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CALGB: N0147
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NCIC CTG: CRC.2
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North Central Cancer Treatment Group

A Randomized Phase III Trial of Oxaliplatin (OXAL) Plus 5-Fluorouracil (5-FU)/Leucovorin (CF) with or without Cetuximab (C225) after Curative Resection for Patients with Stage III Colon Cancer

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DCTD Supplied Agent via Clinical Supplies Agreement (CSA): Cetuximab (NSC #714692) *(Discontinued as of November 25, 2009)*
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Protocol Resources (Contact Information)

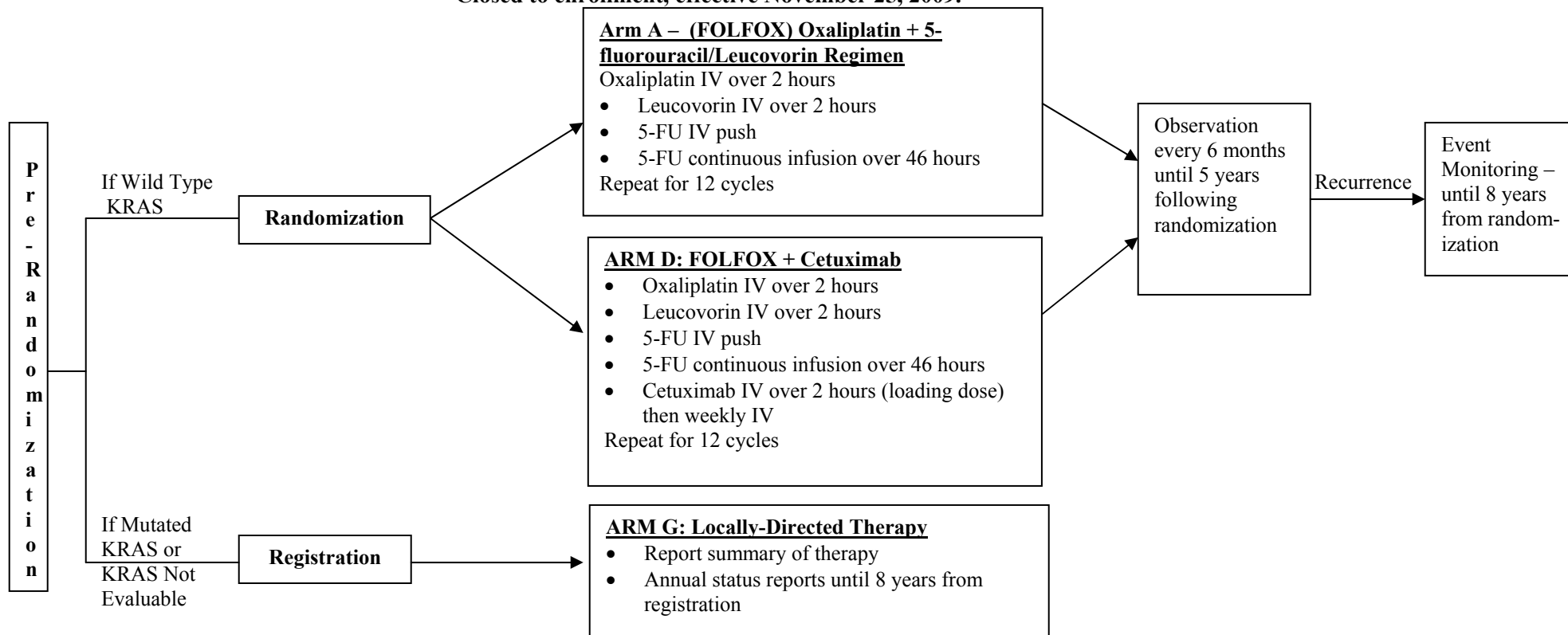|	Questions:	NCCTG Contact:
Add 13	The **study protocol and all related forms and documents** must be downloaded from the N0147 web page of the CTSU Member web site located at https://www.ctsu.org. Sites must use the current form version and adhere to the instructions and submission schedule outlined in the protocol. CTSU sites should follow procedures outlined in the protocol for Adverse Event Reporting and Data Submission.	

	Submit study data directly to NCCTG	NCCTG will manage all data collection processes for this study.
		
		Effective June 1, 2011, all NCCTG sites (until August 31, 2011) and all non-NCCTG sites all data and forms are to be mailed directly to:
		    NCCTG Operations Office
		    Attention: Quality Assurance Office (Study N0147)
		    RO FF 03 24-CC/NW Clinic
		    200 First Street SW
		    Rochester MN 55905
		
		Beginning September 1, 2011, all NCCTG sites will submit all forms via the NCCTG Remote Data Entry System.
		
		Do not copy CTSU on any study data or forms submissions.

Add 3,5,6,8, 10,13	Protocol Document, Regulatory Issues	NCCTG *Research Base* Research Protocol Specialists: Angela C. Patterson-LaBaw Phone: (507) 538-7339 Fax: (507) 284-5280 E-mail: patterson.angela@mayo.edu Patricia A. Aggen Phone: (507) 538-6232 Fax: (507) 284-5280 E-mail: aggen.patricia@mayo.edu
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Index

Schema
Closed to enrollment, effective November 25, 2009.

Add 9,12

Arm A – (FOLFOX) Oxaliplatin + 5-fluorouracil/Leucovorin Regimen
Oxaliplatin IV over 2 hours
- Leucovorin IV over 2 hours
- 5-FU IV push
- 5-FU continuous infusion over 46 hours
Repeat for 12 cycles

ARM D: FOLFOX + Cetuximab
- Oxaliplatin IV over 2 hours
- Leucovorin IV over 2 hours
- 5-FU IV push
- 5-FU continuous infusion over 46 hours
- Cetuximab IV over 2 hours (loading dose) then weekly IV
Repeat for 12 cycles

ARM G: Locally-Directed Therapy
- Report summary of therapy
- Annual status reports until 8 years from registration

Pre-Randomization

If Wild Type KRAS → **Randomization**

If Mutated KRAS or KRAS Not Evaluable → **Registration**

Observation every 6 months until 5 years following randomization

Recurrence →

Event Monitoring – until 8 years from randomization

Add 2

Generic name:	Oxaliplatin	Cetuximab	Fluorouracil, 5-fluorouracil	Calcium leucovorin, Calcium folinate, Citrovorum factor, Folinic acid
Brand name:	Eloxatin	Erbitux	Adrucil	Leucovorin
Mayo abbreviation:	OXAL	C225	5-FU	CF

Add 6,12

*Blood draws for Section 14.2 must be collected following pre-randomization, prior to registration/randomization (all patients) and at the time of the first Observation visit, or the next Observation visit for patients already in the Observation phase (Arm A or Arm D patients only). Site must provide NCCTG patient ID that is given at pre-randomization, with the submitted blood samples and on all applicable forms.

1.0 Background

1.1 Disease Incidence and Prognosis

1.11 Colorectal cancers are among the most common malignant tumors in North Americans. 147,300 new cases and 48,100 deaths are anticipated in the United States alone from this disease in 2002 (1). Most patients present with tumors that appear limited to the locoregional area. Those patients undergo resection with curative intent (2-4). Many previous studies have demonstrated that individuals with stage I and early stage II tumors (Modified Astler-Coller A and B1 [$T_{1-2}N_oM_o$]) have a >90% 5-year survival. Chemotherapy postoperatively is unnecessary for this favorable prognostic group. However, 40-60% of all patients who undergo resection for potential cure have more advanced locoregional disease. Their tumors are classified as either Stage II tumors in which there is invasion to or through the serosa or Stage III tumors, those that have metastases in local/regional lymph nodes (Modified Astler-Coller stage B_2 [$T_{3-4}N_0M_0$] or C [$T_xN_{1-2}M_o$]). In this population the risks of tumor recurrence are significantly higher. The 5-year, disease-free survival for patients with Stage II (Modified Astler-Coller stage B_2) ranges from 75-85%. For all patients with Stage III (Modified Astler-Coller stage C disease), 5-year survival is approximately 50% without postoperative treatment (5-19). This trial will enroll the subset of patients with colon cancer at high risk of recurrence who have disease that is apparently limited to the local/regional area but has spread to lymph nodes (Stage III disease).

1.12 Standard Adjuvant Therapy

Data from several trials have established 5-fluorouracil/leucovorin as the current standard of care for adjuvant treatment of colon cancer. The National Surgical Adjuvant Project for Breast and Bowel Cancers (NSABP) C-04 trial randomly assigned 2,151 Dukes B and C patients to receive one of three postoperative treatments: (1) 5-fluorouracil/high-dose leucovorin (HDCF) on a weekly x 6 schedule for 6 cycles, (2) the same 5-fluorouracil/ high-dose leucovorin schedule with the addition of standard dose oral levamisole (LEV), or (3) 5-fluorouracil/levamisole (20). The addition of levamisole to 5-fluorouracil/high-dose leucovorin in this trial did not appear to be beneficial. Patients randomized to the 5-fluorouracil/high-dose leucovorin-containing arms had a non-significant trend toward improved outcomes compared to those assigned to the non-leucovorin containing arm (5-fluorouracil/lev).

A four-arm intergroup trial (2x2 design) led by the North Central Cancer Treatment Group (NCCTG) randomly assigned patients to 6 months of 5-fluorouracil/lev versus 12 months of 5-fluorouracil/lev, with or without the addition of low-dose leucovorin (21). This trial found 12 months of chemotherapy with 5-fluorouracil/leucovorin provides no additional benefit when compared to 6 months, and that three drugs (fluorouracil/ leucovorin/levamisole) are not superior to two (fluorouracil/levamisole is given for 12 months).

INT 0089 compared four therapeutic strategies: (1) 52 weeks of standard 5-fluorouracil/levamisole, (2) 28 weeks of 5-fluorouracil/low-dose leucovorin administered according to the NCCTG 5-day schedule, (3) six months of 5-fluorouracil/low-dose leucovorin administered according to the NCCTG schedule plus levamisole, and (4) 32 weeks of high-dose weekly leucovorin plus 5-fluorouracil administered according to the NSABP protocol (22). Data presented at the 1998 ASCO meeting, but not yet reported as a manuscript, showed 5-year survival rates ranging from 63-67% for the groups of patients randomized to each of the four study arms. (22) The only statistically significant survival difference occurred in the comparison between the 5-fluorouracil/ levamisole and 5-fluorouracil/leucovorin/levamisole arms. However the three-drug regimen was associated with higher toxicity and there was no survival advantage compared to 5-flourouracil and leucovorin alone. The two most commonly employed 5-fluorouracil/leucovorin treatment programs employ the NCCTG daily x 5 for 28 weeks protocol or the NSABP weekly x 32 weeks protocol both have become widely accepted as the current standard adjuvant regimens for Stage III colon cancer in North America.

1.13   Irinotecan (CPT-11)

Irinotecan is a semisynthetic derivative of camptothecin, a plant alkaloid obtained from the *Camptotheca acuminata* tree (23-25). Irinotecan has greater aqueous solubility, is more active both *in vitro* and *in vivo*, and is associated with less severe and more predictable toxicity than camptothecin (26-29). Irinotecan has demonstrated antitumor activity against metastatic colorectal cancer, both in 5-fluorouracil-refractory patients and in chemotherapy-naive patients (30-35). A number of studies of irinotecan plus 5-fluorouracil have been done in vitro using cell cultures to explore the possibility of synergistic interactions between the two compounds. Mullaney examined the interactions of SN-38 (the active metabolite of irinotecan) plus 5-fluorouracil/leucovorin in HCT-8 cells in a variety of sequences (36). Synergy was noted when SN-38 exposure preceded 5-fluorouracil exposure and antagonism was noted with the reverse sequence. Guichard et al., and Mans et al., reported similar findings (37, 38). Combinations of irinotecan + 5-fluorouracil/leucovorin have been tested in cohorts of patients with no prior therapy for advanced colorectal cancer (39, 40).

1.14   Rationale for Combining Irinotecan with 5-fluorouracil/leucovorin

As documented above, 5-fluorouracil/leucovorin and irinotecan have demonstrated similar clinical activity in patients with colorectal cancer with no prior therapy.

1.15   Phase III Study Supporting the Present Study Regimen

There is mounting evidence that toxicities observed with 5-fluorouracil infusion regimens either coupled with irinotecan or given alone seem to be milder than those observed when 5-fluorouracil is delivered as an IV bolus injection (41). Evidence also suggests the mechanism of action of infusion 5-fluorouracil may differ from that of bolus 5-fluorouracil (42). The combination of irinotecan and 5-fluorouracil/leucovorin in this study is based upon several multicenter Phase III studies from Europe. Douillard and colleagues randomized 387 previously untreated patients with advanced colorectal cancer and ECOG performance score of two or better to 5-fluorouracil/leucovorin with or without irinotecan (40). There were two basic regimens in which 5-fluorouracil was administered as an infusion over either 24 or 48 hours with or without irinotecan. The majority of patients treated with irinotecan were prescribed a schedule of irinotecan 180 mg/m^2 on day one with leucovorin 200 mg/m^2 and 5-fluorouracil 400 mg/m^2 as a loading dose and 5-fluorouracil 600 mg/m^2 as a 22-hour infusion given on day one and repeated on day two every two weeks. The confirmed response rates were 41% in the irinotecan and 23% in the no irinotecan group. Time to progression was also longer in the irinotecan group at a median of 6.7 versus 4.4 months for the group treated without irinotecan. The median survival in the irinotecan group was 17.4 versus 14.1 months for the group treated without irinotecan. When the FDA approved irinotecan + 5-fluorouracil/leucovorin for the indication of first-line therapy of advanced colorectal cancer the every-other-week irinotecan + 5-fluorouracil/leucovorin schedule was approved as an option detailed in the package insert.

Add 2

Since the time that this study was completed investigators in a team led by Aimery de Gramont have attempted to simplify the 5-FU delivery in the trials where a two day 5-FU infusion is employed. There is no pharmacologic rationale for giving patients a loading dose of 5-FU. The de Gramont group has simplified the regimen by preserving the leucovorin infusion, but eliminating the 5-FU bolus on days 1 and 2. With the modified regimen, CPT-11 is administered at 180 mg/m^2 followed by leucovorin 400 mg/m^2. This is followed by 5-FU administered as a 46 hour infusion at 2400 mg/m^2 thus eliminating the need for a clinic visit on day two. (Personal communication, Aimery de Gramont, M.D.) Patients with less than grade 2 toxicity can have their 5-FU dose escalated to 3,000 mg/m^2 at physician discretion. Data using a schedule in which the day 2 loading dose of 5-FU was eliminated suggested that this regimen retained its activity. In this study 106 patients had a response rate of 56%, a median time to progression of 8.5 months, and median overall patient survival of 20.4 months (43). Because of safety and efficacy issues and comparability to an oxaliplatin-containing regimen, we have chosen to use this regimen in this trial.

1.16   Additional Evidence from a Phase III Trial in Advanced Disease

Add 5

A Pharmacia & Upjohn-sponsored multi-center phase III trial in stage IV colorectal cancer compared the irinotecan/5-fluorouracil/leucovorin regimen developed by Saltz, et al. (IFL) with 5-fluorouracil/leucovorin (Mayo Clinic low-dose schedule) and with irinotecan alone (44). The median overall survival was 14.8, 12.6, and 12.0 months for IFL, 5-FU, leucovorin and irinotecan respectively with a p-value of 0.04 for the comparison between the irinotecan + 5-FU/leucovorin vs. 5-FU/leucovorin. Grade 3-4 diarrhea affected 23%, 13%, and 31% of patients. Grade 3-4 neutropenia affected 54%, 66%, and 31% of patients. Seven patients (1%) were reported to have drug related fatal toxicity of which two were on the IFL program although a 7% 60-day death rate regardless of attribution was noted on both of the 5-fluorouracil containing study arms. The Saltz (IFL) regimen was also approved by the FDA for inclusion in the irinotecan package insert along with the regimen described by Douillard noted above for treatment of patients with previously untreated advanced colorectal cancer using the three-drug combination. Subsequent testing of this regimen in N9741, an advanced disease study found a 4.5% 60-day mortality rate compared on IFL compared to a 1.8% 60-day mortality rate on the FOLFOX arm of the trial. In C89803, an adjuvant trial, found a 2.5% 60-day mortality rate with deaths attributed to febrile neutropenia/dehydration and thrombotic events was noted with IFL compared to a 0.9% 60-day mortality rate in the 5-FU/LV arm of the trial (45, 46). Safeguards including dose reductions, careful patient monitoring, cycle interruptions, and aggressive supportive care have been recommended when the IFL regimen is employed. However, whether the efficacy of the three-drug regimen is maintained at these lower doses is currently unknown. Therefore, we will use the modified FOLFIRI regimen described above (without a 5-FU loading dose) rather than the IFL regimen in this study.

1.17   Completed and Ongoing Adjuvant Trials Using Irinotecan + 5-fluorouracil/leucovorin

The US intergroup trial C89803 of irinotecan + 5-fluorouracil/ leucovorin administered according to the IFL regimen compared to 5-fluorouracil/high-dose leucovorin administered according to the NSABP protocol has completed accrual but results are pending. Accrual to a European trial comparing irinotecan + 5-fluorouracil/leucovorin to 5-fluorouracil/leucovorin alone in which 5-fluorouracil is administered by infusion in both treatment arms has also been completed but several years will be needed for the data from this trial to become available.

1.18   Oxaliplatin

Oxaliplatin (trans-*l*-1,2-diaminocyclohexane oxalatoplatinum) is a novel antineoplastic platinum derivative with a 1,2-diaminocyclohexane [DACH] carrier ligand. Although the precise mechanism of action is unknown, platinum compounds are thought to exert their cytotoxic effects through the formation of DNA adducts that block both DNA replication and transcription, resulting in cell death in actively dividing cells as well as the induction of apoptosis. Like cisplatin, oxaliplatin reacts with DNA, forming mainly platinated intra-strand links with two adjacent guanines or a guanine adjacent to an adenine (47-49). However, DACH-platinum adducts formed by oxaliplatin are apparently more effective at inhibiting DNA synthesis (49) and are more cytotoxic than cis-diamine-platinum adducts formed from cisplatin and carboplatin (49). The pharmacokinetics of platinum metabolism was evaluated in patients with advanced cancer who received 130 mg/m^2 oxaliplatin via a 2-hour infusion every 3 weeks for 1 to 5 cycles. (50)

1.19a   Single Agent Activity in Colon Cancer

The efficacy of oxaliplatin monotherapy in patients with advanced colorectal cancer was evaluated in five phase II trials, two with a total of 63 previously untreated patients and three with a total of 139 patients with metastatic disease previously treated with and mostly refractory to 5-fluorouracil (51-54). The objective response rate achieved with oxaliplatin as first-line therapy averaged 18% while that of oxaliplatin as second-line therapy averaged 10%. The response rates are very similar to that observed with either 5-fluorouracil/leucovorin or irinotecan alone.

1.19b   Rationale for Combining Oxaliplatin with 5-cytotoxic properties with fluoropyrimidines particularly 5-fluorouracil, other thymidylate synthase inhibitors such as AG337, and other agents (55, 56).

1.19c   Phase III Studies Supporting the Combination of Oxaliplatin with
          5-Fluorouracil/Leucovorin

The addition of oxaliplatin to 5-fluorouracil/leucovorin results in
treatment responses in patients whose tumors are considered to be
5-fluorouracil refractory (57). In one trial of 115 patients with advanced
colorectal cancer that had progressive disease after treatment with a
bolus schedule of 5-fluorouracil/leucovorin were retreated with
oxaliplatin + 5-fluorouracil/leucovorin. In order to optimize oxaliplatin
delivery, treatment cycles were given every three weeks and the
5-fluorouracil schedule was adjusted to accommodate this interval.
Oxaliplatin was given at the full single-agent dose of 130 mg/m^2/dose.
The dose of 5-fluorouracil administered was decreased by 25% from that
patient's last drug dose. This approach allowed 5-fluorouracil to be
administered with equal dose intensity to that of the every four-week
regimen when the drugs were administered every three weeks. Patients
had measurable disease and scans were independently reviewed. The
response rate was 13%, the median time to progression was 4.3 months,
and the median survival was 10.1 months. Improvement in baseline
performance score was noted in 47% of patients and a decrease in pain
grade was noted in 21% of patients (57).

Oxaliplatin administration has been coupled to 5-fluorouracil infusion
mainly in two ways. In the method developed by de Gramont, patients
are given a loading dose of 400 mg/m^2 5-fluorouracil as a bolus injection
given after a two hours l-leucovorin infusion at a dose of 400 mg/m^2.
The loading dose is then followed by a 22-hour 5-fluorouracil infusion of
600 mg/m^2 via a pump programmed to provide a constant drug infusion
rate. The method for administering the leucovorin and 5-fluorouracil is
identical to that specified in the irinotecan + 5-fluorouracil/leucovorin
regimen known as the Douillard regimen. This program is repeated on
two consecutive days every two weeks. In the method by Levi, patients
are given a variable dose of 5-fluorouracil depending upon the time of
day in a method known as chronomodulation (58-60).

There are two randomized trials of 5-fluorouracil/leucovorin plus or
minus oxaliplatin where the 5-fluorouracil is given according to the de
Gramont or Levi regimens. In the trial using the de Gramont regimen,
410 patients with advanced and previously untreated colorectal cancer
randomized to receive or not receive oxaliplatin 85 mg/m^2 prior to the
5-fluorouracil/leucovorin (61). In the two-drug regimen the response
rate was 29% compared to 55% in the three-drug regimen (p<0.001).
The times to progression were 5.9 and 8.1 months respectively
(p=0.0003). The overall survival may have been influenced by second-
line therapy and was not statistically significantly different between

treatment arms at 14.7 vs. 16.0 months (p=0.1). This study had as its main endpoint a comparison of times to progression and therefore it was not designed to nor did it recruit a large enough number of patients to have the power to show a definitive survival benefit.

In the trial using the Levi regimen leucovorin is given as a chronomodulated infusion via a programmable pump at a dose of 300 mg/m^2 with 5-fluorouracil 700 mg/m^2 daily for 5 consecutive days every 3 weeks (62). 200 patients with advanced and previously untreated colorectal cancer were randomized to receive or not to receive oxaliplatin 125 mg/m^2 as a six-hour infusion prior to infusion of the above-described doses of 5-fluorouracil/leucovorin. In the two-drug regimen the response rate was 16% compared to 53% in the three-drug regimen (p<0.001). The median times to progression were 4.4 and 8.3 months respectively (p= 0.045). The overall survival may have been influenced by second-line therapy and was not statistically significantly different between treatment arms at medians of 19.4 vs. 16.6 months leucovorin (p=0.8).

The NCCTG trial N9741 treated 265 patients with advanced colorectal cancer not previously treated with chemotherapy for advanced disease using the FOLFOX 4 regimen. In this trial IFL was used as the comparator arm. (63) The FOLFOX regimen resulted in a statistically significant advantage in time to progression from a median of 6.9 to 8.8 months (p=.0009), response rate advantage of 29 % versus 38% (p= .03) and a survival advantage of median 14.1 versus 18.6 months (p= .002) over IFL. Toxicity also favored FOLFOX over IFL with significantly less ≥grade 3 nausea 6 versus 15%, vomiting 4 versus 12%, diarrhea 13 versus 33% and 1.8 % versus 4.5% 60-day all cause mortality observed. More paresthesias were observed with FOLFOX over IFL, 18% versus 2%.

In the most recently reported trial (MOSAIC), the FOLFOX4 regimen was compared to the same regimen without the oxaliplatin (LV5FU2) in patients with resected stage II and III colon cancer (ASCO 2003, abstract 1015; virtual meeting presentation). In this randomized phase III study of 2246 patients, a significant improvement in 3-year disease free survival was seen in those receiving FOLFOX4 (77.8% vs 72.9%; p<0.01).

Add 2   1.19d   Cetuximab (C225, Erbitux)

The monoclonal antibody cetuximab is a chimeric antibody directed against the epidermal growth factor receptor (EGFR). Preclinical studies have shown promising antitumor activity of cetuximab against colon cancer. (64) The antitumor activity appears to be attributable to direct inhibition of the EGFR-dependent mitogenic pathway. There also appears to be an inhibitory effect of the secretion of various paracrine growth factors involved in sustaining the proliferation and functional differentiation of intratumoral blood vessels.

The efficacy of cetuximab is reliant on saturating the EGF receptors. Extensive preclinical studies have shown that levels of cetuximab should be approximately 20 times the receptor binding affinity. The lowest dose of cetuximab that is likely to achieve this level of saturation is 30 nmol/L. However, concerns about the distribution and uptake in vivo led to further studies that have shown a dose level of 200 mg/m^2 appears to be more appropriate. (65) No dose limiting toxicities were seen at this dose level. The most common toxicities were fever and chills, asthenia, transaminase elevation, nausea, and skin toxicity.

While approximately 80% of patients will have an adverse event only 12% of these events are classified as grade 3 or greater. (66) Side effects of cetuximab, when used as a single agent, have been reported on approximately 280 patients (Investigator's Brochure 2003, Version 9.0). The most frequently reported events were asthenia (25%), fever (27%), nausea (14%), acne (37%), rash (31%), and headache (14%). Allergic reactions have been mild with only 1% of patients experiencing a grade 4 anaphylactic reaction with single agent cetuximab. Subsequent allergic reactions have been treated successfully with prophylactic antihistamine therapy and an increased infusion time. According to the Full Prescribing Information (package insert) issued in February 2004, severe infusion reactions occurred with the administration of Erbitux in 3% of patients rarely with fatal outcome (<1 in 1000).

The skin reaction to cetuximab appears to be a sterile suppurative form of folliculitis. Folliculitis occurs in more than 80% of patients and tends to affect the face, chest, and back. (66) The skin changes appear to be a result of impaired cell growth and altered differentiation, possibly caused by the upregulation of p27^{Kip1}. (67) Topical treatments with steroids or antibiotics have shown no clear benefit. The folliculitis has not been dose limiting. It has also resolved completely without scarring following cessation of treatment.

A total of 606 subjects treated with cetuximab through February, 2002 were tested for the presence of anti-cetuximab antibodies by analyzing pre- and post-treatment sera using a double antigen radiometric assay. The incidence of an anti-cetuximab immune response in these subjects was 4.1%. When it occurred, the anti-cetuximab response was generally found to be weak (upper limit of normal is 10 ng/ml cetuximab binding). The anti-cetuximab antibodies from two subjects with the highest reactivity (4670 and 6516 ng/ml) did not interfere with the ability of cetuximab to inhibit proliferation in a cetuximab sensitive cell line, suggesting that the antibodies in these sera were non-neutralizing. Levels of reactivity in sera from other subjects were not high enough to perform this type of analysis. In order to determine the specificity of the antibody response, sera from 15 subjects who had a positive anti-cetuximab response were further studied in the double antigen radiometric assay using unlabeled cetuximab as a competitor. This analysis demonstrated that sera from 14 of the 15 subjects contained cetuximab-specific antibodies.

Several different trials combining cetuximab with chemotherapy have been reported, either as published reports or as meeting abstracts. This has included three trials of cetuximab and cisplatin, and one trial each of cetuximab combined with irinotecan, gemcitabine, paclitaxel/carboplatin or doxorubicin. Each of these studies used a weekly dose of cetuximab and most used an initial loading dose followed by a standard weekly dose. In none of the trials reporting toxicity did there appear to be any increased toxicity of the chemotherapy with the addition of cetuximab.

Among the clinical studies assessing the combination of cetuximab and cisplatin a phase I study showed the combination to be well tolerated. (65) In this study cisplatin was given as a fixed dose of 60 mg/m^2 every 4 weeks with an escalating dose of cetuximab up to 400 mg/m^2. The addition of cetuximab to cisplatin did not alter its pharmacokinetics. There was also no change in the clearance of cetuximab compared to its clearance when used alone. Complete saturation of systemic clearance with cetuximab was noted at doses in the range of 200 to 400 mg/m^2. A separate phase I study established the currently recommended doses of cetuximab. (68) In this study three different dose levels of cetuximab were evaluated in combination with a fixed dose of cisplatin. The schedule used for this trial involved a loading dose of cetuximab on week 1. The recommended doses for subsequent phase II and III trials with cetuximab were a loading dose of 400 mg/m^2 followed by 250 mg/m^2 given on a weekly basis. The recommended doses of cetuximab were bases on saturation of the receptors. This study also specifically noted that "combined cetuximab and cisplatin therapy does not appear to induce overlapping toxic effects".

Several preclinical and clinical studies have assessed the combination of cetuximab with irinotecan alone or in combination with 5-fluorouracil and leucovorin. In human colorectal cancer xenografts, the addition of cetuximab to irinotecan enhanced the activity of irinotecan in both irinotecan-sensitive and irinotecan-resistant cell lines. (69) A phase II trial and subsequent phase III trial in patients with irinotecan-resistant metastatic colorectal cancer confirmed the preclinical results. (70,71) In the phase III trial patients receiving irinotecan and cetuximab a 17.9% response rate and median time-to-progression of 126 days was observed, while those receiving cetuximab alone had a 9.9% response rate and median time-to-progression of 45 days. (71) The results from two different trials did not show any apparent increase in irinotecan toxicity with the addition of cetuximab. (70,71)

Add 2

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Add 2

While cetuximab is directed against EGFR, it was noted in the randomized trial of cetuximab with or without irinotecan in patients with irinotecan-resistant tumors that the level of EGFR expression did not correlate with response. (71 [oral presentation ASCO 2003]) This would suggest that current techniques to identify EGFR are inadequate or alternatively that cetuximab is active on other targets in colorectal cancer cells. As such it does not appear that limiting its use to patients with EGFR expressing tumors by current measurement techniques is appropriate.

Add 2,5

The combination of cetuximab with irinotecan, 5-fluorouracil, and leucovorin has been evaluated in several clinical trials. (72, 73) The combination of FOLFIRI and cetuximab was shown to be well tolerated and active. (73) Phase III studies of this combination in patients with metastatic colorectal cancer are planned in Europe.

Add 5

No studies of cetuximab combined with oxaliplatin, 5-fluorouracil, and leucovorin have been reported, however, a large phase III industry-sponsored trial is ongoing. Toxicity had not precluded this study from continuing to accrue. Preclinical assessment of the combination of cetuximab and cisplatin (CDDP) demonstrated activity in a xenograft unresponsive to either agent used alone. (74) The combination of cetuximab and cisplatin in this study led to the disappearance of tumor in all but one mouse. No tumor recurrence was seen during a 6-month observation period. A phase I trial of cetuximab combined with 5-fluorouracil and either cisplatin or carboplatin demonstrated that cetuximab does not appear to alter the toxicity related to the combination of 5-fluorouracil and a platinum agent. (75)

The FDA approved cetuximab for commercial use on February 12, 2004, for the following two indications: (1) use in combination with irinotecan for the treatment of EGFR-expressing, metastatic colorectal carcinoma in patients who are refractory to irinotecan –based chemotherapy and (2) use as a single agent for the treatment of EGFR-expressing, metastatic colorectal carcinoma in patients who are intolerant to irinotecan-based chemotherapy. The effectiveness of cetuximab was based on objective response rates demonstrated in clinical trials of patients whose metastatic colorectal cancer had progressed following treatment with an irinotecan-

containing regimen and those whose disease had progressed following treatment with both an irinotecan-containing regimen and an oxaliplatin-containing regimen (Full Prescribing Information, February 2004).

One trial in particular that helped lead to the approval of cetuximab involved randomizing patients refractory to irinotecan to either cetuximab alone or to cetuximab and irinotecan. Approximately two-thirds (63%) of patients had also previously failed oxaliplatin-based therapy. In this multicenter, randomized, controlled clinical trial 111 patients were randomized to receive to cetuximab alone 218 were randomized to receive cetuximab plus irinotecan. Cetuximab was administered as a 400 mg/m^2 loading dose, followed by 250 mg/m^2 weekly until disease progression or unacceptable toxicity. In the cetuximab plus irinotecan arm, irinotecan was added using the same dose and schedule for irinotecan as the patient had previously failed. An Independent Radiographic Review Committee (IRC), blinded to the treatment arms, assessed both the progression on prior irinotecan and the response to protocol treatment for all patients.

Efficacy was evaluated in an intent-to-treat analysis. Analyses were also conducted in two pre-specified subpopulations: irinotecan refractory and irinotecan and oxaliplatin failures. The results of the study showed that in patients with irinotecan-refractory disease, the addition of cetuximab to irinotecan led to a 26% response rate compared to 15% response rate when used as monotherapy. Similar response rates were seen in patients who had previously received oxaliplatin. The median duration of response in the overall population was 5.7 months in the combination arm and 4.2 months in the monotherapy arm.

Add 2   1.19e   Rationale for current adjuvant trial

This study is designed to compare the activity and toxicity of three different regimens administered over 24 weeks each containing either irinotecan or oxaliplatin or both with 5-fluorouracil/leucovorin given by infusion. The oxaliplatin regimen is a hybrid of the latest modification in the FOLFOX series described by de Gramont. The regimen used in this trial (FOLFOX6.5) uses the FOLFOX6 approach with the exception that the dose of oxaliplatin has been lowered to 85 mg/m^2. The modification is intended to maintain the activity as seen in FOLFOX4, yet remove the need for the day 2 bolus of 5-FU and LV. It will also help to reduce the potential for neuropathy.

Both regimens (FOLFOX, FOLFIRI) employ the identical doses and methods of giving 5-fluorouracil and leucovorin over two days every two weeks. Both of these three drug regimens have been used in adjuvant colon cancer trials in Europe in regimens that employed the loading does of 5-FU but they have not been compared to each other. In the U.S. both 3-drug combinations have been compared to 5-fluorouracil/leucovorin alone. In these trials the 5-fluorouracil/leucovorin has been administered by bolus injection. Because of the apparent advantages in terms of both toxicity and efficacy when 5-fluorouracil is infused rather than

Add 2

administered as a bolus when combined with irinotecan or oxaliplatin, this study will employ the infusion based programs. In the third arm of the study FOLFOX will be administered for 6 two-week cycles followed by FOLFIRI for 6 two-week cycles. In both the European MOSAIC trial and the NSABP C-07 trial the incidence of severe neurotoxicity was less than 15% of patients (76). Additionally most patients recovered from the neurotoxicity within 3 months of drug discontinuation.

There are two potential advantages to this sequential program. This approach will expose patients with residual microscopic disease to all three active drugs in the adjuvant setting. There is the potential for this to improve the cure rate. In addition there is the potential to minimize or even avoid the peripheral neuropathy noted with oxaliplatin containing regimens with this program. Since the usual course of the sensory chronic neuropathy associated with oxaliplatin occurs after 10 or more cycles, the administration of only 6 cycles should allow most patients to avoid this toxicity.

Add 2

Three treatment arms (D, E, F) have been added to incorporate cetuximab (C225) to the chemotherapy regimen. This will assess the ability of cetuximab to enhance the activity of chemotherapy in the adjuvant setting. Based on the promising results from phase II clinical trials, demonstrating increased response and duration of response, when cetuximab is added to chemotherapy, assessment in the adjuvant setting is warranted. The design of this trial will allow an assessment of cetuximab's activity when combined with either FOLFIRI, FOLFOX, or a hybrid regimen of the two chemotherapy combinations.

1.19f   Change of Primary Endpoint

Add 3

In May 2004, the Oncologic Drugs Advisory Committee to the FDA recommended that disease-free survival become an acceptable regulatory endpoint. This was based on data presented from a large pooled analysis showing a very tight relationship between disease-free and overall survival in previous trials in adjuvant colon cancer. Accordingly, we are changing the primary endpoint of our trial to disease-free survival, which will allow the primary analysis to occur one year sooner than with the overall survival endpoint.

1.2   Translational Research – See Appendix XIV for background, aims and methodology and Sections 14.0 and 17.0 for specimen logistics.

1.3   Patient-Completed Questionnaires

We are interested in assessing quality of life and a measure of patient satisfaction with their experience participating on this trial. We use two instruments to measure this: (1) a Linear Analogue Self Assessment Scale and (2) a Completion Questionnaire.

Add 2

Add 2

Add 2

Add 2

Add 2

Add 2

Quality of life has been identified as an endpoint second only to survival in terms of importance for inclusion in cancer clinical trials (77) and the approval of new therapeutic agents (78, 79). Recently the NCCTG demonstrated that the UNISCALE single-item instrument was as reliable, valid, and practical to implement in a community-based clinical trials setting as other longer more involved instruments for measuring overall QOL (80). It is important, however, to include other domains so that further detail is obtainable (78, 79). For this purpose we will use four specific questions using a Linear Analogue Self Assessment (LASA) scale. LASA items have been validated as general measures of global QOL dimensional constructs in numerous settings (80-85). A series of LASA items have been constructed and validated at Mayo for use in cancer patients (86). For these specific items, we will use single-item numeric analogue scales (80-85). This approach has been validated in general by Cleeland et al., in the development of The Brief Pain Inventory (80, 87) and in cancer patients (84, 88, 89) and has been used successfully in numerous NCCTG Oncology Clinical Trials (80). The specific items to be assessed are overall quality-of-life, mental well-being, physical well-being and fatigue. Each of these items will be analyzed as separate individual constructs rather than as a summated psychometrically validated scale.

We will also include in this protocol a small number of items for patients to complete at the end of the trial (i.e., Completion Questionnaire). The goal of these items is to provide data in response to the general concept of 'Was it Worth it' (completion questions). These questions provide a simple means of assessing patients' opinions regarding whether they thought the experience of going through the clinical trial was worth it, would they do it again if they had it to do over, and would they recommend the process to others. These data are intended as both a quality control device and a means to potentially improve patient accrual to and retention on clinical trials.

1.4   Epidemiology Tools

Add 2

Environmental factors appear to play a fundamental role in colorectal carcinogenesis (90-92). However, the molecular events associated with specific exposure agents remain largely undefined. The current study affords an excellent opportunity to further investigate gene-environment and epigenetic-environment interactions among a well-characterized group of colorectal cancer patients, by applying a comprehensive risk factor assessment to the pre-treatment subject evaluation. Collection of colorectal cancer risk factor information has not been standardized across previous studies. Therefore, a core GI cancer risk factor survey (also called the Focused Health Assessment [Patient Questionnaire]) was recently developed at Mayo Clinic Rochester. This data collection instrument takes about 15 minutes to complete and includes assessments of key non-dietary risk factors (see Appendix VII).

Add 1

Since nutritional agents may affect critical cellular pathways in colorectal carcinogenesis, willing subjects will be asked to complete the NCI's Diet History Questionnaire (DHQ) as well. The DHQ is a food frequency questionnaire (FFQ) developed by staff at the Risk Factor Monitoring and Methods Branch (RFMMB). This FFQ consists of 124 food items and includes both portion size and dietary supplement questions. It takes about 15-25 minutes to complete and was designed, based on cognitive research findings, to be easy to use. Data show that the DHQ provides reasonable nutrient estimates, and two studies have been conducted to assess its validity. The first, using a checklist approach, showed that many of the cognitive improvements introduced in the DHQ provided a better measure of frequency than the 1992 Block FFQ (93). The second validation compared the DHQ instrument to both the Block and Willett food frequency questionnaires, using four 24-hour dietary recalls (one in each season) as reference data. This study showed the DHQ to be equal to or superior to the Block and Willett instruments for most nutrients (94).

Add 2

Add 2

DHQ-1 is the current version of the questionnaire distributed by the NCI. The DHQ instrument is currently in use in several NCI epidemiological studies such as The Prostate, Lung, Colorectal, and Ovarian Screening Trial (PLCO) and The Agricultural Health Study (AHS). Data from the Focused Health Assessment (Patient Questionnaire) will also be collected in scannable format to allow for easy, accurate transfer into the study database in timely fashion.

Add 1

Add 3   1.5   VOD (veno-occlusive disease)

VOD, veno-occlusive disease (also known as sinusoidal obstruction syndrome [SOS] or perisinusoidal fibrosis) is a form of toxic liver injury initially observed in patients receiving chemotherapy or undergoing bone-marrow and other transplants, and is characterized clinically by development of hepatomegaly, ascites, and jaundice, and histologically by diffuse damage in the centrilobular zone of the liver (reviewed in Bearman, 1995, and DeLeve et al., 2002). Sequelae of this event include hepatomegaly, splenomegaly, portal hypertension, and esophageal varices.

Add 3

As of April 30, 2004, world-wide patient exposure to oxaliplatin is estimated to be approximately 240,000 post-marketing experience, and approximately 65,000 in clinical trials and compassionate use. A total of 82 reports of hepatobiliary disorders and abnormal biochemical tests (45 from post-marketing experience and 37 from clinical trials and compassionate use. A total of 82 reports of hepatobiliary disorders and abnormal biochemical tests (45 from post-marketing experience and 37 from clinical trials and compassionate use) were received by Sanofi-Aventis (a frequency of approximately 0.02%).

Add 3

Post-marketing experience: As of April 30, 2004, Sanofi-Aventis has received reports of 45 cases of hepatobiliary disorders and biochemical abnormalities possibly related to liver dysfunction. In 6 of the 45 cases, a liver biopsy was performed, and the following morphological changes were reported: peliosis hepatitis (sinusoidal dilatation and congestion) in three cases; severe sinusoidal obstruction with central vein fibrosis in one case (the patient had also received capecitabine) (Tisman et al., 2004); and nodular regenerative hyperplasia (NRH) in two patients who also had liver metastases.

Add 3

A retrospective analysis of 153 patients who underwent surgical resection of liver metastases was recently published; 87 of the patients received chemotherapy (several different regimens) prior to resection, and 66 did not (Rubbia-Brandt et al., 2004). No hepatic lesions were observed in the non-chemotherapy group; whereas, 44 patients in the chemotherapy group displayed histological findings consistent with VOD of the liver. Of the 87 patients who received chemotherapy, lesions were observed in 6/27 receiving 5-FU alone, 4/17 who received 5-FU + oxaliplatin + irinotecan, 20/27 who received 5-FU + oxaliplatin, and 14/16 who received 5-FU + oxaliplatin + irinotecan. There was no information available as to whether these patients had clinically significant consequences or met the clinical diagnosis criteria for VOD of the liver.

Add 3

One instance of a patient with locally recurrent prostate cancer who developed VOD (portal hypertension and ascites, without hepatic metastases) while receiving capecitabine and oxaliplatin post-RT also has been reported (Tisman et al., 2004).

Add 5   1.6   Revision to Study Arms

As of Addendum 5: Following the initiation of N0147 a variety of trials for colorectal cancer have been reported. In particular, the MOSAIC trial (New England Journal of Medicine. 350(23):2343-51, 2004) led to the FDA approval of the combination of Oxaliplatin and Infusional 5-FU/LV (FOLFOX) as the new standard in stage III colon cancer. While none of these new trials have yet provided information on the role of infusional 5-FU combined with irinotecan in the adjuvant setting, results of several recently completed trials are expected in the near future. However, based on the current body of knowledge it appears that FOLFIRI may at best be equivalent to FOLFOX in the adjuvant setting, with little expectation that it will be superior. Based on this information, with the associated expectation that no significant improvement in DFS or OS will be seen in either the FOLFIRI or FOLFOX/FOLFIRI hybrid arm of N0147 compared to FOLFOX, maintaining a comparison of these regimens has become difficult to justify. Given the need to streamline the trial to more rapidly address the potential benefit of adding a biological agent to chemotherapy in the adjuvant setting, N0147 has been modified to a 2-arm trial comparing chemotherapy alone (FOLFOX) to chemotherapy combined with C225. By obtaining an answer to the benefit of a biologic

agent added to chemotherapy more quickly, it will be possible to design the next generation of trials at an earlier time point. Based on the results of trials such as PETACC3 and ACCORD, future trials may wish to readdress the importance of incorporating irinotecan into one or more of the treatment regimens.

1.7   Change of primary analysis population to KRAS wild-type patient subgroup

A growing body of research has shown a correlation between the mutated status of KRAS and response to EGFR inhibitors. Mutations of KRAS and its downstream signaling can adversely affect response to EGFR inhibitors (Moroni M et al. Lancet Oncol 6:279-86). Current available information shows that approximately 40-45% of patients with advanced colorectal cancer have mutations within KRAS making this a potential major determinant of treatment outcome for patients receiving EGFR inhibitors. Retrospective analyses of trials using either panitumumab or cetuximab have shown that there is essential no response to treatment with one of these antibodies in patients with mutated KRAS, while those with wild-type KRAS are likely to respond. In a trial of 463 patients evaluating the potential efficacy of panitumumab in last line therapy, 427 had available KRAS data, of whom. 43% had mutated KRAS (Amado RG, et al. GI Cancer Symposium abstract 278). For patients with wild-type KRAS, 17% responded and 34% had stable disease compared to 0% responders and 12% with stable disease in the mutated KRAS group. When treatment arms were combined, overall survival was longer in patients with wild-type KRAS versus patients with mutated KRAS (HR = 0.67, 95% CI, 0.55 - 0.82). At least three studies with cetuximab now report similar outcomes (De Roock W, et al. ASCO 2007 abstract 4132; Finocchiaro G, et al. ASCO 2007 abstract 4021; Tabenero J, et al. GI Cancer Symposium 2008 abstract 435). Based on these findings it becomes very important that the status of KRAS be assessed in regard to the primary endpoint of this trial.

In addition, based on the outcome of a retrospective review of several recent Phase III trials (CRYSTAL, OPUS), as reported at ASCO 2008, it no longer appears appropriate to use an EGFR inhibitor in patients with tumors expressing mutated KRAS. While these trials have been to date only reported in abstract form, the data in all trials is strongly consistent with the lack of benefit of EGFR inhibition in patients with mutant KRAS tumors. The best data was from the CRYSTAL trial, where in the 540 patients for whom KRAS status was available, in wild-type patients the addition of cetuximab to FOLFIRI resulted in a hazard ration of 0.68, with p-value of 0.017, while in patients with mutant KRAS, the hazard ratio was 1.07, with a p-value of .0.47. (Van Cutsem et al., ASCO 2008, Abstract 4001).

While all of the data to-date has been derived from trials in patients with metastatic colorectal cancer, the change in KRAS occurs at an early stage in the development of the tumor. Based on this early event it would appear that the findings from metastatic colorectal cancer will likely apply to patients treated in the adjuvant setting. Given a reasonable uncertainty of benefit it now appears appropriate to restrict the use of cetuximab and other EGFR inhibitors to patients with wild-type KRAS. As such it does not appear warranted to continue to randomize patients with tumor expressing mutated
KRAS to cetuximab. As of Addendum 9 the trial randomization will be restricted to patients with wild-type KRAS; whereas, patients with KRAS mutations or KRAS

undetermined will be allowed to receive treatment determined by their local physician off study, and these patients will be registered to the trial for long-term follow up.

KRAS will be assessed utilizing the assay developed by DxS Ltd. The DxS kit used will detect 7 KRAS mutations in codons 12 and 13. The methods used in this kit are highly selective and, depending on the total amount of DNA present, can detect approximately 1% of mutant in a background of wild type genomic DNA. The assays have limits of detection of between 5 and 10 copies. These selectivity and detection limits are superior to technologies such as dye terminator sequencing. Technical aspects of the assay are outlined in Appendix XVII.

The DxS assay for KRAS analysis has been used in several European trials. With data from the one of the trials the European Commission recently granted a conditional marketing authorization for Vectibix® (panitumumab) as monotherapy for the treatment of patients with non-mutated (wild-type) KRAS genes after failure of standard chemotherapy regimens. In September 2007 Vectibix received a positive opinion from CHMP (European Committee for Medicinal Products for Human Use) based upon the clinical data supporting the utility of KRAS mutation status as a biomarker for a patient population that has few treatment options available to them. Amgen presented this pivotal data from the "408" study (R.G. Amado et al. (2007) Analysis of KRAS mutations in patients with metastatic colorectal cancer receiving panitumumab monotherapy at ECCO 14 (European Cancer Organisation) European Conference in Barcelona in September 2007, which used the TheraScreen: KRAS Mutation Kit. The study concludes that the efficacy of panitumumab monotherapy in metastatic colorectal cancer seems confined to patients with non-mutated KRAS.

The DxS kit detects the following mutations in codons 12 and 13 against a background of wild type genomic DNA in a real time PCR assay:

Gly12Ala (G_GT>G_CT)
Gly12Asp (G_GT>G_AT)
Gly12Arg (_GGT>_CGT)
Gly12Cys (_GGT>_TGT)
Gly12Ser (_GGT>_AGT)
Gly12Val (G_GT>G_TT)
Gly13Asp (G_GC>G_AC)

With the DxS kit, after DNA extraction, real time PCR assays are performed to detect the target molecule. By comparing control and mutant sample reactions the assay can detect and estimate low levels of mutation. No further sample processing is necessary and the time to result is <3 hours.

DxS TheraScreen: KRAS kit has been CE-marked for professional diagnostic use under the EU registration and the product is CE-marked under the European IVD Directive 98/79/EC.

The DxS analysis of KRAS will be performed in N0147 using the technique outlined in Appendix XVII. The analysis will be performed using the Roche LightCycler 480 platform.

Add 9   1.8   The tolerability and toxicity associated with 5-FU alone adjuvant chemotherapy in older
                    patients has been reported and has been considered only slightly elevated compared to
                    younger patients, the toxicity of multi-drug therapy in the adjuvant setting is not clear.
                    Previously completed trials of multi-drug adjuvant therapy have generally not provided
                    subgroup analysis of toxicities by age. A recently published retrospective analysis from
                    four randomized trials of FOLFOX (3 trials in patients with metastatic colorectal cancer
                    and 1 of adjuvant trial) showed that older age was associated with a modest increase in
                    the rates of hematologic toxicities, and a trend towards increased rate of fatigue and
                    nausea/vomiting. No increases were observed in the rates of severe neurologic adverse
                    events, diarrhea, infection, overall incidence of grade ≥3 toxicity, or 60-day mortality (*J
                    Clin Oncol 24:4085-4091, 2006*). The tolerability of targeted therapy in combination
                    with chemotherapy in the elderly is less clear. The limited availability of any data from
                    trials using cetuximab does not specify the rate of toxicities in those age 70 and older.

                    In an interim toxicity analysis of N0147, performed in April 2008, an increase in toxicity
                    was noted in older patients, particularly in patients on arm D (FOLFOX + Cetuximab).
                    Grade 3+ toxicities in 47% and 66% of patients below the age of 70 in arms A
                    (FOLFOX) and D (FOLFOX + cetuximab), respectively, were observed, compared to
                    rates for patients 70 years of age and older the rates of 57% and 81% for arms A and D,
                    respectively. The differences in toxicity rates in arm D by age were primarily due to
                    gastrointestinal toxicities and included an increase in diarrhea and oral mucositis. In
                    addition, patients with more frequent passage of stool at baseline were more likely to
                    develop grade 3+ diarrhea. These gastrointestinal events in turn led to a higher rate of
                    dehydration and dyspnea. The higher rate of toxicities resulted in greater dose reductions
                    and failure to complete therapy as planned.

                    Based on these findings it is strongly recommended that early dose modification be made
                    for older patients (age 70 or older) with moderate (grade 2+) toxicities, particularly
                    gastrointestinal toxicities. Early intervention with supportive measures (e.g. anti-diarrhea
                    agents, fluid replacement) should also be considered.

Add 10   1.9   Toxicity observed in the elderly

                    At the Fall 2008 NCCTG DMC review of N0147, an increase in deaths in patients in
                    Arm D (FOLFOX + cetuximab), with an elevated proportion of these deaths occurring in
                    patients over the age of 70 was observed. At the time of that review, the rate of "on-
                    treatment" death for patients aged less than 70 on Arm D is 1.1% (10/891) compared to
                    5.1% (9/178) in those aged 70 or greater. For patients under the age of 70, this rate of
                    death was consistent with that reported from other adjuvant trials for resected stage III
                    colon cancer. In regard to the deaths that have occurred there have been several different
                    events that have led to the deaths with no one dominant event. These events have
                    included severe diarrhea and apparent sudden cardiac arrest, as well as several pulmonary
                    events consisting of diffuse lung injury occurring during the later cycles of treatment or
                    within a few weeks of completing the planned therapy. Based on this data, on Oct 24,
                    2008, accrual to the trial for patients aged ≥ 70 was temporarily suspended.

                    Based on a thorough review of the data from N0147, as from multiple other clinical trials
                    involving cetuximab, N0147 is reopening to accrual for patients aged ≥70 with the

implementation of Addendum 10. Addendum 10 involves multiple modifications in an attempt to assure patient safety. This includes adding "Diarrhea Evaluation" as a separate line in the test schedule and requirement that this must be done every 2 weeks. In addition, a specific notation to the protocol stating "Early intervention is important for patient safety, particularly for patients age 70 and older" has been added. A footnote to the table in section 4 has been added making specific reference to Section 8 for appropriate dose modifications and Sections 9.1 and 9.2 for recommending treatment. The wording at the top of the dose modification tables has been changed to read "The following table describes the recommended dose modifications **for patients under age 70 and required dose modifications for patients 70 and older** during a course of therapy and at the start of each subsequent course of therapy. All dose modifications should be based on the worst preceding adverse event. **For patients age 70 and older the required modification or a greater modification (if felt necessary by the treating physician) must be performed with all treatment cycles.** New wording has also been added to the consent form addressing the potential added risk for older patients.

2.0   Goals

2.1   Treatment

Add 2,3,5,9,11   **2.11   Primary Objective**

> 2.111   To compare the disease-free survival (DFS) in patients with stage III ($T_xN_{1-2}M_0$) colon cancer who are KRAS wild-type randomized to 24 weeks of adjuvant chemotherapy with either: (1) Oxaliplatin (OXAL) + 5-fluorouracil/leucovorin (5-FU/LV) (FOLFOX) or (2) FOLFOX + C225.

Add 2,3,5,9,11   **2.12   Secondary Objectives**

> 2.121   To compare the DFS in unselected patients with stage III ($T_xN_{1-2}M_0$) colon cancer randomized to 24 weeks of adjuvant chemotherapy with either: (1) Oxaliplatin (OXAL) + 5-fluorouracil/leucovorin (5-FU/LV) (FOLFOX) or (2) FOLFOX + C225.

> 2.122   To compare the overall survival (OS) in patients with KRAS wild-type tumors, and in unselected patients with stage III (T_x, N_{1-2}, M_0) colon cancer randomized to 24 weeks of adjuvant chemotherapy with FOLFOX with or without C225.

> 2.123   To assess toxicities resulting from the addition of C225 to chemotherapy.

> 2.124   To compare the quality of life, measures of patient satisfaction, nutrition, and cancer risk in patients treated with FOLFOX with or without C225, using four patient-completed questionnaires.

Add 9,11   2.2   Translational Research

2.21   To assess the predictive ability of molecular determinants of EGFR signaling (see Appendix XIV, Specific Aim 1) to predict efficacy of cetuximab for both DFS and OS.

2.22   To prospectively assess genomic instability and mismatch repair status (MMR) (see Appendix XIV, Specific Aim 2) and correlate these markers with DFS and OS.

2.23   To determine the DNA methylation status of CpG islands within the promoter regions of candidate genes (See Appendix XIV, Specific Aims 3 and 4) and its relationship to epidemiologic risk factors and patient outcome.

2.24   To correlate candidate gene expression and pharmacogenetic characteristics (see Appendix XIV, Specific Aim 4) determined from DNA derived from circulating leukocytes with patient outcome.

2.25   To analyze determinants of apoptotic susceptibility (see Appendix XIV, Specific Aim 6) and their association with clinical outcome.

2.26   To characterize the immune profile (see Appendix XIV, Specific Aim 6) and correlate these markers with DFS and OS.

2.27   To assess the prognostic utility of current pathologic parameters (see Appendix XIV, Specific Aim 7) for both DFS and OS.

2.28   To assess various epidemiological factors (see Appendix XIV, Specific Aim 8) and their association with clinical outcome.

3.0   Patient Eligibility

Add 3   3.1   Pre-randomization

3.11   Required Characteristics

3.111   Histologically documented adenocarcinoma of the colon. The gross inferior (caudad) margin of the primary tumor must be ≥12 cm from the anal verge by rigid proctoscopy (i.e., patients with rectal cancer are not eligible). A rigid proctoscopy will be performed in only those settings where it is important to establish if the tumor is a rectal tumor or a colon tumor. Stage III tumor must have been completely resected. Resected Stage IV patients are not eligible. In patients with tumor adherence to adjacent structures en bloc resection must be documented in the operative report. Patients with tumor-related obstruction or colonic perforation are eligible for enrollment.

