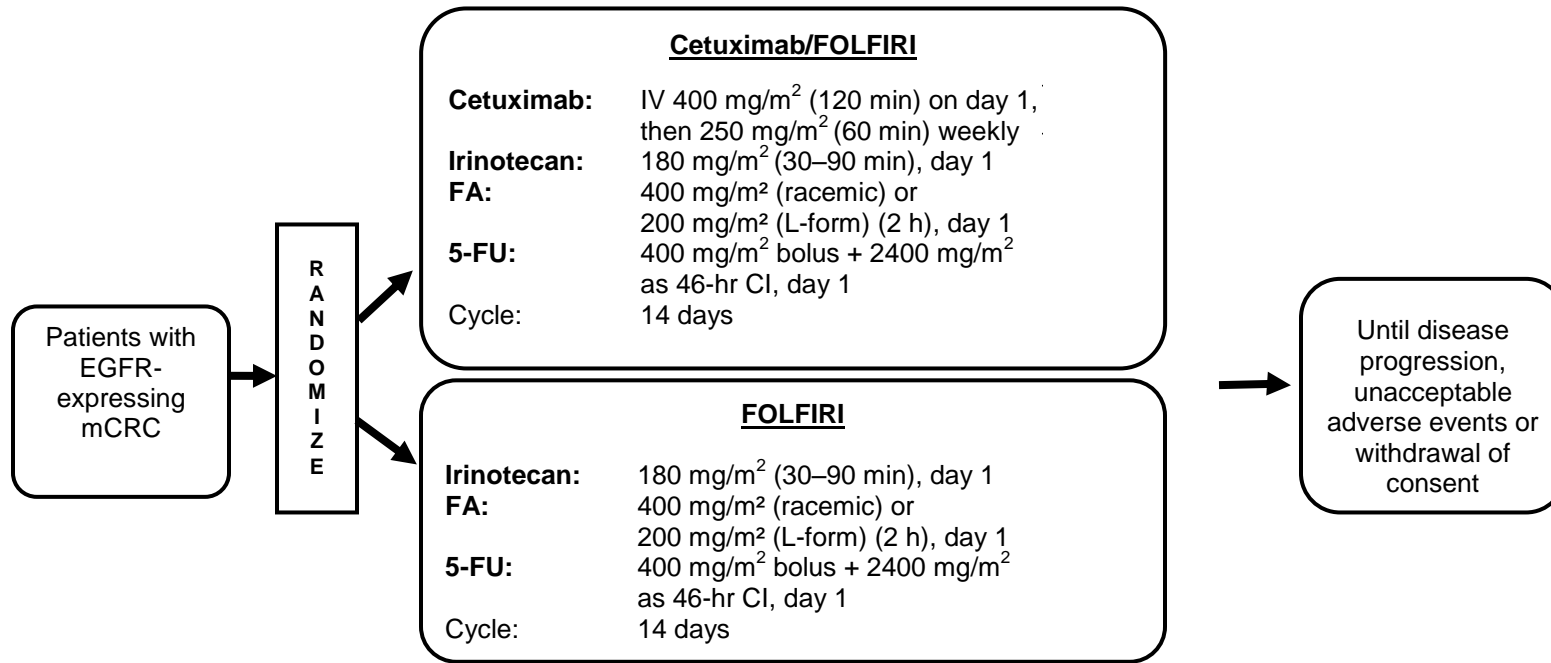


Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

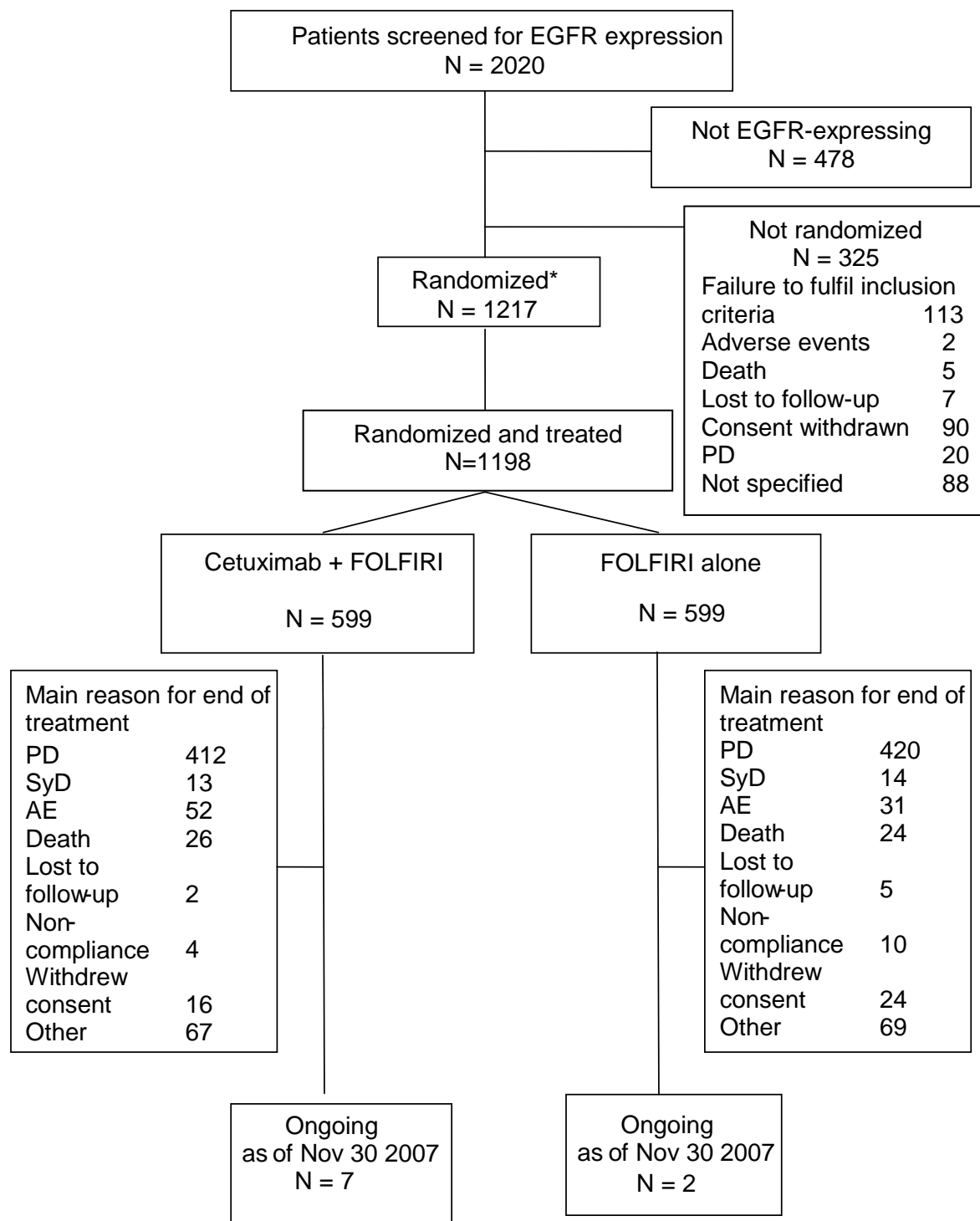
Supplement to: Van Cutsem E, Köhne C-H, Hitre E, et al. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med* 2009;360:1408-17.

Supplementary Figure 1. Study Design



EGFR denotes epidermal growth factor receptor, FA folinic acid, 5-FU 5-fluorouracil, CI continuous infusion, mCRC metastatic colorectal cancer and PD progressive disease.

Supplementary Figure 2. Number of Screened Patients at Each Stage of the Trial



*Safety population (N = 1221) includes 4 patients (2 in each arm) who were treated but not randomized. 19 randomized patients did not undergo treatment: this is common in oncology trials and in this case, while reasons were not specified, it was probably due mainly to intercurrent disease and withdrawal of consent. ITT denotes intention-to-treat, PD progressive disease, SyD symptomatic deterioration (non-imaging proven PD) and AE adverse event.

