Inflammatory Biomarkers, Genetics, and Survival among Colorectal Cancer Patients

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Abstract

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Chronic inflammation significantly increases the risk of colorectal cancer (CRC) through inflammation-mediated mechanisms. Current evidence regarding the connection between circulating inflammatory biomarkers and CRC prognosis is limited to C-reactive protein (CRP) and interleukin (IL)-6. However, other inflammatory biomarkers with biologic relevance, such as monocyte chemoattractant protein-1 (MCP-1), adiponectin, and leptin, are not well understood for their associations with CRC survival. Furthermore, the relevant timing of circulating inflammatory biomarker measurements remains unclear. Existing evidence is limited to pre-diagnostic or pre-operative measures. Circulating markers of inflammation measured after treatment, when CRC survivors are at greatest risk for disease recurrence and metachronous cancers, may be informative in predicting CRC outcomes. Most observational studies of circulating inflammatory biomarkers and

CRC only had a one-time measurement of CRP which may not be representative of a person's average exposure to chronic inflammation. Mendelian randomization utilizes inherited germline genetic markers known to be associated with CRP as non-modifiable markers of long-term susceptibility to chronic inflammation. It can minimize confounding and reverse causation that has hampered previous observational studies. In this dissertation, we aimed 1) to evaluate the association between post-treatment circulating concentrations of inflammatory biomarkers, including CRP, IL-6, MCP-1, adiponectin, and leptin, with overall and disease-specific mortality among stage II-III CRC patients, and 2) to determine the association of genetically explained levels of CRP and CRC survival using the Mendelian randomization approach.

To achieve these aims, we first leveraged the existing data and biospecimens from the Seattle Colon Cancer Family Registry Cohort (SCCFR) and collected data on measures of all five circulating inflammatory biomarkers using stored plasma samples. Hazards ratios for circulating inflammation biomarkers were estimated from Cox proportional hazard regression models. Over the 10-year follow-up, we observed 94 out of 306 CRC patients (31%) died, and 53 (18%) had CRC as their primary cause of death. Elevated levels of CRP, IL-6, MCP-1, and adiponectin were significantly associated with a higher risk of all-cause mortality within 10 years after the blood draw, and CRC-specific mortality within the 1st year of blood draw. In contrast, post-treatment leptin was not associated with overall mortality but was inversely associated with CRC-specific mortality within the first year of blood draw. We observed dose-response effects of post-treatment circulating IL-6 and adiponectin on all-cause mortality over a 10-year follow-up period (p for trend<0.0001). Adiponectin was associated with higher CRC-specific mortality within

the first year of blood draw whereas IL-6 remained significantly associated with a higher risk of CRC-specific mortality over 10 years although the effect sizes attenuated with time. CRP and MCP-1 were also associated with higher all-cause mortality over the 10-year follow-up as well as CRC-specific mortality within the first year of blood draw. However, no association was observed when restricting to patients with CRP<10 mg/L.

We then used the genetic and phenotypic data from the International Survival Analysis in Colorectal Cancer Consortium (ISACC) to construct a genetic risk score for CRP based on 52 genetic variants identified from previous GWAS. We observed that genetically predicted CRP was not associated with CRC-specific survival (HD= -1.15, 95% CI: -2.76 to 0.47 per 100,000 person-year, *P*-value=0.16). No association was observed in subgroup analyses by stage at diagnosis and tumor location.

Our findings support the role of post-treatment chronic inflammation in CRC progression and the potential of using inflammatory markers, particularly IL-6 and adiponectin, to identify subsets of patients at higher risk of overall and disease-specific mortality for targeted CRC surveillance and post-treatment care. Our observational findings of no associations between circulating CRP with CRC mortality when excluding those with CRP>10 mg/L are in line with the Mendelian randomization study results that genetically predicted circulating CRP concentration was not associated with CRC survival among individuals diagnosed with CRC. It suggests that lifetime exposure to chronic inflammation as measured by CRP is not associated with survival among CRC patients.

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DEDICATION

To my husband, Chunfu Xu, who shares his passion for science

-AND-

To my Mom and Dad, thank you for your unconditional love and support throughout the years of schooling and traveling

-AND-

To cancer patients and their family who deserve a good support system

CHAPTER 1. INTRODUCTION

Each year, there are more than 1.8 million individuals diagnosed with colorectal cancer (CRC) and 881,000 CRC-related deaths globally.[1] Among newly diagnosed CRC, more than 70% were localized and regional tumors.[2] Although the prognosis for localized disease is relatively good, only 39% of CRC patients were diagnosed with this early stage and the overall 5-year relative survival following a CRC diagnosis remains poor (65%).[3,4] Limited evidence-based risk stratification tools are available to predict CRC outcomes, especially among patients with regional disease. Identifying novel molecular and genetic markers that could help select patients at higher risk of mortality might guide targeted surveillance and improve the prognosis of the disease.

Inflammation plays an important role in both the development and progression of CRC.[5,6] Evidence linking chronic inflammation and colorectal carcinogenesis suggests several mechanisms that involve DNA damage, activation of transcription factors,[7,8] upregulation of cell proliferation, angiogenesis and migration,[9-11] and inhibition of apoptosis.[7,12,13] Despite plausible links to pathways of colorectal carcinogenesis and progression, epidemiologic studies regarding the connection of circulating inflammatory biomarkers with CRC have been primarily focused on CRC *incidence*.[14-16] Recently, evolving evidence on inflammatory biomarkers and CRC outcomes demonstrated that elevated levels of inflammatory biomarkers, primarily circulating C-reactive protein (CRP) and interleukin-6 (IL-6), are associated with larger tumor sizes, higher likelihood of metastasis, and mortality.[17-19] Other inflammatory biomarkers that were biologically associated with colorectal carcinogenesis are not well studied for their association with CRC survival. In addition, the reported observational studies have focused on either *pre-diagnostic or pre-operative* inflammatory markers. This may direct a better understanding of pathogenesis but may

not directly inform post-treatment prognosis *after* treatment completion, a critical period when CRC patients are at greatest risk for disease recurrence and metachronous cancers.

Beyond limitations in the optimal timing of inflammatory marker measurement, other methodological reasons may contribute to the observed variability in associations between these markers and CRC survival. Specifically, most observational studies of circulating inflammatory biomarkers have been limited by one-time measurement of circulating biomarkers which may not be representative of a person's average exposure to chronic inflammation. They are also subject to inadequate adjustment for unknown/ unmeasured confounders, and potential for reverse causation: whether disease progression precedes or follows the elevation of circulating pro-inflammatory markers. One approach to minimize these biases is *Mendelian randomization* (MR).[20,21] Inherited germline genetic markers known to be genome-wide associated with inflammatory biomarkers from genome-wide association studies (GWAS) [22,23] can serve as non-modifiable measures of long-term susceptibility to chronic inflammation that is not affected by acute inflammatory stimuli. Since genetic alleles are randomized at gamete formation, they are not susceptible to risk factors that occur after conception and are not subject to reverse causation by disease progression.[20,21,24,25] MR has become a common design for observational studies of inflammatory biomarkers in association with cancer risk[26-32] to overcome residual confounding and reverse causality. However, MR studies for cancer survival outcomes are limited.[33]

The *overarching goal* of this study was to investigate the associations between circulating biomarkers (**Chapter 2**) and genetic markers (**Chapter 3**) of post-treatment chronic inflammation and survival among CRC patients. By leveraging existing data and biospecimens from the Seattle Colon Cancer Family Registry (CCFR), we measured circulating concentrations of post-treatment inflammatory biomarkers, namely, CRP, IL-6, MCP-1, adiponectin and leptin, and examined their associations with all-cause and CRC-specific mortality among stage II-III CRC patients (**Chapter 2**). To address potential biases in observational studies, we further evaluated genetically predicted concentrations of circulating CRP in relation to CRC survival in a large consortium of

CRC patients, the International Survival Analysis in Colorectal Cancer Consortium (ISACC, **Chapter 3**).

In the context of MR analysis, the methods for IV estimation were not developed for a censored survival outcome until the last few years.[34] Studies focusing on survival outcomes usually have a highly selected study population: the sampling of study participants is conditional on disease status. The reported SNP-phenotype (i.e. CRP) associations from GWAS might differ from the ones in a selected patient population. In addition, since inflammation is associated with many health conditions, by restricting the study population to CRC patients could lead to selection bias (collider stratification bias) by inducing an association between the genetic variants and factors that would influence both disease risk and mortality. This could potentially violate the assumption on which the IV estimation rests. We also assessed these methodologic issues of MR in the survival context and apply it to real-world data in **Chapter 3**.

This dissertation addresses the link between circulating and genetic markers of chronic inflammation and survival among CRC patients. Results from these studies can help identify novel molecular markers to select patients who are at higher risk of overall and disease-specific mortality, to guide targeted CRC surveillance, and to inform post-treatment care.

CHAPTER 2. ASSOCIATION BETWEEN POST-TREATMENT CIRCULATING BIOMARKERS OF INFLAMMATION AND SURVIVAL AMONG STAGE II-III COLORECTAL CANCER PATIENTS

INTRODUCTION

Inflammation plays an important role in both the development and progression of colorectal cancer (CRC).[5,6] Evidence linking chronic inflammation to colorectal carcinogenesis implicates several mediators.[35,36] These inflammation-modulating factors include cytokines and chemokines released by tumor-infiltrating immune cells and tumor cells. They can activate inflammatory responses and stimulate tumor growth and progression.[36,37] Obesity-induced excess release of adipokines can also lead to low-grade systemic inflammation and establish an inflammatory environment that can contribute to colorectal carcinogenesis and tumor growth.[35] Thus, the upregulation of these inflammatory biomarkers derived from either the tumor microenvironment or adipose tissue may affect CRC outcomes and may be informative in identifying CRC patients who are at higher risk for disease progression and mortality.

Observational studies of circulating inflammatory biomarkers and CRC survival have been primarily focused on the key acute-phase protein, C-reactive protein (CRP), and the major pro-inflammatory cytokine, interleukin (IL)-6. Positive correlations between elevated levels of CRP and IL-6 with larger tumor sizes, metastasis, and mortality among CRC patients have been reported.[17-19] However, these observational studies were limited to measures of either *pre-diagnostic or pre-operative* inflammatory markers. They cannot directly inform prognosis *after* completed treatment, a critical period when CRC patients are at greatest risk for disease recurrence and metachronous cancers.

In addition to CRP and IL-6, other inflammatory markers with biological relevance merit further investigation. Monocyte chemoattractant protein-1 (MCP-1), an essential chemokine, is responsible for the recruitment of monocytes, macrophages, and other

immune cells to the tumor microenvironment.[38] Studies have demonstrated that MCP-1 expression is associated with tumor stage, venous invasion, and metastasis for multiple cancers.[39-41] The association between circulating MCP-1 levels and CRC survival is not well studied.[42] Adipocyte-released hormones- adiponectin and leptin- are strongly associated with obesity, and have also been linked to CRC development.[43] However, there is limited data on their associations with CRC survival.[44]

In this study, we aimed to evaluate post-treatment circulating concentrations of biomarkers of systemic inflammation, including CRP, IL-6, MCP-1, adiponectin, and leptin, in relation to overall and CRC-specific mortality among patients diagnosed with CRC, and to assess whether these associations differ according to patient and tumor characteristics. To accomplish this, we leveraged the existing data and biospecimens from the Seattle Colon Cancer Family Registry (CCFR).

METHODS

Study population

Seattle CCFR is a population-based site of the multi-center international consortium CCFR.[45,46] The details of this consortium have been described elsewhere.[47] Briefly, incident invasive CRC cases were ascertained through the Surveillance, Epidemiology, and End Results (SEER) cancer registry covering 13 counties in western Washington State. All Seattle CCFR participants provided informed consent and completed a standardized telephone interview at enrollment (a median of 9.5 months after diagnosis) to provide information on demographics, personal and family history of cancer, CRC screening, medical history, medication use, and lifestyle factors (e.g. physical activity,

alcohol, and tobacco use). Blood samples were collected after the baseline interview. Paraffin-embedded tumor tissue was obtained from cases according to established CCFR protocols. All study participants were followed up for vital status, date, and cause of death through linkages to SEER and the National Death Index.

Eligible individuals for this study included incident stage II-III CRC cases aged 18-74 years diagnosed between 1998-2007 from whom a blood specimen was obtained after active treatment. A total of 306 eligible participants were identified. The study protocol was approved by the institutional review board of the Fred Hutchinson Cancer Research Center (Seattle, WA).

Biomarker measurement

Blood samples were collected using EDTA-treated vacutainers, processed within 48 hours, and plasma was stored at −70°C until analysis. We measured the circulating concentrations of all five biomarkers (CRP, IL-6, MCP-1, adiponectin, and leptin) using Meso Scale Discovery immunoassays. All assays were performed on never-thawed samples except for CRP, which is stable through freeze-thaw cycles[48] and was measured in samples that had undergone one freeze-thaw cycle. All samples from the same individual were analyzed on the same batch in duplicates. Quality control (QC) samples were randomly plated among the study samples across all batches. Laboratory personnel was blinded to study QC samples and all patients' information including vital status. Study samples were plated randomly regardless of study outcome to reduce bias due to laboratory variation. The inter-plate coefficients of variation (CV) for CRP, IL-6, MCP-1, adiponectin, and leptin were 7.4%, 9.4%, 8.3%, 5.9%, and 3.9%. Intra-plate CVs for each

marker on average were 1.7%, 4.7%, 4.9%, 1.9%, and 3.9%. No samples in this study had biomarker concentrations below the lower limit of detection for any biomarkers. The concentration of leptin for one sample was above the upper limit of the detection range. One sample had an extremely high value, although within the detection range, for MCP-1. These samples were included in the main analysis but were excluded in the sensitivity analysis.

