ALLIANCE FOR CLINICAL TRIALS IN ONCOLOGY

CALGB/SWOG C80702

A PHASE III TRIAL OF 6 VERSUS 12 TREATMENTS OF ADJUVANT FOLFOX PLUS CELECOXIB OR PLACEBO FOR PATIENTS WITH RESECTED STAGE III COLON CANCER

Investigational agent: Celecoxib/placebo, NSC #719627 (Alliance IND #107051), will be supplied by Pfizer, Inc., and distributed by CTEP, DCTD, NCI
Participation limited to U.S. and Canadian sites.

ClinicalTrials.gov Identifier: NCT01150045

Alliance Study Chair, Alliance GI Committee Co-Chair
Jeffrey A. Meyerhardt, MD, MPH
[redacted]

SWOG Study Co-Chair
Anthony F. Shields, MD, PhD
[redacted]

Alliance GI Committee Co-Chair
[redacted]

SWOG GI Committee Chair
[redacted]

Alliance Corr. Sci. Co-Chair
[redacted]

SWOG Corr. Sci. Co-Chair
[redacted]

PPP Co-Chair
[redacted]

Alliance Primary Statistician
[redacted]

SWOG Primary Statistician
[redacted]

Secondary Statistician
[redacted]

Data Manager
[redacted]

Protocol Coordinator
[redacted]

CCTG Champion
[redacted]

NRG Oncology Champion
[redacted]

Participating Organizations
ALLIANCE / Alliance for Clinical Trials in Oncology**, SWOG /** SWOG, **ECOG-ACRIN /** ECOG-ACRIN Cancer Research Group**, NRG /** NRG Oncology, **CCTG /** Canadian Cancer Trials Group

Study Resources

Alliance Protocol Operations Program Office

[redacted]

Adverse Event Reporting

[redacted]

Alliance Biorepository at Ohio State University

[redacted]

Drug Distribution Contact

[redacted]

Alliance Statistics and Data Center

[redacted]

Medidata RAVE® iMedidata Portal

[redacted]

Alliance Patient Registration

[redacted]

CALGB/SWOG C80702 Pharmacy Contact

[redacted]

CALGB/SWOG C80702 Nursing Contact

[redacted]

Protocol-related questions may be directed as follows:	
Questions	**Contact (via email)**
Questions regarding patient eligibility, treatment, and dose modification:	Study Chair, Nursing Contact, Protocol Coordinator, and (where applicable) Data Manager
Questions related to data submission, RAVE or patient follow-up:	Data Manager
Questions regarding the protocol document and model informed consent:	Protocol Coordinator
Questions related to IRB review:	Alliance Regulatory Inbox [redacted]
Questions regarding CTEP-AERS reporting:	Alliance Pharmacovigilance Inbox [redacted]
Questions related to drug supply:	Pharmaceutical Management Branch (PMB)
Questions regarding specimens/specimen submissions:	Alliance Biorepository at Ohio State University

CANCER TRIALS SUPPORT UNIT (CTSU) ADDRESS AND CONTACT INFORMATION

For regulatory requirements:	**For patient enrollments:**	**For data submission:**
Regulatory documentation must be submitted to the CTSU via the Regulatory Submission Portal. (Sign in at [redacted] and select the Regulatory > Regulatory Submission.) Institutions with patients waiting that are unable to use the Portal should alert the CTSU Regulatory Office immediately at [redacted] to receive further instruction and support. Contact the CTSU Regulatory Help Desk at [redacted] for regulatory assistance.	Refer to the patient enrollment section of the protocol for instructions on using the Oncology Patient Enrollment Network (OPEN). OPEN is accessed at [redacted] Contact the CTSU Help Desk with any OPEN related questions by phone or email : [redacted]	Data collection for this study will be done exclusively through Medidata Rave. Refer to the data submission section of the protocol for further instructions.
The most current version of the **study protocol and all supporting documents** must be downloaded from the protocol-specific page located on the CTSU members' website [redacted]. Access to the CTSU members' website is managed through the Cancer Therapy and Evaluation Program - Identity and Access Management (CTEP-IAM) registration system and requires log on with CTEP-IAM username and password. Permission to view and download this protocol and its supporting documents is restricted and is based on person and site roster assignment housed in the CTSU Regulatory Support System (RSS).		
Supplies can be ordered by downloading and completing the CTSU Supply Request Form (available on the protocol-specific page on the CTSU website) and submitting it as instructed on the form.		
For clinical questions (i.e. patient eligibility or treatment-related) see Protocol Contacts, Page 2.		
For non-clinical questions (i.e. unrelated to patient eligibility, treatment, or clinical data submission) contact the CTSU Help Desk by phone or e-mail: CTSU General Information Line – [redacted]. All calls and correspondence will be triaged to the appropriate CTSU representative.		

The pharmacogenomic component of this study is conducted as part of the NIH Pharmacogenomics Research Network, which is funded through a separate U01 mechanism (see http://www.nigms.nih.gov/pharmacogenomics/research_net.html for details).

A PHASE III TRIAL OF 6 VERSUS 12 TREATMENTS OF ADJUVANT FOLFOX PLUS CELECOXIB OR PLACEBO FOR PATIENTS WITH RESECTED STAGE III COLON CANCER

Patient Eligibility

Histologically documented adenocarcinoma of the colon (see §4.1.1)
Completely resected tumors w/ R_0 en bloc resection for tumors adherent to adjacent structures (see §4.1.2)
At least one pathologically confirmed positive lymph node or N1c disease as defined in AJCC version 7 (see §4.1.3)
No evidence of residual involved lymph node disease or metastatic disease at time of registration (see §4.1.4)

Required Initial Laboratory Values

Granulocytes	≥ 1500/µL
Platelet Count	≥ 100,000/µL
Creatinine	≤ 1.5 x ULN
*Total Bilirubin	≤ 1.5 x ULN

* In the absence of Gilbert's syndrome.
If patient has Gilbert's syndrome:

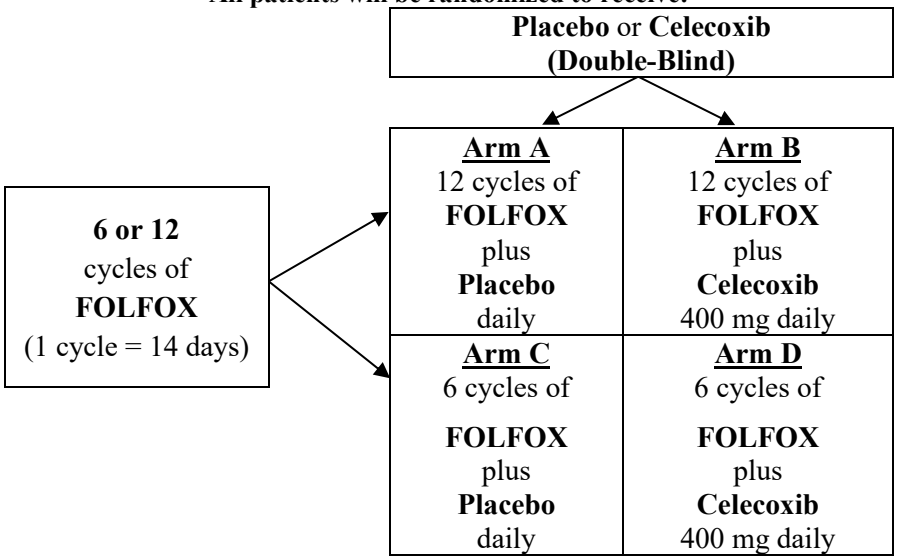
Direct Bilirubin	≤ 1.5 x ULN

Patients with synchronous colon cancers are eligible but patients with synchronous colon and rectal primary tumors are not eligible (see §4.1.5)
Patients are ineligible if they use NSAIDs at any dose more than 2 times a week on average or aspirin at more than 325 mg at least three times per week on average. Low-dose aspirin not exceeding 100 mg/day is permitted (see §4.2)
No previous or concurrent malignancy, except treated basal cell or squamous cell cancer of skin, treated in situ cervical cancer, treated lobular or ductal carcinoma in situ in one breast, or other cancer for which patient has been disease-free for ≥5 years (see §4.3.1)
No neurosensory or neuromotor toxicity ≥ grade 2
No known allergy to platinum compounds
No prior allergic reaction or hypersensitivity to sulfonamides, celecoxib or NSAIDs
No history of upper gastrointestinal ulceration, upper gastrointestinal bleeding, or upper gastrointestinal perforation within past 3 years (see §4.3.5)
No symptomatic pulmonary fibrosis or interstitial pneumonitis
No cardiac risk factors including uncontrolled high BP (systolic > 150), unstable angina, history of documented MI or cerebrovascular accident, or NYHA Class III or IV heart failure
Non-pregnant and not nursing (see §4.4)
ECOG Performance Status: 0-2
Age ≥ 18 years

Schema

2 x 2 Factorial Randomization

All patients will be randomized to receive:



Celecoxib/placebo will be continued for a total of 3 years from the day that study drug was initiated.

Stratification for the duration of adjuvant chemotherapy randomization:

- Number of positive lymph nodes (1-3 vs. 4 or more)

Stratification for the celecoxib randomization:

- Number of positive lymph nodes (1-3 vs. 4 or more)
- Current regular low dose aspirin usage (Yes vs. No)

TABLE OF CONTENTS

1.0 INTRODUCTION

1.1 Colorectal cancer

In the United States, colorectal cancer is the fourth most common malignancy and the second most frequent cause of cancer-related death.[1] In 2008, an estimated 148,810 cases of colorectal cancer were diagnosed and 49,960 people died from this disease. Surgery is the primary modality of treatment for colorectal cancer, and a 'curative intent' resection occurs in 80-85% of patients with non-metastatic disease (stages I-III). Among patients with potentially curable colorectal cancer, pathologic stage (including depth of invasion in the bowel, involvement of regional lymph nodes and distant metastasis) is considered critical in determining prognosis and in whether treatment in addition to surgery is necessary. Overall, 35-40% of colorectal cancer patients have stage III disease at diagnosis (~55,000 people in the United States annually).[2] Of patients with stage III colon cancer, 40-70% will develop cancer recurrence (dependent on substage of stage III disease and other factors) despite curative-intent surgery and postoperative adjuvant chemotherapy.

1.2 Adjuvant therapy for colon cancer

Adjuvant therapy has evolved in the past two decades for stage III colon cancer. In 1990, a National Cancer Institute consensus conference recommended fluorouracil-based adjuvant therapy as standard of care for patients with resected stage III colon cancer based on trials demonstrating a statistically significant 40% improvement in disease-free survival and 33% improvement in overall survival.[3] A pooled analysis of stage III patients participating in seven adjuvant therapy clinical trials demonstrated that such chemotherapy increased the probability of remaining free of tumor recurrence after five years from 42 percent to 58 percent and the likelihood of five-year overall survival from 51 percent to 64 percent.[4]

1.3 Oxaliplatin

Oxaliplatin (trans-*l*-1,2-diaminocyclohexane oxalatoplatinum) is an 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.[5-7] However, DACH-platinum adducts formed by oxaliplatin are apparently more effective at inhibiting DNA synthesis[7] and are more cytotoxic than cis-diamine-platinum adducts formed from cisplatin and carboplatin.[7,8]

1.4 FOLFOX for colon cancer

Oxaliplatin administration has been coupled with 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 administered after a two-hour 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.[9] This program is repeated on two consecutive days every two weeks. A version termed FOLFOX-4 utilizes 85 mg/m^2 of oxaliplatin. In order to ease the administration and reduce the number of clinic visits, a modified version, FOLFOX-6, has been routinely used in clinical practice and recent clinical trials. Patients receive a loading dose of 400 mg/m^2 5-fluorouracil as a bolus injection given after a two-hour leucovorin infusion at a dose of 400 mg/m^2. The loading dose is then followed by a

46-hour 5-fluorouracil infusion of 2,400 mg/m^2 via a pump programmed to provide a constant drug infusion rate.[10-12]

Randomized clinical trials have consistently shown FOLFOX to result in superior response rates and times to disease progression compared to fluorouracil and leucovorin alone when given as first[9,13,14] or second-line[15] treatment of advanced colorectal cancer. 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.[16] 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), a response rate advantage of 29% versus 38% (p= .03), and a median survival advantage of 14.1 versus 18.6 months (p= .002) over IFL. Toxicity also favored FOLFOX over IFL with significantly less grade 3 or greater 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 than with IFL (18% versus 2%).

In 2003, the first analysis of the Multicenter International Study of Oxaliplatin/Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer (MOSAIC) study was presented and showed a statistically significant improvement in disease-free survival with the addition of oxaliplatin to 5-fluorouracil and leucovorin in stage III patients.[17] Soon after this presentation, FOLFOX became increasingly the standard of care for stage III colon cancer adjuvant therapy and the backbone of subsequent adjuvant therapy trials. However, the recent update of the MOSAIC trial demonstrated a 5-year disease-free survival of 66% with FOLFOX compared to 59% with 5-fluorouracil and leucovorin only in stage III patients.[18] Clearly, with 34% of stage III patients eventually manifesting recurrent disease, there is a need for further improvement in outcomes of stage III colon cancer patients. Further, the addition of oxaliplatin to fluoropyrimidine therapy increases the risk of toxicities, including bone marrow suppression and neurosensory symptoms. The latter is particularly problematic for some patients. At the completion of therapy in the MOSAIC trial, 12% of patients had grade 3 peripheral neuropathy and 92% had some level of neuropathy.[19] In a recent update, 15% of patients still had some level of residual neuropathy 4 years after the completion of adjuvant therapy.[18]

1.5 Duration of adjuvant therapy

Trials in the 1990s demonstrated non-inferiority of 6 months of adjuvant therapy to 12 months of therapy. Chau and colleagues conducted a trial to further reduce the timeframe of adjuvant therapy, comparing 3 months of continuous infusion 5-FU to 6 months of monthly Mayo Clinic 5-FU and leucovorin.[20] The investigators reported that overall survival was not appreciably different between the two arms and the probability of 3 months of continuous infusion 5-FU being inferior was extremely low ($p < 0.005$). Given the neuropathy and other toxicities associated with FOLFOX therapy, a trial to test the ability to reduce the total number of cycles of adjuvant FOLFOX is highly relevant. However, in order to test non-inferiority with acceptable confidence bounds, a very large sample size is required. Two trials in Europe that include a hypothesis regarding duration of FOLFOX therapy are currently open and accruing patients. The TOSCA trial in Italy (PI: Alberto Sobrero) is randomizing 3450 high-risk stage II and stage III patients to 3 versus 6 months of FOLFOX with an option for additional randomization to bevacizumab in stage IIIC patients. The SCOT trial in the UK (PI: James Cassidy) is randomizing 9500 stage II and III colon and rectal cancer patients to 3 versus 6 months of FOLFOX/XELOX. In addition, the GERCOR and AIO trials each will be enrolling 2000-2500 patients with stage III colon cancer to a trial of 3 versus 6 months of FOLFOX. As a result, the IDEA (International Duration Evaluation of Adjuvant Chemotherapy) steering committee (PI: Dan Sargent and Axel Grothey) was formed to develop a prospective pooling of

data from each of these and the current trial to test for non-inferiority of 6 treatments versus 12 treatments of adjuvant FOLFOX.

1.6 Cyclooxygenase and colon cancer

Aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) have long been studied as agents that may influence cancer development and progression.[21] In particular, data from observational studies and intervention trials consistently demonstrate that usage of these agents reduces the risk of colorectal adenomas and/or cancer.[22-24] Hypotheses for the mechanism of action of these agents include inhibition of the cyclooxygenase (COX) family of enzymes (increasing arachidonic acid which stimulates the conversion of sphingomyelin to ceramide that mediates apoptosis as well as altering prostaglandin production that decreases angiogenic factors), inhibition of the activation of nuclear factor-κ-B, interference of the binding of peroxisome-proliferator–activated receptor (PPARδ) to DNA and other potential non-COX-mediated pathways.[25] Regardless of the precise mechanism, the data is so consistent that causality is generally accepted.

In the Nurses' Health Study, an over thirty-year ongoing prospective observational study of 121,000 women, a dose- and duration-dependent protective effect of aspirin on colorectal cancer incidence was demonstrated.[26] Among women who regularly used aspirin (at least 2 standard 325 mg tablets per week), the multivariate relative risk (RR) for colorectal cancer was 0.77 (95% confidence interval [CI], 0.67-0.88) compared to non-regular users. A statistically significant risk reduction required more than 10 years of use. The strongest benefit was seen in subjects using more than 14 aspirins per week (RR 0.68 [95% CI, 0.49-0.95]). In a randomized placebo-controlled study of aspirin in patients with prior colorectal adenomatous polyps, aspirin reduced the risk of advanced adenomas at 3 years by 30%.[21] Similarly, in CALGB 9270, a phase III trial of aspirin versus placebo in patients with prior history of colorectal cancer, treatment with aspirin decreased subsequent adenomatous polyp formation by 35% in the 3 year follow-up period.[27]

The discovery of the second isoform of cyclooxygenase, COX-2, resulted in extensive research on the different roles of COX-1 and COX-2 in normal and abnormal cell function.[28,29] Studies emerged that suggested that COX-2 was induced by inflammation and COX-1 was more constitutive, particularly in the gastrointestinal tract. The subsequent conclusion was that inhibitors specific to COX-2 could have more therapeutic specificity with less gastrointestinal toxicity (as well as minimizing inhibition of platelet aggregation by having a more modest effect on thromboxane A_2 synthase). Thus, there was great enthusiasm to develop COX-2-specific inhibitors. In less than 10 years since the initial discovery of COX-2, celecoxib and rofecoxib were approved by the Food and Drug Administration for arthritis.[30]

Given the consistent observational data and two randomized trials on aspirin and colorectal adenomas and/or colorectal cancer, three trials were initiated to test the role of COX-2 inhibitors in polyp prevention.[31-33] The designs of each of these trials were similar. Patients were eligible if they had a colonoscopy in which at least one adenomatous polyp was fully excised (in the celecoxib trials, the polyp had to be 5-6 millimeters in size or multiple). Patients were randomized to placebo or COX-2 inhibitor. Colonoscopies were performed at 1 and 3 years after randomization. Aspirin usage was allowed for cardioprotection, but limited to less than 100 milligrams daily. All the trials demonstrated statistically significant reductions in cumulative incidence of subsequent adenomas at three years, with relative risks ranging from 0.55 – 0.76 (all having upper limits of confidence intervals < 1.0).

Table 1. Polyp prevention trials with COX-2 inhibitors

	Total number of subjects	Treatment arms	Cumulative incidence of adenoma by year 3
Adenoma Prevention with Celecoxib (APC)[33]	2035	Placebo 200 mg celecoxib twice a day 400 mg celecoxib twice a day	61 % 43 % 38 %
Prevention of Colorectal Sporadic Adenomatous Polyps (PreSAP)[31]	1561	Placebo 400 mg celecoxib daily	49% 34 %
Adenomatous Polyp Prevention on Vioxx (APPROVe)[32]	2587	Placebo 25 mg rofecoxib daily	55 % 41 %

A randomized controlled trial, Vioxx in Colorectal Cancer Therapy: Definition of Optimal Regime (VICTOR), evaluating the role of rofecoxib for stage II and III colon cancer was initiated in the United Kingdom in April 2002.[35] The trial was designed to enroll 7,000 patients with stage II or III colorectal cancer who completed adjuvant therapy and were randomized to placebo (1/2 of the patients), 25 mg daily of rofecoxib for 2 years (1/4 of the patients) or 25 mg daily of rofecoxib for 5 years (1/4 of the patients). The trial was terminated in September 2004 due to cardiac concerns with rofecoxib, which eventually led to withdrawal of the drug from the market. Prior to termination, 2,434 patients were enrolled with 1,217 patients randomized to rofecoxib and 1,217 patients randomized to placebo. Patients were only on study medication for a median of 7.4 months (95% CI 3.1-14). Fifteen patients in the rofecoxib group had a cardiothrombotic event during or within 14 days after the treatment period compared to 6 patients in the placebo arm. During the treatment period and within the 2 years after closure of the trial, 21 patients in the rofecoxib arm and 14 patients in the placebo arm had a cardiothrombotic event.[35] The median follow-up of patients was 36.5 months. The hazard ratio for DFS was 0.90 (95% CI, 0.77-1.06) favoring rofecoxib amongst stage II and III patients. Since patients were treated for considerably less time than anticipated (nearly 50% were on study for < 6 months), point estimates for DFS can be considered only hypothesis-generating, but at least support that the point estimate may be beneficial.

1.7 Aspirin and NSAIDs in early stage colon cancer

Fuchs and colleagues reported that consistent users of aspirin in CALGB 89803 have >50% improvement in disease-free survival compared to non-users. A similar impact was demonstrated with use of COX-2 inhibitors.[34] Chan et al. found a 35% reduction in colorectal cancer-specific mortality for women with colon cancer in the Nurses' Health Study who regularly used aspirin after diagnosis.[299] In a randomized study of 635 colorectal cancer survivors (CALGB 9270), Sandler and colleagues found that patients randomized to take a daily dose of 325 mg of aspirin experienced an adjusted reduction in risk of adenomatous polyps of 35%.[27]

1.8 Celecoxib and cardiovascular risks

Celecoxib is more potent than aspirin or other NSAIDs in experimental models of colon tumor formation.[36] Use of celecoxib would have the advantage of permitting patients who are taking aspirin for other indications to be eligible for enrollment in this trial. There have been associations between celecoxib and risk of cardiovascular disease. However, in the Prevention of Spontaneous Adenomatous Polyps (PreSAP) trial utilizing the proposed dose of celecoxib for this intervention (400 mg daily), the risks of cardiovascular events were only modestly above placebo and not statistically significant (HR 1.5 [95% CI, 0.9-2.4] for any cardiovascular event, HR 1.3 [95% CI, 0.6-2.5] for composite endpoint of death from cardiovascular causes, nonfatal MI or stroke, hospitalization for heart failure and HR 0.7 [95% CI, 0.2-2.7] for death from cardiovascular causes). In recent analyses of primary data from 6 randomized trials of celecoxib, the daily dosing of celecoxib appears considerably safer than twice a day dosing. It should also be noted that these studies did not have strict cardiovascular exclusion criteria and thus one can assume that with the exclusion criteria in this trial, the rare but observed differences in cardiovascular events between the intervention and control arm will be attenuated.

Table 2. Event rates per 1000 patient-years and pooled hazard ratios with 95% confidence intervals for the principal composite endpoint of cardiovascular death, myocardial infarction, stroke, heart failure, or thromboembolism for each individual trial, for each dose regimen, and for all the trials combined, adjusted for baseline cardiovascular risk[37].

Study	Median follow-up time (months)	Events/ Participants		Event rate/ 1000 pt years		Hazard ratio	95% CI	Relative weight
		Placebo	Celecoxib	Placebo	Celecoxib			
400 mg qd								
PreSAP	36	12/628	23/933	7.2	9.4	1.3	(0.6, 2.5)	7.9
Selenium/Celecoxib	21	8/410	7/414	11.8	10.3	0.9	(0.3, 2.4)	3.7
Pooled 400 mg qd	***35***	***20/1038***	***30/1347***	***8.6***	***9.6***	***1.1***	***(0.6, 2.0)***	
								200 mg bid
ADAPT	24	18/1083	18/726	8.6	12.8	1.5	(0.8, 2.9)	9.0
APC	37	8/679	20/685	3.9	9.7	2.5	(1.1, 5.7)	5.7
CDME	15	3/47	0/39	54.3	0.0			
Pooled 200 mg bid	***36***	***29/1809***	***38/1450***	***6.9***	***10.8***	***1.8***	***(1.1, 3.1)***	
								400 mg bid
APC	37	8/679	27/671	3.9	13.4	3.6	(1.6, 8.0)	6.2
MA27	5	3/817	6/818	8.7	17.2	1.8	(0.4, 7.3)	2.0
Pooled 400 mg bid	***11***	**11/1496**	**33/1489**	**4.6**	**13.9**	***3.1***	***(1.5, 6.1)***	
Pooled all doses	***31***	**52/3664**	**101/4286**	***7.5***	***11.2***	**1.6**	**(1.1, 2.3)**	

1.9 Rationale for the current trial

This randomized phase III trial will potentially reduce the total amount of cytotoxic chemotherapy and improve upon existing disease-free survival rates. Given the issues of neuropathy associated with oxaliplatin as well as the costs (human, economic and resources) of adjuvant therapy, potential reduction in number of required treatments without impacting on efficacy is a critical question. Furthermore, there are still an appreciable number of patients with stage III colon cancer recurring despite recommended therapy, and thus continued testing of an

agent for superiority of efficacy is critical. Celecoxib will be initiated within two weeks of the start of FOLFOX chemotherapy (i.e., concurrent administration) and continued for 3 years total from the date when celecoxib (or placebo) was started.

1.10 Inclusion of women and minorities

This study will be available to all eligible patients, regardless of race, gender, or ethnic origin. There is no information currently available regarding differential effects of oxaliplatin, 5-fluorouracil or celecoxib-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 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. In CALGB 89803, 12% (148/1264) of patients were classified as minorities by race and 45% (562/1264) of patients were women. No race or gender differences were observed in CALGB 89803.

Accrual Targets

Ethnic Category	**Sex/Gender**					
	Females		**Males**		**Total**	
Hispanic or Latino	66	+	112	=	178	
Not Hispanic or Latino	1034	+	1288	=	2322	
Ethnic Category: Total of all subjects	1100 (A1)	+	1400 (B1)	=	2500	(C1)
Racial Category						
American Indian or Alaskan Native	16	+	21	=	37	
Asian	39	+	49	=	88	
Black or African American	143	+	140	=	283	
Native Hawaiian or other Pacific Islander	11	+	14	=	25	
White	891	+	1176	=	2067	
Racial Category: Total of all subjects	1100 (A2)	+	1400 (B2)	=	2500	(C2)

2.0 OBJECTIVES

2.1 Primary objective

To compare disease-free survival of patients with stage III colon cancer randomized to standard chemotherapy only (FOLFOX) or standard chemotherapy (FOLFOX) with 3 years of celecoxib 400 mg daily.

2.2 Secondary objectives

2.2.1 To contribute to an international prospective pooled analysis that will compare disease-free survival of patients with stage III colon cancer randomized to 6 treatments of adjuvant FOLFOX chemotherapy or 12 treatments of adjuvant FOLFOX chemotherapy (see Appendix VI).

2.2.2 To compare overall survival of patients with stage III colon cancer randomized to standard chemotherapy only (FOLFOX) or standard chemotherapy (FOLFOX) with 3 years of celecoxib 400 mg daily.

2.2.3 To contribute to an international prospective pooled analysis that will compare overall survival of patients with stage III colon cancer randomized to 6 treatments of adjuvant

FOLFOX chemotherapy or 12 treatments of adjuvant FOLFOX chemotherapy (see Appendix VI).

2.2.4 To assess toxicities of celecoxib as maintenance adjuvant therapy in patients with stage III colon cancer.

2.2.5 To assess differences in cardiovascular-specific events with celecoxib versus placebo in a population of stage III colon cancer survivors.

2.2.6 To evaluate differences in toxicities, particularly cumulative peripheral neuropathy, for patients treated with 6 treatments of FOLFOX compared to those treated with 12 treatments of FOLFOX.

2.2.7 See Appendices II, III, and IV for substudy objectives.

3.0 ON-STUDY GUIDELINES

This clinical trial can fulfill its objectives only if patients appropriate for this trial are enrolled. All relevant medical and other considerations should be taken into account when deciding whether this protocol is appropriate for a particular patient. Physicians should consider the risks and benefits of any therapy, and therefore only enroll patients for whom this treatment is appropriate. Although they will not be considered formal eligibility (exclusion) criteria, physicians should recognize that the following may seriously increase the risk to the patient entering this protocol:

- Psychiatric illness that would prevent the patient from giving informed consent.
- Patient is not deemed a candidate for FOLFOX or celecoxib based on overall condition and co-morbidities.
- A medical condition such as active/uncontrolled infection that would make this protocol unreasonably hazardous for the patient in the opinion of the treating physician.
- Inability to take oral medications.

4.0 ELIGIBILITY CRITERIA

All questions regarding eligibility criteria should be directed to the Alliance or SWOG Study Chair. Please note that the Study Chair cannot grant waivers to eligibility requirements.

4.1 Requirements for tumor parameters

4.1.1 Histologically documented adenocarcinoma of the colon. The gross inferior (caudad) margin of the primary tumor must lie above the peritoneal reflection (i.e., patients with rectal cancer are not eligible). Surgeon confirmation that the entire tumor was above the peritoneal reflection is only required in cases where it is important to establish if the tumor is a rectal or colon primary.

4.1.2 Tumors must have been completely resected. In patients with tumor adherent to adjacent structures, en bloc R_o resection must be documented in the operative report or otherwise confirmed by the surgeon. Near or positive radial margin are NOT exclusions as long as en bloc resection was performed. Positive proximal margin or distal margin is an exclusion.

4.1.3 Node positive disease (N1 or N2) as designated in AJCC version 7. Either at least one pathologically confirmed positive lymph node or N1C (defined as tumor deposit(s) in the

subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional lymph node metastases). Patients with resected stage IV disease are not eligible.

4.1.4 No evidence of residual involved lymph node disease or metastatic disease at the time of registration.

4.1.5 Patients with synchronous colon cancers are eligible and staging for stratification will be based on higher N stage of the more advanced primary tumor. However, patients with synchronous colon and rectal primary tumors are not eligible.

4.2 NSAID Use

Patients are ineligible if they plan on regular use of NSAIDs at any dose more than 2 times per week (on average) or aspirin at more than 325 mg at least three times per week, on average. Low-dose aspirin not exceeding 100 mg/day is permitted. Patients who agree to stop regular NSAIDs or higher dose aspirin are eligible and no wash out period is required.

4.3 Patient history

4.3.1 No previous or concurrent malignancy, except treated basal cell or squamous cell cancer of skin, treated in situ cervical cancer, treated lobular or ductal carcinoma in situ in one breast, or any other cancer for which the patient has been disease-free for at least 5 years.

4.3.2 No neurosensory or neuromotor toxicity ≥ grade 2 at the time of registration.

4.3.3 No known allergy to platinum compounds.

4.3.4 No prior allergic reaction or hypersensitivity to sulfonamides, celecoxib or NSAIDs.

4.3.5 No history of upper gastrointestinal ulceration, upper gastrointestinal bleeding, or upper gastrointestinal perforation within the past 3 years. Patients with ulceration, bleeding or perforation in the lower bowel are NOT excluded.

4.3.6 No symptomatic pulmonary fibrosis or interstitial pneumonitis ≥ grade 2.

4.3.7 No cardiac risk factors including:

- Uncontrolled high blood pressure (systolic blood pressure > 150).
- Unstable angina.
- History of documented myocardial infarction or cerebrovascular accident.
- New York Heart Association class III or IV heart failure.

4.4 Pregnancy/nursing status

Non-pregnant and not nursing. Men and women of childbearing potential must agree to employ adequate contraception for the duration of chemotherapy and for as many as 8 weeks after the completion of chemotherapy due to the unknown teratogenic effects of FOLFOX on the developing fetus.

4.5 Age and performance status

4.5.1 ECOG performance status 0, 1, or 2.

4.5.2 Age at least 18 years.

4.6 Required initial laboratory values

Granulocytes	≥ 1,500/μL

Platelet count	≥ 100,000/μL
Creatinine	≤ 1.5 x upper limit of normal
*Total Bilirubin	≤ 1.5 x upper limit of normal

*In the absence of Gilbert's disease. For patients with Gilbert's Syndrome:
Direct Bilirubin ≤ 1.5 x upper limit of normal

5.0 REGISTRATION/RANDOMIZATION, STRATIFICATION, AND DATA AND SAMPLE SUBMISSION

5.1 Cancer Therapy Evaluation Program Registration Procedures

Food and Drug Administration (FDA) regulations and National Cancer Institute (NCI) policy require all individuals contributing to NCI-sponsored trials to register and to renew their registration annually. To register, all individuals must obtain a Cancer Therapy Evaluation Program (CTEP) Identity and Access Management (IAM) account at [redacted] In addition, persons with a registration type of Investigator (IVR), Non-Physician Investigator (NPIVR), or Associate Plus (AP) (i.e., clinical site staff requiring write access to OPEN, Rave, or acting as a primary site contact) must complete their annual registration using CTEP's web-based Registration and Credential Repository (RCR) at [redacted]

RCR uses five person registration types.

- IVR – MD, DO, or international equivalent;
- NPIVR – advanced practice providers (e.g., NP or PA) or graduate level researchers (e.g., PhD);
- AP – clinical site staff (e.g., RN or CRA) with data entry access to CTSU applications (e.g., Roster Update Management System (RUMS), OPEN, Rave,);
- Associate (A) – other clinical site staff involved in the conduct of NCI-sponsored trials; and
- Associate Basic (AB) – individuals (e.g., pharmaceutical company employees) with limited access to NCI-supported systems.

RCR requires the following registration documents:

Documentation Required	IVR	NPIVR	AP	A	AB
FDA Form 1572	✓	✓			
Financial Disclosure Form	✓	✓	✓		
NCI Biosketch (education, training, employment, license, and certification)	✓	✓	✓		
GCP training	✓	✓	✓		
Agent Shipment Form (if applicable)	✓				
CV (optional)	✓	✓	✓		

An active CTEP-IAM user account and appropriate RCR registration is required to access all CTEP and Cancer Trials Support Unit (CTSU) websites and applications. In addition, IVRs and NPIVRs must list all clinical practice sites and Institutional Review Boards (IRBs) covering their practice sites on the FDA Form 1572 in RCR to allow the following:

- Addition to a site roster;
- Assign the treating, credit, consenting, or drug shipment (IVR only) tasks in OPEN;
- Act as the site-protocol Principal Investigator (PI) on the IRB approval; and

- Assign the Clinical Investigator (CI) role on the Delegation of Tasks Log (DTL).

In addition, all investigators act as the Site-Protocol PI, consenting/treating/drug shipment, or as the CI on the DTL must be rostered at the enrolling site with a participating organization (i.e., Alliance).

Additional information is located on the CTEP website at [redacted] For questions, please contact the RCR Help Desk by email at [redacted]

5.2 Cancer Trials Support Unit Registration Procedures

This study is supported by the NCI CTSU.

IRB Approval:

For CTEP and Division of Cancer Prevention (DCP) studies open to the National Clinical Trials Network (NCTN) and NCI Community Oncology Research Program (NCORP) Research Bases after March 1, 2019, all U.S.-based sites must be members of the NCI Central Institutional Review Board (NCI CIRB). In addition, U.S.-based sites must accept the NCI CIRB review to activate new studies at the site after March 1, 2019. Local IRB review will continue to be accepted for studies that are not reviewed by the CIRB, or if the study was previously open at the site under the local IRB. International sites should continue to submit Research Ethics Board (REB) approval to the CTSU Regulatory Office following country-specific regulations.

Sites participating with the NCI CIRB must submit the Study Specific Worksheet for Local Context (SSW) to the CIRB using IRBManager to indicate their intent to open the study locally. The NCI CIRB's approval of the SSW is automatically communicated to the CTSU Regulatory Office, but sites are required to contact the CTSU Regulatory Office at [redacted] to establish site preferences for applying NCI CIRB approvals across their Signatory Network. Site preferences can be set at the network or protocol level. Questions about establishing site preferences can be addressed to the CTSU Regulatory Office by emailing the email address above or calling [redacted]

Sites using their local IRB or REB, must submit their approval to the CTSU Regulatory Office using the Regulatory Submission Portal located in the Regulatory section of the CTSU website. Acceptable documentation of local IRB/REB approval includes:

- Local IRB documentation;
- IRB-signed CTSU IRB Certification Form; and/or
- Protocol of Human Subjects Assurance Identification/IRB Certification/Declaration of Exemption Form.

In addition, the Site-Protocol Principal Investigator (PI) (i.e. the investigator on the IRB/REB approval) must meet the following criteria to complete processing of the IRB/REB approval record:

- Holds an Active CTEP status;
- Rostered at the site on the IRB/REB approval and on at least one participating roster;
- If using NCI CIRB, rostered on the NCI CIRB Signatory record;
- Includes the IRB number of the IRB providing approval in the Form FDA 1572 in the RCR profile; and
- Holds the appropriate CTEP registration type for the protocol.

Additional Requirements

Additional requirements to obtain an approved site registration status include:

- An active Federal Wide Assurance (FWA) number;
- An active roster affiliation with the Lead Protocol Organization (LPO) or a Participating Organization (PO); and
- Compliance with all protocol-specific requirements (PSRs).

5.2.1 Downloading Site Registration Documents:

Download the site registration forms from the protocol-specific page located on the CTSU members' website. Permission to view and download this protocol and its supporting documents is restricted based on person and site roster assignment. To participate, the institution and its associated investigators and staff must be associated with the LPO or a PO on the protocol.

- Log on to the CTSU members' website [redacted] using your CTEP-IAM username and password;
- Click on *Protocols* in the upper left of your screen
  - Enter the protocol number in the search field at the top of the protocol tree, or
  - Click on the By Lead Organization folder to expand, then select *Alliance*, and protocol number *CALGB-80702*;
- Click on *Documents*, select *Site Registration*, and download and complete the forms provided. (Note: For sites under the CIRB initiative, IRB data will load automatically to the CTSU as described above.)

5.2.2 Submitting Regulatory Documents

Submit required forms and documents to the CTSU Regulatory Office via the Regulatory Submission Portal on the CTSU website.

To access the Regulatory Submission Portal log on to the CTSU members' website Regulatory Regulatory Submission.

Institutions with patients waiting that are unable to use the Regulatory Submission Portal should alert the CTSU Regulatory Office immediately at [redacted] in order to receive further instruction and support.

5.2.3 Checking Your Site's Registration Status:

You can verify your site's registration status on the members' side of the CTSU website.

- Log on to the CTSU members' website;
- Click on *Regulatory* at the top of your screen;
- Click on *Site Registration*;
- Enter your 5-character CTEP Institution Code and click on Go.

Note: The status shown only reflects institutional compliance with site registration requirements as outlined above. It does not reflect compliance with protocol requirements for individuals participating on the protocol or the enrolling investigator's status with the NCI or their affiliated networks.

5.3 Registration Requirements

Informed Consent: the patient must be aware of the neoplastic nature of his/her disease and willingly consent after being informed of the procedure to be followed, the experimental nature

of the therapy, alternatives, potential benefits, side-effects, risks, and discomforts. Current human protection committee approval of this protocol and a consent form is required prior to patient consent and registration.

5.4 Patient Registration/Randomization Procedures

The Oncology Patient Enrollment Network (OPEN) is a web-based registration system available on a 24/7 basis. OPEN is integrated with CTSU regulatory and roster data and with the Lead Protocol Organization (LPOs) registration/randomization systems or Theradex Interactive Web Response System (IWRS) for retrieval of patient registration/randomization assignment. OPEN will populate the patient enrollment data in NCI's clinical data management system, Medidata Rave.

Requirements for OPEN access:

- A valid CTEP-IAM account;
- To perform enrollments or request slot reservations: Be on a LPO roster, ETCTN Corresponding roster, or PO roster with the role of Registrar. Registrars must hold a minimum of an AP registration type;
- If a Delegation of Tasks Log (DTL) is required for the study, the registrar(s) must hold the OPEN Registrar task on the DTL for the site; and
- Have an approved site registration for a protocol prior to patient enrollment.

To assign an Investigator (IVR) or Non-Physician Investigator (NPIVR) as the treating, crediting, consenting, drug shipment (IVR only), or receiving investigator for a patient transfer in OPEN, the IVR or NPIVR must list the IRB number used on the site's IRB approval on their Form FDA 1572 in RCR. If a DTL is required for the study, the IVR or NPIVR must be assigned the appropriate OPEN-related tasks on the DTL.

Prior to accessing OPEN, site staff should verify the following:

- Patient has met all eligibility criteria within the protocol stated timeframes; and
- All patients have signed an appropriate consent form and HIPAA authorization form (if applicable)

Note: The OPEN system will provide the site with a printable confirmation of registration and treatment information. Please print this confirmation for your records.

To receive site reimbursement for specific tests and/or bio-specimen submissions, completion dates must be entered in the OPEN Funding screen post registration. Please refer to the protocol-specific funding page on the CTSU members' website for additional information. Timely entry of completion dates is recommended as this will trigger site reimbursement.

Access OPEN at [redacted] or from the OPEN link on the CTSU members' website. Further instructional information is in the OPEN section of the CTSU website at [redacted] For any additional questions, contact the CTSU Help Desk at [redacted]

Treatment is to begin within 14 days of randomization.

No blinded starter supplies will be available for this study. Initial blinded, patient-specific clinical supplies of celecoxib/placebo will be shipped from the Pharmaceutical Management Branch (PMB) to the registering investigator at the time of patient randomization and should arrive within 7 to 10 days of randomization (see Section 9.4).

