Supplementary appendix

This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

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Appendix to Panitumumab and irinotecan versus irinotecan alone for patients with KRAS wild-type, fluorouracil-resistant advanced colorectal cancer (PICCOLO): a prospectively stratified randomised trial

1. Participating centres and principal investigators
2. Laboratory methods
3. Additional clinical data
4. Sensitivity analyses

1) Participating centres and Principal Investigators

Addenbrookes, Cambridge (PI: Charles Wilson); Airedale, Keighley, (PI Michael Crawford); Bradford Royal Infirmary (PI: Andy Conn); Bristol (PI: Stephen Falk); Huddersfield & Halifax (PI: Jo Dent); Charing Cross & Hammersmith, London (PI: Charles Lowdell); Cheltenham & Gloucester (PI: Kim Benstead); Clatterbridge (PI: Sun Myint); Dorset County (PI: Richard Osborne); Eastbourne (PI: Fiona McKinna); Edinburgh Cancer Centre, Western General, Edinburgh (PI: Lesley Dawson); Grimsby (PI: Rajarshi Roy); Hereford County (PI: Nick Reed); Hinchingbrooke, Cambridge (PI: Li Tee Tan); James Cook, Middlesbrough (PI: Nicholas Wadd); Kent and Canterbury Hospitals (PI: Catherine Harper-Wynne); Maidstone (PI: Mark Hill); Mount Vernon Hospital, Middlesex (PI: Rob Glynne-Jones); New Cross Hospital (PI: Mark Churn); Northampton (PI: Craig Macmillan); Nottingham City (PI: Dr Vanessa Potter); Peterborough (PI: Karen McAdam); Poole & Bournemouth (PI: Tamas Hickish); Burton (PI: Prabir Chakraborti); Devon & Exeter (PI: Melanie Osborne); Royal Free Hospital, London (PI: Astrid Mayer); Royal Hampshire (PI: Dr Nolan); Royal Marsden (PI: Ian Chau); Scunthorpe (PI: Abdel Hamid); Swansea (PI: John Wagstaff); Leeds (PI Matt Seymour); South Tyneside (PI: Ashraf Azzabi); Bart’s London (PI: Sarah Slater); St Luke’s Guildford (PI: Gary Middleton); St Mary’s, London (PI: Susan Cleator); St Mary’s/QA Portsmouth (PI: Ann O’Callaghan); St Thomas’s/QUE London (PI: Nick Maisey); Stafford (PI: Selvaraj Giridharan); Swindon (PI: Claire Blesing); Torbay (PI: Nangi Lo); UCL/N.Middx/Harlow (PI: John Bridgewater); Coventry & Warwickshire (PI: Robert Grieve); Velindre, Cardiff (PI: Tim Maughan); Wansbeck, Northumberland (PI: Werner Dobrowsky); West Middlesex (PI: Pippa Riddle); Weston Park, Sheffield (PI: Jonathan Wadsley); Worthing/W.Sussex/Royal Sussex (PI: Andrew Webb); Yeovil, Somerset (PI: Clare Barlow); Ysbyty Gwynedd, Bangor (PI: Catherine Bale); Ysbyty Maelor & Glan Clwyd (PI: Simon Gollins);
2) Laboratory Methods

Formalin-fixed, paraffin-embedded tumor tissue was retrieved, anonymized and encoded with the patient’s trial number at the treating hospital, then sent to the research laboratory. Here, all staff were blind to the patients’ treatment allocation and clinical outcomes. The laboratory processing and storage of samples was under Ethical approval, and the laboratory adheres where possible to GCLP guidelines and participates annually in the UK National External Quality Assessment Service (NEQAS) scheme.

Areas containing the highest density of tumour cells were identified on a hematoxylin and eosin stained section. Six to nine 5 µm sections were used per extraction, depending upon the tumour area per slide. The sections were macrodissected with a scalpel blade, to ensure that only the tumour-rich areas were used. DNA was extracted using the Qiagen QiaAmp Micro Kit (Qiagen, Crawley, UK) following the manufacturer’s instructions. The final DNA was eluted into 25 microlitres of laboratory-grade water and the concentration determined using a Nanodrop ND-1000 spectrophotometer (Labtech International, Uckfield, East Sussex, UK). The DNA was stored at 4°C until required.