Tumor characteristics

Tumor stage according to the American Joint Committee on Cancer (AJCC, 7th edition) was available for most eligible cases. For a subset of CRC cases whose AJCC staging was missing (n=67), we used a standard algorithm to impute from SEER summary staging and clinical TNM staging: stage II (SEER 1 and T3; SEER 3/7 and N0, M0) and stage III (SEER 3/4/7, and N1/N2, M0).[49]

Tumor DNA was extracted from paraffin-embedded formalin-fixed tumor tissues for tumor marker testing. Microsatellite instability (MSI) status was determined based on a 10-marker panel for the majority of cases: tumors were classified as MSI-high (MSI-H) if instability was observed in ≥30% of markers, and as microsatellite stable (MSS) or MSI-low (MSI-L) if otherwise.[50] For other cases, MSI status was assessed using immunohistochemistry testing of MLH1, MSH2, MSH6, and PMS2 expression: patients with positive staining for all markers were considered MSS/MSI-L, and as MSI-H if otherwise.[51,52] Extracted tumor DNA was also tested for p.V600E *BRAF* mutation by using a fluorescent allele-specific polymerase chain reaction assay [53] and for coding sequence in *KRAS* exon 2 using forward and reverse sequencing.[54,55] CpG island methylator

phenotype (CIMP) was determined using a validated five-marker DNA methylation assay (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*).[56,57] We classified tumors as CIMP-positive if three or more of five markers had a percentage of methylated reference ratio ≥10 and as CIMP-negative if otherwise. [58]

Statistical analysis

We first used natural logarithm to transform each marker to ensure the concentrations of all biomarkers were normally distributed. We then categorized each measure using sex-specific quartiles. Pearson correlation coefficients were used to evaluate the correlations between biomarkers.

We used Cox proportional hazards regression to calculate adjusted hazard ratios (HR) and the 95% confidence intervals (CI) evaluating the association of each post-treatment biomarker with mortality. Time to event was defined as days between the date of blood draw to the date of death, last date of contact, or the end of follow-up. We used the International Classification of Diseases-9 (ICD-9) or ICD-10 (depending on the year of linkage) to define CRC-specific deaths, CRC being the primary cause of death (ICD-9: 153.0-153.4, 153.6-153.9, or 154.0-154.1; ICD-10: C18.0-20.0 or C26.0).[59] All regression models were adjusted for *a priori* confounders, including age at blood draw, sex, pre-diagnostic body mass index (BMI), smoking status at baseline, regular use of non-steroidal anti-inflammatory drugs (NSAIDs) at baseline, stage at diagnosis, plasma storage time, and years between diagnosis and blood draw. The proportional hazards (PH) assumption was examined by testing for a nonzero slope of the scaled Schoenfeld residuals as a function of survival time. When the PH assumption was violated for a

confounder, we used the stratified proportional hazards model. When the PH assumption was violated for an exposure variable (i.e. a biomarker), we then used the extended Cox models with time-varying coefficient, which allowed us to calculate interval-specific HRs (95% CIs) for the associations between biomarker and mortality within 1 year, 1-3 years, and more than 3 years after the blood draw. The time intervals were determined based on the Schoenfeld residual plots.

Additionally, sensitivity analyses were conducted excluding 1) patients with extremely high values of MCP-1 (n=1) and leptin (n=1), and 2) patients with CRP>10 mg/L (n=58) to ensure our study results were not driven by extreme values of these biomarkers or by individuals with an acute illness that may have affected their circulating levels of biomarkers.

We performed stratified analyses according to sex, smoking status (never vs ever), BMI (<25, 25-29.9, and ≥30 kg/m_2), stage at diagnosis (II and III), and tumor characteristics (MSI, somatic *BRAF* and *KRAS* mutation status, and CIMP) since circulating concentrations of pro-inflammatory biomarkers are generally higher among females, smokers, and people who are obese, and are likely different by tumor characteristics.[60-64] Tests for interaction were performed by adding the cross-product term of the biomarker and each of these potential effect modifiers in the regression models. All statistical analyses were performed using R 3.6.0 with a two-sided *P*-value <0.05 considered statistically significant.

RESULTS

Study participants in this analysis were on average 55 years old at diagnosis (standard deviation, SD: 12.1 years). They were mostly white (87%), with an equal number of males

and females. Baseline characteristics of the 306 eligible CRC cases are listed in **Table 2.1**. Compared to patients with lower CRP levels, those with elevated concentrations of CRP were more likely to be older at diagnosis, female, obese, ever smokers, regular users of NSAIDs, and more likely to be diagnosed at a later stage. The distribution (sex-specific median and interquartile range [IQR]) and ranges of sex-specific quartiles of each biomarker are listed in **Supplementary Table 2.1 and 2.2**. Pair-wise Pearson correlation coefficients among all markers are summarized in **Supplementary Table 2.3**. CRP was positively correlated with IL-6 (r=0.53), and moderately correlated with MCP-1 (r=0.27) and leptin (r=0.26). No/weak inverse correlations were observed for leptin with IL-6 (r= -0.004) and with adiponectin (r= -0.019).

All-cause mortality

Over the 10-year follow-up, 94 out of 306 CRC patients (31%) died, and 53 (18%) had CRC as their primary cause of death. Elevated concentrations of CRP, IL-6, MCP-1, and adiponectin were significantly associated with a higher risk of all-cause mortality (**Table 2.2**). The HRs ranged from 1.3-2.6 per unit increase in logarithmically transformed biomarkers. In contrast, log-transformed concentrations of leptin were associated with a lower risk of overall mortality although not reaching statistical significance (*P* value = 0.09). We further evaluated the sex-specific quartiles of each marker in relation to all-cause mortality. For IL-6 and adiponectin, dose-response effects were observed (**Table 2.2**). Patients in the highest quartile of IL-6 had an almost 8-fold increase in overall mortality compared to those in the lowest quartile (HR=7.87, 95% CI: 3.47-17.82, P_{trend} <0.0001). Patients in the highest quartile of adiponectin had a 3-fold increased risk of

overall mortality compared to those in the lowest quartile (HR=2.56, 95% CI: 1.34-4.90, P_{trend} =0.0011). Kaplan-Meier survival curves for overall mortality by sex-specific quartiles of circulating IL-6 and adiponectin are shown in **Figure 2.1**. We did not find evidence of dose-response effects for CRP, MCP-1, or leptin.

After excluding cases with CRP>10 mg/L (n=58), CRP, MCP-1, and leptin were no longer associated with overall mortality whereas logarithmic IL-6 and adiponectin remained statistically significantly associated with a higher risk of overall mortality, with HRs (95% CIs) of 3.01 (2.1-4.32) and 1.87 (1.15-3.04), per unit increase in logarithmic biomarker (**Table 2.2**). Stronger dose-response effects were observed for IL-6: patients in the highest quartile had a more than 9-fold increased risk of overall mortality compared to those in the lowest quartile (HR=9.50, 95% CI: 3.54-25.45, P_{trend} <0.0001, **Table 2.2**). Cases in the highest quartile of adiponectin had a 2.3 times higher risk of overall mortality compared to those in the lowest quartiles (HR=2.32, 95% CI: 1.07-5.03, P_{trend} =0.0043, **Table 2.2**). These analyses adjusted for pre-diagnostic BMI. Comparisons between models with and without adjustment of BMI were also shown in **Table 2.2**.

CRC-specific mortality

Since the biomarkers violated the PH assumption for associations with CRC-specific mortality, we used the extended Cox models to calculate interval-specific HRs (95% CIs) for the associations between each logarithmic biomarker and CRC-specific mortality within 1 year, 1-3 years, and more than 3 years after the blood draw. Within the first year after the blood draw, we observed similar but stronger associations of logarithmic markers with CRC-specific mortality as compared to overall mortality: CRP, IL-6, MCP-1, and

adiponectin were associated with increased risks of CRC-specific mortality, with HRs ranges from 1.8 to 3.8 (**Table 2.3**); leptin was inversely associated with CRC-specific mortality (HR=0.44, 95% CI: 0.29- 0.68). However, these effects didn't persist after year 1, except for IL-6. The positive associations between IL-6 and CRC-specific mortality remained statistically significant after 1 year, but the effect sizes attenuated with increasing time since blood draw (**Table 2.3**).

In our sensitivity analysis excluding patients with CRP>10mg/L (n=58), CRP and MCP-1 were no longer associated with CRC-specific mortality, whereas IL-6, adiponectin, and leptin remained statistically significantly associated with CRC-specific mortality within the first year (**Table 2.3**). After the first year, only IL-6 remained significantly associated with a higher risk of CRC-specific mortality, however, the magnitude of the association decreased with increasing time since blood draw.

Stratified analyses

The results of the stratified analyses for association between logarithmic biomarkers and overall mortality according to patient and tumor characteristics are listed in **Table 2.4**. We observed that CRP was associated with higher overall mortality among cases who were normal/underweight (HR=1.76, 95%CI: 1.28-2.42) but not among those who were overweight (HR=1.24, 95% CI: 0.9-1.72) or obese (HR=0.94, 95%CI: 0.63-1.41, $P_{interaction}$= 0.007), and among cases with MSI-high tumors (HR=6.46, 95% CI: 2.04-20.49) but not among those with MSS/MSI-low tumors (HR=1.16, 95% CI: 0.92-1.47, $P_{interaction}$= 0.005). A stronger association between IL-6 and higher risk of overall mortality was observed among patients with *BRAF*-mutant tumors (HR=10.23, 95% CI: 2.46-42.45)

compared to those with *BRAF*-wildtype tumors (HR=2.49, 95% CI: 1.73-3.58, $P_{interaction}$= 0.011), and among cases with MSI-high tumors (HR= 17.47 95% CI: 3.59-85.09) vs those with MSS/MSI-low tumors (HR=1.94, 95% CI: 1.35-2.78, $P_{interaction}$= 0.007). An inverse association between leptin and overall mortality was observed in patients with MSI-high tumors (HR= 0.29, 95% CI: 0.13-0.68) but not among those with MSS/MSI-low tumors (HR=0.85, 95% CI: 0.68-1.08, $P_{interaction}$= 0.005). For other markers, we found no evidence of interactions with patient or tumor characteristics in relation to overall mortality. In the sensitivity analyses restricting to patients with CRP<10 mg/L, we observed similar patterns of effect modification, however, they were no longer statistically significant.

DISCUSSION

In this population-based prospective study of stage II-III CRC patients, we observed dose-response effects of post-treatment circulating IL-6 and adiponectin on all-cause mortality over a 10-year follow-up period. Adiponectin was associated with higher CRC-specific mortality within the first year of blood draw whereas IL-6 remained significantly associated with a higher risk of CRC-specific mortality over 10 years although the effect sizes attenuated with time. CRP and MCP-1 were also associated with higher all-cause mortality over the 10-year follow-up as well as CRC-specific mortality within the first year of blood draw. However, no association was observed when restricting to patients with CRP<10 mg/L. In contrast, post-treatment leptin was not associated with overall mortality but was inversely associated with CRC-specific mortality within the first year of blood draw. These results are all independent of BMI and are discussed by individual biomarkers, below.

CRP and MCP-1

Our study found that elevated CRP and MCP-1 concentrations were associated with a higher risk of overall mortality over the 10-year follow-up period, and higher CRC-specific mortality within the first year of blood draw. These associations were likely driven by patients with CRP≥10mg/L. No associations were observed after excluding these individuals.

Previous studies of circulating **CRP** in relation to CRC outcomes have primarily focused on ***pre-operative*** measures. [18,65-69] These study findings were, however, inconclusive. Chung et al. reported elevated CRP levels were correlated with larger tumor size, lymph node, and liver metastasis, but were not statistically associated with survival after multivariate adjustment.[67] Similar null associations were reported by Volkova et al.[68] Other studies observed that higher concentration of circulating CRP measured before surgery was an independent prognostic factor after multivariate adjustment and associated with overall [18,65,66] and CRC-specific mortality.[69] However, the CRP measures in these studies were crude: either 10 mg/L [18,65] or 5 mg/L [66] was used as the cut-off to dichotomize pre-operative CRP concentrations. Studies using ***post-treatment*** CRP concentrations are very limited. In a population-based multi-ethnic study, Cooney and colleagues observed post-treatment CRP was associated with CRC survival: those in the highest quintile had 80% higher risk of overall mortality compared to those in the lowest quintile, and those in the highest tertile had a two-fold increased risk of CRC-specific mortality compared to those in the lowest tertile.[70] However, BMI was not adjusted in their multivariate analysis. In a study of 467 stage I-III CRC patients, Matsubara et al. reported

higher post-operative CRP (0.9-9.9 mg/L) was univariately associated with worse disease-free survival compared to those in the lower CRP group (<0.9mg/L) and suggested circulating pro-inflammatory biomarkers might contribute to survival and proliferation of residual micrometastases. [71]

Since CRP is correlated with BMI, our main analyses compared models with and without adjustment of BMI. Additional adjustment by BMI did not materially change the effect sizes substantially, although slightly stronger associations were observed. In addition, our stratified analysis by BMI found an association between elevated post-treatment CRP concentrations and overall mortality among normal/underweight individuals but not among patients who were obese. Similar patterns of effect modification by BMI and waist circumference were observed by Swede et al. for the associations between prediagnostic circulating CRP and CRC-specific mortality.[72] Together, these suggest the impact of chronic inflammation on CRC survival is limited to those with normal body weight and may be independent of excess body fat.

Our findings that a higher level of **MCP-1** was associated with an elevated risk of overall and CRC-specific mortality, is consistent with previous findings: cross-sectional studies have shown that circulating levels of MCP-1 are higher among CRC cases compared to healthy controls, and among CRC patients who had deeper local invasion (T stage) and those with distant metastasis (M1).[73,74] One prospective study of 45 CRC patients reported pre-operative serum MCP-1 was associated with less favorable survival.[42] MCP-1 is also expressed at a higher level in human colon carcinoma specimens than in normal mucosa.[75] Its expression levels increase with tumor stage and metastatic potential.[41,73,76,77] In an *in vivo* study, blockage of MCP-1 led to less

macrophage infiltration and eventually reduced the number and size of colon tumors, before and after multiple tumors had developed.