Add 6

Add 6

Note: Evidence of Epidermal Growth Factor Receptor (EGFR) in the resected tumor is **NOT** required.

Add 6

Note: Patients with ≥one synchronous primary colon cancer are eligible. For the purposes of this protocol, staging classifications will be based on the stage of the more advanced primary tumor.

Add 7

Note: Patients with positive radial (serosal, circumferential) margins are eligible as long there is no evidence that the surgeon cut through the tumor; no evidence the tumor invaded adjacent tissues; and the entire specimen was resected by en bloc.

3.112   At least one pathologically confirmed positive lymph node identified.

3.113   There must be no evidence of residual involved lymph node disease. At least one lymph node must be found in the pathologic specimen. To help ensure optimal stratification, the recommended number of identified nodes is four or more.

Add 2,3,6

3.114   ECOG performance status (PS) 0, 1, or 2 (Appendix II).

Add 3,6

3.115   Age ≥18 years.

Add 2,3,6,9,10

3.116   Must be willing to provide blood and tissue samples for eligibility and research purposes, as described in Sections 14.0 and 17.0. NOTE: tumor tissue must be submitted immediately after pre-randomization and ≤42 days following surgery to allow time for central KRAS testing prior to registration/randomization.

Add 9

3.117   Tumor tissue will be made available to NCCTG for centralized KRAS testing prior to registration/randomization.

Add 9

3.118   A pre-randomization pathology review (i.e. KRAS analysis) is required. The site has reviewed and understands the process listed in Section 17.0 and must account for sufficient time to complete pre-randomization and registration/randomization steps.

3.12   Contraindications

3.121   Any of the following:
- Pregnant women
- Nursing women
- Men or women of childbearing potential who are unwilling to employ adequate contraception.

Add 2

Add 2,6

This study involves agents (cetuximab, oxaliplatin, and 5-fluorouracil) whose teratogenic effects on the developing fetus and newborn are unknown.

3.122   Evidence of residual involved lymph node disease. ≥ 1 lymph node must be found in the pathologic specimen. To help ensure optimal stratification, the recommended number of identified nodes is ≥ 4.

Add 9   3.123   Distant metastatic disease at the time of registration/randomization.

               3.124   Prior chemotherapy or radiation therapy for treatment of this malignancy.

Add 2   3.125   Prior therapy with agent(s) directed against EGFR.

Add 2   3.126   Prior allergic reaction (known sensitivity) to chimerized or murine monoclonal
                       antibody therapy or documented presence of human anti-mouse antibodies
                       (HAMA).

               3.127   Previous or concurrent malignancy. Exceptions: Treated basal
                       cell or squamous cell skin cancer, in situ cervical cancer, or
Add 6   lobular carcinoma in situ in one breast; or other cancer for which
                       the patient has been disease-free ≥5 years.

               3.128   Any of the following conditions:

                       • Uncontrolled high blood pressure
                       • Unstable angina
                       • Symptomatic congestive heart failure
                       • Myocardial infarction ≤6 months prior to randomization
                       • New York Heart Association classification III or IV (Appendix III)
                       • Symptomatic pulmonary fibrosis or interstitial pneumonitis
                       • Active uncontrolled bacterial, viral (including HIV or clinically
                         defined AIDS)
Add 6   • Systemic fungal infection

               3.129a Other medical condition which, in the opinion of the treating physician,
                       would make this protocol unreasonably hazardous for the patient.

               3.129b Clinically significant peripheral neuropathy at the time of randomization
                       (defined in the NCI Common Terminology Criteria for Adverse Events,
                       [CTCAE] v3.0 as ≥grade 2 neurosensory or neuromotor toxicity).

               3.129c Concurrent use of other anti-cancer therapy including chemotherapy
                       agents, targeted agents, or biological agents.

Add 3,5   3.129d Known allergy to other platinum compounds.

Add 3,5   3.129e History of gastrointestinal bleeding that has not been appropriately
                       addressed based on the assessment of the enrolling physician.

Add 3,9

3.2   Registration/Randomization

Add 9   3.21   Required Characteristics – Randomization (to Arm A or D)

Add 3   3.211   Randomization must occur ≤56 days post surgery.

Add 9   3.212   KRAS wild-type status determined by central testing.

Add 2,3,9   3.213   Laboratory values obtained ≤28 days prior to randomization:

Add 5,6   • Hgb≥ 9 g/dL
                                   • Absolute neutrophil count ≥LNL, (e.g. 1500/mm^3)
Add 5   • Platelet count ≥100,000/μL
                                   • Creatinine ≤1.5 x UNL
Add 8   • Total bilirubin ≤1.5 x UNL

Add 6   3.214   Negative serum pregnancy test done ≤7 days prior to
                                   randomization, for women of childbearing potential only.

Add 9   3.22   Required Characteristics – Registration (to Arm G)

                           3.221   KRAS mutant status determined by central testing, or KRAS status not
                                   evaluable.

4.0 Test Schedule

All tests and procedures are required for patients who have been randomized to study treatment (e.g. to Arms A and D). Procedures that are required for all patients, including patients registered to Arm G, are noted with a double asterisk (**).

Tests and Procedures	Prior to Registration/Randomization				During Treatment			At Recurrence	Observation[12]
	During Primary Surgery	≤30 days	≤28 days	≤7days	Every 2 weeks	Sixth Cycle of Treatment*	End of Protocol Treatment		Every 6 months until 5 years following randomization
Physical Exam: (Wt, ECOG PS, Ht, & medical history)[1]			X[15]		X[1]				X
WBC, hgb, diff, platelets			X[15]		X[2]				X
Serum Chem[3]			X[15]		X[2]				X
Prothrombin Time (PT)[4]			X[15]		X[2]				
KRAS determination**		X[19,**]							
Chest X-ray or Chest CT[5]		X[17]							X[23]
Abdominal: US or CT or MRI		X[17]							X[23]
Patient Instructions for Preventing and Treating Diarrhea (Appendix IV)		X							
Pulmonary Evaluation[6]					X[6]				
Diarrhea Evaluation[20]					X[20]				
Hemolytic Uremic Syndrome (HUS)Evaluation					X[7]				
Mandatory Translational Research Blood Draw **(Order Kits)** [R,]			X[13,**]						X[21]
Paraffin-embedded Tissue or Slide Collection**	X[8,**]								
Optional Paraffin-embedded Recurrent Tissue or Slide Collection								X[22]	
Patient Completed Questionnaires[9,18] *Questionnaires are not required for patients enrolled following implementation of Addendum 9.*		X[9]				X[9]	X[9]		
Serum Pregnancy Test[11]				X					
Request Letter for Immunohistochemistry (IHC) Test Results (Appendix XV)**	Upon Request (Documentation of when patient requests and receives results of IHC testing is to be noted in the research record.) See Section 14.13 and Appendix XV.								
Dear Participant Letter (Appendix XVI)					X[10]				
VOD (Veno-occlusive disease) Evaluation, (see Section 15.389d)[14]	The evaluation will be done for patients on and off therapy who develop signs and symptoms suggestive of VOD.								
Follow-up colonoscopy									X[16]

Add 12 (Chest X-ray or Chest CT row)
Add 12 (Abdominal row)
Add 12 (Mandatory Translational Research Blood Draw row)
Add 12 (Optional Paraffin-embedded Recurrent Tissue or Slide Collection row)
Add 1,2,3,5,6,7, 8,9,10,11 (VOD row)

* Treatment cycles are two weeks; twelve 2-week cycles will be given, totaling 24 weeks (includes rest periods).
** Required for all patients; i.e. required for patients registered to Arm G as well as those randomized to Arm A or Arm D.

Footnotes continue on the next page

1. Pretreatment medical history should be complete. At subsequent evaluations directed medical histories are acceptable. Ht needed at baseline only. Must include MD, DO, PA, NP, or RN assessment.
2. For all treatment cycles, except cycle 1. Mg is required only for patients on Arm D.
3. Chemistries: Alkaline Phosphatase (U/L), AST (SGOT) (U/L), Total Bilirubin (mg/dL) (µmol/L), Creatinine (mg/dL) (µmol/L), Na, K, Cl, Bicarb CO_2, BUN and Mg. Mg is required only for patients on Arm D. Mg, Na, K, C1, Bicarb CO_2, BUN not required during observation. **In the event of hypomagnesemia, refer to Section 9.7 for ancillary treatment.**
4. Required only for patients taking coumadin as therapy such as for DVT, PE, or cardiac disease. Weekly until stable and therapeutic for 2 consecutive cycles. If taking 1 mg/day for prophylaxis of catheter-related thrombosis, no PT is required although PT should be followed at physician discretion.
5. Use same imaging modality throughout. Documentation of recurrence or new primary is required to be submitted.
6. If patient is experiencing shortness of breath, a chest x-ray and assessment of oxygenation via either finger oximetry or arterial blood gas evaluation are required to confirm the absence or presence of pulmonary infiltrates and/or hypoxia.
7. As clinically indicated. Recommended evaluation of suspected HUS (HCT <25%, PLTs <100,000, creatinine ≥1.6 mg/dL): Evaluation should include CBC differential, platelets, PT, PTT, fibrinogen, FDP (Fibrin degradation products), Anti-thrombin III, Von Willebrand factor, anti-nuclear antibody, rheumatoid factor, Compliment Cascade C3, C4, and CH_{50}, anti-platelet antibodies, platelet-associated IgG, and circulating immune complexes. Renal evaluation should include creatinine, BUN, and urinalysis with microscopic examination. Other laboratory and hematological evaluations as appropriate should also be obtained, including peripheral blood smear and free hemoglobin.
8. See Sections 14.0 and 17.0 for information concerning mandatory paraffin-embedded tissue collection. Normal and tumor tissue preferred. This tissue is required for all patients. Tissue collected must be submitted immediately following pre-randomization and ≤42 days following surgery to allow time for central KRAS testing prior to registration/randomization.
9. **Questionnaires are not required for patients enrolled after the implementation of Addendum 9.**

Administering Patient Completed Questionnaires for patients enrolled prior to implementation of Addendum 9 (August 18, 2008). Instructions for Clinical Research Associates (CRAs) (App V).			
	Time Point		
	Baseline (prior to treatment for cycle 1)	At 6th Cycle	End of Active Treatment
LASA: First 500 patients per arm (App. VI) (Only for pts. enrolled prior to implementation date of Addendum 7)	X	X	X
Brief Food Questionnaire (App. VII)	X		
Completion Questions (App. VIII)			X
Focused Health Assessment (Patient Questionnaire) (App. IX)	X		

10. For patients enrolled prior to the effective date of NCCTG Addendum 2. Letter to be given to patient at the time of the next scheduled evaluation.
11. For females of childbearing potential only. It is recommended that clinical blood draws be taken at the same time (i.e., serum pregnancy test and eligibility bloods both drawn ≤7 days prior to randomization.)
12. The observation phase begins when the patient discontinues or completes all study treatment, with the exception of disease recurrence. (In the case of disease recurrence, patients proceed directly to Event Monitoring.) The first observation visit is 6 months after completion of treatment. Tests and reporting are required, starting from the date active treatment ends and continues until 5 years from randomization or until recurrent disease, whichever occurs first. Patients begin Event Monitoring after the observation phase ends (see Section 18.0).

Add 12

13. This collection is required for all patients and can be collected in conjunction with other required blood draws for this study prior to registration/randomization. See Section 14.0 for information concerning mandatory blood collection. **Include NCCTG ID number provided at pre-randomization on specimen submission form and on the Requisition Form contained in the blood collection kit.**
14. Follow standard clinical practice for evaluation of VOD, including observation of liver and spleen size, history of or actual gastrointestinal bleeding, reversal of portal blood flow visualized by ultrasound, and possibly the development of esophageal varices, ascites and bleeding or jaundice.
15. For female patients, sites may want to consider performing baseline exam and clinical blood work at the same time as serum pregnancy testing, ≤7 days prior to randomization.
16. As of Addendum 6 follow-up colonoscopies are to be done at one year and four years post resection. If a colonoscopy is felt to be clinically unsafe to perform or for other reasons can not be performed, then other forms of colon imaging such as a barium enema or CT colonography are acceptable. If recurrence occurs, the colonoscopy report documenting recurrence is to be submitted per Section 18.0.
17. Allowable within 8 weeks prior to randomization.

Add 13

18. **CANADIAN SITES ONLY -** NCCTG has approved the use of patient completed questionnaires which have been translated into French for the Canadian sites. The Canadian sites are required to transfer data collected via the French questionnaires onto the original English format questionnaires. Any written comments must also be translated into English for data submission. The CRA must initial and date a note on the original English questionnaire stating that data was transferred from the patient-completed French questionnaire. In addition, a second party must verify accuracy of the transferred data on the English questionnaires, and the second party must also initial and date a note on the original English questionnaire stating that the transferred data was verified. The site must retain the patient-completed French questionnaire and a copy of the original English questionnaire submitted to NCCTG. NCCTG will return questionnaires to sites if not submitted on the original English version. **Questionnaires are not required for patients enrolled after the implementation of Addendum 9.**

19. KRAS determination will be performed through a central lab at NCCTG (see Section 17.2). Tumor tissue must be submitted immediately following pre-randomization and ≤42 days following surgery to allow time for central KRAS testing prior to registration/randomization. This tissue is required for <u>all</u> patients. For sites that previously submitted slides instead of blocks, submit an additional 15 five micron (charged) slides and 5 ten micron (uncharged) slides ≤60 days following activation of Addendum 9 (see Section 17.11).

20. Early intervention is important for patient safety, particularly for patients age 70 and older. See Section 8.0 for appropriate dose modifications, Sections 9.1 and 9.2 for recommending treatment, and Appendix IV for instructions to be given to patients.

Add 12

21. This one time collection is required for Arm A or Arm D patients only and can be collected in conjunction with other required blood draws for this study during the first Observation visit, or at the next Observation visit for patients who are already in the Observation phase. See Section 14.0 for information concerning mandatory blood collection. **Include NCCTG ID number on Specimen Submission Form-Blood and on the Requisition Form contained in the blood collection kit.**

Add 12

22. For Arm A or Arm D patients only: If patient has secondary surgical resection following disease recurrence, optional recurrent tissue submission is requested. For patients who have secondary resection for recurrence during Event Monitoring, and have not otherwise been reconsented regarding this additional tissue, the patient should be contacted after site learns of the resection, to request the patient's consent for providing tissue. See Section 17.0 for details.

Add 12

23. CT scans may be performed annually rather than every six months.

R   Specimen kits are research funded. See Section 14.0 and Appendix XIII for blood specimen logistics and the forms packet for Kit Supply Fax Order Form.

Add
3,9,11
5.0   Stratification Factors at the Time of Registration/Randomization

NOTE: The following are required to be collected for all patients at the time of registration/randomization to Arms A, D, or G. These factors are used for stratification for randomization to Arms A or D:

5.1   Positive lymph node involvement: 1-3 vs. ≥ 4.

5.2   Histology: High (poorly differentiated or undifferentiated) vs. low (well or moderately differentiated).

Add 6
5.3   Clinical T Stage: (T1 or T2) vs. T3 vs. T4.

Add 9,13
6.0   Registration/Randomization Procedures (all sponsoring cooperative groups, including NCCTG, also see Appendix XII). Note: Patient enrollment closed as of November 25, 2009.

Add 3
6.1   Pre-Randomization

Add 3
6.11   Prior to the recruitment of a patient for this study, investigators and their institutions must be registered members of the CTSU. Each CTSU investigator or group of investigators at a clinical site must obtain IRB approval for this protocol before they can enroll patients.

Add 2,3
6.12   IRB approval is required for each treating site. A signed Cancer Trials Support Unit (CTSU) IRB Certification Form is to be on file at the CTSU Regulatory Office (fax 215-569-0206). This form can be found at the following Web site: www.ctsu.org/rss2_page.asp. Guidelines can be found under Quick Fact Sheets.

Add 3,5,6
6.13   Contact the CTSU Patient Registration Office by calling 1-888-462-3009 and leave a voice mail to alert the CTSU Patient Registrar that a pre-randomization is forthcoming. To pre-randomize the patient the investigator should complete the following forms:

- CTSU Patient Enrollment Transmittal Form
- N0147 Pre-randomization Eligibility Checklist.

For immediate registration needs, e.g. within one hour, call the registrar cell phone at 1-301-704-2376.

Add 2,3,5,6,11
Update 2

6.14   These forms should be faxed to the CTSU Patient Registrar at 1-888-691-8039 between the hours of 9:00 AM and 5:00 PM, Eastern Time, Mon.-Fri. (excluding holidays). **Patient registration/randomization received after 5 PM will be processed the following business day.** The CTSU registrar will check the investigator and site information provided to insure that all regulatory requirements have been met. The registrar will also check that forms are complete and follow up with the site to resolve any discrepancies. Once investigator eligibility is confirmed and pre-randomization documents are reviewed for compliance, the CTSU Registrar will access the North Central Cancer Treatment Group's on-line registration system to be used on specimen submission and all future forms and obtain assignment of a unique patient ID. The CTSU will convey this information to the enrolling site followed by a confirmation registration e-mail or fax.

Add 3

6.15   Request Letter for Immunohistochemistry (IHC) Test Results (Appendix XV) has been given to the patient.

Add 2,3,5,9,10

6.16   At the time of pre-randomization CTSU personnel will verify the following:
- IRB approval at the registering institution
- Patient eligibility checklist is complete
- Existence of a signed consent form
- Existence of a signed authorization for use and disclosure of protected health information. (USA institutions only)
- Patient has agreed by signature of consent form to allow tissue and blood samples to be used for correlative science studies associated with this study.
- Patient has/has not given permission to store blood and paraffin-embedded tissue for future research of colorectal cancer.
- Patient has/has not given permission to store blood and paraffin-embedded tissue for future research to learn, prevent, or treat other health problems.
- Patient has/has not given NCCTG permission to give their blood and paraffin-embedded tissue to outside researchers.
- Patients will automatically be registered to the mandatory translational research component of this study at the time of pre-randomization.
- Blood draw kit availability checked.

6.2   Registration/Randomization

Add 6,7,9, 10
Update 3

Note: Following pre-randomization, the two site contacts listed on the Pre-Randomization eligibility checklist will be notified by NCCTG (via e-mail) of the patient's KRAS results (wild-type, mutant or not evaluable) for patient assignment. This notification will be sent **≤10 business days from receipt of ALL required pathology materials. ("ALL pathology materials" includes the H&E slides that are required to be submitted. If H&E slides are not submitted, NCCTG will prepare an H&E slide for the KRAS testing, but this will delay the process of reporting KRAS back to the site.)** The site will then need to call CTSU to complete patient registration/randomization.

Add 3,5,6,9

6.21   Must collect translational research blood sample for Section 14.2 following pre-randomization, but prior to registration/randomization.

Add 3,5,9

6.22   Contact the CTSU Patient Registration Office by calling 1-888-462-3009. Leave a voice mail to alert the CTSU Patient Registrar that a registration/ randomization request is forthcoming. For immediate registration/ randomization needs, e.g. within one hour, call the registrar cell phone at 1-301-704-2376. Complete the following forms:

Add 9

- CTSU Patient Enrollment Transmittal Form
- N0147 NCI Cooperative Group Registration Form
- N0147 Registration/Randomization Eligibility Checklist

Add 3,5,6, 11,
Update 2

6.23   Fax these forms to the CTSU Patient Registrar at 1-888-691-8039 between the hours of 9:00 AM and 5: 00 PM, Eastern Time, Monday-Friday (excluding holidays). **Patient registration/randomization received after 5 PM will be processed the following business day.** The CTSU registrar will check the investigator and site information to ensure that all regulatory requirements have been met. The registrar will also check that forms are complete and follow up with the site to resolve any discrepancies.

Add 3,9

6.24   Once investigator eligibility is confirmed and enrollment documents are reviewed for compliance, the CTSU will access the NCCTG on-line registration system and obtain a registration/randomization assignment. The registrar will confirm registration/randomization and convey the patient's assignment by fax.

Add
2,5,9

6.25   Treatment assignment will be calculated using a dynamic allocation procedure that balances the marginal distributions of the stratification factors between the two treatment arms. The factors defined in Section 5.0, together with the randomizing site will be used as stratification factors (Arms A and D only).

Add
3,7,9

6.26   Treatment on this protocol must commence at the accruing membership under the supervision of a CTSU member physician (Arms A and D only).

Add 7,9,11   6.27   Treatment cannot begin prior to registration/randomization (Arms A, D, or G). For Arms A and D, treatment must begin ≤14 days after randomization.

Add 7,9   6.28   Pretreatment tests must be completed within the guidelines specified on the test schedule (Arms A and D only).

Add 7,9   6.29a   All required baseline symptoms must be documented and graded in the patient's medical record (Arms A and D only).

Add 9,10,11   6.29b   Patient Instructions for Preventing and Treating Diarrhea (Appendix IV) has been given to the patient; treating physician has discussed its contents with the patient (Arms A and D only).

Add 10,11   6.29c   Study drug availability checked (Arms A and D only).

Add 2,9   **7.0   Protocol Treatment (Treatment cycles are two weeks, treatment is for twelve 2-week cycles totaling 24 weeks.) Sections 7.1 – 7.3 apply to patients randomized to Arms A or D. Patients registered to Arm G are to be treated per physician discretion, off protocol (see Section 7.4).**

Add 6,9   7.1   All patients randomized to Arm A or Arm D will need a vascular access device. PICC lines are not recommended.

Add 2,5   7.2   Arm A (FOLFOX)

Add 5,6,7   7.21   Oxaliplatin, 5-fluorouracil, and leucovorin (12 cycles): One cycle of therapy is a two-week treatment period. Twelve 2-week cycles totaling 24 weeks are planned.

Add 5,6   <u>As of Addendum 5</u>: Any patients who crossed over from Arms B or C (these two arms were removed as of Addendum 5) to Arm A are to receive a total 24 weeks of protocol therapy.

Add 2,6,9   7.22   On day one of each treatment session patients will receive oxaliplatin 85 mg/m^2 given as a two-hour intravenous infusion. The dose of leucovorin will remain fixed at 400 mg/m^2 as a two-hour intravenous infusion followed by 5-fluorouracil 400 mg/m^2 IV push and 5-fluorouracil 2400 mg/m^2, given as a forty-six to forty-eight hour infusion.

Oxaliplatin + 5-Fluorouracil/Leucovorin (FOLFOX Regimen)*							
Arm	Agent	Dose	Route	Day	Treatment Week	Rest Period	ReRx***
A	oxaliplatin	85 mg/m^2	IV infusion 250 to 500 ml D5W over 120 minutes	1**	Week 1	Week 2	Q 14 days
	leucovorin	400 mg/m^2	In 250 ml D5W IV infusion over 120 minutes.	1**			
	5-fluorouracil	400 mg/m^2	IV push	1**			
	5-fluorouracil	2400 mg/m^2	IV, via an ambulatory infusion pump of choice over 46-48 hours	1** (after leucovorin)			

Add 2,3,8   *   <u>BSA should be recalculated at the beginning of each cycle of treatment</u>
There is no clearly documented adverse impact of treatment of obese patients when dosing is performed according to actual body weight. Therefore, **all dosing is to be determined solely by (1) the patient's BSA as calculated from actual weight or (2) actual weight without any modification unless explicitly described in the protocol**. This will eliminate the risk of calculation error and the possible introduction of variability in dose administration. **Failure to use actual body weight in the calculation of drug dosages will be considered a major protocol deviation.** Physicians who are uncomfortable with administering chemotherapy dose based on actual body weight should not enroll obese patients on this protocol.

** Oxaliplatin may be given concurrently with leucovorin 400 mg/m². Oxaliplatin must not be mixed with normal saline; therefore, when leucovorin and oxaliplatin are given concurrently via a Y-connector, both drugs should be administered in D5W.

Add 2   *** If necessary to accommodate holidays, patient schedule, or other justifiable circumstance, the every 2-week schedule may be modified +/- 3 days.

Add 2,6

7.3   Arm D (FOLFOX + C225)

Add 3,5,6,7

7.31   Cetuximab, Oxaliplatin, 5-fluorouracil, and leucovorin (12 cycles): One cycle of therapy is a two-week treatment period. Twelve 2-week cycles totaling 24 weeks are planned. Cetuximab must always be given **prior** to the prescribed chemotherapy regimen, except when cetuximab must be omitted for toxicities, as specified in Section 8.22. No interval is required between the dose of cetuximab and chemotherapy; however, the patient must be observed for one hour after the cetuximab infusion for any reactions.

Add 6

As of Addendum 5: Any patients who crossed over from Arms E or F (these two arms were removed as of Addendum 5) to Arm D are to receive a total 24 weeks of protocol therapy.

Add 6

7.32   On day one, week one, cetuximab 400 mg/m² is given IV over 120 minutes. On day 8, week 2, and for all consecutive weeks cetuximab 250 mg/m² is given IV over 60 minutes.

Add 6

7.33   On day one of each treatment session patients will receive oxaliplatin 85 mg/m² given as a two-hour intravenous infusion. The dose of leucovorin will remain fixed at 400 mg/m² as a two-hour intravenous infusion followed by 5-fluorouracil 400 mg/m² IV push and 5-fluorouracil 2400 mg/m², given as a forty-six to forty-eight hour infusion.

Add 6,9

Oxaliplatin + 5-fluorouracil/Leucovorin (FOLFOX Regimen)* + Cetuximab							
One Cycle = Two Weeks							
See Footnote 1 below for required premedication prior to cetuximab							
Arm	**Agent**	**Dose**	**Route**	**Day**	**Treatment Week**	**Rest Period**	**ReRx****
Cycle 1 Only							
D	Cetuximab[1,2,3] (give before chemotherapy)	400 mg/m²	IV over 2 hours	1	Week 1	None for cetuximab	Q 14 days
		250 mg/m²	IV over 1 hour (undiluted 2 mg/ml)	8	Week 2		
	Cycle 1: **Give oxaliplatin, leucovorin and 5-fluorouracil as outlined below**						
Subsequent Cycles (cycles 2-12)							
See Footnote 1 below for required premedication prior to cetuximab							
D	Cetuximab[1,2,3,4,5] (give before chemotherapy)	250 mg/m²	IV over 1 hour (undiluted 2 mg/ml)	1,8	Week 1,2	None for cetuximab	Q 14 days
	Oxaliplatin[6]	85 mg/m²	IV infusion 500 ml D5W over 120 minutes	1	Week 1	Week 2	
	Leucovorin	400 mg/m²	In 250 ml D5W IV infusion over 120 minutes	1			
	5-fluorouracil	400 mg/m²	IV push	1			
	5-fluorouracil	2400 mg/m²	IV, via an ambulatory infusion pump of choice over 46-48 hours	1			

Add 3
Add 3

*   BSA should be recalculated at the beginning of each cycle of treatment:
Add 11   There is no clearly documented adverse impact of treatment of obese patients when dosing is performed according to actual body weight. Therefore, **all dosing is to be determined solely by (1) the patient's BSA as calculated from**

Add 6

actual weight or (2) actual weight without any modification unless explicitly described in the protocol. This will eliminate the risk of calculation error and the possible introduction of variability in dose administration. **Failure to use actual body weight in the calculation of drug dosages will be considered a major protocol deviation.** Physicians who are uncomfortable with administering chemotherapy dose based on actual body weight should not enroll obese patients on this protocol.

** If necessary to accommodate holidays, patient schedule, or other justifiable circumstance, the FOLFOX and/or cetuximab schedule may be modified +/- 3 days.

1. In an effort to prevent a hypersensitivity reaction, all subjects must be pre-medicated with Benadryl 50 mg IV 30 - 60 minutes prior to cetuximab administration. Premedication will be administered prior to subsequent doses, but at the Investigator's discretion, the dose of Benadryl may be reduced.

Add 11 2. Refer to Section 15.413 for preparation and administration instructions.

3. The dose and volume of cetuximab to be infused are dependent upon the patient's BSA. The infusion rate of cetuximab must never exceed 10 mg/minute (5 mL/minute).

4. Cetuximab should not be mixed with D_5W and the line should be flushed with normal saline after infusion. If this is not possible, the cetuximab should be given in a separate line.

Add3 5. Cetuximab may be given without the other agent(s) for Day 1 of a new cycle, if the criteria for administering the other agent(s) are not satisfied. However, this dose will be included on the total for the previous cycle. That is, the cycle start date and Day 1 will always coincide with the administration of non-cetuximab agent(s). Once the criteria are satisfied for the non-cetuximab agent(s) for the new cycle, administer Day 1 and Day 8 of treatment (multiple agents) in accord with the treatment schedule. It is recognized that the total dose for cetuximab will be higher for the previous cycle (i.e., 3 administrations versus 2).

Add3 6. Oxaliplatin may be given concurrently with leucovorin 400 mg/m^2. Oxaliplatin must not be mixed with normal saline; therefore, when leucovorin and oxaliplatin are given concurrently via a Y-connector, both drugs should be administered in D5W.

Add 9, Update 3

7.4   Arm G (Locally Directed Therapy)

Patients determined to have mutated KRAS (or KRAS not evaluable) will be assigned to an event monitoring arm in which adjuvant therapy will be determined and assigned by the treating oncologist. The determination of the type of therapy, duration of treatment, and dose modification will be the responsibility of the treating oncologists.

8.0   Dosage Modification Based on Adverse Events

Add 2,3   8.1   Dose Reduction Steps for Arm A (FOLFOX):

The starting dose of 5-fluorouracil is 2400 mg/m^2, over 46 hours and the dose of leucovorin is 400 mg/m^2. The starting dose of oxaliplatin is 85 mg/m^2. Subsequent doses of oxaliplatin and 5-fluorouracil can be adjusted, depending upon individual patient tolerance of treatment. Patients should be carefully monitored for adverse events.

Add 6   **Note: Dose level +1 was removed as of Addendum 5. However, if a patient was increased to dose level +1 prior to Addendum 5 and is tolerating the dose, the patient may remain at this dose level.**

Add 2,3,6

Add 5

Dose Reduction Steps – Arm A - Oxaliplatin + 5-fluorouracil/Leucovorin Regimen *					
	Dose Level + 1	Starting Dose	Dose Level - 1	Dose Level -2	Dose Level – 3**
Oxaliplatin	85 mg/m^2	85 mg/m^2	65 mg/m^2	50 mg/m^2	40 mg/m^2
5-fluorouracil infusion****	3000 mg/m^2	2400 mg/m^2	1900 mg/m^2	1500 mg/m^2	1200 mg/m^2
* Leucovorin dose remains fixed at 400 mg/m^2 (not adjusted).					
**Further dose levels (-4, -5, etc.) will be 20% dose reductions from the previous level.					
****If the 5-FU infusion dose is decreased, the bolus dose of 5-FU should be discontinued for the current cycle and for all future cycles. Leucovorin will still be given.					

Add 5

Add 2,3,8,10

The following table describes the recommended dose modifications for patients under age 70. All dose modifications are required for patients 70 and older, except as noted. Dose modifications are applied during a course of therapy and at the start of each subsequent course of therapy. All dose modifications should be based on the worst preceding adverse event. **For patients age 70 and older the required modification or a greater modification (if felt necessary by the treating physician) must be performed with all treatment cycles.**

Recommended Dose Modifications for Arm A **Oxaliplatin+5-fluorouracil/Leucovorin (FOLFOX Regimen)**		
NCI CTC AE Category/ Version 3.0 NCI Grade (Value)	**Dose Level for Subsequent Cycles Based on Interval Adverse Events**	**At Time of Retreatment**
All Adverse Events ≤1	Maintain dose level.	Maintain dose level.
Blood/Bone Marrow: Neutrophils/Granulocytes Grade 1 (ANC <LLN-1500/mm^3) Grade 2 (ANC <1500-1000/mm^3) Grade 3 (ANC <1000 - 500/mm^3) Grade 4 (ANC <500/mm^3)	Maintain dose level Maintain dose level ↓ 1 oxaliplatin dose level Omit bolus 5-fluorouracil and ↓ both infusional 5-fluorouracil and oxaliplatin 1 dose level	If ANC <1500 at start of cycle, hold and check weekly then treat based on interval adverse event. If ANC <1500 after 4 weeks, discontinue therapy.
Blood/Bone Marrow: Thrombocytopenia (Thromb) Grade 1 (PLT<LLN-75,000/mm^3) Grade 2 (PLT<75,000-50,000/mm^3) Grade 3 (PLT <50,000–25,000/mm^3) Grade 4 (PLT <25,000/mm^3)	Maintain dose level Maintain dose level ↓ 1 oxaliplatin dose level ↓ 2 oxaliplatin dose levels	If PLT <75,000 at start of cycle, hold and check weekly then treat based on interval adverse event. If PLT <75,000 after 4 weeks, discontinue therapy.
Other hematologic adverse events	Dose modifications for leukopenia at the start of subsequent courses of therapy and at time of retreatment are also based on NCI CTCAE criteria (Version 3.0) and are the same as recommended for neutropenia above.	
AGE <70 Gastrointestinal: Diarrhea Grade 1, 2 Grade 3 Grade 4 **Mucositis/Stomatitis** Grade 1, 2 Grade 3 Grade 4 **Vomiting** Grades 1, 2 Grade 3 Grade 4	Maintain dose level ↓ One 5-fluorouracil dose level ↓ Both 5-fluorouracil and oxaliplatin 1 dose level Maintain dose level ↓ One 5-fluorouracil dose level ↓ One 5-fluorouracil dose level Maintain dose level ↓ 1 oxaliplatin dose level ↓ Both 5-fluorouracil and oxaliplatin 1 dose level	For diarrhea, mucositis/stomatitis or vomiting: If Grade ≥2 at start of cycle, hold and check weekly then treat based on interval adverse event. If Grade ≥2 after 4 weeks, discontinue therapy.
AGE >70 Gastrointestinal: Diarrhea Grade 1 Grade 2, 3 Grade 4 **Mucositis/Stomatitis** Grade 1, 2 Grade 3 Grade 4 **Vomiting** Grades 1, 2 Grade 3 Grade 4	Maintain dose level ↓ One 5-fluorouracil dose level ↓ Both 5-fluorouracil and oxaliplatin 1 dose level Maintain dose level ↓ One 5-fluorouracil dose level ↓ One 5-fluorouracil dose level Maintain dose level ↓ 1 oxaliplatin dose level ↓ Both 5-fluorouracil and oxaliplatin 1 dose level	For diarrhea, mucositis/stomatitis or vomiting: If Grade ≥**1** at start of cycle, hold and check weekly then treat based on interval adverse event. If Grade ≥2 after 4 weeks, discontinue therapy.
Other nonhematologic adverse events[1] Grades 1,2 Grades 3, 4	Dose modifications for other nonhematologic adverse events at the start of subsequent courses of therapy, and at time of retreatment are also based on NCI CTCAE criteria (Version 3.0) Maintain dose level ↓ 1 5-FU dose level **Note:** Dose reduction is not required for hypomagnesemia unless symptoms are present. If Grade ≥2 after 4 weeks, discontinue therapy.	

Add 6

Add 6

Add 5

Add 6

Add 6

Add 3,6,10

Add 10

Add 5,6,10

Neurology (do not use CTCAE)	See Section 8.11 for adverse event scale and oxaliplatin dose modifications.
Pulmonary Cough ≥Grade 3; Dyspnea ≥Grade 3 Hypoxia ≥Grade 3; Pneumonitis/ pulmonary infiltrates ≥Grade 3	Hold oxaliplatin until interstitial lung disease is ruled out.
Coagulation[2] Thrombotic microangiopathy (Hemolytic Uremic Syndrome [HUS] HCT <25%, PLTs <100,000, creatinine ≥ 1.6 mg/dL, renal failure) ≥ grade 3	Discontinue oxaliplatin

Add 6

The dose of leucovorin will not be adjusted due to adverse event. It should remain at 400 mg/m² for all courses. Leucovorin will be given immediately prior to each 5-fluorouracil dose; thus, if 5-fluorouracil is delayed, leucovorin will be delayed

Add 6

1. Exceptions: alopecia, fatigue, anorexia, nausea/vomiting if can be controlled by antiemetics, viral infections. 2. Recommended evaluation of suspected HUS: Evaluation should include CBC differential, platelets, PT, PTT, fibrinogen, FDP (Fibrin degradation products), Anti thrombin III, Von Willebrand factor, anti-nuclear antibody, rheumatoid factor, Compliment Cascade C3, C4, and CH_{50}, anti-platelet antibodies, platelet-associated IgG, and circulating immune complexes. Renal evaluation should include creatinine, BUN, and urinalysis with microscopic examination. Other laboratory and hematological evaluations as appropriate should also be obtained, including peripheral blood smear and free hemoglobin.

Add 3   ## 8.11   Oxaliplatin Dose Modifications for Neurologic Adverse Events – Arm A

Oxaliplatin Dose Modifications for Non-CTCAE Neurologic Adverse Events			
	Duration of Adverse Event		**Persistent**[1]
Adverse Events	**1 - 7 Days**	**>7 Days**	**Between Cycles**
Paresthesias/Dysesthesias			
Paresthesias/dysesthesias[2] of short duration that resolve and do not interfere with function (Grade 1)	no change	no change	no change
Paresthesias/dysesthesias[2] interfering with function, but not activities of daily living (ADL) (Grade 2)	no change	no change	↓1 oxaliplatin dose level
Paresthesias/dysesthesias[2] with pain or with functional impairment that also interfere with ADL (Grade 3)	1st time: ↓1 oxaliplatin dose level 2nd time: ↓1 oxaliplatin dose level	1st time: ↓1 oxaliplatin dose level 2nd time: ↓1 oxaliplatin dose level	Stop
Persistent paresthesias/dysesthesias that are disabling or life-threatening (Grade 4)	Stop	Stop	Stop
Laryngeal Dysesthesias (investigator discretion used for grading):			
Grade 0 = none; Grade 1 = mild	No change	↑ duration of infusion to 6 hours	↑ duration of infusion to 6 hours
Grade ≥2 = moderate. (Also recommended is administration of benzodiazepine and patient education. Management of patient if ≥Grade 2 laryngeal dysesthesias occurs while treatment is being administered.) Grade 3 = severe	• Stop oxaliplatin infusion • Administer benzodiazepine and give patient reassurance • At the discretion of the investigator, the infusion can be restarted at 1/3 the original rate of infusion.		

[1] Not resolved by the beginning of the next cycle.
[2] May be cold-induced.

8.2   Dose Reduction Steps for Arm D (FOLFOX + C225):

Add 3,6   8.21   Dose Reduction Steps for **FOLFOX** Regimen, Arm D. (<u>Note that dose reduction steps for Arm D C225 are listed in Section 8.22.</u>)

The starting dose of 5-fluorouracil is 2400 mg/m^2, over 46 hours and the dose of leucovorin is 400 mg/m^2. The starting dose of oxaliplatin is 85 mg/m^2. Subsequent doses of oxaliplatin and 5-fluorouracil can be adjusted, depending upon individual patient tolerance of treatment. Patients should be carefully monitored for adverse events.

Add 6   **Note: Dose level +1 was removed as of Addendum 5. However, if a patient was increased to dose level +1 prior to Addendum 5 and is tolerating the dose, the patient may remain at this dose level.**

Dose Reduction Steps – Arm D - Oxaliplatin + 5-fluorouracil/Leucovorin Regimen (FOLFOX)*					
	Dose Level + 1	Starting Dose	Dose Level - 1	Dose Level -2	Dose Level – 3**
Oxaliplatin	85 mg/m^2	85 mg/m^2	65 mg/m^2	50 mg/m^2	40 mg/m^2
5-fluorouracil infusion****	3000 mg/m^2	2400 mg/m^2	1900 mg/m^2	1500 mg/m^2	1200 mg/m^2
* Leucovorin dose remains fixed at 400 mg/m^2 (not adjusted).					
**Further dose levels (-4, -5, etc.) will be 20% dose reductions from the previous level.					
****If the 5-FU infusion dose is decreased, the bolus dose of 5-FU should be discontinued for the current cycle and for all future cycles. Leucovorin will still be given.					

Add 2, 3, 6 (table left margin)

Add 5

The following table describes the recommended dose modifications for patients under age 70.   All dose modifications are required for patients 70 and older, except as noted. Dose modifications are applied during a course of therapy and at the start of each subsequent course of therapy. All dose modifications should be based on the worst preceding adverse event. **For patients age 70 and older the required modification or a greater modification (if felt necessary by the treating physician) must be performed with all treatment cycles.**

Add 10

A
d
d
3,
6

Add 6

Add
5,6

Add 6

Add 6

Add
3,6,9,
10

Add 10

Recommended Dose Modifications for Arm D **Oxaliplatin+5-fluorouracil/Leucovorin (FOLFOX Regimen Only)** Refer to Section 8.22 for Arm D C225 dose modifications.		
NCI CTC AE Category/ **Version 3.0 NCI Grade (Value)**	**Dose Level for Subsequent Cycles** **Based on Interval Adverse Events**	**At Time of Retreatment**
All Adverse Events ≤1	Maintain dose level.	Maintain dose level.
Blood/Bone Marrow: **Neutrophils/Granulocytes** Grade 1 (ANC <LLN - 1500/mm^3) Grade 2 (ANC <1500 - 1000/mm^3) Grade 3 (ANC <1000 - 500/mm^3) Grade 4 (ANC <500/mm^3)	Maintain dose level Maintain dose level ↓ 1 oxaliplatin dose level Omit bolus 5-fluorouracil and ↓ both infusional 5-fluorouracil and oxaliplatin 1 dose level	If ANC <1500 at start of cycle, hold and check weekly then treat based on interval adverse event. If ANC <1500 after 4 weeks, discontinue therapy.
Blood/Bone Marrow: **Thrombocytopenia (Thromb)** Grade 1 (PLT <LLN-75,000/mm^3) Grade 2 (PLT <75,000 - 50,000/mm^3) Grade 3 (PLT <50,000 - 25,000/mm^3) Grade 4 (PLT <25,000/mm^3)	Maintain dose level Maintain dose level ↓ 1 oxaliplatin dose level ↓ 2 oxaliplatin dose levels	If PLT <75,000 at start of cycle, hold and check weekly then treat based on interval adverse event. If PLT <75,000 after 4 weeks, discontinue therapy.
Other hematologic adverse events	Dose modifications for leukopenia at the start of subsequent courses of therapy and at time of retreatment are also based on NCI CTCAE criteria (Version 3.0) and are the same as recommended for neutropenia above.	
AGE <70 **Gastrointestinal:** **Diarrhea** Grade 1 Grade 2, 3 Grade 4 **Mucositis/Stomatitis** Grade 1, 2 Grade 3 Grade 4 **Vomiting** Grades 1, 2 Grade 3 Grade 4	 Maintain dose level ↓ One 5-fluorouracil dose level ↓ Both 5-fluorouracil and oxaliplatin 1 dose level Maintain dose level* ↓ One 5-fluorouracil dose level ↓ One 5-fluorouracil dose level Maintain dose level* ↓ 1 oxaliplatin dose level ↓ Both 5-fluorouracil and oxaliplatin 1 dose level	For diarrhea, mucositis/stomatitis or vomiting: If Grade ≥2 at start of cycle, hold and check weekly then treat based on interval adverse event. If Grade ≥2 after 4 weeks, discontinue therapy.
AGE >70 **Gastrointestinal:** **Diarrhea** Grade 1 Grade 2, 3 Grade 4 **Mucositis/Stomatitis** Grade 1, 2 Grade 3 Grade 4 **Vomiting** Grades 1, 2 Grade 3 Grade 4	 Maintain dose level ↓ One 5-fluorouracil dose level ↓ Both 5-fluorouracil and oxaliplatin 1 dose level Maintain dose level ↓ One 5-fluorouracil dose level ↓ One 5-fluorouracil dose level Maintain dose level ↓ 1 oxaliplatin dose level ↓ Both 5-fluorouracil and oxaliplatin 1 dose level	For diarrhea, mucositis/stomatitis or vomiting: If Grade ≥**1** at start of cycle, hold and check weekly then treat based on interval adverse event. If Grade ≥2 after 4 weeks, discontinue therapy.

Add 5,6,10	**Other nonhematologic adverse events**[1] Grades 1,2 Grades 3,4	Dose modifications for other nonhematologic adverse events at the start of subsequent courses of therapy, and at time of retreatment are also based on NCI CTCAE criteria (Version 3.0) Maintain dose level ↓ 1 5-FU dose level **Note:** Dose reduction is not required for hypomagnesemia unless symptoms are present. If Grade ≥2 after 4 weeks, discontinue therapy.
	Neurology (do not use CTCAE)	See Section 8.211 for adverse event scale and oxaliplatin dose modifications.
	Pulmonary Cough ≥Grade 3; Dyspnea ≥Grade 3 Hypoxia ≥Grade 3; Pneumonitis/ pulmonary infiltrates ≥Grade 3	Hold oxaliplatin until interstitial lung disease is ruled out.
Add 6	**Coagulation**[2] Thrombotic microangiopathy (Hemolytic Uremic Syndrome [HUS] HCT <25%, PLTs <100,000, creatinine ≥ 1.6 mg/dL, renal failure) ≥ grade 3	Discontinue oxaliplatin

The dose of leucovorin will not be adjusted due to adverse event. It should remain at 400 mg/m² for all courses. Leucovorin will be given immediately prior to each 5-fluorouracil dose; thus, if 5-fluorouracil is delayed, leucovorin will be delayed

Add 6
1. Exceptions: alopecia, fatigue, anorexia, nausea/vomiting if can be controlled by antiemetics, viral infections.
2. Recommended evaluation of suspected HUS: Evaluation should include CBC differential, platelets, PT, PTT, fibrinogen, FDP (Fibrin degradation products), Anti thrombin III, Von Willebrand factor, anti-nuclear antibody, rheumatoid factor, Compliment Cascade C3, C4, and CH$_{50}$, anti-platelet antibodies, platelet-associated IgG, and circulating immune complexes. Renal evaluation should include creatinine, BUN, and urinalysis with microscopic examination. Other laboratory and hematological evaluations as appropriate should also be obtained, including peripheral blood smear and free hemoglobin.
* 1 level dose reduction recommended for patients age 70 and older for grade 2 stomatitis or vomiting

Add 3,6

8.211   Oxaliplatin Dose Modifications for Neurologic Adverse Events – Arm D

Oxaliplatin Dose Modifications for Non-CTCAE Neurologic Adverse Events			
	Duration of Adverse Event		**Persistent**[1]
Adverse Events	**1 - 7 Days**	**>7 Days**	**Between Cycles**
Paresthesias/Dysesthesias			
Paresthesias/dysesthesias[2] of short duration that resolve and do not interfere with function (Grade 1)	no change	no change	no change
Paresthesias/dysesthesias[2] interfering with function, but not activities of daily living (ADL) (Grade 2)	no change	no change	↓1 oxaliplatin dose level
Paresthesias/dysesthesias[2] with pain or with functional impairment that also interfere with ADL (Grade 3)	1st time: ↓1 oxaliplatin dose level 2nd time: ↓1 oxaliplatin dose level	1st time: ↓1 oxaliplatin dose level 2nd time: ↓1 oxaliplatin dose level	Stop
Persistent paresthesias/dysesthesias that are disabling or life-threatening (Grade 4)	Stop	Stop	Stop
Laryngeal Dysesthesias (investigator discretion used for grading):			
Grade 0 = none; Grade 1 = mild	No change	↑ duration of infusion to 6 hours	↑ duration of infusion to 6 hours
Grade ≥2 = moderate. (Also recommended is administration of benzodiazepine and patient education. Management of patient if ≥Grade 2 laryngeal dysesthesias occurs while treatment is being administered.) Grade 3 = severe	• Stop oxaliplatin infusion • Administer benzodiazepine and give patient reassurance • At the discretion of the investigator, the infusion can be restarted at 1/3 the original rate of infusion.		

[1] Not resolved by the beginning of the next cycle.
[2] May be cold-induced.

8.22   Dose Modifications for cetuximab (Arm D)

ALERT: ADR reporting may be <u>required</u> for some adverse events. See Section 10.0.

Cetuximab Dose Modifications			
Cetuximab	**Starting dose (mg/m^2)**	**Dose Level – 1 (mg/m^2)**	**Dose Level - 2 (mg/m^2)**
	400 mg/m^2 (week 1) 250 mg/m^2 weekly	200 mg/m^2 weekly	150 mg/m^2 weekly

Cetuximab dose reductions below the -2 dose level will not be allowed. All dose reductions are permanent, that is, there will not be any re-escalation of cetuximab dose.

If cetuximab is omitted for more than four consecutive infusions for toxicity due to cetuximab, or for an intercurrent illness (e.g., infection) requiring interruption of therapy, the subject should be discontinued from further cetuximab therapy. If toxicities prevent the administration of cetuximab, the subject may continue to receive study therapy without cetuximab.

Add 3

Cetuximab may be given without the other agent(s) for Day 1 of a new cycle, if the criteria for administering the other agent(s) are not satisfied. However, this dose will be included on the total for the previous cycle. That is, the cycle start date and Day 1 will always coincide with the administration of non-cetuximab agent(s). Once the criteria are satisfied for the non-cetuximab agent(s) for the new cycle, administer Day 1 and Day 8 of treatment (multiple agents) in accord with the treatment schedule. It is recognized that the total dose for cetuximab will be higher for the previous cycle (i.e., 3 administrations versus 2).

Add 3

Cetuximab may only be administered if all of the following criteria are met regardless of cycle, providing no criteria for discontinuation are met. If criteria is not met, treatment is omitted, not delayed.
Acne-like rash is ≤Grade 2.
All Grade 3 - 4 hematologic toxicities have resolved to ≤ CTCAE v3.0 Grade 2.

Add 3

All Grade 3 - 4 non-hematologic toxicities have resolved to ≤ CTCAE v3.0 Grade 2, (except fatigue [asthenia], and anorexia).

Add 3

Cetuximab Dose Modifications		
NCI CTC AE Category / Version 3.0 NCI Grade [a]	**Dose Level for Subsequent Cycles Based on Interval Adverse Events [b]**	**At the Time of Retreatment [b]**
Allergy/Immunology **Allergic Reaction** Grade 1: (transient flushing or rash, drug fever <38°C)	Decrease infusion rate by 50% and monitor closely for worsening. Once a cetuximab infusion rate has been decreased due to a hypersensitivity reaction, it will remain decreased for all subsequent infusions. If a patient has a second hypersensitivity reaction with the slower infusion rate, the infusion should be stopped and the patient should receive no further cetuximab treatment for that cycle. The infusion of cetuximab should not exceed 4 hours.	
Grade 2: (rash; flushing; urticaria; dyspnea; drug fever ≥38°C)	Stop the infusion. Administer bronchodilators, oxygen, etc. As medically indicated. Resume infusion at 50% of previous rate once allergic/hypersensitivity reaction has been decreased for all subsequent infusions. If a patient has a second hypersensitivity reaction with the slower infusion rate, the infusion should be stopped and the patient should receive no further cetuximab treatment for that cycle. The infusion of cetuximab should not exceed 4 hours.	
Grade 3: (symptomatic bronchospasm, with or without urticaria; parenteral medication(s) indicated; allergy-related edema /angioedema; hypotension)	Stop infusion immediately. Administer epinephrine, bronchodilators, antihistamines, glucocorticoids, IV fluids, vasopressor agents, oxygen, etc., as medically indicated. Discontinue administration of cetuximab to the patient permanently.	
Grade 4: (Anaphylaxis – Life threatening event)	Stop infusion immediately. Administer epinephrine, bronchodilators, antihistamines, glucocorticoids, IV fluids, vasopressor agents, oxygen, etc., as medically indicated. Discontinue administration of cetuximab to the patient permanently.	

Add 3

Add 3

Add 3	**Blood/Bone Marrow: Neutrophils/Granulocytes** Grade 1 (ANC <LLN - 1500/mm^3) Grade 2 (ANC <1500 - 1000/mm^3) Grade 3 (ANC <1000 - 500/mm^3) Grade 4 (ANC <500/mm^3)	Maintain dose level Maintain dose level Do not administer until ≤Grade 2. Do not administer until ≤Grade 2.	
Add 3	**Blood/Bone Marrow: Thrombocytopenia (Thromb)** Grade 1 (PLT <LLN-75,000/mm^3) Grade 2 (PLT <75,000 - 50,000/mm^3) Grade 3 (PLT <50,000 - 25,000/mm^3) Grade 4 (PLT <25,000/mm^3)	Maintain dose level Maintain dose level Do not administer until ≤Grade 2. Do not administer until ≤Grade 2.	
Add 3	**Other hematologic adverse events**	Dose modifications for leukopenia at the start of subsequent courses of therapy and at time of retreatment are also based on NCI toxicity criteria (CTCAE Version 3.0) and are the same as recommended for neutropenia above.	
Add 5	**Cardiac General Cardiac ischemia/infarction** Grade 3	Discuss with PI prior to re-treating. If appropriate decrease 1 dose level.	Omit cetuximab therapy up to 4 weeks until AE resolves to <Grade 2.
	Grade 4	Discontinue cetuximab	Discontinue cetuximab
	Dermatology/Skin Rash/Desquamation Grade 2 (if tolerable)	Maintain dose level.	Maintain dose level.
Add 3	Grade 3 (or Grade 2 if not tolerable)	1st occurrence: If improvement, maintain dose level. If no improvement, discontinue. 2nd occurrence: If improvement, ↓ 1 cetuximab dose level. If no improvement, discontinue. 3rd occurrence: If improvement, ↓ 1 cetuximab dose level. If no improvement, discontinue. 4th occurrence: Discontinue cetuximab	Omit cetuximab therapy for 1-2 weeks
	Grade 4:	Discontinue cetuximab	Discontinue cetuximab
Add 3,11	**Nail Changes** Grade 2, 3	↓ 1 cetuximab dose level	Omit cetuximab therapy up to 4 weeks until AE resolves to <Grade 2

Add 10,11	**Gastrointestinal - Diarrhea** Grade 2[c]	↓ 1 cetuximab dose level	**For patients 70 or older:** Omit cetuximab therapy up to 4 weeks until AE resolves to <Grade 2
Add 11	Grade 3[c]	↓ 1 cetuximab dose level	Omit cetuximab therapy up to 4 weeks until AE resolves to <Grade 2
	Grade 4	Discontinue cetuximab	Discontinue cetuximab
Add 3,9,10,11	**Other non-hematologic adverse events** Grade 2[c]	↓ 1 cetuximab dose level	**For patients 70 or older:** Omit cetuximab therapy up to 4 weeks until AE resolves to <Grade 2
Add 3,11	Grade 3[c]	↓ 1 cetuximab dose level	Omit cetuximab therapy up to 4 weeks until AE resolves to <Grade 2
	Grade 4	Discontinue cetuximab	Discontinue cetuximab

a –For ≤ CTCAE v3.0 Grade 2 toxicity not described above, maintain dose level of agent.

b –Dose levels are relative to the previous dose. Dose reductions of cetuximab below the –2 dose level will not be allowed. All reductions are permanent, that is, there will not be any re-escalation of the cetuximab dose.

Add 9

c –With the exception of nausea/vomiting (if controlled by antiemetics). For Grade 2 and 3 toxicities felt to be unrelated to cetuximab, the agent may be continued; however, the investigator should use his/her medical judgment.

9.0 Ancillary Treatment

9.1 **Loperamide (Imodium®):** Patients will be instructed to begin taking loperamide at the earliest signs of a poorly formed or loose stool. Loperamide should be taken in the following manner: 4 mg at the first onset of diarrhea, then 2 mg every two hours around the clock until the patient is diarrhea-free for at least 12 hours. Patients may take loperamide 4 mg every four hours during the night.

Add 2,5,6

Note: Effective July 15, 2006, loperamide is no longer provided free of charge by NCI.

9.2 **Atropine:** Diarrhea or abdominal cramping can be treated with atropine (0.25 to 1 mg I.V. as indicated). Patients having recurrent problems with cholinergic symptoms may receive atropine prophylactically (S.C. or I.V.). Additional antidiarrheal measures may be used at the discretion of the treating physician.

