

Molecular effects of Lapatinib in the treatment of HER2 Overexpressing Oesophago-Gastric Adenocarcinoma

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Abstract

Background

Lapatinib, a dual EGFR and HER2 inhibitor has shown disappointing results in clinical trials of metastatic Oesophago-Gastric Adenocarcinomas (OGA), and *in vitro* studies suggest that MET, IGFR, and HER3 confer resistance. This trial applied Lapatinib in the curative neo-adjuvant setting and investigated the feasibility and utility of additional endoscopy and biopsy for assessment of resistance mechanisms *ex vivo* and *in vivo*.

Methods

Patients with HER2 over-expressing OGA were treated for 10 days with Lapatinib monotherapy, and then in combination with 3 cycles of Oxaliplatin and Capecitabine prior to surgery. Endoscopic samples were taken for molecular analysis at: baseline including for *ex vivo* culture +/- Lapatinib to predict *in vivo* response, post Lapatinib monotherapy and at surgery. Immunohistochemistry (IHC) and proteomic analysis was performed to assess cell kinetics and signalling activity.

Results

The trial closed early (n=10) due to an anastomotic leak in 2 patients for which a causative effect of Lapatinib could not be excluded. The reduction in Phosphorylated-HER2 (P-HER2) and P-EGFR in the *ex vivo* treated biopsy demonstrated good correlation with the *in vivo* response at day 10. Proteomic analysis pre and post-Lapatinib demonstrated target inhibition (P-ERBB2, P-EGFR, P-

PI3K, P-AKT, P-ERK) which persisted until surgery. There was also significant correlation between the activation of MET with the level of P-Erk ($P=0.0005$) and P-PI3K:T-PI3K (Total PI3K) ratio ($p=0.0037$). There was no significant correlation between the activation status of IGFR and HER3 with downstream signalling molecules.

Conclusion

Additional endoscopy and biopsy sampling for multiple biomarker endpoints was feasible and confirmed *in vitro* data that MET is likely to be a significant mechanism of Lapatinib resistance *in vivo*.

Introduction

The incidence of Oesophago-Gastric Adenocarcinoma (OGA), has been increasing rapidly (CancerResearchUK, 2015), but 5 yr overall survival remains poor, and it is the 6th most common cause of cancer related death. Locally advanced (non-metastatic) tumours are treated by surgery, and the addition of chemotherapy has improved 5yr overall survival from 23% to 36% (Cunningham et al., 2008). 16.6% of OGAs over-express HER2 (Van Cutsem et al., 2014) and data suggests that this up-regulation occurs early in the process of carcinogenesis at the stage of pre-invasive Barrett's oesophagus and gastric intraepithelial neoplasia (Paterson et al., 2013a, Fassan et al., 2012). Trastuzumab, a monoclonal antibody to HER2, has shown significant activity in HER2 overexpressing gastric cancer, although the clinical use so far has been restricted to the metastatic setting (Bang et al., 2010). Lapatinib, a dual EGFR and HER2 receptor tyrosine kinase inhibitor has also been investigated in metastatic disease but response activity does not appear to be as great as for Trastuzumab. A Phase II monotherapy study showed a very modest response rate to single agent Lapatinib (Iqbal et al., 2011), and when used in combination with chemotherapy (paclitaxel) in the TyTan trial, only modest gains in survival were observed (11m vs 8.9m p=0.1044) (Sato et al., 2014). The TRIO-013/LOGiC trial, a randomised phase III trial evaluating the combination of Capecitabine/Oxaliplatin with or without Lapatinib in the 1st line treatment of metastatic OGAs demonstrated a non-significant increase in overall survival in the Lapatinib arm (overall survival of 12.2m vs 10.5m, p=0.35)(Hecht JR, 2013) whilst the recent Phase 2 trial comparing Lapatinib with or without capecitabine in the second line setting demonstrated a very modest response rates of 11% in combination with chemotherapy and no

responders in the Lapatinib monotherapy arm (Lorenzen et al., 2015). These results point to significant primary resistance mechanisms.

Targetting HER2 in gastric cancer cell lines and xenografts with tyrosine kinase inhibitors (TKIs) or monoclonal antibodies results in inhibition of the phosphorylated receptor and downstream signalling through proliferative pathways such as PI3K (Wainberg et al., 2010, Chen et al., 2012). However, signalling through alternative receptor tyrosine kinases such as MET (Hepatocyte Growth Factor Receptor), HER3, IGF1R (Insulin-like Growth Facotor Receptor-1) were noted to produce resistance to Lapatinib (Zhang et al., 2014). Inhibiting MET or IGFR activation with their corresponding TKI restored Lapatinib sensitivity which was associated with a decrease in downstream activation of Akt and Erk *in vitro*. Similar work on cell lines demonstrated that the use of TKI combinations based on RTK activation status resulted in greater inhibition of proliferation of OGA cell lines (Paterson et al., 2013b). Though these *in vitro* studies shed some insight into the mechanisms of Lapatinib resistance, they may not represent the situation *in vivo*. Studies have explored the use of patient derived xenografts and organoids for drug sensitivity testing (Dobbin et al., 2014, Sachs and Clevers, 2014), but the major disadvantage with these methods is the time required to perform these assays, which means that that results cannot be delivered in a timely manner to inform patient management. Simple and rapid methods need to be developed to provide biomarker evidence to help guide clinical decision making. Options include the use of *ex vivo* treated tumour biopsies, or repeat biopsies after window periods of therapy. This is particularly feasible for OGA which is accessible endoscopically. The Lapatinib in

Early Oesophago-Gastric cancer (LEO) trial assessed Lapatinib as first line therapy in the curative setting for OGA and aimed to:

- 1.) To assess the safety and toxicity profile of Lapatinib with chemotherapy, when used peri-operatively for patients on a radical pathway.
- 2.) To test whether an *ex vivo* assay examining the ability of Lapatinib to suppress HER2 and EGFR could predict *in vivo* response to 10 days of Lapatinib monotherapy.
- 3.) To establish if assays of downstream signalling performed on biopsies taken before and after 10 days of Lapatinib monotherapy could indicate mechanisms of resistance/sensitivity to the drug
- 4.) Correlate the molecular indicators of response to imaging criteria (functional ¹⁸FDG-PET and CT imaging) along with clinical outcome measures such as R0 resection rate, complete pathological response and overall survival.