Our finding on the time-varying associations with CRC-specific mortality for CRP and MCP-1 is worth noting. The higher risk associated with increased CRP level was only evident within the 1st year of blood draw. This suggested a short-term effect of these circulation inflammatory biomarkers in predicting survival outcomes. We also observed that CRP and MCP-1 were no longer associated with mortality after excluding patients with CRP>10 mg/L, suggesting that the prognostic effect of CRP and MCP-1 only applies to CRC patients with surged inflammation. Future studies are needed to validate this finding and expand beyond stage II-III patients to evaluate these associations in a metastatic setting.

IL-6

Several lines of evidence support our findings that higher circulating levels of IL-6 were associated with worse prognosis among CRC patients. Observational studies demonstrated that CRC patients with higher concentrations of serum IL-6 prior to treatment had a two to four times higher risk of overall mortality.[78-83] The long-term effect of IL-6 observed in our study was supported in part by Yeh et al. that CRC patients with IL-6>10 pg/ml had 2.4 times higher risk of 10-year overall mortality compared to those with lower IL-6.[81] *In vitro* studies also demonstrated that IL-6 can stimulate tumor growth in both primary and metastatic colon cancer cell lines.[84] Possible mechanisms may involve the trans-signaling pathway, where the IL-6/soluble IL-6 receptor complex binds to gp130 on tumor cells. These likely activate the downstream intracellular JAK/STAT3

signaling cascade, inducing the transcription of several target genes promoting tumor growth and immunosuppression.[85] Consistently, IL-6-deficient mice, after injected with colon cancer cells, had significantly decreased tumor growth and increased tumor-infiltrating immune cells (i.e. mature dendritic cells, helper T cells, and cytotoxic T-cells) in the tumor microenvironment compared with wild-type mice.[86]

Our study found that the association between circulating IL-6 and overall mortality was stronger in patients with *BRAF*-mutant tumors compared to those with *BRAF* wild-type CRCs. It is supported by Thomsen et al,[78] a study of 393 patients with metastatic CRC, where a more pronounced association between IL-6 and overall mortality was observed in patients with *BRAF* mutation. Although the underlying mechanism is unclear, it is likely that the somatic *BRAF* mutation and elevated IL-6 act in synergy in promoting tumor growth and progression through the BRAF kinase and JAK/STAT3 signaling pathways.

It is noteworthy that the circulating IL-6 levels in our study (median=1.0, IQR: 0.6-1.6 pg/ml) are lower than what has been generally reported in the literature among CRC patients.[19,87,88] This may be because our study population included only stage II-III CRC patients, and circulating IL-6 level increase with tumor stage.[82,89] Also, post-treatment systemic inflammation is likely to be lower compared to that during the pre-operative period when the tumor is present. Tumor-associated inflammation could lead to an elevated level of circulating markers of inflammation.

Adiponectin

Several epidemiologic studies have reported that elevated concentrations of circulating adiponectin were associated with a lower risk of developing CRC.[90-94] However, studies that reported the impact of circulating adiponectin on CRC survival are few and had inconsistent results. One hospital-based study of 344 CRC patients found that adiponectin measured at the time of diagnosis was not associated with CRC survival.[68] Another small study of 60 patients with non-metastatic CRC reported low adiponectin levels measured before treatment was associated with disease recurrence.[95] Chong et al. conducted a prospective study of 621 incident CRC cases and found that pre-diagnostic plasma adiponectin was positively associated with increased risk of both overall and CRC-specific mortality over a median of 9-year follow-up.[44] Patients in the highest vs lowest quartile of adiponectin had 66% higher risk of overall mortality and a nearly two-fold increased risk of CRC-specific mortality. This is similar to our study findings that post-treatment circulating concentrations of adiponectin were associated with a higher risk of overall mortality with dose-response effect over 10 years of follow-up. A more pronounced association with CRC-specific mortality was observed within the first year of blood draw.

Our results suggest that individuals with elevated adiponectin levels who then develop CRC may have tumors through alternative pathways that are associated with adverse disease outcomes. In addition, studies evaluating the link between adiponectin and inflammation have demonstrated the pleiotropic effects of adiponectin.[96] Although it is well-established that circulating adiponectin is inversely associated with visceral obesity, type 2 diabetes, cardiovascular disease, and metabolic syndrome,[97] adiponectin has pro-inflammatory effects in chronic inflammatory and autoimmune diseases, such as

rheumatoid arthritis,[98] inflammatory bowel disease,[99,100] and type 1 diabetes.[101] In patients with these conditions, adiponectin levels are positively associated with other pro-inflammatory markers (i.e. CRP, IL-6). In human colonic epithelial cells, adiponectin exerted proinflammatory effects by inducing chemokine production and it promotes colonic cell proliferation in a dose-dependent manner.[102] In the tumor microenvironment, adiponectin may exert pro-inflammatory properties that facilitate tumor proliferation and metastasis, leading to a worse prognosis.

Leptin

This study observed that elevated concentration of circulating leptin was associated with improved CRC-specific survival within the first year of blood draw but not with overall survival. Although studies on leptin in relation to CRC survival are lacking, this finding is supported by our previous report of long-term CRC survivors: patients who maintained body weight approximately five years after diagnosis had better survival compared to those experienced weight loss. [103] Also, results from clinical trials in the adjuvant setting suggest that overweight and obese patients are, slightly less likely to develop chemotherapy-related toxicities and other complications.[104,105] Since leptin is closely correlated with adiposity, our study results suggest that patients who had higher levels of leptin after treatment were more likely to have higher body fat, less likely to or experience weight loss, and thus were more likely to have better survival.

Our subgroup analyses by pre-diagnostic BMI categories demonstrated that the beneficial effect of post-treatment leptin was the strongest among those who were obese before diagnosis, although the interaction didn't reach statistical significance. This is

further supported by longitudinal studies that pre- to post-diagnosis weight loss was associated with a higher risk of mortality,[106,107] excessive weight loss was associated with increased mortality by two- to three-fold but no association was observed in those gained weights.

In summary, we observed that the most informative biomarkers that were associated with overall mortality were IL-6 and adiponectin whereas CRP and MCP-1 were not useful prognostic markers of chronic inflammation for CRC outcome and leptin was not associated with overall mortality. In addition, IL-6 is a robust marker over the 10 year follow-up period for CRC-specific mortality.

Limitations and strengths

Several limitations need to be considered when interpreting our study results. First, post-treatment inflammatory biomarkers were measured only once; repeated measurements are desired in future studies to better capture intra-individual variabilities. Second, selection bias may be of concern because our study only included cases for whom blood was collected after active treatment (1-3 years after CRC diagnosis). However, the comparison between our study population and all stage II-III CRC patients from the parent study, Seattle CCFR showed similar distributions of baseline patients and tumor characteristics (**Supplementary Table 2.4).** We only included stage II and III CRC patients into this study because 1) stage I CRC patients have relatively high 5-year survival rates (~90%) which can limit the power for survival analysis, and 2) study participation for stage IV patients was relatively low due to high mortality in this group. This limited the generalizability of our findings. Lastly, although this study is well powered

for the main analysis, our subgroup analyses had limited power for subgroups with a limited sample size and need to be interpreted with caution due to the multiple comparisons.

This study also has many strengths. Our study population is well-characterized: standardized questionnaires provided detailed information on potential confounders associated with inflammation; tumor characterizations enhanced our ability to conduct stratified analyses to improve the specificity of our study findings. Circulating biomarkers were measured using standardized and validated assays with quality control procedures. The assays performed extremely well with no values below the assay level of detection and low inter- and intra-plate CVs. The long follow-up time of the Seattle CCFR allowed us to study long-term survival, and to evaluate time-varying associations. In addition, the circulating inflammatory biomarkers in this study were measured after the completion of treatments. We incorporated a lag time between diagnosis and blood draw (1-3 years, median 1.4 years) to preclude acute treatment effects or acute diseases.

Summary

In this population-based study of stage II-III CRC patients, post-treatment circulating CRP, IL-6, MCP-1, and adiponectin concentrations are associated with both all-cause and disease-specific mortality, whereas the observed associations of CRP and MCP-1 appear limited to patients with CRP<10 mg/L. In contrast, post-treatment leptin levels were not associated with overall mortality but were inversely associated with CRC-specific mortality within the first year of blood draw. To the best of our knowledge, this is the first study that directly evaluated post-treatment markers of systemic inflammation in

relation to CRC survival. Although these results need further validation, they support the role of chronic inflammation in CRC progression and the potential of using inflammatory markers, particularly IL-6 and adiponectin, to identify subsets of patients at higher risk for targeted CRC surveillance and post-treatment care.

TABLES AND FIGURES

Table 2.1 Baseline characteristics according to post-treatment C-reactive protein levels, Seattle CCFR 1998-2007

Characteristics [a]	Total (N=306)	C-reactive protein (mg/L)			
		≤1.0 (N=52)	1.1 - 3.0 (N=98)	3.1 - 10.0 (N=98)	>10.0 (N=58)
Age at diagnosis (years), Mean (SD)	55.2 (12.1)	53.3 (13.5)	55.0 (12.0)	55.2 (11.6)	57.2 (11.9)
Female	157 (51)	25 (48)	44 (45)	54 (55)	34 (59)
Race					
White	265 (87)	44 (85)	89 (91)	84 (86)	48 (83)
Black	13 (4)	2 (4)	3 (3)	6 (6)	2 (3)
Asian	17 (6)	4 (8)	3 (3)	4 (4)	6 (10)
Others	11 (4)	2 (4)	3 (3)	4 (4)	2 (3)
Body mass index (kg/m2), Mean (SD)	27.6 (5.5)	24.8 (3.9)	26.5 (4.4)	28.9 (6.1)	29.9 (5.7)
Ever smoker [b]	185 (60)	29 (56)	55 (56)	58 (59)	43 (74)
Regular use of NSAIDs [b,c]	144 (47)	25 (48)	40 (41)	50 (51)	29 (50)
Diagnosed with diabetes [b]	32 (10)	4 (8)	12 (12)	10 (10)	6 (10)
Diagnosed with high cholesterol [b,c]	87 (28)	10 (19)	32 (33)	28 (29)	17 (29)
Stage at diagnosis					
II	143 (47)	29 (56)	44 (45)	43 (44)	27 (47)
III	163 (53)	23 (44)	54 (55)	55 (56)	31 (53)
MSI					
MSS/MSI-low	189 (79)	27 (75)	63 (82)	66 (79)	33 (77)
MSI-high	51 (21)	9 (25)	14 (18)	18 (21)	10 (23)
Missing	66	16	21	14	15
KRAS					
Wildtype	181 (72)	27 (69)	58 (69)	59 (72)	37 (82)
Mutant	69 (28)	12 (31)	26 (31)	23 (28)	8 (18)
Missing	56	13	14	16	13
BRAF					
Wildtype	222 (87)	38 (90)	74 (89)	72 (86)	38 (84)
Mutant	32 (13)	4 (10)	9 (11)	12 (14)	7 (16)
Missing	52	10	15	14	13
CIMP					
CIMP-low/no	171 (81)	22 (71)	57 (84)	60 (84)	32 (76)
CIMP-high	41 (19)	9 (29)	11 (16)	11 (16)	10 (24)
Missing	94	21	30	27	16
Plasma storage time (years), Median [Q1,Q3]	16.7 [16.0, 19.2]	16.7 [16.0, 19.2]	16.6 [16.1, 18.8]	16.6 [16.3, 19.7]	17.0 [16.5, 19.8]
Time between diagnosis and blood draw (years), Median [Q1, Q3]	1.4 [1.0, 3.0]	1.3 [1.1, 1.7]	1.4 [1.2, 2.1]	1.4 [1.2, 1.8]	1.4 [1.1, 1.9]

CIMP: CpG island methylator phenotype; MSI: microsatellite instability; MSS: microsatellite stable; NSAIDs: non-steroidal anti-inflammatory drugs; Q1: 1st quartile; Q3: 3rd quartile; SD: standard deviation.

[a] N (%) unless otherwise specified.

[b] Question on smoking was assessed as "Have you ever smoked at least one cigarette a day for 3 months or longer"; regular use of NSAIDs was defined as ever use of aspirin or ibuprofen-based medications at least twice a week for more than a month; diagnosis of diabetes and high cholesterol was assessed as "Has a doctor ever told you that you had diabetes, also known as diabetes mellitus (not including gestational diabetes/diabetes you had only during pregnancy)?" and "Has a doctor ever told you that you had high cholesterol?", respectively.

[c] There was 1 person who had missing data on regular NSAIDs use and 2 with missing data on the history of high cholesterol.

