patients with advanced esophagogastric adenocarcinoma treated during a 6-year period at a single center. During the period of analysis, the uptake of sequential lines of treatment in the second line and beyond increased, and such an approach was associated with improved survival outcomes.

Background: Although progress has been made in the molecular stratification of esophagogastric adenocarcinoma, the outlook for advanced disease remains poor. The present evaluation of over 500 patients treated at a single European high-volume tertiary center during a 6-year period gives important information on current and developing "real-world" treatment patterns and outcomes. **Results:** The overall survival for the whole cohort was 11.5 months, with a range of treatments used in first-, second-, and third-line settings. Treatment with sequential lines of therapy was associated with better outcomes, although only 39% and 14% of patients subsequently received treatment in the second- and third-line setting, respectively. Treatment within a therapeutic clinical trial was associated with significantly improved survival. **Conclusion:** At present, a substantial proportion of patients with advanced esophagogastric adenocarcinoma will not proceed beyond first-line therapy, and for this group refinement of initial systemic therapies are required to improve outcomes. Although a number of established first- and second-line treatment options are now available, the therapeutic landscape of the disease continues to change, most notably in the application of immunotherapy and increasing interest in establishing evidence-based interventions in the third-line setting and beyond. A small but growing proportion of patients will benefit from sequential treatment approaches incorporating multiple lines of therapy, and improved selection of such patients will be a key challenge for clinicians moving forwards. Data such as these provide an overview of current treatment patterns and outcomes which can be used to inform planning of future research effectively within existing treatment frameworks.

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Keywords: Cancer, Gastric, Chemotherapy, Esophageal, Treatment

Introduction

Esophagogastric (EG) adenocarcinomas represent a challenging health problem globally. Gastric cancer is the fifth most common malignancy worldwide and the third leading cause of cancer mortality.[1] Although the incidence of non–cardia gastric cancers has been decreasing in Western populations, the incidence of distal esophageal and junctional adenocarcinomas has been increasing.[2] Specific to the United Kingdom, this has been reflected by an increasing incidence rate of esophageal cancer, which represents the sixth most common cause of cancer death, accounting for 7800

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deaths annually.[3] Despite recent advances in both genetic characterization and the development of novel targeted agents, the outlook for patients with advanced disease remains poor, with a median overall survival (OS) not extending beyond 12 months in most trials. The treatment paradigms for esophageal and junctional adenocarcinomas compared with gastric cancer in the early disease setting are diverging. However, in the advanced setting, these cancers are still generally considered together in clinical trial populations, and the treatment approaches have been similar.[4-7] The evidence base is well-established for both first- and second-line chemotherapy. The standard reference regimens in the first-line setting consist of a fluoropyrimidine combined with a platinum agent, with the possible addition of either an anthracycline or a taxane.[8-10] Randomized studies of irinotecan, docetaxel, and paclitaxel have all demonstrated a survival advantage compared with best supportive care alone in the second-line setting.[11-13] A benefit has been shown in the first-line setting for the ~20% of *HER2*-amplified cancers through the use of trastuzumab plus chemotherapy. Also, the anti-*VEGFR2* monoclonal antibody ramucirumab has been established both as monotherapy and in combination with paclitaxel in the second-line setting, although it did not show a survival benefit when combined with chemotherapy in the first-line setting.[14-17] Further trials of molecularly targeted agents have proved disappointing, with trials targeting dual-*HER2*, *EGFR*, *MET*, *P13K/mTOR*, and *PARP* inhibition all yielding negative results. Data are now starting to emerge for treatment beyond the second line, with meta-analyses suggesting a modest survival benefit.[18,19] The ATTRACTION-2 study has confirmed the effectiveness of *PD-1* targeting in this context, and it is expected that further agents under investigation will also become available.[20,21] Administration of later lines of therapy is clinically challenging as cancer and chemotherapy-related symptoms often precipitate a deterioration in clinical status, limiting patient tolerance to further treatment. The margins of benefit appear greater for fitter patients and thus consideration of patient suitability and the toxicities of the planned treatment regimen are paramount.[22] A number of groups have reported their institutional experience of advanced EG cancer management, although the included patient numbers have generally been small.[23-25] The colorectal cancer treatment paradigm has been instructive as an exemplar of rational sequencing of multiple lines of therapy to incrementally improve survival outcomes in the advanced disease setting. As the treatment landscape for EG cancer continues to evolve, it can be expected that a greater proportion of patients will subsequently receive sequential lines of therapy and that targeted and immunotherapeutic agents will be increasingly used. At present it is a challenge for clinicians to select patients and rationally sequence available treatment regimens in such a way as to provide optimal benefit. In order to both guide treatment decisions and plan relevant clinical trials in the field it is important to understand current treatment patterns and outcomes in "real world"patient populations.

Materials and Methods

A retrospective analysis was undertaken of consecutively treated patients who had received ≥ 1 cycle of chemotherapy for EG adenocarcinoma in the advanced disease setting at the Royal Marsden Hospital from April 2009 to November 2015. Potential patients were identified through the use of hospital diagnostic coding. Data were collected by review of the electronic patient medical records. The demographic data, treatment, response, and survival outcomes were recorded. Radiologic responses were recorded for each treatment line at the first response assessment point, on completion of treatment, and at any subsequent progression assessment point. Statistical analysis was performed using Stata 13 statistical software.

This retrospective study did not require patient consent to participate. A study protocol outlining the rationale, methods and statistical analysis plan was approved by an internal Committee for Clinical Research prior to commencement and is available on request.

Results

Demographic Data

A total of 511 patients were identified, of whom 384 (75%) were men and 127 (25%) were women. The median age at diagnosis was 66 years (range, 24-90 years). The performance status of patients at cycle 1 of first-line treatment was Eastern Cooperative Oncology

Table 1 Patient Characteristics

Characteristic	n (%)
Total patients	511 (100)
Sex	
Male	384 (75)
Female	127 (25)
Age at diagnosis, y	
Median	66
Range	24-90
ECOG PS	
0	64 (13)
1	276 (54)
2	87 (17)
3	1
Not recorded	83 (16)
Site of primary tumor	
Esophagus	148 (29)
EGJ	173 (34)
Stomach	190 (37)
Disease extent at beginning of first-line treatment	
Locally advanced (unresectable)	68 (13)
De novo metastatic	335 (66)
Relapsed metastatic after radical treatment	108 (21)
HER2 status	
Positive	73 (14)
Negative	296 (58)
Not recorded	142 (28)

Abbreviations: ECOG = Eastern Cooperative Oncology Group; EGJ = esophagogastric junction; PS = performance status.

Group performance status 0 in 64 (13%), 1 in 276 (54%), 2 in 87 (17%), 3 in 1, and not recorded in 83 patients (16%). The site of the primary tumor was the esophagus in 148 patients (29%), esophagogastric junction in 173 (34%), and stomach in 90 patients (37%). The disease extent at the beginning of first-line treatment was locally advanced (unresectable) in 68 (13%), de novo metastatic disease at presentation in 335 (66%), and relapsed metastatic disease after previous radical treatment in 108 (21%) patients. *HER2* status was positive in 73 (14%), negative in 296 (58%), and not recorded in 142 (28%) patients. The patient characteristics are summarized in Table 1.

Treatment

In the first-line treatment setting, 320 patients (63%) received a triplet chemotherapy regimen. These were predominantly platinum/fluoropyrimidine doublets with the addition of either trastuzumab or an anthracycline. Of the 511 patients, 171 (33%) received doublet therapy, predominantly a platinum/fluoropyrimidine doublet, and 20 (4%) received single-agent treatment. Of the 511 patients, 200 (39%) subsequently received second-line treatment. Of these 200 patients, 24 (12%), 68 (34%), and 108 (54%) received triplet-, doublet-, or single-agent treatment, respectively. Of the 511 patients, 71 (14%) subsequently received third-line treatment. Of these 71 patients, 2 (3%), 26 (37%), and 42 (60%) received triplet-, doublet-, or single-agent therapy, respectively. Of the patients treated in the first-line setting, 20% participated in a clinical trial compared with 29% and 36% of those receiving second- and third-line treatment, respectively. Clinical trials in the first-line setting predominantly involved standard chemotherapy with the addition of a targeted agent, such as REAL-3 (EOX [epirubicin, oxaliplatin, capecitabine] with or without panitumumab; 60 participants), RILOMET (ECX [epirubicin, cisplatin, capecitabine] with or without rilotumumab; 12 participants), and JAGUAR (FOLFOX [folinic acid, 5-fluorouracil, oxaliplatin] with or without ipatasertib; 9 participants). The most common trials in the third-line setting were phase I trials (9 participants). The combinations of chemotherapy received and a breakdown of the clinical trials of first-, second-, and third-line therapy are shown in Figure 1.

An increase in the proportion of patients continuing to second-line therapy was observed during the study period. In the first quarter of the study period, 45 of 136 patients (33%) proceeded to second-line therapy. In contrast, in the fourth quarter of the study period, 63 of 135 patients (47%) had proceeded to second-line therapy (Figure 2). The uptake of *HER2* testing over time also increased, with 74% of cases before April 2010 not reported compared with no cases from April 2015 onward (Table 2).

Response

In the first-line setting, the overall best response was complete response in 2%, partial response (PR) in 47%, stable disease (SD) in 29%, and progressive disease (PD) in 22% (Table 3). The overall response rates in the first line were similar between patients with and without confirmed *HER2*$^{+}$ and *HER2*$^{-}$ disease (48% vs. 49%). In the second-line setting, the overall best response was a PR in 21%, SD in 34%, and PD in 45%. In the third-line setting, the best overall response was a PR in 19%, SD in 24%, and PD in 57%.

Figure 1 (A) Breakdown of Treatments Received in First-Line Setting (n = 511). *Including Raltitrexed-Based Regimens and Capecitabine Monotherapy; †Clinical Trials in First Line Included REAL 3 (EOX [Epirubicin, Oxaliplatin, Capecitabine] With or Without Panitumumab; n = 60), RILOMET 1 (ECX [Epirubicin, Cisplatin, Capecitabine] With or Without Rilotumumab; n = 12), JAGUAR (FOLFOX [Folinic Acid, 5-Fluorouracil, Oxaliplatin] With or Without Ipatasertib; n = 9), MET MAB (FOLFOX With or Without Onartuzumab; n = 8), PLATFORM (Randomized Maintenance; n = 6), BRIGHTER (Paclitaxel With or Without Napabucasin; n = 2), and RAINFALL (CX [Cisplatin, Capecitabine] With or Without Ramucirumab; n = 2). (B) Breakdown of Treatments Received in the Second-Line Setting (n = 200). *Included Trastuzumab, Raltitrexed, Irinotecan, and Docetaxel-Based Regimens; †Clinical Trials in the Second Line Included BRIGHTER (Paclitaxel With or Without Napabucasin; n = 11), MK3475 (Paclitaxel vs. Pembrolizumab; n = 5), IMCLONE (Ramucirumab vs. Placebo; n = 5), PEP0206 (Liposomal Irinotecan vs. Irinotecan vs. Docetaxel; n = 5), RAINBOW (Paclitaxel With or Without Ramucirumab; n = 4), and COUGAR (Docetaxel vs. Best Supportive Care BSC; n = 4). (C) Breakdown of Treatments Received in Third-Line Setting (n = 70). *Included Docetaxel and Irinotecan Monotherapy. †Clinical Trials in Third Line Included Phase I Trials (Drug Development Unit; n = 9), JVDF (Ramucirumab Plus Pembrolizumab; n = 4), CHECKMATE 032 (Nivolumab vs. Nivolumab Plus Ipilimumab; n = 3), COG (Gefitinib vs. Placebo; n = 2), and Fibroblast Growth Factor Receptor (AZD4547; n = 2)

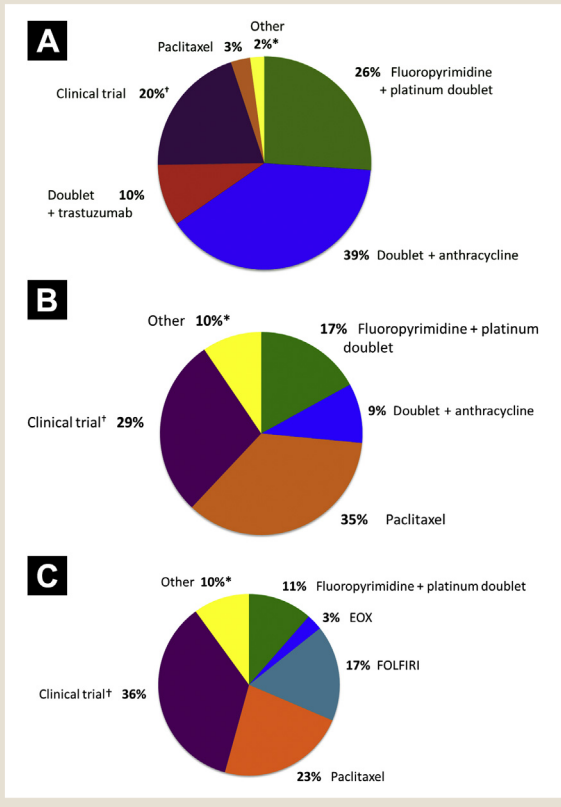
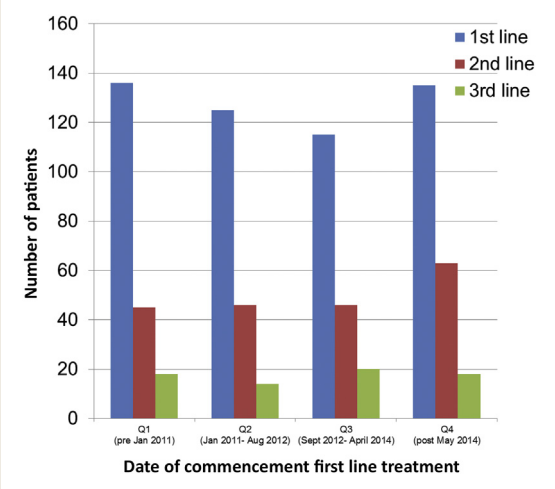


Figure 2 Changes in Uptake of Sequential Treatment Over Time



Survival

The median OS for the whole cohort from the date of diagnosis of advanced disease was 11.5 months (Figure 3A). OS from the beginning of second- and third-line treatment were 6.0 and 4.6 months, respectively (Figure 3B, C). Survival correlated significantly with the number of treatment lines received ($P < .001$; Figure 3D), with a median OS from diagnosis of 8.3, 14.0, 20.1, and 33.0 months for patients receiving 1, 2, 3, or > 3 lines of treatment, respectively. Progression-free survival (PFS) from the initiation of first-, second-, and third-line treatment was 5.5, 3.0, and 1.9 months, respectively (Table 3). No significant difference was found in OS in the advanced setting between patients with relapsed disease after previous radical treatment and those with metastatic disease at diagnosis (12.6 vs. 11.3 months; $P = .10$). PFS with first-line treatment was similar between patients with confirmed *HER2*$^+$ and *HER2*$^-$ disease (5.6 vs. 5.5 months; $P = .11$), although OS was significantly improved for the *HER2*$^+$ patients (15.0 vs. 11.9 months; $P = .02$; Figure 3E). OS was also significantly improved for those patients treated within a therapeutic clinical trial at any line of treatment compared with those who were not (13.5 vs. 10.1 months; $P = .02$).

