

Molecular Determinants of Therapeutic Response to Targeted Therapies in Metastatic Colorectal Cancer

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ABSTRACT

Colorectal cancer is the most common cause of cancer in Australia, causing over 5000 deaths each year. The median survival time for patients with metastatic colorectal cancer (mCRC) is approximately two years. More recently, further modest improvements in survival have occurred due to the use of novel targeted therapies such as Epidermal Growth Factor Receptor antibodies (EGFR-mAb) and bevacizumab, a Vascular Endothelial Growth Factor (VEGF) antibody. The cost to benefit ratio of these new drugs is high, but could be improved by three separate approaches, which are explored in this thesis. Efficacy could be improved by the combination of these drugs with other novel agents to potentiate their action; these drugs could be restricted to use in a population pre-identified by tumour characteristics to have a high chance of benefit; and finally, efficacy could be improved by identifying potentially reversible molecular mechanisms that prevent durable responses to these drugs, leading to acquired resistance.

Currently, EGFR-mAbs are used as single agent therapy or in combination with cytotoxic chemotherapy in patients who do not have mutations in the *KRAS* gene. Mutations in this gene lead to intrinsic resistance to EGFR-mAb therapy. In contrast, acquired resistance to EGFR-mAb therapy is typically due to changes within intracellular signalling pathways, decreasing reliance on the EGFR pathway. The mechanisms of intrinsic and acquired resistance to bevacizumab are less well known, and there are no validated biomarkers to refine clinical use.

There is promising preclinical activity in non colorectal cancer cell lines of the approach of dual targeting of the EGFR using a combination of an EGFR-mAb, with an EGFR tyrosine kinase inhibitor (TKI). This thesis evaluates the effect of an EGFR-mAb and an EGFR-TKI in human colorectal cancer cell lines and the mechanisms governing efficacy, showing that the combination results in synergistic anti-proliferative activity, due to superior vertical inhibition and broader inhibition on downstream signalling pathways. A non-randomised multi-institutional phase II clinical trial of the combination in metastatic colorectal cancer was also performed, demonstrating high rates of clinical activity, restricted to patients with *KRAS* and

BRAF wild type tumours, accompanied by an increase in EGFR related toxicity such as rash.

Second, mechanisms of acquired resistance to EGFR-mAb therapy were determined by generating an *in vitro* model of cetuximab by continuous culture of colorectal cancer cell lines in the presence of cetuximab. The thesis identifies that over expression of a novel candidate secreted protein, REG4 occurs in association with acquired resistance to cetuximab. The thesis also identifies that the resistance phenotype is reversible, which has clinical implications.

Finally, this thesis explores biomarkers to bevacizumab using matched tumour specimens and patient clinical data from the MAX trial, of capecitabine, capecitabine and bevacizumab, or capecitabine, bevacizumab and mitomycin C. This study demonstrated that *KRAS* and *BRAF* mutations are not predictive biomarkers of bevacizumab, but *BRAF* mutations are associated with poor prognosis for overall survival. Using immunohistochemistry, the thesis explored an association between VEGF ligands and receptors with bevacizumab efficacy, and reports on the significant association between VEGF-D over expression and lack of efficacy of bevacizumab on progression free and overall survival.

Thus, molecular mechanisms governing resistance and response to targeted therapy in metastatic colorectal cancer are explored. Different strategies are identified that if validated, may lead to affordable improvements in clinical outcome for patients.

DECLARATION

This is to certify that

- i. The thesis comprises only my original work towards the Doc Med Sci except where indicated in the Preface,
- ii. Due acknowledgement has been made in the text to all other materials used
- iii. The thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices

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PREFACE

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TABLE OF CONTENTS

ABSTRACT	ii
DECLARATION	iv
PREFACE	v
ACKNOWLEDGEMENTS	vi
FUNDING	vii
PUBLICATIONS ARISING FROM THIS THESIS	viii
TABLE OF CONTENTS	1
LIST OF FIGURES	5
LIST OF TABLES	7
ABBREVIATIONS	8

1 INTRODUCTION	11
1.1 BACKGROUND.....	11
1.1.1 Biomarkers	13
1.1.2 Summary.....	14
1.2 TARGETING THE EPIDERMAL GROWTH FACTOR RECEPTOR	15
1.2.1 EGFR related pathways and biology	15
1.2.2 EGFR monoclonal antibodies	18
1.2.3 Mechanisms of action of EGFR monoclonal antibodies	19
1.2.4 <i>In vitro</i> models of innate resistance to EGFR monoclonal antibodies	19
1.2.5 Clinical trials of EGFR monoclonal antibodies in colorectal cancer	22
1.2.6 Biomarkers of resistance and response to EGFR-mAbs	24
1.2.7 EGFR tyrosine kinase inhibitors.....	33
1.2.8 Dual targeting of the EGFR.....	36
1.2.9 Summary.....	44
1.3 ACQUIRED RESISTANCE TO EGFR THERAPY	44
1.3.2 Summary.....	51
1.4 TARGETING ANGIOGENESIS	51
1.4.1 Angiogenic pathways and biology.....	51
1.4.2 Bevacizumab, a VEGF-A monoclonal antibody.....	55
1.4.3 Preclinical models of innate and acquired resistance to bevacizumab.....	60
1.4.4 Biomarkers of response and resistance to bevacizumab	62
1.4.5 Summary.....	69
2 MATERIALS AND METHODS	71
2.1 CHEMICALS AND REAGENTS.....	71
2.1.1 Drugs	71
2.2 CELL CULTURE.....	71
2.2.1 Cell lines.....	71

2.2.2	Maintenance and passaging of cell lines	72
2.2.3	Long term storage of cell lines	72
2.2.4	Mycoplasma testing	72
2.3	CELL GROWTH AND VIABILITY ASSAYS	73
2.3.1	Cell count.....	73
2.3.2	Cell viability assay.....	73
2.3.3	Statistical consideration of synergy and additivity	74
2.4	CREATION OF CETUXIMAB RESISTANT CELL LINES	75
2.4.1	Treatment and passage of cell lines	75
2.4.2	Assessment of resistance	75
2.4.3	Reversal of resistance	75
2.5	FLOW CYTOMETRIC ANALYSIS	76
2.5.1	Assessment of cell cycle distribution using propidium iodide (PI)	76
2.5.2	Assessment of apoptosis using Annexin V	76
2.5.3	Assessment of cell surface EGFR expression using cetuximab.....	77
2.6	WESTERN BLOT ANALYSIS	77
2.6.1	Cell treatment and protein extraction.....	77
2.6.2	Western blot.....	78
2.6.3	Antibodies.....	79
2.6.4	Human phospho-kinase and receptor-kinase antibody arrays.....	79
2.7	DNA AND RNA TECHNIQUES.....	80
2.7.1	RNA extraction.....	80
2.7.2	cDNA synthesis	80
2.7.3	Purification of genomic DNA.....	81
2.7.4	Determination of DNA/RNA concentration	81
2.7.5	Primer Design	81
2.7.6	Real-time PCR.....	82
2.7.7	High Resolution Melt curve analysis for <i>KRAS</i> mutation.....	83
2.7.8	Whole human genome gene expression array.....	83
2.8	REPORTER CONSTRUCTS AND BACTERIAL TECHNIQUES	83
2.8.1	Reporter constructs	83
2.8.2	Luciferase Reporter Assays	85
2.9	STATISTICAL ANALYSIS OF PRECLINICAL DATA	85
2.9.1	Statistical methods	85
2.10	CLINICAL TRIAL OF CETUXIMAB AND ERLOTINIB	86
2.10.1	Design of trial	86
2.10.2	Mutational analysis of <i>KRAS</i> , <i>BRAF</i> , <i>PI3KCA</i> and <i>EGFR</i> genes	88
2.10.3	Statistical analysis.....	89
2.10.4	Study monitoring	90
2.11	THE MAX TRIAL BIOLOGICAL SUB-STUDY	90

2.11.1	The MAX clinical trial design	90
2.11.2	Summary of MAX biological sub-studies.....	93
2.11.3	MAX biological <i>KRAS/BRAF</i> sub-study design	93
2.11.4	MAX biological VEGF ligand and receptor sub-study design	95
2.11.5	Statistical analysis of scoring and correlation with outcomes from trial.....	100
3	DUAL TARGETING THE EGFR: RESULTS AND DISCUSSION	102
3.1	<i>IN VITRO</i> DUAL TARGETING OF THE EGFR: RESULTS.....	102
3.1.1	Human cancer cell line properties	102
3.1.2	Changes in cell growth	104
3.1.3	Changes in cell cycle	113
3.1.4	Changes in apoptosis	113
3.1.5	Changes in downstream levels of phosphorylated proteins	116
3.1.6	Changes in EGFR related MAPK target gene expression	119
3.1.7	Changes in other signalling pathways	123
3.1.8	Validation of changes in STAT3 expression	126
3.1.9	Change in surface EGFR expression	128
3.1.10	Summary of results from <i>in vitro</i> experiments.....	130
3.2	CLINICAL TRIAL OF DUAL TARGETING OF THE EGFR: RESULTS.....	132
3.2.1	Patient Characteristics	132
3.2.2	Efficacy.....	133
3.2.3	Feasibility and safety	138
3.2.4	Toxicity.....	139
3.2.5	Efficacy analysis by skin toxicity and hypomagnesaemia.....	140
3.3	DUAL TARGETING OF THE EGFR: DISCUSSION	145
3.3.1	Dual targeting of the EGFR in <i>KRAS/BRAF</i> colon cancer cell line is synergistic.	145
3.3.2	Broader and increased inhibition of EGFR pathways explains the synergy	147
3.3.3	Changes to surface EGFR do not explain the synergistic effects	148
3.3.4	The combination has a high rate of clinical activity	149
3.3.5	Dual targeting should be restricted to <i>KRAS/BRAF</i> wild type patients.....	150
3.3.6	Implications of the high rate of EGFR related toxicity	152
3.3.7	Financial and toxicity considerations may limit use of this strategy	154
4	ACQUIRED CETUXIMAB RESISTANCE: RESULTS AND DISCUSSION	155
4.1	<i>IN VITRO</i> MODEL OF ACQUIRED CETUXIMAB RESISTANCE: RESULTS	155
4.1.1	Change in sensitivity to cetuximab in resistant cell lines	155
4.1.2	Reacquisition of cetuximab sensitivity of LIM1215 CR-A cells.....	157
4.1.3	Levels of receptor phospho-kinase activation in resistant cell lines	158
4.1.4	Levels of phosphorylated signalling proteins in resistant cell lines.....	161
4.1.5	<i>KRAS</i> mutation status of resistant cell lines.....	161
4.1.6	Change in gene expression in resistant cell lines.....	163

4.2	ACQUIRED RESISTANCE TO CETUXIMAB: DISCUSSION	166
4.2.1	Cells with acquired resistance to cetuximab are cross resistant to erlotinib	166
4.2.2	Compensatory changes in signalling pathways may explain resistance	166
4.2.3	Acquired resistance is not accompanied by <i>KRAS</i> mutations	167
4.2.4	REG4 is a promising molecule for further investigation of resistance	167
4.2.5	Acquired resistance to cetuximab is dependent on exposure to cetuximab	168
5	BIOMARKER ANALYSIS OF MAX TRIAL: RESULTS AND DISCUSSION	170
5.1	BIOMARKER ANALYSIS: RESULTS	170
5.1.1	Characteristics of population available for sub-study analysis	171
5.1.2	Mutation status of <i>KRAS</i> and <i>BRAF</i>	173
5.1.3	Characteristics of patients with <i>KRAS/BRAF</i> mutations	173
5.1.4	Role of <i>KRAS</i> and <i>BRAF</i> as predictive markers for bevacizumab	175
5.1.5	Role of <i>KRAS</i> and <i>BRAF</i> as prognostic markers	180
5.1.6	VEGF A-D and VEGFR1, 2 as predictive factors for bevacizumab efficacy	182
5.1.7	Distribution of angiogenic related factors by treatment arm	187
5.1.8	Correlation of ARF expression levels	187
5.1.9	Correlation of ARFs and baseline characteristics	187
5.1.10	Predictive association of VEGF-D on bevacizumab efficacy (PFS)	188
5.1.11	Predictive association of VEGF-D on OS	191
5.1.12	Predictive association of VEGF-D expression and response rate	195
5.1.13	Prognostic role of VEGF-D on PFS and OS	195
5.1.14	Predictive association of other ARFs on PFS	197
5.1.15	Predictive association of other ARFs status on OS	197
5.1.16	Predictive association of ARFs and response rate	197
5.1.17	Prognostic role of ARFs on PFS and OS	198
5.2	BIOMARKER ANALYSIS: DISCUSSION	205
5.2.1	<i>KRAS</i> and <i>BRAF</i> mutation status	205
5.2.2	<i>KRAS</i> mutations are not predictive of bevacizumab efficacy	205
5.2.3	The role of <i>KRAS</i> mutations as a prognostic biomarker is uncertain	206
5.2.4	<i>BRAF</i> mutations are prognostic for OS	207
5.2.5	The association between ARFs and bevacizumab efficacy	207
5.2.6	Association of VEGF-D with bevacizumab efficacy	208
5.2.7	Association of other ARFs with OS effect of bevacizumab	209
6	CONCLUSION	211
7	REFERENCES	213

LIST OF FIGURES

Figure 1: The EGFR signalling pathway	16
Figure 2: Differential gene expression related to dual targeting of the EGFR	40
Figure 3: EGFR TKI specificity	40
Figure 4: Targets in the EGFR pathway relevant to acquired resistance	46
Figure 5: Angiogenic signalling pathways and ligands	53
Figure 6: Layout of Tissue Micro-Array	97
Figure 7: Properties of human colon cancer cell lines	103
Figure 8: The effect of dual targeting of the EGFR on cell viability	105
Figure 9: Cell number relative to control at 72 hours	107
Figure 10: Change in LIM1215 with dual treatment of the EGFR	108
Figure 11: Effect of dual targeting the EGFR at different concentrations or ratios	110
Figure 12: Change in LIM1215 cell number with higher dose of drug	111
Figure 13: Combination index in LIM1215 cells	112
Figure 14: Change in cell cycle distribution of LIM1215 cells with treatment	114
Figure 15: Change in apoptotic rates in LIM1215 cells with treatment	115
Figure 16: Changes to EGFR signalling intermediaries in LIM1215 with treatment	117
Figure 17: Changes to EGFR signalling intermediaries at higher concentrations in LIM1215	118
Figure 18: Change to pEGFR, pMAPK and pAKT in LIM1215, DiFi, HCA7 cells at 24 hours	120
Figure 19: Change in pEGFR, pMAPK, pAKT in RKO cells with treatment	121
Figure 20: Change in MAPK target gene expression with treatment	122
Figure 21: Correlation of Western blot and multi phospho kinase array	124
Figure 22: Changes in signalling pathways detected by phospho-kinase array	125
Figure 23: Changes in STAT3 reporter activity in LIM1215 cells following treatment	127
Figure 24: Change in surface EGFR expression with treatment	129
Figure 25: Change in total EGFR following treatment	131
Figure 26: Waterfall plot of response, PFS and OS of DUX trial	135
Figure 27: PFS and OS according to KRAS status:	136
Figure 28: PFS and OS according to tumour mutation status	137
Figure 29: Association of skin toxicity and outcome	141
Figure 30: Time to occurrence of skin/Mg related toxicity	141
Figure 31: Outcome according to skin toxicity in KRAS/BRAF WT patients	143
Figure 32: Outcome according to hypomagnesaemia	144
Figure 33: Response rates of cetuximab based treatment of mCRC	151
Figure 34: Characteristics of cetuximab resistant cell lines	156
Figure 35: Reversal of acquired resistance to cetuximab over 2 months	159
Figure 36: Change in receptor activation in resistant cell lines	160
Figure 37: KRAS mutation status and pMAPK changes in resistant cells	162

Figure 38: Change in REG4 expression in resistant cell lines	165
Figure 39: Disposition of patient tumour samples from MAX trial	172
Figure 40: PFS according to KRAS and BRAF status MAX trial.....	176
Figure 41: Outcome in OS according to KRAS/BRAF status in the MAX trial	178
Figure 42: Prognostic effect of KRAS and BRAF on OS	181
Figure 43: VEGF A-D, VEGFR1 and VEGFR2 staining.....	183
Figure 44: Distribution of categorical scores.....	184
Figure 45: PFS according to VEGF-D status MAX trial.....	189
Figure 46: Forest plot for VEGF-D interaction	190
Figure 47: OS and VEGF-D expression	193
Figure 48: Forest plot of hazard ratios for OS according to VEGF-D status.....	194
Figure 49: Prognostic role of VEGF-D for PFS and OS	196
Figure 50: Forest plot of hazard ratios for PFS according to VEGF A-C status	199
Figure 51: Forest plot of hazard ratios for PFS according to VEGFR1 and VEGFR2 status	200
Figure 52: Forest plot of hazard ratios for OS according to VEGF A-C	201
Figure 53: Forest plot of hazard ratios for OS according to VEGFR1,VEGFR2 status	202
Figure 54: Prognostic role of ARF status and PFS.....	203
Figure 55: Prognostic role of ARF status on OS	204

LIST OF TABLES

Table 1: Results of the most important randomised trials of EGFR-mAb therapy in CRC.....	23
Table 2: Outcomes of key trials of EGFR-mAb according to KRAS status.....	25
Table 3: Similarities and differences between EGFR-mAbs and EGFR-TKIs.....	34
Table 4: Key trials of EGFR-TKIs in CRC	36
Table 5: Phase I trials of dual targeting the EGFR	42
Table 6: Randomised trials of bevacizumab in CRC.....	59
Table 7: Possible biomarkers of bevacizumab	63
Table 8: Antibodies used in Western blotting	79
Table 9: Primers used for cDNA expression	82
Table 10: Antibodies and staining methods used for IHC	98
Table 11: Baseline demographics and characteristics of patients on DUX trial	133
Table 12: Response rate, PFS and OS in DUX trial	134
Table 13: Dose intensity of cetuximab and erlotinib in DUX trial	139
Table 14: Adverse events in DUX trial	140
Table 15: Change in gene expression in resistant cell line relative to control	164
Table 16: Median PFS outcomes in intent to treat and sub-study population	171
Table 17: Median OS outcomes in intent to treat and sub-study population	173
Table 18: Demographics and characteristics according to mutation status	174
Table 19: PFS according to KRAS status in the MAX trial	175
Table 20: PFS outcome according to BRAF status MAX trial.....	177
Table 21: Outcome in OS according to KRAS status in the MAX trial	179
Table 22: Outcome in OS according to BRAF in the MAX trial	179
Table 23: Outcome in RR according to KRAS status in the MAX trial	180
Table 24: Outcome in RR according to BRAF status in the MAX trial	180
Table 25: Concordance of expression amongst triplicates of tumour cores	185
Table 26: Inter-rater agreement amongst scorers	185
Table 27: Correlation of expression between primary and metastases of ARFs	186
Table 28: Distribution of ARF expression by treatment arm	187
Table 29: Correlation of ARF expression levels	187
Table 30: Effect of VEGF-D on PFS benefit from bevacizumab	191
Table 31: Effect of VEGF-D status on OS	192
Table 32: Impact of VEGF-D expression on prognosis (PFS)	195
Table 33: Multivariable analysis of VEGF-D expression on PFS	197

ABBREVIATIONS

ADCC	Antibody Dependent Cell-Mediated Cytotoxicity
ARF	Angiogenic Related Factor
ATP	Adenosine Triphosphate
C	Capecitabine
CAF	Circulating Angiogenic Factor
CB	Capecitabine Bevacizumab
CBM	Capecitabine Bevacizumab Mitomycin
CECs	Circulating Endothelial Cells
CEPC	Circulating Endothelial Progenitor Cells
CI	Confidence interval
CoI	Combination Index
CRC	Colorectal cancer
CT	Computed Tomography
DCE-MRI	Dynamic Contrast Enhanced Magnetic Resonance Imaging
DLT	Dose Limiting Toxicity
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DUX	DUal targeting with cetuximab and erlotinib trial
DNA	Deoxyribonucleic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGFR-mAb	Epidermal Growth Factor Receptor monoclonal antibody
EGFR-TKI	Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
FITC	Fluorescein isothiocyanate
FISH	Fluorescent in Situ Hybridization
FKHR	Forkhead homolog 1
FOLFIRI	A Fluorouracil and Irinotecan chemotherapy regimen
FOLFOX	A Fluorouracil and Oxaliplatin chemotherapy regimen
FC γ R	Fragment c γ Receptor

GDP	Guanine Diphosphate
GEF	Guanine Nuclear Exchange Factors
GTP	Guanine Triphosphate
HIF	Hypoxia Inducible Factor
HRM	High Resolution Melt analysis
H&E	Hematoxylin and Eosin
IFL	Irinotecan, Fluorouracil and leucovorin
IHC	Immunohistochemistry
JAK	Janus Associated Kinase
LB	Liquid Broth
M	Molar
MAPK	Mitogen-activated protein kinase
MAX	Mitomycin, Avastin and Xeloda trial
ml	Millilitre
MTD	Maximum Tolerated Dose
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MVD	Microvessel Density
NK	Natural Killer
ng	Nanogram
NRP	Neuropilin
NHMRC	National Health and Medical Research Council
NSCLC	Non small cell lung cancer
mCRC	metastatic colorectal cancer
mTOR	mammalian Target of Rapamycin
OS	Overall Survival
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PE	Phycoerythrin
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PFS	Progression Free Survival
PTEN	Phosphatase and tensin homolog

PMS	Phenazine Methosulfate
PS	Performance Status
QOL	Quality of Life
Ras-GAP	Ras GTPase activating protein
RECIST	Response Evaluation Criteria In Solid Tumours
RR	Response Rate
RNA	Ribonucleic acid
RP2D	Recommended Phase 2 Dose
STAT	Signal Transducer and Activator of Transcription
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline with Tween
TGF- α	Transforming Growth Factor- α
μg	Microgram
μl	Microlitre
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WHO	World Health Organisation

1 INTRODUCTION

1.1 BACKGROUND

Colorectal cancer is the most common cancer affecting Australians, with over 13,000 new cases diagnosed each year and over 5000 deaths per year [1]. For patients with advanced colorectal cancer, only the minority of patients with resectable metastatic disease are curable. Over the last 20 years the use of the cytotoxic chemotherapeutics fluorouracil, oxaliplatin and irinotecan has increased the median overall survival (OS) time from metastatic colorectal cancer from 6 months to close to 20 months in clinical trials [2, 3]. Despite these advances, the 5 year survival rate from colorectal cancer remains less than 20%.

Colon cancer begins as a clonal proliferation within an aberrant crypt focus in the large intestine with progression to adenoma and on to invasive carcinoma [4]. The initial mutation typically occurs at the tumour suppressor gene APC, leading to an accumulation of β -catenin and constitutive activation of Wnt signalling. Approximately 80% of adenomas and colon carcinomas harbour mutations in APC [5]. The development of cancer appears to result from the accumulation of further genetic and epigenetic alterations that was first postulated by Vogelstein and colleagues [6]. Later alterations include mutations to *KRAS*, *SMAD2/SMAD4* and *p53*, which contribute to the transition to carcinoma [7]. Approximately 10 – 15% of colorectal cancer is thought to occur through a different mechanism, in which there is a defective DNA mismatch repair system, characterised by instability of short repetitive DNA sequences, although the pathways mutated are largely the same as in tumours with intact DNA mismatch repair [8].

The majority of patients with colorectal cancer (70 – 80%) present without de-novo metastatic disease, and are potentially cured by resection of the primary tumour and draining lymph nodes. Recurrence of the cancer, typically at metastatic locations, occurs in up to 50% of patients, dependent on molecular and pathological features of the primary tumour [9]. Adjuvant chemotherapy with fluorouracil alone or in

combination with oxaliplatin decreases the risk of recurrence in patients at moderate to high risk of recurrence [10].

In patients who present with unresectable de novo metastatic disease, or with recurrence of their cancer at unresectable sites, no curative options exist. Typically, patients are treated with a combination of cytotoxic chemotherapy agents, such as fluorouracil, oxaliplatin and irinotecan, to reduce tumour growth, improve symptoms, and prolong progression free and overall survival.

Further improvements in progression free survival (PFS) and OS have now been reported for patients with colorectal cancer with the introduction of biological targeted agents that either target the Epidermal Growth Factor Receptor (EGFR) (cetuximab, panitumumab)[11, 12] or angiogenesis through Vascular Endothelial Growth Factor A (VEGF-A)(bevacizumab) [13]. There have been a multitude of alternate novel agents trialed in colorectal cancer since these classes of drugs were released, but none have reported positive results and therefore none have entered into the routine care of patients [14]. There have been no new agents approved by the FDA for use in colorectal cancer since 2004.

The analysis of trials of EGFR targeted therapy demonstrate that a proportion of patients derive no benefit from the addition of this new class of drug to standard chemotherapy [15]. Conceivably this could also be the case with VEGF targeted therapy, but this group has yet to be defined [16]. Furthermore if patients do initially respond to the combination of cytotoxic and targeted therapy, the effect is almost universally a transitory phenomenon, and inevitably the colorectal cancer will begin to grow at some stage after acquisition of resistance to the drugs used in treatment. The median duration of response when EGFR targeted therapy is combined with chemotherapy in first line treatment is less than 12 months [14, 17], and between 4-6 months when used in chemotherapy refractory disease [18-20]. The median duration of response of patients receiving first line chemotherapy in combination with bevacizumab is also less than 12 months [21].

Therefore although the new targeted agents have improved median PFS time and median OS time, their effect has been modest, especially in regard to improvements in

median OS. These drugs are also used at considerable cost to the community. The current drug cost of single agent 5FU is estimated to be to be US \$6 per month, relative to the drug cost of US \$500 per month for FOLFOX or FOLFIRI, and \$2,500 per month if cytotoxic agents and targeted therapies are used for the treatment of a patient for metastatic colorectal cancer. Median OS has risen over this time from 12 months to approximately 24 – 30 months in patients treated on clinical trials [14]. The largest gains in OS have been made by the introduction of oxaliplatin and irinotecan, rather than the more expensive EGFR-mAb or bevacizumab.

1.1.1 Biomarkers

Biomarkers are single traits or a signature of traits that are objectively measured and evaluated as an indicator of a normal biological process, a pathological process, or a pharmacological response to a therapeutic intervention [22].

A prognostic biomarker may predict disease outcome irrespective of treatment. A prognostic marker is a trait that separates a group with respect to the outcome of interest in the absence of treatment, or despite different treatments. A prognostic biomarker is therefore able to be established using data from a series of patients treated either with placebo or with standard treatment.

A predictive biomarker is a characteristic or trait that may predict the response to targeted treatment. A predictive marker can prospectively identify individuals who are likely to have a favourable outcome from a particular treatment [23]. In this way, for each targeted therapeutic agent, future outcomes can theoretically be improved by better patient selection and toxicity and futility minimised. Importantly, a biomarker can be both predictive and prognostic, such as estrogen receptor status in breast cancer, which is both prognostic for survival, and predictive for response to hormonal therapy.

The outcome from clinical trials of targeted therapeutics allows analysis of both clinical data and clinical tissue to identify traits that may be predictive or prognostic. However a single arm or cohort study makes it impossible to isolate any causal effect of the marker on therapeutic efficacy from factors that influence outcome to

treatment. The gold standard for identifying and separating prognostic/predictive factors are a well conducted randomised trial, the availability of samples from the majority of patients to enable a faithful representation of the trial, a prospectively stated hypothesis, the use of a predefined and standardised assay, and upfront power calculation and justification [24].

Unfortunately prospective identification of biomarkers is usually not incorporated into clinical trial design given issues of cost, and there is often lack of knowledge regarding possible biomarkers to allow prospective identification. Furthermore, new targeted agents are always tested initially in single arm phase I and II studies, which will prevent the distinction between a prognostic and predictive factor. Lastly, targeted therapy may interfere or block biological pathways that are not routinely measured by standard pathology laboratories or require specialist equipment. Therefore the data on biomarkers from clinical trials in some areas, such as anti-angiogenic therapy, often are from small, single arm trials that require non standard and poorly reproducible assays to measure particular tumour or patient characteristics and specialised centres. These reports do not enable definitive conclusions, but enable hypothesis generation that can then be used in larger randomised trials.

1.1.2 Summary

Given the high cost, optimizing survival of patients with colorectal cancer requires research into two important questions. Firstly, can there be improved efficacy of these agents by using novel combinations with existing or other new drugs? Secondly, what are the mechanisms of both innate and acquired resistance to these drugs? Restricting use from those patients who have factors that govern immediate innate resistance will enrich and maximise initial response rates (RR). Identification of mechanisms governing acquired resistance may allow novel future strategies to avoid these mechanisms and allow ongoing durable responses to therapy.

This thesis aims to explore methods of optimizing the targeted treatment of the EGFR pathway and the VEGF related pathway in colorectal cancer. The biology of each pathway will be reviewed. Therapeutic options for inhibiting these pathways in colorectal cancer will be discussed, focusing on targeted agents. The concept of maximising the effect of targeting the EGFR pathway by using two drugs that target

the same pathway will be reviewed. Additionally, biological characteristics of the tumour that may predict resistance and response to targeting each individual pathway will also be reviewed.

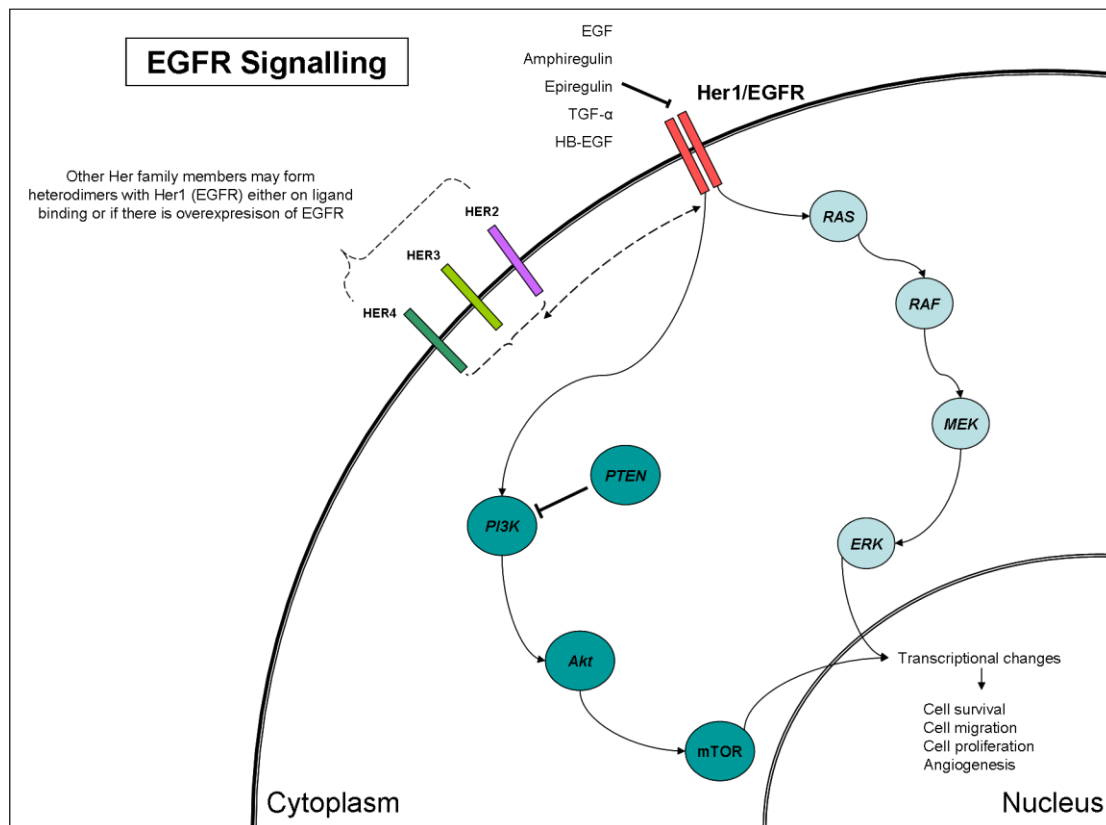
1.2 TARGETING THE EPIDERMAL GROWTH FACTOR RECEPTOR

The EGFR pathway is an important driver of tumour proliferation and invasion that is an appealing target in colorectal cancer. Monoclonal antibodies to the receptor, such as cetuximab and panitumumab have been shown to be effective in metastatic colorectal cancer, but their activity limited to molecularly defined subgroups. The following review summarises the mechanism of action of EGFR monoclonal antibodies, the intrinsic and acquired mechanisms of resistance to therapy, and combination therapy against the EGFR, which has been shown to improve preclinical and clinical outcomes.

1.2.1 EGFR related pathways and biology

The EGFR is a trans-membrane protein that is part of a family of four different but related growth factor receptors [25]: EGFR itself (ErbB1), ErbB2 (HER2/*neu*), ErbB3 (HER3), and ErbB4 (HER4). The EGFR monomer receives signals from the ligand Epidermal Growth Factor (EGF) which in turn triggers homo or heterodimerisation of the receptor with itself or other family members, and then triggers downstream signalling using the intracellular tyrosine kinase portion of the trans-membrane protein [26]. There are several other ligands that can also activate the receptor, including Transforming Growth Factor- α (TGF- α), amphiregulin and epiregulin [26]. Ligand induced dimerisation of the EGFR causes a conformational change to the protein, leading to downstream phosphorylation of several tyrosine residues in the C-terminal (intracellular) domain of the EGFR, including Y992, Y1045, Y1148 and Y1173 [27]. The autophosphorylation at these sites leads to induction of several signal transduction cascades [27] and growth and survival benefits to the cell by altering transcriptional events within the nucleus [28]. The major intracellular signalling pathways activated by the EGFR are thought to be the Mitogen-activated protein kinase (MAPK)[29], and the Phosphoinositide 3-kinase (PI3K) – *AKT* pathway [30] (Figure 1).

Figure 1: The EGFR signalling pathway



1.2.1.1 MAPK and PI3K pathways

The MAPK pathway consists of a cascade of signals transmitted through the serine-threonine selective protein kinases Ras, Raf, MEK and ERK. Ligand-induced EGFR receptor dimerisation and phosphorylation activates Ras guanine nucleotide exchange factors (Ras-GEFs) which catalyze the exchange of GDP for GTP on Ras [31]. In turn this leads to activation of downstream Raf, MEK and ERK. Activated ERK translocates to the nucleus where it phosphorylates MSK1/2. This regulates factors linked to cellular proliferation and survival. This in turn modulates gene expression through phosphorylation and activation of Histone H3, and transcription factors such as AP-1 and CREB, which induce expression of pro-proliferative genes such as *myc*, *fos*, *jun* and *cyclin D2* [32].

The alternate major signalling pathway, the PI3K pathway, transduces signals from PI3K to the down stream protein serine/threonine protein kinase AKT. AKT activates a variety of downstream targets including mammalian Target of Rapamycin (mTOR), Forkhead homolog 1 (FKHR) and Bad that lead to cellular survival and proliferation [30]. Phosphatase and tensin homolog (PTEN) is a negative regulator of PI3K activation, and loss of function leads to up-regulation of the PI3K pathway [33].

1.2.1.2 STAT3 pathway

Although the MAPK and PI3K/AKT pathways have been thought to be the two dominant signalling pathways derived from EGFR stimulation, more recent data suggests that STAT3 is third relevant signalling pathway intimately associated with EGFR activation. STAT3 is a signal transducer in the cytoplasm and additionally acts as a transcription factor in the nucleus. The key activator of STAT3 has been previously ascribed to the inflammatory associated cytokine IL-6, which leads to Janus Associated Kinase (JAK) activity and down stream induction of a variety of genes including VEGF and BCL-xl [34]. However stimulation of the EGFR also leads to STAT3 activation, possibly through Src phosphorylation and consequent down stream cellular changes [35], including TGF- β suppression through Smad7 expression [36]. EGFR-TKIs have been demonstrated to be capable of STAT3 inhibition in other preclinical models [35-37].

1.2.1.3 Oncogenic properties

Under physiological conditions, a variety of ligands cause receptor homo or heterodimerisation which leads to activation of the downstream pathways [38]. In tumour cells there are a variety of means of activation of the EGFR that are different from normal physiological conditions. Firstly, the EGFR may be over expressed on the tumour cell surface relative to normal cellular levels due to gene amplification [39], which can lead to ligand independent receptor dimerisation. Secondly the EGFR can be mutated in either the extracellular domain, with loss of exon 2 to 7 (de₂₋₇), which commonly occurs in glioblastoma [40] or in the intracellular tyrosine kinase domain, such as non-small cell lung cancer and hence be constitutively activated [41]. Lastly, tumours can be stimulated by autocrine loops from excessive secretion of ligands such as amphiregulin and epiregulin [26]. The activated EGFR leads to cellular proliferation, invasion and metastasis through the major signalling pathways already discussed [42]. Clinical correlates of these cellular survival advantages are represented by the poor prognosis observed in EGFR over expressing tumours [43] and the finding that certain EGFR mutations such as de₂₋₇ are associated with poorer prognosis [44].

1.2.2 EGFR monoclonal antibodies

Given the supportive data and preclinical rationale for targeting the EGFR, over the past 10 years, drugs utilising this strategy have moved from preclinical models into clinical trials and routine clinical use in certain cancers. Two separate classes of drugs that target the EGFR are tyrosine kinase inhibitors (EGFR-TKIs), and monoclonal antibodies (EGFR-mAbs).

The EGFR-mAbs that are licensed for use in colorectal cancer are cetuximab (formerly IMC C225, marketed in Australia as Erbitux, Merck-Serono), and panitumumab (formerly ABX-EGF, marketed in Australia as Vectibix, Amgen). Nimotuzumab (Theraloc, Europe) is also an EGFR-mAb approved for treatment of head and neck squamous carcinoma and glioma in some countries.

1.2.3 Mechanisms of action of EGFR monoclonal antibodies

EGFR-mAbs block ligand binding to the EGFR by binding to domain 3 of the EGFR and subsequently inhibit dimerisation of the receptor [26]. EGFR-mAb binding to constitutively activated de₂₋₇EGFR is usually reduced, although the exception to this is mAb 806, which binds to a different epitope of the receptor [45, 46].

Use of an EGFR-mAb leads to a variety of effects in preclinical models, including growth inhibition with G1 phase cell cycle arrest *in vitro* and reduction of tumour xenograft growth *in vivo* [47]. In cell lines with high levels of EGFR, apoptosis is also observed [48, 49]. However this is not case in the majority of colorectal cancer cell lines [50]. There are a multitude of other effects that occur apart from apoptosis that explain the clinical effectiveness of these drugs, and include inhibition of angiogenesis [51, 52], reduction in metastatic potential [53, 54], augmentation of the effects of chemotherapy [55] and through antibody dependent cell-mediated cytotoxicity (ADCC) [56].

ADCC occurs when antigen specific antibodies direct immune effector cells of the innate immune system, natural killer (NK) cells to kill the antigen expressing cancer cell, and had been considered as a possible mechanism of action for EGFR-mAbs [57, 58], as well as other antibodies such as trastuzumab and rituximab [59-61]. Panitumumab, an immunoglobulin G2 antibody, also causes ADCC, but only recruits cells of the myeloid lineage [62]. The relative importance of ADCC in the action of EGFR-mAbs is controversial, as cetuximab and panitumumab have similar clinical efficacy, which does not reflect the increased ADCC activity of cetuximab. Additionally mutations in the *KRAS* gene predict resistance to these agents, suggestive of only a relatively modest role for ADCC.

1.2.4 *In vitro* models of innate resistance to EGFR monoclonal antibodies

Knowledge of the mechanism of action of EGFR-mAbs and the signalling pathways used by the EGFR has led investigators to assess if any cancer cell properties would lead to intrinsic resistance to therapy.

1.2.4.1 KRAS/NRAS

Mutations in the *KRAS* gene confer conformational change within the protein Ras that alter down stream signalling within a cell [63]. As previously discussed, ligand binding to the EGFR results in receptor dimerisation and phosphorylation. This leads to activation of Ras guanine nucleotide exchange factors (Ras-GEFs) which catalyze the exchange of guanine diphosphate (GDP) for Guanine Triphosphate (GTP) on Ras. This dynamic change is reverted to normality by the binding of Ras-GTPase activating proteins (Ras-GAPs) such as NF1 and p120GAP, which activate intrinsic GTPase activity on Ras, thereby returning Ras to its inactive GDP bound state[31, 63]. Mutations in codons 12 or 13 of *KRAS* result in Ras proteins which are insensitive to Ras-GAPs, while mutations in codon 61 inhibit the intrinsic GTPase activity of *KRAS* [63]. The consequence of these mutations is the failure to convert Ras-bound GTP into GDP, and subsequently in the constitutive activation of downstream signalling, independent of external signals (growth factor independence). An Italian group identified that these mutations confer innate resistance to treatment with EGFR-mAb therapy *in vitro* [64].

Although the *NRAS* gene is closely related to *KRAS*, the protein NRas has different signalling and oncogenic properties to KRas. Furthermore the *NRAS* gene is mutated less frequently, and usually in codon 61 [65, 66]. Mutations in *NRAS* are usually mutually exclusive to *KRAS* and *BRAF* [65]. Preclinical models have not yet firmly established its importance in resistance to EGFR mAb.

1.2.4.2 BRAF

An activating mutation in the *BRAF* gene, the V600E mutation on exon 15, results in a valine substitution for glutamine and results in constitutive activation of the MAPK signalling pathway in 8 – 10% of CRC tumours [67, 68]. The mutation occurs in a mutually exclusive manner with *KRAS* mutations in colon cancer [67]. Preclinical work has demonstrated that cell lines with *BRAF* mutations are resistant to EGFR-mAb therapy [64, 69].

There is a difference between the mutation frequency of *BRAF* in microsatellite unstable and microsatellite stable tumours: a high frequency of mutation (up to 50%) occurs in microsatellite unstable tumours as compared to 5 – 10% in microsatellite

stable tumours[70-72]. Mutations of *BRAF* in microsatellite stable tumours are associated with a distinct gene signature indicative of downstream changes in Wnt, TGF- β and MAPK. These changes are distinct from those seen with *KRAS* mutations. In contrast a heterogenous gene signature is observed in *BRAF* mutant tumours that originate from tumours with microsatellite instability [73]. These studies indicate that *BRAF* mutations in microsatellite unstable disease have distinct oncogenic effects compared to those with *BRAF* mutations in microsatellite stable disease.

1.2.4.3 PI3KCA/PTEN

Studies *in vitro* have suggested that *PI3KCA* mutations may confer intrinsic resistance to EGFR-targeted antibodies [50]. Unlike mutations of *KRAS* or *BRAF*, mutations occur at multiple different sites within the gene in tumours, especially in exon 9 (helical domain) and exon 20 (kinase domain) of the gene [74]. Cellular models suggest that mutations within the exon 20 kinase domain are the only mutation with functional significance to cause constitutive downstream AKT activation [74]. Zhao *et al* reported that the gain of function induced by *PIK3CA* exon 20 mutations is independent of Ras binding *in vitro*, whereas exon 9 mutations require Ras-GTP interaction. Consequently exon 20 mutations may be the only mutations of significance in causing innate resistance to EGFR therapy. However this has yet to be proven in cancer cell lines. It is worth noting that mutations with *PI3KCA* are not mutually exclusive with *KRAS* or *BRAF*, and occur in 10 - 15% of cases of advanced colorectal cancer [75, 76].

PTEN is a negative regulator of PI3K mutation, and the loss of PTEN expression has been demonstrated in approximately 30% of sporadic colorectal cancers [77]. Loss of PTEN may occur through mutations of the gene [33], promoter methylation [78], and microRNA suppression [79]. Loss of PTEN leads to over activity of P13Kinase: analogous to a constitutively activating mutation in *PI3KCA*, in causing activation of the downstream pathway through the AKT-mTor pathways that leads to cellular survival and progression [30, 33], and has also been linked to cetuximab resistance [50].

1.2.5 Clinical trials of EGFR monoclonal antibodies in colorectal cancer

Preclinical data regarding intrinsic resistance to the drugs lagged behind the clinical utilisation of the drugs. Initially trials used murine EGFR-mAbs, proving the feasibility of the strategy [80], before dose finding phase I trials [81, 82] and then larger phase III trials demonstrated clinical activity of chimeric and humanised EGFR-mAb [12, 20].

The drugs are given as intravenous infusions either weekly (cetuximab) or every 2 weeks (panitumumab). Cetuximab and nimotuzumab are chimeric mouse/human antibodies, whilst panitumumab is a fully humanised antibody to the EGFR. Although not compared yet in a head to head clinical trial, they have a similar range of side effects and activity, although nimotuzumab has not yet been studied in colorectal cancer [83]. Cetuximab and nimotuzumab have an uncommon but serious side effect of anaphylactoid infusion reactions, thought to arise from cross reactivity of the murine portion of the antibody and previously formed antibodies within the patient. The most common side effects of use of EGFR-mAbs include an acneiform rash, which is reversible upon stopping the drug. Other side effects include diarrhoea, and hypomagnesaemia. All three side effects are thought to be related to the inhibition of a small amount of functional EGFRs, in the skin, gut and kidneys. Relatively large amounts of EGFR are located in the liver, but in an inactive form, leading to no significant hepatotoxicity. The pharmacokinetics properties of EGFR-mAbs are largely governed by EGFR-mAb binding to liver EGFR, internalisation and subsequent clearance [84]. Newer EGFR-mAbs, such as mAb 806 which binds selectively to de₂₋₇ form of the EGFR and the transitional forms of EGFR that occur when over expressed on cancer cells, may lead to clinical activity in the absence of rash due to the lack of these EGFR isoforms being expressed in normal tissues [46].

Conclusive demonstration of efficacy of these drugs, in particular cetuximab and panitumumab have arisen from large randomised trials. These trials have included the combination of an EGFR-mAb with standard chemotherapy in the first line of treatment [11, 17, 85], and with irinotecan in the second [86] or third line [18] of chemotherapy. Additionally these drugs have been studied as single agent therapy in the third line setting [12, 87](Table 1).

In studies of single agent EGFR-mAb therapy, there were relatively low objective RR of 8% - 10% [12, 20]. In both trials the median duration of treatment with active drug was less than 9 weeks. The C017 trial, comparing cetuximab to best supportive care (BSC) reported a statistically significant improvement in OS of 6.1 months versus 4.1 months in the placebo arm. This was not seen in the panitumumab study, presumably because patients in the placebo arm were allowed to cross over to active treatment upon progression, possibly negating survival benefits that may have been present.

Table 1: Results of the most important randomised trials of EGFR-mAb therapy in CRC

Study	Treatment arms	Number of patients	Response rates(%)	p value	Median PFS (months)	p value	Median OS (months)	p value	Ref
<i>First line</i>									
CRYSTAL	FOLFIRI FOLFIRI + C	599 599	38.7 46.9	0.004	8.0 8.9	0.043	18.6 19.9	0.31	[88]
OPUS	FOLFOX FOLFOX+C	168 169	36 46	0.064	7.2 7.2	0.62	NR NR	NR	[17]
MRC COIN	5FU/Cape+Ox 5FU/Cape+Ox+C	815 815	51 53	0.428	8.6 8.6	0.72	17.9 17	0.68	[85]
<i>Second line</i>									
EPIC	Irinotecan Irinotecan+C	650 648	4.2 16.4	<0.001	2.6 4.0	<0.001	9.99 10.71	0.7115	[86]
<i>Third Line</i>									
NCIC CO17	BSC BSC+C	285 287	0 8	<0.001	1.8 1.9	<0.001	4.6 6.1	0.005	[12]
Panitumumab study	BSC BSC+p	232 231	0 10	<0.001	1.8 2	<0.001	NR NR	0.48	[20]

The addition of an EGFR-mAb to chemotherapy has also been shown to improve response rates compared to standard chemotherapy alone. The addition of cetuximab to irinotecan in the second line treatment of colon cancer improved the response rate to 16.4% vs 4.2%, and resulted in an improved median PFS of 4 months versus 2.6 months compared to irinotecan alone [86]. The combination of cetuximab and irinotecan in patients with irinotecan refractory disease in the BOND study demonstrated higher responses in the combination arm (22.9%) compared to cetuximab alone (10.8%) and nearly 3 months improved PFS, suggestive of a degree of resensitisation to the chemotherapy [18].

More recently a larger trial published their results analysing the benefit of the addition of cetuximab to 5-Fluorouracil (5FU) and irinotecan in the first line setting. There was

a modest increase of approximately 10% in the response rate and approximately 1 months improvement in the PFS with the addition of cetuximab, but no change in OS [89].

The addition of cetuximab to the combination of oxaliplatin and 5FU (FOLFOX) in first line chemotherapy was trialed initially in an encouraging preclinical and phase II study [90]. Subsequently, an European trial OPUS reported improved response rates and better progression free survival in patients receiving cetuximab, which was more marked and statistically significant in patients with *KRAS* wild type tumours [17]. A trial comparing the addition of panitumumab to FOLFOX reported similar findings [91].

However a much larger UK trial, MRC COIN [85] was unable to demonstrate statistical superiority of the cetuximab containing arm comparing FOLFOX and cetuximab to FOLFOX alone in response rates, PFS or OS, even in patients with *KRAS* wild type tumours. Similarly the NORDIC FLOX trial found that cetuximab did not add any benefit to an oxaliplatin containing regimen FLOX [92]. Consistent with the MRC COIN study, there was no benefit in either the *KRAS* wild type or mutant tumours. Based on these data, the magnitude of benefit to the addition of EGFR-mAbs to oxaliplatin based chemotherapy seems uncertain. Table 1 summarises the clinical efficacy data with EGFR-mAb therapy seen in randomised trials in unselected populations.

1.2.6 Biomarkers of resistance and response to EGFR-mAbs

Identification of cellular characteristics that predicted resistance to the EGFR pathway, coupled with modest efficacy data led investigators to retrospectively analyse the clinical trials of EGFR-mAb therapy, to identify the groups of patients that harbour those characteristics and associate these with response. This has led to several practice changing findings, with the routine incorporation of *KRAS* screening into clinical practice. Other potential biomarkers such as *NRAS*, *BRAF*, *PTEN* status and *PI3KCA* changes need further validation before routine use.

1.2.6.1 KRAS/NRAS

Mutations within the *KRAS* gene occur in approximately 35 – 40% of patients with metastatic colorectal cancer [15, 87]. Evaluation of randomised studies in advanced colorectal cancer now clearly demonstrates that *KRAS* mutations predict for intrinsic resistance to EGFR-targeted agents whether these agents are used in first-, second- or third- line settings [93]. Most of the testing for *KRAS* is only performed for mutations within codon 12 and 13, but a further 2% of patients have *KRAS* mutations in codon 61, which also seems to be of functional significance [65]. Most mutation testing is performed on patient's archived tissue blocks, usually from previously resected primary tumours. Reassuringly, it is extremely rare to have discordance between *KRAS* and *BRAF* mutations between primary tumour and the metastatic disease [94].

Initial data from small non randomised series confirmed the sound biological hypothesis that *KRAS* mutant patients would not respond to EGFR targeted therapy [95, 96]. The most powerful analysis of the predictive ability of *KRAS* gene status was found when the two large randomised trials of EGFR-mAb were analysed [15, 87] (Table 2).

Table 2: Outcomes of key trials of EGFR-mAb according to KRAS status

Study	Treatment arms	WT/MT	Response rates(%)	p value	Median PFS (months)	p value	Median OS (months)	p value	Ref
First line									
CRYSTAL	FOLFIRI FOLFIRI + C	WT (74%)	43.2 59.3	0.0025	8.7 9.9	0.02	21.0 24.9	NR	[88, 89]
	FOLFIRI FOLFIRI + C	MT (36%)	40.2 26.2	0.46	8.1 7.6	0.7	17.7 17.5	NR	
OPUS	FOLFOX FOLFOX+C	WT (31%)	37 61	0.011	7.2 7.7	0.016	NR NR	NR	[17, 97]
	FOLFOX FOLFOX+C	MT (69%)	49 33	0.106	8.6 5.5	0.019	NR NR	NR	
Third Line									
NCIC CO17	BSC BSC+C	WT (68%)	0 12.8	NR	1.9 3.7	<0.001	4.6 6.1	0.005	[12, 15]
	BSC BSC+C	MT (42%)	0 1.2	NR	1.8 1.8	<0.001	4.6 6.1	0.005	
Panitumumab	BSC BSC+p	WT (67%)	0 17	NR	1.6 2.8	<0.001	7.6 8.1	0.48	[20, 87]
	BSC BSC+C	MT (33%)	0 0	NR	1.6 1.6	0.99	4.9 4.4	0.89	

In the CO17 trial, the benefit of cetuximab was confined purely to the *KRAS* wild type group. Response rate (12.6% vs 1.2%), PFS (3.7 vs 1.9 months) and OS (9.5 vs 4.5 months) were all significantly different in the comparison of the outcomes from *KRAS* wild type patients treated with cetuximab vs *KRAS* mutant patients treated with cetuximab [15]. Similarly, the difference in response rate in the European study of panitumumab (17% vs 0%) and the difference in PFS (12.3 weeks vs 7.3 weeks) were significant [87]. Analysis of the outcomes of *KRAS* wild type vs mutant patients in the first line setting of combining EGFR-mAb therapy with chemotherapy also provide conclusive evidence of the predictive value of *KRAS*. In the both the CRYSTAL trial and PRIME trial, a statistically significant improvement in the overall response rate and median PFS in the EGFR-mAb containing arm was found in the *KRAS* wild type patients, but not those with *KRAS* mutations [89, 91](Table 2).

More recently, an analysis of the effects of codon 12 mutations and codon 13 mutations of *KRAS* suggests that the resistant phenotype of codon 13 mutations may be less than codon 12 mutations [98]. Collated analysis of several trials including the C017 trial demonstrated that patients treated with cetuximab with the G13D mutation had longer overall survival (adjusted hazard ratio (HR) 0.50; 95% Confidence Interval (CI), 0.31-0.81; $p = .005$) and better progression free survival (adjusted HR, 0.51; 95% CI, 0.32-0.81; $p = .004$) relative to other *KRAS* mutations. Patients with G13D mutations in their tumour had made up 14.5% of the total *KRAS* mutation cohort previously analysed together. Although none of the patients had responses to EGFR-mAb therapy, the prolonged PFS and OS of these patients relative to those with G13D mutated tumours indicates a possible different cellular response to EGFR targeted therapy. Indeed preclinical work by the authors demonstrated that codon 13 mutations exhibit weaker in vitro transforming activity than codon 12 mutations.

Currently, clinical guidelines recommend that all patients who are candidates for EGFR-mAb therapy to have their *KRAS* status determined prior to treatment and treatment restricted to those who are *KRAS* wild type [99]. In contrast to the data regarding the predictive role of *KRAS* mutations and EGFR therapy, its prognostic role is uncertain. Earlier studies, including the RASCAL trial, which reported that *KRAS* mutations were associated with increased risk of relapse after resection suggested *KRAS* status was in fact a prognostic factor [100-102]. However most

recent studies suggest this is not the case, including the large CO17 trial [15, 71] with few exceptions [103]. Therefore, although there is some uncertainty, *KRAS* mutations are thought purely predictive biomarkers to EGFR therapy [15].

Mutations in *NRAS* occur in 3 – 5% of patients with metastatic colorectal cancer [65, 68]. Although the larger randomised registration trials of EGFR-mAb have not yet been assessed for *NRAS* mutation status and benefit, a retrospective review of over 300 patients reported that *NRAS* mutations were associated with lower response rates and shorter overall survival [65]. This is similar to an earlier retrospective review of European patients, which reported there were no responses to cetuximab therapy in patients with *NRAS* mutations [104]. Although promising, given the lack of prospective data, and lack of evaluation in randomised studies, *NRAS* mutations cannot be currently recommended as a definitive predictive biomarker for EGFR-mAb therapy.

1.2.6.2 BRAF

Although the data from randomised trials is more limited, tumours with *BRAF* mutations also appear to have intrinsic resistance to EGFR inhibitors, as no patients with *BRAF* mutations in their tumours have ever reported responses when treated with EGFR-mAb [69, 105-107].

An initial paper showed that in a retrospective review of *KRAS* wild type patients, the benefit of panitumumab is confined to those patients with wild type *BRAF* [69]. There were no responses, and shorter PFS and OS in patients with *BRAF* mutations. A larger retrospective review of over 1000 tumours confirmed the significance of *BRAF* in predicting resistance to therapy [65]. A similar pattern was seen in an analysis of 231 tumours from patients treated with first-line cetuximab plus capecitabine, oxaliplatin and bevacizumab in the CAIRO-2 study [108]. Progression free survival (6.6 vs 10.4 months), and overall survival (15.2 vs 21.5 months) was significantly worse in the *BRAF* mutation group. Response rates were also worse in the *BRAF* mutant group, but not significantly (39% vs 48%).

Although three randomised trials that combined EGFR-mAb therapy with chemotherapy in the first line setting were unable to prove statistically significant

associations between *BRAF* mutations and response to EGFR-mAb therapy, they were limited in their analysis by small numbers (~70 patients; 3-4%) of patients evaluable for analysis with the mutation [85, 109, 110]. A further confounding issue in interpreting the data is that *BRAF* mutations are also a strong prognostic marker of inferior survival in colorectal cancer in patients with microsatellite stable disease but not microsatellite unstable tumours [72, 111]. Given this strong prognostic effect, the frequency of *BRAF* mutations decline from approximately 10% in the adjuvant setting, to approximately 5% in the heavily pretreated metastatic patients [65, 105]. Although some series have shown that *BRAF* mutations are not prognostic for relapse from resection of Stage II or III disease [71], this may be explained by the high incidence of *BRAF* mutations in microsatellite unstable colorectal cancer of up to 50% [70-72] in stage II – III disease, which is recognised as a favourable prognostic marker in early-stage colorectal cancer [71]. Given this clinical and *in vitro* data, *BRAF* mutations can be regarded as prognostic in microsatellite stable tumours alone.

In summary, *BRAF* V600E mutations in patients with microsatellite stable tumours carry an association with poor prognosis. Although data from a number of retrospective single arm studies demonstrate that no patients with *BRAF* mutations respond to EGFR-mAb therapy, larger randomised trials of EGFR-mAb therapy in combination with chemotherapy in first line therapy have been unable to confirm an additional and separate predictive role for *BRAF*. Data from the larger randomised CO17 trial is required before the use of *BRAF* mutation testing is standard clinical practice prior to EGFR-mAb therapy.

1.2.6.3 PI3KCA/PTEN

The significance of loss of expression of PTEN and *PI3KCA* mutations is less certain compared to *KRAS* and *BRAF* due to fewer and smaller clinical series investigating this area and different techniques of measurement of these tumour characteristics, particularly PTEN status.

Mutations in *PI3KCA* occur in approximately 10 -15% of patients with colorectal cancer [75, 76, 112]. In an analysis of 92 patients with metastatic colorectal cancer, of 9 (10%) with *PI3KCA* mutations, 1/9 had a response to treatment. However, the correlation of intrinsic resistance and *PI3KCA* mutations has not been universal, with

some studies showing no correlation [95, 113]. These contradictions may arise because of small numbers of patients analysed, the small frequency of mutations, and the multitude of different *PI3KCA* mutations, many of which may have no functional significance [65]. A larger retrospective analysis of over 1000 patients reported a significant association between a lack of response and inferior overall survival to EGFR-mAb in the 9 out of 675 patients who had an exon 20 mutation in the kinase domain [65]. Mutations in exon 9 of *PI3KCA* did not have this association. Further work on larger series such as CO17 data is required to confirm the results [114].

Loss of PTEN has been associated with inferior response to EGFR-mAb and inferior overall survival in retrospective reviews of non randomised trials [105, 115]. A retrospective small series of 27 patients treated with cetuximab tested PTEN expression using immunohistochemistry in the primary tumour [116]. In this study, 0/11 patients who lacked PTEN expression responded to cetuximab, compared to 10/16 patients who had normal expression of the protein. Additionally supportive evidence in a different case series reported that lack of PTEN expression in the metastases correlates with resistance to cetuximab treatment [117]. Another two retrospective series have also shown that there were no responses to cetuximab in patients with a lack of PTEN expression as determined by gene amplification with Fluorescent in Situ Hybridization (FISH) amplification [118, 119].

However the definition of PTEN loss is confounded by different methods of testing, including immunohistochemistry and FISH analysis [68]. Additionally there is no agreed threshold or protocol for detection by immunohistochemistry [65], and there may be discordance between the primary and metastatic tumour in 40% of matched samples[117]. Currently loss of PTEN remains a potential biomarker, but cannot be supported as a predictive biomarker for EGFR-mAb therapy until data from further trials are reported.

1.2.6.4 Fcγ receptor polymorphisms

Recently, two polymorphisms which result in functional changes in the Fragment c γ Receptor (FcγR) bearing immune cells have been identified that may affect the killing properties of the immune cells and mediate ADCC [120]. Two studies have

now been published that correlate changes within the receptor due to polymorphisms with clinical response to cetuximab but with somewhat conflicting results [121, 122].