5.5 Registration to companion studies

There are three substudies within CALGB/SWOG C80702. These correlative science studies **must be offered to all patients** enrolled on CALGB/SWOG C80702 (although patients may opt to not participate). These substudies do not require separate IRB approval. The substudies included within CALGB/SWOG C80702 are:

- CALGB 150911: Correlative science companion studies for CALGB/SWOG C80702 (Appendix II)
- CALGB 60905: Pharmacogenetic companion studies for CALGB/SWOG C80702 (Appendix III)
- CALGB 71002: Cancer prevention companion studies for CALGB/SWOG C80702 (Appendices IV and V)

If a patient answers "yes" to "I agree that my specimen may be used for the research studies described above", question #2 in the model consent, they have consented to participate in the studies described in Appendix II. The patient should be registered to CALGB 150911 at the same time they are registered to the treatment trial (CALGB/SWOG C80702). Samples should be submitted per Sections 5.8.1, 5.8.2, 5.8.3, and 5.8.4.

If a patient answers "yes" to "I agree that my blood may be used for the genetic research studies described above", question #3 in the model consent, they have consented to participate in the studies described in Appendix III. The patient should be registered to CALGB 60905. Samples should be submitted per Section 5.8.5.

If a patient answers "yes" to "I choose to take part in the cancer prevention studies and agree to complete the diet and lifestyle questionnaire", question #1 in the model consent, they have consented to participate in the cancer prevention studies described in Appendices IV and V. Questionnaires and reports should be submitted per Section 5.9.

5.6 Stratification

5.6.1 Stratification for the duration of adjuvant chemotherapy randomization

- Number of positive lymph nodes (1-3 vs. 4 or more)

5.6.2 Stratification for the celecoxib randomization

- Number of positive lymph nodes (1-3 vs. 4 or more)
- Current regular low dose aspirin usage (Yes vs. No)

Patients with N1C only disease (i.e., no positive nodes but N1C disease by AJCC 7 should be stratified to 1-3 nodes).

5.7 Data Submission

As of Update #11 to the protocol, this study will use Medidata Rave® for remote data capture (RDC) of all future data collection. All data originally received by the Alliance and Statistics and Data Center (SDC) (either electronically using the "Print and/or Submit to CALGB" button [i.e. Teleform form] or by mail) has been transferred to Medidata Rave® and can be accessed via the Medidata Rave® system. If necessary, data originally submitted to the SDC electronically (or by mail) can be amended via the Medidata Rave® system.

The Rave system can be accessed through the iMedidata portal at [redacted]. For additional information regarding account setup or training, please visit the training section of the Alliance website. Forms should be submitted in compliance with the table below, and a copy of the All Forms Packet can be downloaded from the Alliance and CTSU websites.

Medidata Rave is a clinical data management system being used for data collection for this trial/study. Access to the trial in Rave is controlled through the CTEP-IAM system and role assignments. To access Rave via iMedidata:

- Site staff will need to be registered with CTEP and have a valid and active CTEP-IAM account; and
- Assigned one of the following Rave roles on the relevant Lead Protocol Organization (LPO) or Participating Organization roster at the enrolling site: Rave CRA, Rave Read Only, Rave CRA (Lab Admin), Rave SLA, or Rave Investigator. Refer to [redacted] for registration types and documentation required.
    - To hold Rave CRA or Rave CRA (Lab Admin) role, site staff must hold a minimum of an AP registration type;
    - To hold Rave Investigator role, the individual must be registered as an NPIVR or IVR; and
    - To hold Rave Read Only role, site staff must hold an Associates (A) registration type.

If the study has a Delegation of Tasks Log (DTL), individuals requiring write access to Rave must also be assigned the appropriate Rave tasks on the DTL.

Upon initial site registration approval for the study in Regulatory Support System (RSS), all persons with Rave roles assigned on the appropriate roster will be sent a study invitation e-mail from iMedidata. To accept the invitation, site staff must log in to the Select Login [redacted] using their CTEP-IAM username and password, and click on the accept link in the upper right-corner of the iMedidata page. Site staff will not be able to access the study in Rave until all required Medidata and study specific trainings are completed. Trainings will be in the form of electronic learnings (eLearnings), and can be accessed by clicking on the link in the upper right pane of the iMedidata screen. If an eLearning is required and has not yet been taken, the link to the eLearning will appear under the study name in iMedidata instead of the Rave EDC link; once the successful completion of the eLearning has been recorded, access to the study in Rave will be granted, and a Rave EDC link will display under the study name.

Site staff that have not previously activated their iMedidata/Rave account at the time of initial site registration approval for the study in RSS will also receive a separate invitation from iMedidata to activate their account. Account activation instructions are located on the CTSU website in the Rave section under the Rave resource materials (Medidata Account Activation and Study Invitation Acceptance). Additional information on iMedidata/Rave is available on the CTSU members' website in the Data Management > Rave section at [redacted] or by contacting the CTSU Help Desk at [redacted]

Form*		Submission Schedule
	Baseline	
C-1953 Report Report	80702 On-Study Operative and Pathology reports CT/X-ray reports	Within one month of registration
	Treatment	
C-1954 C-1955 C-1956 S-067	80702 Treatment Form 80702 Adverse Event Form*** 80702 Follow-up Form 80702 Medication Calendar**	Every 2 cycles (4 weeks) while on FOLFOX, then every 3 months while on celecoxib/placebo alone.
	Follow-up (after end of all protocol treatment)	
C-1956	80702 Follow-up Form	Every 6 months for a maximum of 6 years from the date of registration.
Report	CT/X-ray reports	At time of progression
	Other	
Report Report C-113	Colonoscopy reports Pathology reports CALGB: Notification of Death Form	For patients enrolled to CALGB 71002. See Section 5.9. At time of death

* Use CALGB Remarks Addenda (C-260) if additional comments or writing space is needed. If patient never starts treatment, submit the baseline data, a C-1954 80702 Treatment Form to report the reason for ending treatment, and all follow-up data.

** S-067 80702 Medication Calendar is provided for patient and institutional use. This form does not need to be submitted to the Alliance Statistics and Data Center.

*** Submit AE form until all protocol treatment related events have resolved or until non-protocol treatment begins. If patient death is reported via CTEP-AERS report Grade 5 event on AE form even if patient is off protocol treatment.

This study will utilize the NCI Common Terminology Criteria for Adverse Events version 3.0 for routine reporting on study forms. However, adverse events reported via CTEP-AERS must use CTCAE version 5.0 (see Section 14.0).

5.8 Specimen Submission for Correlative and Pharmacogenomic Substudies

All participating institutions must ask patients for their consent to participate in the components of the correlative **(CALGB 150911)** and pharmacogenomic **(CALGB 60905)** substudies planned for CALGB/SWOG C80702, although patient participation is optional. Rationale and methods for the scientific components of these studies are described in Appendices II and III.

Type of specimen	Pre-treatment
Tissue*	2 paraffin blocks (1 tumor / 1 normal)
EDTA Plasma (lavender top)*	1 x 10 mL
Serum (red top)*	1 x 10 mL
Whole Blood (lavender top)**	1 x 10 mL
[Total blood volume]	[30 mL]

* For patients who answer "yes" to consent question #2 (CALGB 150911).

** For patients who answer "yes" to consent question #3 (CALGB 60905). Note: Specimen is requested to be drawn pre-treatment but can be drawn at any time while the patient is on study.

USE OF THE ALLIANCE BIOSPECIMEN MANAGEMENT SYSTEM (BioMS) IS MANDATORY AND ALL SPECIMENS MUST BE LOGGED AND SHIPPED VIA THIS SYSTEM.

BioMS is a web-based system for logging and tracking all biospecimens collected on Alliance trials. Authorized individuals may access BioMS at the following URL: [redacted] using most standard web browsers (Safari, Firefox, Internet Explorer). For information on using the BioMS system, please refer to the 'Help' links on the BioMS web page to access the on-line user manual, FAQs, and training videos. To report technical problems, such as login issues or application errors, please contact: [redacted] For assistance in using the application or questions or problems related to specific specimen logging, please contact: [redacted]

After logging collected specimens in BioMS, the system will create a shipping manifest. This shipping manifest must be printed and placed in the shipment container with the specimens.

All submitted specimens must be labeled with the protocol number (C80702), patient study ID number, patient's initials and date and type of specimen collected (e.g., serum, whole blood).

A copy of the Shipment Packing Slip produced by BioMS must be printed and placed in the shipment with the specimens.

Instructions for the collection of samples are included below. Please be sure to use a method of shipping that is secure and traceable. Extreme heat precautions should be taken when necessary.

Shipment on Monday through Thursday by overnight service to assure receipt is encouraged. Do not ship specimens on Fridays or Saturdays.

All specimens should be sent to the following address:

[redacted]

5.8.1 Submission of Paraffin Blocks of Archived Colorectal Tissue (CALGB 150911)

For patients who consent to Question #2, tissue blocks will be used for the correlative studies described in Appendix II. Paraffin blocks of tissue obtained from archival colorectal specimens from primary site should be sent to the Alliance Biorepository at Ohio State University (OSU). Submit 1 block of tumor tissue and 1 block of normal tissue.

The Alliance has instituted special considerations for the small percentage of institutions whose policies prohibit release of any blocks. If, due to institutional policy, a block cannot be sent, please call [redacted] to obtain a protocol for submission of representative tissue from your institution.

The goal of the Alliance Biorepository at Ohio State University (OSU) is to provide investigators with quality histology sections for their research while maintaining the integrity of the tissue. All paraffin blocks that are to be stored at the OSU will be vacuum packed to prevent oxidation and will be stored at 4ºC to minimize degradation of cellular antigens. For these reasons it is preferred that the OSU bank the block until the study investigator requests thin sections. Please contact the OSU if additional assurances with your hospital pathology department are required.

5.8.2 Plasma Collection Procedures (CALGB 150911)

For patients who consent to consent question #2, plasma samples will be used for the studies described in Appendix II.

1. Collect blood in 10 mL lavender top tube. After collection, gently mix the blood by inverting the tube 8 to 10 times. Store vacutainer tubes upright at 4°C until centrifugation. Blood samples should be centrifuged within four hours of blood collection.
2. Centrifuge blood samples in a horizontal rotor (swing-out head) for 10 to 15 minutes at 1100-1300 g at room temperature. Warning: Excessive centrifuge speed (over 2000 g) may cause tube breakage and exposure to blood and possible injury. If needed, relative centrifugal force (RCF) for a centrifuge can be calculated. For an on-line calculator tool, please refer to: [redacted]
3. After centrifugation, plasma layer will be at the top of the tube. Mononuclear cells and platelets will be in a whitish layer, called the “buffy coat”, just under the plasma and above the red blood cells.
4. Carefully collect the plasma layer with an appropriate transfer pipette without disturbing the buffy coat layer. Pipette the plasma into the labeled cryovials (recommended cryovials are described in Section 5.8.4). Aliquot volume is to be 500 µL. Close the caps tightly and place on ice. This process should be completed within 1 hour of centrifugation.
5. Check that all aliquot vial caps are secure and that all vials are labeled.
6. Place all aliquots upright in a specimen box or rack in an -80°C or colder freezer. All specimens should remain at -80°C or colder prior to shipping. The samples should not be thawed prior to shipping. Plasma should be shipped on dry ice according to the shipping procedures in Section 5.8.4.

5.8.3 Serum Collection Procedures (CALGB 150911)

For patients who consent to consent question #2, serum samples will be used for the studies described in Appendix II.

1. Collect blood in 10 mL red top tube. After collection, tubes ("vacutainers") should sit upright after the blood is drawn at room temperature for a minimum of 30 to a maximum of 60 minutes to allow the clot to form. Note: Use red top (serum) tubes (silicon-coated)—no additives and not SST (serum separator tubes).
2. Centrifuge the blood sample at the end of the clotting time (30-60 minutes) in a horizontal rotor (swing-out head) for 10-15 minutes at 1100-1300 g at room temperature.
3. Use a pipette to transfer the serum (Recommendation: do not pour). Pipette serum into the labeled cryovials (recommended cryovials are described in Section 5.8.4). Aliquot volume is to be 500 µL. Close the cap on the vial tightly. This process should be completed within 1 hour of centrifugation. Note: Be very careful not to pick up red blood cells when aliquoting. This can be done by keeping the pipet above the red blood cell layer and leaving a small amount of serum in the tube.
4. Check that all aliquot vial caps are secure and that all vials are labeled.
5. Place all aliquots upright in a specimen box or rack in an -80°C or colder freezer. All specimens should remain at -80°C or colder prior to shipping. The samples should not be thawed prior to shipping. Serum should be shipped on dry ice according to the shipping procedures in Section 5.8.4.

5.8.4 Plasma and Serum Processing Procedures

The Alliance strongly recommends the usage of 2 mL cryovials for storage of plasma and serum specimens. Acceptable cryovials include:

Company name	Catalog number
Nalgene	03-337-7Y (through Fisher) NNI No.: 5012-0020
Fisher brand	05-669-57 (through Fisher)
Corning	03-374-21 (through Fisher) CLS430659 (through Sigma) Corning: 430488
VWR	16001-102

All samples should be labeled with the patient's initials, CALGB treatment study number (CALGB/SWOG C80702), patient study ID number, date and time of collection, and sample type. The sample should be shipped on dry ice to the Alliance Biorepository at Ohio State University (OSU).

5.8.5 Whole Blood Submission for the Pharmacogenomic Substudy (CALGB 60905)

For patients who consent to question #3, whole blood will be used for the pharmacogenomic studies described in Appendix III. This sample should be collected prior to the initiation of protocol treatment. Note: Specimen is requested to be drawn pre-treatment but can be drawn at any time while the patient is on study.

Draw 10 mL of venous blood in a lavender top (EDTA coagulant) tube and keep refrigerated until shipped overnight to the Alliance Biorepository at Ohio State University (OSU). Label the tube with the patient's initials, patient study ID number, CALGB treatment number (CALGB/SWOG C80702) and date of collection. The sample should be shipped the same

day on a cold pack by overnight mail to the Alliance Biorepository at Ohio State University (OSU).

5.9 Data Submission for Cancer Prevention Companion Studies (CALGB 71002)

At registration, patients may elect to enroll on the cancer prevention studies (Appendices IV and V) by consenting to question #1. These studies will require patients to complete a Diet and Lifestyle survey and agree to the submission of their colonoscopy and pathology reports as described below.

Diet and Lifestyle Survey:

Surveys will be sent by [redacted] (see below) to individual treatment centers within 2 weeks of registration. Versions of the surveys are available in Spanish and French – please email [redacted] requesting these versions if required for the patient. Patients should complete the first survey within 6 weeks of randomization. Patients should also complete questionnaires 14-16 months from randomization. It is recommended that the surveys be completed during a physician visit, likely during the infusion of chemotherapy. Questions regarding the survey should be directed to [redacted]. Completed surveys should be mailed by the institutional CRA to the following address:

[redacted]

Colonoscopy and Pathology Reports:

According to NCCN and US Multi-Society Task Force guidelines, it is recommended that patients have a colonoscopy performed within 12 months of diagnosis. Clinical considerations should dictate the exact timing (i.e., if the patient's initial colonoscopy at diagnosis was not a complete exam or the patient did not have a colonoscopy at diagnosis, guidelines suggest the first post-treatment colonoscopy be performed sooner than 12 months after surgery – typically after completion of adjuvant FOLFOX). A second post-treatment colonoscopy should be performed within 3 years of the first post-treatment colonoscopy, but may be performed earlier based on findings from the first colonoscopy as well as other clinical conditions. Earlier timing should be dictated by the clinical judgment of the gastroenterologist, oncologist and/or surgeon.

All colonoscopy reports obtained within the first 6 years after randomization should be submitted via Medidata Rave®. If any biopsies were taken during those colonoscopies, those pathology reports should also be submitted via Medidata Rave®. See Section 5.7 for data submission instructions.

6.0 REQUIRED DATA

Pre-Study Testing Intervals

To be completed within 16 DAYS before registration:

- All bloodwork, history and physical, and pregnancy test.

To be completed within 80 DAYS before registration (preoperative scans acceptable):

- CT or MRI abdomen/pelvis or PET-CT scan without evidence of metastatic disease
- CT chest or chest X-ray (PET-CT including chest, is acceptable) without evidence of metastatic disease

	Prior to Registration*	Day 1 of each tx w/ FOLFOX*	During tx w/ Celecoxib Placebo only**	Post-tx Follow Up***
Tests & Observations				
History and Physical Examination	X	A	X	X
Pulse, Blood Pressure	X	A	X	X
Height	X			
Weight/Body Surface Area ¢	X	X	X	X
Performance Status	X	A	X	X
Drug Toxicity Assessment		X	X	X
Celecoxib/Placebo Adherence#		B	B	
Laboratory Studies				
CBC, Differential, Platelets	X	X		
Serum Creatinine and BUN	X	C	F	
AST, Alk Phos., Total Bilirubin	X	C	F	
PT/INR	D	D	D	
Pregnancy Test (UCG) ‡	X			
CEA	X		E	E
STAGING				
Chest x-ray (PA & Lateral) or Chest CT	X		E	E
Abdominal/pelvic Imaging: (CT, MRI or PET-CT scan)	X		E	E
Companion Studies Ψ				
Diet & Lifestyle / Other Meds / Comorbidities Questionnaire	*To be completed w/in first 6 weeks of randomization and 14-16 months after randomization. See Section 5.9.*			
Colonoscopy and Pathology Reports	*See Section 5.9.*			
Tumor Block	*To be collected pre-treatment (see Section 5.8).*			
Whole Blood, Plasma, and Serum	*To be collected pre-treatment (see Section 5.8).*			

* Pre-registration H&P, vital signs, labs and clinical assessments may be used for Day 1 of Cycle 1. For subsequent cycles, H&P and labs may be obtained within 72 hours prior to day of treatment (96 hours if due to holidays).

** Every 3 months until 3 years after initiation of celecoxib/placebo or until disease progression, whichever comes first.

*** Every 6 months until 6 years after randomization or until disease progression, whichever comes first. Once a patient has progressive disease, submission of follow-up forms is still required for survival data. See forms schedule in Section 5.7.

A While on FOLFOX, patients should have a physical exam and report capsule counts of celecoxib/placebo prior to cycles 3 and 5 for study arms C and D and prior to cycles 3, 5, 7, 9 and 11 for study arms A and B. If treatment is held due to toxicity, physical exam and capsule count does not need to be repeated on the day that therapy is resumed.

B Patients will record daily intake of study medication on diary starting when the patient begins taking celecoxib/placebo (no later than cycle 2 day 1 of FOLFOX).

C Prior to cycles 3 and 5 for study arms C and D and prior to cycles 3, 5, 7, 9 and 11 for study arms A and B.

D Only for those patients receiving coumadin/warfarin: PT/INR should be monitored weekly during FOLFOX. During celecoxib monotherapy, PT/INR should be monitored at least every other week for the first month, then as clinically indicated.

E For patients who were randomized to 6 treatments of FOLFOX, the first post-treatment CEA and imaging should occur within 4 months after completion of FOLFOX. For patients who were randomized to 12 treatments of FOLFOX, the first post-treatment CEA and imaging should occur within 6 weeks of FOLFOX (see Section 11.0). Then, for all patients, every 6 months from last scan until at least 3 years after initiation of celecoxib/placebo and then yearly for 3 years, or until disease progression. CEA and scans may be performed +/- 1 month of next testing due date.

F May be obtained 28 days in advance of follow-up appointment

\# Pill counts should include collection and review of monthly diaries as outlined in Section 7.3.

¢ It is not necessary to change the doses of chemotherapy unless the calculated dose changes by ≥10%.

‡ For women of child-bearing potential.

Ψ For those patients who consent to participate in the substudies.

7.0 TREATMENT PLAN

Initiation of treatment must begin within 14 days after randomization. While not strictly an eligibility criterion, it is strongly recommended that patients begin treatment between 21 - 56 days after surgery. For patients who fall outside of this range or for any treatment-related questions, contact the Alliance or SWOG Study Chair.

This is a randomized, double-blind trial. No blinded starter supplies will be available for this study. Initial blinded, patient-specific clinical supplies of celecoxib / placebo will be shipped from the Pharmaceutical Management Branch (PMB) to the registering investigator at the time of patient randomization and should arrive within 7 to 10 days of randomization (see Section 9.4).

One cycle will be defined as 14 days of treatment. Cytotoxic chemotherapy will consist of 6 or 12 cycles of FOLFOX and daily celecoxib/placebo. Celecoxib/placebo will start by Day 1 of cycle 2 of FOLFOX.

7.1 FOLFOX, every 2 weeks.

- **Oxaliplatin** 85 mg/m^2 IV over two hours, **followed by**
- **Leucovorin** 400 mg/m^2 IV over two hours. Alternatively, leucovorin may be administered (via separate infusion containers) concurrently with oxaliplatin, **followed by**
- **5-FU** 400 mg/m^2 IV bolus, then 2400 mg/m^2 continuous IV infusion over 46-48 hours.

Patients receiving oxaliplatin on this study should be counseled to avoid cold drinks, chewing of ice chips, and exposure to cold water or air because the neurotoxicity often seen with oxaliplatin appears to be exacerbated by exposure to cold. The period of time during which the patient is at risk for these cold-induced sensory neuropathies is not well documented. Patients should exercise caution regarding cold exposure during the treatment period. Peripheral sensory neuropathies can occur at any time after receiving oxaliplatin therapy.

Continuous infusion 5-FU should be administered as specified, though early pump shut-offs do not need to be reported on forms unless >10% of dose was not administered.

In the event of a leucovorin shortage, refer to the CALGB 80702 study page, which can be found on the Alliance and CTSU websites.

7.2 Celecoxib or Placebo

Patients will be randomized to celecoxib 400 mg or placebo administered by mouth, once daily. The first dose of celecoxib/placebo will be given by Day 1 of the second cycle of FOLFOX (in clinic). Patients will self-administer celecoxib/placebo at about the same time every day with food. Doses should only be made up if missed within 12 hours of the regularly scheduled dose. Vomited doses should only be made up if the entire capsule can be seen in vomit. Missed doses should be taken with food. Celecoxib/placebo will continue for 3 years from the date of initiation of study drug (i.e. day 1 of celecoxib/placebo) or until progression of disease or unacceptable toxicity.

While celecoxib/placebo does not need to be held around surgical procedures (i.e. port a cath removal or reversal of temporary colostomy), if celecoxib/placebo is held or delayed, treatment should not be held for greater than 28 days. Longer delays require notification of the Alliance or SWOG Study Chair. Repeated delays of > 21 days within 1 year also require notification of Alliance or SWOG Study Chair.

7.3 Adherence

Patients will record study medication (celecoxib or placebo) on diary. Patients will be instructed to bring study medication in original container to clinic visit monthly during FOLFOX treatment, then every 3 months (+/- 1 month) while on study drug treatment. The study team should review the pill diary and perform a pill count. Any extra study medication may be returned to the patient to be finished. A new supply of study medication (200 capsules) will also be dispensed every 6 months while on celecoxib/placebo. Refer to Section 9.4 for drug ordering instruction.

8.0 DOSE MODIFICATIONS AND MANAGEMENT OF TOXICITY

- If a dose reduction beyond Level -3 is required for oxaliplatin, oxaliplatin will be discontinued. Continue celecoxib/placebo and 5-FU/leucovorin.
- If a dose reduction beyond level -3 is required for 5-FU, discontinue FOLFOX. Continue celecoxib/placebo.
- If more than one dose reduction applies, use the most stringent (i.e., the greatest dose reduction.)
- If FOLFOX is delayed due to toxicity for ≥ 4 weeks, counting from the originally scheduled day of treatment that was held, discontinue FOLFOX. Continue celecoxib/placebo.
- If celecoxib/placebo held due to toxicity not deemed related to FOLFOX, continue FOLFOX therapy as scheduled.
- Missed doses of celecoxib/placebo (for any reason) are not made up.

8.1 Dose Levels

Agent	Level 0	Level –1	Level –2	Level –3
Oxaliplatin	85 mg/m^2	65 mg/m^2	50 mg/m^2	40 mg/m^2
5-FU Bolus	400 mg/m^2	320 mg/m^2	270 mg/m^2	230 mg/m^2
5-FU Infusion	2400 mg/m^2 over 46-48 hrs	1920 mg/m^2 over 46-48 hrs	1600 mg/m^2 over 46-48 hrs	1360 mg/m^2 over 46-48 hrs

Leucovorin dose is always 400 mg/m^2. If 5-FU is skipped, leucovorin must also be skipped.

The dose of **celecoxib** is always 400 mg. Celecoxib may be interrupted or discontinued according to the dose modifications provided below, but the dose is not reduced.

8.2 Hematologic toxicities

Dose modifications for hematologic toxicities are based on CBC on Day 1 of each cycle of FOLFOX. CBC may be collected within 72 hours of treatment (or 96 hours if due to holidays).

8.2.1 **For ANC 1000-1199:** Delay FOLFOX until ANC ≥ 1200, then resume at the previous doses of oxaliplatin and 5-FU.

8.2.2 **For ANC < 1000:** Delay FOLFOX until ANC ≥ 1200, then resume with one dose level reduction of oxaliplatin and 5-FU for all subsequent cycles.

8.2.3 **For febrile neutropenia (defined as ANC < 1000 and T ≥ 38.5°C):** Delay FOLFOX until fever has resolved and ANC ≥ 1200, then resume FOLFOX with one dose level reduction of oxaliplatin and 5-FU for all subsequent cycles.

8.2.4 **For platelets 50,000 – 74,999:** Delay FOLFOX until platelets ≥ 75,000, then resume at the previous dose levels of oxaliplatin and 5-FU.

8.2.5 **For platelets < 50,000:** Delay FOLFOX until platelets ≥ 75,000, then resume with one dose level reduction of oxaliplatin and 5-FU for all subsequent cycles.

8.3 Gastrointestinal toxicities

8.3.1 **For ≥ grade 2 diarrhea:** Delay FOLFOX until diarrhea improves to < grade 2. Continue celecoxib/placebo.

- **Following grade 2 diarrhea at any time during a cycle:** Continue FOLFOX at the previous dose levels of oxaliplatin and 5-FU.
- **Following grade 3 diarrhea at any time during a cycle:** Continue FOLFOX with one dose level reduction of 5-FU for all subsequent cycles and the previous dose level of oxaliplatin.
- **Following grade 4 diarrhea at any time during a cycle:** Continue FOLFOX with one dose level reduction of oxaliplatin and 5-FU for all subsequent cycles.

8.3.2 Oral Mucositis

- **For ≥ grade 2 oral mucositis present on Day 1 of a cycle,** delay FOLFOX until mucositis improves to < grade 2. Continue celecoxib/placebo.
- **For grade 2 oral mucositis, at any time during a cycle,** resume/continue FOLFOX at the previous dose level.
- **For grade 3 or 4 oral mucositis, despite optimal management, at any time during a cycle,** resume/continue FOLFOX with one dose level reduction of 5-FU and the previous dose level of oxaliplatin.

8.3.3 **Nausea or Vomiting** Delay FOLFOX until vomiting improves to < grade 2. Continue celecoxib/placebo.

- **For grade ≥ 2 nausea or vomiting present on Day 1 of a cycle despite optimal antiemetic therapy,** delay FOLFOX until nausea and vomiting improve to < grade 2. Continue celecoxib/placebo.
- **For grade 2 nausea or vomiting, at any time during a cycle,** resume/continue FOLFOX at the previous dose levels.
- **For grade 3 nausea or vomiting, despite optimal antiemetic therapy, at any time during a cycle,** resume/continue FOLFOX with one dose level reduction of oxaliplatin and the previous dose level of 5-FU.
- **For grade 4 nausea or vomiting, despite optimal antiemetic therapy, at any time during a cycle, r**esume/continue FOLFOX with one dose level reduction of oxaliplatin and 5-FU.

8.3.4 For upper GI bleeding not associated with thrombocytopenia (platelets < 75,000) or for upper GI ulceration, discontinue celecoxib/placebo. Continue FOLFOX.

8.4 Pulmonary toxicities

For ≥ grade 3 cough, dyspnea, hypoxia, pneumonitis, or pulmonary infiltrates, skip oxaliplatin until interstitial lung disease is ruled out. Continue 5-FU/leucovorin and celecoxib/placebo. Discontinue all protocol therapy if interstitial lung disease is confirmed.

8.5 Thrombotic microangiopathy

For ≥ grade 3 hemolytic uremic syndrome (HUS): Discontinue oxaliplatin. Continue 5-FU/leucovorin and celecoxib/placebo.

8.6 Neurotoxicity

Toxicity Scale for the Sensory Neuropathies Associated with Oxaliplatin (using the Oxaliplatin Specific Neurotoxicity Scale)

	Symptoms
Grade 1	Paresthesias/dysesthesias* of short duration that resolve and do not interfere with function.
Grade 2	Paresthesias/dysesthesias* interfering with function, but not with activities of daily living (ADL)
Grade 3	Paresthesias/dysesthesias* with pain or with functional impairment that also interfere with ADL.
Grade 4	Persistent paresthesias/dysesthesias* that are disabling or life threatening.
	* May be cold-induced

8.6.1 **For grade 2 neurotoxicity persisting between treatments:** Continue FOLFOX with one dose level reduction of oxaliplatin for all subsequent cycles and the previous dose level of 5-FU. Continue celecoxib/placebo.

8.6.2 **For grade 3 neurotoxicity resolving to ≤ grade 2 between treatments:** Continue FOLFOX with one dose level reduction of oxaliplatin for all subsequent cycles and the previous dose level of 5-FU. Continue celecoxib/placebo.

8.6.3 **For grade 3 neurotoxicity persisting between treatments:** Discontinue oxaliplatin. Continue 5-FU/leucovorin and celecoxib/placebo.

8.6.4 **For grade 4 neurotoxicity:** Discontinue oxaliplatin. Continue 5-FU/leucovorin and celecoxib/placebo.

8.6.5 **For pharyngolaryngeal dysesthesia:** Increase the duration of oxaliplatin infusion to 6 hours for all subsequent cycles. See also Section 8.8.1.

8.7 Extravasation

Extravasation of oxaliplatin has been associated with necrosis; if extravasation is suspected, the infusion should be stopped and the drug administered at another site. Extravasation should be treated according to institutional guidelines.

8.8 Allergic Reactions

- **For grade 1 allergic reactions**: Decrease the infusion rate by 50% until symptoms resolve, then resume at the initial planned rate.
- **For grade 2 allergic reactions**: Stop infusion. Administer H_1 and/or H_2 blockers, and/or steroids according to institutional policy. Restart the infusion when symptoms resolve and pretreat before all subsequent doses. Treat according to institutional policy.
- **For grade 3 or grade 4 allergic reactions or anaphylaxis:** Stop the infusion. Discontinue oxaliplatin (if timing consistent with oxaliplatin hypersensitivity). Continue 5-FU and leucovorin and celecoxib/placebo. If grade 3 or 4 allergic reactions or anaphylaxis with first or second cycle, consider leucovorin as offending agent. Discontinue leucovorin and continue FOLFOX (without leucovorin) and celecoxib/placebo.

8.8.1 Oxaliplatin-induced pharyngolaryngeal dysesthesia

Should a patient develop oxaliplatin-induced pharyngolaryngeal dysesthesia, her/his oxygen saturation should be evaluated via a pulse oximeter; if normal, an anxiolytic agent may be given and the patient observed in the clinic until the episode has resolved. Increase the duration of oxaliplatin to 6 hours for all subsequent treatments. Some overlap may exist between the manifestations of pharyngolaryngeal dysesthesia and hypersensitivity reactions. A table comparing the two is presented below.

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

8.9 Cardiovascular toxicities

8.9.1 For grade 3 or 4 cardiac ischemia/infarction: Discontinue all protocol therapy (FOLFOX and celecoxib/placebo).

8.9.2 For grade 3 or 4 cerebrovascular ischemia: Discontinue all protocol therapy (FOLFOX and celecoxib/placebo).

8.10 Venous thrombosis events

For patients who develop venous thrombosis (including deep venous thrombosis or port a cath clots) either during FOLFOX therapy or during celecoxib/placebo only treatment, therapy with either FOLFOX or celecoxib/placebo does not require treatment hold or dose reductions.

8.11 Other non-hematologic toxicities for FOLFOX

For other grade 3 or 4 non-hematologic toxicities considered related to FOLFOX, delay FOLFOX until toxicity resolves to ≤ grade 1, then resume FOLFOX at one dose level reduction of oxaliplatin and 5-FU (if toxicity is hand-foot syndrome or stomatitis, dose reduction of only 5-FU bolus and continuous infusion of 5-FU is permitted). Continue celecoxib/placebo.

8.12 Other non-hematologic toxicities for celecoxib/placebo

For other grade 3 or 4 non-hematologic toxicities considered related to celecoxib/placebo, interrupt celecoxib/placebo for a maximum of 28 days until toxicity improves to ≤ grade 1, then resume celecoxib/placebo at the previous dose.

For recurrence of the same grade 3 or 4 non-hematologic toxicity considered related to celecoxib/placebo, or if toxicity does not improve after 28 days, discontinue celecoxib/placebo. Continue FOLFOX.

For persistent grade 2 non-hematologic toxicity considered related to celecoxib/placebo that the patient finds unacceptable, interrupt celecoxib/placebo for a maximum of 28 days until toxicity improves to ≤ grade 1, then resume celecoxib/placebo at the previous dose. Continue FOLFOX.

For recurrence of unacceptable grade 2 non-hematologic toxicity considered related to celecoxib/placebo, or if grade 2 toxicity does not improve after 28 days, discontinue celecoxib/placebo. Continue FOLFOX.

8.13 Dose modifications for obese patients

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 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 calculating doses based on actual body weight should recognize that doing otherwise would be a protocol violation.

The actual weight on the day of registration or the first day of treatment may be used for cycle 1 unless the change in the weight results in a change in calculated dose ≥10%, in which case the weight on the day of treatment should be used. Over the course of treatment it is not required to change the doses of 5-FU, leucovorin or oxaliplatin due to changes in weight unless the calculated dose changes by ≥10%.

9.0 DRUG FORMULATION, AVAILABILITY, AND PREPARATION

Qualified personnel who are familiar with procedures that minimize undue exposure to themselves and to the environment should undertake the preparation, handling, and safe disposal of chemotherapeutic agents in a self-contained, protective environment.

Discard unused portions of injectable chemotherapeutic agents that do not contain a bacteriostatic agent or are prepared with unpreserved diluents (i.e., Sterile Water for Injection USP or 0.9% Sodium Chloride for Injection USP) within eight hours of vial entry to minimize the risk of bacterial contamination.

For US sites, the total administered dose of cytotoxic chemotherapy may be rounded up or down within a range of 5% of the actual calculated dose.

For Canadian sites, the total administered dose of cytotoxic chemotherapy may be rounded up or down within a range of 5% of the actual calculated dose for oxaliplatin and leucovorin, and 7% for 5FU.

It is not necessary to change the doses of 5-FU, leucovorin or oxaliplatin due to changes in weight unless the calculated dose changes by ≥10%.

9.1 Oxaliplatin [Eloxatin] (NSC #266046)

Availability

Oxaliplatin is commercially available as an aqueous solution in vials containing 50 mg and 100 mg at a concentration of 5 mg/mL. The vials do not contain any preservative and they are intended for single use.

Storage and Stability

Intact vials should be stored at room temperature. Solutions diluted in D5W are stable for 6 hours at room temperature or 24 hours under refrigeration.

Preparation

The calculated dose of oxaliplatin should be diluted for infusion with 250 mL to 500 mL D5W. Oxaliplatin should not be diluted with a sodium chloride solution. Needles, syringes, catheters or IV administration sets containing aluminum should not be used with oxaliplatin. As with other platinum compounds, contact with aluminum may result in a black precipitate.

Administration

Oxaliplatin will be administered by intravenous infusion over 120 minutes prior to or concurrent with leucovorin. Infusion time may be prolonged (up to 6 hours) in patients experiencing pharyngolaryngeal dysesthesia.

Oxaliplatin is unstable in the presence of chloride or alkaline solutions. **Do NOT** mix or administer oxaliplatin with saline or other chloride-containing solutions. **Do NOT** administer other drugs or solutions in the same infusion line. Flush IV lines/catheters with Dextrose 5% in Water both before and after oxaliplatin administration.

Toxicity

The most commonly observed oxaliplatin toxicities include neurotoxicity, GI toxicity, and myelosuppression. Three neurotoxicity syndromes have been seen: acute sensory neuropathy develops within hours to 2 days after oxaliplatin administration. Symptoms include, paresthesias, dysesthesias, and hypothesia of the hands, feet and perioral region. Jaw spasm, abnormal tongue sensation, dysarthria, eye pain and a sensation of chest pressure have also been noted. Acute sensory neuropathy symptoms may be exacerbated by exposure to cold temperature or cold objects. Symptoms are reversible, usually resolving within 14 days and commonly recurring with further dosing. This syndrome has been observed in about 56% of patients receiving oxaliplatin with 5-FU and leucovorin.

Acute pharyngolaryngeal dysesthesia is reported to occur in 1-2% of patients. This syndrome is characterized by a subjective sensation of difficulty breathing or swallowing without laryngospasm or bronchospasm or objective evidence of hypoxia. Avoidance of cold drinks, food and air is suggested in order to minimize pharyngolaryngeal dysesthesia. Antianxiety agents (e.g., lorazepam) may be used to treat pharyngolaryngeal dysesthesias once oxygen saturation has been documented to be normal.

Peripheral neuropathy persisting > 14 days is characterized by paresthesias, dysesthesias, and hypothesia. Abnormalities in proprioception may also be seen. Symptoms of persistent neuropathy may improve upon discontinuation of oxaliplatin.

Various agents have been used in an attempt to minimize neurotoxicity of oxaliplatin (e.g. carbamazepine, Mg+, Ca++). Calcium and magnesium infusions appear to be beneficial in preventing neurotoxicity. Contrary to preliminary findings described in 2007, calcium and magnesium do not appear to interfere with tumor response to FOLFOX. Calcium and magnesium infusions are generally given before and after oxaliplatin, and should not be prepared in the same infusion solution as FOLFOX components.

Gastrointestinal toxicities include nausea, vomiting (oxaliplatin is considered to be moderately emetogenic) and diarrhea.

Neutropenia is reported in 73% of patients receiving oxaliplatin with 5-FU and leucovorin (44% grade 3 or 4). Grade 3 or 4 thrombocytopenia is reported to occur in 4% of patients receiving the combination.

Allergic reactions, similar to those seen with other platinum compounds, have also been observed in patients treated with oxaliplatin. Reactions range from rash to anaphylaxis.

Rarely, oxaliplatin has been associated with pulmonary fibrosis, which may be fatal. Oxaliplatin should be discontinued in the presence of unexplained pulmonary symptoms (e.g. nonproductive cough, dysphagia) or pulmonary infiltrates until interstitial lung disease or pulmonary fibrosis have been ruled out.

Recent reports of oxaliplatin extravasation suggest that tissue necrosis may result and that oxaliplatin should be considered a vesicant. No standard treatment exists for oxaliplatin extravasation although heat and sodium thiosulfate have both been suggested.

Veno-occlusive disease (VOD) of the liver is a rare complication associated with oxaliplatin and 5-FU. Clinical manifestations of VOD include hepatomegaly, ascites, and jaundice. Histologically, VOD is characterized by diffuse damage in the centrilobular zone of the liver. Sequelae of VOD include hepatomegaly, splenomegaly, portal hypertension, and esophageal varices. A recent analysis of resected liver metastases in 153 patients indicated histological findings consistent with VOD in 6/27 patients who received 5-FU alone, 4/17 patients who received 5-FU and irinotecan, 20/27 patients who received 5-FU and oxaliplatin, and 14/16 who received 5-FU, oxaliplatin and irinotecan. The remaining 66 patients had not received chemotherapy prior to resection. There were no such findings in these patients.

For more information on toxicities associated with oxaliplatin, please see the package insert.

9.2 5-Fluorouracil (5-FU; fluorouracil)

Please refer to the package insert for complete product information.

Availability

5-FU is commercially available as a 50 mg/mL solution for injection in 10 mL, 20 mL, 50 mL and 100 mL vials.

Preparation

Inspect for precipitate; if found, agitate or gently heat in water bath. Bolus injections are prepared using undiluted drug.

46-48 hour infusion of 5-FU should be prepared for administration via ambulatory infusion pump according to the individual institution's standards. These solutions may be prepared in D5W or 0.9% NaCl. 5-FU should not be mixed in the same solution with most parenteral antiemetics.

Storage and Stability

Intact vials should be stored at room temperature and protected from light. Slight yellow discolor does not usually indicate decomposition. Stability in ambulatory pumps varies according to the pump, manufacturer of drug, concentration and diluent. Please refer to appropriate reference sources for additional information.

Administration

In this study, 5-FU is administered as a 400 mg/m^2 IV bolus followed by 2400 mg/m^2 by IV infusion over 46 to 48 hours. The bolus is administered after leucovorin, and the 46-48 hour infusion follows immediately after the bolus.

Toxicity

Nausea, diarrhea, vomiting (mild); stomatitis: 5-8 days after treatment initiation; myelosuppression: granulocytopenia (9-14 days); thrombocytopenia (7-14 days); Alopecia; loss of nails; hyperpigmentation; photosensitivity; maculopapular rash; palmar–plantar erythrodysethesias: (42-82% receiving continuous infusion); CNS effects: cerebral ataxia (rare); cardiotoxicity: MI, angina; asymptomatic S–T changes 68%; ocular effects: excessive lacrimation and less commonly, tear duct stenosis.