Table 2 Changes in Uptake of HER2 Testing Over Time

Year of Treatment	HER2 Status		
	Positive	Negative	Not Documented
Before April 2010	8	23	89
April 2010 to March 2011	6	29	34
April 2011 to March 12	11	52	13
April 2012 to March 13	14	56	2
April 2013 to March 14	13	44	3
April 2014 to March 15	15	61	1
April 2015 onward	6	31	0
Total	73	296	142

Discussion

The present comprehensive analysis of treatment and survival for advanced EG adenocarcinoma patients treated within a large volume tertiary referral center in the United Kingdom reflects both the current landscape and developing trends in treatment. The patient population of predominantly men (75%), with a median age of 66 years is typical of the demographics for this disease. Unlike most of the large trial populations, however, the performance status distribution showed that 17% of patients had a recorded performance status of > 2 at baseline and would have typically been excluded from most clinical trials. A platinum doublet with or without an additional third drug was most commonly used in the first line, and single-agent paclitaxel in the second line; however, a substantial proportion of second-line patients were treated with doublet or triplet combination therapies. Most of these cases represented rechallenges of a regimen that had previously been efficacious. This reflects what is often seen in day-to-day practice, in which a few patients maintain sensitivity to platinum/fluoropyrimidine combinations and thus benefit from multiple rechallenges. Emerging data from the field of colorectal cancer have shown the utility of using sequential circulating tumor DNA analysis to investigate dynamic mechanisms of sensitivity and resistance during therapy.[26] Whether such approaches have a role in EG cancer in identifying patients with more intrinsically chemotherapy-sensitive disease suitable for multiple lines of therapy remains an ongoing research question.

Participation in clinical trials was high, with 20%, 29%, and 36% of patients participating in first-, second-, and third-line trials, respectively. This exceeded the reported 14% rate of trial participation in the United Kingdom as a whole, which, in itself, has been purported to exceed that of other countries, with as few as 3% of US cancer patients participating in clinical trials.[27,28] The increase in the use of subsequent lines of treatment reflects the accumulating level-1 evidence base during the study period. Many of the major second-line gastric cancer studies were reported from 2011 to 2014, giving clinicians a greater selection of evidence-based options for later line treatment. Similarly, *HER2* testing trends changed considerably during the analysis period. This coincided with both the landmark ToGA trial results and the funding of trastuzumab in the United Kingdom for *HER2*$^+$ gastric cancer patients, which was approved in November 2010. Before November 2010, 24% of patients beginning first-line treatment underwent *HER2* testing of their tumor. In contrast, after 2010, 89% underwent testing.

The overall response rates were comparable with the reported trial data; however, a significant proportion of patients did not maintain a response for the duration of their treatment. Of the 349 patients with SD or better at the scan assessment of their initial response during first-line therapy, 119 (34%) had documented radiologic PD by the end of treatment and 38 (11%) did not undergo further scanning because of unacceptable toxicity, clinical progression, or death curtailing treatment completion. Thus, although the initial disease control rates have been reasonable, they have often been followed by rapid radiologic progression or clinical deterioration during the same treatment line. Interest is increasing in using maintenance therapies for EG cancer, with a number of ongoing

Table 3 Variables Stratified by Treatment Line

Variable	First Line	Second Line	Third Line	> 3 Lines
Patients, n (%)	511 (100)	200 (39)	70 (14)	15 (3)
Treatment, %				
Triplet	63	12	3	0
Doublet	33	34	37	0
Single	4	54	60	0
Clinical trial participation, n (%)	103 (20)	57 (29)	25 (36)	5 (33)
Median cycles, n	6	3	3	NA
Overall best response, %				
CR	2	0	0	0
PR	47	20	19	0
SD	29	34	24	0
PD	22	41	57	0
PFS, mo	5.5	3.0	1.8	
OS, mo				
Whole cohort	11.5			
According to treatment line received	8.3 (first line only)	14.0 (first and second line)	20.1 (first to third line)	33.0 (>3 lines)

Abbreviations: CR = complete response; OS = overall survival; PD = progressive disease; PFS = progression-free survival; PR = partial response; SD = stable disease.

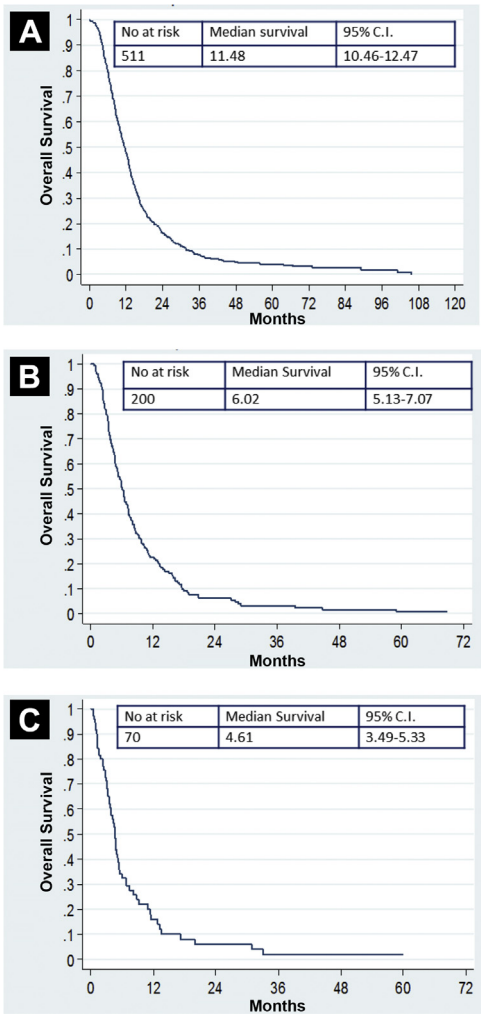
trials using de-escalated chemotherapy or targeted or immunotherapeutic agents to augment and maintain the response to first-line chemotherapy.[29,30] Given that the disease control rates decrease substantially during the course of chemotherapy, this presents a challenge to researchers investigating such maintenance strategies.

The median OS and PFS were also similar to those reported in landmark trials of predominantly European patient populations. However, the OS of 15.0 months for *HER2*$^{+}$ patients was longer than the 13.8 months reported in the ToGA trial and also exceeded that of the chemotherapy plus trastuzumab arm of the recently presented JACOB study (14.2 months).[31] The present real world population differed from the populations in these trials in that 73% of confirmed *HER2*$^{+}$ patients received trastuzumab in the first line, with another 15% receiving it later in their treatment course. This seemingly more efficacious *HER2* targeting may have occurred because the UK National Institute for Health and Care Excellence guidelines allow funding for trastuzumab only for patients who display HER2 immunohistochemistry 3+ staining, a subgroup shown in a ToGA subgroup analysis to have superior survival times. More recent studies have investigated the relationship between *HER2* gene amplification and clinical benefit from trastuzumab.[32,33] These studies showed that the level of *HER2* amplification significantly predicts for sensitivity to therapy, and that the optimal *HER2* amplification ratio predicting for trastuzumab benefit is likely to be considerably greater than the currently mandated definitions of positivity.[32,33] Further refinement of biomarker selection could be necessary to optimize the benefit for this group of patients in the future.

As expected, survival correlated significantly with the number of treatment lines received. An obvious selection bias was present, as patients suitable for sequential treatment are likely to represent a self-selecting fitter group. Also, underlying tumor-biologic factors could render them more sensitive to existing chemotherapy regimens, with the selection of patients suitable for such sequential treatment becoming a more relevant clinical challenge. Previous attempts have been made to define relevant prognostic factors in advanced EG cancer, with ongoing work seeking to identify genetic signatures predictive of the response to both standard chemotherapy agents and immunotherapeutic agents.[34-36] The 70 patients who received third-line or beyond treatment in our cohort were slightly younger (median age, 55 years) and had had a longer PFS with first- and second-line treatment (9.3 and 4.2 months, respectively) compared with the cohort as a whole, factors that have previously been associated with a favorable response to third-line treatment.[37,38] Only 39% of the overall cohort subsequently received second-line therapy. Even within a modern clinical trial, such as the 2018 RAINFALL study, the rate of second-line treatment uptake reached only 51%,[17] underlining the importance of improving first-line interventions for patients who will often only have 1 opportunity to receive systemic therapy. The improved survival for the patients who subsequently received further lines of treatment highlights the positive effect sequential lines of therapy can have for carefully selected patients and should encourage physicians to pursue this approach where appropriate.

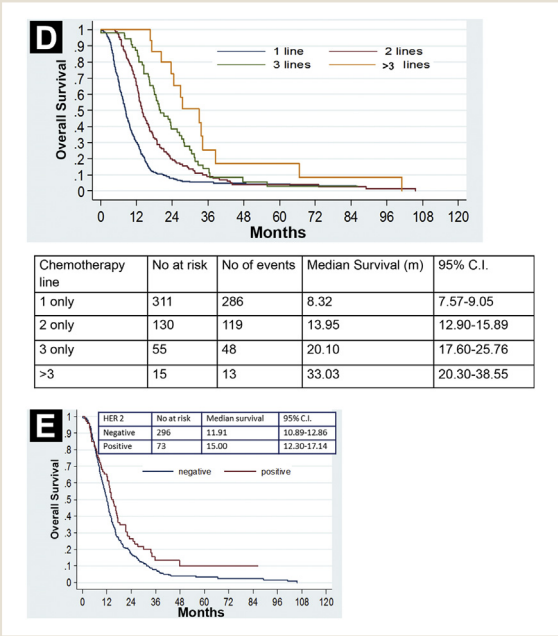
Known variations in treatment and outcome exist in advanced EG cancer globally, with greater uptake of sequential lines of therapy and improved survival in East Asian compared with European populations. Analyses of the treatment patterns in non–trial East Asian populations have reported rates of uptake of second-line chemotherapy ranging from 54% to as great as 80%, and such disparities have important implications for research in the field.[23,24,39] Subgroup analysis of the RAINBOW trial evaluating second-line paclitaxel with or without ramucirumab revealed only a nonsignificant OS benefit for ramucirumab in East Asian patients, despite significant improvements in both relapse rate and PFS.[40] This was likely to be as a result of substantially greater uptake of postprogression treatment lines in this patient group. Thus, the effect of differential uptake of further treatment must be considered when interpreting the survival outcomes in first- and

Figure 3 (A) Overall Survival for Whole Cohort. (B) Overall Survival From Beginning Second-Line Therapy. (C) Overall Survival From Beginning Third-Line Therapy. (D) Overall Survival Stratified by Number of Treatment Lines Received. (E) Overall Survival Stratified by HER2 Status



Abbreviation: CI = confidence interval.

Figure 3 Continued.



Chemotherapy line	No at risk	No of events	Median Survival (m)	95% C.I.
1 only	311	286	8.32	7.57-9.05
2 only	130	119	13.95	12.90-15.89
3 only	55	48	20.10	17.60-25.76
>3	15	13	33.03	20.30-38.55

second-line trials involving both Eastern and Western patient cohorts.[41] The recent ATTRACTION-2 study was performed in East Asian centers only and demonstrated a survival benefit for nivolumab versus best supportive care in the third-line setting, although whether such results are transferable to primarily European populations is as yet unknown.[20] Cancer Genome Atlas data suggested no differences in the distribution of their proposed molecular subtypes between East Asian and Western patients; however, a further study has shown differential expression of genes related to inflammation and immunity between the 2 geographic groups.[42,43] In that analysis, tumors from non-Asian patients were enriched for immune T-cell markers, and tumors from Asian patients were enriched for immunosuppressive T-cell regulatory markers.[43] Such distinct tumor immunity signatures related to T-cell function could affect the immunotherapy response between the 2 geographic groups and emerging data from ongoing global immunotherapy studies should be considered with this in mind. In addition to intrinsic biologic differences in tumor characteristics potentially influencing response, the patient population of such a "third-line" study performed in a Western population is also likely to be significantly different in terms of comorbidity, fitness, and overall treatment tolerance.

Conclusion

As we move into an era in which immunotherapy and targeted agents become more closely integrated into advanced EG cancer treatment pathways it is hoped further survival improvements will be seen, with multiple lines of therapy exposure using these novel agents becoming more commonplace, especially in European practice in which such sequential treatment approaches remain relatively uncommon. It is important to understand current and evolving trends to tailor ongoing research effectively within existing treatment frameworks. Combining clinical and biologic characteristics to refine prognostic and predictive models could identify patients suitable for more prolonged and rationally sequenced treatments in the advanced disease setting.

Clinical Practice Points

- In recent years, the treatment options for advanced esophagogastric adenocarcinoma have expanded, resulting in incremental improvements in survival.
- The present report has provided a comprehensive overview of current patterns and evolving trends in treatment during recent years.
- Of our patients, 39% and 14% subsequently received second- and third-line treatment, respectively, and the uptake of sequential lines of therapy increased during the study period.

- The patients who received such sequential treatment approaches tended to be younger and to have experienced favorable responses to previous lines of therapy, factors previously been associated with improved outcomes in the third-line setting.
- Enrollment in clinical trials was high, and participation in a clinical trial was associated with improved survival.
- As more treatments become available for the management of advanced esophagogastric cancer, patient selection and rational sequencing of chemotherapy, targeted therapy, and immunotherapy drugs will become increasingly important.
- It is clear that a significant proportion of patients will benefit from a sequenced treatment approach incorporating multiple lines of therapy; thus, identifying and selecting such patients will be paramount.
- This will need to incorporate both disease-related biomarkers of treatment response and patient-related clinical characteristics.
- Data such as these highlight the potential benefits that can be gained by using such sequential treatment approaches and should encourage physicians to pursue these approaches, as appropriate.

Disclosure

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Original Research

Clonal diversity of *MYC* amplification evaluated by fluorescent *in situ* hybridisation and digital droplet polymerase chain reaction in oesophagogastric cancer: Results from a prospective clinical trial screening programme

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Oesophagogastric cancer;
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Abstract ***Introduction:*** The *MYC* proto-oncogene is among the most commonly dysregulated genes in human cancers. We report screening data from the i*MYC* trial, an ongoing phase II study assessing ibrutinib monotherapy in advanced pretreated *MYC*- and/or *HER2*-amplified oesophagogastric cancer, representing the first attempt to prospectively identify *MYC* amplifications in this tumour type for the purposes of therapeutic targeting. ***Methods:*** Screening utilising a fluorescent *in situ* hybridisation (FISH) assay for assessment of tumour *MYC* amplification has been instituted. An experimental digital droplet polymerase

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Digital PCR

chain reaction (ddPCR) assay to assess *MYC* amplification in both tumour and circulating-tumour (ct)DNA has been developed and investigated.

