Personalisation of therapy in advanced colorectal cancer

Jennifer Frances Seligmann

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Intellectual property and publication statements

The candidate confirms that the work submitted is her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Input from the supervision team was provided in each chapter, particularly in study conception and design, and interpretation of results. The contribution of JS in each chapter is detailed below, with acknowledgement given for help received. JS was solely responsible for writing of the thesis.

Chapter 2

- Conception and study design
- Dataset creation (from FOCUS and PICCOLO trials)
- Statistical analysis (statistical plan, creation of STATA do-files, data interpretation, creation of figures and tables)

Faye Elliott provided help in creating STATA do-files and statistical advice.

Chapter 3

- Conception and design
- Dataset creation (from FOCUS, COIN and PICCOLO trials)
- Statistical analysis (statistical plan, creation of STATA do-files, data interpretation, creation of figures and tables)

Faye Elliott and David Fisher provided help in creating STATA do-files and statistical advice.

Chapter 4 and 5

- Conception and design
- Laboratory work (identification of suitable samples, RNA extraction, cDNA synthesis, running PCR assays)
- PCR data interpretation
- Statistical analysis (statistical plan, data interpretation)

Gemma Hemmings assisted in RNA extraction. Bart Jacobs and Gemma Hemmings assisted in running of PCR assays and PCR data interpretation. Faye Elliott created the STATA do-files and ran the statistical analysis.

Chapter 6

- Conception and design
- Identification of expanded SNPs
- Laboratory work (identification of suitable samples, germline DNA extraction, tumour DNA extraction, running fluidigm assays)
- Fluidigm data interpretation
- Manual assessment of end-point data for each study participant from the raw trial data
- Statistical analysis (statistical plan and data interpretation)

Philip Chambers and Laura Horsfall provided input in the choice of the expanded SNPs to be investigated. Philip Chambers performed the UGT1A1*28 fragment length analysis and assisted in running the Fluidigm assays and data interpretation. Faye Elliott performed the statistical analysis.

Journal articles

1. *"Combined epiregulin and amphiregulin expression levels as a biomarker of prognosis and panitumumab benefit in RAS-wt advanced colorectal cancer*"

   J Seligmann, F Elliott, B Jacobs, S Richman, S Brown, G Hemmings, J Barrett, S Tejpar, P Quirke and M Seymour.

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2. *"HER3 overexpression as a biomarker of prognosis and panitumumab benefit in RAS-wt advanced colorectal cancer*

   J Seligmann, B Jacobs, F Elliott, A Sibley, S Richman, K Owzar, S Brown, J Barrett, H Hurwitz, P Quirke, A Nixon and M Seymour

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Oral Presentations

"Combined epiregulin (EREG) and amphiregulin (AREG) expression levels as a biomarker of prognosis and panitumumab benefit in RAS-wt advanced colorectal cancer (aCRC)"

Seligmann J, Elliott F, Richman S et al.
European Congress of Pathology. London, 2014

Poster Discussions

"*Exploring the poor outcomes of BRAF-mutant advanced colorectal cancer: Analysis from 2530 patients in randomised clinical trials"*
J Seligmann , D Fisher, F Elliott, et al
American Society for Clinical Oncology Annual Meeting, Chicago, June 2015

"Primary tumour location as a prognostic and predictive factor in advanced colorectal cancer: data from 2075 patients in randomised clinical trials"
J Seligmann, F Elliott, S Richman et al.
European Society for Medical Oncology Annual Meeting, Madrid, September 2014

"Combined epiregulin and amphiregulin expression levels as a biomarker of prognosis and panitumumab benefit in RAS-wt advanced colorectal cancer"
J Seligmann, F Elliott, B Jacobs, et al
American Society for Clinical Oncology Annual Meeting, Chicago, June 2014

Poster presentations

"HER3 overexpression as a biomarker of prognosis and panitumumab benefit in RAS-wt advanced colorectal cancer
J Seligmann, F Elliott, B Jacobs, et al
American Society for Clinical Oncology Annual Meeting, Chicago, June 2015

"The derived neutrophil lymphocyte ratio (dNLR) as a biomarker in advanced colorectal cancer"
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Abstract

Personalisation of therapy is an important goal in modern oncology, however routes for biomarker discovery and validation are challenging with a high level of evidence required prior to application into routine care. Whilst cancer care is stratified by biomarker status in some tumour sites, less progress is evident in advanced colorectal cancer (aCRC).

In this thesis candidate clinical and molecular biomarkers have been tested within the datasets and biobanks of randomised controlled trials in aCRC (FOCUS, PICCOLO and COIN). Specifically, the utility of routine clinical information as biomarkers, the mechanisms of the poor prognosis of *BRAF*-mutant aCRC, predictive markers of efficacy for anti-EGFR agents in *RAS*-wt patients, and testing of pharmacogenomic markers of toxicity and efficacy for irinotecan have been tested.

Routinely measured markers of the systemic inflammatory response, the derived neutrophil-lymphocyte ratio and platelet count, were validated as independent adverse prognostic markers in aCRC and may help identify patients who are not disadvantaged more conservative upfront treatment approach.

It has been demonstrated that the poor prognosis conferred by *BRAF*-mutation is mainly driven by rapid progression following first-line therapy, rather than chemo-resistance. Knowledge of *BRAF*-mutation status therefore provides useful clinical information beyond the context of prognostication and selection for anti-EGFR therapy, with particular implications for treatment sequencing.

mRNA overexpression of EGFR ligands and HER3 were both shown to be promising positive predictive markers for anti-EGFR therapy in aCRC in *RAS*-wt patients. In both studies a population of *RAS*-wt patients who fail to benefit from anti-EGFR agents were clearly identified. Both markers warrant urgent further validation and clinical development.

Therefore further clinical and molecular biomarkers have shown potential clinical utility in aCRC, which all hold promise for routine application and to further personalise treatment in aCRC.

Abbreviations

5FU – Fluorouracil

aCRC – Advanced colorectal cancer

Alk phos – Alkaline phosphatase

AREG - Amphiregulin

AUC – Area under the curve

BMI – Body mass index

BRAF – v-RAF murine sarcoma viral oncogene homologue B

BSC – Best supportive care

CDF – Cancer drug fund

CEA – Carcino-embryonic antigen

CI – Confidence interval

CISH – Chromatin in-situ hybridisation

CMI – Cell mediated immunity

CNV – Copy number variation

CRC – Colorectal cancer

CRP – C-reactive protein

CRUK – Cancer research UK

Ct-DNA – Circulating tumour DNA

CTL – Cytotoxic T-lymphocyte

DCR – Disease control rate

dMMR – Defective mismatch repair

DNA – Deoxynucleic acid

dNLR – Derived neutrophil lymphocyte ratio

DPD – Dihydropyrimidine dehydrogenase

EGF – Epidermal growth factor

EGFR – epidermal growth factor receptor

EREG - Epiregulin

ER – Oestrogen receptor

ESMO – European Society of Medical Oncology

FAP – Familial adenomatous polyposis

FDA – Food and drug agency

FFPE – Formalyn-fixed paraffin-embedded

Fig - Figure

FISH – Fluoresence in-situ hybridisation

FOLFIRI – FU plus irinotecan

FOLFOX – FU plus oxaliplatin

Hb – Haemoglobin

HI – Humoral immunity

HNPCC – Hereditory non-polyposis colon cancer

HR – Hazard ratio

HWE – Hardy-Weinberg equilibrium

IL- - Interleukin

Ir - Irinotecan

KRAS – Kirsten-rat sarcoma

MiRNAs – Micro-RNAs

mGPS – modified Glasgow prognostic score

MHC-1 – Major histocompatibility complex-1

MLH-1 – Mutl homolog1

MoAbs – Monoclonal antibodies

MRC – Medical research council

NCCN – National comprehensive cancer network

NICE – National institute of clinical excellence

NLR – Neutrophil lymphocyte ratio

NRAS – Neuroblastoma rat sarcome

NK – Natural killer

OR – Odds ratio

OS – Overall survival

PCR – Polymerase chain reaction

PET – Positron emission tomography

PFS – Progression free survival

PI3KA – Phosphatidylinositol 3-kinase

Plts – Platelets

PLR – Platelet lymphocyte ratio

P-PS – Post-progression survival

PS – Performance status

PSA – Prostate specific antigen

PTL – Primary tumour location

RCTs – Randomised controlled trial

RFS – Recurrence free survival

RNA – Ribonucleic acid

ROC – Receiver operator curves

RR – Response rate

RT-PCR – Reverse transciptase polymerase chain reaction

SEER – Surveillance, epidemiology and end results program

SMAD – SMAD family member 4

SNP – Single nucleotide polymorphism

TCGA – The cancer genome atlas

TNF-α – Tumour necrosis factor-α

TS – Thymidylate synthetase

UGT – UDP-glucuronosyl-transferases

UK – United Kingdom

VEGF – Vascular endothelial growth factor

WCC – White cell count

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Chapter 1. Introduction

1.1. Personalised medicine

Personalised medicine – the use of patient- and tumour-specific information to select the optimum treatment for individual patients – is an increasingly important approach for many tumour types. This involves applying knowledge of an individual's clinical, histopathological and molecular characteristics to choose the best treatment plan. More accurate prediction of efficacy and toxicity for a wider range of cancer drugs has the potential to improve disease outcomes, avoid toxicity, better patient compliance and improve cost-effectiveness, and could enable oncologists both to make better use of established drugs and to bring forward novel treatments for defined patient subgroups. This is particularly pertinent when several therapeutic approaches are available.

The field of personalised medicine has expanded with the discovery and validation of biomarkers to guide specific clinical scenarios. This is not a new concept in oncology; breast cancer patients have been selected for hormone treatment based upon their oestrogen (ER) and progesterone receptor status since the 1980s. Further progress came with the discovery and validation of HER2 amplification as a positive predictive marker for trastuzumab in the 1990's.(1) Another example was identification of translocation-prone tumour groups such as the BCR-ABL fusion genes in chronic myeloid leukaemia:(2) subsequent use of imatinib, targeted therapy against the ABL kinase domain, has resulted in disease control rates (DCR) of 93% in this population.(3) Therefore the search for cancer biomarkers is an integral part of cancer research.

In its simplest form, personalised medicine may use a single molecule of clear functional significance (e.g. *Kirsten-RAS*[*KRAS]* mutation) to dichotomise the patient population (present; absent) and make a treatment decision (Epidermal growth factor receptor [EGFR]-targeted therapy; not). However, massively parallel gene sequencing now allows whole cancer genomes to be rapidly and accurately sequenced, unveiling the complexity and diversity of gene mutations present in solid tumours. It is hoped that detailed knowledge of this genetic diversity will both steer the development of novel agents and identify new biomarkers of response and toxicity. To date, however, relatively few

biomarkers have been proven sufficiently discriminatory and reliable to guide treatment decisions.

Furthermore this increased understanding of biology has not necessarily translated into the delivery of safe and effective biology-driven cancer treatments: there has been no increase in Food and Drug Administration (FDA) approval of cancer drugs with only half of drugs entering costly phase III trials being approved.(4) The need to utilise biomarkers within the drug development process to predict efficacy and safety of new drugs at an earlier stage is recognised. However the development and commercialisation of biomarker tests is also an expensive and lengthy process.

These issues have been recognised and personalised, or precision medicine, has become a national priority, with creation of the Stratified Medicine Innovation Platform and an investment of £200 million over the next five years.(5) This represents a consortium of government and charities (including Cancer Research UK [CRUK] and Arthritis Research UK), the Medical Research Council (MRC) and the National Institute for Clinical Excellence. It is hoped that communication between industry, academia and government may support innovative ideas by removing potential barriers and aligning priorities.(5)

This thesis will test potential personalisation strategies and novel biomarkers to guide the treatment of advanced colorectal cancer (aCRC). This chapter shall describe classes of biomarkers, and their development, validation and reporting. Current treatment strategies and use of biomarkers, and routes for biomarker development in aCRC will then be discussed.

1.2. Biomarkers

A biomarker is "a characteristic used to measure and evaluate objectively normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention".(6) This term covers a wide variety of data types, including biochemisty laboratory tests, electrocardiograms, and imaging testing such as positron emission tomography (PET) scans.

Biomarkers can therefore inform many different clinical decisions across medical specialties. However to be clinically useful they must fulfill the following criteria:(6)

- Be based upon sound scientific rationale,
- Acceptable to measure,
- Good sensitivity and specificity,
- Have a robust assay
- Be cost-effective.

Biomarkers are divided into broad categories depending upon their application, including screening biomarkers, diagnostic biomarkers, prognostic biomarkers, predictive biomarkers, monitoring biomarkers and toxicity biomarkers. Biomarkers from each category have a role in cancer medicine.

1.2.1. Screening biomarkers

Earlier diagnosis is a key aim of cancer research with an objective of detecting cancer at a curable stage so a screening biomarker could be of great clinical utility. A robust screening biomarker must meet additional criteria: it must detect disease at an early asymptomatic stage, and have a careful balance of sensitivity and specificity. A screening biomarker must be highly specific to avoid false positives and avoidance of unnecessary testing, but be sensitive to ensure that patients aren't falsely reassured. Therefore the optimal balance of sensitivity and specificity will take into account the consequences of producing either a false-positive or a false-negative result. Furthermore to be part of a successful screening programme it needs to be cost-effective, non-invasive and produce better outcomes.

1.2.2. Diagnostic biomarkers

Diagnostic biomarkers will provide information in symptomatic patients and will be used alongside other diagnostic tools. They can be useful in patients with unknown primaries or to aid diagnostic sub-classification.

1.2.3. Prognostic biomarkers

A prognostic biomarker discriminates between patients who will have good or poor outcomes, independent of the treatment they receive. A prognostic marker may identify patients at lower/higher risk of relapse after surgery, or those more/less likely to survive for several years with advanced disease, so can be useful in risk stratification.

Prognostic markers can be used as stratification factors in randomised controlled trials (RCTs) to ensure that the test and control populations are balanced; this ensures that any observed effect between groups is due to the treatment. Such stratification allows for correct interpretation of study end-points in the presence of possible confounders.

Rather confusingly, a poor prognostic marker may also correlate with a poor rate of response or shorter time to progression on treatment, simply because the underlying rate of tumour growth is higher. However, it does not distinguish which treatment will be more or less effective. Therefore, while prognostic biomarkers may sometimes identify patients who do (or do not) need treatment at all, they do not help decide which treatment to use, and therefore have a limited role in personalised medicine.

1.2.4. Predictive biomarkers

Predictive biomarkers, by contrast, correlate directly with the probability of benefit from a specific treatment. An example is ER positivity in breast cancer, which is strongly predictive for benefit from hormonal therapy.(7) Predictive biomarkers are clinically extremely useful, but are much harder to detect and validate than prognostic markers. Differences between a prognostic and predictive biomarker are illustrated in figure 1.1.

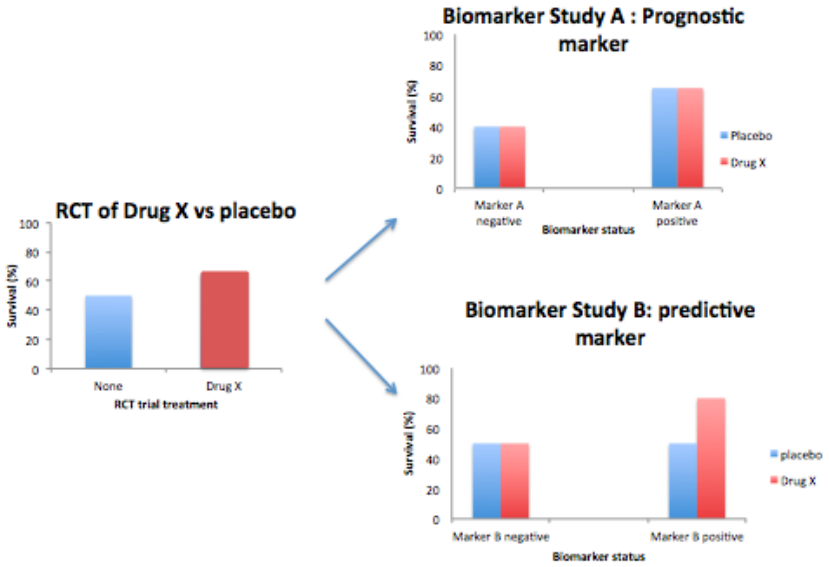

Figure 1-1 Different information provided by a prognostic and predictive biomarker

Drug X was superior to no treatment in a RCT; biomarker studies A and B were then performed to identify candidate predictive biomarkers for Drug X. In Biomarker Study A the biomarker is prognostic: patients who are positive for the prognostic biomarker had

increased benefit from drug X compared to biomarker negative patients, but also had improved outcomes with no treatment. However in Biomarker Study B the biomarker is predictive for benefit from drug X: patients positive for the predictive biomarker will have improved outcomes with drug X compared to biomarker negative patients, but biomarker positive patients not treated with the drug will have similar outcomes to the biomarker negative group (fig. 1.1). This scenario also illustrates potential problems of testing a potential predictive marker in a non-randomised population: without an untreated control group it appears that the prognostic marker positive patients have improved outcomes due to treatment with drug X, but they would do well regardless.

However biomarkers can be prognostic and predictive, such as ER positivity in breast cancers. This should be considered when interpreting results.

1.2.5. Toxicity biomarkers

Toxicity biomarkers are a form of predictive marker that predicts for a patient's likelihood of drug toxicity. Candidates are in most cases genetic polymorphisms for genes encoding proteins involved in drug metabolism or clearance. *UGT1A1*28* and *DYPD* polymorphisms have been proposed as toxicity biomarkers for irinotecan(8) and fluorouracil (5FU)(9) respectively; however, both are still of uncertain clinical utility, so whilst available for routine use their uptake is not universal.

1.2.6. Monitoring biomarkers

Monitoring biomarkers have several potential utilities: monitoring patients following curative treatment to ensure they remain disease-free, surveillance of patients during treatment breaks, or to monitor during treatment to ensure clinical benefit. Similar to screening biomarkers, a monitoring biomarker needs to be both sensitive and specific to ensure correct clinical decisions are made.

1.3. The challenges of biomarker identification and validation

For integration of a biomarker into routine practice there must be great confidence that the biomarker is robust and measures what we think it does; it must demonstrate analytic validity and clinical utility. Analytical validity is the demonstration of quality of the biomarker assay: critically important is reproducibility, ensuring that the same answer will be produced for the same sample within predefined technical variation. Clinical utility is the ability to distinguish two groups biologically that have sufficiently different clinical outcomes.(10)

For this necessary rigour and validation, biomarker development is costly, time-consuming and usually requires collaboration between industry and academia. Very few candidate biomarkers progress to prospective trials, and fewer reach implementation in the clinic; hence this pathway is not efficient.(11)

Guidelines for the process of biomarker validation have been issued by CRUK (CRUK Biomarker Roadmap),(12) and will be described further in Section 1.4.3. The importance of these guidelines are illustrated by the fact that a candidate predictive biomarker that appears promising in pre-clinical and non-randomised studies can often prove non-discriminatory in testing in randomised datasets or prospective trials. Guidelines by the National Comprehensive Cancer Network (NCCN) describe the level of evidence for a biomarker, depending upon strength of association and the level of validation provided.(13)

It is therefore important to understand the stages of biomarker development to ensure that the candidate biomarker is fulfilling each step before validation takes place. Several useful review articles have summarised some of the challenges.(10, 14-17)

1.4. The multi-step process of biomarker development

Biomarker development is a multi-step process that can be broadly categorised into 4 main steps:(10)

- Preclinical exploratory studies for identification of potential biomarkers.
- Development of a robust assay with analytical validity.
- Clinical validation of biomarker and assay in
    - 'Prospectively planned retrospective studies' in randomised trial bio-banks,
    - Prospective trials.

These phases are not distinct, with multiple studies usually performed at different time points. Successful biomarkers will need to overcome challenges at each step before clinical implementation. At each step, every biomarker study requires a pre-defined statistical plan.

1.4.1. Biomarker study design and statistical considerations

A predefined study and statistical plan is crucial to any biomarker study to limit bias and data misinterpretation. Common issues encountered include inappropriate statistical analyses, data over-fitting, multiple hypothesis testing, inappropriate study population difficulty in cut-point determination and overlapping training and validation cohorts.(17)

Ideally a biomarker study population will match the general population as much as possible; retrospective studies may be biased towards patients with available material which may in turn be related to tumour size and patient outcome.(18) To aid interpretation, clear inclusion/ exclusion criteria should be provided and upfront identification of potential confounders.

Consideration of planned sub-group analyses should also be ideally performed prospectively as their results can be over-interpreted: the number of sub-groups, their pre-specification and their ability to detect a statistical interaction should be considered. To compare treatment effects in the sub-groups, interaction tests should be performed rather than p-values: here, the biomarker is treated as a covariate that may affect treatment effect.(19)

Commonly multiple markers will be tested in drug development studies, particularly in the discovery stage. Whilst this is efficient and cost-effective, multiplicity can lead to risk of finding false-positive results.(19) Similarly this can be problematic with post-hoc testing in multiple biomarkers in retrospective series, dealing with interim analyses and multiple endpoints. A solution is to adjust the level of significance by the number of tests performed, the Bonferroni correction.(20) The impact of multiple testing in genome-wide association study (GWAS) and pharmacodynamics studies can be addressed by a post measure assessment of confidence, the false discovery rate, that estimates the number of false-positives based upon the data.(21) Again the need for validation of promising biomarkers in independent datasets is highlighted.

Despite these precautions, false positive results reaching statistical significance may occur; and, conversely, clinically significant associations may fail to meet the statistical significance thresholds required in the context of multiple testing, particularly in studies with small sample sizes. It is therefore important that results are considered in the wider context, for example biological plausibility and consistency with previous evidence. For interpretation of analyses, confidence intervals will be more informative than p-values.

1.4.2. Strategies for biomarker discovery

Strategies for biomarker discovery can be hypothesis-driven, exploiting prior knowledge of molecular pathways to examine molecules likely to impact on drug activity. Alternatively they may exploit "-omic" technologies to interrogate the whole genome, proteome, etc., for groups of markers ('signatures') correlating with drug effects. These studies are usually performed in diseased/ non-diseased, treated/non-treated groups, to identify molecules with discriminatory potential.

With high throughput technology thousands of molecules can be assessed; however this technology suffers from high false-positive rates, thereby slowing the progression of useful biomarkers. One issue may be the lack of clear hypothesis-based clinical question prior to discovery work being performed.(10)

1.4.3. Analytical validity of a candidate biomarker

Analytical validity ensures that the assay will measure the biomarker reliably in the population of interest and be reproducible between laboratories. It is therefore crucial to limit possible bias during assay development and biomarker studies.

Many sources of variations can impact on effect: type of tissue used, collection and storage of samples, fixation, laboratory batch effect (including reagent lots, shifts in instrument calibration), variations on antibody 'work up'.(11)Guidelines have been created to reduce variation by the Biospecimen Reporting for Improved Study Quality(22) and UK NEQAS.(23)

Most biomarker studies are based upon single tumour samples for each study participant, though studies of intra-tumoural heterogeneity suggest that testing from multiple blocks of the same tumour may be necessary.(24) Furthermore the molecular profile of tumours can vary from primary to metastases, and over time: deregulation of EGFR signalling has been shown to occur between primary and metastatic sites.(25) Whilst *KRAS*, *BRAF* and *PIK3CA* mutations show good concordance between primary and metastatic disease (91%, 100% and 94%, respectively), EGFR, *PTEN* and pAKT frequently differ (61%, 66% and 54% respectively).(24, 26-28)

1.4.4. Clinical validation of biomarkers

Clinical validation assesses the strength of association between the assay result and the clinical outcomes. The principle methodology is testing statistical significance or strength of association using receiver operator curve (ROC) analysis.

Validation of prognostic markers should be ideally performed in patient groups not receiving experimental treatment, but for whom clinical information is available, for example the control arm of a randomised controlled trial (RCT).

Ideally predictive biomarker validation is performed in a specifically designed prospective RCT, but this is not usually feasible. Instead the so-called 'prospectively retrospective' approach of performing a biomarker validation experiment to a prospectively-planned statistical plan, but using stored biosamples from a previously performed RCT is

employed. However high quality samples from well annotated series are sparse and therefore only promising biomarkers should be tested in such datasets.(14)

A common issue of testing predictive markers in RCT biobanks is the lack of sufficient power to detect effect, particularly for rare mutations or amplifications. The predictive analysis compares the impact of a drug in two biomarker-defined cohorts, therefore the trial population is divided into four. Hence the smallest detectable difference in treatment effect between the two biomarker groups will be larger than the treatment effect that the original trial had been powered for.(29)

There are a variety of trial designs utilised to test predictive markers prospectively alongside treatment of interest, including population enrichment designs, biomarker by treatment interaction designs, biomarker strategy design, biomarker adaptive threshold designs, adaptive accrual design and enrichment signature designs. The methodology used will depend upon the clinical question, the type of biomarker, the level of evidence for the biomarker, the need to test treatment in a biomarker negative group and projected sample size.(19)

1.4.5. Clinical utility of a biomarker

Clinical utility describes the usefulness of a validated biomarker. This will usually depend upon the clinical situation, availability of effective therapy, magnitude of clinical benefit in biomarker groups and relative value to patient and society. For example, a prognostic marker must provide additional discriminatory clinical information than is already provided by readily available clinical and pathological risk stratification.

Furthermore, a biomarker that has reached the clinic may also have limitations depending upon its setting. For example, prostate specific antigen (PSA) has been approved for monitoring of prostate cancer since 1989; more recently it has been approved for the screening of prostate cancer following research reporting that raised PSA in asymptomatic individuals was associated with increased risk of developing prostate cancer.(30) However this test suffers from several limitations: increased PSA levels are seen in patients with benign prostatic enlargement, inflammation and infection. Additionally PSA levels do not correlate with severity; higher grade tumours are not differentiated from lower grade tumours that may not require active management.(11)

This controversy has been heightened by results of two prospective studies investigating the impact of PSA screening.(31, 32) One study reported that prostate screening led to a 20% reduction in prostate cancer-specific mortality,(31) whereas no effect on survival was demonstrated in the other.(32) Both trials reported that PSA measurement resulted in over-diagnosis and over-treatment of prostate cancer, with resultant unnecessary biopsies, toxicity from perhaps unnecessary treatment and psychological distress.

In England, it is not recommended that the PSA be used as a screening tool in asymptomatic men.(33) Instead a biomarker with greater sensitivity for prostate cancer, which can discriminate between indolent and aggressive disease would be more useful.

1.5. Reporting of biomarker studies

Reporting of results from biomarker studies needs to be thorough and transparent to provide sufficient information for the assessment of the quality of the study and the generalizability of the results. The REMARK guidelines have provided a framework for the structure and content for biomarker papers.(34) Although developed for the reporting of prognostic markers, most principles can be applied into papers studying predictive markers. Additionally the Consolidated Standards of Reporting Trials (CONSORT) have developed a number of initiatives to guide reporting of RCTs, some also relevant to biomarker reporting.(35) CONSORT diagrams should be provided for predictive biomarker reporting from RCTs: these allow the reader to understand the biomarker population within the RCT population and illustrate potential bias with missing samples.

The consequences of poor biomarker reporting are that dramatic but wrong results due to poor study design or inappropriate analysis could attract undeserved attention. This may lead other researchers down incorrect avenues, or worse impact on patient care. This issue may contribute to the current lack of validated biomarkers. Proper reporting will fairly describe limitations to be considered in the study's interpretation and suggestions for future work.(11)

1.6. New technology and integration of new data

Great progress has been made since the initial draft of the human genome was published in 2001.(36) High-throughput technologies have expanded our understanding of cancer pathogenesis in individual patients, with technology covering deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins and metabolites. The worldwide collaborative personal genome project and 1000 genomes project plan to sequence thousands of individual genomes to better understand genomic variability.(37, 38) It is challenging previous held concepts: instead of a single driver mutation, most tumours appear to be driven by a multitude of genetic and epigenetic events.(39)

Results from whole genome sequencing and gene expression profiling can now be available in days and massively parallel technologies have dramatically reduced costs of sequencing,(40) so the integration of this data into routine clinical practice may become reality. For example a gene expression profiling test, the Oncotype Dx, that provides an algorithm that assesses risk and calculates benefit for adjuvant chemotherapy for breast cancer, has been approved by the FDA and the National Institute of Clinical Excellence (NICE).(41)

However several practicalities need consideration prior to routine use of such technology in hospital laboratories. These include capacity to keep updated with rapidly advancing technology, validation of new assays, and necessary expansion of molecular pathology and bio-informatics. Consensus will be needed on choice of sequencing platform, optimal gene depth and coverage for profiling; variable coverage over key genes rather than whole exomes, genomes or transcriptomes. Additionally decision of when to test: at diagnosis of early disease, but then to repeat if metastatic disease is developed? Is there need for repeat biopsy on progression due to tumour evolution with resistance and new mutations and genomic alternation? How many tumour sites should be biopsied, as there can be heterogenous tumours in metastatic disease.(42) Furthermore how will busy clinicians be expected to keep updated with the clinical relevance of each genetic alteration, particularly if mutations with unknown clinical relevance are reported: should there be gene-based reporting or therapeutic based reporting? (43, 44)

These issues will need to be addressed as eventually comprehensive tumour profiling may be more practical and cost-effective than individual gene testing. Routine profiling may

then become standard practice, particularly if the use of expensive treatments are restricted only to those most likely to benefit.(40)

1.7. Personalisation of therapy: patient factors

As well as genetic alterations, patient characteristics may impact on outcomes and influence choice of treatment. There is strong evidence that some patient factors, including performance status (PS),(45) body mass index (BMI),(46) and co-morbidities(47) shall have impact on outcomes. The presence of some co-morbidities may impact on ability to tolerate treatment: for example, diabetic patients with cancer are more likely to be admitted with infective complications during chemotherapy.(48) This presents another opportunity to personalise therapy.

1.8. Examples of personalised medicine in current cancer care

Personalised medicine is making an increasing contribution to routine cancer care in some tumour sites. In breast cancer treatment is guided by numerous biomarkers: ER status to select patients for endocrine therapies,(49, 50) and HER2 overexpression to select patients for trastuzumab,(1, 51, 52) lapatinib,(53) and pertuzumab.(54)

In non-small cell lung cancer (NSCLC) the main driver mutations identified include EGFR, *KRAS*, *FGFR1*, ERBB2, *PIK3CA, ALK, BRAF, ROS1, MEK1, RET, NRAS* and *AKT1*.(44) This knowledge has translated into use of targeted therapies: activating mutations in EGFR are sensitive to EGFR tyrosine kinase inhibitors (TKIs) with response rates of around 70%.(55) Tumours harbouring specific *ALK* gene fusions have approximately 60% response rates to the *ALK* tyrosine kinase inhibitor crizotinib.(56) Crizotinib also has activity in *ROS1* fusion-positive tumours and *MET* mutated tumours.(57) Tumours overexpressing HER2 are responsive to the HER2 TKI afatinib,(58) and the *BRAF* inhibitor vemurafanib has activity in *BRAF*-mut NSCLC.(59)

Multiplex mutational profiling is becoming part of standard practice with mutations being detected in roughly half of unselected lung cancers, and in nearly 90% of lung adenocarcinomas in East Asian never-smokers.(60)

Personalisation of therapy in melanoma is advancing, and this shall be discussed in Chapter 3. This thesis will concentrate on efforts to personalise treatment in aCRC.

1.9. Colorectal cancer

Each year, more than one million people are diagnosed with colorectal cancer (CRC) worldwide, making it the third most common cancer following lung and breast cancer.(61) It is estimated that 54% of bowel cancers are linked to lifestyle and other risk factors, including red meat consumption, low fibre diet, alcohol consumption, smoking and obesity:(62) the incidence of CRC has risen in Asian countries that have seen increasing adoption of a 'western' diet.(63) Other risk factors include older age, African-American ethnicity, previous history of colon polyps, inflammatory intestinal conditions, family history of CRC and inherited syndromes.(64)

Symptoms depend upon the stage of presentation and tumour location, but include change in bowel habit, rectal bleeding, abdominal pain, lethargy and weight loss. Diagnosis is usually by colonoscopy, biopsy, histology, and imaging. Using this information a tumour is staged, using the TNM (65) or Duke's(66) staging system. With this information, and consideration of patient fitness and circumstance, a multidisciplinary team agrees upon a management strategy for an individual patient.

Outcomes are improving, but despite this many patients relapse after initial curative treatment or present with incurable disease: CRC is the second most common cause of cancer related mortality in the United Kingdom (UK).(67) Many patients with early stage disease are cured with resection alone, but for those with more advanced operable disease, or with inoperable aCRC, drug therapies form an important and integral part of treatment.

There are five active agents in aCRC in routine use within the UK, 5-fluorouracil (5FU), oxaliplatin, irinotecan (Ir), cetuximab and bevacizumab. With the routine use of such agents and adoption of an increasingly active approach to surgical management of metastatic disease, the median overall survival (OS) in aCRC has improved from six months to nearly two years in the past 20 years.(68) There is no single proven 'gold standard' regimen and no single sequence for optimum use of these drugs, but specific treatment strategies are selected by individual clinicians using RCT evidence and patient-

specific factors (age; co-morbidity; symptoms; preferences), treatment-specific factors (efficacy; toxicity) and tumour-specific factors (potential future operability; *RAS* mutation status).

Only one predictive marker has been validated in aCRC. *RAS*-mutation status is a negative predictive marker for the anti-EGFR agents cetuximab and panitumumb:(69, 70) only *RAS* 'wild-type' patients are treated with these agents. There is therefore much scope for improvements in precision medicine in aCRC.

This thesis aims to investigate potential new biomarkers that can aid personalisation of therapy in aCRC.

1.9.1. Understanding of the molecular basis of aCRC

Further personalisation of CRC, with hypothesis led studies of novel biomarkers and development of targeted therapies requires understanding of the molecular basis of the disease.

1.9.2. CRC hereditary syndromes

Although it is estimated that 20% of CRCs have a hereditary component,(71) only a small proportion are caused by the autosomal dominant inherited diseases familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). FAP is caused by mutation in the adenomatous polyposis coli (APC) tumour suppressor gene, with subsequent development of multiple colorectal adenomas that usually develop into an invasive carcinoma in the second or third decade.(72) HNPCC (Lynch Syndrome) is due to germline mutation in one of the DNA mismatch repair genes, resulting in early onset cancers in the bowel and other organs.(73) Patients known to harbour these genetic alterations undergo colonic surveillance to detect and remove polyps and cancers at an early stage, however they may undergo prophylactic colectomy.

1.9.3. Development of sporadic CRC

More commonly CRC will develop sporadically from benign adenomatous polyps.(74) Although adenomas are present in approximately 30-40% of the population, just a small proportion will develop into invasive cancer: risk factors for invasion include size over 10 millimetres, villous component and high grade dysplasia.(74) In the majority of CRCs a

series of genetic alterations accumulate as a lesion progresses from benign adenoma to invasive carcinoma – the classical chromosomal instability pathway.(75) Common gene mutations include *APC*, protein 53 (p53), Kirsten rat sarcoma (*KRAS*), v-RAF murine sarcoma viral oncogene homolog B (*BRAF*), SMAD family member 4 (*SMAD4*) and phosphatidylinositol 3-kinase (*PIK3CA*).(75)

However approximately 15% of CRC are associated with inactivation of DNA mismatch repair genes, the microsatellite instability phenotype or deficient mismatch repair (dMMR).(76) Unlike in Lynch syndrome, sporadic CRC associated with dMMR is usually associated with promoter hypermethylation and silencing of the human mutL homolog 1 gene (MLH-1).(77)

Defects in mismatch repair (MMR) can be detected by IHC for the presence of MLH1, MSH2, MSH6 and PMS2 proteins, or alternatively by a polymerase chain reaction (PCR) for MSI. The dMMR phenotype is associated with female sex, larger tumours and right-sided colon location.(78) dMMR is associated with improved OS in early stage CRC,(79) and has been proposed as a predictive marker for lack of efficacy of FU-based adjuvant therapy in stage II CRC.(80)

1.10. Classifications of colorectal cancer

CRC has been traditionally classified morphologically, but wide heterogeneity exists suggesting that additional or alternative classifications may be beneficial for both prognostication and for the prediction of CRC treatment benefit. Proposed classifications include MMR status, molecular sub-typing, epigenetic, gene expression profiling and single gene parameters. Each classification provides its own perspectives on the underlying biology.

1.10.1. Molecular sub-typing of CRC

Wide disease heterogeneity has been shown in aCRC, with many signalling pathways altered, and in different ways, during cancer development. Over the past twenty years major cancer genes and pathways central to CRC development and progression have been identified, including the p53, MAPK, PI3K, TGF-β and WNT pathways.(81) Different driver mutations have been identified depending upon MMR status: dMMR tumours being more

likely to harbour mutations in *BRAF*, *PTEN* and *TGFBR2*, and are predominantly in the proximal colon, show poor differentiation, mucinous histology and increased peritumoral lymphocytic infiltration.(82) In contrast, chromosomally unstable tumours are more likely to be located in the distal colon and develop along the 'classical genetic pathway' of CRC, with mutations in *APC, KRAS, SMAD4* and *TP53*.(82)

CRC genome sequencing studies have revealed greater diversity in the genetic profiles of CRCs. Whole-exome sequencing of 11 dMMR CRCs described around 80 coding sequence mutations, with smaller numbers of commonly mutated driver genes and a larger group of "private mutations" (rare gene mutations usually found in a single family or small population).(83) The Cancer Genome Atlas (TCGA) network subsequently reported comprehensive data on 223 unselected CRCs.(84) Twenty-four genes were identified as commonly mutated including those listed in this section, plus several novel candidate genes such as *SOX9, TCF7L2, ATM, ARID1A* and *FAM123B*. As anticipated hypermutation was identified in nearly 15% and three-quarters demonstrated the expected dMMR phenotype. However the remaining 25% did not display dMMR and instead were associated with DNA polymerase mutations and may represent a new CRC sub-type. The TCGA also reported copy number analysis and reported amplifications of *HER2* and *IGF2*.(84)

1.10.2. Sub-typing by gene expression profiling

Another potential classification of CRC is with gene signatures using expression profiling and hierarchical clustering. Gene expression profiling in samples from a large adjuvant trial suggest six CRC sub-types exist.(85) As well as identifying a *BRAF*-mutant gene signature, a *BRAF* mutant-like population within the *BRAF*-wt population was reported that share similar clinic-pathologic and gene expression features as *BRAF*-mut tumours. This group were associated with proximal primary tumour location (PTL), dMMR, older age and 30% had a *KRAS* mutation. Both populations demonstrated inferior OS than other groups, so this new classification is of prognostic relevance.(85) Additionally a 'MSI-like' gene expression profile has been described that captures the hypermutant tumours described by the TCGA study.(81)

A further gene expression profiling study of 1113 CRC samples proposes instead that five main CRC sub-types exist and that several main biological processes are key determinants of CRC behaviours.(86) Sub-type characteristics each have specific CRC markers and

mutations, histopathological features, grade, gene expression patterns and differing median survival.

A further gene expression study of 88 non-randomised samples of all Duke's stages suggested four sub-types exist with differing outcomes that are independent of stage. Tumour-associated stroma and mucinous histology were pivotal to the proposed classification.(87)

Such studies have been conducted on primary tumours and it is unclear whether such classifications would have relevance in the advanced setting. Also, expression profiling includes only statistically significant genes within a proposed signature; it is likely that only a fraction will have functional relevance

If sub-types were validated identification of high-risk groups may alter therapeutic decisions, such as the need for adjuvant chemotherapy and sub-type specific clinical trials. As demonstrated there is currently a lack of consensus on the optimal sub-type model; such models would require prospective testing before routine adoption, as is being performed in the I-SPY2 trial in breast cancer.(88)

1.11. Management of early stage colon cancer

Detailed discussion on the treatment of early colon and rectal tumours are outwith the scope of this thesis. In brief, patients are treated surgically, with the addition of radiotherapy in most rectal cancers. Depending upon post-operative staging results, patients with stage 3 and 2b tumours with high risk characteristics (for example peritoneal involvement or extramural vascular invasion) are offered adjuvant intravenous 5-fluorouracil or capacitabine, with the addition of oxaliplatin in higher risk patients.(89) Neither EGFR-or vascular endothelial growth factor (VEGF) targeted therapies have demonstrated benefit in the adjuvant setting.(90, 91)

1.12. Current treatment of advanced colorectal cancer

This section will describe current practice for treating aCRC, to identify points in the treatment pathway where further personalisation of therapy would be beneficial.

The European Society of Medical Oncology (ESMO) aCRC guidelines advise that aCRC patients should be stratified into three groups, according to their initial presentation:(92, 93)

- Group 1 – potentially resectable metastases
- Group 2 – non-resectable metastases, high tumour burden and tumour related symptoms
- Group 3 – non-resectable metastases, asymptomatic and less aggressive disease.

It is suggested that for Group 1 and 2 patients early response and progression-free survival (PFS) is the key aim; the management of Group 3 should focus on survival rather than short term outcomes.(93) This classification is based upon both evidence based management strategies and anecdotal experience, rather than being guided by specific biomarkers. Other factors guiding decisions may include dynamics of disease progression, presence of prognostic markers and patient co-morbidity and preference.

1.12.1. Treatment of potentially resectable metastases

For metastatic disease confined to liver or lungs, strategies directed towards resection should be considered in fit patients. The aim of neo-adjuvant chemotherapy is primarily to achieve response, which correlates with success of resection.(94) Evidence for the addition of anti-EGFR agents in *KRAS*-wt patients in this setting is controversial: first-line RCTs have reported improvement in response rates (RR) with anti-EGFR agents compared with chemotherapy alone.(95-97) However the New Epoc trial, designed to test the addition of cetuximab to chemotherapy prior to resection of liver metastases, *KRAS*-wt patients were harmed by the addition of cetuximab to neo-adjuvant chemotherapy (progression free survival [PFS} 14.1 vs 20.5 months, hazard ratio [HR] = 1.5, $p<0.05$),(98) suggesting that initial response may have less longer term importance. Similarly, mixed results are reported for the addition of bevacizumab to neo-adjuvant chemotherapy.(99)

1.12.2. Chemotherapy strategies for aCRC

Table 1.1 describes the landmark chemotherapy trials in aCRC.

Given that there is no 'gold standard' of sequencing, the choice of first line chemotherapy, FU in combination with oxaliplatin (FOLFOX) or irinotecan (FOLFIRI) will depend upon

the individual patient and whether a biological is added. For some, a conservative approach may be appropriate, such as an expectant or single agent upfront strategy,(100, 101) and planned treatment breaks in those with responding or stable disease.(102) Conversely, use of all three active chemotherapy drugs upfront may be useful in carefully selected patients; first-line triplet chemotherapy with FU, oxaliplatin and irinotecan (FOLFOXIRI) has superior RR, PFS and OS than FOLFIRI alone, with increased but manageable toxicity.(103) This regimen has been tested in combination with bevacizumab;(104) the *BRAF*-mut sub-group particularly benefitted from this approach.(105)

Second line therapy is usually reserved for those with good PS and adequate organ function; the regimen is usually changed from first line.

Further trials have investigated the efficacy and optimal integration of the VEGF inhibitor bevacizumab and the anti-EGFR agents, cetuximab and panitumumab.

Trial Name	Description	Patient no	Outcome	Reference
1st line chemotherapy				
De Gramont et al	FOLFOX compared with 5FU alone	420	PFS benefit with FOLFOX	(106)
Saltz et al	FOLFIRI vs Ir vs 5FU	683	OS benefit with FOLFIRI	(107)
Douillard et al	FOLFIRI compared with 5FU alone	387	OS benefit with FOLFIRI	(108)
N9741	FOLFOX vs FOLFIRI	795	OS benefit with FOLFOX	(109)
FOCUS	Sequential single agent vs staged combination vs combination	2135	OS equivalence of initial single agent to combination chemo	(100)
CAIRO	Continuous vs staged combination	675	Equivalence	(101)
COIN	Intermittent vs continuous combination	1630	Non-inferiority of intermittent not proven	(102)
2nd line chemotherapy				
Cunningham et al	Irinotecan plus best supportive care (BSC) vs BSC	179	OS advantage for irinotecan	(110)
Douillard et al	FOLFIRI followed by FOLFOX vs FOLFOX followed by FOLFIRI	226	Equivalent in time to progression	(111)
Rothenburg et al	FOLFOX vs oxaliplatin vs 5FU alone after progression on FOLFIRI	463	FOLFOX superior OS	(112)

Table 1-1 Landmark trials for combination chemotherapy in aCRC

1.12.3. Use of anti-EGFR agents in aCRC

Many CRCs are reliant on EGFR signalling,(84) therefore it is an attractive therapeutic target. There are two main classes of anti-EGFR agents: small molecule TKIs and monoclonal antibodies (MoAbs). TKIs bind to the intracellular protein kinase of receptors, preventing its autophosphorylation of downstream molecules. These agents include gefitinib and erlotinib. MoAbs instead broadly compete with the endogenous EGFR ligands blocking ligand-dependent activation of the EGFR plus induce receptor internalisation and downregulation. These agents include cetuximab and panitumumab. Only the MoAbs have demonstrated consistent benefit in aCRC.

Cetuximab binds with domain III of EGFR partially blocking ligand binding and also preventing dimerization.(113) Panitumumab, a fully humanised monoclonal IgG_2 antibody, has a higher affinity than cetuximab to the EGFR ligand binding site completely blocking the receptors interaction with ligands and preventing resultant downstream signalling.(114)

1.12.3.1. EGFR signalling

The EGFR has six recognised ligands which on binding cause transition from an inactive monodimer to an active homodimer.(115) Extracellular binding of ligands leads to receptor homodimerisation and internalisation by highly regulated clarithrin-mediated endocytosis in usual physiological conditions.(116) However alternative internalisation mechanisms are seen with receptor overexpression or high ligand concentration.(117) Following cellular internalisation, EGFR and ligands are compartmentalised into endosomes with subsequent recycling to the cell surface (figure 1.2).(118)

Homodimerisation starts intrinsic cytoplasmic kinase activity leading to auto- and transphosphorylation of tyrosine residues resulting in downstream signalling cascades. (119) The main downstream pathways are the MAPK and PI3K-AKT pathways (figure 1.2)(120)

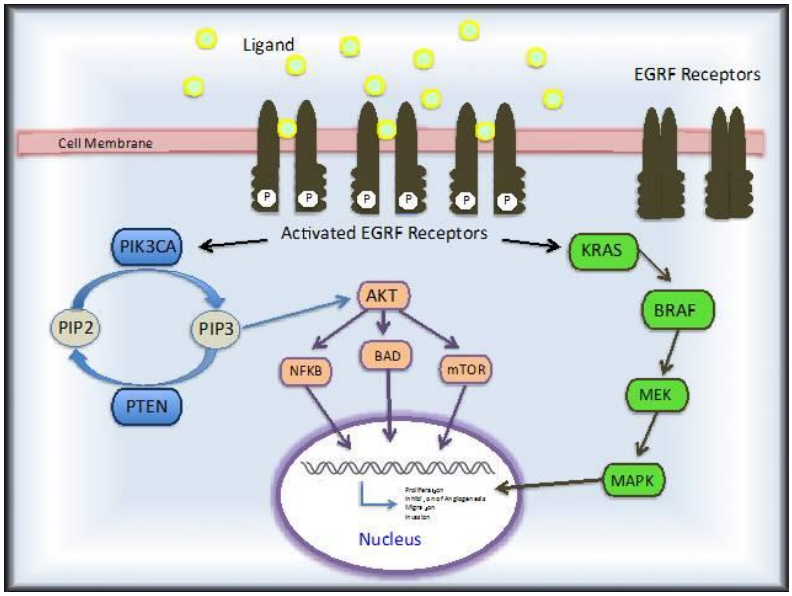

Figure 1-2 Schematic representation of ligand binding leading to EGFR dimerization and phosphorylation with resultant downstream signalling and cellular functions.

This model is likely simplistic as many other factors contribute to EGFR trafficking and signalling; HER2 overexpression can disrupt internalisation and endosomal sorting of EGFR.(121) 'Cross-talk' occurs between HER receptors through formation of heterodimers, resulting in diverse signalling complexes: this shall be explored in Chapter 5.

Whilst activation of the EGFR and consequent downstream signalling results in vital cellular functions, including proliferation, migration, apoptosis and differentiation, (122) upregulation in cancer is associated unregulated proliferation.(123) In contrast to NSCLC, mutations in EGFR are not the main variation in aCRC.

1.12.3.2. Use of anti-EGFR agents in aCRC

Early in the development of anti-EGFR agents it became apparent that a sizable proportion of aCRC patients did not respond. Unlike in NSCLC mutations in the EGFR do not determine sensitivity to these drugs.(124) Instead it was discovered that mutations in exon 2 (codon 12 and 13) of *KRAS*, a small G-protein downstream of EGFR, rendered these agents ineffective.(69, 125, 126) Furthermore prospective-retrospective analysis of expanded mutation testing in *RAS* (*KRAS* exons 3 and 4; *NRAS* exons 2, 3, and 4) in the PRIME trial identified 17% of tumours originally classified as *KRAS*-wt had a mutation.(70) Interaction testing demonstrated clear separation of panitumumab

treatment effect between the newly identified *RAS* mutant and wild-type populations.(70) These agents are now restricted to *RAS*-wt patients, representing a success of personalised medicine in aCRC.

Landmark phase III studies of anti-EGFR agents in *KRAS*-wt patients are described in Table 1.2.

Trial Name	Description	Patient no	Outcome (*KRAS*-wt)	Reference
1st line setting				
CRYSTAL	FOLFIRI +/- cetuximab	666	PFS & OS benefit with cetuximab + FOLFIRI	(95, 97, 127)
PRIME	FOLFOX +/- panitumumab	656	PFS benefit with FOLFIRI + pan. OS benefit with pan in *RAS*-wt.	(70, 96)
COIN	OxFU +/- cetuximab	729	No PFS or OS benefit with cetuximab	(128)
NORDIC VII	Intermittent or continuous OxFU +/- cetuximab	303	No PFS or OS benefit with cetuximab	(129)
2nd line setting				
20050181	FOLFIRI +/- panitumumab	597	PFS benefit with FOLFIRI + panitumumab	(130)
PICCOLO	Irinotecan +/- panitumumab	460	PFS benefit with IrPan	(131)
3rd line setting				
CO.17	Cetuximab vs best supportive care (BSC)	230	PFS & OS benefit with cetuximab	(69, 132)

Table 1-2 Landmark studies of anti-EGFR agents in *KRAS*-wt aCRC patients

However not all *RAS*-wt patients benefit from these treatments: this shall be explored in Section 1.14.

1.12.4. Use of bevacizumab in aCRC

Monoclonal antibodies to VEGF include bevacizumab, aflibercept and cediranib; most experience in aCRC is with bevacizumab. Landmark studies are listed in Table 1.3.

Trial Name/ author	Description	Patient no	Outcome	Reference
1st line setting				
Hurwitz	FOLFIRI +/- bevacizumab	813	PFS & OS benefit with bev + FOLFIRI	(99)
Saltz	OxFU +/- bevacizumab	1401	PFS benefit with FOLFOX + bev	(133)
2nd line setting				
E3200	FOLFOX alone vs FOLFOX + bev vs bev alone	829	OS & PFS benefit with FOLFOX + bev	(134)
Van Cutsem	FOLFIRI +/- aflibercept	1236	OS & PFS benefit FOLFIRI + aflibercept	(135)

Table 1-3 Landmark phase III trials of bevacizumab in aCRC

Bevacizumab has demonstrated activity in aCRC (133, 136-138) but absolute benefits in unselected patients are modest. Bevacizumab in combination with capecitabine may be useful in those unable to tolerate combination chemotherapy.(139) Current controversies include continuation of bevacizumab post-progression(140) and maintenance therapy.(141)

Aflibercept in combination with FOLFIRI is approved by the FDA for patients failing oxaliplatin.(135) Cediranib has been tested head-to head with bevacizumab for first- line treatment; activity was comparable.(142) A further anti-angiogenic regorafinib has also shown activity.(143)

Recent debate surrounds choice of biological agent to be given in combination with chemotherapy for the first-line treatment of *RAS*-wt aCRC: anti-EGFR agents or bevacizumab. Direct comparisons have been made in three RCTs, with mixed results.(144-146) In FIRE-3 a seven month OS benefit was reported with FOLFIRI plus cetuximab compared to FOLFIRI plus bevacizumab (HR = 0.70, p=0.011), but with no significant difference in PFS or response rate (RR), the primary endpoint.(144) The PEAK trial noted improvements for RR and PFS with panitumumab compared with bevacizumab, however CALGB 8045 did not see any difference in effect between the 2 drugs.(146)

1.12.5. Emerging therapies in aCRC

Many agents have demonstrated efficacy in aCRC but are not yet incorporated into routine practice. TAS-102 (a combination of a thymidine-based nucleic acid analogue, trifluridine and a thymidine phosphorylase inhibitor) has demonstrated overall survival (OS) and PFS advantage over placebo in a phase three trial of heavily pretreated aCRC. This effect was seen across all patient sub-groups and was treatment was associated with few adverse events.(147)

Regorafinib, a small molecule multi-kinase inhibitor, has also demonstrated survival benefit in heavily treated aCRC compared with placebo.(143) However 93% of patients treated with regorafinib reported treatment-related adverse events (mainly fatigue and hand-foot skin reaction). Work to identify sub-groups who gain most advantage is underway.

Further testing is warranted to establish optimal integration into the aCRC pathway, along with consideration of cost-effectiveness.

Immunotherapy has for the first time shown benefit in CRC, with testing of PD-1 blockade in dMMR tumours.(148) It was hypothesised that hypermutated tumours would be most susceptible to immunotherapy as mutations encode proteins that are recognised and targeted by the immune system, so immune augmentation could be an effective anti-tumour strategy. For the 13 dMMR CRC patients treated with pembrolizumab (a humanised IgG4 monoclonal antibody PD1-inhibitor), RR was 62% and DCR was 92%. In pMMR CRC minimal effect was observed (RR 0%; DCR 16%). Although this personalised strategy would only benefit a small number of patients with aCRC this is an important development.

Other promising therapeutics include aspirin(149) and vitamin D supplementation.(150)

1.12.6. Current European consensus for the treatment of aCRC

Based upon this evidence, European guidelines currently recommends:(93)

- Upfront *RAS* testing in all patients prior to first-line therapy.
- Doublet chemotherapy plus anti-EGFR agent in *RAS*-wt group 1 and group 2 patients; may be appropriate in some group 3 patients
- *RAS*-mutant patients should be treated with combination doublet plus bevacizumab in the first-line.

Availability of cetuximab and bevacizumab in England is limited by the constraints of the Regional and National Cancer Drug Funds (CDF) approvals.(151) Currently the national CDF approves cetuximab and panitumumab for the first-line treatment of *RAS*-wt patients in combination with FOLFIRI, FOLFOX 4 or OxMdG. Treatment in the third or fourth line is approved for *RAS*-wt patients of PS 0-1, who have not previously been exposed to anti-EGFR agents. Bevacizumab is approved in the second line in combination with doublet chemotherapy; not with FU alone and not as single-agent maintenance therapy.

1.13. Current use of biomarkers in advanced colorectal cancer

Despite an increasing understanding of the molecular basis of CRC, there are only few biomarkers that are routinely utilised to guide treatment management decision.

1.13.1. Validated prognostic biomarkers

Two main prognostic scores have been proposed for aCRC, the Kohne(45) and GERCOR (152)scores. Kohne's study evaluated clinical parameters as prognostic markers in 3825 patients in RCTs treated with 5FU. Clinical parameters associated with poor outcomes were PS over 0, more than one metastatic site, liver metastases, peritoneal metastases; positive markers included rectal primary (compared with colon) and lung or lymph node metastases. Laboratory parameters associated with worse outlook include high platelets (plts), alkaline phosphatase (AlkPhos) and white cell count (WCC), and low haemoglobin (Hb).

A risk score was developed using 4 parameters (PS, no of involved metastatic sites, WBC count and alkaline phosphatase), categorising patients into three prognostic groups: low risk, median OS 15 months; intermediate risk, median OS 10.7 months; high risk, median OS 6.1 months.(45)

The GERCOR score is based upon analysis of 803 patients treated with either first-line FOLFOX or FOLFIRI in RCTs.(152) Significant prognostic factors were PS, number of metastatic sites and LDH. A score using LDH and PS was proposed, categorising patients into three risk groups: low risk, median OS 29.8 months; intermediate risk, median OS 19.5 months; high risk, median OS 13.9 months.

Different prognostic factors may apply to patients commencing second-line therapy. Patients treated in second-line RCT populations are more likely to be poor PS patients and have more metastatic sites, suggesting more advanced disease.(153) For patients commencing irinotecan vs BSC following FU progression, poor PS, recent weight loss, two or more metastatic sites, liver metastases and low Hb were poor prognostic markers.(110) In a second line RCT following FU failure, PS over 0, right primary tumour location (PTL), raised Alk phos, low Hb and low WCC were identified as poor prognostic factors.(154)

For both first and second-line prognostication there are limitations of the described studies for relevance in current oncology practice. Both studies were limited by the missing variables for certain variables, including bilirubin, presence of symptoms and CEA. ECOG PS was dichotomised as 0 vs. 1 or greater, whereas 0/1 compared with 2 or more is more clinically informative. PTL was dichotomised as rectal vs. colon tumours; whereas right colon vs left colon and rectum is more relevant.(155) Kohne's score is less relevant for more effective regimens.

Importantly some molecular markers have been validated as poor prognostic markers in aCRC, such as mutations in *BRAF* and *KRAS*.(156) As molecular testing increasingly becomes routine practice, studies of prognostic markers should include this information.

Other well established molecular markers include aneuploidy(157) and p53 expression.(158) dMMR is a marker of inferior prognosis in aCRC, but meta-analysis of three RCTs suggests that this effect is driven by concurrent *BRAF*-mut status.(159) However as these markers are not routinely assessed in aCRC their utility in patient assessment is limited.

Current suggestions for RCT stratification in aCRC include centre, PS, a lab value (LDH or Alk phos) and 1 vs more metastatic sites; for 2nd line trials, stratification for prior chemotherapy or targeted therapy. Some allowance for non-conventional trial specific factors due to the individual needs of the trial.(153) This guidance is based upon studies described and may need to be revised in view of updated practice.

1.13.2. Validated predictive biomarkers

The only validated predictive marker in aCRC is *RAS*-mutation status to select patients for the anti-EGFR agents.

1.13.3. Validated monitoring biomarkers

Carcinoembryonic antigen (CEA) is secreted from tumour cells into serum and is recommended as a diagnostic, prognostic and monitoring biomarker in CRC.(160, 161) The level of evidence for use of CEA as a monitoring tool is 2C as it has only been studied in observational series.(162)However it is advised that CEA levels be checked prior to chemotherapy as a baseline and tested regularly thereafter, with rising levels suggestive of progressive disease.(161) Caution should be applied in its interpretation: a 'CEA flare' may

occur shortly after commencing chemotherapy, which is not associated with progressive disease.(163) CEA may also be a less useful tool if baseline values are low.(162)

1.13.4. Toxicity biomarkers

There are no biomarkers that are routinely used to predict which patients are at risk of toxicity from specific CRC treatment agents. The evidence for the use of UGT1A1*28 and DYPD as predictors of toxicity will be discussed in Chapter 6.

1.14. Routes to further personalisation of therapy in aCRC

1.14.1. Further predictive biomarkers for anti-EGFR agents

As discussed RCTs of cetuximab and panitumumab have yielded inconsistent results, varying between trials and patient subpopulations from worthwhile benefit to significant harm.(126, 131) An unmutated *RAS* pathway, although necessary, is not sufficient for response to anti-EGFR agents, since many patients whose tumours are wild-type for both *KRAS* and *NRAS* do not respond.(127, 131) Therefore further biomarkers may further refine the target population for anti-EGFR agents.

1.14.1.1. Further candidate predictive biomarkers for anti-EGFR agents

Occurring in nearly half of aCRCs, validation of *RAS* mutations as negative predictive markers was relatively straightforward; validation of further biomarkers has proven difficult with rare alterations being studied in heterogenous populations with, often small, treatment effects.

Candidate biomarkers currently under investigation include further alterations in the MEK-AKT pathway (mutations in *BRAF*, *PIK3Ca* and *PTEN*), EGFR copy number variation(164) and EGFR ligands,(165, 166) interactions with HER and other transmembrane receptors and their ligands,(167) micro-RNA signatures(168) and markers of inflammation.(169)

Some reports suggest that tumours with *KRAS* codon 13 mutations may retain sensitivity to anti-EGFR agents, raising concern that eligible patients may be denied treatment.(170)

However this was refuted in pooled post-hoc analysis of three phase III RCTs (171) but will be prospectively assessed in the ICE CREAM international trial.(172)

1.14.1.2. Alterations in the EGFR and other HER receptors

In other cancers, receptor mutation or overexpression have proven to be predictive markers for targeted agents: EGFR mutation is a predictive marker for gefitinib;(173) HER2 overexpression as a predictive marker for trastuzumab.(1) The frequency of EGFR mutations is lower in CRC than seen in other cancers and has no consistent relationship with outcomes.(174) The relationship between EGFR gene and protein overexpression as a prognostic and predictive marker for anti-EGFR therapies has been tested, with inconsistent findings.

The reported frequency of EGFR protein overexpression in aCRC vastly ranges between studies (18–97%),(175) with different patterns of staining described.(176) EGFR expression in primary colorectal tumors does not correlate with their corresponding metastatic sites.(177) The biological consequence of EGFR protein overexpression is unclear; there is no clear association either with prognosis(178, 179) or anti-EGFR benefit.(180)

EGFR gene copy number variation (CNV) has been investigated as a candidate biomarker. In three studies of cetuximab treated patients increased EGFR CNV was associated with improved outcomes than with normal expression,(164, 181, 182) but a prognostic or predictive effect could not be determined. To complicate further, each study utilised a different mechanism for assessing CNV (CISH [chromatin in-situ hybridisation], FISH [fluorescence in-situ hybridisation], and TaqMan), as such no standardised method for assessment has been validated.

Preclinical work has reported that HER3 and IGF-1 interferes with the biological activity of EGFR in CRC through lateral signalling, so as a single biomarker EGFR CNV may be less useful.(183) This will be discussed further in Chapter 5.

However the EGFR ligands epiregulin and amphiregulin have shown promise as predictive markers for anti-EGFR agents and are hypothesised to be surrogates for tumour EGFR dependence;(166, 184) this shall be further explored in Chapter 4.

The HER family shows interdependence, with the creation of hetero- and homo-dimers between receptors creating multiple signalling complexes. This appears to be particularly important for PI3K/Akt pathway activation.(185) Therefore overexpression of another family member may impact on the efficacy of EGFR-targeted agents by providing 'escape signalling' allowing the tumour to block the pathway being pharmacologically targeted.(186) Overexpression of HER2(167) and HER3(183) has been linked to anti-EGFR resistance, and this shall be explored in Chapter 5. Similarly upregulation of the proto-oncogene *MET* has been linked to anti-EGFR agent resistance.(187, 188) These alterations have been difficult to validate due to the rarity of the amplifications in CRC.

1.14.1.3. Mutations in downstream pathways

The impact of having any other mutation in candidate genes downstream of EGFR has been investigated in *KRAS*-wt patients. In a large series of aCRC patients treated with cetuximab, the presence of a mutation in *BRAF*, *NRAS* or *PIK3CA* was associated with reduced RR, PFS and OS than those with no mutations.(189) This 'all wild type' population had statistically more PFS benefit with panitumumab than *KRAS*-wt patients with any additional mutation in the PICCOLO trial.(131)

BRAF is a negative prognostic marker in aCRC,(156, 190) but its utility as a predictive marker for anti-EGFR therapies is controversial. In several non-randomised series and the PICCOLO trial, *BRAF* mutation was a negative predictive marker for anti-EGFR therapies.(131, 164, 189, 191) Furthermore *BRAF*-mut patients treated with panitumumab in PICCOLO had statistically shorter OS than those treated with irinotecan alone, suggesting a potential harmful interaction.(131) However in biomarker analysis in several RCTs of anti-EGFR agents only prognostic effect was seen.(97) Again validation has been complicated by the low incidence and strong prognostic effect in CRC. The behaviour of *BRAF* mutations in aCRC will be discussed in detail in Chapter 3.

The *PIK3CA* gene is mutated in approximately 20% of CRC, usually occurring in exon 9 or exon 20. Interaction of *PIK3CA* mutations with anti-EGFR agents efficacy remains unproven. Whilst initial work studied grouped both exons together,(192) recent evidence suggests that only exon 9 mutations are relevant.(189)

1.14.1.4. Other potential molecular predictive biomarkers for anti-EGFR agents

MicroRNAs (miRNAs) are non-coding RNAs of 21-13 nucleotides in length that bind to complementary sequences in the 3'-untranslated regions of target mRNAs, blocking transcription. They are involved in important cellular functions, including apoptosis, differentiation and proliferation.

Expression of several miRNAs have been related to CRC prognosis and anti-EGFR efficacy, including miR-7 through regulation of the EGFR(193) and Mir-31-3p,(194) but further validation is required.

1.14.1.5. Clinical and radiological biomarkers for anti-EGFR agent efficacy

The presence of a pustulo-papular skin rash following treatment was identified as a potential indicator of cetuximab or panitumumab response, and is associated with improved RR, PFS and OS.(195) Histologic examination of the skin following treatment shows downregulation of phospho-EGFR, decreased expression of markers of cellular proliferation such as Ki-67 and inflammatory infiltrates.(196) This finding lacks specificity: although patients responding usually develop a rash, those not benefitting could also. Instead the rash may be reflective of adequate plasma drug concentrations. Improved management of cutaneous toxicity also limits the usefulness of this approach.

Early tumour shrinkage and depth of response is proposed as a marker of longer- term benefit from anti-EGFR treatment. The rate of early tumour shrinkage and depth of response were associated with improved survival with chemotherapy, a phenomenon more specific to cetuximab than bevacizumab containing regimens.(197)

1.14.2. Secondary resistance to anti-EGFR agents.

Mechanisms of secondary resistance to anti-EGFR agents have been investigated using post-progression biopsies and circulating tumour DNA (ctDNA). Following progression on anti-EGFR agents, *RAS*-wt patients can develop *KRAS*, *NRAS*, *BRAF* and *PIK3Ca* mutations,(198) amplification of ERBB2 and MET, (187, 188) and changes in the HER ligand axis, with increased amphiregulin, TGF-α and heregulin expression.(199)
Acquired mutations in EGFR exon 12 are also described, rendering cetuximab ineffective but with the possibility of retained panitumumab sensitivity.(200)

Strategies being tested to overcome this include testing re-exposure, particularly using panitumumab following cetuximab resistance,(201) and second generation EGFR MoAbs engineered to induce enhanced antibody-dependent cellular cytotoxicity, or increased receptor internalisation: imgatuzumab has shown preclinical efficacy,(202) but in combination with FOLFIRI was not superior to FOLFIRI plus bevacizumab in a phase II RCT.(203)

1.14.3. Biomarkers for VEGF targeted agents

Currently no validated biomarkers guide patient selection for bevacizumab, although several are under investigation (reviewed in(204)).

Circulating VEGF-A levels have shown discriminatory value for bevacizumab benefit in gastric,(205) breast,(206) and pancreatic cancer(207), but this has not been reproduced in aCRC.(208) Reports of the usefulness of the ratio of $VEGF_{165b}$:$VEGF_{total}$ have also been inconsistent.(209, 210)

Other potential biomarkers for bevacizumab include day 4 circulating endothelial progenitor cells and the proportion of baseline CXCR4-positive circulating endothelial cells,(211), VEGF polymorphisms,(212) and osteopontin(213) but further validation is required.

1.14.4. Chemotherapy

Translational studies from the MRC-FOCUS trial investigated biomarkers for treatment efficacy and toxicity with 5FU, oxaliplatin and irinotecan.(214, 215) One promising marker was Topoisomerase-1 (Topo-1), the molecular target of SN-38: moderate or high levels of Topo-1 as determined by IHC showed the greatest OS benefit of initial combination chemotherapy compared to initial single agent, particularly if irinotecan-based. Topo-1 was also associated with benefit in a series of CRC patients treated with irinotecan based adjuvant chemotherapy.(216) These findings were not validated in CAIRO, with no association seen between Topo-1 expression and response to irinotecan and capecitabine.(217)

Excision cross- complementing gene (*ERCC1* and *ERCC2*) is an excision nuclease that repairs DNA damaged by platinum agents(218) and has been reported to determine sensitivity to platinum agents in lung cancer.(219) A meta-analysis of several small studies

suggested a useful role for ERCC1 in predicting oxaliplatin benefit in CRC,(220) but was not reproduced in biomarker analysis in either FOCUS or CAIRO.(214, 217)

Proposed predictive biomarkers for FU efficacy are thymdylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD). Direct TS inhibition is a major mechanism of 5FU, whilst DPD mediates 5FU catabolism in the liver.

However findings have been divergent and inconsistent, likely exacerbated by heterogenous chemotherapy regimes, patient populations and biomarker measurements between studies. Whilst a meta-analysis suggested that low TS expressing tumours were more sensitive to 5-FU based chemotherapy,(221) this finding was not reproduced in biomarker analysis from the CAIRO or FOCUS trials. (214, 217)

In CAIRO, low DPD expressing tumours were associated with improved outcomes with capecitabine, but this has not been validated independently.(217)

A translational study from the COIN trial tested 260 potentially functionally coding region and promoter variants in genes within the 5-FU, capecitabine and oxaliplatin pathways in 2183 aCRC patients treated with oxaliplatin-fluoropyrimidine. No biomarkers remained significant for efficacy endpoints after correction for multiple testing.(222)

1.14.5. Use of pathway targeted agents successful in other cancers

Another strategy is to test biomarker/ targeted therapy combinations showing action in other cancers. However recognition of an appropriate target and effective treatment in one cancer does not necessarily translate to another. Whilst *BRAF*-mutated melanomas treated with vemurafenib have shown dramatic results (60-80%)(223, 224), disappointingly only 1 of 19 patients with *BRAF*-mutated aCRC responded in a phase I study.(225)

More promising is targeting HER2 overexpressing CRC. Following strong preclinical evidence, the Heracles trial tested heavily treated HER2 overexpressing CRC with trastuzumab plus lapatinib. The study's primary end-point was met with 34% of patients achieving a response.(226) However only a small proportion of aCRC patients express HER2(227) so this strategy is unlikely to make as great a clinical impact as in breast cancer.