The earlier report showed that homozygous carriers of the 158V allele of FC γ RIIIA, (5 of 35 patients tested) had inferior PFS and a trend for inferior response rate as compared to patients heterozygous or homozygous for the alternate 158F allele. Homozygous carriers of the 131R allele of FC γ RIIA (9 of 35 patients) also had an inferior PFS and a trend for an inferior response rate compared to patients heterozygous and homozygous for the alternate allele [121].

In direct contrast however another retrospective study showed that the homozygous carriers of the 158V allele of FC γ RIIIA had a superior PFS time compared to patients with the alternate alleles [122]. Homozygous carriers of 131R allele of FC γ RIIA were associated with inferior PFS in keeping with the earlier report. Importantly this inconsistency in the importance of FC γ RIIIA-V158F allele has also been reported in studies of patients treated with rituximab and trastuzumab [123, 124]. A larger unpublished series from the BMS045 cetuximab study found no statistical association between the FC γ R polymorphisms and response [125]. All these series were not randomised and therefore any results cannot discriminate between prognostic and predictive value of these polymorphisms.

Although the literature across different tumour and drug types supports the association of carriers of the 131R allele of FC γ RIIA with inferior outcome when treated with monoclonal antibodies, this biomarker still requires validation through testing large randomised series before regular clinical application. Given the small nature of the retrospective series and the conflicting results for the FC γ R-V158F polymorphisms, these cannot be currently recommended as biomarkers for EGFR-mAb response. Notably though, newer monoclonal antibodies are designed with low levels of glycosylation, as this leads to enhanced Fc γ receptor binding, possibly lessening the influence of SNP polymorphisms [126].

1.2.6.5 Other predictive factors

If the biomarkers discussed so far were all valid, pretesting patients with colorectal cancer for *KRAS*, *BRAF*, *PI3KCA* and *PTEN* would narrow the proportion of patients

predicted to respond to EGFR-mAb to 40% of all patients. However, taking into account increased response rates with high dose cetuximab [127] only approximately 20 - 25% of patients, will respond to EGFR-mAb therapy [68, 93]. This still leaves somewhere between 20 – 25% of the total treated population which do not benefit from treatment. Conceivably, other predictive factors such as EGFR ligand over expression, or increased EGFR gene copy number as determined by FISH may further refine the selected population to benefit from this therapy.

EGFR expression as measured by IHC is not a reliable method of determining response to EGFR-mAb therapy. This is due to multiple factors including a lack of standardised scoring system, non EGFR related resistance pathways, inter-observer variation, variability of expression dependent on tissue storage and handling, and possible differences between the form of EGFR detected by the IHC and the form of EGFR targeted by the EGFR-mAb [128]. In contrast an increase in *EGFR* gene copy number or EGFR amplification as determined by FISH analysis amplification seems a promising biomarker of response [116, 129, 130]. Responses to EGFR-mAb in three separate retrospective studies were confined to patients with increased *EGFR* gene copy number only. Whilst there are reproducibility issues in using FISH analysis, assessment of gene copy number may have a role in future determination of suitability for EGFR-mAb therapy [131].

Higher levels of expression of the EGFR ligands, epiregulin and amphiregulin were shown on exploratory analysis to be associated with response to cetuximab therapy [96]. An explanation for their relevance is that these ligands are secreted in an autocrine loop by tumour cells, and stimulate the EGFR pathway. These cells may be more susceptible to blockade of the receptor [132]. In this analysis, *EREG* and *AREG*, were shown to be highly over expressed in patients who responded to cetuximab. This initial observation has been supported by retrospective review of two European series, with a relationship between expression of these ligands as measured in formalin fixed tissue with outcome with cetuximab in *KRAS* wild type patients [131, 133, 134]. Gene expression of *EREG* was performed in the C017 trial and was also reported to be significantly associated with outcome to therapy [135]. *KRAS* wild type patients with high levels of gene expression had significantly improved response rates, and significant differences in PFS (5.4 vs 1.9 months) and median OS (9.8 vs 5.1 months).

Despite promising results, there are no current recommendations that testing of these ligands be incorporated into standard testing of patients to determine eligibility for EGFR-mAb therapy.

Interestingly, there may be early indicators of response to EGFR-mAb therapy including rash and magnesium levels. Intriguingly, in both large trials of single agent EGFR-mAb therapy, patients who developed more severe forms of skin toxicity with the use of EGFR-mAb therapy had better outcomes in PFS and OS than those who have minimal or no rash whilst receiving treatment [20, 136]. Rash typically appears after 1 – 2 weeks and reaches maximum severity around 3-4 weeks after beginning treatment [137]. Furthermore, in an analysis of outcomes from patients with *KRAS* wild type tumours treated with panitumumab, those with G2-4 rash without progression at 28 days had superior response rates, PFS, and OS compared to those with only G1 rash [138].

Additionally, the combination of irinotecan with either standard dose cetuximab or dose escalated cetuximab has been compared in the EVEREST trial, and shown to lead to higher response rates and improved PFS in the dose to rash arm of the trial [127]. Rash might indicate local skin EGFR saturation, which may be consistent with tumour EGFR saturation and concentration of EGFR-mAb, which in a proportion of patients is inadequately treated with standard dose EGFR-mAb therapy [137]. However the use of rash as a marker of efficacy has limitations, as rash often occurs in patients who do not subsequently benefit, and clinical benefit is also seen in patients without rash [139].

A further possible surrogate clinical marker of benefit from EGFR-mAb therapy is low magnesium. Hypomagnesaemia is a consequence of EGFR inhibition within the renal tubules leading to magnesium wasting by the kidneys [140]. The mechanism for this is likely to be related to disruption of EGF autocrine/paracrine signalling within the renal tubules. EGF regulates the activity of the Mg^{2+} permeable channel TRPM6 (transient receptor potential cation channel, subfamily M, member 6) and as a consequence inhibition of the EGFR by anti-EGFR antibodies leads to disruption of sorting of the protein to the basolateral membrane of distal convoluted tubule cells in kidney nephrons [141]. In an Italian study, patients with >50% reduction in

magnesium levels compared to basal level had a higher tumour response rate, PFS and longer median OS compared to those with lesser changes [142-144].

1.2.7 EGFR tyrosine kinase inhibitors

Given that there was encouraging activity of EGFR-TKIs in early trials of NSCLC patients [145], it was inevitable that investigators would trial these agents in CRC. However activity in these trials to date has been disappointing and inconsistent in comparison to EGFR-mAb therapy, and there have been toxicity issues when the EGFR-TKIs were combined with chemotherapy [146]. For these reasons the drugs have not been tested in larger randomised trials in advanced colorectal cancer.

1.2.7.1 Mechanisms of action of EGFR Tyrosine Kinase Inhibitors

The EGFR-TKIs have similar mechanisms of action to EGFR-mAbs, but there are important differences that may explain the therapeutic disparity between the agents in colorectal cancer.

EGFR-TKIs block EGFR receptor phosphorylation, leading to similar effects in cell line models to the EGFR-mAbs, such as cell cycle arrest and apoptosis [147], decreased growth of xenografts [148] and reduced angiogenesis [149]. However there are important differences that may influence the spectrum of activity of the different classes of drug (Table 3).

Firstly, the EGFR-TKIs are small molecules that act intra-cellularly by competitive binding within the ATP binding pocket of the EGFR kinase domain, thus preventing ATP binding and receptor phosphorylation [132]. Secondly the EGFR-TKI do not induce ADCC which may potentiate the effect of the EGFR-mAbs via immune cytotoxic effects. Additionally, EGFR-mAbs lead to changes in wild type EGFR expression and receptor internalisation [150], which reduces EGFR signalling [26], whereas EGFR-TKIs either increase or leave EGFR expression levels unchanged [45].

Furthermore, EGFR-TKIs can inhibit ongoing signalling from the EGFR within the nucleus. Nuclear EGFR signalling may be important in propagating oncogenic

signalling and transcription within the nucleus [151]. Levels of nuclear EGFR are associated with poor prognosis in a variety of different cancers [152-154]. EGFR-mAb treatment of colorectal cancer cells leads to an increase in nuclear EGFR signalling but this signalling may be dampened by EGFR-TKIs [155].

Table 3: Similarities and differences between EGFR-mAbs and EGFR-TKIs

Drug Class	EGFR-mAB	EGFR-TKIs
Example	Cetuximab, Panitumumab	Erlotinib, Gefitinib
Similar actions	Cell cycle arrest[47, 156] Apoptosis[48] [156] Decreased angiogenesis[51, 52] Reduced metastasis[53, 54] Augmentation of chemotherapy[55]	Cell cycle arrest[147] Apoptosis[147] Decreased angiogenesis[149] Decreased MMP 9 levels and reduced rate of metastases in xenograft models Augmentation of chemotherapy [157]
Differences	Antibody dependent cytotoxicity[56] Receptor internalisation [150]	
		Nuclear EGFR signalling inhibited[155] Exquisite sensitivity of NSCLC with EGFR mutations to treatment [158] [159]

Lastly, a significant difference between EGFR-TKIs and EGFR-mAbs activity relates to sensitivity of lung cancers that harbour activating mutations within the EGFR tyrosine kinase domain to an EGFR-TKI. These mutations occur in approximately 5-10% of lung adenocarcinomas in caucasians and 30% of lung adenocarcinomas in Asian patients [159]. The two most common mutations found are in frame deletions of exon 19, and a L858R substitution in exon 21 [160]. The mutations within the ATP-binding domain bestow exquisite sensitivity to EGFR TKIs in lung cancer, with response rates typically 70 – 80%, and PFS of 8 – 24 months when an EGFR-TKI is used [158, 159]. The mutations are not predictive for benefit of EGFR-mAbs which is not surprising given mutations likely confer ligand independence [161, 162], and their expression leads to the constitutive activation of downstream survival pathways [162]. Additionally and most importantly, the mutations also confer increased sensitivity to

the EGFR-TKIs, possibly due to reduced ATP affinity at these sites and increased topographic ability for TKI entry into the kinase domain [163].

As previously discussed, these mutations are exceedingly rare in colorectal cancer. An unselected population of 293 colon cancers had 1 EGFR mutation [164], and a different study found 0 in 20 samples[165]. Although a single Japanese study found 4 missense mutations amongst 33 patient samples within the EGFR tyrosine kinase domain [166], they occurred at sites not seen with lung cancer and not known to functionally correlate with sensitivity to EGFR-TKI therapy.

1.2.7.2 Clinical trials of EGFR-TKIs in colorectal cancer

Given that responses to EGFR-TKIs are uncommon in NSCLC patients without mutations within the EGFR [167], and that these mutations are rare in colon cancer it is unsurprising that there is a lack of robust clinical activity in CRC of EGFR-TKIs. Although there is not complete absence of activity in clinical trials of EGFR-TKIs, inconsistency in results and toxicity issues have stopped further clinical use in CRC.

These agents have been tried in first, second and third lines of colorectal cancer either in combination with chemotherapy or as single agents. In single agent trials of erlotinib or gefitinib in chemotherapy refractory patients there were 7 confirmed responses out of a total of 168 pooled patients (4% RR) [168-171] (Table 4).

In trials of combined chemotherapy and EGFR-TKI use, there has been some modest activity, comparable in some cases to previous trials of chemotherapy alone [172-186](Table 4). Additionally the use of EGFR-TKIs has been accompanied by toxicity such as diarrhoea and rash [172, 173], and in some circumstances has led to severe toxicity that led to withdrawal of the EGFR-TKI arm [173]. Other trials have not demonstrated any activity from the EGFR-TKI [187, 188].

Similar to lung cancer, the activity of EGFR-TKIs may be confined to patients who are *KRAS* wild type [189], or who have *EGFR* mutations [165]. It is possible that the activity in these subgroups is buried amongst the non responders. However there was only 1 patient with an EGFR mutation in the only study to investigate this theory, and

they did not have a response to therapy [190]. Furthermore in this study there was no correlation of *KRAS* or *BRAF* status with response rate.

Table 4: Key trials of EGFR-TKIs in CRC

Line of Rx	Trial	Drug	No pts	RR %
3 rd Line	Rothenberg <i>et al</i> , 2005 [168]	Gefitinib 250 – 500mg	115	1%
	Townsley <i>et al</i> , 2006 [169]	Erlotinib 150mg	38	0%
	Keilholz <i>et al</i> , 2005 [171]	Erlotinib 150mg	24	8%
2 nd Line	Kuo <i>et al</i> , 2005 [172]	Gefitinib + IFOX	27	33%
	Meyerhardt <i>et al</i> , 2006 [173]	Erlotinib + CAPOX	32	25%
	Delord <i>et al</i> , 2004 [176]	Erlotinib + CAPOX	17	30%
	Di Bartolomeo <i>et al</i> , 2006 [177]	Erlotinib + CAPIRI	22	10%
	Fisher <i>et al</i> , 2004 [178]	Gefitinib + FOLFOX	22	36%
	Chau <i>et al</i> , 2004 [188]	Gefitinib + Irinotecan	39	11%
	Stebbing <i>et al</i> , 2008 [187]	Gefitinib + 5FU	27	0%
	Hartman <i>et al</i> , 2005 [179]	Gefitinib + FUFOX	18	24%
1 st Line	Fisher <i>et al</i> , 2004 [178]	Gefitinib + FOLFOX	28	78%
	Zeuli <i>et al</i> , 2004 [184]	Gefitinib + CAPOX	12	50%
	Zampino <i>et al</i> , 2005 [185]	Gefitinib + FOLFOX	47	74%

In summary, despite some similar mechanisms of action, EGFR-TKIs have not been as successful in treating patients with metastatic colorectal cancer as EGFR-mAbs. This is possibly because of different mechanisms of action, including a dependence on EGFR mutations to achieve a response, which are rare in colorectal cancer. Whilst there is some activity seen in either single agent treatment or in combination, to date there are no randomised phase III trials to conclusively confirm a lack of clinical benefit. Therefore ultimately the use of these agents has been sidelined by the introduction and widespread testing of EGFR-mAbs which seem in an indirect comparison to be more active.

1.2.8 Dual targeting of the EGFR

Given the non overlapping mechanisms of action of the EGFR-mAbs and EGFR-TKIs, and despite a lack of single agent EGFR-TKI therapy, theoretically the combination may prove more effective than single use of either agent alone in treating colorectal cancer. A parallel example exists in treating breast cancer, where dual

targeting of the HER2 receptor with both an antibody, trastuzumab, and a TKI, lapatinib, has been shown to be more effective in preclinical work and a large randomised clinical trial [191].

1.2.8.1 Preclinical studies

Several groups have investigated preclinical models of the combination of an EGFR-mAb with an EGFR-TKI [192-196]. These studies have demonstrated enhanced growth inhibition of the combination over either agent alone in many but not all cell lines, as measured either by the use of a colorimetric assay such as the MTT assay [193, 194, 196], by cell number [195] or in xenograft models [192, 193, 195].

In a study combining EGFR-mAb and EGFR-TKIs a synergistic effect was demonstrated in cell lines with medium to high expression of EGFR, but not those with low or no EGFR expression [195]. Xenografts of A431 cancer cells have shown impressive reduction in size of tumours as compared to single agent treatment, and in some cases complete remission of the tumour [195] as well as marked reductions in angiogenesis in the xenograft [193]. However not all cell lines demonstrate synergy or additive effects with the combination [195, 197, 198]. Cell lines that have low levels of EGFR, or that have *KRAS* or *BRAF* mutations, demonstrate antagonism in growth inhibition studies [195, 197].

In those studies that demonstrated synergism, the combination of drugs leads to reduced amounts of activated phosphorylated proteins within the EGFR signalling cascade [193, 195]. Levels of pEGFR, pMAPK and pAKT are all reduced compared to single agent treatment alone as detected by Western blotting methods, suggestive of superior suppression of EGFR signalling from the use of the two drugs. In some cell lines such as A431, the combination can lead to the induction of apoptosis as measured by the TUNEL assay [195] or increases in cleaved caspase 7 [193].

1.2.8.2 Explanation of effect

There are at least three plausible explanations for the synergistic benefit of combining an EGFR-mAb with an EGFR-TKI. Firstly, the combination can simply increase blockade of the classical EGFR signalling pathway. Secondly the combination may inhibit a greater number of downstream signalling pathways than either drug alone is

able to inhibit. Lastly complex changes in EGFR surface expression may be responsible for the enhanced effect with subsequent effects on down stream signalling [45, 199].

Given it is rare for any pharmacological agent to completely inhibit its target, the use of two agents that target the same receptor may achieve superior blockade and reduction in signal transduction than either agent alone. Supportive of this theory, preclinical studies show clear additional reductions in signalling in the MAPK and the PI3K pathways when using the combination compared to single agent treatment [193, 195]. Furthermore, clinical data suggests that the ability to maximally inhibit the EGFR with EGFR-mAb is suboptimal at standard doses due to toxicity and saturation of clearance. A recent trial of dose escalated cetuximab achieved higher response rates than standard dose therapy, supportive of a dose response above that currently used [127].

However EGFR-mAbs and EGFR-TKIs may have non-overlapping and alternate mechanisms of action that provide additional growth inhibition. For example, cells treated with high enough doses of an EGFR-TKI for inhibition beyond detection on conventional assays of EGFR tyrosine kinase phosphorylation, still show additive benefit in growth inhibition when combined with an EGFR-mAb [200]. Further supportive data for this hypothesis is that some cetuximab resistant cell lines still retain sensitivity to EGFR-TKIs [193]. In these experiments, cetuximab resistant cell lines SCC-6 and H226 were established that were capable of proliferating in high doses of cetuximab, as compared to their parental lines. The EGFR TKIs gefitinib and erlotinib retained ability to inhibit growth and to inhibit downstream EGFR signalling as manifest by marked decreases in pMAPK and pAKT levels. This suggests that EGFR-TKIs can affect intracellular signalling that is only partially affected by an EGFR-mAb directed at the extracellular domain.

Although an obvious difference between the two classes of drugs is that mutations in the EGFR tyrosine kinase domain bestow sensitivity to EGFR-TKIs, this is extremely unlikely to be of importance in colorectal cancer, where these mutations are rare or non existent [164, 165]. More plausible alternate pathways have arisen from gene expression profiling studies [195]. An analysis of mRNA expression in response to

cetuximab or gefitinib using gene arrays was performed and showed that there were 45 genes that were differentially expressed between the two agents. Differential expression was defined if their level of expression compared to untreated cells had a p value <0.03 calculated by t-test. These included genes involved in cell proliferation (*EMS1*), transcription (*HOXD8*), angiogenesis (*ANG*), and tumour invasion and metastasis (*PLAU*)[201](Figure 2).

Others have also suggested that EGFR-TKIs may be non specific for the EGFR, and affect other pathways, contributing to additional benefit of the combination [193]. Although erlotinib has multiple other kinase targets [202] (Figure 3), there is a 200 fold difference in the dose required to achieve the IC_{50} of the EGFR versus HER2 with gefitinib[203], so this mechanism probably plays a minor role if any.

Another possible explanation for the additive benefit upon combination of the two classes is that the combination leads to changes to EGFR surface expression altering and increasing ADCC. Previously it has been shown that binding of the EGF ligand to the receptor leads to conformational change in the receptor [204], which causes receptor internalization and down regulation [205]. This process has been shown to be inhibited and facilitated by treatment with either an EGFR-TKI or EGFR-mAb respectively [206]. Treatment with an EGFR-mAb leads to decreased amounts of EGFR expression on the cell surface [192, 198]. Treatment with an EGFR-TKI leads to accumulation of inactive EGFR dimers [207] and conformational change of the receptor [45].

Figure 2: Differential gene expression related to dual targeting of the EGFR

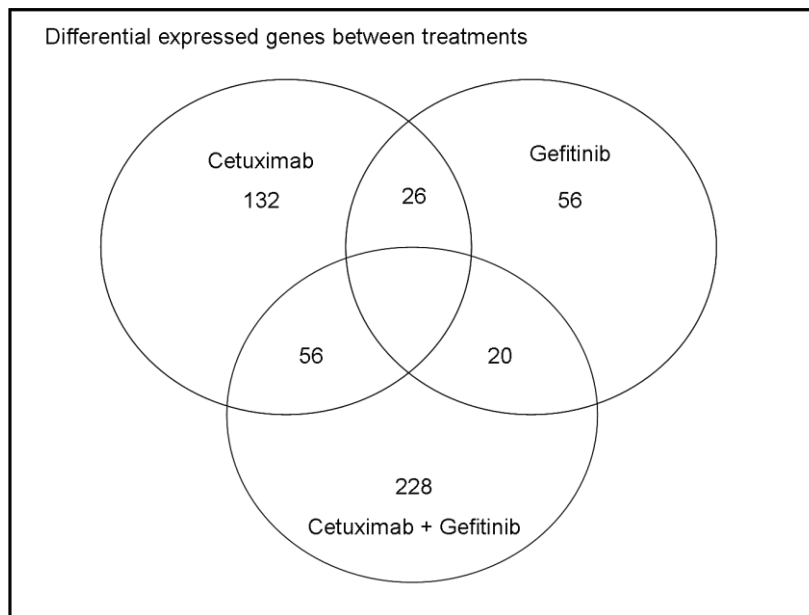


Figure Venn diagram of overlapping gene expression in A431 cells treated with cetuximab, gefitinib, or the combination for 2 hours [201]

Figure 3: EGFR TKI specificity

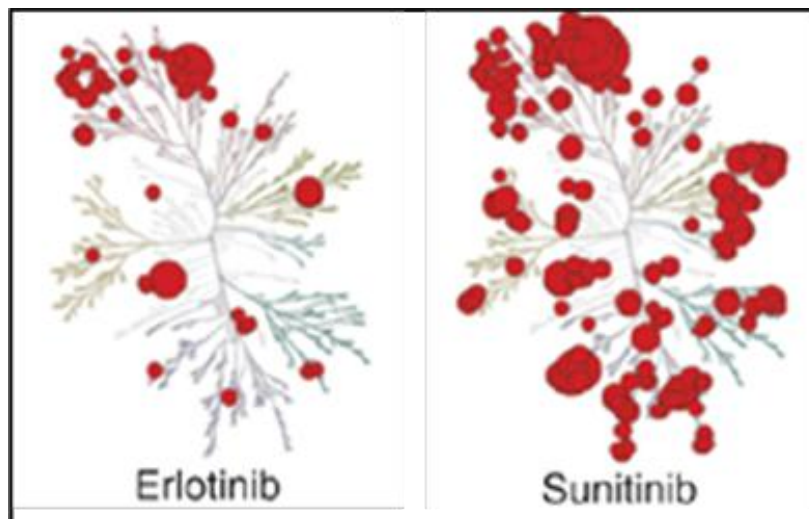


Figure Kinase plot of specificity of erlotinib relative to sunitinib, with spot size inversely proportional to IC_{50} of particular tyrosine kinase, demonstrating relative specificity of erlotinib relative to sunitinib [202]

In a similar study of combined targeting of HER2 in breast cancer, HER2 expression dynamics following treatment with lapatinib (a HER2-TKI) and trastuzumab (a HER2-mAb), or both showed stabilization of the receptor with combined targeting [208]. An accumulation of receptor (HER2) takes place with lapatinib treatment. Conversely, trastuzumab, an antibody to the receptor leads to ubiquitination and degradation of the receptor. Upon combination treatment, the inactive TKI bound HER2 receptors accumulate and form dimers with other Her family members, including EGFR. The accumulation of receptors with lapatinib treatment and enhances trastuzumab binding at the cell surface and subsequent immune mediated trastuzumab cytotoxicity. Given the role of ADCC in antibody therapy in a range of drugs and different cancers [57-61], including panitumumab [62], it is conceivable that such an interaction may be important for the combination of EGFR-mAb and EGFR-TKIs. Contrarily, a study of changes to surface EGFR expression in response to dual targeting of the EGFR with cetuximab and erlotinib demonstrated that there was reduced surface EGFR with combination relative to erlotinib alone in a cholangiocarcinoma cell line [192]. Therefore it is uncertain whether the change in receptor expression level in response in dual targeting differs according to receptor (HER2 vs EGFR), or tumour type.

Further changes in EGFR expression that could mediate response to cetuximab have recently been elucidated [155, 209]. Two papers reported that cetuximab induces accumulation of nuclear EGFR, which can act as a transcription factor and propagate cell growth, separate from the extracellular cetuximab. Nuclear EGFR signalling may be important in propagating oncogenic signalling and transcription within the nucleus [151] and levels of nuclear EGFR in tumour samples are associated with poorer prognosis [153, 154]. However the interaction and implications of an EGFR-TKI on the trafficking of EGFR to the nucleus either alone or in combination with an EGFR-mAb is not clear. Liao *et al* reported increased levels of nuclear EGFR with dual treatment of the cells with cetuximab and erlotinib in DiFi cells [209], but the therapeutic implications are uncertain.

1.2.8.3 Clinical trials of dual targeting of the EGFR

Encouraging preclinical data led investigators to trial the combination of EGFR-mAb with a EGFR-TKI in phase I dose finding studies [201, 210, 211]. A further clinical

trial has been published that assessed the safety and efficacy of the combination in NSCLC [212]. Several further studies are currently recruiting patients, including NCT00895362 and NCT00397384. Information on published trials is included below in Table 5.

Table 5: Phase I trials of dual targeting the EGFR

Trial	Design	EGFR-TKI	EGFR-mAb	No pts	Resp Rate	RP2D
Baselga <i>et al</i> , 2006 [201]	Phase I with increasing doses	Gefitinib 100mg–500mg	Cetuximab 320- 400mg/m ² loading 200-250mg/m ² weekly	35 9 CRC	5/35 patients (14%) 5/9 CRC patients (56%) PR	Cetuximab 400/250mg/m ² and Gefitinib 500mg
Lin <i>et al</i> , 2008 [211]	Phase I with increasing doses	Erlotinib 50-150mg	Cetuximab 400mg/m ² loading 250mg/m ² weekly	21 1 CRC	1/21 (5%) 0/1 CRC (0%)	Cetuximab 400/250mg/m ² and Erlotinib 50mg
Gaurino <i>et al</i> , 2009 [210]	Phase I with increasing doses	Erlotinib 150mg	Cetuximab 100 -250mg/m ² weekly, (no loading dose)	22 2 CRC	0 responses 0/2 CRC (0%)	Cetuximab 250mg/m ² weekly Erlotinib 150mg daily
Ramalingam <i>et al</i> , 2008 [212]	Phase I with increasing doses	Gefitinib 250mg	Cetuximab	13 - all NSCLC	0 responses	Cetuximab 250mg/m ² weekly Gefitinib 250mg daily

RP2D = Recommended Phase 2 dose

A phase I trial completed by a Spanish group used cetuximab and gefitinib in escalating doses recruited 35 patients, of whom 9 had metastatic colorectal cancer evaluable for response [201]. The other patients had either non small cell lung cancer or head and neck cancer. The trial was designed to define the Recommended Phase II Dose (RP2D) for the combination. Patients were recruited in cohorts of either single therapy with either agent, or combination treatment with increasing doses of gefitinib and cetuximab. Encouragingly, 5 of 9 patients (56%) with colorectal cancer treated with the combination achieved a PR. The median PFS in the colorectal cancer patients was 3.2 months. Tumour and skin biopsies were obtained pretreatment and 14 days after beginning treatment and analysed for changes within the EGFR pathway. The

patients treated in the combination cohort had greater decreases in phosphorylation of EGFR, MAPK, and especially AKT as compared to those patients treated with either agent alone. Toxicity was manageable and the RP2D was full dose Cetuximab (400mg/m² IV loading dose followed by 250mg/m² weekly, and Gefitinib 500mg daily.

A similar phase I trial has been published, which was a dose escalation trial of erlotinib with full dose cetuximab to achieve a RP2D of the combination, prior to further combination with bevacizumab in two other cohorts [211]. This trial enrolled 19 patients in the first part of their trial using cetuximab and erlotinib, initially with erlotinib 50mg/day, and then at a higher dose schedule of 100mg/day. Most patients had head and neck squamous cell cancer or non squamous cell lung cancer (NSCLC) and only 1 patient had colorectal cancer. Given 3/8 patients in the higher dose cohort developed a dose limiting toxicity (DLT), the maximum tolerated dose (MTD) was determined to be 50mg/day of erlotinib when combined with full dose of cetuximab (400mg/m² loading, 250mg/m² weekly). The side effects of the combination were similar to those seen with either agent alone, with 3 out of 19 patients developing grade 3 rash. Notably, grade 3 or 4 hypomagnesaemia occurred in 49% of cycles with the higher dose of Erlotinib of 100mg/day, but only 11% when used at 50mg/day. There was no grade 3 or 4 diarrhoea in this study. Activity was limited with only one patient achieving a partial response (renal cell cancer), and two patients achieved durable stable disease.

A further phase I trial with erlotinib and cetuximab has also been published, recruiting 22 patients, of which the majority had lung cancer [210], and 2 patients had colorectal cancer. This trial combined fixed doses of erlotinib at 150mg a day with different doses of cetuximab, without a loading dose of cetuximab. The weekly dose of cetuximab did not exceed 250mg/m² in this trial. The median time of treatment was 5.5 weeks, with 7 of 18 patients with evaluable disease having stable disease. There were no objective responses. There was only one DLT in this group, with grade 3 rash. Unlike the other trials there was a smaller incidence of grade 3 or 4 hypomagnesaemia (11%). The incidence of grade 3 or 4 rash was 9.1% and almost all patients had at least G1 rash. Diarrhoea was not a major problem, with only 4.5%

suffering grade 3 or 4 toxicity. The authors concluded that full dose erlotinib and weekly cetuximab was feasible for phase II evaluation.

More recently a disease focused phase I trial recruited patients with NSCLC only [212] using an escalating dose of cetuximab with gefitinib 250mg/day in order to limit potential toxicity from the combination. Thirteen patients were recruited, in different cohorts each with different doses of cetuximab. Only 1 patient had a grade 3 rash, but 11 of the 13 patients had some degree of skin rash. Grade 3 or 4 hypomagnesaemia was observed in 3 of 6 patients in the cohort who received the highest dose of cetuximab (250mg/m²). None of the 13 patients had an observed response, but four achieved stable disease.

1.2.9 Summary

A review of the literature regarding the biology and treatment of metastatic colorectal cancer highlights several issues. EGFR mAbs such as cetuximab are clinically active in colorectal cancer, but their activity is modest, and limited to a subset of patients who have tumours without *KRAS* or *BRAF* mutations. In contrast EGFR-TKIs are relatively inactive when used alone in colorectal cancer. Preclinical data in non colorectal cancer, encouraging data in early phase I trials, and a completed successful trial of dual targeting of the HER2 receptor in breast cancer guided the investigation into dual targeting of the EGFR in colorectal cancer. This thesis will report the findings of the combination of an EGFR-mAb and an EGFR-TKI in colorectal cancer cell lines, and the mechanisms governing the effectiveness of this therapy. A clinical trial exploring the use of EGFR-mAb and TKI is also reported, with detailed analysis of the intrinsic resistance mechanisms to EGFR-mAb including *KRAS* and *BRAF* mutations.

1.3 ACQUIRED RESISTANCE TO EGFR THERAPY

Despite advances in the selection of colorectal cancer patients for treatment with anti-EGFR antibodies, *KRAS* wild type patients who initially respond to these agents inevitably progress after a few months of treatment. Therefore, an alternate method of improving the efficacy of targeted therapy is to prolong treatment response to

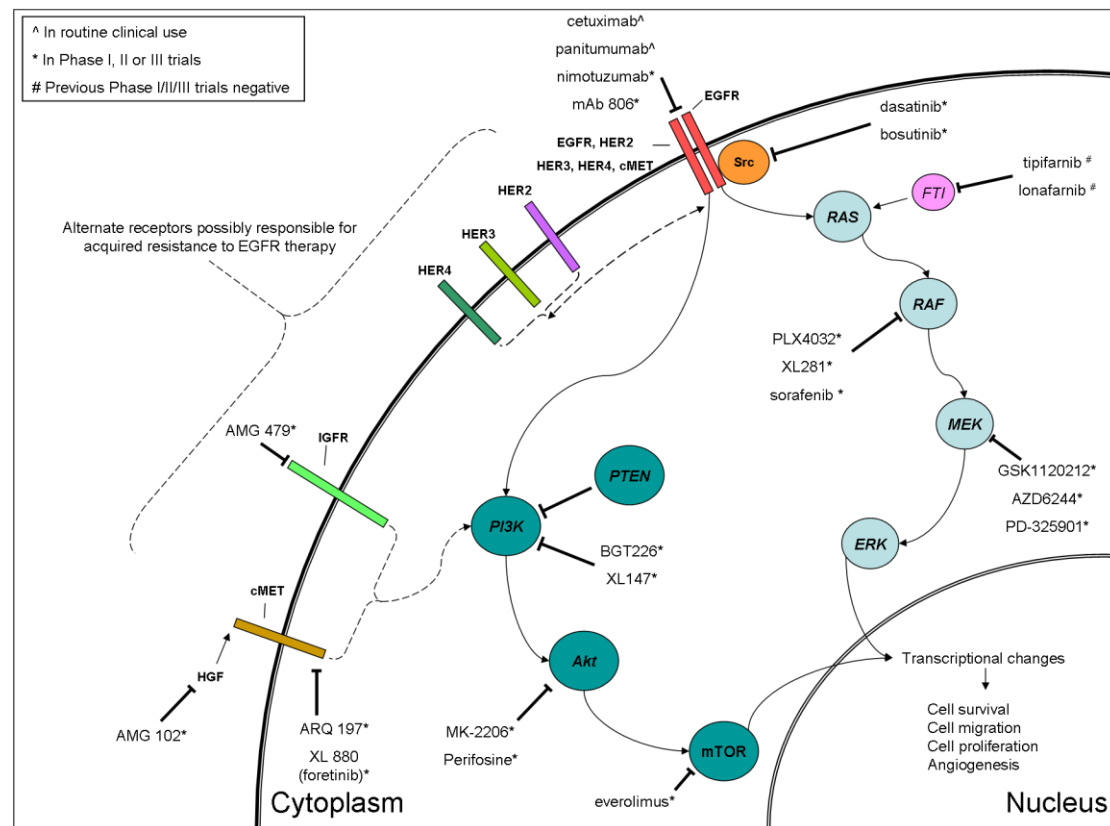
therapy. Understanding the mechanisms of acquired resistance to EGFR-mAbs could guide rational drug development and be used to maximise the effect of EGFR-targeted therapy.

Review of preclinical and clinical data of EGFR targeted therapy in colorectal cancer and in other malignancies points to two fundamental mechanisms of acquired resistance: either activation of compensatory signalling pathways such as *PI3K/mTOR* [213, 214], *Src* [215], *cMET* [216], *IGFR* [217] and other HER family members [218], or the emergence of secondary mutations within the EGFR pathway as observed in patients who become refractory to tyrosine kinase inhibitors of EGFR in lung cancer [41]. Figure 4 illustrates the EGFR pathway, compensatory pathways, and drugs that may overcome acquired resistance.

1.3.1.1 EGFR family member activation

Parallel observations of mechanisms of resistance to HER2 targeted therapy suggest alternate EGFR family member activation may contribute to acquired resistance. Trastuzumab (herceptin) is a monoclonal antibody that targets the HER2 receptor in breast cancer. Acquired resistance due to the formation of alternative HER2 signalling complexes and activation of subsequent downstream signalling has been postulated as a possible mechanism of trastuzumab resistance [219]. This is supported by pre-clinical studies where EGFR inhibitors such as cetuximab can overcome trastuzumab resistance in breast cancer xenografts [220], and by clinical findings where the small molecule dual inhibitor of EGFR and HER2, lapatinib, has efficacy in treating women who have acquired resistance to trastuzumab [221]. Notably, the combination of trastuzumab and lapatinib has also now been shown to be superior to single agent treatment in a recently completed phase III trial [222]. Pertuzumab, a monoclonal antibody that prevents dimerisation of HER2 with other HER family members, has been shown to restore sensitivity to herceptin in breast cancer cell lines and in patients, probably due to altered receptor conformation and signalling changes [223-225]. Collectively, these findings indicate that up regulation of alternate HER family members is a mechanism of acquired resistance to HER2 signalling in breast cancer, establishing the possibility that similar mechanisms may be involved in acquired resistance to EGFR-mAbs in colon cancer.

Figure 4: Targets in the EGFR pathway relevant to acquired resistance



1.3.1.2 HGF/C-Met pathway

C-Met is a trans-membrane receptor tyrosine kinase which is activated upon binding of its ligand, hepatocyte growth factor (HGF). HGF binding induces receptor dimerisation and increases kinase activity of the receptor, triggering downstream signalling via the MAPK and PI3K pathways and ultimately in cell proliferation, migration, and angiogenesis [226]. Evidence for up regulation of c-Met signalling as a mechanism of acquired resistance to EGFR-targeting therapeutics is provided from several studies [214].

First, amplification of the c-MET oncogene has been observed in EGFR mutant lung cancers which have acquired resistance to EGFR inhibitors but which have not developed secondary mutations within the EGFR [216]. Second, Engelman *et al* demonstrated that gefitinib resistant NSCLC cell lines express increased amounts of c-Met, and inhibition of c-Met restores sensitivity to gefitinib [216]. The authors also demonstrated that c-Met signalling drives resistance by activating HER3, and increasing signalling via the PI3K-AKT pathway [216]. Similar findings were observed by Wheeler *et al*, where cetuximab resistant NSCLC cell lines were shown to have increased levels of HER2, Her3 and c-Met, with subsequent heterodimerisation of these receptors, and increased downstream signalling via the PI3K-AKT pathway [218, 227]. The role of c-Met in acquired resistance to cetuximab in colon cancer cells is therefore worthy of investigation. Importantly, antibody-based therapeutics which target HGF (AMG-102), and small molecule inhibitors which target c-Met (ARQ 197, GSK1363089/XL880), are presently in phase I and II clinical trials (NCT00788957, NCT00558207, NCT00726323).

1.3.1.3 IGF/IGFR pathway

Insulin Growth Factor 1 (IGF 1), and Insulin Growth Factor 2 (IGF 2) are synthesised in the liver in response to human Growth Hormone (GH). They act as ligands for the Insulin Growth Factor Receptor (IGFR) [228], which is expressed on all human cells apart from hepatocytes and mature B cells. The IGFR is a transmembrane receptor tyrosine kinase which is autophosphorylated upon binding IGF1 and 2, in turn causing phosphorylation of the proteins IRS-1, -2, -3, -4, and Src Homology and Collagen protein (SHC), which in turn activates *PI3K*/AKT signalling.

A role for IGFR in acquired resistance to EGFR targeting therapies is suggested by a study in A431 cells where exposure to increasing amount of gefitinib resulted in a gefitinib-resistant cell line with increased signalling via the IGF-1R/IRS-1/*PI3K* pathway [229]. The authors also demonstrated that gefitinib resistance was associated with loss of IGF binding proteins which negatively regulate IGFR signalling, and importantly, gefitinib sensitivity was restored upon IGF-1R inhibition [229]. Notably, gefitinib resistant cells were also resistant to erlotinib and cetuximab, suggesting that IGF-1R up regulation may confer resistance to both antibody and small molecule TKI EGFR inhibitors. Finally, the combinatorial inhibition of IGF-1R and EGFR has been shown to enhance the efficacy of EGFR inhibitors in a variety of cancer cells *in vitro* [230-233]. Importantly, as for c-Met, antibody-based therapies which target the IGFR such as AMG 479, are currently in clinical trials including a phase II study in combination with the EGFR-targeting antibody, panitumumab, in advanced colon cancer (NCT00788957).

1.3.1.4 Src

Src is the prototypical member of the Src Family Kinase (SFK) group, which comprises 9 non receptor tyrosine kinases that share similar structures and functions. Src interacts with the EGFR, and is essential for cell signalling via this pathway [234, 235]. Although Src can activate EGFR in the absence of EGF ligand [236], its normal role is to bind and phosphorylate the Y845 residue of EGFR in response to EGF stimulation, increasing MEK and MAPK activity [237, 238].

Notably, the requirement of Src for EGFR-dependent signalling is increased in cell lines that have developed resistance to cetuximab. NSCLC cell lines resistant to cetuximab have increased levels of active SFKs, resulting in hyper-phosphorylation of EGFR Y845 [234]. Although SFKs become active by interacting with many receptors including PDGFR and EGFR, targeting the EGFR with siRNA or an EGFR TKI resulted in decreased SFK activity, suggestive that the primary mechanism of SFK activation in resistant cell lines is via its interaction with EGFR [217]. In NSCLC cell lines resistant to cetuximab, treatment with the src-inhibitor, dasatinib, can re-sensitise cells to cetuximab [217], and reduce downstream signalling via the *PI3K* pathway. Notably, the effect of dasatinib on SFK activity and cell growth was

significantly greater in the resistant line, suggestive of dependence on Src. DiFi colon cancer cells with acquired resistance to cetuximab (DiFi5) also show significantly higher srcY416 phosphorylation at baseline, and show a preferential decrease in Y845 EGFR compared to its parental line in response to dasatinib treatment, suggestive of a greater role of Src in the resistant cell line [37]. The combination of the Src inhibitor, PP2, and cetuximab also preferentially increased apoptosis in the resistant cell line as compared to the parental line [206]. Furthermore, the addition of dasatinib to mAb 806 enhances efficacy of mAb 806 in de₂₋₇EGFR glioma xenografts [239].

Src has also been suggested to facilitate acquired resistance to cetuximab in NSCLC cells by facilitating translocation of the EGFR to the nucleus [155]. Nuclear EGFR has been shown to promote cell proliferation by interacting with transcription factors such as STATs and E2F1 and promoting expression of pro-proliferative genes including cyclin D1 and aurora A [35, 240-242], as well as by inducing PCNA phosphorylation and stabilization [243]. Importantly, the cetuximab resistant NSCLC cells had decreased nuclear EGFR levels and increased membrane levels, and resensitization of cells to cetuximab after treatment with dasatinib [155, 199]. Trials of the combination of cetuximab and dasatinib are planned for colon, head and neck and lung cancer (NCT00835679, NCT00501410).

1.3.1.5 VEGF

In addition to cell proliferation and survival, EGFR signalling can also regulate angiogenesis through modulating the production of pro-angiogenic factors such as VEGF [244]. Up-regulation of proteins which promote tumour angiogenesis may therefore also be a mechanism of acquired resistance to EGFR treatment. In support of this, over expression of VEGF in A431 cells renders the cells resistant to EGFR antibody-based therapy *in vivo* [245]. The same authors also demonstrated that A431 xenografts with acquired resistance to anti-EGFR antibodies express significantly higher levels of VEGF compared to parental controls. Likewise, GEO colon cancer cells rendered resistant to EGFR inhibitors in an *in vivo* xenograft system demonstrated increased VEGF expression and their growth could be inhibited by the VEGFR-1 tyrosine kinase inhibitor, ZD6474 [213]. These findings highlight the importance of angiogenesis in mediating tumour response to EGFR inhibition *in vivo*, and in acquired resistance to these agents. While a potential clinical application of

these findings could be the combinatorial use of EGFR inhibitors with anti-angiogenic therapies, increased toxicity when combining EGFR and VEGF targeted therapies has been observed [246, 247], which may limit the routine clinical use of this combination. Additionally, in a mouse breast cancer model of acquired resistance to trastuzumab, up regulation of VEGF was observed, and tumours were responsive to the VEGF-targeting antibody, bevacizumab [220].

1.3.1.6 Acquired resistance through mutation of the target

In contrast to altering reliance on the target by use of compensatory signalling pathways, alteration of the target itself is also thought an important mechanism of resistance to EGFR targeted treatment, particularly EGFR-TKI therapy. In NSCLC patients, exquisite sensitivity to the small molecule EGFR tyrosine kinase inhibitors (TKI), gefitinib and erlotinib, is observed in those patients who harbour activating mutations of *EGFR*, 90% of which are in exon 19 and 21 of the ATP binding pocket [160, 248]. These activating mutations enhance signalling and reliance on the EGFR pathway, but also confer increased binding and fit of the TKI within the ATP pocket [132]. Patients with these mutations therefore have significantly higher response rates and progression free survival compared with standard chemotherapy, to those patients who lack the mutation [158]. However, patients treated with EGFR TKIs typically develop resistance after several months treatment, which in approximately 50% of cases is due to the acquisition of secondary mutations within the EGFR [41, 249], with the T790M mutation in exon 20 being the most common [159, 250, 251]. This resistance mutation reduces the potency of any ATP-competitive kinase inhibitor such as erlotinib, but may be overcome using newer irreversible inhibitors that use covalent binding [252, 253]. More recently it was shown that the T790M mutation is detectable in the original NSCLC biopsy specimen in 38% of patients [254]. This mutation was present in a very small percentage of tumour cells in the original biopsy, with a likely explanation being that treatment with EGFR TKIs selects for the emergence of the pre-existing T790M subpopulation.

In colorectal cancer, no studies to-date have reported the acquisition of resistance through secondary mutations of the kinase domain. EGFR kinase mutations are rare in untreated colon cancer patients [164], suggesting this may not be a major target for mutation, although the possibility cannot be discounted. Likewise, acquisition of

mutations in *KRAS* and *PI3KCA*, or loss of PTEN expression would be worthy of examination in *KRAS* WT tumours which have developed resistance to EGFR inhibition. The increasing ability to detect and isolate Circulating Tumour Cells (CTCs), which could supplant invasive biopsies that have prohibited much work *in vivo* in this area, coupled with the advent of modern sequencing technologies provides a unique opportunity to comprehensively address these possibilities.

1.3.2 Summary

The mechanisms of acquired resistance to EGFR-mAb therapy probably relate to changes in compensatory pathways, but acquisition and/or emergence of cancer cells with resistant mutations cannot be excluded. Investigation of these mechanisms in colorectal cancer cells will be explored in this thesis.

1.4 TARGETING ANGIOGENESIS

Abnormal angiogenesis is commonly seen in solid tumours, and is distinguished by abnormal and altered vessel morphology, driven by a complex series of factors. Angiogenesis is required for tumours to proliferate and grow beyond a certain size, and therefore presents an attractive target for inhibiting growth of tumours. Bevacizumab, a monoclonal antibody to VEGF-A, is an anti-angiogenic drug shown to improve median PFS in patients with colorectal cancer when used with chemotherapy. Given the high cost of bevacizumab and modest efficacy, a potential way to improve the utility of the drug is to identify tumour or patient biomarkers that predict for response or resistance to the drug. The following section outlines the mechanisms of action of the drug, pathways of resistance, and possible biomarkers of resistance to this therapeutic approach. This information provides a background to a biomarker study using clinical samples from patients treated with the drug as part of this thesis.

1.4.1 Angiogenic pathways and biology

The VEGF family comprises the structurally related ligands that include VEGF-A (commonly referred to as simply VEGF), VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF)(Figure 5). These ligands activate in an overlapping manner

receptors on endothelial cells that mediate changes to tumour vasculature. Collectively the ligands form part of a larger family of Circulating Angiogenic Factors (CAFs). VEGF-A is expressed in a variety of isoforms due to different splicing of VEGF mRNA, with the 165 amino acid isoform the most common, known as VEGF¹⁶⁵. These circulating ligands are not only secreted by tumour cells to activate receptors on endothelial cells in a paracrine fashion, but also by circulating platelets and tumour associated stromal cells [255]. The ligands bind structurally related but functionally different receptors, Vascular Endothelial Growth Factor Receptor (VEGFR) 1, VEGFR2 and VEGFR3 which mediate signalling pathways that govern endothelial cell migration, permeability and proliferation [256].

Additionally, it should be noted that the receptors for these factors are not purely confined to endothelial cells, but also on tumour cells themselves, which may depend on VEGF-A for an autocrine or paracrine growth pathway [257]. Some tumour cells such as breast cancer and haematopoietic stem cells may have intracellular VEGFR, and may promote cell survival by intracrine methods involving intracellular production of VEGF-A [258].

VEGF-A is the predominant signalling ligand within the family, and is over expressed by a variety of tumours. Hypoxia and other stimuli are powerful stimuli for VEGF-A release through mediator molecules such as HIF-1 α and 2 α [259]. VEGF-A principally mediates angiogenesis through binding to VEGFR2, which is expressed at elevated levels on endothelial cells, Circulating Endothelial Precursor Cells (CEPCs) as well as tumour cells [255]. The different isoforms of VEGF-A, VEGF-A₁₂₁ and VEGF-A₁₆₅ have different binding properties to VEGFR2, but the significance of this is uncertain [260]. VEGF-A and VEGFR2 are therefore the main targets of either neutralising antibodies such as bevacizumab (monoclonal antibody to VEGFA), or small molecule tyrosine kinase inhibitors such as sunitinib. The interplay between ligands and receptors is represented in Figure 5.

Figure 5: Angiogenic signalling pathways and ligands

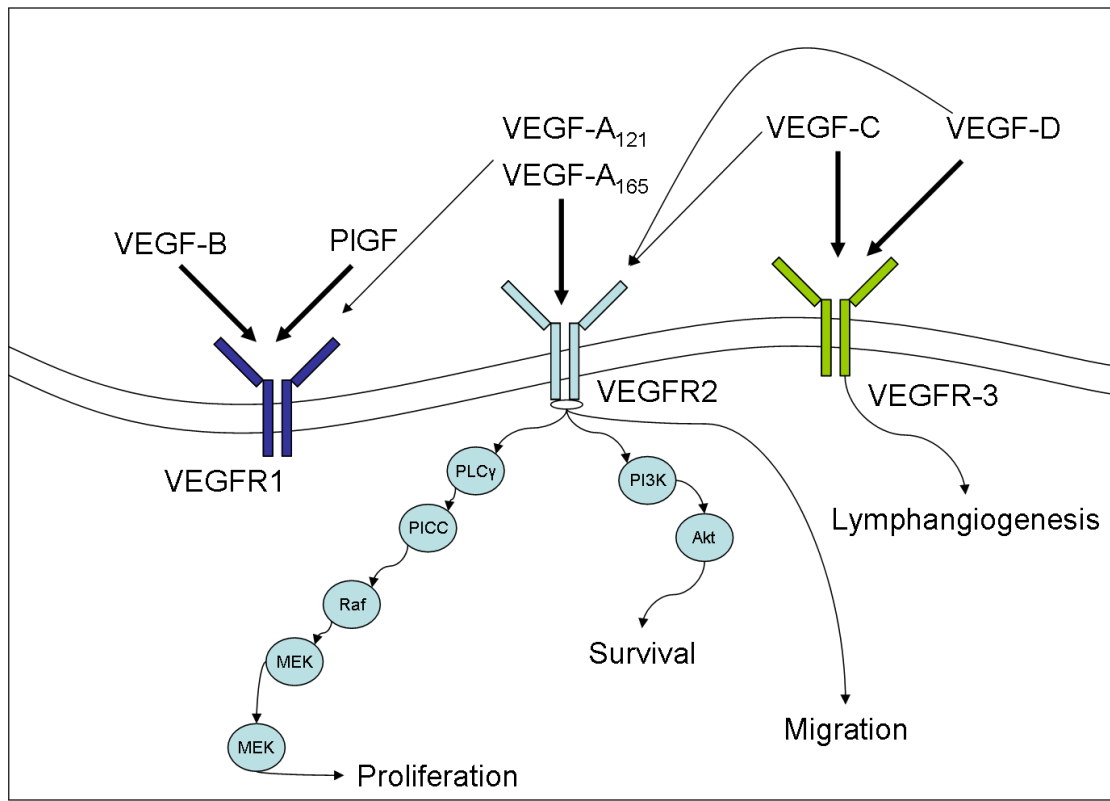


Figure The interaction between ligands and receptors in angiogenesis

The consequence of the binding of VEGF-A to VEGFR2 is increased angiogenesis through increased endothelial cell proliferation and increased permeability of the vasculature increased migration. This in turn increases invasion and survival of tumour cells; and can increase homing of progenitor cells to the site of VEGF release. These functions are mediated through binding to VEGFR2 and dimerisation of the receptor, whereby different intracellular pathways are activated, including the PLCγ-PKC-Raf-MAPK pathway, the PI3KCA-AKT pathway, as well as Src [255].

VEGF-A also binds to VEGFR1, which is expressed nearly 10 fold higher than VEGFR2, however intriguingly has very little intracellular signalling capability, and may instead act as a decoy receptor, binding excess VEGF-A [261]. VEGFR1 is expressed on endothelial cells of the vasculature as well as other cells [256]. Both VEGF-B and PlGF bind to VEGFR1. Circumstantial evidence of VEGFR1's importance in angiogenesis is that the level of its ligand PlGF has prognostic significance in colon and many other cancers [262]. It should also be noted that antibodies to PlGF have been reported to block angiogenesis in tumours that have become resistant to VEGF targeted therapy [263].

VEGF-C and VEGF-D signal predominantly via VEGFR3 expressed on the surface of lymphatic endothelial cells and mediate lymphangiogenesis [264]. However VEGF-C and VEGF-D can also induce angiogenesis through binding to the VEGFR2 receptor [265, 266]. The degree of interaction depends on the proteolytic processing of the ligands to a mature form, which enhances the affinity for VEGFR2 approximately 290 fold [267]. Rissanen *et al* demonstrated that this mature form of VEGF-D had greater angiogenic effects than VEGF-A in *in vitro* experiments [266]. Blockade of VEGFR3 has been reported to augment inhibition of angiogenesis in several models [268, 269].

Aside from the interaction between VEGF family members and VEGFR, there are other important receptors in angiogenesis: Neuropilin 1 and 2 (NRP-1, NRP-2), Tie-2, and the Notch-Delta-like ligand 4. NRP-1 and NRP-2 are co-receptors for VEGFR2, and may enhance the binding and activity of VEGF: blockade of NRP-1 has been reported to be more effective than targeting VEGFR2 alone [270]. Tie-2 is a receptor tyrosine kinase expressed on the vascular endothelium. The ligands angiopoietin-1 (ang-1) and angiopoietin-2 (ang-2), act in concert with VEGF-A to promote

angiogenesis. Newer drugs that block ang-2, including monoclonal antibodies are in development [271]. Notch – delta like ligand 4 (Dll4) signalling pathway is a further pathway that affects angiogenesis. The notch receptor on endothelial cells interacts with Dll4 receptor near the tips of sprouting angiogenesis of vessels, restraining excess budding of the vasculature [272]. Blockade of delta like ligand 4 leads to increased abnormal blood vessels, and combined blockade with VEGF/VEGFR2 inhibitors may be more effective than either drug alone [273].

1.4.2 Bevacizumab, a VEGF-A monoclonal antibody

1.4.2.1 Mechanism of action of bevacizumab

Bevacizumab is a humanised monoclonal antibody to VEGF-A, developed by Genentech, and licensed to Roche, which was approved for treatment of metastatic colorectal cancer by the FDA in 2004. A variety of theories regarding its mode of action have been proposed [256, 274], including reducing vessel growth, changes to vessel function, direct effects on tumour cells and immune alteration. The benefit is probably attributable to a combination of these reasons.

The vasculature of a tumour is often quite different between a primary tumour and its metastases. Additionally different tumour types have different dependence on their vasculature. For example, whereas renal cell cancer is highly vascular and anti-angiogenic drugs have single agent activity, other tumours such as pancreatic and prostate cancer have no benefit from bevacizumab, even when combined with chemotherapy [275, 276]. Therefore bevacizumab may have different actions dependent on the type of tumour and whether it is given as a single agent, or in combination with chemotherapy. Other problems in defining the mechanism of action of bevacizumab are that the tumour itself may change its angiogenic biology with progression and metastasis. Many authors have also stated the concept of an angiogenic switch, which is the certain point in growth of metastases that the tumour no longer depends on its highly vascular surrounds, but instead becomes independent in recruiting and sprouting vessels to sustain further growth [277]. Preclinical models have been the predominant tool by which the mechanism of bevacizumab has been studied, but these are imperfect. Vessels in xenografts may grow more rapidly than

human tumours and are consequently more responsive to anti-angiogenic therapy [256, 278, 279].

Anti-angiogenic therapies such as bevacizumab were originally postulated to alter sprouting angiogenesis: decreased VEGF-A would lead to decreased vessel growth then tumour hypoxia and death. Similar observations have been made with VEGFR2 TKIs, such as sunitinib [256]. Willet *et al* recently reported that bevacizumab alone reduced tumour blood flow as measured by functional imaging in a series of 32 patients receiving neo-adjuvant chemotherapy with bevacizumab then chemoradiotherapy for rectal cancer [280]. However pre-treatment tumour Microvessel Density (MVD) does not correlate with treatment benefit from bevacizumab [281], and there are limited other human studies of pre and post treatment vessel analysis in tumours following single agent bevacizumab treatment to substantiate this theory. Regardless, blockade of VEGF-A can lead to endothelial cell apoptosis, as well as blockade of incorporation of circulating endothelial cell precursor cells (CEPCs). VEGF-A is required for endothelial cell survival in tumour xenografts [282, 283] and its inhibition can lead to elevated apoptotic proteins [284]. VEGF is the most important cytokine for recruitment of CEPCs for their incorporation into the vasculature. Blockade may reduce this process. However xenografts probably over amplify this importance as they depend heavily on CEPCs likely owing to their rapid growth rate [278, 279].

To achieve meaningful decrease in tumour size, alteration of vessel function may be more important than a reduction in vessel number. For example, novel anti-angiogenic therapies have demonstrated that there can be decreases in tumour blood flow and substantial decreases in tumour size despite having an increase in tumour vessel number [285]. The effect of bevacizumab may in part be due to vasoconstriction of vessels due to reduction of VEGF-A related Nitric Oxide release [286]. This effect is readily quantifiable using newer imaging techniques such as Dynamic Contrast Enhanced Magnetic Resonance Imaging (DCE-MRI) and dynamic CT [287], which can both measure tumour blood flow and vessel permeability. Vasoconstriction is also reflected in the hypertension observed as a common side effect of bevacizumab [288].

Other alterations in vessel function that may be related to bevacizumab action include the concept of vessel normalization, as proposed by Jain [289]. Currently there is some controversy over this, related to the relative importance and exact definition of vessel normalisation [256]. In tumours, VEGF-A can lead to excessive vessel permeability, and the formation of vasodilated, tortuous vessels [289]. Bevacizumab and other anti-angiogenic agents such as sunitinib, can reverse this process by causing the redistribution of vessels and increasing blood flow (vessel normalisation). This in turn can improve the delivery of concurrent chemotherapy in some, but not all preclinical tumour models [290]. A clinical observation that is supportive of the role of vessel normalisation is that bevacizumab has no single agent activity in colorectal cancer, and is useful only in combination with chemotherapy [291]. However the exact importance of this mechanism of action is still debated. Although Jain has published several preclinical papers supportive of vessel normalisation following bevacizumab treatment, others have noted that direct vasodilators also have been observed to increase chemotherapy delivery to tumours, and that anti-angiogenic therapy may sensitise the tumour endothelium to chemotherapy [292, 293].

Although the endothelium had previously been thought to be the sole target of VEGF-A and related family members, VEGFR2 as well as NRP-1 and NRP2 co-receptors have been shown to also exist on the tumour cell surface. Hence it follows that the tumour cells themselves are possibly directly affected by an antibody mediated decrease in VEGF-A. Certainly treatment with bevacizumab can decrease the activation of VEGFR2 [294] and direct VEGFR1 antibodies can block growth of breast cancer xenografts independent of any effect on angiogenesis [295]. However the presence of VEGFR2 on tumour cells is controversial, with a recent study reporting that prior studies that showed tumour localization of VEGFR2 were in fact incorrect due to non specific cross reactive antigen staining of tumour cells unrelated to VEGFR2 presence [296]. Furthermore, single agent treatment of cancer cell lines with bevacizumab does not cause growth inhibition, unless in xenograft models or in combination therapy [297, 298]. Therefore the presence and importance of VEGFR2 on tumour cells is controversial.

A final important mechanism of action of bevacizumab relates to the immune system. Bevacizumab is also reported to reduce dendritic cell migration to tumour sites and

maturation, where they normally process and then present tumour antigen to the immune system [299, 300]. This may be an indirect and additional important mechanism of bevacizumab restricting tumour growth.

1.4.2.2 Clinical trials of bevacizumab in colorectal cancer

There have been several large randomised studies that have demonstrated the benefit of bevacizumab in addition to chemotherapy in colon, lung and breast cancer. In particular, the effectiveness of bevacizumab in colon cancer in combination with chemotherapy was recently reviewed in a meta-analysis[301] which reported a significant improvement in PFS HR 0.61 (95% CI 0.45 - 0.83) and OS 0.81 (95% CI 0.73 - 0.90) in favour of those patients receiving first line chemotherapy with bevacizumab. The addition of bevacizumab to first line chemotherapy prolongs PFS and OS for patients in both responders and non responders[302]. The addition of bevacizumab to chemotherapy does not seem to cause significant side effects in most patients, but has been associated with uncommon but significant grade 3-5 side effects, including hypertension (5%), gastrointestinal perforation (2%), arterial thromboembolic events (2%), poor wound healing (1%), and more rarely reversible posterior leuco-encephalopathy [303]. The combination of bevacizumab with oxaliplatin was associated with an increased rate of peripheral neuropathy in the bevacizumab containing arms of recent trials [304, 305].