Primers for PCR amplification and Pyrosequencing analysis were designed using proprietary Pyrosequencing assay design software version 2.0.1.15 (Qiagen, Crawley, UK). PCR reactions contained 12.5µl of Qiagen HotStarTaq Master Mix (Qiagen, Crawley, UK), additional magnesium chloride to a final concentration of 2mM, 200nM each of forward and reverse primers, 20ng of tumour DNA and sufficient water to make a final volume of 25µl. Thermal cycling conditions for all amplicons 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 products were sequenced by Pyrosequencing on a PyroMark ID system (Qiagen, Crawley, UK) following the manufacturer’s protocols. Data was analysed by visual inspection of Pyrograms and by statistical analysis of peak height data.

Table 1 (appendix): PCR and Pyrosequencing primer sequences for amplification and analysis of KRAS codons 12 and 13, KRAS codon 61, KRAS codon 146, NRAS codons 12 and 13, NRAS codon 61, PIK3CA codon 542, PIK3CA codons 545 and 546, PIK3CA codon 1047 and BRAF codon 600.

| Region of interest | PCR primers (5’ → 3’) | Pyrosequencing primer (5’ → 3’) | PCR amplicon length (bp) |
|--------------------|------------------------|-------------------------------|--------------------------|
| KRAS codons 12 and 13 | Fwd: GGCCCTGCTGAAATAAGACTGA <br> Rev: biotin-AGCTGTACGTCAAGGCACTCT | AAACCTTGTGTAAGTTGGA | 80 |
| KRAS codon 61 | Fwd: AATTGATGGGAAACAGTGTCTCT <br> Rev: biotin- TCCTCATGTACTGTCCTCTATT | GGAATTTCTGACACAGACG | 86 |
| KRAS codon 146 | Fwd: TCAGAGCTTACGCAAGAAGTATAGG <br> Rev: biotin-TCGACAGAAGACAGTGTAT | GTGTTACTTACCTGCTCTGT | 100 |
| NRAS codons 12 and 13 | Fwd: CTTCACGCTGGTGAATGACTCGAG <br> Rev: biotin- TGGATTGTCAGTGCTTTTC | CTGGTGTTGGTGGA | 79 |
| NRAS codon 61 | Fwd: biotin-GGACCCTGTGTGTGGACATACTG <br> Rev: TCACACTGTCCTCATGTATTG | CTCTCATGCCACTGTAC | 83 |
| PIK3CA codon 542 | Fwd: biotin-AAAGCAATTTCTACACAGATCC <br> Rev: GCCACCTTACGACTTCTCATAGA | TTCCTCGCTCATGAT | 79 |
| PIK3CA codons 545 and 546 | Fwd: ACAGCTAAAGCAATTTCTACACAG <br> Rev: biotin-TCCTTACGACTTCTCATGAC | GATCCCTCTCCTGAAATC | 95 |
| PIK3CA codon 1047 | Fwd: biotin-TGGACAGAGGCTTTGGAGTAT <br> Rev: TGGTTTTATGGTGTTGGAAGATC | GTGTTCACGACCACCA | 102 |
| BRAF codon 600 | Fwd: TGAACACCTTCACGAAATTTAAG <br> Rev: biotin-TCCAGACAAACTGTCCTGAGTGA | TGATTTTGTGCTAGCTACAG | 91 |
3) Additional clinical data

Table 2 (appendix): Reasons for stopping treatment and post-trial EGFR-mAb therapy

|                    | Irinotecan n | IrPan n |
|--------------------|--------------|---------|
| KRASc.12,13,61-wt  | 230          | 230     |
| Patients stopped treatment | 220          | 207     |
| Reason for stopping |               |         |
| Progression        | 122 (55.5)   | 110 (53.1) |
| Death              | 34 (15.5)    | 36 (17.4)  |
| Toxicity           | 12 (5.5)     | 20 (9.7)   |
| Patient choice / unknown | 52 (23.6)   | 41 (19.8)  |
| Post-trial EGFR-mAb therapy received | 14 (6.4) | 1 (0.5) |

Table 3 (appendix): Breakdown of progression-free survival events

| Progression event          | Irinotecan n (%) | IrPan n (%) |
|---------------------------|------------------|-------------|
| KRASc.12,13,61-wt         | 206 (71.4)       | 193 (66.3)  |
| Radiological progression  | 147 (71.4)       | 128 (66.3)  |
| Clinical progression      | 10 (4.9)         | 12 (6.2)    |
| Death                     | 49 (23.8)        | 53 (27.5)   |
| All-wt                    | 145              | 131         |
| Radiological progression  | 103 (71.0)       | 94 (71.8)   |
| Clinical progression      | 5 (3.4)          | 5 (3.8)     |
| Death                     | 37 (25.5)        | 32 (24.4)   |
| Any-mut                   | 61 (72.1)        | 62 (54.8)   |
| Radiological progression  | 44 (72.1)        | 34 (54.8)   |
| Clinical progression      | 5 (8.2)          | 7 (11.3)    |
| Death                     | 12 (19.7)        | 21 (33.9)   |
Figure 1 (appendix): Subgroup analysis by mutation status: Response Rate

