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Supplementary webappendix

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

Supplement to: Maughan TS, Adams RA, Smith CG, et al, on behalf of the MRC COIN Trial Investigators. Addition of cetuximab to oxaliplatin-based first-line combination chemotherapy for treatment of advanced colorectal cancer: results of the randomised phase 3 MRC COIN trial. *Lancet* 2011; published online June 4. DOI:10.1016/S0140-6736(11)60613-2.

Web Appendix

Title: "The addition of cetuximab to oxaliplatin-based first-line combination chemotherapy for advanced colorectal cancer: results of the randomised phase 3 MRC COIN trial."

Supplementary information: Tumour samples and genetic sequencing

Materials and Methods:

Processing paraffin embedded CRCs and DNA extraction

Sections were cut from paraffin embedded CRCs using a microtome. One 5µm section was stained with Hematoxylin and Eosin and preserved with DPX. A Mirax scanner was used to generate high-resolution images of the sections. Sections containing concentrated pockets of tumour material were macrodissected using a second unstained 5µm section. For sections containing limited regions of tumour, laser capture microdissection (LCM) was performed using 10µm sections cut onto a PALM membrane slide (Carl Zeiss) and incubated at 56°C for 24 hours. Prior to LCM, PALM slides were exposed to UV (254nm) for 30 minutes, incubated in poly-L-Lysine (0.1%w/v) for 5 minutes, and allowed to dry at 60°C for at least 4 hours. Sections were deparaffinised with 100% xylene for 2 minutes (twice), followed by 100%, 95% and 70% ethanol washes for 1 minute. Slides were then dipped 5-6 times in RNase-free distilled water, stained for 1-2 minutes in Mayer's Hematoxylin solution, rinsed for 1 minute in blueing solution, stained for 10 seconds in Eosin Y, washed in 70%, 95%, and 100% ethanol for 1 minute each and air dried. Slides and LCM were viewed with a Zeiss Axiovert S100 inverted microscope using the PALM Robo software (v.1.2.3). DNA was extracted from macrodissected and LCM tumour material using QIAamp DNA Microkits (Qiagen) according to the manufacturer's instruction and eluted in 50µl nuclease-free water.

Pyrosequencing

For codons 12 and 13 of *KRAS*, we used the amplification primers 5'-GGCCTGCTGAAAATGACTGA-3' and 5'-AGAATGGTCCTGCACCAGTAATA-3' together with extension primers 5'-TGTGGTAGTTGGAGCTG-3', 5'-TGTGGTAGTTGGAGCT-3' and 5'-TGGTAGTTGGAGCTGGT-3' as previously described (Ogino et al. 2005). For codon 61 of *KRAS*, we used the amplification primers 5'-CTTTGGAGCAGGAACAATGTC-3' and 5'-CTCATGTACTGGTCCCTCATTG-3' together with the extension primer 5'-ATTCTCGACACAGCAGGT-3', and for codon 600 of *BRAF* we used the amplification primers 5'-TGCTTGCTCTGATAGGAAAATGA-3' and 5'-CAGGGCCAAAAATTTAATCAGTG-3' together with the extension primer 5'-ATTTTGGTCTAGCTACA-3'. Reverse primers were biotinylated and purified by HPLC. All other primers were unmodified and purified by standard SePOP desalting. PCR were performed in 50µl reaction volumes containing 25µl Megamix Gold (Microzone), 10-20ng DNA and 10µM of primers. Thermocycling was performed at 95°C for 10min, followed by 38 cycles of 95°C for 30s, 57°C for 30s, and 72°C for 1min, followed by a final extension of 72°C for 10min. 40µl of each PCR product was used for Pyrosequencing (Biotage). Pyrograms were analysed by two independent observers.