Bevacizumab has now been studied with all contemporary chemotherapy regimens for metastatic colorectal cancer. This includes combination with single agent 5-Fluorouracil regimens, as well as oxaliplatin and irinotecan containing regimens. There are currently no trials that allow a direct comparison between the different bevacizumab containing regimens to allow assessment of superiority between these regimens.

The earlier clinical trials that assessed the additive benefit of bevacizumab to irinotecan, used an older style IFL regimen, which has subsequently fallen out of favour with clinicians because of toxicity issues [21, 306]. The trial which led to the registration of bevacizumab was a North American study that assessed the role of bevacizumab 5mg/kg every two weeks in addition to IFL chemotherapy. The trial enrolled 813 patients, and reported an improvement in PFS of 11 versus 6 months,

and OS of 20 versus 16 months [21]. Similar encouraging data emerged from two other studies when bevacizumab was combined with 5FU and irinotecan [307, 308].

The large randomised trial of bevacizumab in combination with oxaliplatin regimens, the N016966 trial [305], combined bevacizumab with oxaliplatin and either infusional or oral 5FU regimens. However there were no significant differences in response rates between the patients who received bevacizumab and those with standard chemotherapy. Additionally there was no difference in overall survival, and only modest, albeit significant difference in PFS (8 vs 9.4 months, $p = 0.0023$). Given that the combination of bevacizumab to oxaliplatin leads to increased neurotoxicity [304], it is possible that many patients stopped treatment with bevacizumab in this trial earlier than actual tumour progression, losing the benefit of ongoing use of bevacizumab.

Single agent 5FU, whether as a bolus, infusion or oral formulation, has also been tried in combination with bevacizumab with similar improvements in RR and PFS in a large randomised trial [309] and two other smaller studies [13, 310]. The MAX study, which is a focal point of this thesis, was a multi-centre randomised phase II/III study that compared capecitabine monotherapy with capecitabine plus bevacizumab and capecitabine plus bevacizumab plus mitomycin C in patients with previously untreated metastatic colorectal cancer [309]. The primary endpoint was progression-free survival, which was superior in the bevacizumab containing arms. Overall survival was not statistically different between the three arms of the trial. Table 6 shows the outcomes from the randomised trials of bevacizumab.

Table 6: Randomised trials of bevacizumab in CRC

	Number of patients		Response Rate (%)		PFS (months)			OS (months)		
	Without Bev	With Bev	Without Bev	With Bev	Without Bev	With Bev	HR, p value	Without Bev	With Bev	HR, p value
CRC, first line										
MAX Capecitabine [311]	157	158	30	38	5.7	8.5	0.62, $p < .0001$	19.1	18.4	NS
AVF2107, IFL[21]	411	402	35	45*	6.2	10.1	0.54, $p < .001$	15.6	20.3	0.66, $p < .001$
N016966, XELOX/FOLFOX4 [305]	701	700	38	38	8.0	9.4	0.83, $p < .0023$	19.9	21.3	NS
CRC, second line										
E3200, FOLFOX4[291]	290	289	8.6	22.7*	4.7	7.3	0.61, $p < .0001$	10.8	12.9*	0.75, $p = .0011$

1.4.3 Preclinical models of innate and acquired resistance to bevacizumab

Given the multitude of possible mechanisms of bevacizumab action, from vessel growth, function, and direct tumour and immune functions, it is not surprising that there are multiple possible explanations for resistance to the drug. This resistance may be an innate property of the tumour, or acquired after initial response and then inevitable progression. Innate resistance may be due to pre-existing activation of resistance pathways through selective pressures during malignant progression. The resistance mechanisms may be concordant between the innate and acquired resistance, or they may be completely separate. In contrast to evidence surrounding the use of EGFR inhibitors, there is little clinical data regarding possible mechanisms of resistance. Furthermore, given the lack of single agent activity in colorectal cancer, the concept of resistance to bevacizumab is less straightforward than the definition and discussion of resistance to EGFR inhibition. To be precise, ‘resistance’ to bevacizumab implies a characteristic of a patient’s tumour that derives no improvement from the addition of bevacizumab to standard chemotherapy. Acquired resistance implies an initial benefit to the addition of bevacizumab followed by a change in vasculature to no longer be responsive to ongoing bevacizumab usage. This acquired resistance may occur prior to acquired resistance to chemotherapy, at the same time, or may not occur until much later.

Most information is inferred by knowledge about the mechanism of action of bevacizumab and animal and in vivo models across a range of different cancer cell lines. Several mechanisms of resistance are suggested by these laboratory models, including but not restricted to underlying tumour hypovascularity, alternate pre-existing multiple pro-angiogenic pathways that are redundant until therapy is introduced, recruitment of bone marrow derived CEPCs that reverse therapy induced changes in the vasculature, and possible changes to the tumour microenvironment that help sustain tumour angiogenesis and growth.

1.4.3.1 Hypovascularity

Some tumours such as pancreatic cancer, may be inherently hypovascular, and are characterised by large amounts of hypoxic, hypovascular stroma. Bevacizumab in animal models of pancreatic cancer [312], as well as in clinical trials, fails to add any

benefit to standard chemotherapy [276]. Other tumours may have metastases that grow by co-opting the vessels of the host organ, and do not depend on sprouting neo-vasculature. These metastases do not have proliferative endothelial cells, and therefore are likely to not respond to anti-angiogenic therapy, and are examples of innate resistance to angiogenic blockade [312].

1.4.3.2 Alternate angiogenic signalling pathways

An alternate explanation for resistance to anti-angiogenic therapy involves the switch to alternate pro-angiogenic pathways that are not blocked by bevacizumab. These alternate pathways may have pre-existing high importance in sustaining angiogenic growth, or they may be important only in the context of blockade of VEGF-A. For example, late stage breast cancer has higher levels of pro-angiogenic growth factors such as Fibroblast Growth Factor (FGF) 2[313]. This could lead to innate resistance, and may explain why late stage breast cancer has relative resistance to bevacizumab[314]. Xenografts that are treated with VEGFR2 antibodies that initially respond but then begin to grow again demonstrate elevated levels of pro-angiogenic FGF compared to non treated xenografts [292, 315]. Treatment with a FGF-Fc fusion protein abrogates the tumour growth[315]. Other models have implicated not only FGF2 but also Platelet Derived Growth Factor (PDGF)-A as up regulated pro-angiogenic factors which may lead to acquired resistance [316].

Treatment of xenograft models with anti-angiogenic therapy leads to dynamic changes within the tumour and vasculature pro-angiogenic factors become elevated within the tumours as well as recruitment of vascular progenitor cells [316]. Additionally, given that PlGF, VEGF-C and VEGF-D can interact with the VEGFR2, conceivably over abundance of such ligands could lead to innate resistance to treatment with bevacizumab. This has not previously been explored and serves as a hypothesis for resistance that is explored within this thesis.

Other alternate pathways that may be up regulated by the use of bevacizumab include inflammatory pathways, with the Stromal Derived Growth Factor 1 alpha (SDGF1 α), and its receptors as possible mediators of resistance. A report from Duda *et al* demonstrated up regulation of expression of this gene in rectal tumours after 12 days of bevacizumab monotherapy in patients in the study. The rectal cancer was biopsied

pre and post bevacizumab monotherapy, and gene expression measured. Levels of SDGF1 α correlated with the rate of distant metastases in this cohort of rectal cancer patients [317].

1.4.3.3 Stromal and endothelial changes

Functional changes within a tumour such as hypoxia can lead to recruitment of pro-angiogenic tumour associated macrophages [318], as well as CEPCs [319] and can lead to changes within pericytes, protecting the vasculature. The pro-angiogenic monocytes express various factors that promote vessel growth [320]. Signals to recruit the CEPCs are highest around the time of tumour progression and the CEPCs are recruited to propagate the vasculature [321]. Pericytes are required for normal vessel endothelial cell survival [322]: treatment leads to tightly associated pericytes, and vessels are more sensitive to VEGF therapy if pericytes are lacking [323]. The pericyte sheath around the endothelium reacts in a dynamic protective manner upon treatment with anti-angiogenic agents indicative of a reactive protective mechanism that may lead to resistance to the anti-angiogenic therapy [289, 324]. Supportive of this observation, is the report that dual targeting of the pericytes using a PDGF inhibitor and VEGF targeted therapy is more effective than VEGF directed therapy alone in mouse xenograft models [325]. The importance of the microenvironment on the effectiveness of bevacizumab may also hinge on changes in the normally abundant inflammatory myeloid CD11b+Gr1+ cells [326]. In a mouse xenograft model, resistance to bevacizumab was reported to be associated with infiltration of the tumour with these myeloid cells. Dual targeting of the myeloid cells along with bevacizumab was more effective than bevacizumab alone.

1.4.4 Biomarkers of response and resistance to bevacizumab

Disappointingly, despite the interest, and despite the large amount of data so far published, only one of the large randomised trials of bevacizumab in colorectal cancer has been analysed in an attempt to find predictors of efficacy of bevacizumab [281, 327, 328]. The MAX clinical trial had prospectively consented patients for use of their tissue blocks, and therefore is in a strong position to satisfy many of the criteria for investigating biomarkers of bevacizumab [309]. This thesis seeks to investigate the biologically plausible candidates of alternate VEGFR2 ligands such as VEGF-B,

VEGF-C, and VEGF-D, and correlate clinical outcome with biomarker expression from tumour samples obtained from patients on the MAX trial. The association of VEGF-A, VEGFR1, and VEGFR2 expression to clinical outcome is also assessed.

Much of the limited information that has been gathered so far comes from either the randomised trials of bevacizumab in other cancer types, and from the relatively few other small retrospective reports from the same research group, analysing a phase II trial lacking the power and control arm that will derive meaningful differentiation between prognostic and predictive factors [329-332]. A summary of this data is presented in Table 7.

Table 7: Possible biomarkers of bevacizumab

Biomarker	Reference and tumour type	Outcome	Comment
Serum Biomarkers	NSCLC [333]	Pretreatment VEGF-A levels prognostic, but not predictive for outcome	Only one trial showed pretreatment VEGF-A prognostic. Data limited by small trials and problems with ELISA assay. Meta-analysis supports only role as prognostic
Tissue biomarkers	CRC [281] Breast [334] CRC [335]	No correlation of VEGF-A, VEGFR2 or MVD with outcome.	Fairly conclusive lack of evidence for association. Large trials that were randomised were analysed for IHC expression: given no correlation unlikely in future to be used, as other analyses will be either relatively underpowered or single arm trials.
Circulating Endothelial Cells	Breast [280, 336]	Rectal cancer: pretreatment CECs correlate with pCR rate; Breast cancer: pretreatment CECs correlate with OS and PFS.	No consensus to immunophenotype of CECs and difficult logistics of detection. Only small single arm studies with no consistent signal. Not ready for use.
Imaging	Breast [337] [338]	Breast cancer trials: no significant correlation with Ktrans and OS/PFS but trend for decrease in Ktrans with treatment	Small single arm trials, non significant result. Not widely available.
Hypertension	Breast [328, 334]	E2100 trial: OS 38.7 vs 25.3 if G3-4 HT, $p = 0.002$	Promising biomarker shown in E2100 trial to correlate with OS, as well as smaller single arm studies. Perhaps best dynamic biomarker to date
Genetic	Breast [334] Ovarian [339]	E2100 trial: OS HR 0.58 in VEGF-2578 AA genotype, VEGF-1154 A also OS HR 0.62. Smaller ovarian cancer trial demonstrated reduced response rates with some IL8 SNPs but only single arm study	Link between SNPs for VEGF-And VEGF levels not yet conclusive; Although similar results from a variety of trials, not useful currently as uncertainty about exact SNPs and difficulty in routine clinical measurement.

1.4.4.1 Tissue biomarkers

Given the availability of tumour specimens, tumour biomarkers detected via immunohistochemistry from tumour specimens are attractive to investigate as biomarkers of bevacizumab. Previous work has suggested that markers of intratumoural hypoxia correlate with prognosis in colorectal cancer. These include intratumoural levels of VEGF-A and LDH [340, 341], Hypoxia inducible factor (HIF)-1 α [342], and microvascular density [343]. However analysis of a large randomised trial in colorectal cancer failed to demonstrate concordance of MVD with the predictive value of bevacizumab [281].

Although one trial did report a trend for patients with high VEGF-A expression to have superior outcomes with bevacizumab treatment in colorectal cancer [335] this was not seen in two other large studies [281, 334]. A smaller trial of bevacizumab in ovarian cancer suggested that MMP-9 and HIF 1 α expression was associated with a longer PFS, but the trial was too small and non randomised to be powered to draw conclusions from such trends [344]. Other groups have investigated the association of VEGFR2 expression and not found an association with bevacizumab efficacy [334, 335]. Recently the validity of VEGFR2 staining of tumour cells has been questioned, given a recent study demonstrating only endothelial cell expression [296].

To date there have been no studies of in-situ biomarkers to demonstrate correlation with bevacizumab. This may be because of the different expression of angiogenic ligands between early primary tumours and metastatic deposits. Most studies have then correlated primary tumour specimen expression with outcomes in metastatic disease. Not only may there be differences between the two sites in expression of different pro-angiogenic factors, there may be differences in each specimen on the expression level of pro-angiogenic factors [345].

Surprisingly, a review of the literature revealed no studies that evaluated other potential ligands of VEGFR2, such as VEGF B-D. Given the previously described importance of the VEGF-C and VEGF-D ligands for angiogenesis [265-267], this thesis investigated expression of these ligands with bevacizumab efficacy.

1.4.4.2 Circulating angiogenic factors

Given their role as ligands in the VEGFR signalling pathway, circulating angiogenic factors (CAFs) such as VEGF-A have been naturally explored for an association with bevacizumab outcomes. Prior work had reported that pretreatment serum levels of angiogenic factors may be prognostic for survival in patients with a variety of different cancers [346-349]. In particular, low levels of VEGF-A correlate with good prognosis in a lung, colorectal, and breast cancer [346-353]. A similar observation has been made correlating levels of serum VEGF-C and VEGF-D with prognosis [354, 355].

Investigators have examined both pretreatment levels and the change in levels in VEGF-A as a predictive biomarker to bevacizumab. The results are inconsistent and inconclusive, possibly due to differences in definition and differences in technology behind detection of VEGF-A[16].

The amount of active free unbound VEGF-A decreases in response to treatment with bevacizumab, in contrast to total levels of VEGF-A which increases [344, 356-359]. This is possibly because VEGF-A is secreted in response to bevacizumab treatment as part of a feedback loop, but the majority of this becomes bound to bevacizumab and thus inactive [16]. Some studies have reported that VEGF-A levels have increased in response to bevacizumab [360, 361], but these have not used techniques of measurement that are able to distinguish between free and bound VEGF-A. Future studies should be consistent in their measurement of VEGF-A, possibly using immunodepleted plasma, which is able to remove all immunoglobulins and separates the VEGF-A bound to bevacizumab from free VEGF-A [358]. Additionally, measurement of these angiogenic factors is also confounded by the fact that platelets have these factors in them, which could be released and contribute to total VEGF-A levels [362].

The only randomised trial to address the importance of serum levels of VEGF-A is the E4599 trial in NSCLC, which reported a significant correlation between pre treatment VEGF-A levels and response to bevacizumab [333]. This study also demonstrated that although low baseline VEGF-A levels were prognostic for PFS, it was not predictive

for change in PFS upon treatment with bevacizumab. Patients had an increase in VEGF-A upon treatment as their assay did not discriminate between free and unbound levels of VEGF-A, and they did not comment on the magnitude of the change and the predictive benefit. Most recently, a meta-analysis of data from several randomised trials, including E4599, which included a renal cell cancer trial, concluded that serum levels of VEGF-A were prognostic, but not a predictive biomarker for bevacizumab [363].

Smaller studies have examined a multitude of other circulating biomarkers including PlGF, IL-6 [280], FGF 2 [359] and IL-8, VEGFR2 and MMP9 [364] and observed treatment induced changes in each, but all have been too small and non randomised to generate conclusions regarding biomarker utility.

1.4.4.3 Circulating endothelial cells

Circulating endothelial cells (CECs) either arise from vessel walls or in an immature form from the bone marrow as Circulating Endothelial Precursor Cells (CEPCs). They are thought to play a role in the progressive development of tumour vasculature. Many authors have sought to use CECs or CEPCs as a biomarker for drugs that interfere with angiogenesis. Although currently authors have proposed CD45- CD31+ CD146+ as the most specific markers for CECs/CEPCs [365], the field is compromised by a lack of consensus regarding these markers and the logistical difficulties in the timely access of the costly equipment required to sort these cells. Furthermore no study has had large enough numbers or contained a control arm to either have the power or the design to identify predictive biomarkers.

There are also different techniques available to identify CECs/CEPCs, including magnetic bead selection [366], immunophenotyping using flow cytometry [365] or PCR for CD146 mRNA [367]. Given these problems it is not surprising that no large randomised trial has reported CECs/CEPCs as possible biomarkers. A few single arm studies however have utilised these markers, but they have not to date shown results that would encourage further take up and exploration of these markers[368]. These studies include a small trial in rectal cancer [280] where pretreatment CECs correlated with pathological response rates to radiotherapy and bevacizumab and one trial in breast cancer [336], where pretreatment CECs correlated with OS and PFS.

Given the lack of consensus regarding detection and lack of data from randomised trials, CECs/CEPCs are not recommended as biomarkers for bevacizumab efficacy.

1.4.4.4 Genetic biomarkers

A large research effort has focused on the inherited genetic variability in the form of Single Nucleotide Polymorphisms (SNPs) in VEGF-A and its receptor VEGFR2 and their association with bevacizumab efficacy. The SNPs occur both in promoter and untranslated regions of the genes in up to 50% of the population [369]. There may be a direct link between SNPs in the VEGF promoter and levels of VEGF expression [370, 371] and conceivably these SNPs may alter the angiogenic environment that surrounds a tumour by leading to low levels of VEGF, and therefore no benefit from the addition of bevacizumab. Although there have been promising associations between SNPs and outcome with bevacizumab treatment amongst a range of trials, the SNP location has been inconsistent and the biological importance questioned [372].

The largest trial to examine SNPs was the E2100 breast cancer trial where the experimental arm were treated with paclitaxel and bevacizumab, and the control arm with paclitaxel alone [334], which demonstrated that VEGF-2578 AA and VEGF-1154 A SNPS were predictive for better OS in the bevacizumab treatment arm, but were not prognostic in the paclitaxel control arm. Furthermore the SNPs were not predictive for PFS or RR, raising doubts about their validity.

Other studies have also failed to demonstrate the associations of these particular SNPs, instead generating a list of alternate associated changes. These include VEGF-A G634C, ICAM1 T469C from the ECOG 4599 analysis of bevacizumab in lung cancer [373], and VEGFR1 SNPs from the AVITA trial of bevacizumab in pancreatic cancer [372]. SNPs in IL-8, a cytokine implicated in angiogenesis, also possibly play a role. In a single arm trial of patients with ovarian cancer treated with bevacizumab and cyclophosphamide with IL-8 T-251A A/A or A/T genotypes had lower response rates than those with T/T genotypes [339]. Further work is needed to better define the role of polymorphisms in VEGF function and bevacizumab efficacy.

Other molecular markers of interest are *KRAS* and *BRAF* mutations. As discussed earlier, currently *BRAF* mutations, but not *KRAS* mutations, are considered prognostic in colon cancer [15, 97, 100-102, 107, 374, 375]. There is also interplay between the VEGF and EGF pathways of biological significance [376, 377]. Conceivably mutation of either *KRAS* or *BRAF* and constitutive activation could lead to independence from VEGF mediated activation of the MAPK pathway in tumour cells, and relative resistance to bevacizumab. Despite associations with resistance to EGFR targeted therapy, *KRAS* and *BRAF* are not predictive biomarkers for bevacizumab according to the analysis of the pivotal Hurwitz colon cancer trial [327, 378]. This thesis will attempt to provide confirmatory data on these mutations and their relationship to bevacizumab efficacy.

1.4.4.5 Imaging biomarkers

Given the multiple radiological imaging procedures cancer patients undertake to assess the stage of their cancer, it is not surprising that many researchers have sought to use baseline and follow up imaging as biomarkers for anti-angiogenic therapy. The three possible modalities that could undertake this role are Dynamic Contrast Enhanced MRI (DCE-MRI), Dynamic CT, and Positron Emission Tomography (PET). Although these studies have been performed for anti-angiogenic TKIs, no large clinical trials have examined these modalities as biomarkers of bevacizumab efficacy [372].

DCE-MRI is able to measure factors such as K^{trans} which measures the transfer of contrast agent between blood plasma and the extravascular extracellular space, and is dependent on vascular permeability and endothelial surface area, and may change with drugs targeting angiogenesis. However only two small breast cancer trials have used this approach and were unable to demonstrate any significant correlation [337] [338]. Conceivably dynamic CT in the future may provide similar information as DCE-MRI but this is uncertain.

1.4.4.6 Hypertension as a biomarker

Hypertension is a common side effect of patients taking VEGF inhibitors, possibly due to dysregulation of the vasodilator nitric oxide, thus causing vasoconstriction and hypertension [256]. Many trials have now suggested that the dynamic changes in

blood pressure may be a biomarker for benefit of bevacizumab, as well as other anti-angiogenic agents. These include the 39 patient colon cancer trial of fluorouracil, irinotecan and bevacizumab with the PFS (14.5 vs 3.1 months) and RR improved in those patients with grade 2 – 3 hypertension [328]. This study was a single arm retrospective study so is unable to separate prognostic from predictive value of the hypertension, but similar significance was seen in the large randomised E2100 trial in metastatic breast cancer where patients who had grade 3 – 4 hypertension lived significantly longer (OS 38.7 vs 25.3 months) [334]. This effect was also seen in a retrospective study of axitinib, an oral VEGF inhibitor [379]. However different analyses have used different definitions of bevacizumab induced hypertension. Furthermore, a retrospective analysis of six trials in colorectal, breast and renal-cell carcinoma showed that treatment induced hypertension did not have any correlation with clinical benefit from bevacizumab [380]. Further work is required to assess the link between hypertension and outcome with bevacizumab.

1.4.5 Summary

Bevacizumab is an active drug in advanced colorectal cancer, extending median PFS in numerous studies. However the benefit in extending median OS time is inconsistent amongst published trials. Combined with the cost of the drug, a better use of bevacizumab may be amongst a population characterised by a biomarker predicting better efficacy.

A review of the literature reveals that despite numerous studies, there are currently no predictive biomarkers of bevacizumab response or resistance. This includes circulating angiogenic factors, circulating pro-angiogenic cells, tissue expression of VEGF-A, and VEGF/VEGFR1 SNPs. This may be due to the smaller numbers of studies, the small number of patients in each retrospective study leading to lack of power to detect a biomarker, the complexity of the angiogenic signalling pathway, or due to failure to identify and test for the appropriate biomarker. Although tissue expression of VEGF-A, VEGFR1 and VEGFR2 had been assessed as biomarkers, no study had addressed whether alternate ligands for VEGFR2 such as VEGF-B, VEGF-C, or VEGF-D were associated with bevacizumab efficacy.

Although the angiogenic pathway is complex, involving tumour cells, endothelial cells and the stroma, the process is primarily driven by ligand/receptor interaction. Bevacizumab is a monoclonal antibody to the dominant pro-angiogenic signalling ligand VEGF-A, and reduces activation of the VEGFR2 receptor. Several other potential ligands can interact with receptor. Most signalling systems in cells have alternate pathways that may compensate if their primary ligand is reduced or altered. It is possible, that in addition to the interaction between VEGF-A and VEGFR2, VEGF-B, VEGF-C, or VEGF-D can bind to the receptor, and cause angiogenic pathway activation in the absence of bevacizumab mediated decrease in VEGF-A. Patients with high expression levels of these alternate ligands could have ongoing pro-angiogenic signalling despite bevacizumab use.

As part of this thesis, it is hypothesised that tissue expression of VEGF-B, VEGF-C or VEGF-D may be predictive for resistance to bevacizumab in patients recruited to the MAX clinical trial. The predictive and prognostic ability of *KRAS* and *BRAF* were also assessed in this patient population.

2 MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

2.1.1 Drugs

Cetuximab was obtained from Merck (Australia), in a 5mg/ml solution in saline, and stored at 4°C. Erlotinib (E4007, LC Laboratories, USA) was obtained as a powder and dissolved in Dimethyl Sulfoxide (DMSO)(D5879, Sigma-Aldrich, Australia) to form a 5mg/ml stock. Dissolved erlotinib was stored at -20°C.

Several protocols required treatment of human cancer cell lines with either single agent cetuximab, erlotinib or both, with a comparison to an untreated control condition. For these experiments, the drugs were diluted into growth media at the appropriate concentration. Each treatment condition in those experiments received the same concentration of drug vehicle with the appropriate concentration of saline and DMSO.

We chose a dose of 0.1µg/ml of cetuximab and erlotinib in the majority of experiments as it was below the serum levels achievable *in vitro*, and was approximately the IC₅₀ for the LIM1215 cells used in the bulk of our *in vivo* work.

2.2 CELL CULTURE

2.2.1 Cell lines

The colon cancer cell lines LIM1215, HCA7, DiFi, Caco2, HCT-116, HCT-15, A431 and RKO were maintained in Dulbecco's Modified Eagle Medium DMEM F12 1:1 (11320, Gibco Invitrogen, USA), supplemented with 10% fetal calf serum (FCS)(10101-145, Gibco Invitrogen), 5 mM L-glutamine, penicillin-streptomycin (10,000 U/ml and 10 mg/ml, respectively), at 37°C with 5% CO₂. Cell lines were obtained from the Ludwig Institute for Cancer Research, Melbourne - Austin Branch (LIM1215), MD Anderson Cancer Centre (DiFi), and from the American Type Culture Collection.

2.2.2 Maintenance and passaging of cell lines

Cell lines were cultured in media until 80% confluent in T75 flasks (BD Biosciences, USA). Cells were passaged by washing with Dulbecco's Phosphate Buffered Saline (PBS)(14190, Gibco Invitrogen) then incubated with 5ml of TrypLE Express Trypsin replacement (12604, Gibco – Invitrogen) at 37°C until dissociated. A further 5ml of media was then added to the cells, and the cells passaged at a ratio of 1:5 or 1:10, dependent on the cell type and the time that a particular confluence was required. Cell lines were maintained in culture for a period of up to 12 weeks before being discarded.

2.2.3 Long term storage of cell lines

Cells were stored in 2ml cryogenic vials (Corning) in the Ludwig Institute for Cancer Research's liquid nitrogen storage facility at Austin Health. Freezing solution was 10% DMSO (D5879, Sigma-Aldrich), 90% FCS in media. 1ml aliquots of the cells were placed at -20°C for 1 hour, then -80°C overnight, before finally being stored in liquid nitrogen.

To thaw cells, cells were removed from liquid nitrogen and thawed at 37°C. Immediately once thawed, cells were resuspended in 10ml of media, and then centrifuged at 300g for 5 minutes at 4°C to pellet the cells and remove the DMSO. The cells were then resuspended in medium and transferred to a new T75 flask. The medium was changed the following day.

2.2.4 Mycoplasma testing

Cell lines were tested for mycoplasma at four monthly intervals. Media from the cell lines growing in T75 flasks was tested using the Mycoalert Mycoplasma Detection Assay (Cambrex, USA) by Tracy Cardwell.

2.3 CELL GROWTH AND VIABILITY ASSAYS

2.3.1 Cell count

Cell counts were performed by dissociation of cells from their flasks or growth plates using TrypLE Express Trypsin Replacement and subsequent mixing in growth medium to avoid clumping. Cell counts were determined using either a Neubauer haemocytometer or a Beckman Coulter Z2 Cell and Particle Counter (Beckman Coulter, USA).

When using the Coulter Counter to perform cell counts, 100µl of cell suspension was resuspended in 10ml of Normal Saline. The cell suspension was mixed thoroughly prior to analysis by the Coulter counter. The count excluded cells less than 5µm and larger than 30µm in size. The count was performed in triplicates. Quality control was performed at monthly intervals comparing Coulter counter cell counts to haemocytometer cell counts.

2.3.2 Cell viability assay

Cell viability was assessed using the MTS colourimetric viability assay with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) as a substrate (G1112, Promega, Australia). Preparation for the assay required a 2mg/ml mixture of MTS reagent powder with PBS. The pH of the solution was adjusted to between 6.0 and 6.5 using 1M HCL, and the solution filtered using a 0.2µm Acrodisc filter (PN 4612, Pall Corporation, USA) into a sterile, light protected container. The solution was kept at -20C. A 0.92mg/ml solution of Phenazine Methosulfate (PMS)(9625, Sigma-Aldrich) was also prepared by dissolving PMS in PBS. PMS was stored at -20C.

Optimum seeding density to achieve 70 – 80% confluence was assessed in each cell line after 4 days of growth in 100µl of growth media in 96 well plates. Cells were seeded into 96 well plates at the optimum cell number and cultured at 37°C overnight. An additional 100µl of growth media was placed in quadruplicate without any cells.

After 24 hours growth for each cell line, one plate was used for determination of time = 0 (t_0) absorbance values. At this time point, 20 μ l of a 20:1 ratio thawed MTS/PMS solution was added to each well of the $t = 0$ plate and incubated at 37°C for a further 90 minutes. Absorbance was assessed at 490nm using a VersaMax Microplate Reader and Softmax Pro 4.8 software (Molecular Devices, USA). Absorbance at 630nm was also determined as background, and the value subtracted from the 490nm reading. The absorbance of growth medium without cells was further subtracted from this value for the t_0 absorbance value.

At this same time point, media was removed from the alternate 96 well plate and cells were treated in quadruplicate with multiple drug conditions, and the absorbance assessed at 72 hours as above. The relative rate of cell growth for each cell line was factored into the analysis by subtracting the absorbance at t_0 from both the control and treatment groups. All the experiments were repeated on at least 3 independent occasions.

2.3.3 Statistical consideration of synergy and additivity

Calculusyn software (Biosoft, UK), was used to calculate the Combination Index (CoI), as a measure of synergy [381]. The use of the Chou and Talalay Combination Index optimally requires a dose-effect curve which reaches the zero value for survival, which was not strictly the case for each of the cell lines studied. To strengthen our analysis we performed a second calculation of synergy, in accordance with other similar work [198], where we supplemented the Combination Index with the ratio R, where:

$$R = \frac{\%Survival[Combination]}{\%Survival[cetuximab] \times \%Survival[erlotinib]}$$

Where $R < 0.8$ = synergistic, $R 0.8 - 1.2$ = additive, $R > 1.2$ = antagonistic.

2.4 CREATION OF CETUXIMAB RESISTANT CELL LINES

2.4.1 Treatment and passage of cell lines

LIM1215 cancer cells were grown in T25 flasks in growth medium with drug vehicle or cetuximab and serially passaged every 7 days. Two separate flasks of LIM1215 were grown in cetuximab, and designated as LIM1215 Cetuximab Resistant A (LIM1215 CR-A), and LIM1215 CR-B. The cells were initially exposed to cetuximab at a dose of 0.1ug/ml. The dose was doubled each week until a ceiling dose of 100ug/ml was reached after three months. At this point, LIM1215 CR-A and LIM1215 CR-B were maintained in 100µg/ml cetuximab for 12 weeks then frozen down. In parallel to LIM1215 CR-A and LIM1215 CR-B, LIM1215 cells were grown and passaged in growth medium and equivalent volume of drug vehicle to the resistant lines, and was designated LIM1215 CONTROL.

2.4.2 Assessment of resistance

Resistance to cetuximab of the LIM1215 CR-A and LIM1215 CR-B lines was determined with the use of an MTS viability assay as previously described and compared to the LIM1215 CONTROL cell lines

2.4.3 Reversal of resistance

Once the LIM1215 CR-A cell line was demonstrated to be resistant to cetuximab, the effect of growth in the absence of cetuximab was assessed. An aliquot of LIM1215 CR-A cells was taken and placed in a separate T25 flask, and passaged in growth medium with no cetuximab for a period of 3 months. The resistance to cetuximab of these cells was assessed by MTS assay after 1 week, 1 month and 2 months. The cell line was designated LIM1215 Cetuximab Resistant A – Normal median (LIM1215 CR-A-N).

2.5 FLOW CYTOMETRIC ANALYSIS

2.5.1 Assessment of cell cycle distribution using propidium iodide (PI)

Human cancer cell lines were treated for 24 hours in 12 well plates. Cells were harvested by trypsinisation and pelleted by centrifugation at 300g for 3 minutes at 4°C. Cell pellets were resuspended with 400µl of 50µg/ml propidium iodide (PI) diluted in citrate/Triton buffer (0.1% Sodium citrate, 0.1% Triton X-100) (Sigma-Aldrich Corporation, USA) and incubated at 4°C overnight in the dark. PI-generated fluorescence was analysed for each sample using a BD FACS Canto II flow cytometer (BD Biosciences, USA). A total of 10,000 cells (events) were analysed for each sample. Viable cells were analysed by appropriate gating of forward and side scatter. Aggregates and cell debris were excluded from analysis by gating. The percentage of cells in G₀-G₁, G₂-M and S phases were computed using the ModFit Program (Verity Software House, Topsham, ME, USA).

2.5.2 Assessment of apoptosis using Annexin V

Human cancer cell lines were seeded, treated and harvested as described before. Treatment was for 24, 48 or 72 hours. At the completion of the treatment period, 100,000 cells were transferred to ependorf tubes, and centrifuged at 300g for 5 minutes. Supernatant was discarded, and cells resuspended in 1µl of Annexin V Alexa Fluor 488 conjugate (A13201, Invitrogen) and 19µl of Annexin V binding buffer. Annexin V binding buffer consisted of 10mM HEPES, 140mM NaCl and 2.5mM CaCO₂ at pH 7.4. Cells were incubated in the dark at room temperature for 15 minutes. Cell suspensions were then counterstained with PI to achieve a final concentration of 1µg/ml PI before immediate analysis using a BD FACS Canto II with BD FACS Diva software (BD Bioscience). Using appropriate gating and compensation of Fluorescein isothiocyanate (FITC) and PE channels, cells were defined to be apoptotic if Annexin V⁺/PI⁺. Cells were treated in triplicate and the experiment repeated on at least 3 independent occasions.

2.5.3 Assessment of cell surface EGFR expression using cetuximab

Human cancer cell lines were seeded and treated for 24 hours. Cells were pelleted by centrifugation at 300g for 3 minutes. All steps were carried out on ice. Cells were washed in 200µl PBS twice prior to incubation in 50µl of 10µg/ml of cetuximab in 1% human serum albumin (HSA) and PBS. Cells were incubated at 4°C for 1 hour, prior to two washes in 200µl PBS. Cells were then resuspended in 5µg/ml of anti-human IgG Phycoerythrin antibody (P8047, Sigma-Aldrich) in 1% HSA and PBS and incubated at 4°C for 30 minutes. Cells were then washed in 200µl PBS and resuspended in 150µl of FACS-Fix, consisting of 16g of D-Glucose, 12.5ml of 40% formaldehyde in 500ml of PBS. Cells were treated in triplicate and the experiment repeated on at least 3 independent occasions.

Samples were placed on ice, then sorted immediately using the Guava EasyCyte Flow Cytometry System (Millipore, USA), with staining intensity computed using FlowJo Software (TreeStar, USA).

2.6 WESTERN BLOT ANALYSIS

2.6.1 Cell treatment and protein extraction

Human cancer cells grown in 6 well plates were treated as described previously. For determination of levels of intracellular phosphorylated proteins, experiments were conducted in growth medium supplemented with 0.1% FCS. Epidermal Growth Factor (EGF) stimulation of the cells took place for 15 minutes at the end of the time course at a concentration between 40 – 100ng/ml dependent on the cell line.

After treatment, cells were lysed in cell lysis buffer, which consisted of 50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 1% NP-40 IGEPAL), 5% sodium deoxycholate, 1 mM EDTA (pH 8), Complete Protease Inhibitor Cocktail tablet (Roche, Switzerland), and PhosStop Phosphatase Inhibitor Cocktail tablets (Roche). Lysed cells were sonicated for 10 minutes in ice cold water prior to centrifuging for 15 minutes at 17,000g. Supernatant was transferred to a new epindorf for determination of protein concentration and use. Samples were suspended in 1X NuPage Sample Reducing Agent (NP0004, Invitrogen) combined with 1X NuPage LDS Sample Buffer

(NP0007, Invitrogen) before the protein samples were denatured at 95°C for 5 minutes. All experiments were repeated on at least 3 separate occasions.

In experiments designed to assess intracellular phosphorylation of proteins, after treatment, media was removed and cells were lysed directly in their wells with 200µl of 1X NuPage Sample Reducing Agent combined with 1X NuPage LDS Sample Buffer in H₂O. Lysed cells were sonicated for 10 minutes in ice cold water and protein samples denatured at 95°C for 5 minutes. All experiments were repeated on at least 3 separate occasions.

2.6.1.1 Protein quantification using a modified Bradford Assay

Bradford assays were performed to determine protein concentrations using the Bradford Reagent (Bio-rad Laboratories, USA). Absorbance was determined at 595nm using a VERSA_{MAX} Microplate reader (Molecular Devices Corporation, USA). Protein concentrations were determined using a standard curve generated from known concentrations of bovine serum albumin as previously described [382].

2.6.2 Western blot

Equivalent amounts of protein were separated on polyacrylamide NuPage Novex 4-12% Bis-Tris gels (NP0336BOX, Invitrogen) using electrophoresis and transferred to a nitrocellulose membrane using dry blotting with an iBlot Dry Blotting System (Invitrogen). Membranes were blocked with 5% non fat dry milk with 0.1% Tween-20 in Tris Buffered Saline before probing with relevant primary and secondary antibodies. Blots were then washed 3 times for 10 minutes each with TBS/T to remove non bound secondary antibody. Signal detection was performed using luminol-based detection of horseradish peroxidase after probing the membrane with Amersham ECL Western Blotting Detection Reagents (RPN2132, GE Healthcare, USA) on a chemifluorescence imaging system Storm 840 (GE Healthcare) and analysed with ImageQuant software (GE Healthcare). Blots were re-probed with anti-actin (Sigma, 1:10000, Mouse) to control for equal protein loading. ImageQuant software was used to analyse the pixel density of protein bands separated on

membranes. The intensity of each band was then divided by the intensity of the actin band for each sample.

2.6.3 Antibodies

Antibodies used are shown in Table 8.

Table 8: Antibodies used in Western blotting

Antibody	Source	Dilution	Condition	Supplier
tEGFR	Rabbit	1:1000	4°C overnight	D38B1, Cell Signaling,
pEGFR 1173	Rabbit	1:1000	4°C overnight	44-794G Invitrogen
pMAPK	Rabbit	1:1000	4°C overnight	44-680G; Invitrogen
pAKT	Rabbit	1:500	4°C overnight	193H12 Cell Signaling
Actin	Mouse	1:10000	20°C, 1 hour	Sigma
Anti-rabbit IgG HRP linked Antibody	Rabbit	1:2000	20°C, 1 hour	7074 Cell Signaling
Anti-mouse IgG HRP linked Antibody	Mouse	1:2000	20°C, 1 hour	7076 Cell Signaling

All antibodies were stored at -20°C.

2.6.4 Human phospho-kinase and receptor-kinase antibody arrays

The Human Phospho-Kinase Array kit (ARY003, R&D Systems, USA) was used to detect phospho-protein changes in LIM1215 cancer cells treated with cetuximab, erlotinib or the combination. LIM1215 were grown overnight in growth medium supplemented with 0.1% FCS in 6 well plates, and then treated as described previously for 2 hours prior to 15 minutes of stimulation with 100ng/ml EGF. Cells were lysed in accordance with instructions with lysis buffer, and the protein concentration determined with the Bradford assay as described previously.

The Human Phospho-Receptor Tyrosine Kinase Array Kit (ARY001, R&D Systems) was used to assess phospho-protein changes in LIM1215 CONTROL, LIM1215 CR-A, LIM1215 CR-B and LIM1215 CR-A-N cells. Cancer cell lines were grown in growth medium supplemented with 10% FCS in individual 6 well plates for 24 hours

before cell lysis in accordance with the manufacturer's instructions and the protein concentration determined with the Bradford assay as described previously.

For both arrays, at separate instances, the respective array membranes were exposed to lysed protein following the manufacturer's instructions, and following the addition of the provided HRP antibody. Signal detection was performed using the chemifluorescence imaging system Storm 840 (GE Healthcare). Relative protein concentrations of human phospho-kinases were calculated using pixel density analysed with ImageQuant software (GE Healthcare).

2.7 DNA AND RNA TECHNIQUES

2.7.1 RNA extraction

Following completion of experimental time points, medium was removed and cells were washed with 1 x PBS, and harvested by trypsinisation. Cells were pelleted by centrifugation at 300 x *g* for 3 minutes at 4°C. RNA was extracted from a suspension of approximately 1.0×10^6 cells using the High Pure RNA Isolation Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. Briefly, cells were treated with Lysis buffer, vortexed, and transferred to the upper reservoir of the High Pure Filter Tube. Tubes were centrifuged for 15 seconds at 8000 x *g* at room temperature. The filter contents were then incubated with DNase (provided by the supplier) for 15 minutes at room temperature. The filter tube was then washed, and RNA eluted in elution buffer. Extracted RNA was stored at -80°C for long-term storage.

2.7.2 cDNA synthesis

cDNA was generated using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostic, Germany) according to the manufacturer's instructions. Briefly, anchored-oligo(dT)₁₈ primers (50pmol/μl) were annealed to 1μg of mRNA, and mRNA was denatured by incubation at 65°C for 10 minutes. cDNA synthesis was then carried out at 48°C for 60 minutes using dNTP's, reverse transcriptase buffer, and the reverse transcriptase enzyme provided. Reverse transcriptase was

subsequently inactivated by heating at 85°C for 5 minutes, and cDNA products were diluted 1:10 for use in subsequent real-time PCR experiments.

2.7.3 Purification of genomic DNA

Genomic DNA was extracted from LIM1215 CONTROL, LIM1215 CR-A, LIM1215 CR-B using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Briefly, cells were resuspended in proteinase K and buffer before mixing with ethanol and centrifugation at room temperature using the supplied spin columns.

2.7.4 Determination of DNA/RNA concentration

Spectrophotometric analyses with the NanoDrop ND-1000 (Thermo Scientific, USA) were performed on extracted RNA samples to determine their concentration and purity. RNA purity was assessed by the ratio of 260nm/280nm. RNA samples of high purity (260nm/280nm > 1.8) were used in subsequent experiments.

2.7.5 Primer Design

Primers for real-time PCR and sequencing were designed utilising a web-based Primer3 tool designed by Steve Rozan and Helen Skaletsky (<http://Frodo.wi.mit.edu/primer3/>). All primers were between 18 and 27 bases in length, with a GC content of 45 – 55%. Maximum 5' and 3' self complementarity of the primers was set at 3 and 1 respectively. For real-time PCR, PCR products were restricted to <200bp. All primers were obtained from Sigma-Aldrich, and are shown below in Table 9.

Table 9: Primers used for cDNA expression

Primer name	Sequence
FOS F	CAAGCGGAGACAGACCACT
FOS R	ATCAAGGGAAGCCACAGACA
JUN F	TGACTGCAAAGATGGAAACG
JUN R	TGAGGAGGTCCGAGTTCTTG
EGR1 F	ATCCCCGACTACCTGTTTCC
EGR1 R	GTTTGATGAGCTGGGACTGG
GAPDH F	ATGGAAATCCCATCACCATCTT
GAPDH R	CGCCCCACTTGATTTTGG
REG IV F	GAAGACAGAAGGAAGAACTCAGG
REG IV R	CAATGGAGAGAGGGCAGAAG
KRAS F	TTATAAGGCCTGCTGAAAATGACTGAA
KRAS R	TGAATTAGCTGTATCGTCAAGGCACT

2.7.6 Real-time PCR

All real-time PCR reactions were performed using the 7500 Fast Real Time PCR System (Applied Biosystems, USA) and analysed using the 7500 System software, version 2.0.3. Quantitative assessment of specific mRNA was performed using primers designed for the gene in question. All RT-PCR amplification results were normalised against an endogenous control, GAPDH. Each reaction consisted of a total volume of 10µL: 1µL of forward and 1µL reverse primer at 500nM concentrations, 5µL of 2 x SBYR Green PCR Master Mix (Roche Diagnostics, Germany), and 3µL of cDNA.

An initial denaturing step was performed (95°C for 10 minutes) following which 40 amplification cycles were performed for each sample, using a 2-step cycling protocol. The threshold for each amplification reaction was set at approximately 0.1, and the threshold cycle (C_T) value was recorded for each sample. mRNA expression levels were assessed using the ΔC_T method by the subtraction of GAPDH expression.

2.7.7 High Resolution Melt curve analysis for *KRAS* mutation

High Resolution Melt (HRM) curve analysis was undertaken for *KRAS* mutations for the genomic DNA extracted from LIM1215 CONTROL, LIM1215 CR-A and LIM1215 CR-B. Control cell lines were used as positive controls: SW480 DNA for G12V *KRAS* mutations, and HCT 116 DNA for G13D *KRAS* mutations. Appropriate amounts of forward and reverse *KRAS* primer was used in association with 19.5ng of DNA per reaction and the reactions were performed in duplicate PCR thermocycling. HRM analysis was performed on the Roche LightCycler 480 (Roche, Switzerland).

2.7.8 Whole human genome gene expression array

LIM1215 CONTROL, LIM1215 CR-A and LIM1215 CR-A-N were grown in T150 flasks overnight in growth medium and 10% FCS. LIM1215 CONTROL, LIM1215 CR-A and LIM1215 CR-A-N were grown in T150 flasks overnight in medium supplemented with 10% FCS. After 24 hours growth, RNA was extracted from each using the Roche High Pure RNA Extraction kit as previously described. Each RNA sample was labeled using the Affymetrix 3'IVT Express labeling kit (Affymetrix, USA) and hybridized to a Gene Chip U133 Human Genome U133 Plus 2.0 Expression Array (Affymetrix), washed, and scanned according to the manufacturer's instructions by Tim Holloway at Peter MacCallum Cancer Institute. The three resulting cell files were RMA normalized using Affymetrix software and R statistical software (open source), and fold differences in expression between the cell lines computed using log₂ intensities. Probe sets showing log₂ expression fold differences >1 or <-1 were used to assess functional group enrichment in 186 functional categories (assigned based on Gene Ontology annotation), using the binomial distribution test for each category by Georgia Corner.

2.8 REPORTER CONSTRUCTS AND BACTERIAL TECHNIQUES

2.8.1 Reporter constructs

The Apre-Luc reporter (4XAPRE-luciferase) was obtained from Tracy Putockzi, Ludwig Institute for Cancer Research, Parkville. This reporter, containing four repeats

of APRE (IL-6 response element of the rat α -macroglobulin promoter), was constructed by sub-cloning two repeats of the oligonucleotides (5'-TCGACATCCTTCTGGGAATTCTGATCCTTCTGGGAATTCTGGGTAC-3') in front of the minimal *junB* promoter–luciferase gene as previously described [383, 384]. The pRL-TK vector (TK-*Renilla* plasmid)(Promega, USA) was used as an internal control vector to co-transfect cell lines.

2.8.1.1 Transformation

One Shot TOP10 Chemically Competent *E.Coli* (C4040-10, Invitrogen) were used for transformation work. 1 μ l of plasmid DNA was added to 50 μ l of the thawed *E.Coli* and mixed slowly before incubating on ice for 5 minutes. The cells were heat shocked for 30 seconds in a 42°C water bath before further incubation on ice for 2 minutes. Cells were then incubated at 37°C for 1 hour with shaking in 150 μ l of SOC media to induce bacterial growth. 100 μ l of the culture was then spread onto an agar plate containing 100 μ g/ml ampicillin, and incubated at 37°C overnight. The following day, single colonies were picked and inoculated in 5ml of liquid broth (LB) with 100 μ g/ml ampicillin at 37°C overnight with shaking. For long term storage, cultures were frozen at -80°C in LB containing 15% glycerol and 100 μ g/ml ampicillin.

2.8.1.2 Miniprep of plasmid DNA

The QIAprep Miniprep kit (Qiagen, Netherlands) was used for the miniprep of plasmid DNA following manufacturer's instructions. Plasmid DNA was eluted in 50 μ l of buffer EB [10mM Tris-Cl, pH 8.5] provided by the kit.

2.8.1.3 Restriction enzyme digestion of plasmids

Plasmids were digested with the appropriate restriction enzyme at 37°C for 1 hour in a 10 μ l reaction consisting: 2 μ l of plasmid DNA, 2 μ l of enzyme reaction buffer, 1 μ l of digestion enzyme, 15 μ l of water. For agarose gel electrophoresis, 2 μ l of loading dye Orange G was added to 20 μ l of digested plasmid diluted 1:20 in water. Electrophoresis was then performed using a 1% agarose gel (UltraPure™ Agarose; Invitrogen, USA) in TAE buffer with 1 x SYBR® Safe DNA Gel Stain (Invitrogen, USA). Bands were separated by running at 80V for 1 hour. Gels were visualised using CANON Digital camera and Kodak software.

2.8.1.4 Maxipreparation of plasmid DNA

Following verification of plasmid size, 10µl of frozen bacterial stock was inoculated in 200ml of LB with 100µl/ml ampicillin overnight at 37°C with shaking. Plasmid DNA was extracted using the QIAGEN Plasmid Maxi Kit (Qiagen, Netherlands). No changes were made to the manufacturer's instructions. Following purification, DNA concentration was determined by Spectrophotometric analysis as described above for RNA analysis, and stored at -20°C.

2.8.2 Luciferase Reporter Assays

To determine STAT3 reporter activity, cells were co-transfected with the STAT3 reporter (Apre-Luc) and the pRL-TK vector which served as an internal vector control, using the Lipofectamine 2000 Transfection Reagent (11668-027, Invitrogen) according to the manufacturer's instructions. Medium was changed 12-hours post-transfection and the cells were treated with drug vehicle, cetuximab, erlotinib or the combination for 24 hours and stimulated with 100ng/ml of EGF for 22 hours.

After 24 hours treatment, cells were washed with 1 x PBS and were lysed using 200µl of 1 x Passive Lysis Buffer (Promega, USA). Firefly and *Renilla* luciferase activity was determined using the Dual-Luciferase® Reporter Assay System (Promega, USA) according to manufacturer's instructions. Promoter activity was measured using a SpectraMax® L Luminescence Microplate Reader and analysed using the SoftMax® Pro software (Molecular Devices Corporation, USA). Results were expressed as the ratio of Firefly to *Renilla* luciferase activity.

2.9 STATISTICAL ANALYSIS OF PRECLINICAL DATA

2.9.1 Statistical methods

Comparisons between 3 or more groups with parametric data were made with a one way ANOVA test. If the *p* value was significant (<0.05), *post hoc* analysis using a *t*-test comparison between outcomes was made.

2.10 CLINICAL TRIAL OF CETUXIMAB AND ERLOTINIB

2.10.1 Design of trial

2.10.1.1 Description

The Dual targeting with cetuximab and erlotinib (DUX) study was a non randomised, phase II, open label, multicentre study of cetuximab in combination with erlotinib. The trial was conceived at our institution by Assoc. Professor Tebbutt, and protocol written by Dr Arun Azad.

Patients received cetuximab 400mg/m² IV loading, then 250mg/m² IV weekly, along with erlotinib 100mg fixed dose oral daily. The doses were selected with reference to earlier supportive phase I studies [210, 211]. Patients received premedication with an antihistamine prior to the 1st infusion of cetuximab, and additionally were allowed dexamethasone at the discretion of each of the centres. Dose modification criteria were defined in the protocol. Treatment continued until disease progression, unacceptable toxicity, death or withdrawal of patient consent.

2.10.1.2 Ethics

The DUX study was conducted according to a protocol reviewed and approved by the Human Research Ethics Committee of each participating institution. All patients provided informed consent.

2.10.1.3 Eligibility

Patients were eligible if they were 18 years of age or older with a histologically confirmed diagnosis of colorectal cancer, and with metastatic disease that was measurable according to the Response Evaluation Criteria in Solid tumours (RECIST). Patients must have received and failed fluoropyrimidine, oxaliplatin and irinotecan therapy, where failure was defined as radiological progression after therapy for metastatic disease, prior adjuvant therapy or toxicity limiting further therapy. Patients were not allowed to have had previous EGFR targeted therapy. Further inclusion criteria included World Health Organisation (WHO) performance status (PS) of 0 or 1; adequate bone marrow function, including platelets ($>100 \times 10^9$ per l) and neutrophils ($\geq 1.5 \times 10^9$ per l); adequate renal function, including calculated creatinine clearance ≥ 40 ml/min; and adequate hepatic function, including serum total

bilirubin < 1.25 X upper limit of normal range and ALT or AST < 2.5 X upper limit normal range, allowing < 5X upper limit of normal range if liver metastases were present. In addition patients had to have a life expectancy of more than 12 weeks, have no concurrent uncontrolled medical conditions, and no previous malignant disease other than non melanotic skin cancer, carcinoma *in situ* of the uterine cervix or any other cancer treated with curative intent >2 years previously without evidence of relapse.

Patients were required to have had a *KRAS* test performed and the results available, and to give consent for biomarker studies. Although *KRAS* mutations have been associated with resistance to EGFR-mAb therapy, no previous clinical study had assessed if the addition of erlotinib can overcome such resistance. Hence patients with *KRAS* mutations were initially eligible for the study, with a plan for an interim safety analysis and an incorporated early stopping rule for recruitment if there was no responders in the first 10 patients with *KRAS* mutations recruited.

Exclusion criteria included medical or psychiatric conditions that compromised the patient's ability to give informed consent or comply with the study protocol, untreated metastatic disease of the central nervous system, pregnancy or breast feeding or participation in any investigational drug study within the previous 4 weeks.

2.10.1.4 Evaluation and outcomes

Before enrolment each patient was assessed by complete physical examination, full blood count, clotting profile, blood biochemistry, tumour markers (carcinoembryonic antigen (CEA), 12 lead electrocardiogram, contrast-enhanced Computed Tomography (CT) scan of the thorax, abdomen and pelvis, and pregnancy test for women of child-bearing potential.

Subsequently, an adverse event assessment, full blood count and blood biochemistry were repeated every 3 weeks. A physical examination, tumour marker assessment and contrast enhanced CT scan of the thorax, abdomen, and pelvis were repeated every 6 weeks until disease progression.

Toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE), version 3.0, and captured up to 4 weeks after the end of the last administration of cetuximab and erlotinib.

2.10.2 Mutational analysis of *KRAS*, *BRAF*, *PI3KCA* and *EGFR* genes

Given the previous reported association of *KRAS* mutations with resistance to EGFR-mAb therapy [68], *KRAS* mutation status was required before enrolment, but was not performed centrally. One patient had a *KRAS* mutation detected using a pyrosequencing assay. The remaining patients had *KRAS* mutations detected using High Resolution Melting (HRM) analysis for mutations in codon 12 and 13. Mutations detected were confirmed by sequencing by the laboratories performing the HRM.

Mutational analysis of patient tumour samples was undertaken at Peter MacCallum Cancer Institute (Melbourne, Australia) by Dr Renato Salemi and Assoc Professor Alex Dobrovic. Genomic DNA was extracted after review of H&E stained paraffin-embedded tumour tissue sections. Regions with the highest proportion of tumour cells were identified and micro-dissected. Genomic DNA was extracted using a Qiagen DNeasy extraction kit (Qiagen, Germany).

Mutation testing for *BRAF*, *PIK3CA* and *EGFR* were performed centrally using HRM analysis. Only HRM positive samples were sequenced. PCR primers were designed to encompass part of exon 15 of the *BRAF* gene, exons 9 and 20 of the *PIK3CA* gene and exons 19 and 21 of the *EGFR* gene. The *EGFR* primers were previously published (Do *et al*, 2008). Briefly, reactions were performed in a volume of 20 µL comprising 0.5 units of HotStarTaq (Qiagen), 1x PCR Buffer, 2.0mM MgCl₂, 200uM dNTPs, 5uM Syto9 fluorescent dye (Molecular Probes/Invitrogen, Carlsbad, CA), and the appropriate amounts of forward and reverse primers as follows: 400nM of each primer for *BRAF*, *PIK3CA* and *EGFR* exon 21, and 400nM of forward primer and 300nM of reverse primer for *EGFR* exon 19. 5ng of DNA was used per reaction and the reactions were performed in triplicate. PCR thermocycling and HRM analysis was performed sequentially on a Corbett Rotor-Gene 6000 Series (now sold as the Qiagen Rotorgene-Q) as per the following conditions: 95°C for 15 minutes; 95°C for 10

seconds, 65-55°C for 20 seconds with 1°C /cycle touch-down, and 72°C for 30 seconds for 55 cycles; 95°C for 1 minute; and HRM at 70-95°C (0.2°C/sec). Data was analysed using the manufacturer's software.

Control cell lines were used as positive controls: MCF7 for *PIK3CA* exon 9, HCT116 for *PIK3CA* exon 20, H1650 for *EGFR* exon 19, and H1975 for *EGFR* exon 21. For *BRAF* exon 15, a clinical sample harbouring the common V600E mutation was used as the control. Only HRM positive samples were sequenced. All mutated samples were confirmed twice, starting from independent polymerase chain reactions.

2.10.3 Statistical analysis

Data collection was overseen centrally by Ms Effie Skrinos on a database designed to capture and analyse information regarding patient outcome and adverse events.

The primary endpoint of the study was response rate, as assessed by RECIST version 1.1. Secondary end points were OS, PFS, treatment related toxicity, and correlation of *KRAS*, *BRAF*, *PI3KCA* and *EGFR* mutational status with outcomes. A sample size of 50 patients was considered sufficient to demonstrate a true overall response rate of $20\% \pm 10\%$, with 90% probability. The overall response rate (RR), based on each patients best recorded RR and its 95% CI (using the method of Clopper-Pearson) was calculated. The statistical analysis was carried out using the package ACCoRD (Analysis of Censored and Correlated Data, Boffin Software, Australia) by Dr Val Gebiski. Analysis of OS and PFS was carried out using the Kaplan-Meier method and illustrated using PRISM 4.03 for Windows (Graphpad, Software Inc, USA). Descriptive statistics were used for safety evaluations.

Analysis of OS and PFS was carried out using the Kaplan-Meier table method. Median survival and 95% CIs were obtained from the Kaplan-Meier curves. Patients who failed to complete more than 3 weeks of treatment due to toxicity and failed to have a CT scan at 6 weeks were deemed ineligible for assessment of the primary endpoint. Time to progression was determined from the day of study assignment to the date of any progression or last contact. The PFS time of patients without disease progression before the end of the study was censored at the last on-study tumour

assessment date at which the patient was considered to be progression free. PFS was defined as the number of days between the first administration of cetuximab and erlotinib and the first on-study assessment of PD, or death within 90 days after the last tumour assessment or first administration of drug. Deaths were evaluated as progression if they occurred at any time on study or within 90 days after the last on-study tumour assessment. OS was calculated from the day of assignment to death. Patients alive at the final survival analysis were censored using the last contact date. Duration of response (DOR) was defined as the period of time from the initiation of treatment in a patient responding to therapy until documentation of disease progression or death.

Analysis by skin toxicity and hypomagnesaemia was retrospective, not pre-specified and purely exploratory. To minimize lead-time bias and under-reporting of skin toxicity because of early treatment discontinuation, a landmark approach was used that limits the analysis to patients with a PFS time of at least 28 days (when >50% of patients had worst grade skin toxicity severity).

2.10.4 Study monitoring

Interim safety analyses were performed after 10 patients, and then 25 patients were enrolled and had completed at least 6 weeks treatment. After the second analysis, enrolment was restricted to *KRAS* WT patients on the basis of futility in the *KRAS* MT subgroup.

2.11 THE MAX TRIAL BIOLOGICAL SUB-STUDY

The methods used for the biological sub-study adhere to the Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK) guidelines.

2.11.1 The MAX clinical trial design

The MAX (Mitomycin, Avastin and Xeloda) clinical trial was conceived and designed by Dr Niall Tebbutt. Patients (n = 471) were enrolled between 2005 and 2007. Data was collected and analysed by the National Health and Medical Research Council Clinical Trials Centre.

2.11.1.1 Patient eligibility and evaluation

Eligible patients were age 18 years or older, had a histologic diagnosis of colorectal adenocarcinoma, had measurable or nonmeasurable unresectable metastatic disease, were considered suitable for capecitabine monotherapy (by investigator judgment), had an WHO PS of ≤ 2 , and had a life expectancy of at least 12 weeks. No prior chemotherapy was allowed except adjuvant chemotherapy (≥ 6 months before relapse). Adequate bone marrow, renal function, and hepatic function were required.