Table 2.2 Associations between circulating biomarkers of inflammation and overall mortality over 10-year follow up, among all eligible stage II-III CRC cases (n= 306) and among those with CRP < 10 mg/L (n=248)

Markers (N= 306)		All participants (n=306)						CRP < 10 mg/L (n=248)					
		N deaths/ Total	Model 1 [a]			Model 2 [b]		N deaths/ Total	Model 1 [a]			Model 2 [b]	
			HR	95% CI	P for trend	HR	95% CI		HR	95% CI	P for trend	HR	95% CI	P for trend
CRP	**ln(CRP) [c]**		**1.28**	**1.07-1.52**		**1.32**	**1.10-1.59**		**1.06**	**0.82-1.38**		**1.02**	**0.77-1.35**	
	Q1	22/78	1.00	Ref		1.00	Ref	22/78	1.00	Ref		1.00	Ref	
	Q2	13/76	0.47	0.24-0.94	0.08	0.51	0.25-1.02	13/76	0.46	0.23-0.92	0.91	0.45	0.22-0.93	0.67
	Q3	28/76	1.05	0.59-1.85		1.07	0.58-1.95	28/76	1.03	0.58-1.83		0.91	0.49-1.69	
	Q4	31/76	1.29	0.74-2.27		1.38	0.76-2.52	4/18	0.58	0.2-1.71		0.58	0.2-1.71	
IL-6	**ln(IL-6) [c]**		**2.56**	**1.95-3.36**		**2.72**	**2.07-3.56**		**2.88**	**2.01-4.13**		**3.01**	**2.10-4.32**	
	Q1	8/78	1.00	Ref		1.00	Ref	6/75	1.00	Ref		1.00	Ref	
	Q2	17/76	1.96	0.84-4.58	**<0.0001**	2.73	1.14-6.50	15/72	2.39	0.92-6.20	**<0.0001**	3.28	1.23-8.71	**<0.0001**
	Q3	29/76	3.35	1.50-7.47		4.57	1.99-10.46	27/65	5.38	2.18-13.26		6.95	2.73-17.69	
	Q4	40/76	5.51	2.51-12.07		7.87	3.47-17.82	19/36	6.74	2.59-17.53		9.50	3.54-25.45	
MCP-1	**ln(MCP-1) [c]**		**1.89**	**1.13-3.17**		**1.97**	**1.18-3.28**		**1.60**	**0.75-3.4**		**1.65**	**0.78-3.52**	
	Q1	18/78	1.00	Ref		1.00	Ref	14/71	1.00	Ref		1.00	Ref	
	Q2	17/76	0.69	0.35-1.36	0.09	0.69	0.35-1.35	15/67	0.84	0.4-1.77	0.25	0.82	0.39-1.72	0.26
	Q3	29/76	1.28	0.70-2.36		1.33	0.72-2.46	20/60	1.18	0.58-2.41		1.16	0.57-2.36	
	Q4	30/76	1.38	0.74-2.57		1.45	0.78-2.72	18/50	1.38	0.65-2.93		1.37	0.64-2.93	
Adiponectin	**ln(Adip) [c]**		**1.73**	**1.16-2.59**		**1.71**	**1.14-2.58**		**1.88**	**1.16-3.03**		**1.87**	**1.15-3.04**	
	Q1	14/78	1.00	Ref		1.00	Ref	10/61	1.00	Ref		1.00	Ref	
	Q2	17/76	1.20	0.59-2.44	**0.001**	1.20	0.58-2.45	10/60	0.87	0.36-2.11	**0.005**	0.82	0.34-2.01	**0.004**
	Q3	29/76	1.98	1.03-3.79		2.02	1.04-3.91	24/65	2.14	1.01-4.52		2.23	1.04-4.79	
	Q4	34/76	2.55	1.35-4.82		2.56	1.34-4.9	23/62	2.26	1.06-4.83		2.32	1.07-5.03	
Leptin	**ln(Leptin) [c]**		**0.86**	**0.72-1.02**		**0.85**	**0.70-1.03**		**0.98**	**0.79-1.21**		**0.93**	**0.73-1.19**	
	Q1	27/78	1.00	Ref		1.00	Ref	17/66	1.00	Ref		1.00	Ref	
	Q2	23/76	1.01	0.57-1.77	0.15	1.06	0.59-1.89	18/67	1.24	0.63-2.44	0.67	1.28	0.64-2.54	0.39
	Q3	27/76	0.93	0.54-1.59		0.95	0.53-1.68	20/59	1.22	0.63-2.34		1.15	0.57-2.3	
	Q4	17/76	0.62	0.34-1.15		0.59	0.29-1.17	12/56	0.81	0.39-1.72		0.64	0.27-1.51	

CRC: colorectal cancer; CRP: C-reactive protein; IL-6: interleukin 6; MCP-1: monocyte chemoattractive protein 1; HR: hazard ratio; CI: confidence interval.

[a] Model 1 was stratified by stage at diagnosis and adjusted for age at blood draw, sex, smoking status at baseline, regular use of non-steroidal anti-inflammatory drugs (NSAIDs) at baseline, plasma storage time, and years between diagnosis and blood draw.

[b] Model 2 were stratified by stage at diagnosis and adjusted for all covariates in Model 1 plus pre-diagnostic body mass index (BMI)

[c] HRs for overall mortality per unit increase in log-transformed concentrations of each biomarker

Table 2.3 Associations between circulating biomarkers of inflammation and CRC-specific mortality according to time of follow-up since blood draw, overall (n= 302) and among those with CRP < 10 mg/L (n=244)

Markers [a]	HR (95% CI)[b]		
	< 1 year	**1-3 years**	**>3 years**
All (n=302)			
N deaths/Total	17/302	18/280	18/258
CRP (mg/L)	1.75 (1.2-2.56)	1.31 (0.9-1.93)	1.22 (0.83-1.79)
IL-6 (pg/mL)	***5.01 (2.92-8.59)***	***2.66 (1.52-4.66)***	***2.03 (1.1-3.72)***
MCP-1 (pg/mL)	3.78 (1.41-10.08)	1.14 (0.29-4.49)	1.17 (0.31-4.45)
Adiponectin (ug/mL)	***3.16 (1.27-7.86)***	1.83 (0.77-4.35)	0.57 (0.23-1.39)
Leptin (ng/mL)	***0.44 (0.29-0.68)***	1.04 (0.71-1.53)	0.93 (0.63-1.36)
CRP <10 mg/L (n=244)			
N deaths/Total	8/244	12/232	14/216
CRP (mg/L)	0.65 (0.34-1.25)	0.97 (0.53-1.77)	0.99 (0.57-1.72)
IL-6 (pg/mL)	***6.23 (2.97-13.04)***	***3.99 (1.76-9.09)***	***1.74 (0.69-4.34)***
MCP-1 (pg/mL)	5.53 (0.9-34.16)	1.86 (0.32-10.64)	0.54 (0.09-3.09)
Adiponectin (ug/mL)	***5.45 (1.5-19.83)***	2.29 (0.79-6.65)	0.84 (0.31-2.28)
Leptin (ng/mL)	***0.45 (0.23-0.86)***	0.96 (0.58-1.6)	0.9 (0.57-1.44)

CRC: colorectal cancer; CRP: C-reactive protein; IL-6: interleukin 6; MCP-1: monocyte chemoattractive protein 1; HR: hazard ratio; CI: confidence interval.

[a] Natural log-transformed: HRs per unit increase in logarithmic biomarker

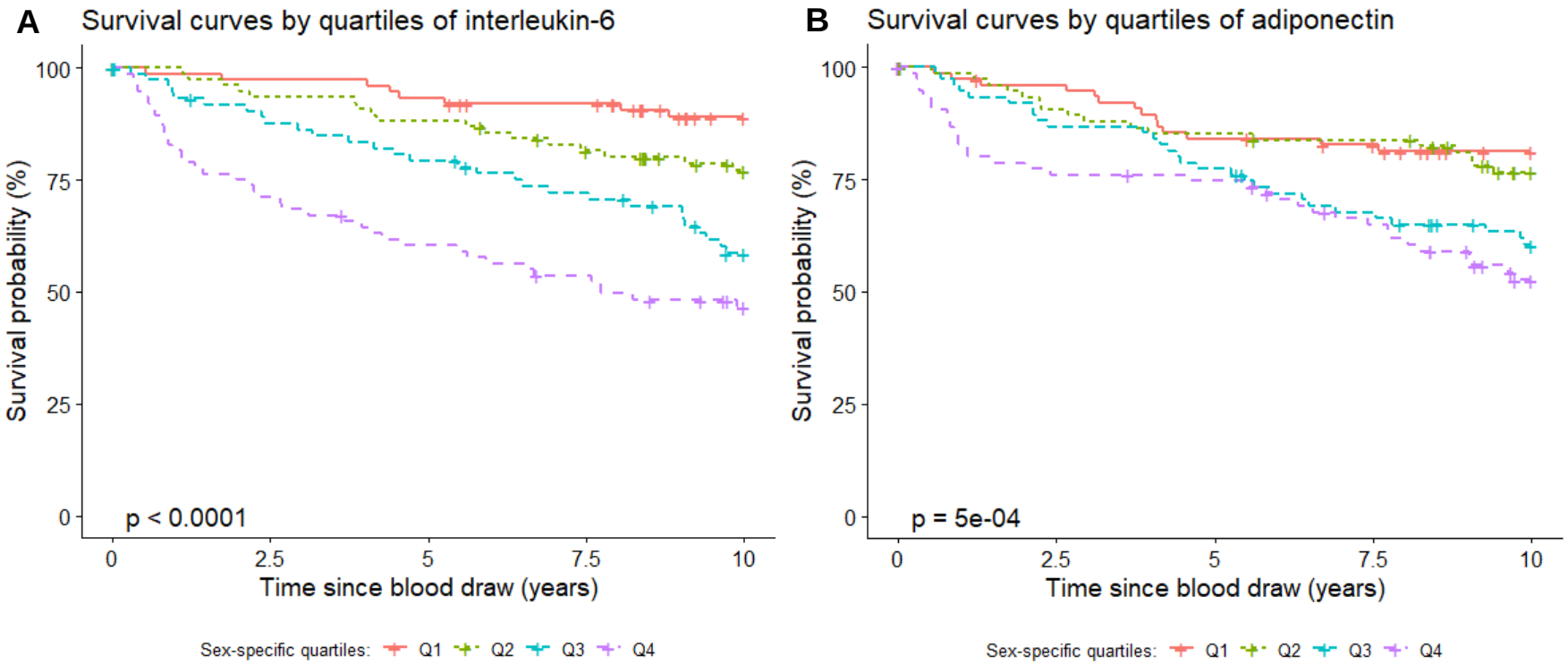
[b] Interval-specific HRs (95% CI) were calculated using extended Cox models with time-varying coefficient to estimate the associations between the logarithmic biomarker and CRC-specific mortality within 1 year, 1-3 years, and more than 3 years after the blood draw. HRs were stratified by stage at diagnosis and adjusted for age at blood draw, sex, pre-diagnostic body mass index (BMI), smoking status at baseline, regular use of non-steroidal anti-inflammatory drugs (NSAIDs) at baseline, plasma storage time, and years between diagnosis and blood draw.

Table 2.4 Associations between circulating biomarkers of inflammation and overall mortality, according to patients and tumor characteristics, among all participants (n= 306)

Characteristics	Events /Total	CRP	IL-6	MCP-1	Adiponectin	Leptin
		HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
Overall	**94/306**	1.32 (1.1-1.59)	2.72 (2.07-3.56)	1.97 (1.18-3.28)	1.71 (1.14-2.58)	0.85 (0.7-1.03)
Sex						
Female	42/157	1.28 (0.93-1.76)	2.46 (1.61-3.76)	2.33 (1.2-4.53)	1.34 (0.77-2.34)	0.69 (0.51-0.93)
Male	52/149	1.31 (1.03-1.65)	3.17 (2.23-4.5)	1.66 (0.69-3.98)	2.31 (1.23-4.35)	1 (0.76-1.32)
BMI						
Normal/Underweight	31/101	**1.76 (1.28-2.42)***	2.37 (1.6-3.53)	1.89 (0.66-5.43)	1.09 (0.46-2.59)	0.81 (0.58-1.14)
Overweight	31/118	1.24 (0.9-1.72)	4.22 (2.3-7.75)	1.13 (0.33-3.95)	1.79 (0.88-3.61)	0.94 (0.65-1.37)
Obese	32/87	**0.94 (0.63-1.41)***	2.3 (1.33-3.99)	2.69 (1.27-5.69)	**2.08 (1.09-3.98)**	**0.66 (0.45-0.97)**
Smoking status						
Ever smoker	65/185	1.28 (1.03-1.59)	2.41 (1.73-3.35)	1.77 (0.98-3.2)	1.73 (1.05-2.88)	0.8 (0.64-1)
Never smoker	29/121	1.52 (1.01-2.28)	3.44 (1.92-6.15)	2.65 (0.77-9.18)	1.86 (0.86-4.02)	0.86 (0.57-1.32)
Stage at diagnosis						
II	42/143	1.3 (0.96-1.76)	2.48 (1.57-3.92)	1.44 (0.74-2.84)	1.91 (1.04-3.5)	0.88 (0.66-1.17)
III	52/163	1.35 (1.05-1.73)	3.15 (2.18-4.56)	2.53 (1.08-5.94)	1.98 (1.09-3.62)	0.78 (0.59-1.03)
***KRAS* mutation**						
Mutant	26/69	0.99 (0.65-1.52)	1.89 (1.03-3.48)	2.38 (0.4-14.16)	1.85 (0.71-4.81)	0.89 (0.59-1.33)
Wildtype	52/181	1.54 (1.2-1.98)	3.66 (2.35-5.69)	1.72 (0.91-3.27)	1.88 (1.11-3.2)	0.75 (0.59-0.97)
***BRAF* mutation**						
Mutant	13/32	2.05 (1.11-3.8)	**10.23 (2.46-42.45)***	1.62 (0.25-10.6)	1.97 (0.6-6.45)	0.64 (0.36-1.15)
Wildtype	66/222	1.24 (0.99-1.55)	**2.49 (1.73-3.58)***	1.8 (0.98-3.31)	1.69 (1.04-2.74)	0.85 (0.68-1.08)
MSI						
MSI-high	11/51	**6.46 (2.04-20.49)****	**17.47 (3.59-85.09)****	2.19 (0.39-12.47)	1.63 (0.4-6.68)	**0.29 (0.13-0.68)****
MSS/MSI-low	63/189	**1.16 (0.92-1.47)****	**1.94 (1.35-2.78)****	1.88 (1-3.51)	1.74 (1.04-2.9)	**0.85 (0.68-1.08)****
CIMP						
High	17/41	1.25 (0.82-1.91)	5.63 (2.04-15.57)	3.56 (0.68-18.55)	4.9 (1.41-17.02)	0.4 (0.23-0.71)
Low	55/171	1.27 (0.99-1.63)	2.64 (1.76-3.95)	1.82 (0.96-3.47)	1.48 (0.85-2.6)	0.78 (0.6-1)

* P for interaction < 0.05
** P for interaction <0.01

Figure 2.1 Kaplan-Meier survival curves



Legend: Kaplan-Meier survival curves comparing overall mortality in stage II-III colorectal cancer patients by sex-specific quartiles of circulating interleukin-6 (A) and adiponectin (B), Seattle Colon Cancer Family Registry, 1998-2007 (n=306). *P* values were based on log-rank tests.

