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Functional Imaging and circulating biomarkers of response to Regorafenib in treatment-refractory metastatic colorectal cancer patients in a prospective phase II study.

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Complete List of Authors:	Khan, Khurum; The Institute of Cancer Research, Molecular Pathology Rata, Mihaela; The Institute of Cancer Research, CRUK Imaging Centre Cunningham, David; Royal Marsden Hospital, Medicine Dow-Mu, Koh; The Institute of Cancer Research, CRUK Imaging Centre Tunariu, Nina; The Institute of Cancer Research, CRUK Imaging Centre Hahne, Jens; The Institute of Cancer Research Vlachogiannis, George; The Institute of Cancer Research, Molecular Pathology Hedayat, Somaieh; The Institute of Cancer Research, Molecular Pathology Marchetti, Silvia; The Institute of Cancer Research, Molecular Pathology Lampis, Andrea; The Institute of Cancer Research Darvish, Mahnaz; The Institute of Cancer Research, Molecular Pathology Lote, Hazel; The Institute of Cancer Research, Molecular Pathology Rana, Isma; Royal Marsden Hospital, Medicine Williams, Anja; Royal Marsden Hospital, Medicine Eccles, Suzan; The Institute of Cancer Research Fontana, Elisa; The Institute of Cancer Research, Molecular Pathology Collins, David; The Institute of Cancer Research, CRUK Imaging Centre eltahir, zakaria; Royal Marsden Hospital, Medicine rao, sheela; Royal Marsden Hospital, Medicine watkins, david; Royal Marsden Hospital, Medicine Starling, Naureen; Royal Marsden Hospital, Medicine thomas, jan; Royal Marsden Hospital, Medicine kalaitzaki, eleftheria; Royal Marsden Hospital, R&D fotiadis, nicos; Royal Marsden Hospital, Radiology begum, ruwaida; Royal Marsden Hospital, Medicine Bali, Maria; Royal Marsden Hospital, Radiology Rugge, Massimo; University of Padova, Department of Diagnostic Sciences temple, eleanor; Royal Marsden Hospital, Medicine Fassan, Matteo; University of Padova, Medical Diagnostic Sciences and Special Therapies, Surgical Pathology and Cytopathology Unit Chau, Ian; Royal Marsden Hospital Braconi, Chiara; The Institute of Cancer Research; The Royal Marsden Hospital, Gastrointestinal Unit Valeri, Nicola; The Institute of Cancer Research, Molecular Pathology
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Title: Functional Imaging and circulating biomarkers of response to Regorafenib in treatment-refractory metastatic colorectal cancer patients in a PROSPECTive phase II study.

Running Title: DCE-MRI and ctDNA as biomarkers of response to Regorafenib

Authors: Khurum Khan[1,2], Mihaela Rata[3], David Cunningham[1,*], Dow-Mu Koh[3], Nina Tunariu[3], Jens C Hahne[2], George Vlachogiannis[2], Somaieh Hedayat[2], Silvia Marchetti[2], Andrea Lampis[2], Mahnaz Darvish Damavandi[2], Hazel Lote[1,2], Isma Rana[1], Anja Williams[1], Suzanne A Eccles[4], Elisa Fontana[1], David Collins[3], Zakaria Eltahir[1], Sheela Rao[1], David Watkins[1], Naureen Starling[1], Jan Thomas[1], Eleftheria Kalaitzaki[1], Nicos Fotiadis[3], Ruwaida Begum[1], Maria Bali[3], Massimo Rugge[5], Eleanor Temple[1], Matteo Fassan[5], Ian Chau[1],Chiara Braconi[1,4], & Nicola Valeri[1,2,†,*]

* These authors contributed equally as senior authors

Affiliations:

[1] Department of Medicine, The Royal Marsden NHS Trust, London and Sutton. United Kingdom.

[2] Division of Molecular Pathology, The Institute of Cancer Research London and Sutton, United Kingdom.

[3] Cancer Research UK Cancer Imaging Centre, Division of Radiotherapy and Imaging, The Institute of Cancer Research and Royal Marsden Hospital, London, UK

[4] Division of Cancer Therapeutics, The Institute of Cancer Research, London and Sutton, United Kingdom.

[5] Department of Medicine (DIMED) and Surgical Pathology, University of Padua, Padua, Italy

† **Corresponding Author:**

Nicola Valeri MD, PhD

Division of Molecular Pathology

The Institute of Cancer Research London and Sutton

15 Cotswold Road, Belmont, Sutton Surrey, SM2 5NG

United Kingdom

Tel: 0044 0208 915 6634

Email nicola.valeri@icr.ac.uk

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ABSTRACT

Objective: Regorafenib demonstrated efficacy in metastatic colorectal cancer (mCRC) patients. Lack of predictive biomarkers, potential toxicities and cost effectiveness concerns highlight the unmet need for better patient selection.

Design: *RAS* mutant mCRC patients with biopsiable metastases were enrolled in this phase II trial. Dynamic contrast enhanced (DCE) MRI was acquired pre and at day 15 post-treatment. Median values of volume transfer constant (K^{trans}), enhancing fraction (EF) and their product KEF (summarised median values of K^{trans} x EF) were generated. Circulating tumour (ct) DNA was collected monthly until progressive disease and tested for clonal *RAS* mutations by digital-droplet PCR. Tumour vasculature (CD-31) was scored by immunohistochemistry on 70 sequential tissue biopsies.

Results: Twenty seven patients with paired DCE-MRI scans were analysed. Median KEF decrease was 58.2%. Of the 23 patients with outcome data, >70% drop in KEF (6/23) was associated with higher disease control rate (p=0.048) measured by RECIST v1.1 at 2 months, improved progression free survival (PFS) [Hazard ratio (HR) 0.16 (95% CI 0.04-0.72), p=0.02], 4-month PFS (66.7% vs. 23.5%) and overall survival (OS) [HR 0.08 (95% CI 0.01-0.63), p=0.02]. KEF drop correlated with CD-31 reduction in sequential tissue biopsies (p=0.04). *RAS* mutant clones decay in ctDNA after 8 weeks of treatment was associated with better PFS [HR 0.21 (95% CI 0.06 - 0.71), p=0.01] and OS [HR 0.28 (95% confidence interval 0.07 - 1.04), p=0.06].

Conclusions: Combining DCE-MRI and ctDNA predicts duration of anti-angiogenic response to regorafenib and may improve patient management with potential health/economic implications.

Significance of the Study

What is already known on this subject?

- Regorafenib is approved as third-line therapy for patients with refractory CRC; however, its use in the clinic has been restricted due to modest clinical benefit in unselected patients.
- Published pre-clinical studies suggested that anti-angiogenic activity of regorafenib is the main pre-determinant of its efficacy but no clinical studies have validated these findings.
- Retrospective analysis of prospective clinical trials failed to identify biomarkers od response to regorafenib that might be implemented in clinical practice.

What are the new findings?

- Regorafenib showed significant activity in patients with marked early anti-angiogenic response, resulting in a longer disease control, better PFS and OS.
- Early (day 15 post-treatment) DCE-MRI predicts response and long term outcome during Regorafenib treatment.
- Sequential analysis of tissue biopsies confirmed that reduction in tumour vasculature as the mechanism underpinning the observed radiological findings.
- Persistent regorafenib-induced anti-angiogenic effect translates into a reduction in circulating tumour (ct) DNA and this might be incorporated into the clinical algorithm for patients' management.

Implications on clinical practice

Implementing the use of DCE-MRI and ctDNA analysis as early biomarkers of response to regorafenib might improve patient selection with clear health/economic implications for patients, health systems and society.

Introduction

Colorectal cancer (CRC) remains a major health burden with significant morbidity and mortality despite recent improvements in its management owing to better screening and therapeutic options[1]. CRC is known to be a biologically heterogeneous disease characterised by the activation of several angiogenic and oncogenic pathways[2]. Regorafenib, a multikinase inhibitor (MKI) with known anti-angiogenic, anti-stromal and anti-oncogenic activities[3], has demonstrated single agent efficacy in patients with treatment refractory metastatic CRC (mCRC)[4, 5]. The use of regorafenib in the clinic is however hampered by the modest efficacy in an unselected patient population, a significant side effect profile and the high drug costs. Consequently, identification of predictive biomarkers of response and resistance to regorafenib is critical for treatment stratification and appropriate patient selection such that treatment benefits could be optimised.

Several efforts are currently ongoing to define gene signatures [6] and bio-markers of response to anti-angiogenic drug in CRC and other cancers[7]; however, ongoing validation will only determine the use of these biomarkers in clinical practice. Whilst recent studies utilising tissue [8] and plasma [9, 10] have attempted to elucidate the response and resistance mechanisms to regorafenib, the search for a clinically useful biomarker has been largely unsuccessful. A growing body of pre-clinical evidence suggests strong anti-angiogenic and pro-apoptotic effects of regorafenib [11, 12, 13, 14] with clinical data demonstrating that drug activity is independent of the tumour's mutational status[8]. These findings strengthen the hypothesis that additional mechanisms other than oncogenic blockade are responsible for the anti-tumour activity of this drug. Dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) may have a useful role in evaluating tumour vascular heterogeneity and early anti-angiogenic effects[15],[16]; moreover, its parameters volume transfer constant (K^{trans}), enhancing fraction (EF), and initial area under the gadolinium concentration-time curve over 60 seconds ($IAUGC_{60}$) have been correlated with micro-vessel

density and in some tumours with degree of VEGF expression [17]. By contrast, diffusion weighted MRI (DW-MRI) offers useful information that reflects tumour cellularity and increase in its quantitative parameter Apparent Diffusion Coefficient (ADC) has been associated with tumour cell death and necrosis[18, 19]. At least two pre-clinical studies demonstrated that regorafenib was able to significantly suppress tumour vascularity when quantified by DCE computed tomography (CT) and MRI modalities respectively in human colon carcinoma xenograft models[14, 20].

In this prospective phase II trial of patients with *RAS* mutant mCRC treated with single agent regorafenib we hypothesised that 1) an early anti-angiogenic and anti-proliferative activity of regorafenib might be detected by multi-parametric DCE-MRI on day 15 of the treatment 2) the depth of anti-angiogenic response detected by a significant drop in DCE-MRI quantitative parameters might correlate with clinical efficacy 3) analysis of sequential tissue and liquid biopsies could be integrated into the biomarker discovery process and shed insights into mechanisms of response to regorafenib.

Material and Methods

Clinical Trial Design

PROSPECT-R trial (clinical trials.gov number [NCT03010722],) is a phase II, open label, non-randomised study of regorafenib in patients with *RAS* mutant, chemo-refractory mCRC (Fig. 1). Patients who were at least 18 years old and had a World Health Organisation (WHO) performance status (PS) of 0-1, were deemed eligible if: all conventional treatment options including fluorouracil, irinotecan, oxaliplatin and at least one anti-VEGF drugs (later trial protocol was amended due to changes in availability of anti-VEGF agents due to funding restrictions in UK) were exhausted; they had metastatic tumour amenable to biopsy and repeat measurements with DCE-MRI. Written informed consent was obtained from all patients. The study was carried out in accordance with the Declaration of Helsinki and approved by National

Institutional review boards [Medicines and Healthcare products Regulatory Agency: 15983/0249/001-0001]. All participants were required to have mandatory pre-treatment biopsies (6 cores targeted towards the MRI identified index lesion), biopsies at 2 months [if response or stable disease by RECIST v1.1 criteria (6 cores)] and at the time of progression (6-12 cores from two suitable progressing metastatic sites). 3 out of 6 cores were snap-frozen; one core was used to establish patient-derived organoids and two cores were formalin fixed and paraffin embedded (FFPE). The results section describes the number of cores used for immunohistochemistry analysis in the current study. Further genomic, transcriptomic and functional analyses are on-going on the remaining cores. Patients with suitable metastatic disease (defined as lesions at least 2 cm in diameter) and no contraindications to MRI underwent multiparametric MRI studies including matched DCE and DWI; images were acquired less than 7 days prior to therapy and at day 15 post-treatment. Treatment consisted of regorafenib 160mg once daily on a schedule of three weeks on and one week off until progression or intolerable side effects. More details on inclusion and exclusion criteria and criteria for patients' withdrawal on the study are provided in the online supplementary material.

MRI data processing:

DCE-MRI data were post-processed using the Magnetic Resonance Imaging Workbench software developed at our institution [21]. The pharmacokinetic analysis was based on the extended Kety/Tofts model in conjunction with a cosine-based arterial input function (AIF) model derived from population-averaged values[22, 23]. DCE-MRI parameters including K^{trans}, $IAUGC_{60}$ and the EF were obtained for pre/post-treatment datasets. K^{trans} estimates were reported for both whole tumour [K^{trans}(all)] and valid voxels only [K^{trans}(nonzeros), i.e. excluding all non-enhancements and non model-fits] in order to address the extended necrosis observed in the cohort. The EF was defined as percentage of the voxels that enhance above the noise floor

out of all tumour voxels. A voxel was considered enhancing when it's post-contrast (Dotarem, Guerbet, France) dynamic intensity signal was at least one standard deviation higher than the mean pre-contrast signal, for a period of 60s post contrast onset. Finally, volume change in tumour enhancement during therapy (such as new necrosis) was accounted for by reporting a composite parameter, KEF, which is the product of summarised median values of KEF= K^{trans} (nonzeros) x EF[24]. For KEF, an ROC curve analysis was performed to establish the cut off able to identify meaningful clinical benefit based on disease control rate (DCR), progression free survival (PFS) and overall survival (OS).

Digital Droplet (dd) PCR

The QX200 ddPCR system (Bio-Rad, Berkeley, California) was used and all reactions were prepared using the ddPCR Supermix with no dUTTP for Probes. All PCR reactions were performed as duplex PCR using the relevant digital PCR assays for the wild-type and the mutation in question. Droplets were generated using the QX200 droplet generator according to the manufacturer's protocols. The PCR reaction was performed in a C1000 Touch Thermo Cycler (Bio-Rad) using the following protocol: 95°C for 10 min followed by 40 cycles of 94°C for 30 sec and 55°C for 1 min, then 98°C for 10 min. Droplets were read in the QX200 droplet reader and analyzed using the Quantasoft software version 1.6.6.0320 (Bio-Rad). Fractional Abundance (FA) was defined as follows: F.A. % = (N*mut*/(N*mut* + N*wt*)) × 100), where N*mut* is the number of mutant events and N*wt* is the number of WT events per reaction. The number of positive and negative droplets was used to calculate the concentration of the target and reference DNA sequences and their Poisson-based 95% confidence intervals. ddPCR analysis of normal control plasma DNA (from cell lines) and no DNA template controls were always included. Samples with very low positive events were repeated at least twice in independent experiments to validate the obtained results as previously described[25].