Supplementary Table 1. Most Common Grade 3 or 4 Adverse Events and Special Adverse Event Categories Shown in Table 3, Presented According to Tumor *KRAS* Mutation Status

MedDRA Preferred term	Patients (%)			
	<i>KRAS</i> wild type		<i>KRAS</i> mutations	
	FOLFIRI alone (N = 176)	Cetuximab + FOLFIRI (N = 173)	FOLFIRI alone (N = 87)	Cetuximab + FOLFIRI (N = 105)
Any	93 (52.8)	139 (80.3)	48 (55.2)	77 (73.3)
Neutropenia	34 (19.3)	50 (28.9)	20 (23.0)	23 (21.9)
Leukopenia	8 (4.5)	12 (6.9)	5 (5.7)	7 (6.7)
Diarrhea	16 (9.1)	32 (18.5)	11 (12.6)	14 (13.3)
Fatigue	9 (5.1)	5 (2.9)	2 (2.3)	10 (9.5)
Rash	0 (0)	13 (7.5)	0 (0)	7 (6.7)
Dermatitis acneiform	0 (0)	10 (5.8)	0 (0)	8 (7.6)
Vomiting	5 (2.8)	8 (4.6)	6 (6.9)	3 (2.9)
Special adverse event categories (all treated patients)				
Acne-like rash*	0 (0)	29 (16.8)	0 (0)	19 (18.1)
All skin reactions*	0 (0)	41 (23.7)	0 (0)	21 (20.0)
Infusion-related reactions	0 (0)	4 (2.3)	0(0)	4 (3.8)

*No grade 4 skin reactions or acne-like rash were reported.

SUPPLEMENTARY METHODS

KRAS mutation analysis

Pathology

Formaldehyde-fixed, paraffin-embedded (FFPE) tumor biopsy samples were sent to a central facility (Merck Serono, Darmstadt) for *KRAS* mutation analysis, either as paraffin blocks or as 3-5 μm paraffin sections mounted on glass slides. Serial sections were cut for pathological analysis (3 μm) and DNA extraction (10 μm , stored at $-80\text{ }^{\circ}\text{C}$ pending isolation). A pathologist confirmed the presence of invasive tumor cells in the tissue through examination of hematoxylin and eosin (HE)-stained sections. If invasive tumor cells could not be confirmed in a particular sample, the biopsy was excluded from further analysis.

DNA extraction

If tumor samples were available only as sections mounted on glass slides, the complete sample was scraped off, using a fresh scalpel for each slide, and transferred into a 1.5 ml tube. For FFPE tissue, DNA was generally extracted from 10 μm sections (3-10 depending on biopsy size). If samples were $<2\text{ mm}$ in diameter, punches of 1.3 mm diameter were taken with a biopsy needle from the paraffin block and transferred into 1.5 ml tubes for extraction.

Paraffin was dissolved by adding 800 μl xylene to each sample followed by gentle mixing. Then 400 μl ethanol (100% v/v) was added and the mixture centrifuged for 2 minutes at 13000 revolutions per minute (rpm) in a microcentrifuge. The supernatant was carefully removed. If any remaining paraffin was visible, the xylene and ethanol extraction was repeated. The pellet was washed with 1000 μl of ethanol (100% v/v), the tubes centrifuged for 2 minutes at 13000 rpm, the supernatant carefully removed and the pellet dried for 10 minutes at $55\text{ }^{\circ}\text{C}$. Genomic DNA was isolated from the dried pellet using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of each isolated DNA was determined by measuring the absorption at 260 nm using a Nanodrop UV/VIS spectrometer. Purity was assessed by determination of the 260/280 nm ratio. The DNA quality was further assessed in a *KRAS*-independent quantitative polymerase chain reaction (qPCR)

amplification. Genomic DNA from cell lines was also isolated using the DNeasy Blood & Tissue Kit, according to the manufacturer's instructions and quantified and quality controlled as for the FFPE material.