Drug Interactions

Leucovorin enhances the cytotoxicity of 5-FU by forming a more stable tertiary complex with thymidylate synthase. Concomitant administration of 5-FU with warfarin has been reported to result in increased INR/prolonged prothrombin time. Patients receiving both drugs should be followed with weekly INRs.

9.3 Leucovorin Calcium (Folinic Acid) (calcium folinate; citrovorum factor; N 5-formyltetrahydrofolate; 5-formyl-FH4; folinic acid)

Please refer to the package insert for complete product information.

Availability

Leucovorin calcium is commercially available in: 50 mg, 100 mg, 200 mg, 350 mg and 500 mg vials for reconstitution, and as a solution for injection in 50 mL vials at a concentration of 10 mg/mL.

In the event of a leucovorin shortage, refer to the CALGB 80702 study page, which can be found on the Alliance and CTSU websites.

Storage and Stability

Intact vials should be stored at room temperature and protected from light. Solutions reconstituted with BWI are stable for at least 7 days at room temperature. Solutions diluted for infusion are stable for 24 hours at room temperature and 4 days under refrigeration.

Preparation

Leucovorin may be reconstituted with Bacteriostatic Water for Injection (BWI), Sterile Water for Injection, or bacteriostatic NaCl or NaCl. Solutions should be further diluted in D5W, 0.9% NaCl or Ringers solution for infusion over two hours.

Administration

Leucovorin will be administered as a 400 mg/m^2 IV infusion over 2 hours after oxaliplatin administration and immediately before 5-FU. Leucovorin may also be administered concurrently with oxaliplatin as a separate IV infusion.

Toxicity

The only adverse reactions associated with leucovorin are allergic reactions. These are extremely uncommon.

9.4 Celecoxib (Celebrex) / Placebo (NSC #719627, Alliance IND #107051)

Availability

Celecoxib (NSC 719627) and matching Placebo will be provided free of charge by Pfizer and distributed by the Pharmaceutical Management Branch (PMB), Cancer Therapy Evaluation Program (CTEP), Division of Cancer Treatment and Diagnosis (DCTD), National Cancer Institute (NCI).

Celecoxib and matching Placebo will be supplied in bottles containing 100 – 400mg capsules (Celecoxib) or 100 – 0mg capsules (Placebo) with a child-resistant cap and a tamper-evident seal. Each blinded, patient-specific bottle will be labeled with:

- The protocol number (i.e., “CALGB-80702”)
- The bottle number (i.e., “Bottle 1 of 2”, “Bottle 2 of 2”)
- The number of capsules (i.e., “100 capsules”)
- The patient ID number (e.g., “999999", where “999999” represents a unique patient identifier assigned at registration)

- The patient initials (i.e., last initial, first initial, middle initial [e.g., "L, FM"])
- The agent identification (i.e., “Celecoxib 400 mg or Placebo”)
- A blank line for the pharmacist to enter the patient’s name
- Administration instructions (i.e., “Take one capsule once daily with food.”)
- Storage instructions (i.e., “Store at room temperature (15ºC to 25ºC, 59ºF to 77ºF).”)
- Emergency contact instructions
- A Julian date

The Julian date indicates the day the bottle was labeled and shipped and is composed of the last two digits of the calendar year (e.g., 2009 = 09, 2010 = 10) and a day count (e.g., January 1 = 001, December 31 = 365). For example, a bottle labeled and shipped on January 1, 2009 would have a Julian date of ‘09001’ and a bottle labeled and shipped on December 31, 2010 would have a Julian date of ‘10365’. The Julian date will be used by PMB for recalls. When a lot expires, PMB will determine the last date the expired lot was shipped and will recall all bottles (i.e., both Celecoxib and Placebo) shipped on or before that date thus eliminating any chance of breaking the blind.

The Alliance for Clinical Trials in Oncology holds the IND (#107051) for celecoxib/placebo for this trial. As such, the Alliance follows NCI CTMB policies and guidelines regarding drug distribution, repackaging and shipment of the drug.

Questions about drug orders, transfers, returns, or accountability should be addressed to the PMB by calling [redacted] Monday through Friday between 8:30AM and 4:30PM Eastern Time or by emailing [redacted] anytime.

Drug Ordering

No blinded starter supplies will be available for this study. Blinded, patient specific clinical supplies will be sent to the registering investigator at the time of randomization and should arrive within approximately 7 to 10 days. This randomization will be performed by the Alliance Statistics and Data Center. The assigned patient study ID number must be recorded by the registering institution for proper bottle dispersion. Once a patient has been registered, the Alliance Statistics and Data Center will electronically transmit a clinical drug request for that patient to the PMB. This request will be entered and transmitted by the Alliance Statistics and Data Center the day the patient is registered and will be processed by the PMB the next business day and shipped the following business day. Shipments within the United States will be sent by FedEx Ground (generally 5 business day delivery) and shipments to Canada will be sent by FedEx (generally one to two day delivery). Thus, if a patient is registered on Monday, the Alliance would enter a clinical drug request for that patient on Monday and PMB would process that request on Tuesday and ship the drug on Wednesday. United States sites could expect to receive their order approximately Tuesday or Wednesday of the following week and Canadian sites could expect to receive their order either Thursday or Friday. Shipments to United States sites can be expedited (i.e., receipt on Thursday in example above) by the provision of an express courier account name and number to the Alliance Statistics and Data Center at the time the patient is randomized.

The initial request will be for **2 – 100 capsule bottles** (a 6 month supply at a dose of one capsule once daily) of Celecoxib or matching Placebo. After five months (one month before needed), sites may reorder an additional **2 – 100 capsule bottles** (a 6 month supply at a dose of one capsule once daily) using the PMB Online Agent Order Processing (OAOP) application [redacted] Access to OAOP requires the establishment of a CTEP Identity and Access Management (IAM) account [redacted] and the maintenance of an “active” account status and a “current”

password. The protocol number (i.e., CALGB-80702), the assigned patient ID number (e.g., "999999"), the patient initials (e.g., "L, FM"), and the number of bottles remaining from the previous shipment should be entered on each order. **Please note that a maximum of six shipments (12 bottles of 100 capsules), a sufficient quantity to complete three years of therapy, will be provided for each patient.**

All drug orders will be shipped directly to the registering physician at the shipping address listed on their most recent Supplemental Investigator Data Form (IDF) on file with CTEP. The registering investigator must maintain an active investigator status with CTEP, DCTD through the annual submission of an FDA Form 1572 (Statement of Investigator), a Curriculum Vitae, a Supplemental Investigator Data Form (IDF), and a Financial Disclosure Form (FDF) [redacted]

CALGB-80702 Shipment Schedule

Patient Randomized with Alliance	Initial e-Order Transmitted by Alliance	Initial e-Order Received and Approved by PMB	Initial Order Shipped by PMB	Initial Oder Received at Site**
Monday	Monday	Tuesday	Wednesday	Tuesday
Tuesday	Tuesday	Wednesday	Thursday	Wednesday
Wednesday	Wednesday	Thursday	Friday	Thursday
Thursday	Thursday	Friday	Monday	Friday
Friday	Friday	Monday	Tuesday	Monday

**** Arrival time approximate / shipments sent by FedEx Ground**

How Supplied

"Celecoxib" and matching "Placebo" are supplied as a size 0 white to off-white opaque hard gelatin capsule for oral administration. Each tamper-evident, child-resistant, 180ml, square, white, opaque, high-density polyethylene (HDPE) bottle contains 100 capsules. For "Celecoxib", each capsule contains 400mg of celecoxib with croscarmellose sodium, edible inks, gelatin, lactose monohydrate, magnesium stearate, povidone, and sodium lauryl sulfate. For "Placebo", each capsule contains croscarmellose sodium, edible inks, gelatin, lactose monohydrate, magnesium stearate, povidone, and sodium lauryl sulfate.

Storage and Stability

"Celecoxib" and matching "Placebo" are shipped at room temperature by US Priority Mail. The capsules should be stored at controlled room temperature (15ºC to 25ºC, 59ºF to 77ºF). The intact bottles of 100 capsules are stable for five years from date of manufacture when stored at controlled room temperature.

Route of Administration

Oral. Celecoxib/placebo at doses of 400mg orally once daily should be administered with food to improve absorption.

Drug Transfers

Bottles may NOT be transferred from one patient to another patient or from one protocol to another protocol. All other transfers (e.g., a patient moves from one participating clinical site to another participating clinical site, the registering investigator for a patient changes) must be approved in advance by the PMB. To obtain an approval for transfer, investigators should complete and submit to the PMB (fax number [redacted]) a Transfer Investigational Agent Form available on the CTEP home page [redacted]. The patient ID number (e.g., "999999"') and the patient initials (e.g., "L,FM") must be entered in the "Received on NCI Protocol No." and the "Transferred to NCI Protocol No." fields in addition to the protocol

number (i.e., "CALGB-80702"). The Julian date / order number (e.g., 10365-9999) should be entered in the "Lot Number" field.

Drug Returns

Only undispensed clinical supplies should be returned to the PMB. When it is necessary to return study drug (e.g., sealed bottles remaining when a patient permanently discontinues protocol treatment, expired bottles recalled by the PMB), investigators should return the study drug to the PMB using the NCI Return Drug List available on the CTEP home page [redacted]. The patient ID number (e.g., "999999"), the patient initials (e.g., "L, FM"), and the Julian date / order number (e.g., 10365-9999) should be entered in the "Lot Number" field. A separate line item is required for each patient ID number (e.g., "999999") being returned. **Dispensed bottles with remaining tablets should be documented in the patient-specific NCI Investigational Agent Accountability Record (i.e., logged is as "returned by patient" and logged out as "destroyed on site") and destroyed on site in accordance with institutional policy.**

Drug Accountability

The investigator, or a responsible party designated by the investigator, must maintain a careful record of the receipt, disposition, and return of all drugs received from the PMB using the NCI Investigational Agent Accountability Record available on the CTEP home page [redacted]. A separate **Oral** NCI Investigational Agent Accountability Record must be maintained for each patient ID number (e.g., "999999") on this protocol. The combination Julian date / order number in the upper right hand corner of the patient-specific bottle label (e.g., 10365-9999) should be recorded as the lot number.

Unblinding Procedures

Unblinding can be done only in the case of an emergency. Follow the directions below to unblind patient treatment. Please note that, if treatment is unblinded due to an emergency, the patient must permanently discontinue all protocol therapy.

Emergency Unblinding Procedures

Examples of emergencies include …

1) a life threatening unexpected adverse event that is at least possibly related to the investigational agent and for which unblinding would influence treatment decisions

OR

2) a medication error, such as an accidental overdose.

Expected adverse events are listed in the "Toxicities" section below.

Contact the Alliance Executive Office on call by calling 773-702-6800, pressing 1 to speak with an operator, and then asking for pager ID 8625 to return the call.

The institution must provide the following information to the Alliance Executive Officer:

- CALGB study ID (i.e., "CALGB-80702")
- Patient study ID number (e.g., "999999")
- Patient initials (e.g., "L,FM")
- Institution name
- Name and telephone number of treating physician
- Name and contact information of person requesting the unblinding procedure
- Name and contact information of person to inform of treatment assignment
- Reason for emergency unblinding

Please remember that emergency unblinding request may be authorized only by an Alliance Executive Officer, and emergency unblinding applies only if unblinding would influence management of the medical situation.

After authorization by a designated Alliance Approving Physician, the treatment assignment will be provided to the contact person.

Toxicities

Celecoxib/placebo is generally well tolerated. The most common toxicity reported in arthritis trials is a headache. Other possible toxicities include peripheral edema, insomnia, dizziness, skin rash, dyspepsia, diarrhea, abdominal pain, nausea, flatulence, back pain, upper respiratory tract infection, sinusitis, pharyngitis, and rhinitis.

The incidence of GI ulcers documented by endoscopy in arthritis trials is 7%. GI bleeding is more likely to occur in patients with a history of peptic ulcer disease and/or GI bleeding. The risk of GI ulceration, bleeding or perforation with celecoxib is increased with concomitant use of aspirin. Chronic use of aspirin (>100 mg/day) or other NSAIDs is not allowed on this trial (see Section 4.2).

As is the case with non-selective NSAIDs, celecoxib may be associated with nephrotoxicity in patients in whom renal prostaglandins are important in maintenance of renal blood flow. Specifically, patients with pre-existing renal dysfunction, heart failure, liver dysfunction or dehydration, elderly patients, or patients taking diuretics or ACE inhibitors are at the greatest risk for significant inhibition of renal blood flow and nephrotoxicity. In addition, long-term use of NSAIDs has been associated with renal injury, including renal papillary necrosis. Unlike other non-selective NSAIDs, celecoxib does not appear to inhibit platelet aggregation.

A safety analysis performed in December, 2004, of several long-term celecoxib trials was conducted following the removal of rofecoxib from the market because of an increased risk of adverse cardiovascular events. The celecoxib analysis resulted in the suspension of drug use for patients enrolled in the Adenoma Prevention with Celecoxib (APC) trial. In this trial, the risk of cardiovascular death, myocardial infection or stroke in the celecoxib groups was 2-3 times higher than the risk in the placebo group. This increased hazard with celecoxib was not observed in another long-term trial.

Drug Interactions

Celecoxib is metabolized in the liver by cytochrome P450 2C9. Drugs that inhibit (e.g., fluconazole) or induce (e.g., rifampin) the 2C9 isoenzyme might be expected to result in increased toxicity or decreased effect of celecoxib, respectively. In addition, celecoxib is reported to inhibit the 2D6 isoenzyme, potentially enhancing the effects of drugs metabolized by this isoenzyme.

The following describes possible drug interactions:

ACE-inhibitors: NSAIDs may diminish the antihypertensive effect of ACE-inhibitors.

Coumadin: Celecoxib does not alter PT/INR; there have been reports of prolonged prothrombin time and bleeding in elderly patients taking both coumadin and celecoxib.

Fluconazole: Concomitant administration of celecoxib and fluconazole results in a two-fold increase in celecoxib levels.

Lithium: Concomitant administration of celecoxib and lithium results in an increase in steady-state lithium levels.

10.0 ANCILLARY THERAPY

10.1 Supportive Care

Patients should receive *full supportive care*, including transfusions of blood and blood products, antibiotics, antiemetics, etc., when appropriate. The use of epoetin products is not allowed. The reason(s) for treatment, dosage, and the dates of treatment should be recorded on Form C-1954 via Medidata Rave®.

10.2 Hormonal/other chemotherapeutic agents

Treatment with *hormones or other chemotherapeutic* agents for cancer treatment may not be administered. Exceptions allowing usage of hormones and other chemotherapeutic agents include, but not limited to, steroid given for adrenal failure, hypersensitivity reactions or other non-cancer related conditions; hormones administered for non-disease-related conditions (e.g., but not limited to, insulin for diabetes); intermittent use of dexamethasone as an antiemetic; or methotrexate or other DMARDs (disease-modifying anti-rheumatic drugs) used for rheumatological conditions."

10.3 Loperamide

For symptoms of diarrhea and/or abdominal cramping that occur at any time during a treatment cycle, patients will be instructed to begin taking loperamide. Loperamide should be started at the earliest sign of (1) a poorly formed or loose stool or (2) the occurrence of 1 to 2 more bowel movements than usual in 1 day or (3) an increase in stool volume or liquidity. Loperamide should be taken in the following manner: 4 mg at the first onset of diarrhea, then 2 mg every 2 hours around the clock until diarrhea-free for at least 12 hours. Patients may take loperamide 4 mg every 4 hours during the night. The maximum daily dose of loperamide is 16 mg/day. Patients should be advised to obtain loperamide at the initial treatment visit so that they have sufficient supply on hand in case antidiarrheal support is required. Additional antidiarrheal measures may be used at the discretion of the treating physician. Patients should be instructed to increase fluid intake to help maintain fluid and electrolyte balance during episodes of diarrhea.

10.4 Anticoagulants

Patients who are taking warfarin or coumadin may participate in this study. For patients receiving coumadin or warfarin, PT/INR should be monitored weekly during FOLFOX. During celecoxib/placebo monotherapy, PT/INR should be monitored at least every other week for the first month, then as clinically indicated. Subcutaneous or low molecular weight heparin is permitted.

10.5 Non-steroidal anti-inflammatory drugs (NSAIDs) and aspirin

While on study, patients should be instructed to avoid NSAIDs or aspirin beyond what is specified in the eligibility criteria (any dose of an NSAID more than 2 times a week on average or aspirin at more than 325 mg at least three times per week on average. Low-dose aspirin not exceeding 100 mg/day is permitted). For a patient requiring acute treatment with NSAIDs or aspirin, the patient should restrict usage to no greater than 7 days of continuous usage, no more frequently than every 3 months. Usage with celecoxib/placebo or other COX-2 inhibitors outside of the study medication is not permitted at any time

10.6 Use of Growth Factors

Growth factor support (G-CSF, GM-CSF, or pegfilgrastim) may be considered for neutropenia at the discretion of the treating physician. Blood products and growth factors should be utilized as clinically warranted and following institutional policies and recommendations. The use of growth factors should follow published guidelines of the American Society of Clinical Oncology 2006 Update of Recommendations for the Use of White Blood Cell Growth Factors: An Evidence-Based, Clinical Practice Guideline. J Clin Oncol 24(19): 3187-3205, 2006.

11.0 CRITERIA FOR RESPONSE, PROGRESSION, AND RELAPSE

To minimize potential bias of differential post-treatment surveillance between the two arms, CEA testing and imaging requires standardization. For patients who have signs or symptoms in which imaging is clinically indicated, the decision of timing of radiographic studies remains at the discretion of the treating clinician. However, for asymptomatic surveillance after the completion of FOLFOX, the timing of CEA testing and radiograph imaging must be similar. As such, for patients randomized to 6 treatments of FOLFOX, the first post-treatment CEA and imaging should occur approximately 4 months after completion of FOLFOX. For patients randomized to 12 treatments of FOLFOX, the first post-treatment CEA and imaging should occur within 6 weeks of completion of FOLFOX. CEA and scan may be performed +/- 1 month of next testing due date.

At the time of each evaluation, patients will be classified in the following manner:

No evidence of disease (NED)

OR

Recurrence of disease (REC): Recurrence must be confirmed by imaging and/or biopsy, with the colonoscopy and pathology reports submitted. Elevated CEA levels only or physical findings only will not be accepted as evidence of recurrence.

12.0 REMOVAL OF PATIENTS FROM PROTOCOL THERAPY

12.1 Duration of Treatment

Patients with documented disease progression at any time during therapy will be removed from protocol treatment.

12.2 Extraordinary Medical Circumstances

If at any time, the constraints of this protocol are detrimental to the patient's health and/or patient no longer wishes to continue protocol therapy, protocol therapy should be discontinued and the study chair and Alliance should be notified. The reason for discontinuation needs to be documented and patient should be asked if he/she can still be followed for recurrence and survival.

13.0 STATISTICAL CONSIDERATIONS

13.1 Sample Size and Power Estimates

The primary endpoint of this trial is DFS measured from study entry (time of randomization) until documented progression or death from any cause. The superiority hypothesis of celecoxib use will be tested in this patient population using the DFS endpoint. Non-exponential survival is expected. Based on the findings from NSABP C07 and the MOSAIC trials, the DFS distribution under the null hypothesis assumes probabilities of failure by year for years 1 through 6 to be 0.10, 0.12, 0.06, 0.05, 0.03 and 0.02, respectively. It is anticipated that 2,500 patients will be enrolled in 3.125 years (800 patients per year) with a follow-up period of 3 years.

The number of expected DFS events at the conclusion of the trial (n=775) is estimated using the method proposed by Schoenfeld. A hazard ratio of 0.79 in favor of celecoxib is assumed; this corresponds to an increase in the probability of being disease-free at 3 years from 0.72 to 0.77. With 775 events observed, this difference can be detected with power of approximately 0.91 (2-sided α=0.05). The stratified log rank test will be used to test the primary hypothesis, with stratification on the number of lymph nodes (1-3; >=4) and aspirin use (no; yes).

Overall survival (OS) measured from study entry (time of randomization) until death from any cause will be studied as a secondary endpoint.

In addition, a prospective international effort is underway to pool patient level data from multiple trials that will test the duration assumption (the IDEA trial; see Appendix VI). Our goal is for the United States Intergroup to contribute 2500 patients with stage III colon cancer to this effort. Data provided to the IDEA trial will be fully de-identified. Alliance Statistics and Data Center at Duke University will maintain the data link between the coded patient numbers provided in the de-identified dataset and the patient study ID numbers. Any interim transfer of data must be approved by the Alliance Data and Safety Monitoring Board.

The ACCENT collaboration reported a 3-year DFS rate of 74.7% in resected stage III colon cancer patients treated since 2004 with fluorouracil, leucovorin, and oxaliplatin[323]. In a prospectively-designed, pooled analysis of six of the most recent large, randomized adjuvant chemotherapy trials conducted between 2007 and 2015, a 3-year DFS rate of 76.0% was reported in stage III patients who received 6 months of FOLFOX (IDEA).[324] Both large-scale, pooled analyses showed improved 3-year DFS rates compared to the rates reported in the original NSABP C-07 and MOSAIC trials: 3-year DFS rate of 72.0%. The current trial, CALGB 80702, enrolled fewer patients with T4 tumor (14.7%) compared to the MOSAIC trial (19.0%).

As a result, it became clear that the originally planned number of events required (775) would not be achievable unless follow-up was extended by multiple more years and that future events would predominantly be deaths from non-colorectal causes. Based on the event rate observed during the most recent 12 months, the trial is projected to observe 775 DFS events between December 2021 and March 2022. To evaluate the possibilities of reporting the trial results in a timely fashion without substantially compromising power, the power calculation was updated by an independent statistician who was blinded to trial data. A reduced number of DFS events, 696, is estimated to provide power of 85% (reduced from 90%) to detect a HR of 0.79 in favor of celecoxib (3-year DFS rate from 0.75 to 0.796 at overall 2-sided α = 0.05). This calculation accounted for the nine previously conducted interim analyses (04/2014, 09/2014, 04/2015, 11/2015, 04/2016, 07/2016, 05/2017, 10/2017, 09/2018), and neither the efficacy nor futility boundary was crossed in any of these interim analyses. At the conclusion of the trial with at least 696 DFS events, if 2-sided stratified log-rank p-value is less than 0.038, then an efficacy in favor of celecoxib can be concluded.

13.2 Interim Monitoring

Formal interim analyses of the primary endpoint, DFS, will begin when approximately 20% ($\geq$ 155/775) of the total expected failures has occurred. Subsequently, interim analyses will be conducted every 6 months to coincide with scheduled meetings of the Alliance DSMB. Three interim analyses are expected during the accrual period and five during the follow-up period. The 2-sided, α=0.05 Lan-DeMets analogue of the O'Brien-Fleming boundaries, will be used to test for efficacy for the celecoxib hypothesis at each interim. Interim analyses are expected when approximately 20%, 29%, 40%, 52%, 64%, 74%, 83%, and 90% of data are available. A futility analysis will also be conducted for the celecoxib hypothesis at each interim analysis. If the adjusted lower confidence bound for the DFS hazard ratio at an interim analysis is greater than 0.79, consideration will be given to curtail further accrual or follow-up. The Alliance Statistics

and Data Center at Duke University will submit quarterly reports to CTEP by electronic means using the Clinical Data Update System (CDUS).

14.0 ADVERSE EVENT REPORTING (AER)

Investigators are required by Federal Regulations to report serious adverse events as defined below (in both the table and text). Alliance investigators are required to notify the Investigational Drug Branch (IDB), the Alliance Central Office, the Study Chair, and their Institutional Review Board if a patient has a reportable serious adverse event. The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 will be utilized until March 31, 2018 for AE reporting. However, CTCAE version 5.0 must be used for serious AE reporting through CTEP-AERS as of April 1, 2018. All appropriate treatment areas should have access to a copy of the CTCAE version 5.0. A copy of the CTCAE version 5.0 can be downloaded from the CTEP web site [redacted]

All reactions determined to be "reportable" in an expedited manner must be reported using the NCI CTEP Adverse Event Reporting System (CTEP-AERS).

Please note: Adverse event reporting on the CALGB 80702 study forms uses CTCAE version 3.0. However, adverse events that require reporting via CTEP-AERS utilize CTCAE version 5.0.

14.1 CALGB/SWOG C80702 Reporting Requirements for <u>all arms of the study</u>:

Phase 2 and 3 Trials: CTEP-AERS Expedited Reporting Requirements for Adverse Events That Occur Within 30 Days[1] of the Last Dose of Treatment Under a CTEP IND or Alliance IND:

	Grade 1	Grade 2	Grade 2	Grade 3		Grade 3		Grades 4 & 5	Grades 4 & 5
				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	Not Required	Not Required	Not Required	Not Required	10 Calendar Days	10 Calendar Days
Possible Probable Definite	Not Required	Not Required	Not Required	Not Required	Not Required	Not Required	Not Required	10 Calendar Days	10 Calendar Days

[1] **Adverse events with attribution of possible, probable, or definite that occur <u>greater</u> than 30 days after the last dose of treatment with an agent under a CTEP IND or Alliance IND require reporting as follows:**
CTEP-AERS 10 calendar day report:
- Grade 4 unexpected events
- Grade 5 expected or unexpected events

March 2005

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:
  - "10 calendar days" - A complete CTEP-AERS report on the AE must be submitted within <u>10 calendar days</u> of the investigator learning of the event.
- Any event that results in persistent or significant disabilities/incapacities, congenital anomalies, or birth defects must be reported via CTEP-AERS if the event occurs following treatment with an agent under a CTEP IND or Alliance IND.
- Use the NCI protocol number and the protocol-specific patient ID provided during trial registration on all reports.

14.2 Additional Instructions or Exclusions to CTEP-AERS Expedited Reporting Requirements for Phase 2 and 3 Trials Utilizing an Agent Under a CTEP IND or Alliance IND:

- CALGB/SWOG C80702 uses a drug under an Alliance IND. The reporting requirements in the table and text should be followed for both arms in this trial.
- All grade 4 events that are unexpected and that are at least possibly related to treatment must be reported via CTEP-AERS within 10 calendar days.
- Grade 4 events that are expected with chemotherapy (FOLFOX) that occur during FOLFOX chemotherapy do not require CTEP-AERS expedited reporting, even if they result in hospitalization.
- Treatment expected adverse events include those listed in Section 9.0; in the package inserts for: 5-FU, leucovorin, oxaliplatin; and the Investigator's Brochure for celecoxib/placebo. Please see below for examples of expected events during FOLFOX chemotherapy:
  - Nausea or vomiting
  - Diarrhea
  - Mucositis
  - Hand foot syndrome
  - Neuropathy
  - Acute pharyngolaryngeal dysesthesia
  - Allergic reaction
  - Dehydration
  - Fatigue
  - Alopecia
  - Hematosuppression (leukopenia, neutropenia, lymphopenia, anemia, and thrombocytopenia)
- Grade 4 events that are expected with chemotherapy (FOLFOX) but unexpected with celecoxib/placebo and that OCCUR DURING CELECOXIB/PLACEBO MONOTHERAPY must be reported via CTEP-AERS within 10 calendar days.
- GRADES 2-5 CARDIOVASCULAR EVENTS (CARDIAC ISCHEMIA, CEREBROVASCULAR ISCHEMIA, VENOUS THROMBOMBOLIC EVENTS, LEFT VENTRICULAR SYSTOLIC DYSFUNCTION, HEART FAILURE) THAT OCCUR WITHIN 30 DAYS OF THE LAST DOSE OF ANY/ALL PROTOCOL TREATMENT MUST BE REPORTED VIA CTEP-AERS WITHIN 10 CALENDAR DAYS.
- All adverse events reported via CTEP-AERS (i.e., serious adverse events) should also be forwarded to your local IRB.
- The reporting of adverse events described above is in addition to and does not supplant the reporting of adverse events as part of the report of the results of the clinical trial (e.g., study forms).
- New primary malignancies should be reported using study form C-1001 via Medidata Rave®. New malignancies, including secondary AML/MDS, should also be reported.
- All new malignancies must be reported via CTEP-AERS whether or not they are thought to be related to either previous or current treatment. All new malignancies should be reported, i.e. solid tumors (including non-melanoma skin malignancies), hematologic malignancies, myelodysplastic syndrome/acute myelogenous leukemia, and in situ tumors.

Secondary Malignancy:

A *secondary malignancy* is a cancer caused by treatment for a previous malignancy (*e.g.*, treatment with investigational agent/intervention, radiation or chemotherapy). A secondary malignancy is not considered a metastasis of the initial neoplasm.

CTEP requires all secondary malignancies that occur following treatment with an agent under an NCI IND/IDE be reported via CTEP-AERS. Three options are available to describe the event:

- Leukemia secondary to oncology chemotherapy (*e.g.*, acute myelocytic leukemia [AML])
- Myelodysplastic syndrome (MDS)
- Treatment-related secondary malignancy

Any malignancy possibly related to cancer treatment (including AML/MDS) should also be reported via the routine reporting mechanisms outlined in each protocol.

Second Malignancy:

A *second malignancy* is one unrelated to the treatment of a prior malignancy (and is NOT a metastasis from the initial malignancy). Second malignancies require ONLY routine reporting unless otherwise specified."

Whenever possible, the CTEP-AERS reports for new malignancies should include tumor pathology, history or prior tumors, prior treatment/current treatment including duration, any associated risk factors or evidence regarding how long the new malignancy may have been present, when and how the new malignancy was detected, molecular characterization or cytogenetics of the original tumor (if available) and of any new tumor, and new malignancy treatment and outcome, if available.

- Death due to progressive disease should be reported as **Grade 5 "Disease progression"** in the system organ class (SOC) "General disorders and administration site conditions." Evidence that the death was a manifestation of underlying disease (*e.g.*, radiological changes suggesting tumor growth or progression: clinical deterioration associated with a disease process) should be submitted.
- Any death occurring within 30 days of the last dose, regardless of attribution to the investigational agent/intervention requires expedited reporting within 24 hours.
- Any death occurring greater than 30 days after the last dose of the investigational agent/intervention requires expedited reporting within 24 hours only if it is possibly, probably, or definitely related to the investigational agent/intervention.
- Pregnancy loss is defined in CTCAE as "Death in utero." Any Pregnancy loss should be reported expeditiously, as Grade 4 "Pregnancy loss" under the Pregnancy, puerperium and perinatal conditions SOC. A Pregnancy loss should NOT be reported as a Grade 5 event under the Pregnancy, puerperium and perinatal conditions SOC, as currently CTEP-AERS recognizes this event as a patient death.
- A neonatal death should be reported expeditiously as Grade 4, "Death neonatal" under the General disorders and administration SOC.

15.0 REFERENCES

1. Jemal A, Siegel R, Ward E, et al: Cancer statistics, 2008. CA Cancer J Clin 58:71-96, 2008
2. O'Connell MJ: Current status of adjuvant therapy for colorectal cancer. Oncology (Huntingt) 18:751-5; discussion 755-8., 2004
3. NIH consensus conference. Adjuvant therapy for patients with colon and rectal cancer. Jama 264:1444-50, 1990
4. Gill S, Loprinzi CL, Sargent DJ, et al: Pooled Analysis of Fluorouracil-Based Adjuvant Therapy for Stage II and III Colon Cancer: Who Benefits and by How Much? J Clin Oncol 22:1797-1806, 2004
5. Petersen LN, Mamenta EL, Stevnsner T, et al: Increased gene specific repair of cisplatin induced interstrand crosslinks in cisplatin resistant cell lines, and studies on carrier ligand specificity. Carcinogenesis 17:2597-602, 1996
6. Saris CP, van de Vaart PJ, Rietbroek RC, et al: In vitro formation of DNA adducts by cisplatin, lobaplatin and oxaliplatin in calf thymus DNA in solution and in cultured human cells. Carcinogenesis 17:2763-9, 1996
7. Woynarowski JM, Chapman WG, Napier C, et al: Sequence- and region-specificity of oxaliplatin adducts in naked and cellular DNA. Mol Pharmacol 54:770-7, 1998
8. Gamelin E, Bouil AL, Boisdron-Celle M, et al: Cumulative pharmacokinetic study of oxaliplatin, administered every three weeks, combined with 5-fluorouracil in colorectal cancer patients. Clin Cancer Res 3:891-9, 1997
9. de Gramont A, Figer A, Seymour M, et al: Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. J Clin Oncol 18:2938-2947, 2000
10. Braun MS, Adab F, Bradley C, et al: Modified de Gramont with oxaliplatin in the first-line treatment of advanced colorectal cancer. Br J Cancer 89:1155-8, 2003
11. Goldstein D, Mitchell P, Michael M, et al: Australian experience of a modified schedule of FOLFOX with high activity and tolerability and improved convenience in untreated metastatic colorectal cancer patients. Br J Cancer 92:832-7, 2005
12. Shimizu T, Satoh T, Tamura K, et al: Oxaliplatin/fluorouracil/leucovorin (FOLFOX4 and modified FOLFOX6) in patients with refractory or advanced colorectal cancer: post-approval Japanese population experience. Int J Clin Oncol 12:218-23, 2007
13. Giacchetti S, Perpoint B, Zidani R, et al: Phase III multicenter randomized trial of oxaliplatin added to chronomodulated fluorouracil-leucovorin as first-line treatment of metastatic colorectal cancer. J Clin Oncol 18:136-47, 2000
14. Grothey A, Deschler B, Kroening H, et al: Phase III study of bolus 5-fluorouracil (5-FU)/ folinic acid (FA) (Mayo) vs weekly high-dose 24h 5-FU infusion/ FA + oxaliplatin (OXA) (FUFOX) in advanced colorectal cancer (ACRC). Proc Am Soc Clin Oncol 21:129a, 2002
15. Rothenberg ML, Oza AM, Burger B, et al: Final results of a phase III trial of 5-FU/leucovorin versus oxaliplatin versus the combination in patients with metastatic colorectal cancer following irinotecan, 5-FU, and leucovorin. Proc Meet Am Soc Clin Oncol 22:252, 2003
16. Goldberg RM, Sargent DJ, Morton RF, et al: A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. J Clin Oncol 22:23-30, 2004

17. de Gramont A, Banzi M, Navarro M, et al: Oxaliplatin/5-FU/LV in adjuvant colon cancer: Results of the international randomized mosaic trial., Proc Am Soc Clin Oncol, 2003, pp Abstract No: 1015
18. de Gramont A, Boni C, Navarro M, et al: Oxaliplatin/5FU/LV in adjuvant colon cancer: Updated efficacy results of the MOSAIC trial, including survival, with a median follow-up of six years. J Clin Oncol 25:Abstract 4007, 2007
19. Andre T, Boni C, Mounedji-Boudiaf L, et al: Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. N Engl J Med 350:2343-51, 2004
20. Chau I, Norman AR, Cunningham D, et al: A randomised comparison between 6 months of bolus fluorouracil/leucovorin and 12 weeks of protracted venous infusion fluorouracil as adjuvant treatment in colorectal cancer. Ann Oncol 16:549-57, 2005
21. Baron JA, Cole BF, Sandler RS, et al: A randomized trial of aspirin to prevent colorectal adenomas. N Engl J Med 348:891-9, 2003
22. Bertagnolli MM: Cox-2 and cancer chemoprevention: picking up the pieces. Recent Results Cancer Res 174:73-8, 2007
23. Cross JT, Poole EM, Ulrich CM: A review of gene-drug interactions for nonsteroidal anti-inflammatory drug use in preventing colorectal neoplasia. Pharmacogenomics J 8:237-47, 2008
24. Chan AT: Aspirin, non-steroidal anti-inflammatory drugs and colorectal neoplasia: future challenges in chemoprevention. Cancer Causes Control 14:413-8, 2003
25. Janne PA, Mayer RJ: Chemoprevention of colorectal cancer. N Engl J Med 342:1960-8, 2000
26. Chan AT, Giovannucci EL, Meyerhardt JA, et al: Long-term use of aspirin and nonsteroidal anti-inflammatory drugs and risk of colorectal cancer. Jama 294:914-23, 2005
27. Sandler RS, Halabi S, Baron JA, et al: A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. N Engl J Med 348:883-90, 2003
28. Xie WL, Chipman JG, Robertson DL, et al: Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. Proc Natl Acad Sci U S A 88:2692-6, 1991
29. Flower RJ: The development of COX2 inhibitors. Nat Rev Drug Discov 2:179-91, 2003
30. Becker RC: COX-2 inhibitors. Tex Heart Inst J 32:380-3, 2005
31. Arber N, Eagle CJ, Spicak J, et al: Celecoxib for the prevention of colorectal adenomatous polyps. N Engl J Med 355:885-95, 2006
32. Baron JA, Sandler RS, Bresalier RS, et al: A randomized trial of rofecoxib for the chemoprevention of colorectal adenomas. Gastroenterology 131:1674-82, 2006
33. Bertagnolli MM, Eagle CJ, Zauber AG, et al: Celecoxib for the prevention of sporadic colorectal adenomas. N Engl J Med 355:873-84, 2006
34. Fuchs C, Meyerhardt JA, Heseltine DL, et al: Influence of regular aspirin use on survival for patients with stage III colon cancer: Findings from Intergroup trial CALGB 89803. J Clin Oncol 23:Abstract 3530, 2005
35. Kerr DJ, Dunn JA, Langman MJ, et al: Rofecoxib and cardiovascular adverse events in adjuvant treatment of colorectal cancer. N Engl J Med 357:360-9, 2007
36. Reddy BS, Rao CV: Colon cancer: a role for cyclo-oxygenase-2-specific nonsteroidal anti-inflammatory drugs. Drugs Aging 16:329-34, 2000
37. Solomon SD, Wittes J, Finn PV, et al: Cardiovascular risk of celecoxib in 6 randomized placebo-controlled trials: the cross trial safety analysis. Circulation 117:2104-13, 2008

38. Hothorn T, Hornik K, Zeileis A: Unbiased recursive partitioning: A conditional inference framework. . Journal of Computational and Graphical Statistics 15:651-674, 2006

39. Breiman L: Random forests. Machine Learning 45:5-32, 1971

40. Hothorn T, Buhlmann P, Dudoit S, et al: Survival ensembles. Biostatistics 7:355-73, 2006

41. Hothorn T, Lausen B, Benner A, et al: Bagging survival trees. Stat Med 23:77-91, 2004

42. RDCT: R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, 2006

43. Gentleman RC, Carey VJ, Bates DM, et al: Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5:R80, 2004

44. Chan AT, Ogino S, Fuchs CS: Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. N Engl J Med 356:2131-42, 2007

45. Markowitz SD: Aspirin and colon cancer--targeting prevention? N Engl J Med 356:2195-8, 2007

46. Brown JR, DuBois RN: COX-2: a molecular target for colorectal cancer prevention. J Clin Oncol 23:2840-55, 2005

47. Eberhart CE, Coffey RJ, Radhika A, et al: Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 107:1183-8, 1994

48. Soumaoro LT, Uetake H, Higuchi T, et al: Cyclooxygenase-2 expression: a significant prognostic indicator for patients with colorectal cancer. Clin Cancer Res 10:8465-71, 2004

49. Sheehan KM, Sheahan K, O'Donoghue DP, et al: The relationship between cyclooxygenase-2 expression and colorectal cancer. Jama 282:1254-7, 1999

50. Uchida K, Schneider S, Yochim JM, et al: Intratumoral COX-2 gene expression is a predictive factor for colorectal cancer response to fluoropyrimidine-based chemotherapy. Clin Cancer Res 11:3363-8, 2005

51. Ogino S, Kirkner GJ, Nosho K, et al: Cyclooxygenase-2 expression is an independent predictor of poor prognosis in colon cancer. Clin Cancer Res 14:8221-7, 2008

52. Zhang H, Sun XF: Overexpression of cyclooxygenase-2 correlates with advanced stages of colorectal cancer. Am J Gastroenterol 97:1037-41, 2002

53. Fux R, Schwab M, Thon KP, et al: Cyclooxygenase-2 expression in human colorectal cancer is unrelated to overall patient survival. Clin Cancer Res 11:4754-60, 2005

54. Masunaga R, Kohno H, Dhar DK, et al: Cyclooxygenase-2 expression correlates with tumor neovascularization and prognosis in human colorectal carcinoma patients. Clin Cancer Res 6:4064-8, 2000

55. Cianchi F, Cortesini C, Bechi P, et al: Up-regulation of cyclooxygenase 2 gene expression correlates with tumor angiogenesis in human colorectal cancer. Gastroenterology 121:1339-47, 2001

56. Tomozawa S, Tsuno NH, Sunami E, et al: Cyclooxygenase-2 overexpression correlates with tumour recurrence, especially haematogenous metastasis, of colorectal cancer. Br J Cancer 83:324-8, 2000

57. Petersen S, Haroske G, Hellmich G, et al: COX-2 expression in rectal carcinoma: immunohistochemical pattern and clinical outcome. Anticancer Res 22:1225-30, 2002

58. Joo YE, Kim HS, Min SW, et al: Expression of cyclooxygenase-2 protein in colorectal carcinomas. Int J Gastrointest Cancer 31:147-54, 2002

59. Yamac D, Celenkoglu G, Coskun U, et al: Prognostic importance of COX-2 expression in patients with colorectal cancer. Pathol Res Pract 201:497-502, 2005

60. Shacter E, Weitzman SA: Chronic inflammation and cancer. Oncology (Huntingt) 16:217-26, 229; discussion 230-2, 2002

61. Yang W, Velcich A, Mariadason J, et al: p21(WAF1/cip1) is an important determinant of intestinal cell response to sulindac in vitro and in vivo. Cancer Res 61:6297-302, 2001