CD31, Ki-67 and Caspase-3 immunohistochemical staining

The immunohistochemical expression of microvascular density (CD31; clone ab28364, Abcam, Cambridge, UK; dilution 1:50), cell proliferation (Ki-67; clone ab16667, Abcam; dilution 1:100), and cell apoptosis (Cleaved Caspase-3 (Asp175) (5A1E) ab9664S, Abcam; dilution 1:100) was examined on consecutive 4-μm formalin-fixed and paraffin-embedded (FFPE) sections of the neoplastic cores. Reactions were performed using the automated Benchmark® XT platform (Ventana Medical Systems, Basel, Switzerland). Appropriate positive and negative controls were run concurrently.

For assessment of tumour microvascular density, CD31-positive micro-vessels were quantified and reported as the average number in 10 random fields at 200x magnification. Ki-67 labelling index was assessed as the average number of proliferating cells in 10 random fields at 200x magnification. Caspase-3 evaluation was categorized as positive or negative.

Statistical Analysis

The Disease Control Rate (DCR) was defined by the sum of complete responses (CR) + partial responses (PR) + stable diseases (SD) using RECIST v1.1. PFS was measured from start of treatment to date of progression or death from any cause. OS was defined as time from start of treatment to death of any cause. Patients without an event were censored at last follow up. Response according to KEF (K^{trans} (nonzeros) x EF) was defined as a drop of >70% from baseline whilst change in CD31 biomarker levels from baseline was calculated as [(8wks-baseline)/ baseline] *100. CD31 change from baseline was explored on a continuous scale and was also dichotomised using the median value.

Response according to KEF parameter and the dichotomised CD31 change from baseline were cross-tabulated with the RECIST measured DCR. Chi^2 or Fisher's

exact tests were employed to explore whether there is an association between them and DCR. Logistic regression was employed to produce odds ratios (ORs) and 95% confidence intervals (CIs). The PFS and OS rates were estimated using the Kaplan-Meier method and survival curves were generated for each group. The log-rank test was used to compare the survival curves and a Cox proportional hazards model was fitted to obtain hazard ratios (HRs) and 95% CIs. The proportional hazards assumption was tested with the use of Schoenfeld residuals.

In our study, despite relatively small study cohort, the changes in K^{trans} and KEF values were noticeably larger (e.g. > 50% reduction in mean and median KEF). Based on results of the 23 analysable patients evaluated by DCE-MRI in our study, our patient sample size by post-hoc analysis (based on Wilcoxon-signed rank test) demonstrated 100% power to detect this difference at a level of significance of 0.05.

*Additiona*l Methods can be found in the Online Appendix

Results

Patients' characteristics and tissue collection

Twenty seven treated patients (63% males) were recruited in the DCE-MRI PROSPECT-R trial and a total of 143 cores were collected by tissue biopsies from 70 metastatic lesions for the current analysis. Right and left sided primary cancers were equally distributed in the study population; other relevant patient characteristics are summarised in Table 1.

Fifty-four tissue cores were obtained from BL biopsies of 27 treated (27 lesions) patients; of the 14 patients with SD at 8 weeks, 24 tissue cores were obtained from 12 (12 lesions) patients (one patient missed the biopsy due to a hospital admission secondary to chest infection and the other developed treatment related rectal wall perforation). A further 65 tissue cores were obtained from 23 evaluable patients (35 lesions in total; 12 patients with two progressing lesions each) with PD (3 patients did

not complete 2 cycles of treatment and 1 came off due to treatment related rectal wall perforation). There was 89% concordance between target DCE-MRI and biopsied metastatic lesions (Appendix Table A1). Two FFPE cores per patient were tested at each time-point. One-hundred and nine plasma samples were tested to track *RAS* mutant clones in 21 corresponding patients; patients were required to have at least one sample available at 2 months following treatment.

Radiological and pathological evidence of early regorafenib induced anti-angiogenic effects

A significant drop in all DCE-MRI parameters was seen after 2 weeks of treatment; median K^{trans}, $IAUGC_{60}$, EF and KEF product decreased by 27.8% [interquartile range (IQR) 6.7-52.6], 57.7% (32.7-67.9), 35.3% (12.4-56.2) and 58.3% (28.3-76.1) (Appendix Table A2). The ROC curve analysis performed for the KEF showed that a 69.21% reduction from baseline had 100% specificity and overall accuracy of 69.57%; for pragmatic reasons a minimum KEF product reduction of 70% was chosen (Appendix Table A3). Matched tissue analysis revealed a strong concordance between a drop in KEF and mean vascular density of tissue, as measured by CD31 count obtained pre-treatment and at 8 weeks in patients with tissue and MR parameter data available (p=0.04). (Appendix Table A4).

Correlation of functional imaging data and CD31 staining with clinical parameters

After a median follow up of 14.3 months [(95% CI 4.9 – not evaluable (NE)], IQR 4.9-not reached (NR)], 23 patients, who had at least 1 cycle of regorafenib and a response assessment by computed tomography (CT) scan at 2 months were analysable. DCR at 2 months, median PFS and median OS were 51.9%, 3.6 months (95% CI 1.9-4.2 months) and 5.8 months (95% CI 4.7-13.3 months) respectively; 77.4% (95% CI 54.0-89.9%), 48.0% (95% CI 24.1-68.5%) and 32.0% (95% CI 11.2-

53.4%) of patients were alive at 4, 6 and 12 months respectively. Patients with >70% drop in KEF (8/27; 2 patients didn't undergo the 2 month scan due to treatment-related toxicities and thus were excluded from the final analysis as per the study protocol) were found to have higher DCR (6/6 vs. 0/6, p=0.05) at 2 months (Appendix Table A5), better PFS [HR 0.16 (95% CI 0.04-0.72), p=0.02], better PFS at 4-months (66.7% vs. 23.5%) and better OS [HR 0.08 (95% CI 0.01-0.63), p=0.02]. For the group with >70% drop in KEF, 6-month and 12-month OS were 100% (95% CI NE) and 75% (95% CI 12.8% - 96.1%) respectively compared to 27.6% (7.2-53.2%) and 13.8% (1.0-42.5%) in the <70% drop in KEF group **(**Fig 2A-B**;** Appendix Figure A1 and Appendix Table A6). In order to address the relative improvement in efficacy with or without KEF drop, we compared the outcomes of all the patients who achieved DCR; PFS was found to be 5.6 vs. 4.2 months [HR 0.30 (95% CI 0.06-1.49), p=0.140) and OS was 15.2 vs. 5.8 months [HR 0.11 (95% CI 0.01-1.06), p=0.057] in this analysis. Interestingly, when the same analysis was repeated with the cut-off chosen by ROC analysis (69.21%), PFS [HR 0.18 (95% CI 0.03-0.91), p=0.038] and OS [HR 0.11 (95% CI 0.01-1.01), p=0.051] were found to be statistically significant despite small numbers.

A decrease in CD31 score at 2 months was associated with higher DCR [OR 30.0 (95% CI 2.22- 405.98), p=0.01], better PFS [HR 0.13 (95% CI 0.03- 0.52), p=0.004] and better OS [HR 0.30 (95% CI 0.08- 1.06), p=0.06] **(**Appendix Fig. A2**).** Examples of KEF drop, RECIST 1.1 response and CD31 scoring at different time-points in a responder (Fig. 3A-C) and non-responder patient (Fig. 3D-F) are provided.

Radiological and pathological analysis of proliferation and apoptosis following regorafenib treatment

Radiological cell kill effects of regorafenib were investigated by examining the changes in ADC on DW-MRI, pre-treatment and at day 15. Matching tissue was scored for cell proliferation (KI-67 index) and apoptosis (caspase 3) at pre-treatment

and 2 months post therapy. Median ADC changes are described in Appendix Table A7. The changes at 2 months in corresponding tissue parameters of cell proliferation was not associated with an improvement in DCR [OR 1.13 (95% CI 0.14-9.0), p=0.91], PFS [HR 1.11 (95% CI 0.35- 3.58), p=0.86] or OS [HR 0.91 (95% CI 0.19-4.42), p=0.91], similarly no significant changes in apoptosis were observed when comparing baseline and 2 months treatment tissue biopsies.

Liquid biopsy as a surrogate marker of response to regorafenib

We hypothesised that regorafenib-induced anti-angiogenic effects would correlate with a reduction in ctDNA. Indeed, in a patient with significant (71%) KEF drop after 2 weeks of treatment (Fig. 4A) and durable RECIST v1.1. response lasting nearly 12 months (Fig. 4B-D), we observed that not only did the KEF reduction correlated with CD31 drop (Fig. 4E) but was also associated with a rapid and marked decrease in *KRAS G12D* ctDNA which persisted for the entire duration of the treatment and increased again when the treatment was halted due to a complication (Fig. 4F). Intriguingly, the changes in CEA lagged behind the changes in ctDNA.

To test this hypothesis we analysed changes in *RAS* mutant clones in sequential liquid biopsies by ddPCR. We examined whether a drop in fractional abundance (FA) was associated with clinical efficacy parameters. We found that the loss of detectable mutant *RAS* clones in ctDNA after 4 weeks was universal to all the examined patients [(n=21) data not shown]. However, a sustained drop in ctDNA was observed in 47.6% of the patients at 2 months and was associated with better median PFS [HR 0.21 (95% CI 0.06 - 0.71), p=0.01] and OS [HR 0.28 (95% CI O.07-1.04), p=0.06] respectively (Fig. 5A and 5B); PFS was 60.0% (after 4 months) and 40.0% (after 6 months) in the groups with decrease in FA. In a multivariate analysis adjusting for KEF reduction, this effect was associated with better PFS [HR 0.23 (95% CI 0.07-0.75), p=0.02].

Despite the small numbers, which precluded any statistical analysis, it was remarkable to observe that patients with a KEF drop >70% and decrease in ctDNA FA had the most durable response to regorafenib (Fig. 5C)

Known biomarkers of benefit from Regorafenib, toxicity profile and clinical outcome in the PROSPECT-R trial.

A previously well conducted study comprising of 208 regorafenib treated patients demonstrated an association between high neutrophil, high platelet, low lymphocyte count and/or high neutrophil lymphocyte ration (NLR) with prognosis [26]. Due to the stringent inclusion criteria of our study, our data distribution did not allow to use the same cut of used in the study be Del Prete and colleagues and median values were used instead. Notwithstanding small numbers and patient selection based on trial inclusion/exclusion criteria, no significant correlation with efficacy was found with any of the above-mentioned factors (Appendix Tables A8 and A9).

Moreover, other clinical factors such as performance status, and number of previously lines of treatment and toxicity were also compared against efficacy in a univariate analysis. Treatment related adverse events were consistent with previously reported data [4] and are summarised in Appendix Tables A10 and A11. As expected, patients who required >50% dose reduction and received less than 2 cycles of regorafenib derived less benefit from the treatment (Appendix Tables A12).

Discussion

This proof of concept phase II translational research study was designed to assess the feasibility of combining imaging, morphological and plasma biomarkers in order to best stratify patients more likely to derive benefit from regorafenib in refractory mCRC. Our study provides the first clinical evidence that regorafenib efficacy is driven by its early anti-angiogenic activity.

It is widely accepted that DCE-MRI can assess tumour vascular function[27]; however, establishing common methodology remains challenging due to the practicalities of technical implementation across different MR platforms and the choice of mathematical models for data analysis. In this study, we have used DCE-MRI acquisition and data analysis in line with international expert recommendations [27]. Whilst a large body of evidence supports the notion that perfusion MRI can be helpful in assisting dose selection and enriching patient populations more likely to respond in early phase clinical trials, most studies have defined an observable anti-angiogenic drug effect based only on the limits of DCE-MRI measurement repeatability rather than also considering the clinical efficacy[28]. Furthermore, as metastases show variable degrees of necrosis and non-enhancement before treatment and drug induced vascular pruning also leads to marked decrease in enhancement within tumors, measuring only the median K^{trans} value is less sensitive to change due to averaging of the voxel values. For these reasons, we calculated the EF and the product of K^{trans} from the enhancing voxels with EF (KEF), which better reflects proportional reduction of vascularity within tumours[24].

In this study, we have evaluated DCE-MRI in a well-defined study population, thus minimizing the bias that may result from patient heterogeneity. The selected DCE-MRI parameter threshold applied for patient stratification is based on both a prior knowledge of the measurement repeatability of our technique [29] as well as clinically validated endpoints of PFS and OS. To our knowledge, this is the first prospective study showing that KEF, a product of K^{trans} and EF, can be used as a parameter of DCE-MRI with high clinical specificity. The KEF measurement was able to identify clinically meaningful responders as early as 2 weeks into treatment with regorafenib with 100% specificity.

The major strength of this study are that it was possible to validate the findings of MRI detected regorafenib-induced suppression of tumour vascularisation by matched tissue analysis using immunostaining of the endothelial marker CD31. We demonstrated that patients with a significant drop in CD31 score on 2-month biopsy had a better PFS and OS. These findings further emphasise the fact that drug activity is due to its anti-proliferative properties.

It is established that genetic and non-genetic mechanisms of tumour heterogeneity allow functional expansion of previously dormant subclones under the selective pressure of chemotherapy in CRC cells [30]. This provides a strong biological rationale for the use of regorafenib given its broad multi-kinase anti-tumour activity. However, the diversity of mechanisms of action of this drug makes it equally challenging to identify predictive biomarkers of clinical utility. Biomarker analysis of CORRECT trial data demonstrated that benefit from regorafenib was independent of the *RAS* pathway mutational status of the tumour, suggesting primarily an antiangiogenic mechanism of action, and that liquid biopsy could be reliably used to characterize clonal mutations[8]. We investigated if the circulating tumour genotype could be used as a biomarker of sustained anti-angiogenic activity to regorafenib by tracking known *KRAS* clonal mutations and performing serial plasma analysis by highly sensitive ddPCR methodology, at clinically relevant time points. A drop in FA was observed in all patients at 4 weeks suggesting a degree of initial anti-angiogenic activity in keeping with an initial drop in radiological parameters; however, this effect was sustained in only a proportion of patients at 2 months. This group of patients with persistent drop at 2 months demonstrated better efficacy with regorafenib suggesting that sustained angiogenic activity was required in order to achieve maintained benefit from therapy. Consistent with the findings from previous studies [25, 31], we demonstrated that ctDNA can be used for tumour genotyping, but beyond this we

proved that it can also be used to monitor efficacy from regorafenib in patients showing initial benefit from the therapy.