Mutant *KRAS* has proven a difficult therapeutic target. *RAS* mutant cells rely heavily on the *RAF/MEK/ERK* cascade.(228) It also plays a role in the maintenance of high levels of Bcl-Xl, an antii apoptotic Bcl-2 family protein. Overexpression of this alone is related to resistance to anti-EGFR agents.(229)

Small molecules have been developed that interfere with GEF binding to lock *KRAS* into an inactive state in mouse models.(230) Further approaches include targeting its post-translational modifications by preventing proper plasma membrane attachment,(230) and inhibiting components of key signalling pathways: in cell lines studies concominant blockade of *RAF* and MEK in *KRAS*-mut CRC reduced cell viability.(231) However thus far this has not translated into an effective treatment.

Difficulties are thought to be due to limited understanding of *RAS*-mediated signalling transduction feedback loops, pathway redundancy, tumour heterogeneity and uncertainty as to how *RAS* proteins activate downstream targets.(232)

A new approach to targeting mutant *KRAS* may be with 'synthetic lethality' or cell check-point therapeutics. These treatments take advantage of the loss of viability resulting from combinations of two separate non-lethal mutations. Several lethal interactions with *KRAS*-mut CRC are seen using small-hairpin RNA screening: deletions of PLK-1, APC and units of the proteasome.(233, 234) If such cancers are treated with check-point inhibitors then cells are unable to arrest the cell cycle for DNA to be repaired, so undergo apoptosis. These agents are in early testing but have shown promising results in solid tumours.(235)

1.14.6. Personalisation of therapy by patient factors

Patient characteristics can also guide treatment decisions, for example gene polymorphisms leading to variations in drug response, increasing age and the presence of co-morbidities.

Polymorphisms of genes involved in drug handling are an important potential source of inter-patient variability. However pharmacogenetic relationships for anticancer drugs are in most cases poorly characterised and inadequately validated, so clinicians are understandably reluctant to incorporate testing into routine clinical practice. The most widely studied is UGT1A1*28 polymorphisms for the prediction of irinotecan toxicity and efficacy.(236) This shall be discussed further in Chapter 6.

The evidence base for treatment strategies for elderly aCRC patients and those with co-morbidities is accumulating. Trial data demonstrates that frailer patients benefit from combination 1st line chemotherapy(237, 238), bevacizumab (139) and anti-EGFR agents (239), but can be at increased risk of toxicity (238). Another study pooling data from five RCTs demonstrated that patients with a PS greater than two have inferior survival and may benefit from initial single rather than combination chemotherapy(238).

Co-morbidity, for example diabetes, can increase risk of toxicity and admission during chemotherapy, and lessen the likelihood of completion.(48) With an ageing population with complex co-morbidities, increasing emphasis must be given to patient factors, including age, specific co-morbidity and immune status to guide management decisions.

1.14.7. Biomarker-stratified trials in aCRC

Further personalisation of treatment in aCRC may be progressed by biomarker stratified clinical trials. The feasibility of a complex prospective biomarker-driven multi-centre trial in aCRC was demonstrated in FOCUS 3.(240) This trial allocated patients into one of four molecular sub-groups based upon prior hypotheses.

In total 244 patients were randomised from 24 centres in the UK and 74% had biomarker results available within 10 weeks of registration. No tested treatment strategy met its efficacy endpoints. Of note, it was observed that patients with low topo-1 (n=30) had improved RR with FU alone than with FOLFIRI, however this did not translate into differences in PFS.

Following demonstration of feasibility The FOCUS 4 trial programme is now open in the UK, and shall be discussed later.

1.15. Further routes to personalisation of CRC treatment in this thesis

There are therefore several avenues to progress the personalisation of therapy in aCRC. Building upon previous evidence and identifying areas of need, several strategies will be investigated in this thesis.

- Use of routine clinical parameters as prognostic and predictive markers in aCRC
- Exploring the poor outcomes of *BRAF* mutant aCRC
- Identifying further predictive markers for anti-EGFR therapy
    - Amphiregulin and epiregulin as predictive biomarkers for panitumumab benefit
    - HER3 as a predictive biomarker for anti-EGFR therapy.
- Identification of pharmacogenomics markers for irinotecan toxicity and efficacy.

Biobanks from two large RCTs shall be utilised, the FOCUS and PICCOLO trials. Additionally for Chapter 3 clinical data from the COIN trial is included.

1.16. Clinical trials

1.16.1. The FOCUS trial

The FOCUS trial recruited 2135 consenting patients with aCRC from 60 centres in the UK between 2000-2003.(100) The trial was designed to compare different sequences of cytotoxic chemotherapy in unpretreated aCRC. Patients were randomly assigned equally between three treatment strategies (fig 1.3). In strategies A (control) and B, first line therapy was FU alone, followed by either single agent irinotecan (A) or combination therapy (B), whereas combination therapy was given first-line in strategy C. In strategies B and C the choice of combination therapy was randomised equally between FU/ irinotecan and FU/ oxaliplatin. Primary outcome measure was OS; secondary outcomes included PFS and RR. Only 0.5% to 1.5% of patients in each arm received anti-EGFR mAb therapy during subsequent salvage therapy.

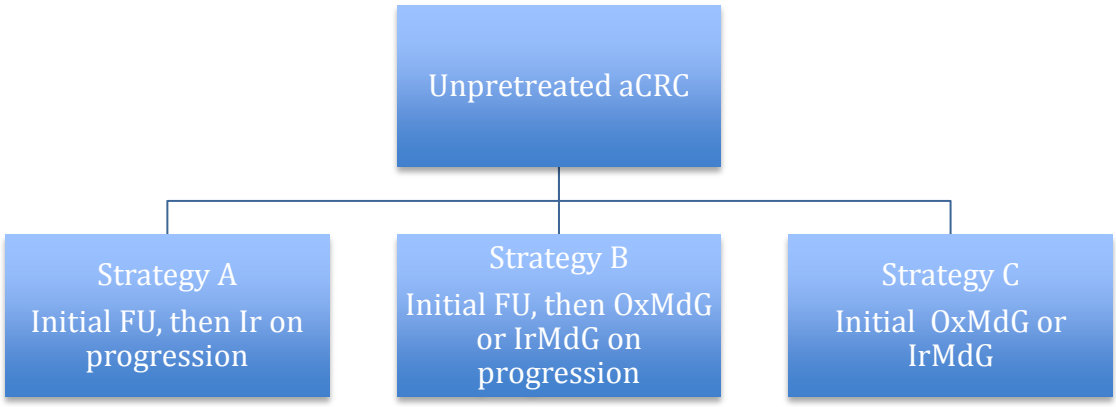

Figure 1-3 FOCUS trial schema

Median survival in the control group was 13.9 months. In each of the other strategies survival was longer, but only the C-Ir (initial FOLFIRI) group met the pre-defined statistical superiority criteria (p=0.01). There was no statistically significant survival advantage to strategy C and B (upfront combination chemotherapy vs deferred combination). No patient characteristics identified patients who benefitted more from upfront than deferred combination chemotherapy. There were no differences in quality of life outcomes between the arms.

PFS and RR were higher with combination chemotherapy than FU alone. This trial challenged the assumption that the maximum tolerated chemotherapy should be given in the 1st line in aCRC.

1.16.2. The PICCOLO trial

In PICCOLO 1196 consenting patients were recruited between 2006-2010.(131, 241).
The trial aimed to answer 2 questions: does the addition of panitumumab to irinotecan (IrPan) improve OS in the second line treatment of aCRC, compared with irinotecan (Ir) alone, and whether the modulation of irinotecan with ciclosporin (IrCs) could reduce toxicity without affecting efficacy. Primary outcome was OS; secondary outcomes included PFS and RR. Initially this was a 3-arm RCT with all patients randomised in equal proportion to three study arms, Ir, IrCs and IrPan (fig. 1.4). However following the

validation of KRAS as a negative predictive marker(69, 125) the need for the application of a molecular selection strategy prior to randomisation was clear and the trial design was modified in mid-2008 (fig. 1.5).

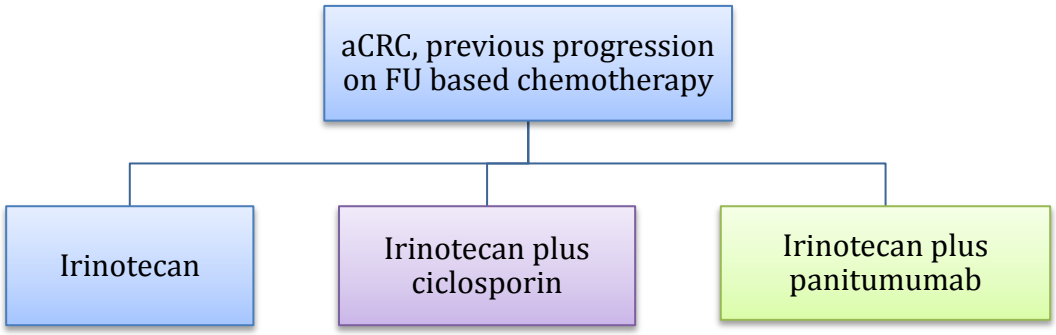

Figure 1-4 Initial PICCOLO trial schema

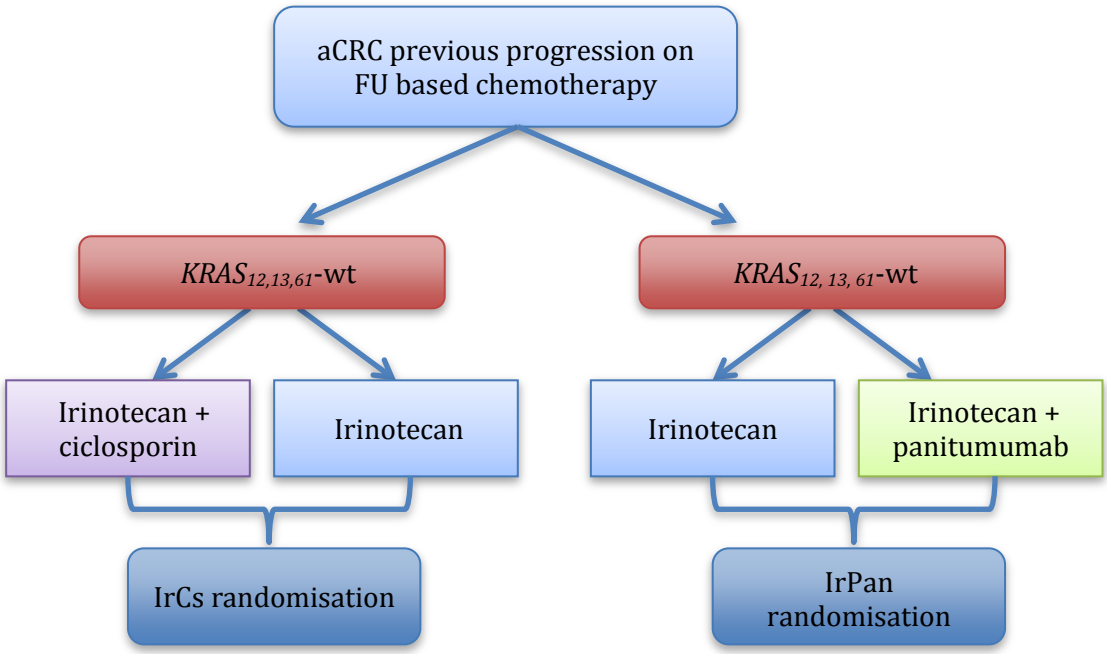

Figure 1-5 Adapted PICCOLO design including molecular stratification

Of the 1198 randomised patients, 460 were included the IrPan vs Ir primary analysis population (KRAS-WT and no previous cetuximab). There was no difference in OS between the IrPan and Ir arms (HR = 0.91, 95%CI 0.73-1.14; p-0.44) but PFS was superior in the IrPan arm (HR=0.78, 95%CI 0.64-0.95; p=0.015).

Pre-planned molecular testing and analyses were performed to test the effect of a downstream mutation in the MEK-AKT pathway (BRAF, NRAS or PIK3CA) on outcome measures, compared with an all-WT population (no downstream mutations). The all-WT population treated with IrPan had improved response rates (43.8%) and PFS (HR=0.68; 95% CI, 0.53-0.86), however still did not achieve OS benefit. Those KRAS-WT patients with a mutation had worse outcomes with IrPan compared with Ir (OS HR = 1.64[1.14-2.34], p=0.028); this was particularly marked for patients with a BRAF-mutation (OS HR=1.84; 95% CI 1.10-3.08).

Therefore PICCOLO did not reach its primary end-point and IrPan could not be recommended as second-line therapy. However there were important translational results that questioned the current opt-in strategy for treatment with anti-EGFR agents for patients with KRAS-wt tumours given the potential detriment. Additionally within the 'all wild-type' group some patients continue to fail to benefit from anti-EGFR agents, suggesting that there may be further, yet unknown, factors contributing to resistance.

Chapter 2. Routine clinical parameters as biomarkers in advanced colorectal cancer.

2.1. Introduction

Biomarkers can provide useful information, aiding risk stratification and guiding treatment decisions, however few are utilised in the routine management of aCRC. Biomarker discovery and validation is therefore a priority for CRC research. With emerging technology, increasing information could be provided to clinicians: integration of this into routine practice and cost-effectiveness shall be challenging.

During the initial patient consultation basic information is collected about the patient and tumour. This chapter will consider whether such readily available data can be utilised to guide treatment decisions in aCRC. In particular the role of the derived neutrophil/lymphocyte ratio (dNLR), platelet count and primary tumour location (PTL) shall be evaluated as biomarkers in aCRC. These markers were selected due to biological rationale and previous evidence, and will be evaluated alongside other clinical and molecular markers to reflect modern oncology practice. Candidate biomarkers shall be tested at different points in the aCRC pathway: both in chemo-naïve patients (FOCUS trial), and following first-line FU failure (PICCOLO).

2.1.1. Advantages of routine blood test results as clinical biomarkers

Biomarkers derived from routine blood tests are readily available, relatively non-invasive, acceptable to patients, and inexpensive. Tumour biomarkers (MEK-AKT pathway mutations, MMR status, grade), may signal likely tumour behaviour, but tissue is often from a historical surgical specimen. Blood tests instead inform of the patient's condition at the point of contact. Repeated measurements during treatment allow for dynamic assessment and perhaps response to therapy, and new information at the point of consideration of future lines of treatment. Both blood-based markers studied in this chapter are proposed to be surrogates for the systemic inflammatory response.

2.1.2. Inflammation

The immune system consists of cells and proteins that recognise and then protect the body from pathogens. It is broadly categorised into the innate and adaptive immune system (figure 2.1). Both are involved in the process of inflammation.

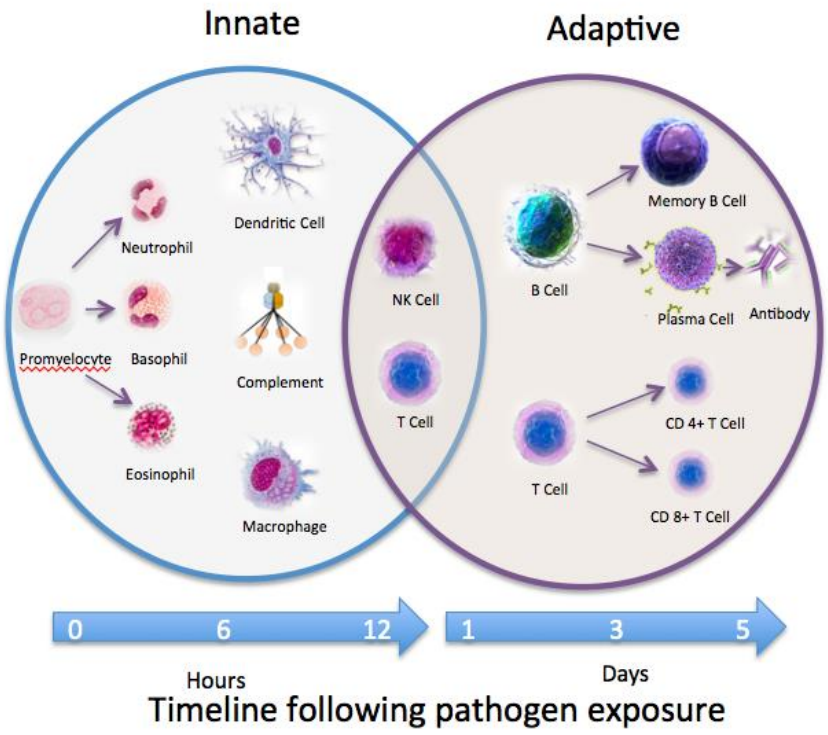

Figure 2-1 Components involved in the innate and adaptive immune response

Inflammation is the body's natural protective response to any insult or injury; detection of the insult leads to an influx of plasma proteins and acute inflammatory cells to the site. The acute inflammatory response initiates healing, but is not always beneficial. Detailed description of components of the immune system, interactions with cancer and its potential manipulation by therapeutics in aCRC are outwith the scope of this chapter, but have been recently reviewed.(242, 243)

Acute inflammation is a complex process, with three main components: alterations in vascular calibre, increased permeability of blood vessels, and emigration of leukocytes from the circulation and subsequent accumulation and activation at the site of injury. Release of chemotactic cytokines attracts specific leucocyte populations and inflammatory cell recruitment.(244, 245) The main initial effector is circulating neutrophils that adhere to the site of injury.(246, 247)

Chemotactically attracted monocytes then differentiate to form tissue macrophages responsible for the release of the cytokines and growth factors driving the local inflammatory response. These inflammatory mediators encourage cellular proliferation and local angiogenesis to enable tissue repair. Additionally inflammatory cascades are triggered by innate immune cells leading to antigen presentation by dendritic cells and macrophages to T-cells, with subsequent activation of the adaptive immune response.

2.1.3. Inflammation and cancer

Virchow made the first observation of the association of tumour growth and inflammation and described leukocyte infiltration within tumours, now widely considered a hallmark of cancer.(248, 249) The role of the inflammatory process has been described at all stages of cancer development and progression. Multiple cancer risk factors have the common principle of chronic inflammation: tobacco, chronic infection, autoimmunity, obesity, dietary factors, and inhaled pollutants.(250)

Inflammation enables many of the cellular and molecular capabilities essential for carcinogenesis: for example, tumour proliferative and survival signalling through IL-6; activation of STAT3 and NK-kB signalling leading to suppression of apoptosis and cell cycle progression.(251, 252) Other consequences of inflammation include genomic instability by increasing rates of DNA damage and compromising DNA-repair processes. Mechanisms include reactive oxygen species released by tissue neutrophils and macrophages leading to DNA breaks;(253) inflammatory cytokines induced expression of activation-induced cytidine deaminase, with consequent mutations in TP53 and MYC;(254) disruption of cell-cycle checkpoints and repression of MMR proteins leading to DNA replication errors and the accumulation of random genetic alterations.(255)

Inflammatory mediators also facilitate the metastatic process: monocytes and IL-4 induced tumour-associated macrophages facilitate tumour invasion, extravasation and metastatic outgrowth.(256, 257)

Patterns of immune response have also been associated with cancer outcomes. Adaptive immunity can be divided further into humoral immunity (HI) and cell-mediated immunity (CMI). HI is associated with TH2 CD4+ lymphocytes, with production of interleukin- (IL-)4, IL-6 and IL-10. CMI is associated with the production of cytokines, interferon-γ (IFN-γ) and (tumour necrosis factor- α) TNF-α by TH1 CD4 T-lymphocytes, with activation of cytotoxic T-lymphocytes (CTLs), natural killer (NK) cells, macrophages and monocytes.

Most cancers are associated CMI suppression(258) and shift towards the TH2 response is associated with tumour survival.(259) Furthermore, activation of the IL-6/ JAK/STAT3 pathway leads to increased expression of various acute phase proteins, such as albumin and C-reactive protein (CRP).

However the immune system also has a role in tumour suppression. The innate immune system recognises tumour specific antigens on the surface of cancer cells: NK cells recognise a lack of major histocompatibility complex-1 (MHC-1) surface molecules on cancer cells and both directly kill such cells and actively recruit other effectors.(260) Recruited macrophages and dendritic cells phagocytose cancer cells and present tumour-associated antigen on their surface, resulting in a specific effector T-cell response designed to eradicate tumour from the body.(261)

However it is hypothesised that cancer cells evolve and undergo a selection process for cells that evade immune system regulation, leading to clinically apparent tumours. The importance of 'immunosurveillance' is evidenced by the increased rate of cancers in immunocompromised patients, including advanced HIV infection and chemically-immunosuppressed transplant recipients.(262)

2.1.4. The inflammatory response in CRC tumours

These complex interactions are relevant in CRC. Chronic gut inflammation may be more critical in CRC carcinogenesis than previously thought. Inflammatory bowel disease is a well-recognised risk factor for CRC, but only accounts for a small proportion of cases. Intestinal inflammation may be more commonly caused by an over-population of 'bad-microflora', with associated pro-inflammatory cytokines, leading to epigenetic changes and recruitment of immune cells that contribute to cancer initiation and progression.(263) Increased IL-6/JAK/STAT3 signalling is a key driver of CRC, implicated in initiation, development and formation of tumours,(264) and associated with a hyperproliferative and invasive phenotype of CRC cells.(265)

CRC tumours with increased immune cell infiltrates have been associated with absence of pathological evidence of early metastatic disease (venous emboli and lymphatic and perineural invasion). Tumours with a 'good immune profile' showed infiltration of markers of T-cell migration, activation and differentiation, but not inflammatory mediators or immunosuppressive markers.(266) Other immune characteristics associated with improved prognosis include the presence of immune effector cells and antigen

presenting dendritic cells, NK cells and markers of T_H1 adaptive immunity.(249, 267, 268) In an independent series of 599 tumour specimens of stage I-IV CRCs, high densities of CD8+ cytotoxic T-lymphocyte infiltrate were associated with earlier stage tumours.(269)

Instead tumour immune characteristics associated with poor outlook include high levels of particular inflammatory cell infiltrates, such as high concentrations of tumour-associated macrophages.(270)

2.1.4.1.Assessing the immune profile of tumours

A CRC tumour immunoscore has been proposed. Genomic and in situ immunostaining analysis in 415 patients reported that immunological data (the type, density and location of immune cells) provided superior prediction of patient survival than current histopathological staging methods.(266) However this requires further validation and has not been routinely adopted.

2.1.5. The effect of the systemic inflammatory response in a cancer patient

A tumour could be considered as a pathogen and as such has the ability to initiate and maintain a host inflammatory response. The clinical consequence of chronic activation of the systemic inflammatory response is cachexia, increased fatigue and decrease in performance status;(271) a cancer patient may also be immunosuppressed due to increasing age and poor nutrition. It is also important to understand how host immune activation interacts with prognosis and response to therapy.

The systemic inflammatory response and the tumour inflammatory response may be entirely separate: both are independently associated with cancer-specific survival,(272) and increased blood neutrophil count (associated with poor prognosis) has been associated with low-grade peritumoral infiltrate.(273)

2.1.5.1.Biomarkers to assess the systemic inflammatory response

Measurement of serum IL-6 level may be a useful biomarker of the host inflammatory response. Activation of IL-6 during the early immune response leads to initiation of the IL-6/ JAK/STAT pathway and regulation of a broad spectrum of target cells, including expression of acute-phase proteins by hepatocytes (CRP and albumin),(274) neutrophil, macrophages T cells and epithelial cells.(275) STAT3 activation leads to cancer cell proliferation, invasion, differentiation and inflammation.(276) IL-6 is elevated in aseptic

inflammatory conditions and associated with a neutrophil-dominated inflammation, then transition to a more sustained adaptive immune response.(277) In CRC, IL-6 has a central role in initiation and persistence of intestinal inflammation(278) and colitis-associated cancers.(279)

However this process may be reflected simply by defects in both the innate immune system with alterations in neutrophils and monocytes, and the adaptive immune system with lymphopenia,(280) and CRP and albumin levels.(281, 282) Several inflammation based prognostic scores have been developed and have been correlated with cancer outcomes, including the neutrophil-lymphocyte ratio (NLR), modified Glasgow Prognostic Score (mGPS – utilising acute phase proteins), platelet lymphocyte ratio (PLR) and the prognostic index. The most extensively studied are the NLR and mGPS.

A high mGPS has been correlated with poor prognosis independent of tumour site,(283) and has been validated in over 30,000 patients across different tumour sites.(284) A meta-analysis of 40,559 cancer patients reported that high NLR was associated with poor survival, compared with low NLR with effect was seen across all disease sub-groups, sites and stages.(285)

Direct comparisons of these scores have been performed, but correlations between the elements of different scores (high levels of CRP, neutrophils, platelets, and low levels of albumin and lymphocytes) are seen.(283)

2.1.5.2.Testing of inflammatory scores in RCT datasets

Inflammation-based scores have not been validated in RCT datasets. Most RCT datasets will not routinely collect all desired components, particularly CRP and lymphocytes, so an alternative marker is required.

This issue was addressed by a useful paper that validated the dNLR.(286) The dNLR utilises the WCC and neutrophil count to create a ratio, presuming that the vast majority of non-neutrophil WCC will be lymphocytes. In over 27,000 patients the area under the ROC curves were 0.65 for the NLR and 0.64 for the dNLR for disease free survival. The authors had recommended different cut-offs for the ratios used in the two scores, with a cut-off for NLR being 4, and 2 for dNLR.

The FOCUS and PICCOLO trials have collected white cell, neutrophil and platelet counts for each trial patient at baseline and at set time-points throughout trial treatment, so this chapter will examine dNLR and platelet count as biomarkers.

2.1.6. The neutrophil-lymphocyte ratio

The NLR has been evaluated as a prognostic marker in a variety of clinical situations including pancreatitis (287) and coronary artery disease, (288) but most evidence is in cancer. A high NLR level is associated with poorer outcomes in nasopharyngeal cancer (289), non-small cell lung cancer (290), breast cancer (291), oesophageal,(292) gastric cancer,(293) renal cell carcinoma,(294) and pancreatic cancer.(295)

2.1.6.1.NLR as a prognostic marker in CRC

The NLR has been tested as a prognostic marker for DFS and OS in surgical series in early CRC: a high NLR is associated with worse outcomes following resection of the primary tumour,(296, 297) following hepatic resection of CRC liver metastases,(298) and poorer OS, time to local recurrence and DFS in locally advanced rectal cancer patients undergoing chemo-radiotherapy.(299)

2.1.7. The systemic inflammatory response and chemotherapy efficacy

The efficacy of chemotherapy drugs may be influenced by a complex interplay of tumour and host immune components, modulating various immune cell populations in different ways, both direct and indirect.(300) The presence of inflammation has been associated with inferior chemotherapy outcomes: high concentrations of tumour associated macrophages are associated with poor response to chemotherapy.(301)
Additionally chemotherapy-induced neutropenia is associated with improved chemotherapy responses.(302, 303) A suggested mechanism is that chemotherapy induces death of myeloid-derived suppressor cells – a heterogeneous group of immature and mature myeloid cells, predominated by neutrophils, increases activity of T cells and subsequent tumour control.(304) Therefore an additional benefit of anti-cancer therapies may be suppression of neutrophil-driven inflammation.

In a study of 182 advanced lung cancer patients treated with first-line palliative platinum chemotherapy, an elevated NLR was associated with reduced RR and shorter PFS and OS, compared with low NLR.(305) High NLR was associated with a lesser chance of clinical

benefit and reduced OS than patients with low NLR, in aCRC patients treated with first line palliative chemotherapy.(306)

One study has assessed the NLR as a predictive marker for chemotherapy. In a RCT of metastatic castration-resistant prostate cancer receiving 1st line chemotherapy, high dNLR was associated with inferior survival, but was not a predictive marker for docetaxel benefit.(307)

Monoclonal antibodies can activate immune cells such as neutrophils, macrophages and NK cells by binding to their Fc regions, resulting in antibody-dependent cytotoxicity: hence their ability to trigger immune cell activation and cause immunogenic cell death may contribute to their efficacy. Polymorphisms in the Fc gamma receptor have been associated with cetuximab-resistance through its inability to induce recruitment and activation of immune effector cells.(308, 309)

2.1.8. Platelet counts and outcomes with aCRC

High platelet count has also been associated with poor outcomes in CRC.(310, 311) As part of the systemic inflammatory response, IL-6 triggers the differentiation of megakaryocytes to platelets in the bone marrow. Tumour-associated thrombocytosis is also induced by the tumour by several mechanisms, including by secretion of VEGF.(312) Platelets may also facilitate metastasis through mediating tumour cell survival in the circulation, and extravasation and angiogenesis in the microenvironment of target sites.(313)

In a study of 1513 surgically treated early CRC patients, high platelets were associated with reduced survival (OS HR = 1.66, $p<0.001$), and with a higher incidence of distant metastatic relapse (HR = 2.81, $p=0.011$), but not risk of loco-regional relapse (0.59, $p=0.32$).(314)

In sub-analysis of COIN C (investigating non-inferiority of intermittent vs. continuous 1st line chemotherapy in aCRC), the only predictive marker of detriment with chemotherapy breaks was a high baseline platelet count: this population experienced a five month reduction in OS with intermittent compared with a continuous strategy (interaction $p=0.0027$).(102) Cancer patients with evidence of a heightened inflammatory response may therefore benefit from continuous intensive therapy.

2.1.9. Markers of the systemic inflammatory response as monitoring biomarkers

The only recommended tumour marker in CRC is CEA, but as described in Chapter 1 this has several limitations. Regular blood tests are performed throughout chemotherapy, so repeated NLR assessments can be made. NLR may reflect changes in tumour behaviour during chemotherapy; lowering values suggesting dampening of the inflammatory response, perhaps secondary to tumour response. Conversely a rising NLR may signal tumour progression and futile treatment.

Early normalisation of the NLR following chemotherapy for aCRC was associated with improved outcomes.(306, 315) A similar NLR pattern was seen during gefitinib treatment in advanced NSCLC,(315) and first-line chemotherapy for advanced bladder cancer.(316) Conversely in a population of aCRC patients treated with first-line FOLFIRI-Bev, although baseline high NLR as an adverse prognostic marker an increase or stable NLR following 2 cycles was associated with a reduced risk of death.(317)

2.1.10. Primary tumour location and CRC outcomes

Another emerging hypothesis is that the location of a CRC primary tumour will impact on its biology, clinical outcomes and potentially, response to treatment. The distal and proximal colon have different embryonic origins (midgut and hindgut respectively), and have differences in bacterial flora and luminal content.(318) Clinico-pathological differences are described in observational studies, with right PTL being associated with increasing age, female sex, peritoneal carcinomatosis and a increased rate of mucinous, poorly differentiated and locally advanced tumours; instead, hepatic and pulmonary metastases more frequent in left PTL.(155, 319)

Studies examining potential differences in biology depending upon PTL have reported that right-sided tumours are more likely to have large chromosomal alterations, be MSI, CIMP-phenotype, and *BRAF*-mutated.(320-322)

An important study has examined biological differences in the colon in detail.(155) Right PTL was associated with higher frequencies of *KRAS*, *BRAF* and *PIK3CA*, regardless of MSI status. Instead HER1 and HER2 amplification and gene expression patterns in keeping with EGFR pathway activation were more likely in distal tumours.

2.1.10.1. PTL as a prognostic marker in aCRC

Many previous studies of prognostic markers in aCRC (including Kohne's score) have compared colon to rectal cancers, with no effect seen.(45) However the more relevant comparison is right colon compared with left colon and rectal cancers.

Study of nearly 78,000 patients from the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) database found that right PTL was associated with worse survival than left colon and rectal cancers, (78 vs. 89 months, p<0.001). This effect was less dramatic following adjustment for relevant confounders (HR = 1.04 [1.02-1.07]).(323) An observational study of over 17,641 CRC patients also reported that right PTL was a poor prognostic marker, but effect was not striking (HR = 1.12).(319) In early aCRC, right PTL was not associated with reduced RFS, but was an independent determinant of shorter survival after relapse (adj HR=1.70, p<0.001).(155)

PTL analysis in 146 patients from the AIO KRK-0104 (1st line CAPIRI+ cetuximab vs CAPOX + cetuximab in aCRC), right PTL was associated was inferior OS (13.0 vs 26.3 months; HR = 0.63, p<0.016), and PFS (5.2 vs 7.8 months; HR = 0.67, p=0.02), than left PTL.(324) In a large analysis of 3 cohorts of previously untreated aCRC (n =2027), right PTL was associated with inferior OS and PFS, independent of mucinous histology and *BRAF*-mutation status.(325)

2.1.10.2. PTL as a predictive marker for chemotherapy benefit

Since right PTLs are hypermutated with hypermethylation (potentially as a consequence of adjuvant chemotherapy) it has been hypothesised that they will be resistant to most current chemotherapeutic regimes. Additionally as active EGFR-signalling is more likely in distal tumours, anti-EGFR agents may only be beneficial in left PTL.(155)

For the 207 *KRAS/BRAF*-WT aCRC patients treated with cetuximab in Missiaglia's study, right PTL had reduced PFS than left PTL (18 vs 30 weeks, p=0.02).(155) In the AOI KRK-0104 *KRAS*-wt population, right PTL patients had reduced OS (p<0.001) and PFS (p=0.007) compared with left, but lesser PTL effect was observed in the *KRAS/ BRAF*-wt population (OS HR = 0.81, p=0.47; PFS HR = 0.60, p=0.23).(324) This hypothesis is yet to be tested in a population randomised to anti-EGFR agent vs. control.

PTL has been tested as a predictive biomarker for bevacizumab: VEGF-A is found in higher concentrations in the left than right colon.(326) In an observational comparison of CapeOx +/- bevacizumab, left PTL patients benefitted from bevacizumab, whilst right tumours did not: PTL/ biomarker interaction was positive for OS [p=0.004), but not PFS [p=0.15]).(327) Patients with rectal cancer had the best outcomes with bevacizumab within the study. These finding were not validated in PTL analysis from two RCTs of bevacizumab (AVF2107 or NO16966).(325)

PTL has not yet been assessed as a predictive marker for standard cytotoxic chemotherapy. In a study of 656 stage III CRC, patients with right PTL had a marked survival advantage with adjuvant chemotherapy, compared to those who did not (48% vs 27%; $p<0.001$), with lesser impact in left PTL (details of this analysis and interaction test not reported).(328)

2.2. Study hypotheses

2.2.1. Derived Neutrophil-lymphocyte ratio

- High dNLR will correlate with shorter OS and PFS, compared to a low dNLR.
- High dNLR will identify patients who will benefit from more intensive chemotherapy (combination vs. single agent).
- Increasing dNLR during chemotherapy will be associated with tumour progression at 12 weeks.

2.2.2. Platelets

- High platelets will correlate with shorter OS and PFS, compared to low platelet status.
- High platelets will identify patients who will benefit from more intensive chemotherapy (combination vs. single agent).

2.2.3. Primary tumour location

- Right PTL will correlate with shorter OS and PFS, compared to left and rectal tumours.
- Right PTL will be associated with lack of benefit to the addition of panitumumab to irinotecan.

2.3. Method

2.3.1. Patient population

This study involves patients from the FOCUS and PICCOLO trials. Consort diagrams for patients included in this biomarker study are shown in Figures 2.2 and 2.3.

In FOCUS all patients are included in the prognostic analysis. For the predictive analyses patients in Arm C (upfront combination chemotherapy) are compared to those in Arms A and B (upfront single-agent chemotherapy). The upfront oxaliplatin vs. irinotecan comparison is limited to Arm C (Fig. 2.2 a. and b.)

In PICCOLO, only patients treated with irinotecan alone (IrCs vs Ir, or IrPan vs Ir randomisations) are included in the prognostic analyses. The predictive analysis compares *KRAS*-wt patients in the IrPan vs Ir randomisation (Fig. 2.3 a and b).

All patients who have dNLR measurements at cycles 1 and 4 are included in the assessment of dNLR as a tumour marker 'NLR dynamic' analysis in both trials.

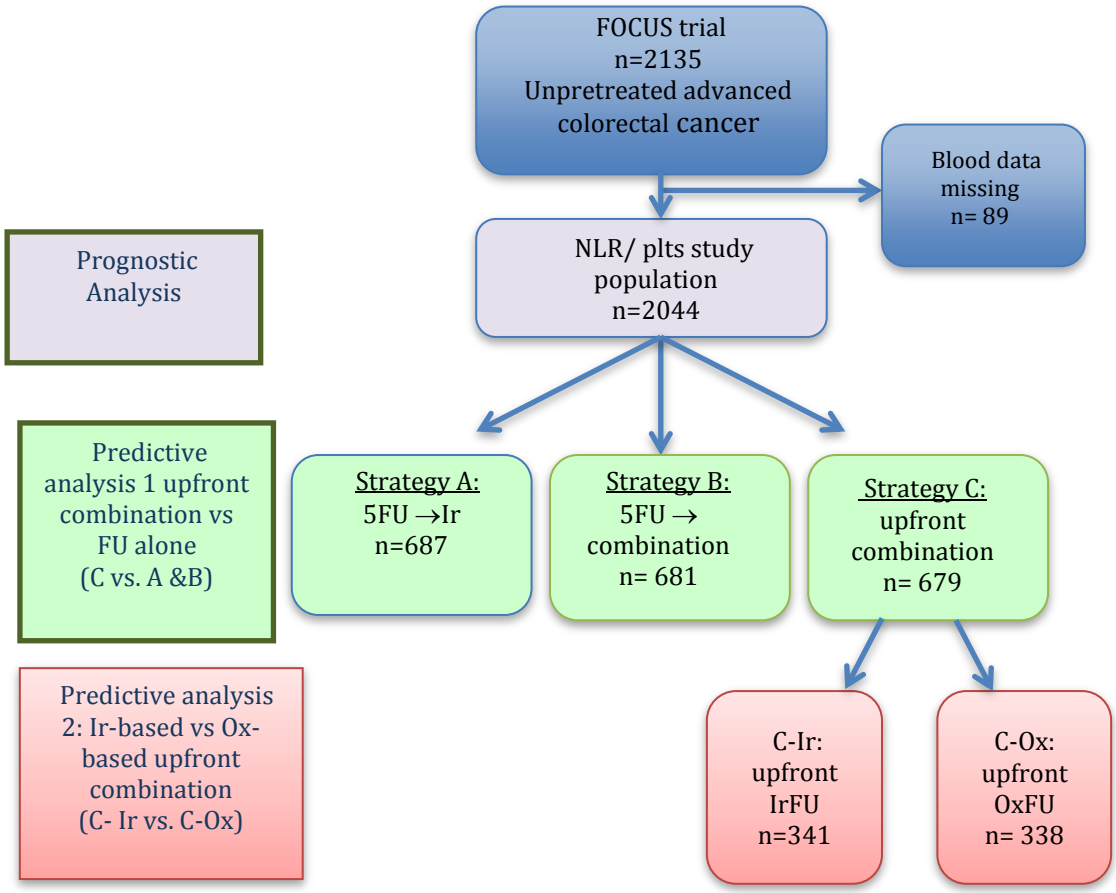

Figure 2-2 Consort diagram of FOCUS patients included in the dNLR/plts analysis

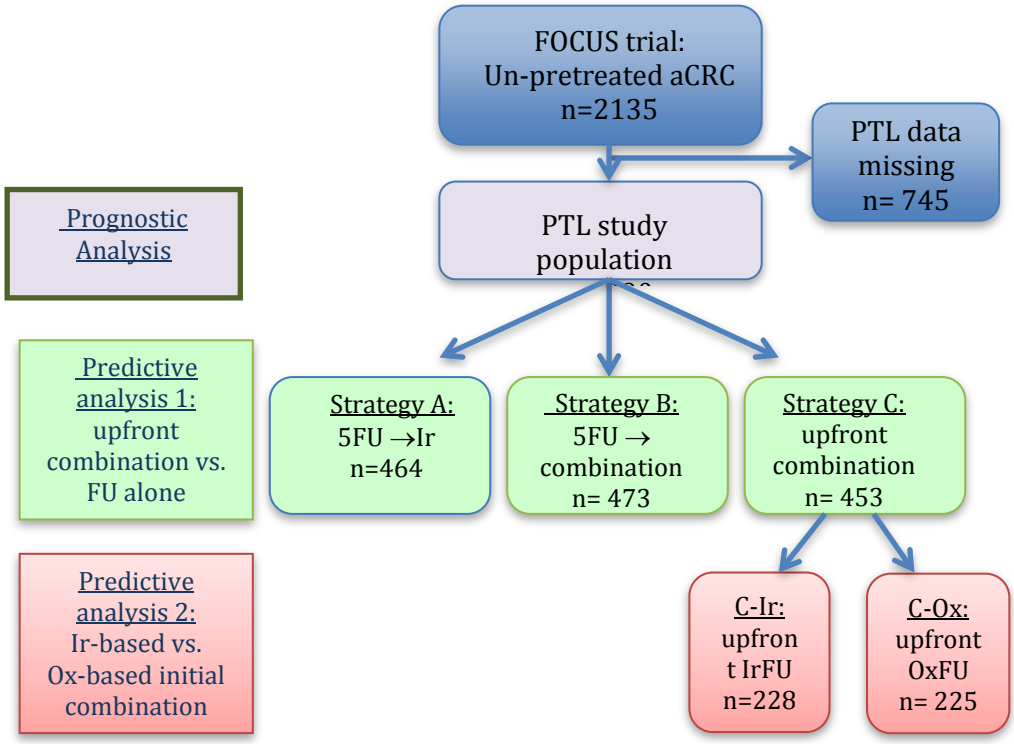

Figure 2-3 Consort diagram of FOCUS trial patients involved in the PTL study

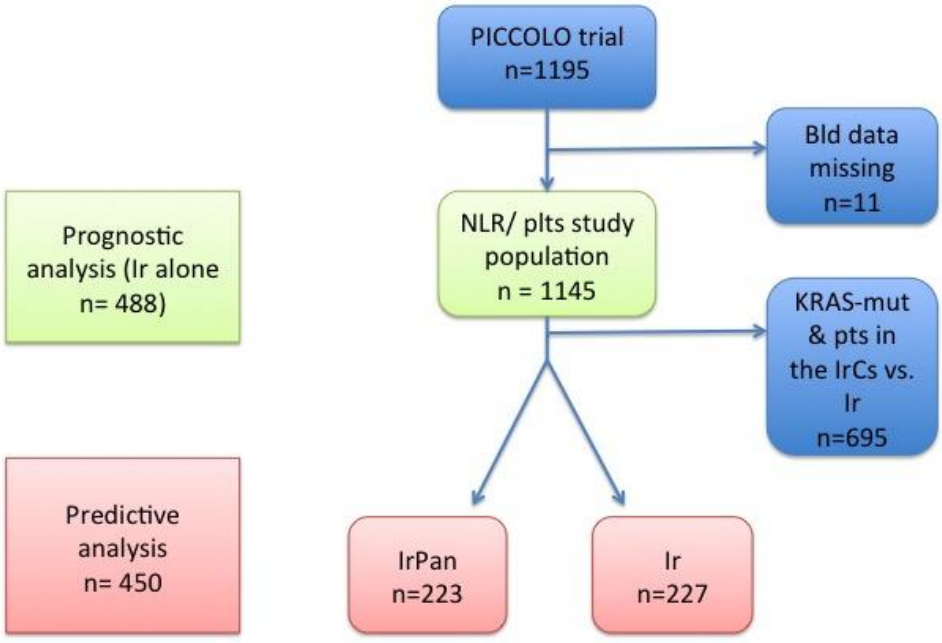

Figure 2-4 Consort diagram of PICCOLO study patients involved in the dNLR/ plts analysis

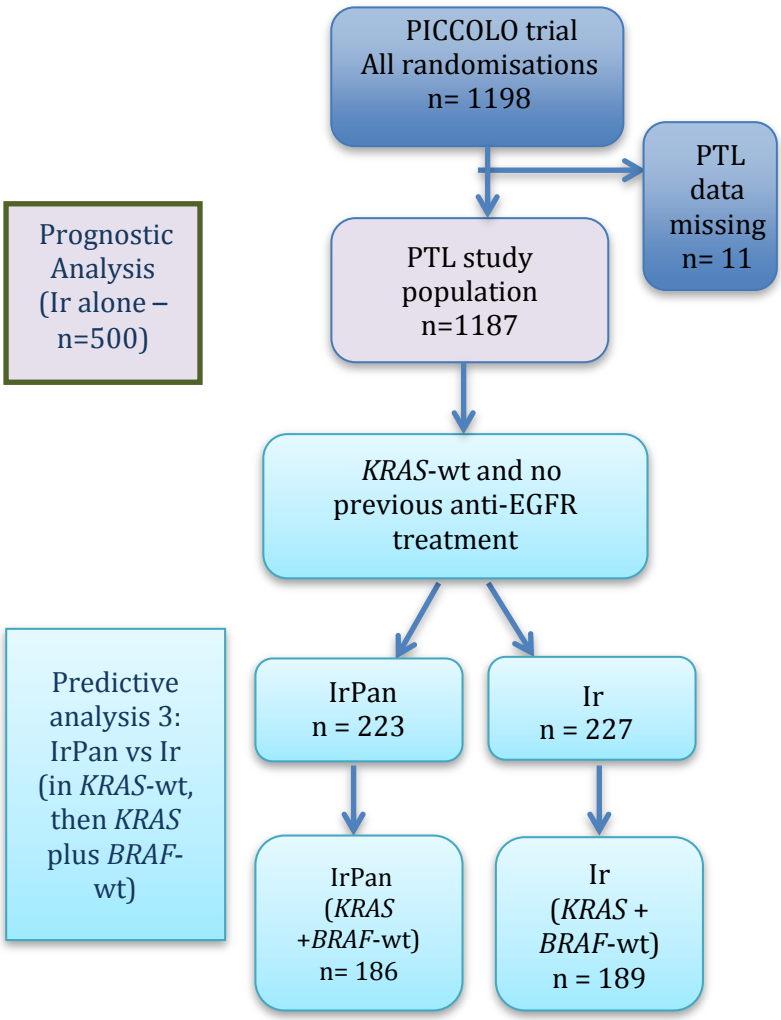

Figure 2-5 Consort diagram of PICCOLO study patients included in the PTL analyses

2.3.2. Study measurements and definitions

2.3.2.1.Neutrophil-lymphocyte ratio measurements

The baseline dNLR was calculated from the pre-treatment FBC (neutrophil count/ total WBC - neutrophil count). The dNLR was primarily assessed as a binary measure, prospectively defined as "high" (≥2.001) and "low" (<2.000), using the binary cut-point validated by Proctor et al.(286) Sensitivity analyses were performed on all pre-defined dNLR cut-points.

For the 'NLR dynamic' analysis, the actual percentage change between the dNLR values from baseline to cycle 4 (approximately 6 weeks) were calculated. Based upon this, patients were assigned to three prospectively defined categories:

- NLR improved (decrease in >25% from baseline to cycle 4),
- NLR rise (>25% increase in dNLR from baseline to cycle 4) and
- NLR stable (<25% increase, and <25% decrease from baseline to cycle 4).

Patients with missing baseline dNLR data were excluded from the prognostic and predictive analysis. Patients with missing follow-up dNLR data were excluded from the 'NLR dynamic' analysis.

2.3.2.2.Platelets

Baseline platelet count was assessed primarily as a binary measure, prospectively defined as high (>400,000u/l) vs low (<400,000 u/l). Patients with missing platelet values were excluded from the prognostic and predictive analysis.

2.3.2.3.Primary tumour location

PTL was prospectively defined as right PTL (to point of the splenic flexure) vs. left PTL (left tumour plus rectum). A secondary analysis examined left colon vs. rectal tumours.

2.3.3. Statistical Analyses

Stata was used for all statistical analyses (*Stata Statistical Software: Release 12 (2011)*, StataCorp. College Station, Texas).

2.3.3.1.Baseline characteristics

Baseline patient characteristics were compared between the high and low dNLR groups, platelet groups and PTL groups using two-tailed T-tests, Wilcoxon rank sum tests (for variables with non-normally distributed frequency distributions) and Pearson Chi-squared tests (for categorical variables).

2.3.3.2.Planned analyses

The primary analyses in both trials was to test the candidate markers as prognostic biomarkers for OS and PFS in the first (FOCUS) and second-line (PICCOLO).

In FOCUS the dNLR, platelets and PTL were tested as predictive biomarkers for upfront combination vs single agent FU chemotherapy, and oxaliplatin-based vs irinotecan-based combination chemotherapy. In PICCOLO the dNLR, platelets and PTL were tested as predictive biomarkers for benefit from the addition of panitumumab to irinotecan.

2.3.3.3.Statistical analyses

Univariate Cox hazard models were estimated for potentially relevant prognostic markers in both trials (FOCUS: age, performance status [PS], previous resection of primary tumour, number of metastatic sites, dNLR, platelet count, alkaline phosphatase, PTL and *KRAS* and *BRAF* mutant status; PICCOLO: PS, previous response to chemotherapy, number of metastatic sites, previous tumour resection, PTL dNLR, platelets, alkaline phosphatase, and *KRAS* and *BRAF* mutant status). Significant factors at univariate analysis were entered into a multivariate Cox model, and HRs and 95% confidence intervals (CIs) were estimated. A sensitivity analysis to investigate the prognostic effect of alternative dNLR cut-points was performed.

For the three predictive analyses testing for interaction between the effects of high vs low dNLR status and treatment was performed on OS and PFS.

For the RR endpoint, odds ratios (ORs) and 95% CIs were estimated from logistic regression models for the effect of dNLR status, and the interaction was analysed in the same way. These analyses were repeated for the effects of high vs low platelets, and right PTL vs left PTL and rectum.

An exploratory analysis was performed to test whether change in dNLR after 6 weeks of chemotherapy is predictive of RECIST response at 12 weeks. Three groups were defined as previously described. Changes in the dNLR between baseline and cycle 4 were compared between 12-week responders and non-responders, and those with clinical benefit and progressive disease. For the primary RR endpoint, ORs and 95% CIs were estimated from logistic regression for the effect of dNLR dynamic groups on 12 week RECIST response. Additionally the sensitivity and specificity of dNLR dynamic group to predict RECIST response was calculated, and a sensitivity analysis to explore the effect of different cut-points.

2.4. Results (1): Clinical biomarkers in the FOCUS trial

2.4.1. Biomarker distributions and patient characteristics

Patient characteristics according to dNLR, platelets and PTL are shown in Table 2.1. As a number of patients had missing PTL data, an analysis was performed to assess whether there were differences in the characteristics of missing cases. Those without PTL data were less likely to have a raised alkaline phosphatase (p=0.04) had fewer metastatic sites (p=0.01), but more likely to have their primary tumour in situ (p<0.001).

2.4.1.1.dNLR population distribution and characteristics

Out of the 2135 patients in the FOCUS trial, 2044 patients had dNLR data available. The dNLR showed a normal distribution (Figure 2.6). Median baseline dNLR value was 2.17 (IQR 1.61-2.97).

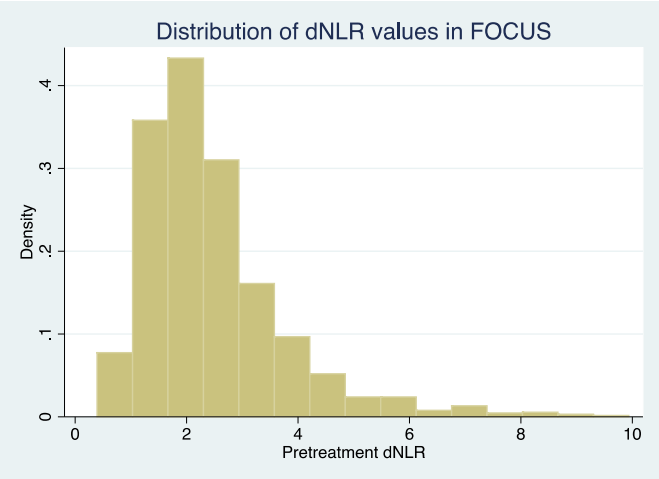

Figure 2-6 Distribution of baseline dNLR within the FOCUS trial

Using the pre-defined cut-point of 2.01, 1153/2044 (56.4%) patients were assigned to the 'high dNLR' group, and 322/891 (43.6%) to the 'low dNLR' population. A higher proportion of patients in the high dNLR group had a primary tumour in-situ, high platelets, raised alkaline phosphatase and were of poor PS (table 2.1).

2.4.1.2.Platelet population distribution and characteristics

Out of 2135 patients in the FOCUS trial, 2073 had baseline platelet data. Platelet distribution was skewed to the right (fig. 2.7), suggesting that the mass of the distribution of platelet values are concentrated on the left of the figure (i.e the lower values). Therefore

as anticipated the median baseline platelet value was 312 (IQR 253-397), lower than the mean (338, sd 126).

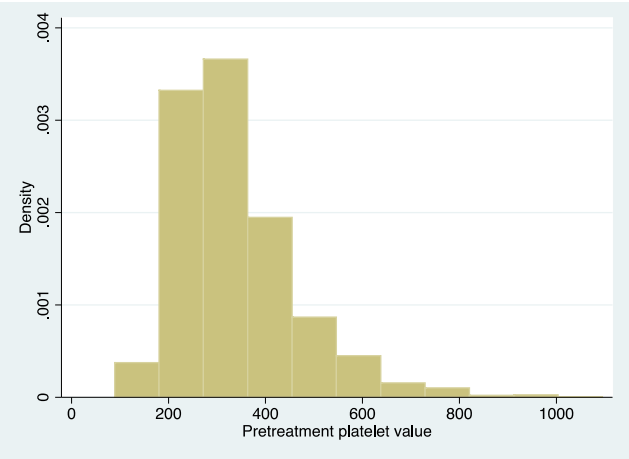

Figure 2-7 Distribution of baseline platelet values within the FOCUS trial

Using the pre-defined cut-point (>400,000 u/l), 510/2073 (24.6%) patients were assigned to the 'high platelet' group, and 1563/2073 (75.4%) to the 'low platelet' group. This group was associated with a higher proportion of patients with high dNLR, high alkaline phosphatase, primary tumour in-site and poor PS (table 2.1) .

2.4.1.3.PTL population distribution and characteristics

In FOCUS, PTL data was available for 1390 patients. In total, 364 (26.2%) of patients had a right-sided tumour (right PTL), and 1026 (73.8%) had a left sided or rectal tumour (left PTL). 454 patients had a rectal tumour (32.7%) and 572 a left colonic tumour (41.1%) (fig. 2.8).

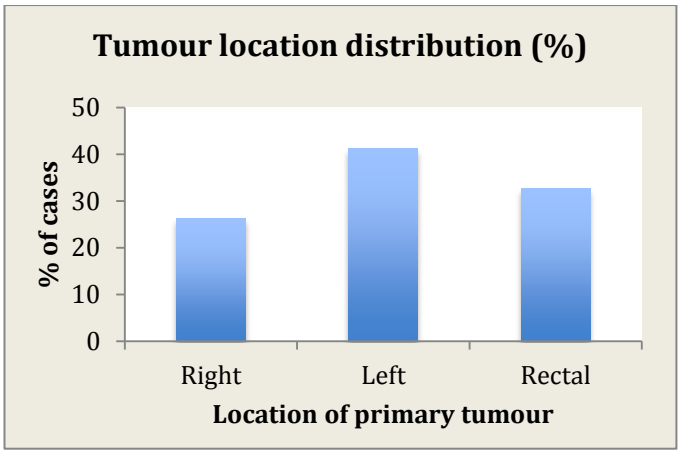

Figure 2-8 Distribution of the PTL study population

Right-sided tumours were more common in females, had a higher incidence of *BRAF* mutations and a higher rate of loss of MLH1 and MSH2, and were more likely to have been resected (Table 2.1).

		dNLR population n=2044			platelets population n =2073			PTL study population = 1390		
		High dNLR (≥2.00)	**Low dNLR (<2.00)**		**High platelets (≥400)**	**Low platelets (<400_**		**Right PTL**	**Left PTL**	**PTL missing**
No of patients		1153 (56.4%)	891 (43.6%)		510 (24.6%)	1563 (75.4%)		364 (26.2%)	1026 (73.8%)	745 (34.9%)
Median age		62	63		60.8	63.0		63.1	62.6	62.1
Sex (n=2044)	Male	777 (55.4%)	628 (44.6%)		303 (60.6%)	1240 (79.4%)		226 (62.1%)	733 (71.4%)	501 (67.3%)
	Female	376 (58.8%)	263 (41.2%)		207 (39.4%))	442 (28.3%)		138 (37.9%)	293 (28.6%)	244 (32.7%)
PS (n=2044)	0-1	1023(88.5%)	849 (95.3%)		434 (85.1%)	1463 (93.6%)		336 (92.3%)	948 (92.4%)	671 (90.1%)
	2	133 (11.5%)	42 (4.7%)		76 (14.9%)	100 (6.4%)		28 (7.7%)	78 (7.6%)	74 (9.9%)*
Resected primary (n=2043)	Yes	794 (68.7%)	737 (82.8%)		309 (60.6%)	1240 (79.4%)		315 (86.5%)	787 (76.7%)	493 (66.4%)
	No	362 (31.3%)	154 (17.2%)		201 (39.4%)	322 (20.6%)		49 (13.5%)	239 (23.3%)	249 (33.6%)
No of metastatic sites (n=2043)	0-1	472(41.9.2%)	383 (44.0%)		194(22.4%)	674 (77.6%)		134 (37.2%)	419 (41.7%)	339 (46.9%)*
	>2	655(58.1%)	487 (56.0%)		300 (25.9%)	859 (74.1%)		226 (62.8%)	585 (58.3%)	384 (53.1%)
High platelets (n=2073)	Yes	376 (32.5%)	125 (14%)		n/a	n/a		91 (25.7%)	226 (22.4%)	226 (30.4%)
	No	779 (67.4%)	766 (85.9%)		n/a	n/a		263 (74.3%)	781 (77.6%)	518 (69.6%)
Primary tumour location (n=1390)	Right	176 (15%)	169 (18.9%)		91 (28.7%)	263 (25.2%)		n/a	n/a	
	Left	551 (47.7%)	446 (50.1%)		226 (71.3%)	781 (74.8%)		n/a	n/a	
KRAS –mut (n=779)	Mutant	169 (51.4%)	160 (48.6%)		75 (49.3%)	258 (43.6%)		106 (51.5%)	208 (43.8%)	28 (35.4%)
	Wild-type	209 (51.9%)	194 (48.1%)		77 (50.7%)	333 (56.4%)		100 (48.5%)	267 (56.2%)	51 (64.6%)
BRAF mutation (n=764)	Mutant	19 (1.6%)	32 (3.4%)		14 (9.0%)	44 (7.3%)		35 (16.7%)	19 (3.9%)	7 (8.6%)
	Wild-type	321 (20.0%)	295 (33.1%)		141 (91.0%)	559 (92.7%)		175 (83.3%)	467 (96.1%)	74 (91.4%)
High alk phos (>400) (n=2028)	Yes	230 (20.0%)	92 (10.4%)		140 (27.5%)	192 (12.3%)		51 (14.1%)	153 (14.9%)	126 (18.1%)*
	No	919 (80.0%)	795 (89.6%)		369 (72.5%)	1364 (87.7%)		312 (85.9%)	871 (85.1%)å	571 (81.9%)

Table 2-1 Baseline characteristics of FOCUS trial patients, by dNLR status, platelet status and PTL status

* = significant p-value between missing cases and PTL population

2.4.2. Prognostic Analysis of Clinical Biomarkers

The prognostic analysis for all tested biomarkers is presented in table 2.2.

	High vs low dNLR		High vs low platelets		R vs L PTL
OS HR	n=2044 (fail = 1769)		n = 2073 (fail = 1796)		n=1389 (fail = 1228)
	1.57(1.43-1.73), p<0.001		1.89 (1.44-2.11), p<0.001		1.22[1.08-1.39],p=0.002
PFS HR	n=2035 (fail = 1978)		n=2064 (fail = 2007)		n=1380, fail (1344)
	1.45 (1.33-1.59), p<0.001		1.59 (1.44-1.77), p<0.001		1.08 [0.96-1.22], p=0.20)

Table 2-2 Prognostic analysis for the effect of the dNLR binary classifier, the binary platelets classifier and PRL on overall survival and progression free survival

2.4.2.1.Prognostic effect of dNLR

A negative prognostic effect of high versus low dNLR status was seen for OS (HR=1.57, [95%CI 1.43-1.73], p<0.001) and PFS (HR=1.45 [1.33-1.59], p<0.001) (table 2.2 and fig 2.9).

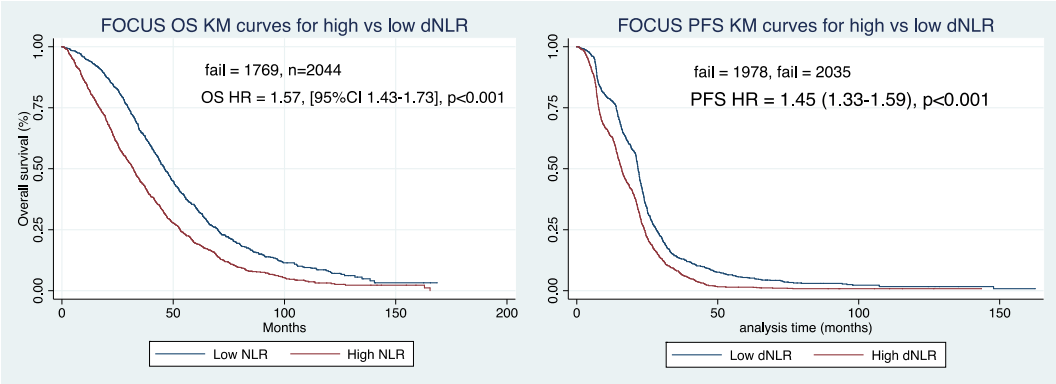

Figure 2-9 a)OS KM curves, and b) PFS KM curves for high vs low dNLR groups in the FOCUS trial

When assessing dNLR as a continuous variable, increasing dNLR levels were associated with inferior OS (HR = 1.23 [1.19-1.26], p<0.001) and PFS (HR=1.17[1.14-1.21], p<0.001).

2.4.2.2.Prognostic effect of platelets

High platelet count was associated with inferior OS (HR = 1.90 [1.71-2.12], p<0.001) and PFS (HR = 1.58[1.43-1.76], p<0.001) than the low platelets (table 2.2 and fig. 2.10).

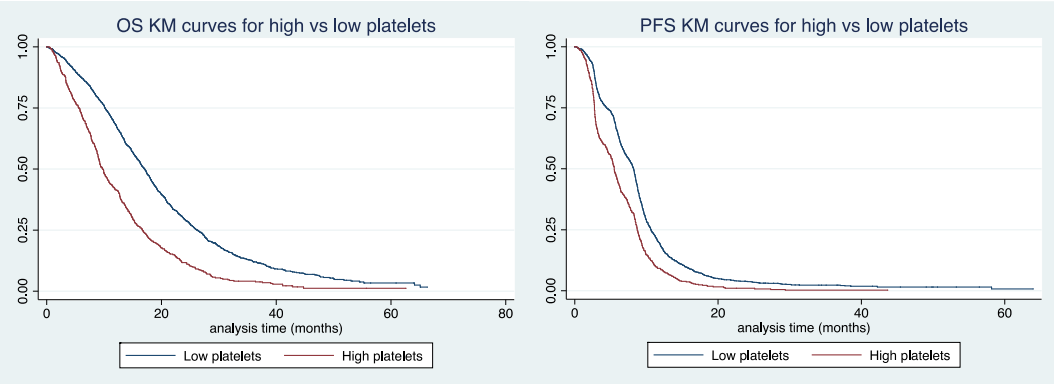

Figure 2-10 a) OS KM curves, and b) PFS KM curves for high vs. low platelets in the FOCUS trial

As a continuous variable, increasing platelets count was a negative prognostic marker for OS (HR = 1.002, p<0.001) and PFS (HR = 1.002, p<0.001).

2.4.2.3.Prognostic effect of PTL

Right sided PTL was a poor prognostic factor for OS (HR=1.22[1.08-1.39],p=0.002), but not PFS (HR = 1.08 [0.96-1.22], p=0.20), compared with left PTL (table 2.2 and fig 2.11).

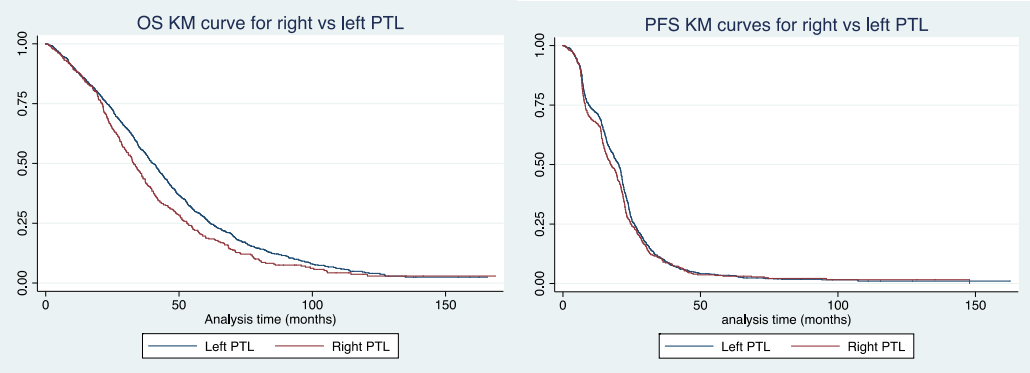

Figure 2-11 a) OS KM curves, and b) PFS KM curves for right vs. left PTL groups in the FOCUS trial

When compared to rectal tumours, left colonic tumours had improved OS (HR = 0.75[0.59-0.95], p=0.015) and PFS (HR = 0.76[0.58-1.00), p=0.05)(data not shown).

2.4.2.4.Multivariate analysis of prognostic factors within the FOCUS trial

Each of dNLR, platelets and PTL were significant prognostic factors within the FOCUS trial, however it is important to understand their usefulness compared with other validated prognostic markers. Other significant factors at univariate analyses were primary tumour in-situ, poor performance status, *KRAS* mutation, *BRAF* mutation, raised alkaline phosphatase and BMI (table 2.3).

Prognostic Factor	No in each population	OS HR (95% CI)	PFS HR (95% CI)
Age*	continuous	1.07 (0.96-1.19), p=0.22	0.94 (0.84-1.04) p=0.25
PS ≥2	180/2135 (8.4%)	1.39 (1.29-1.49), p<0.001	1.19 (1.12-1.29), p<0.001
Primary tumour in situ	514/2044 (25.1%)	1.54 (1.38-1.72),p<0.001	1.34 (1.21-1.49) p<0.001
Right PTL	364/1390 (26.2%)	1.22 (1.08-1.39) p=0.002	1.08 (0.96-1.22) p=0.20
2 or more metastatic sites	1195/2087 (57.3%)	1.40 (1.27-1.54), p<0.001	1.33 (1.22-1.46), p<0.001
KRAS-mut	329/732 (44.9%)	1.21 (1.04-1.42), p=0.014	1.15 (0.98-1.34) p=0.051
BRAF-mut	57/747 (7.6%)	1.53 (1.16-2.04), p=0.003	0.95 (0.72-1.26), p=0.76
dNLR ≥2	1153/2044 (56.4%)	1.57 (1.43-1.73) p<0.001	1.45 (1.33-1.59) p<0.001
Plts ≥400	499/2044 (24.5%)	1.90 (1.71-2.12), p<0.001	1.58 (1.43-1.76),p<0.001
Alkaline phos ≥300	322/2044 (15.7%)	1.98 (1.74-2.24), p<0.001	1.62 (1.44-1.83), p<0.001

Table 2-3 Univariate analysis of prognostic markers in FOCUS for OS and PFS

Multivariate analysis included factors significant at univariate testing. dNLR (adj HR = 1.25, p=0.001), high platelets (adj HR=1.44, p=0.001), high alkaline phosphatase (adj HR = 1.90, p<0.001), *BRAF* mutations (adj HR = 1.62, p<0.001), poor PS (adj HR = 1.23, p=0.003) and ≥2 metastatic sites (adj HR = 1.24, p=0.018) were identified as independent poor prognostic markers for OS.

For PFS, following adjustment high dNLR, platelets, alkaline phosphatase, primary tumour in-situ and over ≥2 metastatic sites were independent prognostic markers.

2.4.3. Clinical markers as predictive biomarkers for FOCUS strategies

Results of the predictive analyses are shown in table 2.4. The tested treatment strategies are:

- Initial upfront combination chemotherapy vs single agent (upfront IrFU or OxFU vs. 5FU alone)
- Irinotecan vs oxaliplatin based upfront combination chemotherapy

2.4.3.1. dNLR as a predictive biomarker for FOCUS strategies

Patients with a high dNLR had an OS benefit with upfront combination compared with FU alone (HR = 0.80 [0.75-0.97], p=0.02), but those with a low dNLR did not (HR = 0.96[0.82-1.13], p=0.68). dNLR/ treatment interaction testing was not significant (p=0.20) (table 2.4 and fig. 2.12). Patients with a high dNLR had PFS benefit with upfront combination chemotherapy rather than single agent FU (HR = 0.68, p<0.001); those with a low dNLR had significant but lesser PFS benefit from combination treatment (HR = 0.78, p=0.01)(interaction p=0.09).