Sequenom

We queried the Catalogue of Somatic Mutations in Cancer (COSMIC) database (http://www.sanger.ac.uk/genetics/CGP/cosmic) for known mutations in KRAS, BRAF and NRAS in CRCs. Two hundred base pairs of sequence upstream and downstream of each mutation was downloaded from Ensembl to design the genotyping assays using the Sequenom MassARRAY Assay Design 3.1 software. In total, three multiplex assays were designed. Assay 1 included KRAS_G35ACT (Forward 5'-ACGTTGGATGCTGTATCGTCAAGGCACTCT-3', Reverse 5'-ACGTTGGATGAGGCCTGCTGAAAATGACTG-3', extension 5'-AACTTGTGGTAGTTGGAGCTG-3'), KRAS_G38ACT (Forward 5'-ACGTTGGATGCTGTATCGTCAAGGCACTCT-3', Reverse 5'-

ACGTTGGATGAGGCCTGCTGAAAATGACTG-3', extension 5'-GCACTCTTGCCTACG-3'), KRAS_A182CGT (Forward 5'-ACGTTGGATGTGGAGAAACCTGTCTCTTGG-3', Reverse 5'-ACGTTGGATGCATGTACTGGTCCCTCATTG-3', extension 5'-GGATATTCTCGACACAGCAGGTC-3'), KRAS_A183CT (Forward 5'-ACGTTGGATGTGGAGAAACCTGTCTCTTGG-3', Reverse 5'-ACGTTGGATGCATGTACTGGTCCCTCATTG-3', extension 5'-ATTGCACTGTACTCCTC-3'), BRAF_T1799A (Forward 5'-ACGTTGGATGTGATGGGACCCACTCCATCG-3', Reverse 5'-ACGTTGGATGTCTTCATGAAGACCTCACAG-3', extension 5'-CCCACTCCATCGAGATTTC-3') and NRAS_G34AT (Forward 5'-ACGTTGGATGGACTGAGTACAAACTGGTGGT-3', Reverse 5'-ACGTTGGATGAGTGGTTCTGGATTAGCTGGA-3', extension 5'-GTGCGCTTTTCCCAACACCAC-3').

Assay 2 included KRAS_G34ACT (Forward 5'-ACGTTGGATGCTGTATCGTCAAGGCACTCT-3', Reverse 5'-ACGTTGGATGAGGCCTGCTGAAAATGACTG-3', extension 5'-TGTGGTAGTTGGAGCT-3'), KRAS_G37ACT (Forward 5'-ACGTTGGATGAGGCCTGCTGAAAATGACTGA-3', Reverse 5'-ACGTTGGATGGCTGTATCGTCAAGGCACTCT-3', extension 5'-CACTCTTGCCTACGC-3'), BRAF_A1781G (Forward 5'-ACGTTGGATGTGATGGGACCCACTCCATCG-3', Reverse 5'-ACGTTGGATGTCTTCATGAAGACCTCACAG-3', extension 5'-CACTGTAGCTAGACCAAAA-3'), NRAS_C181A (Forward 5'-ACGTTGGATGGTGGTTATAGATGGTGAAACCT-3', Reverse 5'-ACGTTGGATGTATTGGTCTCTCATGGCACTG-3', extension 5'-ACAGACTGGATACAGCTGGA-3') and NRAS_A182C (Forward 5'-ACGTTGGATGTATTGGTCTCTCATGGCACTG-3', Reverse 5'-ACGTTGGATGGTGGTTATAGATGGTGAAACCT-3', extension 5'-TGGCACTGTACTCTTCT-3').

Assay 3 included KRAS_G35ACT (Forward 5'-ACGTTGGATGGCTGTATCGTCAAGGCACTCTT-3', Reverse 5'-ACGTTGGATGAGGCCTGCTGAAAATGACTGAA-3', extension 5'-CTCTTGCCTACGCCA-3'), KRAS_A182CGT (Forward 5'-ACGTTGGATGCTCATGTACTGGTCCCTCATTG-3', Reverse 5'-ACGTTGGATGGATGGAGAAACCTGTCTCTTGG-3', extension 5'-ATTGCACTGTACTCCTCT-3'), BRAF_A1781G (Forward 5'-ACGTTGGATGATGGGACCCACTCCATCGAGATT-3', Reverse 5'-ACGTTGGATGTTTCTTCATGAAGACCTCACAG-3', extension 5'-GACTGTAGCTAGACCAAAA-3'), NRAS_G34ACT (Forward 5'-ACGTTGGATGAGTGGTTCTGGATTAGCTGGAT-3', Reverse 5'-ACGTTGGATGGACTGAGTACAAACTGGTGGTG-3', extension 5'-CGCTTTTCCCAACACCAC-3'), NRAS_A182CGT (Forward 5'-ACGTTGGATGGTATTGGTCTCTCATGGCACTG-3', Reverse 5'-ACGTTGGATGCAAGTGGTTATAGATGGTGAAAC-3', extension 5'-ATCCTGGCACTGTACTCTTCT-3').