Supplementary Table 2.1 Median and interquartile range (IQR) for all markers according to sex

Markers	Men (n=149)		Women (n=157)	
	Median	Q1-Q3	Median	Q1-Q3
CRP (mg/L)	2.7	1.3-5.9	4.2	1.4-8.3
IL-6 (pg/mL)	1.0	0.6-1.4	1.0	0.6-1.6
MCP-1 (pg/mL)	116.0	95.9-140.1	118.9	97.3-152.3
Adiponectin (ug/mL)	11.1	7-15.5	17.4	11.4-24.2
Leptin (ng/mL**)**	6.3	2.8-13.3	23.9	10.3-57.4

Supplementary Table 2.2 Sex-specific quartiles of all markers

Markers		Male	Female
CRP (mg/L)			
	Q1	0.1-1.3	0.1-1.4
	Q2	1.3-2.7	1.4-4.2
	Q3	2.7-5.9	4.3-8.3
	Q4	5.9-121.1	8.3-51.3
IL-6 (pg/mL)			
	Q1	0.2-0.6	0.1-0.6
	Q2	0.6-1.0	0.7-1.0
	Q3	1.0-1.4	1.0-1.6
	Q4	1.4-12.6	1.6-12.1
MCP-1 (pg/mL)			
	Q1	53.5-95.9	38.4-97.3
	Q2	97.0-116.0	98.8-118.9
	Q3	116.1-140.1	120.6-152.3
	Q4	141.7-388	152.9-1415
Adiponectin (ug/mL)			
	Q1	2.8-7.0	2.6-11.4
	Q2	7.2-11.1	12.0-17.4
	Q3	11.1-15.5	17.5-24.2
	Q4	15.5-45.7	24.2-67.2
Leptin (ng/mL)			
	Q1	0.3-2.8	0.2-10.3
	Q2	2.8-6.3	11.5-23.9
	Q3	6.3-13.2	24.0-57.4
	Q4	13.8-116.7	60.0-821.3

Supplementary Table 2.3 Pearson correlation coefficient matrix

Markers	CRP	IL-6	MCP-1	Adiponectin	Leptin
CRP	1				
IL-6	**0.528 ****	1			
MCP-1	**0.267 ****	**0.313 ****	1		
Adiponectin	**-0.117 ***	-0.002	0.110	1	
Leptin	**0.262 ****	-0.004	0.047	-0.019	1

* P value < 0.05
** P value <0.0001
All biomarkers were nature log-transformed

Supplementary Table 2.4 Comparison between our study population with the parent study

Characteristics [a]	Our study (N=306)	Parent study [b] (N=1125)
Age at diagnosis (years), Mean (SD)	55.2 (12.1)	56.8 (11.7)
≤50	121 (40)	373 (33)
51-60	61 (20)	262 (23)
61-70	94 (31)	343 (30)
>70	30 (10)	147 (13)
Female	157 (51)	558 (50)
Body mass index (kg/m2), Mean (SD)	27.6 (5.5)	28.1 (6.0)
Underweight/Normal	101 (33)	360 (32)
Overweight	118 (39)	427 (38)
Obese	87 (28)	335 (30)
Ever smoker	185 (60)	664 (59)
Regular use of NSAIDs	144 (47)	515 (46)
Diagnosed with Diabetes	32 (10)	149 (13)
Diagnosed with high cholesterol	87 (28)	334 (30)
Stage at diagnosis		
II	143 (47)	498 (44)
III	163 (53)	627 (56)
MSI		
MSS/MSI-low	189 (79)	828 (82)
MSI-high	51 (21)	183 (18)
Missing	66	114
KRAS		
Wildtype	181 (72)	709 (71)
Mutant	69 (28)	286 (29)
Missing	56	130
BRAF		
Wildtype	222 (87)	895 (86)
Mutant	32 (13)	149 (14)
Missing	52	81
CIMP		
CIMP-low/no	171 (81)	626 (81)
CIMP-high	41 (19)	151 (19)
Missing	94	348

CIMP: CpG island methylator phenotype; MSI: microsatellite instability; MSS: microsatellite stable; NSAIDs: non-steroidal anti-inflammatory drugs; Q1: 1st quartile; Q3: 3rd quartile; SD: standard deviation.

[a] N (%) unless otherwise specified.

[b] Incident stage II-III CRC cases from Seattle CCFR cases

CHAPTER 3. GENETICALLY PREDICTED CIRCULATING C-REACTIVE PROTEIN AND COLORECTAL CANCER SURVIVAL: A MENDELIAN RANDOMIZATION STUDY

INTRODUCTION

Chronic inflammation plays an important role in colorectal cancer (CRC) development and progression.[5] Elevated levels of inflammation after CRC diagnosis may lead to increased expression of proinflammatory mediators and promote tumor growth and progression.[6,37]

C-reactive protein (CRP) is an abundant acute-phase protein produced mainly by hepatocytes in response to pro-inflammatory cytokines.[108] Observational studies on CRC outcomes have revealed positive correlations between elevated levels of CRP with larger tumor sizes, metastases, and mortality among patients diagnosed with CRC.[18,65-69] These studies, however, primarily focused on either *pre-diagnostic* or *pre-operative* measures of CRP. Concentrations of *post-treatment* CRP concentrations may be more informative in predicting CRC outcomes. Most of these observational studies on CRP and CRC survival were unadjusted or insufficiently adjusted for potential confounders, factors related to inflammation and mortality, such as adiposity, use of non-steroidal anti-inflammatory drugs (NSAIDs), and smoking, so that bias may have been present. Furthermore, disease progression itself could lead to enhanced tumor-associated inflammation and elevated levels of circulating pro-inflammatory markers. Thus, it is subject to reverse causation.[109]

Most studies of CRP and CRC only had a one-time measurement of CRP which may not be representative of a person's average level of chronic inflammation over a lifetime. Mendelian randomization utilizes inherited germline genetic markers known to be associated with CRP concentrations and are non-modifiable markers of long-term susceptibility to chronic inflammation. Because of the natural random assortment of

alleles during gamete formation, genetic variants are not affected by environmental factors that occur after conception and are non-modifiable by disease progression.[20,21,24,25] In the past couple of decades, genome-wide association studies (GWAS) have accumulated robust evidence on genetic variants associated with various inflammatory biomarkers, including CRP.[22,23] "Mendelian randomization" has become a common design for observational studies of inflammatory biomarkers in association with cancer risk,[26-32,110] providing a way to minimize reverse causality and residual confounding. However, Mendelian randomization studies for cancer survival outcomes are limited.[33]

In this study, we aimed to test the association of genetically explained levels of CRP with disease-specific survival among CRC patients using a Mendelian randomization approach. As a secondary aim, we evaluated stage- and tumor site-specific associations between genetically predicted circulating CRP and CRC survival. To achieve this, we leveraged the existing data on germline genetic variants and epidemiologic and clinic factors from the International Survival Analysis in Colorectal Cancer Consortium (ISACC).

METHODS

Study population

We included individuals diagnosed with incident, invasive CRC from ISACC, a consortium consists of clinical trials, case-control, and cohort studies from North America and Europe. For this study, we restricted the population to CRC cases who all had available data on genotyping, epidemiologic factors, and survival outcomes, and were of European genetic

ancestry. Further restrictions to patients with data on CRC-specific survival outcome lead to a study population of fifteen studies: Colon Cancer Family Registry (CCFR) [45], Cancer Prevention Study-II (CPS-II) [111], German Darmkrebs: Chancen der Verhuetung durch Screening Study (DACHS) [112], Diet Activity and Lifestyle Study (DALS) [113], Early Detection Research Network (EDRN) [114], European Prospective Investigation into Cancer (EPIC) [115], Health Professionals Follow-up Study (HPFS)[116], Melbourne Collaborative Cohort Study (MCCS) [117], Nurses' Health Study (NHS) [118,119], North Central Cancer Treatment Group (NCCTG) clinical trial N9741 [120], Physician's Health Study (PHS) [121], Prostate, Lung, Colorectal, and Ovarian Study (PLCO) [122,123], UK Biobank (UKB) [124], VITamins And Lifestyle Study (VITAL) [125], and Women's Health Initiative (WHI).[126,127]

The study population is described in **Figure 3.1.** Study-specific details are summarized in **Supplementary Table 3.1**. All studies were approved by their respective Institutional Review Boards and participants provided informed consent for genetic testing and research participation.

Ascertainment of environmental variables and survival outcomes

Demographic and epidemiologic factors were collected using self- or interviewer-administered questionnaires at enrollment (before diagnosis for most studies, after diagnosis for N9741) according to study-specific protocols. A multistep data harmonization process was conducted centrally to define epidemiologic and clinicopathological variables in the same way across studies, as described previously.[128] Information on cancer diagnosis, such as age at diagnosis, tumor location (proximal,

distal colon, or rectum), and stage at diagnosis (local, regional or distant), were obtained from cancer registries and/or medical records.

All study participants were followed up for vital status. Date and cause of death were ascertained through linkages to the National Death Index or cancer registries with dates/cause of death confirmed by death certificates (CCFR, CPSII, DACHS, DALS, EPIC, MCCS, UKB, VITAL) or via active follow-up with dates/cause of death verified by reviewing death certificates and/or medical records (HPFS, NHS, PHS, PLCO, WHI, N9741). Time to event was defined as days between CRC diagnosis and death, last date of contact, or the end of follow-up. We censored all follow-up time at 10 years from the date at CRC diagnosis. Patients who died from causes other than CRC were censored at the time of death. We used the International Classification of Diseases-9 (ICD-9) or ICD-10 (depending on year of linkage) to define CRC-specific deaths (ICD-9: 153.0-153.4, 153.6-153.9, or 154.0-154.1; ICD-10: C18.0-20.0 or C26.0).[59]

Genotyping, quality control (QC) and imputation

Details of genotyping and QC methods have been reported previously.[129-133] Briefly, genomic DNA was extracted from blood or buccal samples using conventional methods. Genotyping was performed using a variety of platforms (**Supplementary Table 3.1**). All genotyping underwent standardized QC procedures including the exclusion of low sample (<98%) and SNP (<98%) call rates, checking for chromosomal anomalies, and discrepancies in self-reported vs. genetic sex, and testing for Hardy–Weinberg Equilibrium. To investigate population structure, we used Plink (v1.9) to conduct principal components analysis (PCA). Due to low number of participants of early ancestries, vast

majority of the study participants were of European ancestry, we restricted our population to only participants with estimated European genetic ancestry based on the PCA to ensure study power. The first two eigenvectors discriminated individuals based on self-identified race (**Supplementary Figure 3.1A**) and were used to select individuals with likely European ancestry. Participants with a value within one standard deviation of the median for the first and second eigenvectors were categorized as European ancestry (**Supplementary Figure 3.1B**) and retained for subsequent analyses. Within the subset of individuals with European ancestry, we recalculated the principal components. The first nine eigenvectors explained 73% of the genetic variation and were subsequently used as covariates in the analysis.

We imputed genotypes to infer unobserved genotypes and increase the density of genetic variants. All samples were first phased using SHAPEIT2 [134] and then imputed to the Haplotype Reference Consortium (HRC) panel [135] using the University of Michigan Imputation Server.[136]

Selection of instrumental variables

The largest GWAS of circulating CRP to date was conducted within the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) of 204,402 individuals of European descent and reported 58 genetic variants that were genome-wide significantly associated with CRP.[23] We included the 52 genetic variants from their main GWAS as instrumental variables. Six variants identified in their BMI-adjusted GWAS were not included because they were either not mapped in the 1000 Genome or not available in our data. We also searched the GWAS catalog, with the most recent update at March 9th,

2020, for SNPs that meet the following criteria: 1) it is associated with CRP at the genome-wide significance level ($P<5x10_{-8}$); 2) study population was of European ancestry; 3) it is not in LD ($R_2<0.3$) with previously selected SNPs; and 4) it had information on effect sizes and standard error. No additional SNPs were identified.

We used the genetic data described above to calculate the CRP genetic risk score (GRS) based on 52 CRP-associated genetic variants. [137] The GRS was calculated by taking the sum of the number of risk (CRP-increasing) alleles for each of the genetic variants weighted by the reported β coefficients from the GWAS. The β coefficients represented a one-unit change in the natural-log-transformed CRP (mg/L) per copy increment in the risk allele (**Table 3.1**). The imputation quality (r_2) of all 52 CRP-associated SNPs in our data was greater than 0.8.

Statistical power

Currently, there is no available statistical power calculation tool for survival outcomes in Mendelian randomization analysis, therefore we decided to take a more conservative approach treating mortality as a binary outcome using logistic regressions. We calculated the statistical power based on the online Power calculations for the Mendelian Randomization tool (https://shiny.cnsgenomics.com/mRnd/).[138] With a type 1 error of 5% and a total of 16,916 CRC cases, we had at least 80% power to detect an odds ratio (OR) of 1.22 for CRC-specific mortality per 1 SD of CRP, assuming the variance of circulating CRP explained by the genetic variance is 5.9%.

We also conducted a simulation study to evaluate power in additive hazard model. Based on our sample size, we have at least 90% power to detect 25% increase in hazard

(HD=0.00675) for every 1 SD increase of CRP assuming the population averaged hazard is around 3808/(16918*10) =0.023 per person*year and the GRS explains 5.9% R-square of CRC.

Statistical Analysis

The genetic variants selected as an instrumental variable in a Mendelian randomization analysis need to meet three assumptions: (1) they are robustly associated with the exposure ("relevance"), (2) they do not share a common cause with the outcome ("exchangeability"), and (3) they affect the outcome only through the exposure ("exclusion restriction").