Exclusion criteria included the following: uncontrolled clinically significant cardiac disease, hypertension, arrhythmias, or angina pectoris; acute myocardial infarction or cerebrovascular accident within 12 months; regular aspirin use greater than 325 mg/d or regular use of nonsteroidal anti-inflammatory drugs; CNS metastases; active bleeding disorders within 3 months; major surgical procedure within 28 days; a serious nonhealing wound, ulcer, or bone fracture; 24-hour urinary protein greater than 2 g in 24 hours; pregnancy; and prior history of other malignancy (except those treated with curative intent > 2 years previously without evidence of relapse).

2.11.1.2 Ethics

The study was performed in accordance with the Declaration of Helsinki. Institutional ethics approval was required, and patients provided written informed consent.

2.11.1.3 Treatment regimens

Patients were randomly assigned to capecitabine (C); capecitabine plus bevacizumab (CB); or capecitabine, bevacizumab, and mitomycin (CBM). Before random assignment, patients were stratified by age (< 65 years $v \geq 65$ years), Eastern Cooperative Oncology Group performance status (0 or 1 v 2), capecitabine dose (1 v 1.25 g/m² twice daily), and institution. Treatment was planned to continue until confirmed disease progression occurred.

The full recommended dosage of capecitabine was 1.25 g/m² twice daily on days 1 to 14 every 3 weeks; investigators could nominate a lower starting dosage (ie, 1 g/m² twice daily) for patients considered at risk of toxicity. Patients with creatinine clearance rates of 30 to 50 mL/min commenced capecitabine at 1 g/m² twice daily. Bevacizumab was administered on day 1 at 7.5 mg/kg every 3 weeks. Mitomycin was

administered on day 1 at 7 mg/m² every 6 weeks, the maximum dose was 14 mg, and mitomycin was administered for only four treatments (ie, 24 weeks) to minimize the risk of HUS. Patients randomly assigned to CBM who continued treatment after 24 weeks received capecitabine and bevacizumab until disease progression occurred.

2.11.1.4 Statistical design and analysis of MAX trial

The primary end point of the MAX study was PFS, defined as documented evidence of disease progression according to RECIST or death as a result of any cause. Two primary comparisons were planned: capecitabine compared with CB, and capecitabine compared with CBM.

A sample size of 450 patients (n = 150 per arm) would provide 80% power to detect an improvement of at least 2.5 months in PFS, from 5.5 months to 8 months (a 33% relative reduction in risk), with 36 months for accrual and 12 months of additional follow-up. A two-tailed, 2.5% significance level for each was used to preserve an overall significance level of 5%. Secondary end points included OS, treatment-related toxicity, RR, and QOL. All comparisons were by intention to treat. All *p* values and confidence intervals were two tailed, without any adjustment for multiple comparisons.

The PFS and OS end points were described with Kaplan-Meier curves measured from random assignment and compared with log-rank tests. Cox proportional hazards models were used to adjust and explore significant prognostic factors for PFS and OS. Prespecified subgroup analyses determined whether treatment effects of combined CB and CBM versus capecitabine on PFS differed significantly in a Cox regression model, to ascertain whether clinical or laboratory characteristics were associated with greater clinical efficacy with bevacizumab. The proportions of patients with complete or partial tumour responses in each treatment group were compared by using χ^2 tests. Analyses used SAS (version 9.2; SAS Institute, Cary, NC) or ACCoRD (Analysis of Censored and Correlated Data).

2.11.2 Summary of MAX biological sub-studies

The biological sub-study of the MAX clinical trial consisted of two separate analyses, for which there were common ethics approval, case selection, acquisition and storage of tumour specimens and blinding. However pre-specified hypotheses differed and the statistical plans were also different.

2.11.2.1 Ethics approval

Patients participating in the MAX clinical trial were provided with the opportunity upon enrolment of consenting to participate in the MAX biological sub-study. If patients consented to participation, archived tissue from their tumour was attempted to be collected by our central site.

2.11.2.2 Acquisition and storage of specimens from patients

Formalin fixed, paraffin embedded samples of tumour tissue from archival specimens collected at the time of diagnosis were retrieved from storage at hospital pathology departments. Tumour blocks were collected centrally at our site and processed by Carmel Murone, who collated data on the type of tumour specimen sent, pathology report, quality and amount of tumour present and patient trial ID.

2.11.2.3 Blinding of assay assessment and trial outcome

Our central site of tumour block storage was blinded to the outcomes of the trial, with each tumour block identified only by trial ID. Additionally, those performing the assays on each of the sub-studies was blinded to outcome. Data regarding trial outcome was kept at a separate site at the National Health and Medical Research Centre (NHMRC).

2.11.3 MAX biological *KRAS*/*BRAF* sub-study design

2.11.3.1 Prespecified hypothesis

Analysis of *KRAS* and *BRAF* mutations were undertaken, based on the rationale discussed in the introduction, on the hypothesis that *KRAS* or *BRAF* mutations are prognostic for inferior PFS and not predictive for resistance to bevacizumab.

2.11.3.2 Extraction of DNA from tumour specimens

A rotary microtome was used to cut 2 x 4µm sections from the paraffin embedded tumour blocks by Dr Carmel Murone. Sections were placed on slides, of which one was used for Hematoxylin and Eosin (H&E) staining. For tumour specimens that consisted of either a tumour biopsy or very small amounts of tumour cells, multiple sections were not cut. Instead, guided by a single H&E slide, and using a TMA Mark II Arrayer, (Beecher Instruments, USA), a core of tumour was extracted.

Genomic DNA was extracted from the formalin-fixed, paraffin-embedded tissue slides or tissue cores with the use of the QIAamp DNA FFPE Tissue Kit (Qiagen) by Dr Jennifer Hardingham at the Basil Hetzel Institute, South Australia. DNA extraction was guided by the location of tumour on the H&E stained slide from the tissue scrolls and sections. Subsequently, HRM analysis for mutations of *KRAS* and *BRAF* was performed using the techniques described earlier.

2.11.3.3 Statistical analysis plan for *KRAS* and *BRAF* analysis

Statistical analysis was undertaken in accordance with a protocol for statistical analysis that was written before the assessment of *KRAS* and *BRAF* mutations were performed by Dr Chee Lee and the author of this thesis, with the analysis undertaken by Dr Chee Lee. All randomly assigned patients for whom data on *KRAS* and *BRAF* mutation status were available were included in the analysis.

The primary objective of the analysis was defined to assess the prognostic value of *KRAS* and *BRAF* mutation by demonstrating that either mutation results in inferior PFS, and to assess the predictive value of either mutation by demonstrating the mutation(s) do not predict for treatment efficacy to bevacizumab in terms of PFS. Progression-free survival (PFS), the primary end point, was defined as the time from randomization until documented evidence of disease progression, the occurrence of new disease or death from any cause. In patients who received study treatment without a progression date or death, PFS was censored on the date of last clinical assessment, tumour assessment or randomization - whichever was the later event. The secondary end points were OS, defined as the time from randomization until death from any cause; and RR defined according to RECIST.

The secondary objectives were to assess the prognostic values of the mutations by demonstrating that the mutations result in inferior OS; to assess the predictive values of the mutations by demonstrating that the mutations do not predict treatment efficacy to bevacizumab in terms of OS, and to assess the predictive values of the mutations by demonstrating that they do not predict treatment efficacy to bevacizumab in terms of response rate.

The PFS of patients according to *KRAS* and *BRAF* status and treatment groups were summarized with the use of Kaplan–Meier curves, and the difference between these groups was compared (C vs CB) with the use of the log-rank tests. A proportional hazards model with treatment covariate (C vs CB), *KRAS* or *BRAF* status, and their interaction was used to assess whether the mutation was predictive of bevacizumab treatment. To assess whether *KRAS* or *BRAF* was an independent prognostic factor, a multivariable proportional hazards regression model was fitted, to data for all patients, with *KRAS/BRAF* status and other protocol pre-specified baseline covariates in the model. Similar methodologies were adopted in assessing the predictive and prognostic values for OS and RR. All reported p values were two sided.

Because of the potential for small numbers of patients in the *BRAF* mutation subgroup (10% predicted mutation rate), an additional analysis similar to the above comparing C vs CBM was planned, in addition to a comparison of C vs the combined grouping of C vs CB+CBM.

2.11.4 MAX biological VEGF ligand and receptor sub-study design

2.11.4.1 Pre-specified hypothesis

Based on our understanding of the angiogenic pathways from the literature, our pre-specified hypothesis was that tumour expression of pro-angiogenic related factors (ARFs) and receptors would be predictive for resistance to bevacizumab. Specifically, we were interested in the association between over expression of VEGF-C and VEGF-D, both of which are ligands for VEGFR2, and resistance to bevacizumab.

2.11.4.2 Creation of Tissue Micro-Arrays (TMAs)

Guided by an H&E stained slide, and using a TMA Mark II Arrayer, 1mm cores of tumour were extracted from paraffin embedded tissue blocks. Cores were extracted in triplicate and inserted in a recipient blank paraffin block. The process was repeated for each patient who had adequate tumour within their paraffin blocks, constructing 10 separate TMAs each consisting of a grid of 12 x 8 cores, representing 32 separate patient samples. Each TMA also had two orientation cores inserted at the site indicated in the figure below. One orientation core was taken from human placental tissue, the other was a core taken from biopsy of a human kidney. There was no negative control on the TMA (Figure 6). The site of each patient's tumour core was recorded in a spreadsheet. After the completion of each TMA, the TMA was inserted into a 37°C chamber overnight to anneal the wax and cores.

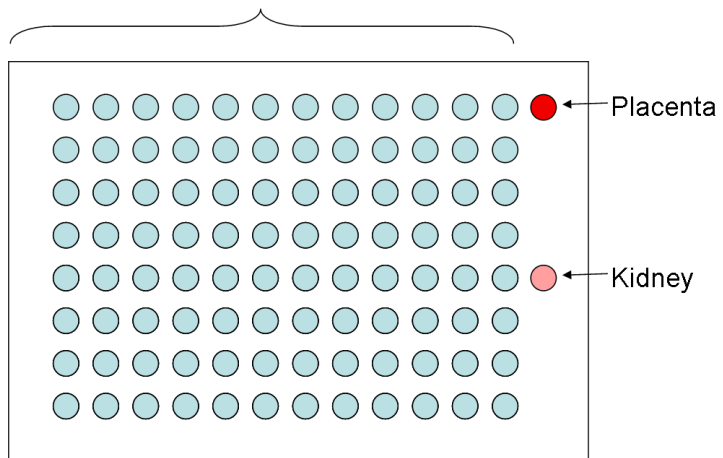
2.11.4.3 Immunohistochemical (IHC) staining of TMAs

We wished to determine tumour expression levels of angiogenic related factors (ARFs) including VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGFR1 and VEGFR2. Therefore a rotary microtome was used to cut a 4µm section from each TMA, which was then stained with H&E. Further 4µm sections were cut from each TMA and placed on microscope slips by Carmel Murone. Each was then stained with antibodies for angiogenic related factors as detailed below. In parallel, separate 4µm sections were stained with the relevant sub-class control Ab or omission of primary antibody as negative controls.

For immunostaining, the slides were deparaffinised by baking at 60°C for 1 hour, followed by 2 washes with Xylene and 2 washes with Ethanol, each for 5 minutes. Slides were rehydrated with a 5 minute wash in deionised H₂O. Antigen retrieval was performed for 30 minutes at 100°C in a water bath using Citrate buffer (Labvision, USA), or for 60 minutes with Dako TRS (S1699, Dako, Denmark) appropriate for each antibody as listed in Table 9. No antigen retrieval was required for the anti-VEGF-B (MAB751) antibody. The slides were cooled for 15 – 20 minutes then washed in tap water and then twice in TBS-T. Endogenous peroxidase was quenched by incubating the slides in 3% H₂O₂ for 10 minutes at room temperature and then washed twice more with TBS-T.

Figure 6: Layout of Tissue Micro-Array

12x8 grid of tumour cores (in triplicates)



The slides were incubated with primary antibody at room temperature or at 37 °C at the appropriate concentration for the appropriate length of time for each antibody as listed below. The slides then had two further washes in TBS-T before exposure to the appropriate secondary antibody at room temperature for the appropriate length of time (Table 10).

Table 10: Antibodies and staining methods used for IHC

Antibody	Company	Antigen retrieval	Concentration	Incubation time	Secondary Ab	Incubation time	Sub-Class Control Ab
VEGF-A	Dako	Dako TRS9	5µg/ml	Overnight	Dako Envision α-mouse Ig-HRP	30 minutes	Mouse IgG1
VEGF-B	R&D Systems	none	5 µg/ml	2hrs, 37 °C	Dako Envision α-mouse Ig-HRP, 37 °C	30 min	Mouse IgG1
VEGF-C	Invitrogen	Citrate	2.5µg/ml	Overnight	Dako Envision α-rabbit Ig-HRP	30 minutes	Rabbit IgG
VEGF-D	Santa Cruz	Citrate	4µg/ml	2 hours	Dako Envision α-rabbit Ig-HRP	30 minutes	Rabbit IgG
VEGFR1	Santa Cruz	Citrate	4µg/ml	Overnight	Dako Envision α-rabbit Ig-HRP	30 minutes	Rabbit IgG
VEGFR2	Santa Cruz	Citrate	4µg/ml	Overnight	Dako Envision α-rabbit Ig-HRP	30 minutes	Rabbit IgG

Sub-Class Control Ab	Company	Antigen retrieval	Concentration	Incubation time	Secondary Ab	Incubation time	Sub-Class
Rabbit IgG	Vectorlabs, USA	Citrate	5µg/ml	Overnight	Dako Envision α-rabbit Ig-HRP	30 minutes	N/A
IgG1	Southern Biotechnology	TRS9	5µg/ml	Overnight	Dako Envision α-mouse Ig-HRP	30 minutes	N/A
IgG1	Southern Biotechnology	none	5µg/ml	2hrs, 37 °C	Dako Envision α-mouse Ig-HRP, 37 °C	30 minutes	N/A

Slides were then washed in TBS-T twice before staining with AEC (3-amino-9-ethylcarbazole; Sigma-Aldrich) chromogen for an interval between 5 – 15 minutes, monitoring for the development of any signal. After washing in water, slides were counterstained with Hematoxylin, washed in water then Scott's Solution (20 g MgSO₄.7H₂O & 3.5g NaHCO₃ per 1 litre dH₂O). Following further washes in tap water, slides were coated with CC/Mount ® (Sigma Aldrich) and then coverslips were applied to each slide.

2.11.4.4 Quality Control for IHC

Quality control was undertaken by undertaking staining of each slide in parallel with the relevant sub-class control of each antibody. Additionally each TMA had human placenta and renal glomeruli on the TMA to provide a relevant positive control.

2.11.4.5 Scoring of IHC expression levels

Scoring was performed independently by two scorers; the author and Dr David Williams, an anatomical pathologist. Each was blinded to the others scores. The staining for each antibody was determined to be between 0 – 3+ based on the intensity and distribution of staining. If greater than 25% of tumour cells were stained, the score was recorded as 0, 1, 2, or 3. However if the intensity of staining was > 0, but the distribution of tumour cells stained between 1 - 25%, the score was recorded as 1, regardless of the intensity of staining. The mean expression score from the assessment of three cores per patient was recorded. When there was duplication on the TMAs of tumour samples from a primary tumour block and an additional metastatic source for a given patient, the expression score from the metastatic site was used. Discrepancies were resolved by consensus on second review between the two scorers. Inter-rater agreement was calculated using a weighted Kappa score between the two scorers.

Variability of expression of ARFs amongst the three cores taken from each tumour block was calculated using three separate weighted Kappa scores comparing each of the expression values against each other in pairs, before determining a weighted mean Kappa score.

Correlation of expression scores from patients who had duplicate tumour specimens on the TMA taken from tumour primary and an additional metastatic source (either lymph node, omental site, or solid organ metastases) was performed using Pearson's correlation with Prism 4.03 software.

2.11.5 Statistical analysis of scoring and correlation with outcomes from trial

All statistical analyses were performed in accordance with a protocol for statistical analysis that was prepared before the assessment of ARFs with patient outcome. The statistical analysis plan was prepared by Dr Chee Lee and the author, and the analysis undertaken by Dr Chee Lee and Professor John Simes. All randomly assigned patients for whom data on IHC expression values were available were included in the analysis.

Prognostic characteristics of patients with tumours eligible for the ARF IHC biomarker trial were compared to the intention to treat population of the MAX trial to ensure the population in the sub-study was representative.

PFS was the primary end point, and was defined in the same manner as for the *KRAS/BRAF* analysis, as was OS and RR, which were secondary endpoints. The primary objective was to assess the prognostic values of ARF expression by demonstrating that ARF family member expression results in inferior PFS, and to additionally assess the predictive values of ARF expression by demonstrating that ARF expression predicts treatment efficacy to bevacizumab in terms of PFS. The secondary objective, was to assess the prognostic values of ARF expression by demonstrating that ARF expression results in inferior OS, and additionally to assess the predictive values of ARF expression by demonstrating that VEGF family member expression predicts treatment efficacy to bevacizumab in terms of OS, as well as response rate.

Categorical scoring of the ARFs was used to separate outcomes into groups of 0 or 1; 2, or 3 for each ARF. The PFS of patients according to ARF expression status and treatment groups were summarised with the use of Kaplan-Meier curves, and the difference between these groups compared (C vs CB) with the use of the log-rank tests. A proportional hazard model with treatment covariate (C vs CB), ARF status

and their interaction was used to assess whether the trend in ARF expression was predictive of bevacizumab treatment efficacy. To assess whether ARF expression status was an independent prognostic factor, a multivariable proportional hazards regression model was fitted to data for all patients with ARF expression and other protocol pre-specified baseline covariates in the model. The analysis was repeated to compare C vs CBM (to allow validation of any observations made in the comparison of C vs CB) and then finally with C vs the combined group of CB + CBM.

The same method was adopted for assessing the predictive and prognostic values for OS and RR, analysing those factors that had reached significance in interaction with PFS. All reported *p* values were two-sided.

3 DUAL TARGETING THE EGFR: RESULTS AND DISCUSSION

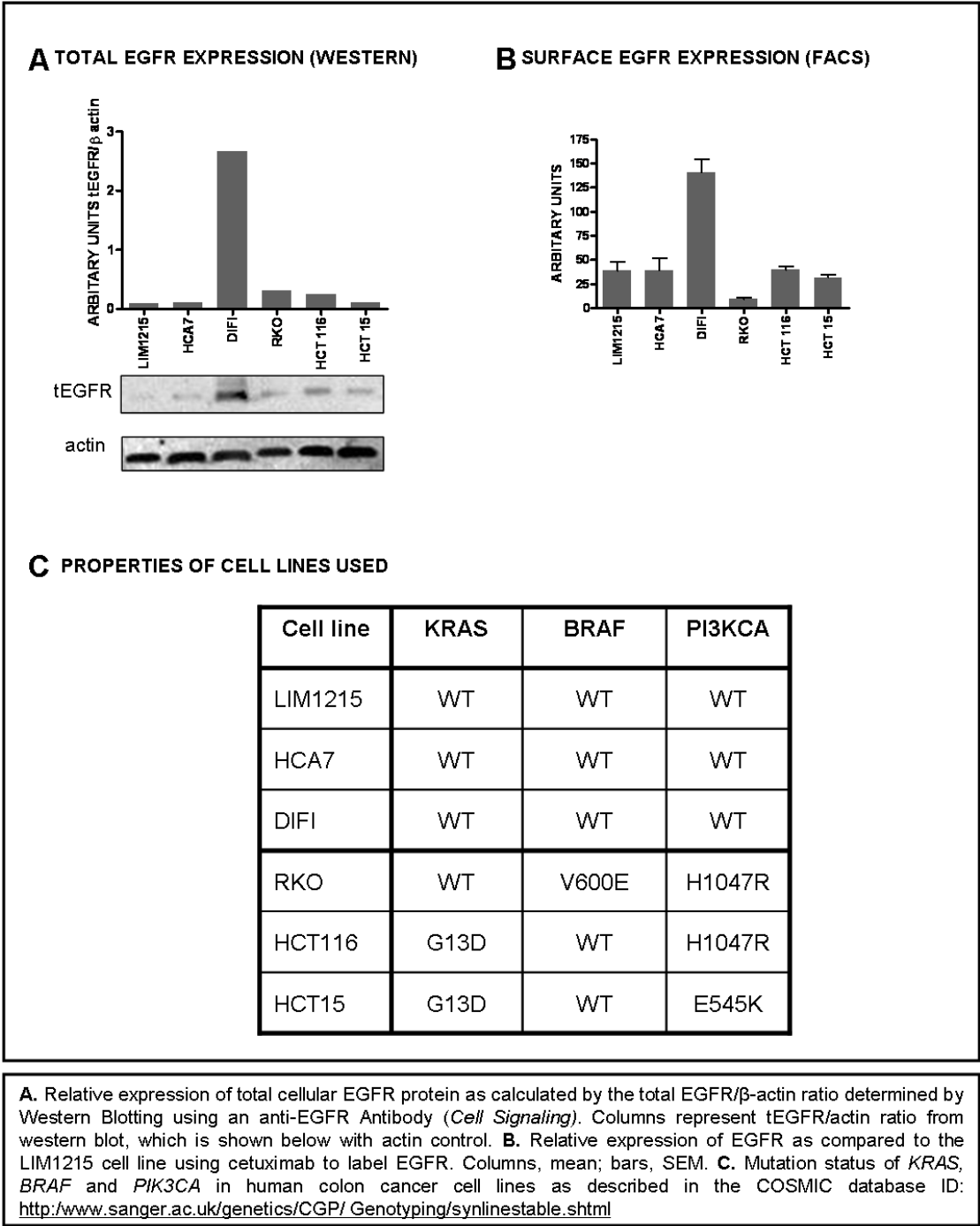
3.1 *IN VITRO* DUAL TARGETING OF THE EGFR: RESULTS

3.1.1 Human cancer cell line properties

Colorectal cancer response to EGFR targeted therapy has previously been characterised as dependent on the mutation status of *KRAS*, *BRAF* and *PIK3CA* both *in vitro* and in *in vivo* studies [12, 50, 68, 69, 95]. Prior to commencing our *in vitro* studies, we selected six colorectal cancer cell lines based on the mutation status of these genes (Figure 7C). Three cell lines were chosen that were wild type for *KRAS*, *BRAF*, and *PIK3CA*: LIM1215, DiFi and HCA7. Additionally three cell lines were chosen that had mutations in one or more of these genes: *KRAS*: HCT116 (*KRAS* and *PIK3CA*), HCT15 (*KRAS* and *PIK3CA*) and RKO (*BRAF* and *PIK3CA*).

Although clinical studies have determined no relationship between colorectal cancer cell surface EGFR expression and cetuximab response[132], other authors have demonstrated that cells with high expression levels of EGFR are more sensitive to EGFR-mAb therapy [385]. The expression level of EGFR of the six colorectal cancer cells lines used in the *in vitro* experiments was determined by Western blot and additionally by FACS analysis for surface EGFR as shown in Figure 8A-B. EGFR was expressed by all cell lines, and was markedly highest in the DiFi cell line as previously described [197].

Figure 7: Properties of human colon cancer cell lines



3.1.2 Changes in cell growth

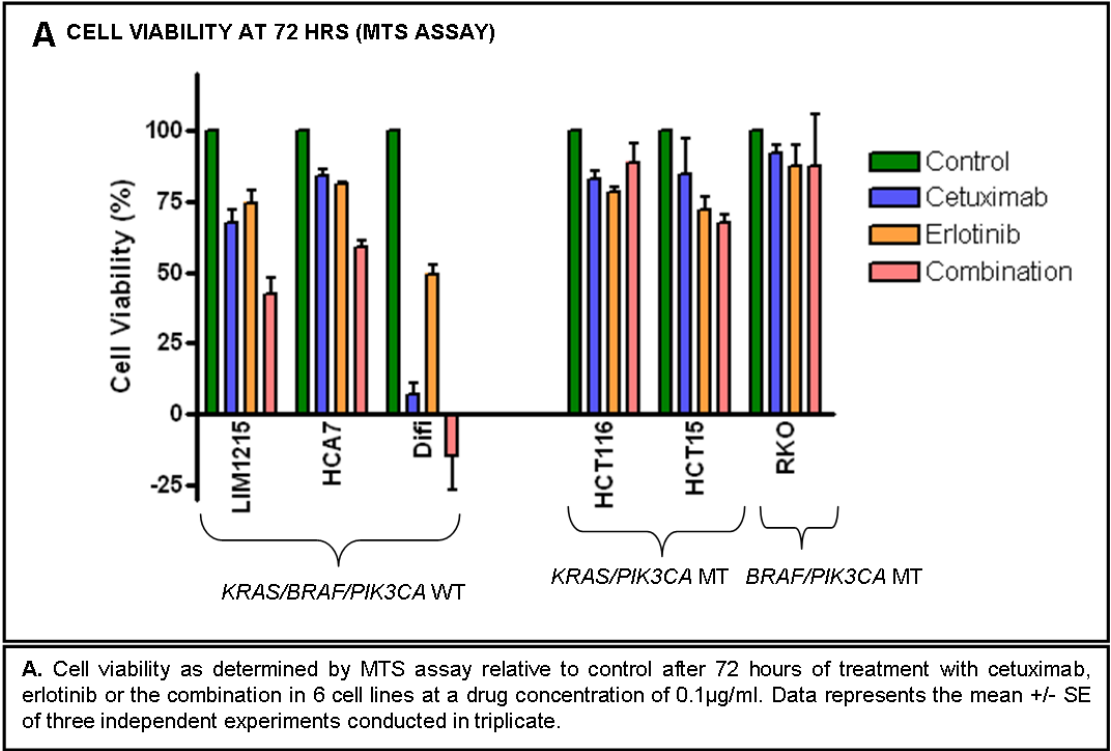
The MTS assay and cell counts were used to investigate the growth inhibitory effects of cetuximab and erlotinib alone and in combination on colorectal cancer cells at a variety of concentrations, after 72 hours of treatment.

After treatment of the six cell lines at a dose of 0.1µg/ml, we made comparisons amongst the treatment conditions and cell lines (Figure 8). In LIM1215, DiFi and HCA7 cell lines, cetuximab alone caused a marked reduction in cell viability at 72 hours (67% mean cell viability in LIM1215 cells, 84% in HCA7 cells and 7% in DiFi cells relative to control). Erlotinib also led to a decrease in cell viability relative to control in these three cell lines (75% mean cell viability in LIM1215, 81% in HCA7, and 50% in DiFi).

Superior reduction in cell viability was observed with dual targeting of the EGFR with the combination of cetuximab and erlotinib (0.1µg/ml each) compared to cetuximab or erlotinib alone in all three cell lines. In LIM1215 cells, the mean cell viability was 67% in cetuximab treated cells, 74% in erlotinib treated cells, but 42% in the cells treated with the combination. In HCA7 cells, the mean cell viability was 84% in cetuximab treated cells, 81% in erlotinib treated cells, and 59% in the cells treated with the combination. In DiFi cells, the combination led to -15% viability relative to control (Figure 8A).

In HCT116, HCT15 and RKO cell lines there was only a mild decrease in cell viability with either cetuximab or erlotinib when administered alone. Although a mild decrease in cell viability in these cell lines with *KRAS* and *BRAF* mutations was observed, it was markedly less than the effect observed in the WT cell lines. Likewise, in contrast to the three cell lines lacking mutations in *KRAS* or *BRAF*, there was no superiority of the combination in the cell lines with mutations in either *KRAS*, *BRAF* or *PIK3CA*.

Figure 8: The effect of dual targeting of the EGFR on cell viability



The superior inhibition of growth of the combination was observed in a separate series of experiments by counting cell number over 72 hours in LIM1215, HCA7 and DiFi cells (Figure 9). The combination led to a significantly greater decrease in cell number of LIM1215 over 72 hours: 54% reduction in LIM1215 (relative to cetuximab or erlotinib alone, $p=0.002$). The combination was also led to significantly greater decreases in cell number with HCA7 ($p=0.01$), and DiFi ($p=0.009$) relative to cetuximab or erlotinib alone.

To further confirm this finding, a time course experiment was performed in which the effects on the growth of LIM1215 cells as measured by cell number were recorded (Figure 10B). The improvement of the combination over the single agent treatment was observable after 48 hours of treatment, with the cetuximab mean cell number 740,000, erlotinib mean cell number 720,000 and combination mean cell number 480,000 at 48 hours ($p=0.003$). The difference in cell number at 72 hours was also significantly reduced in the cells treated with the combination relative to either drug alone ($p=0.003$). Finally the added benefit of the combination over single agent treatment on cell growth was confirmed by light microscopy, 72 hours post treatment (Figure 10A).

3.1.2.1 Treatment effect at higher doses of cetuximab and erlotinib

To demonstrate that the superior inhibition of growth was observable at more than one dose, viability and growth assays were performed at a range of concentrations and ratios of cetuximab:erlotinib. The superior reduction in cell viability was observed at a range of drug concentrations and ratios, all which are within the dose range used by other investigators and are achievable *in vivo* [193, 195](Figure 12A). This includes a range of drug concentrations from 0.05µg/ml – 0.2µg/ml when the ratio of the drugs used in the combination was 1:1. Additionally using a combination of 0.76µg/ml (5nM) cetuximab and 0.21µg/ml (0.5µM) erlotinib that was used in an earlier publication [195] superior reduction in cell viability compared to either drug alone was observed (Figure 12B).

Figure 9: Cell number relative to control at 72 hours

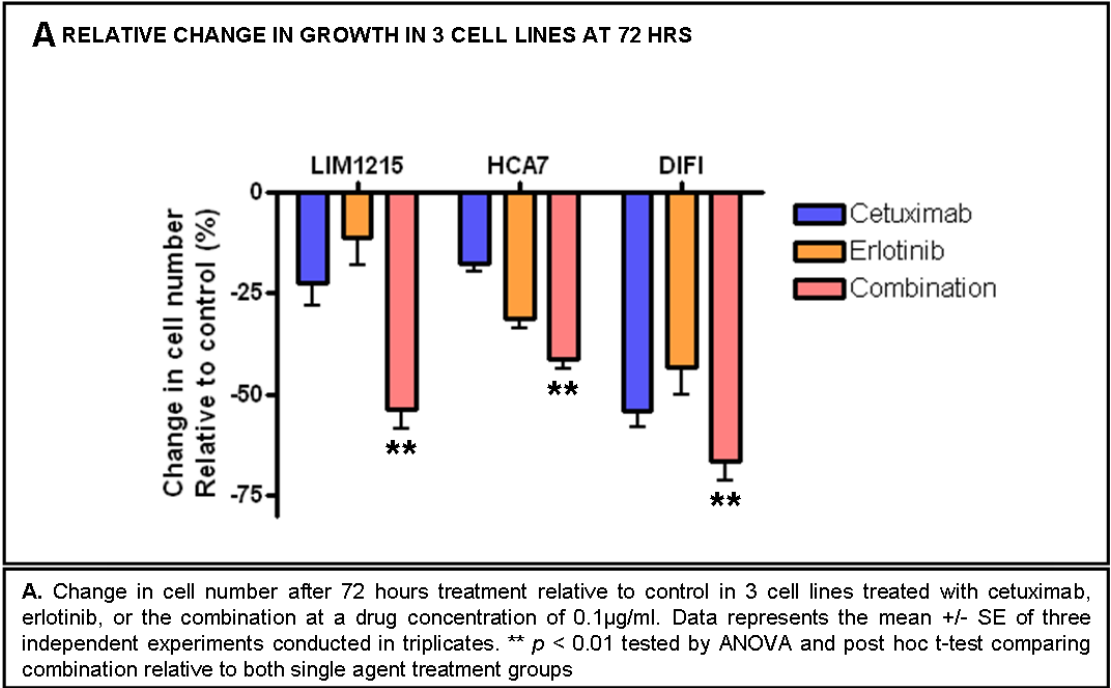
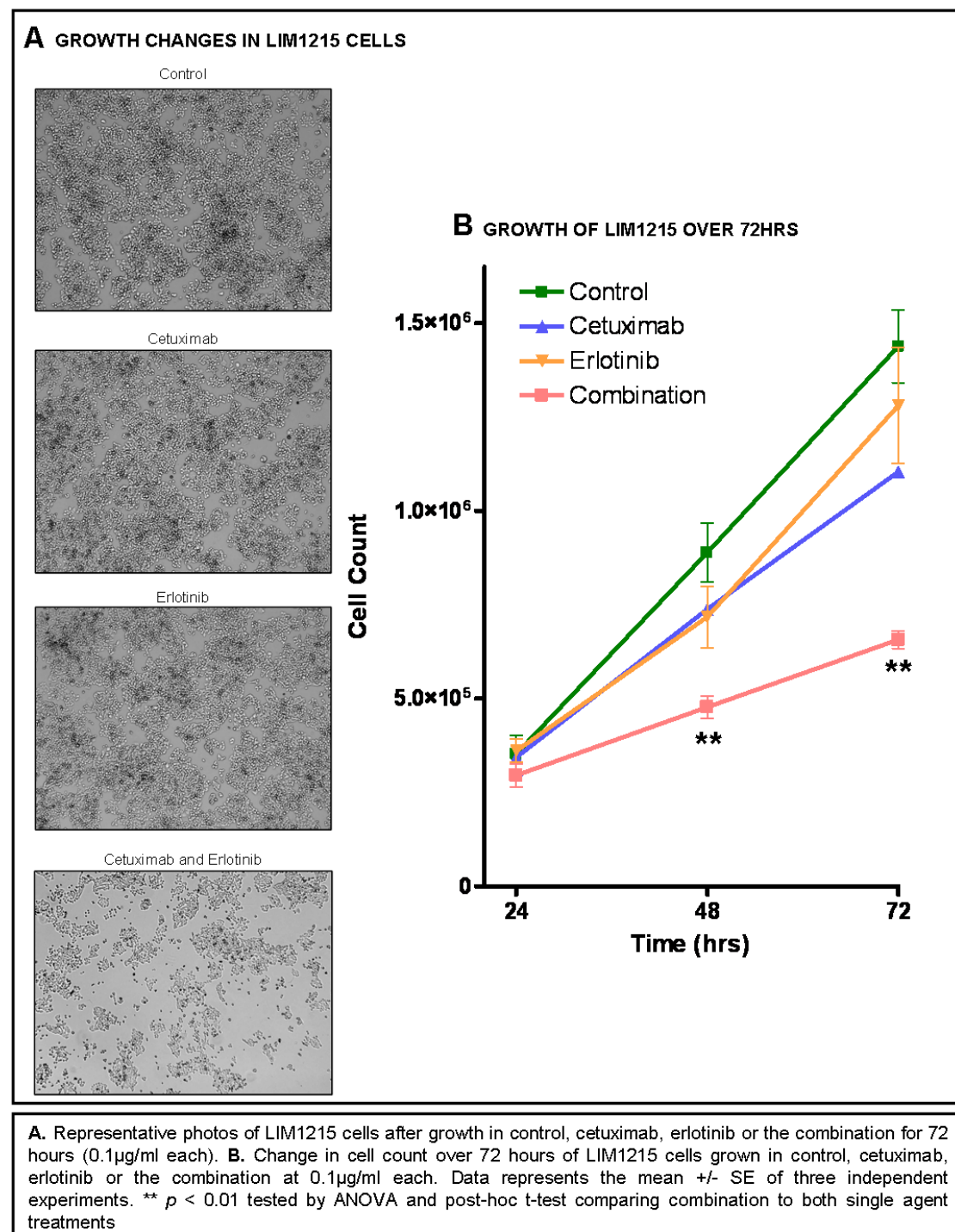


Figure 10: Change in LIM1215 with dual treatment of the EGFR



However MTS assays conducted with higher doses of erlotinib $\geq 0.2\mu\text{g/ml}$, or cetuximab doses $\geq 5\mu\text{g/ml}$ did not demonstrate superiority of dual targeting over single agent treatment alone (Figure 11B and Figure 12).

Finally to exclude experimental design influencing outcomes, and to model daily dosing of erlotinib that occurs with clinical treatment of patients, the MTS assay was undertaken by changing the growth medium with each of the relevant treatment conditions daily over 72 hours and compared to the standard method of performing the assay (Figure 11D). There was no difference in outcomes for cetuximab, erlotinib or the combination with this method compared to the standard experimental design.

3.1.2.2 Dual targeting results in modest synergistic effects on growth inhibition

To model the effect of the combination of the two agents, the Combination Index was computed using CalcuSyn software, where $\text{CoI} < 0.9$ indicates synergism, $\text{CoI} 0.9 - 1.2 =$ additive, and $\text{CoI} > 1$ indicates antagonism [386]. To improve the analysis, LIM1215 cells were used to model the effect size at a range of concentrations. At the dose range between 0.05 and $0.2\mu\text{g/ml}$ the combination index ranged from $0.60 - 0.81$ and the interaction between cetuximab and erlotinib was considered moderately synergistic [387](Figure 13A) The combination also resulted in moderate synergistic anti-proliferative effects in HCA7 and DiFi cells, as shown by a combination index at 50% cell lethality (CoI_{50}) of $0.50 \pm \text{SD } 0.29$ in HCA7 cells and $0.81 \pm \text{SD } 0.36$ in DiFi cells.

For the optimal use of the Chou and Talalay method of calculating the CoI , it is recommended that the dose-effect curve reaches the zero value [198]. If this is not achieved, as was the case in LIM1215 cells, a complementary analysis has been proposed where the CoI is replaced by calculating R , where R represents a fractional effect as represented in Figure 13B. When calculated in this manner, the combination was still shown have a moderate synergistic anti-proliferative effect in all three cell lines, with $R = 0.81$ in LIM1215 cells, $R = 0.55$ in HCA7 cells, and $R = 0.29$ in DiFi cells at a dose of $0.1\mu\text{g/ml}$ for both cetuximab and erlotinib.

Figure 11: Effect of dual targeting the EGFR at different concentrations or ratios

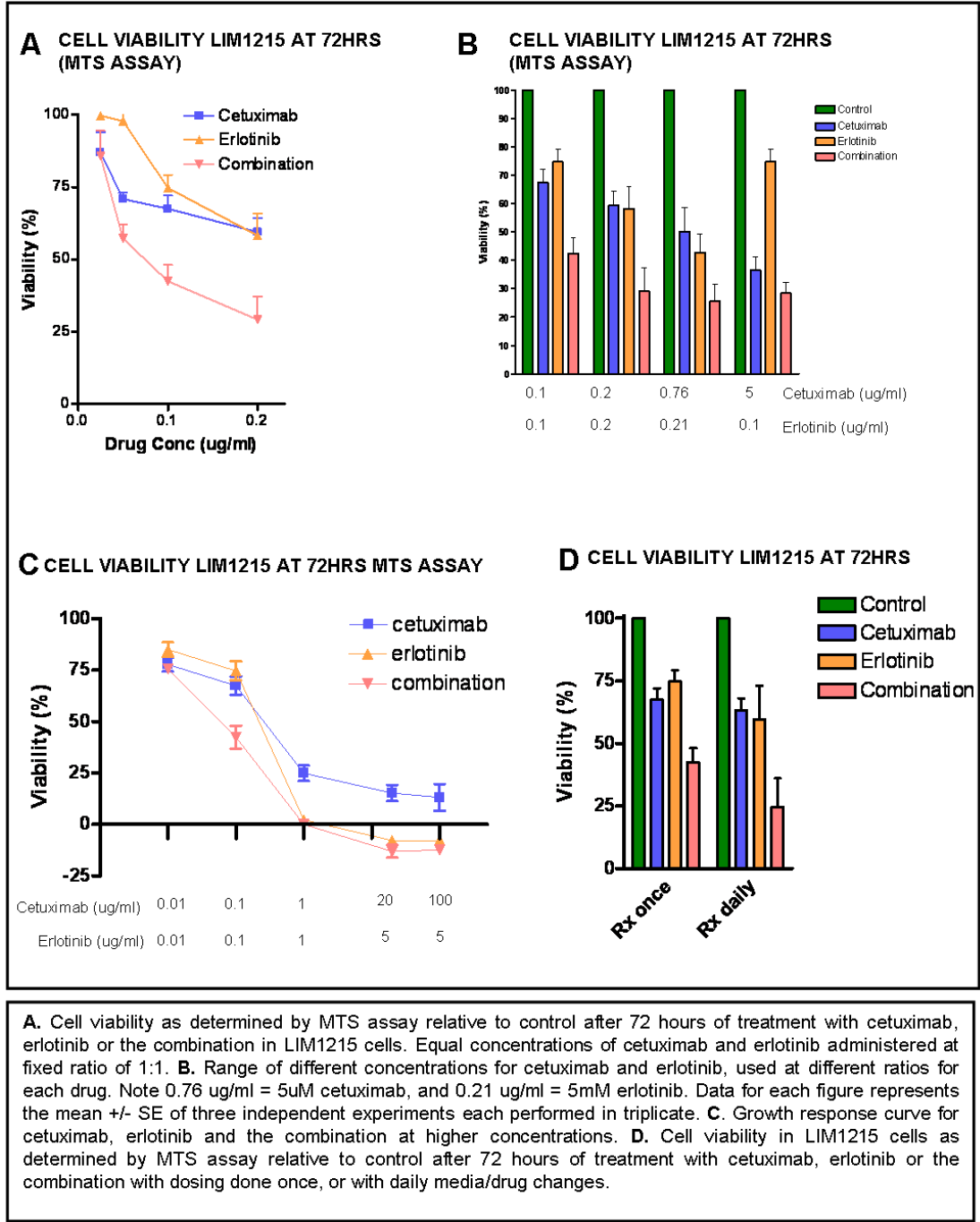


Figure 12: Change in LIM1215 cell number with higher dose of drug

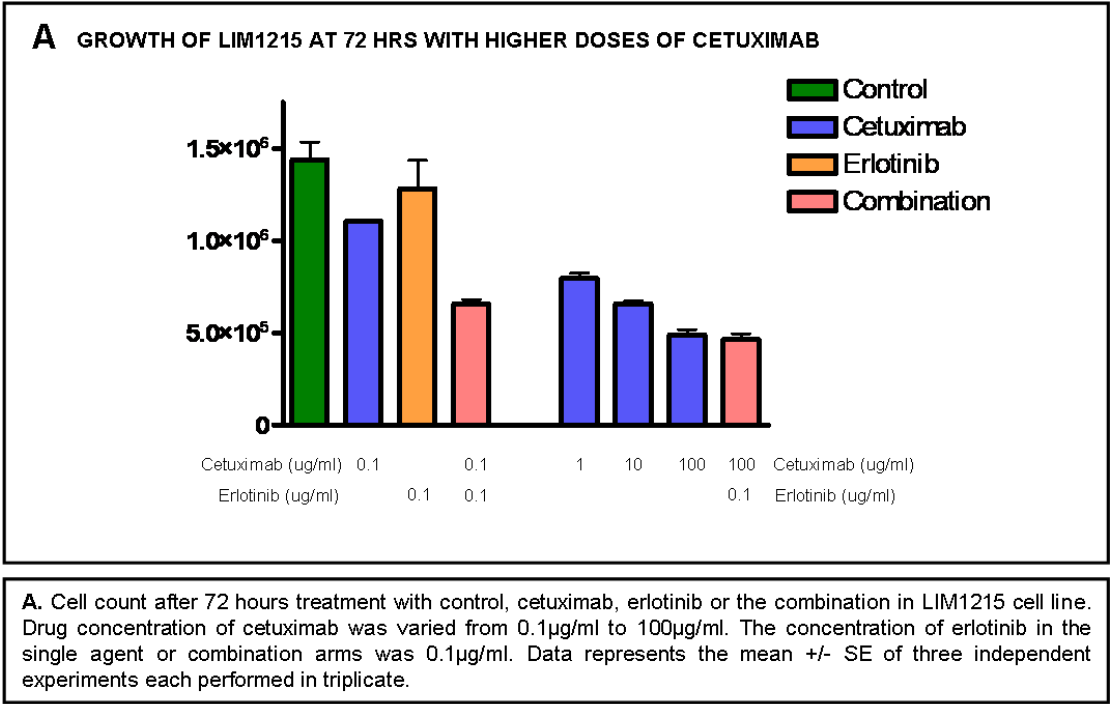
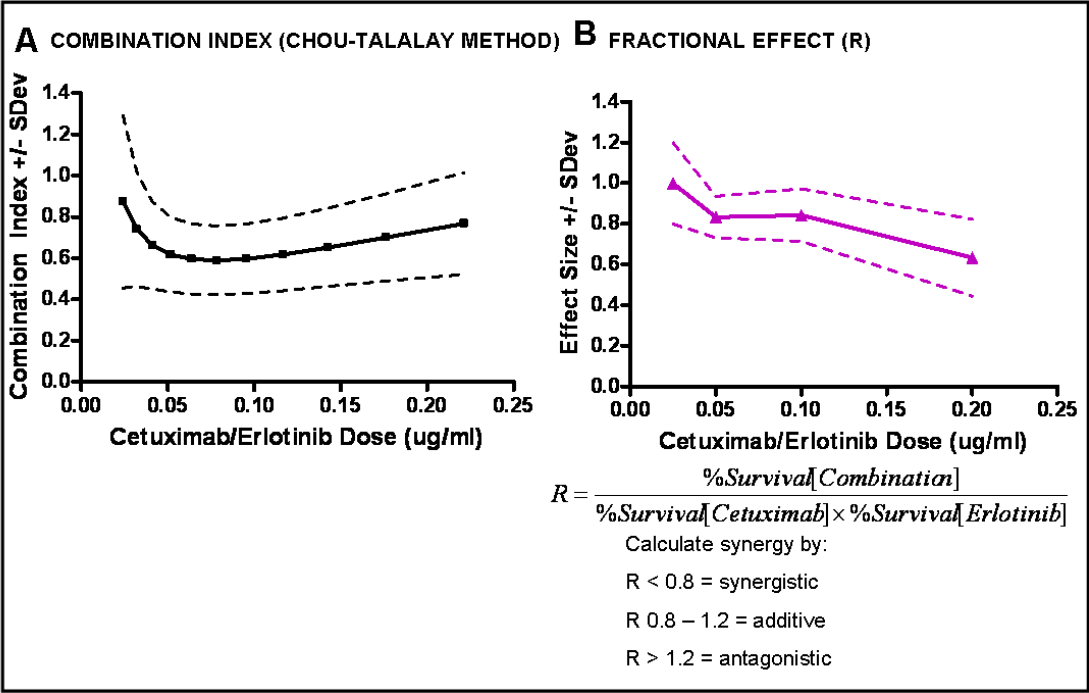


Figure 13: Combination index in LIM1215 cells



A. Combination Index Plot generated by Calcsyn software as determined by Chou and Talalay method with standard deviation. **B.** Fractional effect R (Ratio) as an alternate description of the interaction between cetuximab and erlotinib in LIM1215 cells at different doses of drug, mean of three experiments with standard deviation.

3.1.3 Changes in cell cycle

To investigate the mechanism by which dual targeting leads to synergistic inhibition of cell growth, changes in cell cycle were investigated by PI staining of cells flow cytometry analysis after 24 hours of treatment.

Single agent treatment of LIM1215 cells with either cetuximab or erlotinib led to a decrease in the proportion of cell in S phase (37% in the control cells; 31% in the cetuximab treated cells; 30% in the erlotinib treated cells; and this effect was greater (25%) in the combination arm ($p=0.009$)(Figure 14). In parallel both cetuximab and erlotinib induced a G0-G1 arrest (55% in the control cells; 61% in the cetuximab treated cells; 61% in the erlotinib treated cells), which was greater (69%) in the combination arm, ($p=0.009$).

3.1.4 Changes in apoptosis

Next, to investigate whether increased apoptosis could result from the combination and could explain the decreased cell viability in LIM1215 cells, we assessed apoptosis using flow cytometry of Annexin V/PI labelled cells. 5FU was used as a positive control: cells treated with 50mM of 5FU had an apoptotic rate of 18% after 72 hours as defined by the Annexin V/PI+ subpopulation. Neither cetuximab nor erlotinib led to an increase in apoptosis relative to control over the 72 hour period (7% rate of apoptosis in the control cells, 5% rate of apoptosis in cetuximab cells, 7% rate of apoptosis in erlotinib treated cells). However the combination of cetuximab and erlotinib did not lead to an increase in cells undergoing apoptosis in LIM1215 cells (Figure 15B). To assess if higher doses of cetuximab or the combination led to apoptosis, we treated cells with cetuximab from 0.1 μ g/ml to 100 μ g/ml, as well as the combination of erlotinib 0.1 μ g/ml with cetuximab 100 μ g/ml (Figure 15B) Although there was a non significant trend in increased apoptosis with the higher dose of cetuximab (5% at 0.1 μ g/ml; 9% at 100 μ g/ml), apoptosis did not increase with the addition of erlotinib (10% at 100 μ g/ml cetuximab, 0.1 μ g/ml erlotinib).

Figure 14: Change in cell cycle distribution of LIM1215 cells with treatment

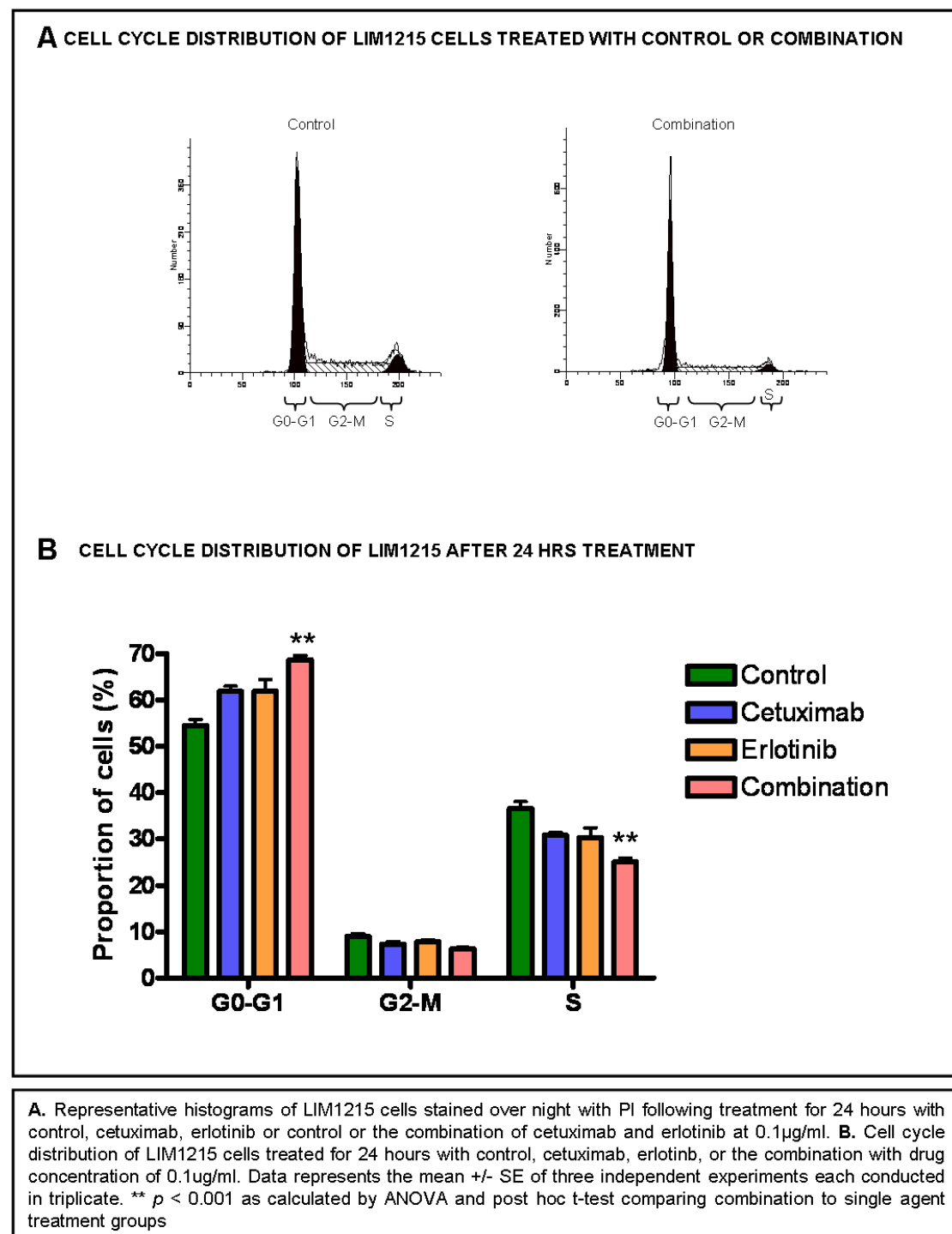
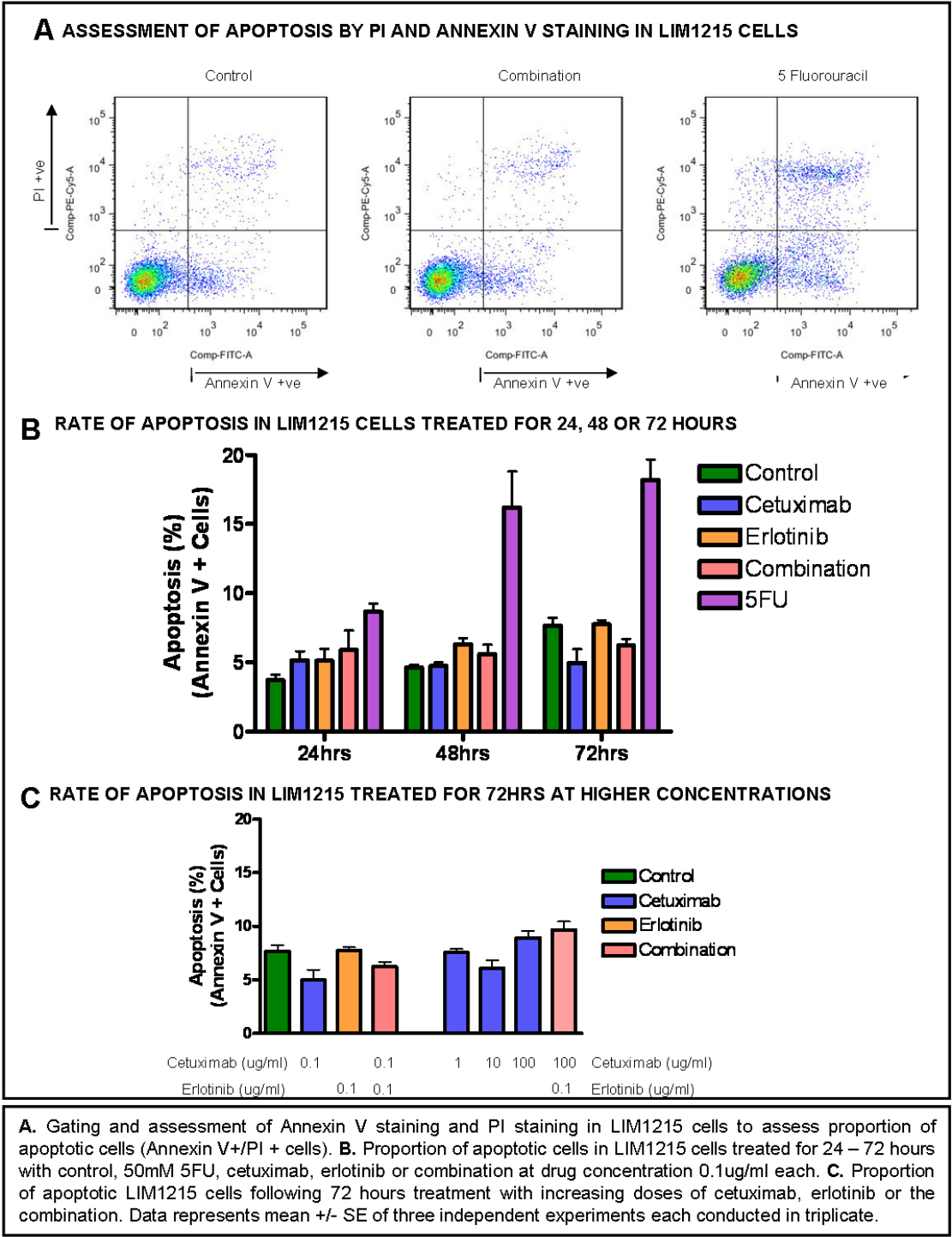


Figure 15: Change in apoptotic rates in LIM1215 cells with treatment



3.1.5 Changes in downstream levels of phosphorylated proteins

Subsequently, to investigate downstream changes within the EGFR signalling pathway, Western blot analysis of key signalling proteins was undertaken after 2 and 24 hours of treatment with cetuximab, erlotinib and the combination.

After 2 hours of treatment, there were clear reductions in pEGFR and pMAPK in LIM1215 cells after treatment with cetuximab or erlotinib alone relative to control (Figure 16). The combination of cetuximab and erlotinib led to further visible decreases in pEGFR and pMAPK relative to either drug used alone.

To gauge changes within the alternate PI3KCA signalling pathway in response to treatment, we attempted to assess levels of pAKT using the same experimental conditions. However the level of pAKT was very low even in LIM1215 cells stimulated with EGF. Therefore, no we draw no conclusions regarding the change in pAKT levels in response to treatment.

To compare the effect of higher doses of cetuximab with that of the cetuximab /erlotinib combination, we treated LIM1215 cells with higher doses of cetuximab and compared the levels of pMAPK and pEGFR to cells treated with the combination (Figure 17). At higher doses of cetuximab there were clear further decreases in pEGFR and pMAPK activity. Although the addition of erlotinib 0.1µg/ml to cetuximab 100µg/ml did visibly further reduce pMAPK activity from that of cetuximab 100µg/ml, this reduction was not significant when assessed by pMAPK/actin intensity over three experiments.