Acknowledging the limitations due to small numbers of patients in our study, we propose that these findings should be validated in larger cohort of patients treated with anti-angiogenic therapies. Due to logistical barriers, it may however not be possible to conduct large scale trials scrupulously designed and statistically powered to address questions of biomarker analysis. The interpretation of our findings thus need to be contextualized; for example, regorafenib is currently unavailable free of charge to patients in the United Kingdom so the use of biomarkers described in this study could significantly reduce the duration of therapy in patients' unlikely to derive benefit. It is conceivable that the health economic assessment might be more favorable with appropriate predictive biomarkers such as those we have identified. Whilst, the search for a positive predictive biomarker may help better application of precision medicine, in a more non-resource-constrained funding environment, based on our findings, patients could be spared from significant drug-related side effects, which again would have health-economic benefits.

In summary, the depth of angiogenic response measured by DCE-MRI and validated by matched tissue IHC analysis correlates with clinical efficacy. The circulating tumour genotype is a potential marker of sustained anti-angiogenic response to regorafenib in patients with known clonal mutations.

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Table 1: Baseline characteristics of participating patients

	No.	*%*
Age, median [range]	63.7 [36.3-79.0]	
Gender		
Female	10	37
Male	17	63
Site of primary		
Rectal	7	26
Left Colon	9	33
Right Colon	11	41
Histology Diagnosis		
Unknown	1	4
Adeno (mucinous)	4	15
Adeno (non-mucinous)	22	81
Stage Diagnosis		
Stage II	5	19
Stage III	5	19
Stage IV	17	62
Radiotherapy to primary		
Yes	4	15
No	23	85
Number of lines in metastatic setting		
1	1	4
2	11	41
3	9	33
4	3	11
5	2	7
6	1	4

Figure Legends

Figure 1: PROSPECT-R Trial design. Patients meeting all inclusion and no exclusion criteria were required to have pre-treatment CT, DCE-MRI, and DW-MRI scans; MRI scans were then repeated on day 15. All patient were also required to have pre-treatment mandatory core biopsy, followed by a core biopsy at 2-months if they had SD or PR. Patients were monitored by CT scans every 2 months until the time of PD and if clinically feasible, they had biopsy of 1 or 2 progressing lesions from PD sites. Plasma samples were collected every 4 weeks until the time of PD. CT=computed tomography; ctDNA= circulating tumour DNA; DCE=dynamic contrast enhanced; DW= diffusion weighted; MRI=magnetic resonance imaging; PD=progressive disease; PR=partial response; SD= stable disease.

Figure 2: Outcome according to radiological parameters in the PROSPECT-R Trial. Kaplan-Meier curves for progression-free survival (**A**) and overall survival (**B**) in patients with or without KEF drop.

Figure 3: Correlation between radiological and pathological findings in the PROSPECT-R Trial. Panels **A-C** demonstrate an example of a patient with durable disease control of 14 months, whilst panel **D-F** shows example of a primary resistance patient (2 months). (**A**) Coronal DCE-MRI (central slice of a liver lesion) showing significant reduction in the median K^{trans} [min^{-1}] with accompanying histogram (whole lesion) at day 15 post-treatment. (**B**) Coronal CT images at baseline, best response (2 months) and at the end of treatment (14 months) for same liver lesion (left) and an abdo-pelvic mass (right). Patient achieved SD by RECIST v1.1. (**C**) Matched IHC analysis demonstrating decrease and subsequent increase in tumour vascularity measured by staining CD31 at 2 months and 14 months respectively. (**D**) Coronal DCE-MRI and accompanying histogram of the liver lesion

showing no significant reduction in the median K^{trans} [min^{-1}] at day 15 post treatment. (**E**) Coronal CT images of the liver showing progression (30% increase) of the same target liver lesion (yellow circle) at baseline and at progression (2 month scan). (**F**) Matched IHC analysis demonstrating no change in tumour vascularity measured by staining CD31 at 2 months. Two separate PD lesions were analysed to take into account tumour heterogeneity; however, no change in vascularity was observed in either of the biopsied lesion.

CT=computed tomography; DCE=dynamic contrast enhanced; IHC-immunohistochemistry; MRI=magnetic resonance imaging; PD=progressive disease; SD= stable disease.

Figure 4: Correlation between radiological, pathological and circulating biomarkers in PROSPECT-R Trial. (**A**) Axial DCE-MRI demonstrating significant reduction (71%) of the median K^{trans}[min^{-1}] in the left pelvic wall recurrence, with accompanying histogram at day 15 post-regorafenib. (**B**) Three dimensional representation of target lesion by CT performed at baseline and at week 31 (best response), demonstrating reduction in lesion volume. (**C**) FDG-PET images performed at 4 months of therapy, showing residual FDG uptake, although significantly less when compared to a historic PET-CT performed 18 months prior to regorafenib therapy. (**D**) Axial CT images demonstrating a maintained RECIST V1.1 PR (45%) to regorafenib for 31 weeks. Images show representative sites of disease including: left pelvis side wall, mediastinal lymphadenopathy, and large lung metastases (yellow circles). Note is made that at the time of progression, left pelvic side wall disease progressed (28%), while the remaining disease had maintained partial response demonstrating the inter-tumoural heterogeneity in resistance to regorafenib. (**E**) Matched IHC analysis demonstrating decrease and subsequent increase in tumour vascularity measured by staining CD31 at 2 months and 12 months respectively. (**F**) Graphical representation of clonal *KRAS* mutation tracked

by ddPCR analysis of ctDNA analysis compared with CEA and total volume of target lesions measured RECIST v1.1 assessment. This demonstrates that an early drop and rise in fractional abundance (FA) of *KRAS* mutation that precedes changes in CEA, both at response and resistance to regorafenib

CEA=Carcino-Embryonic Antigen; ctDNA=circulating tumour; CT=computed tomography; DCE=dynamic contrast enhanced; ddPCR=digital droplet polymerase chain reaction; FA=fractional abundance; FDG-PET=18-Fluoro-deoxyglucose positron emission tomography; IHC=immunohistochemistry; MRI=magnetic resonance imaging; PD=progressive disease; PR=partial response

Figure 5: Outcome according to ctDNA drop after 2 months of treatment in the PROPSECT-R Trial. Kaplan-Meier curves for progression-free survival (A) and overall survival (**B**) in patients with or without ctDNA drop, (**C**) spider plot demonstrating depth and duration of response to regorafenib (evaluated by RECIST v1.1. criteria) according to KEF and ctDNA drop.

Contributions: *Trial Design:* KK, DC, IC, NV. *Enrolment in the Trial:* KK, DC, SR, DW, NS, IC, NV. *Data acquisition:* KK, MR, DMK, NT, JCN, GV, SH, SM, AL, MDD, HL, AW, EAE, EF, DC, ZE, JT, RB, MB, MR, ET, MF, CB, NV. *Statistical Analysis:* EZ. *Writing and final approval of the manuscript:* all the authors.

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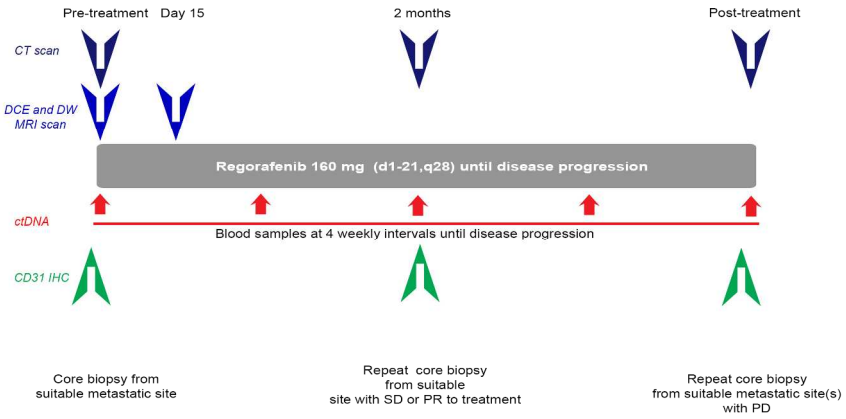
Conflict of Interest: D.C. received research funding from: Roche, Amgen, Celgene, Sanofi, Merck Serono, Novartis, AstraZeneca, Bayer, Merrimack and MedImmune. C.P. has had advisory roles with Sanofi. J.T. has had advisory roles with Amgen, Roche, Sanofi-Aventis, and Merck. A.C. has had advisory roles with Merck-Serono and Roche. He has received research funding from Roche and honoraria from Roche and Merck-Serono. I.C. has had advisory roles with Merck Serono, Roche, Sanofi Oncology, Bristol Myers Squibb, Eli-Lilly, Novartis, Gilead Science. He has received research funding from Merck-Serono, Novartis, Roche and Sanofi Oncology, and honoraria from Roche, Sanofi-Oncology, Eli-Lilly, Taiho. All other authors declare no conflict of interest.



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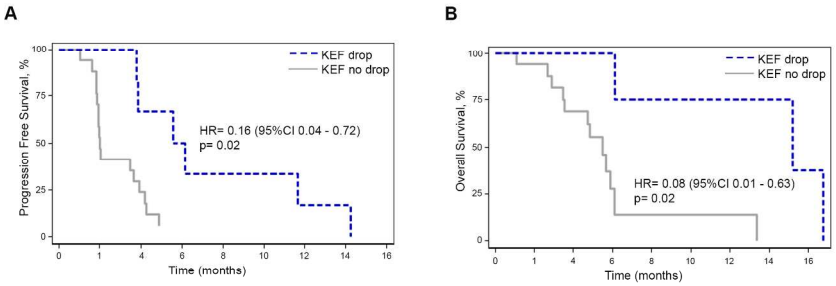

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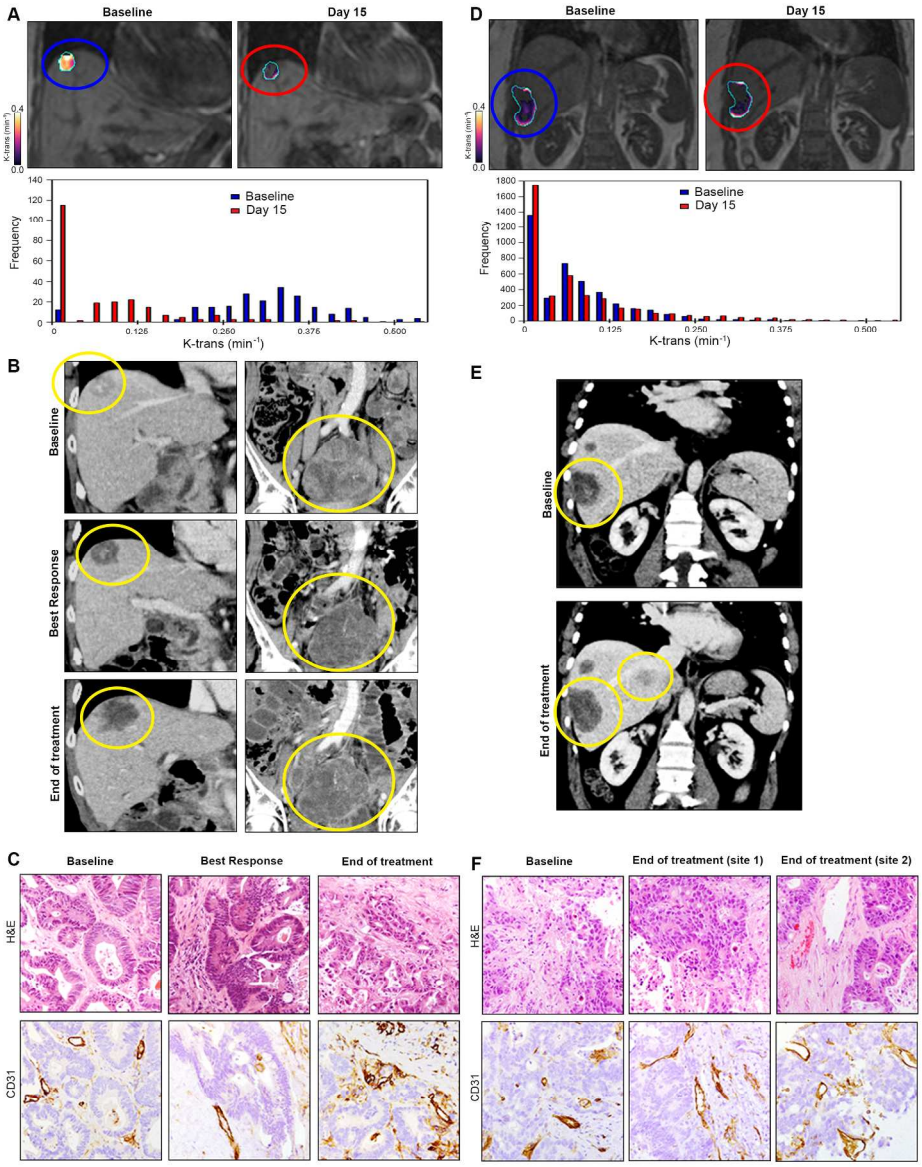