We first verified the "relevance" assumption by evaluating the association between the calculated GRS and circulating CRP concentrations in a subset of 285 CRC cases from Seattle CCFR who had both genotyping and circulating CRP data available.[139] We also estimated the variance explained by the genetic variants (R_2) and calculated the F statistic, a measure of instrument strength, based on the proportion of the variance in the exposure explained by the genetic variants (R_2), sample size (n), and the number of the instruments (k) as described in the formula: $F = \frac{R^2}{R^2+1} * \frac{\mathrm{n-k-1}}{k}$. A "weak" IV is defined as having F<10.[140] For the second "exchangeability" assumption, we identified several epidemiologic and clinicopathological factors that are known to be associated with CRC survival, including smoking, body mass index, NSAID use, tumor location, and stage at diagnosis. We examined their associations with the GRS. The "exclusion restriction" assumption was assessed in a series of sensitivity analyses. We used MR- Egger regression to assess the horizontal pleiotropic effect. The test of a non-zero intercept

indicates whether there are averaged pleiotropic effects.[141] Also, we restricted the instrumental variable to one genetic variant in the *CRP* gene (rs2794520), which by itself explained 1.4% of the variance in circulating CRP concentrations.[23]

We performed the Mendelian randomization analysis using the two-stage regression approach.[34] Because the CRC-specific mortality among CRC patients is common and the hazard ratio is not collapsible over strata of unmeasured and unknown confounders, Cox proportional hazards model cannot be used for causal inference but instead was used in a sensitivity analysis for hypothesis testing. Additive hazards regression models were used, given their collapsibility and interpretability, to calculate hazard differences (HD) and 95% confidence intervals (CI) for the associations between CRP-associated GRS and CRC-specific mortality.[34,142] The "timereg" R package was used for fitting additive hazards models. [143] As a sensitivity analysis, Cox proportional hazards models were also used to test whether there is a causal effect. Potential confounders of CRP-survival associations that violate the "exchangeability" assumption were also included as adjustment variables in the main analysis in addition to age at diagnosis, sex, genotyping platform, study, and the first nine principal components. Also, we evaluated the association between genetically determined CRP and CRC survival using a random-effects inverse-variance weighted (IVW) method,[17] MR-Egger regression,[141] and the estimator from the weighted median approach [144] based on summary statistics on SNP-specific associations with CRC survival. In secondary analyses, we evaluated the associations between genetically predicted concentrations of CRP and CRC-specific mortality by stage at diagnosis and tumor location. All analyses were conducted using R version 3.6.0.

RESULTS

We included a total of 16,918 eligible CRC cases from ISACC in this study **(Figure 3.1**). Study participants were diagnosed at 66.3 (SD=9.9) years of age on average, and 49.7% were female. Over the 10-year study follow up, there were 5,395 (32%) deaths accrued and 3,808 (23%) due to CRC. Study-specific summaries are shown in **Supplementary Table 3.1**. SNP-specific associations with CRP concentrations and with CRC-specific survival are summarized in **Table 3.1**.

We observed strong associations between the GRS and circulating CRP concentrations in a subset of Seattle CCFR participants (N= 285). One unit increase in GRS was associated with elevated circulating CRP concentrations by 1.22 mg/L (95% CI: 0.65 to 1.80, P= 4.33×10^{-5}) on the natural log-transformed scale and explained 5.9% variance of circulating CRP concentrations, similar to the proportion reported in Ligthart et al. Based on a total of 16,918 cases and 52 selected genetic variants, the estimated F statistics was 20.2, indicating a strong instrumental variable for the Mendelian randomization analysis.

Potential confounders and common CRC risk factors were assessed for association with the GRS. BMI was statistically significantly associated with GRS and we included it as an additional adjustment variable in the Mendelian randomization analysis. No other variables were statistically significantly associated with GRS.

Based on the GRS and an additive hazards model, we observed that genetically determined circulating CRP concentration (mg/L) was not associated with CRC-specific survival (HD= -1.15, 95% CI: -2.76 to 0.47 per 100,000 person-year). Although the HD suggested that higher genetically predicted levels of CRP was associated with lower risk

of CRC-specific mortality, it didn't not reach statistical significance (P value= 0.10). IVW, MR-Egger, and weighted median approaches based on summary statistics showed consistent with those based on individual data (**Table 3.2**). Our sensitivity analysis using the Cox proportional hazards model showed a similar association between CRP-associated GRS and CRC survival (HR= 0.90, 95% CI: 0.79 to 1.02, *P* value= 0.10). When we further evaluated this association by stage at diagnosis and tumor location, we found no evidence of association in the subgroups using Cox proportional hazards models whereas the additive model couldn't converge. (**Table 3.3**).

Based on MR-Egger regression, we plotted the SNP-specific associations with CRC survival against coefficients of SNP-CRP associations (**Figure 3.2**). The intercept of Egger regression was not statistically significantly different from zero ($1.28x10^{-07}$, P value= 0.85) when using additive hazards models. This suggested no horizontal pleiotropic effect. The MR-Egger regression using Cox proportional hazards estimates (**Figure 3.2B**) yielded similar results compared to the one using additive hazards models (**Figure 3.2A**). We then restricted the instrumental variable to one genetic variant on the *CRP* gene (rs2794520) and repeated the Mendelian randomization analysis. Similar null association with CRC survival was observed (additive hazards model: HD= -0.049 per 100,000 person-year, P value= 0.88; Cox proportional hazards model: HR= 0.99, 95% CI: 0.94-1.04, P value=0.60).

DISCUSSION

In this large Mendelian randomization study, we observed no association between genetically predicted CRP concentrations and survival among a cohort of individuals

diagnosed with incident invasive CRC. We constructed a weighted GRS as an instrumental variable based on 52 CRP-associated SNPs identified in previous GWAS.[23] Our study results from two-stage regression based on individual genotype data are consistent with the results from the summarized data using IVW, MR-Egger, and weighted median approaches. No associations were observed in subgroups defined by stage at diagnosis and tumor location. Our findings suggest that circulating CRP is not causally associated with survival after a diagnosis of CRC.

Our study provides consistent evidence along with previous studies of CRC incidence and survival. Circulating CRP concentrations measured prior to diagnosis was associated with a 12% increased risk of developing CRC in two meta-analyses of 18 observational studies.[14,16] Conversely a larger multi-consortium Mendelian randomization study with more than 30,400 cases and 22,800 controls demonstrated that genetically determined CRP concentrations are not associated with CRC risk.[110] In observational studies of circulating CRP and CRC survival outcomes, although none were Mendelian randomization studies, the results were inconsistent. Some studies observed that circulating CRP measured before surgery was not statistically significantly associated with survival after multivariate adjustment.[67,68] Other studies observed that pre-operative [18,65,66,69] and post-treatment [70,71] circulating CRP concentrations were associated with CRC survival outcomes. However, the CRP measures in these studies were crude: cut-off used to dichotomize pre-operative or post-treatment CRP concentrations were ≥5mg/L. Individuals in the elevated CRP group are likely driven by acute inflammatory conditions that are not due to chronic inflammation. Similarly in our recent study, circulating concentration of CRP was no longer associated with CRC survival after we

excluded CRC patients who had post-treatment CRP> 10mg/L.[139] In addition, insufficient adjustment of potential confounders (i.e. BMI) may have led to these spurious associations.

In our study, we used genetic variants as proxies of inflammatory biomarkers that can help to address potential biases due to residual confounding and reverse causality, but existing evidence is limited. Slattery et al. evaluated four tag SNPs of the *CRP* gene in relation to CRC survival, however, none were statistically significantly associated with CRC-specific mortality within 5 years after diagnosis.[145] Another study with 421 CRC patients of East Asian ancestry showed that two SNPs from the *CRP* gene were associated with CRC survival: rs3093059 was associated with disease-free survival, whereas rs1205 was associated with CRC-specific survival.[146] Although these two variants were not included in our study, we evaluated rs2794520 at *CRP* locus that is in high LD with these two SNPs. The allele frequencies of these SNPs are twice more common in the East Asian population (ASN) as compared to the European population (EUR): rs3093059 (ASN: 0.14; EUR: 0.07), rs2794520 and rs1205 (ASN: 0.60; EUR: 0.31). This could partially explain the different study findings.

Our study had some limitations. First, in contrast with Mendelian randomization studies of CRC incidence, our study population was restricted to individuals diagnosed with CRC. This could be a potential source of selection bias (also known as collider bias) particularly if CRP is causally associated with increased risk of developing CRC. By conditioning on the collider- CRC risk (selecting only CRC patients into the study population), it can induce an association between genetic variants and risk factors of CRC. However, evidence from the previous Mendelian randomization study showed that

CRP is not causally associated with CRC risk.[110] To further address this potential selection bias, we evaluated the associations between the genetic variants with both potential confounders of CRP and CRC survival associations and common risk factors of CRC risk. BMI was identified as the only variable being statistically significantly associated with the genetic variants in our study population and was adjusted in all analyses. Our exploratory analyses without BMI as an adjustment variable did not change the results materially. Second, the genetic variants shown to be robustly associated with circulating CRP were identified from GWAS of a general population. These associations may be different in a CRC patient population. Although we evaluated this "relevance" assumption in a subset of our study population and observed a strong association between the 52-SNP GRS and circulating CRP concentration in CRC patients, the small sample size had limited statistical power to evaluate SNP-specific associations with CRP. Furthermore, the 52-SNP GRS explained only less than 6% genetic variation of circulating concentrations of CRP. The null results of our study cannot rule out the causal effect of CRP on disease-specific mortality among CRC patients. Lastly, our study population was limited to those with European ancestry, our findings may not be generalizable to other racial/ethnicity groups.

Our study also has many strengths. This is the first study that evaluates circulating biomarkers in relation to cancer survival using a Mendelian randomization approach. The large sample size allowed adequate statistical power to detect associations with moderate effect sizes. The well-characterized study population with individual-level genotype data, detailed epidemiologic, and clinic factors allowed us to compare study results with those based on summary statistics, to evaluate the "exchangeability" assumptions, and to

explore whether the association differed according to the stage at diagnosis and tumor location. A subset of study participants had data on both genotypes and circulating CRP concentrations allowed us to evaluate the “relevance” assumption. By carefully examining the three assumptions, our Mendelian randomization study is less susceptible to confounding and reverse causality compared to observational studies.

In summary, our study found that genetically predicted circulating CRP concentration was not associated with CRC survival among individuals diagnosed with CRC. No evidence of an association was observed in subgroups defined by stage at diagnosis or tumor location. Our study did not find evidence of a causal relationship between circulating CRP after diagnosis and CRC-specific survival.

TABLES AND FIGURES

Figure 3.1 Study population diagram

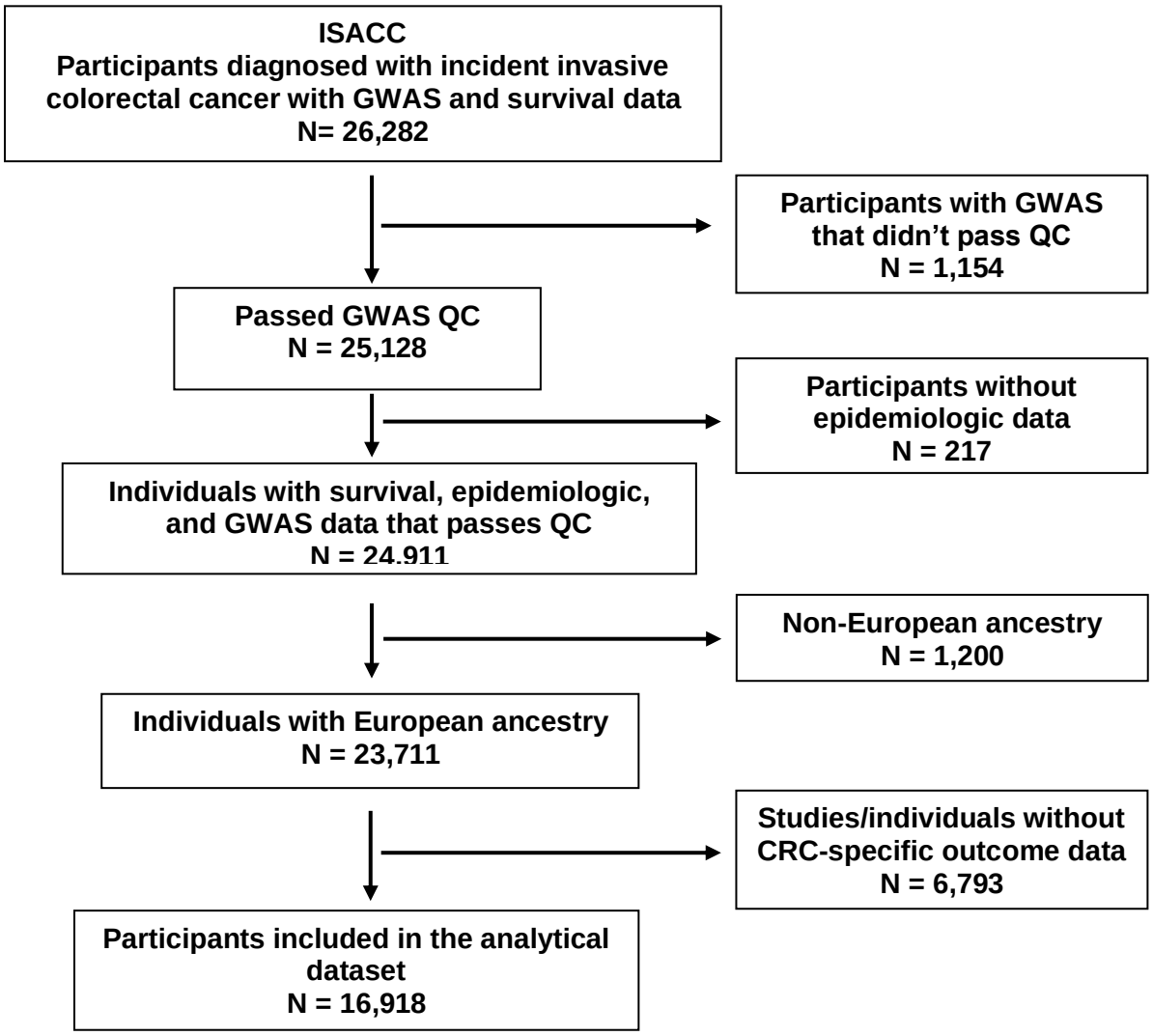


Table 3.1 Association between 52 SNPs and circulating CRP concentrations identified in Ligthart et al.[23] and SNP-survival associations in ISACC