Figure 16: Changes to EGFR signalling intermediaries in LIM1215 with treatment

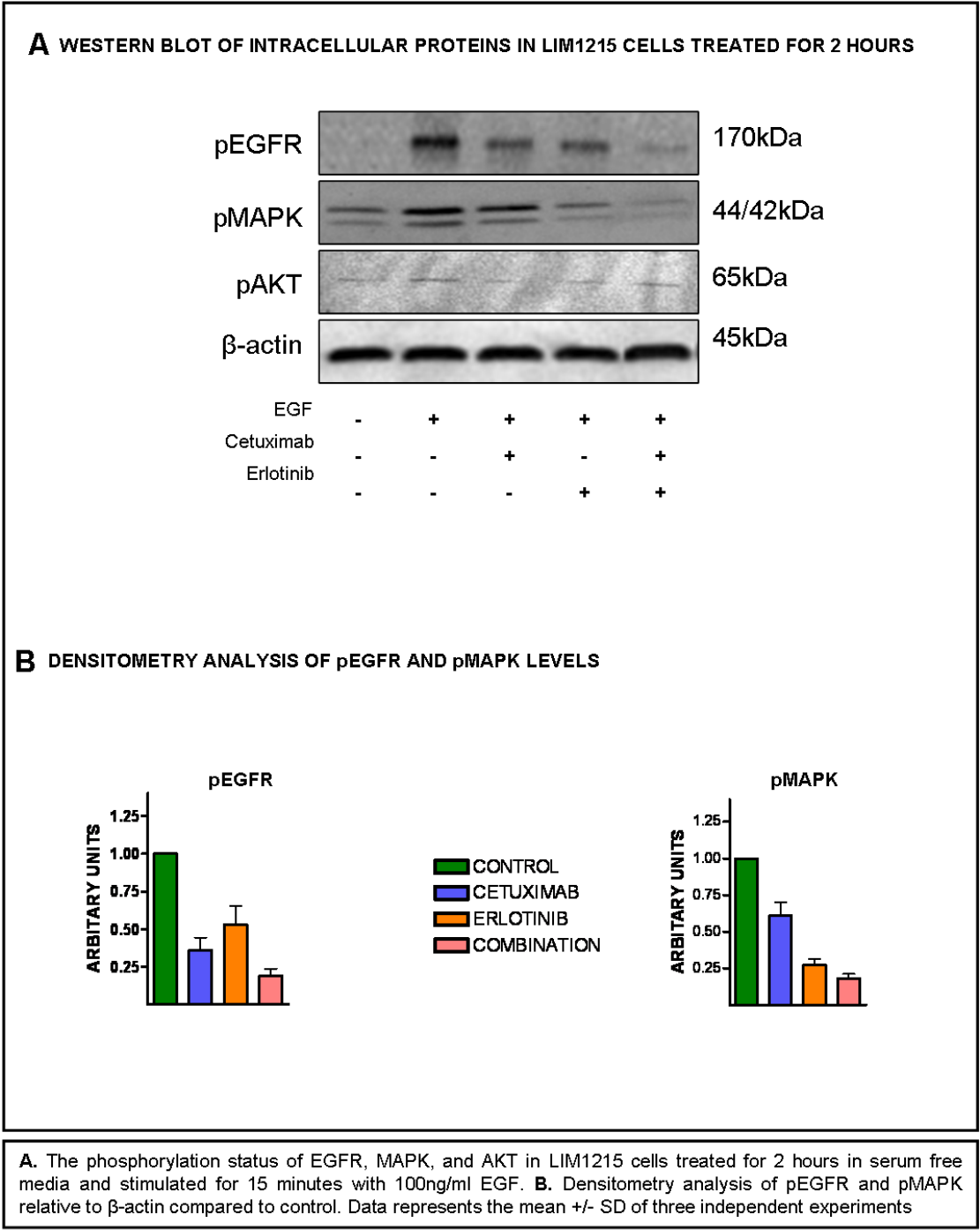
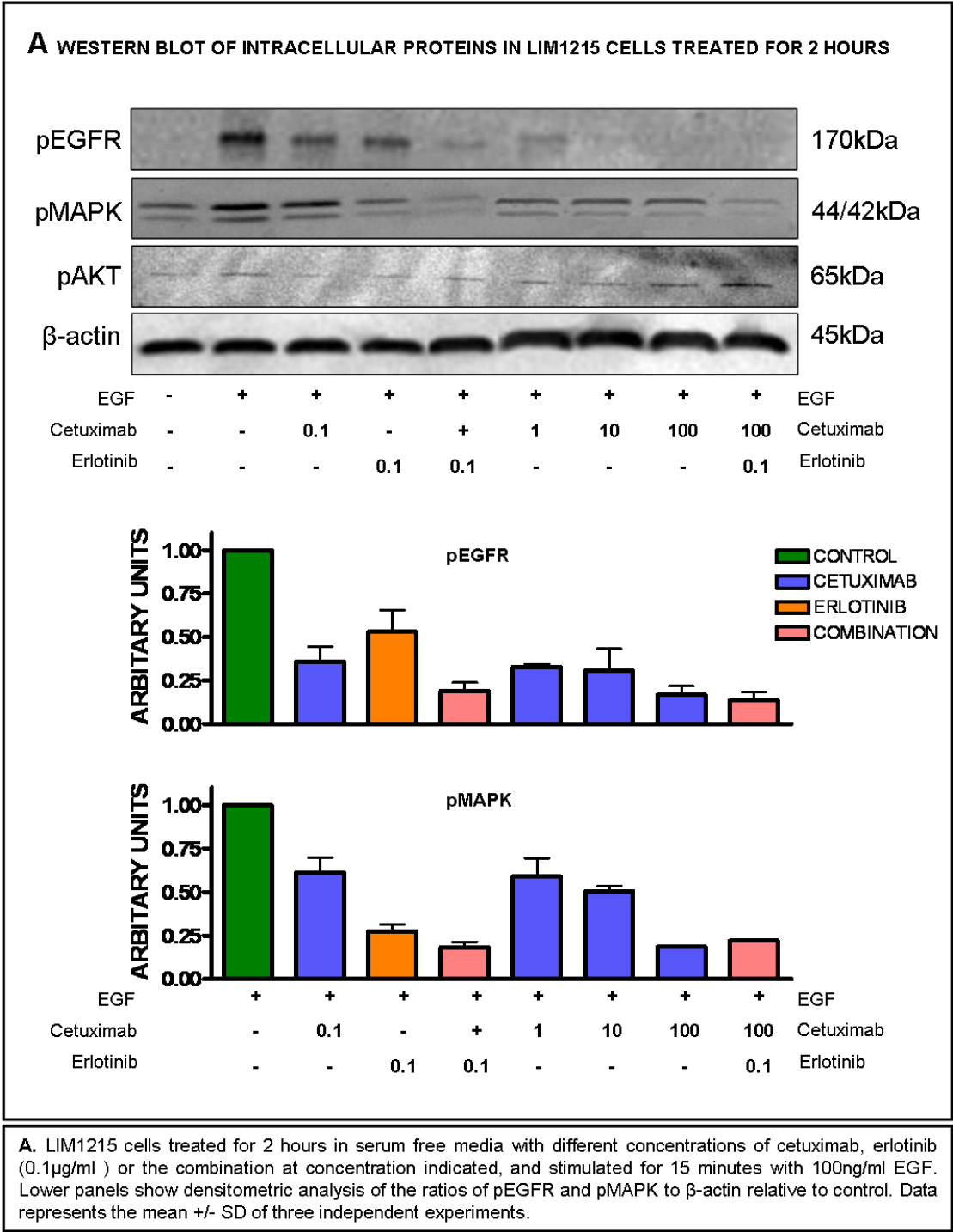


Figure 17: Changes to EGFR signalling intermediaries at higher concentrations in LIM1215



To validate the changes seen in the LIM1215 cell line, western blot analysis was also performed in the HCA7 and DiFi cell lines after treating the cells for 24 hours (Figure 18). As shown in Figure 18, the magnitude of inhibition in pEGFR and pMAPK was significantly greater when treated with the combination relative to either drug alone, for all three cell lines.

In comparison to these 3 cell lines, RKO cells did display any noticeable changes in pMAPK levels following treatment with cetuximab, erlotinib or the combination at 2 or 24 hours, in keeping with the known *BRAF* mutation status of this cell line (Figure 19). Interestingly, there was an increase in pEGFR levels in the RKO cell line after 24 hours treatment in the combination relative to either cetuximab or erlotinib alone.

3.1.6 Changes in EGFR related MAPK target gene expression

To investigate further downstream changes in the EGFR pathway, we subsequently measured the change in expression of MAPK target genes from the Immediate Early gene family (FOS, JUN, and EGR1) in response to single or dual EGFR targeted therapy. In both LIM1215 and DiFi cell lines there were clear decreases in expression levels of these three genes relative to cetuximab or erlotinib treatment alone.

In the LIM1215 cell line the combination resulted in only 3% expression relative to control of both JUN and EGR1. In contrast, cetuximab led to only 44% relative expression of JUN and 43% of EGR1, and erlotinib led to a 67% relative expression of JUN and 21% relative expression of EGR1. Levels of FOS were markedly suppressed by cetuximab and erlotinib alone (7% and 3% respectively), and the combination expression level (2%) was not significantly different (Figure 20).

Cetuximab treatment in the DiFi cell line did not significantly alter the expression level of FOS, JUN or EGR1 (92%, 92%, 106% respectively). However erlotinib led to 38%, 32% and 42% relative expression of these three genes. The combination of cetuximab and erlotinib led to marked further reductions in expression in FOS and JUN (3%, 8% respectively), but not EGR1 expression (Figure 20).

Figure 18: Change to pEGFR, pMAPK and pAKT in LIM1215, DiFi, HCA7 cells at 24 hours

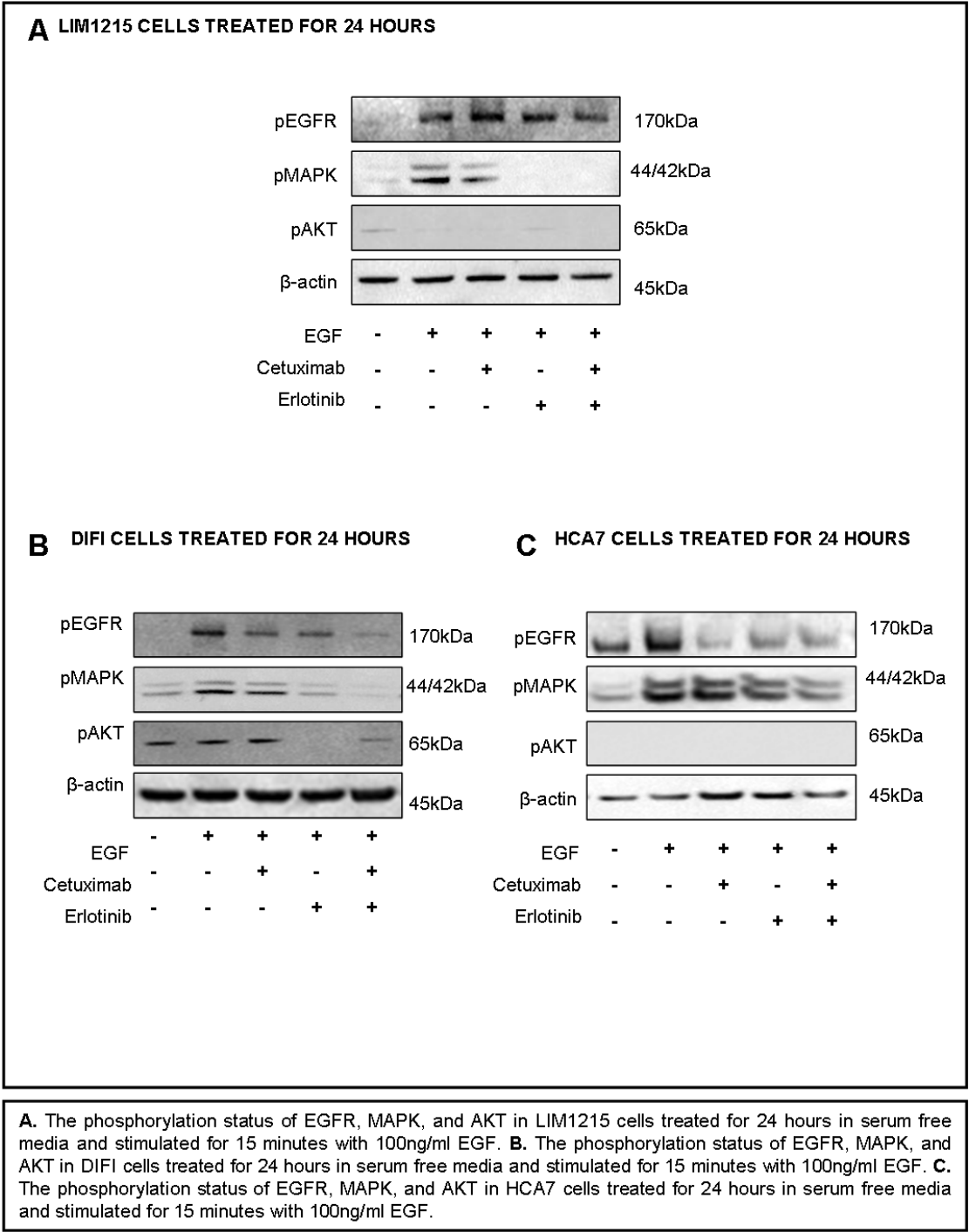


Figure 19: Change in pEGFR, pMAPK, pAKT in RKO cells with treatment

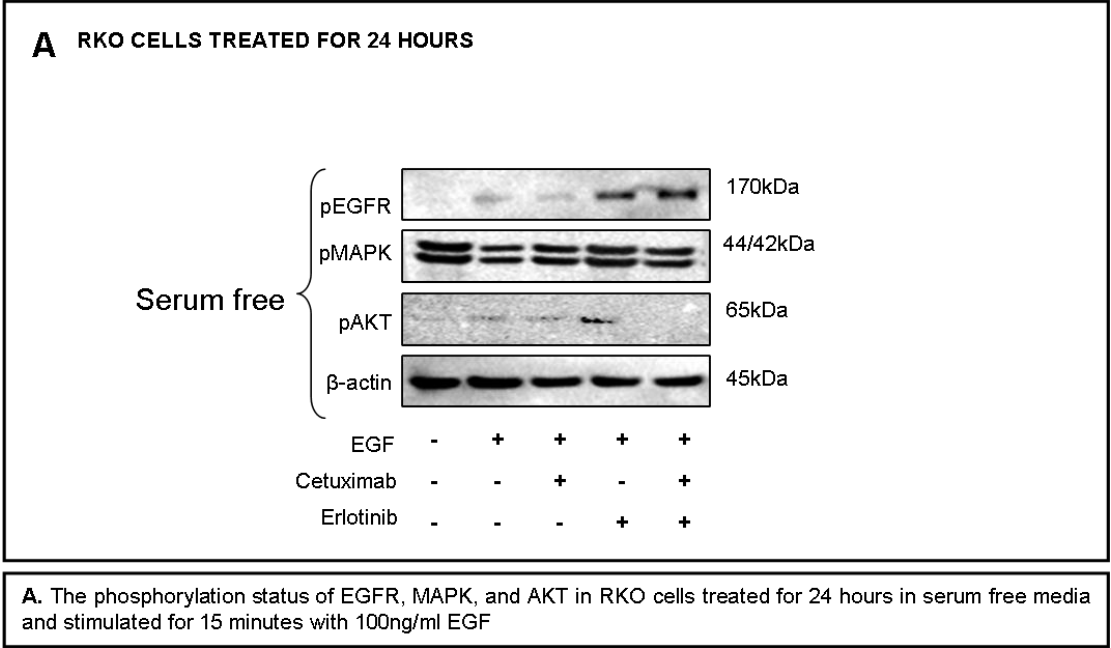
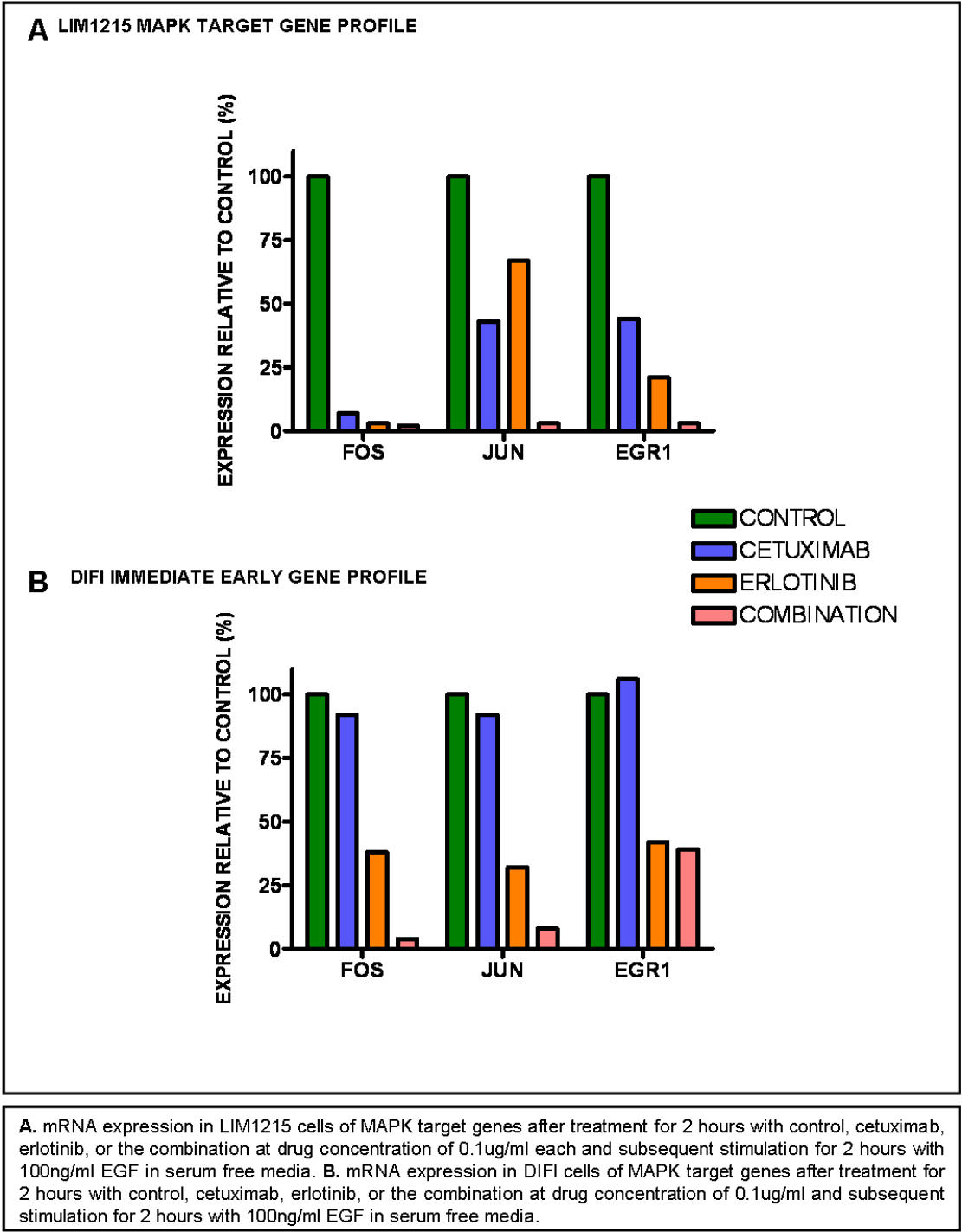


Figure 20: Change in MAPK target gene expression with treatment



3.1.7 Changes in other signalling pathways

In order to better understand the multitude of changes within cancer cell lines in response to dual targeting of the EGFR, we next carried out an analysis to look for change in protein phosphorylation using a human proteome array (R&D Systems), which enabled detection and determination of phosphorylation levels of 46 different proteins.

LIM1215 cells were treated with cetuximab, erlotinib or the combination for 2 hours and stimulated with EGF for 15 minutes before being lysed and phospho-kinase levels determined. To validate the arrays, we compared changes in pMAPK levels from the arrays to that previously obtained by western blot and demonstrated broad concordance (Figure 21). Levels of pMAPK fell with either cetuximab or erlotinib treatment in both experiments. There was a more significant decrease with erlotinib treatment relative to cetuximab. Furthermore both methods demonstrated even further reduction in pMAPK levels with treatment with the combination, greater than cetuximab and erlotinib alone.

There was also greater reduction in pAKT levels in cells treated with the combination in comparison to the either drug alone. Cetuximab led to a 2% reduction, erlotinib led to a 62% reduction and the combination resulted in a 79% reduction in pAKT. This was not able to be validated using western blotting due to inability to consistently detect pAKT levels. The array also demonstrated that levels of pSrc, a proto-oncogene important in EGFR signalling [217], were significantly less in cells treated with the combination (35% reduction), as compared to treatment with cetuximab (0%) or erlotinib alone (+4%)(Figure 22). Notably, there was a significant reduction in pSTAT3 levels in the cells treated with the combination relative to cetuximab and erlotinib alone. Indeed single agent cetuximab and erlotinib alone led to a relative increase in pSTAT3 of 47% and 6% respectively. In contrast, the combination led to a 65% decrease in pSTAT3 levels. There were also greater reductions in other STAT proteins including pSTAT5a, pSTAT5b and pSTAT6 in cells treated with the combination relative to those treated with single agent therapy alone (Figure 22).

Figure 21: Correlation of Western blot and multi phospho kinase array

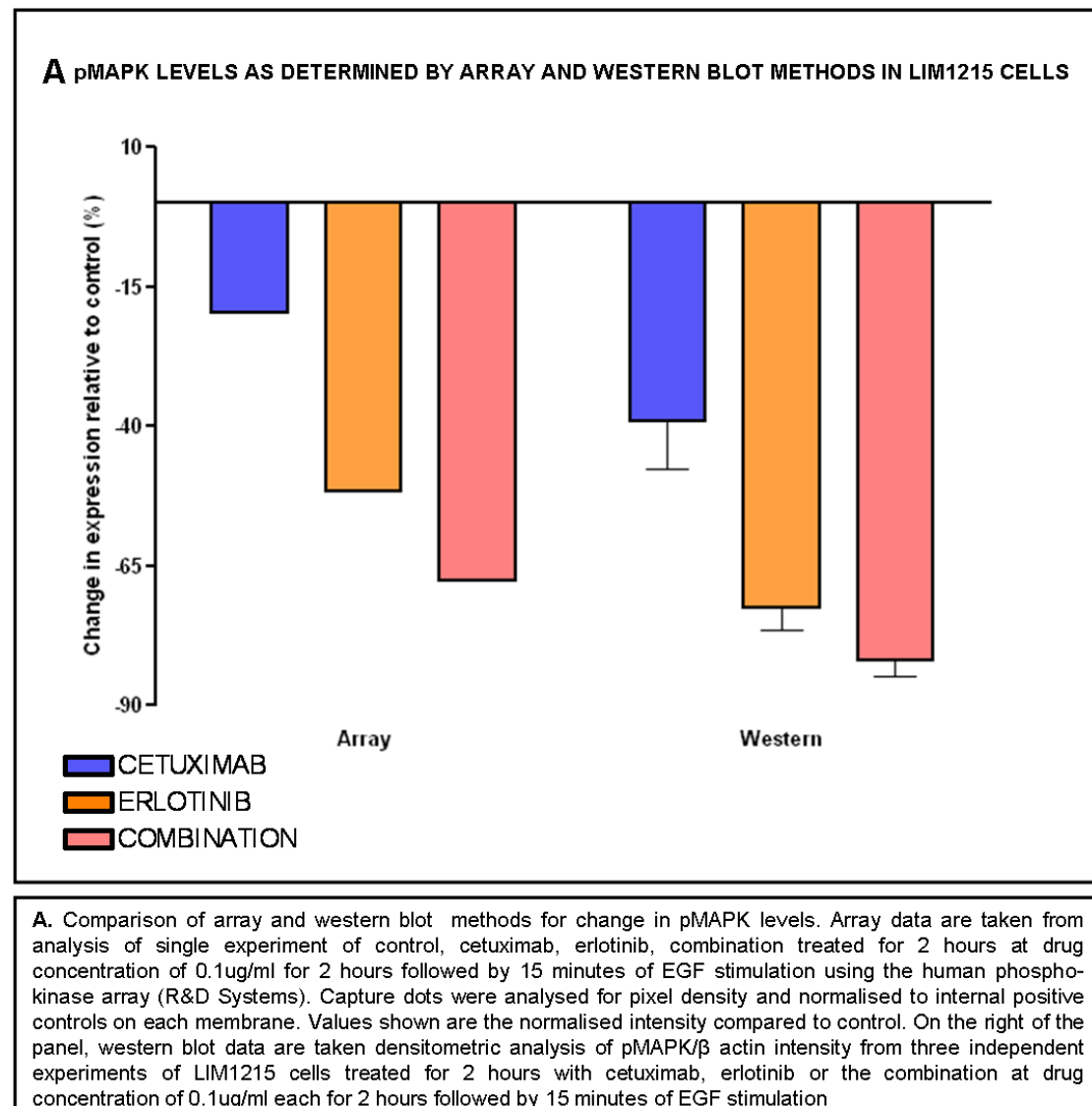
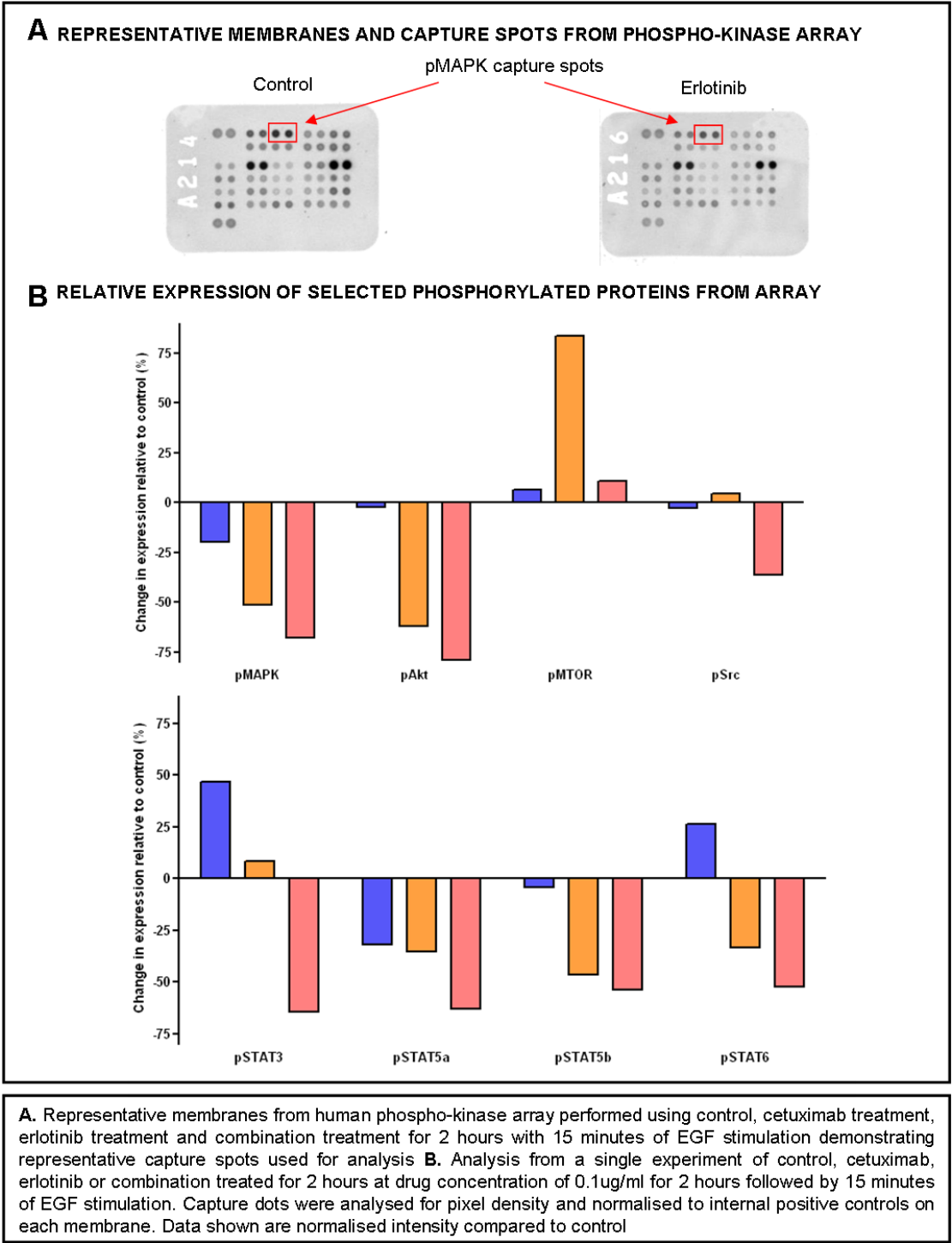


Figure 22: Changes in signalling pathways detected by phopho-kinase array



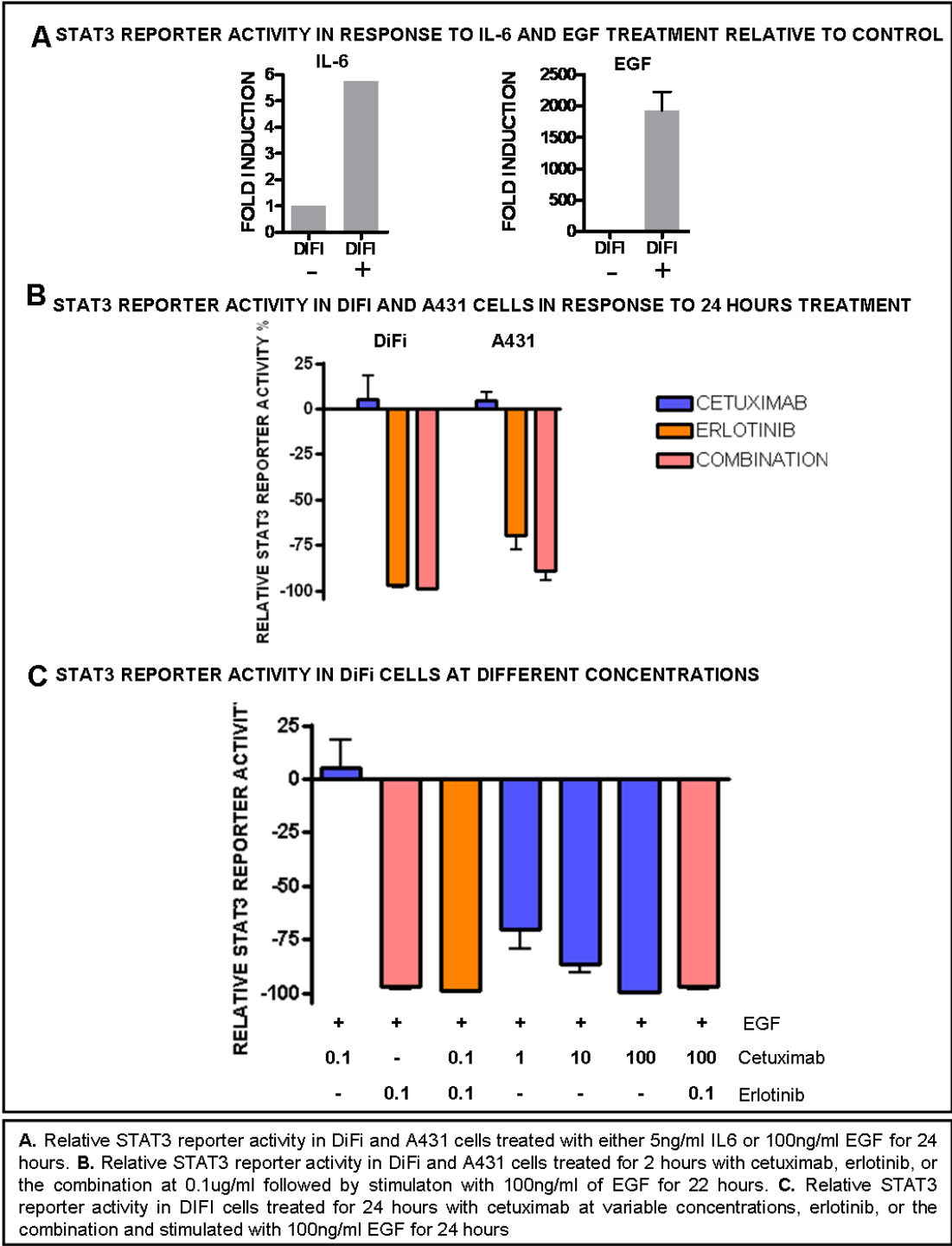
3.1.8 Validation of changes in STAT3 expression

Given the previously established role of STAT3 in modulating angiogenesis and cell motility downstream of EGFR signalling [388, 389], we next attempted to validate the array findings and further quantify changes within the STAT3 pathway following treatment with EGFR inhibition in colon cancer cells.

We used a STAT3 reporter construct, Apre-Luc, to detect changes in STAT3 in response to treatment with cetuximab, erlotinib or the combination. We were unable to transfect the construct or the TK-*Renilla* internal control plasmid into LIM1215 cells despite a variety of different methods. However DiFi cells and A431 cells were both able to be transfected with the plasmids. To establish the functionality of the reporter construct, the cells were transfected with the reporter construct and treated with two known activators of the STAT signalling, IL-6, and EGF. STAT3 reporter activity was significantly increased with both IL-6 and EGF stimulation as shown in Figure 23A.

After treatment with cetuximab, erlotinib or the combination for 24 hours at 0.1 µg/ml, and EGF stimulation with 100ng/ml for 22 hours, STAT3 reporter activity relative to TK-*Renilla* activity was measured (Figure 23B). After 24 hours treatment, cetuximab led to a mild increase in STAT3 activity (5%) in both DiFi and A431 cells compared to control. In contrast, erlotinib led to a 97% decrease of STAT3 reporter activity in DiFi cells and a 69% decrease in A431 cells. Similar to erlotinib treatment, the combination of cetuximab and erlotinib led to a 99% decrease in STAT3 reporter in DiFi cells and 89% decrease in A431 cells. To assess if cetuximab therapy had any effect on the STAT3 pathway, we then assessed STAT3 reporter activity with increasing concentrations of cetuximab in DiFi cells (Figure 23C). There was dose dependent decrease in STAT3 reporter activity with increased doses of cetuximab, with a 70% reduction in activity at 1 µg/ml, 86% reduction at 10 µg/ml and a 99% reduction at 100 µg/ml. Compared to doses of cetuximab of 1 µg/ml and 10 µg/ml, erlotinib still led to markedly greater STAT3 inhibition ($p = 0.05$).

Figure 23: Changes in STAT3 reporter activity in LIM1215 cells following treatment



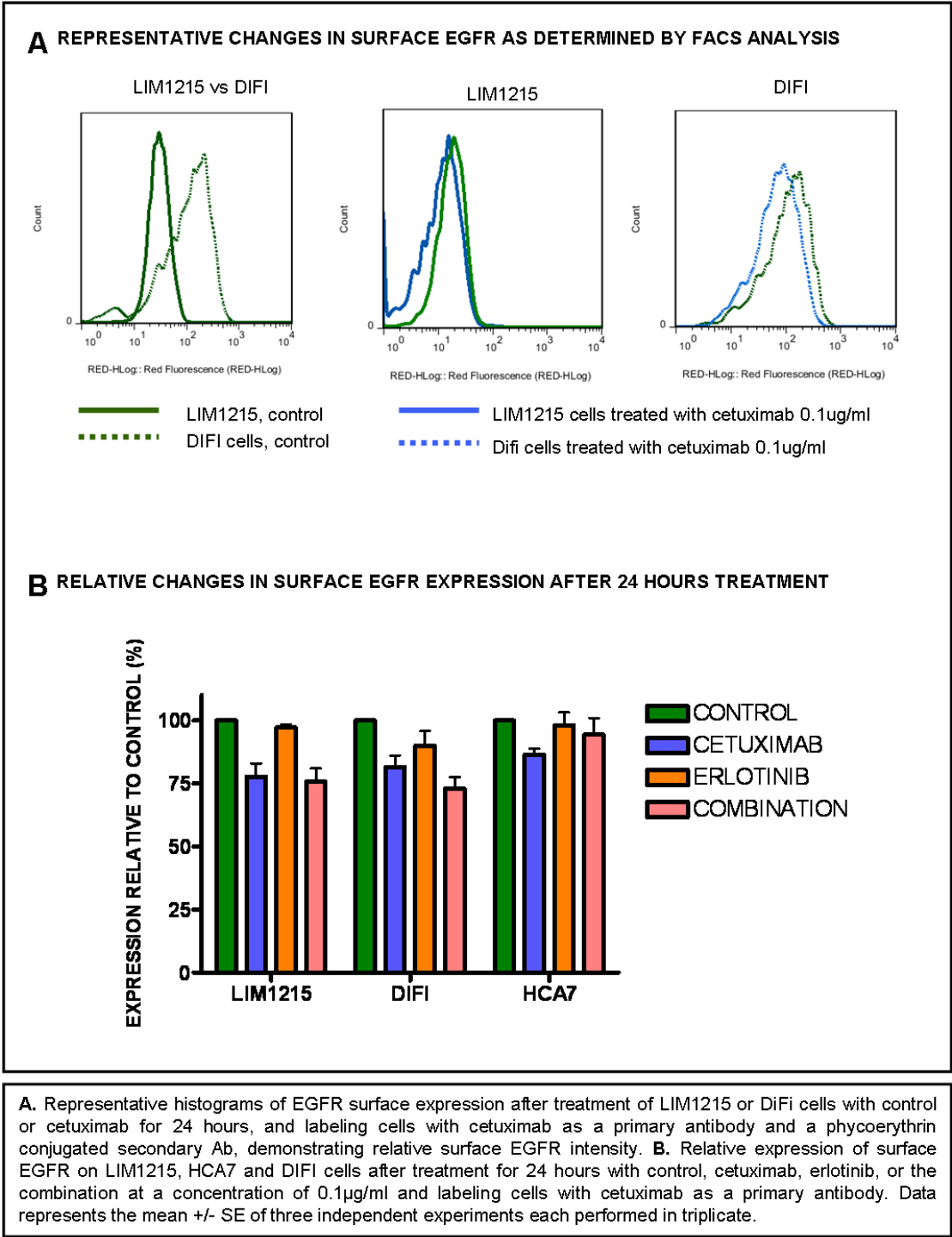
3.1.9 Change in surface EGFR expression

Given that EGFR-mAb therapy of colorectal cancer cells induces down regulation of the EGFR on the cell surface [192], this dynamic may contribute to reduced amounts of binding of the EGFR-mAb to the cell surface and subsequent reduction in cancer cell killing via ADCC [208]. Change in EGFR expression was therefore investigated using flow cytometry and western blot.

LIM1215, HCA7 and DiFi cells were treated with 0.1µg/ml of cetuximab, erlotinib or the combination for 24 hours. For flow cytometry analysis of surface EGFR expression, we used cetuximab as the primary antibody and an anti-human IgG Phycoerythrin (PE) antibody as the secondary antibody. EGFR expression was determined by staining intensity of PE.

Treatment of LIM1215, DiFi and HCA7 with cetuximab resulted in a 22%, 18% and 14% respective decrease in surface EGFR relative to control (Figure 24). In contrast, there was minimal change in surface EGFR expression when the three cell lines were treated with erlotinib (3%, 10%, 2% decrease respectively). The combination led to a decrease in surface EGFR, similar to that induced by cetuximab treatment alone, with a 24% decrease in LIM1215 and 27% decrease in DiFi cell lines. HCA7 cells were the exception to this, with the combination only leading to a 6% reduction in surface EGFR levels. However there was a wider standard error of the mean in this experiment, and this discrepant result should be interpreted accordingly.

Figure 24: Change in surface EGFR expression with treatment

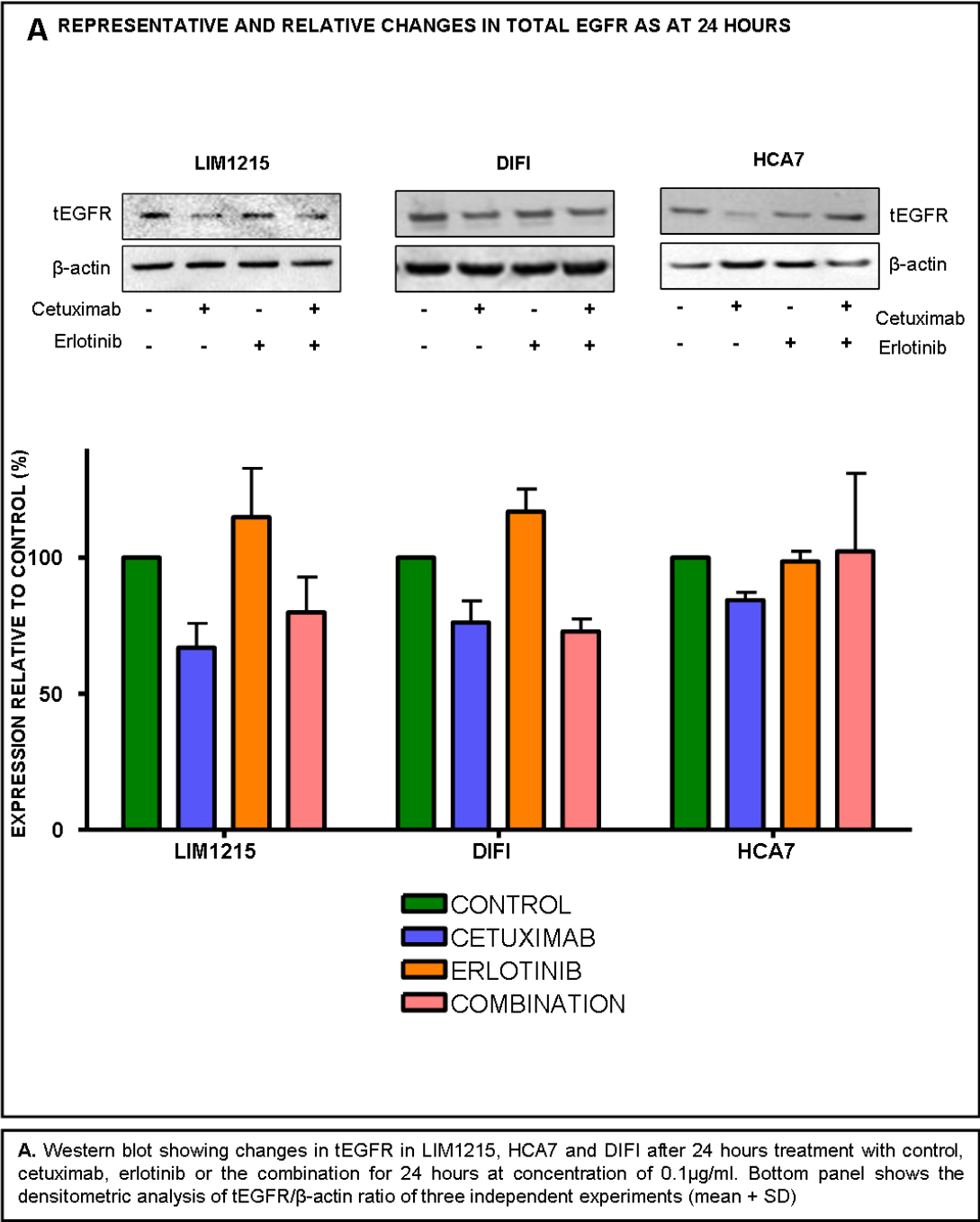


To further validate the observations made with flow cytometry, cellular EGFR levels in the three cell lines was determined using western blot to determine changes to total EGFR expression after 24 hours of treatment. Consistent with the flow cytometry analysis, treatment of the cell lines with cetuximab led to a decrease in EGFR levels, whereas erlotinib had no significant effect on EGFR levels (Figure 25). Change in EGFR following treatment with the combination was similar to that induced by cetuximab alone in LIM1215 and DiFi cells, similar to that seen with flow cytometry. In HCA7 cells the combination seemed to have an effect of preserving total EGFR levels despite treatment. Again in HCA7 cells, similar to the flow cytometry experiment, error bars make interpretation difficult.

3.1.10 Summary of results from *in vitro* experiments

The *in vitro* work confirms observations by others that combined treatment with an EGFR-TKI and EGFR-mAb is an effective strategy that works in colorectal cancer cell lines. The work demonstrated that the enhanced benefit is associated with superior vertical blockade as indicated by more marked inhibition of downstream signalling molecules and MAPK target genes, correlating with higher rates of EGFR related skin toxicity and hypomagnesaemia *in vivo*. The drug combination increased the range of downstream signalling pathways inhibited, as indicated by the selective inhibition of STAT signalling by erlotinib and the combination. This finding is consistent with a previous study demonstrating that a number of genes are differentially regulated by cetuximab and the EGFR-TKI, gefitinib, in A431 cells [195]

Figure 25: Change in total EGFR following treatment



3.2 CLINICAL TRIAL OF DUAL TARGETING OF THE EGFR: RESULTS

Given the preclinical synergy that was demonstrated between cetuximab and erlotinib in colorectal cancer cell lines, and given the previously reported phase I activity of the combination, a non randomised phase II trial was designed to test the clinical efficacy of dual targeting of the EGFR with cetuximab and erlotinib in patients with chemotherapy refractory colorectal cancer.

3.2.1 Patient Characteristics

Between October 2008 and September 2009, 50 patients were enrolled from 4 institutions in Australia. All 50 patients were assessable for safety, with 48 assessable for response. Two patients were ineligible for assessment of the primary endpoint after withdrawing within 3 weeks of commencing treatment due to toxicity.

Baseline patient and disease characteristics are summarised in Table 11. The median age was 63 (range, 39 – 80 years), and 60% of patients were male. All patients had received previous fluorouracil, oxaliplatin and irinotecan. All patients were either PS 0 (20%) or PS 1 (80%). The distribution of metastases and metastatic burden as measured by the sites of metastatic disease were similar to other trials of 3rd line chemotherapy [12].

Given the emerging clinical data on the role of *KRAS*, *BRAF* and *PI3KCA* mutations and lack of response to cetuximab, we determined the mutation profile of these genes amongst the tumour specimens of patients enrolled on the study. Finally, we also determined the mutation status of the *EGFR* gene from the tumours, given the association of EGFR-TKI use and response to these mutations.

There were 11 patients who had mutations in *KRAS* and 8 patients who had mutations in *BRAF*. *BRAF* mutations and *KRAS* mutations were mutually exclusive. There were 4 patients who had mutations in *PI3KCA*: 1 with an additional *KRAS* mutation, 1 with a *BRAF* mutation and 2 with no other concurrent mutations. There were no EGFR

mutations detected in exon 19 or 21 of any of the 50 patients, consistent with previous reports that these mutations do not occur in patients with colorectal cancer.

Table 11: Baseline demographics and characteristics of patients on DUX trial

Characteristic	Number of patients	%
Age		
median	63	
Range	36 – 80	
Gender		
Male	30	60
Female	20	40
ECOG PS		
0	10	20
1	40	80
Disease site of primary		
Colon	30	60
Rectum	20	40
Number of organ sites		
1	12	24
2	20	40
>2	18	36
Site of measurable disease		
Lymph node	24	48
Lung	28	56
Liver	41	82
Peritoneum	8	16
Previous chemotherapy		
5FU	50	100
Irinotecan	50	100
Oxaliplatin	50	100
Anti angiogenic therapy*	14	28
Adjuvant therapy	17	34
Mutation status		
KRAS MUTATION	11	22
BRAF MUTATION	8	16
PI3KCA MUTATION	4	8
EGFR MUTATION	0	0

3.2.2 Efficacy

Efficacy data is summarised in Figures 26, 27, 28 and Table 12. In total 48 patients were assessable for response according to RECIST criteria. Fifteen objective responses were observed (15 PRs, 0 CRs), yielding an overall response rate of 31% (95% CI 19 – 46%). There were no responses in the initial 10/11 patients with *KRAS* mutant tumours, after which the TMC ceased recruitment of patients with *KRAS* mutant tumours. The overall response rate in the *KRAS* wild type patients was 41%

(95% CI 25 - 58%), with 15 confirmed responses out of 37 patients. Patients who were both *KRAS/BRAF* wild type had a RR of 52% (95% CI 34 – 69%), with no responses in those patients with *BRAF* mutations. None of the 2 patients with *PI3KCA* mutations who were *KRAS/BRAF* WT achieved a response.

Median PFS in all patients was 4.6 months (95% CI 2.8 – 5.6 months). Overall PFS time was censored in 4 patients (3 patients related to withdrawal of consent due to patient preference, 1 patient still on study). Median PFS in *KRAS* wild type patients was 5.5 months (95% CI 2.9 – 5.6 months) and 2.7 months (95% CI 1.6 – 5.6 months) in *KRAS* mutant patients. At the time of the analysis (November 2010) 13 patients were still alive and the median OS was 12.1 months (95% CI 8.7 – 15.0 months) after median follow up of 17.4 months. *KRAS* wild type patients had a median OS of 12.9 months (95% CI 8.8 – 16.1 months) and *KRAS* mutant patients had a median OS of 7.3 months (95% CI 4.1 - 12.1 months).

Table 12: Response rate, PFS and OS in DUX trial

	All Patients n = 50 *		<i>KRAS</i> WT n = 39 *		<i>KRAS</i> MT n = 11		<i>KRAS</i> WT / <i>BRAF</i> WT n = 31*		<i>BRAF</i> MT n = 8	
Partial response rate	15	31%	15	41%	0	0%	15	52%	0	0%
Stable disease rate	19	40%	13	35%	7	64%	10	34%	3	38%
Progressive disease	14	29%	10	26%	4	36%	5	16%	5	62%
Median PFS (months)	4.6		5.5		2.7		5.6		2.1	
95% CI	2.8 – 5.6		2.9 – 5.6		1.6 – 5.6		4.6 – 7.0		1.4 – 2.8	
PFS Events	43/50		35/39		11/11		27/31		8/8	
Progression free rate at 6 months	30%		36%		9%		48%		0%	
Median OS (months)	12.1		12.9		7.3		13.2		7.9	
95% CI	8.7 – 15.0		8.8 – 16.1		4.1 – 12.1		10.0 – 17.4		3.1 – 15.2	
Deaths	28/50		26/39		11/11		19/31		7/8	
6 month survival rate	70%		72%		64%		74%		63%	
12 month survival rate	52%		56%		36%		61%		38%	

Table 2. Response rate, PFS and OS outcomes. WT = Wild type. MT = mutant. 2 patients in *KRAS/BRAF* WT group were ineligible for response evaluation after withdrawal from therapy within 3 weeks due to adverse events

* Indicates subgroup where 2 patients who were not eligible for analysis of response evaluation due to withdrawal from therapy

Figure 26: Waterfall plot of response, PFS and OS of DUX trial

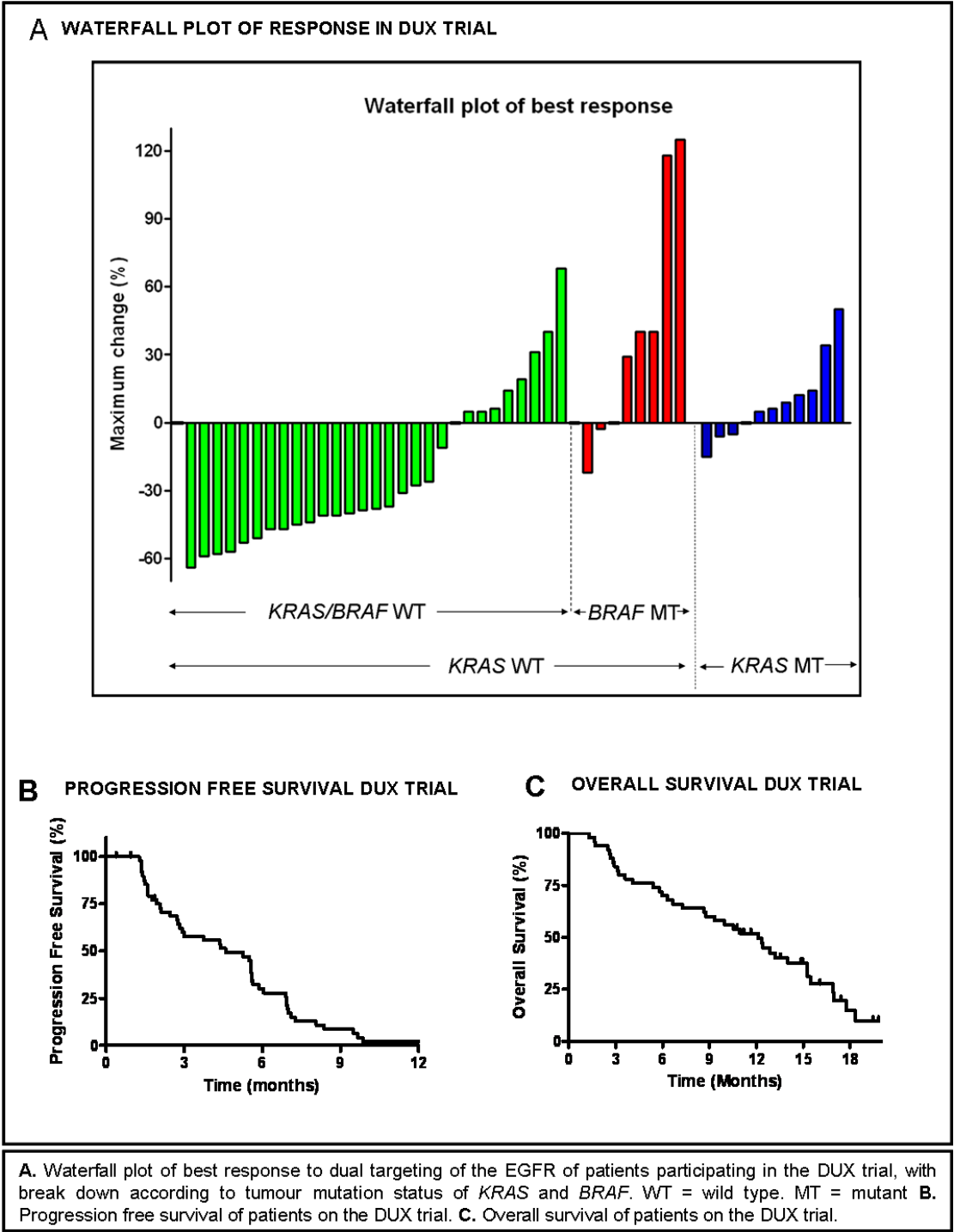


Figure 27: PFS and OS according to KRAS status:

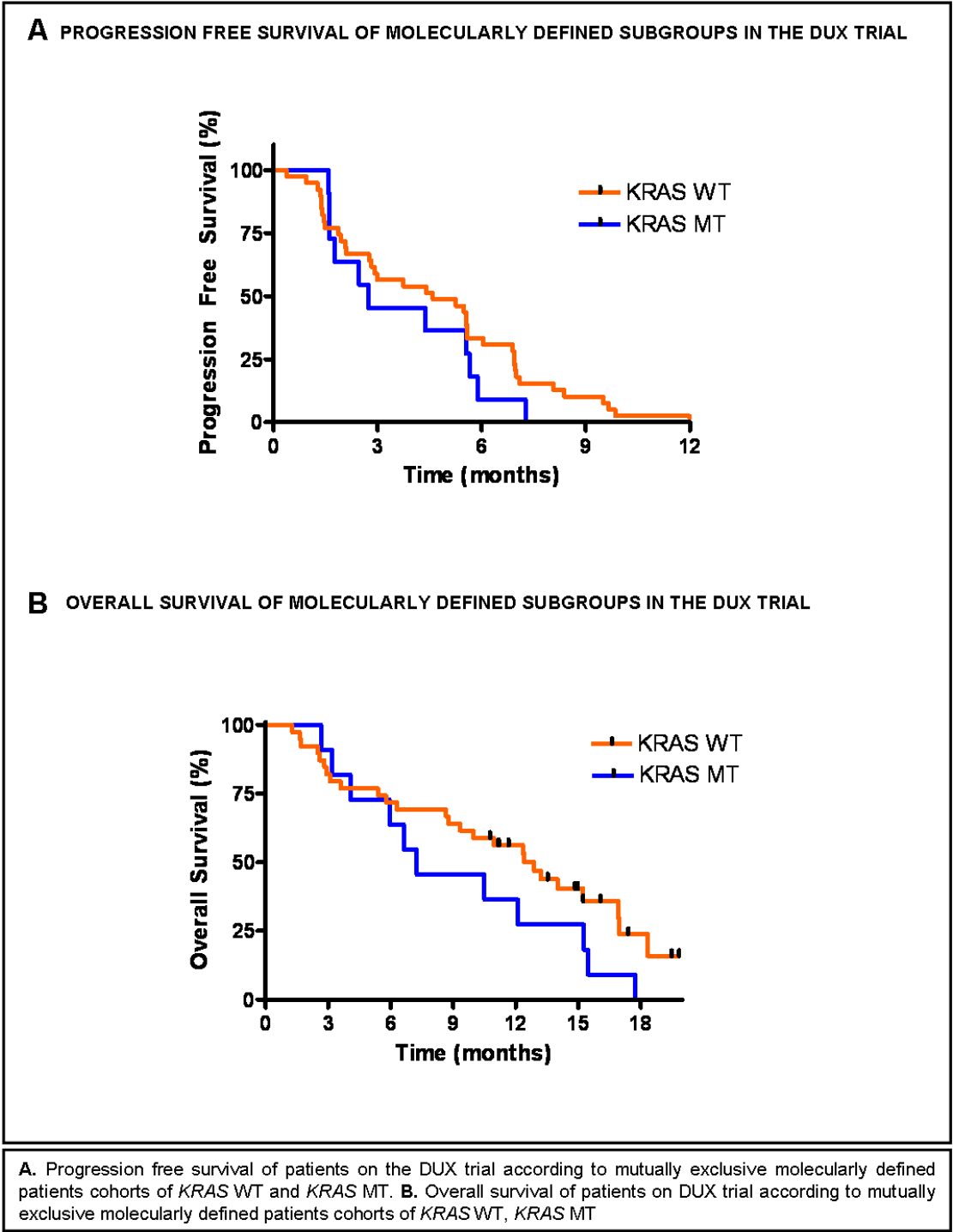
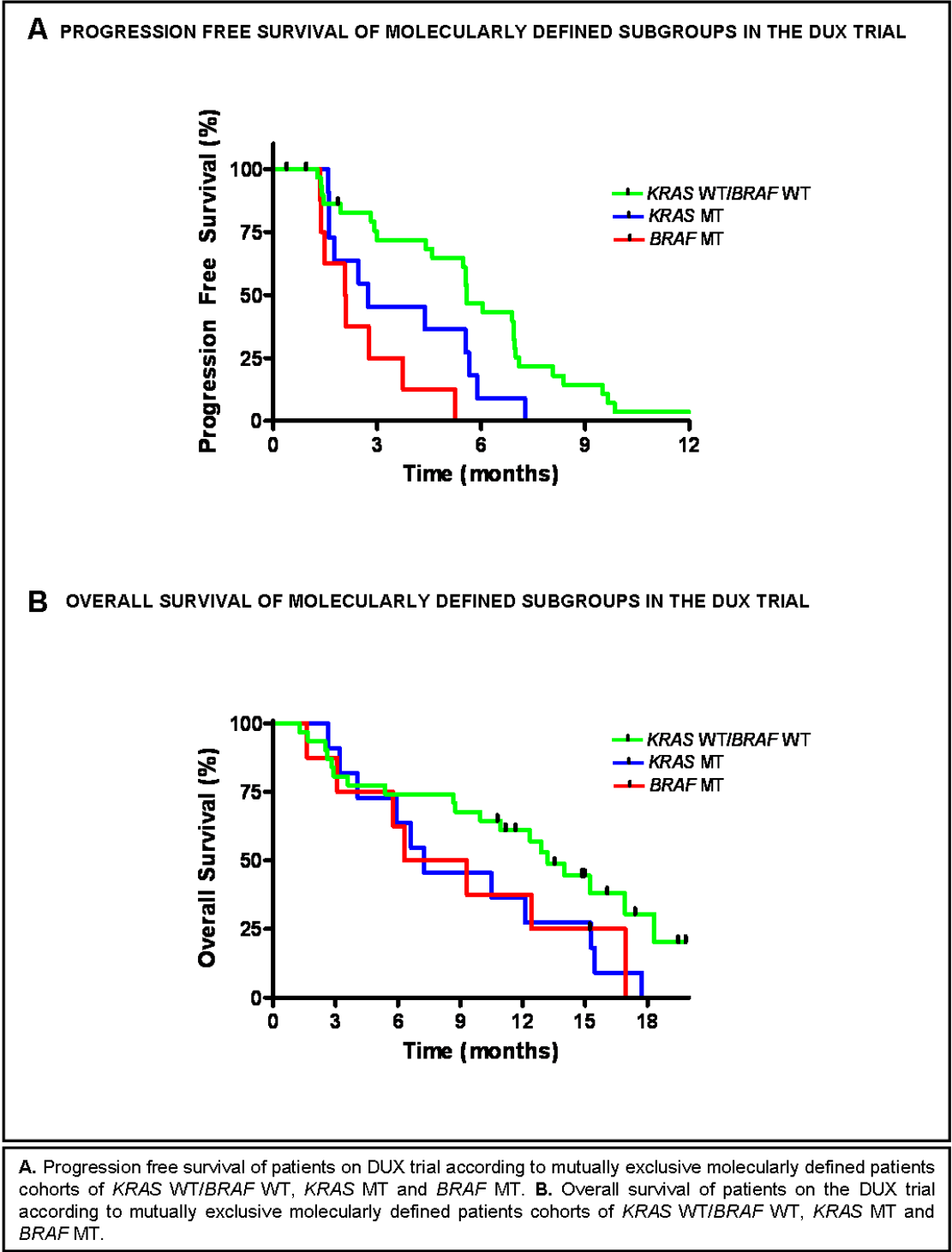


Figure 28: PFS and OS according to tumour mutation status



This was a single arm study, so no formal analysis of predictive interaction between mutation and outcome was undertaken. Additionally, previous reports suggest that *BRAF* is a prognostic factor for overall survival in metastatic colorectal cancer [72, 73]. Therefore the poorer median PFS and median OS in patients with *BRAF* mutations may be due to poor prognosis, rather than absolute lack of benefit from the combination. However as there were no objective responses in this group, it suggests that these patients received little benefit from cetuximab and erlotinib, with median PFS 2.1 months, and median OS 7.9 months. Analysis for outcome of the patients with *KRAS* and *BRAF* wild type tumours demonstrated a PFS of 5.6 months (95% CI 4.6 – 7.0 months) and OS of 13.2 months (95% CI 10.0 – 17.4). This subgroup of patients seemed to derive the greatest benefit of treatment with cetuximab and erlotinib. PFS and OS curves are shown in Figure 27.

3.2.3 Feasibility and safety

There were no treatment related deaths. The median duration of cetuximab treatment was 16.5 weeks, with 70% of patients achieving a relative dose intensity $\geq 80\%$ of cetuximab. Cetuximab treatment was omitted for at least 1 week in 40 patients for a median duration of 2 weeks. Dose reductions of cetuximab were required in 10 patients. The most common cause for delay or dose reduction of cetuximab was skin toxicity (24 patients, 48%). The median duration of erlotinib treatment was 16.5 weeks, with 62% of patients achieving a relative dose intensity $> 80\%$. In the majority of patients (37 patients; 74%) erlotinib was omitted for at least 1 week for a median of 2 weeks, and dose reductions occurred in 18 patients (Table 13). The most common cause for delay or dose reduction of erlotinib was skin toxicity (23 patients, 46%). Four patients withdrew from study due to adverse events (1 fatigue related, 3 with G1- G3 rash related complaints).

Table 13: Dose intensity of cetuximab and erlotinib in DUX trial

Treatment parameter	Cetuximab		Erlotinib	
Duration of treatment				
Median	16.5 weeks		16.5 weeks	
Range	2-38 weeks		2 – 39 weeks	
Median dose intensity	204mg/m ² /week		88mg/day	
Relative dose intensity	No of pts	%	No of pts	%
>90%	21	42%	23	46%
80-90%	14	28%	8	16%
60-80%	13	26%	13	26%
<60%	2	4%	6	12%

3.2.4 Toxicity

37 patients had died at the time of the main analysis. There were no treatment related deaths. Grade 3 - 4 adverse events (AEs) were reported for 68% of patients. Treatment was discontinued due to AEs in 3 patients. The most frequent reasons for discontinuing treatment was skin toxicity in 2 patients.