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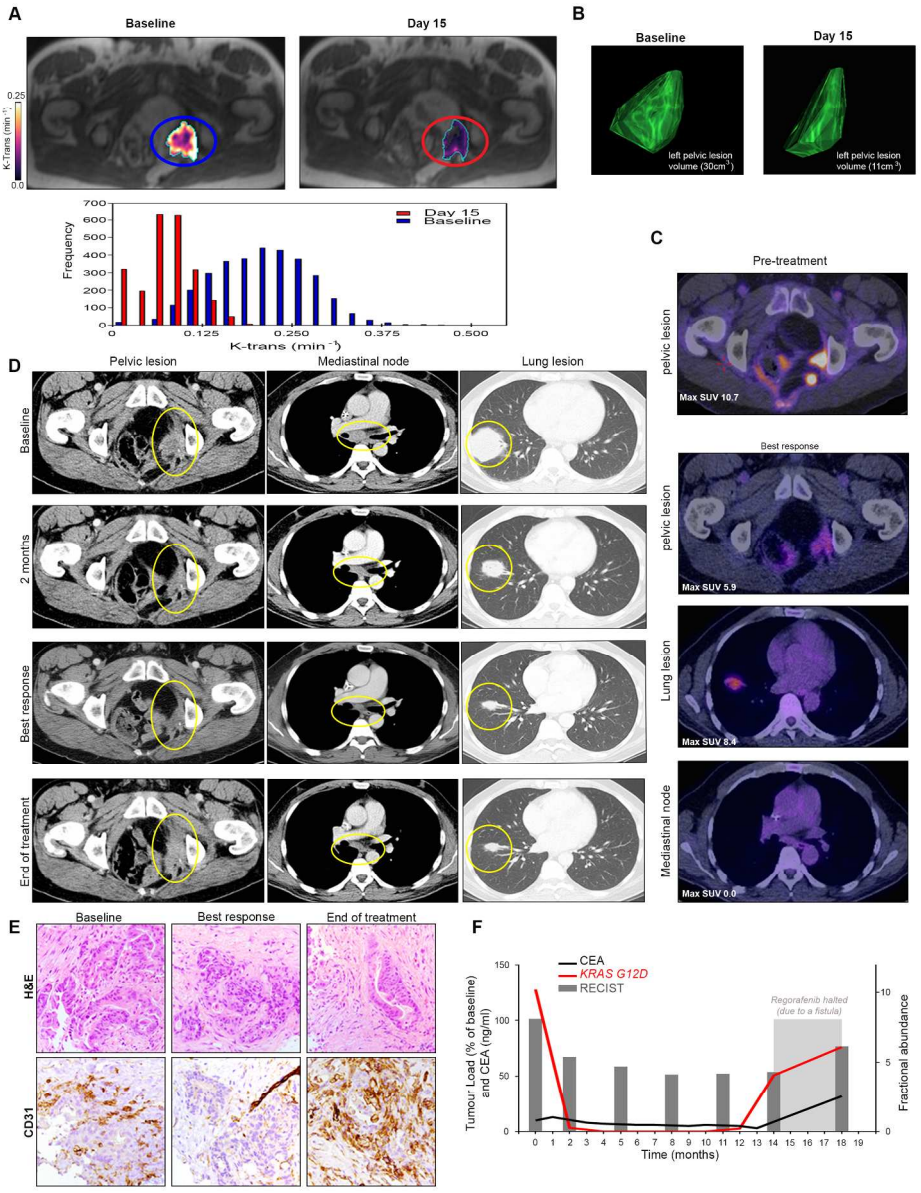

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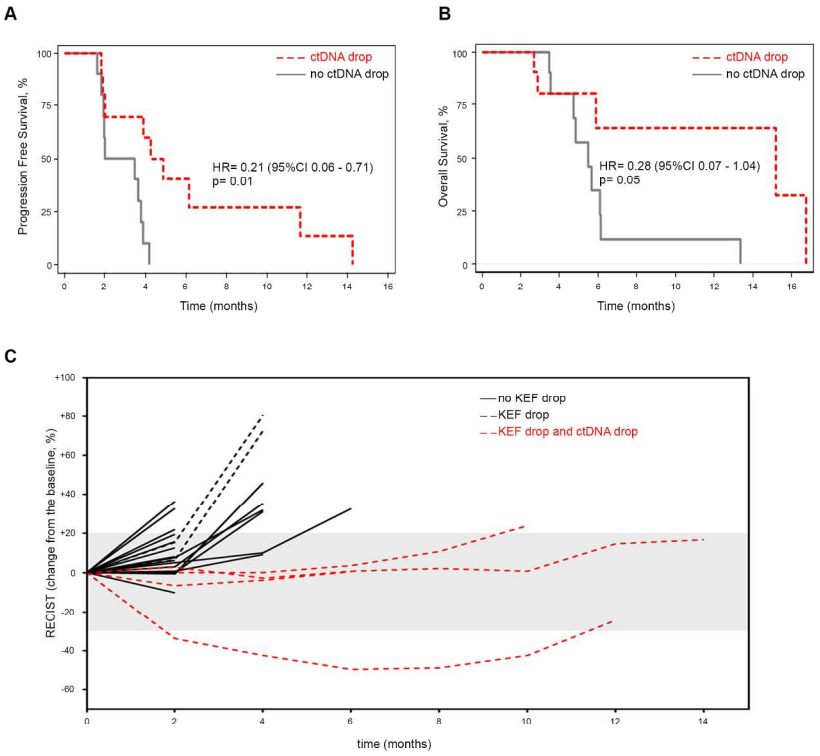

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Appendix to: Functional Imaging and circulating biomarkers of response to Regorafenib in treatment-refractory metastatic colorectal cancer patients in a PROSPECTive phase II study.

Authors: Khurum Khan, Mihaela Rata, David Cunningham, Dow-Mu Koh, Nina Tunariu, Jens C Hahne, George Vlachogiannis, Somaieh Hedayat, Silvia Marchetti, Andrea Lampis, Mahnaz Darvish Damavandi, Hazel Lote, Isma Rana, Anja Williams, Suzanne A Eccles, Elisa Fontana, David Collins, Zakaria Eltahir, Sheela Rao, David Watkins, Naureen Starling, Jan Thomas, Eleftheria Kalaitzaki, Nicos Fotiadis, Ruwaida Begum, Maria Bali, Massimo Rugge, Eleanor Temple, Matteo Fassan, Ian Chau, Chiara Braconi, & Nicola Valeri

INDEX OF SUPPLEMENTARY MATERIAL:

Methods

Study inclusion criteria (as specified in trial protocol):

1. In order to be eligible for registration, all inclusion criteria must be met. A patient must:
   - Understand, be willing to give consent, and sign the written informed consent form (ICF) prior to undergoing any study-specific procedure:
   - Be male or female and ≥ 18 years of age
2. patients with a histologically confirmed diagnosis of metastatic colorectal adenocarcinoma, have RAS MT disease (Patients who are undergoing biopsies for diagnostic purposes will be allowed to participate in the study, as long as the diagnostic test confirms the evidence of RAS mutant disease) and have received the following treatment regimens described below: Previous treatment with fluoropyrimidine-,oxaliplatin-, and irinotecan-based chemotherapy; and progressed following the last administration of approved therapy. Subjects who have discontinued treatment due to unacceptable toxicity will also be allowed into the study.

3. patients with inoperable mCRC who are suitable for treatment with regorafenib as monotherapy and had: a CT or MRI scan (chest, abdomen, pelvis and other suspected sites as applicable) to determine eligibility for recruitment within 4 weeks prior to treatment (hereafter referred to as the “Eligibility scan”)
4. patients who have metastatic disease sites which are amenable to core biopsy (preferably liver, soft tissue or nodal disease, with at least one lesion 1.5cm or more in diameter. If largest lesion 1-1.5cm diameter, eligibility to be discussed with radiologist prior to study entry)
5. patients who have an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1 within 28 days prior to the initiation of study treatment
6. patients who have adequate bone marrow, liver function, and renal function, as measured by the following laboratory assessments conducted within 14 days prior to the initiation of study treatment:

- Total bilirubin < 1.5 times the upper limit of normal (ULN)
- Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) < 2.5 times the ULN in patients with no hepatic metastases and <5 the ULN in patients with hepatic metastatic disease.
- Glomerular filtration rate (GFR) ≥ 30 mL/min/1.73 m2 according to the modified diet in renal disease (MDRD) abbreviated formula.
- Platelet count 100000 /mm3, hemoglobin (Hb) 9 g/dL, absolute neutrophil count (ANC) ^{3} 1500/mm3
- Lipase < 1.5 x ULN
- International normalized ratio (INR) of prothrombin time (PT; PT-INR) and partial thromboplastin time (PTT) ≤ 1.5 times the ULN. Patients who are therapeutically treated with an agent such as warfarin or heparin will be allowed to participate if no underlying abnormality in coagulation parameters exists as per medical history. Weekly evaluation of PT-INR/PTT will be required until stability is achieved (as defined by local standard of care and based on pre-study PT-INR/PTT values). The anti-coagulation therapy will be stopped 48 hours prior to the biopsy and re-commenced 24-48 hours after the procedure on recommendation of the interventional radiologist. Physicians will be strongly encouraged to switch oral coumadin derivatives (e.g. warfarin) to subcutaneous formulations, however if this was not possible due to any clinical reasons or patients preference, they will still be allowed on the study with careful monitoring of their INR and after discussion with the interventional radiologist performing the procedure.

7. If female and of childbearing potential, have a NEGATIVE result on a pregnancy test performed a maximum of 7 days before initiation of study treatment; pregnancy status must be documented prior to the first dose of study treatment

8. If female and of childbearing potential or if male, must agree to use adequate contraception (e.g., abstinence, intrauterine device, oral contraceptive, or double-barrier method) based on the judgment of the investigator or a designated associate from the date on which the ICF is signed until 6 months after the last dose of study drug.

Study exclusion criteria **(as specified in trial protocol)**

A patient who meets **ANY** of the exclusion criteria will **NOT** be eligible for randomization.

A patient must **NOT**

1. have had prior treatment with regorafenib or any other VEGF-targeting kinase inhibitor
2. have had previous or concurrent cancer that is distinct in primary site or histology from colorectal cancer within 2 years prior to recruitment EXCEPT for curatively treated cervical cancer in situ, non-melanoma skin cancer and superficial bladder tumors [Ta (Noninvasive tumor), Tis (Carcinoma in situ) and T1 (Tumor invades lamina propria)].
3. Patients that are participating in another clinical trial involving an investigational medicinal product, unless it is more than 14 days after they have ceased the investigational medicinal product
4. Patients that are participating in another research study involving tumour tissue biopsies planned to take place during the time that the patient is participating in this study
5. Have had a major surgical procedure, open biopsy, or significant traumatic injury within 28 days prior to initiation of study treatment
6. If female and of childbearing potential, be engaged in breast feeding
7. Be unable to swallow oral tablets (crushing of study treatment tablets is not allowed)
8. Arterial or venous thrombotic or embolic events such as cerebrovascular accident (including transient ischemic attacks), deep vein thrombosis or pulmonary embolism within 6 month before the start of study medication (except for adequately treated

catheter-related venous thrombosis occurring more than one month before the start of study medication)

9. Interstitial lung disease with ongoing signs and symptoms at the time of informed consent.
10. Ongoing infection > Grade 2 NCI CTCAE
11. Uncontrolled hypertension (Systolic blood pressure > 140 mmHg or diastolic pressure > 90 mmHg) despite optimal medical management
12. Have congestive heart failure classified as New York Heart Association Class 2 or higher
13. Have had unstable angina (angina symptoms at rest) or new-onset angina < 3 months prior to screening
14. Have had a myocardial infarction < 6 months prior to initiation of study treatment
15. Have cardiac arrhythmias requiring anti-arrhythmic therapy, with the exception of beta blockers, calcium channel blockers or digoxin
16. Have pheochromocytoma
17. Have a known history of human immunodeficiency virus infection
18. Have either active hepatitis B or C or chronic hepatitis B or C requiring treatment with antiviral therapy
19. have an active unstable seizure disorder with last episode of seizure within 4 weeks of starting the trial treatment
20. Have had a hemorrhage or a bleeding event Grade 3 (NCI-CTCAE v 4.0) within 4 weeks prior to the initiation of study treatment
21. Have a non-healing wound, ulcer, or bone fracture
22. Have renal failure requiring hemodialysis or peritoneal dialysis
23. Have persistent proteinuria > 3.5 g/24 h, measured by urine protein:creatinine ratio from a random urine sample (Grade 3, NCI-CTCAE v 4.0)
24. Have a substance abuse, medical, psychological, or social condition that may interfere with participation in the study or evaluation of the study results

25. Have a known hypersensitivity to any of the study drugs, study drug classes, or excipients in the formulation of the study drugs
26. Have history of brain metastases

Subject withdrawal criteria **(as specified in trial protocol)**

Participation in this study is voluntary. Patients will be permitted to withdraw from the study at any time.

If a patient's scheduled dose of regorafenib treatment is delayed for more than 4 weeks for any reason, or if a patient ceases treatment because of toxicity, then the patient will withdraw from this study and will not be asked to undergo the second mandatory or the optional research biopsy, unless the patient has clinically and/or radiologically progressed.

If a patient is not able to have a biopsy because they are no longer considered fit for biopsy, or there is no longer a site suitable for biopsy, then the patient will not be required to undergo further biopsies. This will need to be discussed with the Chief Investigator. These patients will be required to be replaced to complete 30 evaluable patients with paired biopsies; however, the clinical and translational data generated from the withdrawn patients will be reported as part of the study.

If a patient loses capacity to consent during the study, then the patient would withdraw from the study.

Should a patient withdraw from the study, then any biological material and data collected during the study period may still be analysed, unless the patient specifically requests that this does not occur. If the patient consents, serious adverse event data will continue to be collected for 30 days after the last procedure, even if the patient has withdrawn from the

study. Despite treatment withdrawal, patients will continue to be followed in the study. The frequency of follow-up is left to the clinician's discretion.

However, survival status should be ascertained at least once every 3 months which can be conducted over the telephone alone.

MRI data acquisition

All patients were scanned on a MAGNETOM Avanto 1.5T MR scanner (Siemens Healthcare, Erlangen, Germany) Before any post-processing, DCE-MRI liver data were manually registered (2D technique, in house software) to minimise any residual breathing effect. Regions of interest (ROIs) of the imaged target lesion were drawn, slice by slice, by a senior radiologist on high-b-value diffusion weighted images (b900) and translated to the DCE-MRI data. Voxel-wise analysis of the delineated ROIs was performed using in-house written software designed for each imaging technique [1]. For all imaging parameters, the results of each analysed image section were merged to obtain a volume of interest (VOI); the number of image sections (2-10) included in the VOI depended on the lesion size; the median value of VOI imaging parameters for every patient at each time point was reported. The ADC was calculated assuming a mono-exponential fitting algorithm.