Multiplex PCR was performed in 5µl reaction volumes containing 0.5U of Taq polymerase, 5-10ng of genomic DNA, 100nM of PCR primers and 500µM of dNTP. Thermocycling was performed at 95°C for 15 min, followed by 45 cycles of 94°C for 20s, 56°C for 30s and 72°C for 60s, followed by a final extension of 72°C for 3min. Unincorporated dNTPs were deactivated using 0.3U of shrimp alkaline phosphatase at 37°C for 40min, and primer extension was carried out using 7-14µM of each extension primer, 1U of iPLEX termination mix and 1U of iPLEX enzyme. Reactions were cycled at 94°C for 30s, followed by 40 cycles of 94°C for 5s, 52°C for 5s and 80°C for 5s, followed by a final extension at 72°C for 3 min. After the addition of a cation exchange resin to remove residual salt from the reactions, 20µl of water was added and the extension product was spotted onto a matrix pad (3-hydroxypicolinic acid) of a SpectroCHIP (Sequenom). After analysing the SpectroCHIPs using a Bruker MALDI-TOF mass spectrometer, spectra were processed by the SpectroREADER software (Sequenom) and transferred to the MassARRAY Typer 4 Analyser (Sequenom). Genotyping was performed using the MassARRAY RTTM software (Sequenom). Automated calls were validated by manual review of the raw mass spectra.

Sanger Sequencing

Sanger sequencing of codons 12 and 13 of *KRAS* was performed using the primers 5'-AAAAGGTACTGGTGGAGTATTTGA-3' and 5'-CATGAAAATGGTCAGAGAAACC-3', codon 61 of *KRAS* was sequenced using 5'-CTTTGGAGCAGGAACAATGTC-3' and 5'-CTCATGTACTGGTCCCTCATTG-3', and codon 600 of *BRAF* was sequenced using 5'-AACTCTTCATAATGCTTGCTCTG-3' and 5'-TGATTTTTGTGAATACTGGGAAC-3'.

Results:

Tumour samples

We collected paraffin embedded tumour blocks from 1489/1630 patients (91.3%). 173 (11.6%) of blocks contained insufficient tumour material for processing. For the remaining samples, 1245 (94.6%) were from the primary CRC, 51 (3.9%) were from liver metastases and 20 (1.5%) were from lymph nodes..

Somatic profiling

We assayed for somatic mutations in *KRAS*, *BRAF* and *NRAS* using Pyrosequencing and Sequenom technologies. For *KRAS*, we successfully genotyped 1294/1316 samples (98.33%), for *BRAF* we genotyped 1291/1308 samples (98.70%) and for *NRAS* we genotyped 1290/1308 samples (98.62%). We detected the mutations G12A, G12D, G12V, G12C, G12R, G12S, G13C, G13D, G13V, Q61H, Q61L and Q61R in *KRAS*, D594G and V600E in *BRAF*, and G12C, Q61K, Q61L and Q61R in *NRAS*. Overall, *KRAS* mutations were found in 565/1294 samples (43.66%), *BRAF* mutations were found in 102/1291 samples (7.90%) and *NRAS* mutations were found in 50/1290 (3.88%) (see Supplementary Table 7 for mutation frequency by gene for each arm of the trial and Table 8 for data by specific mutation).

Comparison of Pyrosequencing and Sequenom analyses

For *KRAS*, 1091 samples were successfully analysed by both pyrosequencing and sequenom assays. In total, 5803/5860 (99.03%) genotype calls were concordant (ranging from 97.29% to 99.88% depending on which of the 12 mutations was assayed). For *BRAF* V600E, 884 samples were successfully analysed by both technologies. In total, 869/884 (98.30%) genotype calls were concordant. Twenty-six out of 57 samples with discordant *KRAS* calls and 8/15 samples with discordant *BRAF* calls were successfully Sanger sequenced to infer genotype. For the remaining calls, the mutant genotype was selected (since there was an obvious mutant trace via one technology).