rs	Chr: pos*	Closest gene	Count/ Alt allele	Count allele freq	Ligthart et al. SNP-CRP associations			ISACC SNP-survival associations		
					beta**	SE	P value	HD** (per 100,000 person year)	SE	P value
rs2293476	chr1:40036847	*PABPC4*	C/G	0.23	0.030	0.004	8.27E-13	0.124	3.44E-06	0.72
rs1805096	chr1:66102257	*LEPR*	G/A	0.61	0.104	0.004	2.17E-183	-0.121	2.99E-06	0.68
rs4129267	chr1:154426264	*IL6R*	C/T	0.61	0.088	0.004	1.20E-129	-0.474	2.91E-06	0.10
rs2794520	chr1:159678816	*CRP*	C/T	0.66	0.182	0.004	0.00E+00	-0.049	3.17E-06	0.88
rs10925027	chr1:247612562	*NLRP3*	T/C	0.4	0.036	0.004	4.25E-21	-0.759	2.92E-06	0.01
rs1260326	chr2:27730940	*GCKR*	T/C	0.39	0.073	0.004	2.72E-92	0.278	2.95E-06	0.35
rs13409371	chr2:113838145	*IL1F10*	A/G	0.43	0.048	0.004	5.07E-36	-0.209	2.91E-06	0.47
rs13233571	chr7:72971231	*BCL7B*	C/T	0.86	0.057	0.005	2.95E-25	-0.180	4.22E-06	0.67
rs4841132	chr8:9183596	*PPP1R3B*	G/A	0.91	0.065	0.006	2.00E-25	0.442	5.40E-06	0.41
rs10778215	chr12:103537266	*ASCL1*	T/A	0.49	0.033	0.004	1.86E-20	-0.222	2.87E-06	0.44
rs7310409	chr12:121424861	*HNF1A*	G/A	0.61	0.137	0.004	2.54E-299	0.105	2.88E-06	0.71
rs340005	chr15:60878030	*RORA*	A/G	0.62	0.030	0.004	1.01E-15	0.352	2.92E-06	0.23
rs10521222	chr16:51158710	*SALL1*	C/T	0.95	0.104	0.011	2.06E-22	-1.450	6.99E-06	0.04
rs2852151	chr18:12841176	*PTPN2*	A/G	0.4	0.025	0.004	1.36E-11	-0.002	2.94E-06	0.99
rs4420638	chr19:45422946	*APOC1*	A/G	0.82	0.229	0.006	1.23E-305	-0.425	4.16E-06	0.31
rs1800961	chr20:43042364	*HNF4A*	C/T	0.97	0.112	0.011	4.63E-23	-0.742	8.51E-06	0.38
rs469772	chr1: 91530305	*ZNF644*	C/T	0.19	0.031	0.005	5.54E-12	0.242	3.60E-06	0.50
rs12995480	chr2: 629881	*TMEM18*	C/T	0.17	0.031	0.005	1.24E-10	0.329	3.92E-06	0.40
rs4246598	chr2: 88438050	*FABP1*	A/C	0.46	0.022	0.004	5.11E-10	-0.200	2.89E-06	0.49
rs9284725	chr2: 102744854	*IL1R1*	C/A	0.24	0.027	0.004	7.34E-11	-0.434	3.36E-06	0.20
rs1441169	chr2: 214033530	*IKZF2*	A/G	0.53	0.025	0.004	2.27E-11	-0.130	2.81E-06	0.64
rs2352975	chr3: 49891885	*TRAIP*	C/T	0.3	0.025	0.004	6.43E-10	0.161	3.27E-06	0.62
rs17658229	chr5: 172191052	*DUSP1*	C/T	0.05	0.056	0.010	5.50E-09	-0.274	6.67E-06	0.68
rs9271608	chr6: 32591588	*HLA-DQA1*	G/A	0.22	0.042	0.005	2.33E-17	0.094	4.15E-06	0.82
rs12202641	chr6: 116314634	*FRK*	C/T	0.39	0.023	0.004	3.00E-10	0.187	2.94E-06	0.53
rs1490384	chr6: 126851160	*C6orf173*	C/T	0.51	0.025	0.004	2.65E-12	0.175	2.83E-06	0.54
rs9385532	chr6: 130371227	*L3MBTL3*	C/T	0.33	0.026	0.004	1.90E-11	-0.403	3.25E-06	0.21
rs1880241	chr7: 22759469	*IL6*	A/G	0.48	0.028	0.004	8.41E-14	-0.313	2.90E-06	0.28
rs2710804	chr7: 36084529	*KIAA1706*	C/T	0.37	0.021	0.004	1.30E-08	0.298	2.91E-06	0.31
rs2064009	chr8: 117007850	*TRPS1*	T/C	0.42	0.027	0.004	2.28E-14	-0.697	3.03E-06	0.02

rs2891677	chr8: 126344208	*NSMCE2*	T/C	0.46	0.020	0.004	1.59E-08	0.212	3.00E-06	0.48
rs643434	chr9: 136142355	*ABO*	A/G	0.37	0.023	0.004	1.02E-09	-0.041	3.05E-06	0.89
rs1051338	chr10: 91007360	*LIPA*	G/T	0.31	0.024	0.004	2.27E-09	0.514	3.14E-06	0.10
rs10832027	chr11: 13357183	*ARNTL*	A/G	0.33	0.026	0.004	4.43E-12	-0.394	2.96E-06	0.18
rs10838687	chr11: 47312892	*MADD*	T/G	0.22	0.031	0.004	9.12E-13	0.016	3.39E-06	0.96
rs1582763	chr11: 60021948	*MS4A4A*	G/A	0.37	0.022	0.004	2.37E-09	-0.083	2.97E-06	0.78
rs7121935	chr11: 72496148	*STARD10*	G/A	0.38	0.022	0.004	5.28E-09	0.090	3.04E-06	0.77
rs11108056	chr11: 95855385	*METAP2*	C/G	0.42	0.028	0.004	5.42E-14	0.318	3.02E-06	0.29
rs2239222	chr14: 73011885	*RGS6*	G/A	0.36	0.035	0.004	9.87E-20	0.415	3.15E-06	0.19
rs4774590	chr15: 51745277	*DMXL2*	G/A	0.35	0.022	0.004	2.71E-08	0.110	3.07E-06	0.72
rs1558902	chr16: 53803574	*FTO*	A/T	0.41	0.034	0.004	5.20E-20	0.030	2.84E-06	0.92
rs178810	chr17: 16097430	*NCOR1*	T/C	0.56	0.020	0.004	2.95E-08	-0.060	2.83E-06	0.83
rs10512597	chr17: 72699833	*CD300LF; RAB37*	C/T	0.18	0.037	0.005	4.44E-14	-0.048	3.87E-06	0.90
rs4092465	chr18: 55080437	*ONECUT2*	G/A	0.35	0.027	0.004	3.11E-10	-0.154	3.11E-06	0.62
rs12960928	chr18: 57897803	*MC4R*	C/T	0.27	0.024	0.004	1.91E-09	-0.296	3.34E-06	0.38
rs2315008	chr20: 62343956	*ZGPAT*	G/T	0.31	0.023	0.004	5.36E-10	0.118	3.11E-06	0.70
rs2836878	chr21: 40465534	*DSCR2*	G/A	0.73	0.043	0.004	7.71E-26	0.289	3.10E-06	0.35
rs6001193	chr22: 39074737	*TOMM22*	A/G	0.35	0.028	0.004	6.53E-14	-0.678	3.10E-06	0.03
rs75460349	chr1: 27180088	*ZDHHC18*	A/C	0.97	0.086	0.014	4.50E-10	0.477	9.43E-06	0.61
rs1514895	chr3: 170705693	*EIF5A2*	G/A	0.71	0.027	0.004	2.70E-09	0.002	3.26E-06	0.99
rs112635299	chr14: 94838142	*SERPINA; SERPINA2*	G/T	0.02	0.107	0.017	2.10E-10	-2.150	1.22E-05	0.08
rs1189402	chr15: 53728154	*ONECUT1*	A/G	0.62	0.025	0.004	3.90E-09	0.474	3.20E-06	0.14

* Chromosome: position, hg19

** SNP-specific coefficients for association with circulating concentrations of CRP obtained from Ligthart et al, per unit increase in natural log-transformed CRP (mg/L); HD: hazards difference for SNP-specific association with CRC survival per unit increase in the count allele; Abbreviations: ISACC: International Survival Analysis in Colorectal Cancer Consortium; HD: hazards difference; SE: standard error

Table 3.2 Association between CRP genetic risk score and CRC survival, ISACC

	Additive hazards model			Cox proportional hazards model		
	HD* (95% CI)		P value	HR (95% CI)		P value
Using individual-level data						
52-SNP GRS	-1.15	(-2.76, 0.47)	0.16	0.90	(0.79, 1.02)	0.10
Using summary statistics						
IVW	-1.12	(-2.72, 0.48)	0.17	0.90	(0.79, 1.02)	0.10
MR-Egger	-1.29	(-3.68, 1.11)	0.29	0.88	(0.72 ,1.06)	0.18
Weighted median	-0.77	(-3.02, 1.47)	0.50	0.93	(0.77, 1.11)	0.40

* Hazard difference, per 100,000 person-year

Models adjusted for age at diagnosis, sex, body mass index, genotyping platform, study, and principal components

Abbreviations: GRS: genetic risk score; HD: hazard differences; HR: hazard ratio; IVW: inverse-variance weighted;

Table 3.3 Association between genetically determined CRP level and CRC survival by subgroups

52-SNP GRS	Total	Events*	Cox proportional hazards model		
			HR**	95% CI	P value
All	16,918	3,808	0.90	(0.79, 1.02)	0.10
By stage at diagnosis‡					
Local	3,341	142	0.50	(0.24, 1.02)	0.05
Regional	6,420	1,177	0.92	(0.73, 1.17)	0.51
Distant	1,845	1,387	0.97	(0.75, 1.24)	0.79
By tumor location ‖					
Colon	12,000	2,791	0.87	(0.75, 1.00)	0.06
Proximal	6,205	1,365	0.86	(0.69, 1.07)	0.18
Distal	4,879	932	0.98	(0.75, 1.29)	0.91
Rectum	4,729	974	1.02	(0.79, 1.33)	0.85

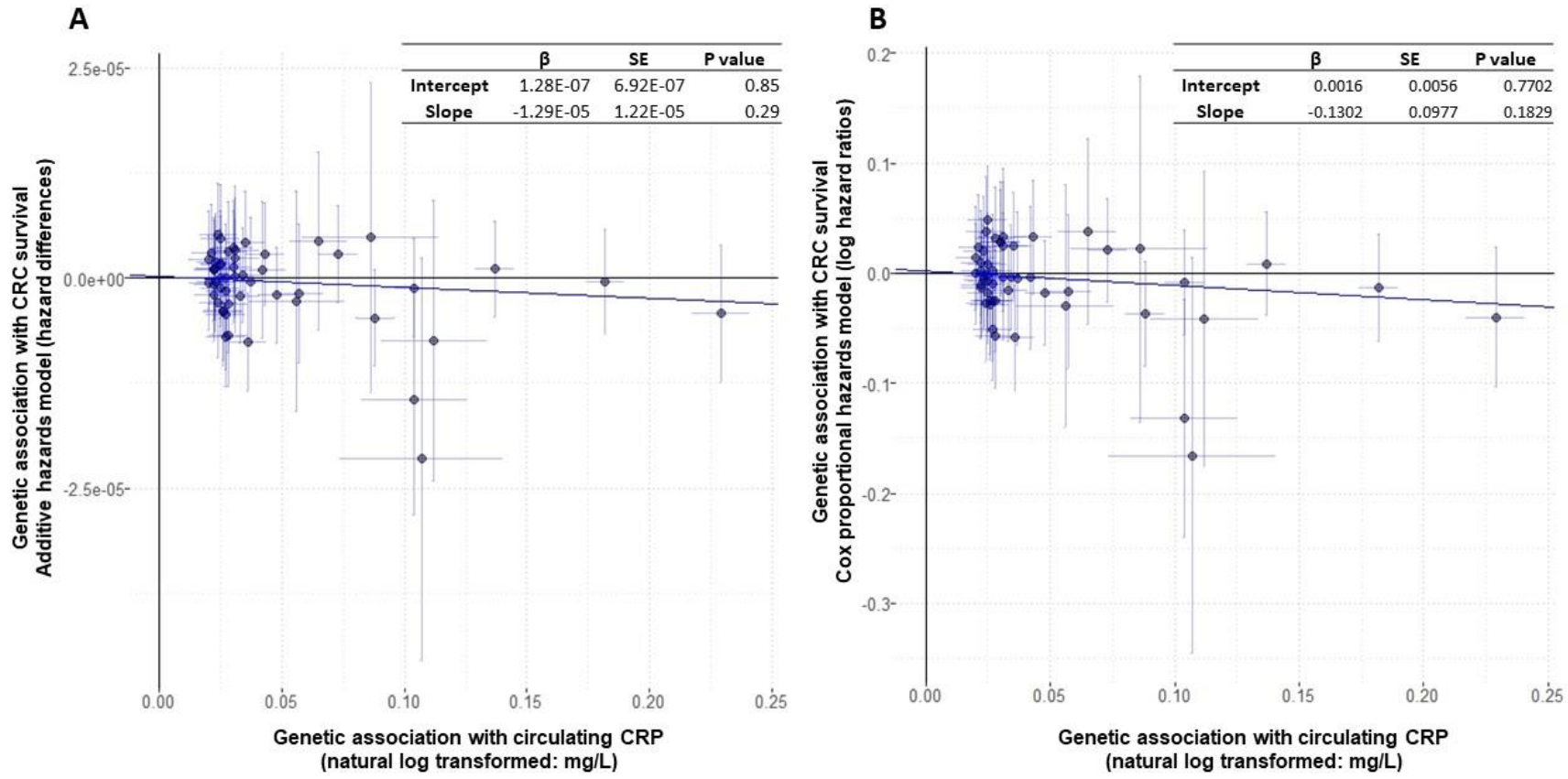
* Death due to CRC within 10-year study follow-up

** Additive models do not converge in subgroup analysis; Cox regression adjusted for age at diagnosis, sex, body mass index, genotyping platform, study, and principal components

‡ Stage at diagnosis was defined using the SEER summary stage

‖ Proximal colon was defined as from the cecum through the transverse colon; distal colon was from the splenic flexure to sigmoid colon, and rectum included the rectosigmoid junction and rectum.