For both dNLR categories, RR was higher for combination than single agent chemotherapy (dNLR high 58.6% vs 34.2%, p<0.001; dNLR low 63.6% vs 43.8%, p<0.001). High dNLR patients were just as likely to respond to initial combination chemotherapy as low dNLR (p=0.21), but were less likely to respond to 5FU alone (p=0.001).

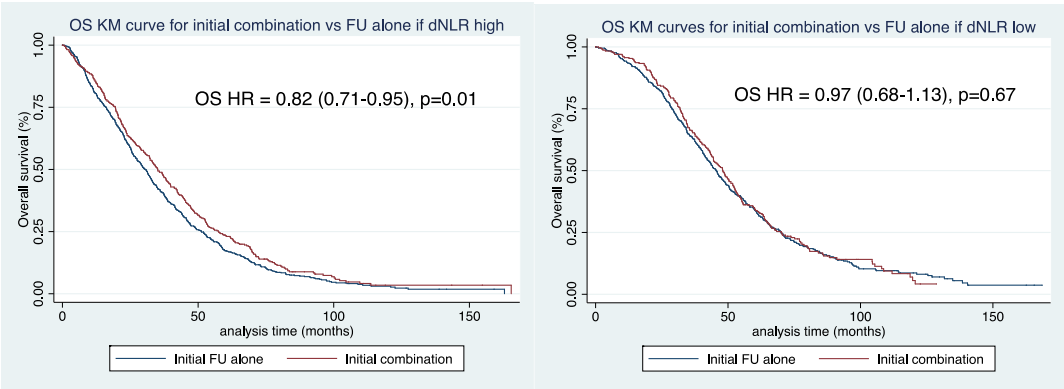

Figure 2-12 OS KM curves for a) high dNLE, and b) low dNLR (interaction p=0.20)

dNLR did not identify patients with differential benefit from a irinotecan compared with an oxaliplatin based regimen for OS (interaction p=0.92) or PFS (interaction p =0.81)(Table 2.4).

	All patients		dNLR study population				Platelets study population				PTL study population		
			dNLR ≥2	dNLR <2			Plts≥400	Plts<400			Right PTL	Left PTL	
Treatment strategy	**Unadjusted HR (95% CI)**	**Int p-value***	**Unadjusted HR (95% CI)**	**Unadjusted HR (95% CI)**	**Int p-value**		**Unadjusted HR**	**Unadjusted HR**	**Int p-value***		**Unadjusted HR**	**Unadjusted HR**	**Int p-value***
OS HR for upfront combination vs single agent	1769 events n =2044	0.051	1043/ 1153	726/ 891	0.20		467/499	1302/1545	0.79		331/363	897/1026	0.30
	0.90 (0.82-1.00)		0.82 (0.75-0.97),p=0.019	0.96 (0.82-1.13),p=0.68			0.94 (0.77-1.14) p=0.51	0.89(0.80-1.01) p=0.07			0.92(0.70-1.19) p=0.52	0.97(0.85-1.12)p=0.71	
PFS HR for upfront combination vs single agent	1978 events n=2035	<0.001	1127/1149	851 events n=886	0.09		494/498	1484/1537	0.68		353/361	991/1019	0.14
	0.73 (0.67-0.81)		0.68 (0.61-0.78),p<0.001	0.78 (0.68-0.91),p=0.001			0.73 (0.61-0.89) p=0.001	0.74 (0.66-0.82),p=0.001			0.67(0.53-0.83)p<0.001	0.79(0.7-0.91)p<0.001	
OS HR for irinotecan5FU vs oxaliplatin5FU	581 events n=68-	0.61	342 /380	239 /300	0.92		158/167	430/521	0.62		112/123	288/329	0.99
	0.96 (0.81-1.13)		0.95(0.77-1.17), p=0.63	0.98 (0.77-1.27),p=0.92			1.07 (0.78-1.47) p=0.66	0.96 (0.79-1.16) p =0.70			0.97(0.67-1.40) p=0.88	0.98(0.78-1.24) p=0.89	
PFS HR for irinotecan5FU vs oxaliplatin5FU	657 events n=678	0.26	370/ 379	287 /299	0.81		165/166	500/520	0.31		118/122	320/328	0.67
	1.09 (0.94-1.27)		1.09(0.89-1.33), p=0.42	1.14 (0.90-1.44),p=0.27			1.27 (0.93-1.74) p=0.13	1.07 (0.89-1.27)			0.97(0.68-1.39)p=0.86	0.86(0.69-1.06) p=0.19	

Table 2-4 Estimated crude HRs and 95% CIs for the effect of tested treatment strategies for high vs. low dNLR, then high vs. low platelets, then right vs. left PTL, including likelihood ratio tests for marker*treatment interactions

2.4.3.2.Platelets as a predictive marker for FOCUS strategies

Platelets were not a discriminative predictive marker for either upfront combination vs. single agent (OS interaction p = 0.79; PFS interaction p = 0.68), or irinotecan vs. oxaliplatin based chemotherapy (OS interaction p=0.62; PFS interaction p=0.31)(table 2.4).

2.4.3.3. PTL as a predictive biomarker for FOCUS strategies

Right-sided PTL did not preclude benefit from any of the tested treatment strategies: upfront combination vs single agent (OS interaction p = 0.30; PFS interaction p = 0.14), or irinotecan vs oxaliplatin based chemotherapy (OS interaction p = 0.99, PFS interaction p = 0.67)(table 2.4).

When comparing outcomes of rectal vs. left colon tumours, there was no differential effect from either strategy: upfront combination vs. single agent (OS interaction p = 0.30; PFS interaction p = 0.08), or irinotecan vs. oxaliplatin based chemotherapy (OS interaction p=0.18; PFS interaction p=0.50)(data not shown).

2.4.4. Exploratory analyses in FOCUS

The exploratory analyses will investigate the effect of alternative cut-points of dNLR, test a combined dNLR/ platelets model, explore the prognostic value of other components of the FBC, and investigate the dNLR as a tumour marker during first-line chemotherapy.

2.4.4.1.Investigating the effect of alternative dNLR cut-points

dNLR is a continuous variable with no natural dichotomisation point, however a binary variable is more practical for clinical application. The dNLR cut-point at 2 was pre-determined based upon previous work,(286) but may not be optimal in this population. The effect of alternative cut point ranges is shown in table 2.5 and figure 2.13. Risk of each dNLR cut-point is compared with that of the low dNLR group (<2.00). Increasing dNLR is associated with progressively worse OS. With a dNLR cut-point of 3.5-4, patients in the high dNLR have a markedly poor outlook (HR OS =2.40 {1.94-2.97, p<0.001). Utilising this cut-point, fewer patients were assigned to the high dNLR group.

dNLR cut point range	High dNLR group	OS HR (95% CI) for high vs low dNLR	PFS HR (95% CI) for high vs low dNLR
	n=1153	1769 events, n=2044	1978 events, n=2035
<2	0	1.0	1.0
2-2.5	384 (33.3%)	1.23 (1.08-1.39), p=0.002	1.17 (1.04-1.33), p=0.010
2.5-3.0	306 (26.5%)%)	1.49 (1.29-1.71), p<0.001	1.50 (1.32-1.72), p<0.001
3.0-3.5	170 (14.7%)	1.82 (1.52-2.16), p<0.001	1.57 (1.33-1.86), p<0.001
3.5-4.0	107 (9.0%)	2.40 (1.94-2.97), p<0.001	2.04 (1.66-2.52), p<0.001
>4	186 (16.1%)	2.08 (1.62-2.69), p<0.001	1.78 (1.39-2.28), p<0.001

Table 2-5 Effect of alternative dNLR cut-point ranges on OS and PFS, compared to low dNLR (<2)

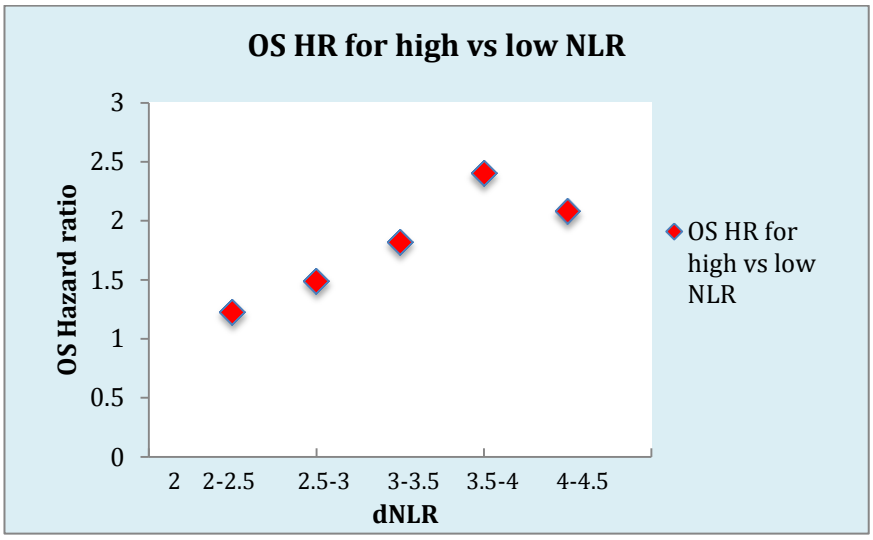

Figure 2-13 Alternative dNLR cut-points, with OS HRs for high vs. low dNLR at tested cut-points

The predictive ability of dNLR for FOCUS strategies with alternative cut-points was tested. At no cut-point was there a significant dNLR/treatment interaction for either strategy tested (data not shown).

2.4.4.2. Testing of a combined dNLR and platelet model

It has been demonstrated that dNLR and platelets have independent effects on survival (section 2.4.2.4), therefore combining information from both blood tests to create a single biomarker may be beneficial, using an 'either dNLR or plts high' vs 'neither high' model. The prognostic analysis is shown in table 2.6, and the predictive analysis in table 2.7.

dNLR/ platelet model	High risk group	OS HR (95% CI) for high vs low dNLR	PFS HR (95% CI) for high vs low dNLR
		1769 events, n=2044	1978 events, n=2035
High dNLR &/or plts	1278(62.5%)	1.75 (1.59-1.93), p<0.001	1.56 (1.43-1.72), p<0.001
Both high dNLR & plts	374 (18.3%)	1.91 (1.69-2.15), p<0.001	1.61 (1.44-1.81), p<0.001

Table 2-6 Prognostic analysis of the two combined dNLR/ platelet models, for OS and PFS

Treatment strategy	High dNLR &/or platelets	Low dNLR and platelets	
	Unadjusted HR (95%CI)	Unadjusted HR (95% CI)	Interaction p-value
OS initial combination vs single agent	0.85 (0.76-0.97), p=0.014	0.97 (0.82-1.16), p=0.78	0.22
PFS initial combination vs single agent	0.67 (0.60-0.76), p<0.001	0.81 (0.69-0.98), p=0.008	0.041
OS IrMdG vs OxMdG	0.97 (0.79-1.19), p=0.77	0.95 (0.72-1.25), p=0.72	0.96
PFS IrMdG vs OxMdG	1.12 (0.92-1.35), p=0.26	1.10 (0.86-1.42), p=0.44	0.91

Table 2-7 Estimated crude HRs and 95% CIs for the effect of tested treatment strategies in high dNLR &/or platelets vs. low dNLR and platelets, including likelihood ratio tests for marker*treatment interactions.

Firstly an 'either dNLR or platelets high' vs. 'neither high' model was investigated. Using that criteria, 1278 (62.5%) patients were assigned to the 'high' group and 766 (37.5%) to the 'low' group.

Having either high dNLR or platelets at baseline was associated with inferior OS (HR = 1.75[1.59-1.93], p<0.001) and PFS (HR = 1.56 [1.43-1.72], p<0.001), compared to patients who were low for both. This effect was maintained following adjustment (adj OS HR = 1.49, p<0.001; adj PFS HR = 1.30, p<0.001).

This joint model was assessed as a predictive marker. For upfront combination vs. single agent chemotherapy, the 'high' group had more benefit from a more intensive strategy than the low group. Whilst the difference in treatment effect between the biomarker groups was not significant for OS (interaction p = 0.22), it was for PFS (interaction p=0.041). For both groups, RR was higher for combination than single agent chemotherapy ('either high' 60.8% vs 33.3%, p<0.001; 'neither high' 61.2% vs 46.7%, p<0.001). The 'either high' group were as likely to respond to initial combination chemotherapy as 'neither high' (p=0.56), but less likely to respond to 5FU alone (p=0.002).

Neither group had differential effect with either an irinotecan compared to an oxaliplatin-based combination (table 2.7).

Next the effect of a 'both high' model was tested: both baseline dNLR and platelet raised vs. all other patients. Using this model, fewer patients were assigned to the 'high risk' group (18.3%). There was a marked prognostic effect of having both markers raised, particularly when the model used the dNLR cut-point of 3.5 (OS HR = 2.26, p<0.001; PFS HR = 2.05, p<0.001)(data not shown). This model did not predict benefit from any FOCUS strategy (data not shown).

2.4.4.3.Prognostic value of other components of the full blood count

To explore the individual effect of neutrophils or lymphocytes, compared to the dNLR and platelets, each FBC component was assessed as a continuous prognostic marker for OS (table 2.8). In a direct comparison, dNLR is a stronger prognostic marker than either neutrophils or lymphocytes individually.

FBC component (n=1809)	Median (IQR)	OS HR
dNLR	2.13 (1.59-2.87)	1.21 (1.17-1.25), p<0.001
Neutrophils	5.3 (4.2-7.0)	1.13 (1.11-1.16), p<0.0house01
Lymphocytes	2.5 2.0-3.1)	1.02 (0.97-1.08), p=0.31
Platelets	309 (251-392)	1.002 (1.001-1.003), p<0.001

Table 2-8 Prognostic analysis of further components of the FBC, treated as continuous variables

2.4.4.4.Change in dNLR and during chemotherapy

The behaviour of the dNLR during chemotherapy was investigated, and whether changes could predict RECIST response at 12 weeks.

The dNLR progressively decreased from baseline at 2 weeks (NLR2), then at 6 weeks (NLR4) from baseline (Fig 2.14). Median dNLR was 2.18 at baseline, 1.67 pre-cycle 2 and 1.5 pre-cycle 4.

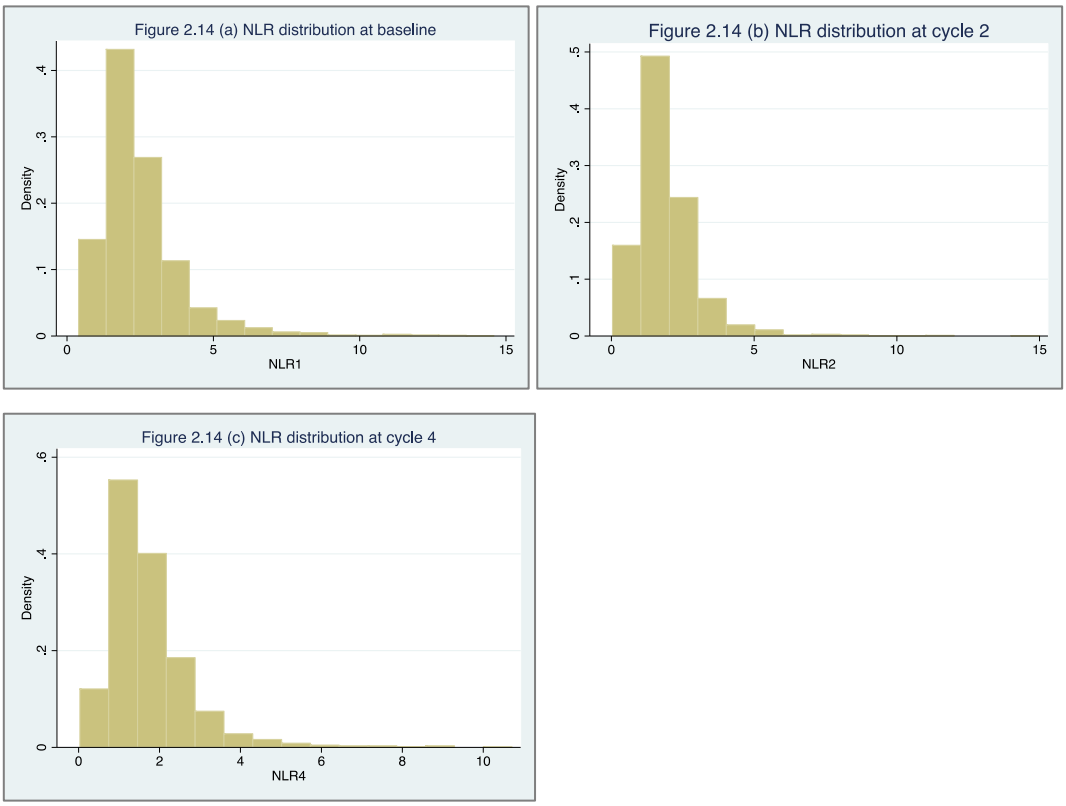

Figure 2-14 dNLR distributions at a) baseline, b) pre-cycle 2, and c) pre-cycle 4

At cycle 4, dNLR information was available in 1804 patients (88.3%) and for those RECIST response data was available for 1709 (94.7%). Utilising the pre-defined model for classifying change in dNLR between baseline and pre-cycle 4, dNLR rose in 183 (8.7%) patients, remained stable in 630 (34.9%), and decreased in 997 (54.9%).

To investigate whether NLR changes were related to response outcomes, the population was divided into baseline high vs. low dNLR as it was hypothesised that these groups may behave differently, and larger relative changes were more likely in the high baseline dNLR group. Patients who had a baseline high dNLR had a median 37.0% drop in dNLR; those with a baseline low dNLR had a median drop of 18%.

Patients with a baseline high dNLR who responded at 12 weeks had a greater decrease in dNLR than those with stable disease (p=0.006). Patients who had clinical benefit at 12 weeks had a greater decrease in dNLR at mid-point than those with progressive disease (p<0.001) (fig. 2.15).

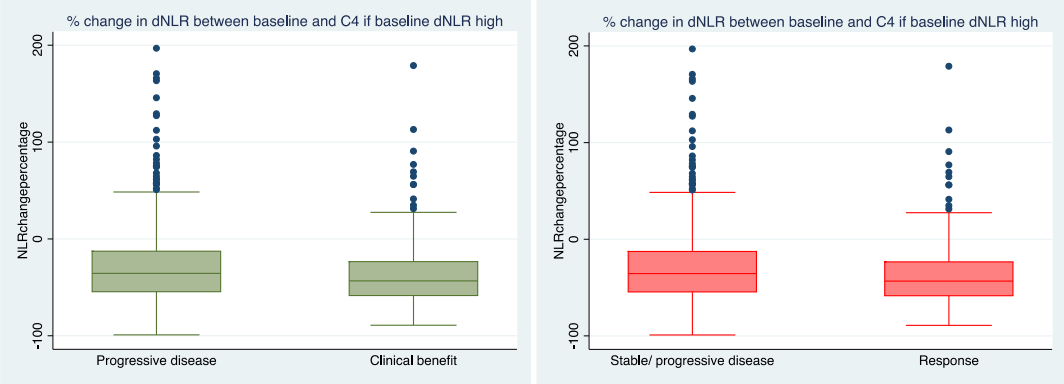

Figure 2-15 Boxplots showing percentage change in dNLR between baseline and cycle 4 in baseline high dNLR patients, a) 12 week responders and stable disease, and b) 12 week clinical benefit vs. progressors

Patients with a low baseline dNLR who had a response at 12 weeks had a greater decrease in dNLR than those with stable disease (p=0.037). Those with clinical benefit at 12 weeks had a greater decrease in dNLR at mid-point than those with progressive disease (p=0.040) (fig. 2.17).

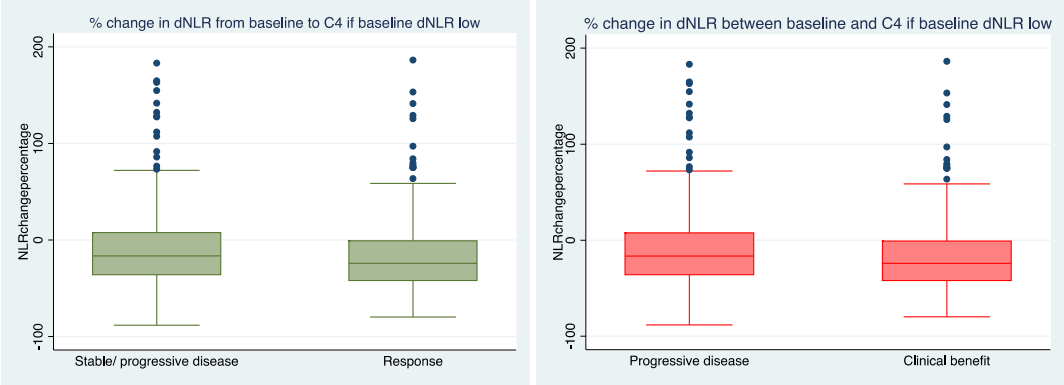

Figure 2-16 Boxplots showing percentage change in dNLR between baseline and cycle 4 in baseline low dNLR patients, a) 12 week responders and stable disease, and b) 12 week clinical benefit vs. progressors

A pre-defined cut-point was then used to assign patients to three 'NLR-dynamic' groups:

- Improved dNLR (>25% decrease in dNLR from baseline to pre-cycle 4),
- Stable dNLR (<25% decrease and <25% increase from baseline to pre-cycle 4)
- Worse dNLR (>25% increase in dNLR from baseline to pre-cycle 4)

Outcomes were compared between falling and stable or worsening dNLR (improved vs. stable or worse), and falling or stable dNLR and rising dNLR (improved and stable vs. worse dNLR), for each of the baseline dNLR groups.

Regardless of baseline dNLR status, rising dNLR during chemotherapy was associated with increased risk of progressive disease at 12 weeks, inferior PFS and OS compared with stable or falling dNLR (table 2.9). These findings were independent of other prognostic markers.

A more pronounced effect of the 'NLR dynamic' model was seen in the baseline high dNLR group: for patients with a rising dNLR only 45.3% had disease control with first-line chemotherapy at 12 weeks.

There were no significant changes in platelets during therapy, and no trends with response (data not shown).

Baseline dNLR low group: effect of changing dNLR during treatment				
NLR kinetic category (n=891)	**12 wk CR/PR OR (95%CI)**	**Clinical Benefit OR (95%CI)**	**PFS HR**	**OS HR**
Improved dNLR n = 341 (54.9%)	RR = 40.5%	DCR = 76.2%	1.01 (0.88-1.67) p=0.86	1.01 (0.86-1.18),p=0.88
	1.53(1.14-2.05), p=0.004	OR = 1.27 (0.13-1.76) p=0.13		
Stable or improved dNLR n =707 (89.8%)	RR = 35.6%	DCR = 74.8%	0.74 (0.59-0.89) p=0.003	0.71 (0.57-0.87) p=0.002
	1.32 (0.86-2.05), p=0.20	1.58 (1.04-2.43) p=0.033		
dNLR rise n = 112 (10.1%)	RR = 29.4%	DCR = 65.2%	1.37 (1.12-1.67), p=0.003	1.41 (1.14-1.75), p=0.63
	0.75 (0.49-1.16) p=0.20	0.63 (0.41-0.96), p=0.033		
Baseline dNLR high group: effect of changing dNLR during treatment				
NLR kinetic category (n=985)	**12 wk CR/PR OR (95%CI)**	**Clinical Benefit OR (95%CI)**	**PFS HR**	**OS HR**
Improved dNLR n = 650 (65.9%)	RR = 32.3%	DCR = 69.8%	0.76 (0.66-0.87) p=0.86	0.83 (0.72-0.95),p=0.009
	1.60(1.18-2.17), p=0.002	OR = 1.48 (1.13-1.96) p=0.005		
Stable or improved dNLR n =914 (92.8%)	RR = 29.9%	DCR = 68.4%	0.59 (0.47-0.77) p<0.001	0.57 (0.45-0.74) p<0.001
	1.91 (1.03-3.54), p=0.040	2.64 (1.63-4.31) p<0.001		
dNLR rise n = 71 (7.2%)	RR = 18.3%	DCR = 45.1%	1.66 (1.30-2.13), p<0.001	1.74 (1.36-2.24), p<0.001
	0.52 (0.28-0.97) p=0.040	0.38 (0.23-0.61), p<0.001		

Table 2-9 Treatment outcomes (ORs for 12 week response; ORs for clinical benefit; HRs for PFS; HRs for OS), depending upon NLR kinetic categories

The most useful clinical application for the 'dNLR kinetic' model would be to detect progression earlier than in routine practice. The sensitivity of the 'dNLR kinetic' model for detecting progressive disease at 12 weeks was 14.3%, but the specificity was 91.7%. This corresponded into a positive predictive value of 42.6% and a negative predictive value of 71.3%. The area under the curve (AUC) for NLR change and clinical benefit was 0.54, suggesting that it was not a discriminative tumour marker (fig. 2.17).

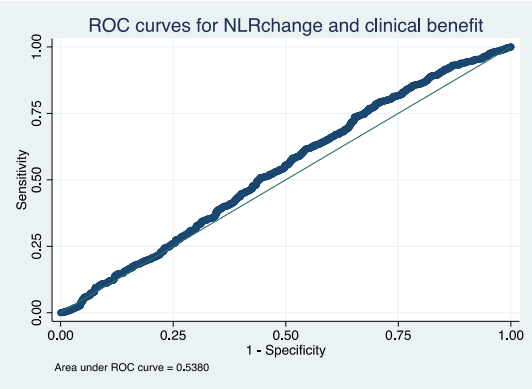

Figure 2-17 ROC curves for dNLR change for prediction of 12 week clinical benefit

2.5. Results: Clinical biomarkers in the PICCOLO trial

2.5.1. Biomarker distributions and patient characteristics

Patient characteristics according to dNLR, platelet and PTL are shown in table 2.10.
dNLR distribution

2.5.1.1.dNLR distribution and characteristics in PICCOLO

Out of the 1196 patients in the PICCOLO trial, 1145 patients (95.7%) had dNLR data available. The dNLR showed a normal distribution (fig. 2.18). Median value was 2.0 (IQR 1.46-2.67), lower than in FOCUS.

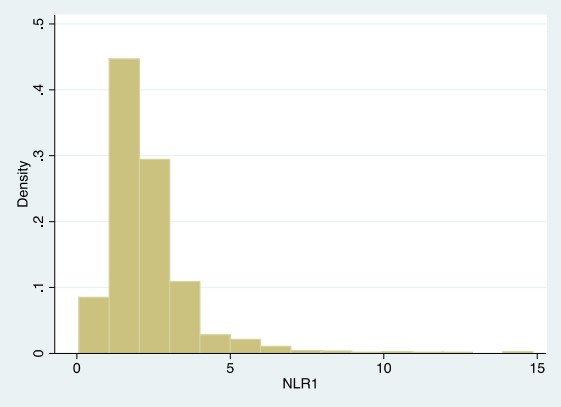

Figure 2-18 Distribution of baseline dNLR values within the PICCOLO trial

Using the pre-defined cut-point, 593/1145 (51.7%) patients were assigned to the 'high dNLR' group (dNLR>2.01), and 551/1145 (48.1%) to the 'low dNLR' population (dNLR<2.00).

A higher proportion of patients in the high dNLR group had their primary tumour in-situ, more than 2 metastatic sites, have raised platelets and alkaline phosphatase and be of a poor performance status, than the low dNLR groupy (table 2.10).

2.5.1.2.Platelet population distribution and

Out of 1196 patients in the PICCOLO trial, 1157 had baseline platelet data. Platelet count was normally distributed (fig. 2.19). Median platelet count was 262 (IQR 1.47-2.68), lower than seen in FOCUS.

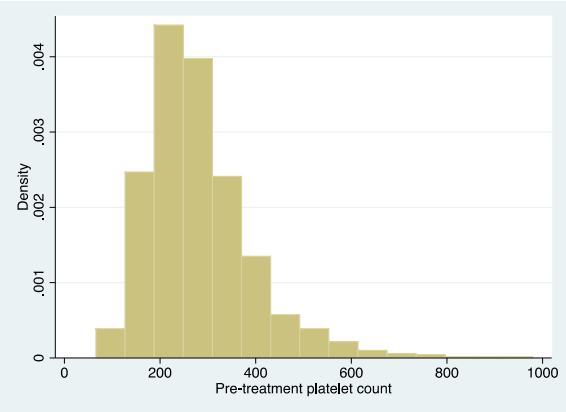

Figure 2-19 Distribution of baseline platelet values within the PICCOLO trial

Using the pre-defined cut-point, 142/1157 (12.3%) patients were assigned to the 'high platelet' group (>400,000), and 1015/1157 (87.7%) to the 'low platelet' group. The high platelet group had a higher proportion of patients with their primary tumour in situ, high dNLR and of female sex, than the low platelet group (table 2.10).

2.5.1.3.PTL population distribution and characteristics

In PICCOLO, PTL data was available for 1179/1196 (98.5%) patients. In total, 362 (30.7%) of patients had a right PTL, and 817 (69.3%) had a left PTL. 441 patients had a rectal tumour (37.4%) and 376 (31.9%) a left colonic tumour.

The right PTL group had a higher proportion of females, *BRAF* mutated tumours, primary tumours resected and fewer patients with previous clinical benefit with chemotherapy than the left PTL population (table 2.10).

		dNLR population n=1144			platelets population n = 1157			PTL study population n =1179	
		dNLR ≥2.00	**dNLR <2**		**Plts ≥400**	**Plts <400**		**Right PTL**	**Left PTL**
No of patients		551 (48.2%)	593 (41.8%)		142 (12.3%)	1015 (87.7%)		362 (30.7%)	817 (69.3%)
Median age		62	63		61.8 (55.1-69.7	62.9 (56.9-70.3)		63.8 (57.6-70.3)	63.8 (56.6-70.3)
PS≥2 (n=1185)	0-1	495(90.4%)	562 (95.7%)		120 (85.1%)	948 (94.3%)		332 (92.5%)	753 (93.1%)
	2	52 (9.6%)	25 (4.3%)		21 (14.9%)	57 (5.7%)		27 (7.5%)	56 (6.9%)
Gender (n=1190)	Male	370 (67.5%)	409 (69.3%)		79 (56.0%)	709 (70.2%)		216 (60.0%)	584 (71.9%)
	Female	178 (32.5%)	181(30.7%)		62 (44.0%)	301 (29.8%)		144 (40.0%)	228 (28.1%)
Resected primary (n=1195)	Yes	378(68.6%)	451 (76.1%)		83 (58.5%)	755 (74.4%)		291 (80.4%)	559 (68.4%)
	No	173 (31.4%)	142 (23.9%)		59 (41.5%)	260 (25.6%)		71 (19.6%)	258 (31.6%)
>2 metastatic sites (n=1149)	Yes	388 (73.2%)	368 (64.0%)		96 (69.6%)	669 (68.3%)		236 (67.2%)	546 (69.6%)
	No	142(26.8%)	207 (36.0%)		42 (30.4%)	310 (31.7%)		115 (32.8%)	238 (30.4%)
Primary tumour location (n=1179)	Right	160 (29.3%)	187 (32.1%)		46 (32.9%)	303 (30.2%)		n/a	n/a
	Left	386 (70.7%)	396 (67.9%)		94 (67.1%)	699 (69.9%)		n/a	n/a
dNLR ≥ 2.00(n=1144)	Yes	n/a	n/a		98 (69.5%)	454 (44.9%)		160 (46.0%)	387 (48.5%)
	No	n/a	n/a		43 (30.5%)	550 (55.1%)		187 (54.0%)	396 (51.5%)
Platelets ≥400 (n=1157)	Yes	98(17.8%)	43 (7.3%)		n/a	n/a		46 (13.2%)	94 (11.8%)
	No	453 (82.2%)	550 (92.7%)		n/a	n/a		303 (86.8%)	699 (88.2%)
Alk phos ≥300 (n=1148)	Yes	176 (15%)	169 (18.9%)		42 (30.0%)	159 (15.8%)		61 (17.6%)	138 (17.5%)
	No	551 (47.7%)	446 (50.1%)		98 (70.0%)	848 (84.2%)		285 (82.4%)	649 (82.8%)
BRAF mutation (n=1068)	Mutant	43 (8.8%)	42 (7.9%)		13 (10.6%)	73 (8.1%)		51 (15.7%)	38 (5.2%)
	Wild-type	447 (91.2%)	486 (92.1%)		110 (89.4%)	834 (91.9%)		274 (84.3%)	689 (94.8%)
RAS mutation (n=969)	Mutant	227 (50.7%)	242 (50.7%)		64 (55.2%)	411 (50.1%)		165 (55.2%)	320 (48.9%)
	Wild-type	221 (49.3%)	235 (49.3%)		52 (44.8%)	409 (49.9%)		134 (44.8%)	335 (51.1%)
Prev clinical benefit (n=2028)	Yes	230 (20.0%)	92 (10.4%)		84 (66.1%)	655 (70.7%)		202 (61.8%)	543 (72.9%)
	No	919 (80.0%)	795 (89.6%)		43 (33.9%)	272 (29.3%)		125 (38.2%)	202 (27.1%)

Table 2-10 Patient characteristics by dNLR status, then by platelet status, then by PTL status

2.5.2. Prognostic Analysis of Clinical Biomarkers

The prognostic analyses for all tested biomarkers are presented in table 2.11. Only patients treated with irinotecan alone are included in this analysis.

	High vs low dNLR		High vs low platelets		R vs L PTL
OS HR	n = 487 (fail =451)		n = 494 (fail = 458)		n=500 (fail =465)
	1.53(1.27-1.84), p<0.001		1.66 (1.27-2.18), p<0.001		1.10 (0.91-1.34), p=0.34
PFS HR	n=484 (fail = 432)		n=491 (fail = 439)		n=495 (fail= 446)
	1.34 (1.12-1.63), p=0.002		1.37 (1.04-1.81), p=0.027		1.08 (0.88-1.31), p=0.45

Table 2-11 Prognostic analysis for the effect of the dNLR binary classifier, the binary platelet classifier, and PTL on overall survival and progression free survival.

2.5.2.1.Prognostic effect of dNLR

A negative prognostic effect of high versus low dNLR status was seen for OS (7.7 vs 12.4mths; HR=1.53, [95%CI 1.27-1.84], p<0.001) and PFS (2.9 vs 5.4 mths; HR=1.34 [1.12-1.63], p=0.002) (table 2.11 and fig. 2.20).

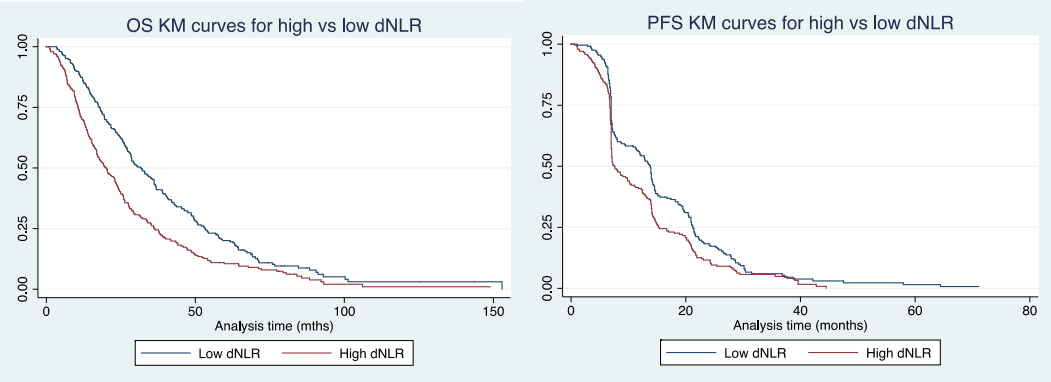

Figure 2-20 a) OS KM curves, and b) PFS KM curves for high vs. low dNLR in PICCOLO

When assessing dNLR as a continuous variable, increasing dNLR levels were associated with inferior OS (HR = 1.12 [1.07-1.17], p<0.001) and PFS (HR=1.17[1.06-1.19], p<0.001).

2.5.2.2.Prognostic effect of platelets

Using the primary dichotomous variable, high platelets was associated with inferior OS (6.5 vs 10.4mths; HR = 1.66 [1.27-2.18], p<0.001) and PFS (2.8 vs 4.7 mths; HR = 1.37[1.04-1.81], p=0.027) than low platelets (table 2.11 and fig. 2.20).

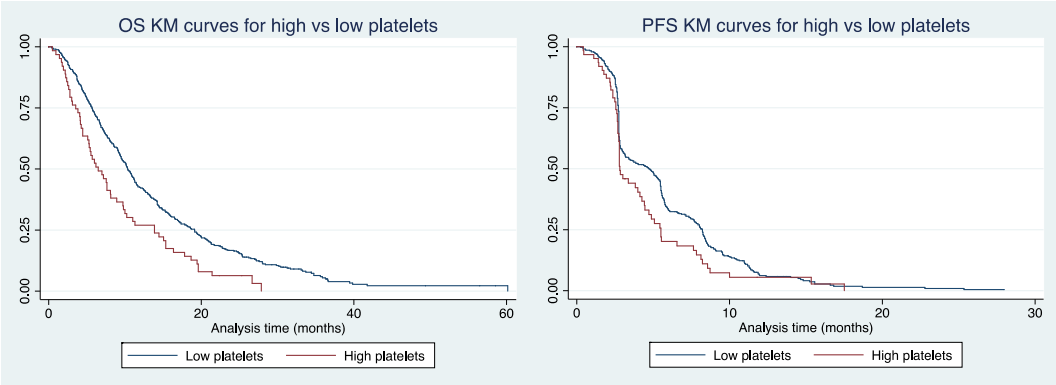

Figure 2-21 a) OS KM curves, and b) PFS KM curves for high vs. low platelets in PICCOLO

As a continuous variable, increasing platelets count was also a negative prognostic marker for OS (HR = 1.001, p<0.001) and PFS (HR = 1.003, p<0.001).

2.5.2.3.Prognostic effect of PTL

Right sided PTL was not associated with either shorter OS (8.5 vs 10.3 mths; HR=1.10[0.91-1.34],p=0.35), or PFS (3.2 vs 4.4 mths; HR = 1.08 [0.96-1.22], p=0.20) than left PTL (table 2.11 and fig. 2.22).

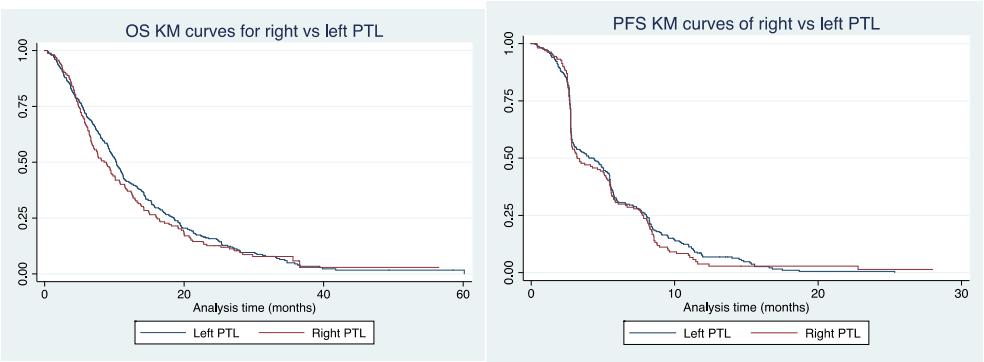

Figure 2-22 a) OS KM curves, and b) PFS KM curves for right vs. left PTL in PICCOLO

Compared to rectal tumours, left colonic tumours trended towards improved OS (10.6 vs 9.7 mths; HR = 0.80[0.64-1.01], p=0.061), but not PFS (3.9 vs 4.1 mths; HR = 1.01 [0.82-1.30), p=0.76)(data not shown).

2.5.2.4.Multivariate analysis of prognostic factors within the PICCOLO trial

dNLR and platelets were significant prognostic factors for patients treated with Ir alone, following progression on 5FU. PTL was not significantly prognostic in the 2nd line.

Other prognostic factors within PICCOLO are shown in table 2.12. Other significant factors were primary tumour in-situ, poor performance status, *BRAF* or *KRAS* mutation status and raised alkaline phosphatase.

Prognostic Factor	Groups	OS HR (95% CI)	PFS HR (95% CI)
Primary in situ	143/511 (27.9%)	1.56 (1.36-1.76),p<0.001	1.43 (1.25-1.63) p<0.001
Right PTL	162/500 (32.4%)	1.10 (0.91-1.34) p=0.37	1.08 (0.96-1.22) p=0.45
≥2 metastatic sites	340/488 (69.6%)	1.36 (1.11-1.67), p=0.003	1.31 (1.07-1.61), p=0.010
No benefit with 1st line chemotherapy	141/463 (30.4%)	1.16 (0.93-1.41), p=0.19	1.15 (0.93-1.42), p=0.18
PS ≥2	32/504 (6.3%)	2.41 (1.68-3.5), p<0.001	2.04 (1.41-2.95), p<0.001
RAS-mut	209/424 (49.0%)	1.24 (1.01-1.51), p=0.035	1.11 (0.91-1.36) p=0.30
BRAF-mut	40/459 (8.7%)	1.17 (0.84-1.64), p=0.34	1.06 (0.76-1.49), p=0.72
dNLR ≥2.00	242/488 (49.6%)	1.54 (1.28-1.85) p<0.001	1.35 (1.12-1.64) p=0.002
Plts ≥400	62/488 (12.7%)	1.66 (1.27-2.18), p<0.001	1.37 (1.04-1.81),p=0.027
Alk phos ≥300	100/488 (20.5%)	2.32 (1.85-2.91), p<0.001	1.70 (1.36-2.14), p<0.001

Table 2-12 Univariate analysis of prognostic markers in PICCOLO

A multivariate analysis was performed, including only factors significant at univariate analysis. In this analysis dNLR (adj HR = 1.35, p=0.006), high high alkaline phosphatase (adj HR = 1.89, p<0.001) and poor performance status (adj HR = 2.74, p<0.001) were independently prognostic for OS.

For PFS, following adjustment high dNLR (adj HR = 1.23, p=0.033), alkaline phosphatase (adj HR = 1.45, p=0.003), and poor performance status (adj HR 1.66, p=0.013) continued to be independent prognostic markers.

2.5.3. Clinical Biomarkers as predictive markers for panitumumab in *KRAS*-wt aCRC

The predictive analysis in PICCOLO shall test whether the clinical biomarkers are predictive of benefit of the addition of panitumumab to irinotecan (limited to the IrPan vs Ir randomisation primary population) (fig. 2.2). Results are shown in table 2.13.

2.5.3.1.Predictive effect of dNLR

Of the 466 patients in the PICCOLO primary analysis, 465 had baseline dNLR data available. dNLR was not a useful predictive marker for panitumumab benefit (OS interaction p= 0.20; PFS interaction p=0.09)(table 2.13).

2.5.3.2.Predictive effect of platelets

High vs. low platelets was not a useful predictive marker for panitumumab benefit (OS interaction p = 0.90; PFS interaction p = 0.40)(table 2.13).

2.5.3.3.Predictive effect of PTL

Given the strong relationship between right-PTL and *BRAF*-mut status, the predictive analysis for PTL was performed in the *KRAS* and *BRAF*-wt population. Right-PTL was not a useful biomarker for panitumumab effect (OS interaction p=0.72; PFS interaction p=0.89)(table 2.13).

When considering rectal vs left sided tumours, PTL was not a useful biomarker for panitumumab benefit (OS interaction p = 0.83; PFS interaction p=0.19)(data not shown).

	All patients		dNLR study population				Platelets study population				PTL study population**		
			High dNLR	Low dNLR			High plts	Low Plts			Right PTL	Left PTL	
Treatment strategy	**Unadjusted HR (95% CI)**	**Int p-value ***	**Unadjusted HR (95% CI)**	**Unadjusted HR (95% CI)**	**Int p-value ***		**Unadjusted HR**	**Unadjusted HR**	**Int p-value ***		**Unadjusted HR**	**Unadjusted HR**	**Int p-value ***
OS HR for IrPan vs Ir	311 events n =458	0.41	203/221	200/ 222	0.73		27/43	349/397	0.90		80/88	245/287	0.72
	0.91 (0.73-1.14)		1.03 (0.79-1.37),p=0.79	0.95 (0.72-1.25),p=0.71			1.01(0.47-2.16) p=0.98	0.89(0.71-1.14) p=0.38			0.87(0.55-1.36) p=0.53	0.96(0.75-1.23)p=0.74	
PFS HR for IrPan vs Ir	398 events n=450	0.010	191/218	196/221	0.98		39/43	349/397	0.40		83/86	261/287	0.89
	0.76 (0.63-0.94)		0.78(0.57-1.02),p=0.06	0.75 (0.57-0.99),p=0.04			0.95(0.49-1.83) p=0.88	0.74 (0.59-0.92),p=0.006			0.70(0.56-0.87)p=0.002	0.67(0.51-0.87)p=0.002	

Table 2-13 Estimated crude HRs and 95% CIs for the effect of IrPan vs. Ir in the high vs. low dNLR populations, the high vs. low platelet populations, then right vs. left PTL populations, including likelihood ratio tests for marker*treatment interactions.

***KRAS* and *BRAF*-wt population

2.5.4. Exploratory Analyses

2.5.4.1.Effect of alternative dNLR cut-points

When the dNLR cut-point was increased, the high dNLR group was associated with worse survival but with fewer patient numbers (table 2.14).

dNLR cut point	Pts assigned to high dNLR group (n=551)	OS HR	PFS HR
<2.00	0	1.00	1.00
2-2.5	158 (28.7%)	1.39 (1.15-1.69), p=0.001	1.34 (1.09-1.63), p=0.004
2.5-3.0	114 (20.7%)	1.49 (1.21-1.85), p<0.001	1.18 (0.95-1.47), p=0.16
3.0-3.5	54 (9.8%)	1.44 (1.08-1.93), p=0.014	1.43 (1.06-1.92),p=0.019
3.5-4.0	40 (7.3%)	2.59 (1.85-3.62), p<0.001	1.94 (1.37-2.74), p<0.001
4.0-4.5	13 (2.4%)	1.35 (0.75-2.41), p=0.31	1.55 (0.86-2.74), p=0.15
>4.5	41 (7.4%)	2.92 (2.10-4.08), p<0.001	2.43 (1.73-3.39), p<0.001

Table 2-14 Assessing alternative dNLR cut-point ranges vs. dNLR low (<2) on OS and PFS in PICCOLO

2.5.4.2.Combining baseline dNLR and platelets information

Again an 'either dNLR or platelets high' vs a 'neither high' model was investigated. Using these criteria, 922 patients were assigned to the 'high' group (79.9%) and 231 (20.3%) to the 'low' group.

'Either high' dNLR or platelets at baseline was associated with inferior OS (HR = 1.59[1.37-1.86], p<0.001) and PFS (HR = 1.36 [1.17-1.59], p<0.001), compared to patients who were high for neither. This model was not predictive for IrPan benefit (data not shown).

Next the effect of both baseline dNLR and platelets being high, compared with the rest of the population was tested. Only 135 patients (11.7%) were assigned to the 'high' group and 1018 (88.3%) to the 'low' group.

The ' both high' grouping was associated with reduced OS (HR = 1.63 [1.36-1.96], p<0.001) and PFS (HR = 1.27 [1.05-1.54], p=0.012), compared to the rest of the population.

2.5.4.3.The dNLR as a tumour marker during chemotherapy

dNLR data was available for 804 patients at cycle 4. Median dNLR at cycle 4 was 1.41, compared with median dNLR 2.0 at baseline. High dNLR at cycle 4 was associated with poor OS (HR = 1.69 [1.43-2.01], p<0.001) and PFS (HR = 1.52[1.28-1.82],p<0.001), compared with a low dNLR.

Patients who had a baseline high dNLR had a median 33.3% drop in dNLR; those with a baseline low dNLR had a median drop of 18.1%.

In the baseline high dNLR population, those who had a response at 12 weeks had a greater decrease in dNLR than those with stable or progressive disease (p=0.015). Those with clinical benefit had a greater decrease in dNLR at mid-point than those with progressive disease at 12 weeks (p=0.041) (figure 2.23).

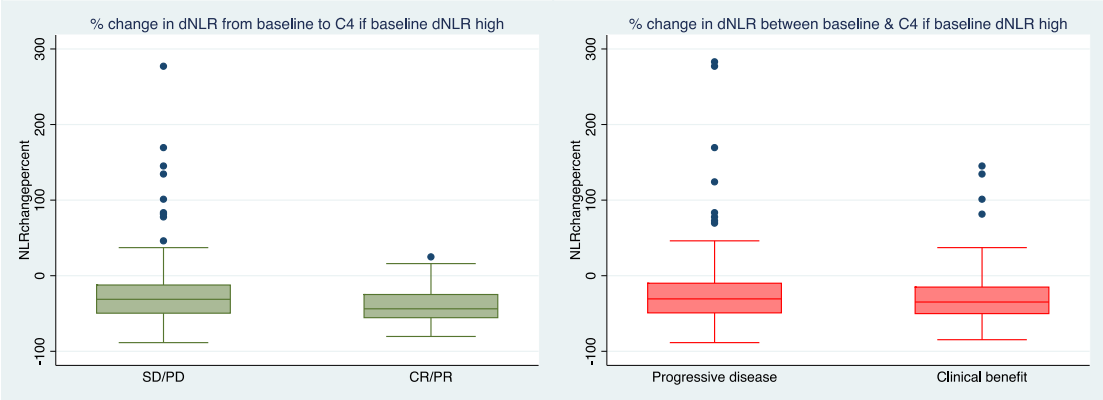

Figure 2-23 Boxplots showing the relationship between percentage change in dNLR from baseline to mid-point and 12 week RECIST response in patients with a high baseline dNLR; a)% change in dNLR in patients with response vs. stable or progressive disease, and b) % change in the dNLR in patients with progressive disease vs. clinical benefit.

For patients in the baseline low dNLR group, there was no significant difference in change dNLR during treatment between responders and those with stable or progressive disease at 12 weeks (p=0.12). Those with clinical benefit had a greater decrease in dNLR at mid-point than those with progressive disease at 12 weeks (p=0.002) (fig. 2.24).

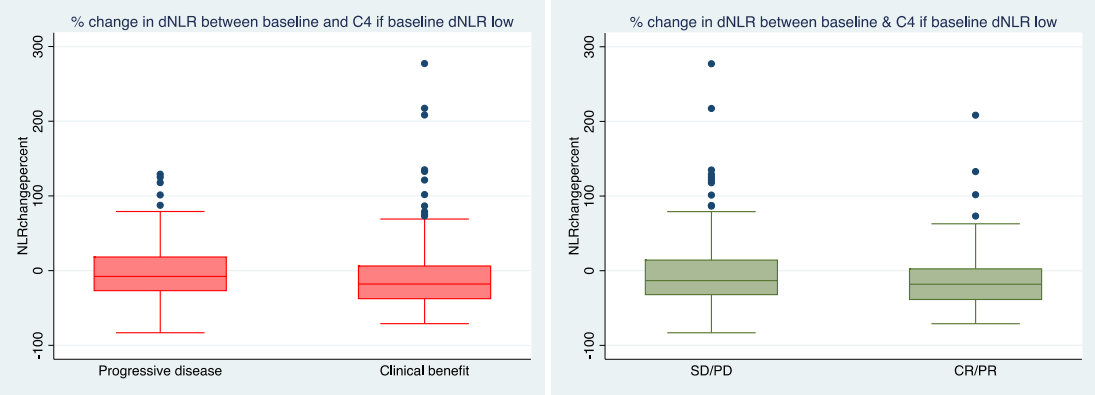

Figure 2-24 Boxplots showing the relationship between % change in dNLR from baseline to mid-point and 12 week RECIST response in patients with a low baseline dNLR: a) % change in those with response vs. stable or progressive disease, and b) % change in those with progressive disease vs. clinical benefit.

As in FOCUS, a pre-defined cut-point was used to assign patients to 3 'NLR-dynamic' groups:

- Improved dNLR (>25% decrease in dNLR from baseline to pre-cycle 4),
- Stable dNLR (<25% decrease and <25% increase from baseline to pre-cycle 4)
- Worse dNLR (>25% increase in dNLR from baseline to pre-cycle 4)

Outcomes were compared between falling and stable or worsening dNLR (improved vs. stable or worse), and falling or stable dNLR and rising dNLR (improved and stable vs. worse dNLR), for each of the baseline dNLR groups.

The 'NLR-dynamic' grouping as a monitoring tool was more useful in those with a baseline high dNLR than a baseline low dNLR; changes in the dNLR in the baseline low dNLR group were not useful for predicting 12 week RECIST response (table 2.15).

Similar to FOCUS, the most useful application was identifying patients most at risk of progression at 12 weeks. Patients with a high baseline dNLR whose dNLR continued to rise during chemotherapy were at most risk of progression. None of these patients had a 12-week RECIST response, and they had a much lower chance of stable disease (OR = 0.33, p=0.018). Conversely those with improving or stable dNLR at the midpoint had similar rates of response and disease control than patients with a low baseline dNLR (table 2.15).

Baseline dNLR low group: effect of changing dNLR during treatment				
NLR kinetic category (n=454)	**12 wk CR/PR OR (95%CI)**	**Clinical Benefit OR (95%CI)**	**PFS HR**	**OS HR**
Improved dNLR n = 171 (37.6%)	RR = 22.2%	DCR = 76.2%	0.97 (0.79-1.19) p=0.80	0.96 (0.78-1.17),p=0.68
	1.51(0.93-2.44), p=0.093	OR = 1.86 (1.22-2.83) p=0.004		
Stable or improved dNLR n =383 (84.4%)	RR = 19.3%	DCR = 69.2%	0.84 (0.64-1.12) p=0.24	0.78 (0.60-1.02) p=0.073
	1.64 (0.78-3.47), p=0.19	1.56 (0.93-2.63) p=0.090		
dNLR rise n = 71 (15.6%)	RR = 12.6%	DCR = 59.4%	1.18 (0.83-1.56), p=0.24	1.27 (0.98-1.65), p=0.073
	0.61 (0.29-1.27) p=0.19	0.63 (0.38-1.07), p=0.090		
Baseline dNLR high group: effect of changing dNLR during treatment				
NLR kinetic category (n=337)	**12 wk CR/PR OR (95%CI)**	**Clinical Benefit OR (95%CI)**	**PFS HR**	**OS HR**
Improved dNLR n = 208 (61.7%)	RR = 18.7%	DCR = 63.4%	0.81 (0.64-1.01) p=0.070	0.94 (0.72-1.22),p=0.67
	1.89 (0.98-3.65), p=0.056	OR = 1.29 (0.82-2.02) p=0.26		
Stable or improved dNLR n =315 (93.4%)	RR = 16.8%	DCR =62.9%	0.48 (0.31-0.76) p=0.002	0.73 (0.46-1.17) p=0.19
	n/a	2.96 (1.21-7.27) p=0.018		
dNLR rise n = 22 (6.6%)	RR = 0%	DCR = 36.3%	2.06 (1.31-3.22), p=0.002	1.35 (0.85-2.15), p=0.19
	n/a	0.33 (0.14-0.83), p=0.018		

Table 2-15 Treatment outcomes (OR for 12 week response; OR for clinical benefit; HR for PFS; HR for OS), depending upon NLR kinetic categories in PICCOLO.

Similar to the FOCUS analysis, change in NLR during therapy has a low sensitivity for detecting progression (16.9%), but a high specificity (89.7%).

ROC curves for changes in NLR for predicting clinical benefit (AUC=0.56) and 12 week response (AUC=0.55) as shown in figure 2.25.

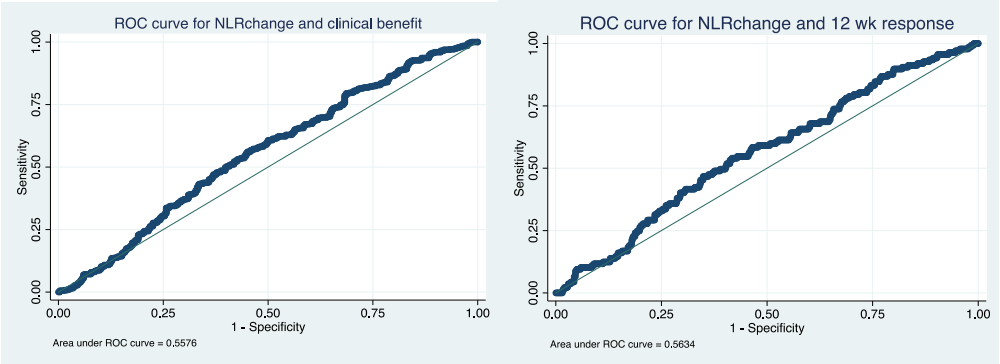

Figure 2-25 ROC curves for dNLR change for the prediction of a)12 week clinical benefit, and b) 12 week RECIST response

2.6. Discussion

In two large RCTs the routine clinical parameters tested provided useful information to aid patient assessment and management at different points of the treatment pathway in aCRC. In particular the dNLR and platelets, markers of the systemic inflammatory response, were strong prognosticators in both the first and second line, and may have value in monitoring response to treatment. These markers also identified low risk patients not disadvantaged by a less intensive first line treatment. PTL was a less useful biomarker in both clinical scenarios.

For previously untreated aCRC patients high dNLR, high platelet count and right PTL were poor prognostic markers for OS. At multivariate testing, only dNLR and platelets retained statistical significance. For PFS, dNLR and platelets were significantly prognostic (at univariate and multivariate testing); PTL was not.

For patients commencing second-line irinotecan in PICCOLO, having progressed on 5FU, dNLR and platelets were poor prognostic markers for OS and PFS, but only dNLR remained significant at multivariate testing. PTL was not a useful prognostic marker in the second line.

This analysis has tested candidate markers alongside available parameters identified by the Kohne(45) and GERCOR(152) scores, and additionally tumour *KRAS* and *BRAF* status, to ensure relevance for modern oncology practice. The strength of effect of each prognostic marker was assessed in both univariate and multivariate models. Using this approach, important prognostic variables in the first-line were (in order of OS adjusted HR), raised alkaline phosphatase, *BRAF*-mut status, platelets ≥400, high dNLR ≥2, 2 or more metastatic sites and poor performance status. The two strongest prognostic categories included only a small proportion of patients (high alkaline phosphatase 15.7%; *BRAF*-mut 7.6%). For prediction of poor PFS, high alkaline phosphatase, high platelets, high dNLR, 2 or more metastatic sites and primary tumour in-situ remained significant.

These findings are in keeping with information from the Kohne and GERCOR scores, and with reports of *BRAF* mutation and alkaline phosphatase as poor prognostic markers in aCRC.(329, 330) The mechanisms of the poor outlook of *BRAF*-mutated aCRC will be discussed in Chapter 3. A high alkaline phosphatase level is likely to represent a high burden of liver metastatic disease with increased liver mass, potentially with a degree of intrahepatic or biliary obstruction.(330)

A limitation of this prognostic model is that it didn't include LDH, identified as a strong marker in both databases. The FOCUS dataset did not find a strong prognostic role for PTL, differing from previous work.(325) Given the strength of evidence of the dNLR and platelet count, a modern prognostic marker panel for chemotherapy-naïve patients should additionally include dNLR, platelets and *BRAF*-mutation status.

The median dNLR and platelets were lower in second-line than first-line patients, and correspondingly a lower proportion of patients were assigned to the high dNLR or platelet category. Given the poor outlook of these markers in first-line therapy, fewer patients with an active systemic inflammatory response may be deemed eligible for further chemotherapy, or will have died during initial treatment. Alternatively the inflammatory response may be most active at the point of diagnosis of advanced disease, and exposure to chemotherapy will have dampened this. This highlights differences between a chemotherapy-naïve population and those fit enough for further treatment. It is likely that the first-line prognostic panel may not be as relevant in this group and a more personalised approach may be appropriate.

For patients commencing second-line chemotherapy, significant prognostic markers OS and PFS in the multivariate model were poor PS, raised alkaline phosphatase and dNLR:

patient characteristics provided more prognostic information than tumour factors. Careful evaluation of patient condition is therefore critical in this setting as those with poor PS, high alkaline phosphatase or dNLR had considerably less PFS and OS benefit.

There is less data providing details of prognostic factors for patients who have failed first-line chemotherapy. In a study examining irinotecan vs. BSC, poor PS, recent weight loss, two or more metastatic sites, low Hb and the presence of liver metastases were significant in multivariate testing.(110) In a second-line trial comparing irinotecan vs. FU, following FU failure PS greater than 1, right PTL, low Hb, low WBC and high Alk phos were associated with poor outcomes.(154)

The evaluated clinical models could not be recommended as predictive biomarkers for the FOCUS or PICCOLO treatments. In FOCUS, patients with a low dNLR had no OS advantage from upfront combination chemotherapy compared with FU alone; patients with a high dNLR did. However dNLR/ treatment interaction was negative so dNLR cannot be recommended as a biomarker for this clinical decision. High dNLR patients had as much chance of responding to first-line combination chemotherapy than low dNLR patients, but had lesser chance with single agent FU. These findings stress that this group benefits from intensive first-line therapy, and challenges previous reports that high dNLR patients have less benefit from chemotherapy.(305, 306)

In COIN the only group to be significantly disadvantaged by intermittent compared continuous first-line chemotherapy were patients with high baseline platelets,(102) however in FOCUS, high platelets did not identify patients benefitting more from intensive chemotherapy.

PTL was not a useful predictive biomarker for any treatment strategy. Right PTL has proposed as a negative predictive marker for anti-EGFR therapies.(155, 324) In PICCOLO, the first randomised assessment of PTL as a predictive marker for anti-EGFR agents, right PTL was not discriminative in either the *KRAS*-wt or the *KRAS*-*BRAF*-wt population: patients with *BRAF*-wt right-sided tumours gained PFS benefit with IrPan compared with Ir alone. These results would not be supportive of clinicians using PTL to guide treatment decisions.

Further exploratory analyses were performed to optimise the biomarker models and investigate dynamic changes in the dNLR during therapy. Firstly the prognostic utility of each component of the FBC was tested to investigate whether neutrophils alone, or

lymphocytes alone, or the ratio was more important. In this model, the dNLR was the strongest marker. This may be a simple representation of shift from a TH2 to a TH1 response, with promotion of neutrophils with corresponding decrease in lymphocytes: this pattern is typically seen in cancer and is associated with worse prognosis.(259)

The dNLR is a continuous variable with smooth distribution and no natural dichotomisation point, but a binary cut-point is more practical for clinical application. This study prospectively defined the cut-point at 2.00, as validated in the Proctor's study.(286) Proctor's paper tested the dNLR in the Scottish Cancer Registry population, including patients with different cancer sites and at different stages. The dNLR measurement was sampled up to 2 years following cancer diagnosis. There are therefore differences between this population and the aCRC population studied in FOCUS so an alternative cut-point may be more appropriate for the aCRC population.

As a continuous marker, rising dNLR was associated with increasingly poor OS so the prognostic effects of alternative cut-points were assessed to identify a more informative cut-point. Progressive rises in the dichotomisation point corresponded with increasing HRs for OS and PFS, compared to the low dNLR population. In FOCUS when a cut-point range of 3.5-4.0 was utilised, the dNLR was a stronger prognostic marker for OS, than the predefined cut-point of 2 (OS HRs of 2.03 vs 1.56, respectively). With this model, fewer patients were assigned to the 'high' group than in the pre-defined model (15.5% vs 56.4%). With a cut-point of 3.5, the dNLR was the strongest prognostic marker for patients commencing first-line chemotherapy, identifying those patients at increased risk. This cut-point range was most associated with increased risk in PICCOLO also.

The optimal dichotomisation point will also reflect the degree of risk that clinicians want to observe: a dNLR cut-point of 3.5 identifies patients at most risk of poor outcomes independent of treatment, rather than moderate risk utilising a lower value. Using this value, the dNLR would be a useful clinical trial stratification factor, identifying a high risk population but having no utility as a predictive marker for any tested treatment strategies.

Platelets and dNLR both had independent prognostic effect for OS and PFS in FOCUS so the use of a clinically usable single combined model was evaluated. Although both may reflect an underlying inflammatory response, different processes may be being captured. An 'either high' vs. a 'neither high' combined dNLR/ platelet model was firstly tested which assigned a high proportion of patients to the 'high' group in both FOCUS (62.5%) and

PICCOLO (79.9%). In both trials this model was a stronger prognostic marker than dNLR alone, and in PICCOLO more discriminative than either marker alone.

'Either high' patients had more PFS benefit from upfront intensive chemotherapy than FU alone, than the 'neither high' (interaction p=0.041). Although lesser effect was seen for OS, 'neither high' patients had no survival advantage with upfront combination chemotherapy. The 'either vs neither' dNLR/platelet model appears to identify a low risk group of patients with a more indolent course who have no survival detriment with a more conservative upfront treatment approach.

Using a model testing the effect of both dNLR and platelets raised, only a small number are assigned to the 'high' group (FOCUS 18.3%; PICCOLO 11.7%) but they had markedly inferior OS and PFS. This model was not a useful predictor for any treatment strategy tested.

Which inflammation score should then be recommended for incorporation into clinical practice? The dNLR and platelets have independent prognostic effects, both when tested in the primary binary model, and in the continuous model. However only dNLR was a significant prognostic marker for both OS and PFS in PICCOLO. For the identification of higher risk patients a dNLR of greater than 3.5 was most discriminative. These patients require upfront intensive treatment and were shown to have less benefit from a gentler approach.

Instead, for the identification of low risk patients the 'either vs neither' model is recommended: patients with both low platelets and low dNLR had a more indolent course and less benefit from intensive first-line chemotherapy. This may aid other routine clinical decisions: a patient and clinician may be more comfortable with an initial expectant approach, gentler upfront chemotherapy and treatment holidays. These approaches could have quality of life benefits for some patients. Additionally, a MDT may be more willing to consider a patient with borderline resectable metastatic disease for surgery if they otherwise in a good prognostic category. Although systemic inflammatory scores are considered alongside other prognostic markers, in both trials host immune markers performed better than tumour specific factors in joint models. This information therefore should be incorporated into the routine assessment of aCRC patients being considered for chemotherapy and into clinical trial stratification, particularly for post first-line studies where host factors were more important than tumour factors.

Comparisons with other inflammatory scores were not possible. Retrospective analyses of RCT datasets are limited by the variables collected, and therefore none of lymphocytes, albumin or CRP data is available. Therefore this study can only advise on application of the dNLR. Importantly this study demonstrates the usefulness of scores of the systemic inflammatory response in clinical practice and this model could be optimised in further datasets.

As blood tests are performed routinely throughout chemotherapy, the dNLR was tested as a tumour marker to identify patients failing midway through treatment. In both trials median dNLR was lower at treatment mid-point than baseline, suggesting chemotherapy acts on the peripheral inflammatory response, additional to its anti-tumour activity. It was hypothesised that a falling dNLR during treatment would correlate with tumour response, as assessed by RECIST at 12 weeks. It was also hypothesised that different dynamic effects would be seen in the baseline dNLR high and low patients, so their data was analysed separately.

In both trials patients with a baseline high dNLR had a greater fall in dNLR during treatment, and the "dNLR dynamic' model was more useful in these patients. In FOCUS stable or falling dNLR mid-way through treatment was associated with an increased chance of response and clinical benefit. Conversely, an increasing dNLR at mid-point was significantly associated with progressive disease at 12 week radiological assessment. In both trials patients with a high baseline dNLR, whose dNLR continued to rise were at high risk of progression during chemotherapy. A rising dNLR may identify failing patients requiring early radiological assessment and consideration of an alternative management plan. In this model, patients who have failed early will have been missed, as they wouldn't have had measurement of dNLR at cycle 4 or a 12-week scan. Therefore this model may underestimate the risk of rising dNLR during therapy.

A candidate monitoring tumour marker requires rigorous testing before clinical application: the ideal marker has 100% sensitivity and 100% specificity for predicting the desired clinical outcome, in this case the dNLR's ability to detect progressive disease at the 12 week assessment. ROC analysis in both trials would not be supportive of the dNLR as a useful monitoring biomarker. In further examination, the dNLR had a low sensitivity for detecting progression in both trials: therefore a clinician cannot presume that a rising dNLR necessarily means chemotherapy is failing. However in both trials specificity (FOCUS – 91.7%; PICCOLO 89.7%) was high, suggesting that if a patient has a low or

improving dNLR then it is unlikely that they are progressing. This is the only setting that the dNLR can be recommended as a monitoring tool.

Currently there is no 'gold standard' of tumour marker for monitoring advanced CRC. As with CEA, dNLR information should be taken into context with the overall clinical picture. A low dNLR may provide reassurance to continue treatment, but should be questioned if there are other features of progression. A high dNLR alone should not prompt an early CT scan, but it would provide supporting evidence alongside other suggestive clinical factors.

In this chapter the dNLR and platelet count are useful prognosticators in the first and second-line treatment of aCRC. Models using these markers can identify both lower risk patients for whom more conservative upfront treatment may be reasonable, and higher risk patients requiring a more intensive approach. Furthermore serial dNLR measurements during treatment may have utility as a monitoring tool.

This work is consistent with and builds upon findings of previous studies reporting that the systemic inflammatory response is important in the assessment and treatment of cancer. Further validation in RCT databases would be valuable to confirm its optimal utility in aCRC routine clinical practice.

2.7. Further work

As these biomarkers are routinely measured, testing in other clinical scenarios may reveal other clinical applications. Testing in RCT datasets of adjuvant chemotherapy would assess whether the dNLR helps in the risk stratification of early CRC; particularly in patients with lower stage tumours not routinely offered chemotherapy. Testing in trials of neo-adjuvant chemotherapy may show that patients with a high NLR benefit more from upfront systemic therapy than a low NLR population. These markers should be tested in other cancer sites, preferably in RCT datasets.

An Australian study is underway aiming to validate and quantify the prognostic value of the host inflammatory response for PFS and OS in bevacizumab treated aCRC patients.