The most commonly reported grade 3 - 4 AEs were rash (48%), hypomagnesaemia (18%), fatigue (10%), and diarrhoea (6%). The most frequent skin reaction was rash (96%), but other EGFR related skin toxicities such as dry skin, skin fissures, paronychia and hand foot syndrome were also observed. Adverse events are listed in Table 14.

Table 14: Adverse events in DUX trial

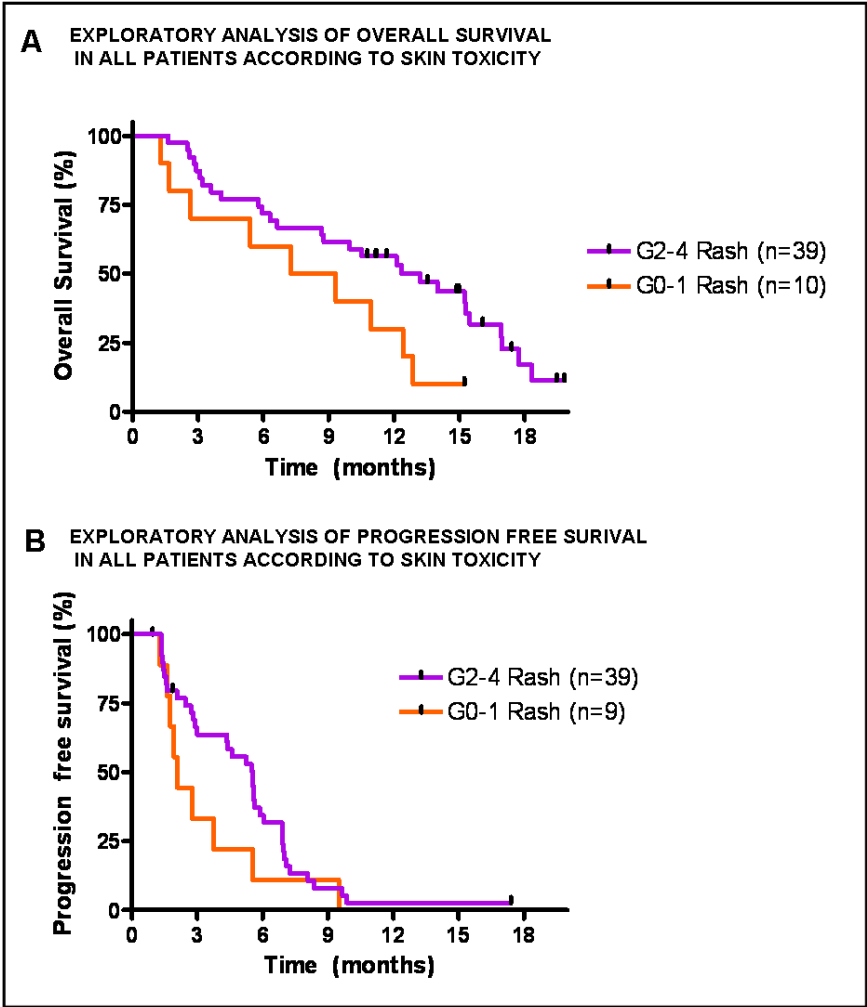
Adverse Event	Grade 3-4 events		All Grades	
	No. of Patients	%	No. of Patients	%
Rash	24	48	48	96
Hypomagnesemia	9	18	19	38
Fatigue	5	10	29	58
Diarrhea	3	6	25	50
Pruritis	3	6	20	40
Pain	1	2	38	76
Dry skin	0	0	14	28
Arthralgia	0	0	16	32
Edema	0	0	11	22
Vomiting	0	0	10	20
Conjunctivitis	0	0	9	18
Anorexia	1	2	8	16
Constipation	1	2	8	16
Nausea	1	2	8	16
Fever	1	2	7	14
Dyspnea	0	0	7	14
Nail Changes	0	0	7	14
Neuropathy	0	0	6	12
Dry eye	0	0	6	12
Rigors/Chills	0	0	5	10
Taste alteration	0	0	5	10

3.2.5 Efficacy analysis by skin toxicity and hypomagnesaemia

Based on previous work that has correlated response to EGFR inhibitors with the degree of skin toxicity [127, 138], outcomes were analysed for patients according to G0-1 vs G2-4 skin toxicity (Figure 29), censoring patients who withdrew from treatment before 28 days, to minimise lead time bias, consistent with the analysis used in previous work [20, 138]. The landmark analysis was performed *ad hoc* and was not pre-specified.

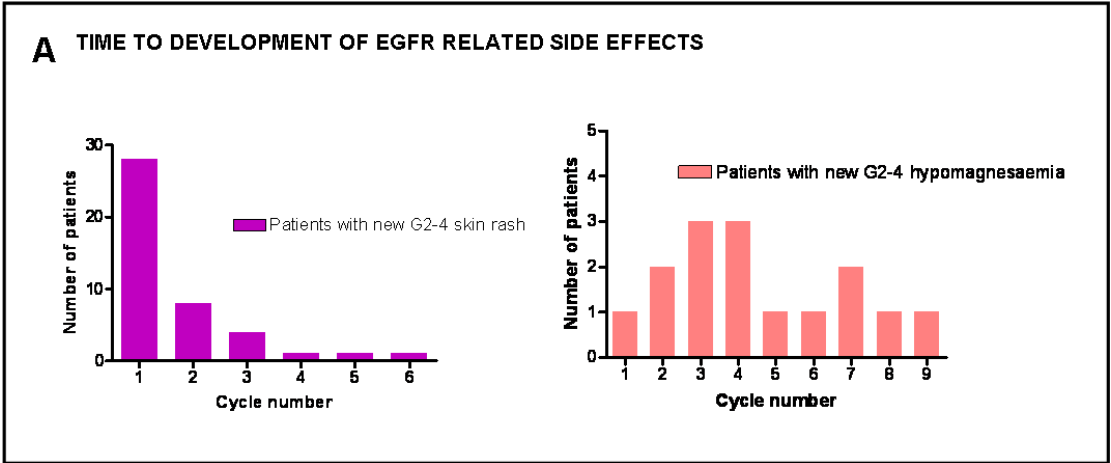
In order to assess if skin toxicity or magnesium toxicity was a surrogate for extended treatment, the median time was calculated to occurrence of G2-4 skin toxicity or hypomagnesaemia (Figure 30). The median time to G2-4 skin rash was 0.7 months. However the median time to G2-4 hypomagnesaemia was 3 months. This latency confounds interpretation of low magnesium as it will bias towards patients with longer survival on the trial.

Figure 29: Association of skin toxicity and outcome



A. Exploratory analysis of OS according to skin toxicity in all patients on the DUX trial. B. Exploratory analysis of PFS according to skin toxicity in all patients on the DUX trial.

Figure 30: Time to occurrence of skin/Mg related toxicity



A. Time to development of G2-4 EGFR related toxicity from patients on the DUX clinical trial

In the patient population eligible for this correlative study, the response rate in those patients with G2-4 skin toxicity was 32% (12 out of 39 patients), compared with 22% (2 out of 9 patients). Overall survival was significantly better in patients who experienced at least G2-4 skin toxicity with a median OS of 13.2 months vs 8.3 months ($p = 0.046$)(HR 0.48, 95% CI 0.14 – 0.98)(Figure 29A). PFS also appeared to favour patients with a worse degree of skin toxicity: 5.5 months vs 2.1 months, but this was not statistically significant (HR = 0.58, 95% CI 0.2 – 1.2)(Figure 29B).

To further assess the strength of skin toxicity as a dynamic marker of response to the combination, the analysis was repeated for patients with *KRAS/BRAF* wild type tumours. Again OS significantly favoured patients with G2-4 skin toxicity: 16.9 vs 6.3 months ($p = 0.0084$)(HR = 0.27, 95% CI = 0.02 – 0.58)(Figure 31). There was a trend towards better PFS in the subgroup who experienced worse skin toxicity, but this was not significant 6.1 vs 3.7 months (HR = 0.69, 95% CI 0.18 – 2.22).

The trial was also analysed for the correlation of G2-4 hypomagnesaemia with outcome in an exploratory analysis, based on work suggesting a correlation in patients treated with cetuximab [142, 143]. Similar to the skin analysis, outcomes were analysed for patients based on the appearance of G0-1 vs G2-4 hypomagnesaemia, censoring patients who withdrew from treatment before 28 days. G2-4 hypomagnesaemia was documented in 15 patients (31%). The response rate was higher in the subgroup with worse hypomagnesaemia, with 8 responders (53% response rate) as compared to 7 responders (21% response rate) in the 35 patients who did not experience G2-4 hypomagnesaemia. Although there was a trend for improved outcomes with G2-4 hypomagnesaemia, the difference in OS between these groups was not statistically significant: 12.9 vs 10.9 months (HR 0.82, 95% CI 0.42 – 1.63), and neither was there any significant difference in PFS 5.6 months vs 2.7 months (HR = 0.54, 95% CI 0.28-1.06)(Figure 32). There was no statistical difference between PFS and OS according to magnesium level in the *KRAS/BRAF* WT group

Figure 31: Outcome according to skin toxicity in KRAS/BRAF WT patients

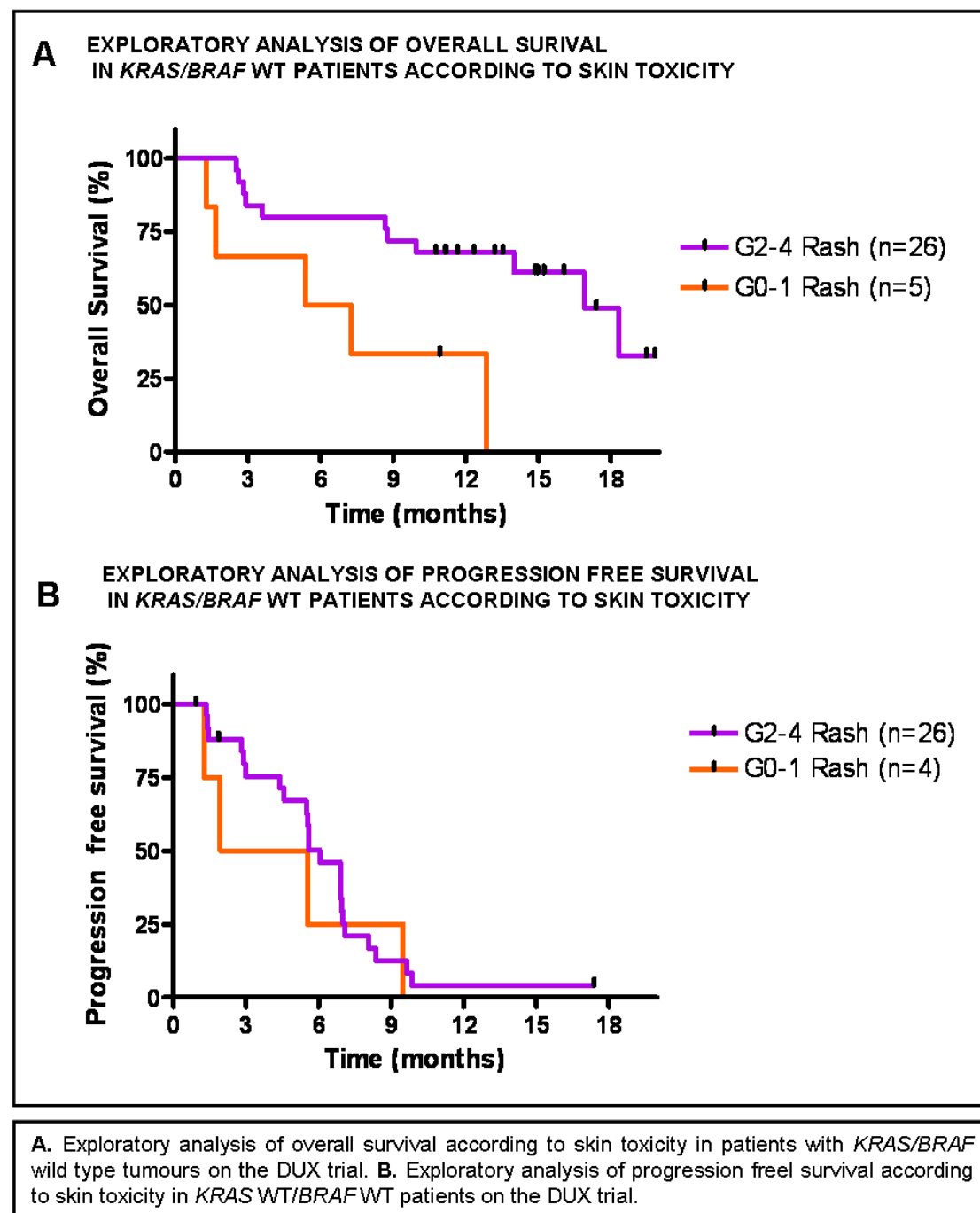
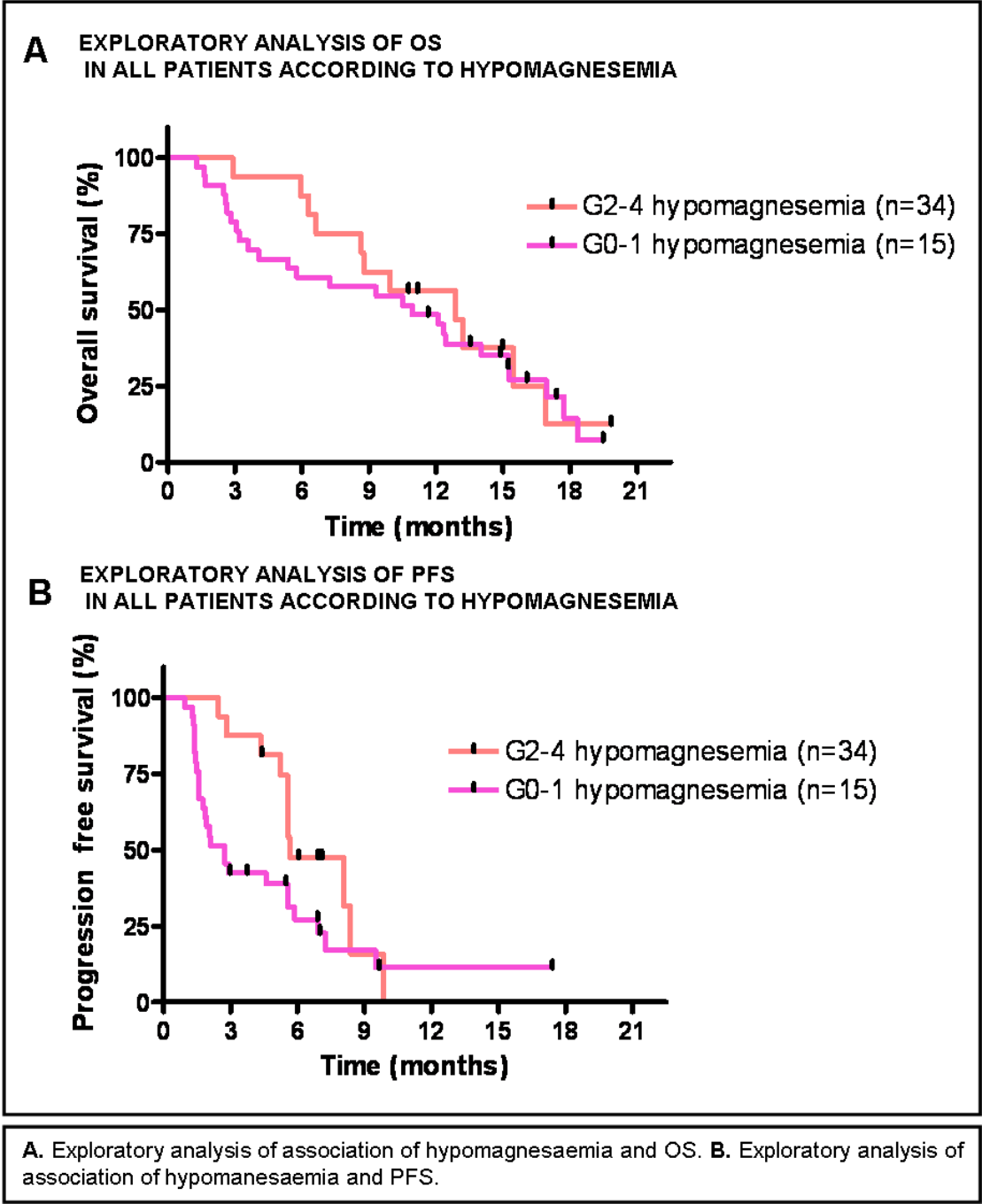


Figure 32: Outcome according to hypomagnesaemia



3.3 DUAL TARGETING OF THE EGFR: DISCUSSION

Given encouraging activity of the combination of EGFR-mAb and EGFR-TKIs in preclinical non colorectal cancer cell lines as well as encouraging early phase I clinical data [195, 201], this thesis explored the activity of the EGFR inhibitors cetuximab and erlotinib in human colon cancer cell lines.

3.3.1 Dual targeting of the EGFR in *KRAS/BRAF* colon cancer cell line is synergistic.

The combination was synergistic in inhibition of growth of colorectal cancer cell lines lacking *KRAS* and *BRAF* mutations. The observation was confirmed in three cell lines, at different concentrations, at different ratios of the drugs, at doses that are achievable *in vitro*, and by using multiple methods (cell number, MTS assay, flow cytometry).

The findings are consistent with a number of previous studies in different cell types [192, 193, 195]. In contrast, a study documenting the interaction between gefitinib and ICR62 (an anti-EGFR-mAb) reported antagonism between the drugs [197]. In this study the authors were unable to demonstrate any enhanced growth inhibition with the combination in DiFi cells, which contrasts to the demonstration of synergy in our work and that of Matar *et al* [195]. A possible explanation for this is methodological differences: a different drug was used by the authors of the negative study (ICR62 vs cetuximab), a different assay was used and higher doses of the drugs used relative to our investigation.

This thesis set out to examine if the combination of cetuximab and erlotinib could overcome intrinsic resistance to cetuximab. Cell lines that had *KRAS* or *BRAF* mutation were resistant to both drugs and there was no enhanced effect with the combination. This observation is consistent with findings by Cunningham *et al* [197], who identified that colon cancer cells resistant to an EGFR-mAb did not gain any benefit from the combination. Although unacknowledged in the initial report, by using the COSMIC database (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>), all of their colon cancer cell lines are identified now as having *KRAS* or *BRAF* mutations,

with the exception of DiFi (discussed earlier), which are now known to confer resistance to EGFR-mAb therapy [64, 69]. Their study therefore supports this report's conclusion that the combination cannot overcome intrinsic resistance governed by either a *KRAS* or *BRAF* activating mutation. These findings also translate into our DUX clinical trial results, where there were no objective responses amongst patients with either *KRAS* or *BRAF* mutations. Whilst this report cannot exclude the possibility that these patients derived some benefit from the use of the combination as it was a non randomised clinical trial, such benefit seems likely to be small or negligible.

We also explored whether cell cycle arrest and or apoptosis contributed to the superior growth inhibition when the combination of drugs was used. Rarely does cetuximab or erlotinib lead to apoptosis in cancer cell lines [26, 48]. Other studies using dual EGFR targeting agents in non colorectal lines have shown modest increases in apoptosis with the use of the combination over single agent therapy [192, 193, 195]. In this study, single agent treatment resulted in minimal effects on apoptosis which was not enhanced by the combination. However the combination did lead to an increase in cell cycle arrest in G1 phase relative to either drug alone.

Given the discrepancy between the lack of apoptosis in the *in vitro* experiments and the high rate of clinical activity as evidenced by tumour shrinkage in the clinical trial of the combination, methodological issues that may have prevented detection of apoptosis were analysed. First, the possibility that the cell lines selected for our study were inherently resistant to apoptosis was excluded as apoptosis was able to be induced with 5FU treatment. Second, the time of analysis for apoptosis was varied. However no change in apoptosis was detected after 1 day and 7 days treatment with cetuximab, erlotinib or the combination in multiple cell lines. Therefore this study demonstrates that the combination does not cause detectable apoptosis in colorectal cancer cell lines *in vitro*. Potential mechanisms by which the combination may induce tumour regression *in vivo* include inhibition of angiogenesis and augmentation of immune mediated cell death [26]. The full exploration of these mechanisms *in vivo* was not possible as tumour tissue pre and post treatment was not available.

3.3.2 Broader and increased inhibition of EGFR pathways explains the synergy

This thesis then explored potential mechanisms underlying the observed synergy of the combination by examining the magnitude and the breadth of EGFR related pathways following combination treatment.

One important mechanism of benefit was that the two agents together exerted a superior blockade of the classical MAPK EGFR related signalling pathway. Greater inhibition of phosphorylation of the EGFR and of MAPK, as well as greater inhibition of the target genes of this pathway (FOS, JUN and EGR1) were observed with the combination. Similar effects were observed across three cell lines. Even at high doses of cetuximab we were able to demonstrate further reductions in pMAPK with the addition of erlotinib. This observation is consistent with those made with non colorectal cell lines by other investigators [192, 193]

Consistent with the conclusion that the combination results in greater inhibition of the EGFR pathway is observation of high rates of EGFR related toxicity that was seen in the clinical trial of the combination. Certainly the 48% rate of grade 3-4 skin toxicity is much greater than would be expected for either drug alone, which are typically between 10 - 15% [390]. Furthermore, the adverse event profile of the combination was consistent with the profile for EGFR-mAb or EGFR-TKI therapy suggesting the predominant *in vivo* effect of the combination is through the EGFR pathways and not through inhibition or activation of novel new pathways unaffected by either drug.

A multi-phospho-kinase array was used to screen and profile signalling changes in response to treatment with either drug alone or in combination. Similar to previously presented work [201] several pathways were differentially regulated, or co-regulated between the two classes of drugs and the combination. Amongst these pathways, there was significant suppression of pSTAT3 expression and STAT3 reporter activity in the combination that was not seen with the use of cetuximab alone. STAT3 is a signalling transducer responsible for angiogenesis, proliferation and cell survival [391] which has been identified by this thesis as an additional signalling pathway that is inhibited by the combination, but not by cetuximab alone.

EGFR-TKIs have been demonstrated to be capable of STAT3 inhibition in other preclinical models [35-37]. In contrast cetuximab alone has not been consistently shown to modify pSTAT3 levels in preclinical studies [392, 393]. Although we did demonstrate suppression of STAT3 with higher doses of cetuximab, a recently published clinical study of high dose cetuximab was unable to demonstrate any such inhibition in serial skin biopsies from skin and tumour specimens [394]. Therefore the addition of erlotinib to cetuximab could potentiate growth inhibition via greater STAT3 suppression. Supportive of this are experiments performed by other groups demonstrating that the combination of a STAT3 inhibitor with an EGFR inhibitor enhances anti-tumour effects over EGFR inhibition alone in human cancer cell lines [35, 395].

3.3.3 Changes to surface EGFR do not explain the synergistic effects

Subsequent to the investigation in changes within downstream signalling pathways, this thesis studied changes to surface EGFR expression to explore if differential effects on the receptor could be responsible for the enhanced growth inhibition, particularly that seen *in vivo*. Changes to surface EGFR expression on a tumour would modulate the ability of the host to utilise ADCC when exposed to EGFR-mAb therapy. The role of ADCC in the clinical effectiveness of EGFR-mAb therapy is controversial [396] but potentially a significant contributor to their *in vivo* effectiveness of EGFR-mAbs [397] that is not typically assessed by *in vitro* studies of the drug. The process of ADCC requires the interaction of Natural Killer (NK) cells or neutrophils with the bound EGFR-mAb. Paradoxically though, the response of cancer cells exposed to EGFR-mAb is to internalise the receptor [192, 199, 398], thereby decreasing the surface binding of the EGFR-mAb and reducing the ability of the host to utilise ADCC to kill the cancer cell. In contrast, treatment with an EGFR-TKI leads to stabilisation of the receptor on the cell surface [45, 192, 398]. One possibility therefore is that the TKI may prevent mAb mediated internalisation of the EGFR, retaining the mAb and EGFR on the cell surface. Therefore the change in receptor expression when the combination is of mAb and TKI could possibly enhance the rate of ADCC.

Therefore this thesis assessed EGFR expression with the combination compared to single agent treatment. Consistent with observations from other investigators, cetuximab led to down regulation of the EGFR on the cell surface and erlotinib did not alter the expression level of the EGFR. However when used together as a combination, there was down regulation of the EGFR, to the same degree as that seen with cetuximab alone. This observation was mirrored in findings using total EGFR levels within the cancer lines in response to treatment. A limitation of these findings is ADCC was not specifically tested *in vitro*.

Other published data analysing surface EGFR expression is consistent with these results [192], and suggest that the combination is unlikely to lead to enhanced ADCC *in vitro*. Interestingly, this seems not to be the case in dual targeting of the HER2 receptor with the combination of trastuzumab (HER2-mAb) and lapatinib (HER2-TKI) in breast cancer cell lines [208]. The authors demonstrated enhanced expression of the HER2 receptor in human breast cancer cell lines in response to dual targeting, and consequent higher rates of ADCC as measured by an *in vitro* assay. The different cancer cell lines (breast vs colorectal), different drugs, and different HER family member receptors (HER2 vs EGFR) could explain this discrepancies.

3.3.4 The combination has a high rate of clinical activity

Given the encouraging preclinical data that we and others had demonstrated using EGFR-mAb and an EGFR-TKI in combination, a clinical trial in advanced colorectal cancer was designed to test the strategy.

There was a high rate of objective responses in the cohort of 50 patients, greater than expected with single agent use of cetuximab. There was an overall response rate of 31% and median PFS of 4.6 months, with responses confined to patients who were *KRAS* wild type (41% response rate, median PFS 5.5 months). Previously, the CO17 trial of single agent cetuximab versus placebo demonstrated a response rate of 8% with median PFS of 1.9 months [12], and a response rate of 13% in patients who were *KRAS* wild type, with a median PFS of 3.7 months [15]. Given the DUX trial was non randomised and did not have any patients with a performance status of 2 enrolled (25% of patients in CO17), definitive conclusions regarding superiority over single

agent cetuximab are premature. However the results do appear very encouraging: a randomised trial is now required to assess superiority over alternate strategies.

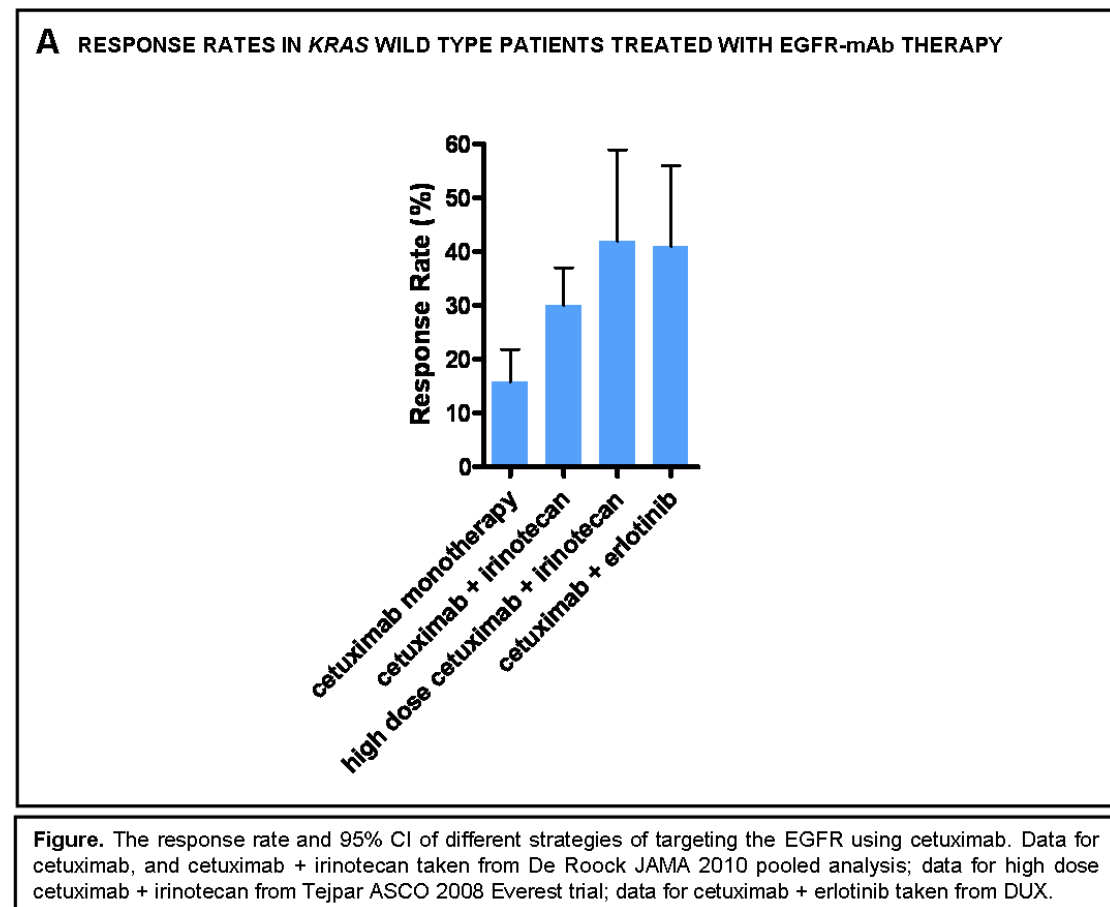
Other strategies to increase the activity of cetuximab in third line treatment of colorectal cancer have been trialed, including the combination of cetuximab with irinotecan [18, 399], and the combination of irinotecan with an increased dose of cetuximab to achieve rash [127]. Both of these strategies have achieved higher response rates than single agent cetuximab alone, but only the latter trial of dose escalated cetuximab achieved response rates similar to our trial (Figure 33).

Notably, the *in vitro* data did not demonstrate that the combination of cetuximab and erlotinib had greater growth inhibitory properties over high dose cetuximab alone. Therefore further formal clinical comparison of the combination compared to high dose cetuximab is planned in the form of a randomised clinical trial.

3.3.5 Dual targeting should be restricted to *KRAS/BRAF* wild type patients

Combined preclinical and clinical data from this thesis suggest that there is little benefit of the combination in patients who have either *KRAS* or *BRAF* mutations. No patients with *KRAS* or *BRAF* mutations had an objective clinical response to treatment with the combination, and both groups had an inferior survival rate relative to patients in the *KRAS/BRAF* wild type group. Outcomes were not analysed according to whether patients had G13D *KRAS* mutations, which has recently been suggested to endow tumours with different clinical characteristics to the more common *KRAS* mutations [98]. Given the trial was only a single arm study, we cannot separate the prognostic role of *BRAF* mutations from predictive properties it may possess for EGFR targeted therapy. However given there were no objective responses with dual targeting, which is consistent with multiple other series [65, 69, 105, 400], there seems no role for using dual targeting of the EGFR in patients with *BRAF* tumour mutations. Therefore, future trials of this strategy should be restricted to patients with *KRAS/BRAF* wild type tumours.

Figure 33: Response rates of cetuximab based treatment of mCRC



Tumours were also analysed for *PI3KCA* mutations, with two documented patients with tumours with *PI3KCA* mutations in exon 10, and two patients with exon 20 mutations, which unlike exon 10 mutations are in the kinase domain and have previously been associated with resistance to EGFR-mAb therapy [98]. None of the four patients with *PI3KCA* mutations had a response to therapy, however two of these patients also had mutations in either *KRAS* or *BRAF*. Given the small number of patients in this trial, meaningful conclusions regarding the significance to therapy cannot be drawn.

3.3.6 Implications of the high rate of EGFR related toxicity

There was a high rate of EGFR related toxicity, including skin rash and hypomagnesaemia in the clinical trial. This raises several issues regarding tolerability, correlation of adverse events with outcome, and implications for dosing strategies with EGFR-mAb therapy.

Forty eight percent of patients experienced Grade 3 – 4 skin toxicity, which is much greater than would be expected with single agent use of cetuximab or erlotinib, with rates typically around 10 – 15% [401, 402]. The rash was typically acneiform; however some patients experienced an erythematous rash and/or pruritis. The maximum grade of skin toxicity occurred early, after a median of 1 cycle of treatment. Patients were treated appropriately with topical steroids, emollient and oral tetracyclines and the majority of patients found the rash tolerable, with only three patients withdrawing because of rash.

Skin toxicity has been previously shown to impact negatively on Quality of Life (QoL) [403] [404], however we did not collect data on QoL in the DUX study. Future trials of the combination of EGFR-mAb and EGFR-TKIs should incorporate QoL data to assess the trade off between toxicity and efficacy. Additionally, given pre-emptive use of oral steroids and doxycycline has been shown to reduce skin toxicity, lessen QoL impairment and not impair efficacy when treating patients with an EGFR-mAb [390], future clinical trials of the combination should consider similar use of pre-emptive measures to lessen patient impact.

Previous analyses have correlated the rate of skin toxicity with increased efficacy of EGFR-mAb in terms of response rate, PFS and OS [138, 139, 405]. There was a similar statistically significant correlation in median OS in patients with G2-4 rash compared to the smaller cohort of patients with G0-1 rash, which was still significant upon restricting analysis to the *KRAS/BRAF* wild type cohort: 16.9 months vs 6.9 months (HR 0.27, 95% CI 0.02-0.58). The severity of rash was an early response indicator of better OS with the combination and is independent of *KRAS* and *BRAF* status. Other authors have reached a similar conclusion [138.].

A plausible explanation for the association of skin toxicity and outcome is that there may be a correlation between affinity of cetuximab for the EGFR within the skin, and within the tumour [406]. This observation would suggest that each patient treated with an EGFR-mAb has a different sensitivity to inhibition of the EGFR pathway, and this sensitivity is similar between the skin and the tumour. This approach was tested in a prospective randomised trial that compared standard dose cetuximab to a strategy of dose escalation to achieve rash in patients with metastatic colorectal cancer. This demonstrated a trend to superior response rates in the dose to rash strategy [127]. A further possibility to explain the importance of skin toxicity is that the concentration of EGFR-mAb is higher in the skin and tumours of patients that respond to therapy [406].

This study and that from Tepjar *et al* [127] suggests that when used at its recommended dose, cetuximab fails to adequately inhibit the EGFR pathway in the majority of patients. A consequence of this is G3-4 rash rates of 10 – 15%, but a response rate of only 10% [98]. An inference of these observations is that cetuximab and panitumumab have a narrower therapeutic window than desirable, and that current dosing strategy ensures only a modest proportion of the activity achievable if higher doses or combination therapy is used whilst accepting skin toxicity as a trade off. Further work is required to compare these strategies in a clinical trial whilst measuring not only tumour related outcomes, but also whilst documenting QoL and tolerability.

3.3.7 Financial and toxicity considerations may limit use of this strategy

Despite demonstrating that the combination has encouraging clinical activity, until further comparative studies are performed, this strategy cannot be recommended for routine clinical use. Furthermore, if the combination therapy does provide superior PFS and OS over cetuximab monotherapy, pragmatic considerations of cost and toxicity burden may limit wide spread use in colorectal cancer.

In regards to cost, the cost of cetuximab and erlotinib is currently AUD \$1400 per week, compared to AUD \$900 per week for cetuximab alone. If the combination is to improve PFS and OS both by a clinically significant 3 months, the extra cost of 3 months of improvement would be approximately \$17,000. This cost per gain in OS would be currently beyond the cost per Quality of Life Year (QALY) ceiling that many Western countries such as Australia would consider subsidising. Erlotinib and cetuximab costs should decrease substantially when eventually off patent, therefore changing the cost benefit ratio.

Additionally, the high rate of skin toxicity means that community oncologists unfamiliar with management may be content to continue using standard dosing of cetuximab whilst accepting inferior outcomes. If validated clinically to be a superior regimen, education regarding management of EGFR related side effects prior to community use should be implemented.

4 ACQUIRED CETUXIMAB RESISTANCE: RESULTS AND DISCUSSION

4.1 IN VITRO MODEL OF ACQUIRED CETUXIMAB RESISTANCE: RESULTS

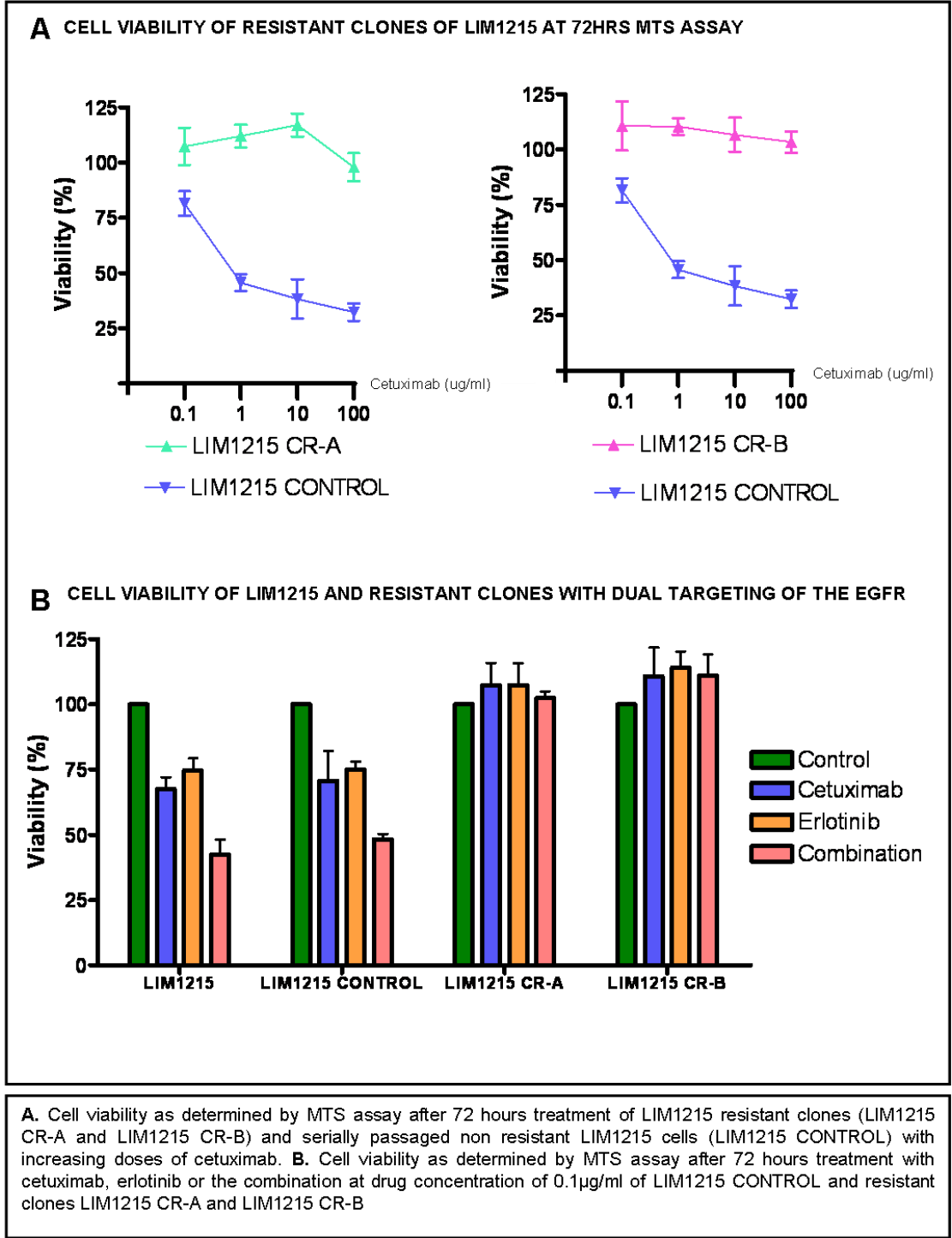
Given that patients who initially respond to EGFR-mAb therapy inevitably progress after several months, this thesis explored mechanisms that underlie this acquired resistance. Previous *in vitro* and *in vivo* data suggests that such mechanisms are either related to either mutation of the target (such as EGFR mutation with EGFR-TKI treatment), or change in compensatory pathways, such as IGFR, c-MET, and Src.

Acquired resistance to cetuximab was modeled by treating the cetuximab sensitive colon cancer cell line LIM1215 continuously with cetuximab for several months. LIM1215 cells were grown in increasing doses of cetuximab over a 6 month period in parallel to generate two new cell lines: LIM1215 Cetuximab Resistant A (CR-A) and LIM1215 CR-B. To eliminate high passage number as a cause of resistance to the drug, LIM1215 cells were also passaged serially alongside the two resistant cell lines in medium with drug vehicle alone, creating a cell line LIM1215 CONTROL.

4.1.1 Change in sensitivity to cetuximab in resistant cell lines

To assess sensitivity to cetuximab, an MTS assay was used to assess growth of the LIM1215 CR-A, CR-B and CONTROL cell lines. The MTS assay was performed after 72 hours growth as previously described. The LIM1215 CONTROL cell line retained sensitivity to cetuximab, with a dose dependent decrease in viability with increasing dose of cetuximab (Figure 34). After treatment with 100µg/ml of cetuximab, viability in LIM1215 CONTROL was 33% of untreated cells. However, in contrast, both LIM1215 CR-A and LIM1215 CR-B were resistant across all doses of cetuximab, with viability of 103% and 98% of control respectively at the 100µg/ml concentration.

Figure 34: Characteristics of cetuximab resistant cell lines



In order to eliminate retention of cetuximab on the resistant cell lines falsely contributing to the appearance of a resistant phenotype, the MTS assay was repeated after 1 week of growth of LIM1215 CR-A in normal growth medium lacking cetuximab and compared to LIM1215 CONTROL (Figure 35). The LIM1215 CR-A cell line was still resistant to cetuximab, even at high doses of the drug (93% viability at 100µg/ml cetuximab), confirming this cell line had acquired resistance to cetuximab. The growth rates of the LIM1215 CR-A, and LIM1215 CR-B cell lines were the same as LIM1215 CONTROL cell line as determined by the ratio of t_{72} / t_0 time points of the MTS assay.

4.1.1.1 Change in sensitivity to erlotinib in resistant cell lines

Given the acquisition of resistance to EGFR-mAb therapy with cetuximab, erlotinib sensitivity was then assessed. The LIM1215 CR-A and CR-B cell lines were cross resistant to erlotinib, with viabilities of 107% and 114% of control respectively after treatment for 72 hours (Figure 32B). In contrast the LIM1215 CONTROL cell line retained sensitivity to erlotinib, with viability 75% after treatment. This was similar to that seen with the LIM1215 cell line (viability 74%).

4.1.1.2 Change in sensitivity to the combination of cetuximab and erlotinib

The MTS assay was also used to assess if the combination of cetuximab and erlotinib could overcome the resistant phenotype of LIM1215 CR-A and LIM1215 CR-B (Figure 34B). Unlike the LIM1215 CONTROL line, there was no benefit of the combination in the two resistant cell lines, with viability in both >100% respectively after 72 hours treatment at 0.1µg/ml. This was in contrast to the reduction in viability in the LIM1215 CONTROL line, where viability reduced from 70% in the cetuximab treated cells and 75% in erlotinib treated cells to 48% viability in cells treated with the combination. The magnitude of the benefit of the combination over single agent drug alone in the LIM1215 CONTROL cell line was similar to that seen with LIM1215 cells treated in earlier experiments (Figure 32B).

4.1.2 Reacquisition of cetuximab sensitivity of LIM1215 CR-A cells

To determine if the acquisition of resistance to cetuximab was permanent, or transitory, over a series of 3 months, a third cell line, LIM1215 CR-A-Normal media

(LIM1215 CR-A-N) was generated. An aliquot of LIM1215 CR-A cells was taken and passaged in medium supplemented with 10% FCS, with no cetuximab. As a control, LIM1215 CR-A was passaged in the presence of 100µg/ml cetuximab. Viability to cetuximab was determined subsequently in each cell line at regular intervals over 3 months (Figure 35).

After 1 week of growth in medium lacking cetuximab, the LIM1215 CR-A-N cells were still resistant to cetuximab, with viability 93% of control at 100µg/ml cetuximab (Figure 35). However after 2 months growth in the absence of cetuximab, LIM1215 CR-A-N was re-sensitised to cetuximab and demonstrated a clear dose dependent decrease in cell viability in response to cetuximab treatment. After treatment with 100µg/ml of cetuximab, cell viability was 46% of control, and not statistically different from LIM1215 CONTROL cells, which had a cell viability of 33% of control at this dose.

4.1.3 Levels of receptor phospho-kinase activation in resistant cell lines

To investigate adaptive changes within the resistant cell lines, a receptor phospho-kinase array was used to screen expression levels of a wide variety of receptor tyrosine kinases on LIM1215 CONTROL, LIM1215 CR-A, LIM1215 CR-B and LIM1215 CR-A-N. Cell lines were grown in normal medium (no cetuximab for 72 hours) before extraction of proteins and analysis. The level of EGFR phosphorylation in both LIM1215 CR-A and LIM1215 CR-B was greater than LIM1215 CONTROL cells (Figure 36). LIM1215 CR-A had a 2.7 fold increase, and LIM1215 CR-B had a 1.9 fold increase in pEGFR levels relative to LIM1215 CONTROL. Interestingly, the LIM1215 CR-A-N cells, resensitised to cetuximab, had a reduced amount of pEGFR, with a 50% reduced amount relative to LIM1215 CONTROL.

Additionally levels of pHER3 were modestly elevated in both LIM1215 CR-A and LIM1215 CR-B (1.6 fold and 1.2 fold respectively). Furthermore, the level of pHER3 in LIM1215 CR-A-Normal was reduced relative to LIM1215 CONTROL (0.42 fold). These results require confirmation in independent experiments using western blotting, and determination of the total amount of receptor.

Figure 35: Reversal of acquired resistance to cetuximab over 2 months

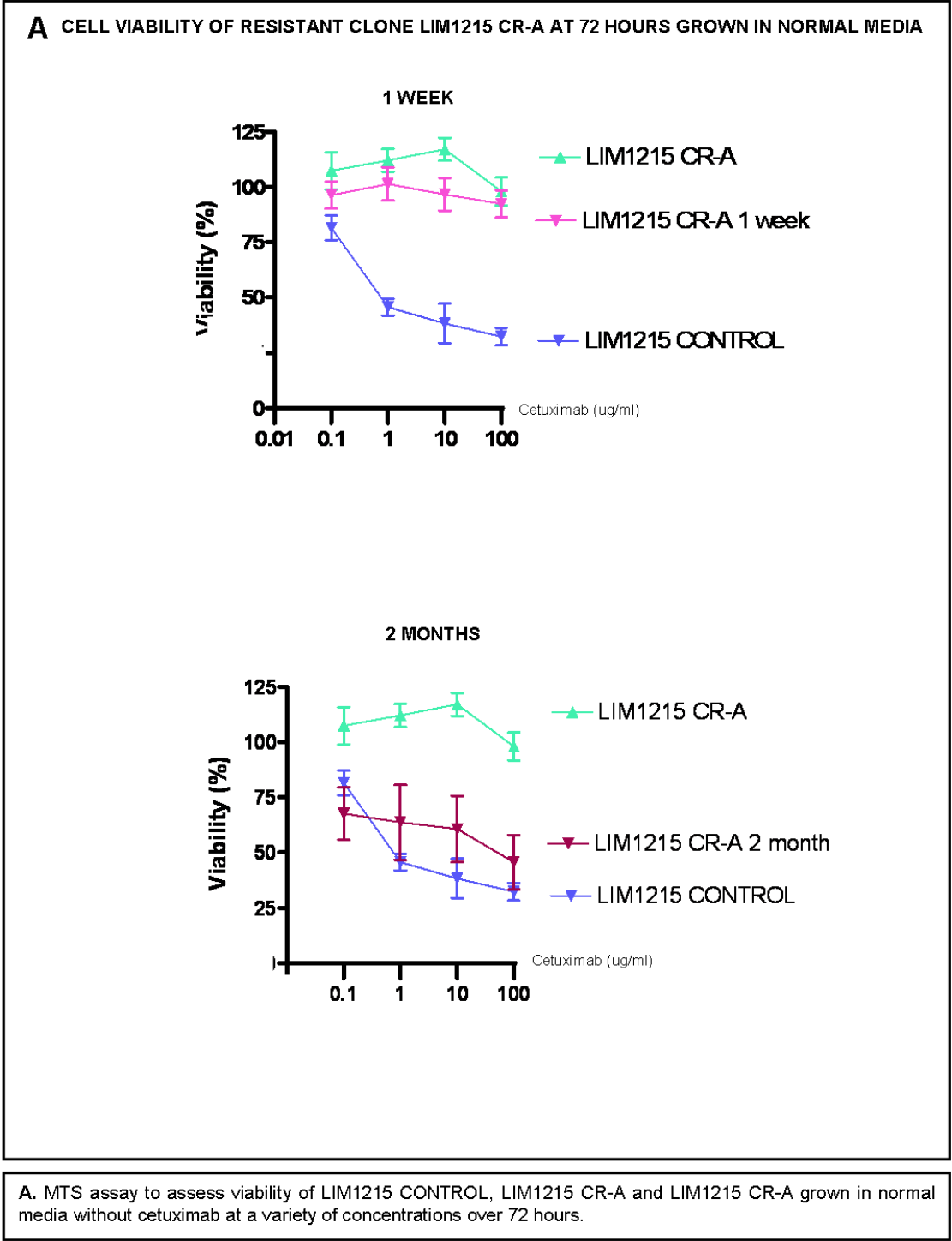
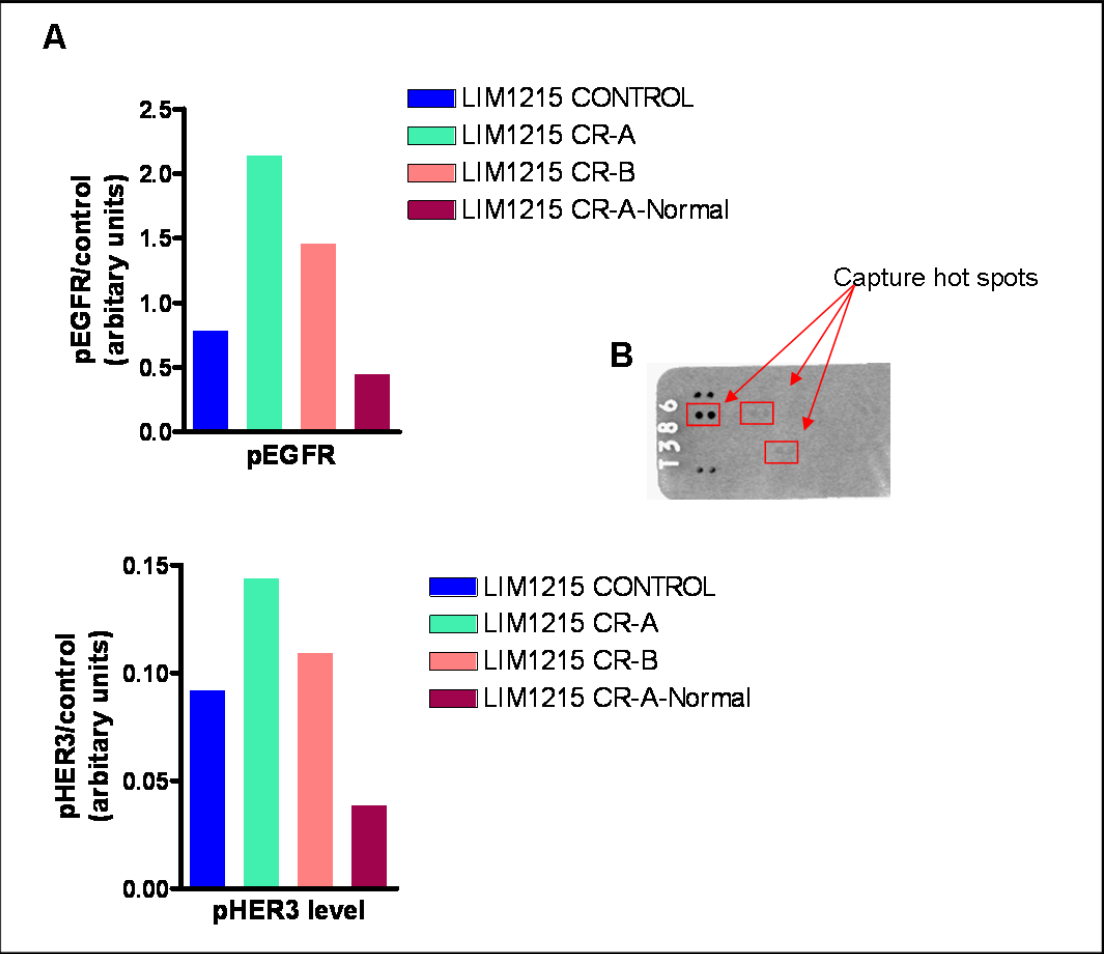


Figure 36: Change in receptor activation in resistant cell lines



A. Change in pEGFR and pHER3 expression assessed by receptor-kinase array analysis of LIM1215 CONTROL, LIM125 CR-A, CR-B, and LIM1215 CR-A-Normal. **B.** Example of an array and protein capture spots used for analysis.

4.1.4 Levels of phosphorylated signalling proteins in resistant cell lines

To compare changes in the downstream EGFR signalling pathway in the resistant cell line, western blot analysis of both LIM1215 and LIM1215 CR-A cell line after treatment with either control, cetuximab, erlotinib or the combination for 24 hours was performed following EGF stimulation.

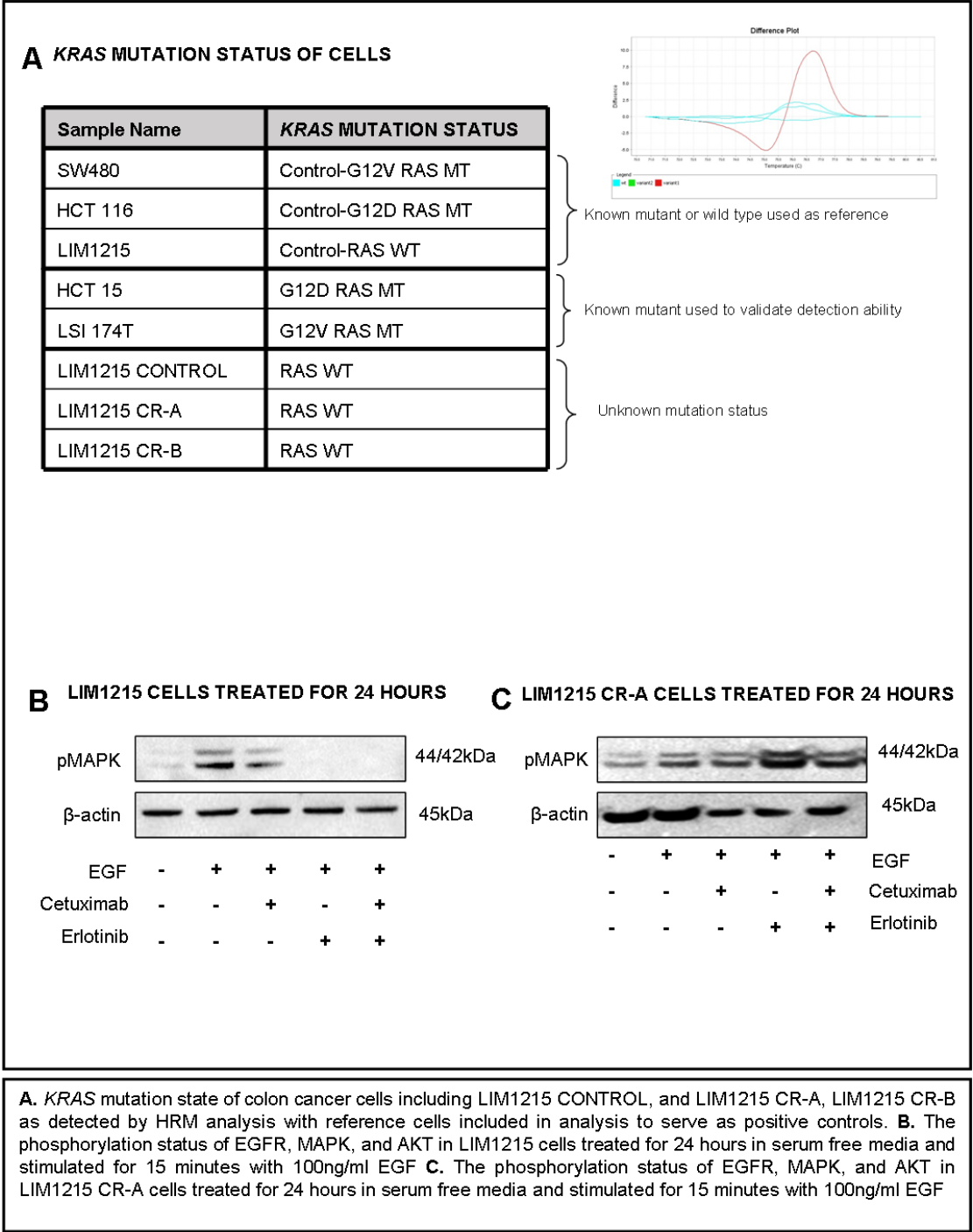
Levels of pMAPK were elevated in the LIM1215 CR-A cell line relative to LIM1215-CONTROL in an initial western blot (Figure 37). With treatment with cetuximab, erlotinib or the combination, there was no decrease of pMAPK levels in LIM1215 CR-A cell line, contrasting with the progressive decrease in pMAPK levels in LIM1215 cells with treatment. In fact, the levels of pMAPK seemed to increase with erlotinib treatment and treatment with the combination. The experiment requires repeating to more accurately assess changes within the EGFR downstream signalling pathway.

4.1.5 *KRAS* mutation status of resistant cell lines

Given that the LIM1215 CR-A cell line had reversible resistance to cetuximab, changes to mutations such as *KRAS* and *BRAF* seemed unlikely to explain the acquisition of resistance to cetuximab. To exclude at least *KRAS* mutations, in a preliminary investigation, HRM mutation analysis was performed to assess for common *KRAS* mutations (codon 12, codon 13).

HRM analysis indicated that the LIM1215 CONTROL, CR-A and CR-B cell lines were *KRAS* wild type, in keeping with the parental LIM1215 cell line (Figure 37). The mutation status of other genes including *BRAF*, *NRAS* and *PIK3CA* were not determined.

Figure 37: KRAS mutation status and pMAPK changes in resistant cells



4.1.6 Change in gene expression in resistant cell lines

Finally, to further investigate changes within the resistant cells, a microarray analysis was undertaken, using Affymetrix U133 Human Genome Arrays (Affymetrix) in which genes differentially expressed between the LIM1215 CR-A cell line and the LIM1215 CONTROL cell line were compared. Analysis of the array data provided a large list of relatively expressed or repressed genes in the resistant cell line that possibly contribute to resistance, and was sorted according to genes that were differentially expressed or repressed 2-fold or greater in the resultant resistant cell line relative to the control cell line (Table 15). Among the genes up-regulated in resistant cells, Regenerating Islet Derived Family, Member 4 (REG4) had a 3.9 log difference in expression relative to LIM1215 CONTROL, and appears twice in the list of the top 10 over expressed genes (Figure 38A).