Results: One hundred thirty-five archival tumour specimens have undergone successful FISH analysis with 23% displaying evidence of *MYC* amplification. Intertumour heterogeneity was observed, with the percentage of cancer cells harbouring *MYC* amplification ranging widely between samples (median 51%, range 11–94%). Intratumoural clonal diversity of *MYC* amplification was also observed, with a significant degree of variance in amplification ratios (Bartlett's test for equal variance $p < 0.001$), and an association between greater variance in *MYC* amplification and improved outcome with prior first-line chemotherapy. ddPCR was most accurate in quantifying *MYC* amplification in tumour-derived DNA from cases with a high proportion (>70%) of amplified cells within the tumour specimen but was not reliable in samples containing a low proportion of amplified cells or in ctDNA.

Conclusions: Our results illustrate the utility of FISH to assess *MYC* amplification prospectively for a biomarker-selected trial by providing reliable and reproducible results in real time, with a high degree of heterogeneity of *MYC* amplification observed. We show that ddPCR can potentially detect high-level *MYC* amplifications in tumour tissue.

1. Introduction

The prognosis for patients with advanced oesophagogastric (OG) cancer is poor, with a median overall survival (OS) of less than 12 months in most clinical trials [1]. There is utility for the targeted agents trastuzumab and ramucirumab in *HER2*-positive first-line and unselected second-line settings, respectively [2–4]. Further studies evaluating targeted agents inhibiting *EGFR*, *MET*, *PI3K/mTOR* and *PARP* have been disappointing, all failing to improve overall survival [5–9]. Therefore, effective biomarker-driven treatment approaches are urgently required to address this area of unmet clinical need.

The *MYC* proto-oncogene is among the most commonly dysregulated genes in human cancer, controlling the transcription of genes involved in multiple oncogenic pathways [10,11]. Effective methods for targeting *MYC* represent an attractive potential therapeutic strategy. As it is a transcription factor that lacks enzymatic activity, *MYC* cannot be directly targeted by small molecule inhibitors or antibody-based therapies [12]. Strategies based on the concept of synthetic lethality, whereby disruption of two or more genes in combination result in a deleterious phenotype, have been investigated to indirectly target tumours displaying *MYC* overactivity [13,14]. Using functional genomic screening of oesophageal cell lines, we previously observed selective decreased cell viability following silencing of Bruton's tyrosine kinase (*BTK*) in *MYC*-amplified cell lines. This was further validated using ibrutinib, a clinical irreversible BTK inhibitor that is also known to target *HER2* [15,16]. In *MYC*-amplified cells, ibrutinib downregulated levels of MYC protein and downstream effectors, and elicited G1 cell cycle arrest and apoptosis. BTK signalling occurs partly via the canonical RAS-RAF-MEK-ERK pathway. ERK is a known mediator of MYC phosphorylation, and a putative mechanism of action for *MYC* and *BTK* interaction was identified through observation of BTK-dependent, ERK-mediated, MYC phosphorylation [16].

As a result of our preclinical observations, we initiated i*MYC* (NCT02884453), an ongoing phase II non-randomised study to assess the efficacy of ibrutinib monotherapy in advanced pretreated OG cancer that represents the first attempt at targeting *MYC* amplification clinically in this tumour type [17]. As there are no established definitions of *MYC* 'positivity' in solid organ tumours, previous studies investigating *MYC* amplification have used fluorescent *in situ* hybridisation (FISH)-based techniques which defined positivity based on established cutoffs derived from *HER2* FISH testing [18]. In parallel, we also developed a novel digital droplet polymerase chain reaction (ddPCR) assay to assess *MYC* amplification in both tissue and circulating-tumour (ct)DNA.

Here we give the first report of the frequency, pattern and heterogeneity of *MYC* amplification in OG cancer from a prospective screening study with clinical outcome correlation. We describe the development of a ddPCR assay using two independent reference probes and assess the correlation of *MYC* amplification detected by FISH and ddPCR in both primary tumour tissue and ctDNA samples.

2. Materials and methods

2.1. Patient samples

Patients treated at the Royal Marsden Hospital, UK, with OG cancer of either squamous cell carcinoma (SCC) or adenocarcinoma (AC) histological subtype being considered for or undergoing systemic anticancer therapy were eligible for screening. Informed written

consent was obtained from all patients within the context of the i*MYC* trial (NCT02884453). At the time of consent, an archival diagnostic formalin-fixed paraffin-embedded (FFPE) tumour sample was obtained for analysis and blood samples were collected in Streck Cell-Free DNA blood collection tubes.

2.2. DNA extraction

FFPE tissue was cut into 5–8 micron sections and macrodissected to ensure >70% tumour content. DNA extraction was performed with the QIAamp DNA FFPE tissue kit (Qiagen) as per manufacturer's instructions. ctDNA was isolated from 4 to 5 mL of plasma using the QIAamp Circulating Nucleic Acid kit (Qiagen) as per manufacturer's instructions. Tumour and ctDNA was quantified using the Qubit dsDNA HS assay (Thermo Fisher Scientific).

2.3. Fluorescent in situ hybridisation

A dual-probe FISH assay was developed and validated to diagnostic quality for the purposes of the study. Screening was performed using a combination of a reference probe mapping to the centromere of chromosome 8 (CEP8) and a *MYC* probe mapping to chromosome 8q24, covering the entire coding region of the *MYC* gene from exons 1–3. Dual-label FISH was performed on each tissue section using standard techniques, and results were reported in a standardised manner, recording both the range and modal ratio of CEP8 and *MYC* signals and, in the case of amplification, the proportion of cells displaying an amplified signal. The results were deemed to be amplified if *MYC*:CEP8 ratio ≥2.5.

2.4. Digital droplet PCR

A challenge in using ddPCR for the detection of gene amplifications is the reliable quantification of changes in the abundances of germline reference loci [19]. Suitable reference genes were identified through analysis of publically available copy number data to calculate copy number ratios of *MYC* versus all possible chromosome 8 reference genes in *MYC*-amplified and non-amplified cancers. For the final analysis, two reference genes were chosen: a chromosome 8 reference gene, *CEBPD*, was selected on the basis of high sensitivity for *MYC* amplification and a chromosome 14 reference gene, *RPPH1*, frequently used in assessment of copy number variation. Data were analysed with respect to each control gene individually and the average ratio of *MYC*:*RPPH1* and *MYC*:*CEBPD* reported for each sample (see supplementary material S1 for additional description of ddPCR assay development and methodology).

2.5. Statistical analysis

Statistical analysis for ddPCR reference probes was carried out using the R statistical package (available at http://www.R-project.org); all further statistical analysis was carried out using PRISM version 7 (available at https://www.graphpad.com/scientific-software/prism). Categorical variables were compared using χ^2 test; continuous variables by Mann–Whitney *U*-test; progression-free survival (PFS) and OS by Kaplan–Meier method. Significance level for statistical tests was set at 0.05.

3. Results

3.1. Prospective patient cohort

Between July 2016 and January 2018, 162 patients were consented for prospective screening as part of the *iMYC* study. Eighty-six percent (139/162) of cases were of AC histological subtype and 92% (149/162) had evidence of distant metastases at the time of enrolment. Of these, 88% of patients had received platinum-based first-line chemotherapy treatment (Table 1).

Table 1
Patient characteristics of i*MYC* prescreening population.

Total number of patients	n = 162
Age in years (median, range)	
At diagnosis	61.2 (36.4–85.5)
At time of ctDNA analysis	62.2 (37.1–85.8)
Sex	
Male	131 (81%)
Female	31 (19%)
Site of primary tumour	
Oesophagus/OGJ	127 (78%)
Stomach	35 (22%)
Histological subtype	
Adenocarcinoma	139 (86%)
Squamous cell carcinoma	23 (14%)
HER2 status*	
Positive	27 (17%)
Negative	135 (83%)
Disease status at enrolment	
Locally advanced	13 (8%)
Metastatic	149 (92%)
First-line treatment received (metastatic patients only)	
Platinum based	131 (88%)
Taxane based	9 (6%)
Other	7 (5%)
No treatment	2 (1%)
ctDNA analysis performed	127 (78%)

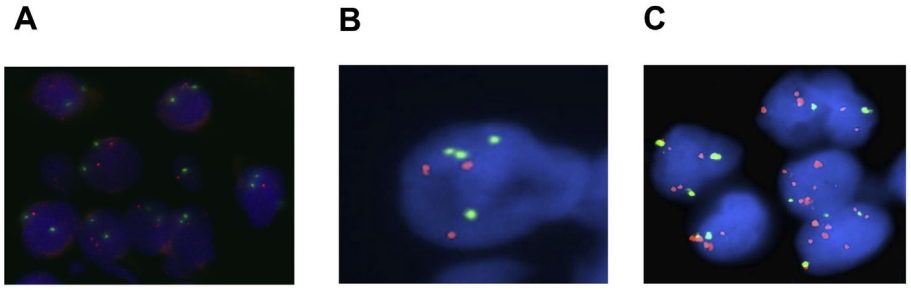
OGJ, oesophagogastric junction.
* Defined as *HER2*:*CEP17* ratio ≥2 by standard DDISH testing.

3.2. Evaluation of *MYC* amplification by FISH analysis of primary tumour FFPE samples

Samples were categorized as follows: 'diploid, copy number neutral' (displaying 2 *MYC* and 2 CEP8 signals per cell) (Fig. 1A), 'polysomatic' (additional signals of both *MYC* and CEP8) (Fig. 1B), '*MYC* amplified' (increased ratio of *MYC* to CEP8 signals) (Fig. 1C) +/− polysomy. Where a range of signal patterns was seen, the most prevalent (modal) pattern within the sample was recorded. Of 135/162 (85%) successfully analysed samples, a 'diploid, copy number neutral' signal pattern was seen in 24 (18%) cases (Fig. 1D). The most commonly observed pattern was polysomy without *MYC* amplification, seen in 80 (59%) cases. Amplification with no evidence of polysomy was observed in 16 (12%) samples and amplification with polysomy was seen in 15 (11%) samples. Intertumour heterogeneity was observed, with the percentage of cancer cells harbouring *MYC* amplification ranging widely between samples (median 51%, range 11–94%) (Fig. 2A). Intratumoural diversity, as manifested by the range of *MYC* amplification present within each specimen, was observed with 22/31 (71%) amplified samples showing a range of amplification ratios present (Fig. 2B–C) and a significant variance in amplification ratios (Bartlett's test for equal variance $p < 0.001$).

3.3. Clinical correlates of *MYC* amplification

We went on to assess association of *MYC* amplification with further histopathological features and response to



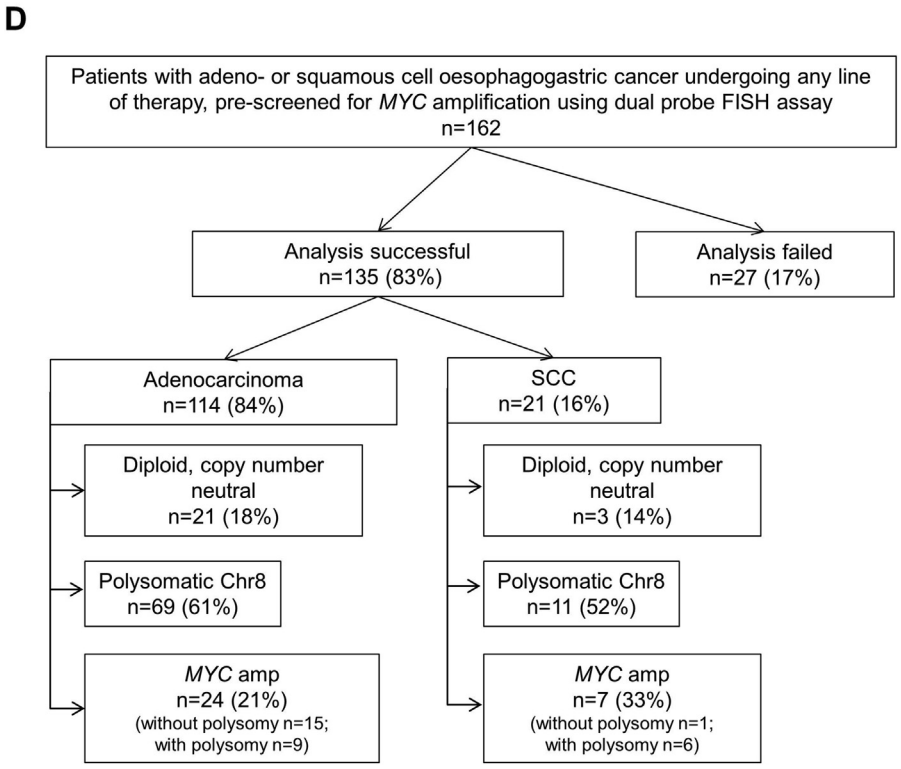


Fig. 1. Patterns of *MYC* amplification in primary oesophagogastric tumour samples detected by FISH (red = *MYC*; green = CEP8). (A) Normal diploid pattern (2 *MYC* and 2 CEP8 signals per cell). (B) Polysomic pattern (additional signals of both *MYC* and CEP8). (C) *MYC*-amplified pattern (increased ratio of *MYC* to CEP8 signals). (D) Flow diagram indicating the results of *MYC* FISH assessment in i*MYC* trial samples, separated based on histology. FISH, fluorescent *in situ* hybridisation.