Add 6

9.3 **Antiemetics:** Patients may receive dexamethasone 10 mg I.V. as a pretreatment antiemetic before irinotecan doses, unless there is a relative or absolute contraindication to corticosteroids (i.e., diabetes, known sensitivity to corticosteroids, severe muscle weakness or myalgias, etc.). Drugs such as Ativan®, Zofran®, Kytril®, or Aloxi may also be used if clinically indicated. As the majority of patients on previous trials have not experienced significant nausea, antiemetics other than Decadron are recommended only for those patients who demonstrate nausea and/or vomiting despite treatment with Decadron.

Add 2

Add 6

9.4   **Anticoagulants:** Patients who are taking Coumadin may participate in this study; however, it is recommended the prothrombin time be monitored carefully (at least weekly). Subcutaneous heparin is permitted.

Add 5,6

9.5   **Pharyngolaryngeal dysesthesias**: Oxaliplatin may cause discomfort in the larynx or pharynx associated with dyspnea, anxiety, swallowing difficulty and is exacerbated by cold. (See Table 3, Section 15.386.) Appropriate therapy includes use of anxiolytics, cold avoidance and monitoring.

9.6   Colony Stimulating Factors

Add 4,6,11

9.61   Use of granulocyte-macrophage colony-stimulating factor (GM-CSF, sargramostim, molgramostim, Leukine®, Leukomax®) is not routinely recommended. Routine prophylactic use of granulocyte colony-stimulating factor (G-CSF, filgrastim, Neupogen®) is not generally recommended. However, prophylactic administration of G-CSF in a patient who is experiencing recurrent difficulties with neutropenia in subsequent cycles, or therapeutic use in patients with serious neutropenic complications such as tissue infection, sepsis syndrome, fungal infection, etc., may be considered at the investigator's discretion, consistent with American Society of Clinical Oncology guidelines (American Society of Clinical Oncology 1994).

Add 6

9.62   Erythropoietin, including darbepoetin alfa, is allowed for patients who meet standardized criteria, as specified in the ASCO guidelines, for its use. Neumega® (oprelvekin) is not allowed in this trial.

Add 4,6,9

9.7   **Hypomagnesemia:** In the event of hypomagnesemia, monitoring of magnesium levels should be performed more frequently than every two weeks during study treatment, and magnesium repletion should be instituted as per routine clinical practice.

Add 9   **10.0   Adverse Event (AE) Reporting and Monitoring.**
                   Note: Adverse event reporting is not required for Arm G.

Add 5,13   10.1   CTCAE term (AE description) and grade: The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events version 4.0 (CTCAE v4.0) will be utilized beginning April 1, 2011 for expedited adverse event reporting only. All appropriate treatment areas should have access to a copy of the CTCAE v4.0. A copy of the CTCAE v4.0 can be downloaded from the CTEP web site (http://ctep.cancer.gov).

Add 5,13   10.11   Adverse event monitoring and reporting is a routine part of every clinical trial. First, identify and grade the severity of the event using the CTCAE. Next, determine whether the event is expected or unexpected (see Section 10.12) and if the adverse event is related to the medical treatment or procedure (see Section 10.13). With this information, determine whether the event must be reported as an expedited report (see Section 10.2). Important: All AEs reported via expedited mechanisms must also be reported via the routine data reporting mechanisms defined by the protocol (see Sections 10.3 and 18.0).

           Expedited adverse event reporting requires submission of an Adverse Event Expedited Reporting System (AdEERS) report(s). Other expedited reporting requirements and systems may also apply. Expedited and routine reports are to be completed within the timeframes and via the mechanisms specified in Sections 10.2 and 10.3. All expedited AE reports must also be sent to the local Institutional Review Board (IRB) according to local IRB's policies and procedures.

           Effective with Addendum 13, and beginning April 1, 2011, expedited AdEERS reporting for this protocol is being updated to CTCAE version 4.0. Therefore:

           1) Events requiring expedited reporting through AdEERS must be reported through the AdEERS system in CTCAE v4.0.

           2) The events reported via AdEERS must ALSO be reported through routine reporting (i.e., case report forms) using CTCAE v3.0.

           3) Routine data collection via case report forms will remain using CTCAE v3.0 for this study.

Add 5,6   10.12   Expected vs. Unexpected Events

           Agent(s) under a CTEP IND:
           • Expected AEs for expedited reporting purposes are listed on the CTEP Agent Specific Adverse Event List (ASAEL), a component of the Comprehensive Adverse Events and Potential Risks List (CAEPR). Refer to Section 15.0 to locate the CAEPR for the CTEP IND agent(s).
           • Unexpected AEs are those not listed in the ASAEL.
           Other agents:
           • The determination of whether an AE is expected is based on agent-specific information provided in Section 15.0 of the protocol.
           • Unexpected AEs are those not listed in the agent-specific information provided in Section 15.0 of the protocol.

Add 5

10.13   Assessment of Attribution

When assessing whether an adverse event is related to a medical treatment or procedure, the following attribution categories are utilized:
Definite - The adverse event *is clearly related* to the agent(s).
Probable - The adverse event *is likely related* to the agent(s).
Possible - The adverse event *may be related* to the agent(s).
Unlikely - The adverse event *is doubtfully related* to the agent(s).
Unrelated - The adverse event *is clearly NOT related* to the agent(s).

Add 5

10.14   Additional instructions for trials that include both investigational agent(s) (those under an IND) and a commercial agent(s):
• When an investigational agent (an agent under an IND) is used in combination with a commercial agent(s) on the same treatment arm, the combination is considered investigational. Expedited reporting will follow the requirements for investigational agents. However, if the event occurs prior to the participant having received any investigational agent, expedited reporting may follow the requirements for commercial agents.

Add 5,6

10.2   Expedited Reporting Requirements

10.21   Phase 2 and 3 Trials Utilizing an Agent under a CTEP IND: AdEERS Expedited Reporting Requirements for Adverse Events That Occur Within 30 Days[1] of the Last Dose of the Investigational Agent
Please also note that important information on additional reporting requirements is located in Section 10.211.

	Grade 1	Grade 2	Grade 2	Grade 3		Grade 3		Grades 4 & 5[2]	Grades 4 & 5[2]
				Unexpected		Expected			
	Unexpected and Expected	**Unexpected**	**Expected**	**with Hospitali-zation**	**without Hospitali-zation**	**with Hospitali-zation**	**without Hospitali-zation**	**Unexpected**	**Expected**
Unrelated Unlikely	Not Required	Not Required	Not Required	7 Calendar Days	Not Required	7 Calendar Days	Not Required	7 Calendar Days	7 Calendar Days
Possible Probable Definite	Not Required	7 Calendar Days	Not Required	7 Calendar Days	7 Calendar Days	7 Calendar Days	Not Required	24-Hour; 3 Calendar Days	7 Calendar Days

[1] **Adverse events with attribution of possible, probable, or definite that occur greater than 30 days after the last dose of treatment with an agent under a CTEP IND require reporting as follows:**
AdEERS 24-hour notification followed by complete report within 3 calendar days for:
• Grade 4 and Grade 5 unexpected events
AdEERS 7 calendar day report:
• Grade 3 unexpected events with hospitalization or prolongation of hospitalization
• Grade 5 expected events

[2] Although an AdEERS 24-hour notification is not required for death clearly related to progressive disease, a full report is required as outlined in the table.

Please see additional instructions and/or exceptions below under section entitled "Additional Instructions or Exceptions."

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Note: All deaths on study require both routine and expedited reporting regardless of causality. Attribution to treatment or other cause should be provided.

- Expedited AE reporting timelines defined:
  - ➢ "24 hours; 3 calendar days" – The investigator must initially report the AE via AdEERS within <u>24 hours</u> of learning of the event followed by a complete AdEERS report within <u>3 calendar days</u> of the initial 24-hour report.
  - ➢ "7 calendar days" - A complete AdEERS report on the AE must be submitted within <u>7 calendar days</u> of the investigator learning of the event.
- Any medical event equivalent to CTCAE grade 3, 4, or 5 that precipitates hospitalization (or prolongation of existing hospitalization) must be reported regardless of attribution and designation as expected or unexpected with the exception of any events identified as protocol-specific expedited adverse event reporting exclusions.
- Any event that results in persistent or significant disability/incapacity, congenital anomaly, or birth defect must be reported via AdEERS if the event occurs following treatment with an agent under a CTEP IND.

Add 13

- **SECONDARY MALIGNANCIES** (defined as "cancer caused by treatment for a previous malignancy", e.g., treatment with radiation or chemotherapy) are to be reported through AdEERS, as noted in Section 10.22. Secondary malignancies are not considered metastasis of the initial neoplasm. Secondary malignancy is unrelated to the first cancer that was treated, and may occur months or even years after initial treatment. Note: Second Primary malignancy (malignancy **not** due to prior treatment) should not be reported through AdEERS.
- Use the NCI protocol number and the protocol-specific patient ID provided during trial registration on all reports.

Add
5,6,13

10.211 Additional Instructions or Exceptions to AdEERS Expedited Reporting Requirements for Phase 2 and 3 Trials Utilizing an Agent Under a CTEP IND:

- All cases of grade ≥3 pulmonary fibrosis or hemolysis with renal failure: Report within 7 calendar days <u>regardless</u> of attribution OR hospitalization.
- All cases of grade ≥3 cardiac events on ALL treatment arms: Report within 7 calendar days <u>regardless</u> of attribution.
- In the rare event when Internet connectivity is disrupted, a 24-hour notification is to be made to NCI by telephone at: 301-897-7497. An electronic report <u>MUST</u> be submitted immediately upon re-establishment of internet connection. Please note that all paper AdEERS forms have been removed from the CTEP website and will NO LONGER be accepted.
- See Section 10.22 for exceptions to AdEERS expedited reporting requirements.
- Refer to Section 10.23 of this protocol for additional expedited reporting requirements.

Add 2,5

10.22   Exceptions to AdEERS Expedited Adverse Event Reporting Requirements

The following grade 3 or 4 adverse events, **including hospitalization for these events**, are specifically excluded from AdEERS expedited adverse event reporting for protocols using oxaliplatin/chemotherapy combination.

CTCAE v3.0 CATEGORY	Adverse Event	Comments
BLOOD/BONE MARROW		
	Hemoglobin	
	Leukocytes (total WBC)	
	Neutrophils/granulocytes	
	Platelets	
CONSTITUTIONAL SYMPTOMS		
	Fatigue (lethargy, malaise, asthenia)	
	Fever in the absence of neutropenia	
DERMATOLOGY		
	Rash: Hand-foot skin reaction	
GASTROINTESTINAL		
	Constipation	
	Dehydration	
	Diarrhea	
	Dysphagia/esophagitis	
	Ileus	**Only Grade 3 GI events are excluded from AdEERS expedited reporting.** **Grade 4 GI events must be reported via AdEERS as stated in Section 10.21.**
	Nausea	
	Obstruction	
	Mucositis/Stomatitis (clinical exam) (oral/pharyngeal mucositis)	
	Mucositis/Stomatitis (functional/symptomatic) (oral/pharyngeal mucositis)	
	Vomiting	
INFECTION		
	Febrile Neutropenia	
	Infection (documented) with grade 3 or 4 neutrophils	
	Infection with unknown ANC	
	Infection without neutropenia	
NEUROLOGY		
	Neuropathy-sensory	
METABOLIC/LABORATORY		
	ALT (SGPT)	
	AST (SGOT)	
PAIN		
	All adverse events	Including abdominal pain/cramping
VASCULAR		
	Thrombosis/embolism (vascular access-related)	
	Thrombosis/thrombus/embolism	

Add 6,10

Note: All deaths on study must be reported using expedited reporting mechanisms regardless of causality. Attribution to treatment or other cause must be provided.

10.23   **Other Required Expedited Reporting**

EVENT TYPE	REPORTING PROCEDURE
Secondary AML/MDS	Reporting for this event required during and after completion of study treatment via AdEERS. **Beginning April 1, 2011, AdEERS will only accept CTCAE v4.0 for this study.** Report these events using "Neoplasms benign, malignant and unspecified (including cysts and polyps)" *and including the appropriate adverse event:* – Leukemia secondary to oncology chemotherapy OR - Myelodysplastic syndrome OR - Treatment related secondary malignancy.

Add
3,6,7,10,13

10.3 Adverse events to be graded at each evaluation and pretreatment symptoms/conditions to be evaluated at baseline per Common Terminology Criteria for Adverse Events (CTCAE) v3.0 grading, using MedDRA v6.0, unless otherwise stated. MedDRA v6.0 Coding for Adverse Events (AEs), dated 10/04/07 is accessible on the N0147 page of the CTSU members web site at https://members.ctsu.org/ under Documents – All.

	Adverse Events/Symptoms	Baseline	Each Evaluation[1]	Non-CTC Grading
	# stools per day	X		
CTCAE v3.0 Category				
Blood/Bone Marrow	Neutrophils	X	X	
	Platelets			
Cardiac General	Cardiac ischemia/infarction (Chest Pain or Heart Attack)	X	X	
Coagulation	Thrombotic microangiopathy (e.g., thrombotic thrombocytopenic purpura [TTP] or hemolytic uremic syndrome [HUS])		X[3]	
Dermatology/skin	Rash/desquamation		X	
Gastrointestinal	Diarrhea		X[4]	
	Nausea	X	X	
	Mucositis/stomatitis (functional/symptomatic) -Oral cavity -Pharynx Mucositis/stomatitis (clinical exam) -Oral cavity -Pharynx		X	
	Vomiting	X	X	
Infection	Febrile neutropenia (fever of unknown origin without clinically or microbiologically documented infection) (ANC <1.0 x 10^9/L)		X	
	Infection (documented clinically or microbiologically) with Grade 3 or 4 neutrophils -Skin (cellulites) -Colon -Catheter – related -Wound -Biliary tree -Lung (pneumonia) -Pleura (empyema) -Bladder (urinary) -Kidney -Urinary tract NOS -Pelvis NOS -Abdominal NOS -Upper aerodigestive NOS -Upper airway (NOS)		X	
Metabolic/ Laboratory	Magnesium, serum-low (hypomagnesemia)	X	X	
Neurology	Laryngopharyngeal dysesthesias Paresthesias/Dysesthesias		X[2]	X[2]
Pulmonary/Upper Respiratory	Cough		X	
	Dyspnea (shortness of breath)		X	
	Hypoxia		X	
	Pneumonitis/pulmonary infiltrates		X	

Add 5 *(row: Cardiac General)*

Add 10 *(row: Gastrointestinal)*

Add 4 *(row: Metabolic/ Laboratory)*

1. See Section 4.0 for schedule of adverse event evaluations.
2. See Section 8.11.
3. As per Section 8.1: Recommended evaluation of suspected HUS (HCT <25%, PLTs <100,000, creatinine ≥1.6 mg/dL): Evaluation should include CBC differential, platelets, PT, PTT, fibrinogen, FDP (Fibrin degradation products), Anti-thrombin III, Von Willebrand factor, anti-nuclear antibody, rheumatoid factor, Compliment Cascade C3, C4, and CH_{50}, anti-platelet antibodies, platelet-associated IgG, and circulating immune complexes. Renal evaluation should include creatinine, BUN, and urinalysis with microscopic examination. Other laboratory and hematological evaluations as appropriate should also be obtained, including peripheral blood smear and free hemoglobin.

Add 10 4. Early intervention is important for patient safety, particularly for patients age 70 and older. See Section 8.0 for appropriate dose modifications, Sections 9.1 and 9.2 for recommending treatment, and Appendix IV for instructions to be given to patients.

Add 6   10.31   Submit the following AEs experienced by a patient:

Add 6   10.311 All Grade 3 and 4 AEs regardless of attribution to the study treatment or
                          procedure, including those required by other expedited reporting forms (e.g.,
                          AdEERS, MedWatch) are submitted via Case Report Forms, using CTCAE
                          v3.0 and MedDRA 6.0, for this trial.

Add 6   10.312 Grade 5 AEs (Deaths)

Add 6   10.3121   Any death within 30 days after the patient's last study treatment,
                                       regardless of relationship to study treatment or procedure.

Add 6   10.3122   Any death more than 30 days after the patient's last study
                                       treatment or procedure that is felt to be at least possibly
                                       treatment related must also be submitted as a Grade 5 AE, with a
                                       CTCAE type and attribution assigned.

Add 6   10.32   Refer to the instructions in the Forms Packet (or electronic data entry screens, as
                   applicable) regarding the submission of late occurring AEs following completion of
                   the Active Monitoring Phase (i.e., compliance with Test Schedule in Section 4.0).

11.0   Treatment Evaluation

11.1   Patients will be evaluated as per Section 4.0.

11.2   At the time of reevaluation, patients will be classified in the following manner:

       11.21   No evidence of disease (NED).

Add
2,6   11.22   Recurrence of disease (REC). Recurrence must be confirmed by imaging and/or
               biopsy, with supporting materials submitted per Section 18.0. Elevated CEA levels
               only or physical findings only will not be accepted. As of Addendum 6, follow-up
               colonoscopies are to be done at one year and four years post resection. If a
               colonoscopy is felt to be clinically unsafe to perform or for other reasons can not be
               performed, then other forms of colon imaging such as a barium enema or CT
               colonography are acceptable. If recurrence occurs, the colonoscopy report
               documenting recurrence is to be submitted per Section 18.0.

Add 10,12   11.221 Secondary Resection. The date of the first resection and extent of resection,
                      post-recurrence (i.e. secondary resection for primary disease), will be
                      collected for Arms A and D only. Pathology, if available, and operative
                      reports are required to be submitted per Section 18.0. Optional recurrent
                      tissue sample is also requested per Section 17.0.

Add 5,9   **12.0   Descriptive Factors at the Time of Registration/Randomization**

12.1   Tumor Characteristics

12.11   Perforation: Yes vs. no.

12.12   Obstruction: Yes vs. no.

12.13   Adherence: Yes vs. no.

13.0   Treatment/Follow-up Decision at Evaluation of Patient

Add 9   13.1   Randomized patients (Arms A and D)

Add
2,13

13.11   If a patient does not receive treatment (and is classified as a cancel), it is necessary to provide follow-up information. The patient will go directly to the event-monitoring phase of the study per Section 18.0. On-study material is to be submitted, which includes tissue and blood specimens. The site must immediately contact NCCTG regarding the cancellation status of the patient.

Add
6,10

13.12   Criteria for Removal from Active Treatment to enter Observation Phase: The primary endpoint of this trial is 5-year disease-free survival. It is imperative that patients proceed to the observation phase of the study after completing active treatment, and be monitored for disease recurrence (per Section 4.0). If the patient discontinues active treatment for any reason (except disease recurrence), they will proceed to the observation phase of the study; otherwise, they will begin the Event Monitoring phase, per Section 13.13. Reasons for discontinuation include:

13.121   Unacceptable toxicity.

13.122   Patient may request to withdraw from the study treatment at any time for any reason.

Add 3,6

13.123   Intercurrent, noncancer-related illness that prevents continuation of therapy.

Add 2

13.124   All reasons for discontinuation of treatment must be documented in an off-study note.

Add
7.10

13.125   Recurrent disease. In this case patients proceed to Event Monitoring.

Add 2

13.13   Upon patient refusal to participate in the observation phase, recurrence of disease, or 5 years from date of study entry, the patient enters the event-monitoring phase until 8 years from date of study entry.

13.14   If a patient is deemed ineligible or in major treatment violation, further treatment is at the discretion of the treating physician. If the patient discontinues treatment,

Add
2.6.7.10

they are to begin the observation phase and then also complete event monitoring. If the patient continues treatment, they will proceed with the study schedule as planned (e.g., test, observation, and follow-up schedules), including submission of all required forms for those study periods. See Sections 4.0 and 18.0. All materials are required to be submitted (e.g., forms, tissue, bloods) regardless of eligibility status.

13.2   Patients Registered to Arm G

13.21   Patients (even if ineligible) will go directly to the event-monitoring phase of the study, which includes annual follow-up per Section 18.0. On-study material is to be submitted, which includes tissue and blood specimens. If patient is classified as a cancel or found to be ineligible, follow-up data must continue to be submitted.

13.22   If a patient does not receive treatment (and is classified as a cancel) it is necessary to provide follow-up information. The patient will go directly to the event-monitoring phase of the study. On-study material is to be submitted, which includes tissue and blood specimens. The site must immediately contact NCCTG regarding the cancellation status of the patient.

14.0 Translational/Pharmacologic Studies:

Add
3,4,6,10

14.1 Paraffin-embedded Blocks **or** Slides: (CTSU Participating Institutions, Including NCCTG)

Note: The blocks/slides used for translational research (Section 14.1) will be the same materials used for Section 17.0, Pathology Considerations for Quality Control. Please refer to Section 17.0 for submission of blocks/slides that will be used according to Section 14.1.

Add 4

14.11 The clinical investigator and the submitting pathologist have the responsibility for submitting representative materials for the goals cited in the protocol.

Add
4,6,10,11,12
Update 3

14.12 For study goals, pathologic assessment of submitted tissue will be done to ensure that all samples processed are representative of overall tumor and also to assess the value of current pathologic parameters in the setting of CRC (see Appendix XIV, Specific Aim 7). In addition, the following translational research areas will also be evaluated in original and recurrent surgical tissue resections: molecular determinants of response to inhibition of EGFR signaling (Specific Aim 1), genomic instability (Specific Aim 2), epigenetics and CpG island methylator phenotype (CIMP) (Specific Aim 3), candidate gene expression and pharmacogenetic analysis (Specific Aim 4), apoptotic susceptibility as determinant of treatment response and prognosis (Specific Aim 5),characterization of immune profile of sporadic colon cancers in relationship to DNA mismatch repair status (Specific Aim 6), and epidemiological factors and clinical outcome in colorectal cancer (Specific Aim 8). Dr. Thomas Smyrk and/or associates, Mayo Clinic Rochester, will score immunohistochemistry results.

Add
2,4,6,7,9,10,11

14.13 Immunohistochemistry (IHC): Numerous IHC (see Appendix XIV) will be used to correlate tumor immunohistochemical expression levels with the patient outcome.

Patients have the opportunity to receive some of their IHC results (i.e., MLH1, MSH2, and MSH6) if desired. IHC results will only be available to patients who complete randomization (to Arm A or D) or registration (to Arm G). Patients who wish to receive results must sign the letter in Appendix XV. The signed letter should then be forwarded directly to:
NCCTG, Attn: N0147
Quality Assurance Specialist
Cancer Center NW Clinic
200 First Street SW
Rochester MN 55905

A copy of the signed letter should also be given to the patient.

Results may take six months or longer to be made available to the treating physician. The NCCTG will communicate IHC results to the local treating physician. It is the responsibility of the local treating physician to share the IHC results with their patient. Documentation of when the patient requests and receives IHC test results is to be noted in the research record.

Add 13

If the patient has died, moved, or the patient's physician relocates, it is up to each treating institution to see that the LMD gets in contact with the patient or the patients family to relay the results. Patients are to contact the local treating physician with any future questions regarding their results. Thereafter, the treating physicians may direct questions to the NCCTG N0147 QAS listed on the protocol resource page.

Add 9,10

14.14   As of Addendum 9, KRAS wild-type status, verified by central review, is required for randomization to protocol treatment. **See Section 17.12 for tissue specimen submission and shipping information.**

IMPORTANT NOTE FOR PATIENTS ENROLLED IN THE STUDY PRIOR TO ADDENDUM 9:
See Section 17.11 for additional tissue specimen submission requirements and shipping information.

Add 6,9,12

14.2   Blood (Mandatory)

14.21   Patient Samples: This study will take advantage of the infrastructure in place for this trial to provide complementary pharmacogenetic and plasma/serum analyses in clinical samples. A 30 mL blood sample will be obtained from each patient following pre-randomization, but prior to registration/randomization. **As of Addendum 12**, an additional 30 mL blood sample will be obtained from each patient (Arm A or Arm D patients only) at their first Observation visit or at the next Observation visit for patients who are already in the Observation phase. From one pre-randomization EDTA tube, genomic DNA will be extracted using standard techniques, quantified, and stored at -80ºC prior to use. Remaining EDTA tubes (from both time points) will also yield plasma and white blood cells, which will be stored frozen at -80ºC. Red top tubes (from both time points) will yield serum, which will be extracted and stored frozen at -80ºC prior to use. Complete patient confidentiality will be maintained for the genomic DNA samples and plasma/serum analyses as only the anonymous laboratory identifier will be available for identification.

Add 11

14.22   Translational Research: See Appendix XIV for specific translational research details utilizing blood product specimens.

Add
4,6,7,8,9,10,11,12

14.23 Submission Logistics (see Appendix XIII)

A small, but sufficient supply of blood kits must be available at the site prior to patient enrollment. Kits will be supplied through Biospecimen Accessioning and Processing (BAP) Shared Resource, Rochester, Minnesota. To order blood kits, use the "Fax Supply Order Form" found in the Forms Packet, and fax as directed on the form. Fill out the site address to where the kits will be shipped on the Fax Supply Order Form. Kits will be sent via FedEx® Ground at no additional cost to the participating institutions. **Allow at least two weeks to receive the kits. Kits will not be sent via rush delivery service unless the participating institution provides their own FedEx® account number or alternate billing number for express service. NCCTG will not cover the cost for rush delivery of kits. Kits will arrive inside the shipping boxes. Do not send any unused kits back to BAP Receiving or the BAP Shared Resource.**

Instructions for blood collection and processing are described in the requisition form contained within the BAP provided kit and prepaid mailer.

Collect 30 mL of whole blood in three vacutainer tubes (draw one tube with no additive {red top} first, followed by two tubes containing EDTA anticoagulant {lavender top}. Prior to mailing, be certain that the specimen tubes are correctly labeled with patient initials, NCCTG patient ID number, protocol number, and date of collection. Include the patient's assigned NCCTG ID number on the Specimen Submission Form – Blood, and on the BAP Requisition Form contained in the blood collection kit. **The <u>Specimen Submission Form – Blood</u> is sent to NCCTG. Forward the <u>original BAP Requisition Form</u> along with the blood samples to BAP Receiving via the kits and prepaid mailers**. The BAP kits will include a smart shipper label (3x5 white barcoded label) affixed to the shipping boxes. The smart shipper label is a pre-addressed return label. Shipping costs will be covered by NCCTG if the shipping box is used for shipping specimens to BAP Receiving. All samples must be collected and shipped **Monday-Thursday ONLY.** Specimens must be shipped the same day as they are collected. Do **NOT** collect or ship blood specimens on Friday, on the weekend, or the day before or day of an observed United States national holiday. NOTE: Retain a copy of the completed BAP Requisition Form for your files.

Add 13

BAP Receiving will forward the samples to the BAP Shared Resource where they will be processed into DNA, and/or plasma and white blood cells, and serum within four (4) hours of receipt and stored in BAP at -80°C. The samples will be transferred to Dr. Sinicrope's laboratory upon request.

15.0   Drug Information

Add 6   15.1   5-Fluorouracil (5-FU) – Commercial Supply - Please refer to the package
                       insert for further information on 5-Fluorouracil.

                       15.11   Preparation and storage: Stable for prolonged periods of time at
                               room temperature if protected from light. Note manufacturer's
                               expiration date. Inspect for precipitate; if apparent, agitate vial
                               vigorously or gently heat to not greater than 140°F in a water bath.
                               Do not allow to freeze.

                       15.12   Known potential toxicities: Myelosuppression, alopecia, diarrhea,
Add 6   mucositis may be dose-limiting. Nausea, vomiting, anorexia,
                               cerebellar syndrome, dermatologic, and ophthalmic reactions also
                               occur. Cardiac ischemia, infarction, or acute cardiomyopathy are
                               possible.

                       15.13   Nursing guidelines:

                               15.131 Monitor complete blood count and platelet count.

                               15.132 Administer antiemetics as indicated.

                               15.133 Diarrhea may be dose-limiting; encourage fluids and treat
                                       symptomatically.

                               15.134 Assess for stomatitis; oral care measures as indicated.

                               15.135 Monitor for neurologic symptoms (headache, ataxia).

                               15.136 Inform patient of potential alopecia.

                               15.137 Those patients on continuous infusion may need instruction
                                       regarding central intravenous catheters and portable
                                       intravenous or IA infusion devices.

                               15.138 5-fluorouracil-induced conjunctivitis is a common problem.
                                       Advise patient to report any eye soreness or redness to the
                                       health care team.

Add 6   15.139 Photosensitivity may occur. Instruct patients to wear sun
                                       block when outdoors.

                       15.14   Drug procurement: Commercially available in 500 mg/10 mL
                               ampules and vials, and 1 gm/20 mL, 2.5 gm/50 mL, and 5 gm/100
                               mL vials.

Add 6

15.2   Leucovorin (CF) – Commercial Supply - Please refer to the package insert for further information on Leucovorin.

15.21   Preparation and storage: All dosage forms are stored at room temperature. At concentrations of 0.5-0.9 mg/mL the drug is chemically stable for at least 24 hours at room temperature under normal laboratory light. The 50 and 100 mg vials for injection are reconstituted with 5 and 10 mL of sterile water resulting in a 10 mg/mL solution. The 350 mg vial is reconstituted with 17 mL of sterile water resulting in a 20 mg/mL solution. Leucovorin (0.5-0.9 mg/mL) is chemically stable for at least 24 hours in normal saline, 5% dextrose, 10% dextrose, Ringer's injection, or lactated Ringer's injection. Leucovorin is also compatible with 5-fluorouracil.

15.22   Known potential toxicities: Nausea, diarrhea, thrombocytosis, rash, hives, pruritus, headache, and wheezing may occur.

15.23   Nursing guidelines:

15.231 Thrombocytosis is a common side effect. Monitor CBCs.

15.232 Observe for sensitization reaction (rash, hives, pruritus, wheezing).

15.233 May potentiate the toxic effects of fluoropyrimidines (5-fluorouracil) therapy, resulting in increased hematologic and gastrointestinal (diarrhea, stomatitis) adverse effects. Monitor closely.

15.24   Drug procurement: Commercially available as parenteral formulations (50, 100, and 350 mg vial).

15.3   **Oxaliplatin (Eloxatin®, OXAL)**

15.31   **Background**: Oxaliplatin, a platinum derivative, is an alkylating agent. Following intracellular hydrolysis, the platinum compound binds to DNA forming cross-links which inhibit DNA replication and transcription, resulting in cell death. Cytotoxicity is cell-cycle nonspecific.

15.32   **Formulation**: Commercially available for injection as:
Solution [preservative free]: 5 mg/mL (10 mL, 20 mL, 40 mL)

15.33   **Preparation, storage, and stability**: Refer to package insert for complete preparation and dispensing instructions. Store intact vials in original outer carton at room temperature and; do not freeze. According to the manufacturer, solutions diluted for infusion are stable up to 6 hours at room temperature or up to 24 hours under refrigeration. Oxaliplatin solution diluted with D_5W to a final concentration of 0.7 mg/mL (polyolefin container) has been shown to retain >90% of it's original concentration for up to 30 days when stored at room temperature or refrigerated; artificial light did not affect the concentration (Andre, 2007). As this study did not examine sterility, refrigeration would be preferred to limit microbial growth. Do not prepare using a chloride-containing solution (e.g., NaCl). Dilution with D_5W (250 or 500 mL) is required prior to administration. Infusion solutions do not require protection from light.

15.34   **Administration:** Refer to the treatment section for specific administration instructions. Administer as I.V. infusion over 2-6 hours. Flush infusion line with D5W prior to administration of any concomitant medication. Patients should receive an antiemetic premedication regimen. Cold temperature may exacerbate acute neuropathy. Avoid mucositis prophylaxis with ice chips during Oxaliplatin infusion.

15.35   **Pharmacokinetic information**:
Distribution: V_d: 440 L
Protein binding: >90% primarily albumin and gamma globulin (irreversible binding to platinum)
Metabolism: Nonenzymatic (rapid and extensive), forms active and inactive derivatives
Half-life elimination: Terminal: 391 hours; Distribution: Alpha phase: 0.4 hours, Beta phase: 16.8 hours
Excretion: Primarily urine (~54%); feces (~2%)

15.36   **Potential Drug Interactions**:
Increased Effect/Toxicity: Nephrotoxic agents may increase Oxaliplatin toxicity. When administered as sequential infusions, observational studies indicate a potential for increased toxicity when platinum derivatives (carboplatin, cisplatin, oxaliplatin) are administered before taxane derivatives (docetaxel, paclitaxel).
Decreased Effect: Oxaliplatin may decrease plasma levels of digoxin.

15.37   Comprehensive Adverse Events and Potential Risks List (CAEPR)
for Oxaliplatin (NSC 266046)

Comprehensive Adverse Events and Potential Risks List (CAEPR)
for
Oxaliplatin (NSC 266046)

The Comprehensive Adverse Event and Potential Risks list (CAEPR) provides a single list of reported and/or potential adverse events (AE) associated with an agent using a uniform presentation of events by body system. In addition to the comprehensive list, a subset, the Agent Specific Adverse Event List (ASAEL), appears in a separate column and is identified with **bold** and *italicized* text. This subset of AEs (ASAEL) contains events that are considered 'expected' for expedited reporting purposes only. Refer to the 'CTEP, NCI Guidelines: Adverse Event Reporting Requirements' http://ctep.info.nih.gov/protocolDevelopment/default.htm#adverse_events_adeers for further clarification. *Frequency is provided based on 1141 patients.* Below is the CAEPR for oxaliplatin.

Version 2.2, March 11, 2010[1]

Adverse Events with Possible Relationship to Oxaliplatin (CTCAE 4.0 Term) [n= 1141]			EXPECTED AEs FOR ADEERS REPORTING Agent Specific Adverse Event List (ASAEL)
Likely (>20%)	**Less Likely (<=20%)**	**Rare but Serious (<3%)**	***Expected***
BLOOD AND LYMPHATIC SYSTEM DISORDERS			
Anemia			***Anemia***
	Disseminated intravascular coagulation		***Disseminated intravascular coagulation***
	Febrile neutropenia		***Febrile neutropenia***
	Hemolysis		***Hemolysis***
		Thrombotic thrombocytopenic purpura	
CARDIAC DISORDERS			
	Atrial fibrillation		***Atrial fibrillation***
	Atrial flutter		***Atrial flutter***
	Paroxysmal atrial tachycardia		***Paroxysmal atrial tachycardia***
	Sinus bradycardia		***Sinus bradycardia***
	Sinus tachycardia		***Sinus tachycardia***
	Supraventricular tachycardia		***Supraventricular tachycardia***
	Ventricular arrhythmia		***Ventricular arrhythmia***
	Ventricular fibrillation		***Ventricular fibrillation***
	Ventricular tachycardia		***Ventricular tachycardia***
EAR AND LABYRINTH DISORDERS			
	Hearing impaired		***Hearing impaired***
	Middle ear inflammation		***Middle ear inflammation***
EYE DISORDERS			
	Conjunctivitis		***Conjunctivitis***
	Dry eye		***Dry eye***
	Eye disorders - Other (amaurosis fugax)		***Eye disorders - Other (amaurosis fugax)***

	Eye disorders - Other (cold-induced transient visual abnormalities)		*Eye disorders - Other (cold-induced transient visual abnormalities)*
	Eyelid function disorder		*Eyelid function disorder*
	Papilledema		*Papilledema*
GASTROINTESTINAL DISORDERS			
	Abdominal pain		*Abdominal pain*
	Ascites		*Ascites*
	Colitis		*Colitis*
	Constipation		*Constipation*
Diarrhea			*Diarrhea*
	Dry mouth		*Dry mouth*
	Dyspepsia		*Dyspepsia*
	Dysphagia		*Dysphagia*
	Enterocolitis		*Enterocolitis*
	Esophagitis		*Esophagitis*
	Flatulence		*Flatulence*
	Gastritis		*Gastritis*
		Gastrointestinal disorders – Other (pneumatosis intestinalis)	
	Gastrointestinal hemorrhage[2]		*Gastrointestinal hemorrhage[2]*
	Gastrointestinal necrosis[3]		*Gastrointestinal necrosis[3]*
	Gastrointestinal ulcer[4]		*Gastrointestinal ulcer[4]*
	Ileus		*Ileus*
	Mucositis oral		*Mucositis oral*
Nausea			*Nausea*
	Pancreatitis		*Pancreatitis*
	Small intestinal obstruction		*Small intestinal obstruction*
Vomiting			*Vomiting*
GENERAL DISORDERS AND ADMINISTRATION SITE CONDITIONS			
	Chills		*Chills*
	Edema face		*Edema face*
	Edema limbs		*Edema limbs*
Fatigue			*Fatigue*
	Fever		*Fever*
	Gait disturbance		*Gait disturbance*
	General disorders and administration site conditions - Other (Hepato-renal syndrome)		*General disorders and administration site conditions - Other (Hepato-renal syndrome)*
	Injection site reaction		*Injection site reaction*
	Non-cardiac chest pain		*Non-cardiac chest pain*
HEPATOBILIARY DISORDERS			
		Cholecystitis	
	Hepatic failure		*Hepatic failure*
	Hepatobiliary disorders - Other (hepatic enlargement)		*Hepatobiliary disorders - Other (hepatic enlargement)*
	Hepatobiliary disorders - Other (veno-occlusive liver disease)		*Hepatobiliary disorders - Other (veno-occlusive liver disease)*

IMMUNE SYSTEM DISORDERS			
	Allergic reaction		*Allergic reaction*
INFECTIONS AND INFESTATIONS			
	Infection[5]		*Infection[5]*
INVESTIGATIONS			
	Activated partial thromboplastin time prolonged		*Activated partial thromboplastin time prolonged*
Alanine aminotransferase increased			*Alanine aminotransferase increased*
	Alkaline phosphatase increased		*Alkaline phosphatase increased*
Aspartate aminotransferase increased			*Aspartate aminotransferase increased*
	Blood bilirubin increased		*Blood bilirubin increased*
	Creatinine increased		*Creatinine increased*
	GGT increased		*GGT increased*
	INR increased		*INR increased*
	Lymphocyte count decreased		*Lymphocyte count decreased*
	Neutrophil count decreased		*Neutrophil count decreased*
Platelet count decreased			*Platelet count decreased*
	Weight gain		*Weight gain*
	Weight loss		*Weight loss*
	White blood cell decreased		*White blood cell decreased*
METABOLISM AND NUTRITION DISORDERS			
	Acidosis		*Acidosis*
	Anorexia		*Anorexia*
	Dehydration		*Dehydration*
	Hyperglycemia		*Hyperglycemia*
	Hyperuricemia		*Hyperuricemia*
	Hypoalbuminemia		*Hypoalbuminemia*
	Hypocalcemia		*Hypocalcemia*
	Hypoglycemia		*Hypoglycemia*
	Hypokalemia		*Hypokalemia*
	Hypomagnesemia		*Hypomagnesemia*
	Hyponatremia		*Hyponatremia*
	Hypophosphatemia		*Hypophosphatemia*
MUSCULOSKELETAL AND CONNECTIVE TISSUE DISORDERS			
	Arthralgia		*Arthralgia*
	Back pain		*Back pain*
	Bone pain		*Bone pain*
	Myalgia		*Myalgia*
	Trismus		*Trismus*
NERVOUS SYSTEM DISORDERS			
	Ataxia		*Ataxia*
	Depressed level of consciousness		*Depressed level of consciousness*
	Dizziness		*Dizziness*
	Dysgeusia		*Dysgeusia*
	Dysphasia		*Dysphasia*

	Extrapyramidal disorder		*Extrapyramidal disorder*
	Headache		*Headache*
	Intracranial hemorrhage		*Intracranial hemorrhage*
	Ischemia cerebrovascular		*Ischemia cerebrovascular*
	Nerve disorder[6]		*Nerve disorder[6]*
	Nervous system disorders - Other (multiple cranial nerve palsies)		*Nervous system disorders - Other (multiple cranial nerve palsies)*
	Peripheral motor neuropathy		*Peripheral motor neuropathy*
Peripheral sensory neuropathy			*Peripheral sensory neuropathy*
	Seizure		*Seizure*
PSYCHIATRIC DISORDERS			
	Anxiety		*Anxiety*
	Confusion		*Confusion*
	Depression		*Depression*
	Insomnia		*Insomnia*
RENAL AND URINARY DISORDERS			
		Acute kidney injury	*Acute kidney injury*
	Hematuria		*Hematuria*
	Renal hemorrhage		*Renal hemorrhage*
	Urinary frequency		*Urinary frequency*
	Urinary retention		*Urinary retention*
REPRODUCTIVE SYSTEM AND BREAST DISORDERS			
	Hematosalpinx		*Hematosalpinx*
	Ovarian hemorrhage		*Ovarian hemorrhage*
	Prostatic hemorrhage		*Prostatic hemorrhage*
	Spermatic cord hemorrhage		*Spermatic cord hemorrhage*
	Testicular hemorrhage		*Testicular hemorrhage*
	Uterine hemorrhage		*Uterine hemorrhage*
	Vaginal hemorrhage		*Vaginal hemorrhage*
RESPIRATORY, THORACIC AND MEDIASTINAL DISORDERS			
		Adult respiratory distress syndrome	*Adult respiratory distress syndrome*
	Allergic rhinitis		*Allergic rhinitis*
	Bronchopulmonary hemorrhage		*Bronchopulmonary hemorrhage*
	Bronchospasm		*Bronchospasm*
	Cough		*Cough*
	Dyspnea		*Dyspnea*
	Hiccups		*Hiccups*
	Pneumonitis		*Pneumonitis*
	Pulmonary fibrosis		*Pulmonary fibrosis*
	Sinus disorder		*Sinus disorder*
	Voice alteration		*Voice alteration*
SKIN AND SUBCUTANEOUS TISSUE DISORDERS			
	Alopecia		*Alopecia*
	Dry skin		*Dry skin*
	Hyperhidrosis		*Hyperhidrosis*
		Palmar-plantar erythrodysesthesia syndrome	*Palmar-plantar erythrodysesthesia syndrome*

	Pruritus		*Pruritus*
	Rash maculo-papular		*Rash maculo-papular*
	Urticaria		*Urticaria*
VASCULAR DISORDERS			
	Flushing		*Flushing*
	Hot flashes		*Hot flashes*
	Hypertension		*Hypertension*
	Hypotension		*Hypotension*
	Phlebitis		*Phlebitis*
	Thromboembolic event		*Thromboembolic event*
	Vascular disorders - Other (hemorrhage with thrombocytopenia)		*Vascular disorders - Other (hemorrhage with thrombocytopenia)*

[1]This table will be updated as the toxicity profile of the agent is revised. Updates will be distributed to all Principal Investigators at the time of revision. The current version can be obtained by contacting PIO@CTEP.NCI.NIH.GOV. Your name, the name of the investigator, the protocol and the agent should be included in the e-mail.

[2]Gastrointestinal hemorrhage includes Anal hemorrhage, Cecal hemorrhage, Colonic hemorrhage, Duodenal hemorrhage, Esophageal hemorrhage, Esophageal varices hemorrhage, Gastric hemorrhage, Hemorrhoidal hemorrhage, Ileal hemorrhage, Intra-abdominal hemorrhage, Jejunal hemorrhage, Lower gastrointestinal hemorrhage, Oral hemorrhage, Pancreatic hemorrhage, Rectal hemorrhage, Retroperitoneal hemorrhage, and Upper gastrointestinal hemorrhage under the GASTROINTESTINAL DISORDERS SOC.

[3]Gastrointestinal necrosis includes Anal necrosis, Esophageal necrosis, Gastric necrosis, Pancreatic necrosis, Peritoneal necrosis, and Rectal necrosis under the GASTROINTESTINAL DISORDERS SOC.

[4]Gastrointestinal ulcer includes Anal ulcer, Colonic ulcer, Duodenal ulcer, Esophageal ulcer, Gastric ulcer, Ileal ulcer, Jejunal ulcer, Rectal ulcer, and Small intestine ulcer under the GASTROINTESTINAL DISORDERS SOC.

[5]Infection includes all 75 sites of infection under the INFECTIONS AND INFESTATIONS SOC.

[6]Nerve disorder includes Abducens nerve disorder, Accessory nerve disorder, Acoustic nerve disorder NOS, Facial nerve disorder, Glossopharyngeal nerve disorder, Hypoglossal nerve disorder, IVth nerve disorder, Oculomotor nerve disorder, Olfactory nerve disorder, Trigeminal nerve disorder, and Vagus nerve disorder under the NERVOUS SYSTEM DISORDERS SOC.

[7]Gastrointestinal perforation includes Colonic perforation, Duodenal perforation, Esophageal perforation, Gastric perforation, Ileal perforation, Jejunal perforation, Rectal perforation, and Small intestinal perforation under the GASTROINTESTINAL DISORDERS SOC.

Also reported on oxaliplatin trials but with the relationship to oxaliplatin still undetermined:

CARDIAC DISORDERS - Heart failure; Left ventricular systolic dysfunction; Myocardial infarction; Pericardial effusion
EYE DISORDERS - Eye pain

GASTROINTESTINAL DISORDERS – Gastrointestinal perforation[7]
INJURY, POISONING AND PROCEDURAL COMPLICATIONS - Injury to superior vena cava; Vascular access complication
INVESTIGATIONS - Cardiac troponin I increased; Lipase increased; Serum amylase increased
METABOLISM AND NUTRITION DISORDERS - Hypercalcemia; Tumor lysis syndrome
MUSCULOSKELETAL AND CONNECTIVE TISSUE DISORDERS - Generalized muscle weakness
NERVOUS SYSTEM DISORDERS - Syncope
RESPIRATORY, THORACIC AND MEDIASTINAL DISORDERS - Hypoxia
VASCULAR DISORDERS - Visceral arterial ischemia

Note: Oxaliplatin in combination with other agents could cause an exacerbation of any adverse event currently known to be caused by the other agent, or the combination may result in events never previously associated with either agent.

Add
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15.38   Nursing guidelines:

15.381 GI toxicity in the form of nausea and vomiting is similar in frequency and severity to that seen with cisplatin. This can be severe, but not always dose-limiting. Monitor for nausea and vomiting and treat accordingly.

15.382 Dose-limiting side effect can be paresthesias of hands, fingers, toes, pharynx, and occasionally cramps which develops with a dose-related frequency (>90 mg/m^2). Duration of symptoms tend to be brief (less than a week) with the first course, but longer with subsequent courses. Phase I patients have reported onset or an exacerbation of paresthesias by touching cold surfaces or exposure to cold. Advise patient of these possibilities and instruct patient to report these symptoms to the health care team.

15.383 These sensory neuropathies developed after subsequent courses with increasing intensity (grade 3 toxicity after fourth course) and with increasing duration. In 63% of the patients tested in phase I at high doses (135-200 mg/m^2), neuropathies became long-term with slow reversal over several months. Disabling walking and handwriting difficulties, as well as mouth and throat dysesthesias and laryngospasms were seen. Instruct patient to report any swallowing difficulties or gait changes.

15.384 OXAL is incompatible with NS. Flush lines with D5W prior to and following OXAL infusion.

15.385 Low back pain is a common side effect, perhaps a form of hypersensitivity reaction. Instruct patient in good body mechanics, advise light massage, heat, etc.

15.386 Laryngopharyngeal dysesthesia (LPD), occurs in about 15% of patients and is acute, sporadic, and self-limited. It usually occurs within hours of infusion, is induced or exacerbated by exposure to cold, and presents with dyspnea and dysphagia. The incidence and severity appear to be reduced by prolonging infusion time. Instruct patient to avoid ice and cold drinks the day of infusion. If ≥Grade 2 laryngopharyngeal dysesthesia occurs during the administration of OXAL, do the following:

- Stop OXAL infusion
- Administer benzodiazepine and give patient reassurance
- Test oxygen saturation via a pulse oximeter

Table 3 Comparison of the Symptoms and Treatment of Pharyngo-Laryngodysesthesias and Platinum Hypersensitivity Reactions		
Clinical Symptoms	Pharyngo-Laryngeal Dysesthesias	Platinum Hypersensitivity
dyspnea	present	present
bronchospasm	absent	present
laryngospasm	absent	present
anxiety	present	present
O$_2$ saturation	normal	decreased
difficulty swallowing	present (loss of sensation)	absent
pruritus	absent	present
urticaria/rash	absent	present
cold-induced symptoms	yes	no
BP	normal or increased	normal or decreased
Treatment	anxiolytics, observation in a controlled clinical setting until symptoms abate or at the physician's discretion	oxygen, steroids, epinephrine, bronchodilators; fluids and vasopressors, if appropriate

15.387 Alopecia is rare with oxaliplatin alone, but is seen with 5-fluorouracil-oxaliplatin combination. Advise patient.

15.388 Mild-moderate diarrhea has been seen -- usually of short duration. Treat accordingly. See Section 9.1 for ancillary treatment.

15.389a Respiratory problems (i.e., pulmonary fibrosis, cough, dyspnea, rales, pulmonary infiltrates, hypoxia, air hunger, and tachypnea) have been observed in patients administered oxaliplatin. In rare cases, death has occurred due to pulmonary fibrosis. Monitor lung sounds frequently for signs of pulmonary toxicity. Discuss with M.D. consideration of holding oxaliplatin until interstitial lung disease is ruled out in patients experiencing respiratory symptoms =Grade 3. Instruct patient to report any SOB, dyspnea, chest pain, rales, or wheezing to the health care team immediately. Report symptoms to the treating M.D. for possible assessment of oxygenation. This can be done via either finger oximetry or arterial blood gas evaluation to confirm the absence or presence of pulmonary infiltrates and/or hypoxia. Treatment choices include no intervention, steroids, diuretics, O_2, or assisted ventilation.

15.389b Acute vein irritation can occur with infusion. Apply heat to arm of infusion if you are using a peripheral line. However, extravasation of drug can cause severe pain, redness, soreness, and exfoliation of the skin in the affected area with loss of affected vein for a long period. If a patient has a problem with pain or sclerosis when chemotherapy is given peripherally, discuss with M.D. the possibility of a central line placement.

15.389c Hemolytic Uremic Syndrome (HUS) may result in kidney damage. Oxaliplatin is to be discontinued in cases where hematocrit is <25%, thrombocytopenia <100,000, and creatinine ≥1.6 mg/dL.

15.389d VOD (veno-occlusive disease) is a rare but serious complication that has been reported in patients receiving oxaliplatin in combination with 5-FU. This condition can lead to hepatomegaly, splenomegaly, portal hypertension and/or esophageal varices. Instruct patients to report any jaundice, ascites, or hematemesis to the MD immediately, as these could be a sign of VOD or other serious condition.

15.389e Patients may experience sleep disturbances, specifically insomnia. Encourage good sleep hygiene, and instruct patient to report any problems with sleep to the MD, to assess for the potential use of sleeping aids.

15.389f Cold-induced transient visual abnormalities can be experienced by patients while receiving OXAL, although the relationship to OXAL has not been completely determined. Instruct patient to report any problems with vision to the MD.

15.389g Extrapyramidal side effects and/or involuntary limb movement has been seen with OXAL administration. Patients may also experience restlessness. Instruct patient to report any of these side effects to the MD.

Add
3,5,6,10,11

15.39   Drug Procurement
        For patients pre-randomized prior to the implementation of Addendum 10:

        Oxaliplatin is an investigational agent supplied to investigators by the Division of
        Cancer Treatment and Diagnosis (DCTD), NCI. Oxaliplatin is provided to the NCI
        under a Cooperative Research and Development Agreement (CRADA) between
        Sanofi-Aventis and the NCI, DCTD. Return any leftover NCI-supplied oxaliplatin
        to NCI by using the NCI Return Investigational Agent Form located under
        "Requisition of Agents" at http://ctep.info.nih.gov/forms/.

        Drug Ordering: Once the patient's eligibility is established and the individual has
        been registered, a supply of oxaliplatin NSC 266046, may be requested by the
        Principal Investigator (or their authorized designees) at each participating institution.
        Pharmaceutical Management Branch (PMB) policy requires that drug be shipped
        directly to the institution where the patient is to be treated. PMB does not permit the
        transfer of agents between institutions (unless prior approval from PMB is obtained).
        Completed Clinical Drug Requests (NIH-986) should be submitted to the PMB by
        fax (301) 480-4612 or mailed to the Pharmaceutical Management Branch, CTEP,
        DCTD, NCI, 9000 Rockville Pike, EPN Rm. 707, Bethesda, MD 20892.

        Drug Accountability: The Investigator, or a responsible party designated by the
        investigator, must maintain a careful record of the inventory and disposition of all
        drugs received from DCTD using the NCI Drug Accountability Record Form. *(See
        the NCI Investigators Handbook for Procedures for Drug Accountability and Storage
        at http://ctep.info.nih.gov/handbook/handbook/default .htm)*

Add 10,11

        For patients pre-randomized following the implementation of Addendum 10:

        Commercial Supply

Add 2,6

15.4   Cetuximab – (IMC-C225) (Erbitux®) (NSC #714692). Please refer to the Full Prescribing
        Information (package insert) for further information on Cetuximab.

Add 8

        To obtain a copy of the investigator brochure for cetuximab, complete and submit the
        "CTSU Request for Clinical Brochure" form located on the CTSU N0147 web site.
        Follow faxing instructions on the form. After the request is processed you will receive
        an electronic copy of the investigator brochure.

        15.41   Formulation and storage:

                15.411 Description

                        Cetuximab, a chimerized antibody of the IgG$_1$ subclass, was originally derived
                        from a mouse myeloma cell line. Cetuximab was genetically engineered by
                        cloning the heavy and light chains of cetuximab and adapting them for
                        expression together with the constant regions of the human kappa light chain
                        and human gamma 1 heavy chain. The chimerization resulted in an antibody
                        with binding affinity to epidermal growth factor receptors (EGFR) greater

Add 5

than the natural ligand epidermal growth factor (EGF). Cetuximab blocks binding of EGF and transforming growth factor alpha (TGFα) to EGFR and inhibits ligand-induced activation of this tyrosine kinase receptor. Cetuximab also stimulates EGFR internalization, effectively removing the receptor from the cell surface for interaction with ligand.

15.412 Toxicology

Pre-clinical toxicology of this drug was performed. A dose-response was demonstrated after a single 15-minute intravenous infusion of cetuximab. Pharmacokinetic studies revealed a dose-response in sera after twice weekly infusions up to 28 days. No evidence of accumulation of antibody was observed. Acute and subacute toxicity studies of single and repeated doses revealed no evidence of treatment-related effects in body weight; food consumption; clinical pathology or gross necropsy data were observed.

A total of 606 subjects treated with cetuximab through February 2002 were tested for the presence of anti-cetuximab antibodies by analyzing pre-and post-treatment sera using a double antigen radiometric assay. The incidence of an anti-cetuximab immune response in these subjects was 4.1%. When it occurred, the anti-cetuximab response was generally found to be weak (upper limit of normal is 10 ng/ml cetuximab binding). The anti-cetuximab antibodies from two subjects with the highest reactivity (4670 and 6516 ng/ml) did not interfere with the ability of cetuximab to inhibit proliferation in a cetuximab sensitive cell line, suggesting that the antibodies in these sera were non-neutralizing. Levels of reactivity in sera from other subjects were not high enough to perform this type of analysis. In order to determine the specificity of the antibody response, sera from 15 subjects who had a positive anti-cetuximab response were further studied in the double antigen radiometric assay using unlabeled cetuximab as a competitor. This analysis demonstrated that sera from 14 of the 15 subjects contained cetuximab-specific antibodies.

Add 6

15.413 Pharmacology

Add 6

Pharmacokinetic modeling has been employed during cetuximab clinical studies with the objective of determining the dosing regimen(s) of cetuximab that maintains drug concentrations within a range associated with zero-order elimination (saturation of clearance). The analyses suggest that complete saturation of the mechanism(s) governing drug elimination occur in the range of 200 to 500 mg/m^2, while doses of cetuximab below 200 mg/rn^2 are not associated with saturation of clearance. Doses of ~ 400 mg/m^2 demonstrated zero-order elimination for at least 96 hours following drug infusion with increasing amounts of trough level drug accumulation. Since this drug accumulation is an expected result when operating under saturating conditions, the model was examined to identify cetuximab dose levels that maintained trough concentrations from initial dosing through all subsequent dosing at or just above Km. The dosing regimen ultimately selected to satisfy these requirements was one that utilized a loading dose of 400 mg/m^2 followed by subsequent maintenance doses of 250 mg/m^2.

Formulation: Cetuximab is an anti-EGFR receptor humane-to-murine chimeric antibody. Cetuximab is expressed in SP2/0 myeloma cell line, grown in large scale cell culture bioreactors and purified to a high level purity using several purification steps including protein A chromatography, ion exchange chromatography, low pH treatment and nonofiltration. Cetuximab is not known to be a vesicant.