The interaction between systemic inflammatory markers and immune modulating drugs would be of value. In particular, assessing the NLR in trials of immune modulating drugs in melanoma or lung cancer may be beneficial.

Although this study has shown trends between changes in the NLR and 12 week radiological response, the model lacked sensitivity. However there is little data supporting the strength of the CEA as a monitoring tool in aCRC: the sensitivity and specificity for detecting progression has only been reported in small series. A prospective study measuring CEA and all components of the inflammatory response measured in routine bloods tests throughout chemotherapy could compare each marker's ability to predict tumour response. Following this, each could be used as a monitoring tool to detect progressive disease off active treatment.

The dNLR and platelet models are thought to be surrogates for the systemic inflammatory response, and understanding the exact underlying immune mechanism underpinning this poor outlook is crucial to identify novel biomarkers and therapeutic targets.

One contributor may be activation of the IL-6/JAK/STAT3 pathway. IL-6 signalling inhibition led to suppression of colitis-associated carcinogenesis in mouse models and in colon cancer cell lines.(331) Current therapies targeting the IL-6/JAK/STAT3 pathway are in early testing. An IL-6 ligand-blocking antibody, (siltuximab) has been shown to be capable of blocking the signal transduction pathway, achieving anti-tumour and anti-inflammatory effect in metastatic renal cell carcinoma.(332) Another IL-6 directed mAb (Tocilizumab) has been reported to treat rheumatoid arthritis and preclinical data suggests efficacy in cancer.(333) Treatments inhibiting JAK/STAT signalling and *STAT3* are also in development.(276) The NLR may identify patients who benefit most from these therapies and should be considered the development of these agents.

To understand the underlying mechanisms, a local study is recruiting aCRC patients prior to commencing first-line chemotherapy. Differences in immune function testing between patients with a high NLR and low NLR will be investigated. Additionally changes in the immune panel during chemotherapy will be tested, and correlated with tumour response.

Blood tests are being collected before treatment, mid-way through chemotherapy and following completion of treatment. Samples are transferred immediately to the laboratory and immune function testing will be performed in batches. Additionally tumour formalin-fixed paraffin-embedded (FFPE) blocks will be collected and immune scoring on whole

sections will be performed and correlated with outcomes. The systemic and tumour immune profiles shall be compared. Biomarkers of interest will be prospectively tested in further datasets.

Challenges of this study are the high volume of blood required for testing (50ml per measurement), the need for immediate transfer to the laboratory and cost (approximately £2000 for 3 serial immune panel tests). Therefore any new biomarker identified would need to considerably add to the information already provided by the current inflammatory score

Chapter 3. Exploring the poor outcomes of *BRAF*-mutant advanced colorectal cancer

3.1. Introduction

As described mutations in *BRAF* confer a poor prognosis in aCRC. However the underlying causes for this phenomenon are unclear. This chapter shall explore this poor prognosis by examining detailed treatment outcomes of patients in FOCUS, COIN and PICCOLO.

3.1.1. Mutations in *BRAF*

The *BRAF* gene is part of the MAPK signalling pathway (Figure 3.1). *BRAF* produces a protein called B-Raf (serine/threonine-protein kinase B-Raf) Mutations in *BRAF* (*BRAF* – mut) are associated with poor outcomes in aCRC independent of treatment. However the mechanism of this poor outlook is unclear.

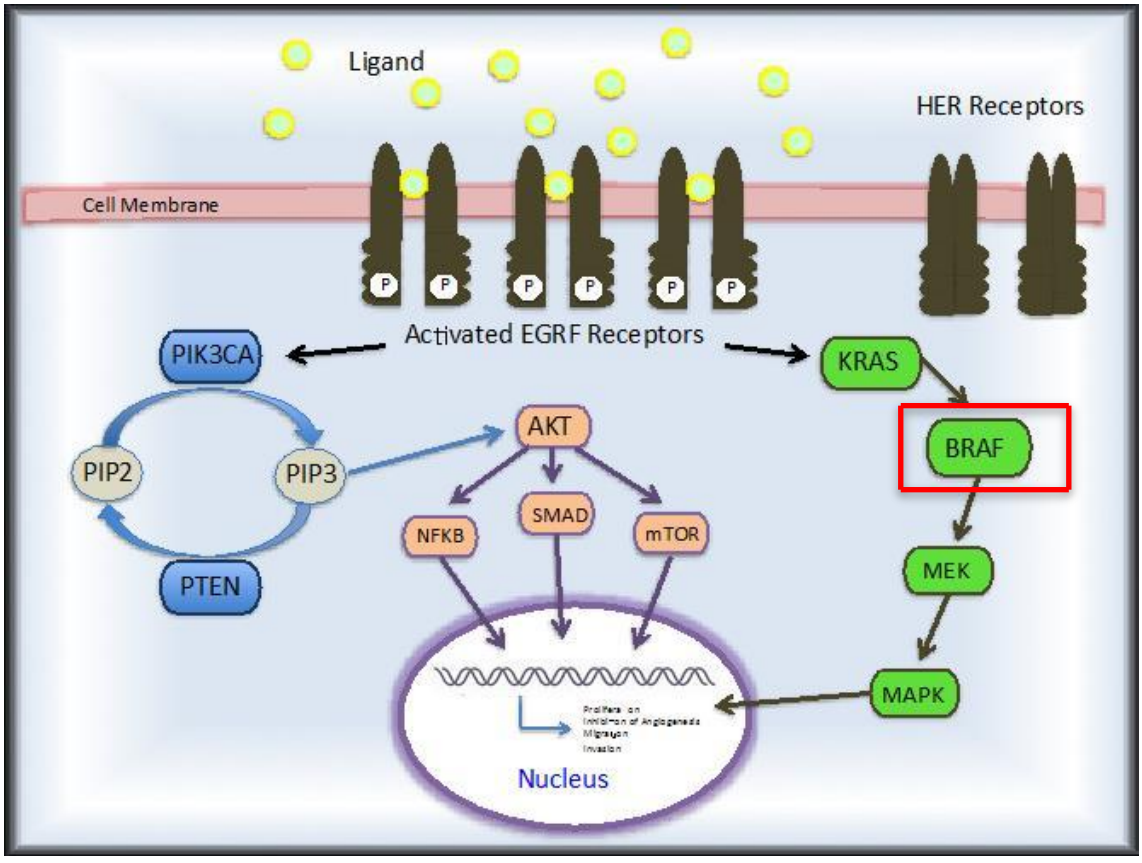

Figure 3-1 Simple representation of the MEK-AKT pathway, highlighting position of *BRAF*. Modified from (334)

3.1.2. The role of *BRAF* in the MAPK signalling pathway

Constitutive activation of the MAPK pathway is a common event in many cancers, leading to proliferative signalling.(249) In normal circumstances MAPK signalling is initiated by ligand binding to the EGFR, leading to receptor dimerisation (fig. 3.1). Subsequently phosphorylated adaptor proteins bind to and activate the *RAS* family of proteins.(335) Next, *RAS* proteins bind to and phosphorylates cytosolic *RAF* dimers,(336) of which there are 3 *RAF* isoforms that are serine/threonine kinases, *BRAF*, *C-RAF* and *A-RAF*. *RAF* activation leads to recruitment of *MEK* and *ERK* and scaffolding proteins to the cell membrane, leading to the phosphorylation of *MEK*, then *ERK* and subsequent nuclear activity.(337) *RAF* plays a critical role in this pathway and mutation provides opportunity for malignant cells to maintain continuous MAPK signalling.

Mutations in *BRAF* were first described by Davies in 2002(338) and are most commonly caused by a valine to glutamic acid change at codon 599 (V600E); mutations of *CRAF* are rare and *ARAF* have not been reported.(339) V600 mutation leads to a conformational change in the G-loop segment of *BRAF*, resulting in its constitutional activation (approximately 500 fold increase) and an ability to bind to *MEK* and *ERK* as a monomer.(337) A consequent increase in *ERK* phosphorylation leads to target gene transcription, resistant to normal negative feedback mechanisms.(340)

Mutations in *BRAF* have been shown to be present in many stages of cancer development and progression: mutations are seen in benign skin naevis(341) and in pre-cancerous colon polyps.(342) However oncogenic *BRAF* alone cannot induce progression to cancer.(342)

BRAF mutations have been described in several cancer types: melanoma, colorectal NSCLC, thyroid, hairy-cell leukaemia, gliomas and multiple myeloma,(343) stressing the importance of the MAPK pathways in oncogenesis across a wide spectrum of cancers. Consequently there has been intense effort to target *BRAF* with small molecules, with most success in metastatic melanoma.

3.1.3. *BRAF* mutations in metastatic melanoma

Mutations in *BRAF* occur in nearly half of all metastatic melanomas; the most common mutation is V600E, followed by V600K, V600D and L597R mutations.(344) Clinico-pathological differences are noted between V600E-mutated and non-V600E mutated patients: non-V600E mutations are associated with increasing age, male sex and chronically sun-exposed areas.(345) There is a decrease in the incidence in any *BRAF* mutation with increasing age: almost all patients under 30 possess a mutation compared with 25% of patients over 70.(345)

Previously the outlook for metastatic melanoma was dismal, but identification of the *BRAF* mutation led to improved pathological understanding of the disease, leading to new therapeutic opportunities. Following disappointing results of clinical trials of sorafanib,(346, 347) a phase I trial of the *BRAF*-enzyme inhibitor vemurafanib reported impressive response rates: of 32 patients treated, 24 experienced a PR, and 2 a complete response (CR).(348) The subsequent RCT of vemurafinib vs. decarbazine in *BRAF*-mut metastatic melanoma was stopped early due to reaching the pre-specified early stopping rule of improved OS and PFS,(224) leading to FDA approval of vemurafinib and the companion diagnostic test in August 2011. Another *BRAF*-inhibitor, dabrafenib, has shown efficacy as a single-agent and in combination with trametinib, a MEK-inhibitor.(349, 350) There is also evidence to support *MEK*-inhibition directly in metastatic melanoma, however in a phase II trial of trametinib overall responses were limited to those naïve of *BRAF*-inhibition,(351) suggesting that acquired resistance to *BRAF*-inhibitors impacts response to single-agent *MEK* inhibition.

Reactivation of the MAPK pathway on progression is a mechanism of resistance to *BRAF*-targeted therapy, with new mutations in *NRAS* and *MEK* identified.(352) Combining *BRAF* and *MEK* inhibition was a logical approach to delay the development of resistance: recent phase III trials in previously untreated *BRAF*-mut patients have demonstrated improvements in all clinical endpoints with the doublet when compared with vemurafenib alone.(353, 354) An ongoing phase II study is looking at this combination for patients with surgically resected high risk V600 mutated melanomas. This molecular understanding of acquired resistance has been possible by studies collecting paired biopsies: prior to treatment and following progression.

3.1.4. *BRAF* mutations in other cancers

The personalisation of therapy for *BRAF*-mut patients is less developed in other cancers. In NSCLC, *BRAF* mutations are present in 1.6-4.9% of adenocarcinomas and of those half are V600 mutations.(343) Unlike melanoma there are no distinct clinico-pathological features and treatment outcomes with first-line platinum treatment compared are comparable with wild-types.(355) Ongoing trials are investigating the role of *BRAF*-inhibition in V600 (NCT01336634) and non-V600 mutated (NCT01514864) NSCLC; additionally the *MEK*1/2 inhibitor selumetinib is under investigation (NCT00888134).

3.1.5. *BRAF* mutations in colorectal cancer

An association between dMMR and *BRAF* mutations in early CRC was first reported by Rajagopalan, also noting that *BRAF* and *RAS* mutations were mutually exclusive.(342) The presence of a *BRAF* mutation in a dMMR tumour only occurs in sporadic CRC, and rules out HNPCC.(356) Instead sporadic tumours arising from MSI are highly associated with *BRAF*-mut and CIMP-positivity.(357, 358) Mutations are highly associated with certain clinic-pathological features: female sex, right PTL, and increasing age, (322) and have a characteristic gene expression profile.(85)

3.1.5.1.BRAF mutations in early stage colorectal cancer

In a molecular sub-study of PETACC-3 (RCT comparing biweekly infusional FU alone or with irinotecan for stage III CRC),(359) *BRAF*-mut occured in 7.9% of early CRCs; the incidence did not significantly vary by tumour stage.

Despite frequent co-existence, dMMR and *BRAF*-mut are associated with differing outcomes: in two large adjuvant trials in stage II/III CRC *BRAF*-mut was associated with poor OS,(322, 360) whereas dMMR instead conferred a favourable outlook.(361) However this was not reproduced in a large adjuvant trial in stage II CRC.(362) The worst prognostic group is the *BRAF*-mut/pMMR group.(322, 360, 363) Of note, *BRAF*-mutant status can still confer poor prognosis in the dMMR population.(363)

The underlying mechanism for this poor prognosis in early-stage cancer is not fully understood. There is, however, no convincing evidence that *BRAF*-mut patients have lesser benefit with adjuvant chemotherapy. In PETACC-3, *BRAF*-mut status was not associated with inferior recurrence free survival (RFS);(322) in QUASAR (RCT comparing adjuvant chemotherapy vs. observation in stage II CRC), *BRAF*-mut status did not predict for lack of

benefit with 5FU adjuvant chemotherapy;(362) and in CALBG 89803, there was a trend towards improved benefit from the addition of Ir to FU/LV chemotherapy in *BRAF*-mut patients.(360)

However, when they do recur *BRAF*-mut cancers display a distinct pattern of metastatic spread: more commonly associated with peritoneal and distant lymph node metastases, and less likely to have lung metastases and are associated with short survival.(364)

3.1.6. *BRAF* mutations in advanced colorectal cancer

Mutations in *BRAF* occur in 8-12% of patients with aCRC(343) and almost all harbour the V600 substitution.(156, 329) They are rarely associated with *RAS* or *PIK3CA* mutations.(156, 338)

Similar to early stage CRC, *BRAF*-mut aCRCs represent a distinct population with typical clinico-pathological features: older patients, proximal primary tumour of high grade, associated with dMMR status, have mucinous histology, and have an increased incidence of peritoneal and distant lymph node metastases, with fewer pulmonary metastases.(159, 364-367)

BRAF-mut aCRC is consistently associated with poor OS in case series(213, 367, 368) and RCTs.(97, 329) Pooled analysis of first-line aCRC trials reports inferior OS compared with *BRAF* wild-types (*BRAF*-wt) (11.4 vs 17.2 months, HR =1.91 $p<0.001$).(159) In a population-based study of 354 patients, following adjustment for prognostic factors, *BRAF*-effect was even more marked (OS HR=10.66, $p<0.001$).(364)

Of note, dMMR is associated with worse prognosis than pMMR in aCRC.(159, 364) Within the larger series, there was no significant interaction between dMMR and *BRAF* status and outcomes; the authors concluded that the poor prognosis of dMMR is driven by *BRAF*-mut status.(159)

BRAF-mut aCRC appears to represent a distinct population with a poor outlook. *BRAF* is therefore an attractive therapeutic target, particularly with successes seen in melanoma.

3.1.6.1.Targeting BRAF in advanced colorectal cancer

Vemurafanib was tested in a phase I study of 21 *BRAF*-mut aCRC patients: only one of 19 patients with evaluable response had a PR. The authors concluded that despite only modest clinical activity, *BRAF* could be a therapeutic target in aCRC.(369)

Preclinical work confirmed that *BRAF*-mutant CRC cell lines were less sensitive to vemurafenib than *BRAF*-mut melanoma.(370) Treatment of *BRAF*-mut CRC cell lines with vemurafenib led to initial suppression of pERK levels, but levels rose again within 24 hours suggesting reactivation of the MAPK pathway. High pEGFR levels were seen following vemurafenib treatment and subsequent inhibition of *EGFR* led to suppression of pERK levels by stopping activation of *RAS*-GTP and the subsequent phosphorylation of *CRAF*. The combination of *RAF*- and *EGFR* inhibition led to greater in vitro and in vivo efficacy compared with *RAF* inhibition alone. Increased levels of pEGFR have not been described in *BRAF*-mut melanoma following *BRAF*-inhibition.

Complementary work confirmed that *BRAF*-mut CRC cell lines were insensitive to single agent *BRAF*-inhibition until knockdown of EGFR. Subsequent clinical activity was observed with combination of vemurafenib with anti-EGFR agents; anti-EGFR agents alone had little anti-tumour effect.(371) The suggested mechanism was that combination therapy 'switched off' EGFR-dependent *Akt* activation.

Dual blockade of the RAF-MAPK pathway with vemurafenib plus a MEK inhibitor was efficacious in melanoma, with decreases in pERK levels strongly associated with tumour response.(372) Combining *BRAF* and *MEK* inhibitors may therefore prove an effective strategy instead in aCRC.

Initial clinical testing of both dual *MEK* and *BRAF* inhibition(373) and dual EGFR and *BRAF* inhibition(374) have been reported. Whilst both demonstrated activity (RR of 12% and 13.3%, respectively), this is not comparable to RR in melanoma. However a triplet combination of dabrafenib, trametinib and panitumumab was more promising: in the 14 patients treated, 40% had a clinical response and a further 40% experienced disease stabilisation, with acceptable toxicity.(374)

BRAF is therefore a complex therapeutic target in aCRC and despite progress there is unlikely to be an approved targeted approach available imminently so optimisation of currently available treatment is important. Further understanding of the mechansim of

poor prognosis and identification of the point of the treatment pathway at which outcomes differ from wild-types may suggest alternative treatment strategies.

3.1.7. Mechanisms of poor prognosis in *BRAF*-mut aCRC

One possible mechanism for poor outcomes is that *BRAF*-mut status may confer resistance to treatments used in CRC. Most data describing potential interaction of *BRAF*-mut status with aCRC treatment is from studies and RCTs investigating anti-EGFR agent efficacy; only limited further information exists for other treatment strategies.

3.1.7.1.EGFR targeted agents

BRAF-mut has been extensively investigated as a negative predictive biomarker for cetuximab and panitumumab treatment in *KRAS*-wt patients. A negative predictive effect was reported in several non-randomised series(189, 191) and a harmful effect of panitumumab was seen in *BRAF*-mut patients in PICCOLO.(131) However molecular analysis in other RCTs reported solely prognostic value.(97, 329)

3.1.7.2.Bevacizumab

There is no evidence that *BRAF*-mut patients have worse outcomes with bevacizumab.(375, 376)

3.1.7.3.Standard chemotherapy regimes

The impact of *BRAF*-mut status on outcomes with standard chemotherapy regimes is less investigated. As discussed there was no evidence of inferior RFS with adjuvant chemotherapy.(360, 362) However poor results with chemotherapy are reported for *BRAF*-mut aCRC patients.(367, 368, 377) In the larger series from MD Anderson, 69 *BRAF*-mut patients were treated with systemic therapy (OxFU or IrFU based, including combinations with monoclonal antibodies) for a median of two lines of therapy, but with no *BRAF*-wt comparison.(377) Median PFS was 6.3 months for first-line treatment, not affected by regimen which the authors felt was inferior to a wild-type population. Survival of *BRAF*-mut patients was 20.2 months, due to inclusion of those with resectable metastatic disease. In a study of 168 patients, inferior PFS with first-line chemotherapy was reported for the 13 *BRAF*-mut patients compared to *BRAF*-wt (4.3 vs 12.5 mths).(368)

However in FOCUS whilst *BRAF*-mut status was associated with markedly inferior OS, the *BRAF*-mut and *BRAF*-wt patients benefitted to a similar extent from the addition of either oxaliplatin or irinotecan to 5FU.(156)

Less information exists regarding the experience of *BRAF*-mut patients on subsequent lines of chemotherapy following first-line progression. The MD Anderson cohort described outcomes following first-line chemotherapy where available: median PFS for *BRAF*-mut patients was 2.5 and 2.6 months for second and third-line treatments respectively, corresponding with the first response assessment.(377)

Therefore for only one class of drug, anti-EGFR antibodies, has *BRAF*-mut been reported to confer lack of benefit,(131) but this finding is inconsistent;(97) given the modest overall impact of these drugs on survival it does not explain the major survival disadvantage seen in *BRAF*-mut patients. One consistent finding is that *BRAF*-mut is associated a greater detriment in overall survival than in progression-free survival. Thus, in the pooled analysis of first-line trials, whilst PFS was modestly inferior in *BRAF*-mut patients than wild-types (6.2 vs 7.7 months, HR = 1.34 $p<0.001$), this small difference contrasted with very markedly inferior OS (11.4 vs 17.2 months, HR =1.91 $p<0.001$).(159) This raises the question whether *BRAF*-mut confers tumour biological changes that lead to accelerated decline following progression on therapy, and it is this rather than drug resistance that drives the poor prognosis.

To investigate this phenomenon further, individual patient data from three large aCRC RCTs has been examined to identify points on the treatment pathway at which *BRAF*-mut outcomes differ from *BRAF*-wt patients treated with standard chemotherapy. Detailed treatment outcomes in two first-line RCTs, behaviour during chemotherapy-free intervals and following disease progression have been tested. Patterns of, and outcomes with second-line therapy will be described. In order to avoid potential interactions of *BRAF* status with anti-EGFR drugs patients treated in trial arms which involved anti-EGFR agents were excluded, and at a time when these drugs were not widely available in the UK for post-trial use.

3.2. Hypothesis

The poor prognosis of *BRAF*-mut aCRC patients is not chiefly driven by chemoresistance. The disparity between PFS and OS detriments in first-line trials instead may suggest that deterioration following progression may underlie the poor outlook of these patients.

3.3. Methods

3.3.1. Patients and Methods

Individual patient data was obtained from selected arms of FOCUS,(100) COIN(102, 128) and PICCOLO,(131) to reflect different clinical uses of standard cytotoxic chemotherapy (without targeted therapy) in aCRC (Figure 3.2).

Full reports of these studies have been previously published.(100, 102, 128, 131) National ethical approval and patients' written informed consent was obtained for all aspects of the clinical and translational research.

3.3.2. Assessment of *BRAF* status

BRAF-status has previously been determined in all three trials; methodology is described in detail in each manuscript.(131, 156, 222) Briefly, DNA was extracted was extracted from FFPE tumour tissue using the QiAMP DNA microkits (Qiagen, Hilden, Germany), following manufacturer's instructions. Primers for amplification were designed using Pyrosequencing Assay Design Software (Biotage AB, Uppsala, Sweden). *BRAF*-analysis was performed using PyroMark ID software (Biotage AB) set to the single nucleotide polymorphism (SNP) allele quantification mode.

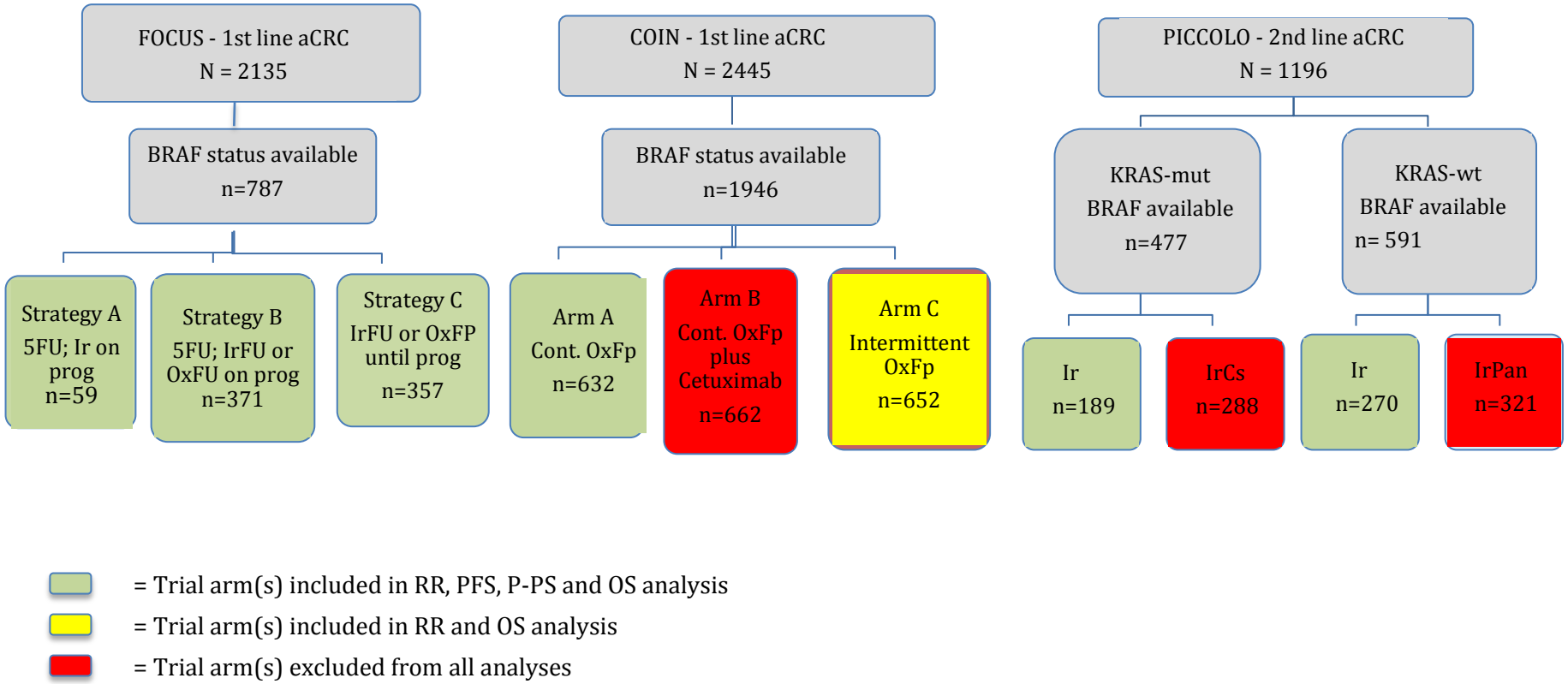

Figure 3-2 Consort diagram of study participants from the FOCUS, COIN and PICCOLO trials

3.3.3. Statistical analysis

Stata was used for all statistical analyses (*Release 12 (2011)*, StataCorp. College Station, Texas). Baseline patient characteristics were compared between *BRAF*-wt patients (with or without other MEK/AKT pathway mutations) and *BRAF*-mut patients using two-tailed T-tests, Wilcoxon rank sum tests (for variables with non-normally distributed frequency distributions) and Pearson Chi-squared tests (for categorical variables).

In addition to overall survival (OS – time from randomisation to death from any cause), three treatment-related clinical endpoints were used: progression-free survival (PFS - time from randomisation to first evidence of progression or death); 12-week RECIST response rate (RR), and disease control rate (DCR).(378) Finally, post-progression survival time was compared (P-PS), defined as time from progression to death in those with a progression event. In COIN arm C, patients with stable/responding disease at 12 weeks then had a planned chemotherapy-free break until progression, making PFS and P-PS inappropriate end-points; therefore, analysis of these end-points in COIN is limited to Arm A.

The prognostic influence of *BRAF*-mut status on survival outcomes (PFS, P-PS and OS) was analysed using Cox proportional hazards modelling and described using HRs and 95% CIs. Additionally, multivariable Cox models were fitted adjusting for other factors previously known to be prognostic in the population or likely to interact with *BRAF*-status. These were: WHO performance status (2 vs. 0/1), primary tumour resected (yes vs no), primary tumour location (PTL) (right colon vs other). In COIN and FOCUS they were additionally: platelet count (< vs ≥ 400,000/μl), peritoneal metastases (present vs absent), MMR status). In PICCOLO they were additionally response to previous therapy and peritoneal metastases. MMR status was unavailable for PICCOLO at time of analysis.

Kaplan-Meier curves were plotted. For response endpoints, ORs and 95% CIs were estimated from logistic regression models for the effect of *BRAF*-mut status. Models were adjusted for the markers previously described.

3.4. Results

3.4.1. *BRAF*-status and other patient characteristics

BRAF-mut status was available for 1284/1630 (78.7%) patients in COIN, 787/2135 (36.9%) in FOCUS and 459/511 (89.8%) in PICCOLO. Prevalence of *BRAF*-mut was consistent with published values (FOCUS 61/787 [7.6%], COIN 130/1284 [10.1%], PICCOLO 40/459 [8.7%]). Baseline characteristics by *BRAF*-status were mostly balanced, but *BRAF*-muts were more likely to have right-sided PTL (58% vs 24%, p<0.001), peritoneal metastases (22% vs 14%, p=0.003) and dMMR (13% vs 3%, p<0.001), and less likely to have lung (24% vs 40%, p<0.001) and liver (68% vs 74%, p=0.040) metastases (table 3.1). There were no differences in demographics from patient involved in this study and those with *BRAF*-mut statuses missing in PICCOLO; in FOCUS and COIN patients with *BRAF*-status missing were less likely to have a right-sided tumour (p=0.006) and to have nodal metastases (p<0.001).

		FOCUS and COIN Study Population (n=2071)		PICCOLO study population (n=459)	
		***BRAF*-mut (n=191)**	***BRAF*-wt (n = 1880)**	***BRAF* mut (n = 40)**	***BRAF*-wt (n = 419)**
Median age		63.4 (IQR 57-71)	64 (IQR 57-69)	63.1 (IQR 56-67)	62.7 (IQR 56-67)
Sex	**Male**	107 (56.0%)	1271 (67.6%)	13 (32.5%)	295 (70.4%)
	Female	84 (44.0%)	609 (32.4%)	27 (67.5%)	120 (28.7%)
	Missing	0 (0.0%)	0 (0.0%)	0 (0.0%)	4 (0.9%)
WHO PS	**0-1**	173 (90.6%)	1750 (93.1%)	39 (97.5%)	386 (84.1%)
	2	18 (9.4%)	130 (6.9%)	1 (2.5%)	26 (5.7%)
	Missing	0 (0.0%)	0 (0.0%)	0 (0.0%)	47 (10.2%)
Resected primary	**Yes**	131 (68.6%)	1326 (70.5%)	36 (90%)	299 (71.5%)
	No	50 (26.2%)	496 (26.4%)	4 (10%)	118 (28.2%)
	Local recurrence	10 (5.2%)	58 (3.1%)	0	1 (0.3%)
Primary tumour location	**Right**	111 (58.1%)	451 (24.0%)	22 (55.0%)	126 (30.1%)
	Left	70 (36.7%)	1327 (70.6%)	17 (42.5%)	284 (67.8%)
	Missing	10 (5.2%)	102 (5.4%)	1 (2.5%)	9 (2.1%)
Previous clinical benefit	**Yes**	n/a	n/a	21 (52.5%)	271 (64.7%)
	No	n/a	n/a	12 (30.0%)	112 (26.7%)
	Missing	n/a	n/a	7 (17.5%)	36 (8.6%)
Peritoneal mets	**Yes**	42 (22.0%)	263 (14.0%)	16 (40.0%)	97 (23.5%)
	No	148 (77.5%)	1603 (85.3%)	24 (60.0%)	311 (75.3%)
	Missing	1 (0.5%)	14 (0.7%)	0	5 (1.2%)
Lung mets	**Yes**	45 (23.6%)	754 (40.1%)	15 (37.5%)	246 (59.7%)
	No	145 (75.9%)	1112 (59.2%)	25 (62.5%)	164 (39.8%)
	Missing	1 (0.5%)	14 (0.7%)	0 (0.0%)	2 (0.5%)
Liver mets	**Yes**	129 (67.5%)	1395 (74.2%)	30 (75.0%)	305 (73.3%)
	No	61 (31.9%)	471 (25.1%)	10 (25.0%)	107 (25.7%)
	Missing	1 (0.5%)	14 (0.7%)	0 (0.0%)	4 (1.0%)
Nodal mets	**Yes**	104 (54.5%)	811 (43.1%)	16 (40.0%)	103 (24.6%)
	No	86 (45.0%)	1055 (56.1%)	24 (60.0%)	311 (74.2%)
	Missing	1 (0.5%)	14 (0.7%)	0 (0.0%)	5 (1.2%)
MMR status	**pMMR**	24 (12.6%)	56 (3.0%)	n/a	n/a
	dMMR	143 (74.9%)	1583 (84.2%)	n/a	n/a
	Missing	24 (12.6%)	141 (7.5%)	n/a	n/a
KRAS status	**WT**	180 (94.2%)	993 (52.8%)	36 (90.0%)	219 (52.3%)
	Mut	8 (4.2%)	857 (45.6%)	0 (0.0%)	172 (41.1%)
	Missing	3 (1.6%)	30 (1.6%)	4 (10.0%)	28 (6.7%)

Table 3-1 Patient characteristics by BRAF status in the study population

3.4.2. *BRAF*-mutation as a prognostic marker for overall survival

BRAF-mut status was a significant prognostic marker for OS in the first line (10.8 vs 16.4 mths; adjusted HR = 1.48, p<0.001)(table 3.2 & fig. 3.3). This effect was seen in both first-line studies (FOCUS 10.8 vs 16.2 mths, HR = 1.55 [1.17-2.04], p=0.002; COIN 9.8 vs 16.6 mths, HR = 1.78 [1.46-2.17], p<0.001).

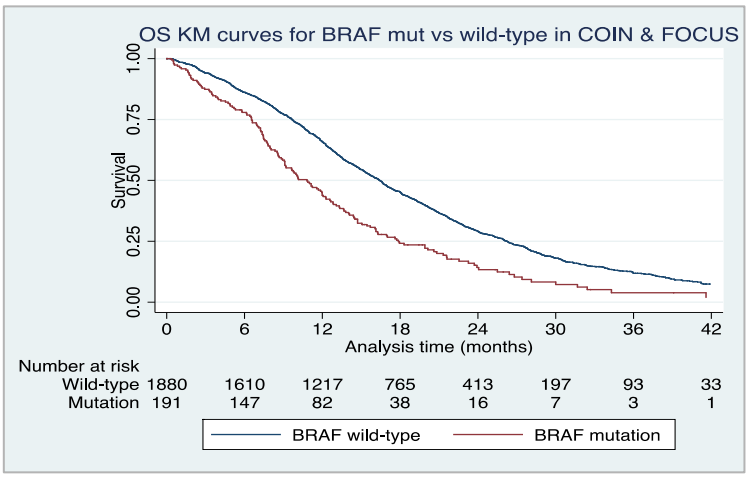

Figure 3-3 OS KM curves for BRAF-mut vs BRAF-wt in 1st line studies

However *BRAF*-mut status did not confer significantly inferior OS for patients commencing second-line treatment (6.9 vs 10.2 mths; HR = 1.17 [0.85-1.65], p=0.33) (table 3.2 & figure 3.4).

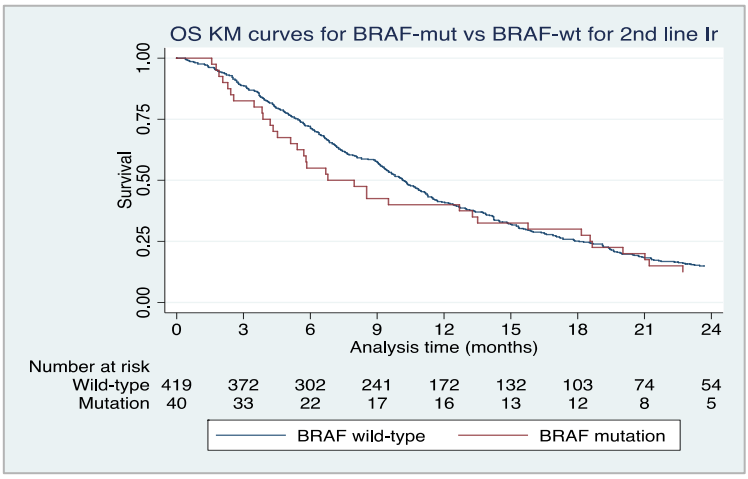

Figure 3-4 OS KM curves for BRAF-mut vs BRAF-wt for patients commencing second-line chemotherapy

Clinical Endpoint	Treatment strategy	Median (IQR) survival (mo)		HR (95% CI)
		BRAF-mut	*BRAF*-wt	
PFS	1st line FU (FOCUS)	n=29 6.5	n=399 6.6	0.96 (0.60, 1.52) P=0.86
	1st line combination (FOCUS & COIN)	n=57 6.0	n=575 6.9	1.12 (0.91-1.36) P=0.26
	2nd line Ir	n=39 3.5	n = 414 4.0	1.06 (0.76, 1.49) p=0.72
P-PS	1st line FU (FOCUS)	n=48 3.5	n=627 8.8	2.13 (1.58, 2.87) P<0.001
	1st line combination (FOCUS & COIN)	n=102 4.4	n=970 9.6	2.00 (1.61, 2.49) P<0.001
	All 1st line strategies	n = 150 4.2	n=1597 9.2	1.69 (1.41-2.06) p<0.001
	2nd line Ir	n=26 5.9	n = 291 6.5	1.13 (0.75, 1.69) p=0.57
OS	1st line (all regimens)	n=191 10.8	n=1880 16.4	1.48 (1.22, 2.17) P<0.001
	2nd line Ir	n = 40 6.9	n= 419 10.2	1.17 (0.85-1.65) p=0.33

Table 3-2 Estimated crude HRs for the effect of BRAF-mut vs BRAF-wt for PFS, P-PS & OS

3.4.3. Effect of *BRAF*-status on treatment outcomes with first-line chemotherapy (FOCUS and COIN)

For patients treated with first-line combination chemotherapy in FOCUS and COIN, *BRAF*-status had only modest impact. Response rates were lower in the *BRAF*-mut patients compared with wild-types (34.6% vs 46.9%, adjusted OR = 0.66[0.44-0.99], p=0.046). However there was no difference in PFS for first-line combination chemotherapy by *BRAF*-status (6.0 vs 6.9 mths; adjusted HR = 1.12[0.91-1.36], p=0.26) (fig. 3.5).

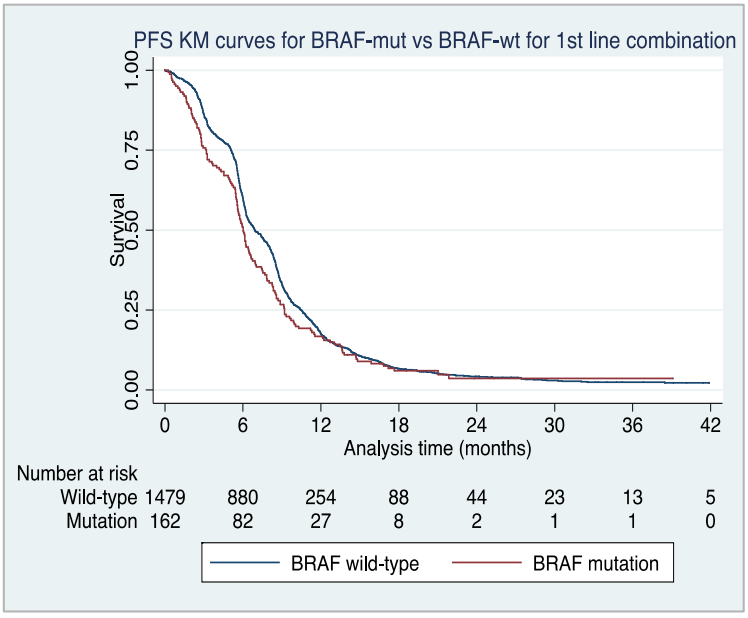

Figure 3-5 PFS KM curves for BRAF-mut vs BRAF-wt patients treated with 1st line combination chemotherapy

Individual data for each trial is shown in figure 3.6. *BRAF*-mut patients did not have inferior PFS when treated with first-line combination chemotherapy in either FOCUS (adjusted HR = 1.07, [0.69-1.67], p=0.37) or COIN (adjusted HR = 1.14, [0.91-1.42], p=0.16) when assessed individually.

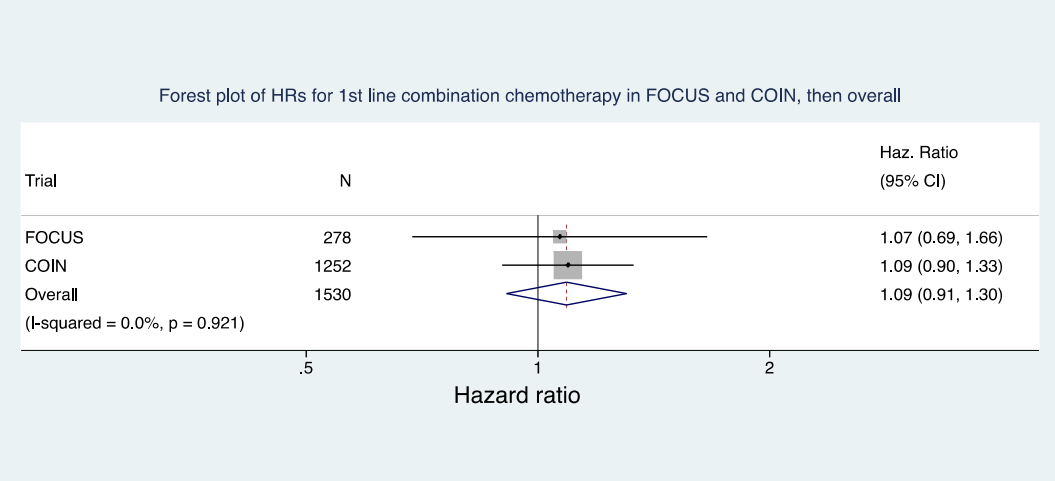

Figure 3-6 Forest plot of PFS HR for 1st line combination chemotherapy in FOCUS & COIN, then overall

There was no evidence of a difference in this effect by chemotherapy regimen (OxFP vs OxCap in COIN (interaction p =0.87). In FOCUS there were too few patients to analyse detailed treatment outcomes by *BRAF*-status for initial OxFU or IrFU.

3.4.4. Effect of *BRAF*-mut status for patients treated with first-line FU

With first-line single-agent 5FU, there was no difference in PFS in *BRAF*-mut compared with *BRAF*-wt patients (6.5 vs. 6.7 mths, adjusted HR=0.96 [0.60-1.52], p=0.86)(table 3.2 & fig. 3.7). There were no significant differences in RR (17.2% vs 21.7% (OR=0.75 [0.28,2.03], p=0.57), or DCR (70.0% vs 76.4% (OR=0.72 [0.28-1.94], p=0.52).

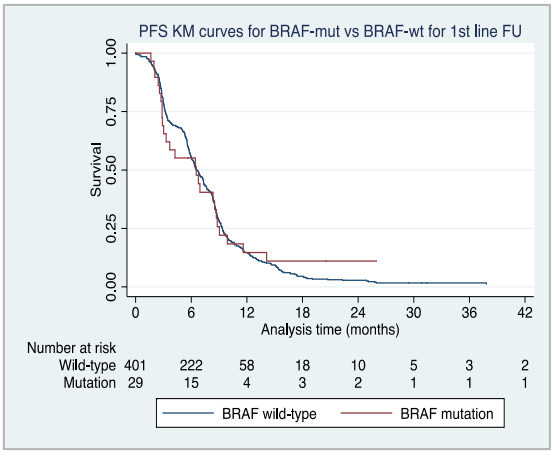

Figure 3-7 PFS KM curves for *BRAF*-mut vs *BRAF*-wt treated with FU alone

These data show that there is a modest reduction in RR in *BRAF*-mut patients treated with oxaliplatin/FP but no difference in PFS after adjusting for prognostic factors. Furthermore, there was no difference in RR or PFS when these patients were treated with first line FP alone.

3.4.5. Experience of *BRAF*-mut patients following first-line chemotherapy: post-progression survival.

Following progression on first-line chemotherapy, *BRAF*-mut patients had marked reduction in P-PS compared with *BRAF*-wt (4.2 vs 9.2 mths, adjusted HR = 1.69[1.41-2.06], p<0.001)(table 3.2 & fig. 3.8).

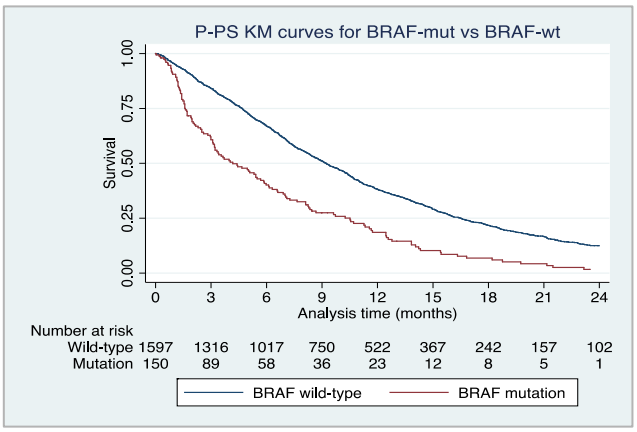

Figure 3-8 Post-progression survival KM curves for *BRAF*-mut vs *BRAF*-wt in FOCUS & COIN

This effect was consistent in both first-line trials (FOCUS, all strategies: 3.5 vs 8.8 mths, HR = 2.13 [1.58-2.87], p<0.0001; COIN 4.5 vs 9.6 mths, HR = 2.00 [1.61-2.49], p<0.0001)(table 3.2 & fig. 3.9).

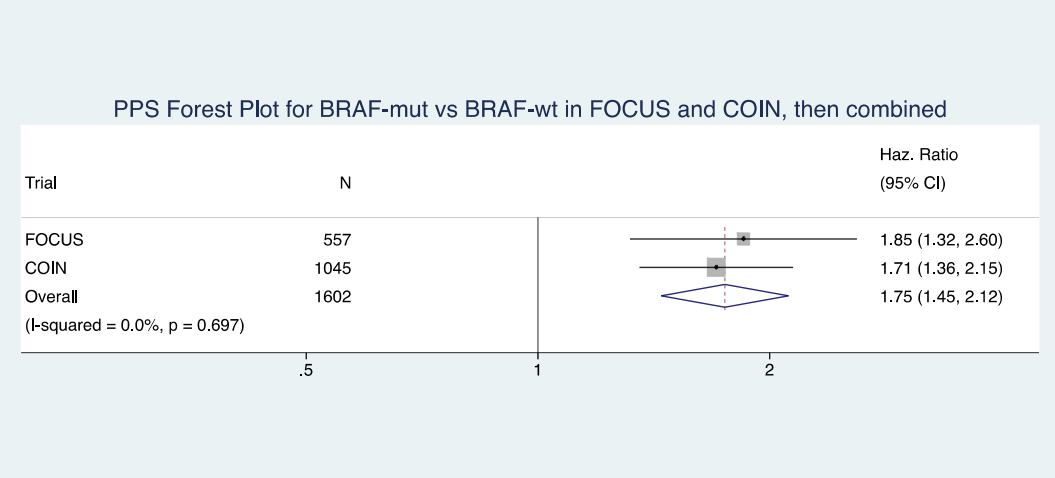

Figure 3-9 Forest plot of P-PS in FOCUS & COIN, then combined

There was no interaction with chemotherapy regimen (FOCUS [OxFU/ IrFU] interaction p=0.44; COIN [OxFU/OxCap interaction p=0.57) or planned post-progression treatment (FOCUS, irinotecan vs doublet p=0.32; OxFU vs IrFU p=0.44). Of other prognostic factors tested in the multivariate model, only primary tumour in-situ was associated with reduced PPS (HR = 1.45 [1.02-2.05], p=0.036).

3.4.6. Impact of chemotherapy-free intervals in *BRAF*-mut patients

In contrast to the higher death rate seen after failure of first-line chemotherapy, there was no evidence that *BRAF*-mut patients fare less well when having a planned treatment break with stable/responding disease. COIN compared continuous or intermittent chemotherapy, and found overall that intermittent chemotherapy was non-inferior for OS (HR=1.04 [0.98–1.10], p=0.16); in *BRAF*-mut patients this was also the case (HR=0.97 [0.80–1.17], p=0.75).

Overall in COIN, progression events in patients during chemotherapy breaks led to shorter PFS (HR=1.27 [1.21–1.33], p<0.001). Interestingly, however, *BRAF*-mut patients were the only molecular sub-group not to have a PFS disadvantage with intermittent chemotherapy (*BRAF*-mut PFS HR=1.09 [0.91–1.31], p=0.33; *BRAF*-wt PFS HR=1.29 [1.22–1.37], p<0.001; interaction p=0.14).

3.4.7. Impact of *BRAF* status on uptake of salvage therapy

To explore the reasons for the markedly inferior 1st-line P-PS in *BRAF*-mut patients, the uptake of post-progression therapies was explored.

In COIN, *BRAF*-mut patients were significantly less likely to receive second-line therapy after first-line progression than *BRAF*-wt (39% versus 60%, p=0.002). However, for those receiving chemotherapy there was no difference in duration spent on second-line chemotherapy (p=0.2).

3.4.8. Outcomes of *BRAF*-mut patients treated with second-line chemotherapy

For patients commencing second-line Ir in PICCOLO there were no differences between *BRAF*-mut and *BRAF*-wt patients for any treatment outcomes: RR (5.0% vs 8.1%; OR = 0.59 [0.22-2.55], p=0.65), DCR (59.3% vs 59.5%; OR = 0.99[0.44-2.23], p=0.98), PFS (3.5 vs 4.0 months; HR = 1.06 [0.76-1.49], p=0.72) (table 3.2 and fig. 3.10).

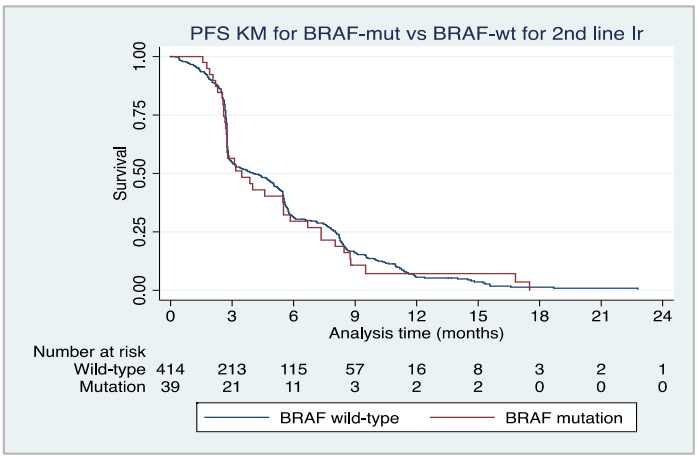

Figure 3-10 PFS KM curves for BRAF-mut vs BRAF-wt for second-line Ir

Following progression on Ir, P-PS was similar in *BRAF*-mut than *BRAF*-wt patients (5.9 vs 6.5months; HR = 1.13 [0.75-1.69], p=0.57) (table 3.2 & figure 3.11). The only factor predicting reduced shorter P-PS in PICCOLO was poor performance status (HR= 2.27 [1.15-4.48], p=0.019).

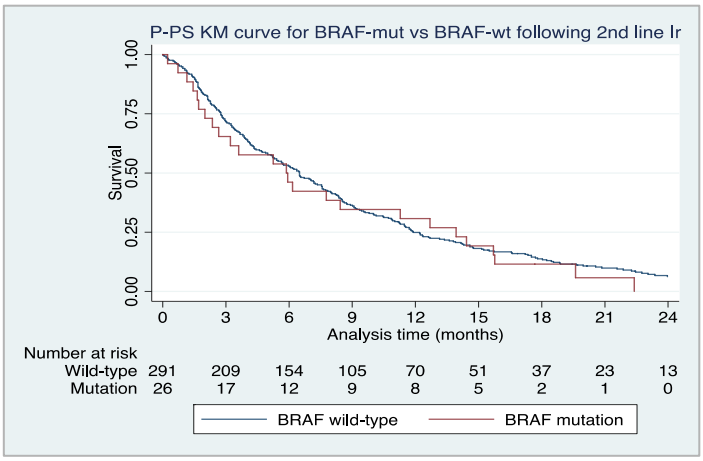

Figure 3-11 P-PS KM curves for BRAF-mut vs BRAF-wt for second-line Ir

3.5. Discussion

This is the largest and most comprehensive clinical series of *BRAF*-mut patients treated with multiple chemotherapy strategies at different points of the aCRC pathway to investigate the mechanism of poor survival and provide clinicians with an evidence base for treatment decisions. The relative rareness of this mutation in aCRC has meant this group of patients has been difficult to study, and to detect a *BRAF*-specific effect.

Within three large RCTs, the point at which the outlook of *BRAF*-mut patients markedly differed from *BRAF*-wt was following progression on first-line chemotherapy. This was seen in both FOCUS and COIN, independent of chemotherapy strategy. Notably no other tested factor was associated with reduced P-PS and thus this appears to be specific to *BRAF*-mut cancers.

To understand this phenomenon patterns of care following progression on first-line chemotherapy were investigated. Fewer *BRAF*-mut patients received second-line chemotherapy in COIN than *BRAF*-wt, but those that did remained on therapy for an equivalent duration. Furthermore there was no significant difference in any treatment outcome according to *BRAF*-status for patients treated with Ir alone in the PICCOLO trial. Therefore, once started, *BRAF*-mut patients benefit equivalently to *BRAF*-wt patients from second-line chemotherapy.

Notably, this accelerated decline cannot be attributed to simply stopping chemotherapy: in COIN *BRAF*-mut patients with stable disease following 12 weeks of chemotherapy were not disadvantaged by a planned chemotherapy-break.

This study would not support the hypothesis that chemoresistance drives the entirety of the poor prognosis of *BRAF*-mut aCRC. Whilst there was reduced activity of first-line chemotherapy compared with *BRAF*-wt in COIN, this was not maintained following adjustment and critically PFS detriment did not mirror the more clinically relevant reduction in survival. No differential effect was seen in any strategy tested in FOCUS.

There is less experience of *BRAF*-mut patients following first-line treatment. Unpublished sub-analysis from the 20050181 Trial (second-line FOLFIRI/panitumumab vs FOLFIRI) primarily describes interactions of *RAS* and *BRAF* mutation status with panitumumab effect.(379) *BRAF*-mut patients treated with FOLFIRI-alone had worse OS (5.7 vs 15.4 mths) and PFS (1.8 vs 5.5mths) than *RAS*-wt counterparts, suggesting a greater *BRAF*-mut effect than seen in PICCOLO. Differences between these trials provide some explanation. This study compares outcomes of *BRAF*-mut patients with *BRAF*-wt, regardless of *RAS*-status to reflect usual practice; in 20050181 outcomes for *BRAF*-mut patients were compared with the dual *RAS* and *RAF*-wt population. There were also important differences in pre- and post-trial therapy: only 67% of patients in 20050181 received first-line oxaliplatin, compared with 95% in PICCOLO. Increased post-trial treatment in 20050181 with anti-EGFR agents (31% vs. 6% in PICCOLO) and oxaliplatin may have

contributed to the particularly good OS in the *RAS-RAF* wt comparator group. Further study of *BRAF*-mut with further lines of chemotherapy would be beneficial.

This report identifies a subset of *BRAF*-mut aCRCs who have a more indolent clinical course, as evidenced by equivalent representation (percentage of *BRAF*-mut patients in the trial) and outcomes in PICCOLO and on post-trial therapy in COIN. Likewise, within the MD Anderson cohort 15% of *BRAF*-mut patients were progression-free at two years.(380) Unfortunately there were no specific clinico-pathological features identifying this group, or marked differences between these first and second line trial populations, however *BRAF*-mut patient in PICCOLO were of good performance status. Such clinical heterogeneity may suggest other molecular drivers, particularly post-progression; study of post-progression biopsies will be essential in identifying the biological mechanism of the rapid decline, and to identify strategies to inhibit these.

This study offers several advantages over previous work. A prospectively defined analysis of *BRAF*-effect allowed for consideration of relevant confounding factors. *BRAF*-mut aCRCs are associated with specific clinico-pathological features: older age, proximal primary tumour, high grade, deficient MMR, mucinous histology and peritoneal and lymph node metastases,(159, 222, 364-367) most of which interact with prognosis. Analysis of *BRAF*-mut patients must consider such factors: highlighted by the fact that the OxFU PFS detriment was less marked (and non-significant) following adjustment. RCT data has allowed accurate and robust measurement of response, progression events, P-PS and post-progression therapies.

A weakness of this, and all current studies of *BRAF*-mut patients, is failure to address outcomes of frail patients: those screened for *MEK-AKT* pathway mutations will be deemed eligible for RCT inclusion or anti-EGFR therapy. Previous work has suggested that patients with *BRAF*-mut CRCs were most likely to be elderly females; this is a population unlikely to be well represented in this or other studies discussed.(365, 366) Clinicians should therefore be cautious when applying RCT evidence to such populations.

Disappointing results of *BRAF*-inhibitors as single agents in aCRC(369) and a growing appreciation of molecular complexity of *BRAF*-mut aCRC(371) suggest that targeted approaches may involve multi-agent combination, with consequent toxicity and expense. There is therefore an urgent need to optimise chemotherapy strategies to improve outcomes. First-line intensive combination treatment with FOLFIRINOX plus bevacizumab

in *BRAF*-mut patients resulted in PFS and OS of 11.8 and 24.1 months respectively, and an acceptable toxicity profile.(105) These were comparable outcomes to *BRAF*-wt patients treated within the TRIBE study,(104) although the benefit of the addition of bevacizumab to this regime is unclear. Given the marked decline we have seen following progression on first line chemotherapy, maximisation of the efficacy of first line treatment with the use of all the active chemotherapy drugs together appears to be an appropriate approach in good performance status patients.

Knowledge of *BRAF*-status offers the clinician useful information and can help guide treatment decisions, outwith the context of anti-EGFR agents. The poor survival of *BRAF*-mut patients is driven by accelerated decline following progression, and a lower probability of receiving further lines of therapy, so clinicians need extra vigilance. Whilst we report some lesser degree of benefit with first line therapy in *BRAF*-mut than wt-patients, this does not seem to drive all of the poor outcome of these patients. However, *BRAF*-mut patients may still enjoy treatment breaks when not progressing, and if treated with second-line chemotherapy are no less likely to benefit than wild-type patients.

3.6. Further work

Further analyses are outstanding for this project as data was unavailable at the point of submission. This data includes post first-progression outcomes in FOCUS by *BRAF*-status and the impact of dMMR status in PICCOLO.

It is planned to do a sub-group analysis looking at interactions between peritoneal metastases, *BRAF*-mut status and outcomes. It is also planned to investigate the outcomes of *RAS*-mutant patients in RCTs, as this mutation sub-group is also associated with inferior prognosis in aCRC.(156) The same analysis plan shall be utilised.

This study has raised some important hypotheses regarding the mechanism of the poor outlook of *BRAF*-mut aCRC patients. The most striking observation is the marked reduction in post-progression survival in *BRAF*-mut patients following first-line chemotherapy, in the absence of treatment with a targeted agent. This appears to be the point at which their outcomes differ from *BRAF*-wt patients. It is therefore important to understand the biology of this accelerated decline: is it due to emergence of new driver mutations or activation of other pathways? Study of paired pre- and post treatment

biopsies would be needed to answer this question. Further treatment targets may be identified which could lead to a targeted approach following progression for fit patients.

Gene expression analysis in early CRC suggested that a *BRAF*-like mutant population exist in the *BRAF*-wt population. As well as having a similar gene expression profile, they had similar survival outcomes to the *BRAF*-mut population. Testing for this gene signature in a study of aCRC would ascertain whether this group exist in the advanced population. If so, investigating whether they follow patterns demonstrated in this work. Again paired pre- and post treatment biopsies would be beneficial to identify whether there is a common driver molecular alteration.

Chapter 4. Combined epiregulin and amphiregulin expression levels as a biomarker of prognosis and panitumumab benefit in *RAS*-wt advanced colorectal cancer

4.1. Introduction

Clinical trial results with the anti-EGFR agents panitumumab and cetuximab in aCRC have been inconsistent, varying in different RCTs and patient sub-populations between worthwhile benefit and significant harm.(126, 131) One consistent finding is that activating mutations in *RAS* (*KRAS* exons 2,3 and 4, *NRAS* exons 2, 3 and 4) confer lack of benefit.(70, 126) However, an unmutated *RAS* pathway, although necessary, is not sufficient for response to anti-EGFR agents: in the PRIME and PICCOLO phase III trials, the response rate of patients with tumours wild-type for all tested codons in *KRAS* and *NRAS* was still less than 50%.(70, 131)

It is plausible that successful EGFR blockade requires upregulation of EGFR signalling in addition to an intact EGFR signal transduction pathway, therefore it would be valuable to identify biomarkers of EGFR-signalling tumour dependence. However, unlike in lung cancer,(381) none of EGFR mutation status, EGFR gene copy number or EGFR protein expression have shown consistent association with anti-EGFR agent efficacy.(181) Instead, another proxy for EGFR-dependence may be EGFR ligand expression.

4.1.1. EGFR ligands

EGFR ligands include epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin (AREG), epiregulin (EREG), heparin binding-epidermal growth factor like growth factor, epigen and betacellulin.(382) All ligands are type I transmembrane proteins cleaved by cell surface proteases leading to release of mature growth factors that bind to and activate the EGFR.(382) On ligand binding the EGFR homodimerises or heterodimerises with ErbB2, ErbB3 or ErbB4, leading to tyrosine kinase domain phosphorylation and activation of downstream pathways (including the RAS/MAPK, PI3/AKT and STAT pathways)(fig. 4.1).(383)

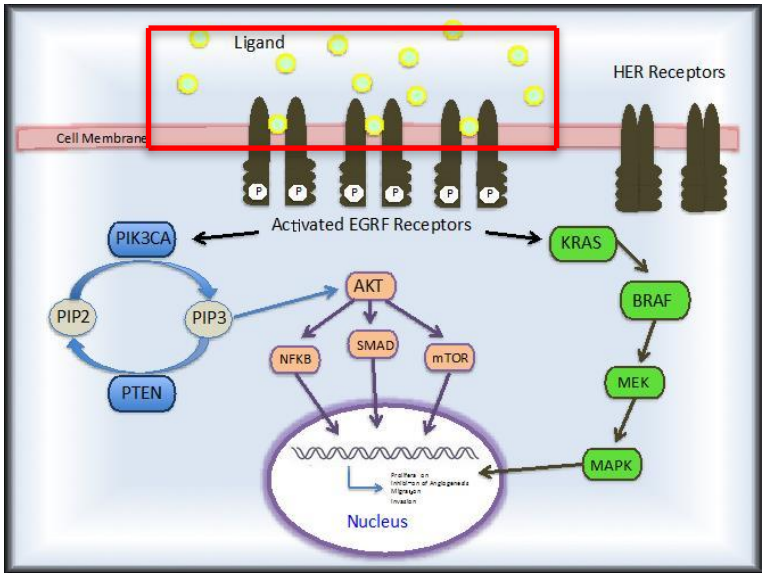

Figure 4-1 Representation of the EGFR pathway, highlighting the role of EGFR ligands. Modified from (334)

Interest in the usefulness of EGFR ligands as positive predictive markers for anti-EGFR therapy was sparked by a GWAS examining biopsies of liver metastases from 80 aCRC patients treated with cetuximab monotherapy, with the aim of discovering genes associated with cetuximab efficacy.(165) Out of a panel of 640 probe sets with moderate to high levels in CRC, the key finding was that high expression levels of the EGFR ligands EREG and AREG were best associated with DCR. Expression levels of other EGFR ligands were not associated with treatment benefit in this,(165) or subsequent studies.(384, 385)

4.1.2. Epiregulin and Amphiregulin

Both EREG and AREG are located on chromosome 4, in close proximity to betacellulin.(382) They are initially expressed as a transmembrane preform, then cleaved by a metalloproteinase and disintegrin enzyme to release a mature active form which binds to the EGFR.(382)

4.1.2.1.Epiregulin

EREG binds to both EGFR and HER4.(386) Whilst EREG has lower affinity to the EGFR than EGF, its binding results in prolonged receptor activation.(387) Compared to EGF there is less ligand-induced EGFR down-regulation and increased receptor recycling.(388) EREG production is through several mechanisms, including establishment of an autocrine signalling loop and paracrine production by surrounding tissue.(389)

EREG is expressed in normal tissue and is implicated in key physiological functions, particularly in the stimulation of and response to inflammation: including angiogenesis and vascular remodelling, cutaneous wound healing, and intestinal epithelial proliferation in response to inflammation.(390)

EREG is overexpressed in several tumour types, including bladder, gastric, breast, lung, head and neck, and colon cancer.(390)

4.1.2.2. Amphiregulin

AREG binds only to the EGFR, in competition with EGF, and is reported to be less effective at inducing EGFR tyrosine phosphorylation at low levels, but with similar activity as other ligands at higher concentrations.(391) AREG is produced by an autoregulated feedback loop and is secreted into the bloodstream by AREG-producing tissue.(392, 393)Following activation on AREG binding, the EGFR is then recycled (rather than internalisation and degradation seen with other ligand activation), favouring accumulation of EGFR at the cell surface.(394)

AREG is expressed in a wide variety of tissues physiologically (including mammary glands, reproductive, vascular, gastrointestinal and respiratory) and participates in many physiological processes.(392) AREG is overexpressed in several human cancers including breast, lung, ovary, prostate, pancreas, stomach and colon.(395) AREG is implicated in several essential malignant processes, including resistance to apoptosis, uncontrolled growth, sustained angiogenesis and tumour invasion and metastasis.(395)

4.1.3. EREG and AREG as predictive biomarkers for anti-EGFR therapy

EREG and AREG are commonly overexpressed in colorectal cancer.(396) In CRC cell line studies, binding of either of these ligands to EGFR leads to autocrine EGFR activation; hence EREG/AREG overexpression may be a proxy for tumour EGFR-dependence.(392, 397)

High mRNA expression of EREG or AREG has been reported to correlate with the efficacy of EGFR-targeted agents in *KRAS*-wt aCRC in non-randomised series.(165, 166, 384, 385) EREG and AREG mRNA expression was measured in FFPE tumour samples from 220 irinotecan-refractory aCRC patients treated with cetuximab. High mRNA expression of EREG and AREG was associated with improved clinical outcomes, but only when limited to

KRAS-wt patients.(166) In *KRAS*-wt patients increasing EREG mRNA expression was associated with improved RR (OR = 1.90[1.27-2.83], p<0.001) and DCR (OR = 1.86 [1.23-3.29], p=0.011), plus improved OS and PFS. Similar results were reported for AREG. In a combined model EREG outperformed AREG, so EREG alone was proposed for clinical development. However in other studies using similar methodology, AREG emerged as the superior predictive marker.(384, 398)

In a recent analysis from the CO.17 trial (BSC with or without cetuximab), *KRAS*-wt patients with high EREG RNA expression had improved OS with cetuximab whilst *KRAS*-wt, low EREG patients did not. With 225 *KRAS*-wt patients and modest power, the biomarker/treatment interaction tests approached but did not reach statistical significance for OS and PFS (p=0.08 and 0.07 respectively).(184)

EREG/AREG mRNA expression was further assessed in 952 patients randomised to first-line line OxFP with or without cetuximab in the COIN trial.(128, 399) EREG superseded AREG in combined predictive models. Within the OxFP arm high EREG expression was associated with improved survival. In patients treated with infusional OxFP plus cetuximab, significant ligand/treatment interactions were reported for both OS and PFS (OS HR 0.33, p=0.011); no ligand/treatment effect was observed when capecitabine treated patients were included.(128) Additionally different dichotomisation points (high EREG vs low EREG) were used to demonstrate statistical EREG/treatment interaction for OS (≥ 80th centile) and PFS (≥ 50th centile).

4.1.4. EGFR ligands as markers of prognosis and predictive markers for panitumumab in PICCOLO

Previous work has provided important indications that EGFR ligand expression may offer a much-needed positive selection strategy for anti-EGFR therapy, and deserves further consideration. The PICCOLO trial biobank provides an excellent opportunity to further study and validate EREG/AREG as positive predictive markers for anti-EGFR agents. However there is need for a novel ligand model and a more comprehensive analysis, considering possible confounders.

4.1.4.1.Considerations for the biomarker study design

The design of this study has been informed by scientific rationale for EGFR activation by EREG and AREG, and builds upon previous work. Firstly there is need to standardise the

methodology used to assess EREG and AREG mRNA expression: the same RT-PCR (reverse transcription polymerase chain reaction) method as used by Jacobs et al will be utilised in this biomarker study.(166)

Previously outcomes for either EREG or AREG have been reported depending upon statistical 'best fit' within the dataset. Since AREG and EREG are commonly but not consistently co-expressed, and either may mediate EGFR activation, it was elected *a priori* to measure both ligands and investigate them as a single putative predictive biomarker in an "either/or" model. As this is a new ligand model it will not be possible to validate ligand cut-points proposed in previous work.

Confounding factors such as MEK-AKT and right PTL may interact with the ligand/treatment model,(70, 131, 155) so the analysis was planned to ensure that any ligand effect is independent of these factors.

4.2. Hypothesis

Benefit of panitumumab will be confined to patients with both wild-type *RAS* ($KRAS_{c.12,13,61}$ and $NRAS_{c.12,13,61}$-wt) and high expression of either AREG or EREG. Patients with a *RAS* or *BRAF* mutation will not benefit from panitumumab regardless of ligand status.

4.3. Methods

4.3.1. Study population

Patients involved in the ligand study have been treated in the IrPan vs Ir comparison in the PICCOLO trial (including patients with *KRAS* mutations/ pre-treatment with EGFR agents) with sufficient tumour material for analysis (fig 4.2).