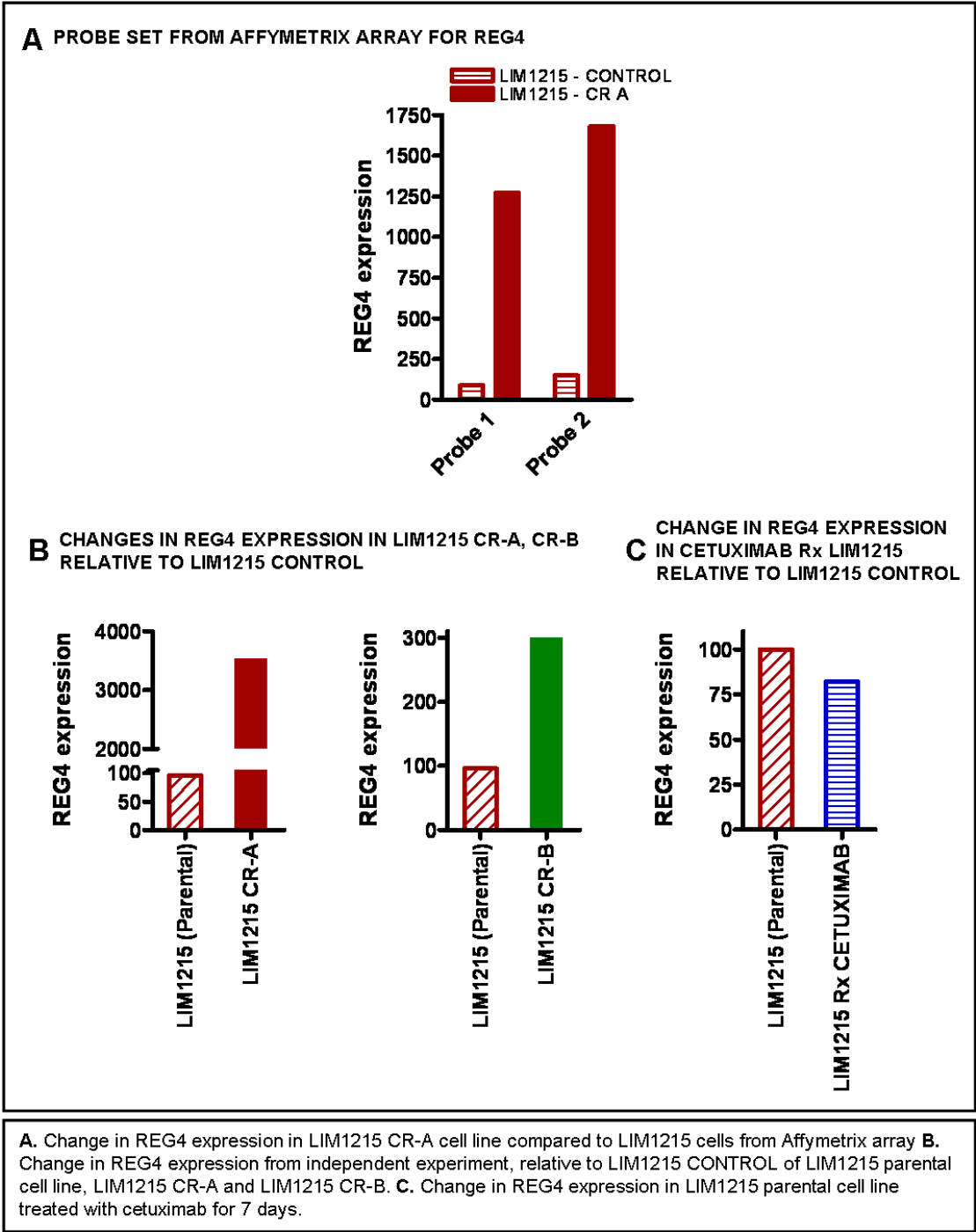
Reg4 is a secreted protein that has previously been shown to be capable of activation of the EGFR pathway, and furthermore Reg4 expression levels are correlated with poor prognosis in colorectal cancer [407-409]. Therefore altered expression of REG4 was further investigated. To validate the gene array experiment, REG4 expression was analysed using quantitative real time PCR with mRNA extracted from LIM1215 CONTROL as well as the parental LIM1215 cell line; LIM1215 CR-A and LIM1215 CR-B cell lines in an independent experiment to that used for the gene array.

Consistent with the array data, REG4 levels were significantly elevated in the LIM1215 CR-A cell line, with 3530% increase over LIM1215 CONTROL cells. REG4 levels were also elevated in the LIM1215 CR-B cell line (350% increase) relative to LIM1215 CONTROL, but not to the same degree as LIM1215 CR-A (Figure 38B). To exclude cetuximab treatment as a cause of high REG4 expression, we separately treated LIM1215 cells with cetuximab 100µg/ml for 7 days and extracted RNA for REG4 expression (Figure 38C). Short term treatment with cetuximab had no effect on REG4 expression, indicating its upregulation was not a transient response to cetuximab treatment.

Table 15: Change in gene expression in resistant cell line relative to control

	at least two fold difference in comparison, up					
	at least two fold difference in comparison, down					
Probe Set ID	Gene Title	Simplified Gene Symbol	fold difference, Res/Ctl	absolute expression level, CtlB 030610 CEL	absolute expression level, ResA 030610 CEL	absolute expression level, AinM 030610 CEL
222771_s_at	myelin expression factor 2	MYEF2	4.13	3.60	7.73	7.62
209120_at	nuclear receptor subfamily 2, group F, member 2	NR2F2	4.07	4.22	8.28	8.18
1554436_a_at	regenerating islet-derived family, member 4	REG4	3.87	6.46	10.31	10.25
232676_x_at	myelin expression factor 2	MYEF2	3.53	5.27	8.80	8.81
223447_at	regenerating islet-derived family, member 4	REG4	3.50	7.21	10.72	10.93
224724_at	sulfatase 2	SULF2	3.33	5.52	8.86	10.03
229464_at	myelin expression factor 2	MYEF2	3.25	4.79	8.05	7.55
219263_at	ring finger protein 128	RNF128	2.90	5.92	8.82	9.65
201963_at	acyl-CoA synthetase long-chain family member 4	ACSL1	2.90	6.03	8.93	9.23
205501_at	phosphodiesterase 10A	PDE10A	2.87	5.54	8.41	8.03
204733_at	kalikrein-related peptidase 6	KLK6	2.79	6.47	9.27	9.38
214240_at	galanin prepropeptide	GAL	2.79	7.79	10.58	9.99
217028_at	chemokine (C-X-C motif) receptor 4	CXCR4	2.74	6.89	9.53	9.18
222484_s_at	chemokine (C-X-C motif) ligand 14	CXCL14	2.70	7.79	10.49	9.43
220253_s_at	low density lipoprotein-related protein 12	LRP12	2.70	3.29	5.99	6.10
218002_s_at	chemokine (C-X-C motif) ligand 14	CXCL14	2.64	7.17	9.82	8.98
205778_at	kalikrein-related peptidase 7	KLK7	2.61	7.01	9.62	9.53
213953_at	keratin 20	KRT20	2.57	8.02	10.59	9.78
219631_at	low density lipoprotein-related protein 12	LRP12	2.50	4.52	7.02	7.32
214023_x_at	tubulin, beta 2B	TUBB2B	2.44	7.49	9.93	8.16
205549_at	Purkinje cell protein 4	PCP4	2.41	6.70	9.11	8.55
232060_at	receptor tyrosine kinase-like orphan receptor 1	ROR1	2.40	3.72	6.11	5.51
209792_s_at	kalikrein-related peptidase 10	KLK10	2.40	10.17	12.57	13.04
229381_at	kalikrein-related peptidase 7	KLK7	2.39	7.30	9.68	9.52
222108_at	adhesion molecule with Ig-like domain 2	AMIGO2	2.37	7.02	9.38	9.64
209937_at	transmembrane 4 L six family member 4	TMS4F4	2.34	5.48	7.82	7.68
223761_at	fibroblast growth factor 19	FGF19	2.28	4.61	6.89	7.62
209201_x_at	chemokine (C-X-C motif) receptor 4	CXCR4	2.27	6.54	8.81	8.24
207275_s_at	acyl-CoA synthetase long-chain family member 4	ACSL1	2.22	5.00	7.22	7.80
222772_at	myelin expression factor 2	MYEF2	2.20	4.70	6.90	6.08
209755_at	hypothetical protein LOC100131795 // nicotinic	LOC100131795	-1.74	7.01	5.27	5.16
241864_x_at	--	--	-1.75	6.31	4.56	4.12
209596_at	matrix-remodelling associated 5	MXRA5	-1.77	5.50	3.73	3.89
213836_s_at	WD repeat domain, phosphoinositide interaction	WIP1	-1.77	8.96	7.19	6.80
1556499_s_at	collagen, type I, alpha 1	COL1A1	-1.80	6.01	4.21	4.10
203827_at	WD repeat domain, phosphoinositide interaction	WIP1	-1.81	8.71	6.90	6.60
222288_at	--	--	-1.81	7.51	5.70	4.28
228423_at	microtubule-associated protein 9	MAP9	-1.82	7.39	5.57	4.71
219522_at	four jointed box 1 (Drosophila)	FJX1	-1.91	7.11	5.20	4.72
235728_at	zinc finger protein 3 homolog (mouse)	ZFP3	-1.97	5.66	3.69	3.69
209656_s_at	transmembrane protein 47	TMEM47	-2.10	10.34	8.24	7.55
213093_at	protein kinase C, alpha	PRKCA	-2.11	9.38	7.27	7.76
204114_at	nidogen 2 (osteonidogen)	NID2	-2.12	6.81	4.69	5.54
205174_s_at	glutaminyl-peptide cyclotransferase	QPCT	-2.12	8.73	6.61	5.79
227425_at	RALBP1 associated Eps domain containing 2	REP52	-2.13	7.61	5.48	4.60
1569532_a_at	MSFL2541	RP11-216L1.1	-2.16	10.37	8.21	7.34
241365_at	--	--	-2.20	4.89	2.89	3.06
215703_at	cystic fibrosis transmembrane conductance regulator	CFT R	-2.25	5.86	3.61	3.83
208937_s_at	inhibitor of DNA binding 1, dominant negative	ID1	-2.33	11.28	8.95	8.51
227725_at	ST6 (alpha-N-acetylneuraminyl-2,3-beta-galactosyltransferase 6)	ST6GALN	-2.38	9.58	7.20	7.61
202437_s_at	cytochrome P450, family 1, subfamily B, polypeptide 1	CYP1B1	-2.46	6.86	4.40	4.62
230645_at	FERM domain containing 3	FRMD3	-2.55	7.78	5.23	5.48
222803_at	phosphoribosyltransferase domain containing 1	PRTFDC1	-2.56	7.75	5.19	4.14
203408_s_at	SATB homeobox 1	SATB1	-2.59	8.44	5.84	6.12
215702_s_at	cystic fibrosis transmembrane conductance regulator	CFT R	-2.81	6.43	3.61	3.75
205042_at	glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylglucosaminase 2	GNB	-3.01	10.14	7.12	5.98
200884_at	creatine kinase, brain	CKB	-3.02	11.47	8.44	7.59
229893_at	FERM domain containing 3	FRMD3	-3.05	9.27	6.22	6.16
209291_at	inhibitor of DNA binding 4, dominant negative	ID4	-3.59	6.92	3.33	3.38
205043_at	cystic fibrosis transmembrane conductance regulator	CFT R	-4.11	10.32	6.21	6.42
209598_at	paraneoplastic antigen MA2	PNA2	-4.88	9.55	4.67	5.06

Figure 38: Change in REG4 expression in resistant cell lines



4.2 ACQUIRED RESISTANCE TO CETUXIMAB: DISCUSSION

4.2.1 Cells with acquired resistance to cetuximab are cross resistant to erlotinib

Although targeted therapies have demonstrated further improvements when combined with or used after standard cytotoxic chemotherapy, the duration of response is typically limited to several months. The mechanisms that govern such resistance fall into two categories. Either the cellular target is altered, or the reliance on the target by the cell is altered. Examples of the former include the emergence of NSCLC cells with T790M mutations resistant to EGFR-TKI therapy [41], whilst examples of the latter include activation of compensatory signalling pathways such as *PI3K*/mTOR [213, 214], Src [215], cMET [216], IGFR [217] and other HER family members [218]. This chapter studied acquired resistance to cetuximab to assess the relative importance of these previously reported phenomena.

LIM1215 cells acquired resistance to cetuximab after several months of exposure to increasing doses of cetuximab. The resistance was demonstrated in two parallel cell lines, LIM1215 CR-A and CR-B. These cells also acquired resistance to erlotinib, and the combination of cetuximab and erlotinib could not overcome the resistant phenotype. In contrast the LIM1215 CONTROL cell line passaged serially alongside these two resistant lines maintained sensitivity to both drugs. Acquired resistance to EGFR-mAb therapy has been modeled in a similar manner by other investigators, but the subsequent cross resistance to EGFR-TKI therapy is variable [193, 410]. In a NSCLC cell line, Kim *et al* demonstrated that a cetuximab resistant line was also resistant to gefitinib [410]. In contrast Huang *et al* generated a head and neck cell line that was resistant to cetuximab, yet sensitive to erlotinib and gefitinib [193]. The reason for the discrepant results is unclear, but may result from the different origin of the cell lines tested in each different series (colorectal, lung, head and neck).

4.2.2 Compensatory changes in signalling pathways may explain resistance

Numerous *in vitro* studies have reported that acquired resistance to EGFR-mAb therapy is governed by changes in compensatory pathways [213-218]. In a preliminary investigation of our resistant cell lines using a screening approach with a

multi-receptor phospho-kinase array, this report identified up regulation of pHER3 and pEGFR expression in resistant lines. Further validation of these changes is required with Western blot techniques, as well as assessment of total receptor number. Additionally, work to determine changes within the PI3K pathway, changes to EGFR dimerisation partners with Her family members, cMET, Src and IGFR is required to better assess the mechanisms governing our cell lines, but was beyond the scope of this project.

4.2.3 Acquired resistance is not accompanied by *KRAS* mutations

An alternate mechanism of acquired resistance to targeted therapy is structural alteration of the target, decreasing binding and effectiveness of the therapy or constitutive activation of the signalling pathway following a mutational event. Whilst such changes to the EGFR have been demonstrated in NSCLC and EGFR-TKI therapy [41], it is unclear whether such changes occur with prolonged EGFR-mAb therapy in colorectal cancer as the acquisition of *KRAS* mutations occurs early in colorectal carcinogenesis [411], and a similar *KRAS* status is found between primary and metastatic disease in untreated patients [412]. Bouchahda *et al* recently published a case report of a patient initially with a *KRAS* wild type tumour who responded to cetuximab, acquiring resistance to cetuximab after several months of therapy [413]. Resection of enlarging metastases at this time demonstrated a new *KRAS* mutation. This novel finding, prompted us to examine the *KRAS* mutation status of the cetuximab resistant cell lines, but we failed to observe an acquisition of mutation in codon 12 and 13. Mutations in codon 61 or other signalling components are possible, however that resistance is reversible suggests a non genetic mechanism.

4.2.4 REG4 is a promising molecule for further investigation of resistance

The resistant cell line LIM1215 CR-A had a wide range of differentially expressed genes relative to LIM1215 CONTROL cells. Amongst the many changes identified, REG4 expression was both highly up regulated relative to the control cell line, and literature review revealed it as a biologically plausible molecule which could contribute to resistance to cetuximab.

REG4 is a secreted protein and a member of the regenerating gene family, which is commonly over expressed in colorectal carcinoma [409], and is detectable in the serum of patients [414]. Several reports in the literature suggest a major role of REG4 in cancer progression, and the EGFR pathway, but none have previously linked REG4 expression with resistance to EGFR-mAb therapy. For example, REG4 stimulates cell growth, migration and invasion of colorectal cancer cell lines [409], correlates with increased peritoneal metastasis in gastric cancer [415], and leads to increased levels of pAKT through stimulation of the EGFR pathway [407]. Treatment of tumour xenografts of cell lines that over express REG4 with a monoclonal anti-REG4 antibody also leads to significant reductions in tumour growth [414].

In summary, REG4 can lead to EGFR pathway activation and consequent cellular proliferative advantages, is measurable in serum and targetable with the use of monoclonal antibody therapy. This report identified the novel association of REG4 over expression with the cetuximab resistant phenotype. Further work is required to validate REG4 as a cause of resistance to EGFR-mAb therapy. Experiments could include measuring REG4 levels in other cetuximab resistant cell lines, aiming to demonstrate the finding is not isolated to a single cell line. Transfection of a vector with over expression of REG4 into a cancer cell line with low levels of REG4 would also allow subsequent evaluation of EGFR-mAb sensitivity. Equally siRNA targeted at REG4 followed by evaluation of change in EGFR-mAb therapy would be important. Clinical measurement of REG4 levels over time of patients treated with cetuximab could also corroborate our *in vitro* observations. If substantiated as a mediator of resistance to EGFR-mAbs, the therapeutic potential of monoclonal antibodies to REG4 could be explored in patients who acquire resistance to EGFR-mAb.

4.2.5 Acquired resistance to cetuximab is dependent on exposure to cetuximab

It has previously been assumed that upon tumour progression whilst treated with an EGFR-mAb that the tumour has acquired permanent resistance to the drug, and the drug class is not used again. However there is scant literature to prove that the resistance is permanent, and that those patients who initially respond to therapy will inevitably progress upon rechallenge.

Given that numerous studies have shown activation of compensatory pathways as a mechanism of acquired resistance, and that such changes are plastic and dependent on EGFR-mAb exposure [214], the consequence of growing the resistant cell line LIM1215 CR-A in growth medium without cetuximab was investigated. Over a 2 month period, these cells regained sensitivity to cetuximab.

A case series in advanced colorectal cancer patients examining the effect of reintroduction of cetuximab corroborates our observation [416]. Five patients who received cetuximab in combination with chemotherapy but subsequently progressed, were given cetuximab again after failure of alternate second line chemotherapy. There was one partial response and two patients had stable disease. Additional support for our observation is provided by the report that reintroduction of an EGFR-TKI in NSCLC in initially responsive patients leads to high rates of clinical activity [417]. In 20 previously responsive patients (median PFS 320 days), gefitinib was reintroduced after a median treatment break of 217 days. There was a 25% response rate, with a median duration of treatment of 120 days. It is possible that the period of time following initial EGFR-TKI discontinuation may relate to the sensitivity of further EGFR-TKI treatment [417, 418]. This chapter's findings may have direct therapeutic implications. A non randomised case series of the strategy (reintroduction of EGFR-mAb to previously responsive patients who have had greater than 3 months break from EGFR-mAb) in metastatic colorectal cancer patients would be worthy of investigation.

5 BIOMARKER ANALYSIS OF MAX TRIAL: RESULTS AND DISCUSSION

5.1 BIOMARKER ANALYSIS: RESULTS

Bevacizumab is a monoclonal antibody that targets VEGF-A. Currently there are no known biomarkers for bevacizumab. Identification of sub groups of patients with metastatic colorectal cancer whom either benefit greatly from the drug or receive no benefit whatsoever would allow refined use, containing costs and potentially limiting side effects.

Based on understanding of the angiogenesis pathway, it was hypothesised that high expression levels of other VEGF family members including VEGF B, C and D would be associated with lack of bevacizumab efficacy as determined by the effect of bevacizumab on improvements in PFS. Given the important role of VEGF-A and VEGFF1 and VEGFR2 in angiogenesis, it was also hypothesised that over expression would correlate with lack of efficacy to bevacizumab. Additionally, given the association between the EGFR and VEGF pathway [376], the association of *KRAS* and *BRAF* mutations with bevacizumab efficacy was explored.

The MAX clinical trial recruited 471 patients between 2005 and 2007 and outcome data has been published [311], demonstrating statistical improvement in PFS in the bevacizumab arms over the single agent capecitabine arm but no improvement in OS. Of 471 patients who underwent randomization, 156 were assigned to receive C, 157 were assigned to receive CB, and 158 were assigned to receive CBM. The median PFS time of patients was 5.7 months for the capecitabine arm (C), 8.5 months for capecitabine/bevacizumab arm (CB), and 8.4 months for the capecitabine/bevacizumab/mitomycin arm (CBM). For C vs CB, the HR = 0.63, 95% CI 0.50 – 0.79; $p < .001$; for C v CBM, the HR = 0.59; 95% CI, 0.47 to 0.75; $p < .001$. After a median follow-up of 31 months, median OS was 18.9 months for capecitabine and was 16.4 months for CBM; these data were not significantly different

At the time of enrolment on the MAX trial, consent to participate in translational studies was obtained from the majority of patients (389 out of 471 patients, 83% of trial population)(Figure 39). From this group, archived formalin fixed paraffin embedded tumour tissue samples were retrieved for 333 patients (71% of trial population). From this number, 314 patients (67%) had DNA extracted for DNA analysis, and 270 patients (57%) had tumour cores inserted into a TMA for IHC analysis. The most common reason for not using patient tumour samples was insufficient tumour material.

The proportion of patients participating in the biological sub-study and the proportion who had specimens suitable for either DNA extraction or IHC is shown in Figure 36.

5.1.1 Characteristics of population available for sub-study analysis

Clinical outcomes were comparable between with no significant difference in primary PFS or secondary OS outcomes between patients from the study population and those evaluated for *KRAS/BRAF* mutation status (Table 17 and Table 18). This holds true for the CB+CBM combined analysis.

Table 16: Median PFS outcomes in intent to treat and sub-study population

Treatment	Median PFS (mths)	ITT POPULATION			Median PFS (mths)	SUB-STUDY POPULATION		
		HR	95% CI	p-value		HR	95% CI	p-value
C	5.7	1.00			6.0	1.00		
CB	8.5	0.62	0.50 - 0.79	<0.0001	8.6	0.69	0.52 - 0.91	0.01
CBM	8.4	0.59	0.47 - 0.75	<0.0001	8.5	0.64	0.48 - 0.86	0.003
CB + CBM	8.4	0.61	0.50 - 0.74	<0.0001	8.5	0.67	0.52 – 0.86	0.001

Figure 39: Disposition of patient tumour samples from MAX trial

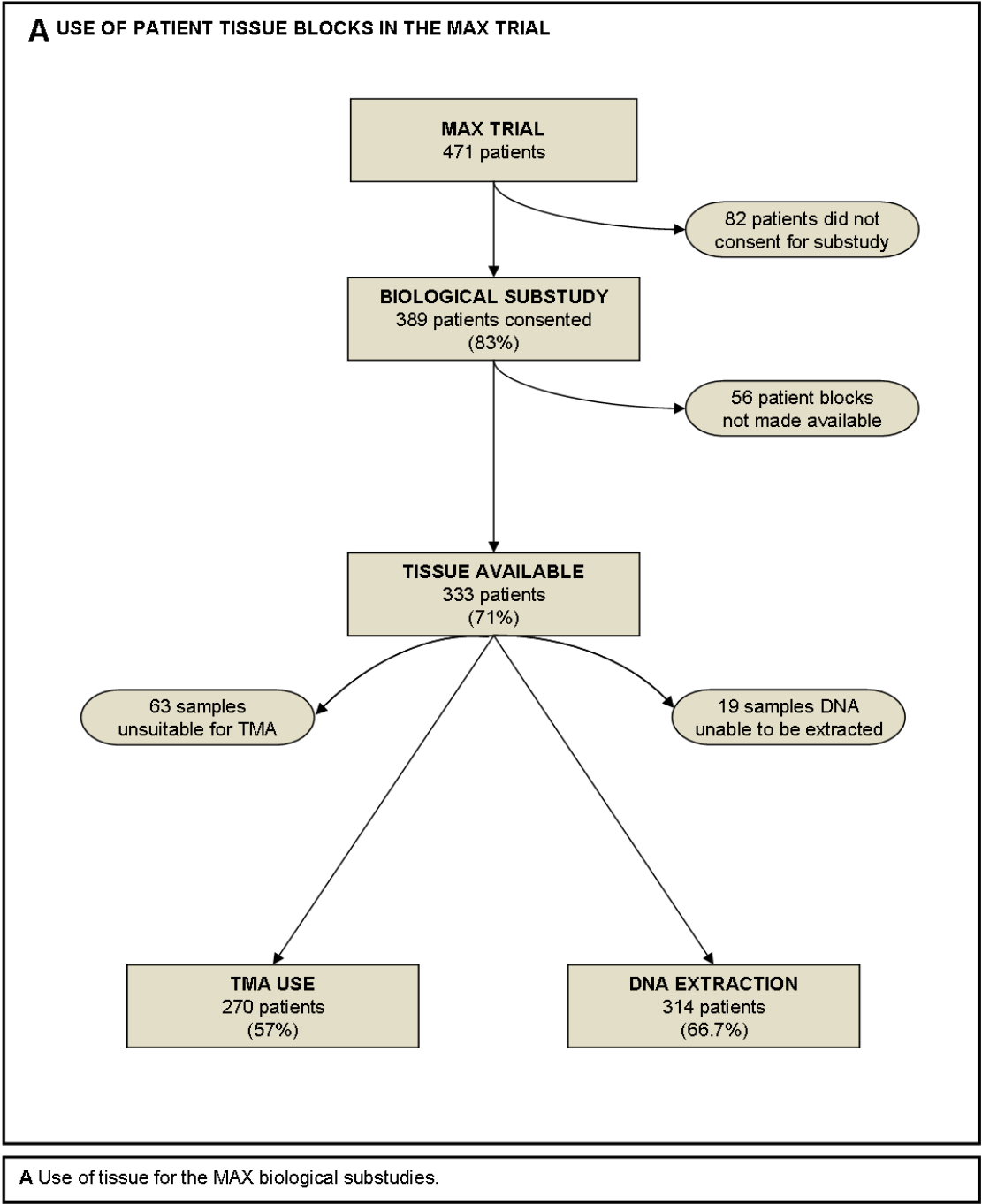


Table 17: Median OS outcomes in intent to treat and sub-study population

Treatment	Median OS ITT POPULATION (mths)				Median OS SUBSTUDY POPULATION (mths)			
		HR	95% CI	p-value		HR	95% CI	p-value
C	18.9	1.00			20.5	1.00		
CB	18.9	0.86	0.66-1.11	0.24	19.8	0.93	0.68-1.28	0.65
CBM	16.4	1	0.78-1.29	0.98	19.4	0.96	0.70-1.32	0.80
CB + CBM	17.3	0.93	0.75-1.16	0.51	19.4	0.94	0.72-1.25	0.69

5.1.2 Mutation status of *KRAS* and *BRAF*

KRAS and *BRAF* status was examined from a total of 314 tumour specimens on the MAX study (66.7%), 103 from the capecitabine (C) group, 111 from the capecitabine/bevacizumab group (CB), and 100 from the capecitabine, bevacizumab and mitomycin group (CBM)(Table 19).

5.1.3 Characteristics of patients with *KRAS/BRAF* mutations

Mutations in *KRAS* were detected in 90 patients (28.7%). Mutations in *BRAF* were detected in 34 patients (10.9%)(Table 19). Patient characteristics were similar between the *KRAS* mutant group and the *KRAS* wild type patients. However in the *BRAF* analysis, there were more patients with *BRAF* mutations with right sided primary (60% vs 25%), a greater number with disease free interval > 12 months (18% vs 30%) and fewer patients who had had prior adjuvant chemotherapy (9% vs 23%) (Table 19).

Table 18: Demographics and characteristics according to mutation status

	ITT (n=471)	KRAS MT % (n=90)	KRAS WT % (n=224)	BRAF MT % (n=34)	BRAF WT % (n=279)
Age (years)					
Median	67	69	68	71	68
Range	32-86	37-85	32-86	36-85	32-86
Sex					
Male	63	60	64	59	64
ECOG performance status					
0	56	59	58	41	60
1	38	38	35	47	34
2	6	3	8	12	6
Capecitabine dosage					
1.25 g/m ² bd	67	72	65	59	68
1.25 g/m2 bd	33	28	35	41	32
Disease-free interval > 12 months					
Yes	27	27	29	18	30
Prior adjuvant treatment					
Chemotherapy	22	20	23	9	23
Radiotherapy	13	8	10	6	10
Primary site					
Right sided (Caecum - Transverse	26	36	26	60	25
Left sided (Descending – Sigmoid)	44	41	49	30	49
Rectum	23	20	21	6	22
Other	6	3	4	6	4
Uncertain	1	0	0	0	0
Primary tumour resected					
Yes	79	88	87	91	86
Any metastases resected					
Yes	10	9	8	3	9
Extent of disease at baseline					
Local disease (colon or rectum)	36	30	31	15	33
Liver metastases	75	78	72	62	75
Lung metastases	39	48	36	24	41
Other metastases	10	11	11	24	10

5.1.4 Role of *KRAS* and *BRAF* as predictive markers for bevacizumab

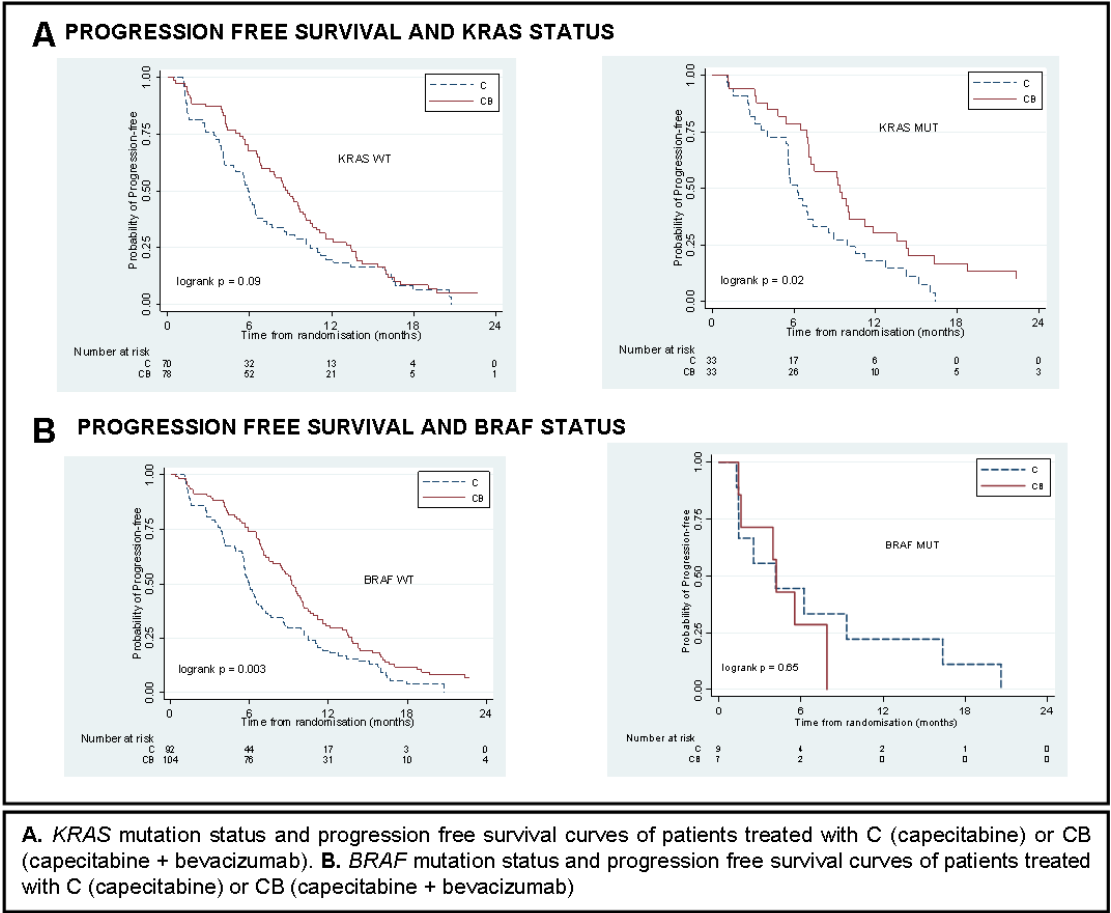
5.1.4.1 Progression free survival

Among patients with *KRAS* wild type tumours, the median PFS was 5.9 months for C (Capecitabine arm) and 8.8 months in CB (Capecitabine/bevacizumab arm)(HR = 0.74; 95% CI, 0.53 to 1.04; $p=0.09$)(Table 20). Among patients with *KRAS* mutant tumours, the median PFS was 6.2 months in the group receiving C and 9.4 months in the group receiving CB (HR 0.53, 95% CI 0.32 to 0.89, $p=0.02$). The additional benefit of bevacizumab on PFS was not significantly greater among the patients with *KRAS* wild type tumours than among those with *KRAS* mutant tumours ($p=0.27$ for the interaction between *KRAS* status and the assigned treatment) (Figure 40).

Table 19: PFS according to *KRAS* status in the MAX trial

	<i>KRAS</i> MT (n= 90)			<i>KRAS</i> WT (n=224)		
Treatment	n	PFS (months)	95% CI	n	PFS (months)	95% CI
C	33	6.2	5.5-7.4	70	5.9	4.1-6.5
CB	33	9.4	7.1-11.2	78	8.8	6.8-10.1
CBM	24	7.5	4.7-8.5	76	8.8	7.5-10.9
CB+CM	57	8.2	7.1-9.5	154	8.8	7.8-9.9

Figure 40: PFS according to KRAS and BRAF status MAX trial



The additional benefit of bevacizumab on PFS was borderline significant among patients with *BRAF* wild type tumours than among those with *BRAF* mutant tumours ($p=0.05$ for the interaction between *BRAF* mutation group and status)(Figure 40B). The median PFS in patients with *BRAF* mutant tumours was 4.1 months and 4.2 months in the group receiving CB (HR 1.29, 95% CI 0.41-4.07, $p=0.66$)(Figure 33B). For *BRAF* wild type tumours, the median PFS was 6.0 months in the group receiving C and 9.2 months in the group receiving CB (HR 0.64, 95% CI 0.48 – 0.86, $p=0.003$). An additional analysis was undertaken, given the small number of patients in this analysis with *BRAF* mutations (16), comparing outcomes in C vs CBM group (27 patients), and confirmed the lack of association of *BRAF* mutation and bevacizumab effect on PFS. Adjustment of pre-defined baseline prognostic factors did not change this result (p interaction = 0.40).

Table 20: PFS outcome according to BRAF status MAX trial

PFS	<i>BRAF</i> MT (n= 34)			<i>BRAF</i> WT (n=279)		
Treatment	n	PFS (months)	95% CI	n	PFS (months)	95% CI
C	9	4.1	1.3-16.4	92	6.0	5.6-6.6
CB	7	4.2	1.4-	104	9.2	7.8-10.1
CBM	18	5.7	2.8-8.6	83	8.6	7.5-10.1
CB+CM	25	5.5	2.9-7.8	187	9.1	8.3-9.9

5.1.4.2 Overall survival

The effect of the addition of bevacizumab on OS was not significantly different between patients with *KRAS* mutant tumours than among those with wild-type tumours ($p=0.98$ for the interaction between *KRAS* status and the assigned treatment). The lack of interaction was also true of *BRAF* status ($p=0.80$)(Table 22, 23)(Figure 41).

Figure 41: Outcome in OS according to KRAS/BRAF status in the MAX trial

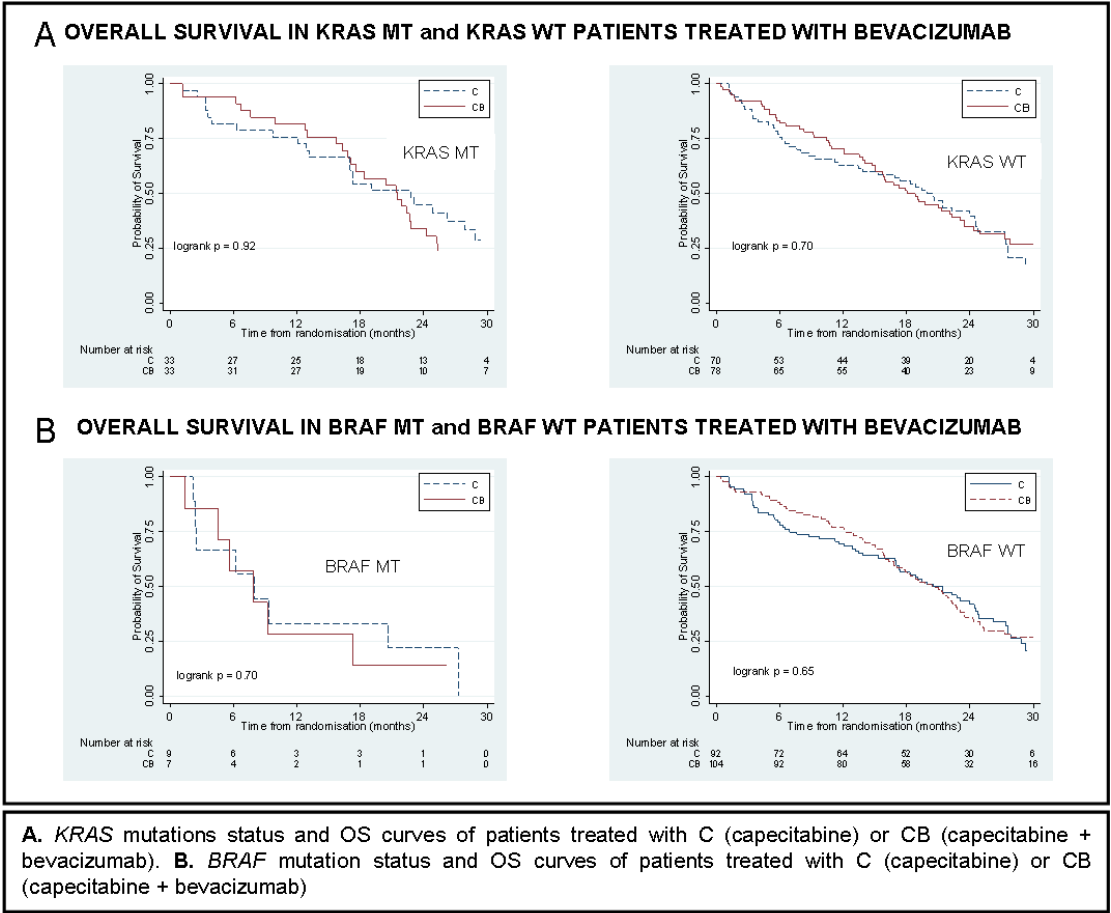


Table 21: Outcome in OS according to KRAS status in the MAX trial

OS	KRAS MT (n= 90)			KRAS WT (n=224)		
Treatment	n	OS (months)	95% CI	n	OS (months)	95% CI
C	33	22.8	13.2-28.9	70	20.0	13.4-24.4
CB	33	21.6	16.8-24.3	78	18.1	15.0-23.1
CBM	24	14.0	10.1-19.4	76	22.2	16.0-24.4
CB+CM	57	17.6	15.1-21.6	154	19.8	16.0-23.1

Table 22: Outcome in OS according to BRAF in the MAX trial

	BRAF MT (n= 34)			BRAF WT (n=279)		
Treatment	n	PFS (months)	95% CI	n	PFS (months)	95% CI
C	9	7.9	2.1-	92	20.5	17.0-24.5
CB	7	7.9	1.4-17.3	104	20.4	16.8-22.7
CBM	18	12.0	3.7-17.0	83	20.8	16.3-23.7
CB+CM	25	9.2	5.4-16.5	187	20.8	17.6-22.4

5.1.4.3 Response rate

Although analysis of the response rate between C vs CB accounting for *KRAS* status appeared to show patients with *KRAS* wild type tumours had a greater likelihood of response to bevacizumab ($p=0.01$ for the interaction between *KRAS* status and assigned treatment)(Table 24), this could not be confirmed when a further analysis comparing C vs CBM arm was undertaken ($p=0.17$ for interaction). The response rate did not vary between the groups based on *BRAF* status ($p=0.39$ for the interaction)(Table 24).

Table 23: Outcome in RR according to KRAS status in the MAX trial

	<i>KRAS</i> MT (n = 90)		<i>KRAS</i> WT (n=224)	
Treatment	n	PR (%)	n	PR
C (n=33)	16/33	48.5%	19/70	27.1%
CB (n=33)	8/33	24.2%	32/78	41.0%
CBM (n=24)	11/24	45.8%	34/76	44.74%

Table 24: Outcome in RR according to BRAF status in the MAX trial

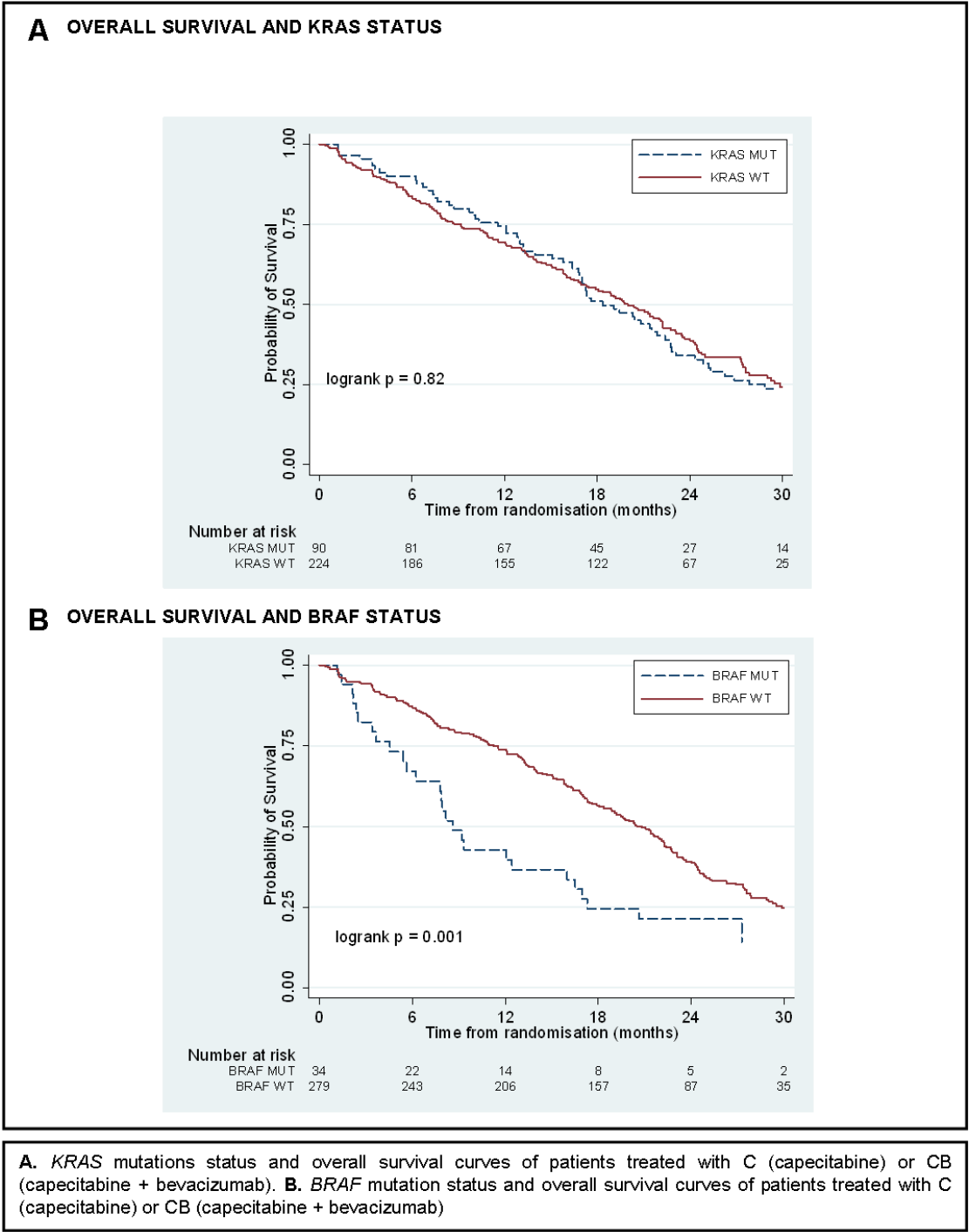
	<i>BRAF</i> MT (n = 90)		<i>BRAF</i> WT (n=224)	
Treatment	n	PR (%)	n	PR
C (n=33)	2/9	22.2%	33/92	35.9%
CB (n=33)	3/7	42.9%	37/104	35.7%
CBM (n=24)	5/18	27.8%	40/83	48.2%

5.1.5 Role of *KRAS* and *BRAF* as prognostic markers

Subsequent to analysis of the role of *KRAS* status and *BRAF* status as predictive markers, progression free and overall survival for all patients according to mutation status was analysed, regardless of the treatment they received to assess prognostic significance.

There was no significant difference between PFS or OS between the *KRAS* wild type tumour group and the *KRAS* mutant group. Median survival in *KRAS* wild type patients was 18.4 months vs 20 months in *KRAS* mutant patients (HR 0.97, 95% CI 0.73-1.28, $p=0.82$)(Figure 42A). However *BRAF* mutations were significantly associated with prognosis on OS, with *BRAF* wild type patient median OS 20.5 months versus 8.6 months in the *BRAF* mutant tumour group (HR 0.52, 95% CI 0.35 – 0.78, $p=0.001$)(Figure 42B). Even with adjustment for other baseline prognostic factors, *BRAF* mutation status was prognostic for overall survival (HR 0.45, 95% CI 0.30 – 0.68, $p<0.0001$). *BRAF* status was not associated with difference in prognosis on PFS between *BRAF* mutant and wild type groups. The median PFS was 8.3 months among the patients with *BRAF* wild type tumours versus 4.5 months among the patients with *BRAF* mutant tumours (HR 0.79, 95% CI 0.54 – 1.16, $p=0.22$).

Figure 42: Prognostic effect of KRAS and BRAF on OS



5.1.6 VEGF A-D and VEGFR1, 2 as predictive factors for bevacizumab efficacy

The association of expression of the ligands VEGFA-D and VEGFR1 and VEGFR2 from tumours of patients in the MAX study was then analysed for interaction with bevacizumab efficacy, using tissue microarrays and immunohistochemistry. 270 patients (57% of the MAX trial) had tumour specimens retrieved and used for the tissue microarray analysis. The patient characteristics of this group were similar to the intention to treat group, as summarised in the *KRAS/BRAF* analysis.

5.1.6.1 Characteristics of TMA staining

TMAs were used for analysis of VEGF A-D, and VEGFR1 and VEGFR2 expression. Staining was scored for intensity as 0, 1, 2, or 3. Examples of TMA staining and scoring for each factor are shown in Figure 40. The distribution of staining expression for each factor is shown in Figure 43.

Figure 43: VEGF A-D, VEGFR1 and VEGFR2 staining

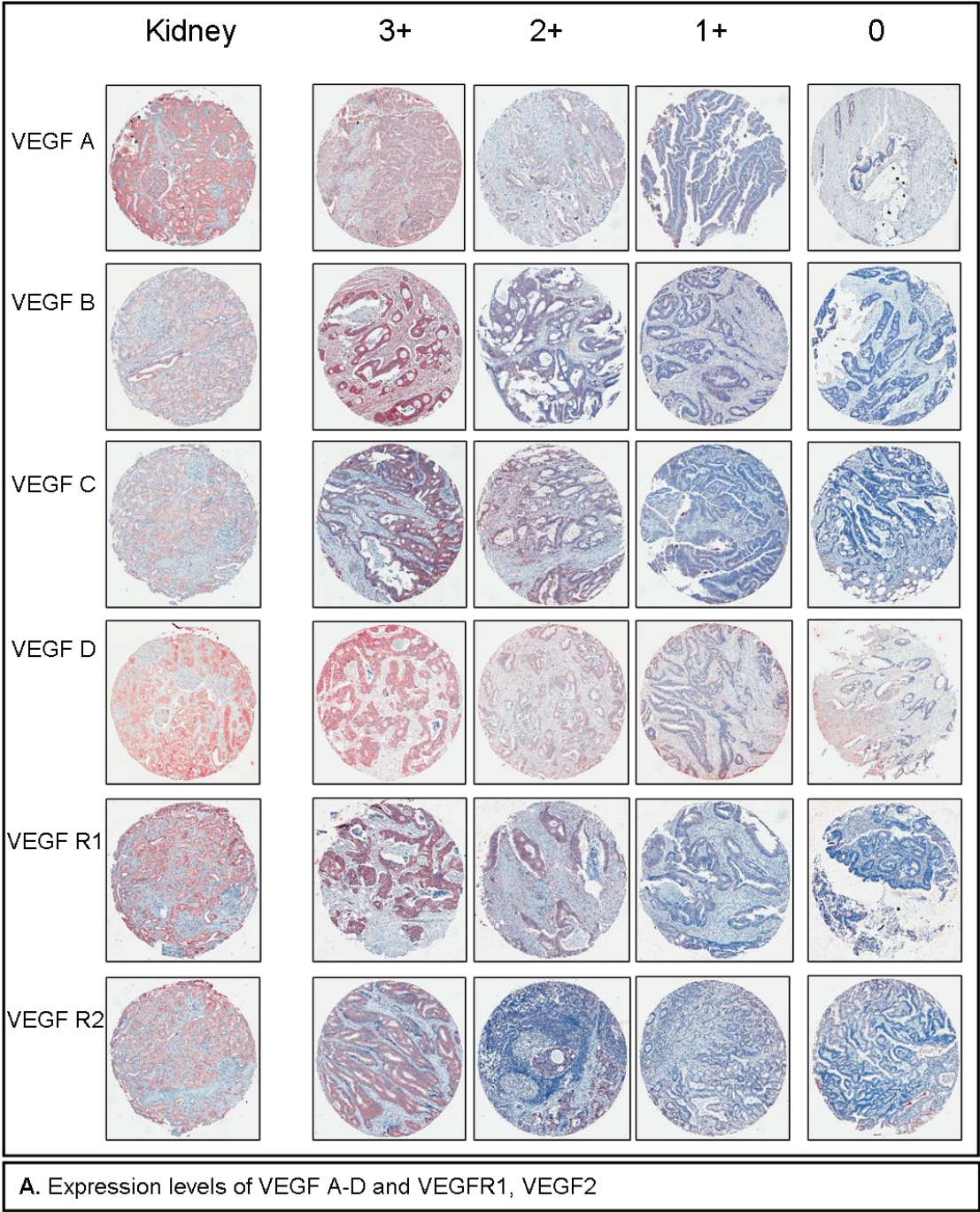
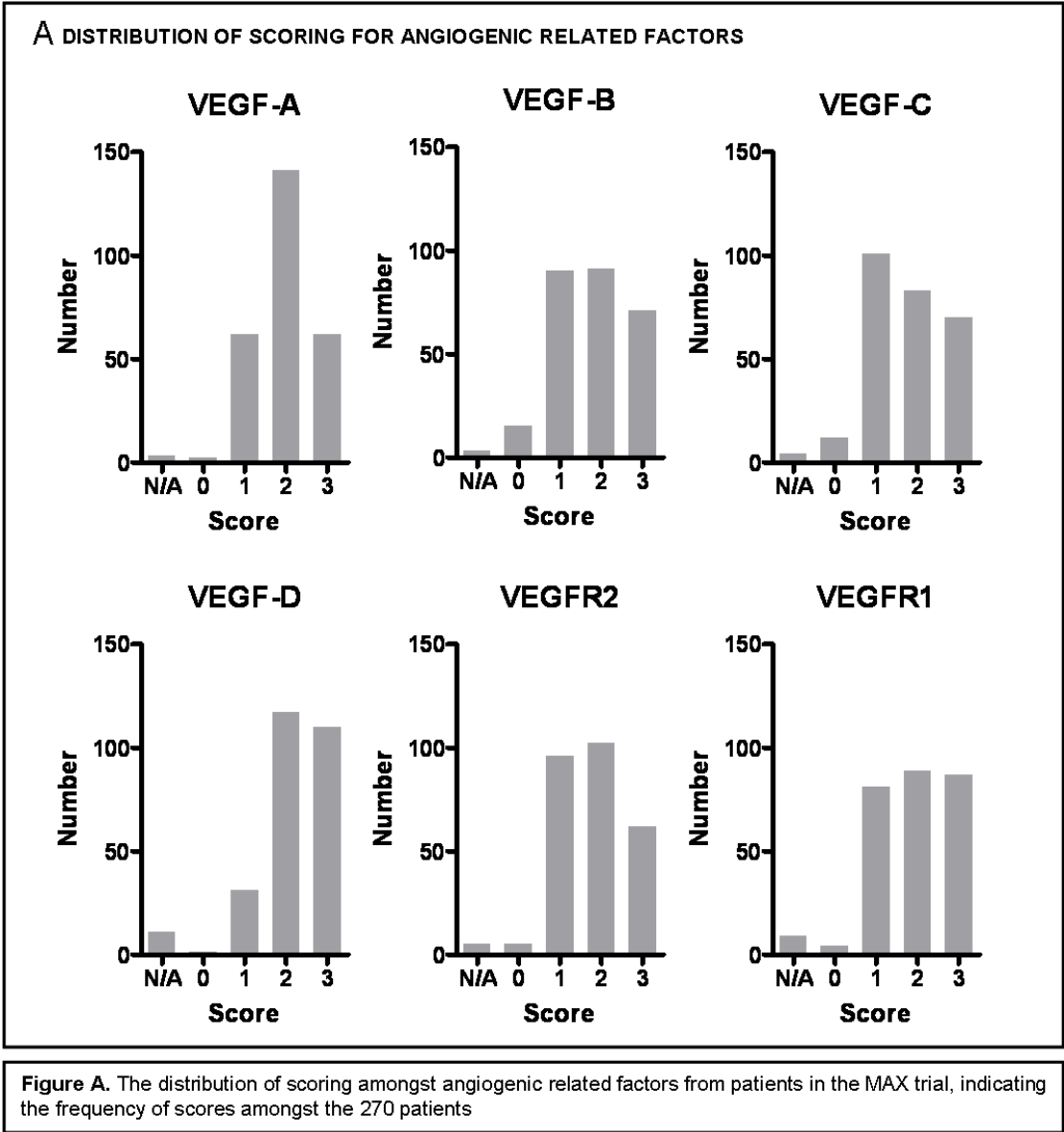


Figure 44: Distribution of categorical scores



5.1.6.2 Quality control issues

Each patient who had tissue available for use in the TMA had three cores inserted into the TMA in order to better represent tissue expression. To investigate if there was heterogenous expression within the tumour blocks the variability of expression of staining amongst the triplicate cores for each factor scored was assessed.

We determined that there was good concordance between expression level amongst the triplicate cores, as determined by weighted Kappa score >0.60 , for all but VEGF-A and VEGF-C, which had moderate concordance with weighted Kappa scores of 0.59 and 0.58 respectively (Table 26). There was concordance amongst expression in the triplicates of between 69% (VEGF-D) to 81% (VEGFR1).

Table 25: Concordance of expression amongst triplicates of tumour cores

IHC	Concordance (%)	Weighted Kappa
VEGF-A	72%	0.59
VEGF-B	70%	0.66
VEGF-C	73%	0.68
VEGF-D	69%	0.58
VEGFR1	81%	0.79
VEGFR2	80%	0.73

Expression levels of ARFs was determined by two independent scorers (the author, and an anatomical pathologist Dr David Williams) to improve consistency and validity of the study. The mean score from the triplicates were then compared and the concordance between scorers assessed. There was good concordance for all the ARFs scored on the TMA, with the weighted Kappa between 0.79 (good) and 0.91 (very good)(Table 27).

Table 26: Inter-rater agreement amongst scorers

IHC	Concordance (%)	Weighted Kappa
VEGF-A	84%	0.82
VEGF-B	82%	0.82
VEGF-C	92%	0.91
VEGF-D	81%	0.79
VEGFR1	91%	0.91
VEGFR2	86%	0.85

5.1.6.3 Correlation of expression between primary and metastatic sites

The TMAs consisted of triplicate cores of patient tumour specimens. The majority of the 270 cores were taken from blocks of resected primary tumour (266, 85%). The other tumour cores were taken from blocks of metastatic tumour (52, 15%)(7 liver, 9 lung, 17 lymph nodes, 17 peritoneal).

There were 36 occurrences where there was tissue available from primary tumour and metastases on the same patients (17 from lymph nodes, 9 from omental biopsies, and 10 from solid organ metastases).

We determined the correlation of expression between the primary tumour block and the metastatic lesion for each of the factors analysed. Pearson's coefficient of correlation, r , is shown below in the table 28 for each factor. VEGF-D expression was significantly correlated between the primary and matched metastatic sample ($r=0.71$, 95% CI 0.50 – 0.84, $p < 0.0001$). Additionally, VEGFR1 expression was significantly correlated between primary specimen and matched metastatic site ($r = 0.73$, 95% CI 0.53 – 0.85, $p < 0.0001$), as was VEGFR2 expression ($r = 0.57$, 95% CI 0.30 – 0.76, $p = 0.0003$). Conversely, there was no significant correlation between VEGF A-C in the primary tumour and matched metastatic tumour specimens (Table 28)

Table 27: Correlation of expression between primary and metastases of ARFs

Primary vs metastases	VEGF-A	VEGF-B	VEGF-C	VEGF-D	VEGFR1	VEGFR2
r	0.106	0.296	0.289	0.710	0.734	0.573
95% CI	-.23 -.042	-0.03-0.56	0.53-0.86	0.50-0.84	0.53-0.85	0.30-0.76
p value	0.53	0.079	0.087	<0.0001	<0.0001	0.0003

Where there were both primary and metastatic tissue available for a particular patient, the cores originating from the metastatic site (or lymph node) were used in preference to the primary tissue to generate expression values for each patient. Expression values for ARFs for the study were taken from 223 (83%) primary tumour and 44 (17%) metastatic tumour samples.

5.1.7 Distribution of angiogenic related factors by treatment arm

There were no significant differences in distribution of the expression level of VEGF A-D, VEGFR1 and VEGFR2 between treatment arms of the study (Table 29).

Table 28: Distribution of ARF expression by treatment arm

	VEGF-A				VEGF-B				VEGF-C				VEGF-D				VEGFR1				VEGFR2			
VEGF/VEGFR expression	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
C arm	0	23	45	20	7	27	35	19	5	38	21	24	0	11	42	33	1	33	23	28	2	39	26	21
%	0	37	32	32	47	30	38	27	42	38	25	34	0	35	35	30	25	41	26	32	40	41	25	34
CB arm	0	20	54	18	4	29	32	27	3	33	33	22	0	12	39	38	2	25	32	31	2	28	40	20
%	0	32	38	29	27	32	35	38	25	33	40	31	0	39	33	35	50	31	36	36	40	29	39	32
CBM arm	2	19	42	24	4	34	24	25	4	30	29	24	1	8	36	39	1	23	34	28	1	29	36	21
%	100	31	30	39	27	38	26	35	33	30	35	35	100	26	31	35	25	28	38	32	20	30	35	34

5.1.8 Correlation of ARF expression levels

VEGF-C expression level was significantly correlated with VEGFR1 expression level ($r=0.69$) and VEGFR2 expression level ($r=0.71$), and less strongly with VEGF-D level ($r=0.54$). VEGFR1 was strongly correlated with VEGFR2 level ($r=0.79$) (Table 30).

Table 29: Correlation of ARF expression levels

	VEGFA	VEGFB	VEGFC	VEGFD	VEGFR1	VEGFR2
VEGFA	1.00					
VEGFB	0.30	1.00				
VEGFC	0.08	0.41	1.00			
VEGFD	0.15	0.38	0.54	1.00		
VEGFR1	0.03	0.39	0.69	0.47	1.00	
VEGFR2	0.11	0.45	0.71	0.47	0.79	1.00

5.1.9 Correlation of ARFs and baseline characteristics

The expression levels of the ARFs were correlated with baseline factors to assess correlation between expression and other significant prognostic factors. There was significant associations (χ^2 $p < 0.05$) between VEGF-B, VEGF-C, VEGFR1, and VEGFR2 and the presence of peritoneal metastasis.

5.1.10 Predictive association of VEGF-D on bevacizumab efficacy (PFS)

Expression levels of VEGF A-D, as well as VEGFR1 and VEGFR2 were correlated with outcome using the statistical methods previously described, and tests for the trend of expression on the interaction with bevacizumab efficacy on PFS performed.

A significant interaction between ARF expression status and bevacizumab efficacy was found for only VEGF-D. This was detected in an analysis of C vs CB, and confirmed on subsequent analysis of C vs CB + CBM, as well as on multivariable analysis accounting for treatment (C vs CBM, and C vs CB+CBM), biomarker expression and treatment by biomarker interaction (HR 1.72, 95% CI 1.03 – 2.87, $p = 0.037$).

The differences between outcome and VEGF-D expression are shown in Table 31, and Figure 45 showing the separation of curves favoring the bevacizumab treatment arms evident in the low expression VEGF-D 0-1 group, but not existent in the high expression VEGF-D groups (VEGF-D 2 and VEGF-D 3). The forest plot in Figure 46 shows that the trend in outcome for VEGF-D expression and benefit from bevacizumab on PFS, with the test for interaction of the trend of expression and benefit significant for the initial analysis of C vs CB, and confirmed with the subsequent comparison of C vs CBM ($p = 0.04$) or C vs CB+CBM ($p = 0.02$)(Figure 46).

In patients with low VEGF-D expression (0 or 1) the median PFS was 5.8 months (95% CI 3.8 – 8.6 months) amongst those receiving capecitabine and 11.6 months (95% CI 5.6 – 25.3 months) amongst those receiving capecitabine and bevacizumab (Table 31). For patients with VEGF-D expression of 2, the median PFS was 6.0 months (95% CI 4.9 – 7.0 months) amongst those receiving capecitabine and 9.54 months (95% 7.0 – 11.8 months) amongst those with capecitabine and bevacizumab. However for patients with VEGF-D expression status of 3, the median PFS was 7.0 months (95% CI 5.6 – 10.1 months) amongst the capecitabine arm, and 8.75 months (95% CI 5.92 – 9.67 months) amongst the capecitabine and bevacizumab arm.