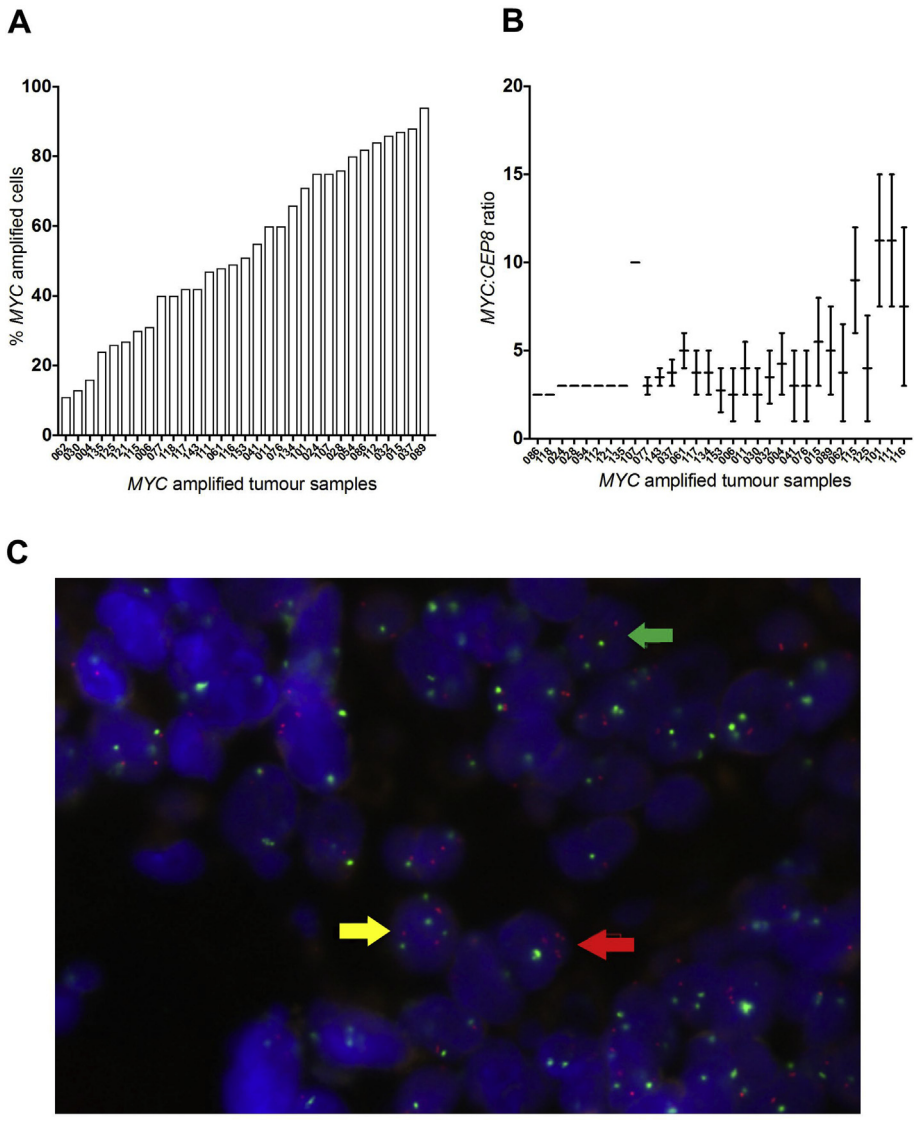


Fig. 2. Intertumoural and intratumoural heterogeneity of *MYC* amplification. (A) Bar chart illustrating proportion of cells displaying *MYC* amplification within each amplified specimen; (B) line chart demonstrating range of *MYC* amplification ratios seen within each amplified specimen; (C) individual sample showing intratumoural heterogeneity of *MYC* amplification patterns; green arrow = normal diploid; yellow arrow = polysomatic; red arrow = *MYC* amplified.

prior systemic therapy. We observed that, although *MYC* amplification was identified in a higher percentage of SCC histological than AC subtypes, the difference was not statistically significant (33 vs 21%; $p = 0.22$ χ^2). We found no difference in *HER2* status, tumour differentiation or presence of signet cells between *MYC*-amplified and non-amplified tumours (Table S1). When considering the potential influence of *MYC* amplification on clinical outcome in patients with advanced disease treated with any first-line systemic therapy (n = 125), we found no significant difference in response rates (68 vs 55%; $p = 0.22$ χ^2), median PFS (22.9 vs 22.1 weeks; $p = 0.55$ log-rank; Fig. 3A) or OS (61.6 vs 63.3 weeks; $p = 0.13$ log-rank; Fig. 3B) between *MYC*-amplified and non-amplified tumours. Given the differences within the screening population in terms of first-line treatment received, we then selected the patient group who had received a standard chemotherapy combination comprising a fluoropyrimidine and platinum agent with or without the addition of an anthracycline (n = 84). For these patients, there was a trend towards improved overall response rates in the *MYC*-amplified cohort (64 vs 45%, $p = 0.09$ χ^2), but amplification status again did not affect median PFS or OS. In an exploratory analysis, we assessed the impact of degree of clonal diversity in *MYC* amplification on survival outcome by undertaking tertile analysis based on the range of *MYC:CEP8* ratios observed within the amplified tumour specimens. We observed that tumour samples with the highest variance in *MYC* amplification

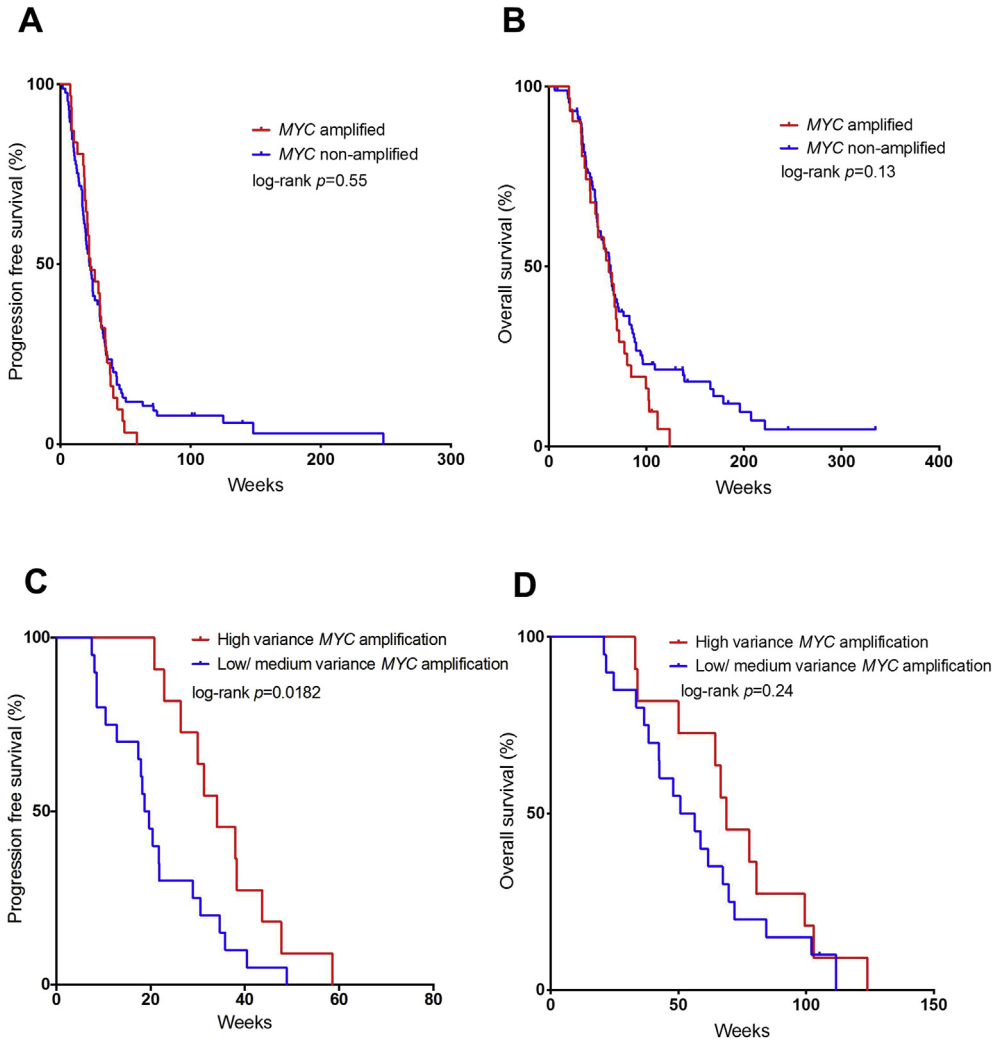


Fig. 3. Influence of *MYC* amplification status on survival. (A) Progression-free survival on first-line systemic treatment for *MYC*-amplified versus non-amplified tumours by FISH (22.9 vs 22.1 weeks; $p = 0.55$ log-rank). (B) Overall survival for *MYC*-amplified versus non-amplified tumours by FISH (61.6 vs 63.3 weeks; $p = 0.13$ log-rank). (C) Progression-free survival for high variance (defined as top tertile of tumours by range of *MYC* amplification observed within specimen) versus low/medium variance (low- and mid-tertile tumours by range of *MYC* amplification observed within specimen) tumours (34.1 vs 18.7 weeks; $p = 0.0182$ log-rank). (D) Overall survival of high variance versus low/medium variance *MYC*-amplified tumours (68.7 vs 50.7 weeks; $p = 0.24$ log-rank). FISH, fluorescent *in situ* hybridisation.

were associated with a significantly longer PFS after first-line treatment (34.1 vs 18.7 weeks; log-rank $p = 0.0182$; Fig. 3C) but no difference in OS was seen (68.7 vs 50.7 weeks; $p = 0.24$ log-rank; Fig. 3D). The proportion of amplified cells within each tumour sample did not appear to influence survival.

3.4. *MYC* amplification assessment by ddPCR in patient samples from the iMYC trial

We then analysed patient samples retrieved as part of the i*MYC* study and, as of October 2018, 105 archival FFPE tumour samples have undergone DNA extraction. Of these, ddPCR analysis was successful in 98 (93%) (Fig. 4A). When comparing *MYC* amplification status as assessed by FISH and ddPCR, we observed a higher median tumour ddPCR ratio in *MYC*-amplified as compared to non-amplified tumours (1.417 vs 1.246; $p = 0.017$ Mann–Whitney *U*-test; Fig. 4B). However, the ddPCR ratios detected in OG tumours were lower than expected compared with cell line data (Fig. S3C), highlighting the difference between cell line and primary samples. Considering that we had observed significant intertumoural and intratumoural heterogeneity of *MYC* amplification, we hypothesised that ddPCR would have greater sensitivity in more highly amplified cases. To address this, we undertook a tertile analysis based on the proportion of amplified cells within the sample. Consistent with our hypothesis, we observed no significant difference in ddPCR ratio between amplified samples containing greater or less than 35% *MYC*-amplified cells (1.432 vs 1.387; $p = 0.3111$ Mann–Whitney *U*-test;

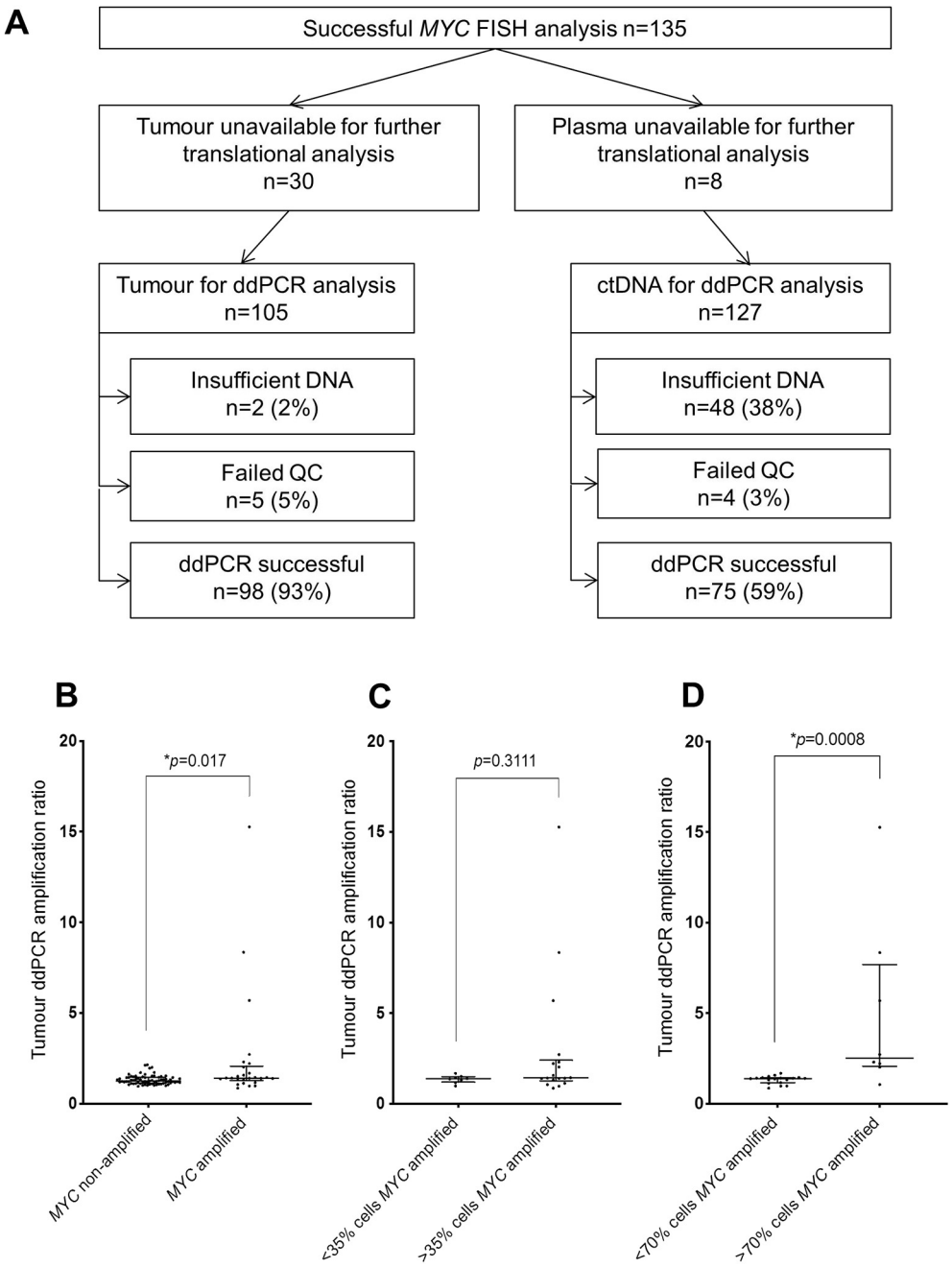


Fig. 4. *MYC* amplification assessment by ddPCR in patient samples from the i*MYC* trial. (A) Flow diagram of tumour and ctDNA ddPCR sample analysis from patients entered into the i*MYC* trial; scatterplots showing (B) significant difference in tumour ddPCR ratio between *MYC*-amplified and non-amplified samples assessed by FISH ($p = 0.017$ Mann–Whitney *U*-test); (C) no difference in tumour ddPCR ratio between *MYC*-amplified samples with< 35% and >35% amplified cells, respectively ($p = 0.3111$ Mann–Whitney *U*-test); (D) a significant difference in tumour ddPCR ratio between *MYC*-amplified samples with <70% and >70% amplified cells, respectively ($p = 0.008$ Mann–Whitney *U*-test). ddPCR, digital droplet polymerase chain reaction; FISH, fluorescent *in situ* hybridisation.

Fig. 4C). By contrast, for samples containing greater or less than 70% amplified cells, the ddPCR ratio was found to be significantly higher in these more amplified samples (2.512 vs 1.396; $p = 0.0008$ Mann–Whitney *U*-test; Fig. 4D), with a receiver operator area under the curve of 0.8958 (95% CI 0.7026–1.089; $p = 0.0015$). Using a ddPCR cutoff ratio of 2.0 resulted in a sensitivity of 87.5% and specificity of 100% in identifying these highly (>70% cells) amplified cases.

ddPCR analysis using ctDNA was possible in 75/127 (59%) plasma samples, as the remainder had insufficient DNA for analysis (Fig. 4A). We observed no difference in plasma ddPCR ratios between *MYC*-amplified and non-amplified tumours (1.547 vs 1.450; $p = 0.5705$

Mann–Whitney *U*-test), and the percentage of cells harbouring *MYC* amplification in the primary tumour did not influence the ddPCR ratio.