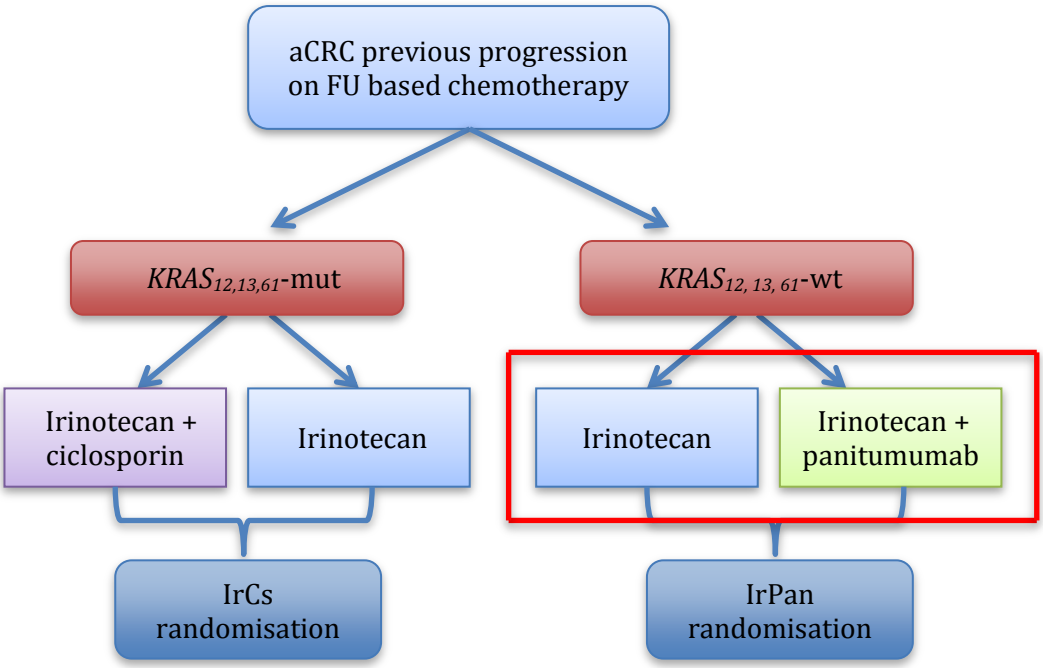

Figure 4-2 PICCOLO trial schema, highlighting patients included in this biomarker study

4.3.2. Laboratory materials and methods

4.3.2.1.Slide preparation

A haematoxylin and eosin stain was performed on a 5µm tumour section to identify area of highest tumour cell density, which would be used for subsequent RNA extraction.

4.3.2.2.RNA Extraction

Five-seven 5 micron slides per tumour were deparaffinised through xylene and graded alcohol to water. Macrodissection was guided by the H&E stained section.

RNA was extracted using the RNEasy FFPE kit (Qiagen, Valencia, CA) according to the manufacturers instructions. Briefly, the macrodissected tissue was digested by proteinase K overnight. DNA was removed using a DNA eliminator column. The remaining sample was washed on a RNeasy MinElute spin column, then eluted into 20 µl RNase-free water. An estimation of RNA concentration was made using a spectrophotometer (Nanodrop, ND) and the RNA stored at -70°C immediately to prevent degradation.

4.3.2.3.cDNA synthesis

cDNA synthesis was performed by firstly adding random primer (Invitrogen, Carlsbad, CA) to RNA and incubating for 10 minutes at 70°C. First strand buffer, DTT and dNTPs were added (all Invitrogen, Carlsbad, CA) and incubated for 2 minutes at 42°C before the addition of of Superscript Reverse Transcriptase II (all Invitrogen, Carlsbad, CA). Following a 50 minute incubation at 42°C and a 70°C inactivation step for 15 minutes, 20µl distilled water was added to give a final reaction volume of 39µl. The resultant cDNA was stored at -20°C.

4.3.2.4.Quantitative real-time PCR (RT-PCR)

AREG and EREG expression levels were measured in duplicate and normalised against expression of 3 reference genes, *GADPH*, *UBC* and *RPL13A*. The geNorm algorithm used in the qBasePlus software (Biogazelle, Ghent, Belgium) was utilised to evaluate the stability of the reference genes. (400)

To correct for inter-run variability, 5 inter-run calibrators were used (5-fold dilution series produced from a mixture of total RNA for CRC cell line HCT116 and human reference RNA (Stratagene, La Jolla, CA).

A 96 well plate was required for each gene being tested and each reference gene. With each gene tested, each sample and standard was added in duplicate along with a negative control.

Briefly, cDNA was diluted in water (1 in 5 dilution), then 5µl of the resulting solution was added to 20µl of stock solution and added to the 96 well plate. Additionally stock solution was added to standards and negative control and added to a labelled plate.

The plate was inserted into the thermocycler (Real Time PCR System, Applied Biosystems, Foster City, CA) and the AREG and EREG gene selected in the detection system (7500 system SDS software). Following 2 mins at 50°C and 10 mins at 95°C, 40 cycles were completed (95°C for 15 seconds then 60 °C for 1 minute). PCR sequences are detailed in the Appendix.

Data analysis was performed using qBasePlus 1.1 software (Biogazelle, Ghent, Belgium). Target-specific PCR efficiencies were calculated from the inter-run calibration standard

curves then utilised to calculated the calibrated normalised relative quantities of ligand expression.(401)

4.3.2.5.Statistical analysis

STATA was used for all statistical analyses (*Stata Statistical Software: Release 12 (2011)*, StataCorp. College Station, Texas). Baseline patient characteristics were compared between treatment arms using two-tailed T-tests, Wilcoxon rank sum tests (for variables with non-normally distributed frequency distributions) and Pearson Chi-squared tests (for categorical variables). Patient characteristics were compared to the whole trial population using the same tests.

Boxplots were produced for raw AREG and EREG expression, and their correlation was estimated using Spearman's coefficient. Data from the two assays were then combined to give a clinically usable single dichotomous classifier by taking the upper/middle tertile cut-point for each ligand and dividing the population into "high expressors" (either EREG or AREG in top tertile) or "low expressors" (neither EREG nor AREG in top tertile). This cut-off was chosen pragmatically, to give high and low groups of similar size.

Three clinical endpoints were used: primary endpoint was PFS; secondary endpoints were OS and RR. PFS and RR data were unchanged from the primary trial analysis, but updated two-year OS data was used in this analysis.

Ligand expression was first assessed as a prognostic marker in patients treated with Ir alone, both using the dichotomous classifier ("high expressors" vs "low expressors") and using each ligand separately as a continuous variable (log-transformed to base 2), in Cox proportional hazards models.

Ligand expression was then assessed as a predictive marker for panitumumab benefit by testing for interaction between the effects of expressor status (high/low) and treatment (IrPan/Ir) on PFS and OS using the likelihood ratio test. Adjustment was performed for significant prognostic factors in the trial population (PS; response to previous therapy). Secondary analysis of predictive effects was performed in patients with *RAS* or *BRAF* mutations.

BRAF mutation and PTL (right colon vs left colon or rectum) were identified as possible confounding factors; therefore survival models were estimated for the joint effects of *BRAF* and ligand, then PTL status and ligand, for the dichotomous classifier and each ligand separately.

4.4. Results

4.4.1. Patient characteristics

Of the 696 PICCOLO patients within the Ir vs IrPan randomisation, 331 had sufficient tumour available for RNA extraction, and subsequent measurement of ligand expression was successful in 323. Baseline characteristics by treatment arm were well balanced and reported in Table 4.1. There were no significant differences in characteristics between the ligand study population and those for whom material was unavailable.

Patient characteristic	Category	Ir (N=171)	IrPan (N=152)	All patients (323)	*RAS*-wt (n=220)
Age at randomisation (yrs)		Mean 62.1 (s.d 10.8)	Mean 61.3 (s.d 11.1)	Mean 61.7 (s.d 10.9)	Mean 61.9 (s.d 11.2)
Sex N(%)	**Male**	106 (62.0)	95 (62.5)	201 (62.2)	133 (60.5)
	Female	65 (38.0)	57 (37.5)	122 (37.8)	87 (39.5)
Ligand expression N(%)	**Low**	93 (54.4)	90 (59.2)	183 (56.7)	121 (55.0)
	High	78 (45.6)	62 (40.8)	140 (43.3)	99 (45.0)
Performance status N(%)	**0-1**	164 (95.9)	147 (96.7)	311 (96.3)	210 (95.5)
	2	7 (4.1)	5 (3.3)	12 (3.7)	10 (4.5)
Previous bevacizumab N(%)	**No**	168 (98.3)	148 (97.4)	316 (97.8)	216 (98.2)
	Yes	3 (1.7)	4 (2.6)	7 (2.2)	4 (1.8)
Previous Oxaliplatin N(%)	**No**	10 (5.9)	11 (7.2)	21 (6.5)	13 (5.9)
	Yes	161 (94.1)	141 (92.8)	302 (93.5)	207 (94.1)
Previous response N(%)	**CR, PR or SD**	100 (58.5)	90 (59.2)	190 (58.8)	135 (61.4)
	PD	50 (29.2)	46 (30.3)	96 (29.7)	57 (25.9)
	Unknown	21 (12.3)	16 (10.5)	37 (11.5)	28 (12.7)
Previous dose modifications N(%)	**No**	64 (37.4)	56 (36.8)	120 (37.2)	85 (38.6)
	Yes	107 (62.6)	96 (63.2)	203 (62.8)	135 (61.4)
Previous chemotherapy N(%)	**No**	63 (36.8)	57 (37.5)	120 (37.1)	78 (35.4)
	Yes	102 (59.7)	95 (62.5)	197 (61.0)	139 (63.2)
	Unknown	6 (3.5)	0 (0)	6 (1.9)	3 (1.4)
***KRAS*$_{c.12,13,61}$ N(%)**	**Wild-type**	137 (80.1)	114 (75.0)	251 (77.7)	-
	Mutant	34 (19.9)	38 (25.0)	72 (22.3)	-
***BRAF*$_{V600E}$ N(%)**	**Wild-type**	150 (87.7)	124 (81.6)	274 (84.8)	173 (78.6)
	Mutant	21 (12.3)	28 (18.4)	49 (15.2)	47 (21.4)
***NRAS*$_{c.12,13,61}$ N(%)**	**Wild-type**	163 (95.3)	142 (93.4)	305 (94.4)	-
	Mutant	8 (4.7)	10 (6.6)	18 (5.6)	-
***KRAS*$_{c.146}$ N(%)**	**Wild-type**	163 (95.3)	145 (95.4)	308 (95.4)	-
	Mutant	8 (4.7)	7 (4.6)	15 (4.6)	-
***PIK3CA*$_{exon\ 9/20}$ N(%)**	**Wild-type**	148 (86.6)	138 (90.8)	286 (88.5)	201 (91.4)
	Mutant	23 (13.4)	14 (9.2)	37 (11.5)	19 (8.6)
No mutations detected		89 (52.1)	68 (44.7)	157 (48.6)	157 (71.4)
Any mutation detected		82 (47.9)	84 (55.3)	166 (51.4)	63 (28.6)
Overall survival time (months)		Median 11.3 (IQR1.7-41.8)	Median 10.1 (IQR 1.5-38.7)	Median 10.7 (IQR 1.3-50.4)	Median 10.9 (IQR 1.4-41.8)
Death event N(%)	**No**	11 (6.4)	9 (5.9)	20 (6.2)	18 (8.2)
	Yes	160 (93.6)	143 (94.1)	303 (93.8)	202 (91.8)
Progression free survival time (months)*		Median 4.3 (IQR 0.9-14.7)	Median 4.9 (IQR 0.7-20.3)	Median 4.4 (IQR 0.4-20.5)	Median 5.1 (IQR 0.6-20.5)
Progression event N(%)	**No**	10 (5.9)	14 (9.2)	24 (7.4)	20 (9.1)
	Yes	161 (94.1)	138 (90.8)	299 (92.6)	200 (90.9)
Best response N(%)**	**CR or PR**	19 (11.1)	37 (24.3)	56 (17.3)	44 (20.0)
	SD or PD	151 (88.3)	113 (74.3)	264 (81.7)	173 (78.6)
	Unknown	1 (0.6)	2 (1.3)	3 (0.9)	3 (1.4)

Table 4-1 Baseline patient characteristics by treatment arm, including mutation status

*****_One patient with zero months progression free survival (PFS) time was excluded from PFS analyses_

Two patients had both a RAS and RAF mutation (BRAF mutant n=49 in study population)

Survival data were available for all study patients. Of these, 299 had a disease progression event (93%) and 303 (94%) patients had died.

A complete set of MEK-AKT mutation (*KRAS*$_{c.12\text{-}13,61,146}$ *NRAS*$_{c.12\text{-}13,61}$ and *BRAF*$_{V600E}$) genotype data was available for all cases. 220/323 (68%) patients were wild-type across all *KRAS* and *NRAS* codons ("*RAS*-wt"). This high proportion is due to the change in trial design in June 2008 meaning that patients with *KRAS*$_{c.12,13,61}$ mutations were not recruited to this randomisation, hence increasing the proportion of *KRAS*$_{c.12,13,61}$-wt patients in this population(fig 4.1). Of the 220 *RAS*-wt patients, 47 (21%) had a *BRAF* mutation. The breakdown of this study population according to mutation status is shown in fig. 4.3.

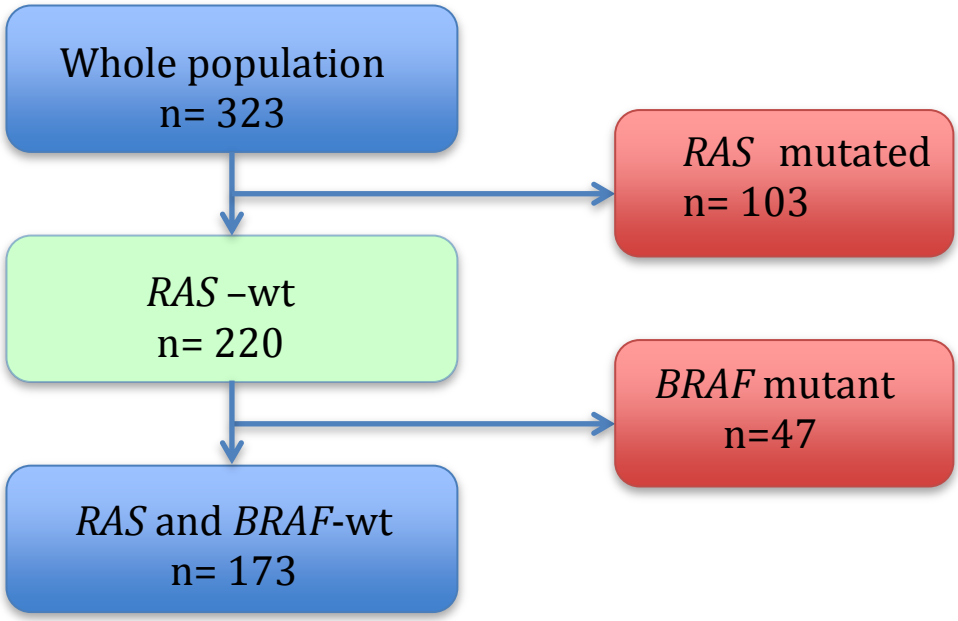

Figure 4-3 Consort diagram of study population

4.4.2. AREG and EREG Distribution

Both ligands showed skewed distributions over the patient population (fig. 4.4), and ligand expressions are log-transformed in subsequent analyses.

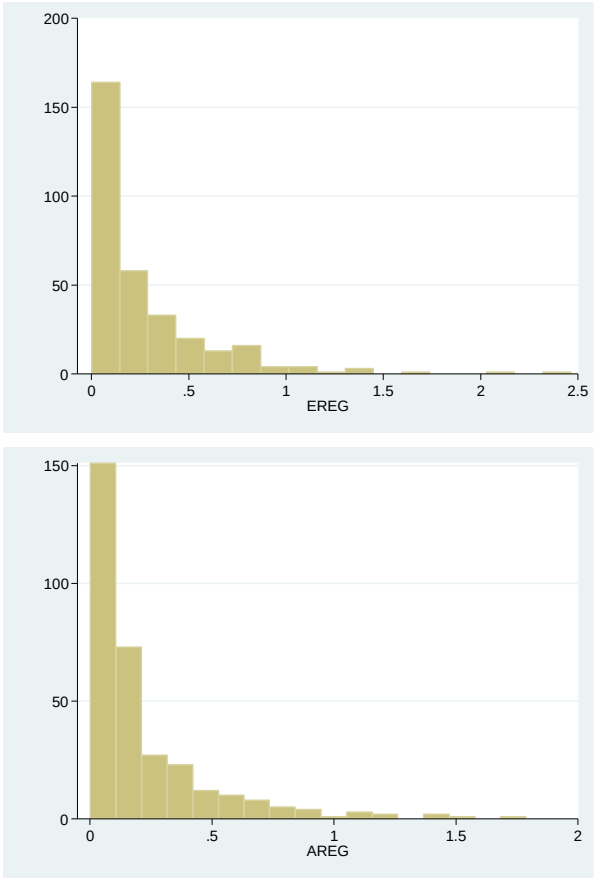

Figure 4-4 Raw ligand distribution for EREG and AREG

EREG and AREG were highly co-expressed (Spearman correlation coefficient, 0.78, p<0.0001) (fig. 4.5).

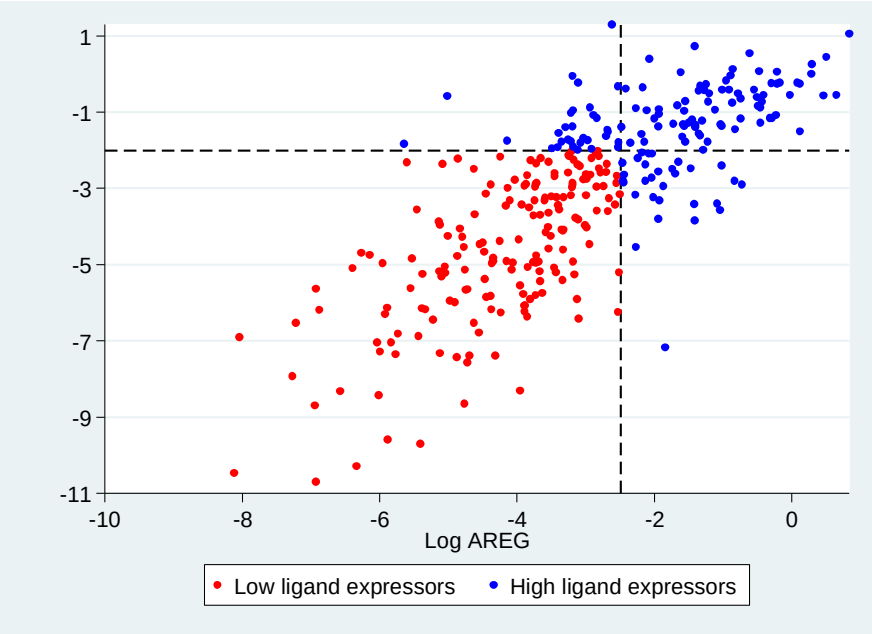

Figure 4-5 Distribution of log transformed EREG and AREG expression. High ligand expressors (top tertile or either or both) are shown in blue, low ligand expressors (bottom tertiles of both) in red.

Using the combined binary classifier, 140/323 (43%) patients were designated as "high expressors" (either ligand in the top tertile), and 183/323 (57%) "low expressors" (neither ligand in the top tertile) (Figure 4.5).

4.4.2.1.EREG and AREG distribution depending upon MEK-AKT pathway mutations

In pairwise comparisons, EREG and AREG expression were not significantly associated with *RAS* mutation status (Wilcoxon rank sum tests: AREG p=0.41; EREG p=0.31). Both ligands were significantly higher in *BRAF*-wt than the *BRAF*-mutated groups (p<0.0001, both comparisons)(fig 4.6).

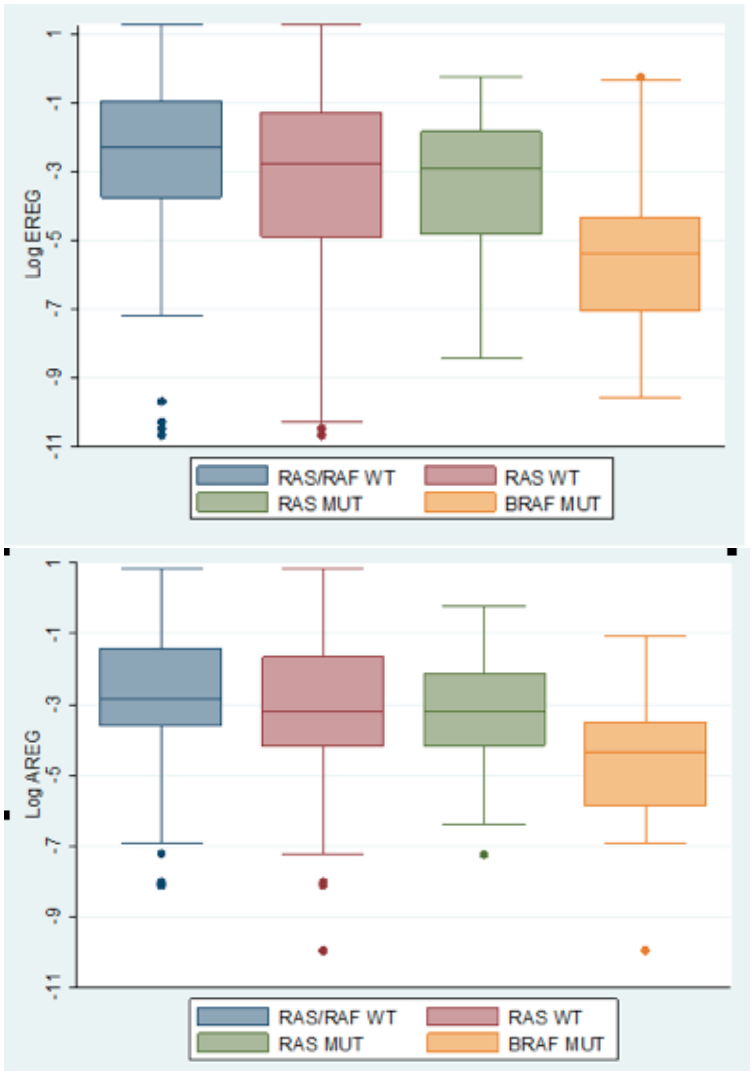

Figure 4-6 EREG and AREG expression depending upon MEK-AKT pathway mutation status

4.4.2.2.EREG and AREG expression and primary tumour location

We investigated the effect of PTL on ligand expression in the *RAS*-wt population as *BRAF*-mut status was associated with low ligand expression, and *BRAF*-mutations are commoner in right-sided tumours. In the *RAS*-wt populations, both EREG and AREG expression was significantly higher in left-sided than right-sided primary tumours ($p<0.001$, both comparisons)(fig. 4.7).

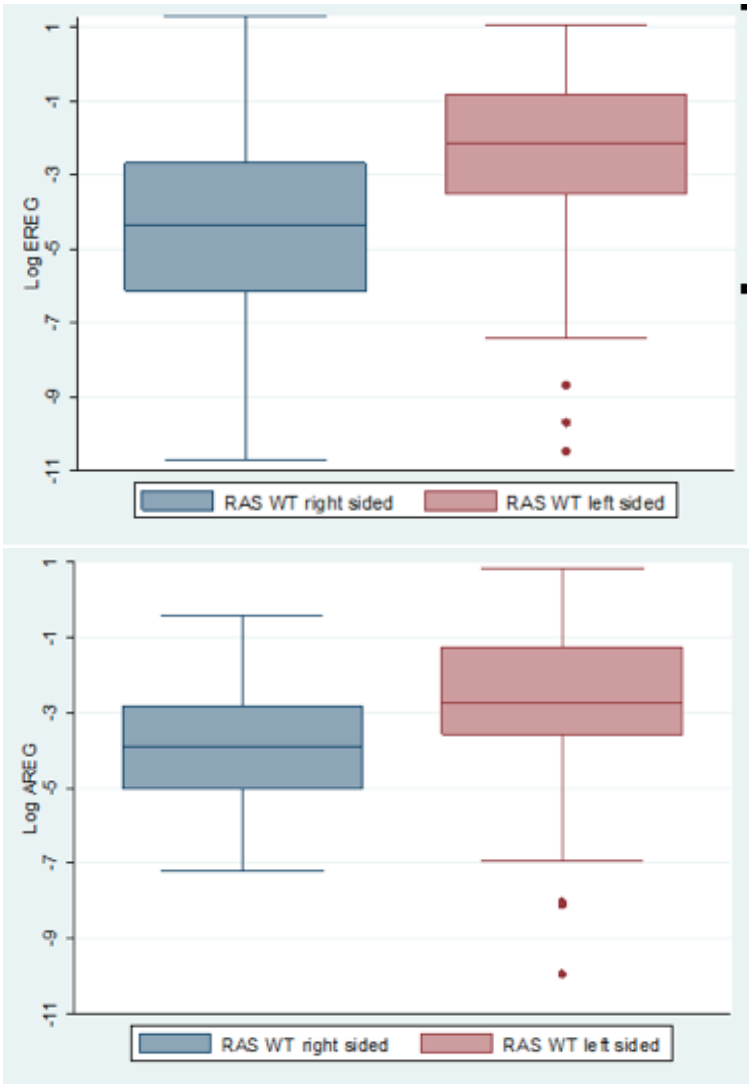

Figure 4-7 a)EREG, and b) AREG expression depending upon primary tumour location

4.4.3. The performance of EREG and AREG as a combined dichotomous marker

The primary analysis of this chapter investigates EREG and AREG as a combined dichotomous marker, dividing the population into "high expressors" (either EREG or AREG in top tertile) or "low expressors" (neither EREG nor AREG in top tertile).

4.4.3.1.Prognostic utility of the combined ligand model

There was no evidence of an effect of high ligand expression on OS (HR 0.79, 95% CI, 0.58-1.09, p=0.15) or PFS (HR 0.93, 95% CI 0.68-1.27, p=0.64), compared with low ligand expressors (table 4.2 and fig 4.8) in patients in the Ir arm of the study.

			Ir		Ir	
			Unadjusted HR (95% CI)	**p-value**	**Adjusted HR (95%CI)***	**p-value**
PFS	**All patients**	**High versus low ligand expression**	0.93 (0.68-1.27)	0.64	0.93 (0.68-1.28)	0.67
		Log AREG	0.97 (0.87-1.07)	0.50	0.94 (0.85-1.05)	0.29
		Log EREG	0.94 (0.86-1.02)	0.16	0.93 (0.85-1.01)	0.10
	RAS-wt	**High versus low ligand expression**	1.03 (0.71-1.50)	0.88	0.99 (0.68-1.46)	0.97
		Log AREG	0.96 (0.85-1.07)	0.47	0.93 (0.82-1.05)	0.23
		Log EREG	0.95 (0.86-1.04)	0.27	0.93 (0.85-1.03)	0.17
OS	**All patients**	**High versus low ligand expression**	0.79 (0.58-1.09)	0.15	0.75 (0.54-1.04)	0.08
		Log AREG	0.94 (0.85-1.03)	0.18	0.91 (0.82-1.00)	0.06
		Log EREG	0.87 (0.80-0.94)	0.001	0.84 (0.77-0.92)	<0.0005
	RAS-wt	**High versus low ligand expression**	0.86 (0.59-1.25)	0.43	0.81 (0.55-1.20)	0.30
		Log AREG	0.95 (0.85-1.06)	0.37	0.92 (0.82-1.04)	0.17
		Log EREG	0.87 (0.79-0.95)	0.002	0.84 (0.77-0.93)	<0.0005

Table 4-2 Prognostic analysis for the effect of EREG and AREG as separate variables and as a combined dichotomous model, on PFS and OS

* *adjusted for performance status, previous response and previous chemotherapy*

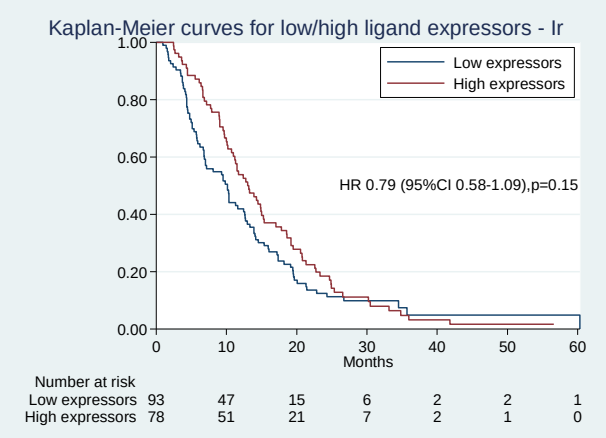

Figure 4-8 OS KM for high vs low ligand expressors (dichotomous model) in the irinotecan arm alone.

4.4.3.2. The predictive utility of the combined ligand model for panitumumab benefit in RAS-wt patients.

As previously stated, the primary hypothesis of this study was that *RAS*-wt patients with high ligand expression would have improved PFS when treated with IrPan compared with Ir alone. Conversely, low ligand expressors would have no benefit from panitumumab, even with intact EGFR signalling. The results were supportive of this hypothesis, as shown in table 4.3 and figure 4.9.

		All patients		Low ligand expression	High ligand expression
	Mutation subgroup*	**Unadjusted HR (95% CI)**	**p-value for interaction**	**Unadjusted HR (95% CI)**	**Unadjusted HR (95% CI)**
PFS**	**RAS WT**	200 events n=219 0.63 (0.47-0.84),p=0.002	0.01	111 events n=120 0.93 (0.64-1.37),p=0.73	89 events n=99 0.38 (0.24-0.61),p<0.0001
	RAS and BRAF WT	156 events n=172 0.53 (0.38-0.74),p=0.0002	0.16	71 events n=78 0.70 (0.43-1.16),p=0.17	85 events n=94 0.41 (0.26-0.66),p=0.0002
	RAS mutated	99 events n=103 1.16 (0.78-1.73),p=0.45	0.62	58 events n=62 0.99 (0.58-1.68),p=0.97	41 events n=41 1.24 (0.65-2.38),p=0.52
	BRAF mutated	46 events n=49 0.99 (0.54-1.80),p=0.96	-	42 events n=44 1.35 (0.73-2.52),p=0.34	4 events n=5 Too few to analyse
OS	**RAS WT**	202 events n=220 1.04 (0.78-1.37),p=0.80	0.11	110 events n=121 1.29 (0.88-1.89),p=0.19	92 events n=99 0.82 (0.54-1.24),p=0.35
	RAS and BRAF WT	157 events n=173 0.94 (0.68-1.29),p=0.70	0.40	70 events n=79 1.10 (0.68-1.79),p=0.69	87 events n=94 0.82 (0.54-1.26),p=0.37
	RAS mutated	101 events n=103 1.38 (0.93-2.05),p=0.11	0.30	62 events n=62 1.13 (0.67-1.89),p=0.65	39 events n=41 1.70 (0.87-3.33),p=0.12
	BRAF mutated	47 events n=49 1.25 (0.70-2.23),p=0.46	0.38	42 events n=44 1.41 (0.75-2.67),p=0.29	5 events n=5 0.24 (0.02-2.67),p=0.24

Table 4-3 Estimated crude HRs and 95% CIs for the effect of treatment on OS and PFS in low ligand expression and high ligand expression stratifying by *RAS* and *BRAF* mutation status, including likelihood ratio tests for ligand*treatment interactions.

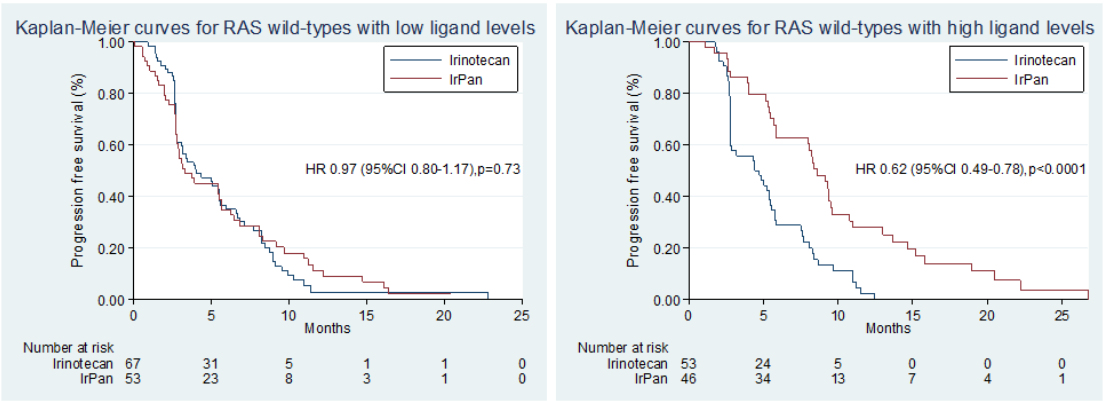

Figure 4-9 PFS Kaplan Meier curves for *RAS*-wt patients a) low ligand expressors, b) high ligand expressors (interaction p = 0.01)

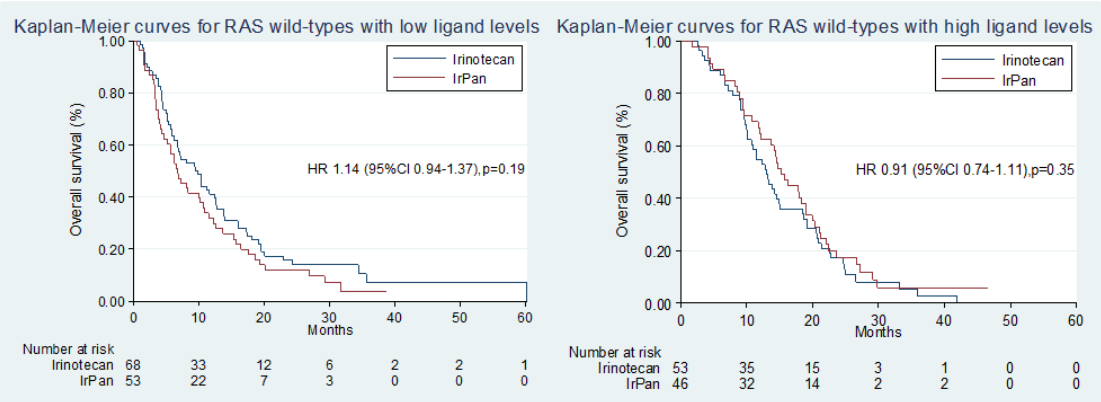

Figure 4-10 OS Kaplan Meier curves for *RAS*-wt patients a) low ligand expressors, b) high ligand expressors (interaction p = 0.11)

For *RAS*-wt high ligand expressors, IrPan had a significant effect on PFS: median 8.3 months (IrPan) vs 4.4 months (Ir); HR=0.38 [0.24–0.61], p<0.0001). However, panitumumab had no effect in *RAS*-wt patients with low ligand expression: median PFS 3.2 months (IrPan) vs 4.0 months (Ir); HR=0.93 [0.64–1.37], p=0.73). The ligand-treatment interaction was significant (p=0.01), and also following adjustment for other prognostic factors (p=0.001, data not shown).

Lesser predictive effect was seen on the secondary endpoints, OS (table 4.3 and fig. 4.10) and RR. Although *RAS*-wt high ligand expressors had improved OS outcomes with IrPan than low ligand expressors (HRs 0.82 vs 1.29, respectively), the ligand-treatment interaction was not significant (p=0.11).

Likewise for *RAS*-wt with high ligand expression, the RR was 47.7% (IrPan) vs 11.3% (Ir), relative risk = 4.22[1.87-9.52], p=0.001. Whilst for *RAS*-wt patients with low ligand expression RR was 18.9% (IrPan) vs 10.5% (Ir), relative risk = 1.81 [0.74-1.16], p=0.17). However ligand-treatment interaction for RR was not significant (p=0.17)(data not shown).

4.4.3.3.The predictive utility of the combined ligand model for panitumumab benefit in other mutation sub-groups.

Whilst the primary analysis was within the *RAS*-wt population, we also considered the predictive effect of the combined ligand model in other mutation sub-groups; *RAS*- and *BRAF*-wt, *RAS* mutant and *BRAF* mutant (table 4.3).

Within the *RAS*- and *BRAF*-wt subpopulation, high ligand expressors had similar outcomes in terms of PFS and OS as the *RAS*-wt group. Again those with low ligand expression did not have improved outcomes with IrPan (HR=0.70[0.43-1.16] p=0.17) compared with Ir alone. However the ligand/treatment interaction was not significant in this sub-group (interaction p=0.16).

We also investigated whether *RAS*-mutant patients with high ligand expression would benefit from panitumumab (table 4.3). However they gained no benefit by any endpoint.

As expected, *BRAF*-mut patients with low ligand expression did not benefit from IrPan compared with Ir. Of note, there were too few *BRAF*-mut patients with high ligand expression to adequately compare treatment effect or ligand/treatment interaction for this mutation sub-group.

4.4.4. The performance of EREG as a single continuous marker

Additional to reporting the combined ligand model we have investigated each ligand individually as a log-transformed continuous variable.

4.4.4.1.Prognostic utility of EREG alone

The prognostic analysis is reported in table 4.2. As a single continuous marker EREG was prognostic for OS (HR=0.87, [0.80–0.94], p=0.001) in patients treated with Ir alone. However it was not prognostic for PFS (HR=0.94, [0.86-1.02], p=0.16).

4.4.4.2.The predictive utility of EREG alone for panitumumab benefit

The predictive analysis for EREG and AREG as individual log-transformed continuous variables is shown in table 4.4.

			All patients	Ir	IrPan	
	Mutation subgroup		**Unadjusted HR (95% CI)**	**Unadjusted HR (95% CI)**	**Unadjusted HR (95% CI)**	**p-value for interaction**
PFS	***RAS* WT**	**Log AREG**	0·86 (0·80-0·93),p=0·0001	0·96 (0·85-1·07),p=0·47	0·79 (0·72-0·87),p<0·0001	0·008
		Log EREG	0·90 (0·84-0·95),p=0·0004	0·95 (0·86-1·04),p=0·27	0·85 (0·79-0·93),p=0·0003	0·08
	***RAS* and *BRAF* WT**	**Log AREG**	0·92 (0·84-1·01),p=0·08	1·0 (0·88-1·13),p=0·96	0·87 (0·76-0·99),p=0·04	0·15
		Log EREG	0·94 (0·88-1·01),p=0·12	1·0 (0·89-1·11),p=0·97	0·92 (0·82-1·02),p=0·11	0·29
	***RAS* mutated**	**Log AREG**	0·91 (0·80-1·05),p=0·20	0·96 (0·78-1·19),p=0·71	0·90 (0·75-1·08),p=0·25	0·46
		Log EREG	0·90 (0·80-1·01),p=0·08	0·90 (0·73-1·10),p=0·29	0·90 (0·77-1·05),p=0·17	0·85
	***BRAF* mutated**	**Log AREG**	0·77 (0·65-0·92),p=0·003	0·88 (0·63-1·24),p=0·47	0·75 (0·61-0·92),p=0·007	0·27
		Log EREG	0·84 (0·71-0·98),p=0·03	0·80 (0·59-1·09),p=0·16	0·82 (0·66-1·02),p=0·08	0·73
OS	***RAS* WT**	**Log AREG**	0·89 (0·83-0·95),0·001	0·95 (0·85-1·06),p=0·37	0·85 (0·77-0·93),p=0·0006	0·07
		Log EREG	0·86 (0·81-0·92),p<0·0001	0·87 (0·79-0·95),p=0·002	0·86 (0·79-0·93),p=0·0005	0·72
	***RAS* and *BRAF* WT**	**Log AREG**	0·96 (0·88-1·05),p=0·40	1·01 (0·89-1·14),p=0·91	0·93 (0·83-1·05),p=0·24	0·26
		Log EREG	0·93 (0·86-1·01),p=0·08	0·92 (0·83-1·03),p=0·15	0·95 (0·85-1·06),p=0·33	0·88
	***RAS* mutated**	**Log AREG**	0·86 (0·75-0·99),p=0·04	0·84 (0·67-1·04),p=0·11	0·90 (0·75-1·08),p=0·27	0·56
		Log EREG	0·91 (0·81-1·02),p=0·12	0·85 (0·69-1·04),p=0·12	0·98 (0·84-1·14),p=0·76	0·27
	***BRAF* mutated**	**Log AREG**	0·82 (0·70-0·95),p=0·01	0·87 (0·65-1·18),p=0·37	0·78 (0·65-0·95),p=0·01	0·74
		Log EREG	0·77 (0·65-0·91),p=0·002	0·74 (0·56-0·97),p=0·03	0·72 (0·56-0·93),p=0·01	0·87

Table 4-4 Estimated crude HRs and 95% CIs for the effect of log AREG and log EREG on PFS and OS in all patients, then Ir, then IrPan stratifying by BRAF and RAS mutation status including likelihood ratio tests for ligand*treatment interaction.

Within the primary analysis population (*RAS*-wt) high EREG expression was associated with improved PFS with IrPan (HR = 0.85 [0.79-0.93] p<0.001 compared with Ir alone (HR = 0.96[0.86-1.04],p=0.27). However the ligand/treatment interaction was not significant (p=0.08). Significant PFS benefit with IrPan with high EREG expression was not seen in any other mutation sub-group. Additionally there was no effect on the secondary endpoints OS or RR regardless of mutation sub-group.

4.4.5. The performance as AREG as a single continuous marker

4.4.5.1.Prognostic utility of AREG alone

As a single continuous marker, AREG was not prognostic for either OS (HR = 0.94[0.85-1.03], p=0.09) or PFS (HR = 0.97 [0.87-1.07], p=0.50) in patients treated with Ir alone (table 4.2).

When the prognostic utility both ligands were tested in a multivariate model, EREG remained prognostic for OS (p=0.001) but not PFS (p=0.11). Again AREG was not a significant prognostic marker.

4.4.5.2.The predictive utility of AREG alone for panitumumab benefit

Within the *RAS*-wt population, high AREG expression was associated with IrPan PFS benefit (HR=0.79[0.72-0.87], p<0.001), but not Ir alone (HR = 0.96[0.85-1.07], p=0.47) (table 4.4). Ligand/treatment interaction was significant (p=0.008). No other mutation sub-group were associated with IrPan PFS benefit according to AREG status.

High AREG expression in *RAS*-wt patients was associated with OS benefit with IrPan (HR = 0.79 [0.72-0.87],p<0.001) but not Ir alone (HR = 0.95[0.85-1.06], p=0.37), however the ligand/treatment interaction fell short of statistical significance (p=0.07). Again significant OS benefit was not seen in other mutation sub-groups.

4.4.6. Effect of possible confounding factors on the ligand models

BRAF-mut and right sided PTL were identified as possible confounders to the ligand model. Indeed expression of both EREG and AREG were lower in the presence of either of these factors (fig. 4.6). Therefore it is necessary to test whether the differential treatment effects by ligand expression in the primary analysis (PFS in *RAS*-wt patients) were instead being driven by these factors.

In total there were 49 *BRAF*-mut patients (15.2%) within this study cohort. This high proportion is due to the population being enriched for *KRAS*-wt patients. In this population treated with Ir alone *BRAF* was prognostic for OS (HR = 2.15 [1.34-3.46],p=0.002) and PFS (HR = 1.61[1.00-2.59], p=0.05) (data not shown).

Within the *RAS*-wt study population, there was not a significant *BRAF* mutation/ treatment interaction for PFS (p=0.21) or OS (0.42) (table 4.5).

		All patients		**Ir**	**IrPan**	
		Unadjusted HR (95% CI)	**p-value**	**Unadjusted HR (95% CI)**	**Unadjusted HR (95% CI)**	**Interaction p-value**
PFS	***BRAF* (mut vs WT)**	1.85 (1.32-2.60)	<0.0005	1.62 (0.99-2.65), p=0.05	2.28 (1.41-3.69),p=0.001	0.21
OS	***BRAF* (mut vs WT)**	2.56 (1.82-3.60)	<0.0005	2.23 (1.36-3.66), p=0.001	2.97 (1.82-4.83),p<0.0005	0.42
PFS	**Site (R vs L)**	1.13 (0.84-1.52)	0.41	0.90 (0.61-1.33), p=0.60	1.25 (0.79-1.97),p=0.34	0.29
OS	**Site (R vs L)**	1.39 (1.04-1.85)	0.03	1.38 (0.93-2.03), p=0.11	1.48 (0.95-2.29),p=0.08	0.81

Table 4-5 Estimated crude HRs and 95% Cis for the effect of BRAF (mut vs WT) and PTL (R vs L) on survival in RAS-wt patients

In total 30.6% of patients had a right-sided primary tumour. In the study population treated with Ir, PTL was not prognostic for OS (HR = 1.38 [0.93-2.03], p=0.11) or PFS (HR = 0.90[0.61-1.33], p=0.60) (table 4.5).

Within the *RAS*-wt study population, there was not a significant PTL/ treatment interaction for PFS (p=0.29) or OS (p=0.81) (table 4.5).

4.4.6.1.The impact of BRAF-mut status on the predictive ligand model

The effect of *BRAF* on the predictive ligand model in *RAS*-wt patients is demonstrated in table 4.6. The primary dichotomous classifier, and the continuous EREG and AREG model are tested.

		All patients		Ir	IrPan	p-value for interaction between ligand and treatment
		Adjusted HR (95% CI)	p-value	Adjusted HR (95% CI)	Adjusted HR (95% CI)	
PFS	Log AREG	0·88 (0·81-0·95)	0·001	0·98 (0·87-1·10),p=0·75	0·82 (0·73-0·91),p=0·0002	0·008
	BRAF	1·60 (1·12-2·27)	0·009	1·59 (0·95-2·64),p=0·08	1·73 (1·04-2·89),p=0·04	
	Log EREG	0·92 (0·86-0·98)	0·009	0·97 (0·88-1·07),p=0·50	0·88 (0·80-0·97),p=0·01	0·11
	BRAF	1·50 (1·04-2·19)	0·03	1·57 (0·93-2·64),p=0·09	1·55 (0·89-2·68),p=0·12	
	High vs. low ligand expression BRAF	0·79 (0·59-1·06) 1·74 (1·23-2·46)	0·11 0·002	1·14 (0·77-1·70),p=0·51 1·71 (1·02-2·86),p=0·04	0·56 (0·36-0·87),p=0·01 2·04 (1·25-3·34),p=0·005	0·005
OS	Log AREG	0·92 (0·85-0·99)	0·03	0·98 (0·87-1·10),p=0·69	0·88 (0·80-0·97),p=0·01	0·09
	BRAF	2·33 (1·63-3·31)	<0·0001	2·19 (1·32-3·63),p=0·002	2·50 (1·50-4·18),p=0·0005	
	Log EREG	0·89 (0·84-0·96)	0·001	0·88 (0·80-0·97),p=0·01	0·90 (0·82-0·99),p=0·04	0·88
	BRAF	2·13 (1·47-3·10)	<0·0001	2·17 (1·30-3·64),p=0·003	2·22 (1·27-3·87),p=0·005	
	High vs. low ligand expression BRAF	0·81 (0·61-1·08) 2·42 (1·71-3·44)	0·15 <0·0001	0·96 (0·65-1·41),p=0·83 2·21 (1·33-3·67),p=0·002	0·64 (0·42-0·99),p=0·04 2·73 (1·65-4·50),p<0·0001	0·10
PFS	Log AREG	0·85 (0·78-0·92)	<0·0001	0·93 (0·82-1·05),p=0·23	0·79 (0·71-0·87),p<0·0001	0·02
	R vs L	0·96 (0·71-1·30)	0·81	0·85 (0·57-1·27),p=0·43	0·96 (0·60-1·53),p=0·87	
	Log EREG	0·89 (0·83-0·95)	0·0002	0·93 (0·84-1·02),p=0·13	0·85 (0·78-0·93),p=0·0003	0·11
	R vs L	0·96 (0·70-1·30)	0·78	0·83 (0·55-1·24),p=0·36	0·94 (0·58-1·53),p=0·80	
	High vs. low ligand expression R vs L	0·72 (0·54-0·97) 1·03 (0·76-1·40)	0·03 0·84	0·99 (0·67-1·45),p=0·95 0·90 (0·60-1·33),p=0·59	0·51 (0·32-0·81),p=0·004 1·00 (0·62-1·62),p=0·99	0·01
OS	Log AREG	0·89 (0·82-0·96)	0·002	0·95 (0·84-1·08),p=0·42	0·85 (0·78-0·94),p=0·001	0·12
	R vs L	1·23 (0·91-1·65)	0·18	1·32 (0·88-1·97),p=0·18	1·23 (0·79-1·93),p=0·36	
	Log EREG	0·86 (0·81-0·92)	<0·0001	0·86 (0·78-0·95),p=0·002	0·87 (0·79-0·95),p=0·001	0·91
	R vs L	1·15 (0·85-1·56)	0·37	1·16 (0·77-1·75),p=0·47	1·18 (0·74-1·88),p=0·48	
	High vs. low ligand expression	0·76 (0·56-1·02)	0·07	0·91 (0·61-1·34),p=0·62	0·61 (0·39-0·95),p=0·03	0·15
	R vs L	1·26 (0·93-1·71)	0·14	1·34 (0·89-2·01),p=0·16	1·21 (0·76-1·94),p=0·42	

Table 4-6 *BRAF* and PTL adjusted HRs and 95% Cis for the effect of log AREG, log EREG and the dichotomous classifier on survival by treatment arm in *RAS*-wt patients.

The dichotomous ligand model continued to be a significant predictor of panitumumab PFS benefit following adjustment for *BRAF* (interaction p=0.005). Furthermore AREG remained significant following adjustment for *BRAF* (interaction p=0.008). However, as in univariate analysis, EREG was not significant (interaction p=0.11).

Following adjustment for *BRAF*, none of the ligands models reached statistical significance for panitumumab OS benefit. Therefore the ligand/treatment PFS effect appears to be independent of *BRAF*-mut status.

4.4.6.2. The impact of primary tumour location on the predictive ligand model

The effect of PTL on the predictive ligand model in *RAS*-wt patients is demonstrated in table 4.6. The dichotomous ligand model remained a significant predictor of panitumumab PFS benefit following adjustment for PTL (interaction p=0.01). Furthermore AREG was significant following adjustment for PTL (interaction p=0.02). However, as in univariate analysis, EREG was not significant (interaction p=0.11). Following adjustment for PTL, no ligand model reached statistical significance for panitumumab OS benefit.

Therefore the ligand/treatment PFS effect appears to be independent of PTL.

4.4.7. Interrogation of the combined AREG/EREG model

The binary ligand model was prospectively defined based upon two factors: to combine data from EREG and AREG as either can activate EGFR through different mechanisms, and to dichotomise at the middle/upper tertile for both and thus generating similar numbered high and low patient groups. However as this is a novel approach, we have performed exploratory analyses to test this model.

4.4.7.1. Interrogation of the combined dichotomous ligand model: testing 'either high vs neither' assumption

The 'either vs neither' model was prospectively proposed based upon the hypothesis that high expression of either EREG or AREG would lead to EGFR upregulation regardless of expression of the other ligand. Hence we have separately looked at patients classified on the basis of both ligands, or just one falling into the top tertile in *RAS*-wt patients (fig 4.11).

Given the small numbers of patients falling into the 'one ligand high' category, this was not split further into high EREG only (n=23) and high AREG only (n=16).

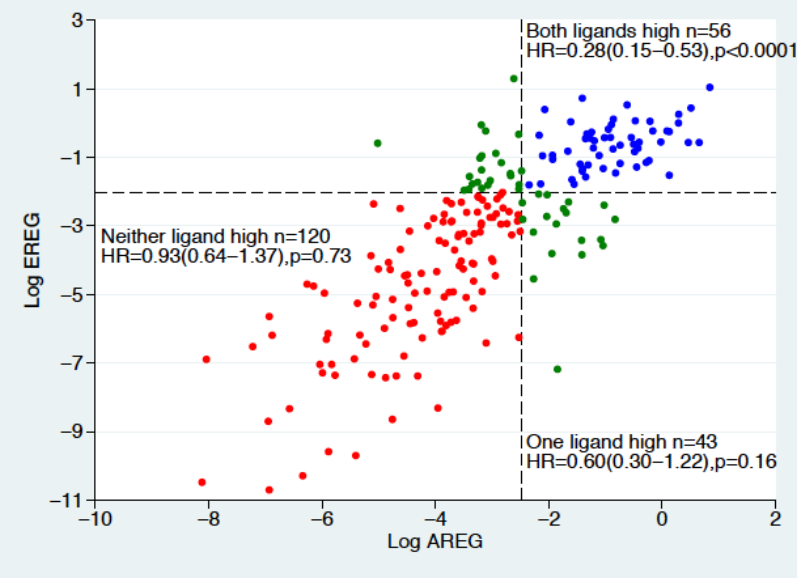

Figure 4-11 Exploratory analysis to assess the contribution of each ligand to the combined model in *RAS*-wt patients.

Those *RAS*-wt patients with high expression of both ligands had a marked PFS benefit from panitumumab (HR = 0.28[0.28-0.53], p<0.0001). For the patients with one ligand within the top tertile, panitumumab PFS benefit was less certain but a wide confidence interval was observed (HR = 0.60[0.30-1.22], p=0.16).

4.4.7.2.Interrogation of the combined dichotomous ligand model: testing the binary cut-point

The cut-point at the middle/upper tertile boundary was prospectively defined based upon the distribution of ligand expression within this population, to create roughly equal groups. Defining an optimal cut-point based upon outcomes was not feasible as this study combines data from both ligands.

This exploratory analysis therefore examines the effect of altering the cut-point for dichotomisation at the 50th, 80th and 90th centile (table 4.7)

Unadjusted HR (95% CI)	Unadjusted HR (95% CI)	p-value for interaction
Low ligand expression (<50th centile for both)	**High ligand expression (>50th centile for either)**	
79 events n=87 1·04 (0·66-1·64),p=0·87	121 events n=132 0·44 (0·30-0·65),p<0·0001	0·02
Low ligand expression (<66th centile for both)	**High ligand expression (>66th centile for either)**	
111 events n=120 0·93 (0·64-1·37), p=0·73	89 events n=99 0·38 (0·24-0·61), p<0·0001	0·01
Low ligand expression (<80th centile for both)	**High ligand expression (>80th centile for either)**	
138 events n=152 0·83 (0·58-1·17), p=0·28	62 events n=67 0·37 (0·21-0·65), p=0·0005	0·04
Low ligand expression (<90th centile for both)	**High ligand expression (>90th centile for either)**	
161 events n=176 0·71 (0·52-0·98), p=0·04	39 events n=43 0·35 (0·17-0·71), p=0·004	0·24

Table 4-7 Estimated crude HRs and 95% CIs for the effect of treatment arm (IrPan vs Ir) n PFS in RAS-wt patients stratified by ligand expression four ways to explosre different cut-offs

None of the exploratory cut-points provided superior discrimination (as defined by ligand/treatment interaction) of the benefitting/ non-benefitting population than the predefined boundary. Notably, a cut-point at the 50th centile assigned more patients to the 'high expressor' group than by the original classification (132 vs 99, respectively) and maintained separation of the benefitting/ non-benefitting populations.

4.4.7.3.Interrogation of the combined dichotomous ligand model: testing the validity of a combined rather than separate ligand approach

When analysed separately, both continuous AREG and EREG acted as significant predictive biomarkers for panitumumab PFS benefit for *RAS*-wt patients (p<0.0001 and p=0.0003 respectively, table 4.3). To assess the contribution of each ligand into the combined model, both were entered into a multivariate model. Resultingly, only AREG retained a significant effect on panitumumab PFS benefit (AREG HR =0.84 [0.71-0.99], p=0.04; EREG HR=0.95 [0.83-1.08], p=0.43).

This analysis is suggestive that if only one ligand was to be further developed then it should be AREG: indeed we observed marked PFS benefit in the 'high AREG' group (HR=0.30 [0.17-0.52],p<0.0001, n=76). In this circumstance this would mean withholding treatment to those who had an isolated high EREG, for whom treatment benefit is uncertain (HR=0.82[0.32-2.09], p=0.68, n=23, data not shown).

4.5. Discussion

This study confirms the utility of EREG and AREG mRNA expression as biomarkers to identify *RAS*-wt patients most likely to benefit from anti-EGFR therapy, and proposes a simple model – 'either high vs neither high' – to combine information from both ligands. Using this model, *RAS*-wt patients with 'neither high' gained no PFS benefit from the addition of panitumumab to chemotherapy, while those with 'either high' gained marked benefit. Lesser differences were seen in the secondary end-points of OS and RR.

Although predictive, the combined ligand model was not significantly prognostic. However, consistent with previous findings,(165, 166, 184, 399) EREG alone was prognostic for improved survival.

It has been hypothesised that EREG and AREG stimulate the EGFR though an autocrine loop with positive feedback.(392) In cell line studies blocking ligand release strongly inhibits autocrine activation of EGFR reducing the rate and persistence of EGFR cell migration;(397) additionally secondary resistance during cetuximab treatment is associated with falling ligand levels.(402) Ligand overexpression is thus a plausible proxy for tumour EGFR dependence, hence a logical positive predictive marker for anti-EGFR therapies.

Following strong signals from non-randomised studies,(165, 166, 384, 385) the predictive utility of ligands for anti-EGFR therapy has been reported in RCTs in first-line,(399) third-line,(184) and now second-line treatment of aCRC, and with both cetuximab(184, 399) and now panitumumab. Additionally, high expression of ligands correlates with anti-EGFR benefit in preclinical studies in squamous cancers.(403) This study provides strong impetus to further develop AREG and EREG into a clinically applicable test.

In the third-line CO.17 trial, *KRAS*-wt patients with high EREG expression had significant benefit with cetuximab over supportive care alone (OS HR=0.46, p<0.001; PFS HR=0.33, p<0.001); conversely no benefit was seen in *KRAS*-wt low EREG expressors (OS HR=0.93, p=0.81; PFS HR=0.7, p=0.21); however, statistical tests for interaction fell just short of statistical significance (OS p=0.08; PFS p=0.07).(184) In the first-line COIN trial, exploratory analysis of the sub-group of *KRAS*-wt patients who received FOLFOX with or without cetuximab showed significant OS benefit with cetuximab only in high EREG

patients (HR=0.33; p=0.0042), with a biomarker/treatment interaction p-value of 0.01.(399)

Similar to validation of *KRAS*(69) then extended *RAS*,(70) several steps are necessary in biomarker development: demonstration of sound scientific rationale, assay validation, optimisation of biomarker model and testing in hypothesis-led studies in intention-to-treat randomised datasets powered to test biomarker/ treatment interaction. The failure of previous work to make clinical impact is likely due to inability to adequately fulfil such steps. This data is thus critical to the development and validation of EGFR ligands as a predictive biomarker for anti-EGFR agents.

Previous work on EGFR ligands has however been vital in informing this study design. The primary analysis utilised a pre-defined combined ligand model with a cut-point at the upper/middle tertile, based upon the ligand expression distribution within the study population. Post-hoc testing of the assumptions to assess whether our model could be improved was also performed.

The *a priori* decision to use data from both ligands was based upon sound scientific rationale and preclinical evidence that either AREG or EREG can activate EGFR.(388, 395, 404) However, they are highly co-expressed so are affected by multicollinearity if treated competitively in a multivariable model. In previous studies using multivariable models, EREG outperformed AREG in some (166, 184, 399) while AREG outperformed EREG in others.(384, 398)

However, this approach is a poor basis for clinical decision-making for those patients whose ligands are not co-expressed. It was therefore prospectively elected to consider both AREG and EREG data using an “either vs neither” approach. Had we instead used a multivariable model, AREG would have emerged as the more useful ligand.

Ligand expression is a continuous variable with smooth linear distribution with clinical end-points, however a dichotomous biomarker is more practical for use in clinical practice. Previous work have utilised minimum p-values to determine the optimal ligand cut-off for study endpoints within their dataset, however no dichotomisation point has been validated. The upper/middle tertile boundaries were chosen for a pragmatic reason: to give similar numbers of patients in the high (either/or) or low (neither/nor) populations for analysis. However, the exploratory analysis of other cut-points suggests

that fortuitously this may be the best discriminator in terms of strength of marker/treatment interaction (p=0.01). A lower threshold (50th centile) would have assigned more patients to the 'high' category whilst still retaining PFS benefit (interaction p=0.02).

An 'either high or neither' approach was utilised as a proportion of patients will have high expression of just one or the other ligand (see fig. 4.4). For those in the top tertile for both EREG and AREG a greater average benefit for IrPan was seen, but the group identified for treatment smaller. Within those in the top tertile for only one ligand, some benefit was seen with IrPan (PFS HR=0.60), but this was not significant likely given the small sample size. This analysis does illustrate that by only using one ligand there is a danger that potential responders would be denied useful treatment.

Other known ligands of EGFR (EGF, TGF-α and HB-EGF) were not tested as they have not been consistently associated with anti-EGFR efficacy.(385, 405) The hypothesis-based approach ensured adequate power to detect a ligand/treatment interaction, avoiding multiple testing.

However if MEK-AKT pathway mutations, or other molecular alterations, are instead driving tumour activity, EGFR activation will be redundant, with correspondingly low ligand expression. In this study, *KRAS*- or *BRAF*-mut status was associated with lower ligand expression and lack of benefit with panitumumab, regardless of ligand expression. One previous study has reported the impact of MEK-AKT pathway mutations beyond *KRAS* on the relationship between ligands and anti-EGFR treatments.(384) Similarly, ligand expression was lower in the presence of a *KRAS* or *BRAF* mutation. Unexpectedly, high EREG expression was associated with cetuximab response in the presence of mutations, as well as in all-wt patients. Other studies saw no treatment effect in *KRAS*-mutant patients (166, 184), in keeping with these findings.

The impact of *BRAF* and PTL status on the ligand-treatment predictive model was considered as both have been shown to interact with anti-EGFR agent effect,(131, 155) as well as adjusting for significant prognostic factors within the trial. Within PICCOLO, *BRAF* was a negative predictive marker for panitumumab benefit in *KRAS*-wt patients, but PTL was not.(406) As expected, ligand expression varied significantly according to *BRAF* and PTL status however an independent predictive effect of ligands was demonstrated.

There were limitations of this study. Sufficient archived tissue was available for only 331(48%) of the 696 patients in the IrPan vs Ir randomisation in PICCOLO. Reassuringly the demographics and outcomes in the study population were consistent with the main trial analysis. As in other aCRC second-line trials of EGFR therapies,(130, 407) the marked PFS benefit seen with panitumumab in *KRAS*-wt patients in PICCOLO did not translate into improved OS, driven in part by shorter survival after progression for patients who had received panitumumab.(131) PFS was therefore chosen as the primary end-point of this study; and it is perhaps no surprise that even though *RAS*-wt high ligand expressing patients had a very marked improvement in PFS with panitumumab, this did not translate into longer OS.

Ligand overexpression potentially represents the first positive predictive marker for cetuximab and panitumumab. Increasingly aCRC treatment decisions will be guided by biomarker status and this study suggests limiting anti-EGFR treatment to *RAS*-wt patients with high ligand expression. Similar to CO-17(184) *RAS*-wt patients with low ligand expression had no benefit from panitumumab; however some clinicians may require a higher level of evidence prior to denying *RAS*-wt patients anti-EGFR treatment. Whilst potentially limiting treatment to 25% of all CRC patients, early exposure to anti-EGFR agents in this biomarker group should be considered. With several agents available for use in the treatment of aCRC, ligand data could impact the decision of whether to treat initially with anti-EGFR agents in *RAS*-wt patients.

Whilst this study serves as clinical validation of AREG and EREG mRNA as positive predictive markers for anti-EGFR agents, further development is now urgently required to bring this potentially important biomarker to routine clinical use. With alternative new agents emerging for use in the treatment of aCRC, it becomes ever more important to ensure that wherever possible our use of anti-EGFR agents is restricted those who will benefit from them, so avoiding the costs and lost opportunities of futile treatment. We propose this combined model as a clinically usable tool and would strongly advise further validation prospectively and in existing datasets.

4.6. Further Work

Further development is urgently needed before the ligand model could be incorporated into routine practice. Firstly, further validation in ideally multiple RCT datasets is required

to define the dichotomisation point between high and low ligand expressers; which patients will be selected for anti-EGFR therapy based upon this biomarker. However this decision will require consensus on what degree of benefit is required to justify treatment with an anti-EGFR drug, and conversely how low a probability of benefit justifies withholding treatment? These clinical decisions will translate into positive and negative predictive values that can then be calculated using independent data sets to establish the optimum cut-point for clinical use.

Additionally consensus on technology used for RNA extraction (dual with DNA, vs RNA alone), the RT-PCR technique (including choice of housekeeping genes) and software used for data analysis requires standardisation before this biomarker could be recommended. RT-PCR is used as a diagnostic tool in other disease areas, particularly in infectious diseases, and so routine use of this technology is feasible.

Alternatively a EREG/AREG protein-based assay would be attractive; immunohistochemistry would allow a high through-put of samples, provide rapid results, require less tumour tissue and would be more cost-effective. However it is unknown whether high mRNA expression will equate to high EREG/AREG protein expression; and also whether protein expression will be predictive of anti-EGFR agent benefit. Furthermore an EREG and/or AREG antibody would need to be reliable and reproducible, and again a consensus would need to be met on how to distinguish high and low expressors. Work is currently underway to investigate this.

Proteonomic techniques could also be utilised to assess EREG/AREG protein expression. Matrix-assisted laser desorption/ionisation-mass spectrometry imaging (MALDI-MSI) is an advanced analytical tool allowing molecular profiling and imaging of several classes of biological compounds directly within tissue sections, thus independent of antibody with high-throughput capabilities. As well as measuring EREG/ AREG, there is capability to measure expression of cell surface receptors and other ligands, providing a more comprehensive characterization than the targeted IHC approach. Whilst this technique has been used to assay the expression level and the tissue distribution of EGFR, AREG and EREG in FFPE human placenta,(408) it has not yet been fully developed using CRC FFPE tissue. Work is currently underway in this area.

Whilst this study has identified patients who have tumours that appear to be driven by EGFR signalling, further work needs to be done to characterise these tumours, particularly

to understand interplay with other ErbB receptors (HER2, HER3 and HER4). The role of HER3 shall be investigated in Chapter 4. Additionally gene copy number analysis may provide useful information about the role of EGFR copy number and response to anti-EGFR agents: a convincing relationship has not so far been demonstrated.(183, 409)

Furthermore, low ligand expression in *RAS* and *RAF*-wt tumours could be indicative of a currently unknown molecular alteration; cell studies suggest that minimal expression of EREG/AREG can be due to aberrant overactivation of *FGFR3*, leading to MEK/ERK activation.(410) Gene sequencing studies in this group may identify alternative tumour drivers and thus more effective therapeutics.

As discussed in Chapter 1, biomarker-stratified clinical trials are feasible and FOCUS-4 is currently recruiting patients to an 'all-wt' cohort, however this data suggests that further molecular stratification by ligand status may be necessary. However, further development of the ligand assay and model is required. Prospective drug trials in this EGFR-driven group is potentially exciting, particularly in the neo-adjuvant and adjuvant setting.

Chapter 5. *HER3* expression levels as a biomarker of prognosis and panitumumab benefit in aCRC

5.1. Introduction

5.1.1. ErbB/ HER receptor axis

The HER family of receptors include ErbB1 (EGFR), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4). Their activation in response to extra-cellular stimulus results in downstream signalling essential for the regulation of diverse cellular processes.(411) The EGFR and HER2 are therapeutic targets in many cancers; for example the EGFR-targeting agents cetuximab and panitumumab in aCRC. As previously discussed, intrinsic and acquired resistance limits the usefulness of these drugs. In this chapter the role of HER3 as a prognostic biomarker in aCRC and a predictive marker for panitumumab shall be examined.

5.1.2. HER-receptor family interdependence

Ligand binding stabilises the extra-cellular domain of HER receptors leading to a conformation that is favourable for dimerisation with other receptors. Following dimerisation the intracellular kinase domain is activated leading to phosphorylation of the c-terminal tail tyrosine kinase residues with subsequent recruitment of signalling molecules and activation of intracellular signalling pathways. This receptor cross-talk is characteristic of the HER receptor family: dimerisation leads to allosteric interaction (binding of a effector molecule/receptor/ protein not at its active site), activating the kinase domain of one receptor by the other without phosphorylation of its active site loop.(412) Instead dimerisation of other tyrosine kinase receptors leads to phosphorylation of the kinase domain producing an active kinase that in turn phosphorylates substrates (and subsequent signalling) until deactivated by phosphatases.(412)

Dimerisation and cross-talk between HER family members allows for diverse combinations of receptor homodimers and heterodimers with potential differences in signalling activity (fig. 5.1).(413) The heterodimers are more active than homodimers,

particularly the HER2-HER3 heterodimer.(413) The functional diversity of this receptor family is widened by the differences in ligand-binding specificity of their extra-cellular domain; the complexity of HER family signalling is not fully understood.