Methods and Materials

Trial Design

This was a 2 centre open label trial of neoadjuvant treatment with Lapatinib alone and then in combination with Oxaliplatin and Capecitabine in HER2 expressing oesophageal or gastric cancers undergoing curative therapy.

All patients provided written informed consent prior to entry on the trial, and the study protocol was approved by a multi-centre research ethics committee, and a

Clinical Trial Authorisation (CTA) was granted (number 12854/0235/001-0001) with Lapatinib, Capecitabine and Oxaliplatin listed as Investigational Medicinal Products.

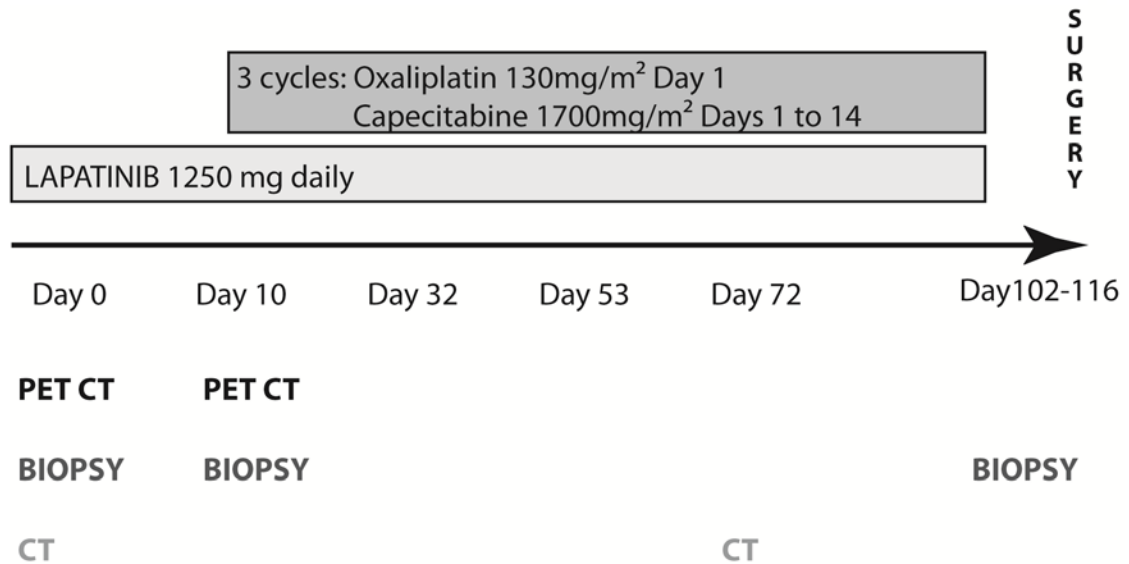


Fig 1 Study design where patients undergo a baseline biopsy and ex vivo sample was taken at D0. Patients then received 10 days of Lapatinib monotherapy after which a repeat biopsy and functional imaging was performed. Patients went on to receive 3 cycles of Oxaliplatin and Capecitabine concurrent with Lapatinib on a 21 day cycle, followed by definitive surgery.

Power Calculation

This is a molecular biomarker study and thus designed to examine molecular rather than clinical endpoints. The primary objective was to compare the molecular response in biopsies taken pre-treatment, which were then treated with Lapatinib *ex vivo* with the molecular response in a biopsy taken after 10 days of oral Lapatinib. It was anticipated that concordant response outcomes (no change or decrease) would

be observed for at least 80% to 85% patients, with a 5% significance (one-sided) level and 80% power, 13 patients would need to be recruited.

Patient Selection

Patients were enrolled with histologically confirmed OGA which over expressed HER2, defined as either 3+ staining intensity for HER2 on immunohistochemistry, or 2+ staining but shown to have HER2 amplification by FISH (Bang et al., 2010). All patients were deemed to have surgically resectable disease on presentation based on CT, PET CT, Endoscopic Ultrasound (oesophageal cases) and staging laparoscopy (gastric cases) and needed to be fit for both chemotherapy and surgery, with adequate haematological parameters ($WBC \geq 3.0 \times 10^9/L$, $Plts \geq 100 \times 10^9/L$, $Hb \geq 9g/dL$), renal function (measured or calculated Cr Clearance $\geq 60ml/min$) and liver function ($Bilirubin \leq 1.5 \times ULN$, $ALT/AST \leq 1.5 \times ULN$, $ALP \leq 2.5 \times ULN$). They needed to be able to swallow oral medication and be over 18yrs of age. Patients with abnormal cardiac function determined by echocardiography/MUGA scan, or a history of interstitial lung disease were excluded. Those who had previously received chemotherapy or Lapatinib were excluded as were those with a known G6PD deficiency. Cases of known HIV, Hepatitis C or B infection were also excluded.