4. Discussion

Based on preclinical work demonstrating a synthetically lethal gene interaction between *BTK* and *MYC* [16], we have prospectively assessed *MYC* amplification for the first time in this tumour type for the purposes of therapeutic targeting with the BTK-inhibitor ibrutinib within a biomarker-selected clinical trial. Our screening results illustrate the utility of cytogenetic analysis by FISH to assess *MYC* amplification in this context by providing reliable and reproducible results in real time. We found *MYC* amplification in OG tumours assessed by FISH to be a relatively common event, occurring in 33% of SCC and 21% of AC tumours, respectively. This is consistent with *MYC* amplification frequencies reported in a recent analysis of oesophageal AC and SCC undertaken by the Cancer Genome Atlas Research Network [20]. We observed a high degree of heterogeneity in amplification patterns, which has previously been described in a retrospective study in this tumour type [21]. Intratumoural heterogeneity in OG cancer, comprising both spatial heterogeneity and temporal heterogeneity along progression from primary to recurrent or advanced disease, is increasingly recognised [22], and heterogeneity of expression of *ERBB2* and *FGFR* genes has been associated with differential responses to targeted therapies in gastric cancer [23,24]. Whether the heterogeneity of *MYC* amplification will impact upon the efficacy of its targeting within our current ongoing clinical trial remains to be seen; however, this will be an important consideration in future efforts to target *MYC* from a clinical perspective.

We observed a trend towards improved response to first-line platinum/fluoropyrimidine-based chemotherapy in *MYC*-amplified tumours, consistent with previously described associations between *MYC* amplification and favourable chemotherapy response in breast and ovarian cancer [25–27]. Although *MYC* status did not influence survival outcomes overall, we identified an association with improved PFS in cases with a higher variance in observed amplification ranges within the specimen. *MYC* amplification has been associated with a tumour-hypoxic molecular signature indicating increased underlying genomic instability [11]. Genomic instability has also been correlated with improved outcomes to platinum-based chemotherapy [28], and it is possible that clonal diversity of *MYC* amplification may be a surrogate for this. However, further work is necessary to clarify the role of *MYC* in mediating platinum response in this disease, and validation of our findings in a larger independent data set will be required.

Given the heterogeneity seen, identifying amplifications from pooled DNA as compared to single cell-based analysis such as FISH is likely to be challenging. Our novel ddPCR assay was developed based on robust identification of suitable reference genes. Although a statistically significant difference in tumour ddPCR ratio was seen between *MYC*-amplified and non-amplified tumours as assessed by FISH, the absolute difference was small. However, ddPCR was able to identify more highly amplified tumour samples (containing >70% amplified cells). Thus, the ability of ddPCR to detect *MYC* amplifications may be limited to those tumour samples displaying a homogenous high-level pattern of *MYC* amplification only. Its application to ctDNA was limited by the relatively small numbers of samples where adequate DNA could be extracted and successfully analysed. The high failure rate encountered is likely to be due, in part, to the nature of the i*MYC* prescreening cohort as patients could have blood taken at any time before, during, or after a line of treatment. Effective systemic treatment can potentially reduce absolute ctDNA levels [29], and thus the yield of extracted ctDNA from plasma was potentially influenced by the clinical context at the time point when the blood was taken. The accuracy of ctDNA ddPCR in detecting genomic amplifications is known to be lower than tumour ddPCR [30], and the high heterogeneity of *MYC* amplification may have contributed to the lower concordance seen [21]. Furthermore, *MYC* amplification has been associated with both aneuploidy and increased intratumoural heterogeneity in other solid tumours and may be an acquired event in tumour evolution from primary to metastatic disease, potentially affecting equivalence of results between primary tumour specimens and ctDNA from blood samples taken at differing time points of disease progression [31,32].

As research into effective *MYC*-targeting treatment continues, robust and reproducible methods of biomarker detection will be necessary, and we show that prospective screening for *MYC* amplification in OG cancer for the purposes of therapeutic targeting is feasible. The FISH assay used has revealed *MYC* amplification to be a common event in this tumour type, with a high degree of heterogeneity of *MYC* amplification patterns observed. We show that ddPCR can potentially be used to detect *MYC* amplifications in tumour samples with a high proportion of amplified cells; however, further work is necessary to optimise this technique in ctDNA. Although a trend towards improved chemotherapy response rates in *MYC*-amplified patients was noted, no clear association between *MYC* status and survival was seen. The clinical implications of *MYC* alterations in OG cancer are therefore likely to lie in the potential to harness *MYC* as a therapeutic target, and the clinical efficacy results of our biomarker-selected trial are awaited.

Statement of author contributions

Study concept and design of the article were performed by MD, LA, IYC and IC. Data acquisition was performed by MD, RLA, RB, DC, DW, NS, SR and IC. Quality control of data and algorithms was performed by MD, IYC, KK and EK. Data analysis and interpretation were carried out by MD, IYC, LA, HB, JHR, RC, SHK, KK, EK, MLC, MT, AW, GB, JS and CJL. Statistical analysis was performed by MD, RC, KK and EK. Manuscript preparation was carried out by MD, LA and IYC. Manuscript editing was carried out by MD. Manuscript review was performed by all authors. All authors were involved in preparation of the manuscript and had final approval of the submitted version.

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Conflict of interest statement

NS has received Research funding from AstraZeneca, BMS and Merck and received honoraria from AstraZeneca. DC has received funding from Amgen, AstraZeneca, Bayer, Celgene, Merck-Serono, Medimmune, Merrimack, Novartis, Roche and Sanofi. IC has served on advisory boards for Eli-Lilly, Bristol Meyers Squibb, MSD, Bayer, Roche, Merck-Serono, Five Prime Therapeutics, AstraZeneca, Oncologie International and Pierre Fabre; received research funding from Eli-Lilly, Janssen-Cilag, Sanofi Oncology and Merck-Serono; received honorarium from Eli-Lilly. IYC has received from research funding from Merck, KGaA and Janssen-Cilag.

Appendix A. Supplementary data

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Detecting and Tracking Circulating Tumour DNA Copy Number Profiles during First Line Chemotherapy in Oesophagogastric Adenocarcinoma

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Abstract: DNA somatic copy number aberrations (SCNAs) are key drivers in oesophagogastric adenocarcinoma (OGA). Whether minimally invasive SCNA analysis of circulating tumour (ct)DNA can predict treatment outcomes and reveal how SCNAs evolve during chemotherapy is unknown. We investigated this by low-coverage whole genome sequencing (lcWGS) of ctDNA from 30 patients with advanced OGA prior to first-line chemotherapy and on progression. SCNA profiles were detectable pretreatment in 23/30 (76.7%) patients. The presence of liver metastases, primary tumour in situ, or of oesophageal or junctional tumour location predicted for a high ctDNA fraction. A low ctDNA concentration associated with significantly longer overall survival. Neither chromosomal instability metrics nor ploidy correlated with chemotherapy outcome. Chromosome 2q and 8p gains before treatment were associated with chemotherapy responses. lcWGS identified all amplifications found by prior targeted tumour tissue sequencing in cases with detectable ctDNA as well as finding additional changes. SCNA profiles changed during chemotherapy, indicating that cancer cell populations evolved during treatment; however, no recurrent SCNA changes were acquired at progression. Tracking the evolution of OGA cancer cell populations in ctDNA is feasible during chemotherapy. The observation of genetic evolution warrants investigation in larger series and with higher resolution techniques to reveal potential genetic predictors of response and drivers of chemotherapy resistance. The presence of liver metastasis is a potential biomarker for the selection of patients with high ctDNA content for such studies.

Keywords: oesophagogastric adenocarcinoma; circulating tumour DNA; somatic copy number aberration; liquid biopsy

1. Introduction

Gastric and oesophageal cancers are challenging health issues, representing the third and sixth leading causes of global cancer mortality, respectively [1]. Advances have been made in the genetic characterisation and development of novel targeted agents for the adenocarcinoma histological subtype; however, the outlook for advanced disease remains poor with median overall survival not extending beyond 12 months in the majority of trials [2]. Recent large-scale sequencing projects have improved insights into the genomic landscape of the disease. The 2014 Cancer Genome Atlas (TCGA) analysis described four different subtypes of gastric cancer, with the most common chromosomal instability (CIN) subtype being characterised by chromosomal instability, aneuploidy, and, in many cases, focal amplification of receptor tyrosine kinases. The genomes of these cancers harbour multiple DNA somatic copy number alterations (SCNAs), defined as deviations in the number of whole chromosomes, chromosome arms, or fragments from the normal number of two copies per cell. With the exception of *p53* mutations, which occur in 70–80% of oesophagogastric adenocarcinomas (OGA) of the CIN subtype, mutations in cancer driver genes are relatively rare in these cancers, and SCNAs are considered the predominant type of genetic driver alterations [3,4]. Common SCNAs identified in CIN tumours in these landmark sequencing studies include amplifications of chromosomal regions harbouring genes encoding for receptor tyrosine kinases, or their ligands such as *ERBB2*, *EGFR*, and *VEGFA*, as well as those involved pathways regulating proliferation (*MYC*) and cell cycle (*CCNE1*, *CCND1*, and *CDK6*). These SCNAs have been implicated as key and, in the case of *ERBB2*/HER2, clinically actionable drivers in OGA [5,6].

The CIN subtype is common among gastric cancers arising proximally from the oesophagogastric junction or cardia [3] and in oesophageal adenocarcinomas [4]. The 'genomically stable' subtype is characterised by few SCNAs and associated with the diffuse histological subtype of gastric cancer that commonly arises more distally from the stomach body [3]. The incidence of noncardia gastric adenocarcinomas is declining in Western populations, whilst that of junctional and oesophageal adenocarcinomas is increasing [7]. These tumours are predominantly of the CIN subtype, and thus detection of SCNAs, in particular the clinically and biologically relevant driver events within these complex profiles, are important for the ongoing development of new biomarkers and therapies.

SCNAs have traditionally been analysed through microarray-based techniques, although more recently improved sensitivity for SCNA detection has been achieved through exome or whole genome sequencing (WGS). However, because of cost, long turnaround times, and intensive bioinformatics analysis requirements, such large-scale genomics analyses are often not feasible. Low-coverage WGS (lcWGS), using a coverage of only 0.1–0.5× (i.e., where only 10–50% of the genome is sequenced), has been shown to be sufficient for reliable detection of SCNAs with recent data showing superior SCNA calling compared to older array hybridisation-based standards [8]. Crucially, lcWGS can also be applied to analyse tumour-derived circulating free (cf)DNA extracted from the plasma of cancer patients [9]. Such liquid biopsies offer clear, practical advantages over conventional biopsies, including the minimally invasive nature of sample acquisition, relative ease of standardisation of sampling protocols, and the ability to obtain repeated samples over time. The latter is of particular interest, as changes in SCNA profiles over the course of treatment may shed light on response and resistance mechanisms to existing chemotherapy agents as well as to novel targeted agents and immunotherapies.

Intratumour heterogeneity is recognised as a major challenge in the delivery of effective molecular targeted treatment in OGA [10,11]. Copy number variation of molecular targets, as assessed in both tumour and cfDNA, has been shown to impact on therapeutic targeting of *ERBB2*, *FGFR*, and *EGFR*, with high level amplifications being associated with more favourable responses [12–14]. Application of targeted genomic sequencing to cfDNA analysis has been shown to allow the detection of mutations that are heterogeneous within OGA [15,16]. Such liquid biopsy techniques may also facilitate tracking of genetic profile changes over time, but this has not been applied to OGAs undergoing systemic therapy.

We applied lcWGS to cfDNA from 30 patients with advanced OGA to investigate whether SCNA analysis can predict responses to first-line chemotherapy and how these profiles may evolve during chemotherapy treatment.

2. Results

The clinical and pathological characteristics of the 30 included patients are summarized in Table 1. Extracted cfDNA concentrations from plasma samples taken at pretreatment baseline ranged from 1.37 to 74.04 ng/mL with a median of 8.88 ng/mL. With a minimum input quantity of 5 ng for lcWGS, sufficient cfDNA was available from all 30 patients. Univariate analysis showed that the presence of the primary tumour in situ was associated with a significantly increased cfDNA concentration (Table 2, 9.66 vs. 4.81 ng/mL, $p = 0.0027$, Mann–Whitney test). The cfDNA concentration was numerically higher in patients with liver metastases vs. those without liver metastases (10.09 vs. 6.80 ng/mL, $p = 0.1306$, Mann–Whitney test), but this was not significant. No other clinical or pathological parameters were associated with pretreatment cfDNA concentration.

Table 1. Clinical characteristics of included patients.

Histopathological Variable		
Number of Cases:		30
Anatomic site of primary:	Gastric	6 (20%)
	OGJ/oesophageal	24 (80%)
Histological subtype:	Intestinal	28 (93%)
	Diffuse	2 (7%)
Clinical stage at presentation:	Locally advanced	3 (10%)
	Metastatic	27 (90%)
HER2 status *:	Positive	6 (20%)
	Negative	24 (80%)
First line chemotherapy:	Platinum/fluoropyrimidine doublet	9 (30%)
	Doublet+ anthracycline	15 (50%)
	Doublet+ trastuzumab	6 (20%)
Metastatic sites: Liver	Yes	16 (53%)
	No	14 (47%)
Peritoneal	Yes	6 (20%)
	No	24 (80%)
Lung	Yes	8 (27%)
	No	22 (73%)
Number of metastatic organ sites:	0–1	22 (73%)
	≥2	8 (27%)
Primary tumour in situ:	Yes	23 (77%)
	No	7 (23%)
CA19-9 secretor:	Yes	15 (50%)
	No	15 (50%)

* defined as HER2 immunohistochemical (IHC) +++ on baseline diagnostic specimen from patient clinical records; OGJ—oesophagogastric junction.

Sequencing was performed with 100 bp single-end reads and a target of 12 million reads per sample. The ichorCNA bioinformatics package [17] was used to reconstruct copy number profiles from sequencing data and to estimate the fraction of cfDNA that was derived from tumour cells (henceforth denoted as circulating tumour (ct)DNA content). Based on ichorCNA analysis, 7/30 cases (23.3%) had ctDNA content of zero, leaving 23 cases (76.7%) in which SCNA analysis could be performed. The seven cases with zero tumour content included all three tumours that were only locally advanced rather than metastatic in this cohort (Cases 2, 152, and 195). The other four (57.1%) cases with zero

tumour content had metastatic disease involving only a single organ site (Cases 52, 66, 119, and 144). The ctDNA content showed a poor correlation with the total cfDNA concentration in the plasma (Figure 1A, Pearson correlation $r^2 = 0.2312$), suggesting that the release of ctDNA from tumour cells and the total amount of cfDNA, which was a mix of DNA from malignant and nonmalignant cells, were largely independent from each other. The presence of the primary tumour in situ (9.1% vs. 0% median ctDNA content, $p = 0.0046$, Mann–Whitney test) and the presence of liver metastases (18.0% vs. 7.2% median ctDNA content, $p = 0.0043$, Mann–Whitney test) significantly correlated with higher ctDNA content (Table 2 and Figure 1B). A greater ctDNA content was also observed in oesophageal and junctional tumours compared to gastric tumours (9.3% vs. 3.3% median ctDNA content, $p = 0.0103$, Mann–Whitney test).