KRAS mutation detection by locked nucleic acid (LNA)-mediated qPCR clamping and melting curve analysis

LNA-mediated qPCR clamping

PCR reactions were performed on a LightCycler[®] 2.0 system using a *KRAS* mutation detection specific program with additional melting curve analysis in a one-step qPCR amplification and detection run using the LightMix k-ras Gly12 assay (TIB MOLBIOL, Berlin, Germany). The methodology is adapted from Chen et al, 2004.¹ The *KRAS* mutation specific primer pair (*KRAS* forward, *KRAS* reverse) amplified a genomic fragment from *KRAS* exon 2. Hybridization probes were designed to be complementary to specific *KRAS* codon 12 sequences. The anchor probe (Anchor FL) was 3'-end labeled with fluorescein, and the sensor probes were 5'-end labeled with LC Red dye and 3'-end phosphorylated to prevent extension. The two different sensor probes covered the mutation hotspot region. Amplification of *KRAS* wild-type DNA was inhibited by temperature stable binding of the *KRAS* wild-type specific LNA (2'-4' bridged ribose backbone) probe.

The mutation analysis was performed in duplicate for each tumor sample. A PCR master-mix was prepared in a cooled reaction tube containing: 7.4 µl PCR-grade water, 1.6 µl 25 mM MgCl₂, 4.0 µl of reagent/primer mix (parameter specific reagents containing both PCR primers, one anchor, two sensor-probes and the LNA), 2.0 µl of qPCR Mastermix (Roche Diagnostics, Penzberg, Germany). The reaction mix (15 µl) was pipetted into a LightCycler capillary and 5 µl of the genomic DNA solution (50 ng) was added to yield a final reaction volume of 20 µl. PCR reactions incorporating 1 ng of DNA derived from cell lines with defined *KRAS* mutations and 1 or 10 ng of DNA from cell lines or cells with wild-type DNA were included as controls in each run. Also, a reagent and device control and a water only control to check for cross contamination were included in each reaction run. The qPCR conditions comprised a pre-cycling incubation of 600 seconds at 95 °C followed by 45 cycles of 95 °C for 10 seconds; 58 °C for 5 seconds and 72 °C for 12 seconds.

Melting curve analysis

After the qPCR run, a melting curve analysis was performed by measuring the fluorescence of the samples with increasing temperature. Initially, the PCR products were denatured at 95°C. Subsequent cooling allowed the anchor-probe and the mutation-specific sensor probes to anneal resulting in increased fluorescence due to fluorescence resonance energy transfer (FRET) between anchor and sensor probe dyes. The melting curve was assessed by heating from 40 °C to 85 °C, with continuous monitoring of fluorescence in the 640 nm/530 nm channel. At a certain temperature, depending on the identity of the *KRAS* mutation, binding of the sensor probes to the DNA strand is disrupted. This results in spatial separation of the FRET partners and as a consequence, in a drop of fluorescence. The change of fluorescence intensity was converted to a melting peak (T_m) by plotting the negative derivative of fluorescent signal corresponding to the temperature ($-dF/dT$). The melting curve analysis program therefore comprised a pre-incubation of 20 seconds at 95 °C followed by 20 seconds at 58 °C followed by 10 seconds at 40 °C followed by heating to 85 °C at 0.3 °C/second.

Different individual *KRAS* mutations displayed different characteristic T_m s and could be identified by comparing the T_m of each sample with the T_m of respective positive control. Prior to assessment of the *KRAS* mutation status in patient samples the method and sensitivity were validated using already characterized cell lines with defined *KRAS* mutations (data available on request). All relevant codon 12/13 *KRAS* mutations previously described as occurring in colorectal tumors could be detected, including nucleotide: 34 G>A (G12S), 34 G>T (G12C), 35 G>A (G12D), 35 G>T (G12V), 35 G>C (G12A), 38 G>A (G13D). Additionally, a seventh mutation rarely occurring in CRC could also be detected: 34 G>C (G12R). Furthermore, the ability of the LNA-mediated qPCR clamping method to detect *KRAS* mutations in an excess of wild-type DNA was confirmed using a dilution series (up to 500-fold excess of *KRAS* wild-type DNA) of DNA derived from SW480 cells (*KRAS* G12V) with HT29 cells (wild-type *KRAS*) and on clinical FFPE CRC biopsies with a small proportion of tumor cells (tumor cell content < 5% as determined by a pathologist).