References

Ogino S, Kawasaki T, Brahmandam M, et al: Sensitive Sequencing Method for KRAS Mutation Detection by Pyrosequencing. J Mol Diagn 7(3):413-421, 2005

Supplementary Information: EGFR Immunohistochemistry Analysis

Results were assessed retrospectively by three reviewers BJ, SS and RA using direct microscopy or Mirax® digital imaging software (initially SS direct microscopy v BJ/RA blind to one another's scores then by BJ/SS together using the Mirax® digital imaging software for a consensus on all discrepant cores).

Supplementary Tables

Table 4: Time on treatment (weeks) among *KRAS* wild-type patients, by drug

	Arm A		Arm B		p-values for Arm B vs Arm A
	Median	**(IQR)**	**Median**	**(IQR)**	
OxMdG	**N=127**		**N=117**		
Oxaliplatin	25.0	(15.3, 30.6)	25.6	(15.1, 30.7)	P=0.89
5FU Infusional	28.9	(15.6, 39.1)	25.6	(14.0, 37.4)	P=0.99
5FU Bolus	19.0	(10.9, 28.7)	19.4	(9.1, 28.1)	P=0.90
Cetuximab			27.7	(15.1, 41.0)	
Overall	**29.3**	**(15.7, 40.1)**	**28.1**	**(15.4, 42.0)**	**P=0.28**
XELOX	**N=240**		**N=245**		
Oxaliplatin	23.0	(12.5, 28.8)	21.7	(12.0, 29.7)	P=0.93
Capecitabine	23.7	(12.3, 31.1)	23.1	(10.9, 34.3)	P=0.95
Cetuximab			24.9	(12.3, 38.4)	
Overall	**24.1**	**(12.6, 31.4)**	**25.1**	**(12.4, 39.3)**	**P=0.15**
p-values for Xelox vs OxMdG					
Oxaliplatin	P=0.031		P=0.037		
Cetuximab			P=0.024		
Overall	**P=0.0058**		**P=0.016**		

Table 5: Dose intensity among *KRAS* wild-type patients during first 24 weeks of treatment

Drug	Arm A		Arm B		p-values for Arm B vs Arm A
	Median	**(IQR)**	**Median**	**(IQR)**	
OxMdG	**N=127**		**N=117**		
Oxaliplatin	80	(70, 87)	78	(69, 87)	P=0.69
5FU Infusional	81	(71, 88)	75	(65, 86)	P=0.016
5FU Bolus	73	(50, 88)	72	(55, 82)	P=0.51
Cetuximab			79	(67, 84)	
Overall	**78**	**(70, 87)**	**73**	**(66, 82)**	**p=0.031**
Xelox	**N=240**		**N=245**		
Oxaliplatin	85	(74, 92)	79	(67, 89)	P=0.0018
Capecitabine	85	(74, 92)	78	(67, 90)	P=0.004
Cetuximab			81	(70, 91)	
Overall	**85**	**(74, 92)**	**79**	**(67, 88)**	**P=0.0021**
p-values for Xelox vs OxMdG					
Oxaliplatin	P=0.018		P=0.19		
Cetuximab			P=0.23		
Overall	**P=0.031**		**P=0.12**		

Note – 24 weeks is roughly the median survival in this group of patients.

Table 6: Grade 3+ toxicities (CTC v3.0) among *KRAS*wt patients over entire treatment period, by Fp regimen received