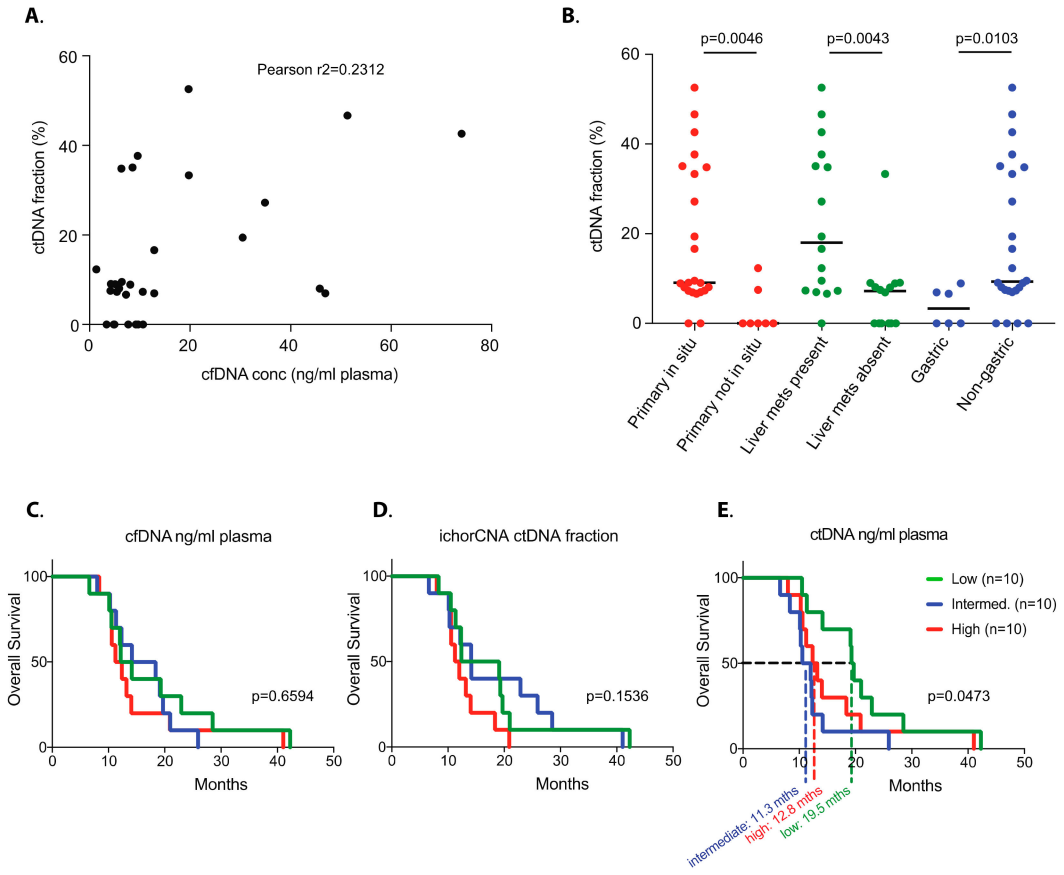

Figure 1. (**A**) No correlation between circulating free (cf)DNA concentration and the tumour-derived cfDNA fraction in 30 plasma samples from patients with treatment naïve metastatic gastro-oesophageal cancers. (**B**) Correlation between selected clinical features and circulating tumour (ct)DNA fraction (line denotes median; *p*-value Mann–Whitney test). (**C**) Kaplan–Meier survival analyses of pretreatment samples grouping by high/intermediate/low cfDNA yield ng/mL plasma, (**D**) ichorCNA ctDNA fraction, and (**E**) ctDNA concentration ng/mL plasma (*p*-values Log-rank (Mantel–Cox) test).

Taken together, copy number profiles could be analysed from cfDNA in 76.7% of cases and three distinct characteristics (primary tumour in situ, presence of liver metastases, and oesophageal/junctional primary tumour location) associated with high ctDNA content, with liver metastases showing the highest tumour fraction of 18% (median).

We next investigated whether any pretreatment cfDNA metrics correlated with overall survival (OS). Neither the total cfDNA concentration extracted from plasma (Figure 1C) nor the ctDNA content estimated by ichorCNA (Figure 1D) correlated with overall survival. However, the absolute ctDNA concentration in the plasma revealed a significant overall survival (OS) difference (Figure 1E). The third

of patients with the lowest absolute ctDNA concentration (mean 0.09 ng/mL) had a median OS of 19.5 months, whereas those with intermediate (mean 0.92 ng/mL) and high (mean 10.12 ng/mL) absolute ctDNA concentrations had median OSs of 11.3 and 12.8 months, respectively.

Table 2. Correlation of cfDNA concentration, median ichorCNA ctDNA fraction, and ctDNA concentration with clinical and laboratory variables (*p*-values Mann–Whitney test).

Histopathological Variable		*n*	Median cfDNA Concentration (ng/mL Plasma)	*p*-Value	Median ctDNA Fraction (%)	*p*-Value	Median ctDNA Concentration (ng/mL Plasma)	*p*-Value
Primary tumour in situ	Yes	23	9.66	0.0027	9.10	0.0046	2.14	<0.0001
	No	7	4.81		0.00		0.00	
Liver metastases present	Yes	16	10.09	0.1306	18.01	0.0043	2.18	0.0099
	No	14	6.80		7.23		0.35	
Primary tumour anatomic site	Gastric	6	8.65	0.8996	3.33	0.0103	0.24	0.1401
	Nongastric	24	9.05		9.31		0.84	
No. of metastatic organ sites	0–1	22	8.31	0.5042	7.77	0.1528	0.47	0.9814
	≥2	8	1.22		14.47		0.58	
HER2 status	Positive	6	11.22	0.3739	8.81	0.4595	2.25	0.1713
	Negative	24	8.32		8.22		0.47	
CA19-9 secretion	Yes	15	9.21	0.9999	8.10	0.5640	0.61	0.7733
	No	15	8.54		9.02		0.78	

We next investigated whether any specific copy number aberrations or chromosomal instability metrics correlated with subsequent responses to chemotherapy (Figure 2A,B). The frequency of copy number gains or losses in 13 responders (based on best radiological response assessment with serial CT scans during treatment) (Figure 2C) was compared to those in 10 nonresponders who had stable or progressive disease as best response (Figure 2D). Frequency plots showed an overall similar appearance in both groups; however, several chromosomes showed alterations that were unique to the responders (Figure 2E) and not present in the nonresponder group (Figure 2F). Gains of chromosomes 2q and 8p were the most frequent (>1/3 of cases) unique aberrations observed only among responders (Figure 2E). A minimal consistent region of 28 Mb on Chr2q encompassing 182 genes was observed in five of 13 cases (34, 63, 68, 134, and 207). These 2q gains were in four cases a single copy number gain relative to ploidy. A 7.5 Mb minimal consistent region on Chr8p encompassing 17 genes (Appendix A Table A1) was detected in six cases (34, 45, 68, 99, 143, and 183), four of which were multiple copies above ploidy. Of the uniquely gained genes, *MCPH1* (microcephalin) is notable as a key regulator of DNA damage response and a repressor of human telomerase reverse transcriptase function [18], and gains of *MCPH1* have been implicated in increased platinum sensitivity in nonsmall cell lung cancer [19] (Figure 2G). Chr8p also harbours *GATA4*, which is frequently gained or amplified in OGA [4,20], but this was located outside the unique region, as gains of *GATA4* were observed in both responders and nonresponders (Figure 2G). Other uniquely altered regions were less frequent and, hence, difficult to assess (Figure 2E). In contrast, only a single loss of a 12 Mb minimal consistent region encompassing 117 genes on Chr1p in four cases (123, 126, 90, and 158) was unique to the nonresponder group (Figure 2F).

Chromosomal instability (CIN) has been associated with poor outcomes and treatment responses in several cancer types [21,22]. We hence assessed whether CIN metrics including the weighted genomic instability index (wGII) [23,24] (Figure 3A), the number of gained or lost chromosomal segments (Figure 3B), or ploidy (Figure 3C), associated with responses or could predict survival in our cohort. None of these metrics showed a significant difference in responders vs. nonresponders or an association with progression-free (Figure 3D–F) or overall survival (Figure 3G–I). Taken together, the presence of Chr2q and 8p gains in pretreatment ctDNA showed an association with chemotherapy responses. In contrast, we could not identify a role of CIN metrics to predict patient outcomes in OGA.

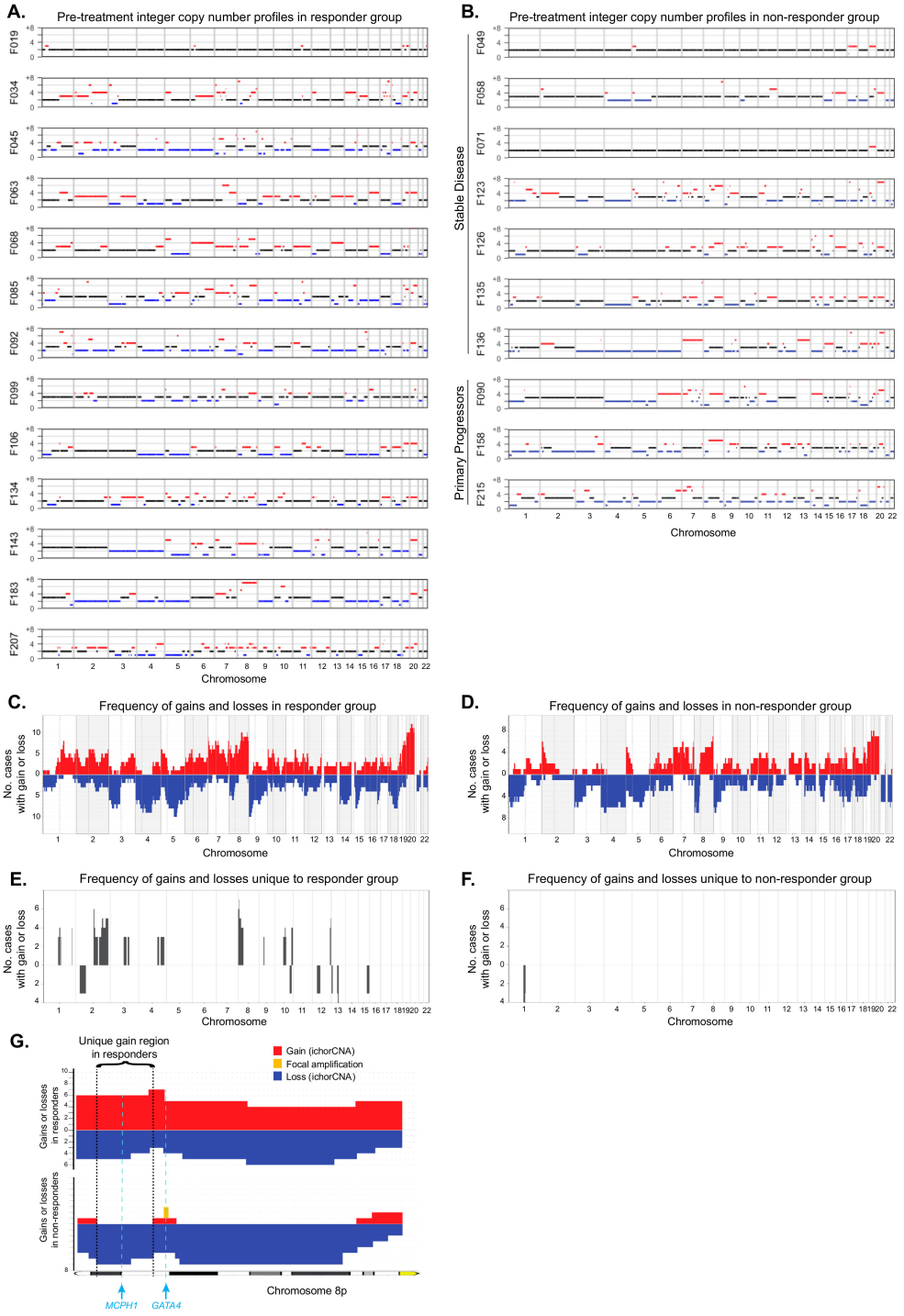

Figure 2. (**A**) Integer copy number profiles (500 kb bins) for pretreatment samples, grouped by subsequent response or (**B**) nonresponse to treatment. Red = gain, blue = loss, and black = ploidy. (**C**) Frequency plots showing the number of cases that show segment gains (red) or losses (blue) in the responder and (**D**) nonresponder groups. (**E**) Frequency plots showing segment gains and losses that are unique to the responder group or (**F**) nonresponder group. (**G**) Frequency of gain (red) and loss (blue) segments of chromosome 8p in the responder group (top) and nonresponder group (bottom). The most frequent region of unique 8p gain is indicated, bounded by dotted lines. The locations of *MCPH1* and *GATA4* are delineated with a blue dashed line. Two additional nonresponder cases showed focal amplifications (orange) of *GATA4*, which were identified with the 50 kb bin method but not the 500 kb ichorCNA analysis.