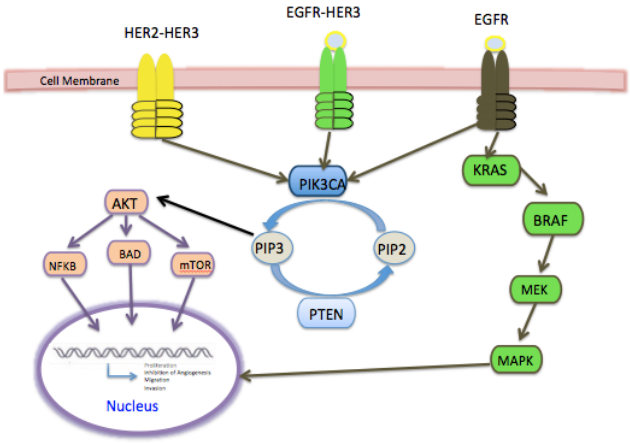

Figure 5-1 Simple representation of downstream signalling involving HER3 heterodimers. Modified from (334)

5.1.3. HER3

HER3 is encoded by the ErbB3 gene that is located on chromosome 12q13. HER3 has three regions: an NH2-terminal extracellular ligand-binding region, a transmembrane domain and an intracellular region.(414) It is physiologically expressed in a wide variety of human tissue.(415) The primary ligands for HER3 are the Neuregulin family, most notably NRG1 (also known as heregulin). Upon ligand-binding the HER3 extracellular domain adopts a structure that is highly favourable for dimerisation with other HER receptors. Inactive HER3 covers its c-terminal tail preventing its allosteric activation function.(416)

HER3 displays several differences from other HER receptors. Firstly its kinase domain lacks catalytic activity and subsequently has minimal tyrosine kinase function. Instead its kinase domain is activated as part of a kinase-domain dimer, not requiring catalytic activity.(417) HER3 cannot generate signalling through homodimerisation as its ligand-associated extra-cellular domain is an obligate heterodimer.(418) Additionally HER3's c-terminal tail becomes transphorphorylated following heterodimerisation, creating docking sites that allow accumulation of downstream signalling proteins including SHC and GRB7, leading to RAS-MAPK pathway activation; HER3 is acting as a signalling substrate. Importantly there are 6 docking sites for PI3KCA, not present on EGFR or HER2, so HER3 may be the main effector of PI3K/Akt pathway signalling.(185) However HER3

can form heterodimers in ligand-independent mechanisms;(419) NRG may only drive a small fraction of the HER3 dimerisation, as the complexes mainly exist as ligand-less monomer. This raises the possibility that HER3 mainly functions as a scaffold for the assembly of signalling complexes.(420)

5.1.4. HER3 in cancer

All members of the HER family have been linked to the development and progression of many types of cancer, either through overexpression or mutation. Given the described interdependence of this receptor family, it is likely that more than one HER protein will be involved in tumour pathogenesis. Unlike EGFR and HER2, HER3 has not been identified as a driver oncogene, but instead an important obligate partner in HER family oncogenesis. In a cell line lacking endogenous HER receptors, EGFR expression alone was not sufficient to promote cellular transformation; however subsequent co-expression of HER3 enabled transformation.(421)

HER3 appears to promote HER2-driven breast cancer; both overexpression of HER3(422) and widespread activation of Akt(423) are seen in HER2 overexpressing breast cancers. Cell line studies have demonstrated cooperation of the receptors, involving heterodimerisation, tyrosine phosphorlylation of HER3, increased PI3K recruitment and an autocrine loop involving heregulin.(424) Furthermore, down-regulation of HER3 in HER2 positive breast cancer cell lines led to G1 arrest, reduced Akt phosphorylation and a reduction of cell proliferation.(425) This suggests that the HER2/ HER3 heterodimer contributes to the pathogenesis and proliferation of HER2-overexpressing breast cancer, particularly as HER2 is unable to directly bind PI3k, activate the PI3K/Akt pathway and initiate an autocrine loop involving heregulin. This is supported by the activity of pertuzumab (a monoclonal antibody that inhibits the dimerisation of HER2 with other HER receptors) in combination with trastuzumab in HER2 positive breast cancer: the Cleopatra trial (trastuzumab +/- pertuzumab in the first-line treatment of metastatic HER2 overexpressing breast cancer) recently reported improved OS and PFS with the combination.(426) Additionally pertuzumab has shown clinical activity in ovarian cancer.(427)

The EGFR/HER3 and HER3/HER4 heterodimers are less well described. Structural analysis of the EGFR/HER3 heterodimer showed HER3 to be an allosteric activator of EGFR on dimerization.(428)

Whilst the critical role of HER3 in oncogenesis is becoming increasing apparent, fewer reports have studied the usefulness of HER3 overexpression as a prognostic marker and of a predictive marker for targeted therapy.

5.1.4.1.HER3 overexpression

The significance of HER3 overexpression has been measured in several studies, across different cancer types and using a range of techniques. Techniques for measuring overexpression include FISH, RT-PCR and protein expression using IHC. Within the studies using IHC, several different antibodies and scoring systems have been utilised. Whilst most studies report clear localisation of HER3 protein at the plasma membrane, others report cytoplasmic staining.

5.1.4.2.HER3 as a prognostic marker

The prognostic significance of HER3 gene and protein overexpression has not been studied extensively. In melanoma increased HER3 expression relates to poor prognosis and blocking melanoma cell lines with a HER3 monoclonal antibody led to reduction in proliferation and invasion.(186) High HER3 protein expression was related to inferior prognosis in gastric cancer,(429) HNSCC,(430) and ovarian cancer.(431) However in biomarker analysis from the Cleopatra trial, high HER3 mRNA related to improved prognosis but high protein expression did not.(432)

5.1.5. The impact of HER3 overexpression on the efficacy of HER-receptor targeted therapy

As the HER receptor family are interdependent, HER3 overexpression may interact with EGFR and HER2 targeted therapy efficacy. Preclinical studies examining this theory have developed two conflicting hypotheses.

5.1.5.1.HER3 overexpression as a negative predictive marker to HER-targeted therapy:

One hypothesis proposes that high expression of HER3 or its ligands may provide escape signalling following successful targeting of a receptor.(186) Preclinical models report that sensitivity to HER family TKI therapy correlates with inhibition of PI3K/Akt pathway signalling,(433) and failure to inhibit this results in drug resistance.(434, 435) Sergina and

colleagues studied the durability of Akt inhibition by gefitinib in HER2-driven cancer cells: following initial inhibition, Akt signalling resumed despite continued drug therapy.(186) Furthermore, whilst TKI treatment led to sustained inhibition of EGFR and HER2 phosphorylation (and of downstream MAPK pathway signalling), phosphorylation of HER3 in response to TKI treatment was transient, and HER3 signalling resumed despite drug treatment and effective suppression of EGFR and HER2. There was no significant expression of HER4 throughout TKI treatment.

The group suggests that drug-refractory HER2 cancers are caused by resistance at the HER3 substrate level, driven by residual HER2 kinase activity, with no evidence for non-HER receptor tyrosine kinase (eg MET) mediating this effect. The biological consequence is continued tumour growth and survival. Of note, the studies supportive of this hypothesis have only considered HER2 driven cancers.

5.1.5.2.HER3 as a positive predictive marker to HER receptor targeted therapy:

An alternative hypothesis is that HER3 expression will predict sensitivity to HER-targeted therapy. Engelman and colleagues report on preclinical models of gefitinib sensitivity in mutant EGFR NSCLC cell lines.(436) Phospho-precipitate patterns were compared between gefitinib-sensitive and resistant cell lines, and then examined interactions between the presence of p85-HER3 interaction and gefitinib sensitvity.

They found that PI3K/Akt pathway was activated in all NSCLC cell lines (EGFR mutant and wild-type) and only inhibited in gefitinib sensitive cell lines. When comparing PI3K immunoprecipitates between gefitinib-sensitive and resistant cells, they saw that HER3 couples to PI3K on gefitinib-sensitive cell lines (both EGFR wild-type and mutant) but not on resistant. In gefitinib-sensitive cell lines, Akt activity was reduced in response to inhibition by gefitinib. In contrast, only minimal HER3 expression was seen in resistant cell lines. Of note, forced expression of HER3 did not increase sensitivity to gefitinib, suggesting that the cell still also relies on EGFR for Akt pathway activation. These finding were in keeping with a further study of gefitinib sensitivity in NSCLC cell lines: the tumours most sensitive to gefitinib had the highest expression of HER3.(420, 437)

A further preclinical study examined signalling pathways that mediate erlotinib sensitivity in pancreatic and colon cancer.(438) They discovered that HER3 is co-expressed with EGFR in erlotinib-sensitive cell lines, but not in erlotinib-resistant lines. Of note, there was

no relationship with expression of other HER receptors and erlotinib sensitivity. Similar to previous work, they noted that knockdown of HER3 in erlotinib-sensitive cells led to suppression of the Akt/mTOR pathway. Both studies concluded that targeting EGFR with gefitinib and erlotinib also downregulates activity of HER3 through disruption of the EGFR/HER3 heterodimer.

Another study in HER2 positive breast cancers treated with lapatinib saw that response was associated with the presence of phosphorylated HER2 and HER3: phosphorylated HER3 was present in 10 out of 12 responders and the authors concluded that co-expression may reflect an activated state.(439)

This data suggests that the EGFR/HER3 heterodimer is an important component of EGFR signalling, likely due to its role in PIK3CA activation. Low tumour expression of HER3 will represent lack of positive feedback, instead suggesting another oncogenic driver; in such tumours EGFR-targeted therapy shall likely be ineffective.

5.1.6. The role of the HER axis in colon cancer

EGFR mutations are oncogenic drivers in some CRCs so overexpression of HER3 may be important. Few studies have tested this specific hypothesis. In a study of CRC cell lines, depletion of HER3 strongly inhibited proliferation and was associated with impaired Akt activation and reduced p-mTOR levels.(440) Additionally blocking HER3 by targeting heregulin, led to inhibition of CRC migration and invasion, and induced apoptosis.(440) Similarly, deletion of HER3 in mouse CRC models led to a dramatic reduction in tumours mediated by reduced PI3k/Akt signalling and caspase-3 mediated apoptosis.(441) Therefore HER3 appears plays a crucial role in CRC PI3K/Akt pathway signalling.

HER3 protein is overexpressed in a significant proportion of CRCs(442-444) and good correlation seen between primary tumours (80%), lymph node metastases (81%) and liver metastases (82%).(178)

One study reported that HER3 protein was overexpressed in 69% of cases. It was not an independent prognostic factor, but was inversely associated with several favourable characteristics: histological grade, tumour size, tumour depth, TNM stage, lymphatic invasion, lymph node and distant metastases. Additionally HER3 overexpression was positively correlated with HER2 protein expression and HER2 gene amplification.(445)

A further study measured HER3 mRNA expression by RT-PCR and protein expression using IHC within the same cohort. HER3 protein overexpression was associated with poor prognosis but mRNA overexpresson was not,(446) similar to HER3 analysis in Cleopatra.(432)

5.1.6.1. HER3 overexpression as a predictive biomarker for anti-EGFR therapy in aCRC

HER3 is a logical candidate biomarker for EGFR-directed therapy in aCRC. Two studies have specifically examined this hypothesis; additionally HER3 has been tested within a panel of potential candidate predictive genes.

Scartozzi and colleagues evaluated HER3 protein expression by IHC in 44 *KRAS*-wt aCRC patients treated with Ir plus cetuximab. In this cohort 52% were deemed high HER3 expressors. Low HER3 expression was associated with improved PFS (6.3 vs 2.8 mths, p<0.001) and OS (13.6 vs 10.5 months, p=0.01), compared with high HER3 expressors.(447) A follow-on paper examined EGFR copy number and IGF-1 in the same cohort. The group proposed that a favourable cetuximab tumour profile was HER3 negative, IGF-1 negative, and had EGFR copy number >2.2.(183) This profile has not been further validated.

Further studies have reported HER3 effect alongside testing several other candidate genes. Strimpakos and colleagues assessed genes in 226 aCRC patients treated with cetuximab-containing regimens.(448) Consistent with PICCOLO, high EREG and AREG expression was associated with cetuximab benefit, but HER3 was not related to any treatment end-points.

An important recent translational study assessed 14 HER-pathway candidate genes (including HER2 and HER3) in 103 patients who had participated in the CALBG 80203 trial of 1st line chemotherapy +/- cetuximab.(449) HER2 and HER3 were strongly co-expressed. When data were pooled from patients treated with or without cetuximab there was a trend toward improved survival with higher HER2 expression (HR = 0.78, p=0.071); however, within the *KRAS*-wt group both HER2 (0.66, p=0.013) and EREG (HR = 0.89, p=0.016) were significantly associated with improved OS. The analysis then looked at differential effects in the chemotherapy alone and chemotherapy/cetuximab treated *KRAS*-wt groups (total n=55). Despite the small sample size, there was a

treatment/biomarker interaction: patients with below-median HER3 expression appearing to gain overall survival benefit from cetuximab whilst those with above-median expression did not (p=0.029). However, this finding was not reproduced with PFS.

To date there are no studies measuring expression of the EGFR/HER3 heterodimer and its relation with prognosis and treatment benefit.

5.1.7. HER2 and HER4 in colorectal cancer

As with HER3, there are few studies reporting the roles of HER2 and HER4 in aCRC. Within FOCUS and PICCOLO 1342 patients were examined for HER2 overexpression both by IHC and FISH.(227) The prevalence of increased HER2 protein overexpression by IHC was 2.2%. HER2 overexpression was strongly associated with *KRAS* and *BRAF*-wt status, and the incidence of raised expression was 5.2% of this population. HER2 was not a significant prognostic marker for OS or PFS in either study. In biomarker analysis from CALB80203, high HER2 expression was associated with improved outcomes;(449) Strimpakos reported a negative prognostic effect.(448)

Bertotti examined the effect of HER2 overexpression on cetuximab efficacy using cetuximab-treated patient xenograft models.(167) HER2 amplification was seen in a sub-set of cetuximab resistant *RAS-RAF –PIK3CA*-wt cases, and concluded that HER2 overexpression was a marker of cetuximab resistance. This hypothesis was tested prospectively in a phase II study of trastuzumb and lapatinib in HER2-positive heavily pre-treated aCRC patients (HERACLES Trial).(450) In total, 646 *KRAS*-wt patients were screened and 4.3% were HER2-positive (3+ or 2+ by IHC, plus FISH positive). The primary end-point was met with 6/18 objective responses, plus stable disease for over 4 months in a further 4 patients. Clinical outcomes were best in the group which had HER2 CNV>20 copies.

HER-4 has been less investigated. Kountokouras reported that HER4 was membranous overexpression by IHC in 18.9% of CRC patients, but had no relation to any treatment end-points.(444)

Therefore the HER axis appears to be important in aCRC and receptor expression may interact with anti-EGFR therapy efficacy. HER3 likely has a pivotal role due to the

biological functions described. This chapter will investigate the role of HER3 as a prognostic biomarker, and a predictive biomarker for panitumumab benefit.

5.2. Hypothesis

The primary hypothesis of this study is that HER3 overexpression will interact with panitumumab efficacy in *RAS*-wt patients. As current evidence is limited and inconsistent, we shall consider both possibilities:

Hypothesis 1

- HER3 overexpression will be a positive predictive marker for panitumumab benefit by identifying tumours reliant on the EGFR/HER3 heterodimer for PI3K/Akt signalling.

Hypothesis 2

- HER3 overexpression will be a negative predictive marker for panitumumab. Despite targeting the EGFR with panitumumab, baseline HER3 overexpression will allow for continued signalling through heterodimerisation with residual EGFR and other HER receptors.

5.3. Methods

5.3.1. Patient population

Patients involved in the HER3 study have been treated in the IrPan vs Ir comparison in the PICCOLO trial (including patients with *KRAS* mutations/ pre-treatment with EGFR agents) with sufficient tumour material for analysis (fig 5.2).

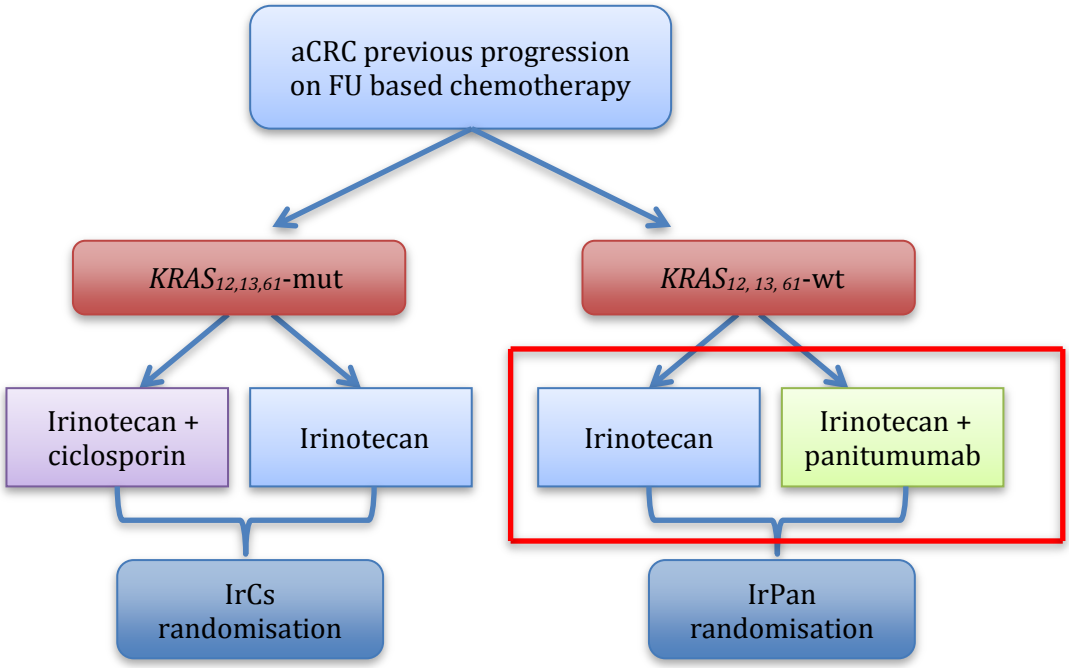

Figure 5-2 Trial schema of the PICCOLO trial demonstrating patients included within this translational study.

5.3.2. Laboratory materials and methods

Slide preparation, RNA extraction and cDNA synthesis were performed as described in Chapter 4.

HER3 expression was assessed by RT PCR, as described in Chapter 4. Again target-specific PCR efficiencies were calculated from the inter-run calibration standard curves, then utilised to calculated the calibrated normalised relative quantities of HER3 expression.(401)

5.3.3. Statistical analysis

STATA was used for all statistical analyses (*Stata Statistical Software: Release 12 (2011)*, StataCorp. College Station, Texas). Baseline patient characteristics were compared

between treatment arms using two-tailed T-tests, Wilcoxon rank sum tests (for variables with non-normally distributed frequency distributions) and Pearson Chi-squared tests (for categorical variables). Patient characteristics were compared to the whole trial population using the same tests.

Boxplots were produced for raw HER3 expression. The primary analysis tested HER3 as a continuous variable (log-transformed to base 2). An exploratory analysis was additionally performed assessing HER3 as a binary variable: similar to the EREG/AREG analysis the population was divided into "high expressors" (HER3 in top tertile) or "low expressors" (HER3 expression in the middle/lower tertile). This cut-point provided superior discrimination in terms of biomarker/ treatment interaction than seen at the median.

Three clinical endpoints were used: primary endpoint was PFS; secondary endpoints were OS and RR. PFS and RR data were unchanged from the primary trial analysis, but updated two-year OS data was used in this analysis.

HER3 expression was first assessed as a prognostic marker in patients treated with Ir alone, both using the continuous variable and the exploratory dichotomous classifier ("high expressors" vs "low expressors"), in Cox proportional hazards models.

HER3 expression was then assessed as a predictive marker for panitumumab benefit by testing for interaction between the effects of expressor status (high/low) and treatment (IrPan/Ir) on PFS and OS using the likelihood ratio test. Adjustment was performed for significant prognostic factors in the trial population (PS; response to previous therapy). Secondary analysis of predictive effects was performed in patients with *RAS* or *BRAF* mutations.

BRAF mutation and PTL (right colon vs left colon or rectum) were identified as possible confounding factors; therefore survival models were estimated for the joint effects of *BRAF* and HER3, then PTL status and HER3, for the primary continuous model and the dichotomous classifier.

Additionally to explore the relationship between HER3 expression and EGFR ligand expression, survival models were estimated for the joint effects of EREG/AREG and HER3 for the primary continuous model and the dichotomous classifier.

5.4. Results

5.4.1. Patient characteristics

Of the 696 PICCOLO patients within the Ir vs IrPan randomisation, 331 had sufficient tumour available for RNA extraction, and subsequent measurement of HER3 expression was successful in 308. Baseline characteristics by treatment arm were well balanced and reported in table 5.1.

Within the study population 285 had a disease progression event (92.5%) and 289 (93.8%) patients had died. Survival data was available for all patients.

Patient characteristic	Category	Ir (N=164)	IrPan (N=144)	All patients (308)	*RAS*-wt (n=209)
Age at randomisation (yrs)		Mean 61.9 (s.d 10.9)	Mean 61.4 (s.d 11.2)	Mean 61.6 (s.d 11.0)	Mean 61.7 (s.d 11.3)
Sex N(%)	Male	103 (62.8)	90 (62.5)	193 (62.7)	127 (60.8)
	Female	61 (37.2)	54 (37.5)	115 (37.3)	82 (39.2)
HER3 expression N(%)	Low	113 (68.9)	90 (62.5)	203 (65.9)	140 (67.0)
	High	51 (31.1)	54 (37.5)	105 (34.1)	69 (33.0)
Performance status N(%)	0-1	157 (95.7)	139 (96.5)	296 (96.1)	199 (95.2)
	2	7 (4.3)	5 (3.5)	12 (3.9)	10 (4.8)
Previous bevacizumab N(%)	No	161 (98.2)	140 (97.2)	301 (97.7)	205 (98.1)
	Yes	3 (1.8)	4 (2.8)	7 (2.3)	4 (1.9)
Previous Oxaliplatin N(%)	No	9 (5.5)	11 (7.6)	20 (6.5)	12 (5.7)
	Yes	155 (94.5)	133 (92.4)	288 (93.5)	197 (94.3)
Previous response N(%)	CR, PR or SD	98 (58.8)	86 (59.7)	184 (59.7)	131 (62.7)
	PD	46 (28.1)	43 (29.9)	89 (28.9)	52 (24.9)
	Unknown	20 (12.2)	15 (10.4)	35 (11.4)	26 (12.4)
Previous dose modifications N(%)	No	62 (37.8)	54 (37.5)	116 (37.7)	83 (39.7)
	Yes	102 (62.2)	90 (62.5)	192 (62.3)	126 (60.3)
Previous chemotherapy N(%)	No	60 (36.6)	54 (37.5)	114 (37.0)	73 (34.9)
	Yes	98 (59.8)	90 (62.5)	188 (61.0)	133 (63.6)
	Unknown	6 (3.6)	0 (0)	6 (2.0)	3 (1.4)
$KRAS_{c.12,13,61}$ N(%)	Wild-type	131 (79.9)	108 (75.0)	239 (77.6)	-
	Mutant	33 (20.1)	36 (25.0)	69 (22.4)	-
$BRAF_{V600E}$ N(%)	Wild-type	145 (88.4)	116 (80.6)	261 (84.7)	164 (78.5)
	Mutant	19 (11.6)	28 (19.4)	47 (15.3)	45 (21.5)
$NRAS_{c.12,13,61}$ N(%)	Wild-type	156 (95.1)	135 (93.8)	291 (94.5)	-
	Mutant	8 (4.9)	9 (6.2)	17 (5.5)	-
$KRAS_{c.146}$ N(%)	Wild-type	156 (95.1)	137 (95.1)	293 (95.1)	-
	Mutant	8 (4.9)	7 (4.9)	15 (4.9)	-
$PIK3CA_{exon\ 9/20}$ N(%)	Wild-type	141 (86.0)	132 (91.7)	273 (88.6)	191 (91.4)
	Mutant	23 (14.0)	12 (8.3)	35 (11.4)	18 (8.6)
No mutations detected		85 (51.8)	64 (44.4)	149 (48.4)	149 (71.3)
Any mutation detected		79 (48.2)	80 (55.6)	159 (51.6)	60 (28.7)
Overall survival time (months)		Median 11.4 (IQR 1.8-41.8)	Median 10.1 (IQR 1.5-31.8)	Median 10.8 (IQR 1.3-50.4)	Median 10.8 (IQR 1.4-41.8)
Death event N(%)	No	11 (6.7)	8 (5.6)	19 (6.2)	17 (8.1)
	Yes	153 (93.3)	136 (94.4)	289 (93.8)	192 (91.9)
Progression free survival time (months)*		Median 4.4 (IQR 0.9-14.7)	Median 4.5 (IQR 0.7-20.2)	Median 4.4 (IQR 0.4-20.5)	Median 5.1 (IQR 0.6-20.5)
Progression event N(%)	No	10 (6.1)	13 (9.0)	23 (7.5)	19 (9.1)
	Yes	154 (93.9)	131 (91.0)	285 (92.5)	190 (90.9)
Best response N(%)**	CR or PR	19 (11.6)	36 (25.0)	55 (17.8)	44 (21.1)
	SD or PD	144 (87.8)	106 (73.6)	250 (81.2)	162 (77.5)
	Unknown	1 (0.6)	2 (1.4)	3 (1.0)	3 (1.4)

Table 5-1 Characteristics of patient population by treatment arm in the whole and limited to the *RAS*-wt population.

A complete set of MEK-AKT mutation ($KRAS_{c.12\text{-}13,61,146}$ $NRAS_{c.12\text{-}13,61}$ and $BRAF_{V600E}$) genotype data was available for all cases. 209/308 (67.8%) patients were *RAS*-wt. Of the 209 *RAS*-wt patients, 45 (21.5%) had a *BRAF* mutation. The breakdown of this study population according to mutation status is shown in fig. 5.3.

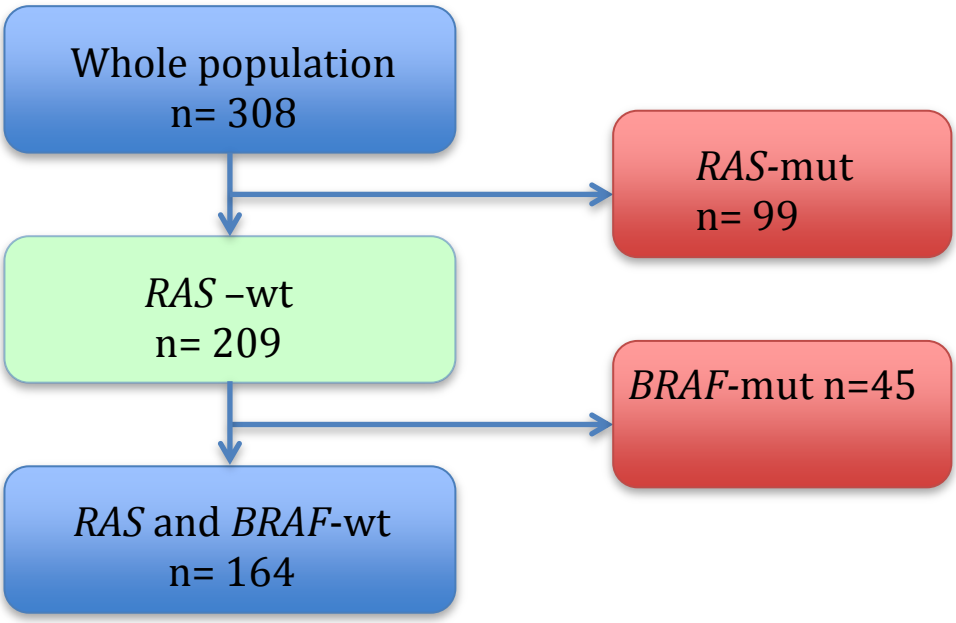

Figure 5-3 Consort diagram of study population

5.4.2. HER3 Distribution

HER3 demonstrated a skewed distribution so subsequent analyses have been log-transformed (to base 2)(fig. 5.4).

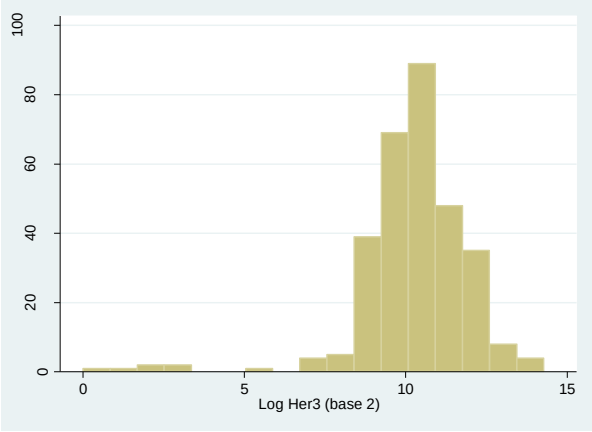

Figure 5-4 Log-transformed HER3 distribution

5.4.2.1.HER3 distribution depending upon MEK-AKT pathway mutations and PTL

In pairwise comparisons, HER3 expression was not significantly associated with *RAS* mutation status (Wilcoxon rank sum tests: HER3 p= 0.46). HER3 was higher in *BRAF*-wt than the *BRAF*-mutated groups (p<0.05)(fig 5.5).

The effect of PTL on HER3 expression was investigated as *BRAF*-mut status was associated with lower HER3 expression, and *BRAF*-mutations are commoner in right-sided tumours. In the *RAS*-wt population, HER3 expression was significantly higher in left-sided than right-sided primary tumours (p=0.02)(fig. 5.5).

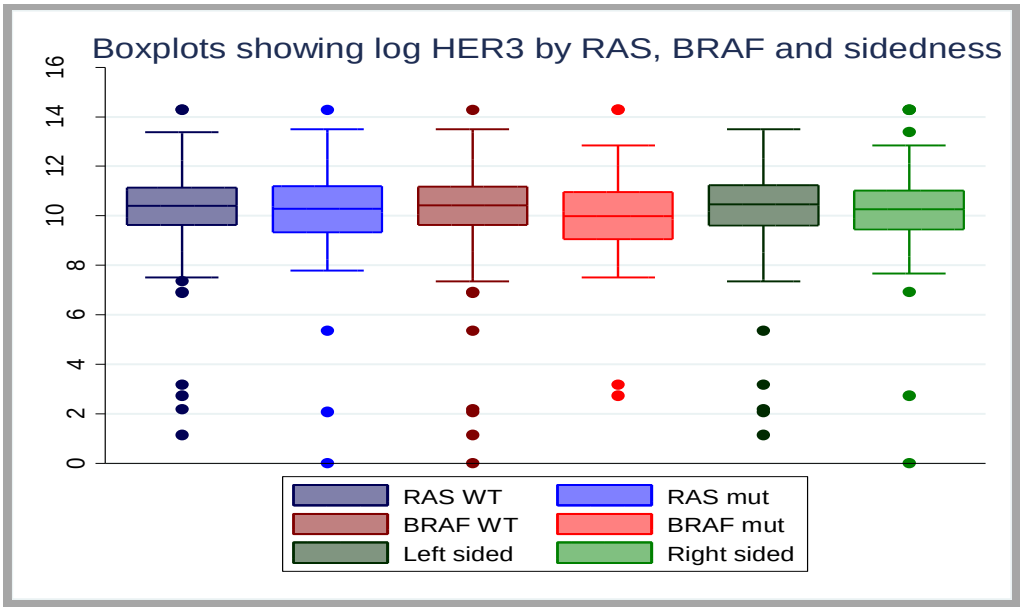

Figure 5-5 HER3 expression levels by *RAS*-status *BRAF*-status and PTL

5.4.2.2.HER3 expression and EREG and AREG expression

HER3 was positively correlated with both continuous EREG and continuous AREG within both the entire study population (both p<0.001) and the *RAS*-wt population (both p<0.001)(fig 5.6). This correlation was not seen in the *RAS*-mut population (EREG p=0.08; AREG p=0.17).

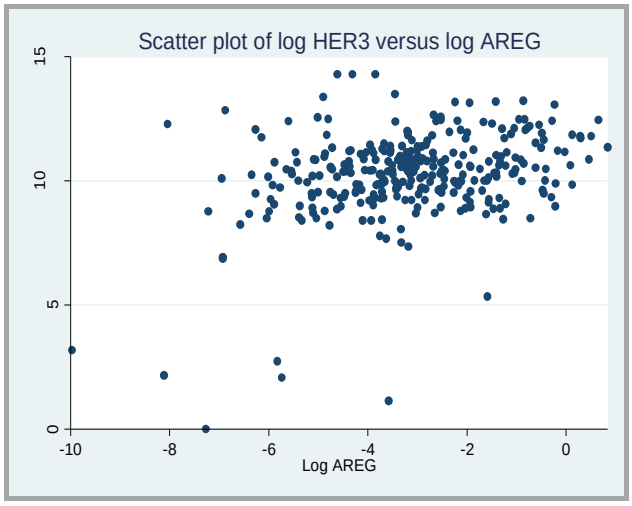

Figure.5-6 Scatter plot of log HER3 vs log AREG

5.4.3. The performance of HER3 as a single continuous marker

The primary analysis investigated HER3 as a log-transformed continuous variable, firstly as a prognostic marker then to assess its predictive utility for panitumumab benefit.

5.4.3.1.Prognostic utility of continuous HER3

The prognostic analysis is reported in table 5.2.

			Ir		Ir	
			Unadjusted HR (95% CI)	**p-value**	**Adjusted HR (95%CI)***	**p-value**
PFS	**All patients**	**High versus low HER3 expression**	1.03 (0.73-1.45)	0.86	0.98 (0.68-1.41)	0.91
		Log HER3	0.93 (0.83-1.05)	0.25	0.90 (0.80-1.02)	0.10
	***RAS*-wt**	**High versus low HER3 expression**	1.09 (0.72-1.66)	0.67	0.98 (0.63-1.52)	0.92
		Log HER3	0.96 (0.82-1.13)	0.65	0.91 (0.77-1.07)	0.25
OS	**All patients**	**High versus low HER3 expression**	0.85 (0.60-1.20)	0.35	0.79 (0.55-1.13)	0.19
		Log HER3	0.91 (0.83-0.99)	0.04	0.88 (0.80-0.97)	0.008
	***RAS*-wt**	**High versus low HER3 expression**	0.99 (0.65-1.51)	0.95	0.93 (0.60-1.44)	0.74
		Log HER3	0.93 (0.83-1.05)	0.25	0.90 (0.80-1.01)	0.07

Table 5-2 Prognostic analysis for the effect of the HER3 dichotomous classifier and log HER3 on overall survival and progression free survival.

* *adjusted for performance status, previous response and previous chemotherapy*

Continuous HER3 was prognostic for OS (HR=0.91, [0.83–0.99], p=0.04) in patients treated with Ir alone, but not for PFS (HR=0.93, [0.83-1.05], p=0.25). Within the *RAS*-wt population HER3 was not a significant prognostic marker for either OS (p=0.25) or PFS (p=0.65).

5.4.3.2.The predictive utility of continuous HER3 for panitumumab benefit

The predictive analysis for continuous log-transformed HER3 is shown in table 5.3. Our hypothesis was that high HER3 expressors would have benefit from IrPan compared with Ir alone; conversely low HER3 expressors would not. This hypothesis was supported by the predictive analysis.

		All patients		Ir		IrPan		
	Mutation subgroup	**n (events)**	**Unadjusted HR (95% CI)**	**n (events)**	**Unadjusted HR (95% CI)**	**n (events)**	**Unadjusted HR (95% CI)**	**p-value for interaction***
PFS	**All patients**	307(285)	0.88 (0.83-0.94),p<0.0005	163(154)	0.93 (0.83-1.05),p=0.25	144(131)	0.87 (0.81-0.94),p<0.0005	0.21
	RAS WT	208(190)	0.82 (0.74-0.90),p<0.0005	114(106)	0.96 (0.82-1.13),p=0.65	94(84)	0.71 (0.61-0.82),p<0.0005	0.001
	RAS mutated	99(95)	0.95 (0.87-1.05),p=0.34	49(48)	0.88 (0.72-1.07),p=0.19	50(47)	0.99 (0.88-1.12),p=0.91	0.37
	BRAF mutated	47(44)	0.79 (0.66-0.94),p=0.009	19(18)	1.03 (0.70-1.53),p=0.88	28(26)	0.77 (0.63-0.93),p=0.006	0.11
OS	**All patients**	308(289)	0.90 (0.85-0.95),p<0.0005	164(153)	0.91 (0.83-0.99),p=0.04	144(136)	0.89 (0.83-0.96),p=0.001	0.74
	RAS WT	209(192)	0.86 (0.80-0.94),p<0.0005	115(106)	0.93 (0.83-1.05),p=0.25	94(86)	0.73 (0.64-0.83),p<0.0005	0.004
	RAS mutated	99(97)	0.94 (0.86-1.03),p=0.17	49(47)	0.83 (0.70-0.98),p=0.03	50(50)	1.01 (0.89-1.14),p=0.93	0.07
	BRAF mutated	47(45)	0.81 (0.70-0.94),p=0.005	19(18)	0.89 (0.61-1.31),p=0.56	28(27)	0.80 (0.68-0.95),p=0.009	0.67

Table 5-3 Unadjusted Hazard ratios and 95% Cis for the effect of log_2 HER3 on PFS and OS in all patients, the *RAS*-wt and *RAS*-mut and *BRAF*-mut

** P-value is from a likelihood ratio test comparing a model including the main effects for log_2 Her3 and treatment (IrPan versus Ir) plus the log_2 Her3*treatment interaction term with a model including only the main effects*

Within the primary analysis population (*RAS*-wt) high HER3 expression was associated with improved PFS with IrPan (HR = 0.71 [0.61-0.82] p<0.0005) compared with Ir alone (HR = 0.96 [0.82-1.13],p=0.65); HER3/treatment interaction was significant (p=0.001). No significant PFS benefit with IrPan with high HER3 expression was seen in any other mutation sub-group.

Additionally there was an OS benefit for the addition of panitumumab to irinotecan in patients with high HER3 expression (IrPan OS HR = 0.73[0.64-0.83], p<0.0005, Ir OS HR = 0.93 [0.83-1.05], p=0.25; interaction p=0.004).

5.4.3.3.The predictive utility of continuous HER3 in other mutation sub-group

The predictive effect of the continuous HER3 model was considered in other mutation sub-groups; *RAS*- and *BRAF*-wt, *RAS* mutant and *BRAF* mutant (Table 5.3).

No other mutation sub-group benefitted from IrPan, regardless of HER3 expression levels. Of note *BRAF*-mut patients appeared to have IrPan PFS and OS benefit (p=0.006 and p=0.009 respectively) with increasing HER3 expression, however interaction testing was negative (p=0.11 and p=0.67 respectively).

5.4.4. The performance of HER3 as a combined dichotomous marker

An exploratory analysis was performed exploring HER3 as a dichotomous marker, dividing the population into "high expressors" (top tertile of HER3 expression) or "low expressors" (HER3 in the middle or lower tertile). HER3 was cut at the upper/middle tertile. This assigned 105 patients to the 'high expressor' group and 203 patients to the 'low expressor' group.

5.4.4.1.Prognostic utility of the dichotomous HER3 model

The prognostic analysis was shown in Table 5.2. Using the dichotomous classifier, high HER3 was not a significant prognostic marker for OS (HR 0.88, 95% CI, 0.60-1.20, p=0.35) or PFS (HR 1.03, 95% CI 0.73-1.45, p=0.86), compared with low HER3 expressors (table 5.2 and fig 5.7) in all patients. HER3 was not prognostic for either OS (p= 0.95) or PFS (p= 0.67) within the *RAS*-wt population.

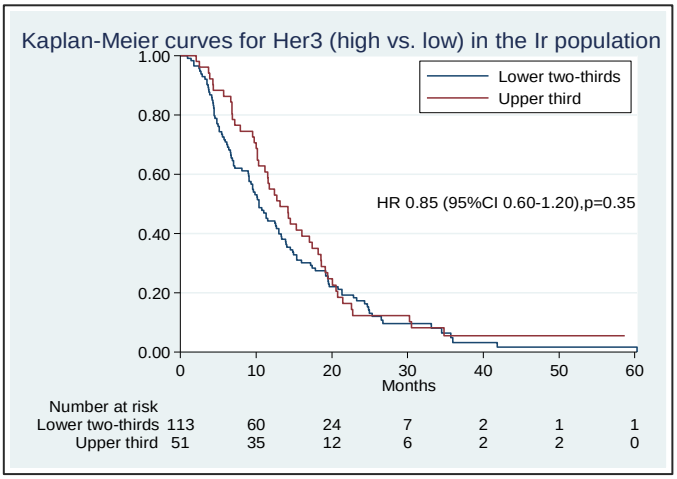

Figure.5-7 OS KM curves for high vs low HER3 expression levels

5.4.4.2.The predictive utility of the HER3 dichotomous model for panitumumab benefit in RAS-wt patients.

The predictive analysis for the exploratory HER3 dichotomous model is reported in table 5.4, figure 5.8 and figure 5.9.

		All patients		Low HER3 expression		High HER3 expression		
	Mutation subgroup*	**Number of events (number of observations)**	**Unadjusted HR (95% CI)**	**Number of events (number of observations)**	**Unadjusted HR (95% CI)**	**Number of events (number of observations)**	**Unadjusted HR (95% CI)**	**p-value for interaction**
PFS	**RAS WT**	190 (208)	0.67 (0.50-0.90) p=0.008	128 (139)	0.96 (0.67-1.38) p=0.84	62 (69)	0.33 (0.19-0.58) p<0.0005	0.002
	RAS and BRAF WT	148 (163)	0.56 (0.40-0.79) p=0.001	97 (106)	0.76 (0.49-1.17) p=0.21	51 (57)	0.36 (0.20-0.67) p=0.001	0.05
	RAS mutated	95 (99)	1.18 (0.78-1.77) p=0.43	60 (63)	1.17 (0.70-1.97) p=0.54	35 (36)	1.26 (0.63-2.52) p=0.51	0.89
	BRAF mutated	44 (47)	1.07 (0.57-1.98) p=0.84	33 (35)	1.29 (0.60-2.74) p=0.51	11 (12)	0.29 (0.06-1.40) p=0.12	0.06
OS	**RAS WT**	192 (209)	1.10 (0.82-1.46) p=0.52	130 (140)	1.56 (1.09-2.23) p=0.02	62 (69)	0.66 (0.40-1.10) p=0.11	0.01
	RAS and BRAF WT	149 (164)	0.98 (0.71-1.36) p=0.92	99 (107)	1.32 (0.86-2.02) p=0.20	50 (57)	0.74 (0.42-1.29) p=0.28	0.14
	RAS mutated	97 (99)	1.36 (0.91-2.04) p=0.14	63 (63)	1.02 (0.62-1.69) p=0.92	34 (36)	2.09 (1.0-4.37) p=0.05	0.09
	BRAF mutated	45 (47)	1.33 (0.73-2.43) p=0.35	33 (35)	1.41 (0.67-2.98) p=0.36	12 (12)	0.41 (0.11-1.57) p=0.19	0.11

Table 5-4 Estimated crude HRs and 95% Cis for the effect of treatment on OS and PFS in low HER3 expression and high HER3 expression stratifying by *RAS* and *BRAF* mutation status, including likelihood ratio tests for HER3*treatment interactions.

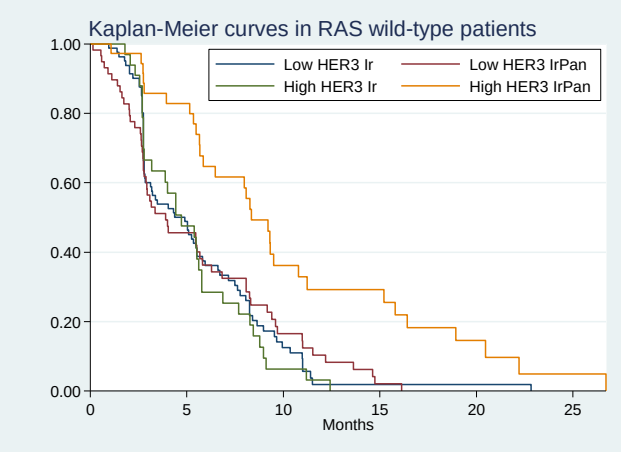

Figure 5-8 PFS KM curves for RAS-wt patients for high HER3 expressors and low HER3 expressors, treated with IrPan vs Ir (interaction p =0.002).

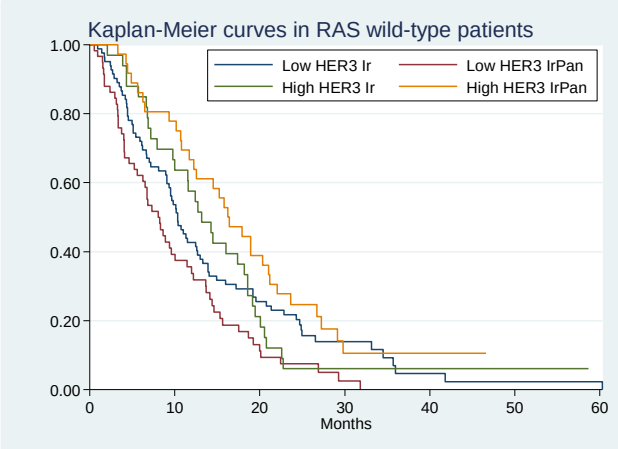

Figure 5-9 OS KM curves for RAS-wt patients for high HER3 expressors and low HER3 expressors, treated with IrPan vs Ir (interaction p=0.01).

For *RAS*-wt high HER3 expressors, IrPan had a significant effect on PFS: median 8.2 months (IrPan) vs 4.4 months (Ir); HR=0.33 [0.19–0.58], p<0.0005). However, panitumumab had no effect in *RAS*-wt patients with low HER3 expression: median PFS 3.3 months (IrPan) vs 4.3 months (Ir); HR=0.96 [0.67–1.38], p=0.84). HER3-treatment interaction was significant (p=0.002), and also following adjustment for other prognostic factors (p=0.001, data not shown).

Additionally HER3/treatment interaction was positive for OS (p=0.01) in *RAS*-wt patients. Of note, patients with low HER3 expression were harmed by treatment with panitumumab (OS HR = 1.56 (1.09-2.23), p=0.02). Instead those with high HER3 expression appeared to benefit from IrPan, but the difference was not significant (OS HR = 0.66 (0.40-1.10),

p=0.11). A lesser HER3 effect was seen for the response rate endpoint, but with high HER3 expressors having a higher likelihood of a CR or PR. (table 5.5)

Relative risks for IrPan versus Ir	Response rate (RR) %		Overall		Low Her3 expression			High Her3 expression			
Mutation subgroup	**Ir**	**IrPan**	**Unadjusted Relative risk (95% CI)**	**p-value**	**RR Ir**	**RR IrPan**	**Unadjusted Relative risk (95% CI)**	**RR Ir**	**RR IrPan**	**Unadjusted Relative risk (95% CI)**	**p-value for interaction**
RAS WT			n=217				n=138			n=68	
	10.8	32.0	2.95 (1.64-5.32)	<0.0005	11.1	24.6	2.21 (1.03-4.75)	12.1	48.6	4.01 (1.50-10.68)	0.34
RAS & BRAF WT			n=170				n=105			n=56	
	12.1	40.9	3.37 (1.85-6.14)	<0.0005	11.4	34.3	3.0 (1.35-6.66)	16.0	54.8	3.43 (1.32-8.89)	0.83
RAS mutated			n=103				n=63			n=36	
	12.0	11.3	0.94 (0.33-2.73)	0.91	6.5	9.4	1.45 (0.26-8.11)	22.2	11.1	0.50 (0.10-2.40)	0.36
BRAF mutated			n=49				n=35			n=12	
	4.8	7.1	1.50 (0.15-15.46)	0.73	9.1	8.3	0.92 (0.09-9.07)	0	0	-	-

Table 5-5 Estimated crude RRs and 95% CIs for response rate by HER3 expression status

5.4.4.3. The predictive utility of the combined ligand model for panitumumab benefit in other mutation sub-groups.

Within the *RAS*- and *BRAF*-wt subpopulation, high HER3 expressors had PFS benefit from IrPan compared with Ir (HR =0.36 [0.20-0.67], p=0.05) as seen in the *RAS*-wt group. Again those with low ligand expression had no improvement with IrPan (HR=0.70[0.43-1.16] p=0.17) compared with Ir alone. The ligand/treatment interaction was of borderline significance (p=0.05). Lesser effect was seen for OS (interaction p= 0.14).

The *RAS*-mut group did not benefit from IrPan treatment regardless of HER3 status. As seen in the continuous predictive analysis, *BRAF*-mut patients in the top tertile for HER3 expression did trend towards improved PFS and OS with IrPan compared to Ir, but again interaction testing was not significant (interaction p=0.06 and p= 0.11 respectively).

5.4.5. The impact of *BRAF*-mut status and PTL on the predictive HER3 model

The effect of *BRAF* and PTL on the predictive HER3 model in *RAS*-wt patients is demonstrated in table 5.6. Both the primary continuous and the dichotomous HER3 models are tested.

		All RAS-wt patients		Ir	IrPan	p-value for interaction between HER3 and treatment
		Adjusted HR (95% CI)	**p-value**	**Adjusted HR (95% CI)**	**Adjusted HR (95% CI)**	
PFS	**Log HER3**	0.82 (0.74-0.91)	<0.0005	0.96 (0.82-1.13),p=0.64	0.74 (0.64-0.86),p<0.0005	0.004
	BRAF	1.72 (1.21-2.43)	0.002	1.54 (0.92-2.57),p=0.10	1.82 (1.09-3.03),p=0.02	
	High vs. low HER3 expression	0.66 (0.49-0.87)	0.004	0.80 (0.54-1.19),p=0.28	0.55 (0.35-0.87),p=0.01	0.13
	BRAF	1.80 (1.27-2.55)	0.001	1.61 (0.96-2.72),p=0.07	1.99 (1.21-3.26),p=0.006	
OS	**Log HER3**	0.86 (0.79-0.93)	<0.0005	0.92 (0.82-1.03),p=0.16	0.77 (0.68-0.87),p<0.0005	0.04
	BRAF	2.56 (1.80-3.63)	<0.0005	2.21 (1.31-3.72),p=0.003	2.56 (1.53-4.29),p<0.0005	
	High vs. low HER3 expression	0.65 (0.49-0.86)	0.003	0.72 (0.48-1.07),p=0.10	0.58 (0.38-0.91),p=0.02	0.54
	BRAF	2.61 (1.83-3.72)	<0.0005	2.35 (1.38-4.01),p=0.002	2.74 (1.66-4.54),p<0.0005	
PFS	**Log HER3**	0.80 (0.73-0.89)	<0.0005	0.93 (0.79-1.09),p=0.37	0.71 (0.61-0.82),p<0.0005	0.003
	R vs L	1.09 (0.80-1.47)	0.59	0.85 (0.56-1.27),p=0.42	1.35 (0.85-2.15),p=0.21	
	High vs. low HER3 expression	0.66 (0.49-0.88)	0.005	0.81 (0.55-1.20),p=0.29	0.52 (0.33-0.81),p=0.004	0.06
	R vs L	1.09 (0.81-1.48)	0.58	0.85 (0.56-1.27),p=0.42	1.31 (0.82-2.10),p=0.25	
OS	**Log HER3**	0.85 (0.78-0.93)	<0.0005	0.92 (0.81-1.04),p=0.18	0.72 (0.63-0.83),p<0.0005	0.008
	R vs L	1.42 (1.06-1.92)	0.02	1.36 (0.91-2.02),p=0.14	1.70 (1.08-2.68),p=0.02	
	High vs. low HER3 expression	0.70 (0.52-0.93)	0.02	0.82 (0.55-1.21),p=0.32	0.55 (0.36-0.85),p=0.007	0.16
	R vs L	1.38 (1.02-1.86)	0.04	1.32 (0.88-1.98),p=0.18	1.66 (1.05-2.61),p=0.03	

Table 5-6 *BRAF* and PTL adjusted HRs and 95% CIs for the effect of log HER3 and the dichotomous classifier on survival by treatment arm in RAS-wt patients.

The primary continuous HER3 model continued to be a significant predictor of panitumumab PFS benefit following adjustment for *BRAF* (interaction p = 0.0004) and PTL (interaction p=0.003). Additionally it continued to predict panitumumab OS benefit following adjustment for BRAF (interaction p=0.04) and PTL (interaction p=0.008).

The exploratory dichotomous HER3 model was not significant following adjustment for *BRAF* and PTL for either PFS or OS.

5.4.6. Effect of EREG and AREG expression on the HER3 models

As shown in Chapter 4, a combined AREG and EREG model was shown to be a predictive biomarker for panitumumab PFS benefit in the *RAS*-wt population. However when treated individually AREG was a superior predictor over EREG as a continuous variable. To explore the relationship between HER3 and EGFR ligands as predictive markers for panitumumab benefit, a joint predictive model with HER3 and AREG has been performed, then a model assigning patients to 4 groups according to the HER3 and combined ligand dichotomous classifiers.

Exploring the continuous model, higher expression of HER3 and AREG continued to be independent predictors of IrPan PFS benefit (interaction p = 0.03 and 0.05 respectively), suggesting that they have independent effects. In the combined model lesser independent effect was seen for OS (interaction HER3 p= 0.07 and AREG p = 0.21) (table 5.7).

		Ir	IrPan	p-value for interaction
		Adjusted HR (95% CI)	**Adjusted HR (95% CI)**	
PFS	**Log HER3**	0.98 (0.83-1.16),p=0.81	0.80 (0.69-0.93),p=0.003	0.03
	Log AREG	0.97 (0.85-1.10),p=0.60	0.83 (0.74-0.92),p=0.001	0.05
OS	**Log HER3**	0.94 (0.83-1.07),p=0.36	0.78 (0.68-0.90),p=0.001	0.07
	Log AREG	0.97 (0.86-1.10),p=0.68	0.90 (0.81-0.99),p=0.04	0.21

Table 5-7 AREG adjusted HRs and 95% Cis for the effect of log HER3 on survival by treatment arm in RAS-wt population.

When dividing the *RAS*-wt population into 4 groups depending upon dichotomous HER3 and ligand status, the greatest panitumumab benefit was in those patients in the top tertile for both markers. This group a marked PFS (HR = 0.24 [0.11-0.51], p<0.005) and OS (HR = 0.36[0.18-0.73], p=0.004) improvement with IrPan compared to Ir alone (table 5.8).

		Low HER3/Low ligand	High HER3/low ligand	Low HER3/high ligand	High HER3/high ligand
PFS	**RAS-wt**	79 events n=86 1.14 (0.73-1.79),p=0.57	26 events n=27 0.61 (0.25-1.44),p=0.26	49 events n=53 0.69 (0.37-1.29),p=0.25	36 events n=42 0.24 (0.11-0.51),p<0.0005
OS	**RAS-wt**	80 events n=87 1.44 (0.92-2.26),p=0.11	24 events n=27 1.54 (0.67-3.55),p=0.31	50 events n=53 1.90 (1.02-3.55),p=0.04	38 events n=42 0.36 (0.18-0.73),p=0.004

Table 5-8 Estimated crude HRs and 95% CIs for the effect of treatment on OS and PFS for 4 groups depending upon dichotomous HER3 and ligand classification in the RAS-wt population.

Conversely, *RAS*-wt patients with neither HER3 nor ligands in the top tertile gained no benefit from panitumumab for either PFS (HR = 1.14[0.73-1.79], p=0.57) or OS (1.44 [0.92-2.26], p=0.11). Those with high HER3 but low ligands had intermediate PFS benefit for IrPan (p=0.26); a similar effect was seen in patients with low HER3 but high ligand was seen (p=0.25). Unexpectedly this group has an OS detriment with IrPan compared with Ir, despite initial PFS benefit; significant in the low HER3 and high ligand group (p=0.04).

5.4.7. Interrogation of the combined dichotomous HER3 model: testing the binary cut-point

The cut-point for the exploratory dichotomous HER3 model at the upper/middle tertile boundary was chosen as it provided the best discrimination in terms of HER3/treatment interaction.

This exploratory analysis demonstrates the effect of altering the cut-point for dichotomisation at the 50th, 80th and 90th centile (table 5.9).

	Unadjusted HR (95% CI)	Unadjusted HR (95% CI)	p-value for interaction
	Low HER3 expression (<50th centile)	**High HER3 expression (>50th centile)**	
PFS	94 events n=102 0.98 (0.64-1.49),p=0.92	96 events n=106 0.51 (0.33-0.78),p=0.002	0.05
OS	95 events n=103 1.41 (0.94-2.14),p=0.10	97 events n=106 0.96 (0.65-1.44),p=0.86	0.16
	Low HER3 expression (<66th centile)	**High HER3 expression (>66th centile)**	
PFS	128 events n=139 0.96 (0.67-1.38),p=0.84	62 events n=69 0.33 (0.19-0.58),p<0.0005	0.002
OS	130 events n=140 1.56 (1.09-2.23),p=0.02	62 events n=69 0.66 (0.40-1.10),p=0.11	0.01
	Low HER3 expression (<80th centile)	**High HER3 expression (>80th centile)**	
PFS	153 events n=167 0.84 (0.61-1.17),p=0.31	37 events n=41 0.20 (0.09-0.44),p<0.0005	0.006
OS	154 events n=168 1.32 (0.96-1.82),p=0.09	38 events n=41 0.47 (0.24-0.92),p=0.03	0.03
	Low HER3 expression (<90th centile)	**High HER3 expression (>90th centile)**	
PFS	168 events n=183 0.75 (0.55-1.03),p=0.08	22 events n=25 0.27 (0.10-0.72),p=0.008	0.13
OS	170 events n=184 1.25 (0.92-1.69),p=0.15	22 events n=25 0.51 (0.21-1.24),p=0.14	0.15

Table 5-9 Estimated crude HRs and 95% CIs for the effect of treatment arm (IrPan vs Ir) on PFS and OS in RAS-wt patients stratified by HER3 expression 4 ways to explore different cut-offs.

5.5. Discussion

In the largest randomised dataset examining HER3 in CRC, HER3 mRNA overexpression does interact with panitumumab effect, and acts as a positive predictive marker. Increasing HER3 mRNA expression was associated with improved PFS and OS in *RAS*-wt patients treated with IrPan, compared with Ir alone. Using the exploratory dichotomous model high HER3 expressors had PFS and OS benefit with IrPan compared with Ir; in contrast, *RAS*-wt low HER3 expressors did not.

High HER3 mRNA expression was a favourable prognostic marker for OS in patients treated with chemotherapy alone, consistent with other studies.(448, 449) This was not seen for PFS, or either end-point in *RAS*-wt patients. The binary HER3 model was not prognostic. In contrast HER3 protein overexpression has been associated with inferior outcomes.(447)

HER3 overexpression is the first biomarker to predict panitumumab OS benefit in PICCOLO. Consistent with other second-line trials of anti-EGFR agents in aCRC, (130, 407)

panitumumab PFS benefit was not mirrored by significant OS improvement. Other tested biomarkers have shown positive biomarker/ treatment interactions only for PFS (*RAS/RAF/PIK3CA*-wt; PFS interaction p=0.02 OS interaction p=0.06), (EREG/AREG; PFS interaction p = 0.01, OS interaction p =0.11). HER3 may identify patients most sensitive to anti-EGFR agents.

Opposing hypotheses are proposed to explain the interaction of HER3 with HER-receptor targeted agent efficacy. HER3 overexpression as a negative predictive marker would provide escape signalling via the PI3k/Akt pathway.(186) Instead HER3 as a positive predictive marker can identify tumours most reliant on EGFR signalling through autocrine feedback loops, and most likely to respond to targeted agents.(436)

HER3 mRNA overexpression has been studied in the CALBG80203 biobank.(449) In contrast to PICCOLO, high expression was associated with lack of cetuximab OS benefit, with lesser effect for PFS. A consistent finding was that high expression of HER3 and EREG were favourable prognostic markers (Ir only arm). This study has advantages of being a hypothesis-led comprehensive analysis in a larger randomised mature dataset, allowing for adjustments for likely confounders. The ability of the CALBG80203 study to demonstrate predictive markers for anti-EGFR agents is limited by small sample size, simultaneous testing of multiple genes and by not demonstrating significant cetuximab effect. Alternatively differential cetuximab and panitumumab effects may be being observed.

In a study of 84 *KRAS*-wt aCRC patients treated with cetuximab and irinotecan, high HER3 protein expression (assessed by IHC) was associated with worse outcomes,(447) also differing from the current data. Difficulties assessing HER3 protein expression by IHC have been described, particularly reproducibility: 3 different studies of HER3 using IHC in ovarian cancer reported widely different frequencies: 53.4%(431) vs 3%(451) vs 85%.(452) The clinical effect of high HER3 gene and protein expression may differ: in two studies HER3 mRNA overexpression was a favourable prognostic marker, but protein expression was not.(432, 446) Therefore the validity of testing HER3 overexpression by IHC is questionable.

Previous work using the PICCOLO biobank has been consistent with expected patterns in aCRC: enhanced IrPan effect in an 'all-wt' group (*RAS/PIK3CA/BRAF*-wt),(70, 189) and EREG/AREG as predictors of panitumumab effect.(166, 184). Patterns of HER3 expression

were as biologically anticipated: lower expression was associated with MEK-AKT pathway mutations and right PTL. With larger sample size, homogenous control arm and consistency of effect seen for both PFS and OS, we are confident that this study identifies HER3 mRNA overexpression as a novel positive predictive marker for anti-EGFR agents. However these important, but contrasting results emphasise need for validation in well-designed hypothesis based studies in further randomised datasets.

Efficacy of targeted agents will not usually be determined by one molecular alteration, so biomarker analyses must consider other possible interactions. *BRAF*-mutation,(191) PTL,(155) and EGFR ligands(166) have been associated with anti-EGFR therapy efficacy. The primary continuous HER3 model was independent of these factors.

As high EREG/ AREG was predictive of IrPan benefit in PICCOLO, and is hypothesised to be a surrogate for tumour EGFR dependence it was important to establish whether HER3 had an independent biological effect. A combined model with continuous HER3 and AREG (the stronger predictive marker) demonstrated independent effect of both for panitumumab PFS benefit; however neither for OS.

Data from both dichotomous classifiers was then combined, dividing the population into 4 according to status of both biomarkers (high vs low). The population with high expression of both had marked panitumumab PFS and OS benefit. Those with one marker high (the other low) had intermediate PFS benefit, regardless of which gene was overexpressed. An unexpected finding was that this cohort trended towards OS harm with panitumumab, despite initial PFS benefit; a significant finding in the low HER3/ high ligand population. This is of great interest as all second-line anti-EGFR studies have failed to prove an OS benefit despite PFS effect.(130, 407) Understanding the behaviour of these tumours following panitumumab progression would be valuable. Although interesting, this was a small sub-group analysis. Importantly, *RAS*-wt patients with low expression of both markers gained no panitumumab benefit for any end-point.

The prospectively planned primary analysis assessed HER3 as a continuous model. This differs to the AREG/EREG analysis that aimed to validate a clinically usable dichotomous ligand model, building upon strong prior evidence. As lesser data exists examining the role of HER3 in aCRC, the continuous model provides more power to examine to test prognostic significance and HER3/panitumumab interactions.

Having demonstrated HER3 effect using the primary model, an exploratory analysis was performed to assess HER3 as a binary measure (high vs low). HER3 is a continuous variable with no natural dichotomisation point so different cut-points were tested, and superior discrimination for biomarker/treatment interaction p-value was at the upper/middle tertile of mRNA expression. Using this model HER3 expression remained predictive of panitumumab PFS and OS benefit. However lesser effect was seen following adjustment for PTL and *BRAF*: this joint model cut the biomarker population into 4, with small numbers within each comparison. However it is likely that further cut-point optimisation may be required.

This was a prospectively designed hypothesis based study investigating the role of HER3 in aCRC, based upon strong scientific hypothesis. Instead multiple testing effects limit studies analysing multiple genes simultaneously. Study of HER2 and HER4 would be desirable; however there is strong co-expression between HER3 and HER2, plus a low prevalence of HER2 overexpression in aCRC(227) so it is unlikely that this would greatly alter HER3 effect described. Preclinical studies did not see interactions with HER4 and anti-EGFR effect. However we cannot discount that the HER2/HER3 heterodimer is driving some signalling.

This study provides further fascinating biological insights into the complex molecular interactions necessary for anti-EGFR agent effect in aCRC and the need for further stratification beyond *RAS*-status.

5.6. Further Work

This study presents strong evidence that HER3 expression is an important component of anti-EGFR activity and testing in other randomised datasets and in prospective studies is advised. Further testing would ideally utilise the same assay to allow establishment of a dichotomisation point for a clinically usable binary marker. Whilst an IHC based HER3 assay is attractive, the relationship between HER3 protein expression and anti-EGFR efficacy requires confirmation and assay development is critical to ensure reproducibility.

From data presented in Chapter 4 and Chapter 5, a panel of biomarkers can identify patients most sensitive to anti-EGFR agents: patients most likely to have marked benefit are *RAS*-wt, EREG/AREG high expressors and HER3 high expressors. In contrast *RAS*-wt patients with low expression of EREG/AREG and HER3 are unlikely to have clinical benefit

with anti-EGFR agents, and alternative treatment should be considered. *RAS*-wt patients with high expression of either EREG/AREG or HER3 appear to gain intermediate benefit to panitumumab, and we do not provide sufficient evidence to deny treatment to these patients. This panel should be assessed in further datasets. It is clear that there are a population of *RAS*-wt patients unlikely to benefit from anti-EGFR therapy.

Testing of HER2 and HER4 mRNA expression levels would be useful, however as discussed it is anticipated that lesser effect would be noted than with HER3. Too few patients with HER2 overexpressing tumours by conventional testing were present to allow a meaningful predictive analysis.(227)

Further preclinical work examining this is warranted; particularly focussing on aCRC. A study of pre- and post treatment samples would be useful to understand the response of HER axis to treatment with cetuximab. Additionally functional studies measuring expression of the EGFR/HER3 and the EGFR/HER2 heterodimer and response to therapy would be beneficial. Phospho-HER3 expression would also be interesting to investigate but requires testing in fresh tissue.

Similar to pertuzumab in HER2-driven cancers, a monoclonal antibody targeting the EGFR/HER3 heterodimer in combination with anti-EGFR agents may be of benefit to some patients.

Chapter 6. Pharmacogenomic predictors of irinotecan toxicity and efficacy

6.1. Introduction

As discussed three main types of chemotherapy are used in aCRC, but there is not a gold standard for sequencing. Each drug causes adverse effects that are significant and highly variable between patients. Prediction and avoidance of the negative effects of treatment is, and will remain, an important goal with the potential to improve patients' experiences, individualise dose levels and avoid early cessation of therapy.

Polymorphism of genes involved in drug handling is an important potential source of inter-patient variability in toxicity and efficacy. However pharmacogenetic relationships for anticancer drugs are in most cases poorly characterised and inadequately validated, so clinicians are understandably reluctant to incorporate testing into routine clinical practice.

Irinotecan is among the anticancer agents with greatest clinical need and greatest potential for benefit from pharmacogenetics, particularly as its metabolism and clearance involves several enzymes and transporters with common functional germline variants. It is commonly used either as a single agent or in combinations, and whilst well tolerated by many patients can causes severe toxicity, particularly diarrhoea.

6.1.1. Irinotecan metabolism

Irinotecan is a semisynthetic derivative of camptothecin. Campothecins interact specifically with topo-1 that relieves torsional strain in DNA by inducing reversible single-strand breaks. Its active lipophilic metabolite SN-38 is formed from irinotecan by carboxylesterase metabolism. SN-38 is inactivated mainly through glucuronidation to SN-38G, which is mediated primarily by UDP-glucuronosyl-transferases (UGTs)(fig. 6.1). The inactive form SN-38G is more soluble and polar and is eliminated principally through biliary excretion.

Other inactive catabolites are produced (APC and NPC) by the action of the CYP3A enzymes. Irinotecan is then actively effluxed out of hepatocytes by ABC transporters.(453) Hence many factors are involved in irinotecan metabolism and clearance.

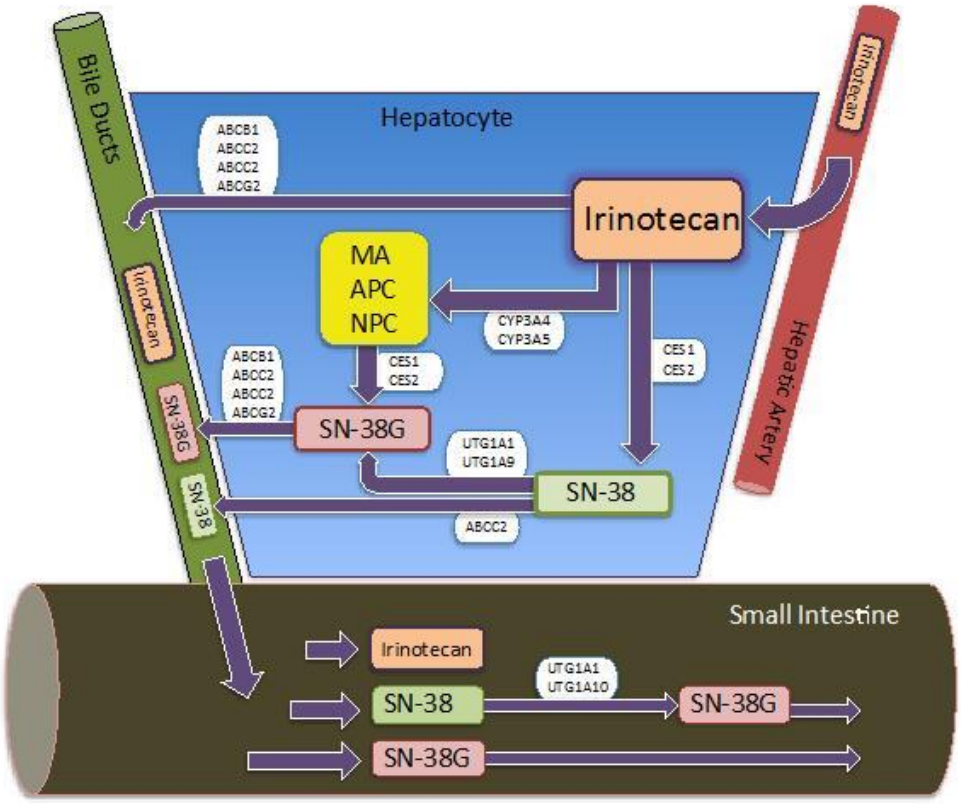

Figure 6-1 Schematic representation of irinotecan metabolism. Adapted from PharmGKB (454)

6.1.2. UGT1A gene

UGT1A is located on chromosome 2q13 and has nine functional isoforms that are expressed differentially through the gastrointestinal system. This UGT1A enzyme sub-family is encoded by a single gene complex comprising of 9 distinct exons corresponding to the N-terminal domain of the isoform that are spliced into 4 shared exons encoding the membrane building C-terminal domain. A promoter region that regulates the tissue-specific expression precedes each unique exon. Certain UGT1A isoforms have demonstrated increased inter-individual variability, most notably UGT1A1, UGT1A9 and UGT1A6.(455) Genetic variations are based upon the number of TA dinucleotide repeats: for example the number of repeats in the UGT1A1 promoter region ranges from 5-8 with 6 repeats being the most widespread (6/6 – wild type) and 7 repeats being the most

common variant, termed UGT1A1*28 (or 7/7 genotype), a TATA box variant. Increasing numbers of TA repeats lead to reduced transcription and enzyme activity.(456)

As well as its role in drug metabolism, UGT1A enzymes are responsible for the metabolism of endogenous substrates including bilirubin. UGT1A1 in particular appears to be the only enzyme capable of bilirubin glucuronidation. A number of genetic conditions are associated with defective bilirubin glucuronidation, most notably Gilbert's syndrome where the genetic defect is UGT1A1*28.(457) Gilbert's syndrome is a frequent (3-10% in Caucasians) but benign bilirubin metabolism disorder that manifests as moderate unconjugated hyperbilirubinaemia, but has no other manifestations. Instead, Criger-Najjar disease is rare and severe and occurs when glucuronidation is totally absent.(458)

There are racial variations in UGT1A1*28 alleic frequency, ranging from homozygosity rates of 8-20% in Caucasians, to less than 3% in Asian populations. Instead, the most frequent variant in the Asian population is UGT1A1*6 and UGT1A1*27; both are associated with markedly reduced enzyme activity.(456)

Given its role in drug metabolism, UGT1A1*28 homozygosity has been identified as a risk factor for adverse drug reactions due to reduced drug clearance.(459)

6.1.3. UGT1A1*28 as a biomarker for irinotecan toxicity

In vitro research demonstrated that UGT1A1 was involved in the glucuronidation of SN-38,(460) and that UGT1A1*28 homozygotes have twice the concentration of plasma SN-38 than wild-types following irinotecan administration.(461) Increased plasma levels lead to myelosuppression and enteric injury, manifesting as diarrhoea. Clinical studies have investigated the association between UGT1A1*28 and irinotecan toxicity; larger studies are summarised in table 6.1. Based upon preliminary reports, in 2005 the FDA approved a genetic test for UGT1A1*28 homozygosity. This was based upon the results of two studies using different regimens, with a total of 132 patients and 13 UGT1A1*28 homozygous patients, of whom 8 experienced toxicity.(8, 462)

Further studies have been performed to further qualify this relationship but results have been difficult to interpret due to small numbers, limited statistical power, heterogenous irinotecan schedules and populations. However meta-analysis of nine studies including 821 patients found a higher risk of haematological toxicity in UGT1A1*28 homozygotes

compared with wild types but only when treated with medium (OR = 3.22, 95%CI =1.52-6.81, p=0.08) and high (OR = 27.8, 95%CI – 4.0-195, p= 0.005) doses of irinotecan.(463) A further recent meta-analysis of 20 small studies (including 1760 patients) found a higher risk for UGT1A1*28 homozygotes compared with wild type for severe diarrhoea (OR = 3.69, CI = 2.00-6.83, p<0.001).(464) However data from three large randomised trials: FOCUS (465), N9741 (466) and PETACC3,(467) together including 2,500 patients concluded that the association to be unclear and at best modest. Perhaps most importantly, whilst UGT1A1*28 may predict modestly increased risk of irinotecan, the majority of patients who experience irinotecan toxicity are not predicted by the test. Therefore, whilst it may have a role as a component of a multivariable tool, it appears to be of little value as a clinical risk tool in isolation.