How Supplied: Cetuximab will be supplied by DCTD, NCI. The product is formulated to 2 mg protein/mL with phosphate buffered saline, pH 7.2 :t 0.2 and aseptically filled into sterile glass vials, 100 mg per 50 cc vial, and stored as a liquid at 2 to 8 *ac*. Each vial contains the following active and inactive ingredients per 1.0 ml: 2 mg of cetuximab, 145 nmol/L sodium chloride, and 10 mmol/L sodium phosphate.

Add 5

Drug Procurement: Cetuximab (NSC #714692) may be requested by the Principal Investigator (or their authorized designee) at each participating institution. Pharmaceutical Management Branch (PMB) policy requires that agent be shipped directly to the institution where the patient is to be treated. PMB does not permit the transfer of agents between institutions (unless prior approval from PMB is obtained). Completed Clinical Drug Request (form NIH-986) should be submitted to the Pharmaceutical Management Branch by fax (301/480-4612) or mailed to the Pharmaceutical Management Branch, CTEP, DCTD, NCI, 9000 Rockville Pike, EPN Room 7149, Bethesda, MD 20892-7422.

Drug Inventory Records: The investigator, or a responsible party designated by the investigator, must maintain a careful record of the inventory and disposition of all agents received from DCTD using the NCI Drug Accountability Record. (See the NCI Investigator's Handbook for Procedures for Drug Accountability and Storage.)

Packaging and Labeling: Cetuximab for injection will be supplied by DCTD, NCI in single-use, ready-to-use 50-mL vials containing 2 mg/mL of product.

Add 5

Handling and Dispensing of Investigational Product: Investigational product should be stored in a secure area according to local regulations. It is the responsibility of the investigator to ensure that investigational product is only dispensed to study subjects. The investigational product must be dispensed only from official study sites by authorized personnel according to local regulations.

Storage Requirement/Stability: Cetuximab must be stored under refrigeration at +2°C to +8°C (+36°F to +46°F). **DO NOT FREEZE CETUXIMAB.** Drug supplies must be kept in a secure, limited access storage area under the recommended storage conditions. Once cetuximab is removed from the vial, the recommended maximum time at room temperature is 8 hours.

Preparation and Administration: Cetuximab will be prepared by ImClone

under appropriate manufacturing conditions as an injectable solution, in single-use, ready-to-use 50-mL vials containing 2 mg/mL of product. Do not shake. Cetuximab requires no dilution. The calculated doses should be placed in a sterile, empty IV bag or glass bottle and administered undiluted through a line with a 0.2 ụm in-line filter within 8 hours of preparation. Patients will receive weekly cetuximab intravenous infusions via either gravity drip, infusion pump, or syringe pump with in-line filtration. Cetuximab should not be mixed with or diluted with other drugs or solutions for infusion such as 5%-glucose. The initial/loading dose is cetuximab 400 mg/m^2 and should be administered over 120 minutes. This initial loading dose will be followed by weekly maintenance infusions IV over 60 minutes. The dose and volume of the study drug to be infused are dependent upon the patient's BSA. The infusion rate must never exceed 10 mg/minute (5 mL/minute).

Cetuximab may be administered via a gravity drip, infusion pump or syringe pump with in-line filtration. Cetuximab requires in-line filtration during administration. The 0.22 11m in-line filters in both the recommended Baxter Healthcare and Abbott Laboratories infusion sets have identical in-line filters composed of polyethersulfone. Calculate and draw the appropriate volume of cetuximab into a sterile syringe based on either the 400 mg/m^2 initial dose or 250 mg/m^2 weekly dose and administer via one of the options detailed below:

1.   In-line Filtration by Infusion Pump or Gravity Drip

        Take an appropriate sterile syringe (min 50 mL), attach a suitable needle, and draw up the required volume of cetuximab solution from a vial. Add the cetuximab into a sterile evacuated container or bag (glass administration containers are not recommended). Repeat this procedure until the calculated volume has been added to the container. Next affix the infusion line with an in-line filter (the cetuximab solution must be filtered with a suitable in-line filter of 0.211m nominal pore size) and prime it with cetuximab before starting the infusion. Use a gravity drip or an infusion pump for administration. Set and control the rate as noted above.

2.   In-line Filtration by Syringe Pump

        Take an appropriate sterile syringe (min 50 mL), attach a suitable needle, and draw up the required volume of cetuximab solution from a vial. Remove the needle and put the syringe into the syringe pump. Take a suitable in-line filter of 0.211m nominal pore size and connect it to the infusion line (Note: one filter per dose should be sufficient but further filers can be used if a filter becomes blocked). Connect the infusion line to the syringe, set and control the rate as described above and start the infusion after priming the line with cetuximab. Repeat this procedure until the calculated volume has been infused.

Studies have been conducted to demonstrate the compatibility of

cetuximab drug product with various infusion systems. Some examples of materials, IV containers, infusion sets and filters tested and recommended for use with cetuximab are listed below. For further examples of approved materials please see Section 3.4.1 in the Investigator Brochure.

Recommended I.V. Containers:

- Evacuated IV Bottle, Model No. 1A8502 (Baxter Healthcare Corporation)
- IntraViaTM IV Bag with PVC Ports, Model No. 2J8002 (Baxter Healthcare Corporation)
- EVATM IV Bag, Model No. 2B8152 (Baxter Healthcare Corporation)
- LifeCareTM IV Bag, Model No. 7951-12 (Abbott Laboratories)

Recommended Infusion Sets:

- Vented Continu-Flo Solution Set™, Model No. 2C541s (Baxter Healthcare Corporation) to be used with an in-line filter set, Model No. 2679 (Abbott Laboratories)
- Vented Paclitaxel Set™ with 0.22-µm downstream high pressure in-line filter, Model No, 2C7553 (Baxter Healthcare Corporation)

Recommended Filters:

- Vented Continu-Flo Solution SetTM, Model No. 2C6541s (Baxter Healthcare Corporation) to be used with an in-line filter set, Model No. 2679 (Abbott Laboratories)
- Intrapur Plus (B. Braun AG) reference number 409 9800
- Poly-lined filtered Extension set (Alaris Medical Systems) reference number C20350

Add 4

Normal saline should be used to clear the infusion set of residual cetuximab. The delivered drug product is > 95% for all recommended infusion sets when flushed with 50 mL of normal saline. Use a separate line of cetuximab infusion.

Safety Precautions: Appropriate mask, protective clothing, eye protection, gloves and Class II vertical-laminar-airflow safety cabinets are recommended during preparation and handling. Opened vials must be disposed of at the investigational center as chemotherapy or biohazardous waste provided documented procedures for destruction are in place. Otherwise, opened vials must be returned to BMS for disposal. For questions regarding cetuximab destruction please contact BMS at 800/743-9224.

Administration of Cetuximab: In an effort to prevent a hypersensitivity reaction, all patients should be premedicated with diphenhydramine hydrochloride 50 mg (or an equivalent

antihistamine) either by IV given 30 - 60 minutes prior to the first dose of cetuximab, or orally given 60 - 120 minutes prior to the first dose of cetuximab. Premedication will be administered prior to subsequent doses, but at the investigator's discretion, the dose of diphenhydramine (or similar agent) may be reduced.

The initial/loading dose of cetuximab is 400 mg/m^2 intravenously (IV) administered over 120 minutes. Patients must be continuously observed during the infusion for signs of anaphylaxis. Vital signs should be checked and recorded prior to the administration of cetuximab, midway through the infusion, at the completion of the infusion, and 1 hour post the infusion.

Following the loading dose, patients will receive weekly treatment with cetuximab IV over 60 minutes. The infusion rate of cetuximab should never exceed 10 mg/minute (5 ml/min or 300 ml/hr). The dose may subsequently be reduced for individual patients, depending on a patient's toxicity. For the weekly doses, vital signs (blood pressure, heart rate, respiratory rate, and temperature) should be checked and recorded prior to the administration of cetuximab, midway through the infusion, at the completion of the infusion, and 1 hour post the infusion. Patients should be closely monitored for cetuximab-related adverse events, especially hypersensitivity reactions, during the infusion and the post-infusion observation hour. For the duration that patients are on cetuximab therapy, adverse event monitoring should be done continuously. Patients will be evaluated for adverse events at each visit and are to be instructed to call their physician to report any adverse events between visits.

In the event that a patient experiences a Grade 2 hypersensitivity reaction, the cetuximab infusion should be stopped, treatment for the hypersensitivity should be administered as necessary, and the patient observed until the reaction resolves or decreases to Grade 1. The infusion should then be resumed at half the original rate of infusion.

In the event of a Grade 1 hypersensitivity reaction, the cetuximab infusion should be decreased to half the original rate of infusion. If the infusion rate is decreased for hypersensitivity reaction, it will remain decreased for all subsequent infusions. If this is not effective at limiting the reaction, removal from the study should be considered.

If a patient experiences a Grade 3 or 4 hypersensitivity reaction, discontinue cetuximab treatment.

CAUTION: Hypersensitivity reactions may occur during or following cetuximab administration. Most hypersensitivity reactions occur with the first infusion of cetuximab, but some patients' first hypersensitivity reactions have been reported following subsequent doses (as far out as the 8th dose). The hypersensitivity reaction may occur during the

infusion, or be delayed until any time after the infusion. As a routine precaution, patients enrolled in this study will be observed closely for any potential adverse events by the medical staff from the start of the cetuximab infusion until at least 1 hour after the end of the infusion in an area with resuscitation equipment and other agents (epinephrine, prednisone equivalents, etc.) available. A nurse must be present in the immediate treatment area throughout the infusion and observation period. A physician must be in close proximity to the patient treatment area. Should a hypersensitivity or infusion reaction to cetuximab occur, the patient must be treated according to the best available medical practices. Patients should be instructed to report any delayed reactions to the investigator immediately.

Add 6,12

15.414 Comprehensive Adverse Events and Potential Risks List (CAEPR) for Cetuximab (NSC #714692)

Comprehensive Adverse Events and Potential Risks list (CAEPR)
for
Cetuximab (NSC 714692)

The Comprehensive Adverse Event and Potential Risks list (CAEPR) provides a single list of reported and/or potential adverse events (AE) associated with an agent using a uniform presentation of events by body system. In addition to the comprehensive list, a subset, the Agent Specific Adverse Event List (ASAEL), appears in a separate column and is identified with **bold** and *italicized* text. This subset of AEs (ASAEL) contains events that are considered 'expected' for expedited reporting purposes only. Refer to the 'CTEP, NCI Guidelines: Adverse Event Reporting Requirements' http://ctep.info.nih.gov/protocolDevelopment/default.htm#adverse_events_adeers for further clarification. *Frequency is provided based on 2282 patients.* Below is the CAEPR for Cetuximab.

Version 2.1, March 31, 2010[1]

Adverse Events with Possible Relationship to Cetuximab (CTCAE 4.0 Term) [n= 2282]			EXPECTED AEs FOR ADEERS REPORTING Agent Specific Adverse Event List (ASAEL)
Likely (>20%)	**Less Likely (<=20%)**	**Rare but Serious (<3%)**	*Expected*
BLOOD AND LYMPHATIC SYSTEM DISORDERS			
	Anemia		
EAR AND LABYRINTH DISORDERS			
	External ear inflammation		
	Tinnitus		

EYE DISORDERS			
	Conjunctivitis		*Conjunctivitis*
	Dry eye		*Dry eye*
	Uveitis		*Uveitis*
	Watering eyes		*Watering eyes*
GASTROINTESTINAL DISORDERS			
	Abdominal pain		*Abdominal pain*
	Cheilitis		*Cheilitis*
	Constipation		*Constipation*
Diarrhea			*Diarrhea*
	Dry mouth		*Dry mouth*
	Dyspepsia		*Dyspepsia*
	Mucositis oral		*Mucositis oral*
Nausea			*Nausea*
	Vomiting		*Vomiting*
GENERAL DISORDERS AND ADMINISTRATION SITE CONDITIONS			
	Chills		*Chills*
	Edema limbs		
Fatigue			*Fatigue*
Fever			*Fever*
	Flu like symptoms		*Flu like symptoms*
	Infusion related reaction		*Infusion related reaction*
	Non-cardiac chest pain		
IMMUNE SYSTEM DISORDERS			
	Allergic reaction		*Allergic reaction*
		Anaphylaxis	
INFECTIONS AND INFESTATIONS			
	Infection[2]		
		Infections and infestations – Other (aseptic meningitis)	
INVESTIGATIONS			
	Neutrophil count decreased		
	Weight loss		*Weight loss*
	White blood cell decreased		*White blood cell decreased*
METABOLISM AND NUTRITION DISORDERS			
	Anorexia		*Anorexia*
	Dehydration		*Dehydration*
	Hypocalcemia		
	Hypomagnesemia		*Hypomagnesemia*
MUSCULOSKELETAL AND CONNECTIVE TISSUE DISORDERS			
	Arthralgia		*Arthralgia*
	Back pain		*Back pain*
	Myalgia		*Myalgia*
NERVOUS SYSTEM DISORDERS			
Headache			*Headache*
	Syncope		
RESPIRATORY, THORACIC AND MEDIASTINAL DISORDERS			
	Allergic rhinitis		*Allergic rhinitis*
	Bronchospasm		

	Cough		*Cough*
	Dyspnea		*Dyspnea*
	Hoarseness		*Hoarseness*
		Pneumonitis	
		Respiratory, thoracic, and mediastinal disorders - Other (non-cardiogenic pulmonary edema)	
SKIN AND SUBCUTANEOUS TISSUE DISORDERS			
	Alopecia		*Alopecia*
Dry skin			*Dry skin*
	Nail loss		*Nail loss*
		Palmar-plantar erythrodysesthesia syndrome	
	Photosensitivity		*Photosensitivity*
	Pruritus		*Pruritus*
	Purpura		
Rash acneiform			*Rash acneiform*
Rash maculo-papular			*Rash maculo-papular*
	Skin ulceration		
	Urticaria		*Urticaria*
VASCULAR DISORDERS			
	Hypotension		*Hypotension*
	Thromboembolic event		*Thromboembolic event*

[1]This table will be updated as the toxicity profile of the agent is revised. Updates will be distributed to all Principal Investigators at the time of revision. The current version can be obtained by contacting PIO@CTEP.NCI.NIH.GOV. Your name, the name of the investigator, the protocol and the agent should be included in the e-mail.

[2]Infection could include all 75 sites of infections under the INFECTIONS AND INFESTATIONS SOC. **Also reported on cetuximab trials but with the relationship to cetuximab still undetermined:**

BLOOD AND LYMPHATIC SYSTEM DISORDERS - Disseminated intravascular coagulation; Hemolysis
CARDIAC DISORDERS - Atrial fibrillation; Atrial flutter; Chest pain - cardiac; Left ventricular systolic dysfunction; Myocardial infarction; Paroxysmal atrial tachycardia; Pericardial effusion; Sinus bradycardia; Sinus tachycardia; Supraventricular tachycardia
EAR AND LABYRINTH DISORDERS - Hearing impaired
EYE DISORDERS - Blurred vision; Extraocular muscle paresis; Eyelid function disorder; Keratitis; Photophobia; Vitreous hemorrhage
GASTROINTESTINAL DISORDERS - Colitis; Dysphagia; Esophagitis; Gastritis; Gastrointestinal disorders - Other (diverticulitis); Gastrointestinal hemorrhage (including Colonic or Gastric hemorrhage or hemorrhage in other sites under the GASTROINTESTINAL DISORDERS SOC); Gastrointestinal perforation (Colonic perforation, Duodenal perforation, or perforation in other sites under the GASTROINTESTINAL DISORDERS SOC); Gastrointestinal ulcer (ulcer includes Duodenal ulcer, Rectal ulcer, or ulcer in other sites under the GASTROINTESTINAL DISORDERS SOC); Ileus; Pancreatitis; Rectal fistula
GENERAL DISORDERS AND ADMINISTRATION SITE CONDITIONS - Edema face; Sudden death NOS
HEPATOBILIARY DISORDERS - Cholecystitis; Hepatic failure

INJURY, POISONING AND PROCEDURAL COMPLICATIONS - Bruising; Wound dehiscence

INVESTIGATIONS - Alanine aminotransferase increased; Alkaline phosphatase increased; Aspartate aminotransferase increased; Blood bilirubin increased; Creatinine increased; Platelet count decreased; Serum amylase increased

METABOLISM AND NUTRITION DISORDERS - Hyperkalemia; Hyperuricemia; Hypokalemia; Hyponatremia; Hypophosphatemia

MUSCULOSKELETAL AND CONNECTIVE TISSUE DISORDERS - Generalized muscle weakness; Musculoskeletal and connective tissue disorder - Other (myasthenia); Musculoskeletal and connective tissue disorder - Other (Sudeck's Atrophy)

NERVOUS SYSTEM DISORDERS - Ataxia; Dizziness; Dysgeusia; Extrapyramidal disorder; Intracranial hemorrhage; Nervous system disorders - Other (cholinergic syndrome); Neuralgia; Peripheral motor neuropathy; Peripheral sensory neuropathy; Seizure; Tremor

PSYCHIATRIC DISORDERS - Agitation; Depression

RENAL AND URINARY DISORDERS - Hematuria; Renal and urinary disorders - Other (acute renal failure)

REPRODUCTIVE SYSTEM AND BREAST DISORDERS - Reproductive system and breast disorders - Other (balanitis); Vaginal inflammation

RESPIRATORY, THORACIC AND MEDIASTINAL DISORDERS - Adult respiratory distress syndrome; Atelectasis; Bronchopulmonary hemorrhage; Epistaxis; Pleural effusion; Respiratory, thoracic and mediastinal disorders - Other (bronchiolitis obliterans-organized pneumonia [BOOP])

SKIN AND SUBCUTANEOUS TISSUE DISORDERS - Hirsutism; Skin hypopigmentation; Skin and subcutaneous tissue disorders - Other (skin fissures)

VASCULAR DISORDERS - Flushing; Hypertension; Lymphedema; Vasculitis

Note: Cetuximab in combination with other agents could cause an exacerbation of any adverse event currently known to be caused by the other agent, or the combination may result in events never previously associated with either agent.

Add 4,5,6   15.42   Nursing guidelines:

15.421   Patients should be closely monitored during the infusion for signs of anaphylaxis and standard resuscitative medications should be available during and for one hour following the C225 infusion.

CAUTION: Infusion reactions may occur during or following cetuximab administration. Most infusion reactions occur with the first infusion of cetuximab, but some patients' first infusion reactions have been reported following subsequent doses (as far out as the 8th dose). The infusion reaction may occur during the infusion, or be delayed until anytime after the infusion. A nurse should be present in the immediate treatment area throughout the infusion and observation period. A physician should be in close proximity to the patient treatment area. Should an infusion reaction occur, the patient should be treated according to institutional guidelines. Patient should be instructed to report any delayed reactions to the investigator immediately.

15.422   Vital signs should be taken prior to, during, post and 1 hour post infusion.

15.423   Patient should be observed for 1 hour following the loading dose and each maintenance dose.

15.424   Premedicate with 50 mg of IV diphenhydramine, or other specific premedications called for in the protocol, prior to each dose.

15.425   Patients should be taught to wear sunscreen and hats and limit sun exposure while receiving treatment.

15.426   Recommend that all infusions be run on a volumetric pump. Infusion rate **MUST NEVER EXCEED 10MG/MINUTE (5 ML/MINUTE)**.

15.427   Monitor CBC and instruct patient to report any signs or symptoms of infection, unusual bruising, or bleeding to the health care team.

15.428   Monitor LFTs.

15.429a   Fever and chills may occur. Discuss with MD about premedication with an antipyretic.

15.429b   Monitor for signs and symptoms of gastrointestinal distress. Administer antiemetics and antidiarrheals as indicated and evaluate their effectiveness.

15.429c   Instruct patient to report rash.

15.429d   Hypomagnesemia is a complication of C225 therapy. Instruct patients to report any of the following signs or symptoms as these may be signs of the disorder. Neuromuscular: muscle weakness, muscle cramps, painful swallowing; CNS: Irritability, combativeness, disorientation, psychosis, vertigo, seizures; Cardiac: irregular and/or fast heartbeat. Any or all of the symptoms may or may not be present in the patient with this condition. If patients present with any of these symptoms, inform MD and a magnesium level should be checked.

15.429e   Rare reports of cardiac toxicities (including MI, chest pain, troponin elevations, and/or cardiomyopathy) have been reported in trials containing 5-FU in combination with C225. The relationship between these events and these drugs is still not determined as of yet. Instruct patient to report any chest pain or shortness of breath to their care provider immediately and seek immediate medical evaluation.

16.0   Statistical Considerations and Methodology

Add
3,4,5,6,7,
9,10
Update 3

16.1   Design: As of June 1, 2005, this study is a two-arm randomized phase III trial comparing the outcomes of patients with stage III colon cancer treated with or without the addition of C225 to a chemotherapy regimen of Oxaliplatin + 5-FU + Leucovorin (FOLFOX). Patients will be randomized equally to either add or not add C225 to the FOLFOX regimen. The primary endpoint of this study is disease-free survival. All analyses will be based on the intention to treat principle, and will include all randomized patients. The total sample size for the trial is 2300 patients to be enrolled to FOLFOX +/- C225. Data from patients enrolled to FOLFOX +/- C225 prior to the removal of the 4 arms (FOLFIRI +/- C225, and FOLFOX/FOLFIRI +/- C225) will be used in the primary efficacy and toxicity comparisons of FOLFOX +/- C225; data from patients enrolled prior to June 1, 2005 to FOLFIRI +/- C225 will not be used in the primary efficacy comparison but will be reported for adverse events. Patients randomized to FOLFOX/FOLFIRI +/- C225 prior to June 1, 2005 who crossed over to FOLFOX prior to the initiation of the FOLFIRI component of their therapy will be included in the primary efficacy comparison; those who do not cross over or who discontinue therapy will be included in safety analyses only. Three hundred forty eight (348) patients were enrolled to arms containing irinotecan and received irinotecan prior to a crossover to FOLFOX +/-C225, thus are unusable for the primary efficacy analysis. Thus, the total planned sample size for the trial is 2,648 patients (2300 + 348).

Revised design as of Addendum 9: Based on the emerging data (Section 1.7) regarding the ability of KRAS mutation to predict a lack of efficacy of EGFR inhibitors (including cetuximab), the protocol has been redesigned to provide sufficient power to detect a significant benefit of the addition of C225 to FOLFOX in the subgroup of patients with KRAS wild-type tumors. In addition, the sample size has been increased A) to account for the expected improved performance of patients treated with FOLFOX alone, from 68% 3-year DFS to 70% 3-year DFS, based on the 72% 3-year DFS observed in MOSAIC stage III patients treated with FOLFOX (reference #107), and the 69% 3-year DFS observed in the FLOX arm of C-07 (reference #108), and B) to accommodate the fact that accrual will actually take approximately 4 years, as opposed to 3, and that this accrual was uneven, averaging 10/month in the first year, 20/month in the 2nd year, 80/month in the 3rd year, and assuming a continued accrual of 80/month in the 4th year.

Based on these 3 factors, the new total sample size for the trial is 2070 KRAS wild-type (WT) patients to be enrolled to FOLFOX +/- C225. The three hundred forty eight (348) patients who were enrolled to arms containing irinotecan and received irinotecan prior to a crossover to FOLFOX +/-C225 remain unusable for the primary efficacy analysis. As of the end of May 2008, N0147 had accrued approximately 2100 usable patients. Based on the assumed 60% prevalence of KRAS WT, the trial will have accrued 1260 KRAS WT patients by the end of May 2008. Thus a total of 810 KRAS WT patients remain to be accrued, as of the end of May 2008. In addition, the estimated 540 KRAS mutant (and KRAS not evaluable) patients who are screened for randomization will be followed, but not treated, on protocol. Thus, the total planned sample size for the primary randomized comparison for this trial is 2070 KRAS WT patients, while in total the trial will enroll approximately 3768

patients (2070 KRAS WT patients, 810 KRAS mutant or not evaluable patients enrolled prior to addendum 9, 540 KRAS mutant or not evaluable patients entered post addendum 9 followed but not treated on protocol, and 348 patients treated with irinotecan who will not be considered in the efficacy analyses).

16.11   Disease-Free Survival: Disease-Free Survival (DFS) is defined as the time from randomization to documentation of disease recurrence or death, whichever occurs first. Patients that do not have a DFS event will be censored for DFS as their last disease assessment date.

16.12   Prior to the implementation of Addendum 5, this trial consisted of two randomizations: one between three chemotherapy arms, and one between C225 or no C225. As indicated in the previous (June 1, 2005) version of the protocol, if one of the two randomizations were to be closed (whether due to efficacy, toxicity or other considerations), then the trial would continue accrual and randomization to the other randomization during the time that an amendment (Addendum 5) was being prepared and processed to modify the protocol. Continuing accrual until implementation of the amendment is appropriate because the two clinical questions being tested through these randomizations are independent of each other, and because the study design remains valid if one of the randomizations is stopped. Per protocol, this protocol has remained open to accrual as the addendum reflecting a two-arm rather than a six-arm study design (Addendum 5) has been prepared and approved.

Sites were instructed to use the following procedures for consenting patients from June 1, 2005 until the new protocol and consent form for Addendum 5 became available. Patients would sign the existing consent form, and may be enrolled provided that the enrolling physician documented in the medical record that: (1) the randomization to one of the randomization factors has been closed, but the other randomization remains open per protocol; (2) since the randomizations and study questions are independent as stated in the protocol, the patient can be enrolled on the study as the official amendment to change the study is being processed; and (3) this has been explained to the patient and he/she understands the changes in the study and agrees to enter the study. After implementation of Addendum 5, patients are to be re-consented to complete the record.

16.2   Accrual: Accrual to this trial should be rapid. The previous intergroup study in this patient population (CALGB C89803) accrued approximately 1250 patients in two years. Prior to its closing, it was accruing over 100 patients a month. With this accrual rate we plan to accrue the required 3768 patients for the trial in 4 years, which includes the time period starting at the implementation of Addendum 2 (the addendum that added C225 to the trial). Per Addendum 9: for the last 12 months, accrual has consistently been between 80-100 patients per month.

Add 5,7,9

16.3 Analysis plans and power: The best historical data available on the outcome of patients with resected stage III colon cancer comes from Intergroup Study 0089, where the 3-year, disease-free survival in stage III patients was 64%. For the purposes of planning this study, we will assume that FOLFOX will provide at least some incremental benefit over this 64% 3-year disease-free survival. Therefore, as the basis for the sample size calculations for the current trial, we assume a 3-year disease-free survival of 68% in the arm with the poorest outcome. We note that this rate is intermediate between the 64% observed in INT 0089, and the 72% rate recently observed for FOLFOX in the MOSAIC trial in stage III patients.

Add 3,5,6

Revised per Addendum 9: Based on the reported 69% 3-year DFS from the NSABP C-07 trial (reference #108), we have increased our expected 3-year DFS rate on FOLFOX to 70%. We additionally assume an accrual period of 4 years with uneven accrual (10/month in 1st year, 20/month in 2nd year, and 80/month in years 3 and 4), a minimum follow-up on all patients of 2 years, and a model for disease-free survival with annual failure rates based on data from INT 0089 and NCCTG 914653 as follows: year 1 12%, year 2 12%, year 3 6%, year 4 5%, year 5 3%, and year 6 2%. The significance values to be used at each analysis (interim and final), are outlined in Section 16.32.

Add 9

The primary analysis for this trial will be a comparison of DFS in the patients who are KRAS wild-type (WT), randomized to FOLFOX +/- C225. A key assumption is the proportion of tumors that will be KRAS WT. No data is available on the prevalence of KRAS WT in the adjuvant setting, however in multiple large series reported in advanced disease the rates have ranged from 57% to 65%. For our sample size calculations we have assumed a rate of 60% KRAS WT. The testing of FOLFOX +/- C225 in the overall population (Section 16.41) will only occur if the initial analysis in KRAS WT patients is significant at two-sided level 0.05.

Add 9

16.31 For the comparison of the DFS in patients with KRAS WT treated with or without C225, a log-rank test will be conducted at two-sided level 0.05. A sample size of 1035 KRAS WT patients per arm, 2070 total, will result in 515 total events, providing 90% power to detect a hazard ratio of 1.33 for this comparison.

Add 5,9

16.32 Alpha levels for interim and final analyses: Three interim analyses will be performed at the time at which 25%, 50% and 75% of the projected total number of events have occurred using a O'Brien-Fleming type stopping boundary (96), truncated at +/-3.5. Specifically, the cut-off values for the log-rank statistics at the four analysis times (three interim and final) are +/-3.5, +/-2.996, +/-2.361, and +/-2.015. It is possible that at the time of these scheduled interim analyses, KRAS status will not be available. If so, then the time points for the interim analyses, and these cut-off values, will be used to apply to entire study population, for consideration of early study termination. Specifically, until KRAS data is available, the timing for interim analysis will be based on 25%, 50%, and 75% of the total of 735 events planned for the DFS comparison in the joint cohort of all non irinotecan-treated patients entered prior to addendum 9, and the KRAS WT patients entered post Addendum 9.

Add 3,5,9

Add 9

16.33   Sample size adjustment post-addendum 9: We anticipate that the accrual to this trial will remain strong after the implementation of the mandatory KRAS testing. However, should accrual not resume to the strong pre-addendum 9 levels, timely completion of the trial remains vital. Therefore, if by month 6 after re-opening, accrual does not exceed 50/month, and by month 7 accrual does not equal or exceed 60/month, the target sample size will be reduced to 1710 KRAS WT patients, which would provide 90% power for a hazard ratio of 1.375 as opposed to 1.33 as specified in section 16.31 above.

16.4   Secondary endpoints and analyses.

Add 3,9

Secondary endpoints include a comparison of DFS between C225 and no C225 in the entire population (excluding the patients treated with irinotecan), as well as comparisons between treatments for overall survival, patient-completed questionnaires, toxicity, and a number of translational endpoints.

Add 9

Note: Questionnaires were discontinued for patients enrolled following implementation of Addendum 9.

Add 9
Update 3

16.41   DFS comparison in all patients: If the initial comparison of DFS between arms in the KRAS WT patients is significant at level 0.05, a second comparison will be conducted between arms including the KRAS mutant (and not evaluable) patients enrolled into the trial prior to addendum 9, also at level 0.05. As this is a closed testing procedure, no alpha adjustment is necessary. This comparison using all 2910 patients will have 90% power to detect a hazard ratio of 1.27 comparing the two treatment arms, based on a total of 735 events.

Update 3

A challenging issue is to determine the appropriate inferential conclusion in situation of the presence of a positive result, at p <0.05, in the entire cohort, but where this difference is driven mainly by the KRAS WT patients. The expected 40% of patients who are KRAS mutant (or not evaluable) will have inadequate power to detect a modest but still possibly meaningful HR in that subgroup. Therefore, a requirement of individual statistical significance at the 0.05 level within each sub-population is inappropriate. The optimal method to determine if KRAS marker status is predictive of C225 benefit is to test for a KRAS status by treatment arm interaction, however, the power for that test is limited. Based on these considerations, in the presence of an overall positive result, will also present results within the two groups of patients defined by KRAS status, and judge the appropriate treatment for each group by both of the individual subgroup treatment effects, the consistency of result across subgroups (judged by interaction testing but also other means), consistency across other secondary endpoints, and additional biological understanding obtained between the time of protocol authoring and final analysis.

Add 3,9

16.42   Overall Survival: Overall survival (OS) is defined as the time from randomization to death, from any cause.

Add 3

16.421   Analysis plans for OS: Analysis plans for OS will parallel those outlined for DFS, including the hypotheses to be tested, the error rates spent for each comparison, and the number of events required prior to analysis. As the event rates for overall survival at 5 years and for DFS at 3 years are very similar in the setting of adjuvant colon cancer (97), the data for OS will be mature after the data for DFS. We expect the data for OS to be mature between 3 and 4 years following the close of accrual.

Add 3,5,6

16.43   LASA: The analyses of the LASA data from this trial will test four specific hypotheses, namely, whether the overall, mental, physical well-being and the level of fatigue differ between the study arms. As indicated below, a sample size of 500 patients per arm will be required for the LASA endpoints. Therefore, after accrual of the initial 500 patients per arm randomized to FOLFOX +/- C225, accrual to the LASA component of the trial will terminate.

All LASA scores are on an ordinal 0-10 scale. The primary LASA endpoint will be the area under the curve (AUC) formed by aggregating the individual LASA scores over time. Secondary LASA endpoints will include the absolute percentage change from baseline value to each evaluation as well as the maximum and minimum value observed during treatment. The LASA items in Appendix VI will be compared across the treatment groups on a per item basis only. No summative score will be calculated or compared from these items. Normality testing via the Shapiro-Wilk (98) procedure will determine whether or not parametric or nonparametric procedures will form the basis for analysis (two-sample t-test, Wilcoxon rank sum).

Add 5,6

Based on complete data from 500 patients per chemotherapy arm, a comparison of the individual LASA items between the two treatment groups would have approximately 90% power to detect a difference of 0.20 standard deviations (a small effect size), using a two-sided procedure and a 5% type I error rate (99). While efforts to minimize missing data will be undertaken, an attained sample size of 350 patients would still provide 90% power to detect an effect size of 0.25 standard deviations.

Missing data will be handled in a number of ways. First, all analyses will be run using only the data that is available. Second, imputation will be carried out by use of last-value-carried-forward (LVCF) and average-value-carried-forward (AVCF) and the analyses run again. Collectively, these three approaches have been demonstrated to be useful for identifying the impact of missing data on results as long as the amount of missing data is no more than 20% (100).

Add 3,5,6

16.44   Toxicity and dose intensity: Toxicity and dose intensity collected for each cycle on the initial 500 patients per arm, i.e. the first 1000 patients randomized to FOLFOX +/- C225. Dose Intensity is defined as the protocol specified dose divided by the actual dose delivered. It will be calculated separately for each drug within a regimen, both by cycle and over the entire course of therapy.

Add 3,5,9

16.45   Completion Questions:

Analysis of the Completion Questions will be bi-focal. First, descriptive analysis pooling over treatment arms will be conducted with the goal of assessing overall satisfaction with the clinical trials process and this trial in particular. If considerable dissatisfaction is documented, it would suggest multiple aspects of the process need to be reassessed. Secondly, we will compare patient satisfaction between treatment arms, using each question individually. With 1035 patients per arm for the primary comparison, each test will have >90% power to detect a difference of 8% in the proportion of "yes" responses between study arms. This calculation allows a 15% no-completion rate for the completion questionnaire.

16.46   Translational endpoints

Add 1,3,6

The details of the statistical analysis plan for the translational endpoints are contained in Appendix XIV. This analysis includes two of the four patient completed questionnaires (i.e., the Focused Health Assessment [Patient Questionnaire] and the Brief Food Questionnaire).

Add 6,13   16.5   Adverse Event Stopping Rule

The study PI and the NCCTG Adverse Event Coordinator review all Serious Adverse Events occurring on both arms of this trial on an ongoing basis. In addition, the independent NCCTG Data Monitoring Committee (DMC) reviews all safety data every 6 months and may convene for a special session when necessary. In addition to this standard safety monitoring we have developed a formal monitoring plan for treatment-related mortality. CTCAE v3.0 will be used to determine grading for these stopping rules.

Death within 60 days is a tool that has proved useful in reviewing adverse event data, by removing any possible subjectivity. It has the caveat of including all deaths, from any cause, even if not related to therapy (105). The following table provides a listing of the death rate seen in other large randomized trials in stage III or II/III colon cancer. Please note that a consistent metric has not been used for all of these trials. Some are a 'pure' 60 day all cause mortality rate, whereas others have excluded deaths not felt to be due to treatment.

Trial	Experimental Arm		Control Arm	
XACT (106)	Capecitabine	0.80%	5FU/LV	1.03%
MOSAIC (107)	FOLFOX	0.54%	LV5FU2	0.54%
N016968 (personal communication)	XELOX	1.07%	5FU/LV	0.97%
NSABP C-07 (108)	FLOX	1.25%	5FU/LV	1.16%
NSABP C-08 (personal communication)	FOLFOX + bev	0.37%	FOLFOX	0.49%

Based on this experience, we consider a rate of death within 60 days of 1.25% on either arm as the upper threshold of acceptability. We will monitor this rate at each notification of a patient death, and at minimum for the semi-annual NCCTG DMC meeting. If at any time the 80% two-sided confidence interval for the rate of death within the first 60 days excludes 1.25% to the right (i.e. includes only values greater than 1.25%), the DMC will immediately be notified to consider suspending accrual pending a determination of the need to enact a protocol amendment or to discontinue the trial; otherwise, accrual continues.

Add 10   16.51   Stopping rule for monitoring rate of early death in patients aged ≥70 on cetuximab arm.

Upon the trial reopening to accrual for patients aged ≥70, the following toxicity monitoring rule will be implemented and continually monitored. Further accrual to patients 70 years of age and older will be halted if ≥2 study-related deaths occur in the next 25 patients from this age group, ≥3 of the next 50, ≥4 of the next 75, or ≥5 of the next 100 or more patients, all based on the date of implementation of Addendum 10.

Add 6   16.6   NCCTG Inclusion of Women and Minorities

16.61   This study will be available to all eligible patients, regardless of race, gender, or ethnic origin.

16.62   There is no information currently available regarding differential effects of oxaliplatin, 5-fluorouracil or irinotecan -based treatment in subsets defined by race, gender, or ethnicity, and there is no reason to expect such differences exist. Therefore, although the planned analysis will, as always, look for differences in treatment effect based on racial and gender groupings, the sample size is not increased in order to provide additional power for ethnic subset analyses.

16.63   To predict characteristics of patients likely to enroll in this trial, we reviewed the total accrual into the most recent GI Intergroup adjuvant colon trial C89803 by membership, gender, and race. This demonstrated that 12% (148/1264) of patients could be classified as minorities by race and that 45% (562/1264) of patients were women.

Planned Gender and Minority Enrollment

Ethnic Category	Sex/Gender			
	Females	**Males**	**Unknown**	**Total**
Hispanic or Latino	36	39	0	75
Not Hispanic or Latino	1625	2068	0	3693
Ethnic Category: Total of all subjects*	1661	2107	0	3768
Racial Category				
American Indian or Alaskan Native	6	11	0	17
Asian	18	25	0	43
Black or African American	114	161	0	275
Native Hawaiian or other Pacific Islander	5	6	0	11
White	1518	1904	0	3422
Racial Category: Total of all subjects*	1661	2107	0	3768

Ethnic Categories:	**Hispanic or Latino –** a person of Cuban, Mexican, Puerto Rican, South or Central American, or other Spanish culture or origin, regardless of race. The term "Spanish origin" can also be used in addition to "Hispanic or Latino." **Not Hispanic or Latino**
Racial Categories	**American Indian or Alaskan Native –** a person having origins in any of the original peoples of North, Central, or South America, and who maintains tribal affiliations or community attachment. **Asian –** a person having origins in any of the original peoples of the Far East, Southeast Asia, or the Indian subcontinent including, for example, Cambodia, China, India, Japan, Korea, Malaysia, Pakistan, the Philippine Islands, Thailand, and Vietnam. (Note: Individuals from the Philippine Islands have been recorded as Pacific Islanders in previous data collection strategies.) **Black or African American –** a person having origins in any of the black racial groups of Africa. Terms such as "Haitian" or "Negro" can be used in addition to "Black or African American." **Native Hawaiian or other Pacific Islander –** a person having origins in any of the original peoples of Hawaii, Guam, Samoa, or other Pacific Islands. **White –** a person having origins in any of the original peoples of Europe, the Middle East, or North Africa.

Add
6.13

16.7   This study will be monitored by the Clinical Data Update System (CDUS) version 3.0. Cumulative CDUS data will be submitted quarterly to CTEP by electronic means. Reports are due January 31, April 30, July 31, and October 31. At the time CTCAE v4.0 became available, this study involved collection of adverse events using CTCAE v3.0. The study continues to collect all routine AE data using CTCAE v3.0; however, all adverse event data submitted via CDUS must use CTCAE v4.0 terminology. CTEP provided mappings will be used to convert v3.0 data to v4.0 prior to submission to CTEP, as agreed upon by NCI and the Mayo Clinic Cancer Center.

Add 6

16.8   In accordance with the NCI's current DMC policy, the NCCTG External Data Monitoring Committee will meet every 6 months in conjunction with the NCCTG semi-annual group meetings to review the progress of this protocol.

17.0   Pathology Considerations for Quality Control

Note: See Section 6.1 (Pre-Randomization procedures) for instructions before submitting pre-randomization pathology materials.

Slides and/or blocks are **mandatory** for pre-randomization KRAS assessment (Section 17.2), central review for confirmation of diagnosis (Section 17.3), and translational studies (Section 14.1 and Appendix XIV).

Pre-randomization tissue requirements for KRAS determination, central review for confirmation of diagnosis and translational studies are included in this section.

As of Addendum 12 (Arm A and Arm D patients only): blocks or slides from secondary resection after first recurrence are **optional** for correlative studies (Section 17.13 and Appendix XIV).

17.1   Slides **or** Blocks

Note: The blocks/slides used for pre-randomization and quality control (Section 17.0) will be the same materials used for Section 14.1, Translational/ Pharmacologic studies (paraffin-embedded blocks or slides). The tissue blocks will be used for the creation of tissue microarrays. The clinical investigator and the submitting pathologist have the responsibility for submitting representative materials for the goals cited in the protocol.

17.11   **Required materials FOR PATIENTS RANDOMIZED PRIOR TO ADDENDUM 9:**

If blocks were previously submitted and the NCCTG Research Base determines there is sufficient tissue for the KRAS testing and for the proposed translational studies currently available at the NCCTG Operations Office, no additional tissue will be required.

If blocks were previously submitted and the NCCTG Research Base determines there is insufficient tissue for the KRAS testing and for the proposed translational studies currently available at the NCCTG Operations Office, an NCCTG Pathology Coordinator will request for submission of additional material.

Before submitting additional materials, please contact the Pathology Coordinator (507 293-3928) prior to sending any blocks or unstained slides. If unstained slides were previously submitted instead of blocks, sequentially cut an additional 15 **five micron** sections and mount on **charged** slides (for translational studies; Section 14.0 and Appendix XIV), then cut 5 **ten micron** sections and mount on **uncharged** slides (for KRAS assessment) within ≤60 days following activation of Addendum 9. **Label the slides with NCCTG patient ID number, accession number, order of cut sections (i.e., 1-15 for the 5 micron slides and 16-20 for the 10 micron slides), and micron thickness of section (either 5 or 10 microns). H&E stain the last 5 micron slide that is cut, which is adjacent to the first 10 micron slide (i.e., slide labeled 15).** Ideally, each slide must have a minimum of 75% tumor tissue on the slide to be deemed adequate for study. **Do not bake or place covers slips on the slides.** See Section 17.13 for shipping information.

Note: the number of slides previously indicated for submission prior to Addendum 9 was insufficient for the correlative studies originally proposed in Appendix XIV. Patient reconsent for submission of these extra materials is NOT required by NCCTG, as these additional materials will be used for studies relative to this trial (i.e., KRAS analysis, correlative studies outlined in Appendix XIV).

Add
3,6,9,10,11,12

17.12   Required materials FOR PATIENTS PRE-RANDOMIZED STARTING WITH ADDENDUM 9:

17.121   Summary Table of **Required** Tissue Biospecimens for This Protocol

Type of tissue biospecimen to submit	**Mandatory or optional**	**When to submit**	**Reason for submission (background/ methodology section)**	**Where to find specific details for biospecimen submission**
ALL diagnostic slides from resection	Mandatory	Immediately following pre-randomization and as soon as possible after surgery (≤42 days)	Confirmation of diagnosis through central review	Section 17.122
Three (four if possible) formalin-fixed paraffin-embedded (FFPE) tissue blocks with corresponding diagnostic H&Es (OR unstained slides with corresponding H&Es)	Mandatory	Immediately following pre-randomization and as soon as possible after surgery (≤42 days)	Pre-randomization requirement, KRAS mutation status of tumor performed centrally	Section 17.123

Add 3,4,6,10

17.122 **ALL** diagnostic tissue slides from the resection **including those that document** extent of disease, radial margin (if positive), those that interface with adjacent mucosa and/or pre-existing adenoma, and normal colonic mucosa- **with corresponding paraffin block (see Section 17.123). (Please note that submission of 1 block or slide is not adequate for this review.)**

   o   At least one slide documenting nodal metastasis.

   o   Slides should be placed in appropriate slide container and labeled with the protocol number, study patient number, and patient initials.

17.123 **Blocks from original resection**

o   At least three blocks, **four blocks** if possible **to correspond with submitted diagnostic H&E slides (see Section 17.122)**.

o   **One paraffin-embedded block with normal colonic mucosa away from tumor (a block at least 10 cm away or from a negative margin) with corresponding H&E.**

IMPORTANT NOTE: IF AN INSTITUTION IS UNABLE TO RELEASE PARAFFIN-EMBEDDED TISSUE BLOCKS, they must be willing to submit 30 tissue sections. Sequentially cut 25 **five micron** sections and mount on **charged** slides (for translational studies, Section 14.0 and Appendix XIV), then cut 5 **ten micron** sections and mount on **uncharged** slides (for KRAS assessment). **Label the slides with NCCTG patient ID number, accession number, order of cut sections (i.e., 1-25 for the 5 micron slides and 26-30 for the 10 micron slides), and micron thickness of section (either 5 or 10 microns). H&E stain the last 5 micron slide that is cut which is adjacent to the first 10 micron slide (i.e., slide labeled 25).** Ideally, each slide must have a minimum of 75% tumor tissue on the slide to be deemed adequate for study. **Do not bake or place covers slips on the slides.**

17.124 The following materials below are **mandatory for pre-randomization/registration** (unless indicated otherwise) and required for shipment:

- ALL Diagnostic Slides and Corresponding FFPE Tissue Blocks (at least 3, 4 if possible)*
- NCCTG Pathology Submission Form
- Surgical Pathology Report
- Operative Report (Optional)

*Note: if institution is not able to release tissue blocks, see Important Note in Section 17.123 for alternate tissue submission.

17.125 Immediately following pre-randomization and as soon as possible after surgery (≤42 days) all institutions (including Mayo and affiliates) are to forward the required materials (diagnostic slides, blocks, and appropriate paperwork) to:
   NCCTG Operations Office
   Attn: PC Office (Study N0147)
   RO_FF_03_24-CC/NW Clinic
   200 First Street SW
   Rochester, MN 55905

Add 12   17.13 **Optional paraffin-embedded tissue blocks/slides <u>requested for correlative</u>**
                   <u>studies STARTING WITH ADDENDUM 12 (Arm A or Arm D patients only):</u>

              17.131 Summary Table of Optional Tissue Biospecimens for This Protocol[1]

Type of tissue biospecimen to submit	Mandatory or optional	When to submit	Reason for submission (background/ methodology section)	Where to find specific details for biospecimen submission
Formalin-fixed paraffin-embedded (FFPE) tumor tissue block and normal tissue block (if available) with corresponding H&Es (OR unstained slides with corresponding H&Es) from secondary surgical resection following metastatic or local disease recurrence.[2]	Optional	≤30 days after site learns of secondary resection for recurrence and obtains patient consent (or ≤30 days following activation of Addendum 12 at your local site if patient has already had metastatic or local disease recurrence and secondary surgical resection reported)	Correlative studies (see Appendix XIV)	Section 17.132

       1 Arm A or Arm D patients only.
       2 Only resected surgical tissue is being requested. Fine needle aspirate and biopsy tissue
         specimens do not need to be submitted.

Add 12   17.132 Submit one formalin fixed paraffin-embedded (FFPE) tumor tissue block
                   with largest amount of resected metastatic or local recurrent invasive
                   tumor (at least 1 cm of tumor for cases of surgical resection) and one
                   FFPE block with normal colonic mucosa away from tumor (if available)
                   from secondary surgical resection following metastatic or local disease
                   recurrence. **A corresponding H&E slide for each submitted block
                   must be provided** to permit quality assessment of the tissue block(s).

                   The FFPE tissue blocks are preferred; however, if an institution is
                   unable to provide the recurrent tumor or normal tissue block from the
                   secondary surgical resection, **cut 30 five-micron sections from each
                   block (i.e., recurrent tumor tissue and normal tissue, if available)
                   and mount on charged glass slides.** Label the slides with NCCTG
                   patient ID number, accession number, and order of sections. **H&E
                   stain every tenth slide (i.e., slides labeled 1, 11, 21). These H&E
                   slides will be reviewed centrally under the research base's
                   protocol for assessing tissue quality. The remaining unstained**

slides will be processed as described in Appendix XIV. For samples containing less than 7 square millimeters of tumor tissue, multiple sections should be mounted onto each slide to ensure that the appropriate amount of tumor tissue is available. Ideally, each recurrent tumor slide must have a minimum of 75% tumor tissue on the slide to be deemed adequate for study. Do not bake or place covers slips on the slides.

Add 12

17.133 **As of Addendum 12,** the following materials below are required for shipment of **optional recurrent or metastatic** resected tissue:

- Paraffin embedded local recurrent or metastatic tumor tissue block from secondary surgical resection with corresponding H&E slide (OR 27 unstained slides with corresponding 3 H&E(s)).
- Paraffin embedded normal tissue block, if available, from secondary surgical resection with corresponding H&E slide (OR 27 unstained slides with corresponding 3 H&E(s)).
- Recurrent Research Tissue Submission Form
- Surgical Pathology Report
- Operative Report *(optional)*

*Notes:
If patient has secondary resection for recurrence during Event Monitoring, and has not been reconsented regarding providing this additional tissue, the patient should be contacted after site learns of the resection, to request the patient's consent for providing their tissue.

If institution is not able to release tissue blocks, see Section 17.132 for alternate tissue submission.

Verify that the appropriate sections of the Recurrent Research Tissue Submission Form are completed and filled in correctly. Enter information from the Recurrent Research Tissue Submission Form into the remote data entry system on the same day the specimen is submitted (see Forms Packet).

Add 12

17.134 Within ≤30 days following secondary surgical resection following metastatic or local disease recurrence (or ≤30 days following activation of Addendum 12 at your local site if patient has already had metastatic or local disease recurrence and secondary surgical resection), all institutions (including Mayo and affiliates) are to forward the required materials (diagnostic slides, blocks, and appropriate paperwork) to:
NCCTG Operations Office
Attn: PC Office (Study N0147)
RO_FF_03_24-CC/NW Clinic
200 First Street SW
Rochester, MN 55905

Add 9,10

17.2 As of Addendum 9, KRAS wild-type status, verified by central testing and review, is required for entry onto this trial. From the tissue specimen submitted in Section 17.1, the submitted H&E slide and the five 10 micron unstained tissue sections mounted on uncharged slides will be used for the KRAS analyses for eligibility determination**.** These slides will be analyzed

for KRAS mutations in the laboratory of Stephen N. Thibodeau, Ph.D, Mayo Clinic Rochester, using the commercially available DxS kit.

Update 3
Add 10

Dr. Thibodeau's laboratory will report pre-randomization KRAS results (wild-type, mutant or not evaluable) for patient assignment within **10 <u>business</u> days from receipt of <u>ALL</u> required pathology materials. ("ALL pathology materials" includes the H&E slides that are required to be submitted. If H&E slides are not submitted, NCCTG will prepare an H&E slide for the KRAS testing, but this will delay the KRAS reporting process.)** KRAS results will be emailed to the two site contacts listed on the Pathology Submission Form (i.e. participating site PI and site contact), the NCCTG Pathology Coordinators and the NCCTG Registration Office. ***Please do not contact NCCTG for KRAS results until at least 7 business days have passed since the submission of all required material.***

Add 3,6,9
Update 3

17.3   The Pathology Coordinator will batch and forward materials to pathologists for central review to confirm diagnosis of specimens received prior to Addendum 9. Thomas C. Smyrk, M.D. and/or associates, Mayo Clinic Rochester, will review materials for all Mayo memberships and their affiliates. James T. Quesenberry, M.D., Thomas A. Webb, M.D., Gist Farr, M.D., and Paul Mazzara, M.D. will review all other materials (non-Mayo-affiliated institutions).

Add
3,4,9,10,12

17.4   As of Addendum 12, if a corresponding H&E wasn't submitted with the block/slides, the NCCTG Operations Office will request a slide to be processed (i.e., cut and H&E stained) from the tumor tissue block and forwarded to Dr. Thomas Smyrk to be reviewed under the research base's protocol for assessing tissue quality for the proposed correlative studies, unless the tumor size is too small. If the tumor tissue is too small, assessment of tissue quality will occur at the time the correlative studies are performed. After the pathologist assesses the tissue quality, blocks will be marked for TMA construction. The tissues and appropriate paperwork will be stored in the NCCTG Operations Office until an appropriate request is submitted for correlative studies, as outlined in Appendix XIV.

Add 12

17.5   The institutional pathologist will be notified in the event that the block may be depleted. The paraffin-embedded blocks will be available to the submitting institution upon specific request to accommodate individual patient management. Sites requesting that tissue blocks be returned for local testing purposes must confirm with appropriate personnel (e.g., local pathologist, laboratories) that no tissue blocks were retained at the local site. Please use retained materials before requesting the NCCTG to return submitted tissue blocks to the site. Original diagnostic slides will be returned to submitting institution with the exception of the slides marked by the NCCTG reviewer for tissue microarrays.

18.0 Records and Data Collection Procedures

With Addendum 10, the protocol re-implemented forms that were discontinued with Addendum 7 ('NCI Cooperative Group Colorectal Cancer - Treatment Form – Subset of Patients' and the 'NCI Cooperative Group Colorectal Cancer – Toxicity Form – Subset of Patients'). The forms and submission schedule provided below will be used. **NCCTG and non-NCCTG sites, alike, will mail case report forms and reports to NCCTG.** Do not copy the CTSU on AdEERS reports. Do not send specimens to the CTSU. **Effective September 1, 2011 – NCCTG sites will submit case report forms via the NCCTG Remote Data Entry System, and the non-NCCTG sites will continue to mail all data and forms to NCCTG (see Protocol Resource page).**

All forms are required for patients who have been randomized to study treatment (e.g. to Arms A and D), except as indicated otherwise. Forms that are required for all patients, including patients registered to Arm G, are noted with a double asterisk (**).

Forms	Active-Monitoring Phase (Compliance with Test Schedule)					Recurrence[2]	Event-Monitoring (EM) Phase		At Each Occurrence	
	Initial Material		Follow-up material							
	Following Pre-Randomization	≤2 wks following Registration/Randomization	Every 2 Cycles	At end of active treatment	Observation phase: every 6 months after ending treatment for 5 years from randomization or until recurrence, whichever occurs first		EM Annually up to 8 years from randomization (or death, whichever occurs first)	Death	ADR/AER	New Primary[11] Or Late Adverse Event
NCI Cooperative Group Registration Form**		X								
Pre-Registration Screening Failure Form +	X[13]									
NCI Cooperative Group Colorectal Cancer - Adjuvant On-Study Form**		X								
Operative & Pathology Reports**	X[1,9]					X[9]				
Operative & Pathology Submission Form						X[9]				
Recurrent Research Tissue Submission Form						X[16,17]				
NCCTG Pathology Submission Form** +	X[1]									
Colonoscopy Report		X[7,9]			X[8,9]	X[8,9]				X[2,8,9]
Colonoscopy Submission Form					X[8,9]	X[8,9]				X[2,8,9]
Paraffin-embedded blocks/slides** +	X[1]									
Translational Research Blood Draw (see Sec.14.0) +	X[5**]				X[5,16]					
NCI Cooperative Group Colorectal Cancer - Specimen Submission Form - Blood	X[5**]				X[5,16]					

Forms	Active-Monitoring Phase (Compliance with Test Schedule) Initial Material — Following Pre-Randomization	≤2 wks following Registration/Randomization	Every 2 Cycles	At end of active treatment	Observation phase: every 6 months after ending treatment for 5 years from randomization or until recurrence, whichever occurs first	Recurrence[2]	EM Annually up to 8 years from randomization (or death, whichever occurs first)	Death	ADR/ AER	New Primary[11] Or Late Adverse Event
NCI Cooperative Group Colorectal Cancer - Treatment Form – Subset of Patients			X^{12}	X^{12}						
NCI Cooperative Group Colorectal Cancer – Adjuvant Disease Treatment Summary Form **Not for Arm G patients**				X^{10}						
NCI Cooperative Group Colorectal Cancer – Adjuvant Disease Treatment Summary Form – **Arm G patients only**				X^{10}						
NCI Cooperative Group Colorectal Cancer – Toxicity Form – Subset of Patients			X^{12}	X^{12}						
NCI- Cooperative Group Colorectal Cancer Toxicity Form-Summary[4]				X^{4}						
NCI-Cooperative Group Colorectal Cancer- Follow Up Form -**Not for Arm G patients**			$X^{2,6}$	X	X	X	X	X		X
NCI-Cooperative Group Colorectal Cancer Follow-Up Form – **Arm G patients only**						X	X	X		
NCI-Cooperative Group Colorectal Cancer Secondary Resection Follow-Up Form – Not for Arm G patients						$X^{9,15}$				
NCI Cooperative Group Colorectal Cancer – Leucovorin Administration Form – **Not for Arm G patients**+				X^{14}						
ADR/AER (See Section 10.0)									X	
Patient-Completed Questionnaires	*Questionnaires are not required for patients enrolled following implementation of Addendum 9 (August 18, 2008).*									
Request Letter for Immunohistochemistry (IHC)Test Results (Appendix XV)**,[3] +	Upon Request. (Documentation of when patient requests and receives results of IHC testing is to be noted in the research record.)									