62. Goldberg Y, Nassif, II, Pittas A, et al: The anti-proliferative effect of sulindac and sulindac sulfide on HT-29 colon cancer cells: alterations in tumor suppressor and cell cycle-regulatory proteins. Oncogene 12:893-901, 1996

63. Castellone MD, Teramoto H, Williams BO, et al: Prostaglandin E2 promotes colon cancer cell growth through a Gs-axin-beta-catenin signaling axis. Science 310:1504-10, 2005

64. Castellone MD, Teramoto H, Gutkind JS: Cyclooxygenase-2 and colorectal cancer chemoprevention: the beta-catenin connection. Cancer Res 66:11085-8, 2006

65. Kuwano T, Nakao S, Yamamoto H, et al: Cyclooxygenase 2 is a key enzyme for inflammatory cytokine-induced angiogenesis. Faseb J 18:300-10, 2004

66. Marrogi AJ, Travis WD, Welsh JA, et al: Nitric oxide synthase, cyclooxygenase 2, and vascular endothelial growth factor in the angiogenesis of non-small cell lung carcinoma. Clin Cancer Res 6:4739-44, 2000

67. Easwaran V, Lee SH, Inge L, et al: beta-Catenin regulates vascular endothelial growth factor expression in colon cancer. Cancer Res 63:3145-53, 2003

68. Takahashi Y, Bucana CD, Cleary KR, et al: p53, vessel count, and vascular endothelial growth factor expression in human colon cancer. Int J Cancer 79:34-8, 1998

69. Yan M, Rerko RM, Platzer P, et al: 15-Hydroxyprostaglandin dehydrogenase, a COX-2 oncogene antagonist, is a TGF-beta-induced suppressor of human gastrointestinal cancers. Proc Natl Acad Sci U S A 101:17468-73, 2004

70. Myung SJ, Rerko RM, Yan M, et al: 15-Hydroxyprostaglandin dehydrogenase is an in vivo suppressor of colon tumorigenesis. Proc Natl Acad Sci U S A 103:12098-102, 2006

71. Yan M, Myung SJ, Fink SP, et al: 15-Hydroxyprostaglandin dehydrogenase inactivation as a mechanism of resistance to celecoxib chemoprevention of colon tumors. Proc Natl Acad Sci U S A 106:9409-13, 2009

72. Backlund MG, Mann JR, Holla VR, et al: 15-Hydroxyprostaglandin dehydrogenase is down-regulated in colorectal cancer. J Biol Chem 280:3217-23, 2005

73. Salama P, Phillips M, Grieu F, et al: Tumor-Infiltrating FOXP3+ T Regulatory Cells Show Strong Prognostic Significance in Colorectal Cancer. J Clin Oncol 27:186-192, 2009

74. Pages F, Berger A, Camus M, et al: Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med 353:2654-66, 2005

75. Koch M, Beckhove P, Op den Winkel J, et al: Tumor infiltrating T lymphocytes in colorectal cancer: Tumor-selective activation and cytotoxic activity in situ. Ann Surg 244:986-92; discussion 992-3, 2006

76. Galon J, Costes A, Sanchez-Cabo F, et al: Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 313:1960-4, 2006

77. Fontenot JD, Gavin MA, Rudensky AY: Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 4:330-6, 2003

78. Hori S, Nomura T, Sakaguchi S: Control of regulatory T cell development by the transcription factor Foxp3. Science 299:1057-61, 2003

79. Ruschoff J, Wallinger S, Dietmaier W, et al: Aspirin suppresses the mutator phenotype associated with hereditary nonpolyposis colorectal cancer by genetic selection. Proc Natl Acad Sci U S A 95:11301-6, 1998

80. McIlhatton MA, Tyler J, Burkholder S, et al: Nitric Oxide Donating Aspirin Derivatives Suppress Microsatellite Instability in Mismatch Repair Deficient and Hereditary Nonpolyposis Colorectal Cancer Cells. Cancer Res 67:10966-10975, 2007

81. Ridker PM: Clinical application of C-reactive protein for cardiovascular disease detection and prevention. Circulation 107:363-9, 2003

82. Pradhan AD, Manson JE, Rifai N, et al: C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. Jama 286:327-34, 2001

83. Fung T, Hu FB, Fuchs C, et al: Major dietary patterns and the risk of colorectal cancer in women. Arch Intern Med 163:309-14, 2003

84. Rifai N, Buring JE, Lee IM, et al: Is C-reactive protein specific for vascular disease in women? Ann Intern Med 136:529-33, 2002

85. Erlinger TP, Platz EA, Rifai N, et al: C-reactive protein and the risk of incident colorectal cancer. Jama 291:585-90, 2004

86. Zhang SM, Buring JE, Lee IM, et al: C-reactive protein levels are not associated with increased risk for colorectal cancer in women. Ann Intern Med 142:425-32, 2005

87. Il'yasova D, Colbert LH, Harris TB, et al: Circulating levels of inflammatory markers and cancer risk in the health aging and body composition cohort. Cancer Epidemiol Biomarkers Prev 14:2413-8, 2005

88. Ito Y, Suzuki K, Tamakoshi K, et al: Colorectal cancer and serum C-reactive protein levels: a case-control study nested in the JACC Study. J Epidemiol 15 Suppl 2:S185-9, 2005

89. Fraser DR: Regulation of the metabolism of vitamin D. Physiol Rev 60:551-613, 1980

90. Garland CF, Comstock GW, Garland FC, et al: Serum 25-hydroxyvitamin D and colon cancer: eight-year prospective study. Lancet 2:1176-8, 1989

91. Braun MM, Helzlsouer KJ, Hollis BW, et al: Colon cancer and serum vitamin D metabolite levels 10-17 years prior to diagnosis. Am J Epidemiol 142:608-11, 1995

92. Tangrea J, Helzlsouer K, Pietinen P, et al: Serum levels of vitamin D metabolites and the subsequent risk of colon and rectal cancer in Finnish men. Cancer Causes Control 8:615-25, 1997

93. Feskanich D, Ma J, Fuchs CS, et al: Plasma vitamin D metabolites and risk of colorectal cancer in women. Cancer Epidemiol Biomarkers Prev 13:1502-8, 2004

94. Wactawski-Wende J, Kotchen JM, Anderson GL, et al: Calcium plus vitamin D supplementation and the risk of colorectal cancer. N Engl J Med 354:684-96, 2006

95. Wu K, Feskanich D, Fuchs CS, et al: A nested case control study of plasma 25-hydroxyvitamin D concentrations and risk of colorectal cancer. J Natl Cancer Inst 99:1120-9, 2007

96. Gorham ED, Garland CF, Garland FC, et al: Optimal vitamin D status for colorectal cancer prevention: a quantitative meta analysis. Am J Prev Med 32:210-6, 2007

97. Lappe JM, Travers-Gustafson D, Davies KM, et al: Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial. Am J Clin Nutr 85:1586-91, 2007

98. Robsahm TE, Tretli S, Dahlback A, et al: Vitamin D3 from sunlight may improve the prognosis of breast-, colon- and prostate cancer (Norway). Cancer Causes Control 15:149-58, 2004

99. Moan J, Porojnicu AC, Robsahm TE, et al: Solar radiation, vitamin D and survival rate of colon cancer in Norway. J Photochem Photobiol B 78:189-93, 2005

100. Ng K, Meyerhardt J, Wu K, et al: Circulating 25-Hydroxyvitamin D Levels and Survival in Patients With Colorectal Cancer J Clin Oncol in press, 2008

101. Shabahang M, Buras RR, Davoodi F, et al: 1,25-Dihydroxyvitamin D3 receptor as a marker of human colon carcinoma cell line differentiation and growth inhibition. Cancer Res 53:3712-8., 1993

102. Evans SR, Schwartz AM, Shchepotin EI, et al: Growth inhibitory effects of 1,25-dihydroxyvitamin D3 and its synthetic analogue, 1alpha,25-dihydroxy-16-ene-23yne-26,27-hexafluoro-19-nor-cholecalcifero l (Ro 25-6760), on a human colon cancer xenograft. Clin Cancer Res 4:2869-76, 1998

103. Matusiak D, Murillo G, Carroll RE, et al: Expression of vitamin D receptor and 25-hydroxyvitamin D3-1{alpha}-hydroxylase in normal and malignant human colon. Cancer Epidemiol Biomarkers Prev 14:2370-6, 2005

104. Andreyev HJ, Norman AR, Cunningham D, et al: Kirsten ras mutations in patients with colorectal cancer: the multicenter "RASCAL" study. J Natl Cancer Inst 90:675-84, 1998

105. Andreyev HJ, Norman AR, Cunningham D, et al: Kirsten ras mutations in patients with colorectal cancer: the 'RASCAL II' study. Br J Cancer 85:692-6, 2001

106. Samowitz WS, Curtin K, Schaffer D, et al: Relationship of Ki-ras mutations in colon cancers to tumor location, stage, and survival: a population-based study. Cancer Epidemiol Biomarkers Prev 9:1193-7, 2000

107. Lievre A, Bachet JB, Le Corre D, et al: KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res 66:3992-3995, 2006

108. Di Fiore F, Blanchard F, Charbonnier F, et al: Clinical relevance of KRAS mutation detection in metastatic colorectal cancer treated by Cetuximab plus chemotherapy. Br J Cancer 96:1166-1169, 2007

109. Khambata-Ford S, Garrett CR, Meropol NJ, et al: Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. J Clin Oncol 25:3230-7, 2007

110. Benvenuti S, Sartore-Bianchi A, Di Nicolantonio F, et al: Oncogenic activation of the RAS/RAF signaling pathway impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies. Cancer Res 67:2643-8, 2007

111. Solomon C, Sebag M, White JH, et al: Disruption of vitamin D receptor-retinoid X receptor heterodimer formation following ras transformation of human keratinocytes. J Biol Chem 273:17573-8, 1998

112. Solomon C, Kremer R, White JH, et al: Vitamin D resistance in RAS-transformed keratinocytes: mechanism and reversal strategies. Radiat Res 155:156-162, 2001

113. Qi X, Tang J, Pramanik R, et al: p38 MAPK activation selectively induces cell death in K-ras-mutated human colon cancer cells through regulation of vitamin D receptor. J Biol Chem 279:22138-44, 2004

114. Stedman L, Nickel KP, Castillo SS, et al: 1,25-dihydroxyvitamin D inhibits vitamin E succinate-induced apoptosis in C3H10T1/2 cells but not Harvey ras-transfected cells. Nutr Cancer 45:93-100, 2003

115. Fan JB, Yeakley JM, Bibikova M, et al: A versatile assay for high-throughput gene expression profiling on universal array matrices. Genome Res 14:878-85, 2004

116. Bibikova M, Yeakley JM, Wang-Rodriguez J, et al: Quantitative expression profiling of RNA from formalin-fixed, paraffin-embedded tissues using randomly assembled bead arrays. Methods Mol Biol 439:159-77, 2008

117. Hoshida Y, Villanueva A, Kobayashi M, et al: Gene expression in fixed tissues and outcome in hepatocellular carcinoma. N Engl J Med 359:1995-2004, 2008

118. Toyota M, Ahuja N, Ohe-Toyota M, et al: CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A 96:8681-6, 1999

119. Yamashita K, Dai T, Dai Y, et al: Genetics supersedes epigenetics in colon cancer phenotype. Cancer Cell 4:121-31, 2003

120. Anacleto C, Leopoldino AM, Rossi B, et al: Colorectal cancer "methylator phenotype": fact or artifact? Neoplasia 7:331-5, 2005

121. Issa JP: CpG island methylator phenotype in cancer. Nat Rev Cancer 4:988-93, 2004

122. Eads CA, Lord RV, Wickramasinghe K, et al: Epigenetic patterns in the progression of esophageal adenocarcinoma. Cancer Res 61:3410-8, 2001

123. Virmani AK, Tsou JA, Siegmund KD, et al: Hierarchical clustering of lung cancer cell lines using DNA methylation markers. Cancer Epidemiol Biomarkers Prev 11:291-7, 2002

124. Widschwendter M, Siegmund KD, Muller HM, et al: Association of breast cancer DNA methylation profiles with hormone receptor status and response to tamoxifen. Cancer Res 64:3807-13, 2004

125. Kang GH, Lee S, Kim JS, et al: Profile of aberrant CpG island methylation along the multistep pathway of gastric carcinogenesis. Lab Invest 83:635-41, 2003

126. Clement G, Bosman FT, Fontolliet C, et al: Monoallelic methylation of the APC promoter is altered in normal gastric mucosa associated with neoplastic lesions. Cancer Res 64:6867-73, 2004

127. Tamura G: Promoter methylation status of tumor suppressor and tumor-related genes in neoplastic and non-neoplastic gastric epithelia. Histol Histopathol 19:221-8, 2004

128. Eads CA, Lord RV, Kurumboor SK, et al: Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. Cancer Res 60:5021-6, 2000

129. Esteller M, Garcia-Foncillas J, Andion E, et al: Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med 343:1350-4, 2000

130. Esteller M, Tortola S, Toyota M, et al: Hypermethylation-associated inactivation of p14(ARF) is independent of p16(INK4a) methylation and p53 mutational status. Cancer Res 60:129-33, 2000

131. Yan PS, al. e: Use of CpG island microarrays to identify colorectal tumors with a high degree of concurrent methylation. Methods 27:162-9, 2002

132. Laird PW: Cancer epigenetics. Hum Mol Genet 14 Spec No 1:R65-76, 2005

133. Ogino S, Nosho K, Kirkner GJ, et al: CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. Gut 58:90-96, 2009

134. Ogino S, Nosho K, Kirkner GJ, et al: A Cohort Study of Tumoral LINE-1 Hypomethylation and Prognosis in Colon Cancer. J. Natl. Cancer Inst. 100:1734-1738, 2008

135. Boland CR, Thibodeau SN, Hamilton SR, et al: A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 58:5248-57, 1998

136. Weidner N: Intratumor microvessel density as a prognostic factor in cancer. Am J Pathol 147:9-19, 1995

137. Jass JR, Biden KG, Cummings MC, et al: Characterisation of a subtype of colorectal cancer combining features of the suppressor and mild mutator pathways. J Clin Pathol 52:455-60, 1999

138. Kawasaki T, Nosho K, Ohnishi M, et al: Correlation of beta-catenin localization with cyclooxygenase-2 expression and CpG island methylator phenotype (CIMP) in colorectal cancer. Neoplasia 9:569-77, 2007

139. Hollis BW: Quantitation of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D by radioimmunoassay using radioiodinated tracers. Methods Enzymol 282:174-86, 1997

140. Platz EA, Leitzmann MF, Hollis BW, et al: Plasma 1,25-dihydroxy- and 25-hydroxyvitamin D and subsequent risk of prostate cancer. Cancer Causes Control 15:255-65, 2004

141. Ogino S, Kawasaki T, Brahmandam M, et al: Sensitive sequencing method for KRAS mutation detection by Pyrosequencing. J Mol Diagn 7:413-21, 2005

142. Weisenberger DJ, Siegmund KD, Campan M, et al: CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet 38:787-93, 2006

143. Eads CA, Danenberg KD, Kawakami K, et al: MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 28:E32, 2000

144. Weisenberger DJ, Campan M, Long TI, et al: Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 33:6823-36, 2005

145. Jung SH, Owzar K, George SL: A multiple testing procedure to associate gene expression levels with survival. Stat Med 24:3077-88, 2005

146. Hajek J, Sidak Z, Sen PK: Theory of Rank Tests (ed 2nd), Academic Press, 1999

147. Rao CV, Cooma I, Rodriguez JG, et al: Chemoprevention of familial adenomatous polyposis development in the APC(min) mouse model by 1,4-phenylene bis(methylene)selenocyanate. Carcinogenesis 21:617-21, 2000

148. Reddy BS, Rao CV, Rivenson A, et al: Inhibitory effect of aspirin on zoxymethane-induced colon carcinogenesis in F344 rats. Carcinogenesis 14:1493-1497, 1993

149. Sano H, Kawahito Y, Wilder RL, et al: Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res 55:3785-9, 1995

150. DuBois RN, Smalley WE: Cyclooxygenase, NSAIDs, and colorectal cancer. J Gastroenterol 31:898-906, 1996

151. Midgley R, McConkey C, Langman M, et al: Victor: a phase III placebo-controlled trial of rofecoxib in colorectal cancer patients following surgical resection. Ann Oncology 19:Abst LBA3, 2008

152. Ulrich CM, Bigler J, Sparks R, et al: Polymorphisms in PTGS1 (=COX-1) and risk of colorectal polyps. Cancer Epidemiol Biomarkers Prev 13:889-93, 2004

153. Drazen JM, Yandava CN, Dube L, et al: Pharmacogenetic association between ALOX5 promoter genotype and the response to anti-asthma treatment. Nat Genet 22:168-70, 1999

154. Helgadottir A, Manolescu A, Thorleifsson G, et al: The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. Nat Genet 36:233-9, 2004

155. Dwyer JH, Allayee H, Dwyer KM, et al: Arachidonate 5-lipoxygenase promoter genotype, dietary arachidonic acid, and atherosclerosis. N Engl J Med 350:29-37, 2004

156. Goodman JE, Bowman ED, Chanock SJ, et al: Arachidonate lipoxygenase (ALOX) and cyclooxygenase (COX) polymorphisms and colon cancer risk. Carcinogenesis 25:2467-72, 2004

157. Iwai N, Katsuya T, Ishikawa K, et al: Human prostacyclin synthase gene and hypertension : the Suita Study. Circulation 100:2231-6, 1999

158. Chevalier D, Cauffiez C, Bernard C, et al: Characterization of new mutations in the coding sequence and 5'-untranslated region of the human prostacylcin synthase gene (CYP8A1). Hum Genet 108:148-55, 2001

159. Nakayama T, Soma M, Watanabe Y, et al: Splicing mutation of the prostacyclin synthase gene in a family associated with hypertension. Adv Exp Med Biol 525:165-8, 2003

160. Tsujita Y, Kinoshita M, Tanabe T, et al: Role of a genetic variation in the promoter of human thromboxane synthase gene in myocardial infarction. Atherosclerosis 153:261-2, 2000

161. Sarraf P, Mueller E, Smith WM, et al: Loss-of-function mutations in PPAR gamma associated with human colon cancer. Mol Cell 3:799-804., 1999

162. Saez E, Tontonoz P, Nelson MC, et al: Activators of the nuclear receptor PPARgamma enhance colon polyp formation. Nat Med 4:1058-61., 1998

163. Murphy GJ, Holder JC: PPAR-gamma agonists: therapeutic role in diabetes, inflammation and cancer. Trends Pharmacol Sci 21:469-74., 2000

164. Lehmann JM, Lenhard JM, Oliver BB, et al: Peroxisome proliferator-activated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs. J Biol Chem 272:3406-10, 1997

165. He TC, Chan TA, Vogelstein B, et al: PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. Cell 99:335-45., 1999

166. Altshuler D, Hirschhorn JN, Klannemark M, et al: The common PPARgamma Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes. Nat Genet 26:76-80., 2000

167. Gong Z, Xie D, Deng Z, et al: The PPAR{gamma} Pro12Ala polymorphism and risk for incident sporadic colorectal adenomas. Carcinogenesis 26:579-85, 2005

168. Landi S, Moreno V, Gioia-Patricola L, et al: Association of common polymorphisms in inflammatory genes interleukin (IL)6, IL8, tumor necrosis factor alpha, NFKB1, and peroxisome proliferator-activated receptor gamma with colorectal cancer. Cancer Res 63:3560-6, 2003

169. Biswas S, Chytil A, Washington K, et al: Transforming growth factor beta receptor type II inactivation promotes the establishment and progression of colon cancer. Cancer Res 64:4687-92, 2004

170. Tak PP, Firestein GS: NF-kappaB: a key role in inflammatory diseases. J Clin Invest 107:7-11, 2001

171. Bours V, Dejardin E, Goujon-Letawe F, et al: The NF-kappa B transcription factor and cancer: high expression of NF-kappa B- and I kappa B-related proteins in tumor cell lines. Biochem Pharmacol 47:145-9, 1994

172. Agarwal A, Das K, Lerner N, et al: The AKT/I kappa B kinase pathway promotes angiogenic/metastatic gene expression in colorectal cancer by activating nuclear factor-kappa B and beta-catenin. Oncogene 24:1021-31, 2005

173. Lind DS, Hochwald SN, Malaty J, et al: Nuclear factor-kappa B is upregulated in colorectal cancer. Surgery 130:363-9, 2001

174. Din FV, Dunlop MG, Stark LA: Evidence for colorectal cancer cell specificity of aspirin effects on NF kappa B signalling and apoptosis. Br J Cancer 91:381-8, 2004

175. Kojima M, Morisaki T, Sasaki N, et al: Increased nuclear factor-kB activation in human colorectal carcinoma and its correlation with tumor progression. Anticancer Res 24:675-81, 2004

176. Klein W, Tromm A, Folwaczny C, et al: A polymorphism of the NFKBIA gene is associated with Crohn's disease patients lacking a predisposing allele of the CARD15 gene. Int J Colorectal Dis 19:153-6, 2004

177. Mozzato-Chamay N, Corbett EL, Bailey RL, et al: Polymorphisms in the IkappaB-alpha promoter region and risk of diseases involving inflammation and fibrosis. Genes Immun 2:153-5, 2001

178. Okamoto K, Makino S, Yoshikawa Y, et al: Identification of I kappa BL as the second major histocompatibility complex-linked susceptibility locus for rheumatoid arthritis. Am J Hum Genet 72:303-12, 2003

179. Hegazy DM, O'Reilly DA, Yang BM, et al: NFkappaB polymorphisms and susceptibility to type 1 diabetes. Genes Immun 2:304-8, 2001

180. Yamashita T, Hamaguchi K, Kusuda Y, et al: IKBL promoter polymorphism is strongly associated with resistance to type 1 diabetes in Japanese. Tissue Antigens 63:223-30, 2004

181. Parker KM, Ma MH, Manyak S, et al: Identification of polymorphisms of the IkappaBalpha gene associated with an increased risk of multiple myeloma. Cancer Genet Cytogenet 137:43-8, 2002

182. Bigler J, Whitton J, Lampe JW, et al: CYP2C9 and UGT1A6 genotypes modulate the protective effect of aspirin on colon adenoma risk. Cancer Res 61:3566-9., 2001

183. Jacobs ET, Martinez ME, Alberts DS: Research and public health implications of the intricate relationship between calcium and vitamin D in the prevention of colorectal neoplasia. J Natl Cancer Inst. 95:1736-7., 2003

184. Newmark HL, Wargovich MJ, Bruce WR: Colon cancer and dietary fat, phosphate, and calcium: a hypothesis. J Natl Cancer Inst 72:1323-5, 1984

185. Walters MR: Newly identified actions of the vitamin D endocrine system. Endocr Rev 13:719-64, 1992

186. Morrison NA, Qi JC, Tokita A, et al: Prediction of bone density from vitamin D receptor alleles. Nature 367:284-7, 1994

187. Durrin LK, Haile RW, Ingles SA, et al: Vitamin D receptor 3'-untranslated region polymorphisms: lack of effect on mRNA stability. Biochim Biophys Acta 1453:311-20, 1999

188. Baker AR, McDonnell DP, Hughes M, et al: Cloning and expression of full-length cDNA encoding human vitamin D receptor. Proc Natl Acad Sci U S A 85:3294-8, 1988

189. Arai H, Miyamoto K, Taketani Y, et al: A vitamin D receptor gene polymorphism in the translation initiation codon: effect on protein activity and relation to bone mineral density in Japanese women. J Bone Miner Res 12:915-21, 1997

190. Jurutka PW, Remus LS, Whitfield GK, et al: The polymorphic N terminus in human vitamin D receptor isoforms influences transcriptional activity by modulating interaction with transcription factor IIB. Mol Endocrinol 14:401-20, 2000

191. Ames SK, Ellis KJ, Gunn SK, et al: Vitamin D receptor gene Fok1 polymorphism predicts calcium absorption and bone mineral density in children. J Bone Miner Res 14:740-6, 1999

192. Gross C, Eccleshall TR, Malloy PJ, et al: The presence of a polymorphism at the translation initiation site of the vitamin D receptor gene is associated with low bone mineral density in postmenopausal Mexican-American women. J Bone Miner Res 11:1850-5, 1996

193. Morrison NA, Yeoman R, Kelly PJ, et al: Contribution of trans-acting factor alleles to normal physiological variability: vitamin D receptor gene polymorphism and circulating osteocalcin. Proc Natl Acad Sci U S A 89:6665-9, 1992

194. Haussler MR, Whitfield GK, Haussler CA, et al: The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. J Bone Miner Res 13:325-49, 1998

195. Kim HS, Newcomb PA, Ulrich CM, et al: Vitamin D receptor polymorphism and the risk of colorectal adenomas: evidence of interaction with dietary vitamin D and calcium. Cancer Epidemiol Biomarkers Prev 10:869-74, 2001

196. Slattery ML, Neuhausen SL, Hoffman M, et al: Dietary calcium, vitamin D, VDR genotypes and colorectal cancer. Int J Cancer 111:750-6, 2004

197. Peters U, Hayes RB, Chatterjee N, et al: Circulating vitamin D metabolites, polymorphism in vitamin D receptor, and colorectal adenoma risk. Cancer Epidemiol Biomarkers Prev 13:546-52, 2004

198. Grau MV, Baron JA, Sandler RS, et al: Vitamin D, calcium supplementation, and colorectal adenomas: results of a randomized trial. J Natl Cancer Inst 95:1765-71, 2003

199. Boyapati SM, Bostick RM, McGlynn KA, et al: Calcium, vitamin D, and risk for colorectal adenoma: dependency on vitamin D receptor BsmI polymorphism and nonsteroidal anti-inflammatory drug use? Cancer Epidemiol Biomarkers Prev 12:631-7, 2003

200. Wong HL, Seow A, Arakawa K, et al: Vitamin D receptor start codon polymorphism and colorectal cancer risk: effect modification by dietary calcium and fat in Singapore Chinese. Carcinogenesis 24:1091-5, 2003

201. Peters U, McGlynn KA, Chatterjee N, et al: Vitamin D, calcium, and vitamin D receptor polymorphism in colorectal adenomas. Cancer Epidemiol Biomarkers Prev 10:1267-74, 2001

202. Ingles SA, Wang J, Coetzee GA, et al: Vitamin D receptor polymorphisms and risk of colorectal adenomas (United States). Cancer Causes Control 12:607-14, 2001

203. Nejentsev S, Cooper JD, Godfrey L, et al: Analysis of the vitamin D receptor gene sequence variants in type 1 diabetes. Diabetes 53:2709-12, 2004

204. Nykjaer A, Dragun D, Walther D, et al: An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. Cell 96:507-15, 1999

205. Malecki MT, Klupa T, Wanic K, et al: Vitamin D binding protein gene and genetic susceptibility to type 2 diabetes mellitus in a Polish population. Diabetes Res Clin Pract 57:99-104, 2002

206. Krylov M, Korotkova TA, Miakotkin VA, et al: [Allele polymorphism of alkaline phosphatase, acid soluble phosphatase, and vitamin D-binding protein genes in postmenopausal osteoporosis]. Ter Arkh 76:61-5, 2004

207. Ishii T, Keicho N, Teramoto S, et al: Association of Gc-globulin variation with susceptibility to COPD and diffuse panbronchiolitis. Eur Respir J 18:753-7, 2001

208. Laufs J, Andrason H, Sigvaldason A, et al: Association of vitamin D binding protein variants with chronic mucus hypersecretion in Iceland. Am J Pharmacogenomics 4:63-8, 2004

209. Pani MA, Regulla K, Segni M, et al: A polymorphism within the vitamin D-binding protein gene is associated with Graves' disease but not with Hashimoto's thyroiditis. J Clin Endocrinol Metab. 87:2564-7., 2002

210. Prosser DE, Jones G: Enzymes involved in the activation and inactivation of vitamin D. Trends Biochem Sci 29:664-73, 2004

211. Omdahl JL, Morris HA, May BK: Hydroxylase enzymes of the vitamin D pathway: expression, function, and regulation. Annu Rev Nutr 22:139-66, 2002

212. Ogunkolade BW, Boucher BJ, Fairclough PD, et al: Expression of 25-hydroxyvitamin D-1-alpha-hydroxylase mRNA in individuals with colorectal cancer. Lancet 359:1831-2, 2002

213. Bises G, Kallay E, Weiland T, et al: 25-hydroxyvitamin D3-1alpha-hydroxylase expression in normal and malignant human colon. J Histochem Cytochem 52:985-9, 2004

214. Bareis P, Bises G, Bischof MG, et al: 25-hydroxy-vitamin d metabolism in human colon cancer cells during tumor progression. Biochem Biophys Res Commun 285:1012-7, 2001

215. Lopez ER, Regulla K, Pani MA, et al: CYP27B1 polymorphisms variants are associated with type 1 diabetes mellitus in Germans. J Steroid Biochem Mol Biol 89-90:155-7, 2004

216. Lopez ER, Zwermann O, Segni M, et al: A promoter polymorphism of the CYP27B1 gene is associated with Addison's disease, Hashimoto's thyroiditis, Graves' disease and type 1 diabetes mellitus in Germans. Eur J Endocrinol 151:193-7, 2004

217. Nickerson D: NIEHS-SNPs, Environmental Genome Project, 2004

218. Bettoun DJ, Burris TP, Houck KA, et al: Retinoid X receptor is a nonsilent major contributor to vitamin D receptor-mediated transcriptional activation. Mol Endocrinol 17:2320-8, 2003

219. Olson JM, Hallahan AR: p38 MAP kinase: a convergence point in cancer therapy. Trends Mol Med 10:125-9, 2004

220. Matusiak D, Benya RV: CYP27A1 and CYP24 expression as a function of malignant transformation in the colon. J Histochem Cytochem 55:1257-64, 2007

221. Ebert R, Schutze N, Adamski J, et al: Vitamin D signaling is modulated on multiple levels in health and disease. Mol Cell Endocrinol 248:149-59, 2006

222. Roff A, Wilson RT: A novel SNP in a vitamin D response element of the CYP24A1 promoter reduces protein binding, transactivation, and gene expression. J Steroid Biochem Mol Biol 112:47-54, 2008

223. Nagasubramanian R, Innocenti F, Ratain MJ. Pharmacogenetics in cancer treatment. Annu Rev Med 2003;54:437-52.

224. Evans WE, McLeod HL. Pharmacogenomics--drug disposition, drug targets, and side effects. N Engl J Med 2003;348:538-49.

225. McWhinney SR, Goldberg RM, McLeod HL. Platinum neurotoxicity pharmacogenetics. Mol Cancer Ther 2009;8:10-6.

226. Gamelin L, Capitain O, Morel A, et al. Predictive factors of oxaliplatin neurotoxicity: the involvement of the oxalate outcome pathway. Clin Cancer Res 2007;13:6359-68.

227. Evans WE, Relling MV. Moving towards individualized medicine with pharmacogenomics. Nature 2004;429:464-8.

228. Weinshilboum R. Inheritance and drug response. N Engl J Med 2003;348:529-37.

229. Botstein D, Risch N. Discovering genotypes underlying human phenotypes: past successes for Mendelian disease, future approaches for complex disease. Nat Genet 2003;33 Suppl:228-37.

230. Consortium TIH. The international HapMap project. Nature 2003;426:789-96.

231. Carlson CS, Eberle MA, Kruglyak L, Nickerson DA. Mapping complex disease loci in whole-genome association studies. Nature 2004;429:446-52.

232. Hinds DA, Stuve LL, Nilsen GB, et al. Whole-genome patterns of common DNA variation in three human populations. Science 2005;307:1072-9.

233. Kennedy GC, Matsuzaki H, Dong S, et al. Large-scale genotyping of complex DNA. Nat Biotechnol 2003;21:1233-7.

234. Lawrence RW, Evans DM, Cardon LR. Prospects and pitfalls in whole genome association studies. Philos Trans R Soc Lond B Biol Sci 2005;360:1589-95.

235. Yeager M, Orr N, Hayes RB, et al. Genome-wide association study of prostate cancer identifies a second risk locus at 8q24. Nat Genet 2007;39:645-9.

236. Thomas G, Jacobs KB, Yeager M, et al. Multiple loci identified in a genome-wide association study of prostate cancer. Nat Genet 2008;40:310-5.

237. Gudmundsson J, Sulem P, Steinthorsdottir V, et al. Two variants on chromosome 17 confer prostate cancer risk, and the one in TCF2 protects against type 2 diabetes. Nat Genet 2007;39:977-83.

238. Gudmundsson J, Sulem P, Manolescu A, et al. Genome-wide association study identifies a second prostate cancer susceptibility variant at 8q24. Nat Genet 2007;39:631-7.

239. Duggan D, Zheng SL, Knowlton M, et al. Two genome-wide association studies of aggressive prostate cancer implicate putative prostate tumor suppressor gene DAB2IP. J Natl Cancer Inst 2007;99:1836-44.

240. Tomlinson I, Webb E, Carvajal-Carmona L, et al. A genome-wide association scan of tag SNPs identifies a susceptibility variant for colorectal cancer at 8q24.21. Nat Genet 2007;39:984-8.

241. Zanke BW, Greenwood CM, Rangrej J, et al. Genome-wide association scan identifies a colorectal cancer susceptibility locus on chromosome 8q24. Nat Genet 2007;39:989-94.

242. Broderick P, Carvajal-Carmona L, Pittman AM, et al. A genome-wide association study shows that common alleles of SMAD7 influence colorectal cancer risk. Nat Genet 2007;39:1315-7.

243. Spinola M, Leoni VP, Galvan A, et al. Genome-wide single nucleotide polymorphism analysis of lung cancer risk detects the KLF6 gene. Cancer Lett 2007;251:311-6.

244. Hunter DJ, Kraft P, Jacobs KB, et al. A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer. Nat Genet 2007;39:870-4.

245. Easton DF, Pooley KA, Dunning AM, et al. Genome-wide association study identifies novel breast cancer susceptibility loci. Nature 2007;447:1087-93.

246. Gold B, Kirchhoff T, Stefanov S, et al. Genome-wide association study provides evidence for a breast cancer risk locus at 6q22.33. Proc Natl Acad Sci U S A 2008.

247. Giovannucci E, Rimm EB, Stampfer MJ, et al: Intake of fat, meat, and fiber in relation to risk of colon cancer in men. Cancer Res 54:2390-2397, 1994

248. Willett WC, Stampfer MJ, Colditz GA, et al: Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women. N Engl J Med 323:1664-72., 1990

249. Giovannucci E, Rimm EB, Ascherio A, et al: Alcohol, low-methionine--low-folate diets, and risk of colon cancer in men. J Natl Cancer Inst 87:265-73., 1995

250. Giovannucci E, Stampfer MJ, Colditz GA, et al: Folate, methionine, and alcohol intake and risk of colorectal adenoma. J Natl Cancer Inst 85:875-884, 1993

251. Baron JA, Beach M, Mandel JS, et al: Calcium supplements for the prevention of colorectal adenomas. Calcium Polyp Prevention Study Group. N Engl J Med 340:101-7., 1999

252. Kampman E, Slattery ML, Caan B, et al: Calcium, vitamin D, sunshine exposure, dairy products and colon cancer risk (United States). Cancer Causes Control 11:459-66., 2000

253. Howe GR, Benito E, Castelleto R, et al: Dietary intake of fiber and decreased risk of cancers of the colon and rectum: evidence from the combined analysis of 13 case-control studies. J Natl Cancer Inst 84:1887-96., 1992

254. Arber N, DuBois RN: Nonsteroidal anti-inflammatory drugs and prevention of colorectal cancer. Curr Gastroenterol Rep 1:441-8., 1999

255. Thun MJ: Aspirin and gastrointestinal cancer. Adv Exp Med Biol:395-402, 1997

256. Giovannucci E, Stampfer MJ, Colditz GA, et al: Multivitamin use, folate, and colon cancer in women in the Nurses' Health Study. Ann Intern Med 129:517-24., 1998

257. Caan BJ, Coates AO, Slattery ML, et al: Body size and the risk of colon cancer in a large case-control study. Int J Obes Relat Metab Disord 22:178-84., 1998

258. Chute C, Willett W, Colditz G, et al: A prospective study of body mass, height and smoking in the risk of colorectal cancer in women. Cancer Causes and Control 2:117-124, 1991

259. Chyou PH, Nomura AM, Stemmermann GN: A prospective study of colon and rectal cancer among Hawaii Japanese men. Ann Epidemiol 6:276-82., 1996

260. Ford ES: Body mass index and colon cancer in a national sample of adult US men and women. Am J Epidemiol 150:390-8., 1999

261. Giovannucci E, Ascherio A, Rimm EB, et al: Physical activity, obesity, and risk for colon cancer and adenoma in men. Ann Intern Med 122:327-34, 1995

262. Le Marchand L, Wilkens LR, Kolonel LN, et al: Associations of sedentary lifestyle, obesity, smoking, alcohol use, and diabetes with the risk of colorectal cancer. Cancer Res 57:4787-94., 1997

263. Lee IM, Paffenbarger RS, Jr.: Quetelet's index and risk of colon cancer in college alumni. J Natl Cancer Inst 84:1326-31., 1992

264. Martinez ME, Giovannucci E, Spiegelman D, et al: Leisure-time physical activity, body size, and colon cancer in women. Nurses' Health Study Research Group. J Natl Cancer Inst 89:948-55., 1997

265. Giovannucci E, Colditz GA, Stampfer MJ, et al: A prospective study of cigarette smoking and risk of colorectal adenoma and colorectal cancer in U.S. women. J Natl Cancer Inst 86:192-9, 1994

266. Giovannucci E, Martinez ME: Tobacco, colorectal cancer, and adenomas: a review of the evidence. J Natl Cancer Inst 88:1717-30., 1996

267. Giovannucci E, Rimm EB, Stampfer MJ, et al: A prospective study of cigarette smoking and risk of colorectal adenoma and colorectal cancer in U.S. men. J Natl Cancer Inst 86:183-91., 1994

268. Giovannucci E: Insulin, insulin-like growth factors and colon cancer: a review of the evidence. J Nutr 131:3109S-20S., 2001

269. Kaaks R, Lukanova A: Energy balance and cancer: the role of insulin and insulin-like growth factor-I. Proc Nutr Soc 60:91-106., 2001

270. Chang TY, Tsai WJ, Chou CK, et al: Identifying the factors and signal pathways necessary for anchorage-independent growth of Ha-ras oncogene-transformed NIH/3T3 cells. Life Sci 73:1265-74, 2003

271. Bonni A, Brunet A, West AE, et al: Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 286:1358-62, 1999

272. Zeng H, Datta K, Neid M, et al: Requirement of different signaling pathways mediated by insulin-like growth factor-I receptor for proliferation, invasion, and VPF/VEGF expression in a pancreatic carcinoma cell line. Biochem Biophys Res Commun 302:46-55, 2003

273. Ogino S, Nosho K, Kirkner GJ, et al: PIK3CA Mutation Is Associated With Poor Prognosis Among Patients With Curatively Resected Colon Cancer. J Clin Oncol 27:1477-1484, 2009

274. Ogino S, Nosho K, Meyerhardt JA, et al: Cohort Study of Fatty Acid Synthase Expression and Patient Survival in Colon Cancer. J Clin Oncol 26:5713-5720, 2008

275. Wolpin BM, Meyerhardt JA, Chan AT, et al: Insulin, the insulin-like growth factor axis, and mortality in patients with nonmetastatic colorectal cancer. J Clin Oncol 27:176-85, 2009

276. Wei EK, Giovannucci E, Fuchs CS, et al: Low plasma adiponectin levels and risk of colorectal cancer in men: a prospective study. J Natl Cancer Inst 97:1688-94, 2005

277. Slattery ML, Boucher KM, Caan BJ, et al: Eating patterns and risk of colon cancer. Am J Epidemiol 148:4-16, 1998

278. Wu K, Hu FB, Fuchs C, et al: Dietary patterns and risk of colon cancer and adenoma in a cohort of men (United States). Cancer Causes Control 15:853-62, 2004

279. Yang WC, Mathew J, Velcich A, et al: Targeted inactivation of the p21(WAF1/cip1) gene enhances Apc-initiated tumor formation and the tumor-promoting activity of a Western-style high-risk diet by altering cell maturation in the intestinal mucosal. Cancer Res 61:565-9, 2001

280. Yang K, Edelmann W, Fan K, et al: Dietary modulation of carcinoma development in a mouse model for human familial adenomatous polyposis. Cancer Res 58:5713-7, 1998

281. Risio M, Lipkin M, Newmark H, et al: Apoptosis, cell replication, and Western-style diet-induced tumorigenesis in mouse colon. Cancer Res 56:4910-6, 1996

282. Meyerhardt JA, Niedzwiecki D, Hollis D, et al: Association of dietary patterns with cancer recurrence and survival in patients with stage III colon cancer. Jama 298:754-64, 2007

283. Colditz GA, Martin P, Stampfer MJ, et al: Validation of questionnaire information on risk factors and disease outcomes in a prospective cohort study of women. Am J Epidemiol 123:894-900, 1986

284. Hu FB, Rimm E, Smith-Warner SA, et al: Reproducibility and validity of dietary patterns assessed with a food-frequency questionnaire. Am J Clin Nutr 69:243-9, 1999

285. Willett WC, Sampson L, Browne ML, et al: The use of a self-administered questionnaire to assess diet four years in the past. Am J Epidemiol 127:188-99, 1988

286. Willett WC, Sampson L, Stampfer MJ, et al: Reproducibility and validity of a semiquantitative food frequency questionnaire. Am J Epidemiol 122:51-65, 1985

287. Willett WC, Stampfer MJ, Underwood BA, et al: Validation of a dietary questionnaire with plasma carotenoid and alpha-tocopherol levels. Am J Clin Nutr 38:631-9, 1983

288. Willett W, Stampfer MJ: Total energy intake: implications for epidemiologic analyses. Am J Epidemiol 124:17-27, 1986

289. Ascherio A, Stampfer MJ, Colditz GA, et al: Correlations of vitamin A and E intakes with the plasma concentrations of carotenoids and tocopherols among American men and women. J Nutr 122:1792-801, 1992

290. Stryker WS, Salvini S, Stampfer MJ, et al: Contributions of specific foods to absolute intake and between-person variation of nutrient consumption. J Am Diet Assoc 91:172-8, 1991

291. Hunter DJ, Rimm EB, Sacks FM, et al: Comparison of measures of fatty acid intake by subcutaneous fat aspirate, food frequency questionnaire, and diet records in a free-living population of US men. Am J Epidemiol 135:418-27, 1992

292. London SJ, Sacks FM, Caesar J, et al: Fatty acid composition of subcutaneous adipose tissue and diet in postmenopausal US women. Am J Clin Nutr 54:340-5, 1991

293. Meyerhardt JA, Heseltine D, Campos H, et al: Assessment of a dietary questionnaire in cancer patients receiving cytotoxic chemotherapy. J Clin Oncol 23:8453-60, 2005

294. Wolf AM, Hunter DJ, Colditz GA, et al: Reproducibility and validity of a self-administered physical activity questionnaire. Int J Epidemiol 23:991-9, 1994

295. Chasan-Taber S, Rimm EB, Stampfer MJ, et al: Reproducibility and validity of a self-administered physical activity questionnaire for male health professionals. Epidemiology 7:81-6, 1996

296. Nosho K, Kawasaki T, Ohnishi M, et al: PIK3CA mutation in colorectal cancer: relationship with genetic and epigenetic alterations. Neoplasia 10:534-41, 2008

297. Itoh N, Semba S, Ito M, et al: Phosphorylation of Akt/PKB is required for suppression of cancer cell apoptosis and tumor progression in human colorectal carcinoma. Cancer 94:3127-34., 2002

298. Ogino S, Meyerhardt JA, Cantor M, et al: Molecular alterations in tumors and response to combination chemotherapy with gefitinib for advanced colorectal cancer. Clin Cancer Res 11:6650-6656, 2005

299. Chan, Ogino, Fuchs, JAMA. 2009 Aug 12;302(6):649-58

300. Purcell, S, Neale, B, Todd-Brown, K, Thomas, L, Ferreira, MA, Bender, D, Maller, J, Sklar, P, de Bakker, PI, Daly, MJ, and Sham, PC. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet, **81**: 559-75, 2007.