	Arm A					Arm B					*p*-value for A vs B	
	OxFU		OxCap			OxFU		OxCap				
	N	(%)	N	(%)	P-value	N	(%)	N	(%)	P-value	OxFU	OxCap
Platelets	2	(2%)	7	(3%)	P=0.72	3	(3%)	6	(2%)	P=0.99	P=0.67	P=0.75
Haemoglobin	2	(2%)	3	(1%)	P=0.99	7	(6%)	7	(3%)	P=0.15	P=0.092	P=0.34
WBC	14	(11%)	0	(0%)	P<0.0001 *	10	(9%)	1	(<1%)	P<0.0001 *	P=0.67	P=0.99
Neutrophils	44	(35%)	10	(4%)	P<0.0001 *	29	(25%)	5	(2%)	P<0.0001 *	P=0.096	P=0.20
Nausea	6	(5%)	16	(7%)	P=0.46	8	(7%)	21	(9%)	P=0.57	P=0.59	P=0.50
Vomiting	4	(3%)	11	(6%)	P=0.59	8	(7%)	15	(6%)	P=0.82	P=0.24	P=0.55
Diarrhoea	17	(13%)	38	(16%)	P=0.53	22	(19%)	57	(23%)	P=0.34	P=0.25	P=0.038 †
HFS/PPE	5	(4%)	9	(4%)	P=0.99	7	(6%)	40	(16%)	P=0.0062 †	P=0.56	P<0.0001 †
Nail changes	0	(0%)	0	(0%)	N/A	4	(3%)	9	(4%)	P=0.99	P=0.051	P=0.0036 †
Skin rash	0	(0%)	0	(0%)	N/A	25	(21%)	56	(23%)	P=0.75	P<0.0001 †	P<0.0001 †
Peripheral neuropathy	31	(24%)	36	(15%)	P=0.026 *	15	(13%)	37	(15%)	P=0.56	P=0.022 *	P=0.99
Hypomagnesaemia	0	(0%)	0	(0%)	N/A	8	(7%)	7	(3%)	P=0.076	P=0.0025 †	P=0.015 †
Anorexia	6	(5%)	13	(5%)	P=0.78	10	(9%)	21	(9%)	P=0.99	P=0.43	P=0.22
Alopecia	0	(0%)	0	(0%)	N/A	0	(0%)	0	(0%)	N/A	N/A	N/A
Pain	15	(12%)	29	(12%)	P=0.94	12	(10%)	35	(14%)	P=0.29	P=0.84	P=0.59
Stomatitis	5	(4%)	2	(1%)	P=0.051	10	(9%)	9	(4%)	P=0.052	P=0.14	P=0.038 †
Lethargy	27	(21%)	45	(19%)	P=0.57	31	(27%)	52	(21%)	P=0.26	P=0.37	P=0.57
Vein pain	0	(0%)	4	(2%)	P=0.30	0	(0%)	1	(<1%)	P=0.99	N/A	P=0.21
Total	**240**	**(100%)**	**127**	**(100%)**		**245**	**(100%)**	**117**	**(100%)**			

† More toxicity in Arm B than Arm A, or in OxCap than in OxFU ($p<0.05$)
* More toxicity in Arm A than Arm B, or in OxFU than in OxCap ($p<0.05$)

Table 7: Mutation frequency by gene

	N samples with mutations/ N successfully analysed (%)					
	Arm A		Arm B		Total	
KRAS	268/ 635	(42.2%)	297/ 659	(45.1%)	565/ 1294	(43.7%)
BRAF	57/ 630	(9.0%)	45/ 661	(6.8%)	102/ 1290	(7.9%)
NRAS	18/ 631	(2.9%)	32/ 659	(4.9%)	50/ 1290	(3.9%)

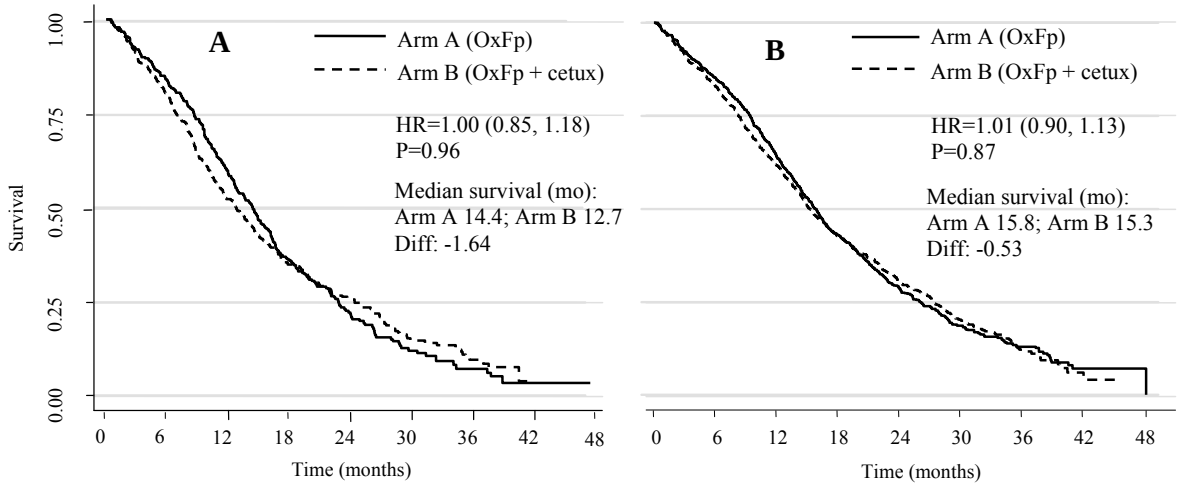
Note – In those samples where genotypes were missing for rare mutations (those with cumulative frequencies <1%), but where all other mutations were successfully tested as wild type, then an overall call of wild type was made at that locus.