** Required for <u>all</u> patients; i.e. required for patients registered to Arm G as well as those randomized to Arm A or Arm D.

Add 13   + Please submit according to guidance provided in this section and in the Forms Instructions.

Add 13   1. Submit one copy to NCCTG per Section 17.0 with other on-study materials.

2. Documentation of recurrence or new primary is required to be submitted.

3. Submit signed request letter to North Central Cancer Treatment Group Research Base, Attention: N0147 QAS, Cancer Center Statistics NW Clinic, 200 First Street SW, Rochester, MN 55905.

4. This form is required to be submitted for patients enrolled ON OR AFTER January 4, 2008, and PRIOR to February 1, 2009.

Add 3,5,6,7,9, 10,12,13   5. Translational blood specimens for Section 14.2 must be collected **following pre-randomization but prior to registration/randomization and at the first Observation visit or next Observation visit for patients already in the Observation phase**. Submit the original BAP Requisition Form along with the blood samples directly to BAP Receiving via the kits and

prepaid mailers, per section 14.23. Maintain a copy of the BAP Requisition Form for your files. Submit the Specimen Submission Form – Blood directly to NCCTG. Include NCCTG ID number (assigned at pre-randomization) on samples, Specimen Submission Form, and on all other applicable forms, (e.g., the BAP Requisition Form contained in the blood collection kit.)

6. Submit at the time a patient ends active treatment due to death or recurrent disease. Also submit this form if a patient is lost to follow-up. See Forms Instructions.

7. Submit colonoscopy report from original diagnosis (if available).

Add 13   8. Submit if colonoscopy is proof of recurrence or new primary. For patients in Event Monitoring who have undergone a colonoscopy as proof of recurrence, please submit report if available and acceptable.

Add 13   9. Pathology, if available, and operative reports are required to be submitted for first resection post-recurrence (Arms A and D only). The report must be accompanied by the respective submission form (from the N0147 Forms Packet) and completed NCCTG Data Submission Cover Sheet.

10. To be completed as soon as possible once the decision has been made to discontinue treatment, or treatment does not start due to patient cancellation after registration/randomization, or following the last cycle of treatment.

11. Documentation (e.g., pathology report, etc.), of new primary required to be submitted.

12. Required for patients enrolled PRIOR to the implementation date of Addendum 7 (January 4, 2008); and for patients enrolled ON OR AFTER February 1, 2009.

13. Complete only if patient is not registered/randomized after pre-randomization completed. Fax this form to NCCTG at fax # (507) 284-1902, Attention: N0147 QAS.

Add 13   14. Required for all patients randomized on or after August 18, 2008 to Arms A and D, to be completed at the end of active treatment. Effective June 1, 2011, this form will be mailed by all sites (both NCCTG and non-NCCTG). Beginning September 1, 2011, NCCTG sites will enter via the Remote Data Entry System, and the non-NCCTG sites will continue to mail (see Protocol Resource page).

15. Submit this CRF only if the patient has developed a first progression (or recurrence) and a secondary resection was performed but not previously reported. Do not complete this form if the secondary resection was

Add 12   reported on the Follow Up Form - Not For Arm G Patients. If patient has not been reconsented to provide tissue from secondary resection for recurrence, the patient should be contacted at this time to request consent to provide their tissue.

Add 12   16. For patients randomized to Arm A or Arm D only.

Add 12,13   17. Submit one copy with tissue to NCCTG per Section 17.0.

Administering Patient-Completed Questionnaires (for patients enrolled prior to implementation of Addendum 9) Instructions for Clinical Research Associates (CRAs) (App V)[3]:			
Form	Time Point		
	Baseline (prior to treatment)	At 6th Cycle	End of Active Treatment
LASA: First 500 patients per arm (App. VI) (Only for pts. enrolled prior to implementation date of Addendum 7)	X	X	X
Brief Food Questionnaire (App. VII)	X		
Completion (App. VIII)			X
Focused Health Assessment (Patient Questionnaire)(App. IX)	X		

1. Focused Health Assessment (Patient Questionnaire) Booklet: CRA must check booklet for completeness and fill out pages 18 and 19 of the booklet.

2. LASA/Completion Questions: CRA must check both forms for completeness and write and fill in the corresponding circles for Patient Study ID and Today's Date in "Office Use Only" section on the bottom left. The date in this section must match the header date. In addition, patient initials must be written on the bottom right of the form above form name, e.g. LASA.

Add 13   3. **CANADIAN SITES ONLY** - NCCTG has approved the use of patient completed questionnaires which have been translated into French for the Canadian sites. The Canadian sites are required to transfer data collected via the French questionnaires onto the original English format questionnaires. Any written comments must also be translated into English for data submission. The CRA must initial and date a note on the original English questionnaire that data was transferred from the patient-completed French questionnaire. In addition, a second party must verify accuracy of the transferred data on the English questionnaires, and the second party must also initial and date a note on the original English questionnaire stating that the transferred data was verified. The site must retain the patient-completed French questionnaire and a copy of the original English questionnaire submitted to NCCTG. NCCTG will return questionnaires to sites if not submitted on the original English version.

19.0   Budget

19.1   Costs charged to patient: Routine clinical care.

Add
2,3,6,9,10,11,1
2

19.2   Drug Availability:
- Oxaliplatin will be obtained commercially for patients pre-randomized following implementation of Addendum 10. If patients do not have insurance coverage for the oxaliplatin, Sanofi-Aventis will provide the agent free of charge for the patient's use. Information regarding the PACT+ program is accessible on the world wide web at http://www.oncology.sanofi-aventis.us/docs/pdf/Pact+SM%20Program%20Services.pdf. **Effective with the implementation of Addendum 10, oxaliplatin is no longer provided by NCI for this study.**

Note: Irinotecan is no longer part of the study as of June 1, 2005.
Effective July 15, 2006, loperamide is no longer provided by NCI for this study.

Note: Cetuximab is no longer part of the study as of November 25, 2009 and is no longer provided by ImClone for distribution by NCI for this study.

19.3   Research funded: Translational studies (Section 14.0 and Appendix XIV) and
Add 9,10   specimen kits for research blood draw.

Add 9   19.4   Research funded: KRAS testing for eligibility purposes (for patients registered/randomized following implementation of Addendum 9).

Add 7

20.0   References

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Appendix I
Consent Form

Add 5,6,10

TITLE: **N0147, A Randomized Phase III Trial of Oxaliplatin (OXAL) Plus 5-Fluorouracil (5-FU)/Leucovorin (CF) with or without Cetuximab (C225) after Curative Resection for Patients with Stage III Colon Cancer**

PARTICIPANTS:
This is an important form. Please read it carefully. Your signature on this form also means that you agree and want to take part in this study.

Add
5,6,9,10,11

Why is this study being done?

This study is being done in patients who have had surgery for colon cancer.

The study is being done to:

- ♦ Find out if adding cetuximab to FOLFOX (a combination of the 3 drugs oxaliplatin, leucovorin and 5-fluorouracil) increases the effectiveness of FOLFOX in preventing your colon cancer from coming back.
- ♦ See whether patients get better results in one of these groups.
- ♦ Compare the side effects in the two groups of patients in the study.
- ♦ We will look at markers in tissue or blood specimens obtained from you to determine whether these markers can be used to predict whether your tumor responds to chemotherapy or the chance that your cancer will come back. These studies are for research purposes and have not been accepted into routine medical practice.

As of June 2008 it became apparent that a change within the genes of the tumor can better help predict who may be likely to benefit from cetuximab. Changes in a gene called KRAS have been shown to greatly lessen or prevent any benefit from the use of cetuximab. Based on this new finding this trial will now treat only new patients that have the normal (wild-type) version of the KRAS gene. In order to determine if you have a normal (wild-type) version of KRAS the assessment of KRAS in this trial uses a test that is a version of one widely used and generally considered reliable outside of clinical trials. However, it is important to note that no test for KRAS assessment has yet been approved by the Food and Drug Administration (FDA).

On the other hand, if the test shows that you have an abnormal (mutated) version of KRAS it means that you may not benefit from cetuximab and therefore will not receive treatment as part of this trial. It is important to note that the changes in the KRAS gene occur only in the tumor and not in normal cells of the body. As such, having a mutated gene for KRAS in your tumor does not mean that you received an abnormal gene from your parents or have the ability to pass the abnormal gene on to your children. The abnormal gene, when present, is only in the tumor.

Patients that have an abnormal (mutated) version of the KRAS gene may receive treatment outside of this study as determined by their physician. This study will not determine or provide treatment for these patients. Instead, yearly reports about the patient's treatment and health will be submitted for this study. Also, if KRAS status cannot be determined using the tissue that has been submitted, patients can receive treatment determined by their physician outside of this study, and yearly reports will be submitted for the study.

Add 12 **The two drug combinations being studied in patients with a normal (wild-type) version of KRAS are listed below.** The drugs listed in Arm A, oxaliplatin (OXAL), 5-fluorouracil (5-FU) and leucovorin (CF) are considered standard of care. The combination of drugs in Arm D is considered experimental in the stage of colon cancer that you have because of the addition of cetuximab:

> **Arm A:** OXAL, 5-FU, and CF
> **Arm D**: Arm A + Cetuximab *(Cetuximab was discontinued as of November 25, 2009)*

> Note: Arms B, C, E and F, which contained the drug irinotecan (CPT-11) are no longer part of the study as of June 1, 2005.

Arm G of this study will include patients with an abnormal (mutated) version of KRAS, and patients whose KRAS status could not be determined from submitted tissue. These patients may receive treatment determined by their physician (treatment is not given as part of this study). Yearly reports will be sent to NCCTG about the treatment that has been given, and the patient's overall health.

It is not possible at this time to know whether treatment given in this study will prevent your cancer from coming back.

Add 7,9 **How many people will take part in the study?**

The plan is to have 3768 people take part in this study.

Add 6,7,9,10,11 **What will happen in the study?**

For all patients enrolled after July 2008: You will first provide samples of your blood and tissue for use in this study. The tissue used will be from your surgery, so you will not have another procedure to collect this tissue. KRAS testing will be done on your tissue to determine whether you will be randomized to one of the two treatment arms (Arm A or D), or whether you will receive treatment outside of the study and have yearly reports submitted for the study (Arm G).

Add 12 Besides KRAS testing, this study also involves other required tests using your blood and tissue blocks from your surgery. The required blood and tissue tests will be done for all patients (Arms A, D, and G). In addition, there is an optional tissue collection if your cancer comes back and you have surgery to remove it. The work to be done on these samples is described later in this consent form. You have the option to learn the result of one of the tests, called 'Immunohistochemistry testing' or 'IHC'. If you wish to receive this result there is a separate letter you will need to sign.

You also have the <u>option</u> to allow your samples to be kept by NCCTG for use in future research outside of this study. Your options regarding future use of your samples outside of this study are described later in this consent form.