DCE protocol: A standard dose of contrast agent (Dotarem, 0.2 ml/kg) followed by 20 ml of saline was delivered by an automatic power injector at 3 ml/s. DCE-MRI data were acquired using a 3D fast field echo sequence with: 14 coronal partitions, slice thickness 5mm, TR/TE = 3/0.89 ms, flip angle = 11°, FOV=400x400 mm^2, matrix=128x128, 1 average, parallel acquisition (Grappa acc. factor 2, ref lines 24). Dynamic scans were preceded by a calibration scan with the same parameters, but at a lower flip angle (2°) and with 7 averages, to enable contrast quantification[2]. For abdominal disease sites, patients were imaged coronally using a sequential breath-hold technique optimised for liver lesions: two image volumes were acquired during each 6 s breath-hold, followed by a 6 s breathing gap; 40

volumes were acquired over 4.18 min[3]. For pelvic disease sites, patients were imaged axially with a free breathing technique: 80 image volumes acquired continuously at 3.3 s/vol for 4.4 min.

DWI protocol: Pelvic (axial plane) and abdominal (coronal plane) DWI data were acquired in free breathing. The DWI parameters for liver acquisition were: 2D echo planar imaging sequence, 20 coronal slices, slice thickness 5 mm, TR/TE=5000/60 ms, FOV=400x400 mm^2, 5 independent acquisitions (no averaging), matrix 128x128, phase partial Fourier 7/8, parallel acquisition (GRAPPA acc. factor 2, ref lines 30), 8 b-values (0,20,40,60,120,240,480,900 s/mm^2), diffusion times δ=14.6 ms and Δ=24 ms, total acquisition time ~2 min/acquisition. Similar axial acquisitions were acquired for the pelvic region.

Isolation of circulating tumour (ct)DNA

ctDNA was extracted from EDTA anti-coagulated blood within 1 h after collection, plasma was separated from the cells by centrifugation (1500*g* for 15 min at 4 °C) followed by a second centrifugation of the supernatant at 1500*g* for 10 min at 4 °C to remove all cell debris. If not used immediately, plasma was frozen at -80 °C until further processing. ctDNA from 2 ml of plasma was isolated by the use of Qiagen blood mini kit (Qiagen, Hilden, Germany) according to manufacturer's protocol.

Statistical Analysis

1) Sample Size (as specified in trial protocol)

Since this study was exploratory in nature and included two (or three) biopsies per patient, we kept the sample size low; to 20 patients with at least two biopsies; i.e. at baseline and at progression. Patients with stable disease or response after 8 weeks were required to have three biopsies. We planed to compare the tumour molecular signatures of patients at commencement of regorafenib with that at the time of progressive disease. It was expected

that 1 to 2 patients would be recruited per month. If any patient refused or withdrew before a second biopsy at the time of disease progression, then that patient was required to be replaced. The sample size was later expanded to 30 patients with paired biopsies through a protocol amendment as half of the patients progressed without any benefit from therapy. Although patients with primary progression would provide valuable information about the reasons behind primary progression but we optimised the sample size in order to have maximum information about the mechanisms of acquired resistance to regorafenib. As indicated in the main body of manuscript, patients meeting the criteria for MRI substudy were included in this cohort analysis.

2) Statistical Analysis of plan on the study (as specified in trial protocol)

The changes in the tumour molecular signature between commencement of regorafenib and development of resistance to the drug will be described at the time of data maturation. Resistance to regorafenib will be defined as the time that the patient ceases regorafenib therapy because of a clinical decision (made by the patient's treating oncologists) to stop treatment due to progressive disease.

Survival endpoints will be analysed using Kaplan Meier methods and median survival presented with 95% confidence intervals. PFS defined as time from start of regorafenib treatment to first progression or death of any cause. OS defined as time from start of regorafenib treatment to death of any cause. Patients who are event free at the time of analysis will be censored.

Candidate genes in circulating free tumour DNA will be tested in the blood samples that are being collected every four weeks. Changes in the candidate genes across time will be described in all patients and also separately for those that achieve disease control and those

that progress (changes up to progression). We will particularly assess whether mutation detection in candidate drivers of regorafenib resistance correlated with primary resistance and whether such candidate resistance drivers become detectable before radiological progression is observed. This may allow the development of minimally invasive therapy stratification biomarkers.

In the patients who have had a tumour biopsy at 8 weeks, the changes in the transcriptomic and genetic signature from baseline to disease control will be described. Disease control rate is defined as partial or complete response or stable disease according to RECIST 1.1.

Objective response rate defined as partial or complete response according to RECIST 1.1 will be summarised as a proportion with 95% confidence interval. Disease control is defined as objective response or stable disease.

Efficacy endpoints (response and survival) will be summarised descriptively by histological growth patterns. Due to small numbers no formal statistical testing will be undertaken.

Changes in the genomic landscape from the time of diagnosis of CRC (using archival tissue) to the biopsy taken before regorafenib treatment and finally until regorafenib resistance has developed will be described. This will provide the first insight into CRC evolution throughout multiple lines of combination chemotherapy, anti-angiogenic treatment and regorafenib therapy. This should provide critical data to define rational re-biopsy strategies throughout CRC patient pathways.

Supplementary Tables

Appendix Table A1: Concordance between DCE-MRI and tissue biopsy. Liver lesions were chosen to optimise the chances of matched tissue analysis as the study involved repeated biopsies and liver is site that can be more conveniently subjected to multiple biopsies. We however didn't find any significant differences in data interpretation depending upon site of metastatic disease. Interestingly, 1 patient with pelvic mass (patient 1), who had >70% drop in KEF was found to have PR with regorafenib. This was the only patient who achieved PR on the current cohort.

Patient ID	Biopsied Target	Biopsy Guidance	Concordance MRI-Tissue Biopsy	Time-lapse between BL MRI and biopsy (less 1 week)
1	left pelvis wall	CT	yes	yes
2	pelvis mass	CT	yes	yes
3	liver: segm 3	US	yes	yes
4	liver: segm 6/7	US	yes	yes
5	pelvic mass	CT	No (MRI of segm8liver)	yes
6	peritoneal	CT/US	No (MRI of segm1liver)	yes
7	liver: segm 5	CT	yes	no (9 days)
8	liver: segm 5	CT/US	yes	yes
9	liver: segm 7	US	yes	yes
10	liver: segm 6	US	yes	yes
11	liver: segm 7	CT	yes	yes
12	liver: segm 5	US	No (MRI of segm8liver)	yes
13	liver: segm 7/8	US	yes	yes
14	liver: segm 8	US	yes	yes
15	liver: segm 3	US	yes	yes
16	liver: segm 6/7	US	yes	yes
17	liver: segm 6	US	yes	yes
18	liver: segm 2/3	US	yes	yes
19	liver: segm 2	US	yes	yes
20	liver: segm 6	US	yes	yes
21	liver: segm 3	US	yes	yes
22	liver: segm 7	US	yes	yes
23	liver: segm 6	US	yes	yes
24	liver: segm 6	US	yes	yes
25	liver: segm 6/7	US	yes	yes
26	liver: segm 6	US	yes	yes
27	liver: segm 7	US	yes	yes

Segm= segment; CT=Computed Tomography; US= Ultrasound; MRI=Magnetic Resonance Imaging; BL=baseline

Appendix Table A2: Summary of changes in DCE-MRI parameters

	K^{trans} *(nonzeros)* *[min^{-1}]*	$IAUGC_{60}$ *[mmol·s]*	*EF [%]*	*KEF [min^{-1}]*
Baseline				
Mean (sd)	0.14 (0.08)	11.91 (6.01)	88.05 (13.14)	0.13 (0.08)
Median (IQR)	0.11 (0.09 - 0.19)	10.96 (7.07 - 15.26)	92.29 (82.75 - 99.31)	0.11 (0.07 - 0.18)
Range	0.06 - 0.37	2.75-27.87	50.81 - 100.00	0.03 - 0.33
C1D15				
Mean (sd)	0.10 (0.07)	5.66 (4.08)	57.86 (23.09)	0.06 (0.05)
Median (IQR)	0.07 (0.07 - 0.10)	5.05 (2.79 - 7.04)	57.94 (41.01 - 77.92)	0.05 (0.03 - 0.07)
Range	0.03 - 0.38	2.04 - 21.97	21.99- 96.87	0.01 - 0.27
Percentage decrease				
Mean (sd)	25.20 (34.56)	49.26 (24.58)	34.31 (24.22)	51.29 (27.42)
Median (IQR)	27.75 (6.74 - 52.56)	57.70 (32.66 - 67.93)	35.33 (12.40 - 56.19)	58.28 (28.28 - 76.14)
Range	-63.22 - 82.32	-1.71 - 77.68	-14.03 - 74.76	-5.55 - 93.76

EF= enhancing fraction; IQR= inter-quartile range; sd=standard deviation; C1D15= cycle 1, day 15, KEF= product of summarised median values of K^{trans} (nonzeros) x EF; $IAUGC_{60}$= initial area under the gadolinium concentration-time curve over 60 seconds

Appendix Table A3: ROC curve analysis to choose clinically appropriate KEF drop

Cut point	*Sensitivity*	*Specificity*	*Correctly Classified*
5.55	100.00%	0.00%	60.87%
-4.63	92.86%	0.00%	56.52%
-10.01	85.71%	0.00%	52.17%
-14.38	85.71%	11.11%	56.52%
-17.87	85.71%	22.22%	60.87%
-18.25	78.57%	22.22%	56.52%
-28.27	78.57%	33.33%	60.87%
-32.49	71.43%	33.33%	56.52%
-41.55	71.43%	44.44%	60.87%
-45.47	71.43%	55.56%	65.22%
-51.59	71.43%	66.67%	69.57%
-53.69	64.29%	66.67%	65.22%
-58.28	57.14%	66.67%	60.87%
-65.25	57.14%	77.78%	65.22%
-66.37	50.00%	77.78%	60.67%
-67.12	50.00%	88.89%	65.22%
-69.21	**50.00%**	**100.00%**	**69.57%**
-70.99	42.86%	100.00%	65.22%
-77.37	35.71%	100.00%	60.87%
-77.94	28.57%	100.00%	56.52%
-78.86	21.43%	100.00%	52.17%
-82.04	14.29%	100.00%	47.83%
-93.76	7.14%	100.00%	43.48%
-93.76	0.00%	100.00%	39.13%

Appendix Table A4: Correlation between KEF and CD31 drop

	Drop in KEF		
CD31	No	Yes	Total
No change	7 *(54%)*	0	7 *(37%)*
Drop (i.e. >5% drop from baseline)	6 *(46%)*	6 *(100%)*	12 *(63%)*
Total	13 *(100%)*	6 *(100%)*	19 *(100%)*

Fisher's exact, p=0.04

Appendix Table A5: KEF (K^{trans} * EF/100) according to RECIST response (70%)

	Responder (CR/PR/SD)		Total
	No (n=9)	Yes (n=14)	(n=23)
No KEF Drop	9	8	17
KEF Drop	0	6	6
Fisher's exact p-value	0.048		
Sensitivity, 95% CI	42.9% (17.7-71.1%)		
Specificity, 95% CI	100% (66.4-100%)		
Accuracy	65.2%		
OR (95% CI)	NE (1.46-NE)		

CI=confidence interval; CR= complete response; n=number; NE= not evaluable; OR= odds ratio; PR= partial response; RECIST=response evaluation criteria in solid tumours; SD= stable disease

Appendix Table A6: KEF (K^{trans} * EF) and correlation with clinical efficacy parameters

	No KEF drop	KEF Drop
PFS		
Events	16/17	6/6
Median PFS (95% CI), months	2.0 (1.8-3.9)	5.6 (3.8-NE)
4 months PFS	23.5% (7.3%- 44.9%)	66.7% (19.5% - 90.4%)
6 months PFS	NE	50.0% (11.9% - 80.4%)
HR (95% CI)	*reference*	0.16 (0.04-0.72), p=0.02
OS		
Events	12/17	3/6
Median OS (95% CI), months	5.5 (3.4-6.1)	15.2 (6.1-NE)
4 months OS	69.0 (40.8% - 85.5%)	100% (NE)
6 months OS	27.6% (7.2% - 53.2%)	100% (NE)
1 year OS	13.8% (1.0% - 42.5%)	75.0% (12.8% - 96.1%)
HR (95% CI)	*reference*	0.08 (0.01-0.63), p=0.02

CI=confidence interval; HR= hazard ratio; PFS= progression free survival; OS=overall survival

Appendix Table A7: Apparent Diffusion Coefficient (ADC) changes on day 15

Number	*Baseline* *[10^{-5} mm^2/s]*	*C1D15* *[10^{-5} mm^2/s]*	*Relative change from baseline* *[%]*	Response
1	101.81	99.03	2.73	0
2	106.83	97.27	8.95	0
3	78.93	100.22	-26.97	0
4	93.77	103.6	-10.48	0
5	108.47	117.04	-7.90	0
6	112.76	114.75	-1.76	0
7	116.47	125.17	-7.47	0
8	85.14	86.24	-1.29	0
9	116.19	119.26	-2.64	0
10	120.72	124.77	-3.35	0
11	112.63	161.71	-43.58	0
12	91.55	103.8	-13.38	0
13	83.82	94.39	-12.61	0
14	118.67	124.74	-5.12	0
15	140.07	150.18	-7.22	0
16	117.57	127.01	-8.03	0
17	98.66	91.16	7.60	0
18	121.47	134.12	-10.41	0
19	124.64	141.39	-13.44	0
20	93.21	118.44	-27.07	0
21	102.08	107.04	-4.86	0
22	102.19	131.36	-28.54	0
23	94.15	111.29	-18.20	0
24	93.5	99.88	-6.82	0
25	95.65	99.01	-3.51	0
26	114.44	124.14	-8.48	0
27	77.73	83.83	-7.85	0

Appendix Table A8: Association of progression free survival with clinical/haematological factors