6.1.1. UGT1A1*28 status and irinotecan efficacy

A further area of interest is the association between genotype and tumour response to irinotecan. As with toxicity, inconsistent results have been reported for the impact of homozygosity on irinotecan efficacy. Initial prospective work conducted in 238 aCRC patients treated with FOLFIRI reported an association of this genotype with improved RR and PFS compared with wild-types.(468) A meta-analysis described that response rates for irinotecan by UGT1A1*28 status were 41% for wild-types, 45% for heterozygotes and 70% for homozygotes.(461)

However, contradictory reports exist. In McLeod's study, homozygote patients treated IrOx had inferior RR than wild-types, which may be reflective of poorer tolerance with increased SN-38 exposure.(236) A meta-analysis of 10 studies did not demonstrate significant association between UGT1A1*28 status and RR or PFS with irinotecan, taking into account dosing heterogeneity between the studies.(469) Again, no relationship with efficacy was demonstrated in the larger studies shown in table 6.1.(215, 468, 470, 471)

Study	No of Ir treated patients	Setting	Regimens	Ir dose	UGT1A1*28 frequency	Association with severe neutropaenia	Association with ≥3 diarrhoea	Association with Ir efficacy	Ref
Braun, 2007	413, Caucasian	1st line palliative RCT	Ir +/- FU	350mg/m^2 or 180mg/m (FOLFIRI)	9.4%	No	No	No	(215)
Kweekel, 2008	218 Caucasian	1st/2nd line	Ir +/- capecitabine	350mg/m^2 or 180mg/m^2 (FOLFIRI)	6.4%	Yes (febrile neut)	No	No	(471)
Cecchin, 2009	250, Caucasian	All lines. Prospective observational.	IrFU	180mg/m^2 (FOLFIRI)	8.8%	Yes (gd 3& 4), but not in MV model	No	No	(468)
Bioge, 2010	262 Caucasian	1st/2nd line palliative RCT	FOLFIRI	180mg/m^2 (FOLFIRI)	10.2%	No	No	No	(470)
McLeod, 2011	221 Caucasian	1st line palliative RCT	IFL & IrOx	IFL:125mg/m^2 IrOx:200mg/m^2	9%	Yes – only in IrOx treated group	No	No	(236)
Ichikawa, 2015	1312 Asian	20% 1st line; 80% 2nd/3rd line palliative. Prospective observational	Ir +/-FU combinations, or Ir plus biological	Multiple regimens: 150 mg/m^2 bi-weekly or FU combin 125-150mg/m^2	11.1%	Yes (gd 3& 4 reported)	Yes	n/a	(472)

Table 6-1 Summary of large studies assessing UGT1A1*28 and irinotecan sensitivity

This data has led to prospective dose escalation studies based upon UGT1A1*28 genotype information. It is hypothesised that the maximum tolerated dose (MTD) in wild-type patients may be higher than currently recommended, and hence they are being under-dosed: wild-type patients were able to tolerate doses 15-19% higher than UGT1A1*28 heterozygotes.(473, 474) However the maximum tolerated dose (MTD) of irinotecan in homozygotes was 28% lower than used in FOLFIRI.(474)

A genotype-directed phase I study was conducted to individualise irinotecan dose based upon UGT1A1*28 status. Each genotype represented three distinct groups with MTD ranging from 400 to 850mg, with no change in toxicity profile. The authors suggest that this work conclusively identifies the role of UGT1A1*28 as a major determinant of safe irinotecan dosing. Homozygous patients were initially given a 20% dose reduction, however all patients (n=3) experienced a dose limiting toxicity. Instead a 40% dose reduction to 400mg was tolerable for 3 weekly Ir dosing. Efficacy was a secondary end-point given the small sample size, but will be explored in a genotype-directed phase II study.(475)

6.1.2. Variants in the UGT1A1 gene and irinotecan sensitivity

Other biologically plausible genetic factors involved in irinotecan metabolism and clearance should be considered. There are further genetic variations in the UGT1A1 sequence such as promoter region variations, and haplotypes or combinations of variants may be important in phenotypic expression.

Additional genotype variation in Gilbert's syndrome has been studied: 300 Gilbert's patients were genotyped for UGT1A and transporter SNPs, along with controls.(476) Gilbert's patients were homozygous for UGT1A1*28, but additionally had variants in other UGT1A genes, including SNPs in UGT1A3, UGT1A6 and UGT1A9, with 76% being homozygous for all tested SNPs. There were no differences in transporter gene variants between Gilbert's and normal subjects, perhaps as expected as impaired glucuronidation underlies the disease. This study concluded that there are more extensive haplotypes of UGT1A gene variants in Gilberts's than previously considered, and also that these variations may also represent a risk factor to drug treatment in a non-Gilbert's population.(476)

The role of variations in extended UGT1A1 SNPs and irinotecan sensitivity has been reported in clinical studies. In 65 irinotecan treated patients, a promoter SNP at position -3156C was a superior predictor of UGT1A1 status than the TA promoter repeat,(477) and was associated both grade 4 neutropaenia and ≥ grade 3 febrile neutropaenia in irinotecan treated patients.(236) This is a common variant, with 10% of Caucasians and African Americans being homozygotes.(478) UGT1A1*93 has also been associated with neutropaenia.(479)

6.1.3. Variants in other UGT genes and irinotecan sensitivity

Additional UGT1A genes including UGT1A7 and UGT1A9, also mediate SN-38 glucuronidation so polymorphism may result in impaired irinotecan excretion (figure 6.1). Homozygosity for UGT1A7*3 or UGT1A9*22 variants were associated with severe diarrhoea following the first cycle of irinotecan. (480) In a separate study these variants were associated with haematological toxicity with irinotecan.(481)

In a prospective study of 167 aCRC patients treated with FOLFIRI, 21 candidate SNPs in the UGT1A gene were tested and associated with toxicity then tested in a validation cohort (n=250). Several UGT1A variants, including UGT1A1828 were associated with toxicity, but in multivariate analysis no UGT1A1 SNPs remained significant. However 3 SNPs located in the central region were associated with neutropaenia.(482)

6.1.4. Other biologically plausible polymorphisms and irinotecan sensitivity

Whilst variants in UGT1A SNPs may reduce efficiency of irinotecan metabolism, polymorphisms of genes involved in irinotecan excretion, may impact on toxicity and efficacy (fig 6.1). In particular variations in transporter gene SNPs are of interest: polymorphisms of ABCB1,(483) ABCC1,(468) ABCG2,(484) SLCO1B1,(479) and CYP3A4(236) have been associated with irinotecan toxicity.

We therefore propose that given the widespread and increasing use of irinotecan, there is a need for a large study population, comprehensively examining the relationship between biologically plausible SNPs in this pathway and irinotecan toxicity and efficacy.

The PICCOLO trial provides an excellent opportunity to perform a wider, methodical, hypothesis-based investigation of irinotecan pharmacogenetics in a large clinical trial biobank of patients treated consistently with single-agent irinotecan schedule, with high-quality clinical data.

6.1.5. Genotyping methods

Classical genotyping assays (such as gel electrophoresis-based procedures, allele-specific PCR and restriction fragment length polymorphism analysis) are generally labour intensive and hence unsuitable for high-throughput analyses. Integrated fluidic circuits can incorporate and significantly reduce fluid-handling steps of experiments plus enhance reliability of workflows. This technology can now be applied to genotyping using the Fluidigm 48.48 SNPtype Genotyping chip.(485) Nanolitre-scale volumes of reagents and samples are channelled through into chambers where distinct genotyping reactions can be run (fig. 6.2). This technology has the potential to run 9216 individual genotyping reactions in one experiment, with considerable savings in time, reagents and total DNA required.(486) In a study comparing the Fluidigm 48.48 SNPtype chip to TaqMan, there was 100% concordance between genotype results for 20 SNPs in 90 samples.(486) In a project genotyping 1698 cases, call rate (assigning a sample to a genotype of a tested SNP) using the Fluidigm 48.48 SNPtype chip was 97.6%.(486) However this technology is not suitable for genotyping all SNPs, such as when variation is due to dinucleotide repeats (for example, UGT1A1*28).

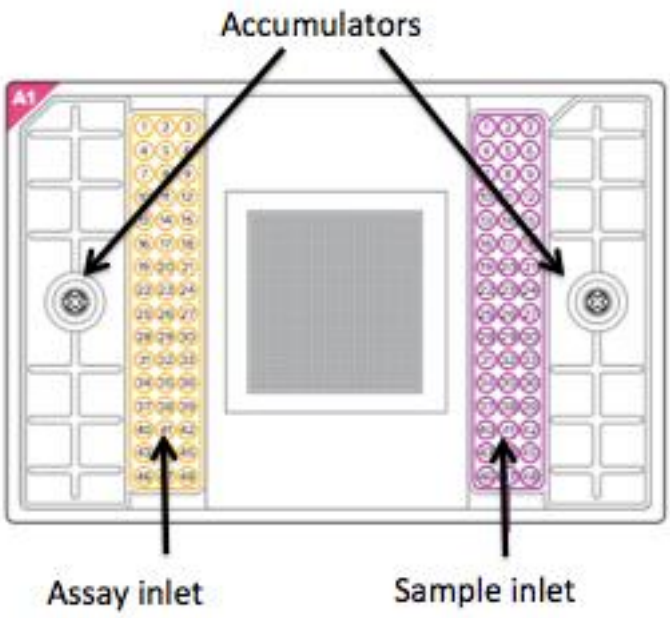

Figure 6-2 Fluidigm 48.48 SNP plate. Modified from (487)

6.2. Study aims

This study aims are to:

- Validate or refute whether UGT1A1*28 homozygosity (7/7 genotype) is associated with a higher rate of irinotecan (Ir) toxicity or efficacy compared with wild type (6/6 genotype) or heterozygotes (6/7 genotype).
- Examine whether other UGT1A genotypes correlate with Ir toxicity and better explain the observed association with UGT1A1*28.
- Discover whether further biologically plausible SNPs are associated with Ir toxicity or efficacy.
- To investigate whether baseline bilirubin level is associated with Ir toxicity and efficacy.

6.3. Methods

6.3.1. Patient Population:

This biomarker study includes patients in the PICCOLO trial treated with single agent Ir (350mg/m^2 every 3 weeks, or irinotecan 300mg/m^2 every 3 weeks if aged>70 or PS2),

and for whom baseline blood samples have been received, or tumour DNA was available (figure 6.3). Of note, patients with a bilirubin over 25µmol/l on trial screening bloods were excluded from the PICCOLO trial. Only patients from the Ir alone arm were included as panitumab and ciclosporin have overlapping toxicities, and hence a causative role of candidiate SNPs in irinotecan toxicity could not be proven.

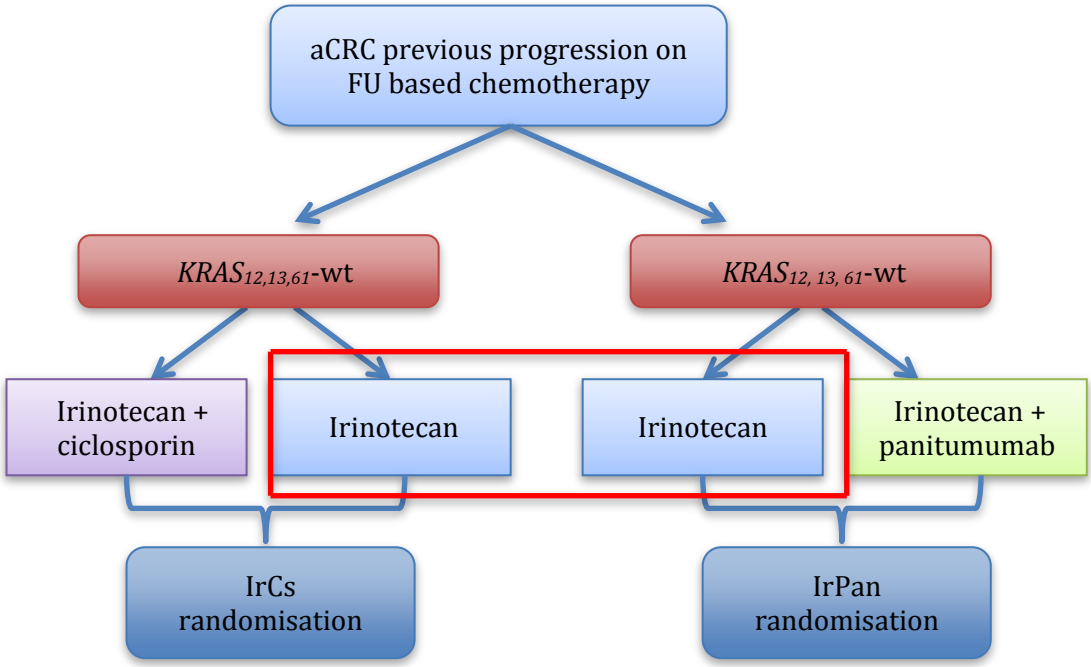

Figure 6-3 PICCOLO trial schema, demonstrating patients involved in this translational study

Assessment of toxicity

Within the trial haematological and non-haematological toxicity were assessed and recorded at the start of each cycle using Common Toxicity Criteria for Adverse Events (CTCAE) version 2.0.(488) Serious adverse events were recorded at any time. Detailed toxicity management was provided in the PICCOLO trial protocol: it was recommended that ongoing toxicity of ≥ grade 2 at the start of a planned chemotherapy cycle incurred a one week delay. Chemotherapy doses were reduced by 20% after an episode of grade ≥ 3 toxicity, or after two delays for grade 2 toxicity.(489)

Toxicity end-points only included events that occurred within the first 12 weeks of treatment. Toxicity data was assessed for each individual study patient from original PICCOLO CRFs by JS (blinded to genotype), assessing each endpoint and using clinical judgement to determine cases of severe toxicity. The primary endpoint was any dose delay

or reduction, any ≥grade non-haematological toxicity or any grade 4 neutropaenia, as this information was accurately recorded and reflects clinically relevant toxicity.(215) Information on the CRFs was used to judge whether a dose delay or reduction was secondary to toxicity. Other end-points are detailed in section 6.3.

6.3.2. Methods for selecting SNPs

Candidate SNPs were selected due to biological plausibility or had previous evidence suggesting a role in Ir sensitivity, and are listed in table 6.2 and table 6.3. Tagging SNPs (ht-SNP) were selected where assessment of candidate SNPs were in the same clusters/ in linkage disequilibrium, or when there would be technical difficulty of SNP genotype assessment.

SNP	Gene	Chromosome name	Chromosome position	Minor Allele	MAF (1000 genomes global (%)	Reason for inclusion	Reference
UGT1A1 gene							
rs887829	UGT1A1	2	234668570	A	31.1	UGT TATA box promoter	(8, 236)
rs3755319	UGT1A1	2	234601965		47.4	ht-SNP; variation associated with impaired bilirubin conjugation	(490)
rs4124874	UGT1A1	2	234665659		49.7	ht-SNP in promoter region; involved in bilirubin conjugation	(490)
rs4148323	UGT1A1	2	234669144	A	5	Associated with low SN-38 AUC	(483)
rs10929302	UGT1A1	2	234665782		26.2	ht-SNP in PBREM region	(490)
Other UGT1A polymorphism							
rs2008595	UGT1A3	2	234637192	G	49.9	htSNP involved in bilirubin conjugation	(490)
rs3806596	UGT1A3	2	234637707	G	49.7	htSNP involved in bilirubin conjugation	(490)
rs3732217	UGT1A4	2	234628270	T	8.4	htSNP involved in bilirubin conjugation	(490)
rs1105880	UGT1A6	2	234601965	C	31.9	htSNP involved in bilirubin conjugation	(490)
rs7577677	UGT1A7	2	234590616	A	29.2	Variant associated with Ir toxicity	(491)
rs7586110	UGT1A7	2	234590527	G	29.2	Functional promoter involved in Ir toxicity	(492)
rs11692021	UGT1A7	2	234591205		29.3	Variant associated with Ir toxicity	(493)
rs17868323	UGT1A7	2	234590970	T	49.8	Variant associated with Ir toxicity	(493)
rs17868324	UGT1A7	2	234590975	G	45.5	Coding exon 1; variant associated with Ir toxicity	(493)
rs1042597	UGT1A8	2	234526871	G	28.7	htSNP involved in bilirubin conjugation	(490)
rs1042605	UGT1A8	2	234527118	G	12.4	htSNP involved in bilirubin conjugation	(490)
rs2741046	UGT1A9	2	234580249	C	14.1	htSNP involved in bilirubin conjugation	(468, 490)
rs2741048	UGT1A9	2	234581748	C	44.5	htSNP involved in bilirubin conjugation	(490)
rs4663871	UGT1A9	2	234581587	A	18.3	htSNP involved in bilirubin conjugation	(468, 490)
rs6731242	UGT1A9	2	234578693	G	17.4	htSNP involved in bilirubin conjugation	(490)
rs13418420	UGT1A9	2	234578762	C	31.0	htSNP involved in bilirubin conjugation	(490)
rs17862856	UGT1A9	2	234582077	A	18.4	htSNP involved in bilirubin conjugation	(490)
rs1823803	UGT1A10	2	234539111	T	36.3	htSNP involved in bilirubin conjugation	(490)
rs2741031	UGT1A10	2	234538716	T	**26.9**	htSNP involved in bilirubin conjugation	(490)

Table 6-2 Details of additional SNPs in the UGT1A gene to be tested in this study:

SNP	Gene	Chromosome name	Position on chromosome	Minor allele	MAF (HAP)	Reason for inclusion	Reference
Transporter genes							
rs1045642	ABCB1	7	87138645	T	39.7	Involved in Ir excretion; variation associated with toxicity	(483)
rs1128503	ABCB1	7	87179601	T	42.2	Involved in Ir excretion; variation associated with increased SN38 levels	(494)
rs2032582	ABCB1	7	87160618	T	34.0	Involved in Ir excretion; variation associated with increased efflux activity	(483)
rs212088	ABCC1	16	16232433	T	16.9	Involved in Ir excretion; variation associated with toxicity	(483)
rs35588	ABCC1	16	16139878	G	43.7	Involved in Ir excretion; variations lead to change in Ir AUC	(495)
rs35605	ABCC1	16	234527118		18.1	Involved in Ir excretion; variations lead to change in Ir AUC	(468)
rs2230671	ABCC1	16	16228242	A	18.7	Involved in Ir excretion; variations lead to change in Ir AUC	(495)
rs3765129	ABCC1	16	16149901	T	10.8	Involved in Ir excretion; variations lead to change in Ir AUC	(495)
rs717620	ABCC2	10	101542578	A	17.6	Involved in Ir excretion; variations lead to increased efflux activity	(483)
rs3740066	ABCC2	10	101604207	A	30.4	Involved in Ir excretion; variations lead to increased efflux activity	(483)
rs562	ABCC5	3	183637845		48.8	Transporter gene; deletions associated with toxicity	(496)
rs425215	ABCG1	21	43707101	G	43.3	Deletions associated with inflammation and Ir toxicity	(496)
rs12721627	CYP3A4	7	99366093		0.1	Key Ir transporter	(497)
rs2740574	CYP3A4	7	99382096	G	20.1	Key Ir transporter	(497)
rs4986910	CYP3A4	7	99358524	O-C-H	0.3	Splice variant – variation alters Ir AUC	(236)
rs776746	CYP3A5	7	99270539	A	31.2	Key Ir transporter	(497)
rs10264272	CYP3A5	7	99262855		4.5	Splice variant- variation alters Ir AUC -	(236)
rs2306283	SLCO1B1	12	21329738	C	40.5	Membrane transporter- variation alters Ir AUC	(495)
rs4149056	SLCO1B1	12	21331549	C	12.3	Membrane transporter – variation alters Ir AUC	(495)
Additional genes involved with bilirubin metabolism							
rs1358503	SEMA3c	7	**80599142**	C		Variation associated with rising bilirubin levels	(498)
rs1517114	C8orf34	8	69389217	O-C-H		Variation associated with rising bilirubin levels	(498)

Table 6-3 Details of further candidate SNPs to be tested in this study

6.3.3. Laboratory methods and assays

6.3.3.1.DNA extraction from blood

Trial blood samples had been previously processed to separate and remove plasma layer, then had been stored at -80^{0}C. DNA extraction was performed according to the manufacturer's instructions using the QIAamp blood kit (Qiagen, Hilden, Germany), including proteinase K digestion and ethanol precipitation. DNA was stored at 4 ^{0}C prior to analysis.

*6.3.3.2. Assessment of UGT1A1*28 genotyping*

The number of TA repeats in the UGT1A1 promoter was determined by PCR-fragment length polymorphism (PCR-FLP). PCR primers were designed using a reference sequence from the UCSC Genome Browser(499) and the online design tool Primer3.(500) Sequences are 5'-3'. The forward primer has a 5' fam label:

- Forward Fam-TCACGTGACACAGTCAAACATT
- Reverse AGAGGTTCGCCCTCTCCTAC

PCR reactions contained 10µl of Qiagen HotStarTaq Master Mix (Qiagen, Crawley, UK. Cat. no. 203445), additional magnesium chloride to a final concentration of 2mM, 200nM each of forward and reverse primers, 2ul of genomic DNA and sufficient water to make a final volume of 20µl. Thermal cycling conditions were 94°C for 12 minutes followed by 40 cycles of 94°C for 10 seconds, 55°C for 20 seconds and 72°C for 20 seconds.

PCR fragment length was determined by diluting PCR reactions 1:1000 in water and running on an Applied BioSystems 3130xl. Fragment length, and therefore genotype, was determined by comparison with reference samples of known genotype.

6.3.3.3. Genotyping for extended SNPs.

Assay primers were designed by Fluidigm based upon the extended SNP list in Table 6.2 and 6.3, and are described in Appendix table 1.

Preparation of the chip, assay mixes and sample pre-mixes (requiring 5.0μL of genomic DNA) was performed as per the manufacturer's instructions.(487) Assay mix and sample mix were pipetted into the chip as shown in figure 6.2.

For the PCR reactions, the chip was loaded into a Fluidigm FC1 Cycler and the SNPtype 48x48 Fast v1.pcl protocol selected and run. When completed, the chip was removed from the FC1 Cycler. The EP1 Reader Data Collection Software was selected, and the chip loaded into the reader, and the genotyping programme selected.

6.3.4. Statistical analysis

6.3.4.1.Study Endpoints:

All end points are dichotomous, that is, each patient was classified as having experienced the toxic event or not.

Primary toxicity measure:

- A dose delay or a dose reduction as a result of chemotherapy toxicity, or non-haematological grade (gd) 3 toxicity (not alopecia), gd 4 neutropaenia, and included patients who have failed treatment by 12 weeks.

Secondary toxicity measures:

- Any ≥CTCAE grade 3 non-haematological toxicity reported within the first 12 weeks of chemotherapy.
- CTCAE grade 4 neutropaenia reported within the first 12 weeks of chemotherapy or admission with febrile neutropaenia.
- Rates of severe toxicity (toxic deaths, grade 4 neutropaenic sepsis, grade 4 toxicity)

Primary efficacy measure:

- RECIST response at 12 weeks (CR/PR vs SD/PD)

Secondary efficacy measures:

- RECIST response at 12 weeks (CR/PR vs SD/PD)
- PFS
- OS

Statistical analyses

The study design was a retrospective analysis of the prospective PICCOLO trial. In total, 371 patients allocated to single agent Ir with a DNA sample were available for the analysis. The polymorphisms selected for analysis are listed in tables 6.2 and 6.3. Each polymorphism was tested to see whether the genotype distributions conform to Hardy–Weinberg equilibrium (HWE).

*6.3.4.2.Replication of association with UGT1A1*28*

Firstly the rate of toxicity in each genotype group separately was be assessed and then the risk of toxicity in UGT1A1*28 homozygotes compared to the risk in the other genotype groups combined. Thirdly the heterozygotes and rare homozygotes were combined and compared with the common homozygotes. Given the sample size, incidence of severe toxicity in the trial and expected 10% frequency of UGT1A*28 homozygosity, there was 80% power to detect a relative risk of 2.3 or more for UGT1A1*28 homozygotes (one-sided test, p=0.05).

6.3.5. Further planned analyses

Genotyping work is still in progress for some samples in this study, and optimisation of extended SNP assays ongoing. The planned analyses for the completed study are described below.

6.3.5.1.Fine mapping of UGT1A region

Linkage disequilibrium will be estimated between the UGT1A polymorphisms. Multiple regression will be used to test for independent associations between genotype and toxicity, including the UGT1A1*28 genotype in the model. In addition haplotype analysis will be carried out by comparing estimated haplotype frequencies between groups with differing outcomes based on the likelihood ratio test. This analysis will identify the pattern of genotypes at this locus that best explains the association with toxicity.

6.3.5.2.SNPs in other genes

For a SNP with common minor allele frequency (~0.3), there will be 80% power to detect a relative risk of 2.0 at a nominal 0.05 significance level and of 2.4 at a significance level of 0.0025, allowing for multiple testing.

6.3.5.3.Predictive model

A predictive model has been developed where genotype data will be sequentially entered into the model, starting with the best-fitting UGT1A genotype, and retained if the model is significantly improved as determined using the likelihood ratio test. The genotype model will then be compared with one containing bilirubin to understand whether genotyping can explain additional variability in toxicity. The predictive model will require further validation in another study.

Poisson regression with robust error will be used to estimate the relative risks and confidence intervals. All regression analyses will be performed using STATA. The analysis of the secondary outcome (response rates) will be similarly conducted. The majority of patients in the trial are self-reported as white-European and so population stratification is unlikely to be an issue; however a sensitivity analysis will be performed where the study population is restricted to those patients self-reporting to be white European.

6.4. Results

There were UGT1A1*28 results for 361 samples (44 tumour DNA and 317 germline DNA) including 20 matched germline and tumour DNA duplicates. Concordance was 96% for these samples. In total, samples and clinical data were available for 333 of the 371 irinotecan treated patients (figure 6.4)

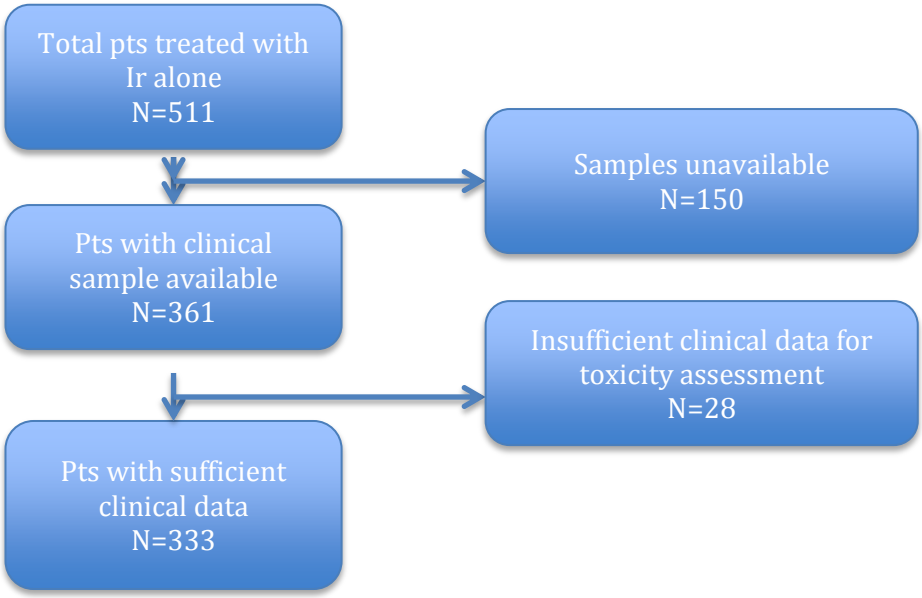

Figure 6-4 Consort diagram of patient's involved in this study

6.4.1. Study population characteristics

Characteristics of the study population are listed in table 6.4.

		Study population (n=371)
Age at randomisation (years)		Mean 62.5 (s.d 10.5)
Sex N(%)	Male Female	244 (66.3) 124 (33.7)
Performance status N(%)	0-1 2	349 (94.1) 22 (5.9)
Previous bevacizumab N(%)	No Yes	359 (96.8) 12 (3.2)
Previous oxaliplatin N(%)	No Yes	16 (4.4) 352 (95.6)
Previous response N(%)	CR/PR/SD PD	236 (69.6) 103 (30.4)
Previous dose modifications N(%)	No Yes	148 (39.9) 223 (60.1)
Previous chemotherapy N(%)	No Yes	173 (47.3) 193 (52.7)
Primary endpoint* N(%)	No Yes Total	198 (56.7) 151 (43.3) 349 (100)
Grade 3 toxicity N(%)	No Yes Total	248 (71.1) 101 (28.9) 349 (100)
Very severe toxicity N(%)	No Yes Total	308 (88.5) 40 (11.5) 348 (100)
Maximum diarrhoea N(%)	0-2 >2 Total	297 (86.1) 48 (13.9) 345 (100)
Grade 2 diarrhoea N(%)	No Yes Total	214 (61.9) 132 (38.1) 346 (100)
Grade 4 neutropaenia or admission with febrile neutropaenia N(%)	No Yes Total	327 (94.2) 20 (5.8) 347 (100)
Cessation of therapy following 12 weeks N(%)	Continued Stopped Total	154 (43.9) 197 (56.1) 351 (100)
Complete or partial response N(%)	No Yes Total	325 (88.8) 41 (11.2) 366 (100)
Disease control rates N(%)	PD CR/PR/SD Total	181 (49.5) 185 (50.5) 366 (100)

Table 6-4 Patient characteristics within this biomarker study

6.4.2. UGT1A1*28 genotype analysis

rs8175347 was successfully genotyped in 333/371 samples (89.7%), consistent with previous work.(236) Fragment length data for each genotype is shown in figure 6.5.

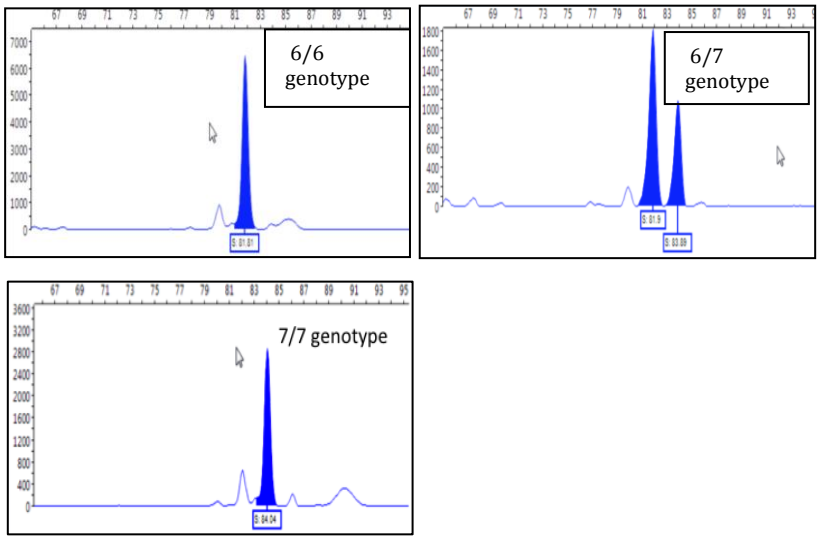

Figure 6-5 Fragment length data for each UGT1A1*28 genotype

The frequency of UGT1A1*28 genotypes in our dataset, and the expected frequency is listed in table 6.5. The number of patients with a 7/7 genotype was lower than expected by HWE (10 vs 27; HWE p<0.001), and instead a larger proportion of patients were heterozygotes than expected (171 vs 136)(table 6.5). The minor allele frequency was 28.6%, similar to seen in other aCRC populations.(215)

Genotype	Observed genotype frequency	Expected (HWE)
6/6 (wild-type)	152 (45.6%)	170 (51.1%)
6/7 (heterozygous)	171 (51.4%)	136 (40.8%)
7/7 (homozygous)	10 (3.0%)	27 (8.1%)

Table 6-5 Actual and expected UGT1A1*28 frequencies within the biomarker population.

Homozygote genotype status was associated with increased bilirubin levels (test for trend across genotype groups p<0.001)(figure 6.6).

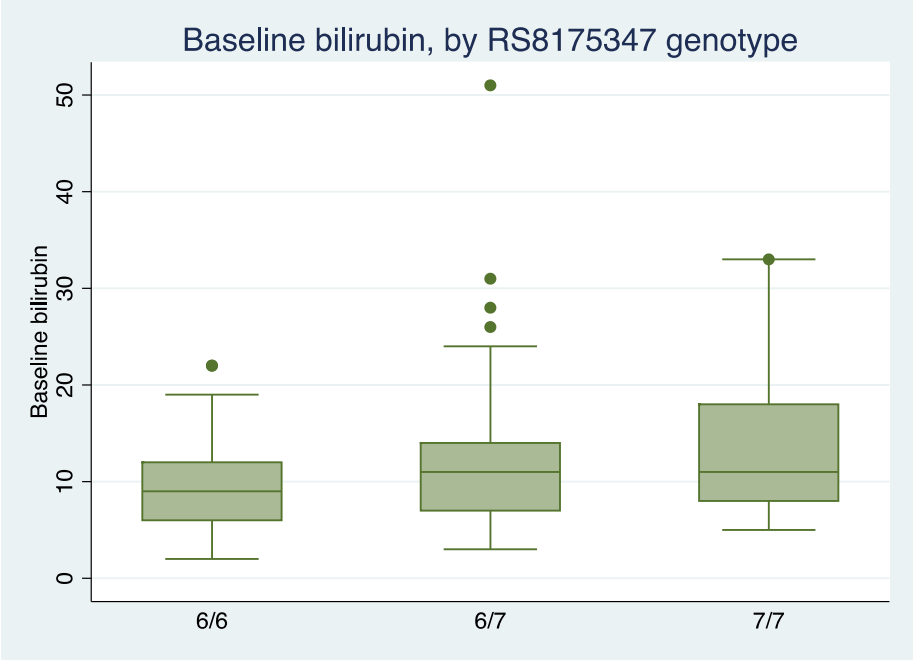

Figure 6-6 Distribution of pre-treatment bilirubin levels, by UGT1A1*28 genotype status

6.4.3. UGT1A1*28 status and study outcomes

The associations between genotype and toxicity and efficacy are reported in table 6.6. ORs for toxicity or efficacy endpoints are reported in table 6.7, comparing risk for heterozygotes to wild-type, then homozygotes to wild-types.

rs8175347 - Number(%)			6/6	6/7	7/7	p-value**
		n*				
Primary endpoint	No	175	78 (54.9)	93 (57.8)	4 (40.0)	0.52
	Yes	138	64 (45.1)	68 (42.2)	6 (60.0)	
Grade 3 toxicity	No	217	97 (68.3)	114 (71.3)	6 (60.0)	0.63
	Yes	95	45 (31.7)	46 (28.7)	4 (40.0)	
Severe toxicity	No	275	129 (90.9)	139 (86.9)	7 (70.0)	0.11
	Yes	37	13 (9.1)	21 (13.1)	3 (30.0)	
Maximum diarrhoea	0-2	264	120 (85.7)	135 (85.4)	9 (90.0)	1.0
	>2	44	20 (14.3)	23 (14.6)	1 (10.0)	
Grade 4 neutropaenia or admission with febrile neutropaenia	No	292	134 (95.0)	150 (94.3)	8 (80.0)	0.16
	Yes	18	7 (5.0)	9 (5.7)	2 (20.0)	
Cessation of therapy following 12 Weeks	Continued	135	54 (37.8)	77 (47.8)	4 (40.0)	0.20
	Stopped	179	89 (62.2)	84 (52.2)	6 (60.0)	
Complete or partial response	No	293	141 (93.4)	144 (86.2)	8 (80.0)	0.05
	Yes	35	10 (6.6)	23 (13.8)	2 (20.0)	
Disease control rates	PD	167	81 (53.6)	80 (47.9)	6 (60.0)	0.53
	CR/PR/SD	161	70 (46.4)	87 (52.1)	4 (40.0)	

Table 6-6 Associations between genotype and each toxicity and efficacy end-point tested

*Totals vary due to missing data **p-values from fisher's exact tests

Outcome	Outcome risk category	Unadjusted Odds ratio (95% confidence interval)		
		6/6 (baseline)	6/7	7/7
Primary endpoint	Yes	1.0	0.89 (0.5-1.40) P=0.62)	1.83 (0.49-6.76) P=0.37
Grade 3 toxicity	Yes	1.0	0.87 (0.53-1.42) P=0.58	1.44 (0.39-5.35) P=0.59
Very severe toxicity	Yes	1.0	1.50 (0.72-3.12) P=0.28	4.25 (0.98-18.46) P=0.05
Maximum diarrhoea	>2	1.0	1.02 (0.53-1.95) P=0.95	0.67 (0.08-5.55) P=0.71
Grade 2 diarrhoea	Yes	1.0	1.02 (0.64-1.63) P=0.93	1.09 (0.30-4.06) P=0.89
Grade 4 neutropaenia or admission with febrile neutropaenia	Yes	1.0	1.15 (0.42-3.17) P=0.79	4.79 (0.85-26.88) P=0.08
Cessation of therapy following 12 Weeks	Stopped	1.0	0.66 (0.42-1.05) P=0.08	0.91 (0.25-3.37) P=0.89
Complete or partial response	Yes	1.0	2.25 (1.03-4.90) P=0.04	3.53 (0.66-18.86) P=0.14
Disease control rates	CR/PR/SD	1.0	1.26 (0.81-1.96) P=0.31	0.77 (0.21-2.84) P=0.70

Table 6-7 Unadjusted ORs for association of each genotype with toxicity and efficacy endpoints

The association between toxicity/efficacy and genotype comparing the homozygous genotype group with the combined wild-type and heterozygous group is reported in tables 6.8 and 6.9. Additionally the risk of toxicity/efficacy for the combined heterozygous and rare homozygous group compared with the wild-types is also reported in table 6.9 (6/7 and 7/7 vs 6/6).

rs8175347 - Number(%)			6/6+6/7	7/7	p-value**
		n*			
Primary endpoint	No	175	171 (56.4)	4 (40.0)	0.35
	Yes	138	132 (43.6)	6 (60.0)	
Grade 3 toxicity	No	217	211 (69.9)	6 (60.0)	0.50
	Yes	95	91 (30.1)	4 (40.0)	
Severe toxicity	No	275	268 (88.7)	7 (70.0)	0.10
	Yes	37	34 (11.3)	3 (30.0)	
Maximum diarrhoea	0-2	264	255 (85.6)	9 (90.0)	1.0
	>2	44	43 (14.4)	1 (10.0)	
Grade 2 diarrhoea	No	191	185 (61.9)	6 (60.0)	1.0
	Yes	118	114 (38.1)	4 (40.0)	
Grade 4 neutropaenia or admission with febrile neutropaenia	No	292	284 (94.7)	8 (80.0)	0.11
	Yes	18	16 (5.3)	2 (20.0)	
Cessation of therapy following 12 weeks	Continued	135	131 (43.1)	4 (40.0)	1.0
	Stopped	179	173 (56.9)	6 (60.0)	
Complete or partial response	No	293	285 (89.6)	8 (80.0)	0.29
	Yes	35	33 (10.4)	2 (20.0)	
Disease control rates	PD	167	161 (50.6)	6 (60.0)	0.75
	CR/PR/SD	161	157 (49.4)	4 (40.0)	

Table 6-8 Outcomes of UGT1A1*28 homozygotes compared with other genotypes

Outcome	Outcome risk category	Unadjusted Odds ratio (95% confidence interval)	
		7/7 versus 6/6+6/7 (baseline)	7/7+6/7 versus 6/6 (baseline)
Primary endpoint	Yes	1.94 (0.54-7.03) P=0.31	0.93 (0.59-1.46) P=0.75
Grade 3 toxicity	Yes	1.55 (0.43-5.61) P=0.51	0.90 (0.55-1.46) P=0.66
Severe toxicity	Yes	3.38 (0.83-13.68) P=0.09	1.63 (0.80-3.34) P=0.18
Maximum diarrhoea	>2	0.66 (0.08-5.33) P=0.70	1.0 (0.53-1.90) P=1.0
Grade 2 diarrhoea	Yes	1.08 (0.30-3.92) P=0.91	1.03 (0.65-1.63) P=0.91
Grade 4 neutropaenia or admission with febrile neutropaenia	Yes	4.44 (0.87-22.63) P=0.07	1.33 (0.50-3.53) P=0.56
Cessation of therapy following 12 weeks	Stopped	1.14 (0.31-4.11) P=0.85	0.67 (0.43-1.06) P=0.09
Complete or partial response	Yes	2.16 (0.44-10.60) P=0.34	2.32 (1.08-5.00) P=0.03
Disease control rates	CR/PR/SD	0.68 (0.19-2.47) P=0.56	1.22 (0.79-1.89) P=0.36

Table 6-9 Unadjusted ORs for UGT1A1*28 homozygotes vs all other genotypes and outcomes, then homozygotes and heterozygotes vs wild-types and outcomes.

*6.4.3.1.UGT1A1*28 genotype status and toxicity outcomes*

When comparing the three genotype groups, there were no statistically significant differences in any toxicity outcome (table 6.6 and table 6.7). There were a higher proportion of homozygotes meeting the primary toxicity endpoint compared with heterozygotes or wild-type patients (60.0% vs 42.2% vs 45.1%, respectively), but this difference was not significant (p=0.52).

There was nearly a trebling in the incidence of severe toxicity in the homozygote group (30%) compared to the heterozygotes and wild-types (13.1% and 9.1% respectively), but this difference was not significant (Fisher's exact test p=0.11; OR=1.5(0.72-3.12) p=0.28 for 6/7 vs 6/6; and OR=4.25(0.98-18.46) p=0.05 for 7/7 vs 6/6).

There was no association between genotype status and severe diarrhoea.

Again there was an increased rate of grade 4 neutropaenia or febrile neutropaenia in the homozygous group, compared to either heterozygotes or wild-type patients (20%, 5.7%

and 5.0%, respectively), but again this difference was not significant (Fisher's exact test p=0.16; OR=4.44(0.87-22.63) p=0.07 for 7/7 vs. other genotypes).

*6.4.3.2.UGT1A1*28 and efficacy outcomes*

Differences were seen in 12-week RR between each genotype group and were of borderline significant (wild-type 6.6%, heterozygotes 13.8%, homozygotes 20.0%, Fisher's exact test p=0.05). Comparing each genotype to wild-type, both heterozygotes (OR=2.25(1.03-4.90), p=0.04) and homozygotes (OR=3.53(0.66-18.86), p=0.14) were more likely to respond but statistical significance at the 5% level was only reached in the heterozygote group. There was no association between DCR rates and genotype group.

Homozygosity was not associated with differences in either PFS (HR = 1.06[0.54-2.06], p=0.87) or OS (HR = 0.81 [0.42-1.57], p=0.53), when compared with wild-types and heterozygotes combined.

Comparing homo- and heterozygotes to wild-type patients, there were no differences in PFS (HR = 0.89 [0.71-1.12], p=0.32) or OS (HR = 0.96 [0.77-1.20], p=0.74).

6.4.4. Effect of baseline bilirubin levels on study outcomes

6.4.4.1.Baseline bilirubin within the study population

Of the 371 patients, baseline bilirubin levels were available for 357: median level was 10 (range 2-51)(fig. 6.7). Patients with screening bilirubin levels greater than 25 had been excluded from the trial.

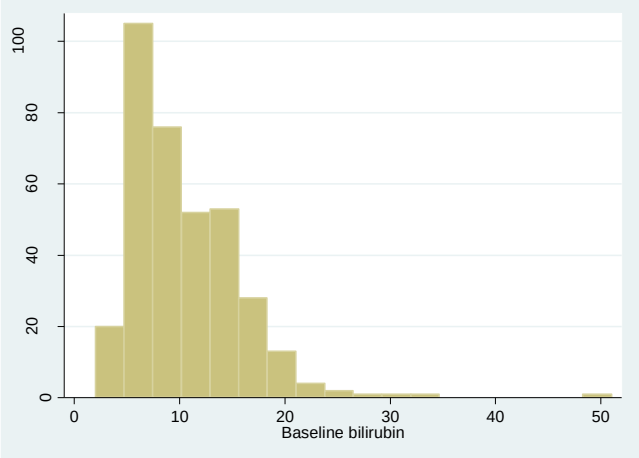

Figure 6-7 Distribution of baseline bilirubin levels

6.4.4.2.Effect of bilirubin levels on outcomes

Using the predefined binary classifier (bilirubin ≥17.0 as high; <17 as low), 40 (11.2%) patients were assigned to the high group, and 317 (88.8%) to the low group. The effect of high vs low bilirubin on outcomes is shown in table 6.10.

In patients with low bilirubin levels the prevalence of the 6/6, 6/7 and 7/7 genotypes were 49%, 49% and 2% respectively compared with 19%, 72% and 8% in patients with high levels (Fisher's exact test p=0.001).

In patients with high bilirubin levels, the proportion of patients with severe toxicity was higher than in patients with low levels, though the difference was not significant (16.2% vs.10.6%, p=0.30). In patients with high bilirubin levels, the proportion of patients with grade 4 neutropeania or admission with febrile neutropaenia was higher than in patients with low levels though again the difference did not reach statistical significance (10.% vs. 4.9%, Fisher's exact test, p=0.14). The high bilirubin group trended towards higher response rates than the low group, but this was not statistically significant (20.0% vs 10.2%, Chi-squared test p=0.07).

			Baseline bilirubin N(%)		
		Number	**<17**	**≥17**	**p-value**
rs8175347	6/6 6/7 7/7	145 164 10	138 (48.8) 138 (48.8) 7 (2.4)	7 (19.4) 26 (72.2) 3 (8.3)	0.001*
rs8175347	6/6 + 6/7 7/7	309 10	276 (97.5) 7 (2.5)	33 (91.7) 3 (8.3)	0.09*
rs8157347	6/6 6/7 + 7/7	145 174	138 (48.8) 145 (51.2)	7 (19.4) 29 (80.6)	0.001**
Primary endpoint	No Yes	194 147	173 (57.1) 130 (42.9)	21 (55.3) 17 (44.7)	0.83**
Grade 3 toxicity	No Yes	244 98	216 (70.8) 89 (29.2)	28 (75.7) 9 (24.3)	0.54**
Very severe toxicity	No Yes	302 38	271 (89.4) 32 (10.6)	31 (83.8) 6 (16.2)	0.30**
Maximum diarrhoea	0-2 >2	292 46	258 (85.4) 44 (14.6)	34 (94.4) 2 (5.6)	0.20*
Grade 2 diarrhoea	No Yes	210 129	183 (60.6) 119 (39.4)	27 (73.0) 10 (27.0)	0.14**
Grade 4 neutropaenia or admission with febrile neutropaenia	No Yes	321 19	288 (95.1) 15 (4.9)	33 (89.2) 4 (10.8)	0.14*
Cessation of therapy following 12 weeks	Continued Stopped	152 191	132 (43.3) 173 (56.7)	20 (52.6) 18 (47.4)	0.27**
Complete or partial response	No Yes	313 40	281 (89.8) 32 (10.2)	32 (80.0) 8 (20.0)	0.07**
Disease control rates	PD CR/PR/SD	171 182	154 (49.2) 159 (50.8)	17 (42.5) 23 (57.5)	0.43**

Table 6-10 Toxicity and efficacy results by bilirubin status (high vs low)

*p-value from Fisher's exact test, **p-value from Pearson Chi-squared test

6.4.5. Fluidigm SNP assay quality control

Results from the preliminary extended SNP analysis are included in this section. Thus far a total of 280 patient samples were included in the extended SNP analysis. It was elected to not include samples that had failed over 14 SNPs, so 206 patients are included in the analysis.

Initial quality control results from 283 samples for multiple SNP testing are shown in table 6. In total 46 additional SNPs were tested. The rationale for the inclusion of each SNP was shown in table 6.1 and 6.2.

Two quality control methods are employed: firstly actual genotype frequency is compared with expected frequency, predicted by the Hardy Weinberg equilibrium (tables 6.11 and 6.12). Next the minor allele frequency (MAF) for each SNP tested within the study population is compared to the reported MAF on the 1000 genomes database: discordance of over 10% is highlighted (tables 6.13 and 6.14).(501)

Eight SNPs failed all assays, and further quality testing was not performed. Eleven SNP frequency distributions did not conform to HWE. The MAF in the population differed from the reference in 8 SNPs: 3 of these SNPs did not conform to HWE.

SNP	Gene	Failed	Missing rate (%)	MAF (%)	HWE p-value	Reason for discordance
UGT1A1 gene						
rs887829	UGT1A1		3	29	0.9	
rs3755319	UGT1A1	Yes				
rs4124874	UGT1A1	Yes				
rs4148323	UGT1A1		3.0%	2.0	0.04	
rs10929302	UGT1A1	Yes				
rs853035	UGT1A1		0	28	**0.01**	Too many homozygotes observed
Other UGT polymorphisms						
rs2008595	UGT1A3		**54**	39	**0.001**	Too few homozygotes observed
rs3806596	UGT1A3		2	45	0.05	
rs3732217	UGT1A4		2	6	**0.02**	Too many homozygotes observed
rs1105880	UGT1A6		0.5	33	0.5	
rs7577677	UGT1A7		1.5	38	**0.02**	Too many homozygotes observed
rs7586110	UGT1A7		0.5	39	**0.01**	Too many homozygotes observed
rs11692021	UGT1A7	Yes				
rs17868323	UGT1A7		**60**	39	1.0	
rs17868324	UGT1A7		0.5	37	0.1	
rs1042597	UGT1A8		1	24	**0.002**	Too many homozygotes observed
rs1042605	UGT1A8		**79**	43	0.6	
rs2741046	UGT1A9		1.5	28	1.0	
rs2741048	UGT1A9		6	37	1.0	
rs4663871	UGT1A9		**15**	21	**0.04**	Too many homozygotes observed
rs6731242	UGT1A9		0.5	18	0.05	
rs13418420	UGT1A9		2	22	**0.02**	Too many homozygotes observed
rs17862856	UGT1A9		4	19	**0.04**	Too many homozygotes observed
rs1823803	UGT1A10		0.5	43	0.3	
rs2741031	UGT1A10		0.5	3	1.0	

Table 6-11 Quality control for extended UGT1A SNP analysis using Hardy-Weinberg Equilibrium to predict genotype frequency.

SNP	Gene	Failed	Missing rate (%)	MAF (%)	HWE p-value	Reason for discordance
Transporter genes						
rs212088	ABCB1		1	20	0.3	
rs1045642	ABCB1		3	50	0.06	
rs1128503	ABCB1		9	44	0.08	
rs2032582	ABCB1		1	44	0.05	
rs35588	ABCC1		**30**	29	0.7	
rs35605	ABCC1	Yes				
rs2230671	ABCC1		**35**	26	0.5	
rs3765129	ABCC1		0.5	18	0.3	
rs717620	ABCC2		0.5	16	1.0	
rs3740066	ABCC2		0.5	33	1.0	
rs562	ABCC5	Yes				
rs425215	ABCG1		2	34	0.4	
rs12721627	CYP3A4	Yes				
rs2740574	CYP3A4		0	5	0.09	
rs4986910	CYP3A4		2	0	-	
rs776746	CYP3A5		1	10	**0.004**	Too many homozygotes observed
rs10264272	CYP3A5	Yes				
rs2306283	SLCO1B1		2	39	**0.005**	Too many homozygotes observed
rs4149056	SLCO1B1		0	14	0.6	
Genes involved in bilirubin metabolism						
rs1358503			0	50	0.3	
rs1517114			**18**	0	-	

Table 6-12 Quality control of extended SNP analysis using HWE to predict genotype frequency – other

O-C-H = only common homozygotes

SNP	Gene	Failed	Missing rate (%)	Minor Allele	MAF (%)	MAF (HAP)
UGT1A1 gene						
rs887829	UGT1A1		3	A	29	31.1
rs3755319	UGT1A1	Yes				47.4
rs4124874	UGT1A1	Yes				49.7
rs4148323	UGT1A1		3.0%	A	2.0	5
rs10929302	UGT1A1	Yes				26.2
rs853035	UGT1A1		0	G	28	40.6
Other UGT polymorphisms						
rs2008595	UGT1A3		**54**	G	39	49.9
rs3806596	UGT1A3		2	G	45	49.7
rs3732217	UGT1A4		2	T	6	8.4
rs1105880	UGT1A6		0.5	C	33	31.9
rs7577677	UGT1A7		1.5	A	38	29.2
rs7586110	UGT1A7		0.5	G	39	29.2
rs11692021	UGT1A7	Yes				29.3
rs17868323	UGT1A7		**60**	T	39	49.8
rs17868324	UGT1A7		0.5	G	37	45.5
rs1042597	UGT1A8		1	G	24	28.7
rs1042605	UGT1A8		**79**	G	43	12.4
rs2741046	UGT1A9		1.5	C	28	14.1
rs2741048	UGT1A9		6	C	37	44.5
rs4663871	UGT1A9		**15**	A	21	18.3
rs6731242	UGT1A9		0.5	G	18	17.4
rs13418420	UGT1A9		2	C	22	31.0
rs17862856	UGT1A9		4	A	19	18.4
rs1823803	UGT1A10		0.5	T	43	36.3
rs2741031	UGT1A10		0.5	T	3	**26.9**

Table 6-13 Comparison of observed MAF/ expected MAF from 1000 genome project in extended UGT1A SNPs

SNP	Gene	Failed	Missing rate (%)	Minor allele	MAF (%)	MAF (HAP)
Transporter genes						
rs212088	ABCB1		1	T	20	16.9
rs1045642	ABCB1		3	T	50	39.7
rs1128503	ABCB1		9	T	44	42.2
rs2032582	ABCB1		1	T	44	34.0
rs35588	ABCC1		**30**	G	29	43.7
rs35605	ABCC1	Yes				18.1
rs2230671	ABCC1		**35**	A	26	18.7
rs3765129	ABCC1		0.5	T	18	10.8
rs717620	ABCC2		0.5	A	16	17.6
rs3740066	ABCC2		0.5	A	33	30.4
rs562	ABCC5	Yes				48.8
rs425215	ABCG1		2	G	34	43.3
rs12721627	CYP3A4	Yes				0.1
rs2740574	CYP3A4		0	G	5	20.1
rs4986910	CYP3A4		2	O-C-H	0	0.3
rs776746	CYP3A5		1	A	10	31.2
rs10264272	CYP3A5	Yes				4.5
rs2306283	SLCO1B1		2	C	39	40.5
rs4149056	SLCO1B1		0	C	14	12.3
Gene involved in bilirubin metabolism						
rs1358503			0	C	50	
rs1517114			**18**	O-C-H	0	

Table 6-14 Comparison of observed MAF and expected MAF from 1000 genome project in other tested SNPs

6.5. Discussion

This study aims to provide a comprehensive analysis of the impact of polymorphisms in SNPs involved in the metabolism and clearance of irinotecan on toxicity and efficacy outcomes. To date published literature has presented inconsistent and divergent results on both outcomes measures, exacerbated by small studies, with heterogenous irinotecan scheduling and chemotherapy combinations. Instead, this study is performed in a large RCT population with well-documented toxicity and efficacy outcomes. Importantly patients have been treated with single agent irinotecan, rather than in combination with 5FU so a causal relationship of candidate SNP polymorphism with irinotecan toxicity can be more convincingly established.

The primary analysis set out to examine the relationship of UGT1A1*28 homozygosity and irinotecan toxicity. Within the PICCOLO population there were no statistically significant interactions demonstrated. In particular there was no association with increased risk of diarrhoea, in keeping with most other large genotyping studies(215, 236, 468, 470, 471) and one meta-analysis,(502) but contrasting to a large study of Asian patients(472) and a further meta-analysis of small studies.(503)

There was a trend observed between UGT1A1*28 homozygosity and increased incidence of grade 4 neutropaenia, but the relationship did not achieve statistical significance. There was a trebling of the rates of severe toxicity in the homozygote population, compared with other genotypes: this relationship had borderline significance when comparing homozygotes to wild-types (p=0.05). The relationship between UGT1A1*28 and neutropaenia appears to be more convincingly demonstrated in the literature: several large studies and meta-analyses reporting this interaction,(236, 468, 471, 502) however not found in others.(215, 470) However on closer examination of positive studies, Kweekel's only found an association with genotype and febrile neutropaenia;(471) Cecchin's study included both grade 3 & 4 neutropaenia, but was not significant in multivariate analysis with other relevant SNPs;(468) McLeod's study found positive association only in the IrOx treated group, not the IrMdG group;(236) and in Hoskin's meta-analysis only patients treated with higher doses of irinotecan were at increased risk.(502)

Furthermore a causal relationship between UGT1A1*28 and irinotecan toxicity cannot be established in previous work as the vast majority of studies have been with in combination with 5-flurouacil, with an overlapping toxicity profile. This study has the advantage of assessing the impact of genotype on irinotecan alone.

Other inconsistencies between studies include comparisons made in the statistical analysis, as 3 risk groups are present for genotyping studies. Some studies report a 3-gene comparison (using Fisher's exact test), others report homozygosity compared with wild-types (chi-squared test). Less frequently reported is homozygotes vs wild-types and heterozygotes, assessing UGT1A1*28 homozygosity as a binary variable (present; absent). However it is important to also understand the risk of toxicity for heterozygotes, which appears to be raised but to not the same degree as heterozygotes, consistent with this work.

In 2005 the FDA approved a genetic test for UGT1A1*28 homozygosity. This was based upon the results of two studies using different regimens, with a total of 132 patients and 13 UGT1A1*28 homozygous patients, of whom 8 experienced toxicity.(8, 462) The extent of the demonstrated clinical validity of this test is therefore questionable. However the FDA renewed the approval in 2010 and currently it is recommended that genotyping is established prior to irinotecan treatment, with dose reduction suggested in homozygote patients. Currently in Europe this test is not recommended by the EMA. Using this

biomarker test UGT1A1*28 risk is presumed to be binary (at risk [7/7]; no risk [6/6 and 6/7]), therefore this comparison should be reported in publications.

Of increasing interest is the relationship between UGT1A1*28 status and irinotecan efficacy. The most important emerging message is that wild-type patients have lesser benefit from irinotecan, which may be due to underdosing with current schedules.(475) This study would be supportive of this hypothesis: wild-type patients were less likely to respond to irinotecan than hetero- or homozygotes (p=0.03). However lesser effect was seen on PFS and OS. This interesting hypothesis is being explored in ongoing prospective trials.

The major limitation of the analysis of UGT1A1*28 in this study was that the observed genotype frequencies were out of HWE: the minor allele frequency of UGT1A1*28 in this study, at 28.6%, was very similar to previous reports. However, in contrast to previous studies the population were strikingly out of HW equilibrium, with a much lower than expected rate of 7/7 homozygotes. As a result, there were only ten 7/7 patients in the population under stduy, which limits the ability to confirm or refute previous reports of the impact of 7/7 genotype on irinotecan toxicity.

There are a number of possible explanations for the low number of 7/7 patients. The genotyping methodology is a standard PCR and the failure rate (10.1%) is comparable to other large studies.(236) Each individual sample's fragment length data was evaluated by an experienced molecular geneticist: it was felt that peak amplification is more likely to be biased towards homozygote selection than heterozygote, so it is unlikely that underreporting of homozygote status contributed significantly.

One possible reason is that the population selected for PICCOLO was depleted of 7/7 patients: could these patients be less likely to receive second-line therapy due to excessive toxicity with first-line chemotherapy? However, homozygote patients treated with OxMdG in FOCUS did not experience excessive toxicity compared to other genotypes.(215) Furthermore there is no evidence that homozygote status is a poor prognostic marker: a meta-analysis of 1524 patients found no statistical difference in survival with UGT1A1*28 homozygosity, compared to either wild-types (HR = 1.01, p=0.92) or heterozygotes (HR = 1.13, p=0.37).(504) It is unlikely that there would be sampling bias against the homozygote group.

Another possible explanation is that the PICCOLO trial protocol excluded patients with baseline serum bilirubin of >25. A strong relationship between homozygote status and increasing bilirubin levels was documented within this study, and so it is likely that a proportion of homozygotes were not eligible for the trial on this basis. However the same restriction was made in the FOCUS trial protocol, and the proportion of homozygotes was 9.4% and was in HWE. It is therefore uncertain how much impact this made. Therefore this disparity cannot be explained.

Certainly the low proportion of homozygote patients has limited the power of this study to detect an interaction with irinotecan toxicity and efficacy. Therefore available samples shall be re-genotyped using the FDA approved UGT1A1*28 genotyping assay.

Although there is clear biological plausibility between UGT1A1*28 homozygosity and increased risk of irinotecan toxicity, multiple genes are associated with drug clearance so it seems unlikely that polymorphism in one SNP confers all of the risk. Another important aim of this study is to explore whether additional polymorphisms of SNPs involved in irinotecan metabolism and clearance can better predict risk, either individually or as part of a 'risk signature'. SNPs examined in this study have been selected due to their biological role in irinotecan metabolism or clearance, or previous evidence implicating an association between polymorphism and irinotecan outcomes.

Initial quality control data for the extended SNP analysis on a limited sample set has been presented in this chapter. Firstly a number of samples (26%) tested failed over 14 SNP assays so were excluded. A high proportion of these samples had low concentrations and/or poor quality DNA; additional DNA has been extracted from remaining WBC pellets or tumour DNA has been sourced where possible. The UGT1A1*28 analysis utilised this re-extracted or resourced DNA.

Eight SNPs assays failed with all samples so further optimisation, including pre-assay amplification, is required. Furthermore eleven SNPs did not conform to HWE; unlike in the primary analysis too many homozygotes were observed. The minor allele frequency differed from reference in eight SNPs. The reference for the expected MAF was ascertained from NCBI resource links, which utilised data from HapMap or the 1000 genomes project.(505) This resource provides details of the population from which genotyping was performed, including number and ethnicity. This study is the largest European population to test many of the SNPs where a discrepancy between observed and expected MAF was

seen (rs853035, rs2008595, rs2741031, rs2741031, rs27405740), so the significance of this inconsistency is less clear.

Another important consideration is the assessment of the contribution of genotyping information for the prediction of irinotecan outcomes alongside other clinical variables. Ichikawa's paper has proposed a nomogram for predicting irinotecan-induced severe neutropaenia in Japanese patients which includes type of regimen, administered dose of irinotecan, gender, age, PS, pre-treated neutrophil count, total bilirubin level alongside UGT1A1*28 genotype.(472)

6.6. Further work

As described further work is necessary to optimise some of the extended SNP assays. Where this is not possible alternative methods of genotyping (for example, SnapShot) shall be explored. It is also planned to re-run the UGT1A1*28 assay on the FDA-approved biomarker test to confirm (or refute) the genotyping results reported in this chapter. Analyses shall then be performed using the planned analysis including the SNPs that have satisfied quality control standards.

Conclusions

The needs for a more personalised approach is aCRC is clear, but there are many challenges and obstacles. Here, the processes and 'best practice' of biomarker discovery and validation have been reviewed and as much as possible studies in this thesis have applied these principles. Each study had a pre-defined statistical plan based upon a biologically plausible hypothesis, adequate sample size, considered potential confounders, and was performed in a randomised population, so allowing for discrimination between prognostic and prediction effects of tested biomarkers. With this approach biomarkers demonstrating effect should be considered for development and potential clinical application.

Chapter 2 has reported that the dNLR and platelet count, both surrogates of the systemic inflammatory response, are independent prognostic markers in aCRC in both the first- and second-line. This model can be utilised in different ways. Using a cut-point over 3 the dNLR identifies a group of high-risk patients who benefit most from upfront combination chemotherapy. Instead patients with lesser evidence of a heightened systemic inflammatory response (low dNLR and low platelets) were not disadvantaged from a more upfront conservative approach. Further validation and testing in other clinical scenarios is warranted. The importance of the systemic inflammatory response in the prognostication and treatment of cancer has again been shown and further translational research may aid in understanding the immunological mechanisms for this strong effect seen.

The usefulness of knowledge of a patient's *BRAF*-status has been demonstrated in Chapter 3. This study reported that rapid decline following first-line chemotherapy appears to be the main driver of the poor prognosis in *BRAF*-mut aCRC patients. However *BRAF*-mut patients have similar benefits with first- and second-line chemotherapy as wild-types, and do not appear to be disadvantaged by treatment breaks. This finding has both clinical and translational significance. Fit *BRAF*-mut patients may benefit from intensive upfront chemotherapy, with the option of treatment breaks following disease control. Further translational work is needed to understand the biological mechanisms of this observed rapid decline: pre- and post treatment biopsies may reveal a further molecular alteration, potentially a therapeutic target.

Chapters 3 and 4 tested mRNA expression of EREG/AREG and HER3 as predictive biomarkers for anti-EGFR therapies. Both chapters clearly demonstrate that a population of *RAS*-wt patients do not benefit from panitumumab and questions the current 'opt-in' strategy. A combined model using data from both markers identified a population most

sensitive to panitumumab. For AREG/EREG this study builds upon previous work, and so clinical validity has been achieved. Instead, this is the first large study to test HER3 mRNA expression levels as a predictive marker for panitumumab, and so testing in further datasets is crucial. For both markers assay development is required, particularly to provide consensus on a dichotomisation point.

Many difficulties relating to biomarker research have been encountered in the irinotecan pharmacogenomics study. Although a standard assay was used for the primary UGT1A1*28 analysis, genotype frequencies in PICCOLO were significantly different from what would be anticipated and so presented results should be interpreted with caution. No reasons for this disparity were found. Similarly it is unclear why many of the extended SNP genotype analyses failed quality control checks. For both, further laboratory work is being performed to optimise assays and confirm genotyping results using an alternative methodology.

Work from this thesis therefore contributes to efforts to further personalise treatment in aCRC. Evidence presented from Chapter 2 and Chapter 3 may influence the routine practice of some oncologists. Instead, the EREG/AREG model requires optimisation and HER3 overexpression further clinical validation as well as assay development, so neither is ready for clinical application.

Further efforts to improve outcomes for aCRC patients are currently ongoing in the UK. The FOCUS 4 trial is a molecularly stratified multi-arm multi-stage phase II/III RCT in aCRC. This study allows for testing of new molecular agents in biomarker-stratified groups, when strong biological or clinical evidence exists linking the biomarker with treatment efficacy. Testing of a novel agent against placebo in these biomarker-enriched populations aims to detect effect in a timely and cost-effective manner.(506)

The Stratification in Colorectal Cancer: from biology to treatment prediction (S:CORT) program aims to expand the understanding of the biology of CRC, validate promising biomarkers in patient samples from national studies and trials. Specific aims are to validate predictive markers to identify patients most likely to respond to oxaliplatin, those who will respond to radiotherapy, identify factors in early CRC that predict risk of invasion and guide surgical management and predictors for efficacy of novel molecularly targeted agents.

With these efforts there is real promise for the application of precision medicine in the routine management in aCRC, with the goal of improving cancer outcomes and quality of life for each patient.