Treatment

Patients initially received 10 days of treatment with Lapatinib 1250mg once a day. This period was used to assess the effect of Lapatinib alone on the tumour. From day 11, patients went on to receive 3 cycles of Oxaliplatin (130mg/m²) and Capecitabine 1700mg/m² whilst continuing Lapatinib. On completion of neoadjuvant therapy, the patient went on to have definitive surgery in the form of an oesophagectomy or gastrectomy depending on the location of the tumour.

Assessment

Toxicity was assessed using the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) Version 3.0. Survival was determined by either confirmed date of death, or censored at the last recorded trial follow up event. The Kaplan Meier method was adopted for the survival analysis.

PET CT scans were performed on day D0 and day D10 after treatment with Lapatinib monotherapy. The fall in the absolute maximum Standard Uptake Value (SUV) and for a region of interest (ROI) of 1.5cm diameter circle at D0 and D10 was reported by an Administration of Radioactive Substances Committee (ARSAC) accredited radiologist. A PET response was defined as a reduction of >35% in the SUV (Lordick et al., 2007). CT scans were performed on patients on D0 and on D72. Tumour response to therapy was evaluated between the two time points using RESCIST 1.1.

Biopsy Analysis

Biopsies at baseline, D10 and surgery were taken and the specimen was split equally into two parts. One, part was fresh frozen and stored in readiness for processing

using the Collaborative Enzyme Enhanced Reactive Immunoassay (CEER) (Prometheus Laboratories Inc, San Diego, USA) and the other part of the biopsy was formalin fixed for H&E and IHC analyses.

At baseline, a further tissue sample was treated with Lapatinib *ex vivo*. This was done by placing the biopsy tissue measuring approximately 0.2cm, on to a mesh placed in a well which was then filled with Dulbecco's Modified Eagles' Medium (Gibco, Invitrogen, Paisley, UK) medium and 10uM Lapatinib (GlaxoSmithKline, Uxbridge, UK) or with a control DMSO (Fisher Scientific, Loughborough, UK) so as to provide an air-liquid interface. This was then incubated at 37°C for one hour, and then fixed in 10% neutral buffered formalin pots, to create paraffin blocks ready for IHC.

All samples were stained by IHC for P-HER2, HER2, P-EGFR, P-Erk, P-Akt, (Cell Signalling), EGFR (Novacastra) and MET (Santa Cruz Biotechnology) using the Leica Bond Max Automated Immunohistochemistry System (Wetzlar, Germany). Staining was scored by 2 independent reviewers using intensity (0=No reactivity or membranous staining of tumour cell, 1=Faint/Barely Perceptible membranous staining of $\geq 10\%$ of tumour cells, 2=Weak to moderate complete, basolateral or lateral membranous reactivity in $\geq 10\%$ of tumour cells, 3=Strong complete, basolateral or lateral membranous reactivity in $\geq 10\%$ of tumour cells)(Koopman et al., 2015). Scores of 2 and 3 were considered positive whereas 0 and 1 were negative, and a response was defined as a positive score becoming negative (Zhang et al., 2014). A response was defined as a change from a positive IHC score (2+ or 3+) to a negative score (0+ or 1+) for P-HER2 and P-EGFR. Proliferation was assessed by

staining for Ki67 (Leica, Wetzlar Germany), and apoptosis assessed by staining for Cleaved Caspase 3 (CC3) (Cell Signalling) and by using the TUNEL assay (Millipore, Consett UK). A score of 1 to 5 was given based on the percentage of tumour cells staining for Ki67 (1=0-20%, 2=21%-40%, 3=41%-60%, 4= 61%-80%, 5=>81%). Scoring CC3 and the TUNEL assay was done by counting all positively staining cancer cells in 5 high power (40x) fields of view by 2 independent scorers, and the average of all 10 scores was taken for the purposes of analysis (Li et al., 2008).

All frozen samples were assayed using the CEER platform (Prometheus Laboratories, San Diego, USA) for receptor tyrosine kinase activation (EGFR, HER2, p95, HER3, IGFR, MET) and downstream signalling. This could not be done with the *ex vivo* biopsy as it was formalin fixed and further material was not available.

Genomic Analysis

Whole genome sequencing was available for one patient's treatment naïve tumour. DNA was extracted from fresh frozen tissue samples using the Qiagen DNA Mini Kit (Qiagen) as per protocol and sequenced on the Illumina HiSeq platform. Paired DNA from blood was sequenced to allow somatic variants to be distinguished from those in the germline. BWA was used for alignment against GRCh37 reference from Ensembl v71. Variant calling was performed with Somatic Sniper (V1.0.2) and a variety of filters was used to exclude low quality calls (Weaver et al., 2014). The single nucleotide variation data was assessed using DAVID Bioinformatics resource Version 6.7 (National Institute of Allergy and Infectious Diseases (NIAID)). Functional annotation was assessed using the Gene Ontology tools and KEGG pathway analysis. ASCAT-NGS v.2.1 (Van Loo et al., 2010, Nik-Zainal et al., 2012) was used to infer

tumour copy number data, and a combination of both copy number and single nucleotide variation data was analysed in cloneHD to assess clonality (Fischer et al., 2014).

Statistical Analysis

For the assessment of the *ex vivo* assay by IHC, a molecular response was defined as a positive P-HER2 or P-EGFR (2+ or 3+) becoming a negative score (0+ or 1+) (Zhang et al., 2014). A Kappa correlation coefficient was calculated to assess agreement between *ex vivo* and D10 *in vivo* response, and between the 2 reviewers for these scores. Wilcoxon Rank Sums analysis was performed, between baseline tumour IHC characteristics (HER2, EGFR, MET) and predefined clinical outcome (RECIST response, complete pathological response, R0 resection).