Table 8: Mutation data by specific mutation

		N samples with mutations/ N successfully analysed (%)					
Gene	**Mutation**	**Arm A**		**Arm B**		**Total**	
KRAS	G12A	23/ 635	(3.6%)	11/ 659	(1.7%)	34/ 1294	(2.6%)
KRAS	G12D	74/ 635	(11.7%)	94/ 659	(14.3%)	168/ 1294	(13.0%)
KRAS	G12V	59/ 635	(9.3%)	82/ 659	(12.4%)	141/ 1294	(10.9%)
KRAS	G12C	23/ 635	(3.6%)	14/ 659	(2.1%)	37/ 1294	(2.9%)
KRAS	G12R	6/ 635	(0.9%)	5/ 659	(0.8%)	11/ 1294	(0.9%)
KRAS	G12S	14/ 635	(2.2%)	20/ 659	(3.0%)	34/ 1294	(2.6%)
KRAS	G13C	3/ 635	(0.5%)	2/ 657	(0.9%)	5/ 1292	(0.4%)
KRAS	G13D	56/ 635	(8.8%)	54/ 659	(8.2%)	110/ 1294	(8.5%)
KRAS	G13V	0/ 635	(0.0%)	1/ 659	(0.2%)	1/ 1294	(0.1%)
KRAS	Q61H	5/ 518	(1.0%)	8/ 541	(1.5%)	13/ 1059	(1.2%)
KRAS	Q61L	2/ 633	(0.3%)	3/ 656	(0.5%)	5/ 1289	(0.4%)
KRAS	Q61R	3/ 633	(0.5%)	3/ 656	(0.5%)	6/ 1289	(0.5%)
BRAF	D594G	7/ 622	(1.1%)	5/ 655	(0.8%)	12/ 1277	(0.9%)
BRAF	V600E	50/ 631	(7.9%)	40/ 660	(6.1%)	90/ 1291	(7.0%)
NRAS	G12C	0/ 621	(0.0%)	11/ 653	(1.7%)	11/ 1274	(0.9%)
NRAS	Q61K	10/ 612	(1.6%)	12/ 634	(1.9%)	22/ 1246	(1.8%)
NRAS	Q61L	2/ 633	(0.3%)	5/ 652	(0.8%)	7/ 1285	(0.5%)
NRAS	Q61R	6/ 633	(0.9%)	3/ 652	(0.5%)	9/ 1285	(0.7%)

Note – Total numbers per locus to do not exactly match those numbers in Table 1 since: (i) for KRAS, four samples contained two independent mutations and four other samples contained uncharacterised mutations and, (ii) for NRAS, one sample contained an uncharacterised mutation.

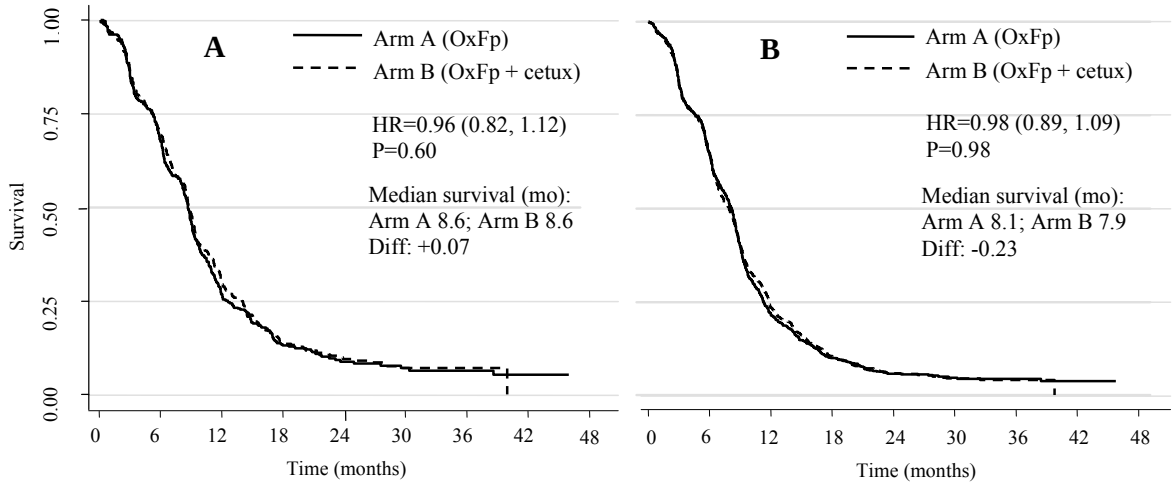
Figure 5: Kaplan Meier overall survival curves for A. patients with "any mutation" (KRAS, or NRAS or BRAF) and B. all randomised patients