301. Falush, D, Stephens, M, and Pritchard, JK. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics, **164**: 1567-87, 2003.

302. Price, AL, Patterson, NJ, Plenge, RM, Weinblatt, ME, Shadick, NA, and Reich, D. Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet, **38**: 904-9, 2006.

303. Agresti, A, *Categorical Data Analysis*. 2002: John WIley & Sons.

304. Nicolae, DL. Testing untyped alleles (TUNA)-applications to genome-wide association studies. Genet Epidemiol, **30**: 718-27, 2006.

305. Nicolae, DL. Quantifying the amount of missing information in genetic association studies. Genet Epidemiol, **30**: 703-17, 2006.

306. Team, RDC, *R: A Language and Environment for Statistical Computing*. 2006, Vienna: R Foundation for Statistical Computing.

307. Gentleman, RC, Carey, VJ, Bates, DM, Bolstad, B, Dettling, M, Dudoit, S, Ellis, B, Gautier, L, Ge, Y, Gentry, J, Hornik, K, Hothorn, T, Huber, W, Iacus, S, Irizarry, R, Leisch, F, Li, C, Maechler, M, Rossini, AJ, Sawitzki, G, Smith, C, Smyth, G, Tierney, L, Yang, JY, and Zhang, J. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol, 5: R80, 2004.

308. Iacopetta, B., K. Kawakami, et al. (2008). Predicting clinical outcome of 5-fluorouracil-based chemotherapy for colon cancer patients: is the CpG island methylator phenotype the 5-fluorouracil-responsive subgroup? Int J Clin Oncol 13(6): 498-503.

309. Curtin K, Slattery ML, Ulrich CM, Bigler J, Levin TR, Wolff RK, Albertsen H, Potter JD, Samowitz WS. Genetic polymorphisms in one-carbon metabolism: associations with CpG

island methylator phenotype (CIMP) in colon cancer and the modifying effects of diet. Carcinogenesis. 2007 Aug; 28(8): 1672-9.

310. Kawakami, K., A. Ooyama, et al. (2008). Low expression of gamma-glutamyl hydrolase mRNA in primary colorectal cancer with the CpG island methylator phenotype. Br J Cancer 98(9): 1555-61.
311. Lynge E, Jensen OM, Carstensen B. Second cancer following cancer of the digestive system in Denmark, 1943-80. Natl Cancer Inst Monogr 1985;68:277-308.
312. Hoar SK, Wilson J, Blot WJ, McLaughlin JK, Winn DM, Kantor AF. Second cancer following cancer of the digestive system in Connecticut, 1935-82. Natl Cancer Inst Monogr 1985;68:49-82.
313. Enblad P, Adami HO, Glimelius B, Krusemo U, Pahlman L. The risk of subsequent primary malignant diseases after cancers of the colon and rectum. A nationwide cohort study. Cancer 1990;65:2091-2100.
314. McCredie M, Macfarlane GJ, Bell J, Coates M. Second primary cancers after cancers of the colon and rectum in New South Wales, Australia, 1972-1991. Cancer Epidemiol Biomarkers Prev 1997;6:155-160.
315. Neugut AI, Lautenbach E, Abi-Rached B, Forde KA. Incidence of adenomas after curative resection for colorectal cancer. Am J Gastroenterol 1996;91:2096-2098.
316. Sandler RS, Halabi S, Baron JA, Budinger S, Paskett E, Keresztes R, Petrelli N, Pipas JM, Karp DD, Loprinzi CL, Steinbach G, Schilsky R. A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. N Engl J Med 2003;348:883-890.
317. Andre T, Boni C, Mounedji-Boudiaf L, Navarro M, Tabernero J, Hickish T, Topham C, Zaninelli M, Clingan P, Bridgewater J, Tabah-Fisch I, de Gramont A. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. N Engl J Med 2004;350:2343-2351.
318. Andre T, Boni C, Navarro M, Tabernero J, Hickish T, Topham C, Bonetti A, Clingan P, Bridgewater J, Rivera F, de Gramont A. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. J Clin Oncol 2009;27:3109-3116.
319. Desch CE, Benson AB, 3rd, Somerfield MR, Flynn PJ, Krause C, Loprinzi CL, Minsky BD, Pfister DG, Virgo KS, Petrelli NJ. Colorectal cancer surveillance: 2005 update of an American Society of Clinical Oncology practice guideline. J Clin Oncol 2005;23:8512-8519.
320. Engstrom PF, Arnoletti JP, Benson AB, 3rd, Chen YJ, Choti MA, Cooper HS, Covey A, Dilawari RA, Early DS, Enzinger PC, Fakih MG, Fleshman J, Jr., Fuchs C, Grem JL, Kiel K, Knol JA, Leong LA, Lin E, Mulcahy MF, Rao S, Ryan DP, Saltz L, Shibata D, Skibber JM, Sofocleous C, Thomas J, Venook AP, Willett C. NCCN Clinical Practice Guidelines in Oncology: colon cancer. J Natl Compr Canc Netw 2009;7:778-831.
321. Rex DK, Kahi CJ, Levin B, Smith RA, Bond JH, Brooks D, Burt RW, Byers T, Fletcher RH, Hyman N, Johnson D, Kirk L, Lieberman DA, Levin TR, O'Brien MJ, Simmang C, Thorson AG, Winawer SJ. Guidelines for colonoscopy surveillance after cancer resection: a consensus update by the American Cancer Society and US Multi-Society Task Force on Colorectal Cancer. CA Cancer J Clin 2006;56:160-167; quiz 185-166.
322. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin 2009;59:225-249.
323. Salem ME, Yin J, Goldberg RM, Pederson L, Wolmark N, Alberts SR, Taieb J, Marshall J, Lonardi S, Yoshino T, Kerr R, Yothers G, Grothey A, Andre T, De Gramont A, Shi Q.

Evaluation of outcomes over time (1998-2009) of patients (pts) with stage III colon cancer receiving adjuvant FOLFOX: Analysis of 7,230 patients from MOSAIC, C07, C08, N0147, AVANT, and PETACC8 trials in the ACCENT Database ASCO GI Meeting (San Francisco, CA). 2018

324. Grothey, A et al. Duration of Adjuvant Chemotherapy for Stage III Colon Cancer. N Engl J Med 2018;378:1177-1188.
325. DeNardo, D.G. and B. Ruffell, Macrophages as regulators of tumour immunity and immunotherapy. Nat Rev Immunol, 2019. 19(6): p. 369-382.
326. Edin, S., et al., The distribution of macrophages with a M1 or M2 phenotype in relation to prognosis and the molecular characteristics of colorectal cancer. PLoS One, 2012. 7(10): p. e47045.
327. Algars, A., et al., Type and location of tumor-infiltrating macrophages and lymphatic vessels predict survival of colorectal cancer patients. Int J Cancer, 2012. 131(4): p. 864-73.
328. Koelzer, V.H., et al., Phenotyping of tumor-associated macrophages in colorectal cancer: Impact on single cell invasion (tumor budding) and clinicopathological outcome. Oncoimmunology, 2016. 5(4): p. e1106677.
329. Pinto, M.L., et al., The Two Faces of Tumor-Associated Macrophages and Their Clinical Significance in Colorectal Cancer. Front Immunol, 2019. 10: p. 1875.
330. Noy, R. and J.W. Pollard, Tumor-associated macrophages: from mechanisms to therapy. Immunity, 2014. 41(1): p. 49-61.
331. Lan, J., et al., M2 Macrophage-Derived Exosomes Promote Cell Migration and Invasion in Colon Cancer. Cancer Res, 2019. 79(1): p. 146-158.
332. Poh, A.R. and M. Ernst, Targeting Macrophages in Cancer: From Bench to Bedside. Front Oncol, 2018. 8: p. 49.
333. van Dalen, F.J., et al., Molecular Repolarisation of Tumour-Associated Macrophages. Molecules, 2018. 24(1).
334. Alexander, J., et al., Histopathological identification of colon cancer with microsatellite instability. Am J Pathol, 2001. 158(2): p. 527-35.
335. Ogino, S., et al., Lymphocytic reaction to colorectal cancer is associated with longer survival, independent of lymph node count, microsatellite instability, and CpG island methylator phenotype. Clin Cancer Res, 2009. 15(20): p. 6412-20.
336. Rozek, L.S., et al., Tumor-Infiltrating Lymphocytes, Crohn's-Like Lymphoid Reaction, and Survival From Colorectal Cancer. J Natl Cancer Inst, 2016. 108(8).
337. Pages, F., et al., International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. Lancet, 2018. 391(10135): p. 2128-2139.
338. Poh, A.R. and M. Ernst, Targeting Macrophages in Cancer: From Bench to Bedside. Front Oncol, 2018. 8: p. 49.
339. Biswas, S.K. and A. Mantovani, Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol, 2010. 11(10): p. 889-96.
340. Murray, P.J., et al., Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity, 2014. 41(1): p. 14-20.
341. Krausgruber, T., et al., IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. Nat Immunol, 2011. 12(3): p. 231-8.
342. Zhang, Y., et al., Crosstalk between colon cancer cells and macrophages via inflammatory mediators and CD47 promotes tumour cell migration. Eur J Cancer, 2013. 49(15): p. 3320-34.

343. Mima, K., et al., Fusobacterium nucleatum and T Cells in Colorectal Carcinoma. JAMA Oncol, 2015. 1(5): p. 653-61.

APPENDIX I

Collaborative Agreement Language

The celecoxib/placebo used in this protocol is provided to CTEP, DCTD, NCI under a Collaborative Agreement (CSA) between the Pfizer, Inc. (hereinafter referred to as "Collaborator") and the NCI Division of Cancer Treatment and Diagnosis. Therefore, the following obligations/guidelines, in addition to the provisions in the Intellectual Property Option to Collaborator [redacted] contained within the terms of award, apply to the use of the celecoxib/placebo in this study:

1. Celecoxib/placebo may not be used for any purpose outside the scope of this protocol, nor can it be transferred or licensed to any party not participating in the clinical study. Collaborator data for celecoxib are confidential and proprietary to Collaborator and shall be maintained as such by the investigators. The protocol documents for studies utilizing investigational Agents contain confidential information and should not be shared or distributed without the permission of the NCI. If a copy of this protocol is requested by a patient or patient's family member participating on the study, the individual should sign a confidentiality agreement. A suitable model agreement can be downloaded from: [redacted]

2. For a clinical protocol where there is an investigational Agent used in combination with (an)other investigational Agent(s), each the subject of different collaborative agreements, the access to and use of data by each Collaborator shall be as follows (data pertaining to such combination use shall hereinafter be referred to as "Multi-Party Data"):

    a. NCI will provide all Collaborators with prior 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 solely 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. Clinical Trial Data and Results and Raw Data developed under a Collaborative Agreement will be made available exclusively to Collaborator, the NCI, and the FDA, as appropriate and unless additional disclosure is required by law or court order.-Additionally, all Clinical Data and Results and Raw Data will be collected, used and disclosed consistent with all applicable federal statutes and regulations for the protection of human subjects, including, if applicable, the *Standards for Privacy of Individually Identifiable Health Information* set forth in 45 C.F.R. Part 164.

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 for Phase 3 studies must be in accordance with the guidelines and policies of the responsible Data and Safety Monitoring Board (DSMB).

6. Any manuscripts reporting the results of this clinical trial must be provided to CTEP by the Group office for Cooperative Group studies or by the principal investigator for non-Cooperative Group studies for immediate delivery to Collaborator for advisory review and comment prior to submission for publication. Collaborator will have 30 days from the date of receipt for review. Collaborator shall have the right to request that publication be delayed for up to an additional 30 days in order to ensure that Collaborator's confidential and proprietary data, in addition to Collaborator's intellectual property rights, are protected. Copies of abstracts must be provided to CTEP for forwarding to Collaborator for courtesy review as soon as possible and preferably at least three (3) days prior to submission, but in any case, prior to presentation at the meeting or publication in the proceedings. Press releases and other media presentations must also be forwarded to CTEP prior to release. Copies of any manuscript, abstract and/or press release/ media presentation should be sent to:

The Regulatory Affairs Branch will then distribute them to Collaborator. No publication, manuscript or other form of public disclosure shall contain any of Collaborator's confidential/ proprietary information.

APPENDICES II, III, IV
COMPANION STUDIES TO CALGB/SWOG C80702

Correlative Science Investigators:

Charles Fuchs, MD, MPH

Heinz-Josef Lenz, MD

Monica Bertagnolli, MD

Sanford Markowitz, MD, PhD

Najjia N. Mahmoud, MD

Peter W. Laird, PhD

Todd Golub, MD

Shuji Ogino, MD, PhD

Bruce Hollis, PhD

Nader Rifai, PhD

Michael Pollak, MD

Pharmacogenomic Investigators:

Federico Innocenti, MD, PhD

Correlative science projects are planned using various biospecimens:

Appendix II correlative science studies:

Tissue

COX-2 expression
p21 expression
Microsatellite instability
VEGF expression and microvessel density
Interleukin (IL-6) expression
β-catenin expression and localization
FOXP3, CD3, CD8, CD45RO
15-PGDH
Next-generation sequencing (NGS) to ascertain tumor mutation status of *RAS, BRAF, PIK3CA, and other somatic events*

Vitamin D receptor (VDR) expression
1-α-hydroxylase expression

Genome-wide expression profiling
CpG island methylation/CIMP
BRAF mutational status
LINE-1 methylation

Blood

Markers of inflammation including CRP, IL-6, sTNFα-R2
Plasma levels of 25-hydroxyvitamin D_3

Appendix III pharmacogenetic studies:

Blood

Genome-wide genotyping and analyses of vitamin D pathway genes (VDR, VDBP, CYP27B1, RXR, CYP24A1)
UGT1A6, CYP2C9, COX-2 (PTSG-2, COX-1 (PTSG-2), PPAR-gamma, thromboxane synthase, NF-kappa B1, prostacyclin synthase, and 5-lipoxygenase genotyping
AGXT 154C>T polymorphisms

Introduction to Companion Studies CALGB 150911 and CALGB 60905

Current concepts and clinical practice regarding prognosis and therapy for patients with colon cancer rest on the gross clinical/pathological staging. Identification of molecular determinants of drug efficacy and toxicity to chemotherapies might become important in the design of individualized chemotherapy based on the individual's molecular tumor and genomic profiles. The goals of these correlative projects are to identify germline variations and gene expression levels and arrays associated with clinical toxicity and outcome in patients treated with FOLFOX therapy with or without celecoxib.

These appendices describe pathways as well as state-of-the-art technologies at the time of protocol activation. However the knowledge base will increase significantly during the course of this study along with development of improved and novel technologies. Analyses of the samples collected within this protocol will incorporate new discoveries and new technologies. Tissue and blood samples will be collected and stored at the Alliance Biorepository at Ohio State University (OSU). The priority of molecular analyses as well as the laboratories chosen for these analyses will be identified by chair (Dr. Fuchs) of correlative science of this protocol who will closely work with participating investigators to assure the highest quality of analyses.

The molecular data generated from the C80702 samples will be sent, in a computer readable format using secure means to the Alliance Statistics and Data Center at Duke University. This transmission is not limited to pre-processed data generated by the lab. The raw (e.g., *.CEL, *.idat or *.sproc) files will be submitted to the Alliance Statistics and Data Center at Duke University. Along with this transmission, the lab will provide a table which at the minimum will provide the following information for each sample received from the repository:

- the Lab ID number that the Statistical Center can use to decode against the registration database to get patient ID number;
- the date at which the specimen was received from the repository;
- the date at which the sample was processed.

Additionally, the lab will also provide the complete results from any quality control measures carried out if requested by the Alliance Statistics and Data Center at Duke University. The lab will report any QC issues encountered directly to the Alliance Statistics and Data Center at Duke University. If a sample had to be redone (e.g., defective or poor quality), the lab will provide the results from both replications and add an appropriate column to the table.

The lab will commit to handle any molecular data generated from Alliance samples in a safe, secure (e.g., HIPAA compliant) and organized fashion.

APPENDIX II

CORRELATIVE SCIENCE COMPANION STUDIES: CALGB 150911

1.0 OBJECTIVES

1.1 To assess molecular features within the tumor that influence the efficacy of celecoxib as adjuvant therapy for stage III colon cancer.

1.2 To assess whether markers of systemic inflammation in blood can predict the efficacy of celecoxib as adjuvant therapy for stage III colon cancer.

1.3 To assess the influence of baseline plasma 25(OH)-vitamin D level on disease-free and overall survival in patients with stage III colon cancer.

1.4 To assess whether tumoral expression of vitamin D receptor (VDR) and 1-α-hydroxylase and KRAS mutational status modifies the relation between baseline plasma 25(OH)-vitamin D level on patient outcome.

1.5 To determine an mRNA expression signature that is predictive of disease-free survival among patients with stage III colon cancer

1.6 To determine an mRNA expression signature that predicts efficacy of celecoxib as adjuvant therapy for patients with stage III colon cancer

1.7 To determine if CpG island methylator phenotype (CIMP) is an independent predictor of DFS in patients with stage III colon cancer

1.8 To determine the association between cytotoxic and memory T cell infiltration and density of immunosuppressive M2 macrophages and survival outcomes in stage III colon cancer and whether the associations are stronger for patient treated with celecoxib who have increased cytotoxic and memory T cell infiltration and lower M2 macrophase density.

1.9 To perform whole-genome sequencing of paired tumor/normal DNA and methylation-sequencing of tumor DNA and obtain a more complete characterization of CRC.

1.10 To conduct a metagenomic and 16S RNA analyses of tumor-colonizing bacteria in FFPE primary tumors.

2.0 BACKGROUND

2.1 Tumoral molecular alterations associated with the efficacy of COX-2 inhibition

Celecoxib, at least in part, prevents colorectal neoplasia through inhibition of cyclooxygenase-2 (COX-2), the rate-limiting step for the conversion of arachidonic acid to prostaglandins and related eicosanoids.[44,45] COX-2 promotes inflammation and cell proliferation,[46] and is overexpressed in the majority of human colorectal cancers.[47,48] Overexpression of COX-2 in tumor tissue has been associated with a poorer prognosis among colorectal cancer patients in some,[48-51] but not all studies.[52,53] In addition, intratumoral expression of COX-2, has been independently associated with tumor differentiation,[52] angiogenesis,[54,55] recurrence,[56] and metastasis.[57] Moreover, COX-2 expression has been correlated with worsened patient survival in some, [48-51,54] but not all studies.[52,53,58,59] In a large prospective cohort study, regular aspirin and NSAID use was found to be associated with a greater reduction in the risk of COX-2 overexpressing colorectal cancer, whereas regular aspirin and NSAID use only modestly reduced the risk of COX-2 negative tumors. Therefore, in the current study of patients with stage III colon cancer, it is hypothesized that the benefit associated with adjuvant celecoxib use will be greater for patients with COX-2 overexpressing cancers.[44]

In light of the interaction between energy excess and inflammation, recent evidence suggests that post-diagnosis celecoxib use could confer a greater survival benefit in colorectal cancer patients whose tumors manifested constitutive activation of energy balance pathways through *PIK3CA* mutation. Among colorectal cancer patients in two large cohort studies, post-diagnosis aspirin use was associated with superior survival in patients with mutant *PIK3CA* (HR = 0.52; 95% CI, 0.30-0.91) but not in those with PIK3CA wild-type CRC (HR =0.91; 95% CI, 0.73-1.14; P for interaction = 0.01). As such, an assessment of the impact of celecoxib on patient survival according to specific somatic mutations, including PIK3C, RAS, and BRAF, among driver event in colorectal cancer pathogenesis would inform future studies and clinical practice. Next generation sequencing (NGS) technology is now routinely used in formalin-fixed paraffin embedded (FFPE) tumor specimens and provides a clinically available cancer genomic assay to detect somatic mutations, copy number variations and structural variants in tumor DNA. Whole exome sequencing can now be routinely performed on tumor/normal pairs from FFPE samples. Extracted DNA is fragmented, adapter-ligated, barcoded, and subjected to solution-phase hybridization with probes designed to enrich selected gene regions. Enriched library fragments are sequenced (2×76 base-paired end) using sequencing-by-synthesis chemistry and the Illumina HiSeq 2500 sequencer. Sequence data are aligned to the specified National Center for Biotechnology Information human reference sequence after low-quality sequences are discarded. Reads that align to more than one region of the reference genome, reads with low alignment scores, and bases with low-quality scores are excluded from variant calling. Sequencing data are analyzed to identify somatic and germ-line point mutations, small insertions/deletions (indels), and copy-number alterations.

Interleukin-6 (IL-6) and its receptor are highly expressed in colorectal carcinoma and colorectal cancer cell lines, but not in normal colon tissue. IL-6 inhibits apoptosis through downstream activation of STAT-1.[60] Administration of NSAIDs block IL-6 mediated STAT-1 activation.[60] This relationship between IL-6 and COX inhibition suggests the importance of examining interactions between IL-6 levels, aspirin and NSAID use, and cancer outcomes. It is hypothesized that the effect of COX inhibition on patient outcome may vary by the level of expression of IL-6 in the tumor. Preclinical models suggest that aspirin and NSAIDs induce the expression of p21, thereby influencing colorectal cancer tumorigenesis.[61] In mice, inactivation of p21 increased tumor formation in a gene-dose-dependent manner.[62] Moreover, inactivation of p21 completely eliminated the ability of NSAIDs to inhibit tumor formation.[61] It is therefore hypothesized that the efficacy of a COX inhibitor on patient outcome may be modified by the level of tumoral p21 expression.

Alternative molecular markers in COX-2-related pathways may also be informative. For example, aspirin may function to disrupt COX-2 through modulation of expression and localization of β-catenin.[63,64] COX-2 has also been hypothesized to mediate its neoplastic influence through the promotion of angiogenesis, potentially through expression of vascular endothelial growth factor (VEGF),[65-67] and as measured by microvessel density.[68] Thus, the association between intratumoral markers such as β-catenin, VEGF, and microvessel density will be examined to determine the importance of these alternative COX-2-related pathways in predicting celecoxib efficacy**.**

Recently, 15-prostaglandin dehydrogenase (15-PGDH), a prostaglandin degrading enzyme, was demonstrated to function as an endogenous inhibitor of the colonic COX-2 pathway and as a tumor suppressor gene.[69,70] Gene knock-out of 15-PGDH conferred near complete resistance to celecoxib colon tumor prevention in mice.[71] 15-PGDH is highly expressed in normal colon mucosa, but expression is lost in human colorectal cancers.[69,72] Preliminary data suggest that normal tissue 15-PGDH expression is an accurate predictor of celecoxib anti-tumor response.[71] Specimens containing normal rectal mucosa from 16 patients were examined on the APC trial, a study that randomized patient at high risk for colon adenoma development to treatment with

celecoxib or placebo. In a subset of these patients, 2 mm biopsies of normal and adenomatous rectal mucosa were obtained. Measurement of pre-treatment 15-PGDH transcript levels by real-time PCR showed a 5.4-fold variation from lowest to highest 15-PGDH mRNA expression across these 16 individuals (median: 3.4, mean: 4.4, range: 2.1-11.4). Post-treatment colonoscopy showed that four of these patients were resistant to celecoxib, as evidenced by development of new adenomas. All of the patients failing treatment had colonic 15-PGDH levels below the median of the cohort (p=0.008).[71] Virtually identical results were obtained when these tissues were examined using IHC or RT-PCR to detect 15-PGDH levels in FFPE tissue. In summary, it was found that 15-PGDH activity can determine sensitivity or resistance to the selective COX-2 inhibitor, celecoxib, and preliminary data from a human clinical trial indicate that low levels of 15-PGDH in disease target tissue predict failure of celecoxib anti-tumor efficacy. These observations imply that measurement of 15-PGDH may be clinically useful in selecting patients most likely to benefit from treatment with COX-2 inhibitors. It is predicted that patients treated with celecoxib in the adjuvant setting who have low levels of 15-PGDH in their normal intestinal tissue at baseline will have reduced DFS and OS compared to patients with high levels of 15-PGDH. In each patient, 15-PGDH levels will be examined by both IHC and gene expression, as described below.

It has been known for many years that lymphocytic infiltrate surrounding primary colorectal cancer is associated with improved prognosis.[73] Although the mechanism remains unclear, the adaptive immune system is thought to play an important role in suppressing the progression of this disease. A high density of $CD8^+$ T cells has been associated with the absence of tumor invasion, earlier stage, and improved patient survival.[74,75] Using CD3 as a universal marker of T cells, the ratio of T-cell density at the advancing tumor margin compared with the central core was recently proposed as having stronger prognostic significance than conventional TNM staging.[76] In addition, $CD45RO^+$ cells include both $CD4^+$ and $CD8^+$ lymphocytes that have been exposed to antigen. Pages et al.[74] subsequently demonstrated that a high density of $CD45RO^+$ cells within the tumor was associated with decreased invasiveness, lower stage, and improved survival. Finally, regulatory T cells (Tregs) suppress the activity of cytotoxic T cells; the most specific Treg cell marker identified to date is the nuclear transcription factor known as FOXP3.[77,78] A high density of tumor-infiltrating $FOXP3^+$ Tregs has been associated with outcome in patients with colorectal cancer.[73] The interrelationship between COX inhibition, T cell infiltrate, and patient survival has not been comprehensively investigated. It is hypothesized that the influence of adjuvant celecoxib on patient outcome may be modified by the inflammatory infiltrative pattern (CD3, CD45RO, CD8 and FoxP3 staining). Greater abundance of tumor-associated macrophages (TAMs), another critical component of tumor immunity, has also been associated with improved CRC survival.[325-329] M1 and M2 types of TAMs have been divergently linked to immune stimulatory and suppressive functions, respectively.[330-333] We will perform tissue-based microbial and immune analyses of CRCs to understand the interaction between the immune system with CRC. Newer in-situ tissue-based immune cell analysis methods preserve spatial information of the tumor microenvironment. Compelling evidence shows differences in the biological effects of immune cells depending on their location in tumor tissue compartments.[334-337] Multiplex immunofluorescence (IF) assays have been optimized for formalin-fixed paraffin-embedded (FFPE) tissue and can identify T cell and macrophage subsets. Multiplex T cell panel includes antibodies for CD3, CD4, CD8, ITGAE (CD103), FOXP3, and KRT (cytokeratins), while macrophage panel includes antibodies for CD68, CD86, CD163, IRF5, MRC1 (CD206), and KRT. Further, a newly-developed Tumor Immune Partitioning and Clustering (TIPC) classification algorithm can assess spatial patterns of tumor and immune interactions in a manner that is independent of tumor morphology and immune cell density.

At the same time, emerging evidence indicates a complex link between gut microbiome, immunity, and intestinal tumorigenesis. Experimental evidence suggests that Fusobacterium nucleatum may promote colonic neoplasia development by downregulating antitumor T cell–mediated adaptive immunity. In a large prospective study of colorectal cancer patients, the presence of F. nucleatum was inversely associated with the density of CD3+ T cells (for a unit increase in quartile categories of CD3+ T cells as an outcome: multivariable odds ratio, 0.47 [95% CI, 0.26-0.87]; P for trend = .006). As such, the presence of Fusobacterium nucleatum in colorectal cancers may modify the tumor inflammatory infiltrate, and, in the process, modify the influence of adjuvant celecoxib on patient outcome.

Lastly, Ruschoff et al. observed, *in vitro*, a marked reduction in microsatellite instability (MSI) during exposure to aspirin or sulindac.[79,80] The effect was reversible, dose dependent, and independent of cyclooxygenase function. The mechanism appeared to be via a genetic selection that enhanced apoptosis in cells undergoing MSI. It is therefore hypothesized that the benefit associated with celecoxib would be greater in microsatellite unstable (MSI-high) tumors.

2.2 Plasma inflammatory factors and colon cancer

Chronic inflammation is characterized by abnormal production of cytokines and inflammatory factors which have been causally linked to obesity, diabetes, and cancer. In humans, cytokines such as C-reactive protein (CRP), interleukin-6 (IL-6), and soluble tumor necrosis factor-α - receptor 2 (sTNFα-R2) not only mediate the inflammatory response, but also serve as potential biomarkers of chronic inflammation and inflammation-related diseases. CRP levels correlate with the metabolic syndrome, triglyceride levels, obesity, fasting glucose, insulin sensitivity,[81] diabetes mellitus,[82] as well as major dietary patterns that have been associated with a higher risk of colorectal cancer.[83] Elevated baseline plasma CRP levels have been associated with an increased risk of colorectal neoplasia in some, though not all studies.[84-88] Two studies found that elevated prediagnostic CRP predicted a 2-fold increase in colorectal cancer,[85,87] while two studies found no association.[86,88] Interleukin-6 (IL-6) is a related inflammatory cytokine that may also be associated with colorectal cancer risk.[87] As described above, administration of NSAIDs block IL-6 mediated STAT-1 activation.[60] This relationship between IL-6 and COX inhibition suggests the importance of examining interactions between IL-6 levels, aspirin and NSAID use, and cancer outcomes. It is hypothesized that stage III colon cancer patients with elevated baseline circulating levels of C-reactive protein (CRP), interleukin-6 (IL-6), and soluble tumor necrosis factor-α-receptor 2 (sTNFα-R2) will experience a greater benefit with use of adjuvant celecoxib.

2.3 Influence of plasma 25(OH)D levels on colon cancer

The best indicator of vitamin D status is plasma 25(OH)D, since it reflects not only skin exposure to ultraviolet-B (UV-B) light and total vitamin D intake, but also cholecalciferol production in the skin and hydroxylation of all sources of cholecalciferol in the liver.[89] Prospective studies have shown that individuals with higher plasma levels of 25(OH)D experience a significant reduction in risk of colorectal cancer when compared to those with low plasma level.[90-95] A meta-analysis of five epidemiologic studies found a 51% decrease in the risk of colorectal cancer associated with plasma 25(OH)D levels in the highest quintile compared to those in the lowest quintile ($P<0.0001$).[96] Furthermore, a randomized placebo-controlled trial of vitamin D and calcium supplementation in postmenopausal women demonstrated a 60% decrease in all-cancer risk (including colorectal cancer) in the intervention arm ($P<0.03$).[97]

In contrast, the influence of vitamin D on survival of patients with established colorectal cancer remains uncertain. A large observational study in Norway found that people diagnosed with colorectal cancer in the summer and autumn, when 25(OH)D concentrations are highest, had a significantly better survival than those diagnosed in the winter.[98, 99] The authors speculated that

a high circulating 25(OH)D at the time of diagnosis, and possibly during initial treatment, may improve cancer prognosis. However, this study was limited by its use of season of diagnosis – an indirect indicator of vitamin D status – as the primary exposure.

In a recently completed prospective analysis of 304 colorectal cancer patients in the Nurses' Health Study (NHS) and Health Professionals Follow-Up Study (HPFS), Ng et al. found that increasing circulating levels of 25(OH)D were associated with a significant reduction in overall mortality (P for trend = 0.02).[100] Compared with levels in the lowest quartile, patients with 25(OH)D levels in the highest quartile had an adjusted hazard ratio (HR) of 0.52 (95% confidence interval [CI], 0.29 to 0.94) for overall mortality. The results remained unchanged after excluding patients diagnosed within five years of blood collection (P for trend = 0.04); the adjusted HR for overall mortality comparing extreme quartiles was 0.45 (95% CI, 0.19 to 1.09). Moreover, the benefit associated with vitamin D appeared greater among patients with more advanced disease. Therefore, the influence of plasma levels of 25(OH)D on disease-free and overall survival will be examined in a large population of patients with stage III colon cancer who are enrolled in a clinical trial of adjuvant chemotherapy.

2.4 Tumoral vitamin D receptor expression and KRAS mutational status

The vitamin D receptor (VDR) and 1-α-hydroxylase (CYP27B1) are frequently expressed in colon cancer cells. Well-differentiated colon cancer cell lines have higher VDR expression,[101] and the antiproliferative effects of vitamin D may only occur in cell lines expressing high levels of VDR.[102] Expression of VDR and CYP27B1 increases in the early stages of colorectal tumorigenesis, but appears to decline in poorly-differentiated tumors and metastases.[103]

Point mutations in the KRAS oncogene occur in approximately 40% of colorectal cancers.[104-106] Recent data indicate that KRAS mutations may be associated with lack of response to epidermal growth factor receptor (EGFR)-targeting agents.[107-110] Interestingly, the vitamin D pathway may interact with KRAS signaling. In a RAS-transformed cell line of human keratinocytes, malignant cells were found to be resistant to the growth-inhibitory effects of $1,25(OH)_2D$.[111,112] Furthermore, VDR expression appears to be down-regulated in KRAS-mutated cell lines,[113] and vitamin D's ability to affect apoptosis may vary by KRAS status.[114]

These data suggest that the improved patient survival associated with higher circulating vitamin D may be greater in patients with tumoral VDR and CYP27B1 overexpression and diminished for those with KRAS-mutated tumors. It will be assessed whether the relationship between vitamin D and patient survival is modified by VDR and CYP27B1 expression and KRAS mutational status.

2.5 Gene expression profiling

A technical challenge facing a more thorough exploration of gene expression profiling approaches to colon cancer outcome prediction has been the lack of suitable patient material for such genomic analyses. Current genome-wide expression profiling methods require frozen tissue for analysis, whereas tissue banks accompanied by long-term clinical outcome are generally populated only by formalin-fixed specimens that are not amenable to microarray-based expression profiling. Even today, the vast majority of patient specimens are formalin-fixed; the collection of frozen tissues has yet to permeate routine clinical practice. Thus, a genomic profiling method suitable for fixed tissues would have the potential for significant translational and clinical impact. Whole genome mRNA expression profiles in colon tumor tissue will be interrogated to identify patterns of genes dysregulated in tumors. Various technologies have been developed to deduce and quantify the transcriptome, including hybridization-or sequence-based approaches. Hybridization-based approaches typically involve incubating fluorescently labelled cDNA with custom-made microarrays or commercial high-density oligo microarrays. Specialized microarrays have also been designed; for example, arrays

with probes spanning exon junctions can be used to detect and quantify distinct spliced isoforms. However, these methods have several limitations, which include: reliance upon existing knowledge about genome sequence; high background levels owing to cross-hybridization; and a limited dynamic range of detection owing to both background and saturation of signals. Moreover, comparing expression levels across different experiments is often difficult and can require complicated normalization methods. In contrast, RNA-Seq is an alternative approach to transcriptome profiling that uses deep-sequencing technologies and provides a far more precise measurement of levels of transcripts and their isoforms than other methods. Investigators at the Broad Institute and the Dana-Farber Cancer Institute have developed a methodology to routine conduct RNA-seq to profile the transcriptome from routine FFPE tumor specimens. These studies will allow a detailed examination of the impact of tumoral gene expression on patient outcome as well as an assessment of how tumoral gene expression may modify the influence of adjuvant celecoxib on patient survival.

2.6 CpG island methylation

Aberrant DNA methylation of CpG islands has been widely observed in both benign and malignant human colorectal tumors and is associated with gene silencing when it occurs in promoter areas. A subset of colorectal tumors has been described to have an exceptionally high frequency of methylation of some CpG islands, leading to the definition of a distinct trait referred to as "CpG Island Methylator Phenotype", or "CIMP".[118] However, the lack of a consistent definition of CIMP has contributed to conflicting reports of its existence.[118-120] An improved panel of five markers to classify CIMP+ tumors has been identified and it was found that CIMP represents a distinct trait with a remarkably tight association with somatic mutation of the BRAF oncogene and that sporadic cases of mismatch repair deficiency occur almost exclusively as a consequence of CIMP-associated methylation of MLH1.

The CpG Island Methylator Phenotype was first proposed by Dr. Jean-Pierre Issa's group in 1999 for a distinct subset of colorectal tumors with an exceptionally high frequency of methylation of "Type C" loci, which were defined as loci methylated in cancer, but not in normal tissues.[118] In essence, CIMP represents concordant methylation of a subset of CpG islands in a subset of tumors. In subsequent studies, the CIMP trait was found to be associated with a variety of clinical, histopathological and epidemiological characteristics,[121] but the initially reported bimodal distribution of methylation frequency was often not observed. Furthermore, several carefully conducted studies concluded that cancer-specific DNA hypermethylation occurs across a continuous frequency spectrum and that the designation of a distinct CIMP subgrouping would be arbitrary.[118-120] These discrepant results stem largely from the use of varying sets of methylation markers used to screen the colorectal tumors.

The initial definition of CIMP was based on concordant methylation of Type C loci, and specifically excluded markers that showed evidence of age-associated methylation in normal tissues, referred to as "Type A" loci.[118] However, the distinction between Type C and Type A loci has not held up particularly well, with some Type A loci showing Type C methylation behavior in other tissues,[122-124] and many Type C loci showing detectable methylation in normal tissues.[122,125-128] More importantly, some authentic Type C loci do not show concordant methylation with classic CIMP markers,[129-131] suggesting that CIMP is not an indiscriminate increase in global CpG island hypermethylation, but may represent one or more distinct defects in epigenetic control, each affecting only a subset of CpG islands in a subset of tumors.[121,132] If this is indeed the case, then unsupervised two-dimensional clustering of large numbers of markers and tumor samples would reveal the existence of these distinct correlated subsets. An automated real-time PCR based MethyLight technology has been established to determine clustered DNA methylation behavior and a final panel of five markers has been determined for CIMP classification. MLH1 showed detectable methylation in normal mucosae using

MethyLight. It is evident that the CIMP+ cases display an increased frequency and intensity of cancer-associated DNA methylation.

Since the assembly of our new five-marker panel contributed to the original CIMP classification, the assessment of its performance compared to our temporary standard could be biased. The new panel of five markers performs very well in all cross-panel comparisons. It outperforms the panel of classic CIMP loci in every comparison. It even gives lower misclassification error than the classic panel against a panel of 14 markers that includes the classic panel, but excludes the new panel.