HER2, p95HER2 and EGFR activation (phosphorylated to total protein ratio) using CEER data was assessed at baseline, D10 and surgery and the Wilcoxon Signed Rank Test was used to assess changes between baseline and D10 as well as baseline and surgery.

Correlation between MET, IGFR and HER3 activation (phosphorylated to total protein ratio) was correlated with the PI3K, P-Erk, and P-Akt activation (absolute levels of P-Erk and P-Akt was used as the total protein level was not available for these to molecules) using the Spearman Rank Correlation.

Results

A total of 10 patients were recruited to the trial. The median age of the 10 patients was 61.7yrs, with a preponderance for males (70%) which is in keeping with the

epidemiology of the disease. 8 of patients entering the trial had a performance status of 0, and the remaining 2 a performance status of 1. The primary site of disease was the gastro-oesophageal junction in 6 of cases and the majority of these tumours were poorly differentiated (Table 1). All patients bar one had a HER2 IHC score of 3+ and only one patient had a tumour with an IHC score of 2, which was subsequently confirmed to be amplified by FISH.

	n=10	
Age (yrs)	Mean	61.7
	Standard Deviation	9.4
	Range	49.7 -75.2
Sex	Male	7
	Female	3
ECOG Performance Status	0	8
	1	2
Site of Primary	Oesophagus	3
	Stomach	1
	GOJ Type 1	1
	GOJ Type II	3
	GOJ Type III	2
HER2 Staining	3+	9
	2+ with FISH amplification	2
Tumour Differentiation	Moderate	4
	Poor	6
T Stage	T2	1
	T3	7
N Stage	N0	7
	N1	2
	N2	1
M Stage	M0	0

Table 1 Demographic data on patient cohort, with primary site, HER2 scoring, tumour differentiation and stage.

The 10 day Lapatinib monotherapy window phase was well tolerated with 100% of patients receiving the intended Lapatinib dose with no \geq grade 3 toxicity. With regards to the neoadjuvant period of chemotherapy with concurrent Lapatinib, 80% of patients received all 3 cycles of chemotherapy. These toxicities are summarised in Table 2.

n=10	
Lapatinib Monotherapy	
Lapatinib monotherapy toxicities	0
Lapatinib and Chemotherapy	
Anastomotic leak	2
Fainting	1
Mucositis	1
Candida infection	1
Nausea	2
Atelectasis	1
Dyspepsia	1
Diarrhoea	2
Gastrointestinal Pain	1
Small Bowel Obstruction	1
Weight Loss	1
Chest Infection	1
Hypoxia	1
Neutropenia	1

Table 2 Adverse events of \geq Grade 3 experienced by patients during their monotherapy window, and concurrent chemotherapy treatment.

The most common adverse effects noted were nausea and diarrhoea. These are in keeping with the known individual toxicities of the drugs, especially Capecitabine and Lapatinib. 3 patients experienced grade 4 toxicity. This included 2 patients who experienced anastomotic leaks. One of these patients died after developing a chest infection. This leak rate of 20% was higher than expected given that both tertiary centres had leak rates in keeping with the national oesophago-gastric audit of 5%,

and as a result the independent data monitoring committee agreed to suspend recruitment to the study after 10 patients had been enrolled (Chadwick G, 2013). Median overall survival was 32.4 months, and median progression free survival was 16.4 months. There were no responses by PET at D10 in the maximal SUV of for the maximal region of interest (ROI 1.5cm² circle). 50% of patients had a partial response on CT after neoadjuvant therapy, and the rest had stable disease.

Molecular Analysis

Immunohistochemistry

Tumour specimens were stained for HER2, EGFR, P-HER2, P-EGFR, MET, as well as downstream targets including P-Erk, P-Akt and examples of staining patterns are demonstrated in Fig 2.