	Group A	Group B
PFS in months according to Platelets		
	<median (261)	**≥median (261)**
Events	8/11	7/12
Median PFS (95% CI), months	3.9 (1.8-5.6)	2.0 (1.8 – 4.9)
HR (95% CI)	*reference*	1.02 (0.42-2.48), p=0.953
PFS in months according to NLR		
	<median (4.46)	**≥median (4.46)**
Events	5/11	10/12
Median PFS (95% CI), months	3.9 (1.8-5.6)	2.0 (1.8 – 4.9)
HR (95% CI)	*reference*	1.50 (0.63-3.59), p=0.364
PFS in months according to line of treatment		
	≤2 lines	**>2 lines**
Events	7/11	8/12
Median PFS (95% CI), months	1.9 (1.6-3.9)	3.9 (1.9-6.1)
HR (95% CI)	*reference*	0. 43 (0.16-1.11), p=0.80
PFS in months according to Performance Status		
	PS0	**PS1**
Events	4/7	11/16
Median PFS (95% CI), months	3.6 (1.8-NE)	3.5 (1.8-4.2)
HR (95% CI)	*reference*	2.27 (0.74-694), p=0.150

CI=confidence interval; HR= hazard ratio; PFS= progression free survival; NLR= Neutrophil/Lymphocyte Ratio; PS=Performance Status

Appendix Table A9: Association of overall survival with clinical/haematological factors

	Group A	Group B
OS in months according to Platelets		
	<median (261)	**≥median (261)**
Events	8/11	7/12
Median OS (95% CI), months	5.8 (4.7-6.1)	13.3 (2.9-NE)
HR (95% CI)	*reference*	0. 72 (0.25-2.12), p=0.554
OS in months according to NLR		
	<median (4.46)	**≥median (4.46)**
Events	5/11	10/12
Median OS (95% CI), months	6.1 (2.7-NE)	5.7 (3.4-6.1)
HR (95% CI)	*reference*	1.52 (0.50-4.67), p=0.463
OS in months according to line of treatment		
	≤2 lines	**>2 lines**
Events	7/11	8/12
Median OS (95% CI), months	4.8 (2.7-NE)	6.1 (3.4-NE)
HR (95% CI)	*reference*	0. 44 (0.14-1.33), p=0.146
OS in months according to Performance Status		
	PS=0	**PS=1**
Events	4/7	11/16
Median OS (95% CI), months	13.3 (2.7-NE)	5.7 (4.7-6.1)
HR (95% CI)	*reference*	2.82 (0.75-10.6), p=0.124

CI=confidence interval; HR= hazard ratio; OS= overall survival; NLR= Neutrophil/Lymphocyte Ratio; PS=Performance Status

Appendix Table A10: Summary of dose adjustments required on the study

Dose reductions*			
Cycle	**Yes**	**%**	**Total**
C1D1	0	0.0%	27
C1D15	1	4.0%	25
C2	14	58.3%	24
C3	5	38.5%	13
C4	2	15.4%	13
C5	2	33.3%	6
C6	0	0.0%	4
C7	0	0.0%	2
C8	0	0.0%	2
C9	0	0.0%	2
C10	0	0.0%	2
C11	0	0.0%	1
C12	0	0.0%	1
C13	0	0.0%	1
C14	0	0.0%	1
Dose Delays†			
Cycle	**Yes**	**%**	**Total**
C1D1	0	0.0%	27
C1D15	0	0.0%	25
C2	4	16.7%	24
C3	6	46.2%	13
C4	1	7.7%	13
C5	0	0.0%	6
C6	1	25.0%	4
C7	0	0.0%	2
C8	0	0.0%	2
C9	0	0.0%	2
C10	0	0.0%	2
C11	0	0.0%	1
C12	0	0.0%	1
C13	0	0.0%	1
C14	0	0.0%	1
Missed days¥			
Cycle	**Yes**	**%**	**Total**
C1D1	11	40.7%	27
C1D15	10	40.0%	25
C2	7	29.2%	24
C3	5	38.5%	13
C4	4	30.8%	13
C5	2	33.3%	6
C6	2	50.0%	4
C7	0	0.0%	2
C8	0	0.0%	2
C9	0	0.0%	2
C10	0	0.0%	2
C11	0	0.0%	1

C12	1	100.0%	1
C13	1	100.0%	1
C14	0	0.0%	1

*****96% and 4% of patients required dose reductions due to non-haematological and haematological toxicities respectively. When dose reduction was required, patients were offered 120mg and 80 mg on first and second dose reductions respectively. Patients came off the study if further dose reduction was required. **†**58% of patients required dose delays due to non-haematological toxicities and 33% for other logistical reasons. **¥**Median days missed on treatment were 6 (4-9) days, mean 7(minimum 1 and maximum 14 days).

Appendix Table A11: Summary of grade 3-5 toxicities on the study

Reported Toxicity	***Grade 3-5***	
	No.	%
Rectal perforation*****	1	4
Skin rash (desquamation)	1	4
Anaemia	2	7
Diarrhoea	2	7
Haemorrhage	2	7
Fatigue	4	15
Mucositis	3	11
Hand foot syndrome	6	22
Infection	6	22

*****This was the only grade 5 toxicity in the reported cohort

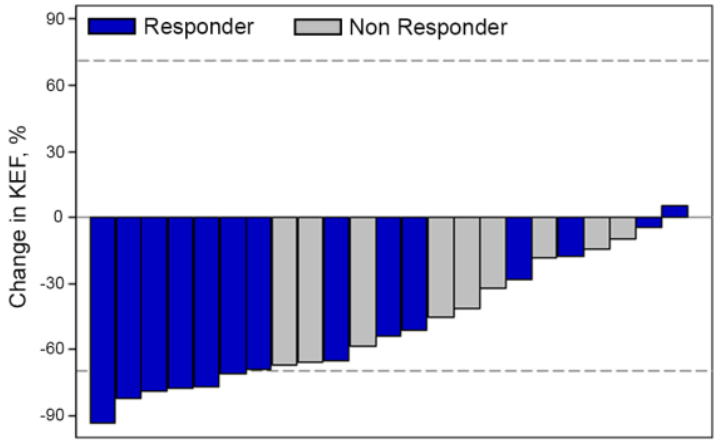
Appendix Table A12: Dose adjustments and efficacy outcomes in patients with >70% KEF drop

	Drop in KEF		
Any dose reduction ≥50%	No	Yes	Total
No	14 *(82.3%)*	2 (33.3%)	16 *(69.6%)*
Yes	3 *(17.7%)*	4 *(66.7%)*	7 *(30.4%)*
Total	17 *(100%)*	6 *(100%)*	23 *(100%)*
			Fisher's exact, **p=0.045**
Any delay	No	Yes	Total
No	13 *(76.4%)*	2 (33.3%)	15 *(65.3%)*
Yes	4 *(23.6%)*	4 *(66.7%)*	8 *(34.7%)*
Total	17 *(100%)*	6 *(100%)*	23 *(100%)*
			Fisher's exact, p=0.131
Number of cycles >2	No	Yes	Total
No	10 *(58.8%)*	0 (0%)	10 *(65.3%)*
Yes	7 *(41.2%)*	6 *(100%)*	13 *(34.7%)*
Total	17 *(100%)*	6 *(100%)*	23 *(100%)*
			Fisher's exact, **p=0.019**

KEF= K^{trans} * EF

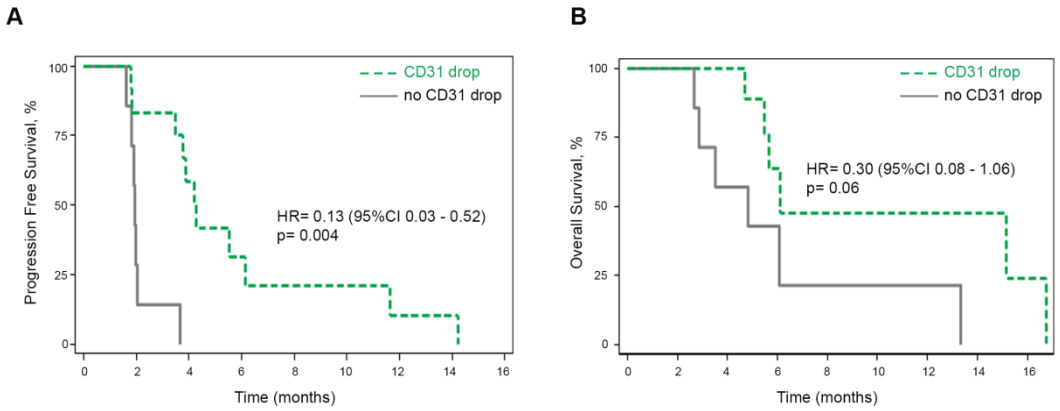
Supplementary Figures

Appendix Figure A1: Waterfall plot representing KEF drop after 15 days of treatment in responders and non- responders patients



Appendix Figure A1: Waterfall plot of drop vs. no drop in KEF (70%) according to disease control rate measured by RECIST v1.1 at 2 months after initiation of therapy; the blue colour key indicates response (defined as stable disease or partial response by RECIST 1.1) and grey key indicates progressive disease.

Appendix Figure A2: Outcome according to CD-31 drop after 2 months of treatment in the PROSPECT-R Trial



Appendix Figure A2: Kaplan-Meier curves for progression-free survival (**A**) and overall survival (**B**) in patients with or without CD-31 drop.

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Title: Functional Imaging and circulating biomarkers of response to Regorafenib in treatment-refractory metastatic colorectal cancer patients in a PROSPECTive phase II study.

Running Title: DCE-MRI and ctDNA as biomarkers of response to Regorafenib

Authors: Khurum Khan[1,2], Mihaela Rata[3], David Cunningham[1,*], Dow-Mu Koh[3], Nina Tunariu[3], Jens C Hahne[2], George Vlachogiannis[2], Somaieh Hedayat[2], Silvia Marchetti[2], Andrea Lampis[2], Mahnaz Darvish Damavandi[2], Hazel Lote[1,2], Isma Rana[1], Anja Williams[1], Suzanne A Eccles[4], Elisa Fontana[1], David Collins[3], Zakaria Eltahir[1], Sheela Rao[1], David Watkins[1], Naureen Starling[1], Jan Thomas[1], Eleftheria Kalaitzaki[1], Nicos Fotiadis[3], Ruwaida Begum[1], Maria Bali[3], Massimo Rugge[5], Eleanor Temple[1], Matteo Fassan[5], Ian Chau[1],Chiara Braconi[1,4], & Nicola Valeri[1,2,†,*]

* These authors contributed equally as senior authors

Affiliations:

[1] Department of Medicine, The Royal Marsden NHS Trust, London and Sutton. United Kingdom.

[2] Division of Molecular Pathology, The Institute of Cancer Research London and Sutton, United Kingdom.

[3] Cancer Research UK Cancer Imaging Centre, Division of Radiotherapy and Imaging, The Institute of Cancer Research and Royal Marsden Hospital, London, UK

[4] Division of Cancer Therapeutics, The Institute of Cancer Research, London and Sutton, United Kingdom.

[5] Department of Medicine (DIMED) and Surgical Pathology, University of Padua, Padua, Italy

† **Corresponding Author:**

Nicola Valeri MD, PhD

Division of Molecular Pathology

The Institute of Cancer Research London and Sutton

15 Cotswold Road, Belmont, Sutton Surrey, SM2 5NG

United Kingdom

Tel: 0044 0208 915 6634

Email nicola.valeri@icr.ac.uk

Keywords: Regorafenib, anti-angiogenic treatment, cancer therapeutics, DCE-MRI, liquid biopsy, colorectal cancer.

Word Count: 3817

ABSTRACT

Objective: Regorafenib demonstrated efficacy in metastatic colorectal cancer (mCRC) patients. Lack of predictive biomarkers, potential toxicities and cost effectiveness concerns highlight the unmet need for better patient selection.

Design: *RAS* mutant mCRC patients with biopsiable metastases were enrolled in this phase II trial. Dynamic contrast enhanced (DCE) MRI was acquired pre and at day 15 post-treatment. Median values of volume transfer constant (K^{trans}), enhancing fraction (EF) and their product KEF (summarised median values of K^{trans} x EF) were generated. Circulating tumour (ct) DNA was collected monthly until progressive disease and tested for clonal *RAS* mutations by digital-droplet PCR. Tumour vasculature (CD-31) was scored by immunohistochemistry on 70 sequential tissue biopsies.

Results: Twenty seven patients with paired DCE-MRI scans were analysed. Median KEF decrease was 58.2%. Of the 23 patients with outcome data, >70% drop in KEF (6/23) was associated with higher disease control rate (p=0.048) measured by RECIST v1.1 at 2 months, improved progression free survival (PFS) [Hazard ratio (HR) 0.16 (95% CI 0.04-0.72), p=0.02], 4-month PFS (66.7% vs. 23.5%) and overall survival (OS) [HR 0.08 (95% CI 0.01-0.63), p=0.02]. KEF drop correlated with CD-31 reduction in sequential tissue biopsies (p=0.04). *RAS* mutant clones decay in ctDNA after 8 weeks of treatment was associated with better PFS [HR 0.21 (95% CI 0.06 - 0.71), p=0.01] and OS [HR 0.28 (95% confidence interval 0.07 - 1.04), p=0.06].

Conclusions: Combining DCE-MRI and ctDNA predicts duration of anti-angiogenic response to regorafenib and may improve patient management with potential health/economic implications.

Significance of the Study

What is already known on this subject?

- Regorafenib is approved as third-line therapy for patients with refractory CRC; however, its use in the clinic has been restricted due to modest clinical benefit in unselected patients.
- Published pre-clinical studies suggested that anti-angiogenic activity of regorafenib is the main pre-determinant of its efficacy but no clinical studies have validated these findings.
- Retrospective analysis of prospective clinical trials failed to identify biomarkers od response to regorafenib that might be implemented in clinical practice.

What are the new findings?

- Regorafenib showed significant activity in patients with marked early anti-angiogenic response, resulting in a longer disease control, better PFS and OS.
- Early (day 15 post-treatment) DCE-MRI predicts response and long term outcome during Regorafenib treatment.
- Sequential analysis of tissue biopsies confirmed that reduction in tumour vasculature as the mechanism underpinning the observed radiological findings.
- Persistent regorafenib-induced anti-angiogenic effect translates into a reduction in circulating tumour (ct) DNA and this might be incorporated into the clinical algorithm for patients' management.

Implications on clinical practice

Implementing the use of DCE-MRI and ctDNA analysis as early biomarkers of response to regorafenib might improve patient selection with clear health/economic implications for patients, health systems and society.