An alternative strategy to evaluate the performance of panels is to compare their associations with characteristics of colorectal cancer that have previously been reported to be associated with CIMP+ status. It is assumed that if this association reflects an important underlying biological relationship, then a superior CIMP classification would result in a tighter association. A significant correlation between CIMP+ and location of the tumor, BRAF, KRAS and MSI status could be demonstrated. Of note, MSI has been associated with an improved survival in colorectal cancer whereas mutations in BRAF have been associated with an inferior survival.[133] Moreover, beyond methylation at CpG islands in promoter regions (CIMP), global DNA methylation, as measured by methylation in repetitive long interspersed nucleotide element-1 (LINE-1) elements, may have an independent effect on patient outcome. Using 643 colon cancers in two independent prospective cohorts, DNA methylation in repetitive long interspersed nucleotide element-1 (LINE-1) elements was quantified using Pyrosequencing, as an indicator of global DNA methylation level.[134] LINE-1 hypomethylation was associated with a significant increase in colon cancer–specific mortality ($P_{trend} < .001$) and overall mortality ($P_{trend} = .002$). The association was independent of MSI, CIMP and BRAF status.[134] As such, it will be interesting to dissect the separate clinical and etiological features associated with mismatch repair deficiency, CIMP, LINE-1 methylation, proximal tumor location, and BRAF mutation status.

A recent study demonstrated that CIMP+ was an independent predictive marker for survival benefit from 5-FU chemotherapy in colorectal cancer.[308] The folate status is critically important to the provision of methyl groups and may be relevant to the CpG-island methylator phenotype (CIMP).[309] Preliminary data suggest that the folate pool in colorectal cancers has been associated with promoter-specific DNA hypermethylation and polymorphisms within the MTHFR gene. Recent data suggested that the haplotype with low enzyme activity of MTHFR is linked with promoter hypermethylations and modifies the risk of the CIMP+ proximal colon cancer development in the Japanese people.[310] Thus, an opportunity exists to perform comprehensive pharmacogenetic investigations and integrate the genetic folate status and DNA repair capacity in conjunction with MSI and CIMP status as predictors of tumor recurrence.

3.0 HYPOTHESES

3.1 The benefit associated with adjuvant celecoxib in patients with stage III colon cancer is significantly modified by tumoral COX-2, VEGF, p21, IL-6, beta-catenin, 15-PGDH, T cell overexpression, higher microvessel density, and microsatellite instability.

3.2 Higher baseline circulating levels of C-reactive protein (CRP), interleukin-6 (IL-6), and soluble tumor necrosis factor-α-receptor 2 (sTNFα-R2) is associated with a greater benefit with use of adjuvant celecoxib in stage III colon cancer.

3.3 Higher baseline levels of plasma 25-hydroxyvitamin D_3 [25(OH)D] are associated with improved disease-free and overall survival in patients with stage III colon cancer.

3.4 The improved patient survival associated with higher circulating 25(OH)D is greater in patients with tumoral VDR and CYP27B1 overexpression, and diminished for those with KRAS-mutated tumors.

3.5 Unique mRNA expression signatures are predictive of disease-free survival among patients receiving adjuvant chemotherapy for stage III colon cancer.

3.6 Unique mRNA expression signatures will predict stage III colon cancer patients most likely to benefit from celecoxib.

3.7 CpG island methylator phenotype (CIMP) is an independent predictor of tumor recurrence in patients with stage III colon cancer.

3.8 Specific spatial patterns of T cell subset infiltration in CRC, as measured by the Tumor Immune Partitioning and Clustering (TIPC) computational algorithm, are associated with better patient survival, and this association is stronger for CRC harboring F. nucleatum. Associations will be stronger for patients treated with celecoxib and increased cytotoxic and memory T cell infiltration and lower M2 macrophase density.

4.0 METHODS

4.1 Tumoral molecular alterations associated with the efficacy of COX-2 inhibition

Tissue blocks procurement: Formalin-fixed/paraffin-embedded tissue blocks and the corresponding pathology slides will be acquired from patients enrolled on C80702 who consent to CALGB 150911. The tissue blocks will be initially inspected by Alliance Biorepository at Ohio State University (OSU) pathologists to ensure that the pathology report and tissue blocks are adequately identified and logged into the CALGB database. After review of the pathology report, a set of blocks will be selected which provide appropriate specimens for a) preparation of routine H and E slides for microdissection and DNA extraction; b) preparation of routine H and E slides for microdissection and RNA extraction for transcriptomic analyses; c) construction of tissue microarrays and immunohistochemical evaluation; d) establishment of a bank of TMA blocks and slides for future molecular and cellular studies; and e) establishment of a DNA bank for future assays.

Tissue DNA and RNA extraction: In the laboratory of Dr. Shuji Ogino (Dana-Farber Cancer Institute, Boston, MA), H&E stained slides will be reviewed, areas comprising at least 70% neoplastic cellularity will be marked and scraped from 15μm unstained slides under direct visualization, and DNA and RNA will be extracted.

Tumoral mutational analysis: We will utilize a cancer genomic assay using Next Generation Sequencing technology to detect somatic mutations, copy number variations and structural variants in tumor DNA extracted from FFPE samples. Extracted DNA is fragmented, adapter-ligated, barcoded, and subjected to solution-phase hybridization with probes designed to enrich selected gene regions. Enriched library fragments are sequenced (2 × 76 base-paired end) using sequencing-by-synthesis chemistry and the Illumina HiSeq 2500 sequencer. Sequence data are aligned to the specified National Center for Biotechnology Information human reference sequence after low-quality sequences are discarded. Reads that align to more than one region of the reference genome, reads with low alignment scores, and bases with low-quality scores are excluded from variant calling. Sequencing data are analyzed to identify somatic and germ-line point mutations, small insertions/deletions (indels), and copy-number alterations.

While coding DNA mutations in CRC have been annotated from whole-exome sequencing data from TCGA, there is limited information on somatic mutations in non-coding regions, which span 98% of the human genome. They include untranslated regions, introns, promoters, regulatory elements, repetitive regions and mitochondrial genomes. Somatic structural variants (large deletions/insertions, inversions, duplications, translocations), pathogen (virus and bacteria) presence, and alternative splicing in CRC also remain widely unexplored for their effects on survival. WGS can cover all of these unexplored alterations and help us to better understand the complete landscape of CRC genomes. We will adopt a high-throughput and high-

output sequencing platform for WGS. Using an Illumina Novaseq, DNA libraries will be produced from tumor and germline samples using the Illumina Nextera DNA library preparation (or a comparable) kit. This kit can be used to prepare up to 96 uniquely indexed paired-end libraries of genomic DNA. The library preparation process will fragment the DNA input (which might vary depending upon the protocol used) and then add adapter sequences onto the template DNA for resultant sequencing on the Illumina platform. The libraries can be then sequenced using 2x100 bp paired-end sequencing to achieve an average depth of coverage of 60 reads for tumor samples (deep coverage) and 30 reads for germline samples (standard coverage). After sequencing, the quality of the raw sequencing data will be assessed using established tools and then the sequencing data will be mapped to the human genome (GRCh38) using established mapping tools, such as BWA.

Assessment for tumoral presence of *Fusobacterium nucleatum*: Tumor genomic DNA is extracted from whole-tissue sections of FFPE tissue blocks and custom TaqMan primer-probe sets (Applied Biosys- tems) for the 16S ribosomal RNA gene DNA sequence of F nucleatum and for the reference gene, SLCO2A1, are used. The primer-probe set for F nucleatum was designed to target the nusG gene of F nucleatum, and it has been demonstrated that the amount of F nucleatum measured by the quantitative PCR assay highly correlates with that measured by using transcriptome sequencing data (Pearson r = 0.97). Each specimen is analyzed in duplicate for each target in a single batch, and we use the mean of the 2 Ct values for each target.

Assessment for tumoral presence of gut microbiota: To have a complete assessment of the diversity and composition of the microbiota colonizing the tumor, we will assay tumor-associated microbiota using shotgun metagenomic and 16S rRNA sequencing. From FFPE tumors, extraction will be performed following procedures specifically adapted for extraction of bacterial DNA from FFPE tumors. DNA will be extracted from up to 12 slides for each sample at 5-10 μm of thickness (one H&E slide will be also obtained). This material is enough to obtain the required amount of total DNA for the assays described below. FFPE slides will undergo paraffin removal prior to DNA isolation. For shotgun metagenomic sequencing, DNA isolation includes a bacterial DNA enrichment step. Three portions of the block (top, middle, and bottom) will be used to cut slides from each sample. To avoid batch effects all samples will be processed within the same batch and with the same lot of reagents. To improve the quality and reproducibility of microbiome analyses, microbial reference materials will be used. In every lane of barcoded samples, it will be used: a ZymoBIOMICS Microbial Community Standard sample as a positive control, and several negative controls: water extraction control, a water PCR control, and a blank paraffin control.

16S rRNA sequencing will be performed following standard procedures, using 12.5 ng of total DNA. All libraries will be multiplexed on a single Illumina MiSeq flowcell using a 250x250 PE sequencing run. FASTQ files will be evaluated using FastQC and MultiQC for quality evaluation and to determine trimming parameters. FASTQ files will be bioinformatically processed with DADA2 package in R following the developer-designed workflow to generate ASV tables, and to assign ASVs to taxa using the SILVA reference database. Host mitochondrial contaminants will be removed based on the taxonomic assignment.

Shotgun sequencing will be performed following standard procedures, using 5 ng of genomic DNA. The Sunbeam pipeline will be used to perform basic quality control: read quality assessment, adapter removal, filtering of low-complexity, and human genomic contamination. Filtered and trimmed reads will be taxonomically classified using Kraken2 and Bracken will be used to re-estimate the abundances at the genus and phylum level.

We will use data from the negative control samples to detect and eliminate bacterial contamination introduced during FFPE block preparation, DNA isolation, or library preparation.

We will test several tools: Decontam, SourceTracker, and methods specifically designed for FFPE microbiome samples.

Standard microbiome data visualization and statistical analysis will be performed on the 16S rRNA and shotgun metagenomic data in R using the phyloseq package. This analysis will include calculation of alpha diversity, visualization of relative abundances, and ordination to visualize of sample distances.

Determination of Microsatellite Instability (MSI): Previously published methods of DNA fragment analysis will be used to determine MSI (Bethesda 10 marker panel).[135] PCR and DNA fragment analysis for all of the markers except for D2S123, D5S346, and D17S250, will be performed in duplicate. "High degree of MSI" (MSI-H) will be defined as having instability in 30% or more of the markers. "MSI-low (MSI-L)" will be defined as having instability in less than 30% of the markers, and "microsatellite stability (MSS)" as having no unstable marker.

Construction of tissue microarrays (TMAs): The use of TMAs allows us to perform a high throughput screen of all available colon cancers in our study population. Construction will be conducted in the OSU. TMAs will be constructed using the Beecher Automated Arrayer. Two 0.6 mm tissue cores each from a tumor and normal mucosa is placed in each TMA block, and four duplicate blocks will also be constructed. Each TMA block will have a total of 400 cores (100 tumors).

Immunohistochemical staining and interpretation: Immunohistochemical analyses will be performed in the laboratory of Dr. Ogino. For p21, β-catenin, IL-6, and VEGF, monoclonal antibodies will be applied to the tumor sections: monoclonal anti-p21 (Pharmingen) dilution 1:50; β-catenin clone 14 (Transduction Laboratories) dilution 1:400; polyclonal antisera (R & D Systems, Abingdon, UK) directed against IL-6, or a polyclonal antisera directed against VEGF 165 (Santa Cruz Antibodies). Biotinylated rabbit anti-mouse antibody (DAKO; code E354) will be used as secondary antibody, and the immunoreaction will be visualized by avidin-biotin complex. Tumors with >10% nuclear expression of p21/WAF1/CIP1 will be considered positive. The κ coefficient between the two observers was 0.62 for p21 ($p<0.0001$; N=179), indicating substantial agreement.

For IL-6, the distribution will be scored according to the numbers of positive cells: none (not stained), 0; focal (<one-third of cells stained), 1; multifocal (<two-thirds of cells stained), 2; and diffuse (most cells stained), 3. The staining intensity will be scored as: none (not stained), 0; mild (between 0 and 2), 1; and strong, 2. The distribution and intensity scores are then added to produce the following grades for the staining: 0, negative; 1 and 2, intermediate; and 3, 4 and 5, positive.

The fraction of cells demonstrating cytoplasmic staining for VEGF will be measured and recorded. Tumors with >10% VEGF staining cells will be considered positive. Microvessel density will be determined in the leading edge of the tumor in an area of apparent highest vessel density following anti-CD31 staining.[136] For this analysis, microvessel density will be coded as a dichotomous variable. Tumors with greater than 28 vessels per 100X field will be considered high.

For β-catenin, normal colonic epithelial cells serve as an internal positive control with membrane staining. Cytoplasmic and nuclear expressions will be recorded separately as either no expression (0), weak expression (1+), or moderate/strong expression (2+). β-catenin activation score will be calculated as the sum of nuclear score (0-2), cytoplasmic score (0-2) and membrane score (0 if membrane staining was positive, +1 if membrane expression was lost), as originally described by Jass et al.[137]. Appropriate positive and negative controls were included in each run of immunohistochemistry. All immunohistochemically-stained slides were examined by one of the investigator unaware of other data. A random sample of 402 tumors was

examined for β-catenin by a second observer unaware of other data, and the concordance between the two observers for β-catenin activation (inactive vs. active) was 0.83 (κ=0.65, p<0.0001).[138] In addition, it was observed that COX-2 overexpression correlated with cytoplasmic β-catenin expression but not nuclear β-catenin, supporting the role of cytoplasmic β-catenin in stabilizing COX-2 mRNA.[138]

For COX-2, monoclonal antibody will be applied to the tumor sections: COX-2 (Cayman), dilution 1:300. A positive and negative control (tumors with known expression status of each of the selected proteins) will be included in each staining batch. COX-2 expression will be scored for both the proportion of cells staining: none (not stained), 0; focal (<33%), 1; multifocal (33-67%), 2; and diffuse (≥67%), 3. The staining intensity will be scored as: none, 0; mild, 1; and strong, 2. The distribution and intensity scores are added: 0, negative; 1 and 2, intermediate; and 3, 4 and 5, positive. In our previous work, the κ coefficient between the two observers was 0.62 for COX-2 (p<0.0001; N=108), indicating substantial agreement.

Multiplex Immunofluorescence (IF): T cells and TAMs in each lesion will be assessed histologically using tissue microarray and a multispectral imaging platform (Vectra 3.0, Akoya Biosciences). Antibodies targeting CD3, CD4, CD8, ITGAE (CD103), and FOXP3 are used in the T cell assay. A pan-cytokeratin antibody (KRT) to positively identify tumor epithelial cells, and 4',6-diamidino-2-phenylindole (DAPI) stain to identify nuclei are used in both assays. After a single section is stained with multiple fluorescent markers, a multispectral image is acquired. Using machine learning algorithms (inForm software, Akoya Biosciences) with pathologist supervision, we phenotype every individual cell and measure number of cells per mm2 (for each cell type) in tumor epithelial areas and stromal areas, separately. In addition, the signal intensity in specific subcellular locations (membrane, cytoplasm, nucleus) is recorded. Multiple QC steps will be taken to ensure the accurate assay performance with appropriate positive and negative control tissues. While we recognize that functional subtypes of T cells do not match perfectly with T cell subsets classified by protein markers, our assay allows for reasonably sensitive detection and quantification of specific T cell subsets.

Macrophages exhibit considerable plasticity and heterogenous phenotypes, which can change according to the local microenvironment (blood or tissue).[338-339] In assaying these cells, previously proposed guidelines will be performed.[340] The M1-M2 polarization of TAMs is a spectrum, and standardized definitions for M1 and M2 TAMs in human tissue samples are yet to be developed.[340] Planned macrophage marker panel includes CD68, CD86,232 IRF5, CD163, MRC1 (CD206), KRT, and DAPI.[341-342] In every TAM, the M1:M2 index [= (CD86 x IRF5) / (CD163 x MRC1)] will be calculated. The higher (or lower) the index, the more M1 (or M2) polarized the TAM is. After assaying available CRCs, M1:M2 indices of all TAMs will be examined, three categories will be created: (1) the highest 30% M1:M2 indices as M1-like TAMs, (2) the lowest 30% as M2-like TAMs, and (3) the remaining 40% as less polarized TAMs. Then, densities of overall, M1-like, and M2-like TAMs will be calculated in tumor epithelial and stromal areas. Raw data on expression level of each marker in each TAM are available if further studies are needed to refine the TAM classification system.

Tumor Immune Partitioning and Clustering (TIPC): Most methods of analyzing immune cells in the tumor microenvironment rely upon calculating an immune cell density or measuring the distance from an immune cell to the nearest tumor cell. While helpful, these measures do not fully capture tumor:immune cell spatial configuration. A novel computational algorithm, TIPC, was recently developed by the laboratory of Dr Shuji Ogino at Harvard Medical School (publication pending) to conduct more effective spatially-informed tumor subtyping. TIPC uses hierarchical (unsupervised) clustering algorithm to group tumors based on their immune-tumor spatial interactions as measured by 6 TIPC metrics that are designed to quantify (1) the degree of partitioning of immune cells in tumor (i.e., intraepithelial) and stromal areas, and (2) the

degree of immune cell clustering/aggregating. TIPC applies a consensus clustering approach to determine the optimal number of clusters and cluster membership. Specifically, multiple repeats (50 is the default setting in TIPC R package) of hierarchical clustering are performed, and tumors which are consistently assigned to the same cluster in most of the runs will be assigned to the same (final) cluster. TIPC can identify CRC subtypes with unique immune cell spatial patterns that are associated with patient survival and key tumor molecular features and which cannot be identified by other known immune cell measures. TIPC can utilize different T cell subpopulations as well as differentially polarized macrophages, and also provides metrics that quantify the robustness and stability of the resulting tumor subtype assignments.

Fusobacterium nucleatum: Based on prior validation,[343] we use custom TaqMan primer/probe sets (ThermoFisher Scientific) for the nusG gene of F. nucleatum will be performed, with the reference human gene SLCO2A1. Cases with detectable F. nucleatum DNA are categorized as above or below the median; cases without detectable F. nucleatum DNA are considered negative.

Quality control for tumor block analyses: In all immunohistochemical analyses, appropriate positive and negative controls will be included in each run of immunohistochemical assay. In addition, a random sample of more than 200 cases will be re-examined by a second pathologist in Dr. Ogino's laboratory to assess inter-rater agreement. A kappa measure of agreement proposed by Kraemer will be used to estimate the agreement between raters for each marker. If agreement is unacceptable, further training and monitor agreement will be instituted in the next set of 200 samples.

Analysis of 15-PGDH: 15-PGDH will be assessed in the laboratory of Dr. Sanford Markowitz. Methods for measuring 15-PGDH in FFPE tissues include IHC using an anti-15-PGDH monoclonal antibody, raised against purified recombinant human 15-PGDH protein by the Markowitz laboratory. Stained slides will be reviewed independently by two GI pathologists, and staining graded semi-quantitatively (scale 0 to +3; 0 = non-reactive, 1+ = weak staining, 2+ = moderate staining, 3+ = strong staining). RNA will be extracted from slides of FFPE colonic tissue and converted to cDNA prior to real-time PCR measurement of 15-PGDH using the Applied Biosystems human 15-PGDH Taqman Probe/Primer kit Hs00168359_ml. Results will be in the form of numerical averages from three independent reverse transcription reactions. The utility of this assay for quantifying 15-PGDH expression levels in FFPE samples from mucosal biopsies as small as 2mm in diameter has been confirmed.[69]

4.2 Circulating inflammatory factors and patient outcome

Plasma will be collected in one 5 mL lavender top tube, centrifuged for 10-15 minutes at 1,300 g and aliquoted into 2 mL cryovials at 0.5 mL/vial. Samples will be frozen and shipped on ice or dry ice. Multiple masked quality control samples will be interspersed among the case samples, and all laboratory personnel will be blinded to patient outcomes.

Plasma levels of C-reactive protein, interleukin-6, and soluble tumor necrosis factor-α-receptor II (sTNF-α-R2) from blood samples obtained at study enrollment will be examined. These inflammatory factors will be measured in the laboratory of Dr. Nader Rifai (Clinical Chemistry Lab, Children's Hospital of Boston). Dr. Rifai is an expert in the measurement of these plasma analytes from large population studies and his laboratory provides high assay precision. Baseline plasma C-reactive protein levels will be measured via a high-sensitivity latex-enhanced immunonephelometric assay on a BN II analyzer (Dade Behring). Plasma interleukin-6 will be measured by a quantitative sandwich enzyme immunoassay technique (Quantikine HS Immunoassay), and sTNF-α-R2 levels by an ELISA kit utilizing immobilized monoclonal antibody to human sTNF-α-R2 (Genzyme). The CVs are 3.8% for CRP, 5.9% for IL-6, and 6.2% for TNF-αR2. In addition, in a cohort of women, the ICC for samples drawn from the same subject one year apart was 0.95 for CRP and 0.66 for TNF-αR2. In a cohort of men, plasma

CRP and IL-6 was also assessed 1-3 years apart in the same participants and yielded ICC's from 0.6-0.85, indicating that plasma measurements of these analytes are relatively stable over time.

The investigators will work closely with Dr. Rifai, who will help interpret the assays and update the team with recent developments. Precision will be monitored by routinely adding approximately 5% of repeated quality control samples as blinded specimens. Should a decrease in precision be noted, the investigators will work with the laboratory to address the problem and suspend additional analysis until the problem is resolved.

4.3 Vitamin D measurement

Plasma will be collected in one 5 mL lavender top tube, centrifuged for 10-15 minutes at 1,300 g and aliquoted into 2 mL cryovials at 0.5 mL/vial. Samples will be frozen and shipped on ice or dry ice. Multiple masked quality control samples will be interspersed among the case samples, and all laboratory personnel will be blinded to patient outcomes. 25(OH)D concentrations will be measured by radioimmunoassay in the laboratory of Dr. Bruce Hollis (Medical University of South Carolina, Charleston, SC), as described previously.[139] In prior analyses, the mean coefficient of variation of the assay was ~10%.[93] For the current trial, plasma 25(OH)D levels will be assessed only at study baseline; nonetheless, our prior studies suggest that within each individual, plasma 25(OH)D remain relatively stable over time. To assess the intraperson stability of 25(OH)D over time, plasma levels of 25(OH)D were measured in 144 men who donated repeated blood specimens four years apart. The Pearson correlation coefficient was 0.70 for the two 25(OH)D measurements ($P < 0.0001$).[140]

4.4 Vitamin D related tumor assays

Vitamin D immunohistochemistry and KRAS mutational status will be performed in the laboratory of Dr. Shuji Ogino.

Tissue Block Procurement: Formalin-fixed/paraffin-embedded (FFPE) tissue blocks and slides will be examined. A set of blocks will be selected for preparation of routine hematoxylin and eosin (H&E) slides for microdissection and DNA extraction for analysis of KRAS mutations. Alliance pathologists will also construct tissue microarrays (TMAs) and Dr. Ogino will perform immunohistochemical (IHC) evaluation of VDR and 1-α-hydroxylase (CYP27B1).

Tissue DNA Extraction: H&E stained slides will be reviewed, areas comprising ≥70% neoplastic cellularity will be marked and scraped from 15μm unstained slides under direct visualization, and DNA will be extracted. DNA of sufficient quality for polymerase chain reaction (PCR) was obtained from >95% of colorectal cancers.

IHC Staining and Interpretation: IHC analyses will be performed in the laboratory of Dr. Ogino. The following antibodies will be applied: polyclonal rabbit anti-VDR (C-20; Santa Cruz, Cat# SC-1008; dilution 1:200) and monoclonal anti-CYP27B1 (1-α-hydroxylase) (clone H-90; The Binding Site # sc-67261; dilution 1:400). The intensity of staining (absent, weak, moderate/strong) will be recorded in each relevant cellular compartment (nucleus, cytoplasm, and/or membrane) as well as fraction of tumor cells with staining. For each antibody, published cell lines have been identified to serve as controls and cells have been embedded into paraffin blocks for incorporation into TMAs. For each antibody tested, positive controls have been successfully stained while noting loss in all negative controls. For each new TMA tested, controls will be included for quality control. In preliminary analysis, 619 colorectal tumors in our laboratory were evaluated for VDR expression, with 233 (38%) showing overexpression. A similar proportion of patients demonstrate 1-α-hydroxylase overexpression.

Quality Control for Tumor Block Analyses: Approximately 5% repeated QC samples will be added as blinded specimens; they will be randomly nested in the sample sets with coded IDs. In all IHC analyses, appropriate positive and negative controls will be included in each run of IHC

assay. In addition, a random sample of >100 cases will be re-examined by a second pathologist in Dr. Ogino's laboratory to assess inter-rater agreement using a kappa measure of agreement (κ) proposed by Kraemer. If agreement is unacceptable, further training and monitor agreement will be instituted in the next set of 200 samples. Thus far, the concordance rate and κ coefficient between the two pathologists is 82% (κ=0.62; n=139) for VDR.

4.5 Whole genome expression

As described above, whole genome mRNA expression profiles in colon tumor tissue will be interrogated to identify patterns of genes dysregulated in tumors. Various technologies have been developed to deduce and quantify the transcriptome, including hybridization-or sequence-based approaches. We propose to conduct RNA-Seq to profile the transcriptome to measure of levels of transcripts and their isoforms. Investigators at the Broad Institute and the Dana-Farber Cancer Institute have developed a methodology to routine conduct RNA-seq to profile the transcriptome from routine FFPE tumor specimens. These studies will allow a detailed examination of the impact of tumoral gene expression on patient outcome as well as an assessment of how tumoral gene expression may modify the influence of adjuvant celecoxib on patient survival.

The ability to discover and implement gene expression signatures on FFPE colon cancer blocks will allow us a unique opportunity to identify genes involved in celecoxib-mediated inhibition of colorectal carcinogenesis. In the future, genes identified in these analyses will be functionally characterized.

4.6 CpG Island Methylator Phenotype (CIMP)

A subset of colorectal adenocarcinomas displays an unusually high frequency and concordance of CpG island hypermethylation. This subset was first described by Toyota et al. as CIMP (CpG Island Methylator Phenotype) colorectal adenocarcinomas.[139] The definition of CIMP was recently refined, and was shown to underlie the majority of sporadic mismatch repair deficient colorectal adenocarcinomas and to be very tightly associated with BRAF mutation.[139] An improved panel of five markers (CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1) has been established to classify the CIMP phenotype, which outperforms other commonly used panels of CIMP in sensitivity and specificity.[142]

CpG Island Methylator Phenotype (CIMP) will be analyzed using the MethyLight procedure at the USC Epigenome Center under supervision by Dr. Peter W. Laird and Dr. Heinz Josef Lenz as described.[142-144] MethyLight technology utilizes real-time PCR analysis for bisulfite-based DNA methylation analysis. The USC Epigenome Center uses a non-methylation-dependent control reaction designed for a consensus Alu repetitive element sequence.[141] This control reaction is a sensitive measure of very low DNA amounts, and is less prone to quantitation errors introduced by gene-copy anomalies, such as aneuploidy, gene amplification, deletions, etc., compared to a single-copy control reaction. MethyLight data are reported as a ratio between the value obtained for a methylation-specific reaction and that obtained for the methylation-independent Alu control reaction, normalized against similar measurements obtained for a fully methylated reference sample. The resulting output is expressed as "Percent of Methylated Reference (PMR)". The USC Epigenome Center has extensive experience with high-throughput DNA methylation analysis, and has established numerous quality control procedures. All reagents are prepared with dedicated or disposable vessels, solutions, and pipettes. Positive displacement pipettes or air-displacement pipettes with aerosol-resistant tips are used. Plate preparation is performed on a dedicated custom-built automated robotic platform based on the Qiagen BioRobot 3000. 10% of samples are repeated in duplicate randomly intermixed with the other samples as an additional quality control. Each bisulfite-converted DNA sample is

evaluated prior to MethyLight analysis using the Alu control reaction to ensure that sufficient bisulfite-DNA amounts are utilized in each CIMP-specific MethyLight assay.

LINE-1 methylation: To accurately quantify global DNA methylation, Pyrosequencing technology will be utilized in Dr. Ogino's laboratory.[134] PCR and subsequent Pyrosequencing for LINE-1 will be performed using the PyroMark kit (Biotage, Uppsala, Sweden). The PCR condition will be 45 cycles of 95C for 20 sec, 50C for 20 sec and 72C for 20 sec, followed by 72C for 5 min. The biotinylated PCR product will be purified and made single-stranded to act as a template in a pyrosequencing reaction, using the Pyrosequencing Vacuum Prep Tool (Biotage). Pyrosequencing reactions will be performed in the PSQ HS 96 System (Biotage). The nucleotide dispensation order will be: ACT CAG TGT GTC AGT CAG TTA GTC TG. Complete conversion of cytosine at a non-CpG site ensures successful bisulfite conversion. The amount of C relative to the sum of the amounts of C and T at each CpG site will be calculated as percentage. The average of the relative amounts of C in the 4 CpG sites will be used as overall LINE-1 methylation level in a given sample. Pyrosequencing to measure LINE-1 methylation has been previously validated.[134]

Complementary to these techniques will be also the use of the Illumina EPIC DNA methylation array. This assay provides a genome-scale interrogation of the human methylome, with sampling of 862,927 CpG sites in the human genome, 2,932 non-CpG sites and 59 known SNPs. EPIC DNA methylation probes assays are located in nearly every gene in the genome (99% of Refseq genes have at least one CpG interrogated), with attention to gene promoters, enhancers, transcription factoir binding sites, and miRNA promoter regions. EPIC probe content includes >90% of the HM450 content. Genomic DNA (250 ng) is bisulfite converted using the Zymo EZ DNA methylation kit. The amount of bisulfite-converted DNA as well as the completeness of bisulfite conversion for each sample are assessed using a panel of MethyLight-based real-time PCR quality control assays. Bisulfite-converted DNA is then repaired using the Illumina Restoration Kit. The repaired DNA is used as a substrate for the Illumina EPIC BeadArrays. After the chemistry steps, BeadArrays are scanned and the raw signal intensities are extracted from the *.IDAT files using the 'noob' function in the *minfi* R package. The 'noob' function corrects for background fluorescence intensities and red- green dye-bias. The beta value is calculated as (M/(M+U)), in which M and U refer to the (pre-processed) mean methylated and unmethylated probe signal intensities, respectively. Measurements in which the fluorescent intensity is not statistically significantly above background signal (detection p value > 0.05) are removed from the data set.

5.0 DATA ANALYSES

The primary efficacy variable for analyses will be disease-free survival (DFS). Secondary efficacy endpoints include recurrence-free survival (RFS) and overall survival.

It is hypothesized that specific tumor alterations will modify the effect of adjuvant celecoxib on patient survival. For the analysis of tumoral alterations (**Objective 1.1**), all patients who had a tumor block available will be included in the analysis. Within each of binary category of COX-2, VEGF, p21, beta-catenin, 15-PGDH, T cell overexpression, higher microvessel density, *PIK3CA* mutation, and microsatellite instability, the effect of celecoxib vs. placebo on DFS and OS will be examined. Cox proportional hazards models will be used to calculate HRs and 95% CIs for DFS and OS, adjusted for other prognostic factors. Tests for statistical interaction will be performed by entering into the model the cross-product term of the relevant biomarker with treatment assignment (celecoxib vs. placebo). Moreover, in Cox models, the main independent effect of each biomarker on DFS and OS will be assessed.

For plasma analytes (**Objective 1.2**: CRP, IL-6, and sTNF-α-R2), whether the effect of celecoxib vs. placebo on DFS differs according to baseline levels of each plasma inflammatory factor will be assessed. For primary analyses of survival, patients who died within three months of plasma collection will be excluded to minimize any bias due to occult cancer recurrence or preclinical illness. For these stratified analyses, each plasma analyte will be categorized into tertiles to maximize statistical power. Within each tertile of the specific plasma inflammatory factor, the effect of celecoxib vs. placebo on DFS and OS will be examined. Cox proportional hazards models will be used to calculate HRs and 95% CIs for DFS and OS, adjusted for other prognostic factors. Tests for statistical interaction will be performed by entering into the model the cross-product term of the plasma level as a continuous variable with treatment assignment (celecoxib vs. placebo).

In addition, the main effect of each plasma inflammatory factor on DFS and OS will be examined. For the main effect, plasma levels will be divided into quintiles for the analysis. Baseline characteristics of patients will be compared according to quintiles of the biomarker using Wilcoxon signed rank tests for continuous variables and chi-squared tests for categorical variables. For primary analyses of survival, patients who recurred or died within three months of plasma collection will be excluded to minimize any bias due to occult cancer recurrence or preclinical illness. In sensitivity analyses, the possibility of reverse causation will be assessed by allowing different lag times between plasma assessment and cancer recurrence or death. The log-rank test and Kaplan-Meier curves will be used to compare DFS OS by quintile of plasma level. Cox proportional hazards models will be used to control for multiple confounders. The two-tailed *P* value for the linear trend test across categories will be calculated using the plasma level as a continuous variable, consistent with prior studies.

The primary efficacy variable for analyses will be disease-free survival (DFS). Secondary efficacy endpoint will be overall survival.

For the main effect of plasma 25(OH)D (**Objective 1.3**), plasma 25(OH)D levels will be divided into quintiles for the analysis. Baseline characteristics of patients will be compared according to quintiles of the biomarker using Wilcoxon signed rank tests for continuous variables and chi-squared tests for categorical variables. For primary analyses of survival, patients who recurred or died within three months of plasma collection will be excluded to minimize any bias in the 25(OH)D level due to occult cancer recurrence or preclinical illness. In sensitivity analyses, the possibility of reverse causation will be assessed by allowing different lag times between 25(OH)D assessment and cancer recurrence or death. The log-rank test and Kaplan-Meier curves will be used to compare DFS OS by quintile of 25(OH)D level. Cox proportional hazards models will be used to control for multiple confounders. The two-tailed *P* value for the linear trend test across categories will be calculated using the 25(OH)D level as a continuous variable, consistent

with prior studies. In secondary analyses, we will examine how the relationship between 25(OH)D level and patient outcome is modified by relevant covariates such as ECOG performance status, treatment assignment, physical activity and body mass index, among others. Tests for statistical interaction will be performed by entering into the model the cross-product term of the plasma 25(OH)D as a continuous variable with the covariate as a continuous or binary variable.

For the analysis of tumoral alterations in vitamin D and related pathways (**Objective 1.4**), all patients who had a tumor block available for analysis of VDR expression, 1-α-hydroxylase, and KRAS mutation and data on circulating 25(OH)D concentration will be included. Tumoral protein overexpression will be defined as a) moderate/strong staining in any fraction of cells, or b) ≥50% of tumor cells with weak staining. All tumors will be categorized as having VDR and 1-α-hydroxylase overexpression versus no overexpression, and KRAS mutated versus wild type. Within each of category of VDR expression, 1-α-hydroxylase expression, and KRAS mutational status, we will examine the influence of plasma levels of 25(OH)D divided into tertiles on DFS and OS. Tertiles will be utilized instead of quintiles of plasma 25(OH)D to maximize the ability to detect an association within each subgroup. Cox proportional hazards models will be used to calculate HRs and 95% CIs for DFS and OS, adjusted for other prognostic factors. Tests for statistical interaction will be performed by entering into the model the cross-product term of plasma 25(OH)D as a continuous variable with the molecular alteration as a binary variable (overexpressed versus not; mutated versus wild type).

In describing analyses related to gene expression, the genes/transcripts from RNA-seq will be referred to as features. The terms feature, feature expression and expression will also be used interchangeably. The maximum number of features is approximately 24,000. In these discussions, K will denote the number features (among the 24,000) which pass the non-phenotypic filters. The expressions, which are summary measures, are obtained following pre-processing. The background correction algorithm implemented by the BeadStudio software will be employed and quantile normalization will be applied to the background adjusted intensities.

Objective 1.5 is to identify the features from whole transcriptome analysis which are associated with DFS. As outliers in both the time-to-event observations and gene expressions may be encountered, the length of follow-up varies among patients, and multiple markers are considered, methods which are robust, are able to incorporate the censoring mechanism, and allow for incorporation of multiplicity adjustments will be employed. The association between the time-to-event endpoint and each of the features will be quantified using the non-parametric rank-covariance measure.[145] This method is a non-parametric counterpart to Cox regression where the time-to-event variable is regressed on the ranks of the expression. The family-wise error rate (FWER) adjusted exact permutation P-values will be calculated using B=10,000 permutation replicates. Features will be considered significant at the 0.05 FWER adjusted level.

We are interested in the association between each feature and DFS. Kendall's tau will be used to quantify these pairwise associations. This quantity is uniquely determined by the copula generating the joint (bivariate) distribution between each feature and DFS and as such is invariant to marginal distributions. It will be assumed that the marginal effects are continuous so as to ensure uniqueness of the generating copula.

An additional objective of gene expression assessment (**Objective 1.6**) is to identify those features for which there is expression by celecoxib interactions with respect to DFS. These analyses will be carried within the framework of two-way multiplicative Cox models.

The analyses outlined for the primary endpoint, will be carried out for OS and AE as well. For the adverse event endpoints, the two-sample Wilcoxon test will be employed to investigate the association between each feature and outcome.[146] For this endpoint, conditional inference tree and random forests methods for binary outcomes will be used.

The classification models for DFS, OS and adverse events in the previous models were based on molecular data. As additional exploratory analyses, the inclusion of clinical or demographic co-variables will be considered.

Functional annotation of discovered signature will be performed by Gene Set Enrichment Analysis (GSEA)16 using the Molecular Signature Database (MSigDB, [redacted]Survival data analyses will be performed using the log-rank test and multivariate Cox regression. All analyses were performed using GenePattern18 [redacted] or the R statistical package [redacted]

For the analysis of CIMP (**Objective 1.7**), the primary efficacy variable for analyses will be disease-free survival (DFS). Secondary efficacy endpoints include recurrence-free survival (RFS) and overall survival (OS).

Baseline characteristics of patients will be compared according to CIMP-high vs. CIMP-low/0 using Wilcoxon signed rank tests for continuous variables and chi-squared tests for categorical variables. The log-rank test and Kaplan-Meier curves will be used to compare DFS and OS by CIMP-high vs. CIMP-low/0. Cox proportional hazards models will be used to control for multiple confounders including MSI, BRAF, and LINE-1 status. In secondary analyses, we will examine how the relationship between CIMP status and patient outcome is modified by relevant covariates such as age, ECOG performance status and treatment assignment, MSI, BRAF, and LINE-1 status among others. Tests for statistical interaction will be performed by entering into the model the cross-product term of the CIMP status (binary variable) with the covariate as a continuous or binary variable. The main independent effect of BRAF and LINE-1 status on DFS will also be assessed using the aforementioned COX models.

For the analysis of immune response (Objective 1.8), the primary efficacy variable for analyses will be disease-free survival (DFS). Secondary efficacy endpoints include recurrence-free survival (RFS) and overall survival (OS). Interactions with celecoxib randomized will also be determined

The prognostic association of TIPC-based CRC subtypes and DFS will be determined, with stratification by celecoxib randomization. In exploratory analyses statistical interaction of TIPC subtype and F. nucleatum level will be assessed.

6.0 STATISTICAL CONSIDERATIONS

The three clinical endpoints are adverse events (e.g., neuropathy), disease-free survival (DFS) and survival (OS). The exact definitions for these outcomes will be equivalent to those specified in the treatment protocol (CALGB/SWOG C80702). The treatment by biomarker (or genotype) interactions will be tested for the celecoxib hypothesis. If an interaction is present, relevant hypotheses will also be tested in the treatment groups of patients receiving and not receiving celecoxib. If no interaction is present, the treatment groups will be combined (celecoxib + no celecoxib) for analysis. Power estimates for specified differences in interaction are given in Table 3. Table 4 contains power estimates for DFS comparisons of biomarker and genotype within treatment group (celecoxib; no celecoxib) by marker prevalence, detectable hazard ratio and the percent of samples available. Table 5 provides power estimates for testing the prognostic value of a marker when no treatment by marker interaction is present. Table 6 illustrates detectable differences in DFS for biomarkers quantified in quartiles. Table 7 illustrates the difference detectable with the specified power for a biomarker quantified in quartiles versus a dichotomous marker; for example, Vitamin D levels measured by plasma 25(OH)D versus K-ras mutational status. Tables 8 and 9 describe the power achieved to detect the illustrated difference in Grade 3+ toxicity rates versus a biomarker quantified in quartiles. Examples are Grade 3+ neutropenia and diarrhea versus body mass index.

Table 3. Approximate power to detect the specified hazard ratio for treatment (no celecoxib; celecoxib) by biomarker interactions with 60% (n=1,500) and 80% (n=2,000) of samples obtained (2-sided α=0.1) assuming exponential survival, a dichotomous biomarker, equal sample sizes per group, and an overall event rate of 0.31 (775/2500).

n (events)	Hazard Ratio-Interaction	Power
	1.5	
1500 (465)		0.34,0.58,0.71
2000 (620)		0.47,0.71,0.81
	1.6	
1500 (465)		0.48,0.71,0.81
2000 (620)		0.63,0.83,0.90
	1.7	
1500 (465)		0.60,0.81,0.88
2000 (620)		0.76,0.91,0.95

Moderate to large interaction hazard ratios are detectable with adequate power.

Table 4. Power estimates for DFS comparisons of a dichotomous biomarker or genotype within treatment groups (e.g., patients receiving celecoxib) by marker prevalence, detectable hazard ratio and the percent of samples available (log rank test, 2-sided α=0.01, 0.05, 0.10). Exponential survival, equal numbers of samples per treatment arm, and an overall event rate of 0.31 (775/2500) are assumed.