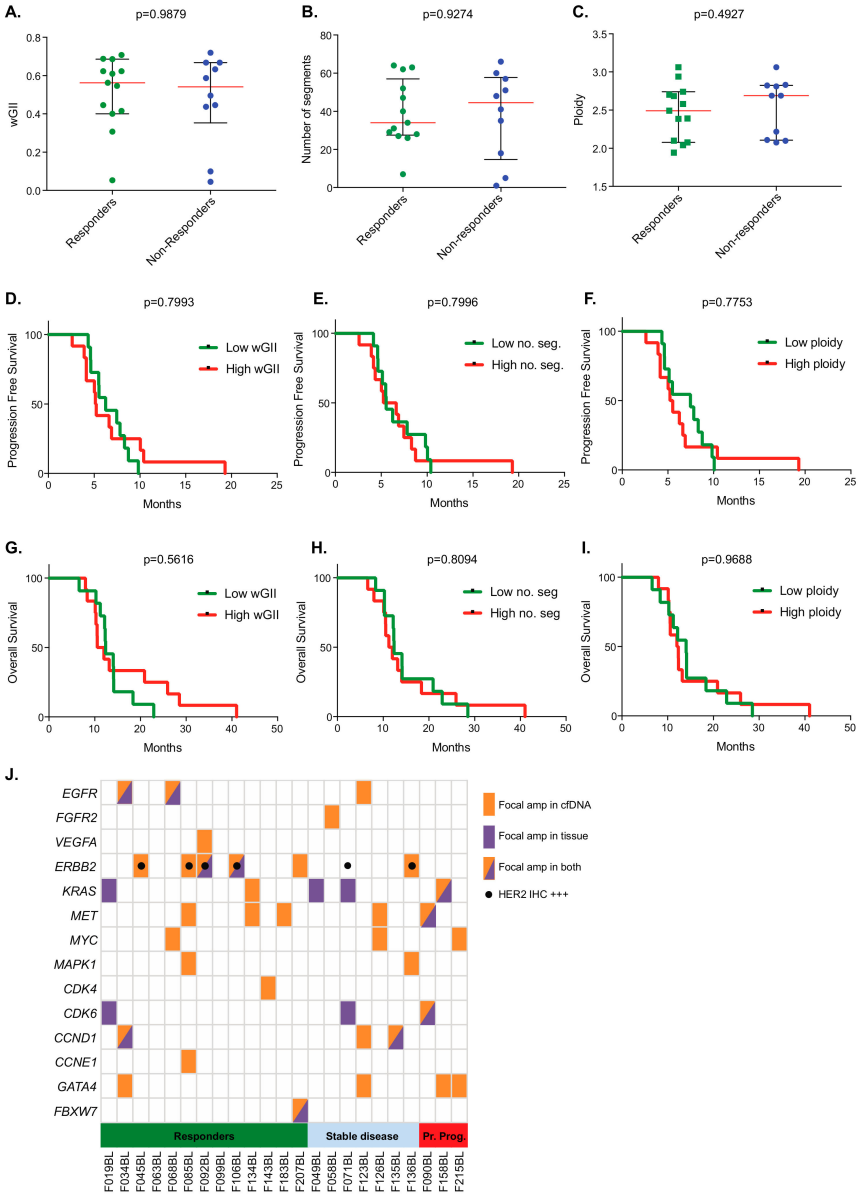

Figure 3. (**A**) Association of pretreatment chromosomal instability (CIN) metrics with subsequent treatment response by comparing analysis of genomic change relative to ploidy using weighted genomic instability index (wGII), (**B**) nonploidy segment number, and (**C**) ploidy between responder and nonresponder groups (line denotes median and interquartile range; *p*-value Mann–Whitney test). (**D**) Kaplan–Meier progression free survival analyses grouping by high/low wGII, (**E**) nonploidy segment number, and (**F**) ploidy. (**G**) Kaplan–Meier overall survival analyses grouping by high/low wGII, (**H**) nonploidy segment number, and (**I**) ploidy. (**J**) Heatmap showing focal gene amplifications (50 kb bins) detected by cfDNA lcWGS at pretreatment (orange) or by archival target sequencing (purple) in each case. Black dots indicate cases classed as HER2+ by immunohistochemistry. Green = responder group, blue = stable group, and red = primary progressor group.

The ichorCNA analysis divides chromosomes into 500 kb large bins to robustly assess the copy number state of these segments. Focal genomic amplifications are often narrow [4] (down to a few dozen kbps) and may have been overlooked as a consequence. Therefore, to further interrogate whether focal amplifications could be detected in the lcWGS data, we applied a 50 kbp bin approach [25]. This revealed narrow high-level amplifications of several OGA driver genes [3,4] (Figure 3J). Any of the high-level amplifications (*EGFR*, *ERBB2*, *KRAS*, *MET*, *MYC*, *MAPK1/ERK2*, *CCND1*, and *GATA4*)

that were observed in two or more cases were detected in both responders and in nonresponders. Several others were only observed once and were, hence, too rare to draw any conclusions. Thus, high-level amplifications detected pretreatment were not associated with chemotherapy responses.

As part of the FOrMAT clinical trial, archival formalin-fixed paraffin-embedded diagnostic or resection samples were sequenced with a custom panel, which targeted 46 genes that had prognostic or predictive significance or were potential targets in existing or upcoming clinical trials [26]. Amplifications of *EGFR*, *CCND1*, *CDK6*, *MET*, *ERBB2*, *KRAS*, and *FBXW7* had been identified in tissue samples from 11 cases (19, 34, 49, 68, 71, 90, 92, 106, 135, 158, and 207). No amplifications were observed in nine cases, and archival target sequencing failed in three cases (45, 58, and 123). cfDNA lcWGS of pretreatment plasma reidentified all gene amplifications found by archival tumour sequencing in eight cases (Figure 3J). Compared to tissue sequencing, ctDNA analysis could not detect *CDK6* and/or *KRAS* amplifications in three cases that had low ctDNA content (Case 19: 9.1%; Case 49: 7.3%; and Case 71: 8.1%). Importantly, in seven cases, cfDNA lcWGS identified additional amplifications of genes that were included in the FOrMAT sequencing panel but for which no amplification was detected in the archival tissue analysis: Case 85 (*MET* and *ERBB2* amplification in plasma), Case 126 (*MET*), Case 134 (*MET*, *KRAS*), Case 136 (*ERBB2*), Case 143 (*CDK4*), Case 183 (*MET*), and case 207 (*ERBB2*). In addition, cfDNA sequencing identified 11 amplifications (in nine cases) of genes that were not covered by the FOrMAT panel including *GATA4*, *VEGFA*, and *MYC*.

Of six cases (45, 71, 85, 92, 106, and 136) that had been classified as HER2-positive based on standard immunohistochemistry testing of archival tissue, cfDNA sequencing detected *ERBB2* amplifications in five cases. Archival tissue sequencing had identified *ERBB2* amplifications in only two of five successfully sequenced cases (Figure 3J). In one case (71), immunohistochemical (IHC) analysis of archival tissue had identified HER2 positivity, but no amplification was detected by either archival tumour sequencing or cfDNA lcWGS. Three of the *ERBB2* amplified cases (85, 92, and 136) had concurrent amplifications in *MAPK1*, *MET*, or *VEGFA* in the cfDNA (Figure 3J).

lcWGS was applied to cfDNA collected at the time of radiological progression, during or after first line treatment, from 20 patients that had detectable ctDNA pretreatment profiles and had a post-treatment sample available. Twelve of these had an initial radiological response with subsequent disease progression (primary responders). Eight showed stable disease or primary progression during chemotherapy (primary nonresponders). In the primary responder group, the ichorCNA ctDNA fraction at progression was significantly lower than at pretreatment (17% vs. 7.6%, $p = 0.02$, Table 3), whereas no significant change was observed in the primary nonresponder group. Only three out of twenty samples taken at progression had a ctDNA content of zero (Cases 68, 99, and 183), showing that ctDNA remained detectable in the majority of tumours. The copy number profiles of the remaining 17 cases (Appendix A Figure A1) were assessed for changes over the course of chemotherapy treatment (Figure 4A). Using the 50 kb bin approach, all focal amplifications present before treatment were reidentified at progression. No new focal amplifications were identified at progression.

Table 3. Comparison of ichorCNA estimated ctDNA fraction at pretreatment and progression of first line chemotherapy (*p*-values Mann–Whitney test).

Paired Pretreatment and Progression Cases		*n*	Median ctDNA Fraction (%)	*p*-Value
All paired cases	Pretreatment	20	15.18	0.1567
	Progression	20	8.72	
Initial radiological response followed by progression to chemotherapy: 'primary responders'	Pretreatment	12	17.00	0.0200
	Progression	12	7.59	
Stable disease or primary radiological progression to chemotherapy: 'primary nonresponders'	Pretreatment	8	11.27	0.7984
	Progression	8	13.58	

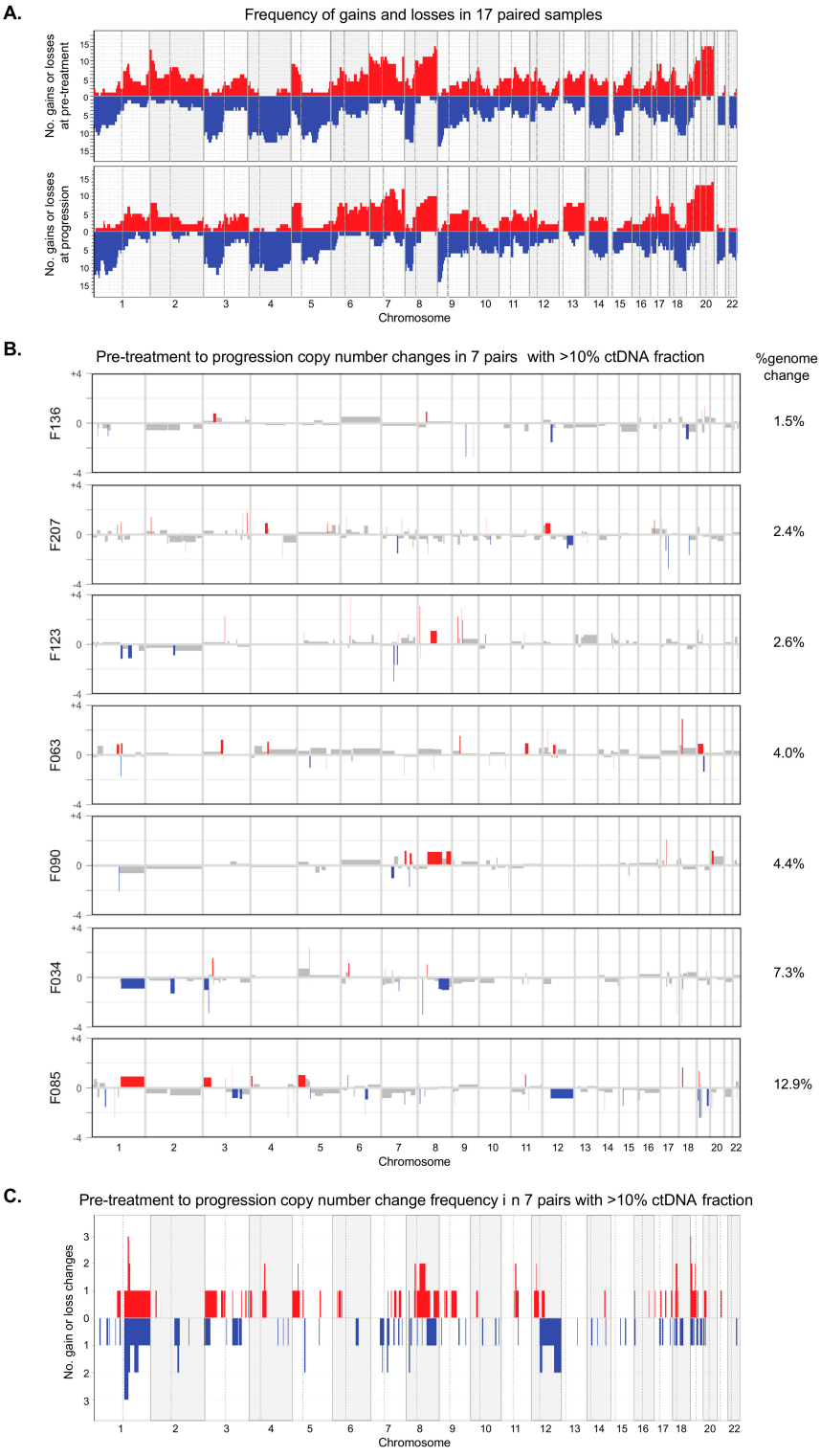

Figure 4. (**A**) Frequency plots showing the number of cases (n = 17) that show segment gains (red) or losses (blue) at pretreatment (top) and at progression (bottom). (**B**) For 7 pairs where both samples had >10% ctDNA fractions, comparative plots show absolute copy number gains and losses at progression relative to pretreatment, ordered by the extent of genomic change. The percent genomic change for each sample is indicated to the right of each plot. Red = gain, blue = loss, and black = no change. A minimum of 0.8 copy number change was required to score a gain or a loss. (**C**) Frequency plot showing the number of cases (n = 7) that show segment gains (red) or losses (blue) at progression relative to pretreatment.

In a second approach, we subtracted the pretreatment absolute copy number (generated with ichorCNA) from the absolute copy number in the matched progression sample to assess which chromosomes changed through chemotherapy. To avoid artefacts from differences in tumour content, this pairwise comparison was only performed in seven cases where tumour content was similar and above 10% at both pre-treatment and progression. Only changes of the integer copy number value exceeding +/−0.8 were considered; this was to enrich for new aneuploidies that had likely occurred in the majority of cells in the tumour and to avoid overinterpretation of changes in small subclones. The SCNA profiles were overall similar before treatment and at progression, but multiple individual segmental and arm-level changes were observed (Figure 4B). The fraction of the genome that changed (defined as the percent of the total genomic length that changed) was higher in responders (median: 5.65%, $n = 4$) than in nonresponders (median: 2.6%, $n = 3$, Figure 4B), but this was not statistically significant. Individual cases showed new gains or losses in multiple chromosomes. However, most of the genomic regions that changed between pretreatment and progression were only observed in a single case, and no large regions were changed in more than two cases (Figure 4C).

3. Discussion

Through use of liquid biopsy, we successfully analysed the SCNA profiles of 76.7% of 30 advanced OGAs. Serial analyses before and after first line chemotherapy were feasible in 85% of cases (17/20) that had detectable ctDNA prior to treatment. This demonstrates proof of concept that lcWGS of cfDNA can reveal genome-wide SCNA profiles in the majority of patients with advanced OGA to, for example, investigate novel prognostic or predictive biomarkers.

We identified several clinical characteristics that should support the selection of patients with predictably higher cfDNA analysis success rates in future studies: the presence of liver metastases was associated with the highest ctDNA concentrations, whilst the ctDNA concentration was also significantly higher if the primary tumour was in situ. This may be the result of more aggressive tumours presenting with synchronous metastatic disease at baseline compared to those with metachronous metastases following resection. All seven cases with zero ctDNA pretreatment either only had locally advanced disease or low metastatic burden. The use of such biomarkers to select OGA patients who are suitable for cfDNA sequencing may allow prioritizing these for liquid biopsy-based genotyping over sequencing of OGA tumour tissue, which has had moderate reported success rates because of technical challenges such as frequent low-tumour content in endoscopic biopsies [26,27]. With readily assessable clinical characteristics to identify suitable patients, cfDNA analysis could become the method of choice to assess amplifications for molecular stratification and, particularly, to longitudinally investigate SCNA evolution.

Neither pretreatment total cfDNA concentration nor ctDNA tumour content correlated with survival; however, a low absolute plasma ctDNA concentration was significantly associated with better OS. A previous gastric cancer case series described an association between baseline cfDNA and both relapse risk and adverse prognosis in the advanced disease setting [28]; however, larger studies are needed to validate the clinical utility of such metrics for optimisation of treatment and surveillance strategies [29].

High chromosomal instability (CIN) has been linked to poorer prognosis and drug sensitivity across a range of cancer types and to drug resistance in vitro [22,30]. Application of several CIN metrics could not identify a correlation with chemotherapy response or survival in our cohort. This could indicate that CIN metrics may perform less well when generated from ctDNA, as this samples a summative copy number profile of the entire cancer population. Alternatively, these metrics may only weakly correlate with aggressiveness and treatment sensitivity and specific genetic aberrations, acquired as a consequence of CIN, which may be more relevant in determining the response and outcome of individual tumours. Although studies of larger cohorts may be able to reveal an association in the future, our results suggest that analysis of these CIN metrics in ctDNA is unlikely to be useful to predict individual patient outcomes in unselected patients undergoing first line chemotherapy.