If your KRAS result is normal (wild-type), you will be put into one of the two study treatment groups by chance (as in the flip of a coin). This is called randomization. A computer will decide which group you will be in. One group will be treated with Oxaliplatin, 5-FU, and CF for 24 weeks. The other group will receive Oxaliplatin, 5-FU, and CF with cetuximab added to the treatment. Both groups will receive study drugs. That is, no placebo (sugar pill) will be used. Treatment will require the placement of an IV tube into a vein under the skin of the chest wall.

```
                              ┌──────────────────────────┐
                              │          Arm A           │
                              │        (FOLFOX)          │
                         ┌──▶ │       OXALIPLATIN        │
                         │    │            +             │
                         │    │     5-FLUOROURACIL       │
                         │    │            +             │
                         │    │       LEUCOVORIN         │
                         │    │                          │
                         │    │       For 24 weeks       │
┌───────────────┐        │    └──────────────────────────┘
│ Randomization │ ───────┤
└───────────────┘        │    ┌──────────────────────────┐
                         │    │          Arm D           │
                         │    │     (FOLFOX + C225)       │
                         └──▶ │       OXALIPLATIN        │
                              │            +             │
                              │     5-FLUOROURACIL       │
                              │            +             │
                              │       LEUCOVORIN         │
                              │            +             │
                              │       CETUXIMAB          │
                              │                          │
                              │       For 24 weeks       │
                              └──────────────────────────┘
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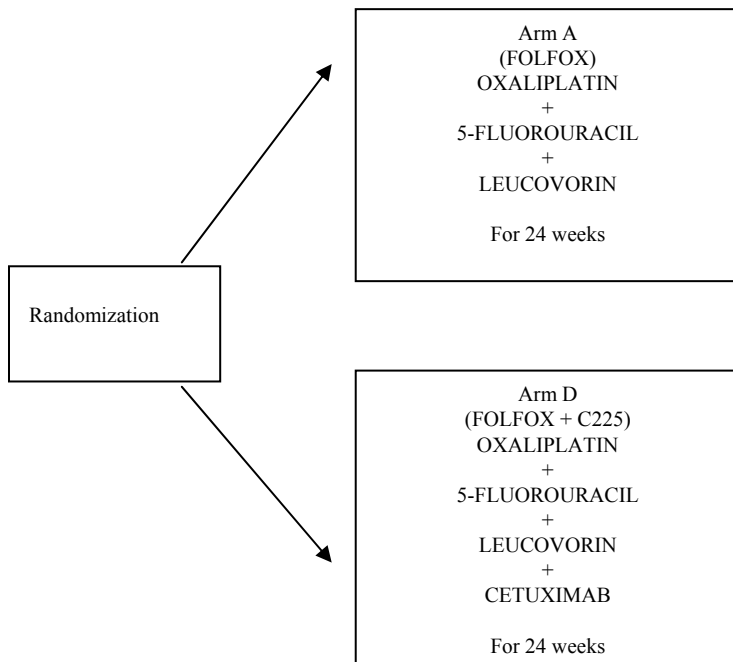
You will have two follow-up colonoscopies. These will be done one year and four years after your surgery.

Add 12

The following table tells you what tests, examinations, and treatments you will have if your KRAS result is "wild-type".

Within 30 days prior to randomization	♦ Chest x-ray (may be done within 8 weeks before randomization) ♦ US, CT or MRI scan (may be done within 8 weeks before randomization) ♦ Discuss patient/physician fact sheet and patient instructions for preventing and treating diarrhea with doctor ♦ Discuss IHC test results letter with doctor
Within 28 days prior to randomization	♦ Routine physical exam ♦ Blood tests to verify eligibility ♦ Collection of tissue blocks/slides
Within 7 days prior to randomization	♦ Blood pregnancy test for females who are able to become pregnant
Groups A and D: Oxaliplatin/5-FU/CF Treatment Cycle	♦ Day 1 treatment with Oxaliplatin and CF, IV (into a vein) for two hours ♦ Day 1 5FU IV (into a vein) injection and then continuing with 5FU IV via a small portable pump for 46 hours ♦ Treatment cycles are two weeks, treatment is for twelve 2-week cycles totaling 24 weeks.
Follow-up (beginning after you have completed study treatment)	♦ Research blood draw done only once during follow-up (Arm A or D only) ♦ Routine physical exam, blood tests, every six months for the next five years or until recurrence, whichever occurs first. ♦ Chest x-ray, or MRI scan every six months or CT at least yearly ♦ Colonoscopy one year and four years after surgery.

Add 9

If your KRAS result is "mutant" or not able to be determined, you will be enrolled to Arm G and may receive treatment determined by your physician, outside of this study. Your physician will send yearly reports to NCCTG about your treatment and how you are doing. You will not need to have any additional procedures as part of this study.

Add 6,9

How long will I be in the study?

This study will have an eight-year follow-up for all patients (Arms A, D, and G).

If you receive study treatment on Arm A or D, the length of time that you receive study treatment will depend on how you do with the drugs and how your cancer acts. Even if you stop taking the study drugs, the study doctors will still want to keep in touch with you as described in the 'Follow-up' row of the table above.

Are there reasons I might leave the research study early?

Taking part in this research study is your decision. You may decide to stop at any time. You should tell the researcher if you decide to stop and you will be informed if any additional tests may need to be done for your safety.

In addition, the researchers may stop you from taking part in this study at any time if it is in your best interest, if you do not follow the study rules you will be given, or if the study is stopped.

Add
6,9,10

What are the risks of the study?

For all patients: Possible risks related to drawing blood may involve discomfort, a risk of bleeding, bruising, or infection at the needle site.

Risks for older patients (age 70 and greater): There is a higher likelihood of severe or life-threatening side effects, or death, for patients 70 years of age and older in both Arm A and Arm D compared to younger patients. However, this risk is greater for those 70 and older who receive treatment as part of Arm D. These side effects may develop shortly after starting therapy or may occur toward the end of the planned 6 months of therapy. The potential side effects from the treatment are outlined in detail below. However, if you experience <u>diarrhea</u> that is not easily controlled with a medication such as Imodium you should contact your doctor right away or go to an emergency room. It is also important to let your doctor know if you start to have any <u>breathing problems</u>. Breathing problems may occur toward the end of the 6 months of treatment or even after you have finished all of the treatment. Finally, should you have any concerns about how the therapy is making you feel it is important that you talk to your doctor. It is very important to identify side effects while they are still mild and treat them so they do not become severe or life-threatening.

(The following risks apply to patients in Arms A and D)
If you receive study treatment on Arm A or D in this study, you are at risk for these side effects. You should talk to the researcher and/or your medical doctor about these side effects. There also may be other side effects that are not known. Side effects may range from mild to life-threatening. Other drugs may be given to make side effects less serious and uncomfortable. As with any medication, allergic reactions are a possibility. Many side effects go away shortly after the study drugs are stopped, but in some cases side effects can be serious, long lasting, or may never go away. Although small, there is always a risk of death.

While you are receiving oxaliplatin you should avoid cold drinks, very cold food, and being in cold air as it may make some of your side effects, such as numbness, worse.

FOLFOX Side Effects (Arm A, Arm D)

Add
6,7,8

<u>Very Likely</u>
- Low white blood cells (may make you more likely to get infections)
- Low platelets (may make you more likely to have bruising or bleeding)
- Anemia (Low red blood cells which may make you feel tired or weak)
- Fatigue or difficulty sleeping
- Nausea and vomiting lasting 24-48 hours following completion of the chemotherapy
- Diarrhea
- Loss of appetite/weight loss
- Mouth sores

- Numbness or tingling of the hands, feet, mouth and/or throat that can be made worse by cold weather, very cold food, or cold drinks
- Joint or muscle pain
- Abdominal pain or cramps
- Irritation of intestines
- Irritation of the esophagus (swallowing tube)
- Infection
- Fever
- Involuntary movement/restlessness
- Dehydration
- Nail changes
- Skin darkening
- Hearing changes or ringing in the ears, which could include ear pain or dizziness
- Change in taste
- Cough or hiccups
- High blood pressure
- Hot flashes/flushes
- Bleeding
- Dizziness or fainting
- Depression
- Allergic reaction or hypersensitivity to the drug infusion

Less Likely
- Flu-like symptoms such as fevers, chills, and muscle aches
- Dry or watery eyes, nasal stuffiness
- Pain or irritation of the vein or skin in the area where the drug is injected
- Allergic skin reaction (hives, welts or wheals)
- Temporary vision changes, especially when exposed to cold
- Eye pain
- Temporary blindness
- Damage or dysfunction of the liver or kidney
- Constipation
- Shortness of breath or wheezing
- Rash or redness of the skin
- Dry skin/itching
- Headache
- Intestinal blockage
- Flatulence (intestinal gas)
- A loss of phosphorus, sodium, magnesium, calcium and/or potassium from the blood that may cause a sense of weakness or muscle cramps. If symptoms occur, replacement may be necessary using either pills or intravenous treatment.
- High or low levels of sugar in the blood (hyperglycemia/hypoglycemia)
- Inflammation of the lungs
- Blistering of the palms of the hands and soles of the feet (hand-foot skin reaction)
- Swelling of the head and neck, arms or legs
- Low blood pressure
- Problems with blood clotting
- Sweating
- Weight gain
- Dry mouth
- Difficulty swallowing (dysphagia)
- Inflammation of the stomach or pancreas
- Heartburn/dyspepsia
- Ulcer
- Anxiety

Add 13

- Voice changes
- Frequent/urgent urination
- Temporary hair loss

Add
6,7,8

Rare, but serious
- Death
- Confusion or memory loss
- Speech impairment, such as slurred speech
- Damage or build-up of scar tissue in lungs, which could interfere with breathing
- Stroke
- There have been 3 deaths reported in older patients who had developed weakness, diarrhea, and low blood pressure. These deaths may have been the result of dehydration that was caused by the diarrhea, and an infection. It is important that the occurrence of diarrhea be promptly reported to your physician.
- A breakdown of red blood cells and kidney failure known as the hemolytic uremic syndrome.
- Heart attack or chest pain
- Lung failure
- Blood clot, which can go to the lungs
- Abnormal heartbeat
- A rare serious side effect, veno-occlusive disease of the liver (VOD), occurred when oxaliplatin and 5-fluourouracil were given in combination. VOD is a disease that sometimes occurs after high-dose chemotherapy or radiation in which the blood vessels that carry blood through the liver become swollen and clogged.
- Disseminated intravascular coagulation (DIC), a condition where abnormal blood clotting and bleeding occurs

Add
7,8,12

Oxaliplatin Side Effects:
Likely:
- Increased blood level of a liver enzyme (ALT/SGPT or AST/SGOT)
- Inflammation (swelling, irritation, or redness) or deterioration of your nerves outside of brain and spinal cord which may cause numbness, tingling, or burning

Add 8,12

Less Likely:
- Irritation or sores in the lining of the mouth or difficulty/limitation in ability to open mouth
- Inflammation in the middle ear
- Seizures
- Destruction of tissue or ulcer somewhere in the digestive tract
- Loss of muscle coordination; awkward, uncoordinated walk or walking difficulties, such as limp
- Pain: stomach, joint, back, bone, muscle, or chest (not heart related)
- Temporary blindness of one eye due to blockage (or decreased blood flow) in the blood vessels leading to that eye
- Inflammation of a vein, or damage to the tissue in the area where the drug is injected
- Skin rash with the presence of macules (flat discolored area) and papules (raised bump)
- Hives
- Sudden reddening or swelling of the face and/or neck
- Depression
- Weight loss
- Chills
- Dehydration
- Fever, including a fever associated with dangerously low levels of a type of white blood cell (neutrophils)
- Hot flashes
- Decreased number of white blood cells (lymphocyte, neutrophil/granulocyte) or total number of white blood cells (leukocytes)
- Destruction of red blood cells

- Abnormal reaction of the body to substances, called allergens, that are contacted through the skin, inhaled into the lungs, swallowed, or injected (allergic reaction)
- Increased blood level of a liver enzyme (GGT), a liver or bone enzyme (alkaline phosphatase), or a liver pigment (bilirubin), which is often a sign of liver problems
- Increased blood level of creatinine (a substance normally eliminated by the kidneys into the urine)
- Increased INR (measure of the ability of the blood to clot properly) which increases the risk of bleeding
- More acid than normal in the blood, including uric acid (a waste material from food digestion)
- Decreased levels of a blood protein called albumin
- Problems with eye lids
- Inflammation around the nerve in the back of the eye, which could lead to problems with vision
- Inflammation of the conjunctiva (the outermost layer of the eye and the inner surface of the eyelids). This is commonly called "pink eye".
- Temporary vision problems caused by the cold
- Confusion
- Feeling of imbalance, lightheadedness, or unsteadiness (dizziness)
- Hearing loss
- Taste changes
- Loss of appetite
- Sinus problems
- Accumulation of fluid in the abdomen (ascites)
- Stomach pain
- Bleeding with a decreased number of blood cells that help to clot blood (platelets)
- Bleeding in the respiratory tract, kidney, prostate, reproductive organs (e.g., vagina, testes), digestive tract or brain
- Inflammation of the esophagus (tube that carries food from the mouth to the stomach), the stomach lining, the small or large bowel (colon), or pancreas (otherwise known as pancreatitis)
- Partial or complete blockage of the small and/or large bowel. Ileus is a functional rather than actual blockage in the bowel.
- Weakness or paralysis (loss of muscle function) caused by damage to peripheral nerves outside brain and spinal cord, including facial, head and neck muscles
- Decreased blood flow to the brain which may lead to stroke
- Problems with speech, such as restless, repetitive, or involuntary movements and rapid speech
- Increases/decreases in blood pressure
- Inability to fall or remain asleep
- Sleepiness
- Blood in the urine
- Difficulty emptying or retaining urine in the bladder
- Inflammation or damage (scarring) to the lung, which could interfere with breathing
- Sneezing
- Cough
- Hiccups
- Sudden constriction of the muscles in the walls of the bronchioles (small airways of the lung)
- Higher risk of blood clots including formations of clots that can break loose, move through the blood stream, and block another blood vessel
- Abnormal heartbeat that could include slow, fast, regular or irregular rhythm. **May be life-threatening, needs immediate attention.**
- Infections (bacterial, fungal, viral, or other unusual infections) that could be life-threatening
- Liver and/or kidney damage or failure

Rare, but serious

Add 8,12
- Formation of blood clots in small blood vessels around the body that leads to a low platelet count
- Gas in the intestinal (bowel) wall
- Inflammation of the gallbladder possibly associated with gall stones
- Sudden or traumatic injury to the kidney
- Swelling and redness of the skin on the palms of the hands and soles of the feet
- Severe potentially life-threatening damage to the lungs, which could lead to fluid in the lungs

Cetuximab Side Effects (Arm D) *(discontinued as of November 25, 2009)*

Add 6,7,12
Likely
- Diarrhea
- Nausea or the urge to vomit
- Fatigue or tiredness
- Fever
- Headache or head pain
- Dry skin
- Acne
- Skin rash with the presence of macules (flat discolored area) and papules (raised bump)

Add 6,7,12
Less Likely
- Lack of enough red blood cells (anemia)
- Inflammation (swelling and redness) of the skin of outer ear and canal
- Noise in the ears, such as ringing, buzzing, roaring, clicking
- Inflammation (swelling and redness) of the conjunctiva (the outermost layer of the eye and the inner surface of the eyelids). Commonly called "pink eye".
- Dry eye
- Inflammation (swelling and redness) of the middle layer of the eye (uvea)
- Excessive tearing in the eyes
- Belly pain
- Inflammation (swelling and redness) of the lip
- Constipation
- Dry mouth
- Heartburn
- Irritation or sores in the lining of the mouth
- Vomiting
- Chills
- Swelling of the arms and/or legs
- Flu-type symptoms (including body aches, fever, chills, tiredness, loss of appetite, cough)
- Reaction that can occur during or following infusion of the drug. The reaction may include fever, chills, rash, low blood pressure, and difficulty breathing.
- Chest pain not heart-related
- Allergic reaction by your body to the drug product that can occur immediately or may be delayed. The reaction may include hives, low blood pressure, wheezing, swelling of the throat, and difficulty breathing.
- Infection
- Decreased number of a type of white blood cell (neutrophil/granulocyte)
- Weight loss
- Decrease in the total number of white blood cells (leukocytes)
- Loss of appetite
- Dehydration (when your body does not have as much water and fluid as it should)
- Decreased blood level of calcium
- Decreased blood level of magnesium
- Joint pain
- Back pain
- Muscle pain

- Fainting
- Stuffy or runny nose, sneezing
- Sudden constriction of the small airways of the lung that can cause wheezing and shortness of breath
- Cough
- Shortness of breath
- Hoarseness
- Hair loss
- Loss of some or all of the finger or toenails
- Increased skin sensitivity to sunlight
- Itching
- Area of bleeding within the skin causing a reddish purple discoloration
- Sore or destruction of skin
- Hives
- Low blood pressure
- Formation of a blood clot that plugs the blood vessel; blood clots may break loose and travel to another place, such as the lung

Add
6,7,12

Rare, but Serious
- Serious, life-threatening allergic reaction requiring immediate medical treatment by your doctor. The reaction may include extremely low blood pressure, swelling of the throat, difficulty breathing, and loss of consciousness.
- Inflammation of the lining of the brain and spinal cord
- Inflammation of the lungs that may cause difficulty breathing and can be life-threatening
- Fluid build-up in the lungs that is not due to a heart problem that can be life-threatening
- Swelling and redness of the skin on the palms of the hands and soles of the feet

Additional risk information for protocols involving chemotherapy and cetuximab in patients with advanced NSCLC
- In clinical trials involving patients with advanced, non-small cell lung cancer, the combination of cetuximab and chemotherapy may increase the risk of life-threatening complications, some of which may be fatal, in elderly patients (65 years old or more), particularly those with pre-existing cardiac disease.

Intravenous Injection Side Effects: If the drug leaks from the vein the shot is given into, it may cause sores on the skin or severe local redness, pain, and/or swelling.

Allergic Reactions: As with any medication, there is the chance of an allergic reaction.

This study may hurt an unborn or breast-fed child. There is not enough medical information to know what the risks might be to a breast-fed infant or to an unborn child of a man or woman who takes part in this study. Men who are able to father a child and women who can become pregnant must use birth control plans while you are receiving study treatment ***and for at least 60 days after the last dose of cetuximab***. Breast-feeding mothers must stop breast-feeding to take part in this study. Women who can become pregnant must have a pregnancy test before taking part in this study. If you are a woman who can become pregnant, blood will be taken from a vein in your arm with a needle within 7 days before you enter the study. You will be told if you are pregnant or not. If you are pregnant, you will not be able to take part in the study. In addition, the effects of cetuximab last up to 2 months (60 days). It is therefore important that if you take part in this study and receive cetuximab that you do not try to become pregnant or father a child for at least 60 days after completing your therapy.

Are there benefits to taking part in this research study?

No benefit can be guaranteed by taking part in this study and the chance of knowing whether or not you will receive any benefits from the study is not able to be accurately predicted.

^{Add}
5,6,7,10

What other choices do I have if I don't take part in this research study?

- The standard recommended treatment for your cancer is to perform the surgery you have already undergone and then to take treatment with FOLFOX chemotherapy for six to seven months.
- All of the drugs in this study are approved for use in people with colon cancer in North America outside of enrollment in this study (although cetuximab is not approved for the stage of colon cancer that is being looked at in this study). Therefore you can get access to standard and alternative treatments without enrolling in this trial.

Add
6,7,9,11,10

Will I need to pay for the tests and procedures?

For all patients: The costs of drawing blood for research purposes and performing KRAS, IHC, and all other research testing, will be covered by the study.

For patients on Arm A or D:
The drug cetuximab will be provided free of charge through NCI; however, you may still need to pay for the cost of having cetuximab made ready for your use.

Oxaliplatin will be provided through NCI for patients enrolled before _____ (*insert local activation of Addendum 10 date)*. For patients enrolled on or after _____ (*insert local activation of Addendum 10 date)*, oxaliplatin will be obtained commercially. If your insurance does not cover the cost of oxaliplatin, Sanofi-Aventis has a program to provide it for you free of charge. Information regarding the PACT+ program is available on the world wide web at
http://www.oncology.sanofi-aventis.us/docs/pdf/Pact+SM%20Program%20Services.pdf.

You and/or your health plan will need to pay for all costs associated with the FOLFOX (5-fluorouracil and leucovorin) treatment. You and your health plan might also have to pay for other drugs or treatments that are given to help you control side effects. Before you take part in this study, you should call your health insurer to find out if the cost of these tests and/or procedures will be paid for by the plan. Some health insurers will not pay for these costs. You will have to pay for any costs not covered by your health insurer.

You may find a National Cancer Institute guide: "Clinical Trials and Insurance Coverage – a Resource Guide" helpful in this regard. You may ask your doctor for a copy, or it is available on the world wide web at http://cancer.gov/clinicaltrials/insurance.

Every effort will be made to ensure that adequate supplies of cetuximab are available free of charge for all who take part. If, however, cetuximab becomes commercially available for how it is used in this study while you are being treated, there is a possibility that you and/or your health plan would be asked to purchase subsequent supplies.

Add 6

What happens if I am injured because I took part in this research study?

It is important that you tell your study doctor, _____ [investigator's name(s)], if you feel that you have been injured because of taking part in this study. You can tell the doctor in person or call him/her at _____ [telephone number]. You will get medical treatment if you are injured as a result of taking part in this study. You and/or your health plan will be charged for this treatment. The study will not pay for this medical treatment.

What are my rights if I take part in this research study?

Add 6

Taking part in this research study is your choice. You may choose either to take part or not to take part in the study. If you decide to take part in this study, you may leave the study at any time. No matter what decision you make, there will be no penalty to you and you will not lose any of your medical care from our institution. We will tell you about new information or changes in the study that may affect your health or your willingness to continue in the study. In the case of injury resulting from this study, you do not lose any of your legal rights to seek payment by signing this form.

Who can answer my questions?

You may talk to Dr. (_____), at any time about any questions or concerns you have on this study.

You can get further information about policies, the conduct of this study, or the rights of research subjects from _____.

You may also call the Project Office of the NCI Central Institutional Review Board (CIRB) at 888-657-3711 (from the continental US only).

Where can I get more information about clinical trials?

You may call the NCI's Cancer Information Service at 1-800-4-CANCER (1-800-422-6237) or TTY: 1-800-332-8615.
Visit the NCI Web site: For clinical trials go to http://cancer.gov/clinicaltrials
                            For cancer information go to http://cancer.gov/cancerinformation

What about confidentiality?

Information from this study may be published or presented at scientific meetings. However, your name and other identifying information will not be sent outside of NCCTG without written permission unless the law allows it. Your medical record will be used by the researchers in this study. Representatives of North Central Cancer Treatment Group (NCCTG) will be able to look at your medical records to check the accuracy of the forms completed for the study. Information from your medical records may also be made available to the Food and Drug Administration, the Cancer Trials Support Unit (CTSU; a research group sponsored by the National Cancer Institute [NCI] to provide greater access to cancer trials), the National Cancer Institute (NCI) and its authorized representatives and its collaborators, NCI Central Institutional Review Board, other U.S. government agencies including the Office for Human Research Protections or other offices within the Department of Health and Human Services, and/or the Office of the Inspector General.

Add
6,9,10,12

What is being done with my blood and tissue samples?
This section applies to all patients – Arms A, D, and G (except for optional recurrent tissue)

Before it is determined which arm of the study you will be in, samples of your blood (about two tablespoons) and tissue blocks from your surgery will be collected. After you go off or complete treatment, samples of your blood (about two tablespoons) will be collected at your next follow-up visit (Arm A or D patients only). These samples are required for the study and will be used to look at characteristics of your tumor that may be the result of your genes. Also, if your cancer comes back (Arm A or Arm D patients only) and you have surgery to remove it, we would like to use leftover tissue samples from this surgery for additional research. You will not need an additional biopsy done. These tissue samples from surgery to remove cancer that has come back are not required, but we strongly encourage you to provide them. Because the research tests in this study are not used for regular medical care, the test results will not be put in your medical record. You will be given the option of learning the results of some testing (IHC) that researchers are doing on part of your colon tumor. If you wish to receive this result there is a separate letter you will need to sign. Your physician will tell you how to get genetic counseling, if this is needed. You are being offered this test result because the test result may be useful in selected families. All results from such tests will be kept confidential and known only to the principal investigators for the clinical and research portions of the study and the lead statistician.

Add
12

Please read the following statement and mark your choice:

If my cancer comes back and I have surgery to remove it, I agree to provide tissue sample(s) to NCCTG for research testing planned as part of this study.

☐ Yes   ☐ No   Please initial here: _____   Date: _____

Add
6,10,11

Will any biological sample(s) be stored and used in the future for other studies by the North Central Cancer Treatment Group (NCCTG)?

Yes, if you agree to let us do so. Another part of this research study is storing samples of your blood and tissue for future research studies. Future research studies may be done to learn more about colorectal cancer or other diseases. The samples may be stored indefinitely. You can decide whether or not your stored samples are used in future research outside of this study. You can still take part in this treatment study without having your samples be used for <u>future</u> research outside of this study.

If you change your mind about allowing your samples to be stored for future research outside of this study, and want the sample to be destroyed or returned to you contact your study doctor.

Your samples will be stored safely at NCCTG and will be given a code (rather than your name) when used in research. This code will allow your samples to be used without anyone knowing that it is yours just by looking at the label.

Your samples will be used only for research and will not be sold. You will not be paid for allowing your samples to be used in research even though the research done on the samples may help to develop new products in the future.

Sometimes blood and tissue are used for genetic research (research about diseases that are passed on in families). Even if your samples are used for genetic research, the findings will not be linked with your medical records and they will not be given to people outside of the research process.

Add 9   ***Please read the following statements and mark your choice:***

1.   I permit my samples (blood and tissue) to be stored and used in future research of colorectal cancer.

☐ Yes   ☐ No   Please initial here: _____   Date: _____

2.   I permit my samples (blood and tissue) to be stored and used in future research to learn about, prevent, or treat any other health problems:

☐ Yes   ☐ No   Please initial here:_____   Date:_____

NCCTG has the right to end storage of the sample without telling you.

Add 6   The samples will be stored at NCCTG. Outside researchers may one day ask for a part of your samples for studies now or future studies.

Add 6   Researchers from universities, hospitals, and other health organizations do research using blood and tissue. They may contact NCCTG and ask for samples for their studies. NCCTG looks at the way that these studies will be done, and decides if any of the samples can be used. NCCTG sends the blood and tissue samples and some general information about you and your health (such as stage/disease, age, sex, etc.) to the researcher. NCCTG will not send your name, address, phone number, social security number, or any other identifying information to the researcher. If you allow your samples to be given to outside researchers, it will be given to them with a code number. If researchers outside NCCTG use the samples for future research, they will decide if you will be contacted and, if so, they would have to contact you through the researchers at NCCTG.

Add 7   3.   I permit NCCTG to give my samples to outside researchers:

Please mark one box:
☐ Yes   ☐ No   Please initial here:_____   Date:_____

Add 6   **I have had an opportunity to have my questions answered. I have also been given a copy of this form. I agree to take part in this research study.**

_____   _____
(Date)   (Printed Name of Participant)

                         _____
                         (Signature of Participant)

_____   _____
(Date)   (Printed Name of Individual Obtaining Consent)

                         _____
                         (Signature of Individual Obtaining Consent)

Appendix II

ECOG Performance Status

Grade

0	Fully active, able to carry on all predisease activities without restriction (Karnofsky 90-100).
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light housework, office work (Karnofsky 70-80).
2	Ambulatory and capable of all self-care, but unable to carry out any work activities. Up and about more than 50 percent of waking hours (Karnofsky 50-60).
3	Capable of only limited self-care, confined to bed or chair 50 percent or more of waking hours (Karnofsky 30-40).
4	Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair (Karnofsky 10-20).
5	Dead

Appendix III

New York Heart Association Classifications

Clinical Evaluation of Functional Capacity of Patients
with Heart Disease in Relation to Ordinary Physical Activity

Class	Cardiac Symptoms	Limitations	Need for Additional Rest*	Physical Ability to work**
I	None	None	None	Full time
II	Only moderate	Slight	Usually only slight or occasional	Usually full time
III	Defined, with less than ordinary activity	Marked	Usually moderate	Usually part time
IV	May be present even at rest, and any activity increases discomfort	Extreme	Marked	Unable to work

*   To control or relieve symptoms, as determined by the patient, rather than as advised by the physician.

**   At accustomed occupation or usual tasks.

Reference: Bruce, R. A.: Mod. Concepts Cardiovasc. Dis. 25:321, 1956. (Modified from New York Heart Association, 1953).

Appendix IV

Patient Instructions for Preventing and Treating Diarrhea

Be aware of your bowel movements. At the first signs that stools are softer than usual, runny or watery, or if you have more bowel movements in a day than normal, begin taking loperamide. **If you do not start taking loperamide right away, severe diarrhea may occur. This can last several days and require hospitalization.** Please follow these directions carefully.

- Take two capsules (4 mg) at the first signs of any changes (see above).

- Continue taking one capsule (2 mg) every two hours until you have returned to your normal pattern of bowel movements for 12 hours. Start taking loperamide again (same doses and frequency) if diarrhea returns.

- During the night, you may take two capsules every four hours instead.

- If you have diarrhea, stop taking any laxatives and avoid dairy products.

- Please call your doctor if you have any questions about taking loperamide, if your diarrhea is not under control after three days, if you are feeling extremely weak, or if you think you are having any side effects.

- Be sure to drink plenty of fluids each day (several glasses of water, fruit juice, soda, soup, etc.). This will help prevent dehydration (note: fluids do **not** cause diarrhea).

- Side effects of loperamide may include tiredness, drowsiness or dizziness. If you experience these effects, avoid driving motorized vehicles or operating machinery.

Appendix V

Administering Questionnaires:
Instructions for Clinical Research Associates (CRAs)

Questionnaires are no longer required to be submitted

Appendix VI – Questionnaires

Linear Analogue Self Assessment (LASA) Scale

As of Addendum 7, the LASA questionnaire will no longer be required for newly enrolled patients.

Brief Food Questionnaire

As of Addendum 9, patient completed questionnaires will no longer be required for newly enrolled patients

Appendix VIII : Questionnaires

Completion Questions

As of Addendum 9, patient completed questionnaires will no longer be required for newly enrolled patients.

Appendix IX
Focused Health Assessment Patient Questionnaire

As of Addendum 9, patient completed questionnaires will no longer be required for newly enrolled patients.

Appendix X
Patient and Physician Fact Sheet

As of Addendum 11, this fact sheet is no longer used.

Appendix XI
NCI/Sanofi-Aventis CRADA

Add 10,11

The agent, oxaliplatin, used in this protocol, has been provided to the NCI under a Cooperative Research and Development Agreement (CRADA) between **Sanofi-Aventis** (hereinafter referred to as Collaborator) and the NCI Division of Cancer Treatment and Diagnosis for all patients pre-randomized prior to activation/implementation of Addendum 10 at participating sites. Oxaliplatin will be obtained commercially for new patients pre-randomized after activation/implementation of Addendum 10 at participating sites. Therefore, the following obligations/guidelines apply to this study:

1. The oxaliplatin supplied by the NCI Division of Cancer Treatment and Diagnosis during the course of this study may not be used outside the scope of this protocol, nor can the oxaliplatin supplied by NCI Division of Cancer Treatment and Diagnosis be transferred or licensed to any party not participating in the clinical study. Collaborator data for oxaliplatin are confidential and proprietary to the collaborator and should be maintained as such by the investigators, even if the supply of oxaliplatin for some patients enrolled on the clinical study is later switched to commercial supply.

2. For a clinical protocol in which an investigational agent has been used in combination with (an)other investigational agent(s), each the subject of different CTAs or CRADAs, the access to and use of data by each Collaborator shall be as follows, even if supply of the investigational agent(s) is later switched to commercial supply (data pertaining to such combination use shall hereinafter be referred to as "Multi-Party Data"):

    a. NCI must provide all Collaborators with written notice regarding the existence and nature of any agreements governing their collaboration with NIH, the design of the proposed combination protocol, and the existence of any obligations that would tend to restrict NCI's participation in the proposed combination protocol.

    b. Each Collaborator shall agree to permit use of the Multi-Party Data from the clinical trial by any other Collaborator to the extent necessary to allow said other Collaborator to develop, obtain regulatory approval, or commercialize its own investigational agent.

    c. Any Collaborator having the right to use the Multi-Party Data from these trials must agree in writing prior to the commencement of the trials that it will use the Multi-Party Data solely for development, regulatory approval, and commercialization of its own investigational agent.

3.   The NCI encourages investigators to make data from clinical trials fully available to
        Collaborator(s) for review at the appropriate time (see # 5). The NCI expects that clinical
        trial data developed under a CTA or CRADA will be made available exclusively to
        Collaborator(s), and not to other parties.

4.   When a Collaborator wishes to initiate a data request, the request should first be sent to
        the NCI, who will then notify the appropriate investigators (Group Chair for cooperative
        group studies, or PI for other studies) of Collaborator's wish to contact them.

5.   Any data provided to Collaborator(s) must be in accordance with the guidelines and
        policies of the responsible Data Monitoring Committee (DMC), if there is a DMC for this
        clinical trial.

6.   Any manuscripts reporting the results of this clinical trial should be provided to CTEP for
        immediate delivery to Collaborator(s) for advisory review and comment prior to
        submission for publication. Collaborator(s) will have 30 days from the date of receipt for
        review. An additional 30 days may be requested in order to ensure that confidential and
        proprietary data, in addition to Collaborator(s) intellectual property rights, are protected.
        Copies of abstracts should be provided to Collaborator(s) for courtesy review following
        submission, but prior to presentation at the meeting or publication in the proceedings.
        Copies of any manuscript and/or abstract should be sent to:

                Regulatory Affairs Branch, CTEP, DCTDC, NCI
                Executive Plaza North, Room 7111
                Bethesda, Maryland 20892
                FAX 301-402-1584

          The Regulatory Affairs Branch will then send them to Collaborator(s).

**Appendix XII
Additional N0147 Information**

1.0 DATA SUBMISSION

☐ Post enrollment data submissions should be accompanied by a NCCTG Data Submission Cover Sheet. Mail all forms to the NCCTG Operations office (see protocol resource page).

☐ See Section 18.0 for records and data collection procedures.

☐ Only current versions of CRFs may be submitted. Please refer directly to the forms located on the Forms Packet on the CTSU Members' Only Website.

☐ The **NCCTG Patient ID number** must be specified on all pages of documents submitted for N0147.

☐ Initials on all Case Report Forms should be the same as they are collected on the registration form: **Last, First, Middle**.

☐ The **Patient Medical Record Number** is your local internal patient number.

Pre-Randomization and Registration/Randomization

☐ Patient not continuing onto Randomization (Arm A or D) or registration (Arm G) must complete and fax the Preregistration Screening Failure form directly to NCCTG (refer to Forms Packet on the CTSU members' web site).

Patient Did Not Receive Treatment (Arms A and D)

☐ If a patient does not receive treatment (and is classified as a cancel), it is necessary to provide follow-up information. The patient will go directly to the event-monitoring phase of the study. On-study material is to be submitted, which includes tissue and blood specimens.

☐ The site must immediately contact NCCTG N0147 QAS regarding the cancellation status of the patient.

Patient Did Not Receive Treatment (Arm G)

☐ If a patient does not receive treatment (and is classified as a cancel), it is necessary to provide follow-up information. The patient will go directly to the event-monitoring phase of the study. On-study material is to be submitted, which includes tissue and blood specimens.

☐ The site must immediately contact NCCTG regarding the cancellation status of the patient (see protocol resource page.

Ineligible Patient

☐ All applicable forms are required to be submitted, even if a patient is deemed ineligible, a cancellation, or in major treatment violation. In such cases, patients are still considered part of the study and proceed to other phases of the trial (per Section 13.0) as this study will be using an "intent to treat" analysis for the primary endpoint.

☐ If a patient is deemed ineligible or in major treatment violation, further treatment is at the discretion of the treating physician.

  ▪ If the patient discontinues treatment, they are to begin the observation phase and then also complete event monitoring.

  ▪ If the patient continues treatment, they will proceed with the study schedule as planned (e.g., test, observation, and follow-up schedules), including submission of all required

forms for those study periods. See Protocol Sections 4.0 and 18.0. All materials are required to be submitted (e.g., forms, tissue, bloods) regardless of eligibility status.

Add 13

Withdrawn Consent or Lost to Follow Up

☐ If a patient withdraws consent, site must submit a letter that clearly states that the patient has withdrawn consent and refuses to participate in the follow up phases (Observation and Event Monitoring), or if a patient is considered Lost to Follow Up, site must submit a letter summarizing the attempts made to contact the patient. A Follow Up Form must also be completed for the next scheduled visit. Mail both the letter and the Follow Up Form to NCCTG (see protocol resource page).

☐ NCCTG to begin the review process. Once accepted by NCCTG, a notification of NCCTG's acceptance will be communicated to the site via an email.

Add 13

Clinical Reports

☐ Copies of reports that are submitted to the NCCTG must include the NCCTG patient ID and protocol number (N0147) on all pages of the report.

☐ The patient's name must be redacted.

☐ Colonoscopy reports, operative reports, and pathology reports must be accompanied by their respective submission forms and a completed NCCTG Data Submission Cover Sheet when submitted to NCCTG. These submission forms are included in the N0147 Forms Packet.

Follow Up Form

☐ This form is to be submitted at the first "Visit" following the end of active treatment (see below). In certain instances noted below, a patient will need this form completed if they have discontinued treatment prior to completing the planned number of cycles per protocol.

☐ If a patient discontinues or refuses treatment and refuses any further study participation, without having had a Follow-Up Form submitted, please submit this form with a Visit number of 1 and the date last known alive as the date of last contact associated with the end of active treatment.

Add 11

☐ If the reason the patient ends active treatment early is disease recurrence or death, a follow up form should be submitted immediately. The visit would be indicated as "OTHER" with a visit number as 0" which indicates "not applicable, occurred during treatment". Patients with disease recurrence enter directly into the Event Monitoring Phase.

Add 11

☐ <u>Observation Phase</u> begins on date active treatment ends or date treatment discontinued due to toxicity, refusal etc. and continues until recurrence or 5 years post registration/randomization (whichever occurs first). Forms are due every six months.

   o First follow-up visit in Observation Phase should be (Arms A and D only):
      ▪ The six (6) month anniversary of the date the patients d/c protocol treatment (date recorded as Last Date Protocol Therapy was Given from the Adjuvant Disease Treatment Summary CRF)
      ▪ Each subsequent follow up assessment in the Observation Phase should be no greater than six (6) months after the last follow up visit date.

Add 11

☐ <u>Event Monitoring Phase</u> begins at disease recurrence or the end of the observation phase and continues for eight years from registration/randomization, recurrence or death (whichever occurs first). Submit the follow-up form annually. Visit number should continue sequentially from the Observation Phase.

- o First follow-up assessment in the Event Monitoring Phase should be:
  - ▪ For Arm G Patients -
    - On the annual anniversary of the Last Date Adjuvant Therapy was Given on the Adjuvant Disease Treatment Summary Form and continue annually.
    - Each subsequent follow up assessment in the Event Monitoring Phase should be no greater than one year after the last follow up visit date.
  - ▪ For Arms A and D Patients –
    - For patient d/c study therapy due to recurrence, the first follow up form with Visit OTHER, 0, is due with the Adjuvant Disease Treatment Summary Form. Follow Up Visit 1, is expected on the annual anniversary of the Last Date Adjuvant Therapy was Given on the Adjuvant Disease Treatment Summary Form
    - For patient's completing the Observation Phase, the first assessment in this phase should be one year from the last follow up assessment in the Observation Phase.
    - Each subsequent follow up assessment in the Event Monitoring Phase should be no greater than one year after the last follow up visit date.

☐ <u>Patients who have started Observation or Event Monitoring Phase</u>: If patient dies or has a recurrence, prior to their next Visit and in a period of time that is shorter than the length of time required between reports (see Protocol Sections 13.0 and 18.0 for details), the visit number will be the next consecutive Visit number and the Visit Date will be the patient's date of death or recurrence date, whichever is applicable.

☐ For patients who have secondary resection for recurrence during Event Monitoring, and have not otherwise been reconsented regarding this additional tissue, the patient should be contacted after site learns of the resection, to request the patient's consent for providing tissue.

☐ If attempted contacts with a patient fail for at least a year, please contact the NCCTG N0147 QAS for further direction.

Adverse Event Reporting

☐ This study will utilize the CTCAE version 3.0 for toxicity and Adverse Event (AE) reporting. MedDRA 6.0 must be used on Case Report Forms. MedDRA v6.0 document "MedDRA v6.0 Coding For Adverse Events (AEs)", document date 10/04/07 is accessible on the N0147 page of the CTSU members web site at https://members.ctsu.org/ under Documents → All.

☐ Your local Institutional Review Board must be informed of all reportable serious adverse reactions.

☐ Submit expedited reports electronically to NCCTG using the CTEP AdEERS application. Do not copy the CTSU on these reports. Refer to the N0147 Protocol Section 10.0 for details.

2.0 SPECIAL MATERIALS or SUBSTUDIES

Add
6,8,9,10,
11,12,13

2.1 Pathology

- Collection of paraffin-embedded blocks or slides is mandatory for pre-randomization quality control purposes and translational research studies. Refer to Section 14.1 (Pharmacologic/Ancillary Studies), 17.0 (Pathology Considerations), and Appendix XIV (Translational Research) of the protocol for details on specimen collection and submission.

- Collection of paraffin-embedded blocks or slides from secondary resection is optional for translational research studies. Refer to Section 14.1 (Pharmacologic/Ancillary Studies), 17.0 (Pathology Considerations), and Appendix XIV (Translational Research) of the protocol for details on specimen collection and submission.

- Mail copies of pathology and operative reports to NCCTG accompanied by a completed NCCTG Data Submission Cover Sheet and respective report submission form (found in the N0147 Forms Packet).

- Tissue banking for future research studies is considered optional.

Add
6,7,9,10,
11,12,13

2.2 Whole Blood

- Baseline (all patients) and one observation follow-up (Arm A and Arm D only) collection of whole blood is mandatory and will be used for translational research studies. Refer to Section 14.0 (Pharmacologic/Ancillary Studies) and Appendix XIII (Blood Specimen Logistics) of the protocol for details. **Note that blood collection kits for blood draws should be available at the time the patient is pre-randomized (all patients) or at the time of the patient's first or next Observation visit (patients randomized to Arm A or Arm D only) (necessitating that each site should have blood kits pre-ordered.)** To request a kit, download the BAP Fax Supply Order Form for Ordering Specimen Kits posted under the Site Registration Documents Section of the CTSU N0147 Web page and fax as directed on the form.

- Forward the original BAP Requisition Form along with the blood samples to Biospecimen Accessioning and Processing (BAP) Receiving via the kits and prepaid mailers. All samples must be collected and shipped **Monday-Thursday ONLY.** Specimens must be shipped the same day as they are collected. Do **NOT** collect or ship blood specimens on Friday, on the weekend, or the day before or day of an observed United States national holiday. NOTE: Use kit mailing labels for shipment to BAP Receiving. Maintain a copy of the BAP Requisition Form for your files.

- The NCCTG Data Submission Cover Sheet and Specimen Submission Form – Blood, are to be completed for all patients and mailed to NCCTG with other on-study material.

- Banking of blood samples for future research studies is considered optional.

Add
1,6,9,10

2.3 Epidemiology

The patients' cancer risk and dietary history will be performed using the Brief Food Questionnaire and the Focused Health Assessment (Patient Questionnaire) tools. Refer to Section 1.4, Appendix VII, and Appendix IX of the protocol for details. *Note: Questionnaires are not required for patients enrolled following implementation of Addendum 9 (August 18, 2008).*

Add
5,6,9,10

2.4 Other: Patient-Completed Questionnaires

Note: Questionnaires are not required for patients enrolled following implementation of Addendum 9 (August 18, 2008).

Please respond immediately to any correspondence regarding previously submitted questionnaires.

3.0   ADVERSE EVENT (AE) REPORTING (Arms A and D only)

3.1   Serious Adverse Event Reporting

- This study will utilize the CTCAE version 3.0 for toxicity and Adverse Event (AE) reporting. A link to the CTC guidelines is available on the CTSU registered member Web site. Investigators should employ definitions of adverse events as described in Section 10.1 of the protocol. All reporting should be conducted within the time frames specified in Section 10.2 of the protocol. MedDRA 6.0 must be used on Case Report Forms. MedDRA v6.0 Coding for Adverse Events (AEs), dated 10/04/07 is accessible on the N0147 page of the CTSU members web site at https://members.ctsu.org/ under Documents - All.

- Your local Institutional Review Board must be informed of all reportable serious adverse reactions.

- Submit expedited reports electronically to North Central Cancer Treatment Group using the CTEP AdEERS application. Do not copy the CTSU on these reports. Links to the NCI Guidelines for expedited adverse event reporting and the AdEERS application are available on the adverse events tab of the CTSU member web site (https://members.ctsu.org/) and exclusions are listed in Section 10.22. AdEERS will automatically courtesy copy WorldWide.Safety@bms.com.

Add 13

- In compliance with the NCI/CTEP mandate (dated May 28, 2010), expedited adverse event reporting requirements were converted from CTCAE v3.0 to CTCAE v4.0 (affected sections 10.1, 10.11, and 10.23) while routine data collection via Case Report Forms will remain using CTCAE v3.0 (clarifications added to sections 10.3, 16.5, 16.7, and Appendix XII). Effective April 1, 2011, expedited reporting via AdEERS must use CTCAE v4.0 while the remainder of the data collection for legacy trials will continue to use CTCAE v3.0.

Add
3,5,6,13

3.2   Secondary AML/MDS reporting:

CTSU investigators will submit the NCI Secondary AML/MDS Report Form and supporting documentation as noted in Section 10.23 of the protocol. Reporting for this event required during and after completion of study treatment via AdEERS. Beginning April 1, 2011, AdEERS will only accept CTCAE v4.0 for this study.

Appendix XIII
Blood Specimen Logistics

N0147, A Randomized Phase III Trial of Oxaliplatin (OXAL) Plus 5-Fluorouracil (5-FU)/Leucovorin (CF) with or without Cetuximab (C225) after Curative Resection for Patients with Stage III Colon Cancer

Add 2,3,5,6,9, 10,12

1. Kits will be supplied by the Biospecimen Accessioning and Processing (BAP) Shared Resource. Participating institutions may obtain kits by submitting the FAX Supply Order Form (see forms packet) to BAP. All sections of the form/specimen collection labels must be completed in order to expedite processing of the request. **Mark the tubes with the NCCTG patient registration ID provided at pre-randomization (e.g., 9000000), NCCTG N0147, patient initials, and date of collection.**

Add 7,10,12

2. A small, but sufficient supply of specimen collection kits should be ordered prior to patient entry (all patients) and prior to Arm A or Arm D patient's first Observation visit (or next Observation visit for patients who are already in the Observation phase at the time of Addendum 12 activation). Allow at least two weeks to receive the kits. BAP will not be able to forward kits to you by express mail unless the participating institution provides their own FedEx® account number or alternate billing number for express service. **NCCTG will not cover the cost for rush deliver of kits**.

Add 5.6.7.9.10.12

3. The appropriate type and number of collection tubes and a requisition form will be contained within each specimen collection kit. The requisition form contained within the kit outlines specimen collection and processing details.

    Please fill in the NCCTG patient demographics, including the NCCTG patient registration ID (e.g., 9000000), on all copies of the requisition form where indicated. Missing information will result in delays. **Forward the original BAP Requisition Form along with the blood samples to Biospecimen Accessioning and Processing (BAP) Receiving** via the kits and prepaid mailers, per Section 14.23. **Note: Use kit mailing labels for shipment to BAP Receiving.** Maintain a copy of the BAP Requisition Form for your files.

Add 11

4. See the "Non-paraffin Biospecimens" contact on the Protocol Resources page of the protocol with questions regarding specimen collection and processing.

Add 6

5. Courier services will not be used. All specimens will be shipped Federal Express Priority Overnight.

Add 6.9.11.12

6. All samples must be collected **Monday-Thursday ONLY.** Specimens must be shipped the same day collected. Do **NOT** collect or ship blood specimens on Friday, on the weekend, or the day before or day of an observed United States national holiday. Transportation services are frequently not available and the specimens may be compromised by delayed arrival at BAP.

Add 6,13

7. The Specimen Submission Form – Blood must be sent to NCCTG.

Translational Research

Background and Significance

Colorectal cancer (CRC) is the fourth most incident malignancy in the United States and ranks second only to lung cancer as a cause of cancer-related death [1]. Surgery remains the primary treatment modality and resection with 'curative intent' is possible in approximately 85% of patients [2]. The finding of regional lymph node metastases (stage III disease) at surgery warrants the use of adjuvant chemotherapy. Three distinct therapeutic classes of chemotherapy (fluoropyrimidines, platinum complexes and topoisomerase I inhibitors) have demonstrated activity in patients with advanced CRC and clinical data has shown improved outcome with combination therapy [3-6]. As with all anticancer agents, a subset of patients receiving these drugs will have systemic toxicities which include both serious acute and chronic symptoms. These side effects can, in some instances, be an obstacle for further therapy and are a major contributor to the cost of cancer care. In addition, treatment failure due to drug resistance mechanisms is common among patients receiving these agents. Regardless, adjuvant chemotherapy for node-positive disease has been shown to reduce recurrence rates and to significantly prolong patient survival compared to observation [7]. In this regard, 60-65% of stage III colon cancer patients receiving current adjuvant chemotherapy will not develop metastasis and will be cured of their disease. While pathological staging is the most important prognostic variable, these staging criteria do not precisely define which patients are at highest risk of metastases after the primary tumor has been removed. Furthermore, there are currently no prospective tools for identifying patients at risk for suboptimal treatment outcome after therapy or excessive toxicity [8]. Therefore, despite treatment, 35-40% of stage III patients will recur and die from their disease [9].

Tumor characteristics used to determine prognosis include tumor stage and to a lesser extent, histopathological features. Stage-related variables measure the extent of disease (depth of invasion of bowel wall, presence or absence of metastasis in regional nodes or distant sites, number of involved nodes) and represent modifications (TNM staging system [10], the Gunderson-Sosin [11] and Astler-Coller [12]) of the staging system proposed by Dukes in 1932 [13]. Histopathological variables (tumor differentiation or grade, extramural vascular and lymphatic invasion) also have been evaluated in an effort to improve prognostication. However, conflicting results have been obtained with some but not other studies demonstrating a significant association with survival of either colon or rectal cancer patients [14-18]. In addition, tumor staging systems have the limitation of only being applied after pathologic examination of the resected primary tumor and lymph nodes. Consequently, investigators have examined genotypic or phenotypic characteristics in colon carcinomas that may be of additional prognostic value. These studies have focused on markers of cell proliferation or on features that may contribute to metastatic capacity in colon cancer such as DNA ploidy and S-phase fraction [19-23], expression of proteases or their receptors (i.e. metalloproteinases or urokinase and the uPAR) [24, 25], and the expression of plasma membrane glycoproteins which may contribute to cell adhesion (CD44, sialyl Lewis a) [26, 27]. Unfortunately, most of these reports represent single marker retrospective or small prospective investigations, making definitive analysis difficult. In addition, interpretation of these studies often has been hampered by poor quality clinical databases, lack of standardization and/or quality control of the laboratory methodologies, or variability of statistical methodologies [28].

The Colorectal Cancer Working Group of the College of American Pathologists recently noted that the findings of these previous studies have not been incorporated into the design of prospective therapeutic trials [28]. Yet, despite these gaps in investigative analysis, clinical observation continues to show that there is considerable stage-independent variability in clinical outcome, further underscoring the need for identifying cellular and molecular biomarkers in large prospective trials. The identification and validation of such markers has several advantages including: 1) risk stratification, i.e., selection of patients for adjuvant

therapy and identification of low risk groups not likely to benefit, thereby, sparing them unnecessary toxicity; 2) identification of patients at high probability of recurrence despite established adjuvant therapy who might be candidates for novel therapies; 3) genetic profiling of tumors enabling individualized treatment to include targeted therapies producing potentially greater efficacy and less toxicity; and 4) gaining further insight into mechanisms of tumorigenesis facilitating drug development and prevention strategies. Such an approach would represent a paradigm shift from the historical use of the same cytotoxic agents for all patients with a given stage of CRC.

The current trial offers a unique opportunity for translational research. The study design includes two distinct treatment arms on which patients with wild type *KRAS* receive modified FOLFOX6 (mFOLFOX6; 5-fluorouracil (5-FU), leucovorin, and oxaliplatin) with or without cetuximab. We propose a strategy for correlative science as a companion to North Central Cancer Treatment Group (NCCTG) N0147 that will analyze cellular and molecular characteristics of primary colorectal cancers. We will determine their prognostic utility either individually or in combination in a prospective phase III adjuvant therapy trial in patients with node-positive colon carcinoma (Stage III; MAC C). Potential prognostic markers have been selected based on strong preliminary retrospective data and will be accomplished using the laboratory expertise of Mayo Clinic and collaborative investigators. These studies fall into eight broad categories:

1) molecular determinants of response to inhibition of EGFR signaling,
2) genomic instability (microsatellite instability, centrosome amplification and chromosome instability, telomere shortening),
3) epigenetics and CpG island methylation phenotype (CIMP),
4) candidate gene expression and pharmacogenetic analysis (microRNA expression profile, gene identification by transcriptional profiling and pharmacogenomics studies),
5) apoptotic susceptibility as determinant of treatment response and prognosis (pro-apoptotic BH3-only proteins as novel prognostic and predictive markers, Bcl-2 family, caspase-3 as a surrogate marker of apoptotic susceptibility, and cyclooxygenase-2 [COX-2] expression),
6) characterization of the immune profile of sporadic colon cancers in relationship to DNA mismatch repair status,
7) pathological assessment (tumor grade, tumor border configuration, peritumoral host lymphoid response), and
8) epidemiological factors and clinical outcome in CRC (methylator phenotype in relation to family history and environmental exposures, body mass index, obesity-associated circulating biomarkers, and circulating vitamin D [25-hydroxyvitamin D] concentrations).

The pathological assessment study aim will provide for the pathologic examination of accrued tissue to: a) ensure that all samples processed for Specific Aims 1-6 are representative of the overall tumor, and b) to assess the prognostic utility of current pathologic parameters for CRCs. Specific statistical considerations for each aim are presented at the conclusion.

SPECIFIC AIM 1: Molecular Determinants of Response to Inhibition of EGFR Signaling

Background and Significance: Epidermal growth factor receptor (EGFR) is a member of the ErbB/HER family of transmembrane tyrosine kinase receptors, which is involved in controlling cell growth, differentiation, and proliferation by triggering both the Ras–RAF–mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol-3-kinase (PI3K)– phosphatase and tensin homolog (PTEN)–Akt pathway [29]. Epidermal growth factor receptor constitutive activation leads to malignant transformation, angiogenesis, and metastatic dissemination. In colorectal cancer, where EGFR is frequently overexpressed, cetuximab and panitumumab, two monoclonal antibodies (MAbs) that recognize the extracellular domain of the receptor leading to its inactivation, are utilized for the treatment of metastatic disease. To date, several studies demonstrated that EGFR protein

expression detected by immunohistochemistry (IHC) in cancer specimens is insufficient to determine response to cetuximab therapy [30-33]. Thus, patient selection or exclusion for cetuximab treatment is unsupported based on EGFR IHC expression alone, demonstrating the need for identifying other markers or a combination of markers of cetuximab response.

Variations of gene copy numbers (GCN), either in terms of gains or losses, reflect the many different routes taken by individual tumors to disrupt/escape mechanisms governing normal cellular behavior. These genomic aberrations have been successfully investigated by fluorescence *in situ* hybridization (FISH) in a number of malignancies. Importantly, three recently published studies have reported on enhanced sensitivity to anti-*EGFR* MAbs in colorectal cancer patients harboring an increase of mean *EGFR* GCN by FISH [34-36]. In most solid tumors, including non–small cell lung cancer and colorectal cancer, the best characterized mechanisms underlying increased *EGFR* GCN are gene amplification and chromosome 7 polysomy [34-37]. Generally, amplification is representative of high-level genomic gain and it is readily identifiable by FISH. Conversely, polysomy mirrors variable degrees of chromosomal gains, making the evaluation of subtle changes of GCN somewhat more arbitrary. Historically, the retrieval of relevant cutoff points for gene amplification and chromosome polysomy to predict outcome after targeted therapies represents a major challenge. In the field of breast cancer, this problem is well illustrated by the ongoing debate over the classification of *HER2*-positive tumors to predict trastuzumab efficacy by FISH analysis [38].

Besides the full-length transmembrane form of EGFR, normal and malignant cells synthesize soluble EGFR (sEGFR) isoforms that lack the transmembrane and cytoplasmic domains of the receptor (reviewed by [39, 40]). These sEGFR proteins are generated by alternate mRNA splicing/processing events of the holoreceptor and have been shown to result in several sEGFR mRNA transcripts encoding 60-kilodalton (kDa) [41], 80-kDa [42], 110-kDa/140-kDa [43], and 115-kDa [44] sEGFR isoforms. Functionally, sEGFR isoforms have been shown to decrease cellular proliferation *in vitro* [45-47]. The mechanism(s) responsible for growth inhibition may involve competitive binding of ligands to sEGFRs [48-50], and/or the formation of sEGFR/EGFR heterodimers that are capable of inhibiting the holoreceptor's intracellular kinase activity [49, 51]. Consequently, sEGFR isoforms may function as dominant negative regulators of full-length EGFR signaling cascades, much as insulin-like growth factor binding proteins (IGFBPs) regulate the bioavailability of insulin and, hence, the activity of insulin receptors.

Given the ability to quantify circulating sEGFR using immunoassay methods that involve minimal risk and discomfort to patients, serum sEGFR isoforms are being investigated as potential biomarkers for a variety of cancers, including lung, breast, ovarian, and endometrial [52-62]. A limited number of studies have examined sEGFR concentrations in CRC patient sera [63-65]. Zampino et al., showed higher sEGFR concentrations at baseline were associated to best objective response in patients (n=42) treated with gefitinib/FOLFOX6 for 10 cycles followed by gefitinib maintenance [65]. Also, Abdel-Aziz and coworkers recently showed that sEGFR can differentiate between CRC patients (n=48) and normal participants (n=20) with a sensitivity of 71% at 100% specificity [63]. Finally, Spindler and colleagues demonstrated there were significant differences between pretreatment sEGFR concentrations in controls (n=126) and patients (n=118), higher baseline sEGFR concentrations were correlated with increased OS, and sEGFR increased with cetuximab cetuximab/irinotecan treatment and the magnitude of increase correlated with increased skin toxicity [64].

In addition to EGFR protein expression and GCN, and sEGFR serological concentrations, constituents of the EGFR signaling cascade and other HER family members are being assessed as potential biological markers that may affect or predict response to anti-EGFR treatment.

Numerous studies have shown that patients with *KRAS* mutations do not derive benefit from anti-EGFR monoclonal antibody treatment. In support of this, a recent American Society of Clinical Oncology Provisional Clinical Opinion addresses the use of *KRAS* status testing in metastatic CRC to predict anti-EGFR treatment response [66]. *KRAS* mutations are detected in approximately 40%- 50% of metastatic CRC, yet, even in patients with wild-type *KRAS*, response rates with anti-EGFR monoclonal antibodies are <30% [67, 68], suggesting a role for additional mechanisms of resistance. Recent studies also have shown the oncogene *BRAF* may be significant in determining response to anti-EGFR treatment [68-70]. A selective mutation in *BRAF* (V600E) appears to be of importance. As with *KRAS* mutations, the mutation in *BRAF* results in the gene being constitutively activated. Preliminary studies indicate *BRAF* mutations occur in 10-12% of patients with colorectal cancer. *BRAF* mutations appear to be independent of *KRAS* mutations. Given evidence that *BRAF* mutations block response to cetuximab in patients with metastatic disease [68-70], it will be important to determine the role of *BRAF* in the adjuvant setting. Furthermore, *PIK3CA* mutations [33, 71] loss of PTEN expression [33, 71-75], and more recently, alterations in expression of the EGFR ligands, epiregulin (*EREG*) and amphiregulin (*AREG*) [76-79], have been implicated as being potential predictive markers of resistance to EGFR inhibitors. Furthermore, recent *in vitro* studies have shown that long-term trastuzumab treatment in HER2-expressing cell lines resistant to trastuzumab can alter expression of other HER family members and can sensitize cancer cells to other HER family-directed therapies, such as gefitinib and cetuximab [80, 81]. Thus in the context of this study, other HER family member expression, in addition to EGFR expression, will be assessed for alterations in expression levels in tumor tissue collected prior to treatment and at the time of recurrence.

Finally, in multiple studies of small molecule and antibody EGFR inhibitors, a rash in response to therapy has been correlated with improved clinical outcome. Several CRC clinical studies have shown a strong correlation between the intensity and severity of the skin rash and clinical activity of either cetuximab or panitumumab therapy, either alone or in combination with other cytotoxic chemotherapy [31, 32, 82-86]. Before *KRAS* mutation data became available, this was the best marker for those most likely to benefit from therapy targeting EGFR. The mechanism underlying the correlation between skin toxicity and tumor response is currently unclear. EGFR inhibitors prevent ligand-induced activation and downstream signaling which results in growth arrest and induction of apoptosis in cells that are dependent on EGFR for survival, through the inhibition of downstream pathways such as the Ras–RAF–MAPK pathway, the PI3K-PTEN-Akt pathway, and the stress-activated protein kinase pathway that involves both protein kinase C (PKC) and the Janus kinase/signal transducer and activator of transcription (Jak/STAT) pathway [87]. Genetic polymorphisms in genes encoding these various proteins in the EGFR pathway may predict rash from cetuximab therapy or may be correlated with patient outcome.

Specific Aims:

a) To analyze molecular alterations that may predict therapeutic response to the anti-EGFR antibody, cetuximab, in stage III colon cancers from this large, adjuvant therapy trial. Specifically, we will analyze *KRAS*, *BRAF, sEGFR,* and *PIK3CA* mutations, *EREG* and *AREG* gene expression, as well as EGFR (HER1), HER2, HER3, HER4, sEGFR, PTEN, Akt, and MAPK protein expression.

b) To evaluate the clinical usefulness of *EGFR* GCN by FISH in predicting outcome to cetuximab. To this end, we will evaluate whether previously generated cutoff points [34, 36] can be validated in our independent series. Second, in view of the important intra-patient and inter-patient variability of *EGFR* GCN, we will attempt to define an optimal cutoff point on this data

set. Furthermore, using multivariate analysis, we will explore the combined use of *EGFR* GCN with the *KRAS* mutation status of the tumor.

c) To determine if pretreatment serological sEGFR concentrations correlate with sEGFR tissue expression and to determine if sEGFR concentrations and/or circulating EGFR ligands (e.g., EGF, TGF-alpha, HB-EGF, amphiregulin, epiregulin) prior to treatment and/or during observation are associated with patient outcome in stage IIII colon cancer patients treated with mFOLFOX6 plus cetuximab.

d) To determine gene expression profiles in colon cancers with wild type *KRAS* and favorable (remain disease-free) versus unfavorable (i.e., recurrence or death) patient outcomes. Similarly, gene expression profiles will be analyzed in cancers with mutant *KRAS* showing favorable versus unfavorable outcomes.

e) To determine genetic polymorphisms in genes encoding additional proteins in the EGFR pathway (e.g., *EGF, EGFR, Akt, STAT, PKC, Src*) that correlate with rash development from cetuximab therapy. Secondary aims will correlate genotype with outcome, accounting for *KRAS* mutation status.

Materials and Methods:

a) *Nucleotide Sequence Analysis*: Genomic DNA will be extracted using the QIAamp Mini kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. Mutational analyses *KRAS*, *BRAF*, *and PIK3CA* will be performed using available DNA isolated from tumor specimens. *KRAS* mutations will be analyzed using the commercially available DxS kit (see Section 1.7 of the protocol for additional information). The *BRAF* V600E mutation will be assessed as previously described [88]. The sEGFR mutations will be assessed using the MALDI-TOF MS platform (SEQUENOM, Inc., San Diego, CA) and the iPLEX Gold reaction [see Specific Aim 4(C)]. For *PIK3CA,* the majority of mutations occur in exon 9 (codons 542 and 545) and in exon 20 (codon 1047). Polymerase chain reaction (PCR) fragments will be cleaned with QIAquick PCR Purification Kit (Qiagen), sequenced on an ABI 3100A Capillary Genetic Analyzer (Applied Biosystems), and analyzed in both sense and antisense directions for the presence of heterozygous mutations. Analysis of the DNA sequence will be performed using Sequencher v4.2 (Gene Codes, Ann Arbor, MI) followed by visual analysis of each electropherogram. Appropriate positive and negative controls were included for each of the exons evaluated. Mutational analyses will be performed without knowledge of clinical outcome, including tumor response.

b) *Immunohistochemistry (IHC):* Epidermal growth factor receptor protein expression will be determined using the EGFR pharmDx™ Kit (Dako, Carpinteria, CA) per the manufacturer's protocol in the Tissue and Cell Molecular Analysis (TACMA) Shared Resource, Mayo Clinic Rochester. Tissue samples will be considered EGFR positive if at least 1% of malignant cells stain for EGFR. IHC for sEGFR (EGFR isoform D) will be performed in the laboratory of Nita J. Maihle, Ph.D., Yale University School of Medicine, as previously described [89]. All other IHCs will be performed in the TACMA Shared Resource, Mayo Clinic Rochester, using an immunoperoxidase system:

      –   Phosphorylated EGFR
      –   HER2
      –   HER3
      –   HER4
      –   PTEN
      –   Total and phosphorylated Akt
      –   Total and phosphorylated MAPK (Erk1/2)

Whole tissue sections or slides from tissue microarrays (TMAs; see Specific Aim 7) will be incubated with appropriate commercially-available primary antibodies. Isotype-matched antibodies will be used as negative controls. Positive controls will include normal colon mucosa and normal endometrium. The intensity of IHC staining will be scored as 0 (negative), 1 (weak), 2 (medium), and 3 (strong). The extent of staining will be scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%), according to the percentage of cells staining positive. The sum of the intensity and extent scores will be used as the final staining score. Tissues having a final staining score larger than 2 will be considered positive. Final staining score of 2-3 will be considered 'low' and 4-7 considered 'high' [90].

c) *EGFR Analysis by FISH*: Serial sections (4-5 μm) will be prepared from formalin-fixed paraffin-embedded tissue. *EGFR* GCN will be investigated using the LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen probes (Vysis). Following deparaffinization and dehydration, pretreatment and enzyme digestion will be done using the SPoT-Light Tissue Pretreatment Kit (Zymed) according to the manufacturer's recommendations. Probes will be applied to target area and codenatured at 85°C for 5 min before overnight hybridization at 37°C. Posthybridization washes will be done in 0.4x SSC/0.3% NP40 at 73°C for 2 min and in 2x SSC/0.1% NP40 for 1 min. Slides will be counterstained with 4',6'-diamidino-2-phenylindole and will be stored at –20°C before evaluation. Captures will be acquired using a Zeiss Axioplan 2 microscope (Zeiss) equipped with a charge-coupled device camera and a MetaSystem Isis software (MetaSystem).

FISH Interpretation: Probe signals will be enumerated in individual nuclei if they are bright, distinct, and easily assessable against a dark background relatively free of fluorescent particles and haziness. The number of hybridization signals representing genes and chromosome centromeres will be determined observing four to five tumor areas selected in proximity of relevant histologic features identifiable on the H&E slide. At least 20 representative nuclei, distributed in two to three microscope contiguous fields within an individual tumor area, will be selected for scoring. The number of gene copies and the number of centromeres per nucleus will be individually recorded for at least 100 nuclei. Chromosome polysomy will be defined as ≥3 gene signals per nucleus paralleled by similar increases in chromosome centromere signals. *EGFR* amplifications will be defined according to cutoff points previously reported in non–small-cell lung cancer [91].

d) *Serum sEGFR and Circulating EGFR Ligand Concentrations*: Serum concentrations of sEGFR will be determined using blood samples collected at baseline and during observation that have been centrifuged, aliquotted, and stored at –80°C. Maihle and colleagues (Yale University School of Medicine) have identified a 110-kDa soluble EGFR (p110 sEGFR) protein in human sera and have developed a well characterized, sensitive acridinium-linked immunosorbent assay (ALISA) for determining sEGFR concentrations in body fluids [92, 93]. Multiple masked quality control samples will be interspersed among the case samples, and all laboratory personnel will be blinded as to test or control samples and clinical information. The mean coefficient of variation of this ALISA has been shown to be <10% [92]. EGFR ligand

concentrations will be determined in plasma or serum as validated ELISAs become commercially available.

d) *Gene Expression Profiling and Data Analysis:* Microarray technology will be used to identify gene expression profiles to classify tumors into prognostic groups using the U133 2+ chip from Affymetrix (see Materials and Methods in Specific Aim 4(A) for additional details). A predictive analysis for microarrays (PAM) will be used to identify a subset of these genes whose expression profile could accurately predict those tumors that had a good outcome from those with a poor outcome.

e) *Genetic Polymorphisms:* Genes encoding additional proteins in the EGFR (e.g., *EGF, EGFR, Akt, STAT, PKC, Src*) pathway will be identified, and using linkage disequilibrium (LD), common polymorphisms will be identified from published and available online sources (including HapMap, SeattleSNPs). Algorithms for selecting single nucleotide polymorphisms (SNPs) to represent each LD "bin" will be followed to maximize the coverage of the genes. Genotyping of DNA from only those patients exposed to cetuximab will be performed in the laboratory of Dr. Robert Diasio (see Materials and Methods in Specific Aim 4(B) for additional details) or the Mayo Genotyping Shared Resource, under the direction of Dr. Julie Cunningham.

SPECIFIC AIM 2: Genomic Instability

A. Microsatellite Instability (MSI):

Background and Significance: Two distinct pathways of colorectal carcinogenesis have been described and include the chromosomal instability pathway and the mutator pathway. Unlike the chromosomal instability pathway that is characterized by allelic loss, the mutator pathway is characterized by increased mutation rates with instability of short, tandemly repeated DNA sequences known as microsatellites. There are two principle mechanisms of MSI: methylation and germline mutation. The main cause of MSI in patients with sporadic colon cancer is hypermethylation of the human mutL homolog 1 (*hMLH1*) gene resulting in loss of protein expression and consequently, defective mismatch repair (dMMR). This epigenetic silencing of *hMLH1* is estimated to occur in up to 15% of sporadic colon cancers [94]. In individuals with the syndrome of hereditary nonpolyposis colon cancer (HNPCC), MSI occurs secondary to germline mutations in *hMLH1*, human mutS homologs 2 and 6 (*hMSH2*, *hMSH6*), or human postmeiotic segregation increased 1 and 2 (*hPMS1*, *hPMS2*) genes [95]. In fact, alterations in *hMLH1* and *hMSH2* account for the majority of HNPCC cases as defined by the Amsterdam criteria [95]. Specific antibodies to hMLHI and hMSH2 proteins enable the expression of these proteins to be determined in tissue sections using a rapid, cost effective and sensitive immunohistochemical assay [96].