	Detectable Hazard Ratio	1.4	1.5	1.6
Marker/ Genotype Prevalence	**n (number of events)**			
0.5				
	750 (233)	0.49,0.72,0.82	0.69,0.87,0.92	0.84,0.94,0.97
	1000 (310)	0.65,0.84,0.90	0.83,0.94,0.97	0.94,0.98,0.99
0.2				
	750 (233)	0.30,0.53,0.65	0.46,0.69,0.79	0.61,0.81,0.88
	1000 (310)	0.41,0.65,0.76	0.61,0.81,0.88	0.76,0.91,0.95
0.1				
	750 (233)	0.15,0.33,0.45	0.23,0.45,0.58	0.33,0.57,0.69
	1000 (310)	0.21,0.42,0.55	0.33,0.57,0.69	0.46,0.69,0.79

Adequate power is achieved to detect moderate to large hazard ratios for dichotomous biomarkers or genotypes with prevalence close to 50%. The power estimates at 50% prevalence are approximately correct for biomarkers or genotypes with prevalence ≥ 30% and ≤ 70%. For biomarkers or genotypes with prevalence near 20% and, in particular, 10%, larger differences are detectable with adequate power.

Table 5. Power estimates for DFS comparisons of a dichotomous biomarker or genotype by marker prevalence for all patients, detectable hazard ratio, and the percent of samples available (log rank test, 2-sided α=0.01, 0.05, 0.10). Exponential survival and an overall event rate of 0.31 (775/2500) are assumed.

	Detectable Hazard Ratio	1.2	1.3	1.4	1.5
Marker/ Genotype Prevalence	**n (number of events)**				
0.5					
	1500 (465)	0.27,0.50,0.62	0.59,0.80,0.88	0.85,0.95,0.97	0.96,0.99,0.99
	2000 (620)	0.37,0.62,0.73	0.75,0.90,0.94	0.94,0.98,0.99	0.99,0.99,0.99
0.2					
	1500 (465)	0.15,0.34,0.47	0.37,0.61,0.73	0.62,0.82,0.89	0.82,0.93,0.96
	2000 (620)	0.22,0.44,0.56	0.51,0.74,0.83	0.78,0.91,0.95	0.92,0.98,0.99
0.1					
	1500 (465)	0.08,0.21,0.32	0.18,0.39,0.52	0.034,0.58,0.70	0.51,0.74,0.83
	2000 (620)	0.11,0.27,0.38	0.26,0.49,0.62	0.47,0.71,0.80	0.67,0.85,0.91

Adequate power is achieved to detect moderate to large hazard ratios for dichotomous biomarkers or genotypes with prevalence close to 50%. The power estimates at 50% prevalence are approximately correct for biomarkers or genotypes with prevalence ≥ 30% and ≤ 70%. For biomarkers or genotypes with prevalence near 20% and, in particular, 10%, larger differences are detectable with adequate power.

Table 6. Approximate DFS hazard ratios (maximum versus minimum hazard under "least favorable configuration") detectable with 70%, 80%, and 90% power for comparisons of a continuous biomarker categorized by quartiles for all patients and the percent of samples available (2-sided α=0.01, 0.05, 0.10). The chi-square non-centrality parameters (ncp) are 20.66, 16.74, 14.16, respectively, 70%, 80% and 90% power, with α=0.01; 16.21, 12.73, 10.46, respectively, for α=0.05; and 8.67, 10.77, and 14.02, respectively, for α=0.1. Exponential survival and an overall event rate of 0.31 (775/2500) are assumed.

	Detectable Hazard Ratio (DFS)		
Power	0.7	0.8	0.9
n (number of events)			
1500 (465)	1.64,1.53,1.47	1.72,1.60,1.54	1.82,1.70,1.64
2000 (620)	1.54,1.45,1.40	1.60,1.50,1.45	1.68,1.58,1.53

With 60 and 80% of samples studied, adequate power is achieved to detect moderate to large hazard ratios for biomarkers categorized by quartiles.

Table 7. Difference in median DFS detectable for a continuous marker categorized by quartiles (Biomarker 1) versus a second dichotomous biomarker (Biomarker 2) with approximate powers of 0.44, 0.68, 0.78, respectively, for 2-sided α=0.01, 0.05, 0.10, with 60% of patients submitting samples and approximate powers of 0.61, 0.81, 0.88, respectively, for α=0.01, 0.05, 0.10, with 80% of patients submitting samples (logrank test). Exponential survival, equal sample sizes per group, and an overall event rate of 0.31 (775/2500) are assumed.

	Median DFS (years)			
Biomarker 1	Quartile(1)	Quartile(2)	Quartile(3)	Quartile(4)
Biomarker 2				
Absent	8.0	9.0	10.0	11.0
Present	9.0	8.0	7.0	6.0

Moderate to large interaction hazard ratios are detectable with adequate power.

Table 8. Power estimates to detect the difference illustrated in Table 9 for a continuous marker categorized by quartiles versus toxicity (< Grade 3+; ≥ Grade 3+) for 60% (ncp=9.2) and 80% (ncp=12.3) of patients submitting samples (chi-square, 2-sided α=0.01, 0.05).

	Significance Level	
n	α=0.01	α=0.05
1500 (60% of patients)	0.67	0.85
2000 (80% of patients)	0.82	0.93

Table 9. Difference in proportions of patients by a continuous marker categorized in quartiles versus toxicity (< Grade 3+; ≥ Grade 3+) that is detectable with power given in Table 8. A Grade 3+ toxicity prevalence of 0.3 is assumed.

Biomarker	Quartile(1)	Quartile(2)	Quartile(3)	Quartile(4)	**Total**
Adverse Event					
< Grade 3+	0.1875	0.18	0.175	0.1625	0.7
≥ Grade 3+	0.0625	0.075	0.075	0.0875	0.3
Total	0.25	0.25	0.25	0.25	1.0

For gene expression analyses, we will assume that 775 events are expected in the clinical study. This corresponds to an event rate of 0.31. We will assume that 70% will provide consent and usable samples for these analyses. The corresponding sample size and number of events to be used for the power calculations are 1750 and 542 respectively. For these simulations, we will assume that the DFS distribution is mixture of exponentials, with rates -log(0.72)/3 and -log(0.77)/3 respectively, and that the censoring distribution is uniform. The parameters on the latter are chosen so as to set the expected event rate is 0.31. The power, at the two-sided 0.01 FWER Bonferroni adjusted level, is illustrated in Table 10 based on a Kendall tau coefficient of 0.11, 0.12 and 0.13 under the Gaussian, Frank and Gumbel copulas. Each illustration is based on 10,000 Monte Carlo simulations. As we have pointed out, we will sharpen these FWER bounds through permutation resampling for the analyses.

Table 10. Power illustration for the gene expression study at the two-sided 0.05 FWER level

tau	Copula	Power
0.11	Normal	0.79
0.12		0.91
0.13		0.97
0.11	Frank	0.82
0.12		0.92
0.13		0.98
0.11	Gumbel	0.54
0.12		0.71
0.13		0.84

A molecular classification model will be constructed using conditional inference trees with binary splits.[38] These trees will allow for direct incorporation of the censoring mechanism, which is important given that the length of follow-up varies among patients, as well as adjustment for multiplicity. The overall error rate will be adjusted at the 0.2 level using permutation resampling. The terminal nodes of these trees will be considered "risk" groups (classified by the trees). Kaplan-Meier estimates of the hazard profiles for these risk groups will be produced and presented graphically. We will also consider the random forests for survival data.[39-41] One of the key advantages of the two methods is that they implicitly incorporate interactions among the variables.

7.0 STATISTICAL SOFTWARE AND COMPUTING HARDWARE

The statistical environment R along with extension packages from the Bioconductor project will be used for carrying out the statistical analyses.[42,43] When appropriate, bindings with other languages such as Python and C++ will be used. The CALGB Bioinformatics Unit maintains a dedicated 4-way quad-core (16 cores) AMD Opteron 8384 processors with 64GB of RAM, 3TB of usable hard-drive space with RAID 10 redundancy running the Debian GNU/Linux stable AMD64 operating system. The machine can be connected to the CALGB SAN through an iSCSI interface for additional storage. Drs. Niedzwiecki and Owzar also have access to an 8-way Socket F AMD Opteron server with dual core 3.0 Ghz 2222SE processors (total of 16 cores) with 64GB of RAM running Debian GNU/Linux stable AMD64 administered by the Duke Comprehensive Cancer Center Information Science (CCIS). By virtue of their academic affiliations with Department of Biostatistics and Bioinformatics, at the Duke University Medical Center (for whom Dr. Owzar serves as the chair of the computing committee), will have access to departmental computing resources as well. These include two 8-way Socket F AMD Opteron servers each with dual core 3.0 Ghz 2222SE processors (total of 16 cores) with 64GB of RAM and one 4-way Socket F AMD Opteron server each with dual core 3.0 Ghz 2222SE processors (total of 8 cores) with 32GB of RAM. Additionally, through a collaborative agreement with the Duke HPC group, Drs. Niedzwiecki and Owzar also have access to GPU (graphics processor unit) computing facilities.

APPENDIX III

PHARMACOGENETIC COMPANION STUDIES: CALGB 60905

1.0 OBJECTIVES

1.1 To assess the influence of genetic variations in the cyclooxygenase and related pathways on the efficacy of celecoxib as adjuvant therapy for stage III colon cancer

1.2 To assess the influence of germline variation in vitamin D pathway genes on disease-free and overall survival in patients with stage III colon cancer.

1.3 To investigate the potential association between the AGXT 154C>T polymorphisms and neuropathy in the Caucasian population.

1.4 To identify specific SNPs and/or copy number variations that are associated with the prevalence of oxaliplatin-related peripheral neuropathy.

1.5 To identify specific SNPs and/or copy number variations that are associated with outcome, severe toxicity, and risk of cancer.

2.0 BACKGROUND

Candidate gene, pathway analyses and whole genome scans are common approaches for the identification of germline polymorphisms that contribute to a given phenotype. A candidate gene approach focusing on drug metabolizing enzymes and drug targets is proposed. In addition, a genome wide single nucleotide polymorphism (SNP) and copy number variant (CNV) scan will be performed to provide more definitive assessment of the genomic contribution to variation in drug effect (see below).

2.1 Genetic variation in COX-2 and related pathways

It is hypothesized that germline polymorphisms in COX related pathway will influence the effect of celecoxib on patient survival. Aspirin and NSAIDs may influence colorectal carcinogenesis, in part, through the inhibition of cyclooxygenase-2 (COX-2; PTSG-2) and COX1 (PTSG-1).[147,148] COX is the rate-limiting enzyme for the metabolic conversion of arachidonic acid to prostaglandins and related eicosanoids. COX-2 promotes inflammation and cell proliferation and is overexpressed in human colorectal cancer. Polymorphisms in COX-2 (PTGS2) have been associated with the risk of colorectal cancer.[149,150] In a randomized trial of adjuvant rofecoxib in colorectal cancer patients that was terminated early, 3 COX-2 SNPs (rs10911907, rs11583191, and rs2179555) which lie 5' to the COX2 gene significantly influenced the effect of rofecoxib.[151] The effect of rofecoxib was significantly greater in patients with at least one variant allele at each of the three SNP sites (p, interaction = 0.04). Similarly, COX-1 polymorphisms have been linked to the colorectal adenoma risk and appear to modify the influence of aspirin/NSAID use on adenoma risk.[152] 5-lipoxygenase (5-LOX) represents an alternative pathway to COX in arachidonate metabolism. Polymorphism in the promoter region of 5-LOX (ALOX5) has been associated with increased inflammation,[153,154] levels of C-reactive protein,[155] aspirin-intolerant asthma,[153] and colorectal cancer.[156] Prostacyclin synthase is a downstream enzyme from COX. A variable number tandem repeat polymorphism in the prostacyclin synthase gene has been associated with altered transcriptional activity and increased risk of hypertension and stroke.[157-159] Thromboxane synthase is an alternative pathway that is downstream from COX. The T(-386)G polymorphism has been associated with an increased risk of myocardial infarction.[160]

PPARγ, a member of the steroid receptor/transcription factor family, is a critical regulator of adipogenesis and a target of the adenomatous polyposis coli (APC) gene.[161-163] PPARγ may also be a potential target of aspirin and NSAIDs.[164,165] Animal and clinical studies demonstrate that PPARγ also has a role in insulin signaling, insulin resistance, development of type 2 diabetes and may function as a tumor suppressor gene.[163] A common variant in the PPARγ gene (Pro12Ala) reduces the promoter affinity by approximately 50% and is associated with a reduced risk of type 2 diabetes.[166] Carriers of the PPARγ 12Ala variant allele were at reduced risk of colorectal neoplasia.[167,168] Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor involved in the regulation of inflammation, apoptosis, and carcinogenesis.[169,170] Various proinflammatory cytokines (IL-1-beta, IL-8, TNF-alpha) activate NF-κB, and, in turn, activated NF-κB promotes colorectal cell proliferation and other inflammatory genes including COX-2.[171-175] NF-κB is constitutively activated in human colorectal cancer, and aspirin-induced suppression of NF-κB impairs colorectal tumorigenesis. Polymorphisms in NF-κB have been associated with the increased risk of inflammatory disorders,[176-178] diabetes mellitus,[179,180] and myeloma.[181]

The genes involved in celecoxib metabolism will also be examined. CYP2C9 metabolizes both celecoxib and NSAIDs, and variant CYP2C9 genotypes modified the effect of aspirin on adenoma risk in one study.[182] Thus, the efficacy of celecoxib on colon cancer recurrence may be influenced by the metabolism of the agent.

2.2 Germline variations in the vitamin D pathway

The pathway through which vitamin D exerts transcriptional effects is complex. An editorial in the Journal of the National Cancer Institute recommended that future investigations consider the interrelationships of vitamin D and associated genetic polymorphisms within the vitamin D pathway.[183] In CALGB/SWOG C80702, we will examine the joint effect of vitamin D with polymorphisms in five critical genes in the vitamin D pathway.

The cellular effects of 1-25-dihyroxycholecalciferol [$1,25(OH)_2D$] – the active metabolite of vitamin D – are principally mediated through the vitamin D receptor (VDR), which regulates the transcription of genes involved in cellular differentiation and inhibition of proliferation.[184,185] Well-differentiated human colon cancer cell lines have higher VDR expression[101] and the antiproliferative effects of vitamin D only occur in cell lines expressing high levels of VDR.[102] Several common polymorphisms (FokI, ApaI, TaqI and BsmI) have been identified in the VDR gene, although functional studies of these polymorphisms have shown contradictory results.[186-194] Moreover, associations between these polymorphisms and the risk of colorectal neoplasia remain inconsistent.[195-202] To better define genetic variation in the VDR gene, Nejentsev developed high-resolution SNP, haplotype and linkage disequilibrium (LD) maps of VDR in a multiethnic cohort.[203] A total of 24-26 TagSNPs were required to tag the three haplotype blocks ($r^2 \geq 0.8$). The authors observed that FokI was not in LD with any other common SNP in their study. In addition, ApaI, TaqI and BsmI missed a large fraction of common variation. Therefore, a comprehensive examination of VDR, plasma 25(OH)D, and patient outcome will be performed using these 26 TagSNPs.

Vitamin D binding protein (VDBP, GC) transports vitamin D and its plasma metabolites to target tissues and may also be involved in intracellular metabolism of vitamin D.[204,205] Two common VDBP polymorphisms (Glu432Asp and Thr436Lys) have been associated with osteoporosis,[203] COPD,[204] chronic mucus hypersecretion,[208] diabetes,[204,205] and Grave's disease.[209] In contrast, genotyping in 24 unrelated Caucasian individuals of 33 other SNPs with a minor allele frequency >5% revealed little LD across VDBP. Consequently, the investigators will focus on the Glu432Asp and Thr436Lys VDBP polymorphisms.

1-α-hydroxylase (CYP27B1) catalyzes the 1α-hydroxylation of $25(OH)_2D$ to $1,25(OH)_2D$.[210] Beyond the proximal renal tubules, the enzyme is present in both normal human colonic mucosa and colorectal adenocarcinomas.[210-214] 1-α-hydroxylase polymorphisms have been associated with the risk of Addison's disease, Hashimoto's thyroiditis, Graves' disease, and type 1 diabetes mellitius.[215,216] We will examine five SNPs with a minor allele frequency >5% (all noncoding) identified in a multiethnic cohort.[217]

Retinoid X receptor (RXR) functions as a heterodimer with the VDR, forming a VDR/RXR complex that regulates transcription of several target genes.[211,218] Seven SNPs with a minor allele frequency >5% (6 noncoding and one coding, Ser327Ile) will be examined in a multiethnic cohort.[219]

25-hydroxyvitamin D-24-hydroxylase (CYP24A1) initiates degradation of $1,25(OH)_2D$ and 25(OH)D. CYP24A1 mRNA and protein levels are significantly upregulated in cancers relative to normal tissues, suggesting CYP24A1 may be an oncogene.[220] Polymorphisms in the promoter region of CYP24A1 enhance both basal and vitamin D_3-stimulated promoter activity.[221] Six SNPs in the CYP24A1 promoter will be examined.[222]

2.3 AGXT 154C>T polymorphisms and neuropathy

There is increasing evidence which suggests that germline polymorphisms related to anticancer therapeutics metabolism, transport, and resistance correlate with drug response; furthermore, germline polymorphisms related to therapeutic targets and/or therapeutic pathways might also help predict therapeutic outcomes.[223,224]

This study offers an excellent opportunity to evaluate the role of genetic variants in relevant genes influencing the pharmacology of 5-fluorouracil, oxaliplatin, and celecoxib, which might influence the eventual drug response and/or toxicity of these agents.

The introduction of oxaliplatin to 5-fluorouracil has had a major impact on the treatment of colorectal cancer. However, the efficacy of oxaliplatin-based therapy is often compromised because of the substantial risk for severe toxicities, including neurotoxicity.[225] Oxaliplatin has a direct "pharmacologic" effect on the excitability of sensory neurons and muscle cells that has not previously been described with other platinum agents. Neurotoxicity can result in both acute and chronic debilitation. Moreover, colorectal cancer patients treated with oxaliplatin more often discontinue therapy due to peripheral neuropathy than for tumor progression, potentially compromising patient benefit. Numerous methods to prevent neurotoxicity have so far proven unsuccessful.[225] In order to circumvent this treatment-altering side effect, while taking advantage of the antitumor activities of oxaliplatin, efforts to identify mechanism-based biomarkers are underway.

The neurotoxic effects of oxaliplatin are not seen with exposure to the cytotoxic metabolite, DACH platinum, but rather with the oxalate metabolite. A promising biomarker for risk of oxaliplatin-associated peripheral neuropathy is AGXT, which encodes alanine-glyoxylate aminotransferase. Gamelin et al. proposed that key components of the oxalate synthesis pathway could be associated with platinum-drug neurotoxicity. In a study of 145 patients treated with oxaliplatin, a C to T change in AGXT nucleotide 154 was associated with risk of chronic neurotoxicity.[226] Patients with a C/C genotype (~70% of Caucasians) had a significantly lower incidence of grade 2+ neurotoxicity (~5%) compared to the C/T and T/T patients (~30% of patients, expected incidence of grade 2+ neurotoxicity would be 30%). AGXT is a major enzyme of glyoxylate and oxalate metabolism and 154C>T has a partially reduced activity. These patients seem unable to cope with a brutal inflow of oxalate after oxaliplatin infusion, whose elimination becomes predominantly urinary. Its intracellular concentration increases and it interferes with sodium channels and generates acute neurotoxicity. Our primary hypothesis is

that patients with AGXT 154 C/T or T/T will have a significantly greater incidence of grade 2+ peripheral neuropathy, compared with 154 C/C patients.

Finally, in addition to specific hypothesis testing for the above candidate genes, this study will also provide the framework for hypothesis generation investigations of genotype and/or haplotype in additional candidate genes of putative importance to drug response of the agents being evaluated in this study.

2.4 Genome-wide association studies

Most pharmacogenetic analyses have taken a candidate gene approach that utilizes biological data to guide the selection of drug response genes in a pathway. This approach is limited by our knowledge of the mechanisms underlying the phenotypes. In the case of drug response phenotypes, most candidate gene studies have focused on drug metabolizing enzymes and transporters, thus limiting the chance of discovering causal SNPs not involved in mediating drug levels.[227,228] In contrast, a genome-wide approach collects SNP data across the entire human genome and has significant power to detect common variants that confer a modest risk for a complex phenotype.[229] Genome-wide studies capitalize on the large number of SNPs (more than 10 million available in dbSNP) that have been localized and validated across the genome, a majority of which have resulted from the HapMap project.[230] This valuable collection of publicly available, validated SNPs has provided the framework for performing genome-wide association studies. Recent technological advancements in genotyping platforms have also enabled the development of genome-wide associations. Searching the whole genome in an association study requires genotyping of anywhere between 10^5 to 10^6 markers across the genome.[231-234] Until recently, this approach was fiscally prohibitive and impractical. However, new gene chip platforms from Affymetrix and Illumina have made large-scale genotyping feasible and cost effective. The Illumina Infinium OmniExpress chip that will be used in this study has the capacity to genotype over 555,000 SNPs simultaneously. In addition, there are 4,300 SNPs in regions of copy number variations (CNVs), thus allowing for the detection of CNVs as well. This new capability represents a paradigm shift in the number of genotypes that can be evaluated in any given individual with one genotyping assay and provides a platform for the identification of novel genes involved in the response to and toxicity associated with 5-fluorouracil, oxaliplatin, and celecoxib.

An increasing number of reports of significant findings from genome-wide association studies in cancer are being published. To date, these have all focused on SNPs associated with risk of developing cancer, and include studies in prostate,[235-239] colorectal,[240-242] lung,[243] and breast cancer.[244-246] The success of these studies illustrates the power and validity of this approach for identifying genetic causes of disease. The relatively large size of CALGB/SWOG C80702 and robust response and toxicity phenotype data make it an ideal sample set for whole genome analysis. The identification of SNPs that contribute to response and toxicity of the three widely used drugs studied in CALGB/SWOG C80702 will lead to additional studies to understand the mechanism for these associations and to investigate the application of genetic information for the optimization of cancer therapy. The whole genome SNP data will also be useful for biological associations, including those detailed in the correlative science portions of this protocol.

3.0 HYPOTHESES

3.1 Genetic variation in COX-2 and related pathway genes will influence the efficacy of adjuvant celecoxib on cancer recurrence.

3.2 Germline variation in vitamin D pathway genes (VDR, VDBP, CYP27B1, RXR, CYP24A1) will impact disease-free and overall survival in stage III colon cancer patients. Moreover, polymorphisms in these genes modify the effect of plasma 25(OH)D on patient outcome.

3.3 Patients with AGXT 154 C/T or T/T will have a significantly greater incidence of grade 2+ peripheral neuropathy, compared with 154 C/C patients.

3.4 Novel SNPs and/or copy number variations will be identified that are associated with the prevalence of oxaliplatin-related peripheral neuropathy as well as DFS and OS.

3.5 Genome-wide analyses will elucidate novel genes and pathways associated with risk of recurrence, survival, safety, and efficacy of treatments.

3.6 Genome-wide analyses will allow the ascertainment of the genetic basis of risk of sporadic CRC through participation in larger case-control studies of CRC predisposition.

4.0 METHODS

DNA will be available from patients who consented to the pharmacogenomic companion study (CALGB 60905) and provided blood samples (10 mL in EDTA tube). Samples will be banked by the Alliance Biorepository at Ohio State University (OSU). DNA will be isolated and DNA quality will be assessed by UV spectrophotometry and by agarose gel electrophoresis. Phenotypic data will be extracted from research databases by the Alliance statistical group.

4.1 Genetic analysis of candidate genes

For genetic analyses of the candidate genes such as COX-2, vitamin D (VDR, VDBP, CYP27B1, RXR, CYP24A1), and AGXT pathway genes, assays will be performed using previously published methods such as Taqman allelic discrimination or pyrosequencing assays. If more efficient alternative genotyping methods become available in the future, the PET committee will change the genotype approach to optimize resources. Output from the genotyping platforms is entered in a database and forwarded to the Alliance Statistics and Data Center at Duke University where it will be correlated with clinical outcomes.

4.2 Whole-genome genotyping

Illumina's HumanHap550 Genotyping BeadChip enables whole-genome genotyping of over 555,000 single nucleotide polymorphisms (SNPs) loci efficiently and accurately on a single BeadChip. The HumanHap550 BeadChip is powered by the Infinium™ II assay, which uses a single-tube, whole-genome amplification method that does not require PCR and enables intelligent SNP selection using tagSNPs. TagSNPs are loci that can serve as proxies for many other SNPs. The information and power from a larger number of SNPs can be gathered by genotyping only a subset of loci. TagSNPs on the HumanHap550 BeadChip were selected from the recently completed International HapMap Projected. The Illumina's HumanHap550 Genotyping BeadChip is one of the platforms that might be used for this study. However, additional platforms, including high-throughput resequencing, might also be used to interrogate the germline genomic variation of patients.

5.0 DATA ANALYSES

5.1 Genetic variation of COX and related pathways

For analyses of germline variation in COX-2 and related pathway genes (objective 1.1), there are several strategies for choosing variants to genotype in specific candidate genes, ranging from selection of a single SNP with proven or potential functional significance, to haplotype- or linkage-disequilibrium tagging approaches. We have chosen the strategy for each gene that we feel can be best justified at this time, taking note of the fact that this is a fast-moving research area. Thus, for most candidate genes, the SNP(s) of proven function, or SNPs with probable function that have been previously associated with the disease, have been chosen. For the most part, the choices are conservative on a gene-by-gene basis, and are the variants that are most likely to interact with the environmental factors proposed, given current knowledge. Although some of the proposed SNPs have been investigated in functional studies, the functional roles of many other SNPs, especially those in the noncoding regions, have been minimally studied.

Candidate SNPs as Effect Modifiers of Celecoxib Efficacy: All polymorphisms will be individually evaluated and categorized as variant or wild type. Baseline characteristics of patients will be compared according to genotype. We will also investigate whether COX-2 and related pathway genotypes modify the relationship between celecoxib use (vs. placebo) and patient outcome. Cox proportional hazards models will be used to calculate multivariable-adjusted HRs and 95% CIs for DFS and OS by celecoxib vs. placebo for each SNP: those with the variant genotype and those with wild type. Tests for statistical interaction will be performed by entering into the model the cross-product term of celecoxib vs. placebo with the genotype as a binary variable. The main effect of each genotype on DFS and OS will also be examined. The main effect analyses will also utilize Cox proportional hazards models to calculate multivariable-adjusted HRs and 95% CIs for DFS and OS by genotype. Multivariate models will be adjusted for confounding variables.

5.2 Germline variations in the vitamin D pathway

For analyses of germline variation in vitamin D pathway genes (objective 1.2), there are several strategies for choosing variants to genotype in specific candidate genes, ranging from selection of a single SNP with proven or potential functional significance, to haplotype- or linkage-disequilibrium tagging approaches. We have chosen the strategy for each gene that we feel can be best justified at this time, taking note of the fact that this is a fast-moving research area. Thus, for most candidate genes, the SNP(s) of proven function, or SNPs with probable function that have been previously associated with the disease have been chosen. For the most part, our choices are conservative on a gene-by-gene basis, and are the variants that are most likely to interact with the environmental factors proposed, given current knowledge. Although some of the proposed SNPs have been investigated in functional studies, the functional roles of many other SNPs, especially those in the noncoding regions, have been minimally studied. In addition, in future analyses, an agnostic approach will be employed and genome-wide association studies will be conducted to identify SNPs that may be associated with plasma 25(OH)D concentrations and patient survival.

Candidate SNPs as Main Effects: All polymorphisms will be individually evaluated and categorized as variant or wild type. Baseline characteristics of patients will be compared according to genotype. The main analyses will utilize Cox proportional hazards models to calculate multivariable-adjusted HRs and 95% CIs for DFS and OS by genotype. Multivariate models will be adjusted for confounding variables. It will be examined whether genetic variation in the vitamin D pathways is associated with vitamin D levels using linear regression. All patients who have available plasma 25(OH)D and genotype data will be included; analyses will be age- and season of blood draw-adjusted. We will also investigate whether vitamin D pathway

genotypes modify the relationship between vitamin D levels and patient outcome. Cox proportional hazards models will be used to calculate multivariable-adjusted HRs and 95% CIs for DFS and OS by tertile of 25(OH)D for each SNP: those with the variant genotype and those with wild type. Tertiles will be used instead of quintiles for these analyses in order to maximize power. Tests for statistical interaction will be performed by entering into the model the cross-product term of 25(OH)D as a continuous variable with the genotype as a binary variable.

5.3 AGXT 154C>T polymorphism and neuropathy

The primary statistical objective for the pharmacogenetic companion of this study is to investigate the potential association between the AGXT 154C>T polymorphism and neuropathy in the Caucasian population.[226] Specifically, it is hypothesized that the presence of the T allele is associated with higher incidence oxaliplatin-related peripheral neuropathy.

The primary statistical endpoint will be the realization of a grade 2 or higher neuropathy event. Let D denote the corresponding event and pi=P(D=1) denote the relative prevalence of the event in the population. The study will be powered assuming that the model is non-decreasing with respect to the T allele. In other words, it is assumed that pi0<=pi1<=pi2, where pi0=P(D=1|CC), pi1=P(D=1|CT) and pi2=P(D=1|TT). Assuming Hardy-Weinberg, the event probability is expressible as the mixture pi=(1-q)^2* pi0 + 2*q(1-q)*pi1+q^2*pi2.

The clinical study plans to contribute 2,500 patients to this intergroup study. It is assumed that 85% of these patients will provide consent and usable samples for this companion study. The analysis population will consist of those self-reported as Caucasian on the CRF form. The expected proportion for this population is at least 0.85. The companion study will be powered based on a sample size of 1806 (=2500*0.85*0.85).

The putative allelic relative frequency for the risk T allele is q=0.3. The power of the Cochran-Armitage test, at the two-sided 0.05 level, assuming a dominant model (DOM: pi1=pi2=pi0*GRR) and an additive model (ADD: pi1=pi0*GRR, pi2=pi0*GRR*GRR) is illustrated in Table 1 for GRR>1. Here GRR denotes the Genotype Relative Risk.

pi	GRR	Power	
		DOM	ADD
0.30	1.15	0.42	0.73
	1.20	0.62	0.92
	1.25	0.80	0.98
0.33	1.15	0.46	0.78
	1.20	0.69	0.95
	1.25	0.85	0.99

Table 1: Power illustration for the genotype test, at the two-sided 0.05 level, assuming a dominant or additive model.

It is noted that this is a hypothesis of association (gene by outcome) regardless of the number of cycles of oxaliplatin received. Any potential effect due to amount of therapy received will be examined using regression techniques.

Secondary SNPs of interest include potential predictive markers of outcome (OS or DFS) with respect to celecoxib (e.g., COX-2 and pathways) and germline variation in the vitamin D pathway.

The addition of other important clinical and demographic co-variables will be considered. Multivariable models, with molecular, clinical and demographic variables, will be constructed using conditional inference trees and random forests. All secondary and exploratory objectives will be tested at an unadjusted two-sided level of 0.05.

5.4 Genome-wide association studies

5.4.1 Pre-processing

For pre-processing (QC and genotype calls) the Illumina chips, we will use the commercial program Bead Studio developed by Illumina. Although, Illumina does not provide a Linux port of Bead Studio, one can run the software on VMWARE running on a Linux host. A two CPU dual core (four cores) AMD Opteron Socket F workstation with 16GB of RAM

will be available for this purpose (the statistical analyses will be carried out on a Linux server with four quad-core Opteron CPUs (16 cores) with 64GB of RAM [expandable to 128GB if needed]).

5.4.2 Analyses to assess genotyping quality and population stratification

Initial quality studies will be conducted to identify SNPs that have generated sufficiently poor quality genotype data and should be removed from analyses. Call rate, patterns of missing data, and departures from Hardy-Weinberg equilibrium (HWE) assessed using an exact test will all be scrutinized to identify markers that will not be used in analysis. In general, SNPs with call rates <95% and those with highly significant departures from HWE ($p<10^{-7}$) will not be included in analyses. Non-random patterns of missing data are sometimes encountered in data generated on high-throughput genotyping platforms; the most common non-random missing data problem is that heterozygous genotypes are more likely to be assigned as missing than either homozygous genotype. We will perform analyses using blind duplicates as well as analyses assessing the relationship between heterozygous call rates and missing data to identify any SNPs in which data are clearly not missing at random. Depending on the number and degree of difficulty observed, we will either remove problematic SNPs from analysis, or assign quality scores to reflect the extent of the non-random missing data.

Additional preliminary quality control analyses will be conducted to ensure that the sample does not include duplicated samples or closely related individuals. These analyses can be rapidly conducted using PLINK.[300] Duplicated samples (or unrecognized identical twins) will be reduced to a single sample for further analyses. Although we do not expect to have closely related individuals included in this sample, only one member of any set of first-degree relatives will be included in subsequent analysis.

Population structure that is not appropriately recognized and accommodated can lead to both false positive and false negative results in association studies. We will conduct studies using structure[301] to estimate ancestry proportions using 10,000 SNPs chosen for having no pairwise LD with unrelated individuals from the HapMap CEU, YRI and CHB+JPT samples used to model the ancestral populations. Substantial previous research has shown this to be a rapid and effective approach to defining historical geographic ancestry. Although self-identified race/ethnicity is usually highly correlated with estimated historical geographic ancestry, there are often a few individuals who appear to be misclassified with self-defined labels, and it is the genetically defined ancestry that is critical to correctly accommodate to ensure robust results from association studies. Each individual will then have estimates of European, African and Asian ancestry. For individuals with high ancestry proportion for a single group (>98%), we will conduct further analyses with eigenstrat[302] using all SNPs to determine whether there are additional important sources of variation among individuals leading to detectable stratification by allele frequencies (reflecting, for example, differences in ethnic make-up within individuals of European descent from different U.S. cities from which subjects for the trial were obtained). Primary analyses, described below, will be conducted within groups defined by historical geographic ancestry. Secondary analyses will be conducted using logistic regression with ancestry proportions (and any additional stratification identified using eigenstrat) as covariates.

5.4.3 Feature discovery

The association between the genotype call (say AA, AB or BB) for each autosomal SNP and the clinical outcome [for example, adverse event (AE) or no AE] will be investigated within the framework of 2 by 3 contingency table stratified by ancestry. Fisher's exact test (i.e., randomized conditional counterpart to Fisher's test for 2 x 3 tables)[303] will be used for carrying out inference on these tables. A feature (SNP) will be considered significant if the

corresponding nominal unadjusted two-sided P-value is less than 0.05/K, where K is number of features which pass the pre-processing step. Needless to say, this approach may be conservative. It does however guarantee strict type I error control.

For the sake of discussion, let B denote the risk allele with an assumed relative allelic frequency of q. Under the Hardy-Weinberg equilibrium assumption, the genotypes AA, AB or BB will have relative genotypic frequencies of $(1-q)^2$, $2q(1-q)$ and q^2, respectively. Let D denote the binary clinical outcome (D=1 if the AE event occurs or =0 otherwise) and define the probability of an AE occurrence given the copies of the risk allele on the genotype, to be denoted by G, as $p_g=P[D=1|G=g]$, for g=0,1 or 2. The relationship between the event probability p=P[D=1] in the general population is then expressible as the following mixture $p=(1-q)^2p_0+2*q*(1-q)p_1+q^2p_2$. The effect size in the context of genome-wide association studies is typically quantified using the genotype relative risk (GRR) whose definition depends on the disease model. Under the recessive disease model, $p_0=p_1$ and $p_2=GRRp_0$ while under the dominant disease model $p_1=p_2=GRRp_0$. Finally, under the multiplicative (log-additive model), $GRR=p_1/p_0=p_2/p_1$. Under these disease model, the event probability in the population, p, can then be reformulated as the mixture $p=(1-q)^2p_0w_0+2*q*(1-q)p_0w_1+q^2p_0w_2$, where $w=(w_0,w_1,w_2)=(1,1,GRR)$, for the recessive model, $=(1,GRR,GRR)$ for the dominant model and $=(1,GRR,GRR^2)$ for the multiplicative model.

The clinical study plans to contribute 2,500 patients to this intergroup study. It is assumed that 85% of these patients will provide consent and usable samples for this companion study. The analysis population will consist of those self-reported as Caucasian on the CRF form. The expected proportion for this population is at least 0.85. The companion study will be powered based on a sample size of 1806 (=2500*0.85*0.85). The power, at the two-sided 0.05/600000 level (i.e., assume K=600,000 autosomal SNP markers pass through the pre-processing step) is 0.9, for a range of relative allele frequencies (q) assuming the event probability is P[D=1]=0.3 under recessive, dominant and multiplicative models assuming HWE.

5.4.4 Submission of molecular data

The laboratory of Dr. Yusuke Nakamura will submit the Illumina *.idat image files using secure means to the Alliance Statistics and Data Center at Duke University. The lab will also submit a table along with this transmission, which at the minimum will provide the following information for each sample received from the repository.

The lab ID number provided by the repository.

The experimental ID, a concatenation of the plate, well and replicate information, generated by the lab.

The idat file names (the file string name will contain the Lab ID).

The md5sum signature of the idat files to ensure data integrity.

The date the specimen was received from the repository.

The date the sample was analyzed by the RIKEN laboratory.

Additionally, the lab will also provide the complete results from any quality control measures carried out. If a sample had to be redone (e.g., defective or poor quality array), the lab will provide all replicate idat files and add an appropriate column to the supplementary table. The molecular data generated for this aim may not be shared with other investigators or used for any analysis not specified in the protocol until a formal approval from the Alliance Statistics and Data Center at Duke University is obtained.

5.4.5 Secondary objectives

Logistic regression models and conditional inference trees (or more generally conditional random forests) will be used to construct multi-variable models based on the SNPs identified as interesting. These models also allow for inclusion of other potentially relevant clinical and demographic variables.

The Illumina Human610 Quad chip contains 184.064 SNPs in regions with common copy number variants (CNVs). Given the complex structure of CNVs, it is not always clear how to define the genotype of a CNV. Instead of categorizing copy numbers into genotypes, we will estimate relative genomic abundance probe intensities. This approach allows for the consideration of other CNVs beyond deletions, including duplications and combinations of both. For notational brevity, we shall refer to these as CNV markers.

For each objective, the association between each CNV marker and the clinical AE endpoint, will be assessed using the Wilcoxon two-sample test. The family-wise error rate will be controlled at the 0.05 level using permutation resampling (based on B=100,000 replicates).

Regression methods, as in the case of the SNP markers, will be employed to construct multivariable models based on the CNV markers.

Secondary relevant clinical endpoints include other adverse events (e.g., proteinuria and hypertension), progression-free and overall survival. For censored time-to-event outcomes, the stratified log-rank test will be primarily used for assessment of significance.

A risk analysis will be carried out by comparing the genotypic distributions of the SNPs from the CALGB/SWOG C80702 data to those from controls (thought to not to have cancer). The SNP data from the controls will be obtained from public databases.

In addition to conducting analyses on all features directly assessed on the high-throughput platform used in these studies, we will also interrogate all additional HapMap SNPs that are not in strong pairwise LD with any genotyped SNP but for which there is sufficient multi-locus LD to SNPs on the high-throughput platform. TUNA (Testing UNtyped Alleles) is a robust approach for conducting such analyses that provides inexpensive in silico follow up to the initial analysis and allows us to more efficiently design any follow up genotyping studies.[304,305] For example, use of Illumina HumanHap300 enables direct testing of 270K-450K SNPs, and indirect testing of 750K-1.5M additional SNPs (i.e., these SNPs are so highly correlated with SNPs that are directly tested for association that testing them would provide little additional information). The ranges given above bracket the expectations for different human populations, with European populations at the high end of the range, and populations of recent African descent at the lower end. Use of TUNA enables interrogation of an additional 100K-250K SNPs that are neither on the platform nor highly correlated with any individual SNP on the platform. Note that use of TUNA will facilitate comparisons to genome-wide association studies on potentially related phenotypes (e.g. clinical trials of the same or related drugs) conducted using other high-throughput platforms or candidate gene studies utilizing SNPs not directly genotyped on the high-throughput platform chosen for our studies.

Finally, we note that the methodology field for the analysis of genome-wide SNP data is in its infancy. We will consider the employment of "newer" methods if they are deemed to be statistically sound and enable us to better interrogate, and more importantly, understand the data.

5.4.6 Statistical software

The R statistical environment[306] and Bioconductor[307] packages will be used for all of the primary statistical analyses relating features to phenotypes. Specialized statistical genetics software, including PLINK,[300] structure,[301] eigenstrat,[302] and TUNA[304,305] will be used for some of the quality or secondary analyses, and R will be used for logistic regression analyses allowing for ancestry covariates.

APPENDIX IV

CANCER PREVENTION COMPANION STUDIES (DIET AND LIFESTYLE): CALGB 71002

The influence of diet and other exogenous factors on disease-free survival, overall survival and treatment-related toxicity among patients with stage III colon cancer will be assessed. Patients enrolled on CALGB/SWOG C80702 will be asked to complete a food-frequency questionnaire within the first 6 weeks of start of randomization and 14-16 months after randomization. The questionnaire has been extensively validated among large populations and provides comprehensive data on 131 food items and over 100 micronutrients. The instrument will also ascertain leisure-time physical activity, cigarette smoking, height and weight, aspirin and non-steroidal anti-inflammatory drug use, vitamin/supplement use, and alternative medicine use. It is suggested that patients are given the questionnaire in clinic, completed in clinic/infusion and then mailed back by the research assistant/nurse. Patients who recur are not required to complete the questionnaire.