RECIST response rate by treatment arm in patients in the primary analysis population. “All-wt” = no mutations detected; “Any-mut” = any mutation detected.

*KRAS_{12,13,61}-mut: patients randomised prior to the protocol amendment in June 2008 and genotyped retrospectively (see main paper Figures 1 and 2)*
Figure 2 (appendix): Survival after progression

(A) All-wt patients (B) Any-mut patients. Patients whose progression event was death are classed as an event at time zero. Please view this figure alongside the PFS and OS figures shown in the main paper, Figure 5A-D.
4) Sensitivity analyses

a) All-mut status definition sensitivity analysis
In the main analysis, patients with an incomplete set of genetic data are included in the “All-wt” group provided their status is wt at every codon for which data is available. In Table 4 (Webappendix), these data are shown in the top row, and for comparison, in the second row, the data for patients with a full set of data confirming wild-type status at all 12 loci under investigation (n=237). Data are given for each efficacy endpoint for this patient group and the tests for interaction with “any-mut” status.

Table 4 (appendix): Sensitivity analysis for missing mutation status

|                      | OS Hazard ratio* (95% CI) | PFS Hazard ratio* (95% CI) | RECIST Response |
|----------------------|---------------------------|---------------------------|----------------|
| All-wt as defined in main analysis (no mutations detected, n=323) | 0.92 (0.73, 1.16) | 0.68 (0.53, 0.86) | 20/163 (12.3%) 70/160 (43.8%) |
| Confirmed wt at every locus (n=237) | 0.98 (0.74, 1.29) | 0.61 (0.46, 0.82) | 17/124 (13.7%) 48/113 (42.5%) |
| Any-mut (n=137) | 1.64 (1.14, 2.34) | 1.20 (0.83, 1.74) | 7/67 (10.4%) 9/70 (12.9%) |
| Interaction p-value (any-mut vs. confirmed wt at every locus) | 0.071 | 0.012 | 0.025 |

* For IrPan vs. irinotecan. A hazard ratio <1 indicates improved survival with IrPan compared to irinotecan alone.

b) PIK3CA sensitivity analysis
In the main analysis, mutations at PIK3CA exon 9 or 20 are grouped along with mutations in the RAS-RAF-MEK pathway for inclusion in the “Any-mut” group, shown here in the second row (n=137) of Table 5 (Webappendix). However the role of PIK3CA in EGFR signalling is less direct. The sensitivity analysis presented here shows treatment impact when the 26 patients with only a PIK3CA mutation are not included (third row, n=111). Also shown are the treatment effects in patients with only a PIK3CA mutation, although numbers here are extremely small, and the confidence intervals correspondingly wide.

Table 5 (appendix): Sensitivity analysis for PIK3CA mutations

|                      | OS Hazard ratio* (95% CI) | PFS Hazard ratio* (95% CI) | RECIST Response |
|----------------------|---------------------------|---------------------------|----------------|
| All-wt (n=323) | 0.92 (0.73, 1.16) | 0.68 (0.53, 0.86) | 20/163 (12.3%) 70/160 (43.8%) |
| Any-mut as defined in main analysis (any mutation including PIK3CA, n=137) | 1.64 (1.14, 2.34) | 1.20 (0.83, 1.74) | 7/67 (10.4%) 9/70 (12.9%) |
| Any mutation in BRAF, NRAS or KRAS c.146 (n=111) | 1.75 (1.17, 2.61) | 1.16 (0.77, 1.75) | 4/49 (8.2%) 9/62 (14.5%) |
| PIK3CA exon 9 mutation: (n=19) | 1.49 (0.43, 5.17) | 1.80 (0.57, 5.67) | 3/13 (23.1%) 0/6 (0%) |
| PIK3CA exon 20 mutation: (n=7) | 0.77 (0.14, 4.30) | 0.42 (0.05, 3.80) | 0/5 (0%) 0/2 (0%) |