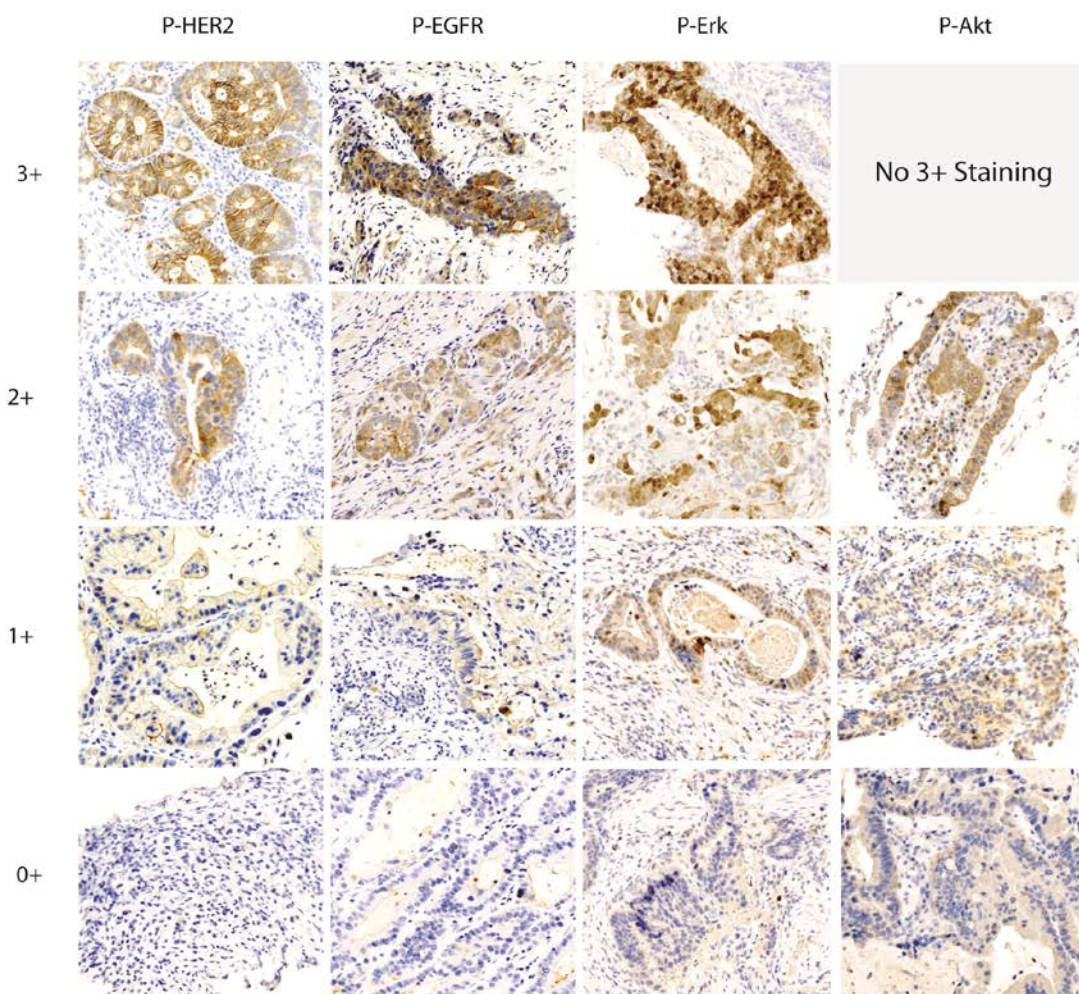


Fig 2 Immunohistochemistry for the phosphorylated species for the RTKs P-HER2, P-EGFR and the downstream molecules P-Erk and P-Akt. Examples of staining performed using the Axio Slide Scanner (Oberkochen, Germany) at 40x and images at a 35% digital zoom. No 3+ staining was observed for P-Akt.

Assessment of the *ex vivo* assay

The *ex vivo* assay was used to determine whether it is possible to predict *in vivo* response after 10 days of Lapatinib monotherapy.

There was good agreement between the 2 independent scorers. 3 samples were inadequate specimens and so paired data was available for 9 cases for P-HER2 and 8 for P-EGFR. The *Ex vivo* and D10 molecular response correlated 89% of the time for P-HER2 and 100% for P-EGFR which meets the pre-defined aim of 75%.

Comparison of molecular and clinical outcomes

As an alternative to the *ex vivo* culture assay, the IHC results on biopsies sampled over time were correlated to clinical outcome measures to assess if any of these assays could be a potential biomarker for response to Lapatinib therapy.

The baseline score for HER2, EGFR and MET (potential resistance mechanism) was related to clinical outcome measures. Our hypothesis was that stronger HER2 and EGFR staining tumours would be more responsive to Lapatinib, whereas tumours with strong MET expression would be resistant to therapy. Wilcoxon-Rank Sum Analysis failed to show any association between baseline HER2 scores and the clinical outcomes by RECIST response, R0 resection rate, or complete pathological response ($p=1.000$, $p=0.660$, $p=0.606$ respectively). Nor was there any associations with baseline EGFR score ($p=0.535$, $p=0.087$, $p=0.702$) or MET scores ($p=0.399$, $p=0.615$, $p=0.615$ respectively). On target response did not correlate with progression free survival or overall survival based on an IHC response (HER2 based response OS $p=0.2278$ PFS $p=0.6650$; EGFR based response OS $p=0.3938$, PFS $p=0.3173$) or CEER

response (HER2 based response OS p=0.4126, PFS p=0.2216; EGFR based response OS p=0.2922, PFS p=0.1486).

P-HER2		<i>Ex vivo</i>	
		No Change	Response
<i>In vivo</i>	No Change	6	0
	Response	1	2

Kappa Score for P-HER2

Between scorers (weighted) 0.5902
 Between *ex vivo* and D10 IHC score 0.7273

Correlation between *ex vivo* and D10 *in vivo*

88.9%

P-EGFR		<i>Ex vivo</i>	
		No Change	Response
<i>In vivo</i>	No Change	7	0
	Response	0	1

Kappa Score for P-EGFR

Between scorers 0.832
 Between *ex vivo* and D10 IHC score 1

Correlation between *ex vivo* and D10 *in vivo*

100.0%

Table 3 Comparison of the *ex vivo* response to Lapatinib for P-HER2 and P-EGFR, with that seen after 10 days of *in vivo* Lapatinib monotherapy.

CEER proteomic analysis

The target effect of Lapatinib was examined at baseline, D10 and surgery using the CEER assay (Fig 3). There was a significant drop in the P-HER2:T-HER2, and P-EGFR:T-EGFR ratios after only 10 days of Lapatinib monotherapy ($p=0.0039$ and $p=0.0195$ respectively). All 10 patients demonstrated reductions in HER2 activation by day 10 and 9 of 10 patients had a reduction in EGFR activation. This reduction in growth factor signalling persisted to the time of surgery and EGFR signalling remained significantly inhibited ($p=0.0391$), despite a 30 day washout period between completing neoadjuvant therapy and surgery. HER2 signalling (PI3K, P-AKT, P-ERK) continued to be lower at the time of surgery compared to baseline, although the difference did not reach significance ($p=0.4961$). The truncated form of HER2, p95HER2 lacks the extracellular domain, and significant inhibition was again noted at D10, which persisted through to surgery ($p=0.0078$ and $p=0.0234$ respectively).