Numbers at risk:

Arm A	340	285	198	108	46	19	7	1	0	815	688	513	297	151	73	32	4	1
Arm B	366	290	187	108	65	27	13	0	0	815	669	490	294	160	76	28	3	0

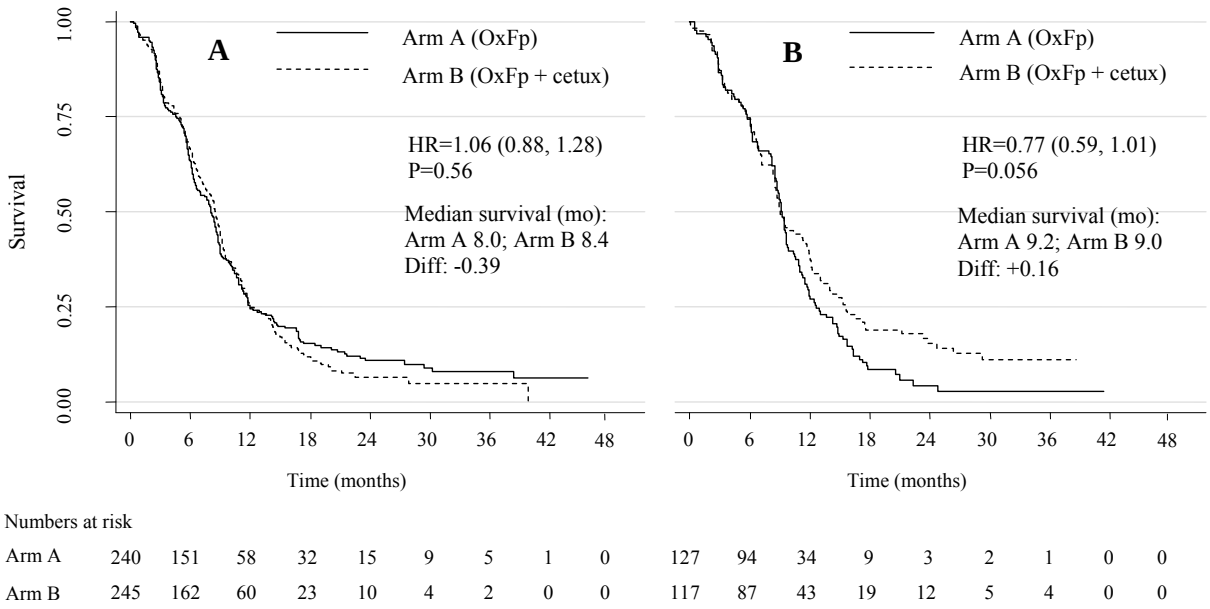
Figure 6: Kaplan Meier progression free survival curves for A. patients with *KRAS*-wt tumours B. all randomised patients



Numbers at risk:

Arm A	367	245	92	41	18	11	6	1	0	815	522	171	70	29	15	8	1	0
Arm B	362	249	103	42	22	9	6	0	0	815	517	186	71	31	15	7	0	0

Figure 7: Kaplan Meier progression free survival curves for KRAS-wt patients receiving A. OxCap chemotherapy B. OxFU chemotherapy



Numbers at risk

Arm A	240	151	58	32	15	9	5	1	0	127	94	34	9	3	2	1	0	0
Arm B	245	162	60	23	10	4	2	0	0	117	87	43	19	12	5	4	0	0