For patients with evaluable ctDNA, multiple SCNAs could be identified in genes that were currently clinically relevant or might become relevant to future practice. In samples with detectable ctDNA, we identified all amplifications that had been found by previous targeted sequencing of matched FFPE tissue samples [26]. In seven cases, lcWGS found an additional nine focal amplifications in genes that had been analysed by targeted sequencing in tissue (*ERBB2*, *MET*, *KRAS*, and *CDK4*) and where no amplification had been called. In three cases where tumour tissue sequencing failed, amplifications in *ERBB2*, *FGFR2*, *EGFR*, and *CCND1* were identified in ctDNA. Furthermore, lcWGS revealed multiple additional amplifications of potentially targetable driver genes such as *VEGFA*, highlighting the advantage of whole genome approaches over predetermined targeted sequencing gene sets.

Concurrent pretreatment amplifications of *MAPK1*, *MET*, or *VEGFA* with *ERBB2* were seen in 3/6 HER2-positive cases. These may potentially influence variability of outcomes to HER2-targeted therapy, as amplifications of *MET* and *MAPK1* have previously been implicated in trastuzumab resistance [31,32]. However, the limited numbers in this cohort precluded meaningful survival analyses.

Comparison of pretreatment SCNA profiles revealed gains of chromosomes 2q and 8p in cases that subsequently responded to treatment, and these gains were absent in nonresponders. These need to be investigated in larger cohorts to assess their potential role as predictive biomarkers. The uniquely gained region on chromosome 8p harbours the DNA damage regulator *MCPH1*, which has been suggested to increase sensitivity to platinum chemotherapy [19]. This is, therefore, a candidate gene for further investigation. Identifying predictive biomarkers of chemotherapy response is an unmet need. To date, the most extensive study of genetic predictors of therapy response using targeted sequencing of tumour tissue in advanced OGA failed to identify any biomarkers of response to platinum-based chemotherapy [27].

Both ctDNA detection and lcWGS were possible from plasma samples taken at the timepoint of progression on first line chemotherapy, with 17/20 (85%) cases having detectable ctDNA. SCNA profiles were relatively stable between the pretreatment and progression samples, but segmental and whole chromosomal arm changes were detected in seven cases where pair-wise comparisons were quantifiable. As it was unlikely that multiple subclones within a cancer would all gain or lose the same chromosomal regions, these copy number changes suggested that there had been major shifts in the clonal composition of the tumour cell populations, where one or a few subclones became dominant whereas others had been lost. lcWGS may, therefore, be a useful technology for the investigation of resistance landscapes in larger cohorts. The lack of recurrent copy number change events at progression in this study may be a result of the small evaluable cohort, but equally it is feasible that chemotherapy resistance may be driven by point mutations. Use of a higher resolution technique that will allow the combined analysis of SCNA and mutations (such as whole exome cfDNA sequencing) may be warranted, with patient selection based on the presence of liver metastases to maximise successful sequencing rates and cost efficiency. Longitudinal cfDNA analysis has become a favoured method to interrogate resistance mechanisms during treatment, such as the tracking of known oncogenic *RAS* mutations in colorectal cancer [33]. Dynamic cfDNA testing should be equally applicable to monitor resistance to therapy in OGA.

The potential clinical application for this technique lies in the feasibility of biomarker stratification on the basis of lcWGS cfDNA sequencing, circumventing some of the limitations related to tumour heterogeneity in OGA [13]. Furthermore, sequential lcWGS of cfDNA is a low-cost method for continuing to investigate genetic changes associated with chemotherapy response in larger series or for early detection of resistance mechanisms to novel agents in clinical trials. Preliminary proof of concept for the use of longitudinal cfDNA analyses to predict response and resistance to HER2-targeting treatment has already been described [34]. *ERBB2* copy number alterations detected by targeted sequencing were found to be associated with both innate and acquired trastuzumab resistance. Additionally, mutations in genes, including *PIK3CA*, *ERBB2*, and *ERBB4*, were also associated with resistance, highlighting the benefit of combined mutation identification and SCNA analysis

in interrogating drug resistance mechanisms. Detection of relevant gene amplifications in cfDNA has been already shown to be clinically important for patient selection and therapeutic targeting of *FGFR* in gastric cancer [13]. However, plasma contains multiple components in addition to cfDNA that could also be utilised to realise the full potential of the liquid biopsy. Promising techniques under investigation in OGA include the enumeration and characterization of circulating tumour cells (CTCs), which have been associated with both prognosis [35] and treatment response [36]. In prostate cancer, mRNA extracted from CTCs has been used to identify splice variants of the androgen receptor that are prognostic for taxane therapy [37]. Furthermore, CTCs from small cell lung cancer have been successfully cultured ex vivo in order to screen for targeted therapy sensitivity and relevant biomarkers [38,39]. As an alternative to CTCs and cell-free nucleic acids, exosomes may also provide a means for tumour profiling, including in OGA [40].

As novel targeted and immune-modulating therapies are introduced into clinical management of OGA, there will be a need for stratification of patients in order to guide personalised treatment. The use of genome-wide analysis to interrogate key driver events and genomic evolution over time will be important in refining the effective biomarker stratification of such treatments moving forward. It is possible that a combination of lcWGS cfDNA sequencing with CTC or exosome analyses will facilitate maximal clinical utility to be gained from liquid biopsy approaches in order to guide treatment decisions. Ultimately this may support precision medicine in both trial and routine clinical practice settings by avoiding the cost, delay, and clinical complications of repeated invasive biopsy procedures.

4. Methods

4.1. Trial Design and Sample Collection

The FOrMAT (Feasibility of a Molecular Characterisation Approach to Treatment, Chief Investigator: N Starling ClinicalTrials.gov NCT02112357) study enrolled patients with advanced gastrointestinal malignancies treated at the Royal Marsden from February 2014 to November 2015 [26]. The trial was approved by the UK National Ethics Committee (approval number: 13/LO/1274RM), and all patients provided written informed consent. As part of the tissue collection component of the trial, blood samples were obtained at trial entry and at the timepoint of response assessment CT scans during treatment. The trial recruited 71 advanced OGA cancer patients in total. The clinical trial database was interrogated to identify 30 patients with a diagnosis of locally advanced inoperable or metastatic OGA. These patients had undergone baseline research blood sampling prior to commencement of treatment, and had sequential bloods spanning at least the full course of comparable first-line systemic chemotherapy. This consisted of a platinum/fluoropyrimidine doublet in all cases, plus or minus anthracycline or, in the case of *ERBB2*-positive tumours, trastuzumab. cfDNA was extracted from plasma samples taken at a baseline pretreatment timepoint for all patients. To assess the evolution of SCNA profiles through treatment, lcWGS was additionally performed on cfDNA collected at the time of radiological progression during or after first line platinum and 5FU-based combination chemotherapy from 20 patients that had detectable ctDNA pretreatment profiles and had a post-treatment sample available.

4.2. Circulating Free (cf)DNA Extraction and Quantification

Plasma was separated within 2 h of blood draw and frozen at −80 °C. The QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) was used to isolate cfDNA from 3–4 mL plasma according to manufacturer's instructions. cfDNA within a size range of 100 to 700 bp was quantified using a Bioanalyzer High Sensitivity chip (Agilent, Santa Clara, CA, USA), encompassing the predominant three cfDNA fragment peaks [41].

4.3. Low-Coverage Whole Genome Sequencing (lcWGS)

For the majority of cases, 10 ng of input DNA was used for sequencing, although 5 ng was used in some cases with limited yield [42]. Libraries were prepared using the NEBNext Ultra DNA Library Prep kit (NEB, Ipswich, MA, USA), which were pooled and sequenced on an Illumina HiSeq2500 in Rapid mode single read 100 bp.

4.4. Somatic Copy Number Aberration (SCNA) Analysis

Sequencing reads were aligned to the human reference genome (hg19) using Bowtie (v1.2.9) [43], and resultant bam files were deduplicated using Picard MarkDuplicates (http://picard.sourceforge.net; v.2.1.0). Reads were subsequently assigned to nonoverlapping 500 kb bins and normalized to correct for GC content and mappability bias using the HMMcopy suite (http://compbio.bccrc.ca/software/hmmcopy/) [44]. IchorCNA [17] was used to quantify tumour fraction in cfDNA from lcWGS without prior knowledge of somatic single nucleotide variants (SSNVs) or SCNAs present in the primary tumour sample. IchorCNA segmented data were normalised using the best-fit tumour content and ploidy solution in order to compare samples. To compare multiple samples, data were uniformally segmented using interpolate.pcf, which was part of the copynumber package in R (http://bioconductor.org/packages/copynumber/) [45]. Cohort frequency plots were generated using the copynumber plotFreq function. Seg files were viewed as a heat map using the Integrated Genome Viewer (IGV) software (Broad Institute, Cambridge, MA, USA; v.2.3.97), allowing comparison of genomic SCNA profiles across multiple samples with the ability to zoom in to areas of interest in order to investigate genes located within this genomic region [46]. Focal SCNAs were identified by assigning mapped reads to 50 kb bins using the method described by Baslan [25]. SCNAs were assessed in IGV by two independent observers and recorded for all patients.

4.5. Survival Analyses by Pre-Treatment Circulating DNA Metrics

Tertile survival analyses were undertaken according to three circulating DNA metrics: (1) total cfDNA concentration extracted from plasma, (2) ctDNA content estimated by ichorCNA, and (3) absolute ctDNA concentration in the plasma, calculated by multiplying the total cfDNA concentration with the ichorCNA ctDNA content. In each case, the 30 samples were classified into 'low', 'medium', and 'high' tertiles for each metric, and the overall survival trend was analysed using the log-rank method.

4.6. Data Availability

Sequence reads have been deposited in the European Genome Phenome Archive (ID: submission ongoing—will be updated as soon as ID assigned).

5. Conclusions

SCNA profiles were successfully analysed through the use of lcWGS applied to cfDNA extracted from pretreatment baseline plasma samples in 23/30 (76.7%) cases. The presence of liver metastases, primary tumour in situ, and oesophageal or junctional primary tumour site were associated with higher pretreatment ctDNA content, and a lower baseline ctDNA concentration was associated with subsequent improved overall survival. Concordance was noted with prior targeted tumour sequencing results. Additionally, lcWGS revealed additional amplifications of potentially targetable driver genes, highlighting the advantage of whole genome approaches over predetermined targeted sequencing gene sets. ctDNA detection and lcWGS were possible from plasma samples taken at the timepoint of progression on first line chemotherapy, with SCNA profiles successfully analysed in 17/20 (85%) cases. Although SCNA profiles were relatively stable between pretreatment and progression, segmental and whole chromosomal arm changes were detected in seven cases where pair-wise comparison was quantifiable. Such shifts in the clonal composition of tumour cell populations during chemotherapy

warrant further investigation as a possible dynamic means of investigating resistance landscapes in OGA.

Author Contributions: Conceptualization, M.D., L.J.B., and M.G.; Formal analysis, M.D., L.J.B., A.W., and M.G.; Funding acquisition, N.S. and M.G.; Investigation, M.D., L.J.B., C.C., S.M., and B.G.; Methodology, L.J.B. and I.A.; Project administration, M.G.; Resources, S.-Y.M., I.R., R.B., N.M., S.R., D.W., I.C., D.C., and N.S.; Writing—original draft, M.D., L.J.B., and M.G.; Writing—review & editing, N.S.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Genes in frequently gained region of chromosome 8p in responders.

CSMD1
LOC100287015
MCPH1
ANGPT2
CLDN23
MFHAS1
ERI1
MIR4660
PPP1R3B
LOC157273
TNKS
MIR597
LINC00599
MIR124-1
MSRA
PRSS55
RP1L1

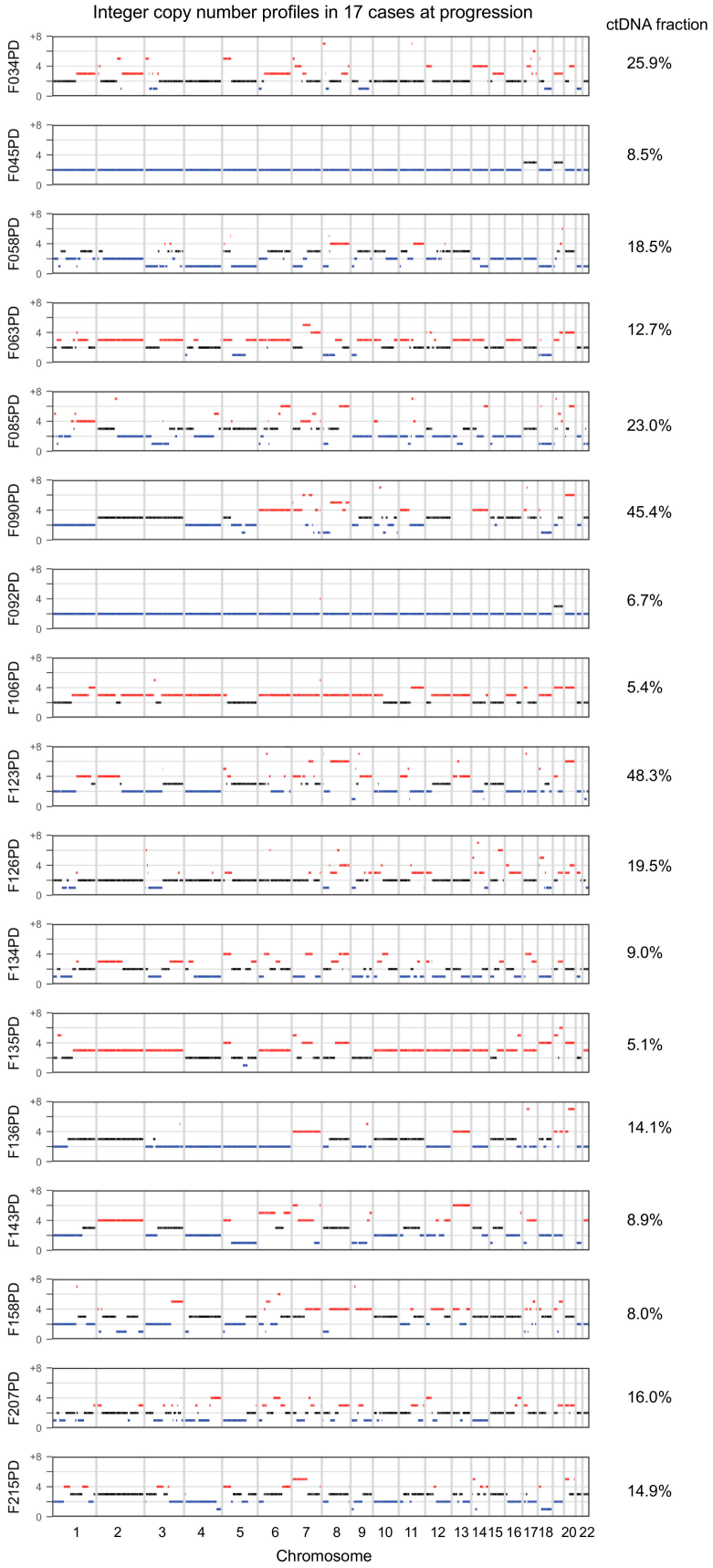

Figure A1. Integer copy number profiles for the 17 paired non-zero ctDNA cases at progression. ichorCNA ctDNA fraction is indicated for each sample.

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