There was a reduction in P-PI3K:T-PI3K activation which mirrors the response in HER2 and EGFR, with significant dampening in signalling between baseline and both D10 as well as surgery ($p=0.03$ and $p=0.01$ respectively). Only phosphorylated assays were available for Akt and Erk, and though there was a reduction in median levels, this did not reach significance. This maybe because it was not possible to normalise the phosphorylated receptor level to the total protein content (assay not available at the time) and as such outliers tended to skew the data.

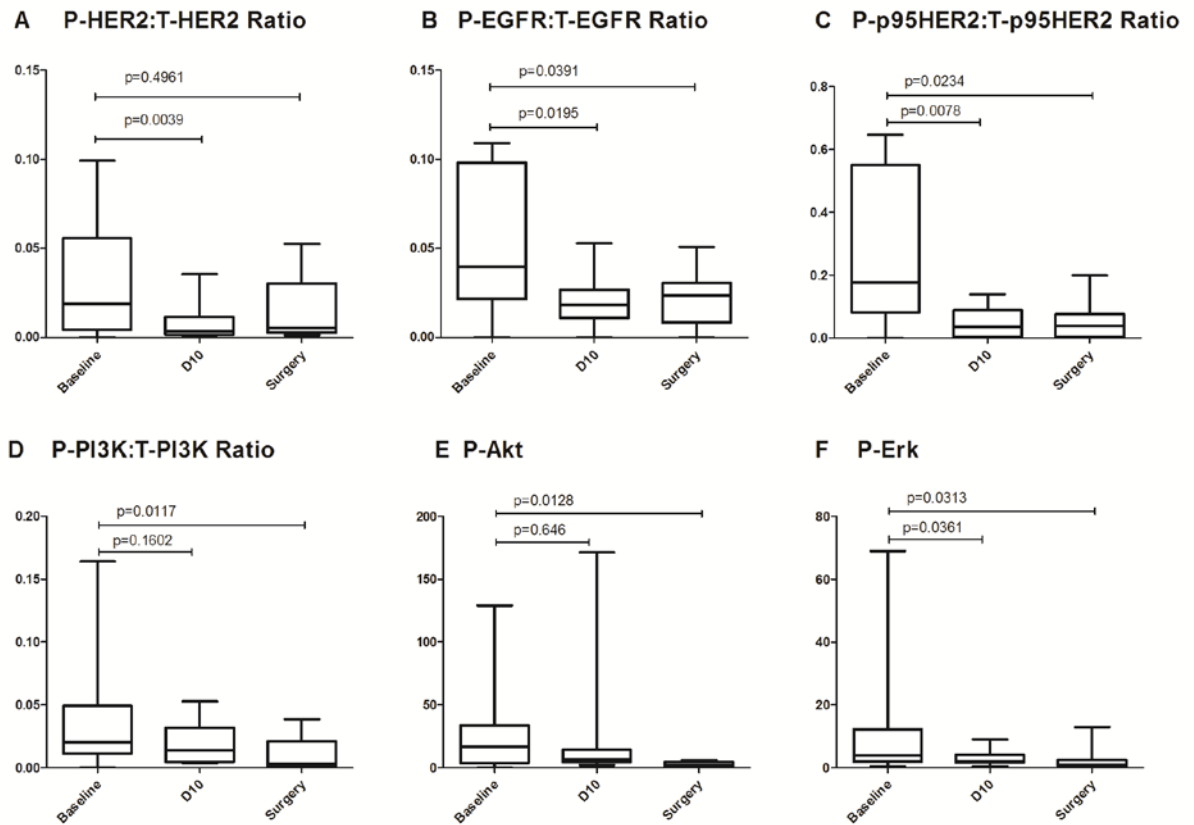


Fig 3 Phosphorylated:Total (P:T)receptor ratios for HER2 (A), EGFR (B) and p95HER2 (C), along with the downstream molecules P-PI3K:T-PI3K ratio (D), P-Akt (E), and P-Erk (F) at baseline, D10 and surgery.

The correlation between the activation of RTKs and downstream signalling effectors was examined (Fig 4). There was a significant correlation between the activation of C-Met with the level of P-Erk ($P=0.0005$) and P-PI3K:T-PI3K ratio ($p=0.0037$). There was no significant correlation between the activation status of IGFR and HER3 with downstream signalling molecules, and no significant correlation was seen between the activation status of any receptor and P-Akt. This would suggest that activation of C-Met may be a mechanism of Lapatinib resistance *in vivo* with an R^2 value of 0.914 for P-Erk (Fig 4 Ai) and 0.84 for P-PI3K:T-PI3K (Fig 4 Aii).