Figure 3.2 Scatter plots of MR-Egger regression



Legend: Scatter plot of SNP-specific associations with CRC survival against coefficients of SNP-CRP associations among CRC cases from ISACC using A) additive hazards models and B) Cox proportional hazards models. The slope of

the regression line provides an estimate of the association between genetically predicted CRP and CRC survival; the intercept is an estimate of the average pleiotropic effect across all the genetic variants.

Supplementary Table 3.1 Study-specific characteristics

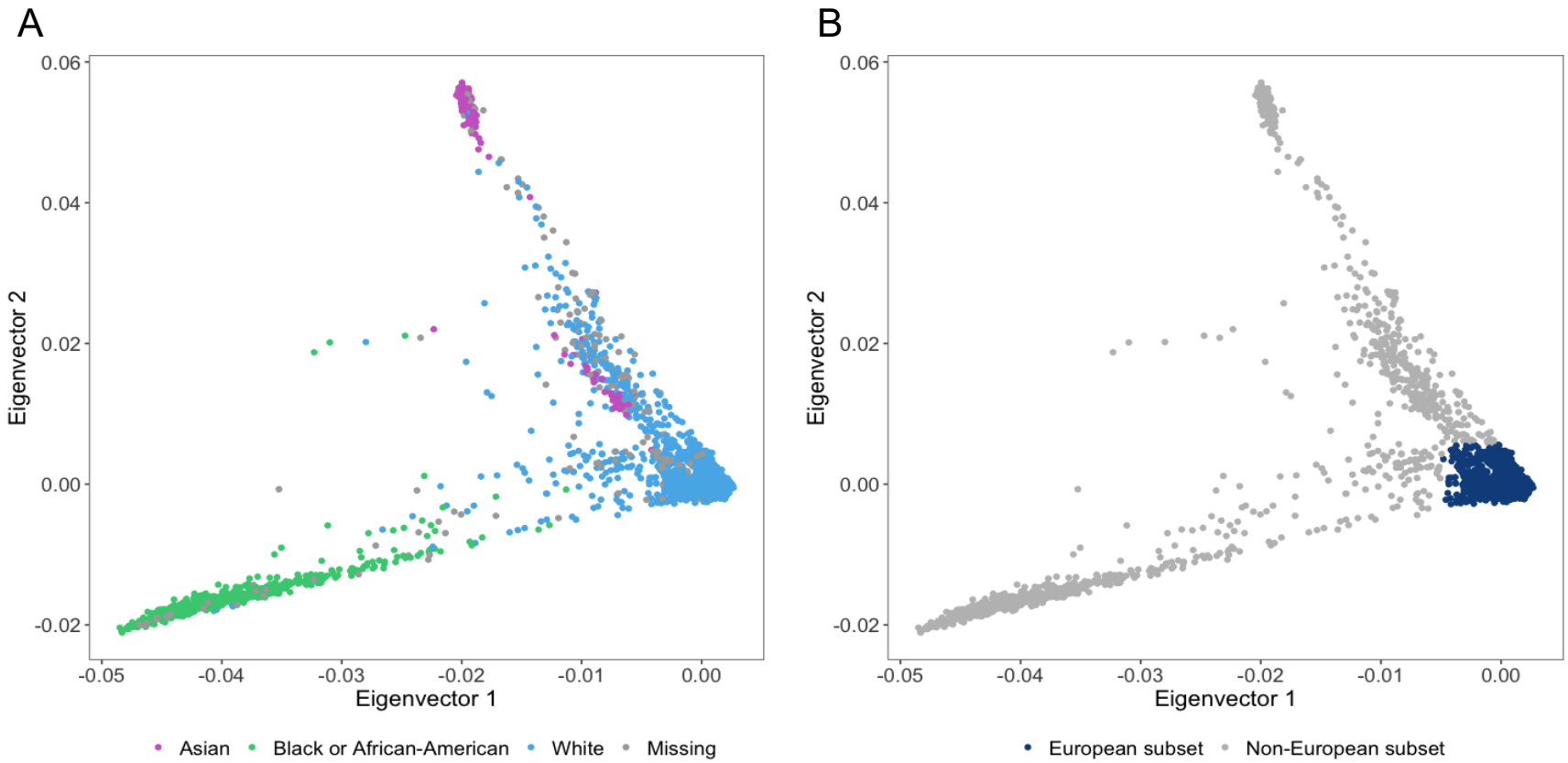
Study Abbrev.	Study Name	Study Design	Location	Genotyping platform	N	Any deaths N (%)*	CRC deaths N (%)*	Age at dx Mean (SD)	Female (%)	Follow-up years, median (IQR)	Survival ascertainment method**
CCFR	Colon Cancer Family Registry	Case-control	USA, Australia, Canada	Illumina Human 1M or Human1M-Duo, Illumina Omni-1Quad, Illumina OncoArray, Custom Affymextrix Axiom array (1.3M SNPs)	2453	821 (33.5)	518 (21.1)	58.7 (10.5)	1154 (47)	12.9 (6.1 - 15.7)	Registry linkage
CPSII	Cancer Prevention Study - II	Cohort	USA	Illumina OncoArray + Custom iSelect array, Custom Affymetrix Axiom Array (1.3M SNPs)	816	312 (38.2)	184 (22.5)	76.4 (5.9)	418 (51.2)	5.2 (2.5 - 8.2)	Registry linkage
DACHS	Darmkrebs: Chancen der Verhutung durch Screening Study	Case-control	Germany	Illumina HumanCytoSNP, Illumina HumanOmniExpress, Illumina OncoArray	2659	725 (27.3)	537 (20.2)	68.5 (10.6)	1080 (40.6)	3.3 (2.4-5.0)	Registry linkage
DALS	Diet Activity and Lifestyle Study	Case-control	USA	Illumina HumanCytoSNP, Illumina 550K, Illumina 610K	1098	351 (32)	210 (19.1)	65.1 (9.8)	492 (44.8)	4.9 (3.5 - 6.6)	Registry linkage
EDRN	Early Detection Research Network	Case-cohort	USA	Illumina OncoArray + Custom iSelect Array	190	19 (10)	14 (7.4)	61.6 (12)	78 (41.1)	3.4 (2.3 - 5.4)	Registry linkage
EPIC	European Prospective Investigation into Cancer	Cohort	Sweden	Illumina HumanOmniExpress + ExomeChip	1821	588 (32.3)	469 (25.8)	63.4 (8.3)	991 (54.4)	3.4 (1.4 - 6.5)	Registry linkage
HPFS	Health Professionals	Cohort	USA	Illumina HumanOmniExpress, Illumina	344	151 (43.9)	79 (23)	71.8 (8.7)	0	7.1 (3.4 - 12.4)	Active f/up

	Follow-up Study			HumanOmniExpress + ExomeChip							
MCCS	Melbourne Collaborative Cohort Study	Cohort	Australia	Illumina OncoArray, Custom Affymetrix Axiom Array (1.3M SNPs)	751	151 (20.1)	77 (10.3)	70.2 (9)	359 (47.8)	11.4 (6.6 - 15.5)	Registry linkage
N9741	N9741	Clinical trial	USA	Illumina HumanOmniExpress + ExomeChip	426	405 (95.1)	366 (85.9)	60.7 (11.2)	177 (41.5)	1.6 (0.9 - 2.6)	Active f/up
NHS	Nurses' Health Study	Cohort	USA	Illumina HumanOmniExpress, Illumina HumanOmniExpress + ExomeChip	587	199 (33.9)	153 (26.1)	69.1 (8.6)	587 (100)	7.6 (3.1 - 12.8)	Active f/up
PHS	Physician's Health Study	Cohort	USA	Illumina HumanOmniExpress	323	170 (52.6)	125 (38.7)	70.6 (9.6)	0	5.7 (2.1 - 13.1)	Active f/up
PLCO	Prostate, Lung, COlorectal, and Ovarian Cancer Screening Trial	Case-control	USA	Illumina HumanCytoSNP, Illumina HumanHap300 and HumanHap240S, Illumina 610K	972	260 (26.7)	174 (17.9)	69.6 (6.3)	416 (42.8)	5.3 (2.7 - 8.5)	Active f/up
UKB	UK Biobank	Cohort	UK	Custom Affymetrix Axiom Array (1.3M SNPs)	2877	731 (25.4)	539 (18.7)	64.5 (6.4)	1204 (41.8)	3.1 (1.8 - 4.9)	Registry linkage
VITAL	VITamins and Lifestyle Study	Cohort	USA	Illumina HumanCytoSNP	270	109 (40.4)	67 (24.8)	69.8 (6.5)	124 (45.9)	4.9 (2.5 - 7.3)	Registry linkage
WHI	Women's Health Initiative	Cohort	USA	Illumina HumanCytoSNP, Illumina 550K, Illumina 610K	1331	403 (30.3)	296 (22.2)	71.6 (7.2)	1331 (100)	3.8 (1.5 - 7.1)	Active f/up

*All death events were censored at 10 years since diagnosis

**Registry linkage involves National Death Index, state cancer registries, state death records, or population registers with the cause of death verified by death certificates; Active f/up involves death certificate and/or medical record review

Supplementary Figure 3.1 Principal components analysis



Legend: Principal components analysis (PCA) used to define European genetic ancestry. (A) PCA plot colored by the self-identified race. Values within one standard deviation of the median for the first and second eigenvectors were used to define individuals with European genetic ancestry. (B) individuals defined as European genetic ancestry are highlighted in blue. All others were excluded from further analysis.

CHAPTER 4. CONCLUSIONS AND FUTURE DIRECTIONS

The projects included in this dissertation are both conceptually and methodologically innovative studying the association between both circulating and genetic markers of inflammation and CRC survival. Specifically, it is, to our knowledge, the *first epidemiologic evidence* testing the association between *post-treatment* levels of five circulating inflammatory biomarkers with CRC survival outcomes. It also addresses the methodological challenges in its application of the Mendelian randomization method in the context of survival outcomes. It is the first Mendelian randomization study using additive hazards models to appropriately evaluate the association between genetically explained levels of inflammatory biomarkers and CRC survival.

Leveraging the well-characterized population of stage II-III CRC patients from the Seattle CCFR, we measured the concentrations of five inflammatory biomarkers (CRP, IL-6, MCP-1, adiponectin, and leptin) using existing plasma samples collected after treatment completion. Post-treatment circulating concentrations of CRP, IL-6, MCP-1, and adiponectin were associated with a higher risk of both overall mortality over 10 years follow-up. For CRP and MCP-1, these associations were likely driven by patients with CRP>10mg/L. No associations were observed after excluding these individuals. IL-6 and adiponectin were robustly associated with overall mortality with statistically significant dose-response effects. The associations between these inflammatory biomarkers and CRC-specific mortality were only observed within the first year of blood draw, except IL-6. Its association with CRC-specific mortality remained statistically significant over the 10-year follow-up period. In contrast, circulating concentrations of leptin were not associated

with overall mortality but were inversely associated with CRC-specific mortality within the first year of blood draw. These results from Chapter 2 suggest that IL-6, by itself or in combination with other markers of inflammation, may serve as a risk stratification tool to identify subsets of patients who are at a higher risk for disease progression. This can inform targeted CRC surveillance and post-treatment care.

Chapter 3 leveraged the largest study population of CRC cases from the International Survival Analysis in Colorectal Cancer Consortium (ISACC). Using the Mendelian randomization approach, we observed no association between genetically predicted CRP concentrations and CRC-specific survival. Our study results from two-stage regression based on individual genotype data, by constructing a weighted GRS based on 52 CRP-associated SNPs identified in previous GWAS[23], are consistent with the results using summary statistics (such as IVW, MR-Egger, and weighted median approaches). No associations were observed in subgroups defined by tumor stage at diagnosis and tumor location. Our findings suggest that circulating CRP is not causally associated with survival after a diagnosis of CRC, which is consistent with previous studies of CRC incidence and survival. Based on our findings on IL-6 from Chapter 2, future studies could use the Mendelian randomization approach to evaluate genetically predicted levels of IL-6 in relation to disease-specific survival. Existing studies demonstrated that genetic variants at coding regions of *IL6* and *IL6R* loci were associated with circulating concentration of IL-6[147] and that blockade of the interleukin-6 receptor (IL6R) with a monoclonal antibody (tocilizumab) can reduce systemic and articular inflammation. [148,149] Tocilizumab was licensed for the treatment of rheumatoid arthritis.[150] By applying the Mendelian randomization approach, future studies could use genetic

variants in the gene *IL6R* to evaluate IL-6 receptor inhibition for secondary prevention of CRC. This may help provide insight into therapeutic interventions among CRC patients.

The identification of genetic and phenotypic inflammatory markers associated with CRC survival may help identify subgroups of CRC patients who are at a higher risk of disease progression, and who are more susceptible to chronic inflammation and therefore perhaps are more likely to benefit from anti-inflammatory drugs (such as aspirin) for secondary prevention. Future studies can use these genetic and circulating markers of inflammation together with environmental factors to build a prognostic model among CRC patients for not only survival outcomes but also disease recurrence. Our study findings could advance the goal of developing better risk stratification tools for targeted CRC surveillance, improved post-treatment care, and ultimately to enhance patients' outcomes.

VITA

Xinwei Hua was born and grew up in Ma'anshan, China. She has lived and traveled to many places and has called Seattle home in the past six years. She earned her Bachelor of Engineering degree in Bioengineering at the South China University of Technology from Guangzhou, China and earned her Master of Public Health in Epidemiology from Emory University in 2012. During the global coronavirus pandemic in 2020, she earned a Doctor of Philosophy at the University of Washington and will start her post-doctoral fellowship at Massachusetts General Hospital in Boston.

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