Human colorectal cancers with dMMR are characterized by distinct genotypic and phenotypic alterations that may also have prognostic implications. A previous retrospective NCCTG study lends support for this phenotypic variation. Thibodeau et al [97] analyzed 508 human CRC samples for MSI by evaluating 11 microsatellites on chromosomes 5, 8, 15, 17 and 18. They found that tumors with high-frequency MSI (MSI-H) were more commonly proximal (p=0.001), of lower tumor stage (p=0.01), DNA diploid (p=0.03) and from female patients (p=0.005) compared to low-frequency MSI (MSI-L) or microsatellite stable (MSS) tumors (defined in Preliminary Data). Reports regarding MSI and clinical outcome when matched for stage are less definitive. In a study of 607 CRC patients aged 50 or younger, Gryfe et al [98] found that MSI-H status was significantly

associated with patient survival (p<0.001), but a similar study by Watanabe et al [99] failed to detect a significant difference in clinical outcome based upon MSI status. Loss of DNA MMR leads to tumor cell resistance by desensitizing cells to specific DNA-damaging agents, including cisplatin [100, 101]. However, cisplatin analogs with a diamminocyclohexane carrier ligand, such as oxaliplatin, do not elicit such resistance [100, 102, 103]. HCT116 colon cancer cells carry an *hMLH1* mutation and are relatively insensitive to 5-FU treatment by clonigenic survival assay [104]. However, when chromosome 3, which contains an intact *hMLH1* gene, is transferred to HCT116 cells, MMR function is restored and the cells display sensitivity to 5-FU. Similarly, RKO colon cancer cells with hypermethylation of *hMLH1* are rendered sensitive to chemotherapeutic agents by treatment with the demethylating agent 5-azacytidine [105]. In contrast, colon cancer cell lines with defective MMR appear to be more sensitize to the topoisomerase I and II inhibitors camptothecin (CPT-11) and etoposide, respectively [106]. A retrospective analysis has been performed using patient materials from NCCTG and the National Cancer Institute of Canada (NCIC). Specifically, patients with MSI-H colon cancers did not benefit from adjuvant 5-FU-based therapy in contrast to MSI-L and MSS patients [107]. These results suggest that the presence of MSI may negate the survival advantage conferred by 5-FU treatment of stage III colon cancers. To date, there are no published studies that have examined MMR function relative to cetuximab sensitivity or response in human colorectal cancer; therefore, this study provides a unique opportunity for this assessment.

Preliminary Data:

a) MSI and Immunohistochemistry (IHC): Lindor et al [96] tested the predictive value of hMLH1 and hMSH2 protein expression by IHC for delineating the microsatellite subgroups as defined by the National Cancer Institute (NCI) consensus conference [108]. NCI microsatellite status is based on the examination of stability of 5 loci wherein if 0/5 show instability the tumor is microsatellite stable (MSS), 1/5 it is microsatellite low (MSI-L) and ≥ 2/5 it is microsatellite high (MSI-H). Using these criteria Lindor et al [96] found that the predictive value of normal protein expression for a MSS/MSI-L phenotype was 96.7%, and the predictive value of loss of expression of either protein was 100% for an MSI-H phenotype.

b) MSI and survival: A retrospective analysis of an NCCTG study of 570 Stage II and III tumors from patients randomized to either resection or 5-FU-based adjuvant treatment post resection was performed [107]. Using the NCI criteria for determining MSI, a total of 95 (16.7%) MSI-H patients were identified. With a median follow-up of 7 years, it was shown that MSI-H patients had better overall survival rates compared to MSS patients in the absence of chemotherapy (p=0.004). Adjuvant chemotherapy had a beneficial effect on overall survival among MSS patients (p=0.02), but MSI-H patients receiving chemotherapy did not benefit and actually showed a trend toward decreased survival (p=0.07).

Specific Aim: To determine the MMR status of Stage III colon cancers and to examine its relationship with recurrence-free and overall disease-specific survival rates a) overall, and b) in *KRAS* wild-type patients who are randomized to 24 weeks of adjuvant chemotherapy with mFOLFOX6 versus mFOLFOX6 plus cetuximab.

Materials and Methods:

a) *Immunohistochemistry (IHC)*: IHC for hMHL-1, hMSH-2, and hMSH-6 expression will be performed in the TACMA Shared Resource using commercially available antibodies. Briefly,

5 micron sections will be deparaffinized and rehydrated through xylene and graded alcohol washings. After antigen retrieval using 1mM EDTA (pH=8.0), samples will be incubated with the appropriate primary antibody (MLH-1 [clone G168-15, Biocare Medical], MSH-2 [clone FE11, Biocare Medical], and MSH-6 [clone BC/44 Biocare Medical]). Slides will be subsequently processed using the Envision + (DAKO) kit. Isotype-matched antibodies will be used as negative controls and appropriate positive controls will include adjacent normal colorectal mucosa stained for each protein. Appropriate control slides will be included in all slide runs. IHC slides will be scored as previously described in the Materials and Methods section in Specific Aim 1. Only those tumors showing loss of protein expression will undergo molecular testing to determine MSI status as described below.

b) *DNA Extraction for MSI evaluation*: DNA will be extracted from paraffin-embedded tissues as previously described [97] using the QIAmp Tissue Kit (Qiagen, Inc.). In tumors, only those areas containing >70% tumor cells will be used. The corresponding normal control DNA for each patient will be extracted from 5 mL peripheral blood using an automated platform (AutoGen FlexStar Qiagen chemistries) and quantified by ultraviolet (UV) absorbance and purity assessed by 260/280 optical density (OD) ratio. All DNA samples will be quantitated to 250 ng/uL in TE buffer. One 5 mL whole blood sample typically yields 100 -150 ug (400 – 600 uL at 250 ng/uL) of high molecular weight DNA that is 100-200 kb in size.

c) *MSI evaluation*: Paired normal DNA (from patient peripheral blood) and tumor DNA will be analyzed for microsatellite instability with six dinucleotide microsatellite markers (D5S346, TP53, D18S34, D18S49, D18S61, and ACTC) and one mononucleotide repeat (BAT 26). PCR and gel electrophoresis will be performed as described by Thibodeau et al [109]. Primers for PCR amplification of 10 different variable length-repeat segments tagged with a fluorescent marker will allow for the analysis of the PCR products using the Applied Biosystems 377 Automated Sequencer with GeneScan and Genotyper Software. PCR products will be denatured and run on a non-denaturing high-resolution 10% polyacrylamide gel. Tumors will be classified as MSH-H if 40% markers demonstrated instability, as MSH-L if <40% demonstrated MSI and as MSS if no markers exhibit MSI [97, 108].

B.   **Centrosome Amplification and Chromosomal Instability:**

Background and Significance: The orderly duplication of the centrosome and the chromosomes once in each cell cycle and the formation of a bipolar mitotic spindle are key cell cycle events leading to normal cell division. The nuclear and centrosome cycles are synchronized by checkpoints that prevent DNA reduplication prior to karyokinesis and prevent centrosome reduplication prior to anaphase. Amplification of centrosomes has been documented in many types of human tumors [110, 111]. Centrosome amplification in cancer is an indicator that centrosome duplication has become uncoupled from the cell cycle and can lead to aneuploidy by increasing the rate of mis-segregation of chromosomes during mitosis. The degree of centrosome amplification has been shown to correlate with the extent of chromosome instability (CIN) in human breast tumors and to occur prior to invasion [112]. CIN is defined as the flux in karyotype and as such measures the rate of change in chromosome number. The correlation between centrosome amplification and CIN demonstrated in breast cancer indicates that it is possible that centrosome amplification in fact drives CIN in the development and evolution of tumors [112]. However, since unrestrained CIN is likely to be self-limiting, the successful tumor may develop a mechanism, such as centrosome coalescence, to reduce the deleterious effects of centrosome amplification. Of note, fractional allelic loss which is a measure of CIN has been associated with adverse patient outcome in CRC [113]. In addition, many studies including those from investigators at Mayo Clinic using

the NCCTG colon cancer specimens have shown that aneuploidy is associated with shorter survival rates relative to diploid colon cancers [114].

Preliminary Data.   Our preliminary data show that centrosomes are amplified in aneuploid colon cancers. Centrosomes are both larger and more numerous in malignant epithelial cells compared with normal colonic crypt epithelium.

Specific Aims: 1) To determine if the level of centrosome amplification is positively correlated with the level of chromosomal instability (CIN) in colon cancers. 2) To determine if CIN level is prognostic of patient outcome. Centrosome amplification has been described mainly in solid tumors with chromosomal instability and aneuploidy. These features are less common in MSI-H relative to MSS colon cancers. If correlations are in MSS tumors, all MSI-H patients will be examined as well.

a) *Centrosome amplification*: Structural centrosome amplification will be assessed by immunofluorescence microscopy using image analysis to measure size and number of centro-somes per cell [115]. Preliminary data in human breast cancers indicates that centrosome number ranges from 0-10. Antibodies against the centrosome protein pericentrin (Covance) will be used to label centrosomes. Measurements will be based on average values of at least 50 tumor cells. The average signal from five fibroblast centrosomes will be used to normalize the values for each tissue.

b) *FISH analysis.* Chromosomal instability for chromosomes 3, 7, and 18 will be measured using fluorescence in situ hybridization of chromosome enumeration probes for these chromosomes [113]. Chromosome 7 is frequently gained and chromosome 18 frequently lost early in colorectal tumorigenesis. Chromosome 3, on the other hand, does not seem to be frequently lost or gained, and should serve as an indicator of overall tumor ploidy [116-118]. FISH probes to pericentromeric regions of chromosomes 3 (CEP3), 7 (CEP7), and 18 (CEP18) (Vysis, Downers Grove, IL) will be hybridized to sectioned tissue according to published methods [112, 113].

C.   **Telomere Shortening:**

Background and Significance: Telomeres are the caps of linear chromosomes that function to maintain chromosome stability. With progressive cell division, telomeres shorten; for healthy cells, this eventually leads to regulated cell senescence and apoptosis. Telomere shortening is a genetic event associated with aging and can be measured in constitutional DNA from peripheral blood and nearly all somatic cell DNA. In breast cancer patients, patients with node positive disease and shorter peripheral blood telomeres have increased survival rates compared to those node positive patients with longer telomeres[119]. CRC cell lines exhibiting telomere lengthening and increased telomerase expression have been reported to lead to increased resistance to chemotherapy possibly by leading to chromosomal stability and immortalization of the cancer cells [120]. The increased drug resistance, thus far seen in CRC cell lines in association with telomere lengthening, could be the explanation for the worsened prognosis seen in breast cancer patients with nodal disease and peripheral telomere lengthening. This association of peripheral telomere lengthening with poorer overall survival being connected to multidrug resistance conferred by telomere lengthening of the DNA of the cancer itself, would likely necessitate that constitutional telomere length reflects the same pattern of telomere dynamics as that ongoing in the tumor, i.e., longer telomeres in the peripheral blood would occur synchronously with longer telomeres in the tumor. For CRC, we have preliminary data indicating that patients with longer telomeres in their rectal cancer DNA also have

longer peripheral telomere lengths in comparison to peripheral telomere lengths in DNA from rectal cancer patients with shorter tumor telomeres.

Constitutional telomere shortening versus lengthening and its association with survival or response to chemotherapy has not been studied in colorectal cancer (CRC) patients. We hypothesize that: (a) patients with peripheral telomere shortening will have different response rates to FOLFOX versus FOLFOX + cetuximab and increased survival compared to patients with peripheral lymphocyte telomere lengthening and (b) that specific polymorphisms in telomere maintenance genes that contribute to telomere length variability will be associated with differences in responsiveness to FOLFOX versus FOLFOX + cetuximab, and ultimately to survival in CRC. As a secondary aim, we will evaluate for associations between the tumor molecular phenotype based on *KRAS* mutation; EGFR, MSI and CIMP status compared to peripheral telomere length and telomere maintenance repair (TMR) gene polymorphisms.

Specific Aims:

a) To determine if constitutional telomere length is a prognostic indicator in Stage III CRC patients enrolled in N0147. Specifically, we will measure telomere length in peripheral lymphocyte DNA from chemo-radiotherapy naive Stage III CRC patients treated with either FOLFOX or FOLFOX and the EGFR inhibitor cetuximab. We will then test for an association between telomere length and three year survival and overall survival from CRC. Finally, we will evaluate whether potential telomere length variation-associated survival differences seen in CRC are modified according to: 1) the *KRAS* mutation, EGFR, MSI and CIMP tumor status, and 2) treatment with FOLFOX versus FOLFOX + cetuximab.

b) To determine if polymorphisms in TMR genes may serve as a prognostic indicator in Stage III CRC patients enrolled in N0147. Specifically, we will genotype the Stage III CRC patients in this study with approximately 300 SNPs localized to TMR genes known to be associated with variation in telomere length. We will then test for an association between telomere maintenance genes and three year and overall survival from CRC. Finally, we will next evaluate whether the association of TMR polymorphisms with survival differences seen in CRC are modified according to: 1) *KRAS* mutation, EGFR, MSI and CIMP status of the tumor, and 2) treatment with FOLFOX vs FOLFOX + cetuximab.

Materials and Methods:

a) *DNA extraction from peripheral blood lymphocytes (PBLs)*: Genomic DNA will be extracted as described in Materials and Methods (b) in Specific Aim 2(A).

b) *Quantitative PCR assessment of telomere length*: Telomere length can be measured by several techniques including Southern Blot, Q-FISH (fluorescence *in-situ* hybridization), combined telomere FISH and immunostaining and quantitative FISH. We have chosen to use quantitative PCR because it is a well-tested assay [121] that has the benefits of being a high throughput PCR based test. This is an inexpensive test that is practicable, affordable, and reliable, having a relatively stringent intra and inter- assay variability of 6%.

All PCR will be performed on the ABI Fast Real-Time 7900HT (Applied Biosystems, Foster City, CA). We have utilized this technology to assess telomere length in PBL, normal colonic epithelium and CRC tissue from over 100 CRC cases and controls and believe that we will be able to successfully accomplish assessment of PBL (constitutional) DNA telomere length in these samples.

c) *TMR gene selection:* To identify our list of genes involved in TMR, we used these three strategies:

Set A: 152 yeast genes that have been reported as being involved in the regulation of telomere length were extracted from two recently published articles [122, 123]. We used release 53 of HomoloGene database to search for human homologues, resulting in 66 human genes.

Set B: The database of the commercial pathway analysis software developed by Ingenuity Systems, Inc., that includes curated gene information was searched to extract telomere related genes. A keyword search resulted respectively into 49 genes.

Set C: The genes included in Set B are part of two networks; first: Cellular Assembly and Organization, Cellular Function and Maintenance, Cell Cycle; and second: Cell Cycle, Cellular Assembly and Organization, Cellular Development. The first network includes 35 of the 49 genes, whereas, the second network includes only 12 genes. An additional 36 genes not identified by the keyword search are also part of this network bringing to a total of 82 unique genes included in both networks. The gene list was extended by including upstream genes that match the criteria that the protein product of the upstream gene has to be an enzyme, growth factor, transcription regulator, translation regulator, kinase, peptidase or transporter. The function of the gene product was also constrained to activation, inhibition, transcription, regulation of binding, protein-protein interaction, and DNA-protein interaction. This extension resulted in 413 genes defining Set C. It should be noted that the large majority of these genes are not annotated as being involved in TMR.

The sum of these three gene sets was combined to establish a list of genes with higher confidence to be associated to TMR.

d) *Genotyping:* We will use DNA from peripheral blood lymphocytes of the controls to perform the Illumina Golden Gate Assay. This specific aim will utilize 1536 SNPs for genes relating to TMR. The Illumina GGGT assays encompass primer extension, ligation and universal PCR in very highly-plexed reactions (384-1536plex).

SNP Genotyping Quality Control: Although the SNP genotyping methods with the Illumina assay are highly reliable and accurate, we will carry out several procedures in order to assess the quality of our genotype data. We will assess genotyping reproducibility by genotyping ~2% of the samples in duplicate by the redundant plating of 8 samples on each plate. We will also assess reproducibility across DNA plates by placing DNA of a CEPH trio on each plate, allowing us to have samples genotyped at least four times per SNP. Genotype data will be further evaluated for quality by computing SNP-specific and person-specific call rates, tests of Hardy Weinberg equilibrium (HWE) among the controls, and assessment of duplicate genotypes.

SPECIFIC AIM 3: Epigenetics – CpG Island Methylator Phenotype (CIMP)

Background and Significance: Colorectal cancers are characterized by genetic instability in the forms of chromosomal instability (CIN) or microsatellite instability (MSI) due to loss of DNA mismatch repair (MMR; see Specific Aim 2). In addition to these two well-described paths to colon cancer, recent data indicate that some colon cancers have neither CIN nor MSI [124]. The

mechanism of MSI in sporadic colon cancers is due to epigenetic changes marked by DNA methylation of the *hMLH1* MMR gene [125]. Epigenetic changes are common in cancers, and dense DNA methylation in colon cancers has been described and is referred to as the CpG island methylator phenotype (CIMP) [125]. Epigenetic silencing of genes and inactivating mutations produce similar outcomes utilizing different mechanisms [126], and therefore, epigenetic inactivation is an alternative pathway to genetic instability.

In a recent report [127], an inverse relationship between CIN and CIMP were found, confirming that these distinct mechanisms of genetic instability rarely overlap [128, 129]. Important clinicopathological differences exist between tumors arising from these distinct pathways. Compared with CIN cases, CIMP cases show a predilection for the proximal colon and are more common in older patients, especially females. CIMP cases also have a distinct histology including poor differentiation, frequent mucin production, and increased tumor infiltrating lymphocytes. CIMP colon cancers also have different genetic characteristics, including the finding of *BRAF* and *KRAS* mutations in CIMP cases with fewer *APC* and *p53* mutations. Some cases have been found to show extensive DNA methylation and CIN, as well as cases with both MSI and CIN. It remains to be seen whether these represent yet other subgroups, or are due to technical problems.

The CIN and MSI pathways of colon tumorigenesis have markedly different prognoses, ranging from favorable in the case of MSI to poor in tumors showing CIMP but without MSI. Specifically, CIMP-high MSI colon cancers have been shown to have a better stage-adjusted prognosis compared to CIMP negative cases or MSS cases [130]. There is also accumulating evidence that MSI colon cancers are resistant to 5-fluorouracil given as adjuvant chemotherapy, which likely also extends to CIMP cases. Recent data presented by Cheng et al. [127] provide evidence for a third pathway of colon carcinogenesis. This pathway involves CIMP-high tumors with *KRAS* mutations, and occasional *BRAF* mutations, that appear to have the worse prognosis compared to CIMP-high non-MSI or CIMP-high MSI cases. Colon cancers arising via this 3rd pathway also show lower responsiveness to chemotherapy. Recently, colon cancers with *KRAS* mutations (most of which belong to the CIMP group) have been shown to have a very low response rate to antibodies against the epidermal growth factor receptor (EGFR) such as cetuximab [131]. This is especially relevant to this colon cancer adjuvant study, N0147, in which *KRAS* wild type patients will be randomized to receive mFOLFOX6 with or without cetuximab following potentially curative surgery.

At the present time, the panel of methylation markers is not standardized, although studies have evaluated promoter hypermethylation in panels of CpG islands of several known genes including *p16*, *hMLH1*, *THBS1*, *MINT1*, *MINT31*, *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*[125, 132, 133]. However, recent data identified three distinct groups of colon cancers named CIMP1, CIMP2, and CIMP-negative [134]. Genetically, these three groups correspond to very distinct profiles. CIMP1 are characterized by MSI and *BRAF* mutations and rare *KRAS* and *p53* mutations. CIMP2 is associated with a high rate of *KRAS* mutations and rare MSI, *BRAF*, or *p53* mutations. CIMP-negative cases have a high rate of *p53* mutations and lower rates of MSI or mutations of *BRAF* or *KRAS* mutations. The three groups are independent of age, gender, or stage, but CIMP1 and CIMP2 are more common in proximal tumors.

Finally, O^6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair gene which removes promutagenic O^6-methylguanine (O^6-MeG) residues from DNA and is considered an important predictive factor for chemoresistance in human cancers [135-139]. Mutations in *MGMT* have rarely been found, and it appears that MGMT inactivation is primarily manifested through hypermethylation-induced silencing of its promoter in colorectal and other cancers [137, 140-145]. Studies in cultured cells indicate that the candidate core region of the *MGMT* promoter involves 2

methylation-sensitive regions. The first of these regions is upstream of exon 1, termed the Mp-region, and includes the minimal promoter. The second region is downstream of Mp, termed as the Eh-region, and contains several enhancer elements required for transcription of certain growth regulatory genes [142, 146-149].

Specific Aim:

a) We aim to: 1) determine the frequency of aberrant promoter hypermethylation of the classical CIMP panel (*p16, hMLH1, THBS1, MINT1, and MINT31*) and the more recent CIMP 5-gene marker panel (*CACNA1G, IGF2, NEUROG1, RUNX3*, and *SOCS1*) in MSI-H and MSI-L tumors versus a matched subset of MSS stage III colon cancers; 2) compare CIMP status with *KRAS*, *BRAF*, and *p53* mutations, and 3) determine whether the methylator phenotype correlates with clinicopathological features and can predict recurrence and/or patient survival rates. Methylation studies will be performed on all MSI-H and MSI-L colon cancers which appear to occur at a frequency of 15% and 3%, respectively, in sporadic cases. Results will be compared to a matched group of MSS cases.

b) We aim to determine the specific patterns of DNA methylation in the candidate core regions of the *MGMT* promoter in colon cancers, and to compare this to MGMT protein expression, *KRAS* and *BRAF* mutations, and other clinical features by analyzing methylation level in discrete regions of the *MGMT* promoter.

Materials and Methods: The presence or absence of MSI will be determined as previously described under Specific Aim 2(A). MSI-H is estimated to occur in 15% of sporadic colon cancers and an equal number of MSI-L and MSS cases will be identified and matched for age, gender, and tumor site. A total of 4 ten-micron slides and 5 five-micron slides will be provided to Ajay Goel, Ph.D., at Baylor University Medical Center at Dallas, Dallas, TX, for DNA extraction, CIMP analysis and validation as outlined below. (Note: these same tissue specimens will be used for RNA isolation, microRNA profiling and validation, see Specific Aim 4A.)

a) *Methylated CpG island amplification*: CpG island methylator phenotype (CIMP) will be assessed using an established 5-gene marker panel (*CACNA1G, IGF2, NEUROG1, RUNX3*, and *SOCS1*), with results reported as CIMP-positive (CIMP+) or CIMP-negative (CIMP-), as previously described by Weisenberger et al.[150]. Methods will be identical to, or generally similar as, procedures used in the USC Epigenome Center. Extracted DNA will be treated with sodium bisulfite prior to MethyLight analysis. Sodium bisulfite treatment results in the conversion of unmethylated cytosine residues to uracil, while methylated cytosine residues are resistant to this conversion. The resulting methylation-dependent sequence change forms the basis for the real-time PCR-based MethyLight analysis. Sodium bisulfite conversion is performed by an overnight incubation using the Zymo EZ DNA methylation kit (Zymo Research, Orange, CA). This bisulfite-conversion protocol consists of 16 cycles of 95 °C for 30 seconds followed by 50 °C for 1 hour. MethyLight analysis will be performed as described elsewhere [151]. Two types of reactions will be used. Methylation-specific reactions contain primers and probe sequences specific for bisulfite-converted methylated DNA (methylated cytosines remain cytosine, unmethylated cytosines are represented by uracil/thymine). Bisulfite-specific control reactions lack CpG dinucleotides, and are thus impervious to variations in DNA methylation status. The ALU-C4 control reaction is used to normalize for quantity and quality of the template DNA [150]. The ratio of methylated reaction to control reaction represents the primary measurement. This ratio is further normalized against a similar ratio for a fully methylated, M.SssI-treated reference DNA sample [151]. The resulting ratio is

expressed on a percentage scale, and is referred to as a Percent of Methylated Reference (PMR). These methylation data will be reported as PMR values.

Other methylation markers will include *p16*, *ESR1*, *THBS1*, *MGMT* and *hMLH1*, which will be determined as previously described [125, 132 , 152]. These latter loci were selected based on their methylated CpG islands detected in a population-based study of colon cancers [132]. One hundred nanograms of methylated CpG island amplification PCR products will be blotted onto nylon membranes and hybridized using ^{32}P-labeled probes. Thirty nanograms of a DNA fragment from p16 exon 1 and MINT clones will be labeled by random priming and will then be used as probes. Each sample will be blotted in duplicate. Each filter includes mixtures of a positive control and a negative control. The filters will be exposed to a phosphor screen for 24-72hr and will be developed by using a PhosphoImager (Molecular Dynamics). A detailed protocol for methylated CpG island amplification is available at www.med.jhu.edu/methylation.

b) *Methylation-specific PCR (MSP) and Bisulfite-PCR*: Methylation status will be determined by MSP [125] or bisulfite-PCR followed by restriction digestion [125, 153]. In brief, 2 µg of genomic DNA will be treated with Na-bisulfite for 16 hr. After purification, a 2 uL aliquot will be used as a template for PCR reactions. In brief, 20-40 uL of the amplified products will be digested with restriction enzymes that distinguish methylated from unmethylated sequences, then electrophoresed on 3% agarose or 5% polyacrylamide gels and visualized by ethidium bromide staining. Primer sequences, conditions for PCR, and restriction enzymes used are available at www.med.jhu.edu/methylation/ primers.html. Both MSP and bisulfite-PCR provide semiquantitative results. The loci selected for analysis are unmethylated (<1%) in normal tissues. Therefore, any tumor showing >5% methylation will be considered positive. Because the tumors were not microdissected and contain a variable amount of contaminating normal tissues, no attempt will be made to distinguish levels of methylation beyond positive/negative.

c) *Combined bisulfite restriction analysis assays for MGMT methylation*: We designed the combined bisulfite restriction analysis (COBRA) to examine both the "minimal promoter" (Mp-region) and the "enhancer" (Eh-region) of the O^6-methylguanine-DNA methyltransferase (*MGMT*) promoter (Fig. 1*a*). Primer sequences for "Mp-region" and "Eh-region" for the *MGMT* promoter region were: (*a*) Mp-F (5′-GAGGATGYGTAGATTGTTTTAG-3′) and Mp-R (5′-AAACCRAAAACCTAAAAAAAAC-3′), and (*b*) Eh-F (5′ GTTTTTAGAAYGTTTTGYGTTT-3′) and Eh-R (5′-CCTACAAAACCACTCRAAACTA-3′), generating fragment lengths of 163 and 145 bp, respectively.

SPECIFIC AIM 4: **Candidate Gene Expression and Pharmacogenomic Analysis**

A. **MicroRNA Expression Profile and Association with Therapeutic Outcome**

Background and Significance: MicroRNAs are 18- to 25-nucleotide, noncoding RNA molecules that regulate the translation of many genes [154]. Since their discovery [155, 156], microRNAs have been found to regulate a variety of cellular processes including apoptosis [157-159], differentiation [155, 156, 160], and cell proliferation [161]. MicroRNAs may also have a causal role in carcinogenesis [162-164]. MicroRNA expression levels are altered in most tumor types [165, 166], including colon tumors [165, 167-169]. Experimental manipulation of specific

microRNAs modulates tumor development in mouse-model systems [161, 170-172]. The prognostic potential of microRNAs has also been demonstrated for chronic lymphocytic leukemia [173], lung cancer [174], pancreatic cancer [175], and neuroblastomas [176].

If aberrant microRNA expression is causal to carcinogenesis, inhibiting specific microRNAs may have therapeutic implications. Modified antisense oligonucleotides can easily be designed to specifically inhibit microRNA function [177]. Antagomirs are one type of antisense oligonucleotide that has proven effective at inhibiting microRNA function *in vivo* in mice [178]. The ease of designing specific inhibitors of microRNA function makes them candidates for therapeutic targets. Given the therapeutic and prognostic potential for microRNAs in cancer, we will evaluate microRNA profiles of colon tumors and paired nontumorous tissue to study their potential role in tumor formation, diagnosis, and response to chemotherapy in colon carcinoma.

Specific Aims: To identify microRNA expression patterns associated with colon adenocarcinomas, prognosis, or therapeutic outcome. MicroRNA microarray expression profiling of tumors and paired non-tumor tissues will be performed in a cohort of colon adenocarcinomas from participants in this study, N0147. We will evaluate associations between microRNAs with tumor status, TNM staging, prognosis, and response to adjuvant chemotherapy. Associations will be validated in a second, independent cohort using quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays. MicroRNAs will be identified that are differentially expressed in tumors and microRNA expression patterns associated with survival using cancer-specific death as the end point.

Materials and Methods:

a) *Tissue Collection and RNA Isolation*: Up to approximately 1200 pairs (~600 pairs for testing and ~600 pairs for validation) of primary colon tumor and adjacent nontumorous tissues from participants in this study, N0147, will be analyzed. Tissues were obtained at surgical resection and were paraffin embedded. A total of 4 ten-micron slides and 5 five-micron slides will be provided to Ajay Goel, Ph.D., at Baylor University Medical Center at Dallas, Dallas, TX, for RNA isolation, microRNA profiling and validation as described below. (Note: these same tissue specimens will be used for DNA extraction, CIMP analysis and validation, see Specific Aim 3.)

b) *RNA Isolation and MicroRNA Profiling*: RNA from paraffin-embedded tissue sections will be extracted using standard TRIZOL (Invitrogen, Carlsbad, California) methods. Briefly, 5 µg of total RNA will be labeled and hybridized to each microRNA microarray) containing quadruplicates of 389 human microRNA probes. Tumor/nontumorous pairs of tissues will be profiled at the same time. Slides will be scanned using a PerkinElmer ScanArray LX5K scanner (Perkin Elmer, Waltham, Massachusetts).

c) *Microarray Analysis*: The data will be preprocessed using statistical software to remove probes with higher background intensities than foreground and probes with inconsistent measurements across the quadruplicates. The data will be normalized by locally weighted scatter plot smoothing LOESS and imported into Biometric Research Branch (BRB) array tools 3.5.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html) for subsequent microarray. Probes with values missing from more than 20% of the arrays will be removed from the analysis. This filtering method was decided a priori to eliminate probes whose microRNAs expression levels were

thought to be unreliable. Class comparison analysis using paired *t* tests will identify microRNAs that are differentially expressed in tumors (*P*<.001). Class prediction algorithms in BRB array tools will be used to determine whether microRNA microarray expression patterns could accurately differentiate between tumor and paired nontumor tissue. For these analyses, 3 nearest neighbors and nearest centroid algorithms will be arbitrarily chosen and percent accuracy reports the percentage of tissues that are correctly identified. These algorithms will also be used for qRT-PCR data. To initially search for microRNAs associated with poor survival, tumor:nontumor (T:N) microRNA expression ratios will be analyzed using microarray data. Tumor:nontumor expression ratios for microRNAs will be created by subtracting the $\log_2$ nontumor from the $\log_2$ tumor expression values. MicroRNAs missing more than 25% of T:N ratios will be filtered out. Expression data will be dichotomized into clearly defined high and low groups to examine associations with microRNA expression and survival. Tumor:nontumor expression ratios will be dichotomized with the highest tertile classified as high and the lower 2 tertiles classified as low. This cutoff was set based on associations within the test cohort prior to analyzing the validation cohort. Once set, this high-low cutoff was used universally throughout this study. To analyze associations with tumor expression and nontumor expression with survival using microarray data, the array data had to be normalized based on the day of microarray profiling to remove systematic bias introduced from the day-to-day variability observed in the microarray data acquisition. To do this, for each given day, the highest one-third expressing values were labeled high and the lowest two-thirds were labeled low, consistent with the predetermined cutoff that was used for this study.

d) *Quantitative RT-PCR*: Quantitative RT-PCR of microRNAs will be performed using Taqman MicroRNA assays (Applied Biosystems, Foster City, California) according to the manufacturer's instructions with the 7500 real-time RT-PCR system (Applied Biosystems, Foster City) using expression levels of the small nuclear RNA, U6B, as the normalization control. All assays will be performed in duplicate (*miR-20a, miR-203*) or triplicate (*miR-21, miR-106a, miR-181b*). Quantitative RT-PCR for *miR-21, miR-106a*, and *miR-181b* will be performed by an investigator who will be blinded to the survival outcomes and clinical data for members of the validation cohort.

e) *In Situ Hybridization (ISH)*: ISH will be performed with probes for human *miR-21*, scramble, and U6 (Exiqon, Woburn, Massachusetts) with a modified version of the manufacturer's protocol for formalin-fixed paraffin-embedded tissue written by W. Kloosterman (http://www.exiqon.com/uploads/LNA_52-_FFPE_miRNA_in_situ_protocol.pdf) on human colon tissue. Modifications will include the use of polyclonal rabbit anti-DIG/HRP-conjugated antibody and DakoCytomation GenPoint Tyramide Signal Amplification System (DakoCytomation, Carpinteria, California), and VECTOR NovaRed substrate (Vector Laboratories, Burlingame, California). Images will be taken on an Olympus BX40 microscope using the Olympus DP70 digital camera and DP controller software (Olympus, Champaign, Illinois).

B. **Gene Identification by Transcriptional Profiling:**

Background and Significance: The identification of molecular markers that can predict disease recurrence or patient survival would be useful in establishing a more accurate staging classification system. Such prognostic markers can be used to better categorize those patients that have a higher risk of tumor recurrence or disease progression. Prognostic markers would enable a more tailored and molecularly targeted treatment approach to be utilized by clinical oncologists. Studies in other tumor systems have shown that the molecular characteristics exhibited by tumors can serve as

predictors of a patient's disease-free and overall survival [179-182]. In colorectal tumors, the status of the DNA mismatch repair system has been shown to influence both time-to-recurrence and patient survival [183], and suggests that an alternative treatment approach be tested in these patients [107]. Studies have suggested that loss of 8p and/or 18q might serve as a prognostic indicator, though results appear to be variable among different reports [99, 183]. To date, however, no definitive markers have emerged that can distinguish those patients that will benefit from adjuvant chemotherapy from those that will not.

In addition, a genome-wide association study is currently underway in pancreatic cancer at the Mayo Clinic. In the first stage, 400 patients were examined with a genome-wide 550K Illumina SNP platform and 21 SNPs showed genome wide significance ($p<10^{-6}$). A second set of patients (N=600) will have results released in October 2009, which will be used for replication. A third replication of approximately 800 patients will be utilized for the final stage. From this study, it is anticipated that highly replicated polymorphisms associated with survival prediction in pancreatic cancer will be identified. From this data, we would examine these polymorphisms for association with survival in colon cancer, since survival factors could include overlapping mechanisms between both malignancies (immune surveillance, regulation of response to tumor signals, response to therapy, etc.).

Specific Aims:

a) To identify and determine the prognostic significance of a series of markers derived from high density transcriptional profiling of stage III (Dukes' C) colon cancers that have exhibited both a favorable and a poor clinical outcome. Early stage tumors will form the foundation of the preliminary studies in which transcriptional expression profiling has been used to identify candidate transcripts that correlate with outcome. Such markers could then be used to help clinicians risk stratify patients based upon the aggressiveness and recurrence potential of their tumors. Ultimately, this information will enable the development of tailored treatment protocols for specific disease stages and tumor types.

b) To determine the effect of SNPs associated with altered survival in pancreatic cancer in colorectal cancer. We aim to identify the top 10-20 SNPs that consistently associate with survival in pancreatic cancer, and genotype all patients in N0147 for an overall survival effect, adjusted for treatment arm and other identified confounders in univariate analysis.

Materials and Methods: From these cases, total RNA will be extracted from both normal and tumor tissue and gene expression analysis will be performed utilizing Affymetrix high density arrays (U133 set). Data derived from these experiments will be analyzed to identify markers/expression patterns that can predict outcome. Verification of these candidate genes will be performed by RT-PCR, within the original tumors evaluated by the gene expression arrays. All confirmed genes will be considered possible candidate markers.

After this initial preliminary data is obtained, the identified markers will be analyzed in approximately 400 stage III colon cancers from N0147 to test the overall hypothesis of this project, namely that the expression profile of a colon cancer that is associated with a cure. In addition to identifying these differences, a secondary goal of this work is to develop profile marker sets to help predict outcome for colon cancer patients and perhaps design treatment approaches based on this profile.

a) *Tissue collection and RNA extraction.* Tissue samples will be obtained from surgical specimens that are paraffin embedded. For further processing, tumor will be manually dissected to enrich for areas with >70% tumor. Normal colonic epithelium, a minimum of 8 mm from the tumor

margin, will be microdissected to enrich for epithelial cells, minimizing stromal and muscular tissue contamination as much as possible. RNA will be processed from tumor and normal tissue samples. Specifically, microdissected tissue from 10 μM sections of paraffin-embedded tissue specimens will be placed into Rneasy RLT buffer (Qiagen, Valencia, CA) and RNA extracted using the Rneasy extraction technique. RNA will be quantitated by spectrophotometry and the quality assessed by gel electrophoresis (1.2% agarose/TBE gel containing 5 mM guanidium isothyocyanate (Invitrogen) and 2x GelStar (Biowhitaker Molecular Application, Rockland, ME)). RNA samples with 28S/18S ratios > 1 will be used for analyses.

b) *Transcriptional profiling*: Gene expression profiling will be performed using Affymetrix Human Genome U133 Set - a series of two microarrays containing 39,000 transcripts that represent approximately 33,000 genes. Both quantitative and qualitative expression will be measured.

Validation: We will attempt to validate the expression array data obtained in preliminary results in the large population of stage III colon cancer patients to be studied in this protocol. We propose to generate and to compare microarray data on patients with recurrence or colon cancer-specific death versus an equal number of patients alive and free of recurrence at 4 years follow-up.

a) *Tissue microarrays (TMAs)*: TMAs will be constructed from 0.6 mm cores of formalin-fixed paraffin-embedded human colon tissues. TMAs will contain multiple tumor and normal cores from each of the 30-40 patients samples used for the gene expression arrays.

b) *In situ hybridization (ISH)*. Radioactive ISH will be performed by methods described in Deeds *et al.* [184]. ^{35}S-labeled riboprobes will be generated by *in vitro* transcription of PCR products amplified from sequence-confirmed IMAGE clones with T3 and T7 primers. TMAs will be utilized for the ISH experiments. ISH signals will be graded from 0 to 3 with the liver tissue as reference (grade 0= no signal, 1= weak signal, 2= moderate signal and 3= strong signal). Grading will also be based on the number of positive cells as either diffuse (positive cells all over the tissue) or focal (sparsely positive cells). Pooled results from the same tissues and the calculated percentage of each grade in these tissues will also be used to determine grading.

c) *Quantification of Gene Expression by Lightcycler*: Clones will be further analyzed by quantitative PCR in normal/cancer pairs. RNA, extracted as described above, will be transcribed into cDNA by SuperScript II reverse transcriptase according to manufacturer's protocol (Invitrogen, Carlsbad, CA). All PCR reactions will be performed using a LightCycler real-time fluorescence detection system (Roche Diagnostics, Indianapolis, IN). For each PCR run, a master mix will be prepared on ice, including 10 pmole of each primer, 3-4 mM of $MgCl_2$, and 1xSYBR Green I solution (Roche Diagnostics, Indianapolis, IN). Two μl of each RT product will be added to 18 μl of the PCR master-mix. The thermal cycling conditions will be optimized for each of the targets of interest. As it is difficult to assess the precise amount of total RNA added to each reaction (based on absorbance) and its quality, we will quantitate a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous RNA control, and each sample will be normalized on the basis of the control copy number. The primers for this gene are: *forward-ACCACAGTCCATGCCATCAC* and *reverse-TCCACCACCCTGTTGCTGTA*.

C.   **Pharmacogenomic Studies:**

Background and Significance: The variability in tumor responses and adverse events in patients treated with chemotherapy agents is strongly influenced by genetics. A recent publication authored by the Gastrointestinal Scientific Leadership Council of the Coalition of Cancer Cooperative Groups argues that pharmacogenomic studies to identify genetic markers that predict toxicity or tumor response are a key priority [185], since they have the potential to alter clinical practice by individualizing the treatment of colon cancer. There are three distinct therapeutic classes, fluoropyrimidines, platinum complexes, and EGFR inhibitors, that have demonstrated activity in patients with advanced CRC disease and preclinical support for improved outcome with combination therapy is evident [3-6]. Despite similar treatment regimens for a given tumor stage/grade, individual patients still experience varying outcomes ranging from greater toxicity to suboptimal treatment outcome. Given this continued clinical observation, it would seem that the identification of a biochemical or molecular predictive test is necessary to aid in the individualization of drug therapy.

Indeed, many approaches have been taken to identify patient or tumor characteristics that are predictive for response to therapy or survival. However, most studies have evaluated a specific target in isolation rather than taking a comprehensive approach [8] and therefore have not had a significant impact on current therapeutic decision making for colorectal cancer. Recent clinical studies suggest that genomic solutions are worthy of more definitive evaluation. For example, variant alleles in the thiopurine methyltransferase gene can predict severe hematological toxicity after azathioprine, 6-mercaptopurine, or related agents [186]. A similar association is seen with dihydropyrimidine dehydrogenase (DPD) where specific mutations alter functional enzyme activity and give an increased risk of hematological, gastrointestinal, and/or neurotoxicity from 5-FU therapy [187, 188]. In addition, genetic variants in thymidylate synthase (TS) and members of the excision repair enzyme family have been associated with therapeutic response to 5-FU and oxaliplatin, respectively [189-191].

The current trial offers a unique opportunity for addressing the question of genetic variants and patient toxicity and/or outcome. Patients on this study will receive FOLFOX (i.e., 5-FU, leucovorin, and oxaliplatin), which is the standard-of-care therapy administered to over 60,000 CRC patients per year in the United States. Thus, the findings from this study will be applicable to a large number of patients. The study design includes two distinct arms on which patients receive FOLFOX with or without cetuximab. The expected toxicity profile will thus be different for the individual study arms allowing questions to be asked regarding association between a marker and risk of toxicity. The availability of uniform information on toxicity, tumor response, and patient survival makes it a powerful framework on which to ask pharmacogenetic and protein expression questions. In addition, the large size of this study provides us with an unparalleled opportunity to sufficiently power the analysis to detect the effects of rare SNPs. As defined below, we will be using a candidate gene approach to fully examine the role of allelic variants.

1.   **5-Fluorouracil (5-FU) Pharmacogenetics**:

A growing body of evidence suggests that the intratumor protein expression and gene expression of drug-metabolizing enzymes or DNA repair enzymes may have important implications for anticancer drug efficacy [192-194]. However, whether the pharmacogenetic variations are useful in predicting drug response to and survival in relation to specific chemotherapy regimens remains unknown. Determination of significant

associations between gene expressions and defined clinical endpoints (e.g., survival and response) may improve the prediction of treatment success and, thereby, lead to the tailoring of chemotherapy.

A genome-wide screening analysis using cell lines concluded that the heritability of 5-FU cytotoxicity was greater than for many common human phenotypes [195]. Despite evidence for such strong genetic components in this response, only a few genetic variations that affect 5-FU toxicity have been identified. There are three elements that seem to be involved in the modulation of 5-FU: thymidylate synthase (TS), methylenetetrahydrofolate reductase (MTHFR), and dihydropyrimidine dehydrogenase (DPD).

The cellular target of 5-FU chemotherapy is TS. In colorectal cancer, high TS mRNA and protein are associated with poor objective response and reduced patient survival [196, 197]. TS expression appears to be regulated by a highly polymorphic tandem repeat in the TS promoter enhancer region (TSER) [198, 199]. This polymorphism is of significance as greater *in vitro* transcription occurs with the triple repeat than is observed for the double repeat. In colorectal cancer, the clinical significance of TS expression as a predictor of response to fluoropyrimidines is considered as "evidence level I" [200]. Nevertheless, Bertino et al. [201] have questioned whether the measurement of TS expression is "ready for prime time?" In clinical practice, TS expression is still not used to predict response to colorectal cancer treatment. This is because the value of the measurement of TS expression to predict response has not been validated by large prospective clinical studies.

5-FU activity is also influenced by the cellular folate pools which are influenced by MTHFR. A 'C' to 'T' missense mutation at nucleotide 677 of the *MTHFR* gene has been associated with altered tissue folate levels and increased toxicity from 5-FU-based treatment in breast cancer patients [202]. Studies in CRC patients, however, have led to variable conclusions regarding the ability of these polymorphisms to predict toxicity and/or tumor responses; and a recent meta-analysis concluded that *MTHFR* polymorphisms were not reliable predictors of patient responses [203].

High DPD expression (the enzyme that inactivates 5-FU) is associated with drug resistance while low activity identifies patients at risk for severe toxicity [187, 204]. DPD expression is highly variable and several polymorphisms have been described including *DPYD*2* (allele frequency 1%), *DYPD*5* (28%), *DPYD*6* (5%) and *DYPD*9A* (34%) [205]. The association of these polymorphisms with toxicity has yet to be investigated.

Despite over two decades of work devoted to identifying polymorphisms in the genes that encode the proteins that metabolize 5-FU and then assessing whether these polymorphisms are associated with toxicity in patients treated with 5-FU/leucovorin, we have made only limited progress in identifying predictors of response [206]. These findings, coupled with the observation that 5-FU sensitivity is highly heritable [195], suggest that additional genetic factors that affect the toxicity of 5-FU remain to be discovered. In addition to performing studies on the enzymes that metabolize 5-FU in this large phase III setting, we will assess how checkpoint signaling proteins that respond to the DNA damage inflicted by 5-FU affect 5-FU toxicity, a completely unexplored area.

5-FU, like other agents that disrupt dNTP supplies and cause DNA damage, activates the Rad9-Hus1-Rad1 (911)•ATR•Chk1 checkpoint signaling pathway [207-209]. Once activated, the various components of this pathway block the firing of origins for DNA replication, slow DNA replication, prevent cells from exiting G2, regulate DNA repair, and

stabilize stalled replication forks. Consistent with the roles of these proteins in orchestrating cell cycle arrest and DNA repair, cells with defects in checkpoint activation are sensitive to agents that damage DNA and cause replication stress, including many genotoxic anticancer drugs. Importantly, cells that lack or are depleted of Chk1 are more sensitive to the antiproliferative effects of 5-FU [207-209]. The challenge of identifying polymorphisms that are associated with toxicity is further compounded by the fact that 5-FU/leucovorin is almost always combined with other agents in modern chemotherapy regimes, thus making it even more difficult to tease out the effects of specific polymorphisms on toxicity. Although this clearly complicates matters, it also underscores what is most clinically important: the ability to identify patients most at risk when given standard-of-care combination therapies.

Multiple studies have linked Rad17 and the 911 complex to various cancers and cancer-associated phenotypes. In the vast majority of head and neck squamous cell tumors, Rad17 levels are very low and there is loss of heterozygosity of the *RAD17* locus, and the frequent loss of one *RAD17* allele [210]. In contrast, the *RAD9A* gene is amplified and upregulated by methylation in breast cancer [211], and Rad9, Hus1, and Rad17 are overexpressed in many tumors, including ovarian, lung, breast, testicular, prostate, thymoma, and colon, with overexpression associated with more aggressive tumors and/or poorer prognoses [212-219]. Further indications that these checkpoint proteins are associated were found in the only two studies that have examined any of the genetic polymorphisms that occur in these checkpoint genes. In one study of Japanese patients with lung cancer, a polymorphism in *RAD9A* (His239Arg) is present in 16% of patients with adenocarcinoma compared to ~1% of matched controls and patients with squamous cell lung carcinoma [220]. Similarly, a study to identify low penetrance risk factors for breast cancer tentatively identified an intronic and coding SNP that was associated with an increased risk of breast cancer in Spanish women [221]. Taken together, these results strongly suggest that these checkpoint signaling proteins have roles in tumorigenesis. That role in cancer—and the roles of polymorphisms in these genes—remains unknown.

Here we propose that the genetic variability that is inherent in the genes that encode Rad17 and the 911 complex may have major impact on the toxicity of FOLFOX because the 911 complex protects cells from death induced by *both* 5-FU and oxaliplatin (Fig. 11). By focusing on a genotoxin-activated signaling/repair complex that is important for facilitating the survival of cells treated with both chemotherapy agents, we predict that we will find novel genetic markers that associate with FOLFOX toxicity.

Fig. 11. Rad9-deficient ES cells are sensitive to oxaliplatin. *Rad9*−/− ES cells stably expressing wild-type (WT) Rad9 or transfected with empty vector were plated as single cells, allowed to adhere, and exposed to the indicated concentrations of oxaliplatin for 24 h, washed, and allowed to form colonies.
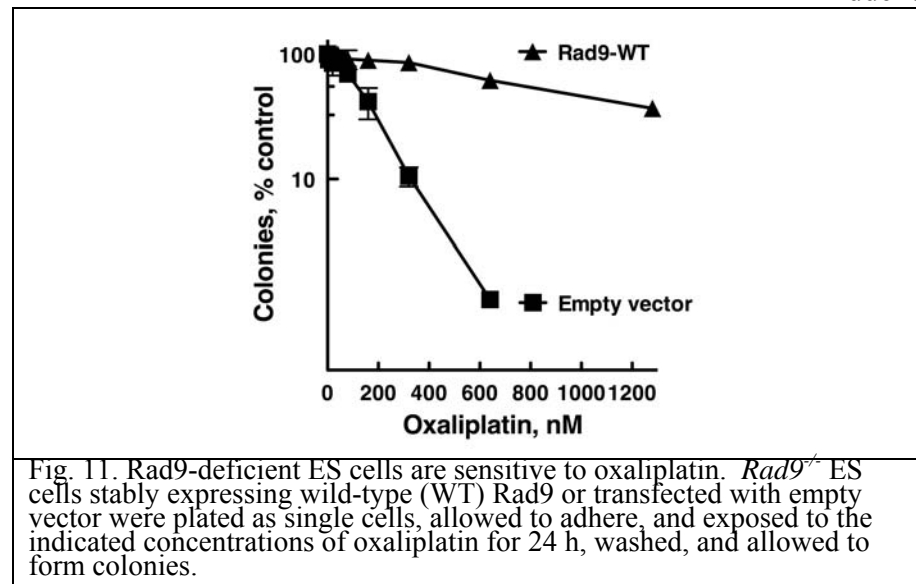
Specific Aim: To identify molecular signatures associated with response to treatment and clinical outcome following curative tumor resection in stage III colon cancer patients treated with oxaliplatin plus 5-FU/leucovorin with or without cetuximab.

a) To assess the prevalence of the identified polymorphisms in the fluoropyrimidine and folate metabolic pathways and DNA repair genes.

b) To identify expression patterns of genes involved in the fluoropyrimidine and folate metabolic pathways and DNA repair genes.

c) To determine the methylation patterns of genes involved in the fluoropyrimidine and folate metabolic pathways and DNA repair genes.

d) To assess whether selected SNPs in the genes that encode Rad17 and the 911 complex are associated with toxicity in colon cancer patients treated with FOLFOX.

Assessment of fluoropyrimidine and folate metabolic pathway SNPs, CpG island methylation and quantitative gene expression (QGE) will be conducted using a matrix-assisted laser desorption / ionization time-of-flight mass spectrometry (MALDI-TOF MS) platform and MassARRAY® Typer `4.0 Software (SEQUENOM, Inc., San Diego, CA) in the laboratory of Robert Diasio, Ph.D., Mayo Clinic Rochester. Assessment of the Rad17 and 911 complex SNPs will be performed under the direction of Larry Karnitz, Ph.D., Mayo Clinic Rochester, and will be conducted either in conjunction with Dr. Robert Diasio's SNP studies or using Illumina GoldenGate Genotyping assays in the Genotyping Shared Resource. These methods are described in more detail below.

2. **Oxaliplatin Pharmacogenetics:**

Although the pharmacology of oxaliplatin is less well defined, all platinum agents appear to be influenced by intracellular levels of the glutathione S-transferase (GST) family [222]. This multigene family is a key component of detoxifying pathways and is responsible for conjugation of reactive radicals. In addition, genetic variation in XRCC1 [191] and XPD [190] have been associated with response to oxaliplatin.

Specific Aim: To evaluate the deletion in *GST M1* and a SNP in *GST P1* to determine the influence of detoxification genotype on cytotoxicity and outcome [223]. In addition, genetic variants in XRCC1 and XPD will be identified and correlated with toxicity events and treatment response.

Materials and Methods:

a) *Patient samples*: This study will take advantage of the infrastructure in place for this trial to provide complementary pharmacogenetic and serum analyses in clinical samples. A 30 mL blood sample in three vacutainer tubes (two tubes containing EDTA anticoagulant [lavender top], and one plain tube [red top]) will be obtained from each patient at study registration. From EDTA tubes, genomic DNA will be extracted using standard techniques, quantified by fluorescence spectroscopy and stored at −80ºC prior to use. EDTA tubes will also yield plasma, and red top tubes will yield serum, which will both be processed and stored frozen at -80ºC prior to use. Complete patient confidentiality will be maintained for the genomic DNA samples and serum analyses as only the anonymous unique laboratory identifier will be available for identification of individual samples. However, this laboratory identifier can be linked backed to appropriate patient information via a secured password-restricted database. We will also obtain formalin fixed, paraffin embedded tumor samples from each patient to: 1) extract DNA after pre-registration and prior to registration/randomization in order to determine *KRAS* mutation status and 2) perform IHC.

b) *Tumor DNA and RNA extraction*: Tumor and normal tissue samples will be microdissected from 1 cm^2 areas on the unstained, 10 um thick slides and scraped into microfuge tubes. The QIACUBE system (cat # 9001292; Qiagen Inc., Santa Clarita, CA) for full automation of nucleic acid purifications using QIAGEN spin kits will be utilized for the extraction of DNA and RNA from biospecimens. This will eliminate the need for manual steps and help achieve consistency in the extraction performance and yield, while avoid pipetting errors. Sample preparation follows the same steps as the manual procedure (i.e., lyse, bind, wash, and elute). For the purification of genomic DNA from FFPE tissue sections we will utilize the QIAamp DNA FFPE Tissue Kit (cat #56404; Qiagen Inc., Santa Clarita, CA), according to the manufacturer's instructions. This kit uses special lysis conditions to release DNA from tissue sections and to overcome inhibitory effects caused by formalin crosslinking of nucleic acids. For the methylation studies, purification of bisulfite converted DNA will be performed using the EpiTect Bisulfite Kit (cat #59110; Qiagen Inc., Santa Clarita, CA) according to the manufacturer's instructions. EpiTect Bisulfite Kits enable complete conversion of unmethylated cytosines to uracils and subsequent purification in less than 6 hours. This method allows protection against DNA degradation and ensures high conversion rates of over 99%. RNA purification from FFPE sections will be performed using the RNeasy FFPE Kit (cat #74404; Qiagen Inc., Santa Clarita, CA), according to the manufacturer's instructions. This method reverses formaldehyde modification of RNA and releases RNA from tissue sections while avoiding further RNA degradation. Additionally, the use of gDNA Eliminator spin columns allows the removal of genomic DNA contamination. These methods have been successfully

employed for the analysis of tissue DNA from several thousand FFPE blocks and, in our experience, the amount and purity will be more than sufficient to complete the full spectrum of proposed laboratory assays. For RNA, we expect that further optimization of the extraction methods may be needed to obtain high-quality materials for the proposed gene expression analyses (which represent one component of the proposed translational). However, given our promising experience to date, we do not foresee this as an insurmountable obstacle.

c) *QGE, SNP genotyping, CpG site specific methylation detection*: The proposed methodologies for performing the assays will be performed according to established protocols in the Diasio laboratory, Mayo Clinic Cancer Center, using the MassARRAY system. Based on our experience and others, this approach will allow efficient, high throughput for the large number of samples proposed (n=3768 cases target enrollment) without compromising sensitivity or specificity of the analyses. MassARRAY, in combination with MALDI-TOF mass spectrometry technology, offers a suite of quantitative and qualitative applications for molecular testing at the genotyping (iPLEX Gold assay), gene expression (MassARRAY QGE) and methylation (MassARRAY Epityper) levels, which are the current core of translational research. Major strengths of this approach include the follow up of whole genome studies, mainly in the areas of SNP genotyping, DNA methylation and gene expression analysis. The MassARRAY Epityper for CpG site specific methylation detection allows the assessment of individual methylation ratios for CpGs within a target sequence in a range between 10-90%, with a standard deviation of 5% (scalable for investigating a few or several hundred regions over multiple samples). Long reads up to 600 bp in one reaction enable discovery of differential methylation within large promoter regions. The methodology is suitable for a range of sample types, including FFPE samples. The iPLEX Gold assay for genotyping on the MassARRAY® Platform provides an ideal balance of multiplexing (up to 40 SNPs in a single well) and throughput (up to 384 samples in parallel) that allows fine-mapping and SNP validation studies with greater than 99.7% accuracy and direct mass detection of the molecule of interest, as well as the capability of discovering failed PCR or tri-allelic SNPs. The MassARRAY QGE allows quantitative gene expression of up to 24 multiplexed targets per reaction from a wide variety of samples. This platform allows investigating a few or several hundred regions over multiple samples with >10-1,000 times greater sensitivity over real-time PCR and has proven to offer high precision over a large dynamic range with accurate quantitaion through a comprehensive data normalization set. Additional advantages of the MassARRAY QGE include its suitability for post-array validation; splice variant analysis; biomarker characterization; disease association studies; copy number variation; loss of heterozygosity; high rate of first-pass assay success (100%) with standardized PCR conditions; use of ~50-100 times less total RNA (start with 5 pg); examination of 20-200 genes for larger studies; comparison of absolute levels of gene expression within the same biological sample; rapid assessment of optimal reference gene sets for data normalization; detection of as little as a single molecule; differentiation of 10% change in expression levels; and high precision (~3% RSD) over a large dynamic range.

i) **QGE:** Measurements of candidate gene expression will be conducted using a MALDI-TOF MS platform (SEQUENOM, Inc., San Diego, CA). This platform will be used to quantitatively determine the level of expression of genes in the pyrimidine catabolic and anabolic pathways, colon cancer associated genes, Folate, Methionine, one carbon metabolism genes and DNA repair and oxaliplatin associated sensitivity and resistance genes. This approach allows multiplexing of 16 genes/well for the determination of the number of cDNA molecules per sample with MassARRAY QGE-iPLEX technology. The data will be normalized with a set of internal control genes. QGE analyses will be performed using a Competitive PCR and MassARRAY technique using the Matrix for Pre-PCR methods. QGE Analyzer Software will be used for data analysis. cDNA samples will

be run at a 1:10 dilution with a 10 plex panel of control genes to be used in normalization with a competitor range of 1x10-8 M to 1x10-18 M. Reports can be generated with information such as plate summary (ID, date, user, number of assays, samples, plexing level, number of calls, call rate, and status of calls), allelotype (average frequency of allele 1 and 2, frequency error, assay status, primer frequency, and pause frequency), allelotype correction for heterozygous skewing, assay type count (number of assays for the selected data), best call probability, call probability, description count (count of each call status for the selected data), genotype area (genotyping data for the selected experiment and additional information derived from peak area data), and QC data.

ii) **SNP Genotyping**: A SNP genotyping strategy will be employed utilizing a MALDI-TOF MS platform (SEQUENOM, Inc., San Diego, CA) and the iPLEX Gold reaction to screen for genetic variants in pyrimidine catabolic and anabolic pathways. This is a universal method for detecting insertions, deletions, substitutions, and other polymorphisms in amplified DNA. This approach allows multiplexing of 36 SNPs per well. The flexibility of this platform allows the incorporation of any number of SNPs in multiplexed pools (~1-40 SNPs/pool), which offers the option to include novel SNPs reported in future literature. The first step in the post-PCR processing of iPLEX Gold reactions is to neutralize unincorporated dNTPs in amplification products using shrimp alkaline phosphatase (SAP). The SAP cleaves a phosphate from the unincorporated dNTPs, converting them to dNDPs and rendering them unavailable to future reaction. Next, iPLEX Gold reaction cocktail (primer, enzyme, buffer, mass-modified nucleotides) is added to the amplification products. The amplification products and iPLEX Gold reaction cocktail are thermocycled to process the iPLEX Gold reaction, which involves the enzymatic addition of mass-modified nucleotides into the diagnostic site. In the reaction mixture, all four mass-modified nucleotides (A, T, C, and G) are present. During the iPLEX Gold reaction, the primer is extended by one of the nucleotides, which terminates the extension of the primer. Using a DNA polymerase that incorporates nucleotides, the iPLEX Gold reaction produces allele-specific extension products of different masses depending on the sequence analyzed. Prior to mass spectrometry, the products of the iPLEX Gold reaction are desalted and transferred onto a SpectroCHIP by the MassARRAY nanodispenser. The SpectroCHIP is then analyzed by the MassARRAY analyzer compact using the the Typer 4.0 Software. The Typer software is a suite of modular applications that enable the determination of the SNP genotype or allelotype (frequency) of reaction products analyzed on a MassARRAY MALDI-TOF MS system. Additionally, the number of VNTR in the promoter enhancer region of the TYMS gene will be detected utilizing a DHPLC methodology previously developed and published by our laboratory [224].

iii) **CpG Site Specific Methylation Detection:** Quantitative CpG site specific methylation detection in candidate genes will be analyzed using a MALDI-TOF MS platform (SEQUENOM, Inc., San Diego, CA). This approach will be used to screen for aberrant methylation patterns of genes in the pyrimidine catabolic and anabolic pathways. Addition this approach will be utilized to evaluate the risk predictive value of the traditional CIMP versus the recent CIMP five gene panel [133]. This technique involves a bisulfite-treatment-based method for the discovery and CpG site specific quantitative analysis of DNA methylation using base-specific cleavage. In this cleavage reaction, the reverse strand is cleaved by RNase A at specific bases (U or C). Cleavage products are generated for the reverse transcription reactions for both U (T in DNA) and C in separate reactions. The reactions are then transferred onto a 384-Spectro-CHIP bioarray. Analyses of the cleavage products are performed on the Sequenom MassARRAY Analyzer, resulting in a distinct signal pair pattern from the methylated and non-methylated template DNA (C/T) variations,

which appear as G/A variations in the cleavage products, resulting in a mass difference of 16 Da per CpG site. The EpiTYPER software generates quantitative results for each cleavage product, enclosing either one CpG site or an aggregate of multiple CpG sites (known as a "CpG unit") depending on the cleavage site. For methylation detection in the DPYD promoter region, a pyrosequencing technique will be utilized to analyze two CpG islands detected in the DPYD gene. The main advantage of pyrosequencing technology over conventional bisulfite sequencing is the fact that quantitative DNA methylation information can be obtained from whole PCR products, without the need for cloning and sequencing of a large number of clones to obtain statistically relevant information.

d) *Investigated genes for QGE, SNP genotyping, CpG site specific methylation*: Investigated genes will include, but not limited to, <u>colorectal cancer associated genes </u>(e.g. AKT1, APC, BAX, BCL2, KRAS, BRAF, PDGFRB, PIK3CA, EGF, EGFR, STAT, PKC, Src, and TP53); <u>Pyrimidine Metabolism associated genes</u> (e.g., DPYD, DPYS, BUP1, NME2, NME7, UMPS, UPP1, TK, TYMS and MTHFR); <u>Folate Biosynthesis Genes</u> (e.g. ALPI, ERCC2 and ERCC3); <u>One Carbon Metabolism Genes</u> (e.g. MTFMT, MTHFR, and TYMS); <u>DNA Repair and Oxaliplatin Associated Sensitivity and Resistance Genes</u> (e.g. ERCC-1/ERCC1-XPF, Bcl-2; Bax,, RAD9,POL32, MMS4), <u>Apoptotic genes </u>(e.g. CD40LG, CD40, CD27, BAD, BAX, BCL2, BCL2A1, CASP1 and CASP); <u>Cell cycle genes</u> (e.g. ABL1, ATM, CDK2, CDK4, CDK5R1, CHEK1,,CHEK2, RAD1, RB1, B2M and HPRT1), <u>Angiogenesis genes </u>(e.g. AKT1, ANGPT1, COL4A3, TYMP, EGF, HAND2, MDK, MMP2, MMP9, NOTCH4, TGFA, TNF, VEGFA and VEGFC); <u>Epigenetic chromatin modulation genes </u>(e.g. KDM1, AURKA, AURKB, DNMT1, DNMT3A, DNMT3B, HAT1, HDAC1, HDAC10, HDAC11); <u>Methylation genes</u> including the traditional and new CIMP genes (e.g. SOCS1, CACNA1G, CDKN2A, IGF2, MLH1, RUNX3, CRABP1, MGMT, NEUROG1, PAX2, MINT1, hMLH1, MINT2, MINT31 and P16); <u>Cancer drug resistance and metabolism genes </u>(e.g. ABCB1, BRCA1, BRCA2, CDK2, CDKN2A, CYP1A1, CYP2C19, CYP2C9, CYP2D6, CYP3A4, CYP3A5, DHFR, EGFR, ERBB2).

e) *IHC for CpG site specific methylation:* We will analyze protein expression to verify loss of expression for highly methylated genes. IHC will be performed using commercially-available antibodies and according to manufacturer's recommendations. In general, the IHC staining will be scored as described in the Materials and Methods in Specific Aim 1.

f) *Illumina GoldenGate Genotyping for RAD17-911 complex SNPs:* The genotyping will be performed under the direction of Dr. Larry Karnitz (Mayo Clinic Rochester), and in collaboration with Dr. Richard Weinshilboum and the Mayo Advanced Genomics Technology Center, which runs the Genotyping Shared Resource (GSR) core facility. The purified DNAs will be robotically aliquoted into 96-well plates. Each plate will contain 88 patient samples. As controls, each plate will also contain 2 randomly selected duplicates of the patient samples, and duplicates of a trio of CEPH DNAs (2 parents, 1 child). Genotyping will be performed using Illumina GoldenGate Genotyping assays, which employ primer hybridization, extension and ligation in 48-, 96-, 144-, 192-, and 384-multiplexing options. Following PCR amplification using specifically labeled fluorescent primers, the products will be hybridized to Illumina VeraCode beads, which will be analyzed with the Illumina BeadXpress reader. BeadStudio (Illumina) software will be used for automated genotype clustering and calling according to standard protocol [225]. Only SNPs that show duplicate concordance >95%, are in Hardy-Weinberg equilibrium, and have call rates in excess of 90% will be used for the association studies. Because we believe the Set 1 SNPs with functional effects (see below) may be the most informative and interesting, if any of the assays for these SNPs do not meet these

standards, we will use TaqMan allelic discrimination assays to perform those assays.

i) Selection of Polymorphisms for Analysis:

Set 1: All coding SNPs with minor allele frequency (MAF) ≥ 0.005 that affect expression and/or function of Rad17 or the 911 complex will be included in our genotyping assays. Coding SNPs to be analyzed will be based on information available in the public databases. To date, 24 coding SNPs in these 4 genes (i.e., 5 SNPs for *RAD17*, 8 SNPs for *RAD9A*, 5 SNPs for *HUS1,* and 6 SNPs for *RAD1*) have been identified.

Set 2: Polymorphisms that lie outside coding sequences can also affect phenotypes by altering the binding of transcription factors, the stability of the mRNA, the splicing of the mRNA, the rate of protein translation, etc. Because of the vast number of noncoding polymorphisms, even in genes as compact as *RAD9A* (6 kbp), it is not economically feasible to analyze whether each polymorphism is associated with toxicity. However, because adjacent polymorphisms are often inherited as haplotype blocks due to linkage disequilibrium (LD), it is possible to select tagging SNPs that represent these blocks of polymorphisms.

ii) Discovery Study:

We will use a two-tiered approach to search for associations between SNPs and FOLFOX toxicities. In the first step, we will perform a discovery analysis, where we will use all Set 1 and Set 2 SNPs and look for association with adverse events occurring at a rate of > 2% and any grade ≥3 adverse event. For this discovery study we will use a statistical significance cut-off of p < 0.10. Assuming 10 AEs are considered for each of 96 SNPs, the total number of tests will be 960, which by chance alone would result in 96 positive associations to be brought forward to the validation study. In order to maintain 80% power for the validation study, 2000 patients in the validation study will be required (see below). Thus we expect approximately 1600 patients (selected at random) will be available for the discovery study. Using a p-value cut-off of 0.10 for the discovery study, this provides greater than 85% power for detecting differences in the rate of adverse events of 5% vs. 20% or 20% vs. 40% for SNPs with prevalence as low as 2%. Given the low expected prevalence of many of the SNP markers to be tested, the primary comparison will be between patients with both dominant alleles vs. patients with either one or both rare alleles.

iii) Validation Study:

All SNPs with a positive association with adverse events at the 0.10 level in the discovery analysis will be brought forward to the validation study in 2000 patients. The tests in the validation study will be at the 0.05 level. 2000 patients was selected to provide 80% power for testing at the 0.05 level for association between SNPs and adverse events at a magnitude of for example 5% vs 20% or 20% vs 40% (for a SNP with prevalence of 2%) which are considered the minimal differences required for clinical relevance. SNPs with higher prevalence will have greater power for detecting an association. The power for SNPs with lower prevalence is obviously lower, however, for a SNP with prevalence 1%, we still have 80% power to detect a difference in AE rates of 5% vs. 30%, which is likely the magnitude needed for clinical relevance for such a rate SNP. This discover/validation testing plan assures an overall false positive rate of 5% for each SNP considered in this analysis for

association with any toxicity (roughly, 100 SNPs * 10 AEs = 1000 tests, discovery level test requires p< 0.10 leaving 100 SNP/AE associations by random, validation level test requires p < 0.05 leaving 5 false positive SNP/AE associations out the 100 SNPs considered).

SPECIFIC AIM 5: **Apoptotic Susceptibility as Determinant of Treatment Response and Prognosis**

A. **Pro-Apoptotic BH3-only Proteins as Novel Prognostic and Predictive Markers**

Background and Significance: We showed for the first time that a higher level of expression level of pro-apoptotic BH3-only proteins (Bim, Puma, Bad, Bid) in primary stage II and III human colon cancers are independently associated with more favorable survival compared to tumors with lower level expression in 5-fluorouracil-based adjuvant trials [226, 227]. These data [228-230] support experimental evidence that these BH3 proteins serve a tumor suppressor role [231-234]. Therefore, we propose to validate these results in this study, NCCTG N0147. A critical question, however, is whether the prognostic effect observed is actually due to a predictive effect which we could not address since relatively few patients were randomized to observation alone arms. The utility of the BH3-only proteins as predictors of the benefit of 5-FU-based therapy is a highly relevant question with considerable importance to cancer treatment.

Inhibition of the epidermal growth factor receptor (EGFR) induces apoptosis that is inhibited by pro-survival Bcl-2, and sensitivity was restored by treatment with a small molecule Bcl-2/Bcl-x_L antagonist. The pro-apoptotic BH3-only protein BIM was found to be up-regulated after erlotinib or gefitinib treatment through both transcriptional and post-translational mechanisms, and suppression of BIM by siRNA induced resistance to erlotinib [235-237]. Blockade of MEK-ERK1/2 signaling, but not blockade of PI3K or AKT, was critical for BIM activation. Using RNA interference, BIM was shown to be essential for gefitinib-induced killing of NSCLC cells. Taken together, these data indicate that BIM activation is essential for tumor cell killing and that shutdown of the EGFR-MEK-ERK signaling cascade is critical for BIM activation.

Autophagy is a degradation process whereby cellular proteins and organelles are delivered to lysosomes for degradation [238-240]. Autophagy is a mechanism of adaptation to cellular stress and may; therefore, confer protection from cell death [239]. Tumor suppressor proteins, such as BH3-only proteins, can induce autophagy [241-244]. Beclin-1 is an essential autophagy protein that is bound to and inhibited by Bcl-2 or Bcl-x_L via its BH3 domain. Recent data indicate that beclin-1 expression may confer prognostic information in stage III colon cancer patients [245].

Specific Aims:

a) To validate the prognostic impact of BH3-ony protein expression in stage III colon cancers from a large, adjuvant therapy trial.

b) To determine whether expression of the the BH3-only protein BIM, or regulators of autophagy (beclin-1) can predict response to cetuximab in colon cancers from patients treated in this adjuvant study, N0147.

Materials and Methods:

a) *Immunohistochemistry (IHC)*: IHC for BH3-only proteins has been previously described in our recent publications [226, 227]. Staining for Beclin-1 has been described by Li et al [245] and is being performed currently in our laboratory.