The validation of the method included testing against specific cell lines with defined *KRAS* mutations, determination of the sensitivity in dilution series of mutated and wild

type DNAs as well as comparative analysis, using an alternative technique (DxS *K-RAS* Mutation Detection Kit, DxS Ltd, Manchester, UK), of the *KRAS* mutation status in 43 paraffin-embedded formalin-fixed colorectal cancer tissue samples from a pilot study for *KRAS* testing.

1. Chen CY, Shiesh SC, Wu SJ. Rapid detection of K-ras mutations in bile by peptide nucleic acid-mediated PCR clamping and melting curve analysis: comparison with restriction fragment length polymorphism analysis. *Clin Chem* 2004;50:481-9.

Appendix

The following investigators from 189 centres in the listed countries screened patients for enrolment in the CRYSTAL trial: *Argentina*, M. Chacon (Instituto Alexander Fleming, Buenos Aires), M.G. Pallotta (Hospital Italiano de Buenos Aires, Buenos Aires), E. Roca (Hospital de Gastroenterología Dr. Carlos Bonorino Udaondo, Buenos Aires), C. Silva (Hospital Británico, Buenos Aires); *Australia*, J. Dickson and C. Karapetis (Flinders Medical Centre, Bedford Park), T. Price (The Queen Elizabeth Hospital, Woodville), N. Tebbutt (Austin Hospital, Heidelberg), G. van Hazel (Mount Medical Centre, West Perth and Sir Charles Gairnder Hospital, Nedlands), R. Ward (St Vincent's Hospital, Darlinghurst); *Austria*, P. Balcke (Allgemeines Krankenhaus St. Pölten, St. Pölten), C. Dittrich (Ludwig-Boltzmann-Institute for Applied Cancer Research, Wien), W. Eisterer and E. Wöll (Univ.-Klinik f. Inn. Medizin, Innsbruck), K. Gattringer (A.ö. Bezirkskrankenhaus Kufstein, Kufstein), R. Greil (St. Johannis Spital-und Landkrankenhaus, Salzburg), P. Kier (Donauspital im SMZ-Ost der Stadt Wien, Wien), H.J Neumann (A.ö. Krankenhaus d. barmherzigen Brüder, St. Veit a.d. Glan), J. Thaler (Klinikum Kreuzschwestern Wels GmbH, Wels); *Belgium*, G. D'Haens (Imelda Ziekenhuis, Bonheiden), C. Focan (Cliniques Saint Joseph, Liège), B. Neyns (AZ VUB Dienst, Jette), M. Peeters (University Hospital Gent, Gent), M. Rasschaert (St. Vincentius Hospital, Antwerpen), E. Van Cutsem (University Hospital Gasthuisberg, Leuven), J.-L. Van Laethem (Erasme Hospital, Bruxelles), J. Van Ongeval (AZ Sint-Lucas, Gent), J.B. Vermorken (University Hospital Antwerp, Edegem); *Brazil*, C. Cabral (Associação de Combate ao Câncer em Goiás, Goiânia), A. Cosme de Oliveira (HCFMUSP, São Paulo), A. del Giglio (Faculdade de Medicina do ABC, Santo André), J. Fleck (Irmandade Santa Casa de Misericórdia de Porto Alegre, Porto Alegre); *Bulgaria*, N. Ivanova (Multiprofile Hospital for Active Treatment, Pleven), G. Kurteva (Specialized Hospital for Active Treatment in Oncology, Sofia), A. Tomova (District Dispensary for

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