1.0 OBJECTIVES

1.1 Assess the influence of diet, body mass index, physical activity and other lifestyle habits on disease-free and overall survival among patients with stage III colon cancer.

1.2 Assess the influence of baseline plasma C-peptide, insulin-like growth factor binding protein-1 (IGFBP-1), insulin-like growth factor-1 (IGF-1), IGFBP-3, and adiponectin on disease-free survival in patients with stage III colon cancer.

1.3 Assess whether tumoral expression of phospo-Akt (pAkt) and fatty acid synthase (FASN) and KRAS and PI3K mutational status modifies the relation between measures of energy balance (e.g., dietary insulin index, obesity, physical activity, plasma C-peptide) and patient survival.

1.4 In exploratory analyses, assess the influence of diet, obesity, physical activity, and other lifestyle habits on the risk of toxicity associated with chemotherapy.

2.0 BACKGROUND

Epidemiologic and scientific research indicates that diet and other lifestyle factors have a significant influence on the risk of developing colon cancer. Consumption of red meat [247,248], alcohol [249,250], calcium [251,252], fiber [253], aspirin [254,255], and folic acid [250,253,256], obesity [257-264], physical activity [261,262,264], and cigarette smoking [259,265-267] are among factors that have been suggested to influence the risk of developing colorectal cancer.

Not until recently have there been data accessing the influence of these factors on patients with established cancer. In CALGB 89803 (trial of adjuvant therapy for stage III colon cancer comparing 5-FU/LV to irinotecan/5-FU/LV), a self-completed questionnaire was utilized to access diet, physical activity, smoking, medication use and family history. Multiple important findings have stemmed from this trial. However, these data require confirmation. Furthermore, the trial utilized an adjuvant therapy regimen that is now not standard of care and thus the influence of these factors on patients receiving FOLFOX would be very important.

3.0 PROPOSED HYPOTHESES

3.1 Lower dietary insulin index and regular physical activity are associated with an improved disease-free survival, and the benefit of lower dietary insulin index and regular physical activity are greater among tumors with wild-type KRAS, wild-type PI3K, and reduced expression of pAkt and FASN.

In prospective and retrospective studies, obesity is associated with an increased risk of colon cancer, whereas regular physical activity confers a reduced risk. Recently, several studies among patients with stage II and III colon cancer suggest that obesity has been associated with a reduced disease-free and overall survival, whereas physical activity was associated with an improved patient survival, including a recent study conducted within an

adjuvant chemotherapy trial in stage III colon cancer patients (CALGB 89803). Recent hypotheses have linked physical activity, obesity, and adipose distribution to circulating insulin and free insulin-like growth factor 1 (IGF-1),[268,269] which is determined by the integrated actions of circulating IGF-1 and IGF binding proteins (BPs). Indeed, colon cancer risk is elevated in individuals with higher circulating levels of insulin or C-peptide (a marker of insulin secretion) and IGF-1 or IGF-1/IGFBP-3 ratio. Pre-clinically, insulin stimulates pathways that increase levels of free IGF-1, and both insulin and IGF-1 promote cell proliferation and inhibit apoptosis in colon cancer cells.

The relation between hyperinsulinemia and colon cancer suggests that a diet inducing an elevated insulin response may contribute to tumor growth. A Western pattern diet has been associated with hyperinsulinemia and hypertriglyceridemia. Among stage III colon cancer patients participating in CALGB 89803, Meyerhardt et al. found that increasing consumption of a Western pattern diet was associated with a significant increase in cancer recurrence and mortality. Brand-Miller et al. have therefore developed a novel insulin index for foods, which represents the incremental area under the insulin curve after feeding 1000 kjoules of a test food, divided by the insulin response to 1000 kjoules of white bread. A database for the insulin index of foods with the Willet dietary questionnaire that will allow the calculation of a dietary insulin index for each patient has been developed and validated. The insulin index will be used to assess the relation with patient outcome in the clinical trial.

Several studies suggest that insulin and IGF-1 act synergistically with activation of the K-ras/MAP-kinase pathway.[270-272] Inactivation of ras blocks insulin- and IGF-1-induced cell proliferation, and K-ras cannot transform mouse fibroblasts that are devoid of the IGF-1 receptor. It is hypothesized that the relation between dietary insulin index, physical activity and patient survival may be stronger among patients with K-ras-wild-type tumors. Similarly, the growth-promoting and anti-apoptotic effect of sedentary lifestyle, and insulin appears to be mediated principally through activation of phosphatidylinositol 3-kinase (PI 3-kinase), which in turn activates Akt/protein kinase B (PKB) via phosphorylation. Phosphorylation of Akt (phospho-Akt) results in cell proliferation and escape from apoptosis. Approximately 20% of patients have mutations in PI3K, and it was recently found that such mutations confer a reduced survival in a large population of stage I-III colon cancer patients.[273] Moreover, 45% of colorectal cancers overexpress phospho-Akt. Finally, fatty acid synthase (FASN) is the major enzyme required for the anabolic conversion of dietary carbohydrate to fatty acids. FASN is overexpressed in ~25% of colon cancers and was associated with a significantly improved survival among colon cancer patients. Moreover, the effect of FASN was significantly modified by obesity.[274] In light of these data, we will assess whether expression of phospho-Akt and FASN or mutations in PI3k and KRAS modify the influence of dietary insulin index and exercise on colon cancer recurrence and mortality.

3.2 Elevated baseline plasma C-peptide, IGF-1 and leptin and reduced plasma IGFBP-3 and adiponectin are associated with reduced disease-free survival among patients with stage III colon cancer.

Among stage I-III colon cancer patients, Wolpin et al. recently found that higher prediagnosis plasma C-peptide (a long term measure of insulin secretion) and reduced plasma IGFBP-1 (inversely associated with insulin secretion) conferred a significant increase in cancer-specific and all-cause mortality, independent of plasma levels of IGF-1 and IGFBP-3 (an antagonist to IGF-1).[275] Ongoing trials are assessing antibodies to the IGF-1 receptor (IGF1R) in patients with metastatic colorectal cancer. Further, plasma adiponectin has been associated with the risk of developing colorectal cancer and is a

marker of insulin sensitivity.[276] Therefore, the influence of plasma levels of these factors and cancer recurrence and mortality among patients with stage III colon cancer will be examined. In exploratory analyses, it will also be assessed whether the effect of the plasma factors are modified by tumoral mutations in KRAS, PI3K, and expression of pAkt and FASN.

3.3 Higher intake of a Western dietary pattern, as manifested by higher red meat and total fat intake and lower n-3 polyunsaturated fatty acids, fruit and vegetable intake, is associated with increased cancer recurrence and mortality.

Western-style diets have been hypothesized as contributing to the development of colon cancer.[277] A factor analysis was conducted and two major dietary patterns: "prudent" and "Western" were identified. The prudent pattern was characterized by higher intakes of fruits, vegetables, legumes, fish, poultry, and whole grains, while the Western pattern, by higher intakes of red and processed meats, sweets and desserts, french fries, and refined grains. Increasing consumption of a Western diet was associated with an increased risk of colon cancer whereas increasing consumption of a prudent diet was associated with a reduced risk.[83,278] In mouse models, a Western-style diet accelerates colon cancer progression and mortality.[279-281] Dietary factors have been associated with the risk of developing colon cancer but the influence of diet on patients with established disease is unknown. The association of dietary patterns with cancer recurrences and mortality was examined in 1009 patients with stage III colon cancer who were enrolled in a randomized adjuvant chemotherapy trial (CALGB 89803). A higher intake of a Western dietary pattern after cancer diagnosis was associated with a significantly worse disease-free survival (colon cancer recurrences or death). Compared with patients in the lowest quintile of Western dietary pattern, those in the highest quintile experienced an adjusted hazard ratio (AHR) for disease-free survival of 3.25 (95% confidence interval [CI], 2.04-5.19; P for trend <.001). The Western dietary pattern was associated with a similar detriment in recurrence-free survival (AHR, 2.85; 95% CI, 1.75-4.63) and overall survival (AHR, 2.32; 95% CI, 1.36-3.96]), comparing highest to lowest quintiles (both with P for trend <.001). The reduction in disease-free survival with a Western dietary pattern was not significantly modified by sex, age, nodal stage, body mass index, physical activity level, baseline performance status, or treatment group.[282]

The influence of a Western pattern diet on the outcome of stage III colon cancer patients will be examined in the clinical trial. Moreover, the influence of pre- and post-diagnosis intakes of red meat, n-3 polyunsaturated fatty acids, and fruits and vegetables on survival will be examined.

3.4 In exploratory analyses, the effect of diet on chemotherapy-induced toxicity will be examined.

The influence of Western and prudent pattern diets, physical activity, and dietary insulin index on chemotherapy-based toxicity will be assessed and, in additional secondary analyses, other dietary determinants for toxicity will be explored.

4.0 METHODS

4.1 Assessment of diet and lifestyle factors

In this companion study, patients participating in the treatment trial will be asked to complete a 131-item validated, food-frequency questionnaire within first 6 weeks of randomization and 14-16 months after randomization. The questionnaire proposed, designed by Dr. Walter Willett and colleagues for the Nurses' Health Study, has been extensively validated among both health professional and lay populations, and provides comprehensive data on over 100 micro-nutrients,

with and without supplement use. This questionnaire can be self-administered. Within the questionnaire, a series of questions about leisure-time physical activity, smoking habits, alcohol intake, and other habits that have also been validated in large populations will be included. Height and weight will also be obtained as part of the adjuvant therapy. A similar study was initiated in the preceding CALGB adjuvant therapy trial (CALGB 89803) and more than 90% of eligible patients completed the questionnaire.

Validation of the Semi-quantitative Food Frequency Questionnaire: The current version of the questionnaire consists of 131 food items plus vitamin and mineral supplement use that collectively account for over 90% of the intake of the nutrients assessed.[283-287] For each food, a commonly used unit or portion size (e.g., one egg or slice of bread) is specified, and participants are asked how often, on average over the past year, they consumed that amount of each food. There are nine possible responses, which range from never to six or more times per day. The nutrient intakes will be computed by multiplying the frequency of consumption of each food by the nutrient content of the specified portions, using composition values from Department of Agriculture sources supplemented with other data, including the components of specific vitamins and breakfast cereals. All nutrients will be adjusted for total energy intake by the residuals method.[288]

In 1980, the food frequency questionnaire was administered twice to 173 individuals at an interval of approximately one year, and four one-week diet records for each subject were collected during that period. Diet records probably are the best measures of current, short-term food intake. Since the seven-day record provides information for a relatively short period of time, four one-week diet records in different seasons were collected. The mean calorie adjusted intakes from the four one-week diet records and those from the questionnaire were well-correlated.[285-287] In the 1986 diet validation study, the correlation between folate calculated from the semi-quantitative food frequency questionnaire (SFFQ) and red cell folate level was 0.55.[250] Nutrients calculated from the expanded SFFQ were correlated with other corresponding biochemical indicators: plasma beta-carotene (r = 0.30-0.42),[289,290] plasma vitamin E (r = 0.41-0.53),[289,290] adipose linoleic acid (r = 0.35-0.37),[291,292] adipose trans fatty acid (r = 0.51),[291,292] and adipose N-3 fatty acids (r = 0.48-0.49).[291,292] To evaluate further the capability of the revised 131-item questionnaire to discriminate among subjects, Willett and colleagues asked 127 individuals to complete two weeks of diet records and the semi-quantitative food frequency questionnaire in 1986. The mean calorie adjusted intakes from the diet records and those from the questionnaire were well-correlated.[285]

The validity of this 131-item SFFQ will be separately assessed in 200 patients with colorectal, breast, or neuroendocrine cancer undergoing treatment with cytotoxic chemotherapy.[293] The Pearson correlation coefficients for various carotenoids as measured by the questionnaire, with the corresponding measurements in plasma specimens, ranged from 0.33 to 0.44 (all $P < .001$), adjusted for total energy intake, body mass index, age, sex, smoking status, and total plasma cholesterol. Similarly, the adjusted correlation between self-reported total vitamin E intake and plasma alpha-tocopherol was 0.34 ($P < .001$). Correlations between questionnaire and plasma measurements of trans-fat, eicosapentaenoic acid, and docosahexaenoic acid were 0.55, 0.29, and 0.42 (all $P < .001$), respectively. These levels of correlation were consistent with those reported in similar studies of self-reported diet in otherwise healthy populations. Thus, among patients with cancer receiving cytotoxic chemotherapy, questionnaire-based measurements of various micronutrients and dietary factors appeared to predict meaningful differences in the corresponding measurements in plasma specimens.

These data indicate that the proposed self-administered dietary questionnaires provides highly informative and biologically relevant measurement of a wide variety of nutrients, thus allowing one to address the dietary hypotheses outlined in the specific aims.

In terms of other measures from the survey, Wolf et al. reported on a detailed validation study of the physical activity questionnaire among a sample of 325 participants in the parallel Nurses' Health Study II (NHS II) (241 random cohort sample and 84 random sample of African American participants).[294] Participants completed four 1-week activity recalls and four 7-day activity diaries over one year and then repeated the NHS II activity questionnaire. For the total activity score, the correlations of the last activity questionnaire with the diaries was 0.64 for the total cohort sample and 0.59 for the African American sample. Within the Health Professionals Follow-up Study, a parallel study of men, validity of the physical activity questionnaire was assessed among 238 randomly selected participants by comparisons with four 1-week activity diaries, four 1-week activity recalls, and resting and post exercise pulse rates.[295] Correlations with the activity diaries were 0.41 for inactivity (sitting) and 0.58 for vigorous physical activity. Vigorous activity assessed by the questionnaire was correlated with resting pulse (r = -0.45) and post-exercise pulse (r = -0.41).

4.2 Analysis of the Growth Factor Blood Markers

Serum will be collected in a red top tube prior to start of any chemotherapy. The blood is allowed to clot for 30 minutes at room temperature. The clotted blood is centrifuged for 10-15 minutes at 1,300 g and serum is aliquoted into two 2 mL cryovials, frozen and stored. Assays for C-peptide, IGFBP-1, IGF-1, and IGFBP-3 will be performed in the laboratory of Dr. Michael N. Pollak (Lady Davis Research Institute of the Jewish General Hospital, McGill University). Plasma levels of C-peptide were assayed by RIA (Linco Research), an assay with little or no cross-reactivity with proinsulin. IGFBP-1, IGF-1, and IGFBP-3 ELISAs will be assayed using reagents from Diagnostic Systems Laboratory (Webster, TX). The mean intra-assay CVs for C-peptide, IGFBP-1, IGF-1, and IGFBP-3 were each <10%.[275] Plasma adiponectin will be measured in the laboratory of Dr. Nader Rifai (Children's Hospital of Boston) by competitive RIA using a commercial reagent set (Linco Research), utilizing a highly purified antibody (intra-assay CVs 2-6%). All assays will be carried out by laboratory personnel blinded to patient outcome. In addition, masked quality control duplicate samples will be interspersed among the case samples.

4.3 Tumor based analyses

pAkt and FASN immunohistochemistry and *KRAS* and *PI3K* mutational status analyses will be obtained from Next Generation Sequencing (NGS) as described above.

Tissue Block Procurement: Formalin-fixed/paraffin-embedded (FFPE) tissue blocks and slides will be examined. A set of blocks will be selected for preparation of routine hematoxylin and eosin (H&E) slides for microdissection and DNA extraction for analysis of KRAS and PI3K mutations. Alliance pathologists will also construct tissue microarrays (TMAs) for immunohistochemical (IHC) evaluation of pAkt and FASN.

Tissue DNA Extraction: H&E stained slides will be reviewed, areas comprising ≥70% neoplastic cellularity will be marked and scraped from 15µm unstained slides under direct visualization, and DNA will be extracted. DNA of sufficient quality for polymerase chain reaction (PCR) will be obtained from >95% of colorectal cancers.

In addition, among 450 resectable colon cancers (stage I to III) in two independent prospective cohorts, the PIK3CA mutation was detected in 82 tumors (18%) by pyrosequencing. Compared with patients with PIK3CA wild-type tumors, those with PIK3CA-mutated tumors experienced an increase in colon cancer-specific mortality according to univariate analysis (HR = 1.64; 95% CI, 0.95 to 2.86), which persisted after adjusting for other known or potential risk factors for cancer recurrence (including MSI; multivariate HR = 2.23; 95% CI, 1.21 to 4.11). The effect of PIK3CA mutation on cancer survival seemed to differ according to KRAS mutational status. Among patients with KRAS wild-type tumors, the presence of PIK3CA mutation was associated

with a significant increase in colon cancer-specific mortality (HR = 3.80; 95% CI, 1.56 to 9.27). In contrast, PIK3CA mutation conferred no significant effect on mortality among patients with KRAS-mutated tumors (HR = 1.25; 95% CI, 0.52 to 2.96).[270]

IHC Staining and Interpretation: IHC analyses will be performed in the laboratory of Dr. Ogino. For FASN immunohistochemistry, primary antibody against FASN (BD Biosciences, Mississauga, ON, Canada) (dilution 1:100) will be applied for 60 min at room temperature. Then, Multilink secondary antibody (BioGenex) (20 min) and then streptavidin horseradish peroxidase (BioGenex) will be applied (20 min). Sections were visualized by diaminobenzidine (DAB) (5 min) and methyl-green counterstain. FASN expression will be interpreted as negative, weak (1+), positive (2+), and strongly positive (3+), using normal colonic epithelial cells and adipose tissue as reference. Appropriate positive and negative controls are included in each run of immunohistochemistry. All immunohistochemically stained slides will be interpreted by a pathologist blinded to other data. Random samples of 246 colorectal cancer have previously been examined for FASN by a second observer unaware of other data, and the concordance between the two observers was 0.93 for FASN (= 0.57, P < .0001). Using a database of 647 patients with colon cancer, FASN overexpression was detected in 84 tumors (13%) by immunohistochemistry.[274] FASN overexpression was associated with a significant reduction in colon cancer-specific mortality (adjusted HR, 0.41; 95% CI, 0.19 to 0.89). Notably, the effect of FASN expression on mortality differed according to body mass index (BMI; P(interaction) = .019); the adjusted HR of overall mortality for FASN overexpression was 0.63 (95% CI, 0.39 to 1.02) among patients with BMI less than 27.5 kg/m^2 and 2.91 (95% CI, 1.19 to 7.12) among those with BMI ≥27.5 kg/m^2. Moreover, the adverse effect of moderate overweight/obesity on overall survival was limited to FASN-positive tumors (adjusted HR, 4.10; 95% CI, 1.14 to 14.8; BMI ≥27.5 kg/m^2 v < 27.5 kg/m^2).

For phospho-AKT, monoclonal antibody will be applied to the tumor sections: phospho-Akt (Ser473: Cell Signaling Technology) dilution 1:50. A positive and negative control (tumors with known expression status of each of the selected proteins) will be included in each staining batch. Using the method of Itoh et al., phospho-Akt will be recorded as: 0, nearly no positive cells; 1+, 5-25% of tumor cells showing reactivity; 2+, 25-50% of cells showing reactivity; or 3+, >50% showing reactivity.[297] Tumors scoring 0-1 are considered as normal whereas those with 2-3 are considered as over-expression. In a prior analysis, 46% of colorectal cancers showed a high level (2+ or 3+) of phospho-Akt.[297,298]

Quality Control for Tumor Block Analyses: Approximately 5% repeated QC samples will be added as blinded specimens; they will be randomly nested in the sample sets with coded IDs. In all IHC analyses, appropriate positive and negative controls will be included in each run of IHC assay. In addition, a random sample of >100 cases will be re-examined by a second pathologist in Dr. Ogino's laboratory to assess inter-rater agreement using a kappa measure of agreement (κ) proposed by Kraemer. If agreement is unacceptable, further training and monitor agreement will be instated in the next set of 200 samples.

4.4 Data Analyses

The primary efficacy variable for analyses will be disease-free survival (DFS). Secondary efficacy endpoints include recurrence-free survival (RFS) and overall survival.

Exposure definitions: For all dietary exposures (**Objective 1.1**) including dietary insulin index, intakes will be categorized into energy-adjusted quintiles, consistent with our previous studies. In addition, physical activity will be categorized into categories of MET-hours as previously defined in prior work. Body mass index (kg/m^2; Aim 2) will be divided into World Health Organization categories of underweight, normal weight, overweight and obesity.

For the main effect of plasma analytes (**Objective 1.2**; C-peptide, IGFBP-1, IGF-1, IGFBP-3, and adiponectin), plasma levels will be divided into quintiles for the analysis. Baseline characteristics of patients will be compared according to quintiles of the biomarker using Wilcoxon signed rank tests for continuous variables and chi-squared tests for categorical variables. For primary analyses of survival, patients who recurred or died within three months of plasma collection will be excluded to minimize any bias in the due to occult cancer recurrence or preclinical illness. In sensitivity analyses, the possibility of reverse causation will also be assessed by allowing different lag times between plasma assessment and cancer recurrence or death. The log-rank test and Kaplan-Meier curves will be used to compare DFS and OS by quintile of plasma level. Cox proportional hazards models will be used to control for multiple confounders. The two-tailed P value for the linear trend test across categories will be calculated using the plasma level as a continuous variable, consistent with prior studies. In secondary analyses, we will examine how the relationship between a specific plasma analyte level and patient outcome is modified by relevant covariates such as ECOG performance status, treatment assignment, physical activity and body mass index, among others. Tests for statistical interaction will be performed by entering into the model the cross-product term of the plasma level as a continuous variable with the covariate as a continuous or binary variable.

It is hypothesized that specific tumoral alterations modify the effect of measures of energy balance (e.g., dietary insulin index, obesity, physical activity, plasma C-peptide) on patient survival. For the analysis of tumoral alterations (**Objective 1.3**), all patients who had a tumor block available for analysis and data on the relevant exposure or plasma analyte will be included. All tumors will be categorized as having FASN and pAkt overexpression versus no overexpression, and KRAS and PIK3CA mutated versus wild type. Within each of binary category of FASN expression and pAkt expression, and KRAS and PIK3CA mutational status, we will examine the influence of an exposure or plasma analyte divided into tertiles on DFS and OS. Tertiles will be utilized instead of quintiles of to maximize the ability to detect an association within each subgroup. Cox proportional hazards models will be used to calculate HRs and 95% CIs for DFS and OS, adjusted for other prognostic factors. Tests for statistical interaction will be performed by entering into the model the cross-product term of the relevant exposure or plasma analyte as a continuous variable with the molecular alteration as a binary variable (overexpressed versus not; mutated versus wild type).

In exploratory analyses (**Objective 1.4**), the influence of diet and other factors on toxicities associated with adjuvant therapy will be assessed. Data from the questionnaire obtained during adjuvant therapy will be used for these analyses. Using logistic regression models, odds ratios and 95% CIs for the specific toxicity will be the measure of association with an exposure, adjusted for multiple potential confounders simultaneously.

APPENDIX V

CANCER PREVENTION COMPANION STUDIES (COLONOSCOPY): CALGB 71002

Colon cancer survivors experience multiple risks beyond their initial surgical and chemotherapy treatments. Survivors can continue to have issues with neuropathy that may take years to resolve, if ever. Even in the absence of a familial syndrome, they have an increased risk of subsequent colorectal adenomatous polyps, new primary colorectal tumors and other primary tumors compared to the general population. CALGB/SWOG C80702 is collecting a risk set of data that can also be utilized to study these late effects in colon cancer survivors who have completed therapy. The database provides a unique opportunity to examine the impact of energy balance factors, medications and diet, which have previously been studied in a non-cancer population, in colon cancer survivors.

1.0 OBJECTIVES

1.1 Estimate the incidence density rate for each of second primary cancers and adenomatous polyps in colon cancer survivors.

1.2 Explore the impact of celecoxib, dietary factors, energy balance, physical activity, and vitamin D on second primary cancers and adenomatous polyps in colon cancer survivors.

1.3 Explore the role of celecoxib, physical activity, and vitamin supplementation on the incidence of peripheral neuropathy and time course of improvement/resolution.

1.4 Assess the influence of diet, body mass index, physical activity and other lifestyle habits on disease-free and overall survival among patients with stage III colon cancer (see Appendix IV).

2.0 BACKGROUND

2.1 Second primary cancers in colon cancer survivors

The rate of second cancers after the diagnosis of colon cancer has been poorly defined. Older series have suggested colon cancer survivors have an increased risk of small bowel, bowel, cervical, uterine, ovarian and prostate cancers, compared to the general population.[311-314] While common risk factors may explain these observations, many of these studies were conducted prior to the recognition of genetic syndromes that link some, but not all, of these cancers to colon cancer. More defined collection of these data would facilitate understanding the true rates of second primary cancers in colon cancer survivors. Further, one needs to understand whether these cancers relate to an underlying genetic predisposition and/or modifiable diet/lifestyle/other exposure risk factors.

2.2 Polyps in colon cancer survivors

The rates of detection of adenomatous polyps after the diagnosis of colorectal cancer range from 20-30% within the first 3 years after diagnosis.[315,316] Further, survivors of colorectal cancer have an increased lifetime risk of colorectal polyps and cancer above the general population.

2.3 Cumulative neuropathy from oxaliplatin

While oxaliplatin has had definitive influence on disease-free survival as adjuvant therapy for colon cancer, it adds toxicities that can persist well past the treatment period. At the completion of therapy in the MOSAIC trial, 12% of patients had grade 3 peripheral neuropathy and 92% had some level of neuropathy.[317] In a recent update, 15% of patients still had some level of residual neuropathy 4 years after the completion of adjuvant therapy.[318] Multiple agents have been tested to influence the development and/or severity of the cumulative neuropathy with limited efficacy.

3.0 METHODS

3.1 Utility of prevention-related studies embedded in a treatment trial

CALGB/SWOG C80702 provides an ideal opportunity to study factors influencing secondary cancers and long-term treatment-related toxicities of therapy. The primary interventions and the correlative science companion studies, including molecular markers (tumor and host-related) and dietary and lifestyle exposures, are testing these factors' influence on disease-free survival related to the index cancer. However, given the relatively high long-term cure rate of patients, rates of later adenomatous polyps, secondary cancers and oxaliplatin-related neurotoxicity are important considerations for colorectal cancer survivors. While there is a wealth of data on the influence of diet, lifestyle, aspirin/COX inhibitors, anthropometrics and other exposures on the risk of developing colorectal cancer (see protocol background and Appendix IV), most of those observational studies exclude patients with prior colorectal cancer. Thus, those data report only on the influence of these factors on an initial diagnosis of colorectal cancer. Whether such factors have a similar impact on subsequent polyps or malignancies (colorectal and non-colorectal) is not known. Potential research questions that arise in considering this issue include:

1. In a patient whose colon cancer has low COX-2 expression by IHC, does celecoxib lower the risk of subsequent polyps in those patients similar to those whose tumors had higher expression of COX-2?
2. Does physical activity reduce the rate of breast cancer in female colon cancer survivors by similar magnitude as in observational studies reporting breast cancer and physical activity in an initially non-cancer population?
3. What is the influence of vitamin D levels on the risk of polyps in colorectal cancer survivors?

Further, while one of the goals of CALGB/SWOG C80702 is to explore ways to minimize oxaliplatin-related toxicities (by testing reduction in number of cycles), it will be important to explore other exposures that may minimize oxaliplatin-related neuropathy. In Appendix III of the protocol (Vitamin D pathway) and Appendix IV of the protocol (Diet and Lifestyle), we propose to study whether AGXT 154 C/T or T/T polymorphisms (Appendix IV) and/or physical activity (Appendix IV) influences the incidence of grade 2+ peripheral neuropathy. Other hypotheses may also arise over the course of CALGB/SWOG C80702 that will be important to test, with the availability of tumor blocks, blood samples and diet/lifestyle questionnaires in this population.

3.2 Ascertainment of endpoints

For this companion study, we propose to collect data on (1) polyps and new primary colorectal cancer in colon cancer survivors, (2) secondary cancers in colon cancer survivors, (3) cumulative neuropathy after exposure to oxaliplatin and (4) disease-free and overall survival as defined in the treatment protocol.

For the first endpoints, we will request colonoscopy reports for all patients who consent to Question #1. Guidelines on the timing of colonoscopies vary across organizations. In 2005, ASCO recommended a repeat colonoscopy at 3 years and, if normal, every 5 years thereafter.[319] NCCN recommends a colonoscopy within 1 year of diagnosis and if normal, within 3 years and then every 5 years thereafter.[320] The United States Multi-Society Task Force on Colorectal Cancer also recommended colonoscopy within 1 year of diagnosis, then 3 years later if normal and then every 5 years thereafter.[321] For all of these guidelines, the timing should be influenced by findings as well as other factors (including whether the patient has high-risk features like hereditary nonpolyposis colorectal cancer syndrome, inflammatory bowel disease and other conditions). Further, symptoms may dictate the use of colonoscopy earlier than the proposed

time frames. As such, in CALGB/SWOG C80702, we will strongly recommend adherence to the NCCN and US Multi-Society Task Force guidelines (which should result in at least 2 colonoscopies within the first 4 years after diagnosis).

For the second endpoint (other cancers besides colorectal cancer), the follow-up form will inquire about other cancers. For patients who enroll on this substudy and develop other cancers in the 6-year follow-up period, we will request pathology records and documentation to confirm self-reports of cancer as well as staging.

For the third endpoint (cumulative neuropathy), toxicity forms during therapy and follow-up and recurrence data forms will be collected. Neuropathy will be graded based on the CTCAE Version 4.0:

Grade 1: asymptomatic; loss of deep tendon reflexes or paresthesia.

Grade 2: moderate symptoms; limiting instrumental activities of daily living.

Grade 3: severe symptoms; limiting self-care activities of daily living.

Grade 4: life-threatening consequences; urgent intervention indicated

Disease-free and overall survival are defined in Section 13.0 of the protocol. Appendix IV addresses the methods for diet, lifestyle and medication use as related to disease-free and overall survival.

3.3 Exposures

In CALGB/SWOG C80702, we will be collecting exposure data ranging from the primary intervention (celecoxib versus placebo) to tumor markers to blood markers to the diet and lifestyle questionnaire data. The questionnaire will be administered at 2 time points – within the first 6 weeks after randomization and within 14-16 months after randomization (~ 1.5 years after surgery). For these prevention substudies, exposures that are of particular interest include, but are not limited to:

1. Celecoxib versus placebo
2. Plasma vitamin D
3. Physical activity
4. Dietary pattern
5. Smoking history
6. Celecoxib or placebo (primary randomization in 80702)
7. Adiposity (body mass index and waist:hip ratio)
8. Vitamin intake (MVI as well as individual vitamins, including B6 & E)
9. Alcohol intake

4.0 DATA ANALYSIS

Data on the rates of adenomatous polyps, new primary colorectal cancer and new other primary cancers are drawn from older literature. Given the changes in incidences in certain cancers over the past several decades,[322] it will be important to define the rates of these events in current colorectal cancer survivors. We will collect data as described above. Alliance plans to incorporate similar data collection in other treatment trials. Exposure data will be stored in a central database that will allow for further studies of the influence of such host factors in cancer survivors.

Testing the exposures on the rates of polyps and new cancers will depend on the rates obtained in CALGB/SWOG C80702. For example, to estimate the incidence of second primaries, we expect to obtain follow-up colonoscopy data on 80% of patients without recurrence. Since 33% of patients are

expected to recur by year 4, 50% (n=1,250) is a conservative estimate. Two to 4% of these patients (25 to 50 patients) are expected to develop second primary cancers over the follow-up period. The incidence density rate will be estimated by the number of observed second primaries divided by the number of person years follow-up. Person-years follow-up is expected to be approximately 7,500 assuming 1,250 patients are followed for 6 years. The 95% confidence interval for the incidence density rate will be computed assuming a Poisson distribution for the number of observed secondary cancers and adjusting by the person-years follow-up.

The incidence of adenomatous polyps will be studied in the same patient population. Twenty to 30% of patients are expected to develop polyps within 3 years. If 80% of non-recurring patients have data on colonoscopy, we expect 250-375 pts with polyps. Approximately 50% of patients with colonoscopy data will receive treatment with celecoxib. Thus, the incidence density estimates by treatment with celecoxib will be based on approximately 625 patients per treatment group, with each patient (theoretically) followed for 6 years (3,750 person-years). The 95% confidence intervals for the incidence density estimates will be computed within each celecoxib treatment group as described above and compared.

In regard to neuropathy from oxaliplatin, an example of such an analysis is provided in Appendix III in which the AGXT 154 C->T polymorphism can be tested as impacting neuropathy after oxaliplatin.

In general, primary to any analyses, we will consider power for testing an exposure based on the number of events available (similar to methods utilized in prospective cohort studies). If power is inadequate for a specific comparison of interest, we will explore the inclusion of similar data from other trials for the analysis, if appropriate. Using logistic regression models, odds ratios and 95% CIs, we will measure associations with an exposure, adjusted for multiple potential confounders simultaneously.

APPENDIX VI

IDEA PROSPECTIVE POOLED ANALYSIS

1.0 DESIGN

IDEA is a prospective pooled analysis of individual patient data from multiple randomized trials conducted worldwide in the setting of stage III colon cancer. Each trial has randomized patients between 3 and 6 months of either FOLFOX or XELOX treatment. Specifically, for patients treated with FOLFOX, patients will be randomized to either 6 or 12 bi-weekly FOLFOX treatments. For patients treated with XELOX, patients will be randomized to either 4 or 8 three-weekly treatment cycles. Some trials have included a second randomization in a factorial design, others have included both stage II and III patients, and one trial (SCOT) has included rectal cancer patients. The primary IDEA analysis will include only stage III colon cancer patients, and in trials where a second randomization was present, the arms will be pooled over the second randomization. The primary endpoint is disease free survival, as defined below.

1.1 Disease-Free Survival

Disease-Free survival (DFS) is defined as the time from date of randomization to documentation of 1st disease recurrence or death due to any cause, whichever occurs first. Patients who fail to return for evaluation after beginning therapy will be censored for DFS on the last day of therapy. Patients who are lost to follow-up after completing therapy will be censored for DFS on the date they were last confirmed to be disease free.

2.0 ACCRUAL

It is anticipated that 6 trials world-wide will contribute patients. The specific trials, and accrual goals (for stage III colon cancer patients) are indicated in the table below.

	TOSCA	SCOT	Alliance/ SWOG	GERCOR/ PRODIGE	HORG	ACHIEVE
Expected accrual	2350	4400	2500	2000	1000	1500
Accrual period	6/2007 – 3/2013	3/2008 – 11/2013	6/2010 – 6/2015	5/2009 – 6/2013	10/2010 – 12/2014	8/2012- 8/2014

3.0 POWER CONSIDERATIONS

The 3 year DFS rate for patients with stage III disease has been highly consistent over the last decade, at 72% from both MOSAIC and N0147. A sample size of 10,500 patients, based on an expected accrual duration of 4.5 years, 1.5 years minimum follow-up, and an expected 3 year DFS rate in the control (6 month) group of 72%, is expected to provide 3390 DFS events required to provide 90% power to declare non-inferiority of the 3 month arm when the true hazard ratio between arms is 1.0. This design has a type one error rate of 0.025 if the true hazard ratio between arms is 1.12. This hazard rate, in an exponential survival model, corresponds to a decrease in the 3 year DFS rate on the 3 month arm to 69.2%. In this design the critical driver of the analysis timing is the number of DFS events, as the precise accrual and follow-up patterns will differ by study and are impossible to precisely anticipate.

4.0 ANALYSIS PLAN

4.1 Primary efficacy analysis

The primary IDEA efficacy analysis will consist of estimating the hazard ratio for DFS comparing 3 versus 6 months of therapy using a Cox proportional hazards regression model.

The Cox regression will be stratified for the initial study that each participant was enrolled to. Individual IDEA trials may also have study specific sub-questions, however to protect the integrity of each individual trial, the study-specific sub-question randomization will be pooled for the IDEA analysis. The primary analyses will be modified intention to treat, including patients in their randomized arm regardless of the actual treatment or duration of treatment received, with only patients who receive no therapy whatsoever excluded from the analysis. This is the result of the potential bias of a per-protocol analysis, which may be typically considered for a non-inferiority trial, in that patients on the 6 month arm who complete 3 or fewer months of therapy may be a biased sub-group to include with patients randomized to the 3 month arm as would be done in a per-protocol analysis.

The timing of the analysis will be event driven. Given that this is a non-inferiority trial, and there is no expectation of superior results for the reduced duration therapy, an interim analysis to conclude non-inferiority will not be conducted. A single interim analyses to reject H1 and conclude inferiority of 3 months of therapy is planned, at 50% of the protocol specified pooled number of events. Because this interim will occur after most (if not all) trials have closed to accrual, we will use the Lan-Demets implementation of the O'Brien-Fleming stopping boundaries for futility to be able to declare non-inferiority. The significance values to be used at each analysis (interim and final), are outlined in Section 4.2.

4.1.1 Test statistic

The formal test statistic for the analysis will be a Z-statistic generated using the log-hazard ratio for the covariate of randomization to the 3 month or 6 month arm from a univariate Cox regression model, stratified by trial. If $\delta = -\ln(HR)$, and $\delta_0 = -\ln(1.12)$, then $Z = (\delta_0 - \delta) / SE(\delta)$.

4.1.2 Critical values.

Based on two analyses occurring at 50% and 100% of expected events, the critical Z-values to reject the alternative hypothesis (and thus conclude that non-inferiority is not attained) are Z <-0.254 and Z <-1.947. This corresponds, approximately, to hazard ratios comparing the 3 to 6 month arm of > 1.106 and 1.047 respectively for the 2 analyses to reject the alternative hypothesis of non-inferiority. Under the null hypothesis of inferiority (true HR = 1.12), the trial will terminate for futility (inability to conclude non-inferiority) at the 50% of events mark with approximately 60% probability. The interim analyses will be conducted by an independent statistician and presented to an IDEA-specific DSMB. As it will be difficult to precisely coordinate the timing of the interim and final analyses to a specific number of events, the specific cut-off values for terminating the trial at the interim analyses will be based on the O'Brien-Fleming family boundaries at the actual number of events observed.

4.2 Assessing treatment effect heterogeneity:

At both the interim and final analyses, individual trial HRs with confidence intervals will be plotted using a forest plot. Potential heterogeneity in treatment effects will be assessed by the procedures specified in Section 4.2.1 and 4.2.2. If heterogeneity in the in treatment effect across studies is detected, subgroup analysis (excluding outlier trial(s)) and meta-regression analysis will be conducted to assess the robustness of the primary efficacy results and investigate the trial characteristics (trial design, enrolling country, etc.) which may contribute to the heterogeneity.

4.2.1 Primary assessment by likelihood ratio test:

Primary assessment by likelihood ratio test: Two stratified (by study) Cox models will be fit to the data, 1) Full model: with both treatment assignment and trial-specific indicator (dummy variables) as predictors; and 2) Reduced model: with only treatment assignment as

the predictor. The likelihood ratio test will be used to test whether the full model significantly fits the data better than reduced model. A p-value less than 0.05 will be taken to indicate significant trial-specific heterogeneity of treatment effect. In that case, the trial specific treatment effect estimates (hazard ratios) and p-values associated with each of the trial indicator will be further examined to detect the specific heterogeneity (i.e., differences between trials).

5.0 SECONDARY ENDPOINTS AND ANALYSES.

Secondary endpoints include overall survival, toxicity, and treatment compliance with the randomized regimen. A formal statistical analysis plan will be developed prior to actual conduct of the analyses for secondary endpoints.

5.1 Definition of Overall Survival

Overall survival (OS) is defined as the time from start of therapy to death, from any cause.

5.2 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 the data for OS will be mature after the data for DFS. We expect the data for OS to be mature between 4 - 6 years following the close of accrual.

5.3 Treatment Compliance

Treatment compliance will be estimated as the proportion of patients receiving the protocol-defined length of treatment, i.e. 3 months and 6 months of FOLFOX/XELOX for patients randomized on to 3 vs. 6 months of therapy, respectively. 95% confidence interval will be calculated. Mantel-Haenszel Chi-squared test (stratified by study) will be used to compare treatment compliance between treatment groups. The proportion of patients receiving therapy by cycle will also be compared.

6.0 PRE-SPECIFIED SUBGROUP AND SENSITIVITY ANALYSES.

6.1 Modified per-protocol analysis

The primary endpoint of DFS will be compared between treatment groups in the modified per-protocol population, defined as all patients randomized and who received at least 3 months of therapy. Patients on either arm who receive fewer than 3 months of therapy will be excluded from this analysis.

6.2 Stratification of Subgroup analyses

Subgroup analyses stratified by N-stage, T-stage, therapy (FOLFOX vs. XELOX), age (< 70 vs. > 70), and tumor sidedness (right vs. left for those trials collecting site of disease): Interaction testing will be performed between treatment assignment and N-stage, N-stage and the choices of initial therapy (FOLFOX vs. XELOX). Stratified HRs and 95% confidence intervals within pre-defined subgroups will be presented graphically via forest plots.