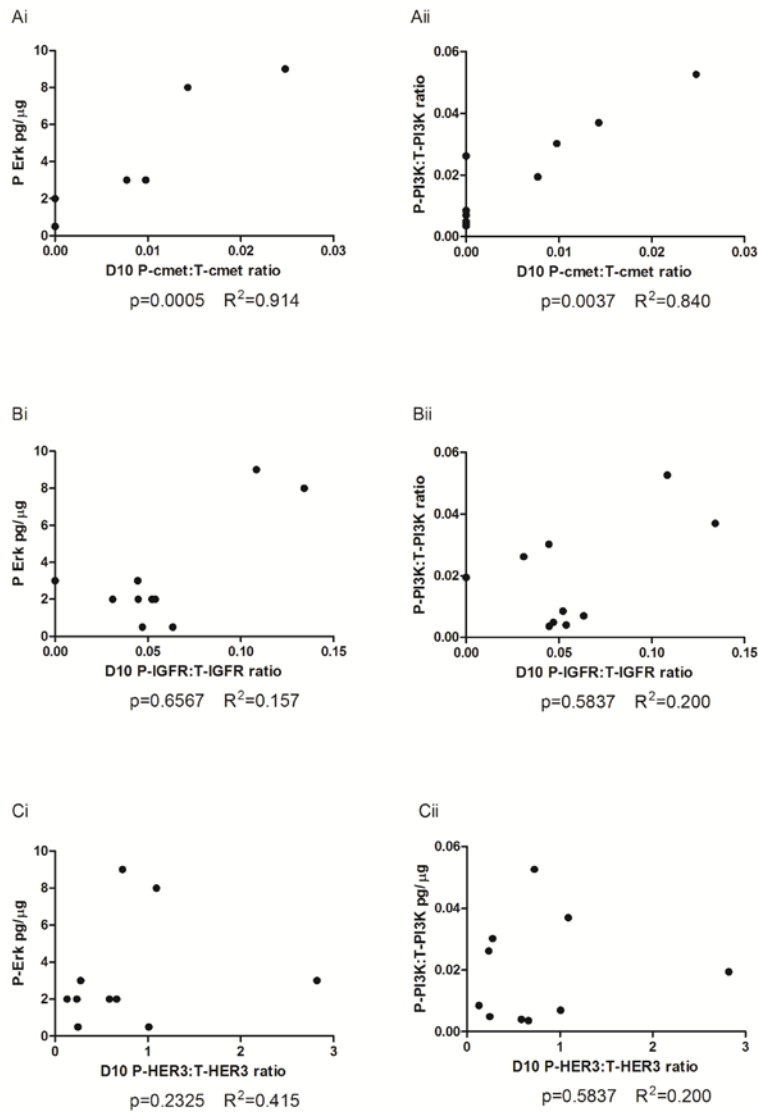


Fig 4 The ratio of phosphorylated to total protein amount representing the activation status of c-met (A), IGFR (B) and HER3 (C), plotted against the activated downstream signalling molecules P-Erk (i), and P-PI3K:T-PI3K (ii) ratio.

Whole genome sequencing from a treatment naïve sample was available for one of the 4 patients, with persistent MAPK and PI3K pathway activation, which was related to MET activation (Fig 4Ai and 4Aii). There were amplifications in EGFR and HER2

(total copy number 3 for both with a single copy in the minor allele), but the greatest copy number increase was seen with MET with a total copy number of 4. KEGG output using single nucleotide variation data revealed significant clustering in the Phosphatidylinositol signalling and Wnt pathways, although no mutations were observed in the growth proliferative canonical parts of these pathways.

Discussion

There are 7089 new cases of gastric cancer per year in the UK and 8332 for oesophageal cancer in the UK (<http://info.cancerresearchuk.org>). In the ToGa trial, which is the largest assessment of HER2 positivity in this patient group, 16.6% of tumours were HER2 positive. This would mean that 2560 patients a year in the UK would be HER2 amplified. At present, the ToGa trial has established the use of Trastuzumab in the metastatic setting (Bang et al., 2010), but there is no trial evidence to support the use of HER2 targeted therapies in patients undergoing treatment with curative intent. The use of Lapatinib has been unsuccessful in prolonging survival in metastatic patients with HER2 overexpressing OGA (Satoh et al., 2014, Iqbal et al., 2011, Hecht JR, 2013, Lorenzen et al., 2015).

The LEO trial assessed the use of Lapatinib in the peri-operative setting where median overall survival was 32.4 months. This compares to previous trials where surgery alone and in combination with peri-operative chemotherapy demonstrated median overall survival rates of 18-24 months (Cunningham et al., 2006, Allum et al., 2009). Lapatinib monotherapy was well tolerated and the main grade 3 toxicity resulted from the combination with chemotherapy. Grade 3 toxicities such as nausea/vomiting and mucositis occurred at a similar rate between the LEO patients

and those seen with ECX alone in the MAGIC trial at around 20% and 10% respectively (Cunningham et al., 2006). Rates of grade ≥ 3 diarrhoea was higher within the LEO trial which is in keeping with the fact that both Lapatinib and Capecitabine can induce diarrhoea individually, and was similar to the rates seen in the TRIO-013/LOGiC trial (Hecht JR, 2013). The most significant toxicities were the 2 anastomotic leaks, which is a much higher rate than observed in the UK national audit (Chadwick G, 2013), which was 7.4% for oesophagectomies and 4.4% for gastrectomies. Although with such small numbers in the trial it is difficult to draw conclusions, a drug effect could not be excluded for this serious complication which resulted in death of one patient, especially in view of the persistent inhibition of HER2 and EGFR at the time of surgery.

One of the study endpoints was to examine if an *ex vivo* assay could help predict treatment response. This was done using an organ culture, in which a whole biopsy is treated with Lapatinib for an hour, and then fixed in formalin for IHC analysis. The *ex vivo* assay accurately predicted *in vivo* P-HER2 response in 88.9% of cases, and for 100% of the cases for P-EGFR. This met the predefined study target of concordance of 75%. This approach may warrant further consideration in other clinical trials.