Figure 45: PFS according to VEGF-D status MAX trial

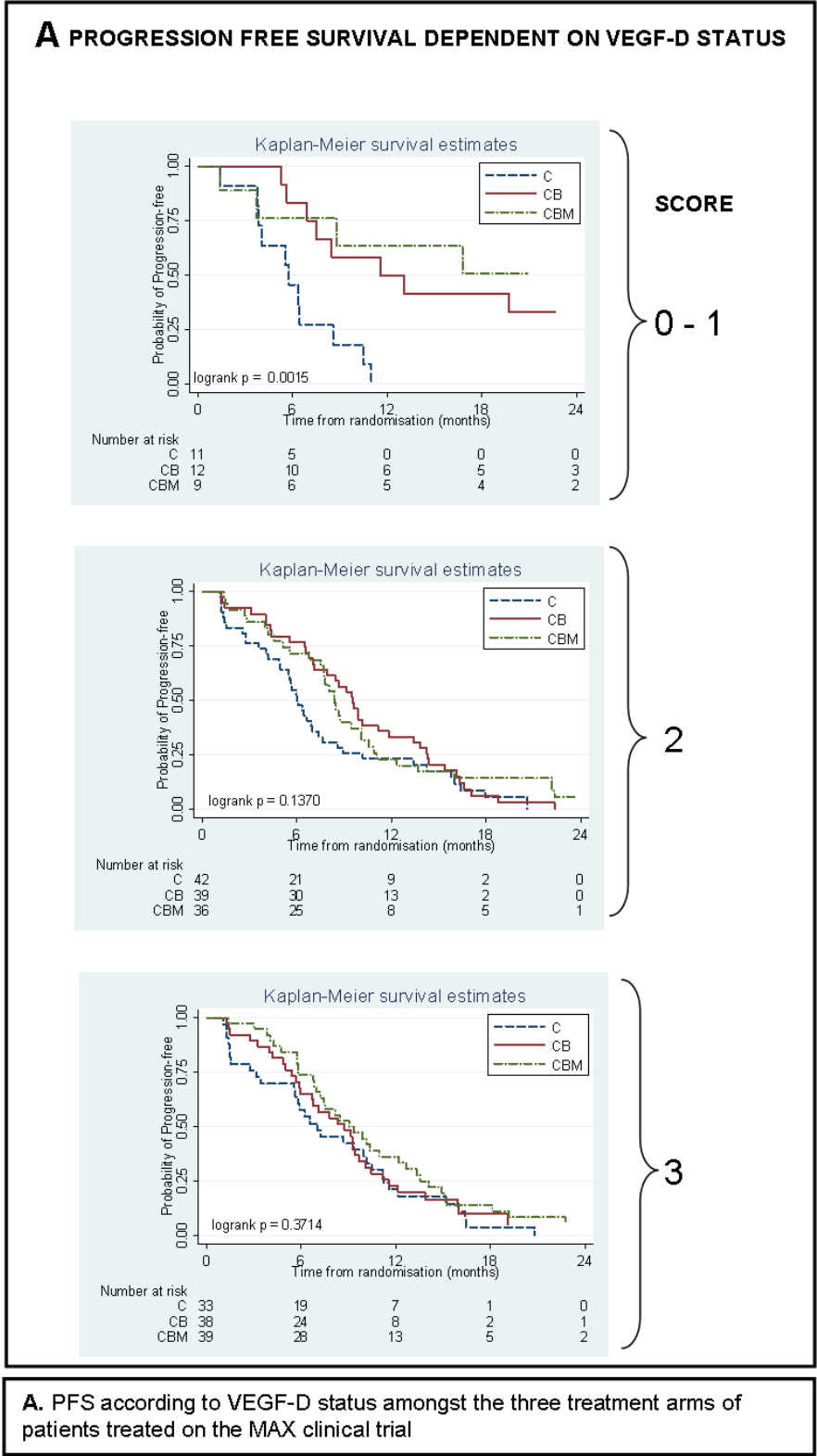
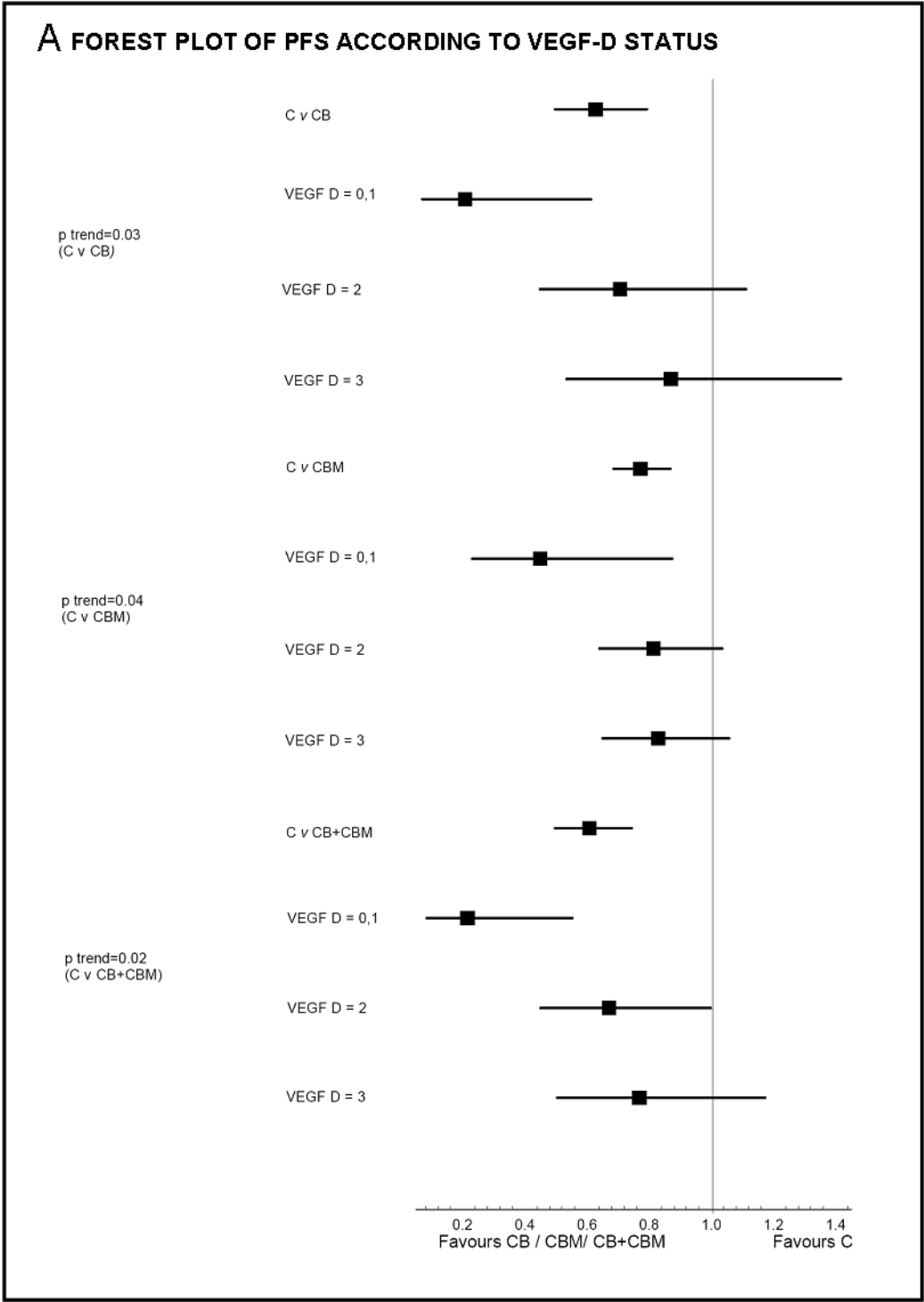


Figure 46: Forest plot for VEGF-D interaction



A. Forest plot demonstrating Hazard Ratios for PFS according to VEGF-D status

Table 30: Effect of VEGF-D on PFS benefit from bevacizumab

Score	Treatment	n	Median PFS (months)	95% CI	
0-1	C	11	5.76	3.75	8.55
	CB	12	11.55	5.63	25.33
	CBM	9	-	1.35	.
2	C	42	5.99	4.93	6.97
	CB	39	9.54	7.01	11.84
	CBM	36	8.39	7.50	10.10
3	C	33	7.01	5.56	10.13
	CB	38	8.75	5.92	9.67
	CBM	39	9.34	6.97	12.17

In a multivariate model testing for biomarker-treatment interactions the only significant predictive biomarker was VEGF-D where lower scores were associated with greater treatment effects: test for interaction $p=0.016$ unadjusted and $p=0.05$ adjusted for other prognostic factors. However, the global test for interaction when considering all 6 biomarkers together with VEGF-D was not statistically significant ($p=0.25$) so this evidence should still be regarded as exploratory.

5.1.11 Predictive association of VEGF-D on OS

The interaction between the trend in ARF expression and the effect of bevacizumab on OS was then analysed. This was a secondary endpoint of the biological sub-study. Similar to the interaction with PFS, the trend in VEGF-D expression was significantly associated with the effect of bevacizumab on OS, with low expression of 0 or 1 associated with significantly better OS when bevacizumab was used, in contrast to minimal effect of bevacizumab when VEGF-D expression was high (2 or 3)(Figure 47).

In patients with low VEGF-D expression (0 or 1) the median OS was 18.9 months (95% CI 5.6 – 27.9 months) amongst those receiving capecitabine and 25.3 months (95% CI 14.7 - months) amongst those receiving capecitabine and bevacizumab (Table 32). For patients with VEGF-D expression of 2, the median OS was 20.6 months (95% CI 17.0 – 24.8 months) amongst those receiving capecitabine and 22.4 months (95% 17.1 – 34.7 months) amongst those with capecitabine and bevacizumab. However for patients with VEGF-D expression status 3, the median OS was 24.5

months (95% CI 9.7 – 31.0 months) amongst the capecitabine arm, and 19.7 months (95% CI 15.1 – 24.2 months) amongst the capecitabine and bevacizumab arm. The differences are shown in Figure 47, showing the separation of curves favouring the bevacizumab treatment arms evident in the VEGF-D 0,1 group, but not existent in the VEGF-D 2 and VEGF-D 3 group.

Table 31: Effect of VEGF-D status on OS

Score	Treatment	n	Median OS (months)	95% CI	
0-1	C	11	18.88	5.56	27.86
	CB	12	25.33	14.70	-
	CBM	9	-	3.39	.
2	C	42	20.63	17.04	24.84
	CB	39	22.37	17.11	34.70
	CBM	36	19.61	13.13	24.57
3	C	33	24.51	9.7	31.0
	CB	38	19.05	13.85	23.06
	CBM	39	19.7	15.07	24.24

The effect of the addition of bevacizumab was significantly greater amongst the patients with VEGF-D expression of 0 or 1, than among those with VEGF-D expression of 2 or 3 ($p=0.01$ for the test for interaction). A further comparison of C vs CB+CBM confirmed the predictive association between bevacizumab and VEGF-D expression status (C vs CBM, $p = 0.07$; C vs CB+CBM, $p = 0.01$)(Figure 47 and Figure 48). Whilst this association was significant when examined singularly of other biomarkers, the association was not significant in a multivariate model.

Figure 47: OS and VEGF-D expression

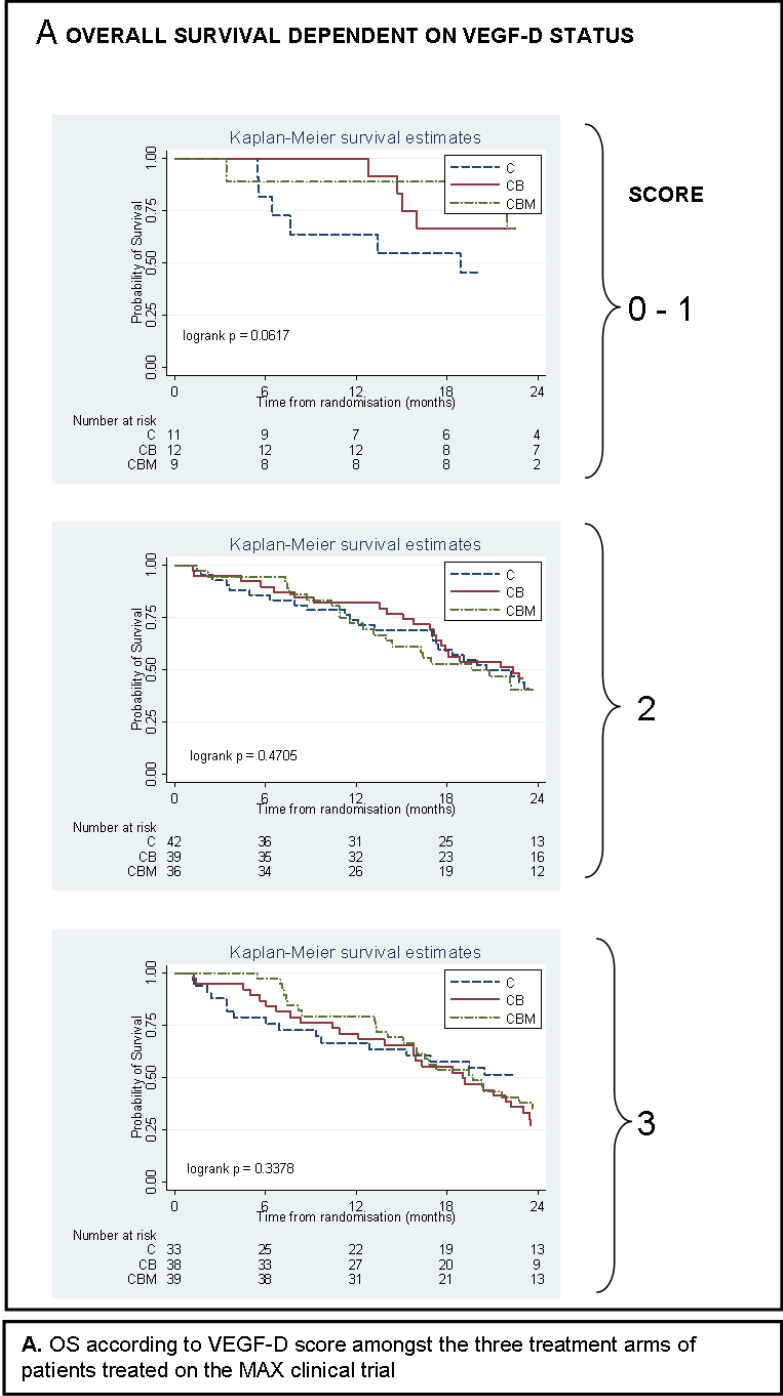
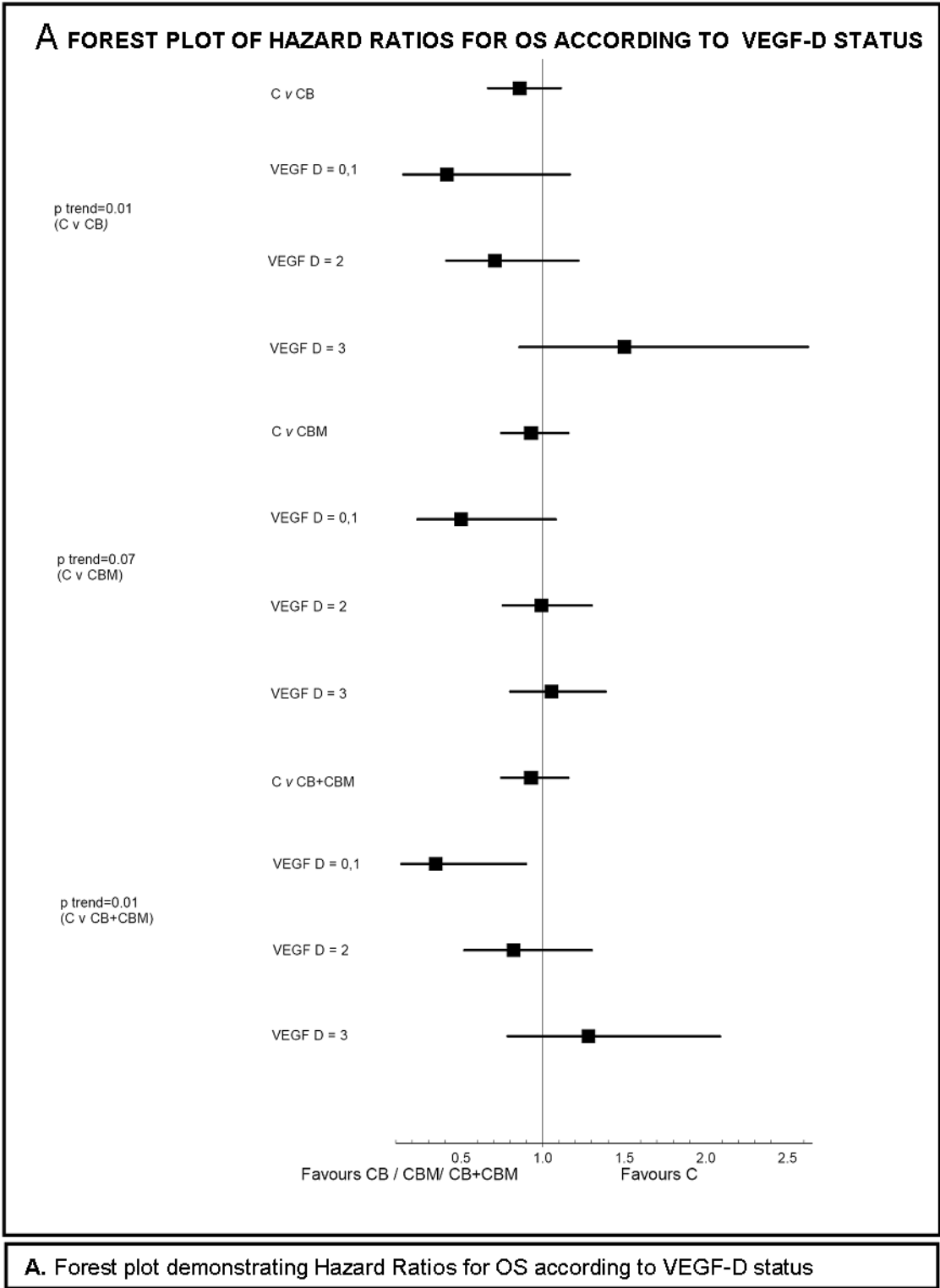


Figure 48: Forest plot of hazard ratios for OS according to VEGF-D status



5.1.12 Predictive association of VEGF-D expression and response rate

Finally, to further assess the predictive role of VEGF-D related to the efficacy of bevacizumab, we examined the interaction between the expression level of VEGF-D and the effect of bevacizumab on response rate. There was no significant interaction between the expression levels of VEGF-D, and the influence of bevacizumab on response rate.

5.1.13 Prognostic role of VEGF-D on PFS and OS

Following assessment of the interaction between ARF expression and bevacizumab efficacy, the association between the expression level of the ARFs and both PFS and OS was examined, regardless of treatment received (C, CB and CBM groups included). Whilst VEGF-D expression level was not prognostic for PFS including on multivariate analysis, VEGF-D expression level was prognostic for OS ($p=0.05$) with lower levels associated with better survival. This takes into account treatment with bevacizumab, including CB and CBM arm. When tested in the C only arm VEGF-D expression was not prognostic for OS, but the numbers analysed are too small to make conclusions from in this subgroup.

Table 32: Impact of VEGF-D expression on prognosis (PFS)

C, CB and CBM arms											
VEGF-D expression	n	Median PFS (mths)	95% CI (months)		Progression free at 12 mths	95% CI (%)		HR	95% CI of HR		p-value
0,1	32	8.55	5.76	13.06	35.6%	19.5%	52.0 %	1.00			0.03
2	117	7.89	6.78	8.91	26.4%	18.7%	34.7 %	1.74	1.12	2.69	
3	110	8.45	6.97	9.67	27.0%	19.0%	35.7 %	1.63	1.05	2.53	
C arm only											
VEGF-D expression	n	Median PFS (mths)	95% CI (months)		Progression free at 12 mths	95% CI (%)		HR	95% CI of HR		p-value
0,1	11	5.76	3.74	8.55				1.00			0.43
2	42	5.99	4.93	6.97	23.2	11.8	36.9	0.68	0.34	1.35	
3	33	7.01	5.56	10.13	21.2	9.4	36.3	0.62	0.31	1.24	

Figure 49: Prognostic role of VEGF-D for PFS and OS

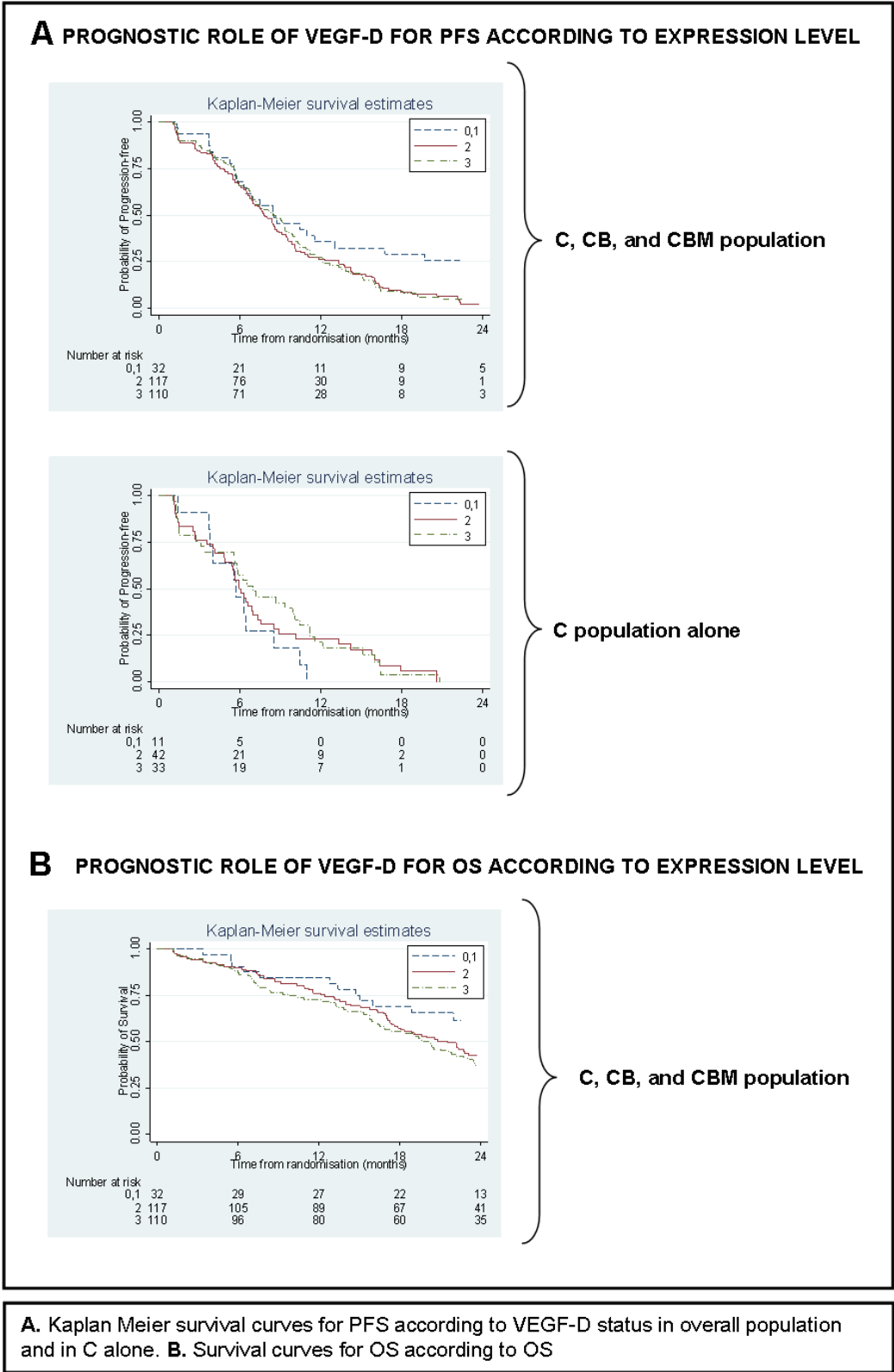


Table 33: Multivariable analysis of VEGF-D expression on PFS

Multivariate analysis in C, CB and CBM groups

VEGF-D	HR	95% CI of HR		p-value
0,1	1.00			0.048
2	1.69	1.06	2.63	
3	1.54	0.99	2.41	

5.1.14 Predictive association of other ARFs on PFS

The expression of other ARFs including VEGF-A, VEGF-B, VEGF-C, VEGFR1 and VEGFR2 was not significantly associated with the benefit of bevacizumab efficacy on PFS. The forest plots in Figures 50 and 51 display the hazard ratios and confidence intervals in the group C vs CB in addition to the *p* value for test for the interaction between the trend and the effect of bevacizumab efficacy on PFS.

5.1.15 Predictive association of other ARFs status on OS

The association between the trend in ARFs other than VEGF-D and bevacizumab effect on OS were also examined. Interestingly, the trend in VEGF-B, VEGF-C, VEGFR1 and VEGFR2 were all significantly associated with the effect of bevacizumab on OS (Figures 52 and 53) when assessed in a model of C vs CB and C vs CB+CBM. The only significant biomarker that remained significant (in a step-down procedure) was VEGFR1 (test for interaction $p=0.001$ unadjusted and $p=0.001$ adjusted for significant prognostic factors). However this is despite no significant association of these factors with PFS benefit of bevacizumab, so it is uncertain how VEGFR1 expression may influence OS and not PFS.

5.1.16 Predictive association of ARFs and response rate

Finally, to further assess the predictive role of other ARFs on the efficacy of bevacizumab, we examined the interaction between the expression level of the ARFs and the effect of bevacizumab, on response rate. There was no significant interaction between the expression levels of any of these ARFs on the influence of bevacizumab on response rate.

5.1.17 Prognostic role of ARFs on PFS and OS

We studied the association of ARF status and prognosis for PFS and OS, but apart from VEGF-D impacting on PFS as discussed earlier, none of the other factors had a significant prognostic role (Figures 54 and 55).

Figure 50: Forest plot of hazard ratios for PFS according to VEGF A-C status

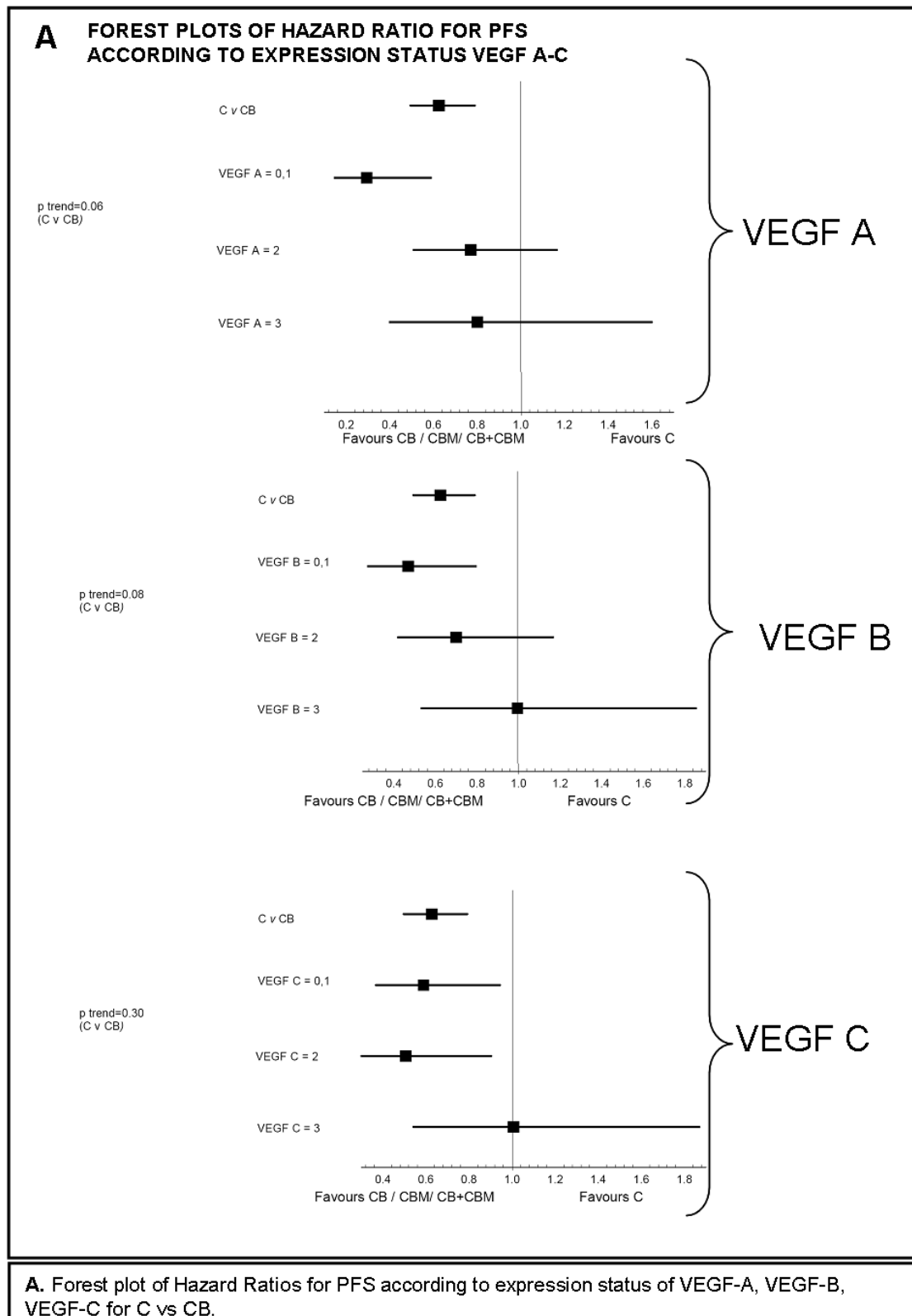


Figure 51: Forest plot of hazard ratios for PFS according to VEGFR1 and VEGFR2 status

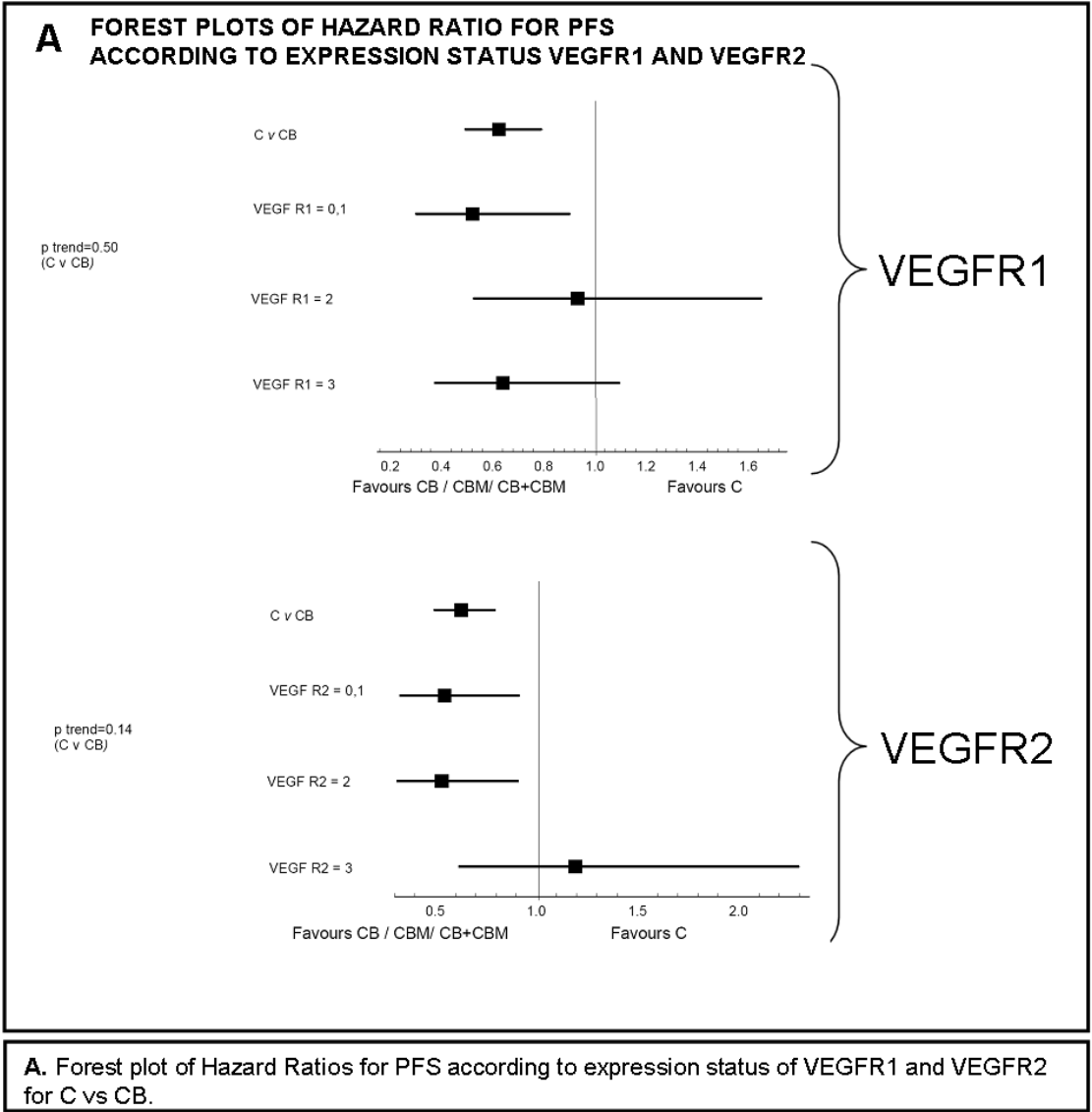


Figure 52: Forest plot of hazard ratios for OS according to VEGF A-C

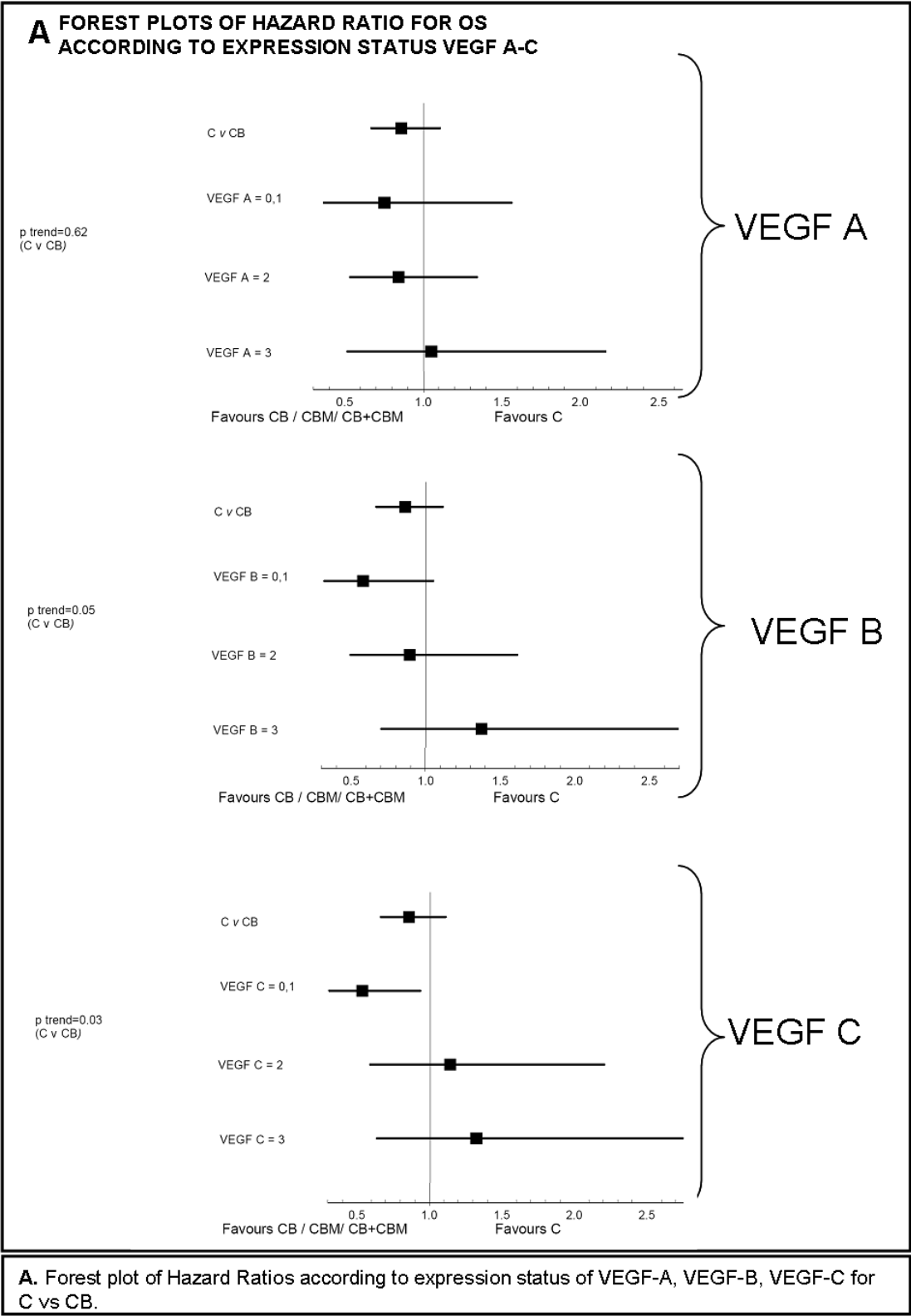


Figure 53: Forest plot of hazard ratios for OS according to VEGFR1,VEGFR2 status

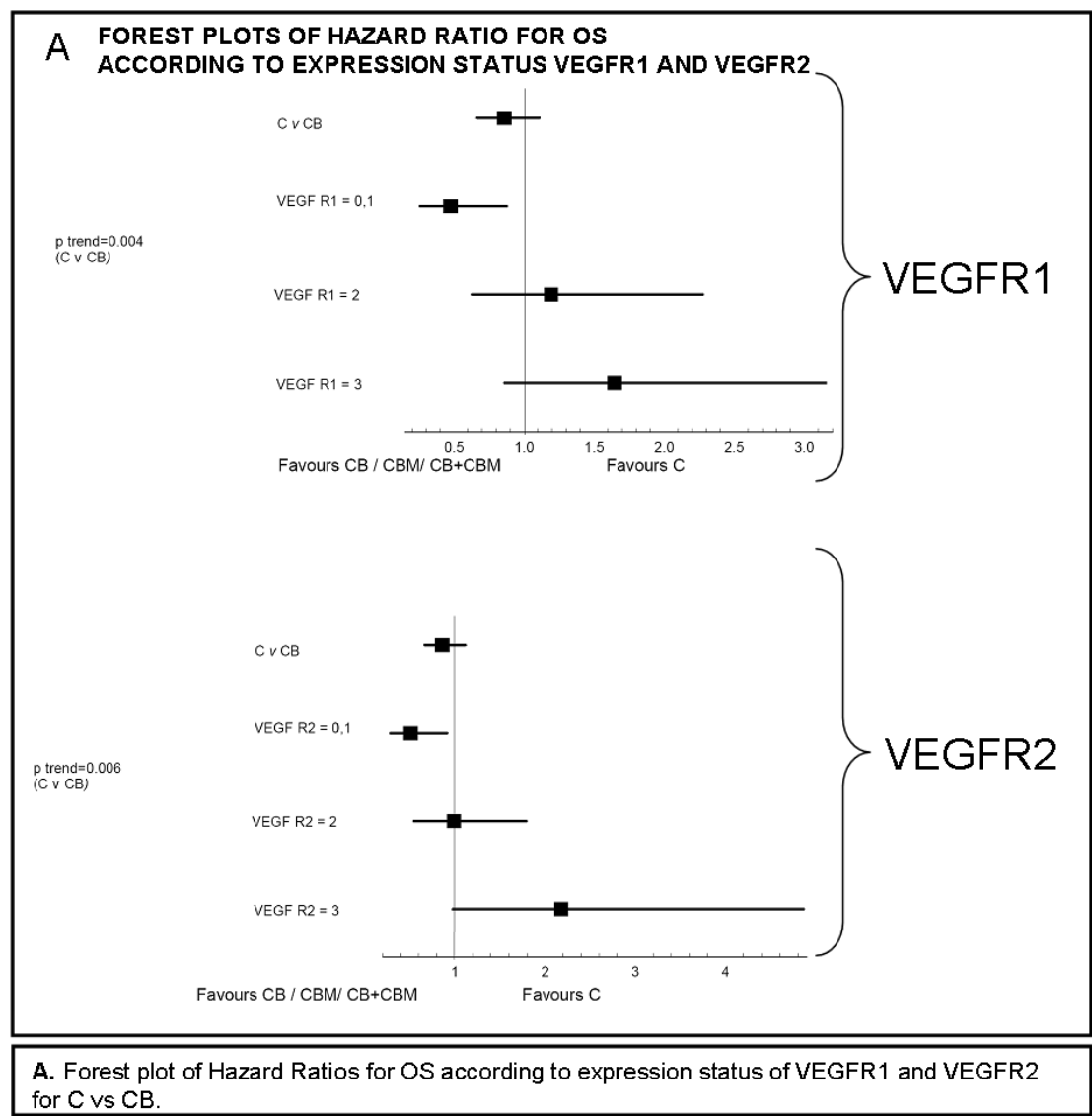


Figure 54: Prognostic role of ARF status and PFS

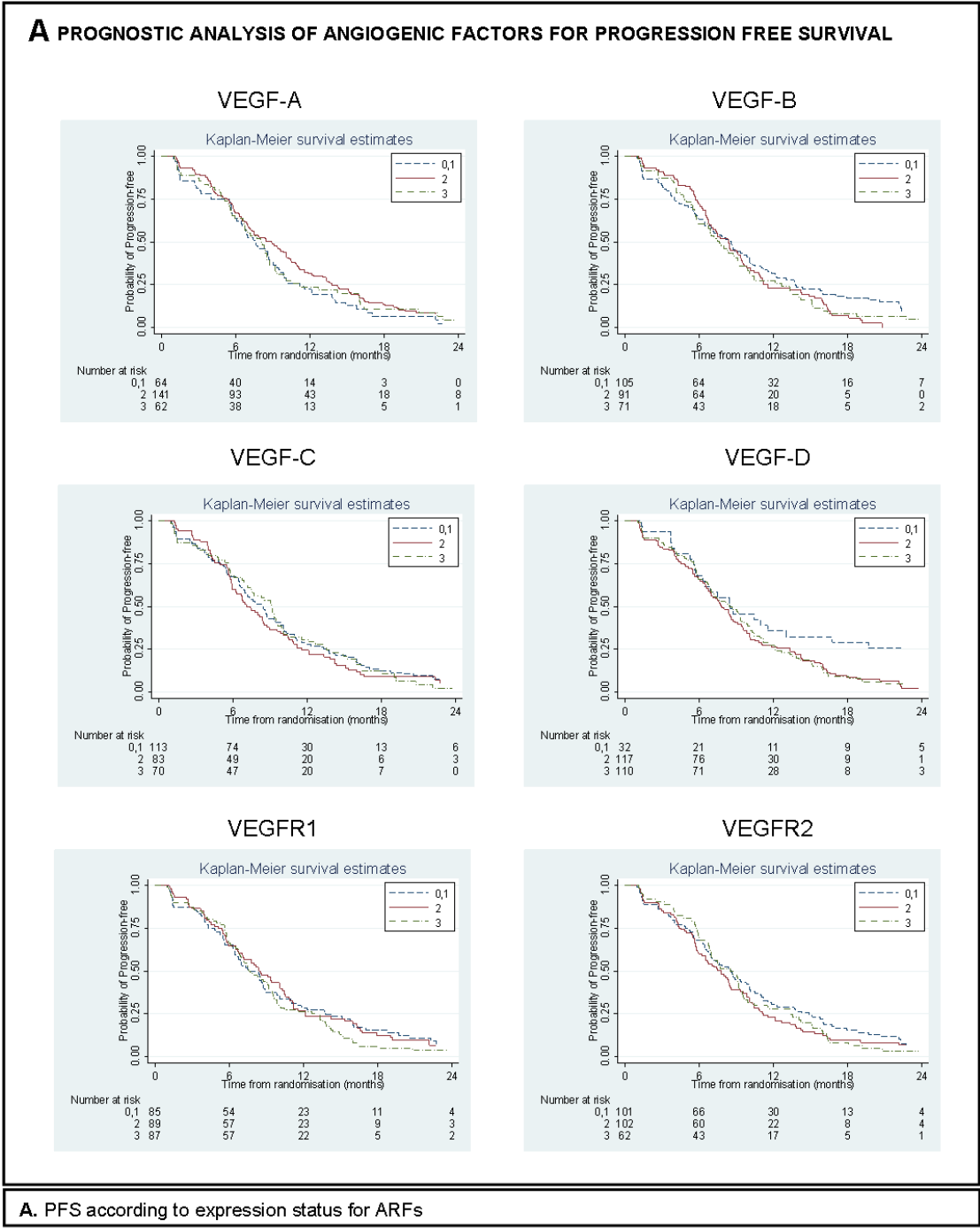
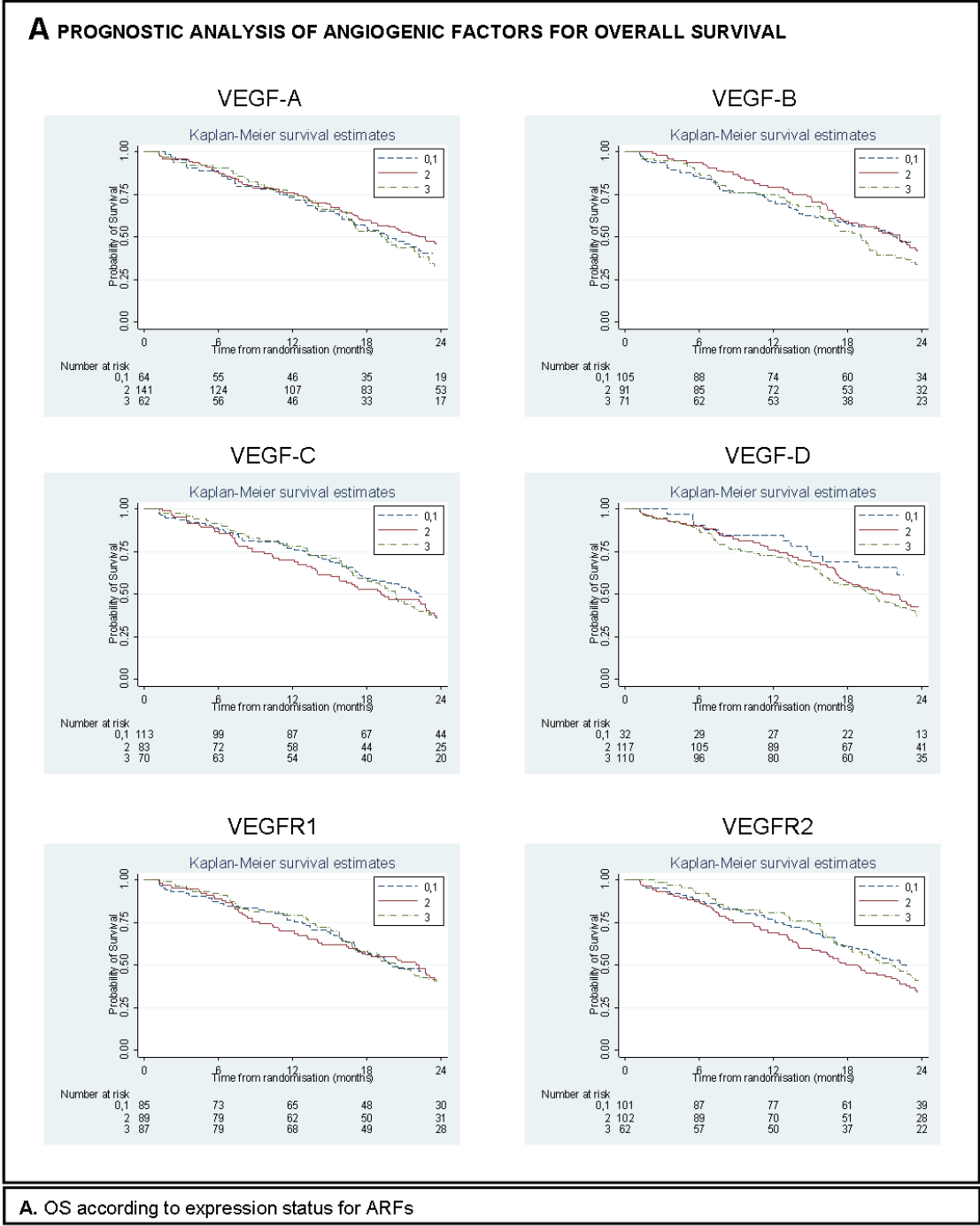


Figure 55: Prognostic role of ARF status on OS



5.2 BIOMARKER ANALYSIS: DISCUSSION

A large representative biomarker study evaluating biomarkers of bevacizumab from a phase III randomised trial was performed. Previous biomarker studies from randomised trials have been limited by the availability of only 18%[335], 34%[281, 378] or 54% [334] of specimens for analysis. The group of patients available for the biomarker study were representative of the trial population with similar prognostic characteristics between the groups, limiting the effect of selection bias on the results.

5.2.1 *KRAS* and *BRAF* mutation status

The mutation status of *KRAS* and *BRAF* genes was obtained from the tumours of 67% of patients from the MAX trial, which evaluated the benefit of adding bevacizumab to capecitabine, with or without mitomycin C. We detected a *KRAS* mutation rate of 28.7% and *BRAF* mutation rate of 10.9%. Although the *KRAS* mutation rate is slightly lower than some studies, it is within the range of results previously published [65, 327, 378]. Additionally, the analysis of MAX samples did not include *KRAS* mutation testing in codon 61, which may account for a slightly lower frequency than other series.

5.2.2 *KRAS* mutations are not predictive of bevacizumab efficacy

Using samples from patients treated on the MAX trial, the ability of *KRAS* and *BRAF* mutations to predict the efficacy of bevacizumab on altering PFS was assessed. Improvements in PFS with OS with bevacizumab were consistent regardless of *KRAS* or *BRAF* mutation status.

This is similar to an earlier analysis performed by Hurwitz *et al* [327, 378] using tumour samples and trial outcomes from the randomised trial of irinotecan, fluorouracil, leucovorin (IFL) with or without bevacizumab [21]. Only 31% of the tumour samples were used for the analysis. In this study the *KRAS* mutant group had a median PFS of 5.5 months in the IFL + placebo group compared to a median PFS of

9.3 months in the IFL + bevacizumab group. In the *KRAS* wild type group the median PFS was 7.4 months for the IFL + placebo group, and 13.5 months for IFL + bevacizumab group. There was improvement in OS with the addition of bevacizumab regardless of *KRAS* mutations status. The findings from this study that *KRAS* mutations do not predict efficacy of bevacizumab improvement in PFS and OS are reinforced by our work.

Interestingly, the study by Hurwitz *et al* reported that there was no increase in response rate with bevacizumab in the *KRAS* mutant group, with response rate of 41.2% in the IFL + placebo group and 43.2% in the IFL + bevacizumab group. The response rate in the *KRAS* wild type group was 37.3% in the IFL + placebo arm, and 60% in the IFL + bevacizumab arm. The lack of increase in response rate in the *KRAS* mutant group was similar in our study, and also not statistically significant. In the *KRAS* mutant group, the response rate was 48% in the capecitabine arm, but 33% in the CB+CBM arm. In the *KRAS* wild type group, the response rate was 27% in the C arm, but 43% in the CB+CBM arm. We explore the similarity by pooling our results with those from Hurwitz *et al*, and showed that the response rate is significantly greater in the *KRAS* wild type group who receive bevacizumab (32% vs 49%, $p=0.0015$), but not significantly different in the *KRAS* mutant group (44% vs 37%, $p=0.36$). The significance of the lack of benefit of bevacizumab increasing response rate in the *KRAS* mutant group is uncertain, as there was no interaction with the more important end points of PFS and OS, which have also been demonstrated to be improved in an unselected population by bevacizumab regardless of tumour response rate [302].

5.2.3 The role of *KRAS* mutations as a prognostic biomarker is uncertain

KRAS mutations did not appear to have a prognostic effect in the MAX trial, consistent with several other studies [15, 378]. However the prognostic significance of *KRAS* is uncertain as there have been alternate large studies showing that *KRAS* is prognostic including the MRC COIN study in metastatic colorectal cancer [85], and the RASCAL study[101]. Given this uncertainty and discordant results, the role of *KRAS* mutations as a prognostic biomarker in metastatic colorectal cancer is likely to be weak.

5.2.4 *BRAF* mutations are prognostic for OS

BRAF mutations were not predictive for bevacizumab efficacy as measured by PFS, OS or RR. This reinforces the similar conclusion reached by Ince *et al* [327]. However the V600E *BRAF* mutation did have a prognostic effect for OS, demonstrated by a median OS in our study of 8.6 months versus 20.5 months in the *BRAF* wild type group (HR 0.45, 95% CI 0.35 – 0.68, $p < 0.001$). The prognostic effect of *BRAF* has been demonstrated in several other studies, in both Stage II and III colorectal cancer [72], as well as metastatic disease [88, 108, 109, 111]. The poor OS in patients with *BRAF* mutant tumours underscores the necessity of developing effective treatments for this subgroup, especially in light of the recent failure of the *BRAF* inhibitor PLX4032 to lead to responses in this subgroup [419].

5.2.5 The association between ARFs and bevacizumab efficacy

This thesis investigated the prognostic and predictive value of the VEGF ligand family members VEGF A-D, and VEGFR1 and VEGFR2 (referred to as angiogenic related factors ARFs), each grouped in 3 levels of scores: 0,1 vs 2 vs 3. Treatment was considered in 2 groups: C vs CB, and C vs CB + CBM, with similar results obtained.

The study used a large representative cohort of 270 tumour samples from the randomised MAX clinical trial (57% of 471 patients). Expression levels were scored by two independent reviewers, one of whom was a anatomical pathologist, blinded to each others scores and to trial outcomes. There was good to very good inter-rater agreement of scoring as determined by a weighted Kappa assessment of > 0.79 between the two assessors. When discrepancies did arise, they were resolved by consensus agreement.

The majority of archived patient tumour samples were from primary tumours (83%), which may not accurately represent the expression level of ARFs in metastatic disease. Whilst there are conflicting reports regarding the correlation of expression of VEGF-A levels from primary and metastatic tissue, there are no studies examining this question for VEGF-B, VEGF-C, VEGF-D, VEGFR1 or VEGFR2 expression. VEGF-A levels have been reported to vary dependent on the location of the

metastases [420, 421] and may be higher in colorectal metastases [422] compared to primary disease, and may vary dependent on the location of the metastases. However Kuramochi *et al* demonstrated that VEGF-A mRNA levels in primary tumours are significantly correlated with matched liver metastases [423]. In our study, there was significant correlation between the matched primary and metastases for VEGF-D, VEGFR1 and VEGFR2. There was not significant correlation of VEGF A-C expression between primary and metastatic samples.

There is no consistent and validated scoring system to define expression levels of the ARFs [335]. Even for VEGF-A, there is an inconsistency of scoring methods and cut-points. The H score, derived from the sum of staining intensity multiplied by the percentage of stained tumour cells has been used for some biomarker studies [281, 334, 335], whereas categorical scoring has been used by others [424, 425].

Moehler *et al* classified as VEGF-D positive any tumour with more than 25% of tumour cells stained (with weak to strong positivity) [355]. In contrast Geroge *et al* defined positivity as >20% of tumour cells staining, and Onogawa *et al* classified VEGF-D positivity by immunoreactivity in >10% of the cancer cells [426, 427]. White *et al* instead graded tumour expression by 0 (absent), 1(weak), 2 (moderate) and 3 (strong) staining intensity, defining positive expression as at least moderate staining intensity [428]. Given the inconsistent approaches in the literature, this study used a simplified scoring system that was designed to be easily replicated. Where intensity was documented as >0, if the percentage of tumour cells was less than 25%, the intensity score was recorded as 1. This was a rare occurrence, occurring in 2.8% (VEGF-A), 5% (VEGF-B), 4.9% (VEGF-C), 5.3% (VEGF-D), 5.8% (VEGFR1), and 4.4% (VEGFR2) of occasions when a core was scored.

5.2.6 Association of VEGF-D with bevacizumab efficacy

Prior to commencing the tissue biomarker study, the literature regarding the angiogenic signalling pathway and VEGF family members was reviewed. VEGF-A, VEGF-C and VEGF-D have all been shown to bind to VEGFR2, triggering angiogenesis [264, 266, 267]. This study explored the hypothesis that depletion of functional VEGF-A levels by the use of bevacizumab, would leave VEGF-C and

VEGF-D to continue signalling unimpeded via VEGFR2, and that over expression of these ligands would be associated with resistance to bevacizumab.

The trend in expression of VEGF-D is predictive for benefit of bevacizumab on PFS and OS, consistent with the pre-specified hypothesis. In a multivariate model testing for biomarker-treatment interactions, the only significant predictive biomarker was VEGF-D, where lower scores were associated with greater treatment effects. Patients with tumours that had over expression of VEGF-D received less benefit from bevacizumab on PFS relative to those patients who had little or no expression. This is the first time that a biomarker has been identified that significantly predicts for the benefit of the drug on PFS in colorectal cancer. This is also significant as VEGF-D is a plausible biomarker, with a known interaction with VEGFR2 and capability of inducing angiogenesis.

A biomarker study such as this does require validation. To further strengthen the relationship, the association should be tested on an independent cohort, such as the NO16966 study [335], but logistically this may prove difficult due to lack of tumour availability for staining. Secondly, the findings could be confirmed by staining with independent anti-VEGF-D antibody. Finally, the association between VEGF-D over expression and resistance to bevacizumab could be strengthened by modeling in xenograft experiments using cell lines be modeled in xenograft experiments by examining the response following over and under expression. If validated, the utility of bevacizumab could be improved by restricting use to patients with low levels of VEGF-D, enhancing PFS and OS, whilst reducing the overall cost burden to the community.

5.2.7 Association of other ARFs with OS effect of bevacizumab

The only significant biomarker that remained significant on a test for interaction in a multivariate analysis (in a step-down procedure) was VEGFR1 expression. This was despite a lack of association of these factors with PFS effect from bevacizumab. The significance of this result is uncertain, as this was a secondary endpoint of the study, and a chance association with OS is a possibility. Furthermore, although statistically significant, the strength of the association is clinically less certain due to the hazard

ratio < 2. Furthermore, other studies have previously investigated the association of VEGFR1 and VEGFR2 with bevacizumab efficacy, but not found any association [334, 335]. Given the lack of interaction with PFS of these factors, the importance of the interaction with OS is uncertain.

These results indicate that VEGF biomarkers may play an important role in predicting greater treatment effects of bevacuzimab on PFS and overall survival for patients with lower expression of these biomarkers. VEGFD appears particularly important when considering it was the main factor identified on the PFS outcome which is a more sensitive (and hence probably more reliable) indication of treatment effect. However, the role of VGFR1 is also important to assess further, as there was some evidence of different treatment effects on survival according to biomarker status (global test for interaction) and it was the most significant variable in those multivariate analyses.

6 CONCLUSION

This thesis aimed to explore methods of maximising the efficacy of the two classes of targeted therapies currently used in treating patients with metastatic colorectal cancer. EGFR-mAb therapy and the anti-VEGF-A antibody bevacizumab provide modest treatment benefits to patients, however refinement in their use may improve overall survival times, and limit spiraling treatment costs.

The *in vitro* and *in vivo* combination of an EGR-mAb with an EGFR-TKI in colorectal cancer was assessed. Synergistic anti-proliferative growth effects that translated to high response rates in *KRAS/BRAF* wild type patients were documented. The mechanism behind the synergy was identified as a greater degree of vertical inhibition of the EGFR pathway, represented by high rates of EGFR related toxicity, and potentially by greater breadth of suppression of the pathway, through the decrease in STAT3 levels which are not affected by cetuximab as a single agent at the doses used in our *in vitro* studies. The activity was sufficiently high to encourage a randomised clinical trial of the strategy, restricted to *KRAS/BRAF* wild type patients.

Cell lines with acquired resistance to cetuximab were created, that were also cross resistant to EGFR-TKI therapy. Changes in compensatory pathways are likely to explain the mechanism of resistance rather than acquisition of *KRAS* mutations. REG4 is highly up-regulated in the cell lines, and a candidate for further investigation in *in vitro* and *in vivo* studies. Additionally this thesis demonstrated that the resistant cell lines reacquire sensitivity to cetuximab. Based on supportive literature, this novel finding encourages the development of a small non randomised clinical trial of the strategy.

Lastly we evaluated biomarkers of efficacy with bevacizumab in colorectal cancer using tumour samples and outcome data from the MAX clinical trial were explored. *KRAS* and *BRAF* mutations do not predict efficacy of bevacizumab, but *BRAF* mutations are prognostic for overall survival. VEGF-D expression was identified as a predictive biomarker for improvement in PFS and OS with bevacizumab. This is biologically plausible, and the first statistically significant biomarkers discovered for

the drug. The observation requires further validation, ideally in a prospective randomised clinical trial of bevacizumab.

In summary, the strategies identified provide potential avenues for incremental improvements in the treatment of colorectal cancer. Whilst further work is required to validate these approaches, the thesis demonstrated that identifiable molecular mechanisms govern response and resistance to targeted therapy in colorectal cancer. Harnessing this knowledge may lead directly to improvements in outcomes for patients.

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