Introduction

Colorectal cancer (CRC) remains a major health burden with significant morbidity and mortality despite recent improvements in its management owing to better screening and therapeutic options[1]. CRC is known to be a biologically heterogeneous disease characterised by the activation of several angiogenic and oncogenic pathways[2]. Regorafenib, a multikinase inhibitor (MKI) with known anti-angiogenic, anti-stromal and anti-oncogenic activities[3], has demonstrated single agent efficacy in patients with treatment refractory metastatic CRC (mCRC)[4, 5]. The use of regorafenib in the clinic is however hampered by the modest efficacy in an unselected patient population, a significant side effect profile and the high drug costs. Consequently, identification of predictive biomarkers of response and resistance to regorafenib is critical for treatment stratification and appropriate patient selection such that treatment benefits could be optimised.

Several efforts are currently ongoing to define gene signatures [6] and bio-markers of response to anti-angiogenic drug in CRC and other cancers[7]; however, ongoing validation will only determine the use of these biomarkers in clinical practice. Whilst recent studies utilising tissue [8] and plasma [9, 10] have attempted to elucidate the response and resistance mechanisms to regorafenib, the search for a clinically useful biomarker has been largely unsuccessful. A growing body of pre-clinical evidence suggests strong anti-angiogenic and pro-apoptotic effects of regorafenib [11, 12, 13, 14] with clinical data demonstrating that drug activity is independent of the tumour's mutational status[8]. These findings strengthen the hypothesis that additional mechanisms other than oncogenic blockade are responsible for the anti-tumour activity of this drug. Dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) may have a useful role in evaluating tumour vascular heterogeneity and early anti-angiogenic effects[15],[16]; moreover, its parameters volume transfer constant (K^{trans}), enhancing fraction (EF), and initial area under the gadolinium concentration-time curve over 60 seconds ($IAUGC_{60}$) have been correlated with micro-vessel

density and in some tumours with degree of VEGF expression [17]. By contrast, diffusion weighted MRI (DW-MRI) offers useful information that reflects tumour cellularity and increase in its quantitative parameter Apparent Diffusion Coefficient (ADC) has been associated with tumour cell death and necrosis[18, 19]. At least two pre-clinical studies demonstrated that regorafenib was able to significantly suppress tumour vascularity when quantified by DCE computed tomography (CT) and MRI modalities respectively in human colon carcinoma xenograft models[14, 20].

In this prospective phase II trial of patients with *RAS* mutant mCRC treated with single agent regorafenib we hypothesised that 1) an early anti-angiogenic and anti-proliferative activity of regorafenib might be detected by multi-parametric DCE-MRI on day 15 of the treatment 2) the depth of anti-angiogenic response detected by a significant drop in DCE-MRI quantitative parameters might correlate with clinical efficacy 3) analysis of sequential tissue and liquid biopsies could be integrated into the biomarker discovery process and shed insights into mechanisms of response to regorafenib.

Material and Methods

Clinical Trial Design

PROSPECT-R trial (clinical trials.gov number [NCT03010722],) is a phase II, open label, non-randomised study of regorafenib in patients with *RAS* mutant, chemo-refractory mCRC (Fig. 1). Patients who were at least 18 years old and had a World Health Organisation (WHO) performance status (PS) of 0-1, were deemed eligible if: all conventional treatment options including fluorouracil, irinotecan, oxaliplatin and at least one anti-VEGF drugs (later trial protocol was amended due to changes in availability of anti-VEGF agents due to funding restrictions in UK) were exhausted; they had metastatic tumour amenable to biopsy and repeat measurements with DCE-MRI. Written informed consent was obtained from all patients. The study was carried out in accordance with the Declaration of Helsinki and approved by National

Institutional review boards [Medicines and Healthcare products Regulatory Agency: 15983/0249/001-0001]. All participants were required to have mandatory pre-treatment biopsies (6 cores targeted towards the MRI identified index lesion), biopsies at 2 months [if response or stable disease by RECIST v1.1 criteria (6 cores)] and at the time of progression (6-12 cores from two suitable progressing metastatic sites). 3 out of 6 cores were snap-frozen; one core was used to establish patient-derived organoids and two cores were formalin fixed and paraffin embedded (FFPE). The results section describes the number of cores used for immunohistochemistry analysis in the current study. Further genomic, transcriptomic and functional analyses are on-going on the remaining cores. Patients with suitable metastatic disease (defined as lesions at least 2 cm in diameter) and no contraindications to MRI underwent multiparametric MRI studies including matched DCE and DWI; images were acquired less than 7 days prior to therapy and at day 15 post-treatment. Treatment consisted of regorafenib 160mg once daily on a schedule of three weeks on and one week off until progression or intolerable side effects. More details on inclusion and exclusion criteria and criteria for patients' withdrawal on the study are provided in the online supplementary material.

MRI data processing:

DCE-MRI data were post-processed using the Magnetic Resonance Imaging Workbench software developed at our institution [21]. The pharmacokinetic analysis was based on the extended Kety/Tofts model in conjunction with a cosine-based arterial input function (AIF) model derived from population-averaged values[22, 23]. DCE-MRI parameters including K^{trans}, $IAUGC_{60}$ and the EF were obtained for pre/post-treatment datasets. K^{trans} estimates were reported for both whole tumour [K^{trans}(all)] and valid voxels only [K^{trans}(nonzeros), i.e. excluding all non-enhancements and non model-fits] in order to address the extended necrosis observed in the cohort. The EF was defined as percentage of the voxels that enhance above the noise floor

out of all tumour voxels. A voxel was considered enhancing when it's post-contrast (Dotarem, Guerbet, France) dynamic intensity signal was at least one standard deviation higher than the mean pre-contrast signal, for a period of 60s post contrast onset. Finally, volume change in tumour enhancement during therapy (such as new necrosis) was accounted for by reporting a composite parameter, KEF, which is the product of summarised median values of KEF= K^{trans} (nonzeros) x EF[24]. For KEF, an ROC curve analysis was performed to establish the cut off able to identify meaningful clinical benefit based on disease control rate (DCR), progression free survival (PFS) and overall survival (OS).

Digital Droplet (dd) PCR

The QX200 ddPCR system (Bio-Rad, Berkeley, California) was used and all reactions were prepared using the ddPCR Supermix with no dUTTP for Probes. All PCR reactions were performed as duplex PCR using the relevant digital PCR assays for the wild-type and the mutation in question. Droplets were generated using the QX200 droplet generator according to the manufacturer's protocols. The PCR reaction was performed in a C1000 Touch Thermo Cycler (Bio-Rad) using the following protocol: 95°C for 10 min followed by 40 cycles of 94°C for 30 sec and 55°C for 1 min, then 98°C for 10 min. Droplets were read in the QX200 droplet reader and analyzed using the Quantasoft software version 1.6.6.0320 (Bio-Rad). Fractional Abundance (FA) was defined as follows: F.A. % = (N*mut*/(N*mut* + N*wt*)) × 100), where N*mut* is the number of mutant events and N*wt* is the number of WT events per reaction. The number of positive and negative droplets was used to calculate the concentration of the target and reference DNA sequences and their Poisson-based 95% confidence intervals. ddPCR analysis of normal control plasma DNA (from cell lines) and no DNA template controls were always included. Samples with very low positive events were repeated at least twice in independent experiments to validate the obtained results as previously described[25].

CD31, Ki-67 and Caspase-3 immunohistochemical staining

The immunohistochemical expression of microvascular density (CD31; clone ab28364, Abcam, Cambridge, UK; dilution 1:50), cell proliferation (Ki-67; clone ab16667, Abcam; dilution 1:100), and cell apoptosis (Cleaved Caspase-3 (Asp175) (5A1E) ab9664S, Abcam; dilution 1:100) was examined on consecutive 4-µm formalin-fixed and paraffin-embedded (FFPE) sections of the neoplastic cores. Reactions were performed using the automated Benchmark® XT platform (Ventana Medical Systems, Basel, Switzerland). Appropriate positive and negative controls were run concurrently.

For assessment of tumour microvascular density, CD31-positive micro-vessels were quantified and reported as the average number in 10 random fields at 200x magnification. Ki-67 labelling index was assessed as the average number of proliferating cells in 10 random fields at 200x magnification. Caspase-3 evaluation was categorized as positive or negative.

Statistical Analysis

The Disease Control Rate (DCR) was defined by the sum of complete responses (CR) + partial responses (PR) + stable diseases (SD) using RECIST v1.1. PFS was measured from start of treatment to date of progression or death from any cause. OS was defined as time from start of treatment to death of any cause. Patients without an event were censored at last follow up. Response according to KEF (K^{trans} (nonzeros) x EF) was defined as a drop of >70% from baseline whilst change in CD31 biomarker levels from baseline was calculated as [(8wks-baseline)/ baseline] *100. CD31 change from baseline was explored on a continuous scale and was also dichotomised using the median value.

Response according to KEF parameter and the dichotomised CD31 change from baseline were cross-tabulated with the RECIST measured DCR. Chi^2 or Fisher's

exact tests were employed to explore whether there is an association between them and DCR. Logistic regression was employed to produce odds ratios (ORs) and 95% confidence intervals (CIs). The PFS and OS rates were estimated using the Kaplan-Meier method and survival curves were generated for each group. The log-rank test was used to compare the survival curves and a Cox proportional hazards model was fitted to obtain hazard ratios (HRs) and 95% CIs. The proportional hazards assumption was tested with the use of Schoenfeld residuals.

In our study, despite relatively small study cohort, the changes in K^{trans} and KEF values were noticeably larger (e.g. > 50% reduction in mean and median KEF). Based on results of the 23 analysable patients evaluated by DCE-MRI in our study, our patient sample size by post-hoc analysis (based on Wilcoxon-signed rank test) demonstrated 100% power to detect this difference at a level of significance of 0.05.

*Additiona*l Methods can be found in the Online Appendix

Results

Patients' characteristics and tissue collection

Twenty seven treated patients (63% males) were recruited in the DCE-MRI PROSPECT-R trial and a total of 143 cores were collected by tissue biopsies from 70 metastatic lesions for the current analysis. Right and left sided primary cancers were equally distributed in the study population; other relevant patient characteristics are summarised in Table 1.

Fifty-four tissue cores were obtained from BL biopsies of 27 treated (27 lesions) patients; of the 14 patients with SD at 8 weeks, 24 tissue cores were obtained from 12 (12 lesions) patients (one patient missed the biopsy due to a hospital admission secondary to chest infection and the other developed treatment related rectal wall perforation). A further 65 tissue cores were obtained from 23 evaluable patients (35 lesions in total; 12 patients with two progressing lesions each) with PD (3 patients did

not complete 2 cycles of treatment and 1 came off due to treatment related rectal wall perforation). There was 89% concordance between target DCE-MRI and biopsied metastatic lesions (Appendix Table A1). Two FFPE cores per patient were tested at each time-point. One-hundred and nine plasma samples were tested to track *RAS* mutant clones in 21 corresponding patients; patients were required to have at least one sample available at 2 months following treatment.

Radiological and pathological evidence of early regorafenib induced anti-angiogenic effects

A significant drop in all DCE-MRI parameters was seen after 2 weeks of treatment; median K^{trans}, $IAUGC_{60}$, EF and KEF product decreased by 27.8% [interquartile range (IQR) 6.7-52.6], 57.7% (32.7-67.9), 35.3% (12.4-56.2) and 58.3% (28.3-76.1) (Appendix Table A2). The ROC curve analysis performed for the KEF showed that a 69.21% reduction from baseline had 100% specificity and overall accuracy of 69.57%; for pragmatic reasons a minimum KEF product reduction of 70% was chosen (Appendix Table A3). Matched tissue analysis revealed a strong concordance between a drop in KEF and mean vascular density of tissue, as measured by CD31 count obtained pre-treatment and at 8 weeks in patients with tissue and MR parameter data available (p=0.04). (Appendix Table A4).

Correlation of functional imaging data and CD31 staining with clinical parameters

After a median follow up of 14.3 months [(95% CI 4.9 – not evaluable (NE)], IQR 4.9-not reached (NR)], 23 patients, who had at least 1 cycle of regorafenib and a response assessment by computed tomography (CT) scan at 2 months were analysable. DCR at 2 months, median PFS and median OS were 51.9%, 3.6 months (95% CI 1.9-4.2 months) and 5.8 months (95% CI 4.7-13.3 months) respectively; 77.4% (95% CI 54.0-89.9%), 48.0% (95% CI 24.1-68.5%) and 32.0% (95% CI 11.2-

53.4%) of patients were alive at 4, 6 and 12 months respectively. Patients with >70% drop in KEF (8/27; 2 patients didn't undergo the 2 month scan due to treatment-related toxicities and thus were excluded from the final analysis as per the study protocol) were found to have higher DCR (6/6 vs. 0/6, p=0.05) at 2 months (Appendix Table A5), better PFS [HR 0.16 (95% CI 0.04-0.72), p=0.02], better PFS at 4-months (66.7% vs. 23.5%) and better OS [HR 0.08 (95% CI 0.01-0.63), p=0.02]. For the group with >70% drop in KEF, 6-month and 12-month OS were 100% (95% CI NE) and 75% (95% CI 12.8% - 96.1%) respectively compared to 27.6% (7.2-53.2%) and 13.8% (1.0-42.5%) in the <70% drop in KEF group **(**Fig 2A-B**;** Appendix Figure A1 and Appendix Table A6). In order to address the relative improvement in efficacy with or without KEF drop, we compared the outcomes of all the patients who achieved DCR; PFS was found to be 5.6 vs. 4.2 months [HR 0.30 (95% CI 0.06-1.49), p=0.140) and OS was 15.2 vs. 5.8 months [HR 0.11 (95% CI 0.01-1.06), p=0.057] in this analysis. Interestingly, when the same analysis was repeated with the cut-off chosen by ROC analysis (69.21%), PFS [HR 0.18 (95% CI 0.03-0.91), p=0.038] and OS [HR 0.11 (95% CI 0.01-1.01), p=0.051] were found to be statistically significant despite small numbers.