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Appendix

1. STATA do-files used in Chapter 2

a) STATA file for FOCUS analyses

```
clear
cd "/Users/medjcri/Documents/NLR project"

insheet using "FOCUSWorkbook1csv.csv", comma
keep trialno ratiocategory
drop if trialno==.
bysort trialno:assert _N==1
save "FOCUSratiocategory.dta", replace

insheet using "/Users/medjcri/Documents/NLR project/FOCUStrialcsv.csv", clear
bysort trialno:assert _N==1
save FOCUStrial.dta, replace

use "/Users/medjcri/Documents/BRAF/FOCUS_all_variables.dta", clear
bysort trialno:assert _N==1
merge trialno using "FOCUSratiocategory.dta"
tab _merge
drop _merge
sort trialno
merge trialno using "FOCUStrial.dta"
tab _merge
drop _merge

**generating variables

foreach temp of varlist liv-oth {
gen `temp'_1=0 if `temp'=="No"
replace `temp'_1=1 if `temp'=="Yes"
}

gen number_met_sites=liv_1+nod_1+lung_1+peri_1+oth_1
foreach temp of varlist liv-oth {
gen `temp'_1=0 if `temp'=="No"
replace `temp'_1=1 if `temp'=="Yes"
}


gen metsite1 =.
replace metsite1 = 0 if number_met_sites==0
replace metsite1 = 0 if number_met_sites==1
replace metsite1 = 1 if number_met_sites==2
replace metsite1 = 1 if number_met_sites==3
replace metsite1 = 1 if number_met_sites==4
replace metsite1 = 1 if number_met_sites==5

gen metsite2 =.
replace metsite2 = 0 if number_met_sites==0
replace metsite2 = 0 if number_met_sites==1
replace metsite2 = 0 if number_met_sites==2
replace metsite2 = 1 if number_met_sites==3
replace metsite2 = 1 if number_met_sites==4
replace metsite2 = 1 if number_met_sites==5


gen gender = .
replace gender = 0 if sex== "Male"
replace gender = 1 if sex== "Female"
label define gender_lbl 0 "male" 1 "female"
tab gender sex
drop sex


recode who (1 = 0) (2 = 1)(3 = 1)
label define who_lbl 0 "PSlow" 1"PShigh"
label values who who_lbl
```

```
gen primresect = .
replace primresect = 0 if resect== "No"
replace primresect = 1 if resect== "Yes"
label define primresect_lbl 0 "no" 1 "yes"
tab primresect resect
drop resect

gen agecat = age
recode agecat (1/69 = 0) (70/99 = 1)
label define agecat_lbl 0 "<70" 1">70"
label values agecat agecat_lbl

gen agecat2 = age
recode agecat2 (1/50 = 1)(51/70 = 0)(70/90 = .)
label define agecat2_lbl 0 "<50" 1"50-70"
label values agecat2 agecat2_lbl

gen agecat3 = age
recode agecat3 (1/50 = .)(51/70 = 0)(70/90 = 1)
label define agecat3_lbl 0 "50-70" 1 "70+"
label values agecat3 agecat3_lbl

gen analysisirvsox1 = .
replace analysisirvsox1 = 1 if regimen== "B"
replace analysisirvsox1 = 1 if regimen== "C"
replace analysisirvsox1 = 0 if regimen== "D"
replace analysisirvsox1 = 0 if regimen== "E"
label define analysisirvsox1_lbl 1 "irinotecan based chemo" 0 "Oxaliplatin based chemo"
label values analysisirvsox1 analysisirvsox1_lbl


gen apcat1 = ap
recode apcat1 (0/299 = 0)(300/2000 = 1)
label define apcat1_lbl 0 "low ap" 1 "high ap"
label values apcat1 apcat1_lbl

gen basewbc = wbct1st
recode basewbc (0/9.99 = 0)(10/100 =1)
label define basewbc_lbl 0 "low WBC" 1 "high WBC"
label values basewbc basewbc_lbl

gen NLR1 = neutt1st/(wbct1st - neutt1st)
drop if NLR1<0
drop if NLR1== .
drop if NLR1>15

gen NLRcateg1 = NLR1
recode NLRcateg1 (0/2.000001 = 0) (2.0000002/40 = 1)
label define NLRcateg1_lbl 0 "low NLR" 1"high NLR"
label values NLRcateg1 NLRcateg1_lbl

gen platcat1 = platt1st
recode platcat1 (1/400 = 0) (401/1500 = 1)
label define platcat1_lbl 0 "low platelets" 1">high platelets"
label values platcat1 platcat1_lbl

gen bestresp = .
replace bestresp = 0 if bestrespall== "Stable disease"
replace bestresp = 0 if bestrespall== "Progressive disease"
replace bestresp = 1 if bestrespall== "Partial Response"
replace bestresp = 1 if bestrespall== "Complete Response"
label define bestresp_lbl 0 "SD/PD" 1 "CR/PR"
label values bestresp bestresp_lbl
tab bestresp bestrespall

gen clinben = .
replace clinben = 1 if bestrespall== "Stable disease"
replace clinben = 0 if bestrespall== "Progressive disease"
replace clinben = 1 if bestrespall== "Partial Response"
replace clinben = 1 if bestrespall== "Complete Response"
label define clinben_lbl 0 "progressed" 1 "clinben"
label values clinben clinben_lbl


gen treatfail12wk = .
```

```
replace treatfail12wk = 1 if bestresp12wk== "Stable d"
replace treatfail12wk = 0 if bestresp12wk== "Progress"
replace treatfail12wk = 1 if bestresp12wk== "Partial"
replace treatfail12wk = 1 if bestresp12wk== "Complete"
recode treatfail12wk (.=0)
label define treatfail12wk_lbl 0 "progressed" 1 "clinben"
label values treatfail12wk treatfail12wk_lbl


gen bestrespall212wk = .
replace bestrespall212wk = 0 if bestresp12wk== "Stable d"
replace bestrespall212wk = 0 if bestresp12wk== "Progress"
replace bestrespall212wk = 1 if bestresp12wk== "Partial"
replace bestrespall212wk = 1 if bestresp12wk== "Complete"
recode bestrespall212wk (.=0)
label define bestrespall212wk_lbl 0 "SD/PD" 1 "CR/PR"
label values bestrespall212wk bestrespall212wk_lbl


gen progressed = .
replace progressed = 0 if pfsevent== "No"
replace progressed = 1 if pfsevent== "Yes"
label define progressed_lbl 0 "No" 1 "Yes"
label values progressed progressed_lbl

gen died = .
replace died = 0 if death== "No"
replace died = 1 if death== "Yes"
tab died death
label define died_lbl 0 "No" 1 "Yes"
label values died died_lbl

gen analysisirvsox = .
replace analysisirvsox = 1 if regimen== "B"
replace analysisirvsox = 1 if regimen== "C"
replace analysisirvsox = 0 if regimen== "D"
replace analysisirvsox = 0 if regimen== "E"
label define analysisirvsox_lbl 1 "irinotecan based chemo" 0 "Oxaliplatin based chemo"
label values analysisirvsox analysisirvsox_lbl


gen treatmentintensity = .
replace treatmentintensity = 0 if regimen== "A"
replace treatmentintensity = 0 if regimen== "B"
replace treatmentintensity = 0 if regimen== "D"
replace treatmentintensity = 1 if regimen== "C"
replace treatmentintensity = 1 if regimen== "E"
label define treatmentintensity_lbl 0 "initial single agent" 1 "initial combination"
label values treatmentintensity treatmentintensity_lbl

gen combination = .
replace combination = . if regimen== "A"
replace combination = 0 if regimen== "B"
replace combination = 0 if regimen== "D"
replace combination = 1 if regimen== "C"
replace combination = 1 if regimen== "E"
label define combination_lbl 0 "deferred combination" 1 "initial combination"
label values combination combination_lbl

gen htm = ht/100
gen bodymi = wt/(htm*htm)

gen bmi = wt/ (ht*ht)

*demographic information

codebook NLRcateg1

summarize age if NLRcateg1==0
summarize age if NLRcateg1==1

tab who if NLRcateg1==0
tab who if NLRcateg1==1
tab who NLRcateg1, chi
```

```
tab primresect if NLRcateg1==0
tab primresect if NLRcateg1==1
tab primresect NLRcateg1, chi

tab platcat1 if NLRcateg1==0
tab platcat1 if NLRcateg1==1
tab platcat1 NLRcateg1, chi

tab braf_cat if NLRcateg1==0
tab braf_cat if NLRcateg1==1
tab braf_cat NLRcateg1, chi

tab site_cat2 if NLRcateg1==0
tab site_cat2 if NLRcateg1==1
tab site_cat2 NLRcateg1, chi

tab apcat1 if NLRcateg1==0
tab apcat1 if NLRcateg1==1
tab apcat1 NLRcateg1, chi


tab metsite1 if NLRcateg1==0
tab metsite1 if NLRcateg1==1
tab metsite1 NLRcateg1, chi

histogram NLR1, bin(15)


*Prognostic analysis

stset stime, failure(died==1)
stcox NLRcateg1
sts graph, by (NLRcateg1)
stcox NLRcateg1 primresect who platcat1 braf_cat site_cat2 metsite1 kras121361 apcat1


stcox NLR1

stcox agecat
stcox metdis
stcox primresect
stcox who
stcox platcat1
stcox braf_cat
stcox site_cat2
stcox agecat2
stcox agecat3
stcox apcat1
stcox metsite1
stcox metsite2


stset ptime, failure(progressed==1)
stcox NLRcateg1
sts graph, by (NLRcateg1)
stcox NLRcateg1 who primresect platcat1 metsite1 apcat1

**predictive analyses - treatment intensity


stset stime, failure(died==1)
stcox combination
stsum, by(combination)



stset stime, failure(died==1)
stcox combination if NLRcateg1==1
stsum, by(combination)

stset stime, failure(died==1)
stcox combination if NLRcateg1==0
```

```
 xi: stcox i.NLRcateg1*i.combination
estimates store a
xi: stcox i.combination i.NLRcateg1
lrtest a


stset ptime, failure(progressed==1)
stcox combination
stcox combination if NLRcateg1==1

 stset ptime, failure(progressed==1)
stcox combination if NLRcateg1==0

 xi: stcox i.NLRcateg1*i.combination
estimates store a
xi: stcox i.combination i.NLRcateg1
lrtest a

tab treatmentintensity bestresp if NLRcateg1==1, chi2
tab treatmentintensity bestresp if NLRcateg1==0, chi2
logistic bestresp treatmentintensity if NLRcateg==1
logistic bestresp treatmentintensitab ty if NLRcateg==0

 xi: logistic i.NLRcateg1*i.treatmentintensity
estimates store a
xi: logistic i.treatmentintensity i.NLRcateg1
lrtest a

**predictive analysis - Ir vs Ox

stset stime, failure(died==1)
stcox analysisirvsox1


stset stime, failure(died==1)
stcox analysisirvsox2
stcox analysisirvsox2 if NLRcateg1==1
stcox analysisirvsox2 if NLRcateg1==0



 xi: stcox i.NLRcateg1*i.analysisirvsox2
estimates store a
xi: stcox i.analysisirvsox2 i.NLRcateg1
lrtest a


stset ptime, failure(progressed==1)
stcox analysisirvsox2
stcox analysisirvsox2 if NLRcateg1==1
stcox analysisirvsox2 if NLRcateg1==0


 xi: stcox i.NLRcateg1*i.analysisirvsox2
estimates store a
xi: stcox i.analysisirvsox2 i.NLRcateg1
lrtest a


****tumour marker analysis

gen NLR4 = neutt4th/(wbct4th - neutt4th)
drop if NLR4<0

histogram NLR4, bin(15)

gen NLRcat4 = NLR4
gen NLRcateg4 = NLR4
recode NLRcateg4 (0/2.000001 = 0) (2.0000002/40 = 1)
label define NLRcateg4_lbl 0 "low NLR" 1"high NLR"
label values NLRcateg4 NLRcateg4_lbl

stset stime, failure(died==1)
stcox NLRcateg4
stset ptime, failure(progressed==1)
```

```
stcox NLRcateg4


gen NLRchange = (NLR4-NLR1)/NLR1
gen NLRchangepercent = NLRchange *100
gen NLRchangecat1 = 2 if NLRchange<=-0.25
replace NLRchangecat1 = 1 if NLRchange>-0.25 & NLRchange<0.25
replace NLRchangecat1 = 0 if NLRchange>=0.25 & NLRchange~=.
label define NLRchangecat1_lbl 0 "NLRdecrease" 1 "NLRstable" 2 "NLRincrease"
label values NLRchangecat1 NLRchangecat1 _lbl

gen dynamic = .
replace dynamic = 0 if NLRchange<=-0.25 & NLRcateg1==0
replace dynamic = 1 if NLRchange<=-0.25 & NLRcateg1==1
replace dynamic = 2 if NLRchange>-0.25 & NLRchange<0.25 & NLRcateg1==0
replace dynamic = 3 if NLRchange>-0.25 & NLRchange<0.25 & NLRcateg1==1
replace dynamic = 4 if NLRchange>=0.25 & NLRchange~=. & NLRcateg1==0
replace dynamic = 5 if NLRchange>=0.25 & NLRchange~=. & NLRcateg1==1
label define dynamic_lbl 0 "NLRimproved0" 1 "NLRimproved1" 2 "NLRstable0" 3"NLRstable1" 4"NLRrise0" 5"NLRrise1"
label values NLRchangecat1 NLRchangecat1 _lbl


codebook NLRchangecat1
codebook dynamic


gen NLRimproved = NLRchangecat1
recode NLRimproved (1=0)(2=1)
label define NLRimproved_lbl 0 "no improvement" 1 "improvement"
label values NLRimproved NLRimproved_lbl

gen NLRfavourable = NLRchangecat1
recode NLRfavourable (2=1)
label define NLRfavourable_lbl 0 "not favourable" 1 "favourable"
label values NLRfavourable NLRfavourable_lbl

gen NLRworse = NLRchangecat1
recode NLRworse (0=1)(2=0)(1=0)
label define NLRworse_lbl 0 "no deterioration" 1 "deterioration"
label values NLRworse NLRworse_lbl

gen dynamicworse= dynamic
recode dynamicworse (1=0)(2=0)(3=0)(4=1)(5=0)

tabulate NLRimproved treatfail12wk
tabulate NLRworse treatfail12wk

tabulate NLRimproved bestrespall212wk
tabulate NLRworse bestrespall212wk

logistic bestrespall212wk NLRimproved
logistic bestrespall212wk NLRfavourable
logistic bestrespall212wk NLRworse
logistic bestrespall212wk NLRimproved if NLRcateg1==1
logistic bestrespall212wk NLRimproved if NLRcateg1==0
logistic bestrespall212wk NLRfavourable if NLRcateg1==1
logistic bestrespall212wk NLRfavourable if NLRcateg1==0
logistic bestrespall212wk NLRworse if NLRcateg1==1
logistic bestrespall212wk NLRworse if NLRcateg1==0


logistic treatfail12wk NLRimproved
logistic treatfail12wk NLRfavourable
logistic treatfail12wk NLRworse
logistic treatfail12wk NLRimproved if NLRcateg1==1
logistic treatfail12wk NLRimproved if NLRcateg1==0
logistic treatfail12wk NLRfavourable if NLRcateg1==1
logistic treatfail12wk NLRfavourable if NLRcateg1==0
logistic treatfail12wk NLRworse if NLRcateg1==1
logistic treatfail12wk NLRworse if NLRcateg1==0

logistic dynamicworse0 treatfail12wk
tab dynamicworse0 treatfail12wk

stset ptime, failure(progressed==1)
```

```
stcox NLRfavourable

stset ptime, failure(progressed==1)
stcox NLRworse

stset ptime, failure(progressed==1)
stcox NLRimproved


stset stime, failure(died==1)
stcox NLRfavourable

stset stime, failure(died==1)
stcox NLRworse

stset stime, failure(died==1)
stcox NLRfavourable

***15% difference in NLR c4 (same analyses used for 20%, 30%)

gen NLRchange = (NLR1-NLR4)/NLR1
gen NLRchangepercent = NLRchange *100
gen NLRchangecat15 = 0 if NLRchange<=-0.15
replace NLRchangecat15 = 1 if NLRchange>-0.15 & NLRchange<0.15
replace NLRchangecat15 = 2 if NLRchange>=0.15 & NLRchange~=.
label define NLRchangecat15_lbl 0 "NLRdecrease" 1 "NLRstable" 2 "NLRincrease"
label values NLRchangecat15 NLRchangecat15 _lbl

codebook NLRchangecat15

gen NLRimproved15 = NLRchangecat15
recode NLRimproved15 (1=0)(2=1)
label define NLRimproved15_lbl 0 "no improvement" 1 "improvement"
label values NLRimproved15 NLRimproved15_lbl

gen NLRfavourable15 = NLRchangecat15
recode NLRfavourable15 (2=1)
label define NLRfavourable15_lbl 0 "not favourable" 1 "favourable"
label values NLRfavourable15 NLRfavourable15_lbl

gen NLRworse15 = NLRchangecat15
recode NLRworse15 (0=1)(2=0)(1=0)
label define NLRworse15_lbl 0 "no deterioration" 1 "deterioration"
label values NLRworse15 NLRworse15_lbl

tabulate NLRimproved15 treatfail12wk
tabulate NLRworse15 treatfail12wk
tabulate NLRfavourable15 treatfail12wk

tabulate NLRimproved15 bestrespall12wk
tabulate NLRworse15 bestrespall12wk

logistic bestrespall12wk NLRimproved15
logistic bestrespall12wk NLRfavourable15
logistic bestrespall12wk NLRworse15

logistic treatfail12wk NLRimproved15
logistic treatfail12wk NLRfavourable15
logistic treatfail12wk NLRworse15


stset ptime, failure(progressed==1)
stcox NLRfavourable15

stset ptime, failure(progressed==1)
stcox NLRworse15

stset ptime, failure(progressed==1)
stcox NLRimproved15


stset stime, failure(died==1)
stcox NLRfavourable15

stset stime, failure(died==1)
```

```
stcox NLRworse15

stset stime, failure(died==1)
stcox NLRfavourable15



gen NLR

gen NLRchangecat1 = 2 if NLRchange<=-0.25
replace NLRchangecat1 = 1 if NLRchange>-0.25 & NLRchange<0.25
replace NLRchangecat1 = 0 if NLRchange>=0.25 & NLRchange~=.
label define NLRchangecat1_lbl 0 "NLRdecrease" 1 "NLRstable" 2 "NLRincrease"
label values NLRchangecat1 NLRchangecat1 _lbl

**effect of alternative cut-points

gen NLRcut15 = NLR1
recode NLRcut15 (0/1.500001 = 0) (1.5000002/40 = 1)
label define NLRcut15_lbl 0 "low NLR" 1"high NLR"
label values NLRcut15 NLRcut15_lbl
drop if NLRcut15==.
drop if NLRcut15<0

gen NLRcut25 = NLR1
recode NLRcut25 (0/2.500001 = 0) (2.5000002/40 = 1)
label define NLRcut25_lbl 0 "low NLR" 1"high NLR"
label values NLRcut25 NLRcut25_lbl
drop if NLRcut25==.
drop if NLRcut25<0


gen NLRcpt3 = NLR1
recode NLRcpt3 (0/3.00001 = 0) (3.000002/40 = 1)
label define NLRcpt3_lbl 0 "low NLR" 1"high NLR"
label values NLRcpt3 NLRcpt3_lbl
drop if NLRcpt3==.
drop if NLRcpt3<0

gen NLRcpt35 = NLR1
recode NLRcpt35 (0/3.50001 = 0) (3.500002/40 = 1)
label define NLRcpt35_lbl 0 "low NLR" 1"high NLR"
label values NLRcpt35 NLRcpt35_lbl
drop if NLRcpt35==.
drop if NLRcpt35<0

gen NLRct4 = NLR1
recode NLRct4 (0/4.00001 = 0) (4.000002/40 = 1)
label define NLRct4_lbl 0 "low NLR" 1"high NLR"
label values NLRct4 NLRct4_lbl
drop if NLRct4==.
drop if NLRct4<0

tab NLRcut15

stset stime, failure(died==1)
stcox NLRcut15


stset ptime, failure(progressed==1)
stcox NLRcut15

tab NLRcut25

stset stime, failure(died==1)
stcox NLRcut25

stset ptime, failure(progressed==1)
stcox NLRcut25

tab NLRcpt35

stset stime, failure(died==1)
stcox NLRcpt35
```

```
stset ptime, failure(progressed==1)
stcox NLRcpt35

tab NLRct4

stset stime, failure(died==1)
stcox NLRct4
stcox NLRct4 who platcat1

stset ptime, failure(progressed==1)
stcox NLRct4

***Platelets analysis

*demographic information

codebook platcat1

summarize age if platcat1==0
summarize age if platcat1==1

tab who if platcat1==0
tab who if platcat1==1
tab who site, chi


tab primresect if platcat1==0
tab primresect if platcat1==1
tab primresect site, chi

tab metsite1 site, chi
tab braf_cat site, chi
tab apcat1 site, chi
tab NLRcateg1 site, chi


summarize age if NLRcateg1==0
summarize age if NLRcateg1==1

tab who if NLRcateg1==0
tab who if NLRcateg1==1

tab group if NLRcateg1==0
tab group if NLRcateg1==1

tab analysisirvsox if NLRcateg1==0
tab analysisirvsox if NLRcateg1==1

tab treatmentintensity if NLRcateg1==0
tab treatmentintensity if NLRcateg1==1

tab primresect if NLRcateg1==0
tab primresect if NLRcateg1==1

tab metdis if NLRcateg1==0
tab metdis if NLRcateg1==1

tab platcat1 if NLRcateg1==0
tab platcat1 if NLRcateg1==1

tab braf_cat if NLRcateg1==0
tab braf_cat if NLRcateg1==1

tab site_cat2 if NLRcateg1==0
tab site_cat2 if NLRcateg1==1

tab apcat1 if NLRcateg1==0
tab apcat1 if NLRcateg1==1

tab agecat2 if NLRcateg1==0
tab agecat2 if NLRcateg1==1

tab agecat3 if NLRcateg1==0
tab agecat3 if NLRcateg1==1
```

```
tab metsite1 if NLRcateg1==0
tab metsite1 if NLRcateg1==1
tab metsite1 NLRcateg1, chi
histogram NLR1, bin(10) normal kdensity



*Prognostic analysis

stset stime, failure(died==1)
stcox platcat1
sts graph, by (platcat1)
stcox NLRcateg1 primresect who platcat1

stset ptime, failure(progressed==1)
stcox platcat1
sts graph, by (platcat1)
stcox who primresect platcat1 NLRcateg1

*treatment intensity analysis

stset stime, failure(died==1)
stcox treatmentintensity if platcat1==1

stcox treatmentintensity primresect NLRcateg1 who if platcat1==1

stset stime, failure(died==1)
stcox treatmentintensity if platcat1==0
stcox treatmentintensity primresect who NLRcateg1 if platcat1==0

 xi: stcox i.platcat1*i.treatmentintensity
estimates store a
xi: stcox i.treatmentintensity i.platcat1
lrtest a



stset ptime, failure(progressed==1)
stcox treatmentintensity if platcat1==1
stcox treatmentintensity primresect who NLRcateg1 if platcat1==1

 stset ptime, failure(progressed==1)
stcox treatmentintensity if platcat1==0
stcox treatmentintensity primresect NLRcateg1 who if platcat1==0

 xi: stcox i.platcat1*i.treatmentintensity
estimates store a
xi: stcox i.treatmentintensity i.platcat1
lrtest a



*lr vs ox analysis

stset stime, failure(died==1)
stcox analysisirvsox2 if platcat1==1
stcox analysisirvsox2 if platcat1==0
  xi: stcox i.platcat1*i.analysisirvsox2
estimates store a
xi: stcox i.analysisirvsox2 i.platcat1
lrtest a

 stset ptime, failure(progressed==1)
stcox analysisirvsox2 if platcat1==1
stcox analysisirvsox2 if platcat1==0


  xi: stcox i.platcat1*i.analysisirvsox2
estimates store a
xi: stcox i.analysisirvsox2 i.platcat1
lrtest a
```

```
**other prognostic markers

tab platcat1

stset stime, failure(died==1)
stcox platcat1

stset ptime, failure(progressed==1)
stcox platcat1

tab brafstatus

stset stime, failure(died==1)
stcox brafstatus

stset ptime, failure(progressed==1)
stcox brafstatus

tab primresect

stset stime, failure(died==1)
stcox primresect

stset ptime, failure(progressed==1)
stcox primresect

tab site_cat2

stset stime, failure(died==1)
stcox site_cat2

stset ptime, failure(progressed==1)
stcox site_cat2

tab metdis

stset stime, failure(died==1)
stcox metdis

stset ptime, failure(progressed==1)
stcox metdis

tab who

stset stime, failure(died==1)
stcox who

stset ptime, failure(progressed==1)
stcox who

**predictive analyses - treatment intensity


stset stime, failure(died==1)
stcox treatmentintensity


stset stime, failure(died==1)
stcox treatmentintensity if NLRcateg1==1
stsum, by (treatmentintensity)
stcox treatmentintensity platcat primresect who if NLRcateg1==1

stset stime, failure(died==1)
stcox treatmentintensity if NLRcateg1==0
stsum, by (treatmentintensity)
stcox treatmentintensity platcat primresect who if NLRcateg1==0


 xi: stcox i.NLRcateg1*i.treatmentintensity
estimates store a
xi: stcox i.treatmentintensity i.NLRcateg1
lrtest a


stset ptime, failure(progressed==1)
```

```
stcox treatmentintensity
stcox treatmentintensity if NLRcateg1==1
*sts graph, by (analysisintensity, if NLRcateg1==1)
stcox treatmentintensity platcat primresect who if NLRcateg1==1

 stset ptime, failure(progressed==1)
stcox treatmentintensity if NLRcateg1==0
*sts graph, by (analysisintensity, if NLRcateg1==1)
stcox treatmentintensity platcat primresect who if NLRcateg1==0

 xi: stcox i.NLRcateg1*i.treatmentintensity
estimates store a
xi: stcox i.treatmentintensity i.NLRcateg1
lrtest a

tab treatmentintensity bestresp if NLRcateg1==1, chi2
tab treatmentintensity bestresp if NLRcateg1==0, chi2
logistic bestresp treatmentintensity who primresect if NLRcateg==1
logistic bestresp treatmentintensity who primresect if NLRcateg==0

 xi: logistic i.NLRcateg1*i.treatmentintensity
estimates store a
xi: logistic i.treatmentintensity i.NLRcateg1
lrtest a

 ***plts + NLR analysis

 drop if NLRcateg1== .
drop if NLRcateg1<0

gen NLRplat = 1 if platcat1==1 |NLRcateg1==1
recode NLRplat (.=0)

codebook NLRplat
tab NLRplat

stset stime, failure(died==1)
stcox NLRplat
sts graph, by (NLRplat)
stcox NLRplat primresect who  braf_cat site_cat2 metsite1 kras121361 apcat1



stset ptime, failure(progressed==1)
stcox NLRplat
stcox NLRplat primresect who  braf_cat site_cat2 metsite1 kras121361 apcat1

stset stime, failure(died==1)
stcox treatmentintensity if NLRcateg1==1
stsum, by (treatmentintensity)
stcox treatmentintensity platcat primresect who if NLRcateg1==1

stset stime, failure(died==1)
stcox treatmentintensity if NLRcateg1==0
stsum, by (treatmentintensity)
stcox treatmentintensity platcat primresect who if NLRcateg1==0


 xi: stcox i.NLRcateg1*i.treatmentintensity
estimates store a
xi: stcox i.treatmentintensity i.NLRcateg1
lrtest a


stset ptime, failure(progressed==1)
stcox treatmentintensity if NLRplat==1
stcox treatmentintensity if NLRplat==0
stcox treatmentintensity platcat primresect who if NLRcateg1==0

 xi: stcox i.NLRplat*i.treatmentintensity
estimates store a
xi: stcox i.treatmentintensity i.NLRplat
lrtest a
```

```
***missing variables generation. then use demographics
gen sitemissing =.
replace sitemissing = 1 if site_cat==.
replace sitemissing = 0 if site_cat==1
replace sitemissing = 0 if site_cat==0
replace sitemissing = 0 if site_cat==2
```

B) STATA file for PICCOLO analyses

```
cd "/Users/medjcri/Documents/NLR project"

clear
cd "/Users/medjcri/Documents/BRAF"

insheet using "IrPan_dataset.csv", comma
keep patientno
gen trialno=patientno
drop if trialno==.
drop patientno
save "PICCOLONLRmerge.dta", replace


use "/Users/medjcri/Documents/BRAF/PICCOLO_data_allvariables_all pop.dta", clear
replace trialno="1397" if trialno=="01397"
destring trialno, replace
merge trialno using "PICCOLONLRmerge.dta"
tab _merge


gen agecat = age
recode agecat (1/69 = 0) (70/99 = 1)
label define agecat_lbl 0 "<70" 1">70"
label values agecat agecat_lbl


recode whof04 (1 = 0) (2 = 1)(3 = 1)
label define whof04_lbl 0 "PSlow" 1"PShigh"
label values whof04 who_lbl

recode prevrespf04 ( 2= 0) (3=.) (9876 = .)
label define prevrespf04_lbl 0 "progression" 1 "clinical benefit"
label values prevrespf04 prevrespf04_lbl


recode prevoxali (2/4 = 0)
label define prevoxali_lbl 0 "no" 1 "yes"
label values prevoxali prevoxali_lbl

recode prevmod (2/4 = 0)
label define prevmod_lbl 0 "no" 1 "yes"
label values prevmod prevmod_lbl



gen platcat1 = plts_vc1
recode platcat1 (1/399 = 0) (399/3000 = 1)
label define platcat1_lbl 0 "low platelets" 1">high platelets"
label values platcat1 platcat1_lbl

gen primaryan1 = 1 if kras==2 & prevcetu==2

***define NLR
clear
cd "/Users/medjcri/Documents/BRAF"


use "/Users/medjcri/Documents/BRAF/PICCOLO_data_allvariables_all pop.dta", clear
replace trialno="1397" if trialno=="01397"
destring trialno, replace
drop if trialno==.
bysort trialno:assert _N==1

gen NLR1 = neuphil_vc1/(wbc_vc1-neuphil_vc1)
```

```
gen NLRcateg1 = neuphil_vc1/(wbc_vc1-neuphil_vc1)
recode NLRcateg1 (0/2.000001 = 0) (2.000002/40 = 1)
label define NLRcateg1_lbl 0 "low NLR" 1"high NLR"
label values NLRcateg1 NLRcateg1_lbl
drop if NLRcateg1==.
drop if NLRcateg1<0


drop if randtrt==2
drop if randtrt2==3

label define dcr_lbl 1 "Clinical benefit" 0 "Progressive disease"
label values dcr dcr_lbl

label define crpr_12w 1 "Response" 0 "Stable or progressive disease"
label values crpr_12w crpr_12w_lbl

**NLR demographics

codebook NLRcateg1
summarize agecat if NLRcateg1==0
summarize agecat if NLRcateg1==1

tab who if NLRcateg1==0
tab who if NLRcateg1==1

tab randtrt2 if NLRcateg1==0
tab randtrt2 if NLRcateg1==1

tab prevresp if NLRcateg1==0
tab prevresp if NLRcateg1==1


tab prevoxali if NLRcateg1==0
tab prevoxali if NLRcateg1==1


tab prevbevac if NLRcateg1==0
tab prevbevac if NLRcateg1==1



tab prevmod if NLRcateg1==0
tab prevmod if NLRcateg1==1

tab braf_cat_sr if NLRcateg1==0
tab braf_cat_sr if NLRcateg1==1

tab site if NLRcateg1==0
tab site if NLRcateg1==1


tab mesperi
tab mesperi if braf_cat_sr==0
tab mesperi if braf_cat_sr==1

tab liver
tab liver if braf_cat_sr==0
tab liver if braf_cat_sr==1

tab lung
tab lung if braf_cat_sr==0
tab lung if braf_cat_sr==1

tab lung
tab lung if braf_cat_sr==0
tab lung if braf_cat_sr==1

tab abln
tab abln if braf_cat_sr==0
tab abln if braf_cat_sr==1

tab exabln
tab exabln if braf_cat_sr==0
```

```
tab exabln if braf_cat_sr==1

tab sex
tab sex if braf_cat_sr==0
tab sex if braf_cat_sr==1

tab kras121361_sr if braf_cat_sr==0
tab kras121361_sr if braf_cat_sr==1

tab nras1213_sr if braf_cat_sr==0
tab nras1213_sr if braf_cat_sr==1

tab pik3ca_sr if braf_cat_sr==0
tab pik3ca_sr if braf_cat_sr==1


*Prognostic analysis

gen fuptimemonths = (fuptime/12)
stset fuptime, failure(died==1)
stcox NLRcateg1 if randtrt2==1
stcox NLRcateg1 who braf_cat_sr prevresp if randtrt2==1
sts graph, by (NLRcateg1)

gen pfuptimemonths = (pfuptime/12)
stset pfuptimemonths, failure(f_pfs==1)
stcox NLRcateg1
sts graph, by (NLRcateg1)
stcox NLRcateg1 who prevresp braf_cat_sr if randtrt2==1

*Predictive analysis

stset fuptime, failure(died==1)
stcox randtrt2 if primaryan1==1
stcox randtrt2 if primaryan1==1 & NLRcateg1==1
stcox randtrt2 if primaryan1==1 & NLRcateg==0


 xi: stcox i.NLRcateg1*i.randtrt2 if primaryan1==1
estimates store a
xi: stcox i.NLRcateg1 i.randtrt2 if primaryan1==1
lrtest a

 stset pfuptime, failure(f_pfs==1)
stcox randtrt2 if primaryan1==1
stcox randtrt2 if primaryan1==1 & NLRcateg1==1
stsum, by (randtrt2)
stcox randtrt2 if primaryan1==1 & NLRcateg1==0
stsum, by (randtrt2)

xi: stcox i.NLRcateg1*i.randtrt2 if primaryan1==1
estimates store a
xi: stcox i.NLRcateg1 i.randtrt2 if primaryan1==1
lrtest a

tab crpr if randtrt2==1 & primaryan1==1 & NLRcateg1==1
tab crpr if randtrt2==2 & primaryan1==1 & NLRcateg1==1

tab crpr if randtrt2==1 & primaryan1==1 & NLRcateg1==0
tab crpr if randtrt2==2 & primaryan1==1 & NLRcateg1==0

logistic crpr randtrt2 if primaryan1==1 & NLRcateg1==1
logistic crpr randtrt2 if primaryan1==1 & NLRcateg1==0

xi: logistic i.NLRcateg1*i.randtrt2 if primaryan1==1
estimates store a
xi: logistic i.NLRcateg1 i.randtrt2 if primaryan1==1
lrtest a

**other prognostic factors

tab who if randtrt2==1

stset fuptime, failure(died==1)
stcox who if randtrt2==1
```

```
stset pfuptime, failure(f_pfs==1)
stcox who if randtrt2==1


tab platcat if randtrt2==1

stset fuptime, failure(died==1)
stcox platcat if randtrt2==1

stset pfuptime, failure(f_pfs==1)
stcox platcat if randtrt2==1

tab prevresp if randtrt2==1

stset fuptime, failure(died==1)
stcox prevresp if randtrt2==1

stset pfuptime, failure(f_pfs==1)
stcox prevresp if randtrt2==1



stset fuptime, failure(died==1)
stcox prevmod if randtrt2==1

stset pfuptime, failure(f_pfs==1)
stcox prevmod if randtrt2==1


tab braf_cat_sr if randtrt2==1

stset fuptime, failure(died==1)
stcox braf_cat_sr if randtrt2==1

stset pfuptime, failure(f_pfs==1)
stcox braf_cat_sr if randtrt2==1


tab site if randtrt2==1

stset fuptime, failure(died==1)
stcox site if randtrt2==1

stset pfuptime, failure(f_pfs==1)
stcox site if randtrt2==1

***platelets predictive

stset fuptime, failure(died==1)
stcox randtrt2 if primaryan1==1
stcox randtrt2 if primaryan1==1 & platcat1==1
stcox randtrt2 if primaryan1==1 & platcat1==0


 xi: stcox i.platcat1*i.randtrt2 if primaryan1==1
estimates store a
xi: stcox i.platcat1 i.randtrt2 if primaryan1==1
lrtest a

 stset pfuptime, failure(f_pfs==1)
stcox randtrt2 if primaryan1==1
stcox randtrt2 if primaryan1==1 & platcat1==1
stsum, by (randtrt2)
stcox randtrt2 if primaryan1==1 & platcat1==0
stsum, by (randtrt2)

xi: stcox i.platcat1*i.randtrt2 if primaryan1==1
estimates store a
xi: stcox i.platcat1 i.randtrt2 if primaryan1==1
lrtest a

tab crpr if randtrt2==1 & primaryan1==1 & platcat1==1
tab crpr if randtrt2==2 & primaryan1==1 & platcat1==1
```

```
tab crpr if randtrt2==1 & primaryan1==1 & platcat1==0
tab crpr if randtrt2==2 & primaryan1==1 & platcat1==0

logistic crpr randtrt2 if primaryan1==1 & platcat1==1
logistic crpr randtrt2 if primaryan1==1 & platcat1==0

****tumour marker analysis

gen NLR4 = neuphil_vc4/(wbc_vc4 - neuphil_vc4)
drop if NLR4<0

gen NLRcat4 = NLR4
gen NLRcateg4 = NLR4
recode NLRcateg4 (0/2.000001 = 0) (2.0000002/40 = 1)
label define NLRcateg4_lbl 0 "low NLR" 1"high NLR"
label values NLRcateg4 NLRcateg4_lbl

stset fuptime, failure(died==1)
stcox NLRcateg4
stset pfuptime, failure(f_pfs==1)
stcox NLRcateg4


gen NLRchange = (NLR4-NLR1)/NLR1
gen NLRchangepercent = NLRchange *100
gen NLRchangecat1 = 2 if NLRchange<=-0.25
replace NLRchangecat1 = 1 if NLRchange>-0.25 & NLRchange<0.25
replace NLRchangecat1 = 0 if NLRchange>=0.25 & NLRchange~=.
label define NLRchangecat1_lbl 0 "NLRdecrease" 1 "NLRstable" 2 "NLRincrease"
label values NLRchangecat1 NLRchangecat1 _lbl

gen NLRimproved = NLRchangecat1
recode NLRimproved (1=0)(2=1)
label define NLRimproved_lbl 0 "no improvement" 1 "improvement"
label values NLRimproved NLRimproved_lbl

gen NLRfavourable = NLRchangecat1
recode NLRfavourable (2=1)
label define NLRfavourable_lbl 0 "not favourable" 1 "favourable"
label values NLRfavourable NLRfavourable_lbl

gen NLRworse = NLRchangecat1
recode NLRworse (0=1)(2=0)(1=0)
label define NLRworse_lbl 0 "no deterioration" 1 "deterioration"
label values NLRworse NLRworse_lbl

recode crpr_12w (.=0)

logistic crpr_12w NLRimproved
logistic crpr_12w NLRfavourable
logistic crpr_12w NLRworse

recode response_12wpd (.=0)
logistic response_12wpd NLRimproved
logistic response_12wpd NLRfavourable
logistic response_12wpd NLRworse

****Alternative cut-points for baseline NLR
gen NLR2025 = .
replace NLR2025 = 0 if NLR1<=1.999
replace NLR2025 = 1 if NLR1>=2.0 & NLR1<=2.5

gen NLR2530 = .
replace NLR2530 = 0 if NLR1<=1.999
replace NLR2530 = 1 if NLR1>=2.5 & NLR1<=3.0

gen NLR3035 = .
replace NLR3035 = 0 if NLR1<=1.999
replace NLR3035 = 1 if NLR1>=3.0 & NLR1<=3.5

gen NLR3541 = .
replace NLR3541 = 0 if NLR1<=1.999
replace NLR3541 = 1 if NLR1>=3.5 & NLR1<=4.0

gen NLR4045 = .
```

```
replace NLR4045 = 0 if NLR1<=1.999
replace NLR4045 = 1 if NLR1>=4.0 & NLR1<=4.5

gen NLRmax = .
replace NLRmax = 0 if NLR1<=1.999
replace NLRmax = 1 if NLR1>4.5

stcox NLR2025
stcox NLR2530
stcox NLR3035
stcox NLR3541
stcox NLR4045
stcox NLRmax
```

Appendix 2: STATA files used in Chapter 3

a) STATA files for FOCUS and COIN analyses

```
use "/Users/medjcri/Documents/BRAF/COINFOCUSdataset.dta", clear

drop if trt==2                    // COIN Arm B is not used in any part of this work
label var trial "Trial"

gen who2 = (who==2) if !missing(who)
gen resect = (tstat==1) if !missing(tstat)
recode plt (400/max=1 ">=400,000/`micro'l") (min/400=0 "<400,000/`micro'l"), gen(plt400)


*** Findings(1): BRAF-mut frequency
tab braf trial, col


*** Findings(1): BRAF-mut status was associated with inferior OS in the 1st line (median 10.8 vs 16.4mths)
stset ostime, fail(death) scale(30.4375)
stsum, by(braf)
stcox braf                                                                                                    // Total
available N=2071
stcox braf trial who2 resect plt400 right mperi msi          // Adjusted incl. MSI: N=1608 (78%)
stcox braf trial who2 resect plt400 right mperi                    // Adjusted incl. MSI: N=1865 (90%)

* Forest plot
ipdmetan, study(trial) hr forestplot(nowt boxsca(250) lcols((e(N)) "N") ///
        range(.6 2.75) ysize(2.5)) : stcox braf
                        // Unadjusted (for info only)

ipdmetan, study(trial) hr forestplot(nowt boxsca(250) lcols((e(N)) "N") ///
        range(.6 2.75) ysize(2.5)) : stcox braf who2 resect plt400 right mperi msi                    // Adjusted, incl.
MSI

ipdmetan, study(trial) hr forestplot(nowt boxsca(250) lcols((e(N)) "N") ///
        range(.6 2.75) ysize(2.5)) : stcox braf who2 resect plt400 right mperi                    // Adjusted, excl.
MSI

* Kaplan-Meier plot (unadjusted)
sts graph, by(braf) xlabel(0(6)42) tmax(42) risktable(, order(1 "Wild-type" 2 "Mutation")) ///
        legend(order(1 "Wild-type" 2 "Mutation")) xtitle("Analysis time (months)") ytitle("Survival") title("")


*** Findings(2): BRAF-mut status had modest impact on PFS & response endpoints
gen resp12 = dstat12<3
gen dcr = dstat12<4

* 1st line doublet/combination (COIN + FOCUS C)
tab resp12 braf if firstline| trial==2, col
logistic resp12 braf if firstline | trial==2
logistic resp12 braf who2 resect plt400 right mperi msi if firstline | trial==2
logistic resp12 braf who2 resect plt400 right mperi if firstline | trial==2

tab dcr braf if firstline | trial==2, col
logistic dcr braf if firstline | trial==2
logistic dcr braf who2 resect plt400 right mperi msi if firstline | trial==2
```

```
logistic dcr braf who2 resect plt400 right mperi if firstline | trial==2

stset pfstime, fail(pfsfail) scale(30.4375) if(firstline | trial==2)
stsum, by(braf)
stcox braf                                                                  // Total available N=1641
stcox braf who2 resect plt400 right mperi msi                  // Adjusted incl. MSI: N=1283 (78%)
stcox braf who2 resect plt400 right mperi                              // Adjusted incl. MSI: N=1530 (93%)

sts graph, by(braf) xlabel(0(6)42) tmax(42) risktable(, order(1 "Wild-type" 2 "Mutation")) ///
        legend(order(1 "Wild-type" 2 "Mutation")) xtitle("Analysis time (months)") ytitle("Survival") title("")


ipdmetan, study(trial) hr forestplot(nowt boxsca(250) lcols((e(N)) "N") ///
        range(.6 2.75) ysize(2.5)) : stcox braf
                                // Unadjusted (for info only)

ipdmetan, study(trial) hr forestplot(nowt boxsca(250) lcols((e(N)) "N") ///
        range(.6 2.75) ysize(2.5)) : stcox braf who2 resect plt400 right mperi msi                  // Adjusted, incl.
MSI

ipdmetan, study(trial) hr forestplot(nowt boxsca(250) lcols((e(N)) "N") ///
        range(.6 2.75) ysize(2.5)) : stcox braf who2 resect plt400 right mperi                  // Adjusted, excl.
MSI



* 1st line FU alone (FOCUS A and B)
tab resp12 braf if !firstline, col
logistic resp12 braf if !firstline
logistic resp12 braf who2 resect plt400 right mperi msi if !firstline
logistic resp12 braf who2 resect plt400 right mperi if !firstline

tab dcr braf if !firstline, col
logistic dcr braf if !firstline
logistic dcr braf who2 resect plt400 right mperi msi if !firstline
logistic dcr braf who2 resect plt400 right mperi if !firstline

stset pfstime, fail(pfsfail) scale(30.4375) if(!firstline)
stsum, by(braf)
stcox braf                                                                  // Total available
N=430
stcox braf who2 resect plt400 right mperi msi                  // Adjusted incl. MSI: N=325 (76%)
stcox braf who2 resect plt400 right mperi                              // Adjusted excl. MSI: N=335 (78%)

sts graph, by(braf) xlabel(0(6)42) tmax(42) risktable(, order(1 "Wild-type" 2 "Mutation")) ///
        legend(order(1 "Wild-type" 2 "Mutation")) xtitle("Analysis time (months)") ytitle("Survival") title("")


*** Findings(2): Following progression, BRAF-mut patients had shorter post-progression survival
gen ppstime = ostime-pfstime if pfsfail

* 1st line doublet/combination (COIN + FOCUS C)
stset ppstime, fail(death) scale(30.4375) if(firstline | trial==2)
stsum, by(braf)
stcox braf                                                                  // Total available N=1377
stcox braf who2 resect plt400 right mperi msi                  // Adjusted incl. MSI: N=1083 (79%)
stcox braf who2 resect plt400 right mperi                              // Adjusted excl. MSI: N=1296 (94%)

* 1st line FU alone (FOCUS A and B)
stset ppstime, fail(death) scale(30.4375) if(!firstline)
stsum, by(braf)
stcox braf                                                                  // Total available
N=370
stcox braf who2 resect plt400 right mperi msi                  // Adjusted incl. MSI: N=296 (80%)
stcox braf who2 resect plt400 right mperi                              // Adjusted incl. MSI: N=306 (83%)

* Combined (all COIN and FOCUS arms)
stset ppstime, fail(death) scale(30.4375)
stsum, by(braf)
stcox braf                                                                  // Total available N=1747
stcox braf who2 resect plt400 right mperi msi                  // Adjusted incl. MSI: N=1379 (79%)
stcox braf who2 resect plt400 right mperi                              // Adjusted excl. MSI: N=1602 (92%)

* Forest plot
ipdmetan, study(trial) hr forestplot(nowt boxsca(250) lcols((e(N)) "N") ///
```

```
        range(.6 2.75) ysize(2.5)) : stcox braf
                                // Unadjusted (for info only)

ipdmetan, study(trial) hr forestplot(nowt boxsca(250) lcols((e(N)) "N") ///
        range(.6 2.75) ysize(2.5)) : stcox braf who2 resect plt400 right mperi msi                    // Adjusted, incl.
MSI

ipdmetan, study(trial) hr forestplot(nowt boxsca(250) lcols((e(N)) "N") ///
        range(.6 2.75) ysize(2.5)) : stcox braf who2 resect plt400 right mperi                        // Adjusted, excl.
MSI

* Kaplan-Meier plot (unadjusted)
sts graph, by(braf) xlabel(0(3)24) tmax(24) risktable(, order(1 "Wild-type" 2 "Mutation")) ///
        legend(order(1 "Wild-type" 2 "Mutation")) xtitle("Analysis time (months)") ytitle("Survival") title("")

*** BRAF-mut status as a significant prognostic marker for OS
stset ostime, fail(death) scale(30.4375) if(firstline!=1)

* Combined, n=1714 (i.e. EXcluding FOCUS Strategy C (Arms C or E) patients)
stset ostime, fail(death) scale(30.4375) if(firstline!=1)

ipdmetan, study(trial) hr forestplot(nohet nowt boxsca(250) lcols((e(N)) "N") ///
        range(.6 2.75) ysize(2.5) spacing(2.5)) : stcox braf
                                        // n=1714
ipdmetan, study(trial) hr forestplot(nohet nowt boxsca(250) lcols((e(N)) "N") ///
        range(.6 2.75) ysize(2.5) spacing(2.5)) : stcox braf who2 resect plt400 right mperi msi                //
n=1334 (78%)

sts graph if _rsample, by(braf) xlabel(0(6)42) tmax(42) risktable(, order(1 "Wild-type" 2 "Mutation")) ///
        legend(order(1 "Wild-type" 2 "Mutation")) xtitle("Analysis time (months)") ytitle("Survival") title("")


* Combined, n=2071 (i.e. INcluding FOCUS Strategy C (Arms C or E) patients)
stset ostime, fail(death) scale(30.4375)

ipdmetan, study(trial) hr forestplot(nohet nowt boxsca(250) lcols((e(N)) "N") ///
        range(.6 2.75) ysize(2.5) spacing(2.5)) : stcox braf
                                        // n=2071
ipdmetan, study(trial) hr forestplot(nohet nowt boxsca(250) lcols((e(N)) "N") ///
        range(.6 2.75) ysize(2.5) spacing(2.5)) : stcox braf who2 resect plt400 right mperi msi                //
n=1608 (78%)

sts graph if _rsample, by(braf) xlabel(0(6)42) tmax(42) risktable(, order(1 "Wild-type" 2 "Mutation")) ///
        legend(order(1 "Wild-type" 2 "Mutation")) xtitle("Analysis time (months)") ytitle("Survival") title("")



*** BRAF-mut status as a significant prognostic marker for PPS

gen ppstime = ostime-pfstime if pfsfail
stset ppstime, fail(death) scale(30.4375)

ipdmetan, study(trial) hr forestplot(nohet nowt boxsca(250) lcols((e(N)) "N") ///
        xlabel(.5 1 2 4) range(.6 4) ysize(2.5) spacing(2.5)) : stcox braf
                                                                        // n=1747
ipdmetan, study(trial) hr forestplot(nohet nowt boxsca(250) lcols((e(N)) "N") ///
        xlabel(.5 1 2 4) range(.6 4) ysize(2.5) spacing(2.5)) : stcox braf who2 resect plt400 right mperi msi
        // n=1379 (79%)

sts graph if _rsample, by(braf) xlabel(0(3)24) tmax(24) risktable(, order(1 "Wild-type" 2 "Mutation")) ///
        legend(order(1 "Wild-type" 2 "Mutation")) xtitle("Analysis time (months)") ytitle("Survival") title("")
*/

*3 trial merged demographics using Faye's merged file

summarize age if braf==0
summarize age if braf==1


tab ps
tab ps if braf==0
tab ps if braf==1
tab ps braf, exact

tab resect
```

```
tab resect if braf==0
tab resect if braf==1
tab resect braf, chi

tab prevrespf04
tab prevrespf04 if braf==0
tab prevrespf04 if braf==1
tab prevrespf04 braf, chi

tab site
tab site if braf==0
tab site if braf==1
tab site braf, chi

tab mesperi
tab mesperi if braf==0
tab mesperi if braf==1
tab mesperi braf, chi

tab MSIstatus
tab MSIstatus if braf_cat_sr==0
tab MSIstatus if braf==1


tab liver
tab liver if braf==0
tab liver if braf==1
tab liver braf, chi

tab lung
tab lung if braf==0
tab lung if braf==1
tab lung braf, chi

tab lung
tab lung if braf_cat_sr==0
tab lung if braf==1

tab abln
tab abln if braf_cat_sr==0
tab abln if braf_cat_sr==1

tab exabln
tab exabln if braf_cat_sr==0
tab exabln if braf_cat_sr==1
tab exabln braf, chi


tab sex
tab sex if braf==0
tab sex if braf==1
tab sex braf, exact

tab kras121361_sr if braf==0
tab kras121361_sr if braf==1
tab kras121361_sr braf, exact


tab nras1213_sr if braf_cat_sr==0
tab nras1213_sr if braf_cat_sr==1

tab pik3ca_sr if braf_cat_sr==0
tab pik3ca_sr if braf_cat_sr==1
```

b) STATA files for PICCOLO analyses

```
clear
cd "/Users/medjcri/Documents/BRAF"

use "/Users/medjcri/Documents/BRAF/PICCOLO_data_allvariables_all pop.dta", clear
replace trialno="1397" if trialno=="01397"
```

```
destring trialno, replace
drop if trialno==.
bysort trialno:assert _N==1

gen agecat = age_rand
recode agecat (1/69 = 0) (70/99 = 1)
label define agecat_lbl 0 "<70" 1">70"
label values agecat agecat_lbl

recode whof04 (1 = 0) (2 = 1)(3 = 1)
label define whof04_lbl 0 "PSlow" 1"PShigh"
label values whof04 who_lbl

recode prevrespf04 ( 2= 0) (3=.) (9876 = .)
label define prevrespf04_lbl 0 "progression" 0 "clinical benefit"
label values prevrespf04 prevrespf04_lbl

recode mesperi (2=0)

recode prevoxalif04 (2/4 = 0)
label define prevoxalif04_lbl 0 "no" 1 "yes"
label values prevoxalif04 prevoxalif04_lbl

gen fuptimemths = (fuptime/30.5)
gen pfuptimemths = (pfuptime/30.5)
gen postprogOS = (fuptime-pfuptime) if f_pfs==1
gen postprogOSmths = (postprogOS/30.5)

recode mesperi (9876=.)

gen MSIstatus =.
replace MSIstatus = 0 if mlh1ihc_sr=="P" & msh2ihc_sr=="P"
replace MSIstatus = 1 if msh2ihc_sr=="N"
replace MSIstatus = 1 if mlh1ihc_sr=="N"

recode prevresect (2=0)



gen rightperit =.
replace rightperit =1 if site==1 & mesperi==1
recode rightperit(.=0)

drop if randtrt== 2
drop if randtrt== 3

**demographic info

codebook braf_cat_sr

summarize age_rand if braf_cat_sr==0
summarize age_rand if braf_cat_sr==1

tab whof04
tab whof04 if braf_cat_sr==0
tab whof04 if braf_cat_sr==1
tab whof04 braf_cat_sr, chi

tab prevrespf04
tab prevrespf04 if braf_cat_sr==0
tab prevrespf04 if braf_cat_sr==1
tab prevrespf04 braf_cat_sr, chi

tab site
tab site if braf_cat_sr==0
tab site if braf_cat_sr==1
tab site braf_cat_sr, chi

tab mesperi
tab mesperi if braf_cat_sr==0
tab mesperi if braf_cat_sr==1
tab mesperi braf_cat_sr, chi

tab MSIstatus
tab MSIstatus if braf_cat_sr==0
```

```
tab MSIstatus if braf_cat_sr==1


tab liver
tab liver if braf_cat_sr==0
tab liver if braf_cat_sr==1
tab liver braf_cat_sr, chi

tab lung
tab lung if braf_cat_sr==0
tab lung if braf_cat_sr==1
tab lung braf_cat_sr, chi

tab lung
tab lung if braf_cat_sr==0
tab lung if braf_cat_sr==1

tab abln
tab abln if braf_cat_sr==0
tab abln if braf_cat_sr==1

tab exabln
tab exabln if braf_cat_sr==0
tab exabln if braf_cat_sr==1

tab sex
tab sex if braf_cat_sr==0
tab sex if braf_cat_sr==1

tab kras121361_sr if braf_cat_sr==0
tab kras121361_sr if braf_cat_sr==1

tab nras1213_sr if braf_cat_sr==0
tab nras1213_sr if braf_cat_sr==1

tab pik3ca_sr if braf_cat_sr==0
tab pik3ca_sr if braf_cat_sr==1

****BRAF status and response12weeks

recode crpr (.=0)
tab crpr braf_cat_sr, chi2
logistic crpr braf_cat_sr

recode crpr_12w (.=0)
tab crpr_12w braf_cat_sr, chi2
logistic crpr_12w braf_cat_sr
logistic crpr_12w braf_cat_sr whof04 mesperi prevrespf04 prevresect MSIstatus site

recode response_12wpd (.=0)
tab response_12wpd braf_cat_sr, chi2
logistic response_12wpd braf_cat_sr

***survival analyses


stset fuptimemths, failure(died==1)
stsum, by (braf_cat_sr)
stcox braf_cat_sr
sts graph, by(braf_cat_sr) xlabel(0(3)24) tmax(24) risktable(, order(1 "Wild-type" 2 "Mutation")) ///
        legend(order(1 "Wild-type" 2 "Mutation")) xtitle("Analysis time (months)") ytitle("Survival") title("")


sts graph, by(braf_cat_sr)
stsum, by(braf_cat_sr)


stset pfuptimemths, failure(f_pfs)
stsum, by (braf_cat_sr)
stcox braf_cat_sr
stcox braf_cat_sr site prevresp prevresect mesperi whof04
sts graph, by(braf_cat_sr) xlabel(0(3)24) tmax(24) risktable(, order(1 "Wild-type" 2 "Mutation")) ///
        legend(order(1 "BRAF wild-type" 2 "BRAF mutation")) xtitle("Analysis time (months)") ytitle("Survival")

        stsum, by(braf_cat_sr)
```

```
drop if postprogOS==0
stset postprogOSmths, failure(died==1)
stsum, by (braf_cat_sr)
stcox braf_cat_sr
stcox braf_cat_sr whof04 site mesperi prevresect prevresp


sts graph, by(braf_cat_sr) xlabel(0(3)24) tmax(24) risktable(, order(1 "Wild-type" 2 "Mutation")) ///
            legend(order(1 "BRAF wild-type" 2 "BRAF mutation")) xtitle("Analysis time (months)") ytitle("Survival")



***site demographic information

codebook site

summarize age_rand if site==0
summarize age_rand if site==1


tab whof04 if site==0
tab whof04 if site==1


tab prevrespf04 if site==0
tab prevrespf04 if site==1


tab kras121361_sr if site==0
tab kras121361_sr if site==1

tab braf_cat_sr if site==0
tab braf_cat_sr if site==1

tab nras121361_sr if site==0
tab nras121361_sr if site==1

tab pik3ca_sr if site==0
tab pik3ca_sr if site==1

codebook site_lcr

summarize age_rand if site_lcr==0
summarize age_rand if site_lcr==1

tab whof04
tab whof04 if site_lcr==0
tab whof04 if site_lcr==1

tab prevrespf04
tab prevrespf04 if site_lcr==0
tab prevrespf04 if site_lcr==1

tab kras121361_sr if site_lcr==0
tab kras121361_sr if site_lcr==1

tab braf_cat_sr if site_lcr==0
tab braf_cat_sr if site_lcr==1

tab nras121361_sr if site_lcr==0
tab nras121361_sr if site_lcr==1

tab pik3ca_sr if site_lcr==0
tab pik3ca_sr if site_lcr==1

tab prevoxalif04 braf_cat_sr

***site  prognostic analysis

gen fuptimemths = (fuptime/12)

stset fuptimemths, failure(died==1)
stcox site
sts graph, by(site)
```

```
stset pfuptime, failure(f_pfs)
stcox site
sts graph, by(site)

gen postprogOS = (fuptime-pfuptime) if f_pfs==1
drop if postprogOS==0
stset postprogOS, failure(died==1)
stcox site
sts graph, by(site)

***site predictive analysis


stset fuptime, failure(died==1)
stcox randtrt2 if site==1

stset fuptime, failure(died==1)
stcox randtrt2 if site==0



 xi: stcox i.site*i.randtrt2
estimates store a
xi: stcox i.randtrt2 i.site
lrtest a

***RAS analysis chemotherapy alone

tab crpr any_ras, chi2
logistic crpr any_ras

tab crpr_12w any_ras, chi2
logistic crpr_12w any_ras

tab response_12wpd any_ras, chi2
logistic response_12wpd any_ras

stset fuptimemths, failure(died==1)
stcox any_ras
sts graph, by(any_ras)
stsum, by(any_ras)

stset pfuptimemths, failure(f_pfs)
stcox any_ras
sts graph, by(any_ras)
stsum, by(any_ras)


drop if postprogOS==0
stset postprogOSmths, failure(died==1)
stcox any_ras
stcox any_ras whof04 site
sts graph, by(any_ras)
stsum,by(any_ras)

**PIK analysis chemotherapy alone

tab pik3ca_sr
tab crpr pik3ca_sr, chi2
logistic crpr pik3ca_sr

tab crpr_12w pik3ca_sr, chi2
logistic crpr_12w pik3ca_sr

tab response_12wpd pik3ca_sr, chi2
logistic response_12wpd pik3ca_sr

stset fuptimemths, failure(died==1)
stcox pik3ca_sr
sts graph, by(pik3ca_sr)
stsum, by(pik3ca_sr)

stset pfuptimemths, failure(f_pfs)
stcox pik3ca_sr
```

```
sts graph, by(pik3ca_sr)
stsum, by(pik3ca_sr)

drop if postprogOS==0
stset postprogOSmths, failure(died==1)
stcox pik3ca_sr
stcox pik3ca_sr whof04 site
sts graph, by(pik3ca_sr)
stsum,by(pik3ca_sr)
```

Appendix 3: PCR primers used in Chapters 4

Gene	Sequence
EREG	CCACATATTATTTCTGCAGATGGTACAGAAATCGAA AAAGTAAAGAACCAAAGAAGGAATATGAGAGAGTT ACCTCAGGGGATCCAGAGTTGCCGCAAGTCTGAAT
AREG	ATTCACGGAGAATGCAAATATATAGAGCACCTGGA AGCAGTAACATGCAAATGTCAGCAAGAATATTTCG GTGAACGGTGTGGGGAAAAGTCCATGAAAACTCAC
GADPH	TCAGACACCATGGGGAAGGTGAAGGTCGGAGTCAA CGGATTTGGTCGTATTGGGCGCCTGGTCACCAGGGC TGCTTTTAACTCTGGTAAAGTGGATAT
UBC	TAGTTCCGTCGCAGCCGGGATTTGGGTCGCAGTTCT TGTTTGTGGATCGCTGTGATCGTCACTTGACAATGC AGATCTTCGTGAAGACTCTGACTGGTAAGACCATCA CCCTCGAGGTTG
RPL13A	CTTGGGGACAGCATGAGCTTGCTGTTGTACACAGGG TATTTCTAGAAGCAGAAATAGACTGGGAAGATGCAC AACCAAGGGGTTACAGGCATCGCCCATGCTCCTCAC CTGTATTTTGTAATCAGAAATAAATTGCTTTT

Appendix 4. Extended SNP analysis PCR primers used in Chapter 6

	Gene	Allele	Allele detected by ASP1*	ASP1	Allele detected by ASP2G	ASP2
rs887829	UGT1A1	AG	A	GTGAACAAGTTAGGCTTCTTTTCCAA	G	GTGAACAAGTTAGGCTTCTTTTCCAG
rs3755319	UGT1A1	GT	G	GCTCATCTTTCCCTTTTGACTTCAAC	T	GCTCATCTTTCCCTTTTGACTTCAAA
rs4124874	UGT1A1	AC	A	CTTTGATGTTCTCAAATTGCTTTGTTCAA	C	CTTTGATGTTCTCAAATTGCTTTGTTCAC
rs4148323	UGT1A1	GA	G	CGTCTTCAAGGTGTAAAATGCTCC	A	ACGTCTTCAAGGTGTAAAATGCTCT
rs10929302	UGT1A1	AG	A	CCCAGCCCACCTGTCT	G	CCCAGCCCACCTGTCC
rs853035	UGT1A1	AG	A	GGTGGCGGGTCTCCA	G	GGTGGCGGGTCTCCG
rs2008595	UGT1A3	AG	A	CCACTTCAGAGAGAGTCCTCTT	G	CCACTTCAGAGAGAGTCCTCTC
rs3806596	UGT1A3	AG	A	TGGCTCAGTGACAAGGTAATTAAGAT	G	TGGCTCAGTGACAAGGTAATTAAGAC
rs3732217	UGT1A4	CT	C	GAAGACCATGTTGGGCATGATC	T	GAAGACCATGTTGGGCATGATT
rs1105880	UGT1A6	CT	C	CTTTGCTGAGCGATCATTCCTG	T	ACTTTGCTGAGCGATCATTCCTA
rs7577677	UGT1A7	AC	A	GGTCAGCAGTAGACACACATATAGT	C	GGTCAGCAGTAGACACACATATAGG
rs7586110	UGT1A7	GT	G	CAGGTTCTATCTGTACTTCTTCCACG	T	CAGGTTCTATCTGTACTTCTTCCACT
rs11692021	UGT1A7	CT	C	CAAGTGCATGATGTGGTTCCG	T	CAAGTGCATGATGTGGTTCCA
rs17868323	UGT1A7	GT	G	ACTTATTTTTTTCAAATTGCAGGAGTTTGTTTAAG	T	ACTTATTTTTTTCAAATTGCAGGAGTTTGTTTAAT
rs17868324	UGT1A7	AG	A	CAAAACAACTCTCCTTTAAGTATTCTACTAATTTTT	G	CAAAACAACTCTCCTTTAAGTATTCTACTAATTTTC
rs1042597	UGT1A8	CG	C	TCTTCGCCAGGGGAATAGC	G	GTCTTCGCCAGGGGAATAGG
rs1042605	UGT1A8	AG	A	GTTTGGGATAGTCCAAAACAAAGTCT	G	GTTTGGGATAGTCCAAAACAAAGTCC

SNP	Gene	Allele	Allele detected by ASP1	ASP1	Allele detected by ASP2	ASP2
rs2741046	UGT1A9	CT	C	GACCTTGAAGGTTCAGAAAGATAAAGTAAG	T	GACCTTGAAGGTTCAGAAAGATAAAGTAAA
rs2741048	UGT1A9	AC	A	CATTTCCTCTGGGGCGGT	C	ATTTCCTCTGGGGCGGG
rs4663871	UGT1A9	AG	A	AAGGGCAGTTTTATAAAATTTGCTACTGAT	G	AGGGCAGTTTTATAAAATTTGCTACTGAC
rs6731242	UGT1A9	CT	C	CCTACTGTGCACTAGAAGCCG	T	CCTACTGTGCACTAGAAGCCT
rs13418420	UGT1A9	CT	C	TTGGCATGTTATATGTGTTATATACTGTATTATCAC	T	TTGGCATGTTATATGTGTTATATACTGTATTATCAT
rs17862856	UGT1A9	AG	A	ATTTTTTGTCTTTATGAATAGGGCCA	G	ATTTTTTGTCTTTATGAATAGGGCCG
rs1823803	UGT1A10	CT	C	ATTCTGTCCAGTGCAACAAATATTCC	T	GAATATAATTCTGTCCAGTGCAACAAATATTCT
rs2741031	UGT1A10	CT	C	GAGCTTTACCAAATTAATTGATCTCAACAAAC	T	AGAGCTTTACCAAATTAATTGATCTCAACAAAT
rs1045642	ABCB1	CT	C	TCCTTTGCTGCCCTCACG	T	TCCTTTGCTGCCCTCACA
rs1128503	ABCB1	CT	C	TCTGCACCTTCAGGTTCAGG	T	CTCTGCACCTTCAGGTTCAGA
rs2032582	ABCB1	GT	G	AGTTTGACTCACCTTCCCAGC	T	ATATTTAGTTTGACTCACCTTCCCAGA
rs212088	ABCC1	CT	C	CCCAAAGCCTAGAGGCCAC	T	CCCAAAGCCTAGAGGCCAT
rs35588	ABCC1	AG	A	GCCAATGGCACAGCGT	G	GCCAATGGCACAGCGC
rs35605	ABCC1	CT	C	TAGACGGCAAATGTGCACAG	T	TAGACGGCAAATGTGCACAA
rs2230671	ABCC1	AG	A	CGGGAGCTGGGAAGTCA	G	CGGGAGCTGGGAAGTCG
rs3765129	ABCC1	CT	C	GGCGACCCTGGGATCAG	T	GGCGACCCTGGGATCAA
rs717620	ABCC2	AG	A	CTGGACTGCGTCTGGAACA	G	TGGACTGCGTCTGGAACG
rs3740066	ABCC2	AG	A	ACCTACCTTCTCCATGCTACCA	G	CCTACCTTCTCCATGCTACCG
rs562	ABCC5	CT	C	ATGCAACGCTGACCATTCAAC	T	CATGCAACGCTGACCATTCAAT
rs425215	ABCG1	CG	C	GCCACCACACCTCCTAGATC	G	GCCACCACACCTCCTAGATG

SNP	Gene	Allele	Allele detected by ASP1	ASP1		ASP2
rs12721627	CYP3A4	CG	C	GCCACCACACCTCCTAGATC	G	GCCACCACACCTCCTAGATG
rs2740574	CYP3A4	AG	A	CAGCCATAGAGACAAGGGCAA	G	AGCCATAGAGACAAGGGCAG
rs4986910	CYP3A4	CT	C	CATGTTCATGAGAGCAAACCTCG	T	TCATGTTCATGAGAGCAAACCTCA
rs776746	CYP3A5	AG	A	GTGGTCCAAACAGGGAAGAGATAT	G	TGGTCCAAACAGGGAAGAGATAC
rs10264272	CYP3A5	CT	C	CCCCTTTGTGGAGAGCACTAAG	T	CCCCTTTGTGGAGAGCACTAAA
rs2306283	SLCO1B1	CT	C	CTTACAGTTACAGGTATTCTAAAGAAACTAATATCG	T	CTTACAGTTACAGGTATTCTAAAGAAACTAATATCA
rs4149056	SLCO1B1	CG	C	CACGAAGCATATTACCCATGAACG	G	CACGAAGCATATTACCCATGAACA
rs1358503	SEMA3c	CT	C	GTAATATGCAAAGCACCCTGTGG	T	TGTAATATGCAAAGCACCCTGTGA
rs1517114	C8orf34			AGGGAGTATGATAATTTTCCTGATAGCC		AGGGAGTATGATAATTTTCCTGATAGCG