b) *IHC Scoring*: Staining intensity (0/1/2/3+) and percent tumor cell positivity (grouped into quartiles) will be determined and categorized at light microscopy [227]. Intensity and immunopercent will be multiplied to yield an immunoscore. All specimens will be analyzed and scored by a pathologist without knowledge of clinical information. Dr. Thomas Smyrk (Mayo GI pathologist) will establish scoring criteria, to be agreed upon before analysis, and will oversee this aspect. Slides will be scanned by a cytotechnologist using a slide scanner and the images will be digitally captured at 480 x 752 pixel resolution and at x40 magnification (BLISS system, Bacus Labs).

B.   Bcl-2 Family:

Background and Significance: Anti-cancer drugs exert their cytotoxic effects against tumor cells by inducing apoptosis. Therefore, susceptibility to apoptosis is a critical determinant of the efficacy of anti-cancer therapies. Understanding and predicting this susceptibility holds promise for improving therapeutic efficacy. Apoptotic cell death is genetically regulated by the *Bcl-2* gene family [246]. The anti-apoptotic Bcl-2 and Bcl-X_L proteins confer resistance to multiple anti-cancer drugs whereas the pro-apoptotic *Bax* gene sensitizes tumor cells to these therapies. Specifically, *Bax* has been shown to enhance apoptosis induction by 5-FU and other anti-cancer drugs in human colon cancer cells [247, 248]. Furthermore, the ratio of Bcl-2 to Bax has been shown to predict chemosensitivity to 5-FU in carcinoma cell lines [249]. In addition, Bcl-X_L anti-sense oligonucleotides were shown to sensitize human colon cancers cells to 5-FU [250]. The mechanism by which Bcl-2 confers resistance to a wide range of cytotoxic drugs appears due to its inhibition of cytochrome c release from mitochondria [251]. Anti-cancer drugs activate the mitochondrial apoptotic pathway which is accompanied by caspase activation, mitochondrial release of cytochrome c, and translocation of the pro-apoptotic *Bax* protein from the cytosol to the mitochondrial membrane [252]. Bcl-2 has been shown to delay or inhibit these processes. Furthermore, Bcl-2 anti-sense oligonucleotides can block Bcl-2-mediated drug resistance *in vitro* and *in vivo,* and clinical trials to evaluate their efficacy in patients with lymphoma are ongoing. Studies by ourselves [253] and others [254, 255] have shown that Bcl-2, Bcl-X_L and Bax are overexpressed in human colorectal cancers. Data also indicate that Bax [254, 255] and Bcl-2 [253, 256] expression may confer prognostic information in colon cancer patients. At a median survival of 69 months, UICC stage III primary colon cancers with high level Bax expression had better survival rates than did tumors with low Bax expression (p=0.009) [254]. In some studies, Bcl-2 protein expression has been paradoxically associated with more favorable outcome in colon cancer patients [253, 256, 257]. Furthermore, we found that the prognostic utility of Bcl-2 was most evident in untreated, proximal stage II colon cancers that were DNA diploid [253]. Interestingly, Bcl-2 protein expression has been reported to occur more frequently in MSI-H versus MSS human colon cancers [258]. In addition, the ratio of Bcl-2 to Bcl-X_L was shown to be prognostic in human breast cancers [259]. The aforementioned studies regarding the prognostic utility of these markers in colon cancer patients have generally been relatively small, retrospective studies that included different tumor stages.   Therefore, evaluation of these markers in relation to

clinical outcome in a prospective study of same stage colon cancer patients is seriously needed and represents an aim of this proposal.

Specific Aim: To evaluate anti- (Bcl-2, Bcl-X_L) and pro-(Bax) apoptotic Bcl-2 family members in relation to disease-free and overall patient survival rates after 5-FU-based adjuvant therapy for stage III colon cancer. The ratio of Bcl-2 to Bax and to Bcl-$_{XL}$ and their association with clinical outcome will also be determined.

Materials and Methods: Bcl-2, Bcl-X_L and Bax expression will be determined using commercially available antibodies and immunohistochemical methods as previously described in Specific Aim 1. Procedures for scoring of IHC are outlined below. In brief, tumor cell percent cell positively and staining intensity will be determined and dichotomous values assigned as discussed below. A weighted score will then be computed for each marker (product of % cell positively and intensity).

C. Caspase-3 As a Surrogate Marker of Apoptotic Susceptibility

Background and Significance: Caspases are endogenous cell death proteases that respond to pro-apoptotic stimuli (caspase-8, 9, 10) and catalyze the activation of effector caspases (caspase-3, 7) which perform proteolytic cleavage events involved in apoptosis [251]. Anti-cancer drugs engage the mitochondrial apoptotic pathway, which involves release of cytosolic cytochrome c with activation of Apaf1 and caspase-9, and subsequent caspase-3 activation [251]. Caspase-3 is the main effector caspase of the both mitochondrial and death receptor-mediated apoptotic pathways. Once cleaved through activation of the apoptotic cascade, caspase-3 is a marker of cellular commitment to apoptosis that precedes nuclear DNA fragmentation and apoptotic morphology which are late events in the cell death pathway [260]. While DNA fragmentation has been considered the standard for the detection of apoptosis in individual cells, positive TUNEL staining can also be found in necrotic cells. Evidence indicates that the basal level of apoptotic activity in a tumor may predict the magnitude of the apoptotic response to anti-cancer drugs or radiation [261, 262]. The predictive and prognostic utility of tumor apoptotic rates remains controversial and its clinical utility have been limited by the low frequency and narrow ranges of apoptotic indices and the small numbers of patients studied [253]. Despite these limitations, we [253] and others [263] have shown that apoptotic indices in human colon cancers are associated with shorter patient survival rates. Our finding was limited to tumors of the left colon which had higher rates of aneuploidy and p53 expression [253].

Cleaved caspase-3 expression signals impending apoptotic cell death and may increase the sensitivity while maintaining specificity for the detection of apoptosis in tissue specimens [264]. Gown et al [264] exposed tumor cells in culture to apoptotic stimuli and detected cleaved caspase-3 proteins in cells with apoptotic nuclear morphology by fluorescence microscopy. The strong caspase-3 signal remained detectable after these cells were formalin fixed and paraffin embedded. These data suggest that caspase-3 expression may be a highly specific and sensitive method for detection of individual apoptotic cells in archival tumors.

Specific Aim: To determine and compare the frequency of caspase-3 expression with late apoptotic morphology and to determine the association of caspase-3 staining with clinical outcome in stage III colon cancer patients.

Materials and Methods: Caspase-3 expression will be analyzed by IHC as previously described [265]. We will evaluate the usefulness of three commercially available antibodies (Santa Cruz, Cayman Chemicals and Zymed) on a subset of samples prior to proceeding with the larger patient cohort. Before incubation with the primary antibody, slides will be subjected to antigen retrieval in a solution of 10 mM citrate buffer, pH 6.0 for 10 minutes in a microwave oven at 700W, and cooled down in this buffer to room temperature. A polyclonal goat anti-caspase-3 antibody (Santa Cruz Biotechnology, Inc., CA) has been evaluated and is applied to tissue sections at a dilution of 1:50 and incubated overnight at 4°C. Tonsil and colon tissue sections will be used as positive controls for caspase-3 expression. Caspase-3 staining will be scored as outlined below.

Immunochemistry and Scoring: Each tissue marker will be analyzed on coded material independently of the standard histopathologic assessment. Marker expression will be evaluated for intensity of staining (weak, moderate, or strong) and percent of tumor cells positive, and will be recorded as dichotomous variables.

The percentage of immunopositive tumor cells will be grouped into the following categories (0; < 5%, 1, 6-25%; 2, 26-50%; 3, 51-75%; > 75%), as previously described [266]. This system is widely utilized for analysis of protein expression in tissue sections. Staining intensity will be categorized as (0/1/2/3) and a weighted score (product of percent tumor cell positivity and intensity score) will be calculated (0-12) [266]. Tumor specimens will be scored by examiners who are blinded to clinical outcome.

D. **Cyclooxygenase-2 (COX-2) Expression:**

Background and Significance: Cyclooxygenase-2 (COX-2) is an inducible enzyme that regulates the biosynthesis of prostaglandins (PGs) and related eicosanoids [267]. PGs, particularly PGE_2, are increased in colorectal neoplasms relative to adjacent normal mucosa [267]. COX-2 is induced in the setting of inflammation and in certain epithelial neoplasms, including colorectal cancers. In this regard, COX-2 is overexpressed in the tumor cell cytoplasm in 80-90% of human colon cancers [268]. Interestingly, we [269] and Karnes et al [266] at Mayo Clinic have shown that human colon cancers with defective DNA MMR have reduced rates of COX-2 expression relative to MSS cases. In a study of 76 colorectal cancer patients, increased dichotomized COX-2 expression was associated with more advanced tumor stage, increased tumor size, and positive lymph node status [270]. Moreover, a higher level of COX-2 expression (versus the lowest level) was associated with reduced patient survival rates [270]. COX-2 overexpression has also been associated with adverse outcome in carcinomas of the breast [271], lung [272], and esophagus [273], and ovary [274]. However, these studies have been retrospective and generally involve tumors of different stages. The mechanism by which COX-2 may adversely affect patient outcome is unknown, but may relate to its role in apoptosis resistance, metastasis, or angiogenesis [275]. Colon cancer-induced angiogenesis is regulated by mechanisms that include COX-2 mediated modulation of pro-angiogenic factors, including VEGF [276].

Specific Aim: To determine the correlation between COX-2 protein expression and localization with clinicopathological variables, MSI status, and patient survival rates. COX-2 overexpression may confer resistance to 5-FU based adjuvant therapy and may, therefore, be associated with poorer survival rates.

Materials and Methods: COX-2 expression will be determined by IHC. Briefly, after dewaxing and rehydrating, samples will be blocked in 3% peroxide/methanol. After antigen retrieval (microwave for 4 minutes in PBS, pH=7.4), samples will be avidin and biotin blocked (Vector). The COX-2 antibody (Cayman Chemical) will be applied at a dilution of 1:500 for 2 hours at room

temperature. After washing, slides will be processed using the Vectastain Elite ABC kit (Vector) and developed with 3,3'-diaminobenzidine (Research Genetics). For COX-2, the primary monoclonal antibody (MAb) against human COX-2 (Cayman Chemical, Ann Arbor, MI) recognizes a 19-amino acid sequence at the carboxy terminus that is absent in COX-1. After rinsing in PBS, the biotylinated secondary immunoglobulin G antibody is applied for 30 minutes at RT. Slides are then rinsed in PBS, and avidin conjugated to horseradish peroxidase (ABC reagent) is applied for 45 minutes at RT. The chromogen 3,3' diaminobenzidine (Research Genetics, Huntsville, AL) will be added, and the color reaction observed at light microscopy. The reaction will be stopped by immersing slides in deionized water, and slides counterstained with hematoxylin and then mounted.

As a control for nonspecific staining, a COX-2-specific blocking peptide (Cayman Chemical), derived from the human *COX-2* complementary DNA sequence, will be mixed with the COX-2 MAb and applied to sections. As a negative control, the primary MAb will be omitted and PBS applied while maintaining all other steps of the procedure.

SPECIFIC AIM 6: Characterization of the Immune Profile of Sporadic Colon Cancers in Relationship to DNA Mismatch Repair Status

Background and Significance: Approximately 15% of sporadic colon cancers show MSI due to defective DNA mismatch repair secondary to epigenetic inactivation of the *hMHL1* gene [277]. MSI colon cancers show distinct phenotypic features include proximal site, poor differentiation, and increased tumor infiltrating lymphocytes (TILs) [109, 277-281]. Several studies by ourselves [280-284] and others [285, 286] have shown that patients with MSI colon cancers have a better stage-adjusted prognosis compared to microsatellite stable (MSS) cancers. However, the mechanism underlying their better prognosis remains elusive. In a prior study, we found that increased TILs were significantly associated with higher apoptosis to proliferation ratios suggesting that immune activation may limit tumor growth [280]. We hypothesize that the anti-tumor immune response generated by MSI tumors, as shown by their increased TILs [109, 278, 280], is a critical factor in limiting their progression and can account for their better prognosis. While most TILs are T lymphocytes based upon their expression of the pan T lymphocyte marker CD3[+] [287] a comparison of the specific T cell subpopulations that infiltrate MSI versus MSS cancers has not been adequately performed.

Studies by ourselves [280] and others [288] indicate that the anti-tumor immune response can influence prognosis in colon cancer. Colon cancers expressing higher levels of markers of adaptive immunity including CD3+,CD8+, granzyme B and CD45RO+ were associated with reduced rates of relapse [289]. Cytotoxic CD8+ T cells secrete granzyme B, an inducer of apoptosis, which can serve as a measure of cytotoxic activity [290, 291]. However, it is unknown whether these T cell markers are differentially expressed based upon tumor MSI status. We propose to compare markers of adaptive immunity (CD3+, CD4+, CD45R0+) and the cytotoxic T cell (CTL) response (CD8+, granzyme B) in MSI versus MSS cases. We hypothesize that CD8+ cytotoxic T lymphocytes will be increased in MSI versus MSS colon cancers and may contribute to their better prognosis. In contrast, regulatory T cells (Tregs) appear to interfere with the host-mediated immune anti-tumor response and may interfere with CTL-induced tumor cell death [292-296]. We will test the hypothesis that MSI-H tumors have a reduced Treg density and therefore, show a decreased capacity for immune evasion. Tregs (CD4[+]CD25[+]FOXP3[+]) express the nuclear transcription factor forkhead box P3 (FOXP3) that is required for their development and function [297, 298]. FOXP3 is regarded as a specific marker of Tregs [297] and its protein expression can be evaluated by IHC. Recent data indicate that COX-2, which is upregulated at sites of inflammation, can induce FOXP3

expression [299, 300] suggesting an important link between COX-2 and immune suppression [301, 302]. Induction of FOXP3 by COX-2/PGE$_2$ has been shown to be reversed by treatment with NSAIDs *in vitro* [300]. Accordingly, we will analyze the association between COX- and FOXP3+ Tregs in colon cancers. We will also study the functional impact of T cell infiltration by measuring the inflammatory cytokines IL-6 and IL-17 within the tissues [303]. Pending resources and in addition to the lymphocyte markers described above (e.g., CD3, CD4, CD8, granzyme B, FOXP3, CD45RO), we will also plan to assess tumor cell surface proteins representing human leukocyte antigen (HLA) class I, HLA class II, and beta-2 microglobulin (B2M), as well as the transporter 2 (TAP2) and large multifunctional peptidase 7 (LMP7) components of the antigen processing machinery, reflecting cytotoxic, regulatory and mixed-function T cells, by IHC.

Specific Aim: To compare the immune profiles of MMR deficient and proficient cancers by analyzing T lymphocyte subsets related to immune rejection or immune evasion that may explain differences in prognosis. We have a unique opportunity to conduct this study given the large number of colon cancers to be analyzed for DNA mismatch repair (MMR) protein expression in this study.

Materials and Methods:

a) *Patient population*: Surgically resected stage III colon carcinomas from patients treated in this study, NCCTG N0147. Of the approximately 3700 cases available for MMR protein expression, 15% of these tumors would be expected to show loss of a MMR protein consistent with defective MMR. An equal number of colon cancers with intact MMR expression matched for age category and gender will be used as a comparison group.

b) *Tissue Microarrays*: We will construct tissue microarrays from all cases as described in Specific Aim 7 and as previously described [226]. A GI pathologist will select tumor blocks and will mark the areas of tumor and normal mucosa on each block for coring.

c) *Immunohistochemistry (IHC)*: IHC will be performed in the TACMA Shared Resource, Mayo Clinic Rochester, using an immunoperoxidase system. Slides from tissue microarrays (TMAs; see Specific Aim 7) will be incubated with commercially-available antibodies against CD3, CD4, CD8, granzyme B, CD45RO, IL17, IL-6, FoxP3, HLA class I, HLA class II, B2M, TAP2, and LMP7. COX-2 staining will be performed as described in Specific Aim 5(D). Isotype-matched antibodies will be used as negative controls.

d) *IHC Scoring*: The density, i.e. average number of lymphocytes positive for each marker over the total TILs, will be quantified manually in 5 high-power fields in each of tumor epithelium and peritumoral stroma, scored separately, for each of the 3 tumor cores per case. We will calculate the CD8+/CD4+ ratio as well as the ratios of CD3+, CD4+, and CD8+ to FoxP3+, in addition to the COX-2/FoxP3 ratio. TMAs will be scanned using the BLISS system (Bacus Labs) that enables scoring at the time of microscopic review [226]. In general, all TMAs will be scored as described in the Materials and Methods in Specific Aim 1 by clinical pathologists blinded to clinical data.

SPECIFIC AIM 7: Pathological Assessment

Background and Significance: : In colorectal cancer, pathologic features were among the first variables studied with reference to biologic behavior and have been the subject of numerous

studies. These studies, however, have employed different approaches to the analysis of pathological features, have been retrospective and have failed to have adequate quality control. In addition, these studies have used different approaches to staging. Because tumor stage is the strongest prognostic indicator in CRC, all other factors of possible importance must be analyzed against staging parameters in order to demonstrate independent prognostic value. Therefore, the prognostic significance of many of the most familiar and widely reported pathologic variables is unclear and remains to be defined by a prospective study. After staging the tumors using the internationally accepted TNM staging system [304] and the standards of pathological evaluation proposed by the College of American Pathologists [305], samples will be assessed for the following by an expert gastrointestinal pathologist under strict quality control. Unless otherwise specified, all evaluations will be done from a routine hematoxylin and eosin stain on a formalin fixed, paraffin embedded tissue section. To ensure that the aforementioned marker analyses (Specific Aims 1-5) are representative of the overall tumor, an attempt will be made to include only those sections that have >70% tumor. A central review of all tumor specimens will be performed by a Mayo Clinic pathologist. This review will be performed on H&E-stained tissue sections.

Tissue microarrays.   We plan to construct colon cancer microarrays to be utilized for immunohistochemistry (IHC) [306]. NCCTG Research Base GI pathologists, will select representative areas of tumor and normal tissue from H&E-stained sections made from each paraffin block from which cores will be taken. Two to 5 cylinders of 0.6 mm diameter tissue will be taken from each block from previously selected representative areas and arrayed into a new recipient paraffin block with a custom-built precision instrument (Beecher Instruments, Silver Spring, MD). Serial sections (4-6 μm) will then be cut from the multitumor tissue microarray block and applied to 3-aminopropyltriethoxysilane (APES)-coated slides (Sigma). Expertise for making tissue microarrays exists at Mayo Clinic in the Division of Pathology and in the molecular genetics laboratory of Dr. Stephen Thibodeau, and in the TACMA Shared Resource. Use of tissue microarrays will assist in preserving the valuable tissue resource of the NCCTG.

A.   **Tumor Grade:**

Background and Significance: A number of grading systems for colorectal carcinoma have been proposed in the literature, but a single widely accepted, uniformly employed standard for grading is lacking. Among the suggested grading schemas, both the number of grades and the criteria for distinguishing among them vary markedly. Regardless of the complexity of the criteria, however, most systems stratify tumors into three or four grades as follows: Grade 1 (well-differentiated), Grade 2 (moderately differentiated), Grade 3 (poorly differentiated) and Grade 4 (undifferentiated). Ultimately, however, histologic grading is largely subjective and is associated with a significant degree of interobserver variability [307]. Yet, despite these issues, histologic grade has repeatedly been shown to be an independent prognostic factor in multivariate analysis [308-311]. Specifically, it has been demonstrated that high tumor grade is an adverse prognostic factor. In the majority of studies documenting the prognostic utility of tumor grade, the number of grades has been collapsed into a two-tier stratification schema (i.e., high grade and low grade) for data analysis. In the two-tier system, low grade has included both well-differentiated and moderately differentiated adenocarcinomas and high grade has included poorly differentiated and undifferentiated cancers. In general practice, a two-tiered grading system would also be expected to reduce interobserver variability since the widest variations in grading occur with stratification of low grade tumors into well- or moderately-differentiated categories and diagnosis of poorly differentiated or undifferentiated tumors is more consistent [312]. In light of its proven prognostic value as well as its relative simplicity and reproducibility, the use of a two-tiered grading system for colorectal carcinoma would be advisable but, unfortunately, is not common practice.

Specific Aim: In this study, tumors will be graded according to a two tier system (low grade or high grade) by the NCCTG reviewing pathologist. A multivariate analysis will be performed to determine the independent prognostic significance of tumor grade in stage III colorectal cancer. In addition, correlation with other parameters such as MSI status will be determined.

B.   Tumor Border Configuration:

Background and Significance: For CRC, the growth pattern of the tumor at the advancing edge (tumor border) has been shown to have prognostic significance that is independent of stage and may predict liver metastasis. Specifically, an irregular, infiltrating pattern of growth, as opposed to a pushing border, has been demonstrated to be an adverse prognostic factor by multivariate analyses [313, 314]. In a study by Jass et al [313] interobserver variability among pathologists evaluating tumor border configuration was found to be about 30% if no specific definitions of infiltrating growth were provided. Concordance was found to improve to 90% when the following diagnostic criteria were employed [313]: 1) inability to define limits of invasive border of tumor and/or to resolve host tissue from malignant tissue on naked eye examination of the slide; and 2) "streaming" of tumor through the full thickness of the muscularis propria without stromal response and/or dissection of mesenteric adipose tissue by small glands or irregular clusters or cords of cells and/or perineural invasion on microscopic examination.

Specific Aim: In this study tumor border configuration will be analyzed according to the above criteria by the NCCTG reviewing pathologist. The guidelines and rationale for tumor border evaluation will be provided. The tumor border will be assessed as either infiltrating or pushing. Tumor border configuration will be correlated with outcome to determine the independent prognostic significance of this feature.

C.   Peritumoral Host Lymphoid Response:

Background and Significance: Lymphocytic infiltration of tumor or peritumoral tissue is indicative of a host immunologic response and has been shown by multivariate analysis in several studies to be a favorable prognostic factor [310, 313]. However, other studies have either failed to confirm the prognostic significance of a peritumoral lymphoid reaction [314] or have demonstrated its significance only by univariate analysis [315]. The specific features that are considered indicative of a host immunologic reaction to tumor include perivascular lymphocytic cuffing in the muscularis propria, perivascular lymphocytic cuffing in the pericolonic fat or subserosa, lymphocytic infiltration at the tumor edge, and a "Crohn's-like" lymphoid reaction (i.e., transmural peritumoral lymphoid follicle formation).

Specific Aim: In this study the peritumoral host lymphoid response will be analyzed by the NCCTG reviewing pathologist. The guidelines and rationale for evaluation of the host lymphoid response will be detailed. Peritumoral host lymphoid response will be assessed as either present or absent and will be correlated with outcome to determine the independent prognostic significance of the presence (or absence) of this feature.

SPECIFIC AIM 8: Epidemiological Factors and Clinical Outcome in Colorectal Cancer

A. **Methylator Phenotype in Relation to Family History and Environmental Exposures:**

Background and Significance: Because dietary factors, lifestyle habits and pharmaceutical agents are involved in methyl group donation, such environmental factors can influence DNA methylation leading to gene inactivation [316]. Epidemiological data including diet and migration studies clearly indicate that environmental factors are important in colon carcinogenesis and influence colorectal cancer incidence [317]. Studies indicate that approximately 15% of sporadic colon cancers are MSI-H due, in nearly all of these cases, to hypermethylation of the *hMLH1* gene [94] and many of these patients have been reported to demonstrate the CIMP phenotype [125]. Interestingly, aberrant methylation of *p16INK4a* was significantly associated with female gender indicating that epigenetic changes are related to certain patient characteristics [318]. Most studies of MSI status in relation to clinicopathological features and patient outcome have been small and retrospective, non population based, and have included hereditary nonpolyposis colorectal cancer (HNPCC) cases within the larger group of MSI-H patients. The relationship between tumor MSI status and colorectal cancer family history was studied using PCR-based analysis of 7 microsatellite loci. Lothe et al [319] found that 31% of patients with a strong family history of colorectal cancer, but not meeting criteria for a hereditary syndrome, exhibited changes at one or more loci compared to 17% of patients without such history. However, a recent study failed to find significant association between family history and MSI status in sporadic colorectal cancer patients [320]. Obtaining detailed family history data on CIMP+ relative to CIMP- cases in a large prospective trial remains critical to further elucidating the phenotype of these patients and may aid in their identification and risk stratification.

Environmental exposure, nutritional factors, and gene-environment interactions including epigenetic changes are likely to be very important in the etiology of most colorectal cancers [317]. In a prospective study utilizing a large population-based cohort (n = 41,836) of postmenopausal women, Limburg et al [321] found that cigarette smoking was associated with an increased risk of incident (RR-1.17; 95% C.I.=1.00-1.36) and fatal (RR=1.31; C.I. 0.98-1.74) colorectal cancer in a multivariate analysis. Furthermore, ever smokers had higher rates of proximal CRC (p=0.03) than incident distal CRCs. Interestingly, CRCs with MSI-H were reported to be more common among smokers in case-control [316] and case-case [317] studies. Of note, sporadic colon cancers that are MSI-H demonstrate hMLH1 hypermethylation and are associated with proximal location and female gender. Cigarette smoking has been shown to upregulate DNA methyltransferase [318] and may also promote hypermethylation in extra-colonic tissues [152]. Therefore, these data suggest a potential link between cigarette smoking and/or tobacco-derived carcinogens and DNA methylation.

Specific Aim: To determine associations between molecularly-defined CRC subtypes, based on CpG island methylation patterns (as well as additional molecular markers described under other specific aims), (1) family history of colorectal neoplasia and (2) common environmental exposures (i.e., smoking, exogenous estrogens, physical activity level, etc.). In addition to analysis of CIMP in MSI-H and MSI-L tumors and the matched MSS patients, patients with at least one first degree affected relative with colorectal cancer diagnosed at age <50 or 2 or more affected first and second degree relatives will also undergo testing for CIMP. Patients meeting Amsterdam criteria for Lynch Syndrome or Familial Colorectal Cancer Type X, or those with the phenotype of familial adenomatous polyposis, will be excluded from these analyses.

Materials and Methods: All patients will be screened for MSI status as previously outlined under Specific Aim 2 (A) and for methylation status, as set forth in Specific Aim 3 (A). To address the specific aim outlined here, we will have each study participant complete a cancer family history

questionnaire and a self-administered Cancer Risk Factor survey. These instruments will be
electronically scored.

B. Body Mass Index (BMI) in CRC:

Background and Significance: While obesity is associated with an increased risk of developing
colon cancer, its influence upon the prognosis of patients with established colon cancer remains
undefined. We analyzed body mass index [BMI (kg/m^2)] in patients with TNM stage II and III
colon carcinomas (n= 4,381) enrolled in 7 randomized trials of 5-fluorouracil-based adjuvant
chemotherapy. Among patients, 868 (20%) were obese (BMI >30 kg/m^2) of which 606 were class
1 (BMI 30-34 kg/m^2) and 262 were class 2,3 (BMI >35 kg/m^2). Obese versus normal-weight
patients were more likely to be younger, to have distal tumors, show proficient DNA mismatch
repair, and increased lymph node metastases (N2: 28% versus N1: 22%; p <0.017). By univariate
analysis, class 2,3 obesity was associated with shorter DFS [HR 1.23 (95% CI 1.01, 1.49),
p=0.037)]. The prognostic impact of class 2,3 obesity was more evident in males [DFS: HR 1.39
(95% CI: 1.04, 1.85); p= 0.027; OS: HR 1.42 (95% CI: 1.07, 1.88); p= 0.015] than in females, and
was independently associated with worse OS [HR 1.35 (1.02, 1.79); p=0.039] versus normal weight
patients. These data suggest that obesity-related biological factors can influence clinical outcome
and have important implications for cancer prevention and treatment.

Specific Aim: To validate the association of body mass index (BMI) with colon cancer recurrence
and mortality and to determine its association with gender and DNA mismatch repair status.

Materials and Methods: Patient height and weight were recorded at study entry and BMI (kg/m^2)
will be calculated by dividing the patient's squared height (meters) into the weight (kilograms).
BMI categories will be created on the basis of WHO classifications and prior analyses [322, 323]
with the following definitions: obese (BMI $\geq$30 kg/m^2); overweight (BMI, 25 to 29.9 kg/m^2);
normal-weight (BMI, 20 to 24.9 kg/m^2), and underweight (BMI < 20 kg/m^2).

C. Obesity-associated Circulating Biomarkers in CRC

Background and Significance: Since peripheral blood specimens are being prospectively
collected on all participants within NCCTG N0147, we will have a unique opportunity to study the
biological mechanism underlying the effect of obesity upon tumor aggressiveness. To date, studies
are conflicting with regard to whether obesity associated with prognosis [322, 324-326], and
mediators of the effect of obesity upon cancer prognosis remain poorly defined. Hyperinsulinemia
and perturbations in the insulin-like growth factor (IGF) axis are plausible biological mechanisms
in that high BMI, sedentary lifestyle, and consumption of a Western diet lead to elevated levels of
circulating insulin and low levels of circulating insulin-like growth factor binding protein (IGFBP)-
1 [327-330]. In contrast, these factors have little effect on circulating levels of other components of
the IGF axis, such as IGF-I and IGFBP-3 [331]. Among patients with resected CRCs, recent data
indicate that higher levels of prediagnosis plasma C-peptide and lower levels of plasma IGFBP-1
were associated with increased mortality and may therefore, represent the link between lifestyle
factors and the clinical behavior of CRC [332]. We propose to test the hypothesis that increased
levels of circulating C-peptide and reduced levels of circulating IGFBP-1 in patients with resected
stage II and III colon cancers can explain the association of BMI with worse clinical outcome.

Specific Aim: To analyze obesity-associated factors including circulating C-peptide (marker of insulin secretion) and insulin-like growth factor binding protein-1 (IGFBP-1) in relation to BMI and clinical outcome in patients with resected colon cancers.

Materials and Methods: Blood samples will be collected upon study registration and during observation at the respective institutions and will be sent to the NCCTG Research Base at Mayo Clinic Rochester. The stability of these biomarkers during the period of transport has been previously documented [333]. Samples will be centrifuged, divided, and stored frozen at −80ºC. Circulating levels of IGF-I, IGF-II, IGFBP-3, and C-peptide in either plasma or serum will be assayed using enzyme-linked immunosorbent assays (ELISA) using kits and reagents purchased from Diagnostic Systems Laboratory (Webster, TX) in the laboratory of Dr. Michael Pollak (McGill Univ., Montreal, Canada). All assays will be carried out by laboratory personnel who will be blinded to patient outcome. Each sample will be assayed in duplicate. In reported studies, the mean intra-batch coefficients of variation calculated from the quality-control samples were 7%, 5%, 9%, and 10% for IGF-I, IGF-II, IGFBP-3 and C-peptide, respectively [334-337].

D. Circulating Vitamin D (25-Hydroxyvitamin D) Concentrations in CRC

Background and Significance: Epidemiological studies have shown lower rates of CRC incidence in areas with higher sunlight exposure, suggesting a role for UV-related vitamin D conversion in skin [338, 339]. However, the influence of circulating 25(OH)D on the outcome of patients with established CRC is largely unknown. In a study of 304 participants in the Nurses' Health Study (NHS) and the Health Professionals Follow-Up Study (HPFS) with CRC, higher prediagnosis 25-Hydroxyvitamin D [25(OH)D] levels were associated with a significant improvement in overall survival. In the HPFS, the relative risk for CRC was 0.66 (95% CI, 0.42-1.05) across quintiles of total vitamin D intake, [340] and, in the NHS, women in the highest quintile of vitamin D intake had a 67% reduction in CRC risk [341].

According to the National Center for Health Statistics, as many as 36% of Americans are vitamin D deficient with the decline believed to be related to combined changes in BMI, milk intake, and sun protection (Looker AC Am J Clin Nutr 2008). Interestingly, data indicate that obesity is associated with lower levels of 25(OH)D [342-344], suggesting that vitamin D deficiency could contribute to the adverse prognostic impact found for BMI. Vitamin D and its analogues have been shown to exert anti-metastatic effects in animal models of colon cancer [345]. Many cell types [346-348] possess vitamin D receptors, which induce differentiation and inhibit proliferation, invasiveness, angiogenesis, and metastatic potential when activated by $1,25(OH)_2$ vitamin D. Normal and malignant colonic cells possess 1-alpha-hydroxlase activity [349, 350] and can thus convert circulating 25(OH)vitamin D to the active $1,25(OH)_2$vitamin D. We hypothesize that subnormal circulating vitamin D levels will be associated with adverse clinical outcome as indicated by increased rates of colon cancer recurrence and/or shorter survival. We will study this relationship in a phase III colon cancer adjuvant therapy trial (NCCTG N0147) comparing FOLFOX with or without cetuximab in over 3700 patients. The patient population of the NCCTG is predominantly from the upper Midwest, so patients will be from similar geographic latitude.

Specific Aim: To determine the association of circulating Vitamin D (25-Hydroxyvitamin D) levels with BMI, the IGF axis, and colon cancer recurrence and mortality in stage IIII colon cancer patients from an adjuvant chemotherapy trial.

Materials and Methods: Circulating levels of 25(OH)D will be determined using blood samples that have been centrifuged, aliquotted, and stored at –80°C. Circulating 25(OH)D concentrations will be measured by radioimmunoassay, as described previously [351], and performed in the laboratory of Dr. Bruce Hollis (MUSC). Multiple masked quality control samples will be interspersed among the case samples, and all laboratory personnel were blinded as to test or control samples and clinical information. The mean coefficient of variation of the assay has been shown to be approximately 10% [352, 353]. Analyses will be adjusted for patient gender.

SPECIFIC AIM 9: Statistical Considerations

General Statistical Methods: The validity of the normality assumption for each variable will be assessed via methods such as box plots, stem and leaf plots, histograms, and q-q plots. Transformations may be used to achieve normality. In general, methods of determining the relationships among markers will be guided by the underlying distributions. Categorical and nonparametric methods will be used as needed. Graphical methods will be used to illustrate the relationships.

Time to event analyses will be conducted using Kaplan-Meier, log rank, and proportional hazards (Cox regression) methods. Graphical techniques and formal tests such as those proposed by [354] will be used to assess validity of assumptions and goodness of fit of the proportional hazards model. Overall survival will be defined as the time from surgery until the date of death from any cause while disease free survival will be defined as the time from surgery until the time of tumor recurrence or death, whichever is first. Survival distribution curves will be estimated by the method of Kaplan and Meier.

All power calculations assume the use of two-sided tests with alpha=0.05. Survival power calculations were made assuming an overall 5 year survival of 70%, three years of accrual, and five years of follow-up. Power and confidence interval tables for proportions were made using the normal approximation.

Protocol N0147 proposes a very large number of translational analyses. The strength of these analyses is greatly enhanced by the mandatory nature of biospecimen (blood and tissue) collection that was instituted from the initiation of this protocol. As of Addendum 11, with more than 3200 total enrolled patients, biospecimens are available on approximately 98% of enrolled patients. However, multiple testing and limited tissue resource are both issues that must be addressed.

Based on the history and pattern of accrual to N0147, enrolled patients can be separated into 5 cohorts of patients that bear relevance to the translational analyses:

Cohort A: Patients enrolled to arm A before the introduction of C225 into the trial, all patients enrolled to arms B and E, and patients enrolled to arms C and F who received any irinotecan. These are patients who will not be used in any efficacy analyses, and will be useful primarily for prognostic factor hypotheses. Cohort size: approximately 350 patients.

Cohort B: WT *KRAS* patients randomized into arms A and D while concurrent randomization was in place but prior to the introduction of *KRAS* testing. These are patients for whom retrospective *KRAS* testing was performed. These patients will be included in the primary efficacy analysis and will be useful for all predictive factor analyses. Cohort size: approximately 1260 patients.

Cohort C: MT *KRAS* patients randomized into arms A and D while concurrent randomization was in place but prior to the introduction of *KRAS* testing. These are patients for whom retrospective *KRAS* testing was performed. These patients will not be

included in the primary efficacy analysis but are very valuable to test for predictive factors as they were randomized between A and D. Cohort size: approximately 840 patients.

Cohort D: *KRAS* WT patients randomized into arms A and D after the introduction of *KRAS* testing. These patients will be included in the primary efficacy analysis. Cohort size: approximately 810 patients.

Cohort E: *KRAS* MT patients registered into arm G after the introduction of *KRAS* testing. These patients will not be included in the primary efficacy analysis, and were not randomized. They will be useful primarily for prognostic factor hypotheses. Cohort size: approximately 540 patients.

Cohort	Size	Patient Groups	Inclusion in Prognostic/Predictive Testing	Inclusion in Primary Efficacy Analysis
A	350	• Arm A prior to C225 • Arms B and E • Arms C and F receiving CPT-11	Prognostic	No
B	1260	• WT *KRAS* from Arms A and D, or from C and F but with no CPT-11, with retrospective *KRAS* testing	Predictive Prognostic	Yes
C	840	• MT *KRAS* from Arms A and D with retrospective *KRAS* testing	Predictive Prognostic	No
D	810	• WT *KRAS* from Arms A and D with prospective *KRAS* testing	Predictive Prognostic	Yes
E	540	• WT *KRAS* from Arms A and D with prospective *KRAS* testing	Prognostic	No

Prior to any analyses, markers will first be classified as hypothesized to be:
1) Prognostic only
2) Prognostic and predictive overall (independent of *KRAS* status)
3) Prognostic and predictive in *KRAS* WT patients only

A marker in class 2 would be a marker that would be hypothesized to provide an alternative population stratification to predict C225 responsiveness to *KRAS*. A marker in class 3 would be a marker hypothesized to provide additional stratification of the *KRAS* WT population into responsive and non-responsive patients. Markers in class 1 will be testing on patients in cohorts A and E. Markers in class 2 will be tested in cohorts B and C. Markers in class 3 will be tested in patients in cohorts B and D.

To preserve the tissue resource, and to protect against type-I error due to the multiple (and likely ever expanding) number of markers that will be examined, a sequential testing strategy will be used. For a marker in any class with 40-60% prevalence, 600 patients will be used for initial testing. For markers in class 1, this will consist of 600 patients in Cohorts A and E, selected at random. For markers in class 2, this will consist of 600 patients in cohorts B and C, equally

distributed to arms A and D, and with the same *KRAS* WT/MT ratio as the overall trial. For markers in class 3, this will consist of 600 patients in cohorts B and D, equally distributed to arms A and D, with preference to patients from cohort D.

Power calculations are based on the following assumptions. Assume an overall 3 year DFS (primary endpoint) of 70%. Assume a 4 year accrual period, and 3 years minimum follow-up. Also assume all definitive tests will be using two-sided log-rank tests at level 0.05. For prognostic markers, 600 patients provides 85% power to detect a hazard ratio of 1.50 between prognostic groups. Prognostic markers that are positive for association with DFS at a p-value of 0.05 in the initial set of 600 patients will be tested in a subsequent set of an additional 600 patients, also at level 0.05. Only prognostic markers that are associated with outcome in both 600 patient sets will be considered 'validated'. Note the combined cohort size for cohorts A + E is expect to be approximately 890 patients, and as such, some patients from cohort C or D (depending on availability) will need to be added to these analyses to confirm the prognostic effects in the second testing sets.

Power for predictive marker hypotheses is more difficult. As such, a less stringent criterion will be used to allow predictive markers to proceed to the second set of testing. Predictive markers will be tested for association between marker and outcome through interaction testing (likelihood ratio tests using Cox models with and without treatment by marker terms) on the initial 600 patients, at a two-sided level 0.25. The use of 600 patients provides 74% power to detect a HR of 1.6 for the DFS endpoint using a two-sided level 0.25 test. Markers that are positive for a predictive association treatment for DFS at level 0.25 in the initial testing will then be tested on an additional 1500 patients which will provide 80% power to detect an interaction of magnitude HR = 1.6 using a two-sided level of 0.05 test. Note that the combined size of both cohorts B + C and B + D is approximately 2100 patients, thus for markers where confirmatory testing for predictive ability is conducted, the entire N0147 cohort will be used.

These calculations assume an ideal scenario of a marker with a prevalence of 40-60%. For prognostic markers, the option exists to increase the sample size for both the initial and confirmatory sets based on the expected prevalence of the marker. The sample size will be based on that necessary to provide 80% power for a hazard ratio for the prognostic marker of 1.5. For a marker with prevalence 20%, the required sample size based on the same assumptions as above is 1000 patients for each of the test and validation sets. However, based on tissue availability, we will limit the sample size for testing solely prognostic markers at 900 patients for initial testing, based on the expected sample availability from cohorts A and E of approximately 900 patients.

Power for predictive factor testing for low prevalence markers is even more problematic; in this case based on pragmatic issues we will pursue such markers with the same 600 patient initial exploration/1500 patient validation strategy as for higher prevalence markers. Ultimately, we hope to be able to combine the translational data from N0147 with the data from the PETACC-8 trial which had an almost identical design to allow greater power for particularly the predictive marker hypotheses.

For the association of biomarkers in N0147 with non-survival endpoints such as toxicity or other baseline patient or tumor characteristics, the power is substantially improved. Even for a low prevalence marker of approximately 15%, a sample size of 600 patients provides power to detect a difference in a binary outcome of 20% (such as the proportion with or without grade 3+ toxicity of 20% in one marker define group and 40% in the other marker group) of at lest 95%.

A variety of statistical methods will be used for the different analyses. Correlation of dichotomous outcomes and marker status will be tested using chi-squared tests; odds ratios and associated 95% confidence intervals will be reported as estimates of the association. Univariate logistic regression models will be used to explore the association between continuous variables and binary outcomes; multivariate logistic regression will be used to adjust for known risk factors (performance status, stage, age, etc). Associations between marker status and time-to-event variables (DFS, overall survival) will be illustrated graphically using Kaplan-Meier curves, with hypothesis testing using the log-rank test for univariate associations and Cox regression modeling for multivariate associations.

Specific Statistical Considerations for Each Aim

Aim 1a) The analyses for molecular alternations to predict response to therapy is a predictive marker hypothesis, thus these analysis are class 2 analyses in the nomenclature presented, and will be conducted on patients enrolled to cohorts B and D, using the strategy of an initial 600 patient testing set with a 1500 patient validation set. It is expected that in fact all approximately 2100 patients from cohorts B and D will be tested given the inclusion of *KRAS* in this set of markers.

Aim 1b) The analysis of EGFF CIN by FISH is a predictive marker hypothesis (class 2). The initial set of 600 patients will be used to 1) attempt to validate the previously generated cutpoints, and b) define optimal cutpoints, which if promising, will be validated on the remaining 1500 patients in cohorts B and D.

Aim 1c) The analysis of serological EGFR expression is a prognostic marker hypothesis, but only in cetuximab treated patients. As such, patients treated with cetuximab in cohorts B and D will be used. While in theory, patients from cohort C could be used, cetuximab will only be used in practice (if at all) in *KRAS* WT patients, thus we will not include patients from cohort C in this analysis. Power calculations and analysis plans are otherwise identical to those for prognostic markers in Class I.

Aim 1d) Gene expression will be analyzed for patients with recurrence or death (25% - 30% of the patients) and in a matched sample of 4-year disease free patients. Microarray Suite 5.0 (Affymetrix, Santa Clara, CA) software will be used to scan and quantitatively analyze the scanned image. The per-chip summaries provided by MAS 5.0 will be used for initial assessment of the results. The primary analysis will be based, however, on the model-based methods of Chu et al [355]. These methods include all of the chips in the experiment in a single analysis with the treatment design itself as a part of the model. Permutation test methods will be used to calibrate the final significance values for between group comparisons. Genes will be prioritized for further study based on statistical significance as well as biological relevance. Additional statistical analysis can be performed as necessary, while other methods for final data reduction and relationship modeling may include hierarchical and K-means clustering, linear regression plots, and Principle Component Analysis.

Aim 1e) The primary analysis association of genetic polymorphisms with rash will be based on χ^2 tests treating rash as a dichotomous variable (grade 0-1 rash vs. grade ≥ 2 rash) and protein expression (positive or negative). Power considerations are as stated previously for non-survival points. The sample size of 600 patients from the initial testing will be used to test association between protein expression and rash using univariate

approaches, and will allow the development of a possible multi-protein expression signature that will then be validated in a subsequent set of 600 patients.

Aim 2a)   The analyses for specific aim 2 relate to prognostic factor hypothesis. Given the previously demonstrated prognostic importance of MMR status, we plan to complete MMR testing on all patients. Patients will be classified as dMMR or pMMR for the primary analysis. Based on attained sample size of approximately 3800 patients, this analysis will have exceptional power (>99%) to detect a hazard ratio of 1.4 comparing DFS in patients with dMMR versus pMMR status.

Aim 2b)   The correlation between centrosome amplification and CIN will be assessed using Pearson's correlation coefficient. 600 patients provides >95% power to detect a correlation of 0.3. The prognostic ability of CIN status will be assessed as a class 1 marker initially in 600 MSS patients. If the CIN status if demonstrated as a prognostic marker in MSS patients, CIN status will then be assessed in all MSI-H patients.

Aim 2c)   We plan to compare telomere length among patients with and without recurrence using a t-test with a 2-sided 5% type I error rate. The telomere length in base pairs (4157 conversion factor) will be transformed to the natural log, since the natural log of the telomere length in base pairs has been shown in CRC to be approximately normally distributed and generally better behaved than the skewed distribution of the Telomere-to-Single Copy Gene (T/S) ratios. The relationships between continuous variables and other pathologic and translational endpoints will be examined, using first generalized additive model (GAM) of the mgcv package in R, and then logistic regression. Fisher's exact test or chi-square tests will be performed to examine the relationship between categorical variables such as *KRAS* mutation, EGFR, MSI, and CIMP tumor status. Results will be quantified in terms of odds ratios (ORs) with the corresponding 95% confidence intervals (CIs). For survival analysis, the survival period will be calculated from the date of CRC diagnosis to the last recorded date of follow-up or death. Kaplan-Meier (product-limit) method will be used to estimate survival functions, and the log-rank test will be performed to reveal differences in survival between groups. The Cox proportional hazard regression will also performed for both categorical and continuous variables in order to determine significance of each variable on survival and estimate their effects (Hazard Ratios, 95 % HR) with examination of proportional hazard assumption. Telomere length will be considered a class I marker.

The primary statistical analysis for each genotyping stage is as follows. To test for associations between telomere length and each SNP, we will use linear regression. Each SNP will be modeled as an additive effect of the number of minor alleles. This model is selected for its biological plausibility as well as for the fact that is demonstrates good power to detect genetic association, even when the true genetic model follows a different one. We will also consider modeling each gene as a "whole". Specifically, we will model the main effects for all independent (r^2 <0.25) SNPs from a gene in a multivariate regression model. This approach does not require phase information and has been shown to have greater power than haplotype analyses. To evaluate model fit and assumptions, we will use standard regression diagnostics, including the analysis of leverage values and influential statistics. We will also check the normality assumption of telomere length. Transformations will be used if we observe a departure from normality. Population stratification is always a concern for any molecular epidemiology study. Further, we will use the program STRUCTURE to assess whether subpopulation structure is present.

Aim 3)   The proportions of hypermethylation of the genes of interest will be estimated alone with 95% confidence intervals within each of the three patient groups. The frequencies of hypermethylation for each gene will be compared between matched MSI-H, MSI-L, and MSS tumors using Chi-square tests. For the primary comparison of interest, MSI-H vs. MSS tumors, based on an expected total sample of 570 per MSI-H patients, there is >90% power to detect a 10% difference in prevalence between two groups. The prognostic ability of CIMP status will be assessed as a class 1 marker.

Aim 4a) Randomized complete or incomplete block designs will be utilized to ensure sample and assay processing order and plates are balanced with respect to study groups [356]. Data will be analyzed on the log base 2 scale. In addition to the laboratory quality control steps, data quality will be assessed via per-specimen box and whisker plots in order to examine global shifts in intensity due to run order and other experimental factors. The nature of biases, e.g., existence and (non)linearity, between specimens will be assessed via residual MVA plots (Minus Versus Average) plots [357, 358] which place model residuals on the y-axis and model predicted values on the x-axis with a smoother for each specimen and help visualize any abundance-dependent biases in the data. There is debate in current literature as to the proper normalization algorithm for high density microRNA microarrays [359-363]. Our group has extensive experience in developing model-based normalization algorithms [358, 364-367]; assumptions will be assessed and the proper model-based normalization algorithm applied. The quadruplicate spots per miRNA will be summarized via robust mean after normalization.

Linear mixed effects models and contrast statements will be utilized to examine differences between tumors and paired non-tumor tissues and effects of TNM staging accounting for paired specimens. Survival methods (Kaplan-Meier, log-rank, Cox regression) will be used to assess relationship of tumor miRNAs with prognostic (relationship with overall survival) and predictive (response to chemotherapy) outcomes. Analyses will be performed overall and in *KRAS* wild-type patients who are randomized to 24 weeks of adjuvant chemotherapy with mFOLFOX6 versus mFOLFOX6 plus cetuximab. Covariates will be included in models as necessary. False discovery rates will be calculated in order to determine significance [368].

Aim 4b) Randomized complete or incomplete block designs will be utilized to ensure sample and assay processing order and plates are balanced with respect to study groups [356]. The perfect match only data will be analyzed on the log base 2 scale. In addition to the laboratory quality control steps, data quality will be assessed via per-specimen box and whisker plots in order to examine global shifts in intensity due to run order and other experimental factors. The nature of biases, e.g., existence and (non)linearity, between specimens will be assessed via residual MVA plots (Minus Versus Average) plots [357, 358] which place model residuals on the y-axis and model predicted values on the x-axis with a smoother for each specimen and help visualize any abundance-dependent biases in the data. Data will be normalized using fastlo [364], a model-based intensity dependent algorithm similar to (but computationally faster than) cyclic loess. The quadruplicate spots per miRNA will be summarized via robust mean after normalization.

Gene expression data will be available for approximately 2000 subjects (25-30% of subjects with recurrence or death and a matched sample of 4-year disease free patients).

The subjects will be randomly split into training and test sets within good/poor outcome cohorts; 1/3 will be used for training and 2/3 for testing [369]. Differential expression and predictive/prognostic model building will be performed in the training set. Results will be verified in the testing set.

Linear mixed effects models and contrast statements will be utilized to examine differences between tumors and paired non-tumor tissues and effects of TNM staging accounting for paired specimens. Survival methods (Kaplan-Meier, log-rank, Cox regression) will be used to assess relationship of tumor mRNAs with prognostic (relationship with overall survival) and predictive (response to chemotherapy) outcomes. Analyses will be performed overall and in *KRAS* wild-type patients who are randomized to 24 weeks of adjuvant chemotherapy with mFOLFOX6 versus mFOLFOX6 plus cetuximab. Covariates such as staging information will be included in models as necessary. False discovery rates will be calculated in order to determine significance [368].

We will evaluate if multi-gene signatures result in improved predictors of outcome and response to treatment over and above tumor stage and *KRAS* mutation status. Non-informative genes will be removed by deleting the 60% of genes with the lowest variance across all samples. To evaluate the correlation across genes, initial steps will include examination of pairwise correlations, (unsupervised) hierarchical clustering, and principle components analysis. If these analyses identify pairs or clusters of highly correlated gene sets, then only a representative member or the average expression of the gene set will be used for building a predictive signature. Variable selection and prediction strategies will be applied to the remaining genes. Random Forests [370] for regression trees will be used to determine the most important genes. Alternatively, we may modify Dr. Efron's empirical Bayes framework for predicting a continuous time-to-event outcome for use in the linear regression setting [371]. If outcome is dichotomized (e.g. good/poor outcome, responder/non-responder) variable selection strategies including but not limited to Random Forests, Evaporative Cooling and decision trees [372, 373], diagonal linear discriminant analysis, support vector machines and K-nearest neighbors, and the shrinkage methodology of prediction analysis CLanC (classification to nearest centroids) [374] which has been shown to perform better than PAM (Prediction Analysis for Microarrays) [375] will be considered. In these analyses, cross validation and other model validation strategies will be used to mitigate the potential for over-fitting the model using the extensive panel of variable sets or variables to be studied. The generalized R2N index and the probability of concordance (c index) will be used to quantify the predictive ability of the Cox proportional hazards models via bootstrapping [376]. Calibration curves and over optimism in the c index and the Dxy rank correlations will be reported to quantify the amount of over fitting present in the final models. In the current scenario, calibration is the ability of the final predictive model to make unbiased estimates of good/poor outcome or response to treatment.

We will perform model verification in the test set of samples. For significant genes in the training set, relationships will be assessed using the same models with coefficients fixed to assess fit in the training cohort. For example, to validate differential expression between paired tumor/normal tissues for a given gene, the gene observations in the test sample will be the dependent (y) variable, tumor/normal status will be the independent (x) variable, and the estimate of difference observed in the training subjects will be held fixed. The R2 and c index from this test model will be compared to the R2 and c index

of the training model. Similar R2 and c index values indicate a verified result; test values that are much lower than the training values indicate a result that is not verified. The predictive models will be formally verified in a similar manner; the final predictive models will be fit to the test sample holding the parameter estimates from the training cohort fixed, and the R2 and c index values will be compared. Finally, for models that are verified, a final fit to the combined training plus test samples will be reported.

Aim 4c)   The association between SNPs and AEs will be tested through chi-squared tests for SNPs of prevalence ≥2% and by Fisher's exact tests for lower prevalence SNPs. All statistical tests will be 2-sided. Logistic regression models will be used to test for the association between SNPs and AEs adjusted for baseline factors of age, gender, race, treatment status (cetuximab or no cetuximab), and any other SNPs (such as MTHFR, TYMS, ERCC1, DPYD, XRCC1, and GSTM1) found to influence toxicity in the other planned pharmacogenomics studies proposed in this study. With the majority of patients being Caucasian, we will run the analysis adjusting for self reported race and along with a subset analysis with only the Caucasian subjects, due to possible population stratification and difference in allele frequencies between races.

Aim 5)   BH3, Bc1-2 family, caspase 3, and cox-2 expression will be treated as a class 2 marker, to explore the possible prognostic and predictive importance of this marker. As such, it will follow the standard class 2 testing strategy outlined above.

Aim 6)   We will compare the density of individual T cell markers and COX-2, analyzed as a continuous variable, in tumors with defective versus intact DNA MMR using a 2 sample t-test (or nonparametric equivalent). Cased will be matched by age, and gender. As such, we have at least 80% power to detect a 25% effect size (e.g. difference in the T cell marker mean densities divided by the pooled standard deviation) between defective versus intact MMR cases, using a two-sided t-test at level 0.05. We will also examine the prognostic (and predictive) significance of each marker separately within both patient groups. As such these markers will be treated as class 2 markers and analyzed accordingly.

Aim 7)   Survival methods (Kaplan Meier, log-rank, Cox regression) will be used to assess the relationship between tumor grade and overall survival. Chi-square tests will be used to assess the relationship between tumor grade, tumor border configuration, and peritumonal host lymphoid response. The large sample size provides exceptional power to explore these associations in the entire patient cohort.

Aim 8a)   The relationship between family history and MSI or CIMP status will be assessed with Chi-square tests. We expect approximately 570 MSI-H and MSI-L subjects, matched to 625 MSS subjects, plus those with family history. This sample size will provide >90% power to detect a 10% difference in proportions. Associations between environmental exposures and molecularly-defined CRC subtypes will be explored using the Chi-square test, two-sample t-test, and/or multivariable regression models, as appropriate.

Aim 8b)   Data on cancer recurrence or death was monitored and recorded during 5 years of follow-up. In the analysis of BMI and prognosis, the primary study endpoint will be disease-free univariate and multivariate Cox regression models [377], respectively, after stratifying by study. These models will be used to determine the simultaneous

impact of BMI and other potential confounders on DFS. BMI will be considered as a prognostic factor of DFS, if the 2-sided p-value is <0.05.

Aim 8c)   Circulating biomarkers will be treated as class 1 markers. For the analysis they will be categorized according to quartiles for survival analysis, where the 1st quartile will be the reference group. For this reason, and due to the greater availability of plasma or serum as opposed to tissue, 820 patients will be used for these analyses. The Kaplan-Meier method will be used to describe the distribution of DFS and overall survival (OS). Univariate and multivariate Cox proportional hazards modeling will be used to calculate hazard ratios and confidence intervals [377]. With a total sample size of 820 patients (205 in the reference group (quartile 1) vs. 614 otherwise), we would have at least 80% power to detect a 5 year survival rate difference of 10%, with a 2-sided significance level of 0.05. Multivariate Cox models will adjust for BMI and clinicopatholgical variables (stage, histologic grade, gender, etc.). In a secondary analysis, we will correlate the circulating biomarkers versus each other and BMI.

Refer to Statistical Section for Specific Aim 8 (B) regarding analysis of survival. In addition, we will correlate 25(OH)D levels with BMI, the IGF axis, C-peptide levels, and with clinicopathological variables.

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Appendix XV
Request Letter for Immunohistochemistry (IHC) Test Results

Add 6   This letter is to inform you of an option to learn the results of some testing that will be conducted on part of your colon tumor. This testing is called tumor Immunohistochemistry testing. This is not a test to diagnose cancer, but rather a test that tells you something more about the cancer that you already have.

You are being offered this test result because the test result may be clinically useful in selected families. The fact that you are getting this invitation to learn of results does not imply that your particular result will be medically helpful; we are offering to give results to anyone who is interested. Only a small percentage of people have abnormal results.

Add 6,7   You have the choice of learning about some of your Immunohistochemistry testing. The Immunohistochemistry (IHC) results will tell you whether or not certain proteins, MLH1, MSH2, and MSH6, are present in your tumor tissue. If one or more of these proteins are missing, there may be an increased risk for Hereditary Non-Polyposis Colon Cancer (HNPCC). Studies show that loss of MSH2 and MSH6 are usually related to HNPCC. Loss of MLH1 also can be related to HNPCC, but is more

Add 2,3,5   often not hereditary. If you are interested in learning the results, please sign and return this letter to your treating physician, otherwise please disregard this letter. Your treating physician will forward the request directly to the North Central Cancer Treatment Group Research Base, Attention: N0147 Quality Assurance Specialist, Cancer Center Statistics NW Clinic, 200 First Street SW, Rochester, MN 55905. NCCTG will be processing tissue in large groups (batches) for maximum efficiency. Therefore, it may be some time before this information is available to the treating physician to share with the patient. We anticipate that this may take up to 6 months, but may be longer due to accrual and tissue submission rates. The requested information will be given to your treating physician who will contact you to arrange a meeting time to discuss the results. Your research record will contain notes stating that you have requested to see your test results and also that the results have been given to you.

Add 9,10,11   It is the responsibility of the local treating physician to share your IHC results with you. You are to contact your treating physician with any future questions regarding your IHC results.

Your treating physician will decide if your results should be given to you through a genetic counselor that may be able to better help explain the possible risks and benefits of learning this confidential information.

IHC is not considered genetic testing. IHC testing looks at the effects of genes, but not at the genes themselves. For those with suspected hereditary conditions, the option of gene testing does exist, but is not done as part of these IHC tests. Your physician and/or geneticist will work with you if you are a candidate for genetic testing and were interested in knowing more.

Please note that useful test results will not be found in all study participants.

Add 10

☐   Yes, I want to receive the results of my Immunohistochemistry testing.

_____ _____ 9 -0 __ __-__ __ __
(Date)   (Printed Name of Participant)   (NCCTG Registration Number)

            _____ _____
                (Signed Name of Participant)   (Patient's Local/Institutional
                                                                              Identification Number)

Add 10   Results will be sent in a form letter to:

Physician Name: _____

Medical Center/Facility **_____**

Street Address: _____

City/State/Zip Code _____

Phone: _(_____)_____

E-mail: _____

Appendix XVI
(Dear Participant Letter)

Add 6   **[For patients enrolled prior to September 1, 2004 <u>only</u>]**

Dear Participant:

Add 10   You are receiving this letter because you are a participant in N0147, A Randomized Phase III Trial of Irinotecan (CPT-11) and/or Oxaliplatin (OXAL) Plus 5-Fluorouracil (5-FU)/Leucovorin (CF) after Curative Resection for Patients with Stage III Colon Cancer.

We are writing to let you know that changes have been made to the study. However, these changes will not affect you and will only involve people enrolled under the modified protocol.

The study has been changed to find out if adding a new drug called cetuximab (C225) to chemotherapy makes the chemotherapy work better. Half of the people entered after the date of this letter will, by chance, receive cetuximab in addition to one of the three chemotherapy combinations you are receiving as part of this study. The other half will get the chemotherapy without cetuximab.

Cetuximab is a newly approved drug that helps some people with cancer of the colon and rectum that has spread and is not curable. It is only approved for use in people whose cancer has continued to grow after they have received the chemotherapy drug called CPT-11 (irinotecan). There have been no trials so far to determine if cetuximab helps prevent cancer from coming back. We are, therefore, adding cetuximab to this study, as described above, to see if it helps prevent cancer from coming back. Because cetuximab has side effects that can be severe or even rarely lead to death, we are not offering it to everyone on the study. We also do not yet know if it will help lessen the risk of the cancer from coming back. That is why we are going to look at how it might improve the benefit of chemotherapy in this study.

We again want to stress that this change will not involve you or make any changes in the care and treatment you will receive by taking part in this study. If you have any questions about this change in the study, we encourage you to speak with your doctor.

Clinical trials remain an important tool for finding ways to prevent cancer and improve health for all Americans. We greatly appreciate each person's participation in this important cancer trial. We remain confident that this trial ultimately will provide valuable information to permit progress in the treatment of colorectal cancer. An analysis of the study data will be done at a later time, and we expect to publish these findings in the years ahead.

Sincerely,

Instrumentation Guide for the DxS KRAS Kits

For Use by NCCTG Research Base Only

Introduction

The DxS KRAS mutation test kit (KR-21/22) has been validated for diagnostic use in Europe on the ABI 7500. The research use only kit (KR-03/04) has also been validated for use on the ABI 7500. However, it is possible to use these kits, for non-diagnostic purposes, on other instruments with additional validation by the user.

The kit is specific to 96 well block format instruments only. We do not support use on 384 well blocks or instruments that use non-block formats.

These notes cover the most widely used 96 well block instruments; the ABI platforms and the Roche LightCycler 480.

ABI Platforms

The kits can be used for research purposes on 96 well block ABI instruments such as the 7900 using the protocol given in the instructions for use. The KR-21/KR-22 kit is validated for diagnostic use on the ABI 7500 only.

LightCycler 480

The LightCycler 480 requires a slightly different protocol and the instructions given below should be followed for research use of the kits on this instrument. The kit has not been validated for diagnostic use on the LightCycler 480.

LightCycler 480 Master Mix Setup and Plate Layout

The eight master mixes should be set up according to the instructions for use. These mixes should be plated out, as described in the instructions for use, into a LightCycler 480 multiwell plate 96 (Catalogue Number: 04 729 692 001). Add 5 μl of template to the wells and seal with LightCycler 480 sealing foil (Catalogue Number: 04 729 757 001). Spin the plate and load into the LightCycler 480.

Instrument Setup

1. Start a new experiment and under the 'Run Protocol' tab select Mulitcolour Hydrolysis Probe from the detection format drop down menu. Click the 'Customise' button and deselect all the dyes except HEX and FAM.

2. Change the reaction volume to 25.

3. The cycling conditions must be as given in the instructions for use. Follow steps 4-6 of this document to set up the cycling parameters.

4. Under Programs click the '+' button to increase the number of programs to 2.

5. The first program should have 1 cycle and the 'Analysis Mode' set to none. Under 'Temperature Targets' set the temperature to 95°C, the 'Acquisition Mode' to none and the 'Hold' to 00:04:00.

6. The second program should have 45 cycles and the 'Analysis Mode' set to quantification. Under 'Temperature Targets' click the '+' button to increase the number of steps to 2. The first step

should have a temperature of 95°C, an acquisition of none and hold of 00:00:30. Set the second temperature to 60°C, single acquisition and hold of 00:01:00. Use the ramp rates given by the software.

7. Use the 'Subset Editor' and 'Sample Editor' buttons to setup the plate layout.

8. Select the 'Experiment' button and click the 'Start Run' button to save the experiment and start the cycling.

Data Analysis
To analyse the data use the following protocol.

1. Click the 'Analysis' button and choose 'Abs Quant/2nd Derivative Max' from the 'Create New Analysis' window.

2. Click the downward arrow next to the 'Colour Comp' button. Select a colour compensation file. A colour compensation run for FAM and HEX must have been performed prior to using the KRAS kit to produce the colour compensation file.

3. Click the downwards arrow next to the bottom right button that allows a choice between 'High Sensitivity' and 'High Confidence'. Choose 'High Confidence'.

4. Click the 'Filter Comb' button and choose the HEX filter (523-568). Check the wells for amplification of the exogenous control.

   a. If the exogenous control assay gives a positive result continue with the analysis.

   b. If the exogenous control assay has failed but the FAM reaction has worked well continue with the analysis as the FAM reaction has out-competed the exogenous control reaction.

   c. If both the FAM and exogenous control reactions have failed the data must be discarded, as there may be inhibitors present, which could lead to false negative results.

5. Click the 'Filter Comb' button and choose the FAM filter (483-533). Click the 'Calculate' button to obtain Ct values.

6. Export the Ct values and calculate the ΔCt values as follows

   [sample mutation assay Ct] – [sample control assay Ct] =ΔCt

7. The control Ct value must be greater than or equal to 20 to avoid overloading the assay.

8. The mixed standard ΔCt value should be as given in Table 1, but variation of ±2 is acceptable.

9. Use the 1% ΔCt values (Table 1.) as the cutoff for positive or negative. If the sample ΔCt is higher than the 1% value the sample is classed as negative or below the limits of the kit. If the sample value is lower than the 1% value, the sample is classed as positive. Samples that give a ΔCt close

to the 1% value should be repeated in triplicate and all replicates should be in the positive zone for the sample to be classed as mutation positive. Samples with a mutation Ct of greater than 38 should also be repeated in triplicate and all replicates should be in the positive zone for the sample to be classed as mutation positive.

Assay Performance Characteristics

The mutation assay performance characteristics are given in Table 1.

The positive control for each assay is a mixed standard containing 7 synthetic constructs (one positive control for each mutation assay), in 2 ng/µl of genomic DNA. The mixed standard ΔCt values, given in Table 1, should be used to ascertain that the assays are working correctly.

Deviations of ±2 from these values are acceptable.

All assays have been shown to detect 1% mutant. The 1% ΔCt values have been determined over a range of DNA concentrations and set to prevent the calling of false positives. The LOD in Table 1 is the amount of DNA required for reproducible detection of 1% mutant DNA. The approximate control assay Ct is given for this level of DNA.

Table 1. Assay performance characteristics

Assays	Mixed Standard ΔCt	1% ΔCt	LOD	Control Ct
12ALA	1.5	5.7	2	31
12ASP	3.9	8	5	29.5
12ARG	2.4	7.4	2	31
12CYS	1.5	6.2	2	31
12SER	3.7	10.5	10	28.5
12VAL	1.7	6.5	2	31
13ASP	5.3	10	10	28.5

The data interpretation section in the instructions for use describes the cross talk pattern. This should be used to interpret data from the LightCycler 480 where there are 2 apparent mutations within a sample.

**N0147 Quick Reference Guide for Submitting Pathology Materials
for Patients Enrolled after Addendum 9**

<u>PATHOLOGY MATERIALS TO NCCTG:</u>
**All of the materials listed under Pathology Materials Checklist below must be submitted
immediately following pre-randomization and as soon as possible after surgery or reporting of
KRAS results may be delayed.**

Pathology Materials Checklist
- NCCTG Pathology Submission Form
- Surgical Pathology Report
- Operative Report (Optional)
- ALL diagnostic slides*
- At least one slide documenting nodal metastasis.
- 3-4 paraffin embedded (PE) tissue blocks (corresponding to diagnostic slides, if possible)
- One PE block with normal colonic mucosa away from tumor (a block at least 10 cm away or from a negative margin).
- Corresponding H&E slides for the all submitted blocks and/or unstained slides

*Diagnostic slides should be from the resection including those that document extent of disease, radial margin (if positive), those that interface with adjacent mucosa and/or pre-existing adenoma, and normal colonic mucosa.

Additional Notes:
- Label slides with the following:
  NCCTG Patient ID
  PE block accession number
  Micron thickness of section (i.e., 5 or 10 micron)
  Order of cut section (i.e., 1-30)
- Please make sure the contact information on Pathology Submission Form is filled out **completely, correctly,** and is **legible**. If the contact information is incomplete, incorrect, or illegible, KRAS results may be delayed.
- If you are submitting blocks and their corresponding H&E's, it is **not** necessary to submit the 25 unstained slides cut at 5 micron and the 5 unstained slides cut at 10 micron. We will query you later if we need more material. **However, if you are only able to submit the 25 unstained slides cut at 5 micron and the 5 unstained slides cut at 10 micron, make sure you send a corresponding H&E that matches these unstained slides as well.**

- Pathology materials should be sent to:
  NCCTG Operations Office
  Attn: PC Office (Study N0147)
  RO_FF_03_24-CC/NW Clinic
  200 First Street SW
  Rochester, MN 55905

Add 11 **Contacts for Pathology questions:**

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