A decrease in CD31 score at 2 months was associated with higher DCR [OR 30.0 (95% CI 2.22- 405.98), p=0.01], better PFS [HR 0.13 (95% CI 0.03- 0.52), p=0.004] and better OS [HR 0.30 (95% CI 0.08- 1.06), p=0.06] **(**Appendix Fig. A2**).** Examples of KEF drop, RECIST 1.1 response and CD31 scoring at different time-points in a responder (Fig. 3A-C) and non-responder patient (Fig. 3D-F) are provided.

Radiological and pathological analysis of proliferation and apoptosis following regorafenib treatment

Radiological cell kill effects of regorafenib were investigated by examining the changes in ADC on DW-MRI, pre-treatment and at day 15. Matching tissue was scored for cell proliferation (KI-67 index) and apoptosis (caspase 3) at pre-treatment

and 2 months post therapy. Median ADC changes are described in Appendix Table A7. The changes at 2 months in corresponding tissue parameters of cell proliferation was not associated with an improvement in DCR [OR 1.13 (95% CI 0.14-9.0), p=0.91], PFS [HR 1.11 (95% CI 0.35- 3.58), p=0.86] or OS [HR 0.91 (95% CI 0.19-4.42), p=0.91], similarly no significant changes in apoptosis were observed when comparing baseline and 2 months treatment tissue biopsies.

Liquid biopsy as a surrogate marker of response to regorafenib

We hypothesised that regorafenib-induced anti-angiogenic effects would correlate with a reduction in ctDNA. Indeed, in a patient with significant (71%) KEF drop after 2 weeks of treatment (Fig. 4A) and durable RECIST v1.1. response lasting nearly 12 months (Fig. 4B-D), we observed that not only did the KEF reduction correlated with CD31 drop (Fig. 4E) but was also associated with a rapid and marked decrease in *KRAS G12D* ctDNA which persisted for the entire duration of the treatment and increased again when the treatment was halted due to a complication (Fig. 4F). Intriguingly, the changes in CEA lagged behind the changes in ctDNA.

To test this hypothesis we analysed changes in *RAS* mutant clones in sequential liquid biopsies by ddPCR. We examined whether a drop in fractional abundance (FA) was associated with clinical efficacy parameters. We found that the loss of detectable mutant *RAS* clones in ctDNA after 4 weeks was universal to all the examined patients [(n=21) data not shown]. However, a sustained drop in ctDNA was observed in 47.6% of the patients at 2 months and was associated with better median PFS [HR 0.21 (95% CI 0.06 - 0.71), p=0.01] and OS [HR 0.28 (95% CI O.07-1.04), p=0.06] respectively (Fig. 5A and 5B); PFS was 60.0% (after 4 months) and 40.0% (after 6 months) in the groups with decrease in FA. In a multivariate analysis adjusting for KEF reduction, this effect was associated with better PFS [HR 0.23 (95% CI 0.07-0.75), p=0.02].

Despite the small numbers, which precluded any statistical analysis, it was remarkable to observe that patients with a KEF drop >70% and decrease in ctDNA FA had the most durable response to regorafenib (Fig. 5C)

Known biomarkers of benefit from Regorafenib, toxicity profile and clinical outcome in the PROSPECT-R trial.

A previously well conducted study comprising of 208 regorafenib treated patients demonstrated an association between high neutrophil, high platelet, low lymphocyte count and/or high neutrophil lymphocyte ration (NLR) with prognosis [26]. Due to the stringent inclusion criteria of our study, our data distribution did not allow to use the same cut of used in the study be Del Prete and colleagues and median values were used instead. Notwithstanding small numbers and patient selection based on trial inclusion/exclusion criteria, no significant correlation with efficacy was found with any of the above-mentioned factors (Appendix Tables A8 and A9).

Moreover, other clinical factors such as performance status, and number of previously lines of treatment and toxicity were also compared against efficacy in a univariate analysis. Treatment related adverse events were consistent with previously reported data [4] and are summarised in Appendix Tables A10 and A11. As expected, patients who required >50% dose reduction and received less than 2 cycles of regorafenib derived less benefit from the treatment (Appendix Tables A12).

Discussion

This proof of concept phase II translational research study was designed to assess the feasibility of combining imaging, morphological and plasma biomarkers in order to best stratify patients more likely to derive benefit from regorafenib in refractory mCRC. Our study provides the first clinical evidence that regorafenib efficacy is driven by its early anti-angiogenic activity.

It is widely accepted that DCE-MRI can assess tumour vascular function[27]; however, establishing common methodology remains challenging due to the practicalities of technical implementation across different MR platforms and the choice of mathematical models for data analysis. In this study, we have used DCE-MRI acquisition and data analysis in line with international expert recommendations [27]. Whilst a large body of evidence supports the notion that perfusion MRI can be helpful in assisting dose selection and enriching patient populations more likely to respond in early phase clinical trials, most studies have defined an observable anti-angiogenic drug effect based only on the limits of DCE-MRI measurement repeatability rather than also considering the clinical efficacy[28]. Furthermore, as metastases show variable degrees of necrosis and non-enhancement before treatment and drug induced vascular pruning also leads to marked decrease in enhancement within tumors, measuring only the median K^{trans} value is less sensitive to change due to averaging of the voxel values. For these reasons, we calculated the EF and the product of K^{trans} from the enhancing voxels with EF (KEF), which better reflects proportional reduction of vascularity within tumours[24].

In this study, we have evaluated DCE-MRI in a well-defined study population, thus minimizing the bias that may result from patient heterogeneity. The selected DCE-MRI parameter threshold applied for patient stratification is based on both a prior knowledge of the measurement repeatability of our technique [29] as well as clinically validated endpoints of PFS and OS. To our knowledge, this is the first prospective study showing that KEF, a product of K^{trans} and EF, can be used as a parameter of DCE-MRI with high clinical specificity. The KEF measurement was able to identify clinically meaningful responders as early as 2 weeks into treatment with regorafenib with 100% specificity.

The major strength of this study are that it was possible to validate the findings of MRI detected regorafenib-induced suppression of tumour vascularisation by matched tissue analysis using immunostaining of the endothelial marker CD31. We demonstrated that patients with a significant drop in CD31 score on 2-month biopsy had a better PFS and OS. These findings further emphasise the fact that drug activity is due to its anti-proliferative properties.

It is established that genetic and non-genetic mechanisms of tumour heterogeneity allow functional expansion of previously dormant subclones under the selective pressure of chemotherapy in CRC cells [30]. This provides a strong biological rationale for the use of regorafenib given its broad multi-kinase anti-tumour activity. However, the diversity of mechanisms of action of this drug makes it equally challenging to identify predictive biomarkers of clinical utility. Biomarker analysis of CORRECT trial data demonstrated that benefit from regorafenib was independent of the *RAS* pathway mutational status of the tumour, suggesting primarily an antiangiogenic mechanism of action, and that liquid biopsy could be reliably used to characterize clonal mutations[8]. We investigated if the circulating tumour genotype could be used as a biomarker of sustained anti-angiogenic activity to regorafenib by tracking known *KRAS* clonal mutations and performing serial plasma analysis by highly sensitive ddPCR methodology, at clinically relevant time points. A drop in FA was observed in all patients at 4 weeks suggesting a degree of initial anti-angiogenic activity in keeping with an initial drop in radiological parameters; however, this effect was sustained in only a proportion of patients at 2 months. This group of patients with persistent drop at 2 months demonstrated better efficacy with regorafenib suggesting that sustained angiogenic activity was required in order to achieve maintained benefit from therapy. Consistent with the findings from previous studies [25, 31], we demonstrated that ctDNA can be used for tumour genotyping, but beyond this we

proved that it can also be used to monitor efficacy from regorafenib in patients showing initial benefit from the therapy.

Acknowledging the limitations due to small numbers of patients in our study, we propose that these findings should be validated in larger cohort of patients treated with anti-angiogenic therapies. Due to logistical barriers, it may however not be possible to conduct large scale trials scrupulously designed and statistically powered to address questions of biomarker analysis. The interpretation of our findings thus need to be contextualized; for example, regorafenib is currently unavailable free of charge to patients in the United Kingdom so the use of biomarkers described in this study could significantly reduce the duration of therapy in patients' unlikely to derive benefit. It is conceivable that the health economic assessment might be more favorable with appropriate predictive biomarkers such as those we have identified. Whilst, the search for a positive predictive biomarker may help better application of precision medicine, in a more non-resource-constrained funding environment, based on our findings, patients could be spared from significant drug-related side effects, which again would have health-economic benefits.

In summary, the depth of angiogenic response measured by DCE-MRI and validated by matched tissue IHC analysis correlates with clinical efficacy. The circulating tumour genotype is a potential marker of sustained anti-angiogenic response to regorafenib in patients with known clonal mutations.

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Table 1: Baseline characteristics of participating patients

	No.	*%*
Age, median [range]	63.7 [36.3-79.0]	
Gender		
Female	10	37
Male	17	63
Site of primary		
Rectal	7	26
Left Colon	9	33
Right Colon	11	41
Histology Diagnosis		
Unknown	1	4
Adeno (mucinous)	4	15
Adeno (non-mucinous)	22	81
Stage Diagnosis		
Stage II	5	19
Stage III	5	19
Stage IV	17	62
Radiotherapy to primary		
Yes	4	15
No	23	85
Number of lines in metastatic setting		
1	1	4
2	11	41
3	9	33
4	3	11
5	2	7
6	1	4

Figure Legends

Figure 1: PROSPECT-R Trial design. Patients meeting all inclusion and no exclusion criteria were required to have pre-treatment CT, DCE-MRI, and DW-MRI scans; MRI scans were then repeated on day 15. All patient were also required to have pre-treatment mandatory core biopsy, followed by a core biopsy at 2-months if they had SD or PR. Patients were monitored by CT scans every 2 months until the time of PD and if clinically feasible, they had biopsy of 1 or 2 progressing lesions from PD sites. Plasma samples were collected every 4 weeks until the time of PD. CT=computed tomography; ctDNA= circulating tumour DNA; DCE=dynamic contrast enhanced; DW= diffusion weighted; MRI=magnetic resonance imaging; PD=progressive disease; PR=partial response; SD= stable disease.

Figure 2: Outcome according to radiological parameters in the PROSPECT-R Trial. Kaplan-Meier curves for progression-free survival (**A**) and overall survival (**B**) in patients with or without KEF drop.

Figure 3: Correlation between radiological and pathological findings in the PROSPECT-R Trial. Panels **A-C** demonstrate an example of a patient with durable disease control of 14 months, whilst panel **D-F** shows example of a primary resistance patient (2 months). (**A**) Coronal DCE-MRI (central slice of a liver lesion) showing significant reduction in the median K^{trans} [min^{-1}] with accompanying histogram (whole lesion) at day 15 post-treatment. (**B**) Coronal CT images at baseline, best response (2 months) and at the end of treatment (14 months) for same liver lesion (left) and an abdo-pelvic mass (right). Patient achieved SD by RECIST v1.1. (**C**) Matched IHC analysis demonstrating decrease and subsequent increase in tumour vascularity measured by staining CD31 at 2 months and 14 months respectively. (**D**) Coronal DCE-MRI and accompanying histogram of the liver lesion

showing no significant reduction in the median K^{trans} [min^{-1}] at day 15 post treatment. (**E**) Coronal CT images of the liver showing progression (30% increase) of the same target liver lesion (yellow circle) at baseline and at progression (2 month scan). (**F**) Matched IHC analysis demonstrating no change in tumour vascularity measured by staining CD31 at 2 months. Two separate PD lesions were analysed to take into account tumour heterogeneity; however, no change in vascularity was observed in either of the biopsied lesion.

CT=computed tomography; DCE=dynamic contrast enhanced; IHC-immunohistochemistry; MRI=magnetic resonance imaging; PD=progressive disease; SD= stable disease.

Figure 4: Correlation between radiological, pathological and circulating biomarkers in PROSPECT-R Trial. (**A**) Axial DCE-MRI demonstrating significant reduction (71%) of the median K^{trans}[min^{-1}] in the left pelvic wall recurrence, with accompanying histogram at day 15 post-regorafenib. (**B**) Three dimensional representation of target lesion by CT performed at baseline and at week 31 (best response), demonstrating reduction in lesion volume. (**C**) FDG-PET images performed at 4 months of therapy, showing residual FDG uptake, although significantly less when compared to a historic PET-CT performed 18 months prior to regorafenib therapy. (**D**) Axial CT images demonstrating a maintained RECIST V1.1 PR (45%) to regorafenib for 31 weeks. Images show representative sites of disease including: left pelvis side wall, mediastinal lymphadenopathy, and large lung metastases (yellow circles). Note is made that at the time of progression, left pelvic side wall disease progressed (28%), while the remaining disease had maintained partial response demonstrating the inter-tumoural heterogeneity in resistance to regorafenib. (**E**) Matched IHC analysis demonstrating decrease and subsequent increase in tumour vascularity measured by staining CD31 at 2 months and 12 months respectively. (**F**) Graphical representation of clonal *KRAS* mutation tracked

by ddPCR analysis of ctDNA analysis compared with CEA and total volume of target lesions measured RECIST v1.1 assessment. This demonstrates that an early drop and rise in fractional abundance (FA) of *KRAS* mutation that precedes changes in CEA, both at response and resistance to regorafenib

CEA=Carcino-Embryonic Antigen; ctDNA=circulating tumour; CT=computed tomography; DCE=dynamic contrast enhanced; ddPCR=digital droplet polymerase chain reaction; FA=fractional abundance; FDG-PET=18-Fluoro-deoxyglucose positron emission tomography; IHC=immunohistochemistry; MRI=magnetic resonance imaging; PD=progressive disease; PR=partial response

Figure 5: Outcome according to ctDNA drop after 2 months of treatment in the PROPSECT-R Trial. Kaplan-Meier curves for progression-free survival (A) and overall survival (**B**) in patients with or without ctDNA drop, (**C**) spider plot demonstrating depth and duration of response to regorafenib (evaluated by RECIST v1.1. criteria) according to KEF and ctDNA drop.

Contributions: *Trial Design:* KK, DC, IC, NV. *Enrolment in the Trial:* KK, DC, SR, DW, NS, IC, NV. *Data acquisition:* KK, MR, DMK, NT, JCN, GV, SH, SM, AL, MDD, HL, AW, EAE, EF, DC, ZE, JT, RB, MB, MR, ET, MF, CB, NV. *Statistical Analysis:* EZ. *Writing and final approval of the manuscript:* all the authors.

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