The analysis comparing baseline predictors of sensitivity, namely IHC staining for HER2 and EGFR staining intensity, and biomarkers of resistance (C-MET staining) failed to show any correlation with clinical outcomes, namely CT response by RECIST criteria, R0 resection rates or complete pathological response. This is in keeping with the ToGa trial (Bang et al., 2010) where baseline HER2 scoring did not correlate with

sensitivity to anti-HER2 directed therapy. The reasoning for this is thought to be the large amount of heterogeneity observed in staining patterns in Upper GI adenocarcinomas when compared to other cancers. Indeed, similar heterogeneity in staining was observed in this study, highlighting the need to use robust and validated scoring systems such as those used in this study (Koopman et al., 2015).

Further samples at baseline, D10 and surgery were analysed using the CEER assay, which has been previously been shown to be much more sensitive for determining activation levels compared to IHC (Lee et al., 2013a). The window design of this trial also confirms that 10 days of monotherapy can give information of on target effects of the drug, as well as assaying potential primary resistance mechanisms. Compared to other methods of assaying drug sensitivity, such as establishment of cell lines and organoids, repeat endoscopic biopsies after a window period is relatively simple, cheap and effective method of getting molecular response data in a timeframe that can be clinically relevant for the patient. The effect of Lapatinib was first examined on its primary targets, namely HER2 and EGFR. After 10 days of Lapatinib monotherapy, there was a significant reduction in the proportion of activated receptors for both HER2 and EGFR. This is particularly important as it appears to precede any metabolic response as no response was observed by PET imaging over this same time period. There was also significant inhibition of p95, the truncated form of HER2 which lacks the extracellular domain. In breast cancer, resistance to Trastuzumab has been partly attributed to p95 (Duchnowska et al., 2014), as the antibody is unable to bind the protein due to the lack of an extracellular domain, but there was significant inhibition of p95 noted in this study by Lapatinib at day 10, which continued through to surgery. This highlights that repeat biopsy after a

window period of monotherapy can provide the earliest evidence of on target action for Lapatinib, and the drug also significantly inhibits p95.

A 4-6 week wash out period prior to surgery was used to ensure that the patients had recovered fully from the neoadjuvant therapy prior to definitive surgery. This period has typically been used with cytotoxic chemotherapy as this usually allows for complete marrow recovery. Similar pre-operative washout periods have also been used with anti-HER2 agents such as Trastuzumab in breast cancer, and have not been associated with an increased operative morbidity (Gianni et al., 2010). The CEER data demonstrated that despite a washout period of several weeks, there was still significant reduction in EGFR signalling at the time of surgery, with a further non-significant reduction in HER2 signalling. Although the small numbers make it impossible to draw any definitive conclusion, it is possible that this persistent inhibition of growth factor signalling pathways at surgery may have contributed to the anastomotic leak rate. It should therefore be considered whether the washout period required for neo-adjuvant therapy using Lapatinib needs to be longer than typically used for traditional chemotherapeutics. This is particularly noteworthy given that there are current trials open to recruitment using Lapatinib in a similar context with a similar wash out period (Cunningham, 2012).

Not only is there evidence of receptor deactivation in response to drug but a decrease in the activation of downstream molecules such as PI3K, P-Erk and P-Akt. This supports the on-target action of Lapatinib on these tumours reducing EGFR and HER2 mediated signalling by PI3K and with a trend for reduction of the MAPK pathway.

Preclinical studies have pointed to RTK crosstalk from METas being an important mechanism of resistance to Lapatinib and other HER2 directed therapies (Liu et al., 2014, Zhang et al., 2014, Paterson et al., 2013b, Shattuck et al., 2008, Chen et al., 2012, Ha et al., 2014, Lee et al., 2013b). This study provides some of the first *in vivo* evidence that, MET appears to be the most important RTK in activating proliferative pathways (PI3K and MAPK) in the presence of Lapatinib inhibition of EGFR and HER2. This was demonstrated by the CEER assay where the activation of the MAPK and PI3K pathways after 10 days of Lapatinib monotherapy was significantly correlated with the activation of MET, but not with IGFR or HER3 (Fig 4). Larger studies would need to be undertaken to fully understand whether this molecular crosstalk has implications on clinical outcome as this trial was not powered to detect this. Our cohort is also not large enough to adequately correct for differences in staging, or to perform a multiple regression analysis in order to correlate clinical outcomes and molecular findings.

Whole genome sequencing was available for one of the patients with persistent mitogenic signalling in the presence of Lapatinib, which appeared to be due to MET activation. The main canonical pathways for PI3K and MAPK were intact in keeping with previous genomic studies in OGA have suggested that point mutations do not tend to drive proliferation (Weaver et al., 2014, Dulak et al., 2013). Copy number data from this same patient demonstrated HER2 and EGFR amplification (total copy number of 3 for each), but interestingly, the most amplified of the RTKs was MET. This supports the finding that activation of these mitogenic pathways at D10 is more likely to be due to RTK cross talk from MET by amplification.

These *in vivo* findings suggests that MET mediates Lapatinib resistance and is particularly noteworthy given recent interest in using MET inhibitors. These trials have failed to demonstrate efficacy of single agent MET TKIs ((Shah et al., 2013) and MET targeting antibodies (Cunningham et al., 2015), so it is likely that combination therapies should be considered with anti-HER2 therapies used in combination with anti-MET agents in the context of well designed stratified trials (Ha et al., 2014).

This study demonstrated that 10 days of monotherapy followed by CEER analysis can reveal *in vivo* evidence of target activity in a simple, rapid and cheap manner, and in addition may provide clinically useful information about patients who may be resistant, providing the information for clinicians to make management decisions in real time, and to select appropriate targeted therapy in a truly personalised manner.

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